Department of Physiology

Institute of Neuroscience and Physiology

Sahlgrenska Academy at University of Gothenburg

GLIAL HEMICHANNELS

A NEW ROUTE FOR CHEMICAL COMMUNICATION IN BRAIN

MALIN STRIDH

UNIVERSITY OF GOTHENBURG

2008

ISBN: 978-91-628-7468-1

ABSTRACT

The extracellular neurochemistry determines normal brain function and the faith of neurons after insults such as stroke. This thesis concerns the effect of extracellular events related to intense neuronal stimulation and stroke, i.e. over-activation of glutamate-receptors and dramatically decreased extracellular Ca^{2+}-concentrations, on efflux of neurotoxic and neuroprotective substances. The use of cultured slices of rat hippocampus enabled parallel analysis of efflux in combination with determination of delayed nerve cell death after brief (5 min) overactivation of NMDA-receptors or omission of extracellular Ca^{2+} for 15 min. Efflux by NMDA-receptor stimulation was selective and dominated by N-acetylaspartate, the antioxidant glutathione, phosphoethanolamine, taurine and hypotaurine. The efflux induced by concentration at and above 60 μM NMDA was paralleled by delayed neurotoxicity 24 h later. The efflux pathway is still unknown but does not appear to involve hemichannels, the Ca^{2+}-calmodulin dependent kinase II or NO-synthesis.

Efflux activated by omission of extracellular Ca^{2+} for 15 min caused an efflux pattern from cultured slices that was dominated by glutathione but lacked N-acetylaspartate, indicating efflux originating from glial cells. This efflux was blocked by gap junction blockers, carbenoxolone, flufenamic acid and endothelin-1, which indicated efflux from activated so called hemichannels (half gap junctions). The involvement of hemichannels was further strengthened by the inhibitory effect of a mimetic/blocking peptide for Cx43, the major connexin-protein in astroglial cells. Inhibitors of other putative channels, the $P2X_7$-receptor and pannexin hemichannels, were without effect. Volume regulated channels were probably not involved as hypertonic medium did not reduce the efflux stimulated by omission of extracellular Ca^{2+}. The efflux was mainly of glial origin as cultured slices in which neurons had been degenerated showed similar efflux pattern by omission of Ca^{2+}. These results together showed that omission of extracellular Ca^{2+} activate opening of glial connexin hemichannels. Omission of extracellular Ca^{2+} did not induce delayed nerve cell death as long as glutamate uptake was intact. However, using glutamate uptake blockers revealed that opening of glial hemichannels resulted in glutamate efflux which caused delayed neurotoxicity and efflux of N-acetylaspartate, i.e. effects similar to that induced by NMDA-receptor overactivation. In another set of experiments the efflux induced by Ca^{2+}-omission from primary astroglial cultures was characterized. Using inhibitors for $P2X_7$-receptors, gap junctions and connexin hemichannels demonstrated efflux of the neuroprotective substance adenosine via connexin hemichannels. It was also shown that curcumin, an agent which activate a transcription factor which in turn induce transcription of a multi-fold of antioxidant genes, dramatically increase both efflux and intracellular levels of glutathione.

The main finding of the work is that opening of astroglial connexin hemichannel cause efflux of neuroprotective substances. However, opening of hemichannels in conditions with reduced capacity for glutamate uptake, such as stroke, can cause additional neurotoxicity.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hjärnan är det mest komplexa biologiska struktur vi känner till och dess funktion är till stora delar fortfarande okänd. En sak vet man dock, den normala hjärnans funktion är till stor del beroende av den kemiska sammansättningen av den vätska som finns i det extracellulära utrymmet, det vill säga i mellanrummet mellan cellerna i hjärnan. Även om det oftast är nervceller man förknippar med hjärnans funktion, är det faktiskt en annan celltyp det finns flest av, nämligen astrocyterna. Astrocyter tillhör en grupp celler som går under benämningen gliaceller. Ordet glia kommer från det grekiska ordet för lim och länge trodda man att astrogliacellerna var ”limmet” som höll ihop nervcellerna. Idag vet man att astrocyter fyller många fler funktioner än så. Det har till exempel visats sig att astrocyterna kan hjälpa till att förse nervcellerna med näring och att de kan känna av och svara på ändringar i nervcellsaktivitet runt sig. En speciell egenskap som astrocyterna har, är att de är sammankopplade i stora nätverk med hjälp av så kallade gap junction. Dessa kanaler, som utgörs av proteiner vid namn connexiner, möjligör transport av många viktiga ämnen och signalmolekyler mellan cellerna. Det finns även connexinkanaler som inte binder samman celler utan öppnar sig ut mot det extracellulära utrymmet. Dessa halva gap junctions kallas för hemikanaler.

Denna avhandling fokuserar på hur den extracellulära kemin påverkas av situationer som kan uppkomma vid stroke och vid intensiv neuronal signalering, närmare bestämt överaktivering av glutamatreceptorer i hjärnan och låga halter av extracellulärt kalcium. Vi har genom studier på odlade hjärnskivor visat att stimulering av glutamatreceptorer av NMDA-typ ger ett kraftigt utflöde av antioxidanten glutation, den neuronspecifika aminosyran N-acetylaspartat och ett flertal andra aminosyror. Stimulering av NMDA-receptorer orsakade en fördröjd skada på neuronen i hjärnskivorna och graden av cellskada 24 h efter försöket korrelerade intressant nog med utflödet av glutation och N-acetylaspartat. När vi utsatte de odlade hjärnskivorna eller odlade astrocyter för drastiskt reducerade kalcium-nivåer extracellulärt fann vi återigen ett utflöde av glutation och flera aminosyror. Däremot ökade inte utflödet av N-acetylaspartat från hjärnskivorna, vilket tyder på att utflödet främst kommer från astrocyterna. Behandlingen orsakade ingen cellskada. Däremot när hjärnskivorna utsattes för minskat extracellulärt kalcium samtidigt som astrocyternas glutamat-återupptagsmaskineri hämmats, orsakade behandlingen en cellskada som liknade den som uppkom 24 h efter stimulering av NMDA-receptorerna. En kraftig ökning av de extracellulära glutamatkoncentrationerna kunde också påvisas. Utflödet som orsakades av låga extracellulära kalciumnivåer blockerades av antagonister mot gap junction kanaler och beror till största sannolikhet på öppning av halva gap junctions, hemikanaler.

Frisättning av glutation har visat sig ha nervskyddande egenskaper. Det är möjligt att hemikanalsöppning och frisättning av glutation kan vara ett sätt att hjälpa neuronen att klara sig under situationer av oxidativ stress. Vi har visat att man kan öka både de intracellulära mängderna och frisättningen av glutation med hjälp av curcumin som finns i gurkmeja. Astrocyterna frisätter även ett annat ämne med skyddande egenskaper vid lågt extracellulärt kalcium, nämligen adenosin. Dessa resultat leder till slutsatsen att hemikanalsöppning kan ha en skyddande effekt på nervceller genom att förse dem med glutation och adenosin. Om astrocyternas funktion däremot är störd och de inte kan ta upp glutamat lika effektivt som normalt, kan hemikanalsöppning och glutamatutflöde leda till överaktivering av NMDA-receptorer och nervcellsdöd.

LIST OF PUBLICATIONS

The thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I. Mattias Tranberg, Malin H. Stridh, Yifat Guy, Barbro Jilderos, Holger Wigström, Stephen G. Weber and Mats Sandberg
NMDA-receptor mediated efflux of N-acetylaspartate: physiological and/or pathological importance?
Neurochemistry International (2004) Dec; 45(8):1195-204

II: Malin H Stridh, Mattias Tranberg, Stephen G. Weber, Fredrik Blomstrand and Mats Sandberg
Stimulated efflux of amino acids and glutathione from cultured hippocampal slices by omission of extracellular calcium: likely involvement of connexin hemichannels
Journal of Biological Chemistry (2008) Feb; doi:10.1074/jbc.M704153200

III: Malin H Stridh, Stephen G. Weber, Fredrik Blomstrand, Michael Nilsson and Mats Sandberg
Stimulated efflux of adenosine via astroglial connexin hemichannels
Submitted to Neuroscience letters

IV. Malin H Stridh, Stephen G. Weber, Fredrik Blomstrand, Michael Nilsson and Mats Sandberg
Characterization of glutathione efflux from astroglial connexin hemichannels
Manuscript

Related work not included in the thesis:

Mattias Tranberg, Malin H. Stridh, Barbro Jilderos, Stephen G. Weber and Mats Sandberg
Reversed phase HPLC with UV-detection for the determination of N-acetylaspartate and creatine.
Analytical Biochemistry (2005) Aug; 343(1): 179-82

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACSF	artificial cerebrospinal fluid
AMP	adenosine mono-phosphate
AQP	aquaporin
ATP	adenosine tri-phosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BBG	brilliant blue G
BSO	buthionine sulfoximine
CaMKII	Ca^{2+}/calmodulin-dependent kinase II
CBX	carbenoxolone
CFTR	cystic fibrosis transmembrane conductance regulator
CNS	central nervous system
Cx	connexin
EAAT	excitatory amino acid trasporter
ET-1	endothelin-1
FFA	flufenamic acid
GFAP	glial fibrillary acidic protein
GLAST	glutamate and aspartate transporter
GLT-1	glutamate transporter 1
GSH	glutathione (reduced form)
GSSG	glutathione disulfide (oxidized form)
GZA	glycyrrhizic acid
HPLC	high pressure liquid chromatography
LDH	lactate dehydrogenase
L-NAME	L-N^G-Nitroarginine methyl ester
MCT	monocarboxylate transporter
Mrp	multidrug resistance protein
NAA	N-acetylaspartate
NCAM	neural cell adhesion molecule
NMDA	N-methyl-D-aspartate
Nrf2	nuclear factor E2-related factor-2
Panx	pannexin
PDC	L-trans-pyrrolidine-2,4-dicarboxylic acid
PEA	phosphoethanolamine
PI	propidium iodide
SNARE	soluble NSF attachment receptor
TBOA	DL-threo-β-benzyloxyaspartic acid
TNF-α	tumour necrosis factor α
VDAC	voltage dependent anion channel
VGLUT	vesicular glutamate transporter
VRAC	volume regulated anion channel

INTRODUCTION

Cellular organisation of the central nervous system

The brain is the most complex biological structures we know about today. It is composed of several different cell types, all of which are vital to the proper function of the brain. There are two classes of cells in the brain, nerve cells and glia cells. Neurons constitute the main signalling units, but the most abundant cells in the brain are the glia cells.

The glial cells can be divided into three different classes with diverse functions. The oligodendrocytes are the myelin-producing cell, responsible for insulating the axons and ensuring a fast and correct signal transmission. The microglia are the immunocompetent cells of the central nervous system (CNS) and can be described as sensors of pathological events (Kreutzberg 1996). Normally, microglia reside in a resting state and are engaged in monitoring the extracellular space. They can rapidly become activated in response to changes in their microenvironment caused by for instance viral and bacterial infections and physical injuries (Raivich 2005). Pathological activation of microglia has implicated in a wide range of conditions such as cerebral ischemia, Alzheimer's disease, prion diseases and multiple sclerosis, for review see (Nakamura 2002).

Astrocytes

The third class of glial cells consists of the most abundant cells in the brain, the astrocytes. They are estimated to represent over 50% of the total cell number in the cerebral cortex of mammals (Bass et al. 1971; Tower and Young 1973). Astrocytes were named after the stellate structure revealed by staining for the astrocytic cytoskeletal protein glial fibrillary acidic protein (GFAP). Recent studies using microinjection of dye into single astrocytes have uncovered a rather different appearance. In fact, astrocytes are more bush-like than star-shaped, with many fine protrusions arranged in specific domains. These microdomains are arranged with minimal overlap between different astrocytes (Bushong et al. 2002; Wilhelmsson et al. 2004).

The word glia originates from the greek word for glue and the glial cells was originally described as the cement that holds the neurons together in the brain. Now it is known that the astrocytes perform an array of different functions in the brain and the list of functions assigned to astrocytes is growing rapidly. The astrocytes in the brain do not constitute one

homogenous population. Instead, several studies suggest the existence of subpopulations of cells with different electrophysiological characteristics, glutamate receptor expression and gap junction coupling (Matthias et al. 2003; Steinhauser et al. 1992; Wallraff et al. 2004).

The astrocytic network

Astrocytes have been shown to form large networks via gap junctions (Binmoller and Muller 1992; Dermietzel et al. 1991; Fischer and Kettenmann 1985; Rouach et al. 2002a). Due to the extensive gap junctional coupling between these cells, it has been suggested that astrocytic functions should be viewed from the perspective of groups of communicating cells instead of single cells acting on their own (Giaume and McCarthy 1996). Functions assigned to this network include transport of energy substrates from the blood-brain interface to the brain parenchyma (Giaume et al. 1997; Morgello et al. 1995) and propagation of Ca^{2+}-waves. Intracellular Ca^{2+}-oscillations are a vital part of the astrocytes intra- /intercellular signalling system and can be elicited either spontaneously (Aguado et al. 2002; Parri et al. 2001) or by a number of triggering factors including mechanical stimuli and activation of metabotropic glutamate receptors (Chen et al. 1997; Deitmer et al. 1998; Venance et al. 1997; Zur Nieden and Deitmer 2006). The intracellular Ca^{2+}-oscillations is propagated in the astrocytic network either by diffusion of the intracellular second messenger molecule inositol triphosphate (Sanderson et al. 1994) or by an extracellular pathway triggered by for example connexin dependent ATP-release (Cotrina et al. 1998). These Ca^{2+}-oscillations functions as the molecular mechanism for integration within the astroglial syncytium and between glial and neuronal circuits. Ca^{2+}-signals travelling within astrocytes can for instance link neuronal activity to local circulation by triggering release of vasoactive compounds from astrocytic end-feet on to brain capillaries (Mulligan and MacVicar 2004; Zonta et al. 2003).

A role in the dissipation and homeostasis of K^+ ions has also been suggested as a main function of the astroglial syncytium (Orkand et al. 1966; Rose and Ransom 1997; Walz 2000). The importance of gap junctions in K^+ buffering have however been questioned since mice lacking coupled astrocytes still show a large capacity for K^+ redistribution (Wallraff et al. 2006).

Astrocytes form contacts with microvessels in the brain via specialized structures called perivascular endfeet. These structures are an important part in the formation and regulation of the blood brain barrier as thoroughly reviewed by Abbott (Abbott 2005). The endfeet express,

for instance, the potassium channel Kir 4.1 and the water transport channel aquaporin 4 (AQP4), proteins that presumably take part in the process of activity dependent volume regulation (Nagelhus et al. 2004; Price et al. 2002).

Astrocytes are in contact with both the brain vasculature and the neurons and appear to take an active part in supplying energetic metabolites to neurons in several different ways. The astrocytes have, in light of their extensive intercellular coupling, been suggested to operate as an metabolic syncytium by sharing their glucose and energetic intermediates, including lactate (Tabernero et al. 1996). This metabolic network is regulated by the gap junctional permeability of the cells and with that, factors that affect gap junctional coupling also affects metabolic trafficking (Giaume et al. 1997).

Chemical interaction between astrocytes and neurons

Glutamate and glutamine

The extracellular concentration of glutamate must be kept under strict control to avoid over-activation of glutamate receptors which can result in excitotoxicity, i. e. nerve cell death following uncontrolled ion influx via glutamate receptors. Astrocytes, which have their processes closely wrapped around glutamatergic synapses, reduces the extracellular glutamate concentration by an efficient up take machinery consisting of at least two glutamate transporters. The glutamate transporters predominantly expressed by glia are GLAST/EAAT1 and GLT-1/EAAT2, for review see (Gegelashvili and Schousboe 1998), with GLT-1 being the dominant transporter in the mature brain (Guillet et al. 2002). A large proportion of the glutamate is then converted to glutamine by the astrocyte specific enzyme glutamine synthetase (Martinez-Hernandez et al. 1977). Since glutamine is not neuroactive, it can be released to the extracellular space where it serves as a primary neuronal glutamate precursor (Broer and Brookes 2001). In addition to preventing excitotoxic damage to the neurons, this rapid removal of extracellular glutamate is important to keep the signal to noise ratio high during glutamatergic signalling.

Astroglial glutamate transporters usually operates to clear the extracellular space of glutamate, but during periods of elevated extracellular K^+ the transporters can reverse their operation and instead release glutamate (Szatkowski et al. 1990). Reversal of glutamate carriers is thought to contribute substantially to the extracellular glutamate that accumulates during severe brain ischemia (Rossi et al. 2000).

Lactate

Another mechanism by which astrocytes contribute to neuronal metabolism is described by Magistretti and co-workers and is coupled to astrocytic glutamate uptake. In short, glutamate uptake, stimulated by neuronal firing, causes the intracellular Na^+ levels to increase due to the fact that glutamate is cotransported with Na^+. The increase in intracellular Na^+ activates the Na^+ / K^+ -ATPase and the pump fuelled by ATP provided by membrane-bound glycolytic enzymes triggers glycolysis, i.e. glucose utilization and lactate production (Pellerin and Magistretti 1994). Lactate is then released from the astrocytes, presumably via the monocarboxylate transporter MCT-1, and taken up by the neurons via MCT-2 (Broer et al. 1997). The neurons metabolize the lactate into pyruvate that enters the mitochondria to serve as an energy fuel.

Glutathione

Glutathione (γ-Glu-Cys-Gly) is the major water soluble antioxidant in the brain. Its reducing capacities was described in already 1921 (Hopkins 1921) and the tripeptide structure was resolved almost decade later by Ben Nicolet (Nicolet 1930). It is present in the brain in millimolar concentrations and is distributed among all cells type. Glutathione exists in a reduced form (GSH) and an oxidized, dimeric form (glutathione disulfide, GSSG). In the brain, the predominant form is reduced glutathione with a ratio of 99:1 (GSH:GSSG) (Cooper et al. 1980; Folbergrova et al. 1979).

Synthesis of glutathione

In the cells, glutathione is synthesized in two steps by the action of two consecutive enzymes. First glutamate and cysteine is linked to form the dipeptide γ-glutamylcysteine (γ-GluCys). This step is carried out by γ-GluCys-synthetase. In the next step γ-GluCys is combined with a glycine in a reaction catalyzed by glutathione synthetase to form glutathione. Both of the enzymes in the process use ATP as a cosubstrate. Synthesis of glutathione is regulated by a feedback loop where glutathione inhibits the γ-GluCys-synthetase, thus ensuring that synthesis and consumption is in balance (Richman and Meister 1975). The transcription of the enzymes involved in glutathione synthesis is controlled by the nuclear factor E2-related factor-2 (Nrf2), which in turn can be activated by dietary compounds such as curcumin, sulforaphane and resveratrol (see further below).

Extracellular glutathione

Glutathione is present in the extracellular space in concentrations in the low micromolar range (Yang et al. 1994) and it has been shown in co-culture experiments that presence of astrocytes can increase neuronal glutathione (Bolanos et al. 1996). Glutathione synthesis depends on the intracellular availability of its building blocks, glutamate, glycine and cysteine. These amino acids are not present at high concentrations outside the cells due to the fact that both glutamate and glycine are neurotransmitters and that cysteine in high concentrations can have neurotoxic effects (Janáky et al. 2000). Glycine also functions as a co-agonist of the NMDA-receptor and potentiates NMDA-receptor mediated responses (Johnson and Ascher 1987). Since astrocytes and neurons preferentially use different substrates for their glutathione synthesis astrocytes are able to support the neuronal synthesis by exporting glutathione. Astrocytes prefer to use glutamate and cystine as glutathione precursors, in contrast to neurons that rely on extracellular cysteine and glutamine (Dringen and Hamprecht 1998; Dringen et al. 1999; Kranich et al. 1998; Kranich et al. 1996; Sagara et al. 1993). This differential use of precursors makes it possible for astrocytes to produce glutathione without competing for substrate with the neurons and then release it to the extracellular space. In the extracellular space, glutathione is converted by the ectoenzyme γ-Glutamyl transpeptidase to the dipeptide CysGly and a γ-Glutamyl peptide (Meister et al. 1981; Tate and Meister 1974). Data suggest that the CysGly dipeptide generated by γ-Glutamyl transpeptidase activity serves as a precursor for neuronal glutathione synthesis (Dringen et al. 1999), but whether it is the dipeptide itself that is taken up by the neurons or if it is hydrolyzed in the extracellular space by a neuronal ectopeptidase to cysteine and glycine is not fully known. Astrocytes also contribute with the other substrate for neuronal glutathione synthesis by their release of glutamine.

Glutathione as an antioxidant

Glutathione is a very important of the cellular defence against accumulation of reactive oxygen species. It can react directly with radicals such as superoxide radical anions, nitric oxide or hydroxyl radicals via non-enzymatic processes (Clancy et al. 1994; Singh et al. 1996; Winterbourn and Metodiewa 1994). It can also function as an electron donor in the reduction of peroxides, a reaction catalyzed by glutathione peroxidases (Chance et al. 1979). The final product of oxidation of glutathione is glutathione disulfide (GSSG). Glutathione disulfide is a substrate for the enzyme glutathione reductase. This enzyme transfers electrons from NADPH to GSSG, thus regenerating glutathione.

Glutathione as a neuromodulator

Glutathione is considered as a possible neurohormone (Guo et al. 1992; Janaky et al. 1999) based on the fact that it is present in the extracellular space and that it binds specifically to extracellular receptors in the brain (Guo and Shaw 1992; Lanius et al. 1994), which appear to be linked to Na^+ ionophores as glutathione causes Na^+-dependent depolarization in the neocortex in vitro (Shaw et al. 1996). It has also been shown that glutathione is an endogenous ligand of glutamate receptors with capability of modulating central excitability (Ogita et al. 1995; Regan and Guo 1999; Steullet et al. 2006). With these data in mind, glutathione might be added to the list of glia-derived transmitters as have been suggested for glia-derived glutamate, D-serine and ATP/adenosine (Martin et al. 2007; Miller 2004; Vesce et al. 2001; Volterra and Steinhauser 2004).

Glutathione in redox regulation

The redox state of a cell is determined by the balance of its oxidizing components and its reducing equivalents. It is important for the cell to keep the concentrations of reactive oxygen species, free radicals and other oxidants low to avoid oxidative damage to proteins, lipids and nucleic acids. However, below their toxic threshold, reactive oxygen species, free radicals and other oxidants may have signalling functions, for review see (Gabbita et al. 2000). This often includes oxidative changes of kinases and phosphatases, which in turn may affect transcription factors leading ultimately to a changed expression profile. One example of such oxidation-mediated signalling is the Nrf2-ARE system discussed in a section below.

Glutathione and the Nrf2-ARE system

The production of reactive oxygen species is an inevitable consequence of cellular metabolism and can lead to DNA damage and protein and lipid oxidation. To counteract these deleterious effects, animal cells have developed several defence mechanisms including phase II detoxification enzymes and antioxidant proteins. The antioxidant responsive element (ARE) is a regulatory element found in the promoter regions of several genes encoding so called phase II detoxification enzymes and antioxidant proteins, including NAD(P)H, quinine oxidoreductase, glutathione-S-transferases and glutamate-cysteine ligase (Mulcahy et al. 1997; Rushmore et al. 1990).

The cytosolic transcription factor Nrf2 is under normal conditions kept in an inactive state by binding to the cytoskeleton-associated protein Keap1 (Itoh et al. 1999; Kobayashi et al. 2002). The interaction between Nrf2 and Keap1 can be antagonized by electrophilic agents suggesting that the Nrf2-Keap1 complex is capable of sensing oxidative stress (Itoh et al. 1999). Once released from the inhibition by Keap1, Nrf2 is translocated from the cytosol to the nucleus where it binds to the ARE-sites (Alam et al. 1999; Moi et al. 1994). Keap1 also have an important role in terminating the Nrf2 mediated transcription. Keap1 has been shown to translocate into the nucleus independently of Nrf2 and terminates transcription by escorting Nrf2 out of the nucleus (Sun et al. 2007). In the cytosol, Keap1 targets Nrf2 for proteosomal degradation by binding to it and recruiting the complex into the E3 ubiquitine-ligase complex for ubiquitination (Stewart et al. 2003; Sun et al. 2007). This intricate signalling system is highly conserved in vertebrate cells (Kobayashi et al. 2002).

Glutathione and Nrf2 activating agents

Several plant derived substances have been shown to activate the Nrf2-ARE system. Keap1 is rich in cysteine residues, which contain sulfhydryl groups (Itoh et al. 1999), and therefore it is likely that the mechanism of many of the Nrf2 inducers act by separating Nrf2 from Keap1 by reacting with these cysteine residues. Curcumin, the bioactive component of turmeric *(Curcuma longa)*, have been shown to potently induce Nrf2-mediated transcription (Balogun et al. 2003). In the same study, similar effects were seen by another natural antioxidant, caffeic acid phenetyl ester (CAPE). Both these substances contain electrophilic, unsaturated carbonyl groups that are capable of reacting with thiols and curcumin is able to relieve inhibition mediated by Keap1 in a coexpression model (Balogun et al. 2003). Two other plant derived Nrf2 inducers are resveratrol, found in grapes, and sulforaphane, found in broccoli (Chen et al. 2005; Kraft et al. 2004; Thimmulappa et al. 2002). Both sulforaphane and curcumin have been proven efficient when it comes to reduce cellular damage after ischemic insults, a condition known to cause increased levels of oxidative stress (Al-Omar et al. 2006; Wang et al. 2005; Zhao et al. 2006).

Transport of glutathione by multidrug resistance proteins

Multidrug resistance proteins (Mrps) are ATP-driven export pumps that mediate export of organic anions (Kruh and Belinsky 2003). Mrps fulfil several essential transport functions, depending on the expressing cell type. Typical Mrp substrates include glutathione-S-conjugates, glutathione disulfide (GSSG), conjugates of glucuronate cyclic nucleotides and

nucleotide analogues (Homolya et al. 2003; Konig et al. 1999; Kruh and Belinsky 2003). There is substantial evidence for expression of Mrp1 (Decleves et al. 2000; Hirrlinger et al. 2001) and Mrp3-5 (Ballerini et al. 2002; Hirrlinger et al. 2002a) in astrocytes, both *in vivo* and in cultures. In astrocytes Mrp1 but not Mrp5, have been shown to mediate export of GSH and GSSG (Hirrlinger et al. 2001; Hirrlinger et al. 2002b; Minich et al. 2006).

Adenosine

Adenosine is a neuromodulator with many effects in the brain. It has been shown to increase in the extracellular space during pathological conditions such as epileptic activity (Dunwiddie 1999), hypoglycemia and hypoxia /ischemia (Hagberg et al. 1987; Rudolphi et al. 1992; Schubert et al. 1994). Most of the effects of adenosine are conveyed via 4 main receptor subtypes in combination with different intracellular transducing pathways (Fredholm et al. 2001) that in turn have effects on diverse targets, from ion channels to gene transcription. The experiments so far have mainly been focused on effects of adenosine on neurons and its neuroprotective actions via presynaptic A1 adenosine receptors (Arrigoni et al. 2005; Fowler 1990). Ischemic preconditioning involves adenosine signalling and the reduction in ischemic injury was found to be mediated by A1-receptor activation (Heurteaux et al. 1995). During hypoxia, astrocytes have been shown to release adenosine. This downregulates the synaptic activity via the A1 adenosine receptor, a mechanism proposed to be neuroprotective during transient hypoxia (Martin et al. 2007).

Recent studies suggest that not only neurons, but glial cells as well, are affected by activation of adenosine receptors. For example, astroglial reactivity that follows different disorders can be induced via activation of A2a receptors (Brambilla et al. 2003). Likewise microglial activation and production of cytokines such as TNF-α can be reduced via these receptors (Boucsein et al. 2003).

N-acetylaspartate

The amino acid derivative N-acetylaspartate is a substance first discovered in the brain of rats in 1956 (Tallan et al. 1956). It is a divalent anion at physiological pH and is mainly located in the central nervous system with small amounts detected in the peripheral nervous system. The highest concentration (up to 10 mM) is found in mammalian and avian brain while the concentrations in the peripheral nervous system and retina are five-fold lower (Miyake and Kakimoto 1981; Nadler and Cooper 1972). It has been shown to be located primarily in

neurons, but a small fraction is found in oligodendrocytes (Koller and Coyle 1984; Moffett et al. 1991; Nadler and Cooper 1972). The concentrations of N-acetylaspartate increase uniformly throughout the brain and the peripheral nervous system during development (Florian et al. 1996; Koller and Coyle 1984; Miyake and Kakimoto 1981; Tallan 1957). The function of N-acetylaspartate in the brain is elusive, but several theories and suggestions have been made. These suggestions include functions as a myelin precursor (D'Adamo et al. 1968; D'Adamo and Yatsu 1966), energy substrate (Mehta and Namboodiri 1995), neuromodulator and/or neurotransmitter (Akimitsu et al. 2000), N-acetylaspartylglutamate precursor (Baslow 2000) and osmoregulator (Baslow 2002). In spite of the lack of conclusive functional data, it is interesting to note that the levels of N-acetylaspartate have been shown o be decreased after stroke, in Alzheimer´s disease, multiple sclerosis and Huntington´s disease as well as a number of other neuropathologies (Tsai and Coyle 1995). Efflux of N-acetylaspartate has been reported in microdialysis studies after anoxia (Sager et al. 1999). N-acetylaspartate efflux has also been detected after depolarisation and in hypoosmotic medium (Davies et al. 1998; Taylor et al. 1994), but the efflux pathways have not been resolved.

Efflux pathways that contribute to extracellular neurochemistry

Ca^{2+} -dependent vesicular release

Vesicular release of glutamate and other transmitters is the main release pathway in neurons. Vesicular release of transmitters from glial cells has been a more controversial topic. However, Ca^{2+}-dependent release of glutamate have been reported from both cultured astrocytes and acute hippocampal slices (Bezzi et al. 1998; Parpura et al. 1994). In addition, recent findings show that glutamate can stimulate exocytotic release of ATP from cultured astrocytes (Pangrsic et al. 2007). Intracellular elevation of Ca^{2+} was shown to be sufficient and necessary to cause glutamate release (Parpura et al. 1994). Incubation of the cells with either the Ca^{2+}-chelator BAPTA (Araque et al. 1998; Bezzi et al. 1998), or thapsigargin (Araque et al. 1998), an inhibitor of a Ca^{2+}- ATPase specific for internal stores, led to a reduction in the evoked release of glutamate indicating that Ca^{2+} release from internal stores is the predominant source of Ca^{2+} in this type of release. Ca^{2+}-dependent release is in neurons mainly associated with SNARE-dependent vesicular release and there is evidence of such a release machinery in astrocytes as well. Astrocytes express SNARE proteins known to mediate exocytosis such as synaptobrevin II, syntaxin I and cellubrevin (Parpura et al. 1995) as well as vesicular glutamate transporters (VGLUTs) and vacuolar H^{+}-ATPase (Fremeau et al. 2002).

Swelling induced opening of anion-channels

Several different anion channels have been linked to swelling induced release of amino acids, inorganic anions and ATP. The most studied of these channels are the volume-regulated anion channels (VRACs), a type of anion channel found in essentially all cells. Although the channels, also termed volume-sensitive outwardly rectifying (VSOR) Cl^- channels or volume-sensitive organic osmolyte and anion channels (VSOAC),are well characterized biophysically, the molecular identity of these proteins is still not known (Nilius and Droogmans 2003; Okada 2006). The most uniform feature of these channels is the characteristic outwardly rectifying chloride current that develops in cells swollen by exposure to hypotonic media, for references see (Jentsch et al. 2002; Okada 1997; Strange et al. 1996). Astrocyte swelling have been shown to cause efflux of glutamate, aspartate and taurine via VRACs (Kimelberg et al. 1990) and this has led to a hypothesis stating that this efflux reduce intracellular osmolarity and thereby swelling via water efflux. However, this process can contribute to excitotoxicity during pathologies characterized by marked astrocytic swelling, such as stroke and closed head trauma (Feustel et al. 2004; Kimelberg 1995). The intracellular pathways regulating the activity of these channels are poorly understood, but a recent study suggest the involvement of ATP and two protein kinase C (PKC) isoforms in regulating VRAC function and efflux of glutamate from cultured astrocytes (Rudkouskaya et al. 2008).

$P2X_7$ receptors

Astrocytes express a multitude of receptors and among them are purine receptors of the $P2X_7$-type (Kukley et al. 2001). $P2X_7$ receptors are activated by extracellular ATP and upon activation, they open large channels (North and Surprenant 2000). These channels are permeable to substances up to 900 Da, but the permeability characteristics of $P2X_7$-receptors seem to vary with the expressing cell type. In some cell types the receptors allows only passage of smaller molecules or exhibit ion selectivity (Markwardt et al. 1997; Soltoff et al. 1992; Surprenant et al. 1996). It has been shown that activation of $P2X_7$-receptors can result in release of ATP from C6 glioma cells (Suadicani et al. 2006) and glutamate from cultured astrocytes (Duan et al. 2003). Another feature of the $P2X_7$-receptors is the response amplification observed in low divalent cation medium (Bianchi et al. 1999; North and Surprenant 2000). Recent studies suggest a close association of $P2X_7$-receptors and pannexin hemichannels (Locovei et al. 2007; Pelegrin and Surprenant 2006). This could in part explain the very different permeability characteristics seen in different $P2X_7$ expressing cell types.

Connexin hemichannels

Hemichannels or connexons are the terms used for unpaired gap junction channels. They are composed of hexamers of connexin subunits and in their open state they connect the intracellular space of the cell with the extracellular surroundings. The connexin gene family consists of 20 members in rodents (Willecke et al. 2002) and the most prevalent form in vertebrate tissues is Cx43 (Goodenough et al. 1996). In mammalian brain at least eight connexins have been identified and the predominant astroglial forms are Cx43 and Cx30 (Nagy and Rash 2000; Rouach et al. 2002a; Theis et al. 2005). The major oligodendroglial connexin is Cx32 (Nagy and Rash 2000) and microglia have been reported to express Cx36 (Dobrenis et al. 2005) and Cx43 (Eugenin et al. 2001). The latter form of connexin is, however, only detected after microglial activation by interferon-γ and lipopolysaccharide or tumor necrosis factor-α (TNF-α) (Eugenin et al. 2003; Eugenin et al. 2001) and is not detected when microglia is co-cultured with astrocytes (Faustmann et al. 2003; Rouach et al. 2002b).

Connexin hemichannels have a large pore diameter (~ 1,2 nm) that allows diffusion of substances up to 1 kDa. Substances that have been shown to pass through connexin hemichannels include several cytosolic metabolites and signalling molecules, such as ATP, glutamate, glutathione, prostaglandin E_2 and NAD (Bruzzone et al. 2001; Cherian et al. 2005; Cotrina et al. 1998; Rana and Dringen 2007; Stout et al. 2002; Ye et al. 2003). In a recent study, glucose and glucose derivatives was added to the list of substances that permeate Cx43 channels, as shown by uptake of a fluorescent glucose-derivate (Retamal et al. 2007a).

Since connexin hemichannels are large, relatively unselective pores that connect the cytosol to the extracellular space, uncontrolled and/or prolonged opening of such channels could have detrimental effects on cell survival. The opening of such channels must therefore be strictly regulated. A number of regulatory mechanisms have been found, including closure by high concentrations of extracellular divalent cations, in particular Ca^{2+} (Contreras et al. 2003; Valiunas and Weingart 2000). Opening probability is increased by positive membrane potentials (Contreras et al. 2003), metabolic inhibition (Contreras et al. 2002; John et al. 1999), reduced cellular redoxpotential (Retamal et al. 2006; Retamal et al. 2007b) and, most recently, the proinflammatory cytokines TNF-α. and IL1-β (Retamal et al. 2007a). Intracellular pH is another factor influencing the opening of hemichannels. Intracellular acidification causes closure of hemichannels (Trexler et al. 1999) and the mechanism behind

this effect is a direct pH-dependent effect on the C-terminus of the Cx43 subunit (Duffy et al. 2004; Hirst-Jensen et al. 2007).

Both hemichannels and gap junction channels consisting of Cx43 are regulated by phosphorylation and it seems like the Cx43 subunits exist in three different states, non-phosphorylated, Cx43-P and Cx43-PP (Cooper and Lampe 2002). Phosphorylation of connexin has been suggested to close hemichannels and this suggestion is supported by a recent study that shows the involvement of PKC in regulating size selectivity in Cx hemichannels (Bao et al. 2007).

Pannexin hemichannels

The most recent player in the field of efflux pathways from astrocytes are pannexin hemichannels or pannexons. Pannexins were discovered to be the mammalian orthologs of the invertebrate gap junction protein innexin (Baranova et al. 2004; Panchin et al. 2000) and have been proposed to be able to form gap junction channels (Bruzzone et al. 2003; Vanden Abeele et al. 2006). Connexin and pannexin show no sequence homology, but share several structural features (Panchin et al. 2000). The tissue expression of pannexin and connexin overlap considerably (Baranova et al. 2004; Bruzzone et al. 2003; Ray et al. 2005).

One feature that distinguish pannexin hemichannels from their connexin counterpart is that pannexin channels have been shown to lack gating by extracellular Ca^{2+} (Bruzzone et al. 2005). Whether the pannexins are regulated by phosphorylation like the connexins is not known. However, both pannexin (Panx) 1 and 3 have been shown to be N-linked glycosylated, a post-translational modification not reported for any of the connexins (Penuela et al. 2007). The presence of complex carbohydrates on the extracellular-loop regions of these pannexins can be predicted to interfere with formation of intercellular channels. This, taken together with the failure to form robust intercellular channels when transiently expressed in N2A cells (Penuela et al. 2007) and lack of evidence of gap junction formation other than in the paired oocyte expression system, point towards other functions for the pannexins than those of the connexins. One suggestion is that rather than being a redundant system of gap junction proteins, they exert a physiological function as hemichannels (Dahl and Locovei 2006) . Hemichannels composed of pannexins is mechanosensitive and can mediate efflux of ATP and interleukin-1β (Bao et al. 2004; Pelegrin and Surprenant 2006). Opening of Panx1

hemichannels have also been implicated in the neuronal death after ischemia (Thompson et al. 2006).

Voltage dependent anion channels (VDACs)

The presence of large conductance anion channels (>400 pS) have been described in the plasma membrane of cultured astrocytes (Sonnhof 1987) and cultured rat Schwann cells (Bevan et al. 1984) that resembles the type of voltage-dependent anion channels found predominantly in the outer mitochondrial membrane. At least one type of plasmalemmal VDAC (BR1-VDAC) have been identified on astrocytes *in situ* in bovine brain (Dermietzel et al. 1994). VDACs have been shown to release ATP after cell swelling (Sabirov et al. 2001), but seems to not be involved in the swelling-induced release of excitatory amino acids (Abdullaev et al. 2006).

NMDA-receptor mediated anion efflux

Microdialysis studies have shown that during certain pathological conditions, such as ischemia, deep hypoglycaemia and prolonged epilepsy, the efflux of the anionic amino acid phosphoethanolamine increase (Hagberg et al. 1985; Lehmann 1987; Sandberg et al. 1986b). In an *in vitro* setup this efflux was shown to be parallel to efflux of another organic anion, glutathione, and was found to be dependent on NMDA-receptor activation and extracellular calcium (Wallin et al. 1999). The pathway mediating this efflux is not known.

Cystic fibrosis transmembrane conductance regulator (CFTR)

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cyclic AMP-gated Cl^--channel that belongs to the ATP binding cassette protein superfamily. It is expressed in cultured astrocytes (Ballerini et al. 2002) and has been associated with facilitated extracellular transport of ATP (Schwiebert 1999). Function of the CFTR in CNS is poorly understood, but this channel type is permeable to larger organic anions as well as Cl^- and has been suggested to mediate export of glutathione in airway epithelial cells (Linsdell and Hanrahan 1998). An interesting discovery is the interaction between gap junction communication and CFTR activation (Chanson et al. 1999; Chanson and Suter 2001).

AIMS

The extracellular neurochemistry determines normal brain function and the faith of the neurons after insults such as stroke. This thesis concerns the effect of extracellular events related to intense neuronal stimulation and stroke, i.e. over-activation of NMDA-receptors and dramatically decreased extracellular Ca^{2+}-concentrations, on cellular efflux pathways of neurotoxic and neuroprotective substances.

The specific aims of the thesis were:

I: To investigate the temporal and chemical efflux profiles caused by NMDA-receptor over-activation and reduced extracellular Ca^{2+}-concentrations from cultured hippocampus slices.

II: To investigate the cellular origin of the efflux by analysis of the neurospecific amino acid N-acetylaspartate and by using neurodegenerated cultured hippocampus slices and primary astrocyte cultures.

III: To investigate if hemichannels are involved in the stimulated efflux.

IV: To investigate if hemichannel opening by reduced extracellular Ca^{2+}-concentrations is neurotoxic

V: To investigate how basal and stimulated efflux of glutathione relate to changes in intracellular levels

METHODS

Organotypic hippocampus cultures (Paper I and II)

Organotypic cultures of hippocampal tissue were prepared using the interface method according to Stoppini (Stoppini et al. 1991). In brief, hippocampi of eight to nine days old Sprague-Dawley rat pups were dissected and cut in 400 µm thick slices using a McIlwain tissue chopper. The slices were transferred to a Petri dish containing Gey´s balanced salt solution with 0.45 g/l of D-glucose. Four slices were put on a porous membrane insert (Millicell CM; Bedford, MA, USA) in 6-well plates with 1.3 ml culture medium. Slices were cultured for 12-14 days at 36 ºC in a humidified atmosphere containing 5% CO_2 and 95% air. Culture medium, 1.2 ml, was changed twice a week. Slice cultures with a low number of neurons were prepared by incubating slice cultures with 300 µM NMDA for 24 h three to four days prior to efflux experiments. The slices were cultured in medium containing Basal medium Eagle and Earl's basal salt solution (50 and 20 %, respectively), horse serum (23 %), penicillin/streptomycin (25 U/ml), L-glutamine (1 mM) and D-glucose (41.6 mM).

Comments:

Organotypical hippocampal cultures can be kept alive, with preserved cytoarchitecture, for several weeks (Bahr 1995; Gahwiler et al. 1997). This stability makes the model suitable for studies of prolonged events (i.e., days to weeks) such as synaptogenesis, excitotoxicity and slow degenerative processes associated with aging and age-related disorders. Aditionally, it is easy to gain access to the cells with different pharmacological tools. The cultured slices have been shown to resemble the adult *in vivo* hippocampus in many aspects. For instance, they maintain their glutamate receptors and other synaptic components such as synaptophysin and NCAMs as well as structural and cytoskeletal components for at least up to 30 days in culture (Bahr et al. 1995). However, in some aspects they show a more immature/different phenotype than *in vivo*. It has been shown that they retain a more immature pattern of lactate dehydrogenase isozymes (Schousboe et al. 1993) and this might suggest an incomplete transition from anaerobic to aerobic glycolysis. They also lack the developmental increase in N-acetylspartate seen *in vivo* (Baslow et al. 2003). Concerning the glial cells in the cultured slice it is important to note that the astrocytes do not retain their layer-specific distribution (Derouiche et al. 1993) as shown by staining for glutamine synthetase. *In vivo*, the staining is layer-specific and perisynaptic with the highest immunoreactivity found in well-defined termination zones of glutamatergic hippocampal afferents. This distribution is not present in

cultured hippocampal slices, which might indicate that the laminated organisation of glutamine synthetase expression is in the hippocampus is dependent on neuronal activity. The oligodendrocytes have a distribution and phenotype corresponding to the *in vivo* situation (Berger and Frotscher 1994).

Primary astrocyte cultures (Paper III and IV)

Primary cultures of astrocytes were prepared from the hippocampi of newborn (P1-P2) Sprague-Dawley rats as described previously (Hansson et al. 1984; Nodin et al. 2005). In brief, the rats were decapitated and the hippocampi were carefully dissected. The tissue was mechanically passed through a nylon mesh (80 μm mesh size) into culture medium consisting of minimum essential medium (MEM) supplemented to the following composition: 20% (v/v) fetal calf serum, 1% penicillin-streptomycin, 1.6 times the concentrations of amino acids and 3.2 times the concentration of vitamins (in comparison to MEM), 1.6 mM L-glutamine, 7.15 mM glucose and 48.5 mM $NaHCO_3$. The cells were grown in 35 mm wells at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed after three days in culture and thereafter three times per week. Cells were used after 14–19 days in culture when a confluent monolayer had been formed. For the efflux experiments the cells were cultured in 35 mm Petri dishes.

Comments:

Primary cultures are cell cultures prepared directly from animal tissues. The cells are harvested from newborn animals and it is therefore important to recognize that the results obtained using these cultures probably reflect the immature phenotypes of the cells. The cells are grown in medium which contains fetal bovine serum, containing an undefined mixture of growth factors, which may also influence the cells to retain their immature properties. In the case of astroglial cell cultures, the degree of reactivity must also be considered. The preparation of the cell cultures does, in itself, resemble a traumatic injury and may therefore induce a much higher degree of reactivity than what is exhibited by cells *in situ*.

The benefits of using primary cultures of astrocytes are many. Since the cells grow in monolayers, it is easy to access all cells when drugs are added to the incubation medium. It is a clean system where the results reflect the properties of a single cell type. However, to get reproducible results, it is important to make sure that the cells are in a confluent state before they are used in experiments and that the contamination of microglia is low.

HPLC-analysis of glutathione and amino acids (Paper I, II and IV)

Glutathione and amino acids were determined using *o*-phtaldialdehyde (OPA) derivatization and fluorescence detection essentially as described earlier (Lindroth and Mopper 1979; Sandberg et al. 1986a). A solution of β-mercaptoethanol, Na_2-EDTA and NaN_3 (final concentration 20, 1 and 5 mM respectively) was added to the samples and standards to keep GSH in its reduced form as well as to prevent bacterial growth. The OPA-solution was prepared weekly and consisted of OPA (40 mg) dissolved in methanol (400 µl), β-mercaptoethanol (40 µl), borate buffer (2.0 ml, 0.8 M, pH 12) and H_2O (1.6 ml). Every two days β-mercaptoethanol (10 µl) was added to the solution. Amino acids were derivatized (25 µl of sample mixed with 25 µl OPA solution) in the autosampler before injection. The amino acid derivatives were separated on a Nucleosil C_{18} column (200 x 4.6 mm; Macherey-Nagel, Germany) with a mobile phase consisting of NaH_2PO_4 (50 mM, pH 5.28) and methanol in a gradient from 25-95 % methanol. A flow rate of 1 ml/min was used. Detection was carried out by excitation at 333 nm and emission over 418 nm.

Comments:

Precolumn derivatization of the sample with *o*-phtaldialdehyde allows fluorescence detection of glutathione and amino acids, making the method highly sensitive. However, the derivatization with *o*-phtaldialdehyde is limited to primary amines and can therefore not be used to analyze secondary amino acids such as proline. This method does not discriminate between oxidised and reduced glutathione due to the addition of β-mercaptoethanol in the reagent solution. However, the main part of the glutathione released after NMDA stimulation have been shown to be in the reduced form (Wallin et al. 1999) and the reduced form have also been found to be predominant in the brain (Cooper et al. 1980; Folbergrova et al. 1979).

HPLC-analysis of purine catabolites (Paper III)

Chromatography of purine catabolites was performed using a HPLC pump coupled to a UV detector. All separations were performed at room temperature. Sample injection was made using an autosampler. Analysis of purine catabolites were carried out as described earlier (Hagberg et al. 1987). In brief, samples were run on a column (ACE 5 C18; 4.6 mm in diameter, 150 mm in length) packed with C18 coated particles (5 µm). Sample volumes of 40 µl were injected and the purine catabolites were eluated with a buffer containing 94 % 10 mM

$NH_4H_2PO_4$ (pH 5.50) and 6% methanol. The UV-absorbance was measured at 254 nm. Identification of the peaks was carried out by adding known amounts of each compound to the samples. Quantification was determined by external standardisation and standards were run at three different concentrations. The resulting linear standard curve (peak area vs. concentration) was used to calculate the concentration in the samples. Standards were run before and after each sample set.

Comments:

The advantage of this method is that it is a straight forward, isocratic method and it does not require any special sample preparation. However, the disadvantage to this method is that it is based on UV absorbance which is less sensitive than, for example, fluorescence detection. Another disadvantage is that many of the drugs used in these studies also absorb in the UV range of the spectrum, which may complicate analysis.

HPLC-analysis of N-acetylaspartate (Paper I and II)

Separation of N-acetylaspartate was carried out at room temperature using a TSK-GEL ODS-80T column (250 x 4.6 mm; 5µm particle size Tosoh, Tokyo, Japan). The mobile phase consisted of 50 mM NaH_2PO_4 (pH 2.15) and was degassed with N_2 before use. The flow rate was 1 ml/min and N-acetylaspartate was detected by absorbance at 210 nm. To improve the peak shape, the samples were mixed with HCl (0.2 M) in a ratio of 6:1 (sample/HCl) prior to injection. Sample injection volume was 90 µl. The N-acetylaspartate peak was identified and quantified using external standards and by the addition of known amounts of N-acetylaspartate to the samples.

Comments:

The most commonly used method for HPLC-based analysis of N-acetylaspartate is that described by Koller and co-workers (Koller et al. 1984). However, this method is not optimal for detection of N-acetylaspartate in buffers with high K^+ and therefore, a reversed-phase method developed to function in saline sample buffers was used (Tranberg et al. 2005). One drawback with this method is the low pH of the buffer needed to keep N-acetylaspartate neutral. Low pH, in combination with the fact that pure aqueous buffers are not recommended

for silica columns, can increase the rate of silica hydrolysis. This, in turn, may greatly shorten the lifespan of the column.

Efflux protocol for slice cultures (Paper I and II)

The slices were incubated for 30 minutes in serum-free medium followed by another 30 minutes period of incubation in ACSF before the beginning of the efflux experiments. The efflux experiments were carried out by transferring the inserts with the slices to a 6 well plate kept in a water-bath set at 36 ºC (for details see (Tranberg et al. 2004). The atmosphere inside the plate was kept at 60 % O_2, 35 % N_2 and 5 % CO_2 by directing a flow of gas into a water filled container inside the plate and performing the incubation with the lid on. All solutions were equilibrated with a gas-mixture of 60 % O_2, 35 % N_2 and 5 % CO_2 (Pomper et al. 2001). The efflux experiments were carried out by incubating the slices with ACSF (400 µl) on top of the membrane for 5 min. The fluid was then removed and filtered before immediate HPLC analysis or storage in – 20 °C (maximally two weeks). This incubation procedure was repeated 9 times (45 min in total) with Ca^{2+} omission during the 5, 6 and 7th incubation periods (20-35 min). All inhibitors were present during the second 30 minutes preincubation period and the whole incubation period (50 min in total before Ca^{2+} removal). After the experiments, the slices were cultured in culture medium with added propidium iodide (PI). When NMDA-stimulation was used instead of Ca^{2+}- omission, 60 µM NMDA was added during the fifth incubation.

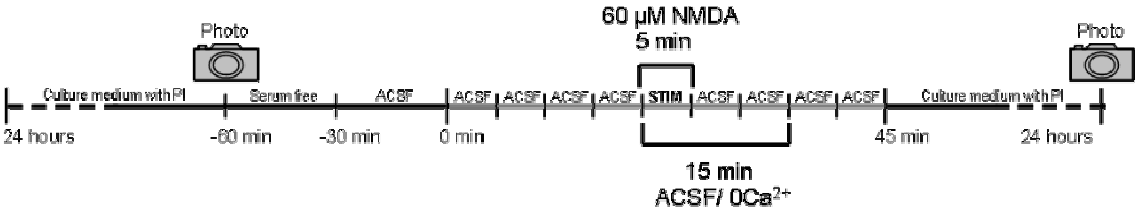

Fig 1. Time scale of efflux experiments in combination with analysis of delayed nerve cell death in cultured hippocampal slices. (PI, propidium iodide)

Efflux protocol for primary cell cultures (Paper III and IV)

The efflux protocol for the primary cell cultures resembles the protocol for the organotypical slices with a few modifications. The cells were incubated in ACSF for 30 minutes before the start of the experiment. Inhibitors used in the experiments were added during this incubation.

The experiments were carried out by incubating the cells with ACSF or ACSF/ $0Ca^{2+}$ (400 µl) for 10 min. The fluid was then removed and filtered before immediate HPLC analysis or storage in – 20 °C (maximally two weeks). The incubation procedure was repeated 7 times (70 min in total) with Ca^{2+} removal occurring during the fourth and fifth incubation period. All inhibitors were present during a 30 min preincubation period and the whole incubation period (60 min in total before Ca^{2+} removal). All solutions were equilibrated with a gas-mixture containing 5 % CO_2 to reach a pH of ~7.4. Thereafter, all solutions were put in an incubator in a humidified atmosphere of 95% air and 5% CO_2 at 36.5 ºC for at least 30 min. After the seventh incubation, the cells were scraped in to 800 µl of 0.3 M $HClO_4$ and sonicated. After centrifugation at 11000 g the supernatant was removed and filtered (Acrodisc, 0.2 µm, Pall Corporation, Ann Arbor, MI, USA).

Comments on the efflux models:

These protocols for measuring efflux offer an easy way of measuring release of substances from cells and slice cultures and it offers an opportunity to measure both efflux and delayed cell death. It is easy to gain access to all of the cells when using cell cultures. However, when it comes to slice cultures attention must be paid to ensure that the incubation time is long enough to let inhibitors and other drugs penetrate the slice. It is also difficult to assess whether it is possible to gain access to the inner part of the slice or if the resulting efflux originates from the outer cell layers only. An additional drawback with this method is the low temporal resolution that, in combination with the large incubation volume used in the experiments, makes it difficult to follow quick changes in efflux rates.

Determination of intracellular concentrations of glutathione and amino acids: (Paper IV)

Intracellular glutathione and amino acids were extracted after the efflux experiments by addition of 400 µl of 0.3 M $HClO_4$ to the wells. The cells were scraped off the bottom of the well and the samples were sonicated. After centrifugation at 11000 g the supernatant was removed and filtered (Acrodisc, 0.2 µm, Pall Corporation, Ann Arbor, MI, USA). The supernatant was used to determine the cellular content of glutathione and amino acids.

Evaluation of cell toxicity

Propidium iodide uptake assay (Paper I and II)

To evaluate cell toxicity in the slice cultures, we used propidium iodide uptake as a measurement of cell death. Propidium iodide is a cell impermeable dye that becomes fluorescent when it binds to DNA. It does not enter cells with intact plasma membranes and therefore the amount of fluorescence can be correlated to the amount of cell damage. Propidium iodide was added (final concentration of 2 μM) to the slice cultures 24 h prior to the efflux experiment. Before starting the experiments, the slices were photographed using a digital camera (Olumpus DP50) coupled to an inverted fluorescence microscope (Olympus IX70) equipped with a rhodamine filter. Photographs were captured using Studio Lite and View Finder Lite software (Pixera Corporation, Los Gatos, USA). To calculate cell death in the slices, the slices were photographed again 24 h after the experiments and the photographs were converted to grayscale. Then the CA1, CA3 areas and part of the background (~ 10 % of total) were encircled and the fluorescence intensity of each area was measured by Scion Image software (Scion Corporation, Frederick, MI, USA). The fluorescence intensities obtained in slices before the efflux experiments were subtracted before calculation as described earlier (Tranberg et al. 2004). The fluorescence intensity measured 24 h after adding 300 μM NMDA to the culture medium was used as a value of maximal nerve cell death (Vornov et al. 1998). Histologic degeneration has been shown to be limited to neurons 24 h after NMDA exposure and consistent with the PI staining (Vornov et al. 1991). The fluorescence intensity in incubated slices above that of controls (i.e. non-incubated slices), was expressed as the percentage of maximum fluorescence intensity. The formula used for calculating the percentage of maximum fluorescence intensity was as follows:

Fluorescence intensity (% of max) = ((Incubated – Control) / (Max – Control)) * 100

where
Incubated = the fluorescence intensity in incubated slices 24 h after the efflux experiments,
Max = fluorescence intensity in slices subjected to 300 μM NMDA for 24 h,
Control = fluorescence intensity of non-incubated slices 24 h after the efflux experiments.

The observed cell death after NMDA-treatment correlated well with a decrease in the neuronal amino acids GABA and N-acetylaspartate.

Comments:
The use of propidium iodide as a marker of cell death has been thoroughly evaluated and has been found to correlate well with other methods of cell death determination (Noraberg et al. 1999). This method allows for an analysis of the regional differences in vulnerability in the hippocampus, since fluorescence in the different layers of hippocampus can be calculated separately.

However, uptake of propidium iodide has also been used to measure channel/pore opening in the plasma membrane (Hur et al. 2003; Kondo et al. 2000). This could possibly lead to an over-estimation of the cell death when used in an experimental paradigm that includes opening of channels in the membrane. In the studies in this thesis, this has been avoided by excluding the propidium iodide during the experimental conditions that facilitates channel opening and by subtracting the pixel intensity of the slice-photos taken before incubation from the photos taken after the experiment.

Lactate dehydrogenase-release assay (Paper III and IV)
To evaluate cell toxicity in primary astrocyte cultures during the efflux experiment, lactate dehydrogenase (LDH) release was measured and analyzed using the cytotoxicity detection kit (Roche Diagnostics, Germany). This colorimetric assay measures the activity of lactate dehydrogenase, a cytosolic enzyme, which is released by cells with damaged plasma membranes. The amount of enzymatic activity detected in the culture supernatant correlates to the amount of lysed cells. The percentage of cytotoxicity was calculated as follows:

Cytotoxicity (%) = (sample LDH amount – background control) / (total LDH amount – background control) * 100

where
sample LDH amount = absorbance in culture supernatant
background control = absorbance in culture medium
total LDH amount = absorbance in sample where all cells have been lysed with Triton X-100 (2%) + *sample LDH amount*

Comments:

Lactate dehydrogenase is a cytosolic enzyme that is present in large amounts in the cells and is easily released upon damage of the plasma membrane. It is a relatively stable enzyme and the use of 96-well plates and a plate reader allows for the rapid screening of a large number of samples. The spontaneous release of lactate dehydrogenase is lower than for other enzymes used in cytoxicity assays (Korzeniewski and Callewaert 1983). It has also been shown to correlate well with other methods of assessing cell toxicity/viability such as propidium iodide uptake (Noraberg et al. 1999).

Protein determination

Protein content in the cell cultures was measured using the bicinchoninic acid method (Smith et al. 1985). Determination of the protein content in the slices were carried out as described by (Whitaker and Granum 1980). In both cases, bovine serum albumin was used as standard.

Statistics

All data were expressed as mean ± SEM and p values >0.05 were considered statistically significant. When multiple values were compared, ANOVA followed by Tukeys post hoc tests were used.

SUMMARY OF RESULTS

Paper I: NMDA-receptor mediated efflux of N-acetylaspartate: physiological and/or pathological importance?

In this paper, an efflux protocol allowing investigation of changes in the extracellular chemistry in hippocampus cultures in parallel with measurement of delayed nerve cell death 24 h later is presented. Organotypical hippocampus cultures, grown on porous membranes, are incubated with artificial cerebrospinal fluid on top of the membrane in 5 min fractions (Paper I, Fig. 1). Incubating the slices with fluid on top of the membrane, instead of below, speeds up diffusion of released substances, NMDA and inhibitors. Stable efflux rates were obtained incubating the slices in a gas mixture containing 60 % O_2 / 5% CO_2.

Using this setting, incubation of cultured hippocampal slices in artificial cerebrospinal fluid containing 60 μM NMDA for 5 min caused a pronounced delayed efflux of glutathione, N-acetylaspartate and phosphoethanolamine. Addition of NMDA also generated an increased efflux of taurine and hypotaurine, while the efflux rates of amino acids such as glutamate, aspartate and asparagine remained unchanged (Paper I, Fig. 2). The efflux peaked after NMDA was omitted form the medium and lasted throughout the remaining sample period (25 min).

Incubation of slices in the NMDA-receptor antagonist MK-801 (30 μM) completely abolished the efflux. Both addition of kainate (300 μM) and incubation in high K^+ (50 mM) failed to generate a significant efflux of N-acetylaspartate. The NMDA-induced efflux of N-acetylaspartate, glutathione and amino acids was totally dependent on extracellular Ca^{2+} (Paper I, Fig. 4).

Transient application of NMDA (30 μM and above for 5 min) caused a delayed neurotoxicity, detected by propidium iodide staining. The cell damage increased gradually with time, reaching significant cell damage in the CA1 area after 8 h. Treatment with 300 μM kainate for 5 min induced an injury similar to treatment with 60 μM NMDA while incubation in high K^+ (50 mM) for 5 min was non-toxic after 24 h (Paper I, Fig. 5).

Additional data on NMDA-receptor mediated efflux

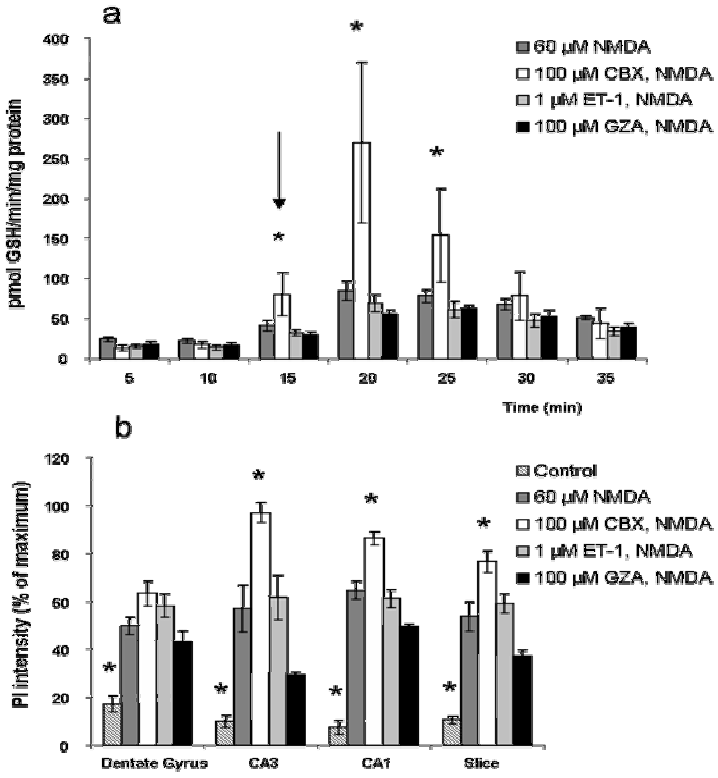

Fig 2a: Efflux of glutathione induced by 5 min incubation in 60 µM NMDA was greatly enhanced by carbenoxolone (CBX), but not by endothelin-1 (ET-1). NMDA was applied for 5 min (10-15 min, see arrow). Star mark significant difference from treatment with 60 µM NMDA for 5 min.

b: Incubation with CBX also generated an increase in cell damage 24 h after NMDA application. This increase in delayed cell death was not observed by treatment with ET-1

The efflux of glutathione is likely neuronal as it is paralleled by N-acetylaspartate. The pathway for efflux is still unknown but it is not likely to be due to opening of connexin or pannexin hemichannels as neither carbenoxolone nor endothelin-1 reduced efflux. Interestingly, carbenoxolone potentiated both the efflux and the cellular damage induced by brief NMDA exposure.

Since the NMDA-receptor mediated efflux was dependent of Ca^{2+}-influx, the slices were incubated with KN-93 (10 µM) to investigate the involvement of Ca^{2+}/calmodulin-dependent kinase II (CaMKII). The slices were also incubated with L-N^G-Nitroarginine methyl ester (L-NAME, 100 µM), an inhibitor of neuronal NO-synthase in an attempt to pinpoint the signalling pathway downstream of NMDA-receptor activation. Neither of the two inhibitors showed any significant effect on the efflux.

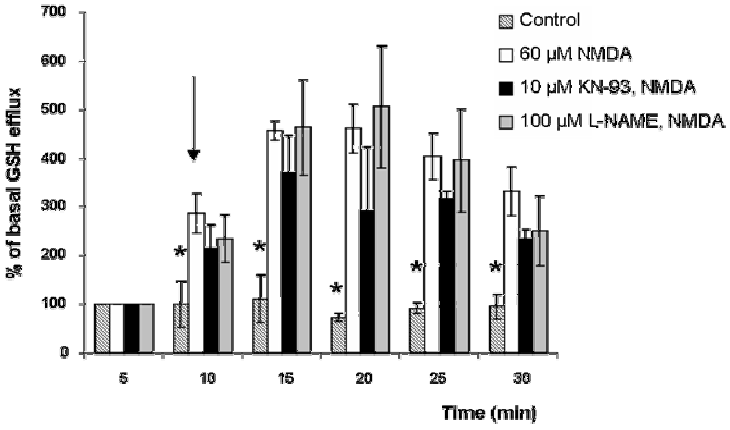

Fig 3. Inhibitiors of CaMKII (KN-93) or NO-synthase (L-NAME) did not alter the glutathione efflux stimulated by NMDA-receptor activation in cultured hippocampal slices. NMDA was applied for 5 min, see arrow. Star mark significant difference from treatment with 60 µM NMDA for 5 min.

Paper II: Stimulated efflux of amino acids and glutathione from cultured hippocampal slices by omission of extracellular Ca^{2+}: likely involvement of connexin hemichannels

Incubation of cultured hippocampus slices for 15 min in artificial cerebrospinal fluid (ACSF) without added Ca^{2+} generated an increase in the efflux rates of glutathione, phosphoethanolamine, taurine and hypotaurine while the efflux of glutamate and valine remained unchanged (Paper II, Fig. 1 and 2). If both major divalent cations (Ca^{2+} and Mg^{2+}) were omitted from the incubation medium, the efflux of glutathione and amino acids was dramatically increased. In addition to the increased efflux, omission of both Ca^{2+} and Mg^{2+} elicited an efflux of glutamate, which was not detected after omitting Ca^{2+} alone (Paper II, Fig. 3).

The efflux induced by Ca^{2+} omission was inhibited by several different types of gap junction/hemichannel blockers including endothelin-1 and the Cx43 mimetic peptide Gap26, but the pannexin mimetic peptide ^{10}Panx1 did not affect on the increased efflux rates (Paper II, Fig. 5). Inhibition of $P2X_7$-receptors also failed to decrease the efflux induced by Ca^{2+} omission (Paper II, Fig. 6).

To address the cellular origin of the efflux, experiments were carried out on slice cultures that had undergone extensive neurodegeneration. Efflux from these slices was unchanged compared to that from normal slice cultures, indicating that the detected efflux is of non-neuronal origin (Paper II, Fig. 7).

Incubation in ACSF without added Ca^{2+} for 15 min did not affect the viability of the slice cultures. However, Ca^{2+} omission in combination with inhibited glutamate uptake resulted in increased extracellular glutamate and delayed cell damage (Paper II, Fig. 8). The pattern of cell damage correlates well with the pattern obtained after NMDA-receptor over activation, observed in paper I, and is most likely a result of extracellular glutamate causing over-activation of NMDA-receptors.

Paper III: Stimulated efflux of adenosine via astroglial connexin hemichannels

The main result in this study was the finding that astrocytes in culture release adenosine after 15 minutes of stimulation with Ca^{2+}- free artificial cerebrospinal fluid (Paper III, Fig. 1b). An increased efflux was also detected for hypoxanthine while the efflux rates of xanthine and inosine were not significantly altered (Paper III, Fig. 1). The increased extracellular levels of adenosine was not due to extracellular breakdown of ATP, since incubation with an ectonucleotidase inhibitor (ARL 67156, 50 μM) did not significantly reduce the extracellular adenosine concentration (Paper III, Fig. 2b).

As in paper II, the efflux induced by Ca^{2+} omission was blocked by gap junction inhibitors, but not by blocking $P2X_7$-receptors (Paper III, Fig. 2b).

Paper IV: Characterization of glutathione efflux from astroglial connexin hemichannels

This study focus on efflux of glutathione from primary astrocyte cultures and effects of different extracellular Ca^{2+} concentrations, prolonged depolarisation and changed intracellular glutathione concentrations. The efflux profile after Ca^{2+} omission was similar to the efflux from cultured hippocampus slice presented in paper II, except for glutamate efflux that was increased in the astrocyte cultures (Paper IV, Fig. 1). As in paper II and III, the stimulated efflux by omission of Ca^{2+} was not affected by the $P2X_7$-receptor inhibition (Brilliant Blue G, 100 nM), but inhibited by the gap junction blocker carbenoxolone (100 μM) and the Cx43 mimetic peptide Gap26 (300 μM) (Paper IV, Fig. 2).

The threshold concentration of Ca^{2+} for inducing stimulated efflux was found to be 0.1 mM as glutathione efflux was observed at 0.1 mM Ca^{2+} but not at 0.2 or 0.3 mM Ca^{2+}. Depolarisation of the cultured cells by 50 mM K^+ alone or in combination with 0.1 mM Ca^{2+} did not significantly change the efflux of glutathione stimulated by 0.1 mM Ca^{2+} (Paper IV, Fig. 3).

Both the basal and stimulated efflux of glutathione could be increased by adding curcumin (30 μM) to the culture medium. Curcumin is a potent activator of the transcription factor Nrf2 which in turn stimulates the transcription of a number of antioxidant genes. Inhibition of the glutathione synthesis with buthionine sulfoximine (BSO) led to the opposite effect, i.e. a marked reduction of the basal and stimulated efflux after Ca^{2+} omission in addition to the lowered intracellular levels of glutathione (Paper IV, Fig. 4).

DISCUSSION

Efflux routes

The results presented in this thesis are based on analysis of the extracellular neurochemistry. Elevated concentrations are interpreted as reflecting mainly efflux, although reduced uptake may contribute to increased levels of some substances (see further below). This assumption is based on the fact that all analysed substances have a high intra- to extracellular ratio. The first part of the results concerns a mainly neuronal efflux pathway mediated via activation of the NMDA-receptor and the second part deals with an efflux of astroglial origin elicited by reduced extracellular Ca^{2+} concentrations.

The different efflux profiles- what comes out and what doesn´t?

In paper I we showed that NMDA-receptor activation elicited an efflux dominated by the neuronal amino acid derivate N-acetylaspartate, glutathione, phosphoethanolamine and taurine and hypotaurine while increased efflux was not detected for amino acids such as glutamate, asparagine and aspartate. To exclude the possibility that the efflux was merely a consequence of plasma membrane disruption, the efflux pattern after NMDA-receptor stimulation was compared to that of cells treated with water for 5 min. Incubation with water caused a massive efflux of glutamate and at least ten more UV absorbing peaks were detected showing that NMDA-mediated efflux is selective compared to membrane burst.

Omission of extracellular Ca^{2+} was similar to the NMDA-receptor activation in that it also caused an increased efflux of glutathione, phosphoethanolamine, taurine and hypotaurine from cultured hippocampal slices (paper II). However, unless both Ca^{2+} and Mg^{2+} were omitted from the incubation medium, no efflux of N-acetylaspartate could be detected. No increased glutamate efflux was observed after Ca^{2+} -omission in the slices, but when primary astrocyte cultures were subjected to reduced extracellular Ca^{2+}-concentrations, a pronounced glutamate efflux could be detected (paper IV). The use of a glutamate uptake blocker, L-trans-pyrrolidine-2,4-dicarboxylate (PDC), in the incubation medium of the slices resulted in elevated extracellular glutamate levels (paper II). This indicates that glial cells in the slice do release glutamate in response to reduced extracellular Ca^{2+}-concentrations, but that the uptake machinery is efficient enough to prevent elevated extracellular concentrations in the slice model. When incubating the slices with another glutamate uptake blocker, DL-threo-β-benzyloxyaspartic acid (TBOA), extracellular glutamate was only increased when both Ca^{2+}

and Mg^{2+} were omitted. This apparent discrepancy is not easy to explain, but it may relate to the different pharmacotoxic profiles of the uptake blockers (Martin et al. 2005). Due to the fact that PDC is a transportable inhibitor of glutamate uptake, it can be argued that the increase in extracellular glutamate is caused by heteroexchange (Waagepetersen et al. 2001). In our slice system, however, heteroexchange is not likely to be the cause of glutamate efflux during Ca^{2+} -omission as no effect was observed on baseline glutamate levels when the slices were incubated in PDC.

In primary astrocyte cultures, a significant efflux of adenosine was also detected after omission of extracellular Ca^{2+}. This efflux was not significantly reduced by inhibition of ectonucleotidase activity by incubation with ARL-67156, indicating that the detected increase in extracellular adenosine not primarily originates from extracellular breakdown of ATP.

NMDA-receptor mediated efflux is not likely to be a hemichannel mediated process.

The pathway mediating the NMDA-receptor activated efflux, presented in paper I, is still elusive, but highly interesting as this efflux correlated well with neurotoxicity 24 h later. The efflux is dependent on Ca^{2+} influx via NMDA-receptors since both addition of the NMDA-receptor blocker MK-801 and omission of extracellular Ca^{2+} from the medium inhibited the efflux. In an earlier study performed by our group, the efflux of glutathione after NMDA-receptor activation was abolished when the protein kinase C inhibitor polymyxin B was added to the slices (Wallin et al. 2003). However, other protein kinase inhibitors such as staurosporine or H9 showed no effect indicating that the effect of polymyxin B was not due to inhibition of protein kinase C. Polymyxin B has been reported to affect not only protein kinase C but also Ca^{2+}-activated K^{+}-channels and calmodulin (Hegemann et al. 1991; Varecka et al. 1987; Weik and Lonnendonker 1990). In the same study, it was shown that inhibition of calmodulin attenuates the efflux after NMDA-receptor activation. Calmodulin stimulates several enzymes including Ca^{2+}/calmodulin dependent kinase II (CaMKII), calcineurin and neuronal NO-synthase (Abu-Soud et al. 1994; Walters and Johnson 1988). Inhibition of two of the downstream enzymes in the calmodulin signalling pathway, neuronal NO-synthase and CaMKII, however, proved to have no effect on the NMDA-receptor mediated efflux of glutathione (Fig. 3).

Efflux of amino acids have been reported after opening of VRACs (Kimelberg et al. 1990). The efflux pattern generated by hypoosmotic medium is, however, different from both efflux

types described in this thesis in that the predominant compound released during hypoosmotic conditions is taurine. To test for the possibility of efflux being induced by cell swelling, both experimental paradigms were carried out in hyperosmotic medium (120 and 240 mM sucrose). Since incubation in hyperosmotic medium did not block neither the NMDA-receptor mediated efflux nor the efflux induced by Ca^{2+} -omission, it is unlikely that swelling induced efflux contributes to the observed changes in extracellular composition.

A potential candidate for mediating the NMDA-receptor activated efflux is the pannexin hemichannel (see section in introduction). A recent study, using acutely isolated hippocampal neurons, has shown that ischemia opens a type of channel with pharmacological and electrophysiological characteristic of pannexin hemichannels (Thompson et al. 2006). Pannexin-1 is expressed in pyramidal neurons and pannexin hemichannels are capable of mediating efflux of substances in the same size range as connexins (Bao et al. 2004; Bruzzone et al. 2005; Bruzzone et al. 2003).

However, blocking putative connexin and/or pannexin hemichannels in the slices using carbenoxolone or endothelin-1 did not inhibit the efflux elicited after NMDA-stimulation (Fig 2a). Addition to the incubation medium of carbenoxolone, which potently inhibits both connexin and pannexin channels (Bruzzone et al. 2005; Davidson and Baumgarten 1988; Goldberg et al. 1996; Locovei et al. 2007), did instead increase the efflux drastically. Incubation with carbenoxolone during the efflux experiment (75 min in total) also increased the delayed cell death in the slice 24 h after the NMDA-stimulation. Whether this effect is related to blocking gap junctions/hemichannels in the slice or not is unknown. Carbenoxolone has been shown to accelerate NMDA-induced cell death in co-cultures of hippocampal neurons and astrocytes (Zundorf et al. 2007). Although carbenoxolone is supposed to lack effects on neuronal excitability according to one study (Kohling et al. 2001), it has been shown to cause an increase in action potential threshold in cultured neurons (Rouach et al. 2003). Other reports show effect of carbenoxolone on several ion channels and receptors including voltage-gated Ca^{2+}-channels (Armanini et al. 2003; Salvi et al. 2005; Vessey et al. 2004). Conclusions from results obtained with carbenoxolone as the only gap junction/hemichannel blocker must therefore be drawn with caution.

Glutamate-receptor induced opening of Ca^{2+} -dependent chloride channels permeable for larger substances such as acetate and methanesulphonate has been demonstrated in cultured

cortical neurons (Backus and Trube 1993). These channels could potentially be the mediators of the NMDA-receptor mediated efflux, but due to a lack of specific pharmacological tools the contribution of Ca^{2+} -dependent chloride channels has not yet been evaluated.

Evidence in support of connexin hemichannels as the mediators of efflux stimulated by Ca^{2+}-omission.

The efflux induced by Ca^{2+}-omission is, as shown in paper II, strictly dependent on the absence of extracellular divalent cations. Omission of both major divalent cations (Ca^{2+} and Mg^{2+}) from the incubation medium, however, caused a dramatic enhancement of the glutathione efflux. When Ca^{2+} was substituted by Mg^{2+} in the incubation medium, no increased efflux of glutathione could be detected. These results of the cation dependency fit well with other studies on regulation of connexin hemichannels by extracellular divalent cations (Ebihara et al. 2003; Ye et al. 2003).

Other pieces of evidence supporting the theory that connexin hemichannels are the channels responsible for the efflux induced by Ca^{2+}-omission comes from the inhibitory effects of a battery of gap junction/hemichannel inhibitors. In paper II to IV, we show that the conventional gap junction/hemichannel blocker carbenoxolone and flufenamic acid blocks the efflux while the structural analogue of carbenoxolone, glycyrrhizic acid, have no effect. In addition to the conventional blockers the endogenous peptide endothelin-1, which has been shown to inhibit gap junction coupling by acting on the intrinsic signalling pathways (Blomstrand and Giaume 2006; Blomstrand et al. 1999; Blomstrand et al. 2004), blocks the efflux to a similar extent as carbenoxolone and flufenamic acid.

The use of connexin and pannexin mimetic/blocking peptides allowed us to further characterize the efflux pathway. Connexin mimetic peptides were originally designed to mimic the docking gate of gap junction channels and were used in attempts to define the functional domains of different connexins (Dahl et al. 1994). The Gap26 peptide contains amino acids 64-76 of the first extracellular loop of Cx43 and has been shown to specifically inhibit hemichannels when applied for 30 min at 0.25mg/ml (~160 μM) (Braet et al. 2003; Leybaert et al. 2003). Gap junction blocking effects by mimetic/blocking peptides are achieved first after prolonged incubation periods, if they are achieved at all (Leybaert et al. 2003; Wang et al. 2007). It is speculated that this might be because the peptides interfere with the formation of new gap junction channels rather that blocking already existing ones (Evans

and Boitano 2001). The connexin mimetic Gap26 peptide has in several other studies shown to inhibit hemichannel mediated processes such ATP efflux induced by Ca^{2+}-omission from endothelial cells in cornea (Gomes et al. 2005) and dye uptake induced by oxygen-glucose deprivation in cardiac myocytes (Shintani-Ishida et al. 2007). The use of the pannexin mimetic peptide, ^{10}Panx1, is based on the same principles as the use of the connexin mimetic peptides. It is a ten amino acids long peptide that has been used to potently inhibit $P2X_7$-mediated dye uptake without altering other aspects of $P2X_7$- receptor activation in several cell lines including 1321-N1 astrocytes transfected with a rat $P2X_7$ expression vector (Pelegrin and Surprenant 2006). In our case the effects of the peptides were clear-cut both in the cultured slices and in the primary astrocyte cultures. The connexin mimetic peptide, Gap26, inhibited the efflux as efficient as the other gap junction/hemichannel blockers used. In contrast to the effects of Gap26, incubation with the pannexin mimetic peptide ^{10}Panx1 did not significantly affect the efflux elicited by Ca^{2+}-omission.

Pannexin channels are only weakly affected by flufenamic acid and they are not regulated by external cations (Bruzzone et al. 2005). The finding (paper II) that efflux was induced by omission of extracellular Ca^{2+}, robustly blocked by flufenamic acid and unchanged by the pannexin mimetic peptide, indicate that pannexin hemichannels are less likely candidates for mediating the efflux of glutathione and amino acids caused by reduced extracellular Ca^{2+}-concentrations.

Purine receptors of the $P2X_7$-type are ionotropic receptors activated by ATP. These receptors show response amplification in solutions with low concentrations of divalent cations (Bianchi et al. 1999; North and Surprenant 2000) and there have been reports on possible cross-reactivity with conventional gap junction/hemichannel blocking agents (Suadicani et al. 2006). Therefore it was important to evaluate $P2X_7$-receptor involvement in the efflux caused by reduced extracellular Ca^{2+}-concentrations. Two different $P2X_7$-receptor inhibitors, oxidized ATP and Brilliant Blue G, were employed in the efflux experiments in paper II to IV and neither of them inhibited the efflux of glutathione. Strangely, in the slice studies, we found a slight, but significant, potentiation of the efflux both by incubation with the $P2X_7$-receptor agonist 3-O-(4-benzoylbenzoyl)ATP (BzATP) and by the antagonist oxidized ATP. The stimulating effect of the antagonist is confusing, but effects on transporter functions by oxidized ATP have been reported previously and can not be ruled out as possible explanations for the observed change in efflux (Henke et al. 1998). No potentiating or inhibitory effects by

$P2X_7$-receptor inhibition using BBG were observed in the primary astrocyte cultures (paper III and IV). Overall the data strongly favour that $P2X_7$-receptor activation is not involved in the efflux pathway stimulated by omission of extracellular Ca^{2+}.

In conclusion, astroglial hemichannels are the most plausible candidates for mediating the efflux stimulated by a reduction in extracellular Ca^{2+}-concentrations. This interpretation is supported by the fact that astrocytes express high levels of Cx43 while the pannexin expression in the hippocampus is localized mainly to post synaptic structures (Huang et al. 2007; Zoidl et al. 2007).

Possibility of combined efflux pathways

Omission of both Ca^{2+} and Mg^{2+} caused an efflux of N-acetylaspartate from cultured slices, as observed after NMDA-receptor stimulation (paper II). Interestingly, efflux of N-acetylaspartate was also observed when omission of extracellular Ca^{2+} was combined with the glutamate uptake blocker PDC. During both of these situations the extracellular glutamate concentrations increase most likely due to hemichannel opening. A likely scenario is that the increased extracellular glutamate levels activate NMDA-receptors in the slice and thereby initiates the NMDA-receptor mediated efflux described in paper I. This could explain why the increased efflux of N-acetylaspartate is not observed during Ca^{2+}-omission only.

During efflux mediated by Ca^{2+}-omission in the slices, there was a small residual efflux after blocking the hemichannels with carbenoxolone (paper II). This residual efflux was not observed in the slices that had undergone neuronal degeneration caused by prolonged NMDA-treatment (paper II) or in the astrocyte cultures (paper IV) which indicates a neuronal component of the efflux induced by Ca^{2+}-omission that is not mediated by connexin or pannexin hemichannels. This putative neuronal efflux pathway is unknown but it should be pointed out that reduction in extracellular Ca^{2+} can have effects on other transport pathways. For example, it has been demonstrated that omission of extracellular Ca^{2+} elevates extracellular taurine by interaction with the carrier system (Molchanova et al. 2005).

During which physiological/pathological circumstances are these efflux pathways activated?

One fundamental issue concerning hemichannel opening *in vivo* is if extracellular Ca^{2+} -concentrations can be reduced to levels that stimulate opening.

In the mammalian brain, external Ca^{2+}-concentrations range between 1.5 to 2.0 mM (Hansen 1985). The intracellular concentrations, on the other hand, are as low as 50-100 nM which creates an outside-to-inside gradient of 15000-40000:1. Activation of processes that causes Ca^{2+}-entry into the cells, for instance activation of receptors permeable to Ca^{2+}, can cause dramatic fluctuations in extracellular Ca^{2+}-concentrations. As an example, ionophoretic application of glutamate in rat motor cortex generates a decrease in extracellular Ca^{2+} from a baseline level of 1.25 to 0.08 mM (Pumain and Heinemann 1985). This decrease is most likely caused by a translocation of Ca^{2+} from the extracellular space into the cytosol and was found to be mostly due to voltage-dependent Ca^{2+}-channels. During anoxia, the external Ca^{2+} levels in murine cortex have been reported to drop down to 0.06 mM (Hansen and Zeuthen 1981). From computer-based simulations it seems likely that physiological activity can generate changes in extracellular Ca^{2+} well within the limits of what has been used to open connexin hemichannels *in vitro* (Egelman and Montague 1999). This is supported by the development of new Ca^{2+}-sensitive electrodes, which shows that earlier techniques for measuring extracellular Ca^{2+} -concentrations greatly underestimates the earlier reported changes in Ca^{2+}- concentrations (Fedirko et al. 2006). In our studies, we find significant efflux of glutathione after incubating astrocyte cultures in 0.1 mM Ca^{2+}, but this concentration did not cause efflux in the slice model.

A plausible explanation for the discrepancy of extracellular Ca^{2+}-thresholds observed in our studies is that the astroglia cells in the monolayer are directly subjected to the new Ca^{2+}-concentrations in the ACSF whereas it takes time to reduce the Ca^{2+}-concentrations in the interior of the cultured slice. It is therefore likely that the Ca^{2+}-concentrations inside the slice are considerably higher than in the incubation medium which may give a falsely high threshold for stimulation of hemichannel opening.

There are several studies presenting evidence of connexin hemichannels opening under physiological cicrcumstances (Bruzzone et al. 2001; Kamermans et al. 2001; Plotkin and Bellido 2001). For example, expression of Cx43 was shown to regulate cell volume in response to fluctuations in extracellular physiological Ca^{2+} (1.8 to $\leq$ 1.6 mM) in an otherwise isoosmotic situation (Quist et al. 2000). One interesting finding in this context is that both carbenoxolone and endothelin-1 significantly reduced the basal efflux of glutathione (paper II), which may be indicative of hemichannel activity under physiological concentrations of extracellular Ca^{2}.

Localized fluctuations in Ca^{2+} in the vicinity of signalling glutamatergic neurons

In brain, the extracellular space is confined to narrow clefts of 20-50 nm width located between the densely packed neurons and glial cells (Vanharreveld et al. 1965). In acute hippocampus slices stimulation of the Schaeffer collaterals have been shown to generate decreases in extracellular Ca^{2+} of about -0.15 mM in standard artificial cerebrospinal fluid (Fedirko et al. 2007). These data indicates that in the extracellular space surrounding active synapses calcium concentrations can drop fast and may, highly speculative, facilitate hemichannel opening in an activitydependent manner.

Possible physiological/patophysiological roles of efflux mediated by Ca^{2+}-omission.

In paper II, we show that the combination of reduced extracellular Ca^{2+} with inhibition of the glial glutamate transporters caused a delayed cell damage with the characteristic pattern of excitotoxic injury. The combination of low extracellular Ca^{2+} and impaired glutamate uptake occur *in vivo* for example during anoxia as discussed above (Hansen and Zeuthen 1981). Indeed, opening of neuronal hemichannels have been proposed to participate in the neurodegenerative process in excitotoxicity (Thompson et al. 2006). The use of hemichannel specific antagonists (the mimetic peptides may be the best choice available) *in vivo* would be very interesting in order to evaluate the isolated contribution of hemichannels and not the combined effect of both hemichannels and gap junctions, as is the case with the use of most antagonists and connexin knock out animals.

Astrocytic export of glutathione is proposed to be a mechanism through which astrocytes can support neurons with antioxidants (Dringen et al. 1999). This efflux has mainly been attributed to multidrug resistance proteins, in particular mrp-1 (Hirrlinger et al. 2001; Hirrlinger et al. 2002b). Efflux of glutathione via hemichannels, as shown in paper II and IV and by Dringen and coworkers (Rana and Dringen 2007) may be one additional efflux pathway.

In paper III, we present evidence of hemichannel mediated increase in the extracellular levels of another neuroactive compound, adenosine, in addition to glutamate and glutathione. Adenosine is a neuromodulatory compound which reduces glutamate release via presynaptic A1 receptors (Arrigoni et al. 2005; Fowler 1990). The extracellular levels of adenosine have previously been thought to mainly originate from extracellular breakdown of ATP, but in

paper III we showed that the increase in extracellular adenosine stimulated by Ca^{2+} -omission was insensitive to inhibition of ectonucleotidase activity. The efflux of adenosine showed the same characteristic features as the efflux of glutathione induced by Ca^{2+} -omission, i.e. it is blocked by extracellular divalent cations, carbenoxolone and Gap26, while inhibition of $P2X_7$-receptors have no effect on the efflux. Unfortunately, it was not possible to evaluate the effect of the pannexin mimetic peptide in this setup due to interference with the UV-absorbance detection method.

From a functional point of view it is interesting to relate these results to a recent report from Nedergaard´s group concerning a putative function of hemichannels. In that study, it was demonstrated that extracellular adenosine, suggested to originate from efflux of ATP via Cx43 hemichannels, is a key factor by which hypoxic preconditioning may reduce excitotoxicity under stroke-like conditions (Lin et al. 2008).

Can these pathways be manipulated?

Since efflux of both glutathione and adenosine can have neuroprotective functions in the brain it would be attractive to elevate the efflux in situations which encompass neurodegeneration, i.e. after stroke and in Parkinson´s and Alzhemiemer´s diseases. Enhancing the efflux of glutathione has proven neuroprotective *in vitro*, presumably through the glutathione shuttling mechanism described in the introduction (Dringen et al. 1999). In paper IV, we show an increased efflux of glutathione from cultured astrocytes after treatment with curcumin, a substance that increase the transcription of several key enzymes in the cellular antioxidant defence system via the transcription factor Nrf2 (Balogun et al. 2003).

Some of the most potent Nrf2 activators known are plant-derived compounds present in our everyday food. The substance used in paper IV, curcumin, is for instance the active ingredient of turmeric (*Curcuma longa*) and another potent Nrf2 activator, sulphoraphane, is found in broccoli. In addition to inducing expression of enzymes in the glutathione synthesis chain, Nrf2 activation has been shown to upregulate some components in the glutathione efflux pathways, including the multidrug resistance protein-1 (Hirrlinger et al. 2002b; Shih et al. 2003). The efflux pathway stimulated by Ca^{2+}-omission does, however, not seem to be upregulated as the efflux rates of phosphoethanolamine and glutamate are unchanged by curcumin treatment (paper IV).

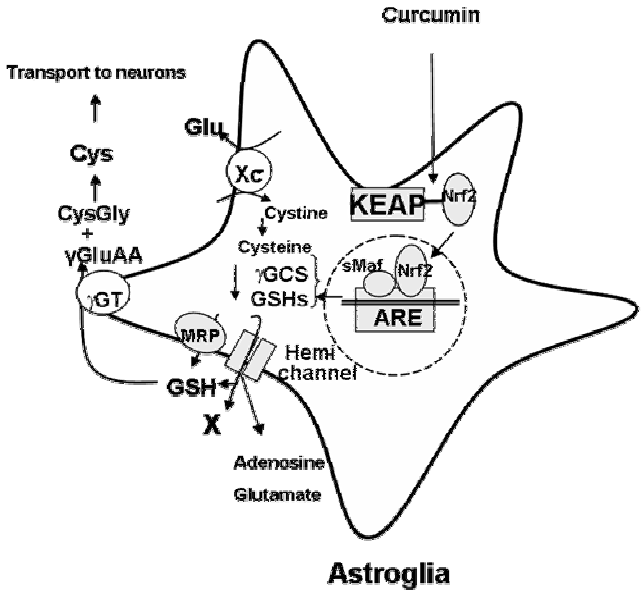

Fig. 4 A schematic drawing showing the the mechanism by which curcumin can increase the synthesis and export of glutathione (GSH) via the Nrf2/KEAP pathway. The astroglial contribution to the "GSH shuttle"; i.e. GSH export via hemichannels and multidrug resistance proteins (MRP) and extracellular degradation of GSH by γ-glutamyltranspeptidase (γ-GT) is depicted to the left

Nrf2 mediated stimulation of glutathione synthesis and efflux offer interesting possibilities when it comes to finding new therapeutic strategies for dealing with the consequences oxidative insults. Several studies have already confirmed the protective effects of curcumin and sulphoraphane administration in experimental ischemia and increased oxidative load (Al-Omar et al. 2006; Wang et al. 2005; Zhao et al. 2006). From a therapeutic standpoint, it is promising that the protective effects of curcumin could be achieved even when the substance is administred 24 h after the injury (Al-Omar et al. 2006). The glutathione stimulating effect of curcumin could also have implications for Parkinson´s disease, a condition thought to at least partially depend on oxidative stress caused by glutathione depletion (Jagatha et al. 2008).

Are functional hemichannels only an artefact due to culturing?

Most of the studies on hemichannel function have been carried out in primary cultures, connexin expressing cell lines or in Xenopus oocytes. The most common criticism against the concept of functional hemichannels has been the lack of evidence of functional connexin hemichannels in more *in vivo*-like preparations. Our study on hemichannel-mediated efflux in cultured hippocampus slices is a step in the direction of unravelling the question of whether functional hemichannels are present in tissues as well as in cell cultures. Further evidence of this is presented by Ransom and coworkers who showed hemichannel activity in CNS white matter by measuring glutamate release from acute preparations of optic nerve (Ye et al. 2003). A recent *in vivo*-study on astroglial control of blood flow suggests the involvement of Cx43 channels in the vasodilation signalling conduit after cortical neuronal activation (Xu et al.

2008). Even though no definite conclusions on the contributions of gap junctions and hemichannel respectively is drawn in this study, it is interesting to note that the incubation time of the mimetic peptides used is not sufficient to cause inhibition of gap junctions which would suggest effect on hemichannels primarily (Leybaert et al. 2003). To fully address the question of whether functional hemichannels are present *in vivo*, better and more selective pharmacological tools are needed. The use of connexin knock out animals presents many opportunities to study connexin functions, but the risk of affecting the entire transcriptome by knocking out connexin function complicates the interpretation of data obtained with such animals (Kardami et al. 2007).

CONCLUSIONS

I: NMDA-receptor activation causes a Ca^{2+}-dependent efflux of glutathione, N-acetylaspartate, phosphoethanolamine and taurine, which can be correlated to the amount of delayed cell death in cultured hippocampus slices. The efflux pathway is still unknown.

II: Reduced extracellular Ca^{2+} -concentrations stimulate efflux of glutathione, glutamate, PEA, taurine, hypotaurine and adenosine from astrocytes in primary cultures and cultured hippocampus slices.

III: Efflux mediated by reduced extracellular Ca^{2+} -concentrations occurs through connexin hemichannels.

IV: The NMDA-receptor mediated efflux is most likely a pathologic response while the hemichannel-mediated efflux might have signalling and supportive functions.

V: The efflux of glutathione from astrocytes can be enhanced by activation of the nrf-2/ARE-mediated transcription signalling pathway.

ACKNOWLEDGEMENTS

The work in this thesis was performed at the Department of Physiology, Institute of Neuroscience and Physiology at the Sahlgrenska Academy, Göteborg University and I am deeply thankful to all my colleagues for their friendship and support. It sure has been a pleasure.

There are some people I would like to acknowledge in particular:

Professor Mats Sandberg, my dear supervisor. Without you there would be no thesis, that´s for sure. Thank you for putting up with all my maybe not so clever ideas and for letting me try things my own way. Thank you also for keeping my head out of the clouds most of the time and for making sure that at least some of the work got done. I have learnt so much from you and my time here that I would have to write a second thesis if I were to put it all in words.

Fredrik Blomstrand, my second supervisor and my guide in the astrocytic labyrinth. Thank you for introducing me to the exciting and messy field of connexin channels and for all the help on my manuscripts etc.

Michael Nilsson, my third supervisor. Thank you for your enthusiasm and interesting discussions on the benefits of yellow spices and broccoli.

Barbro Jilderos, for excellent technical support and for taking such great care of my slices. I also want to thank you for being a wonderful collegue and for the great cooperation during the endless efflux experiments.

Mattias Tranberg, thank you for being a great coworker and room-mate and friend. It really hasn´t been the same since you moved out of our office.

Lena Olofsson, you are the best when it comes to handling administrative stuff! Thank you for all your help and the nice coffe/tea breaks and department trips.

All my collegues at the former Department of Medical Biophysics, both past and present:
Holger Wigström, Li Rui, Karim Abbas, Fen-Sheng Huang, Mikhail Dozmorov, you have all made my time here at the department so much more enjoyable. I cannot thank you enough for providing me with such an interesting company throughout the years.

Ann Fagerberg for all the moments shared in frustration over the sometimes very obnoxious HPLC-pumps. Thanks for helping me out on more than one occasion!

Tina Eklöf, my new office-mate, deserves a special thanks for putting up with my strange music and weird behaviour during the writing of this thesis.

I would also like to thank my “foster-family” in the scientific world up on the third floor. It has ment a lot to me to be able to sneak in on yor coffe breaks. Sometimes it gets very lonesome down in the cellar.
Eric Hanse for your enthusiasm and scientific excellence and for allowing me to tag along on a lot of great things from exciting kayak expeditions to conferences.

My Andersson for being the best friend/conference-travel buddy/coworker anyone could possibly have. And, of course, for being one of the smartest, most stylish and human people I know.
Therese Abrahamsson for being a good friend and for all the nice coffe/tea breaks. I promise to swing by Paris someday soon.
Joakim Strandberg, Ilse Riebe Jonny Daborg and Line Löken, thank you for great company, good times and interesting discussions. Work is so much more enjoyable when one has collegues like you.

My friends at the Botanical Institute, **Anna Stina Sandelius, Conny Liljenberg and Karin Larsson**. You have been such a great source of scientific inspiration for me and I still miss the afternoon tea at Botan.

Of course I want to thank my wonderful friends:
Malena for helping me taking care of Gello when I didn´t have time leave the office and for nice lunches and coffes and the occasional beer/wine. I am so looking forward to our Scotland trip soon.
Tara for being the best friend anyone can wish for and for all the fun back in the good old days. I miss our "just go for a coffe" dates dearly.
Fredrik A for all the nice breakfasts and lunches and for being such a great friend for a long time.
Jeanette, Jens, Therese H for being such great friends. I value your company immensely.
All the people att Gunnesby stall for your friendship for looking after my horse allt the times I got stuck at work.

Joel for being the bravest, coolest and most caring person I know and for everything else.

Fredrik for all and everything, all the time we have known each other and for all your support. However, I am not happy with you for ditching me and Gello for St. Andrews ;-)

My horse **Reggello** deserves to be mentioned here, because without him I would probably not have gotten out of the office at all the last couple of months and by now, I would have gone completely mad.

And last, but by no means least, I want to thank my family, my father **Cenneth**, my mother **Berit** and my sister **Linnea**. To my sister who also has chosen the path of becoming a PhD-student in neuroscience I just want to offer one advice: Don´t start writing your thesis too late! And good luck! And of course, it is so nice to have someone in my family that I can discuss work with.

To my parents I just want to add: This is your thesis, as much as it is mine. You did all the preparatory work, I just did the rest. Love you!

The work on which this thesis is based was supported by the Swedish Research Council/Medicine, Parkinsonfonden, Åhlén-stiftelsen, the National Institutes of Health (GM 44842), Fredrik and Ingrid Thurings-, Edit Jacobsons-, Magnus Bergvalls- and Edit Jacobssons Foundations.

REFERENCES

Abbott NJ. 2005. Dynamics of CNS barriers: evolution, differentiation, and modulation. Cell Mol Neurobiol 25(1):5-23.

Abdullaev IF, Rudkouskaya A, Schools GP, Kimelberg HK, Mongin AA. 2006. Pharmacological comparison of swelling-activated excitatory amino acid release and Cl- currents in cultured rat astrocytes. J Physiol 572(3):677-689.

Abu-Soud HM, Yoho LL, Stuehr DJ. 1994. Calmodulin controls neuronal nitric-oxide synthase by a dual mechanism. Activation of intra- and interdomain electron transfer. J Biol Chem 269(51):32047-50.

Aguado F, Espinosa-Parrilla JF, Carmona MA, Soriano E. 2002. Neuronal activity regulates correlated network properties of spontaneous calcium transients in astrocytes in situ. J Neurosci 22(21):9430-44.

Akimitsu T, Kurisu K, Hanaya R, Iida K, Kiura Y, Arita K, Matsubayashi H, Ishihara K, Kitada K, Serikawa T and others. 2000. Epileptic seizures induced by N-acetyl-L-aspartate in rats: in vivo and in vitro studies. Brain Res 861(1):143-50.

Al-Omar FA, Nagi MN, Abdulgadir MM, Al Joni KS, Al-Majed AA. 2006. Immediate and delayed treatments with curcumin prevents forebrain ischemia-induced neuronal damage and oxidative insult in the rat hippocampus. Neurochem Res 31(5):611-8.

Alam J, Stewart D, Touchard C, Boinapally S, Choi AMK, Cook JL. 1999. Nrf2, a Cap'n'Collar Transcription Factor, Regulates Induction of the Heme Oxygenase-1 Gene. J Biol Chem 274(37):26071-26078.

Andersson M, Blomstrand F, Hanse E. 2007. Astrocytes play a critical role in transient heterosynaptic depression in the rat hippocampal CA1 region. J Physiol 585(3):843-852.

Araque A, Parpura V, Sanzgiri RP, Haydon PG. 1998. Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. Eur J Neurosci 10(6):2129-42.

Armanini D, Calo L, Semplicini A. 2003. Pseudohyperaldosteronism: pathogenetic mechanisms. Crit Rev Clin Lab Sci 40(3):295-335.

Arrigoni E, Crocker AJ, Saper CB, Greene RW, Scammell TE. 2005. Deletion of presynaptic adenosine A1 receptors impairs the recovery of synaptic transmission after hypoxia. Neuroscience 132(3):575-580.

Backus KH, Trube G. 1993. Single-channel activity in cultured cortical neurons of the rat in the presence of a toxic dose of glutamate. Eur J Neurosci 5(2):174-85.

Bahr BA. 1995. Long-term hippocampal slices: a model system for investigating synaptic mechanisms and pathologic processes. J Neurosci Res 42(3):294-305.

Bahr BA, Kessler M, Rivera S, Vanderklish PW, Hall RA, Mutneja MS, Gall C, Hoffman KB. 1995. Stable maintenance of glutamate receptors and other synaptic components in long-term hippocampal slices. Hippocampus 5(5):425-39.

Ballerini P, Di Iorio P, Ciccarelli R, Nargi E, D'Alimonte I, Traversa U, Rathbone MP, Caciagli F. 2002. Glial cells express multiple ATP binding cassette proteins which are involved in ATP release. Neuroreport 13(14):1789-92.

Balogun E, Hoque M, Gong P, Killeen E, Green CJ, Foresti R, Alam J, Motterlini R. 2003. Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. Biochem J 371(Pt 3):887-95.

Bao L, Locovei S, Dahl G. 2004. Pannexin membrane channels are mechanosensitive conduits for ATP. FEBS Lett 572(1-3):65-8.

Bao X, Lee SC, Reuss L, Altenberg GA. 2007. Change in permeant size selectivity by phosphorylation of connexin 43 gap-junctional hemichannels by PKC. Proc Natl Acad Sci U S A 104(12):4919-24.

Baranova A, Ivanov D, Petrash N, Pestova A, Skoblov M, Kelmanson I, Shagin D, Nazarenko S, Geraymovych E, Litvin O and others. 2004. The mammalian pannexin family is homologous to the invertebrate innexin gap junction proteins. Genomics 83(4):706-16.

Baslow MH. 2000. Functions of N-acetyl-L-aspartate and N-acetyl-L-aspartylglutamate in the vertebrate brain: role in glial cell-specific signaling. J Neurochem 75(2):453-9.

Baslow MH. 2002. Evidence supporting a role for N-acetyl-L-aspartate as a molecular water pump in myelinated neurons in the central nervous system. An analytical review. Neurochem Int 40(4):295-300.

Baslow MH, Suckow RF, Gaynor K, Bhakoo KK, Marks N, Saito M, Duff K, Matsuoka Y, Berg MJ. 2003. Brain damage results in down-regulation of N-acetylaspartate as a neuronal osmolyte. Neuromolecular Med 3(2):95-104.

Bass NH, Hess HH, Pope A, Thalheimer C. 1971. Quantitative cytoarchitectonic distribution of neurons, glia, and DNa in rat cerebral cortex. J Comp Neurol 143(4):481-90.

Berger T, Frotscher M. 1994. Distribution and morphological characteristics of oligodendrocytes in the rat hippocampus in situ and in vitro: an immunocytochemical study with the monoclonal Rip antibody. J Neurocytol 23(1):61-74.

Bevan S, Gray PT, Ritchie JM. 1984. A calcium-activated cation-selective channel in rat cultured Schwann cells. Proc R Soc Lond B Biol Sci 222(1228):349-55.

Bezzi P, Carmignoto G, Pasti L, Vesce S, Rossi D, Rizzini BL, Pozzan T, Volterra A. 1998. Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. Nature 391(6664):281-285.

Bianchi BR, Lynch KJ, Touma E, Niforatos W, Burgard EC, Alexander KM, Park HS, Yu H, Metzger R, Kowaluk E and others. 1999. Pharmacological characterization of recombinant human and rat P2X receptor subtypes. Eur J Pharmacol 376(1-2):127-38.

Binmoller FJ, Muller CM. 1992. Postnatal development of dye-coupling among astrocytes in rat visual cortex. Glia 6(2):127-37.

Blomstrand F, Giaume C. 2006. Kinetics of endothelin-induced inhibition and glucose permeability of astrocyte gap junctions. J Neurosci Res 83(6):996-1003.

Blomstrand F, Giaume C, Hansson E, Ronnback L. 1999. Distinct pharmacological properties of ET-1 and ET-3 on astroglial gap junctions and Ca2+ signaling. Am J Physiol Cell Physiol 277(4):C616-627.

Blomstrand F, Venance L, Siren AL, Ezan P, Hanse E, Glowinski J, Ehrenreich H, Giaume C. 2004. Endothelins regulate astrocyte gap junctions in rat hippocampal slices. Eur J Neurosci 19(4):1005-15.

Bolanos JP, Heales SJ, Peuchen S, Barker JE, Land JM, Clark JB. 1996. Nitric oxide-mediated mitochondrial damage: a potential neuroprotective role for glutathione. Free Radic Biol Med 21(7):995-1001.

Boucsein C, Zacharias R, Farber K, Pavlovic S, Hanisch U-K, Kettenmann H. 2003. Purinergic receptors on microglial cells: functional expression in acute brain slices and modulation of microglial activation in vitro. European Journal of Neuroscience 17(11):2267-2276.

Braet K, Aspeslagh S, Vandamme W, Willecke K, Martin PE, Evans WH, Leybaert L. 2003. Pharmacological sensitivity of ATP release triggered by photoliberation of inositol-1,4,5-trisphosphate and zero extracellular calcium in brain endothelial cells. J Cell Physiol 197(2):205-13.

Brambilla R, Cottini L, Fumagalli M, Ceruti S, Abbracchio MP. 2003. Blockade of A2A adenosine receptors prevents basic fibroblast growth factor-induced reactive astrogliosis in rat striatal primary astrocytes. Glia 43(2):190-4.

Broer S, Brookes N. 2001. Transfer of glutamine between astrocytes and neurons. J Neurochem 77(3):705-19.

Broer S, Rahman B, Pellegri G, Pellerin L, Martin JL, Verleysdonk S, Hamprecht B, Magistretti PJ. 1997. Comparison of lactate transport in astroglial cells and monocarboxylate transporter 1 (MCT 1) expressing Xenopus laevis oocytes. Expression of two different monocarboxylate transporters in astroglial cells and neurons. J Biol Chem 272(48):30096-102.

Bruzzone R, Barbe MT, Jakob NJ, Monyer H. 2005. Pharmacological properties of homomeric and heteromeric pannexin hemichannels expressed in Xenopus oocytes. J Neurochem 92(5):1033-43.

Bruzzone R, Hormuzdi SG, Barbe MT, Herb A, Monyer H. 2003. Pannexins, a family of gap junction proteins expressed in brain. Proc Natl Acad Sci U S A 100(23):13644-9.

Bruzzone S, Guida L, Zocchi E, Franco L, De Flora A. 2001. Connexin 43 hemi channels mediate Ca2+-regulated transmembrane NAD+ fluxes in intact cells. Faseb J 15(1):10-12.

Bushong EA, Martone ME, Jones YZ, Ellisman MH. 2002. Protoplasmic Astrocytes in CA1 Stratum Radiatum Occupy Separate Anatomical Domains. J Neurosci 22(1):183-192.

Chance B, Sies H, Boveris A. 1979. Hydroperoxide metabolism in mammalian organs. Physiol Rev 59(3):527-605.

Chanson M, Scerri I, Suter S. 1999. Defective regulation of gap junctional coupling in cystic fibrosis pancreatic duct cells. J Clin Invest 103(12):1677-84.

Chanson M, Suter S. 2001. Regulation of gap junctional communication in CFTR-expressing pancreatic epithelial cells. Pflugers Arch 443 Suppl 1:S81-4.

Chen CY, Jang JH, Li MH, Surh YJ. 2005. Resveratrol upregulates heme oxygenase-1 expression via activation of NF-E2-related factor 2 in PC12 cells. Biochem Biophys Res Commun 331(4):993-1000.

Chen J, Backus KH, Deitmer JW. 1997. Intracellular calcium transients and potassium current oscillations evoked by glutamate in cultured rat astrocytes. J Neurosci 17(19):7278-87.

Cherian PP, Siller-Jackson AJ, Gu S, Wang X, Bonewald LF, Sprague E, Jiang JX. 2005. Mechanical Strain Opens Connexin 43 Hemichannels in Osteocytes: A Novel Mechanism for the Release of Prostaglandin. Mol Biol Cell 16(7):3100-3106.

Clancy RM, Levartovsky D, Leszczynska-Piziak J, Yegudin J, Abramson SB. 1994. Nitric oxide reacts with intracellular glutathione and activates the hexose monophosphate shunt in human neutrophils: evidence for S-nitrosoglutathione as a bioactive intermediary. Proc Natl Acad Sci U S A 91(9):3680-4.

Contreras JE, Saez JC, Bukauskas FF, Bennett MV. 2003. Gating and regulation of connexin 43 (Cx43) hemichannels. Proc Natl Acad Sci U S A 100(20):11388-93.

Contreras JE, Sanchez HA, Eugenin EA, Speidel D, Theis M, Willecke K, Bukauskas FF, Bennett MV, Saez JC. 2002. Metabolic inhibition induces opening of unapposed connexin 43 gap junction hemichannels and reduces gap junctional communication in cortical astrocytes in culture. Proc Natl Acad Sci U S A 99(1):495-500.

Cooper AJ, Pulsinelli WA, Duffy TE. 1980. Glutathione and ascorbate during ischemia and postischemic reperfusion in rat brain. J Neurochem 35(5):1242-5.

Cooper CD, Lampe PD. 2002. Casein kinase 1 regulates connexin-43 gap junction assembly. J Biol Chem 277(47):44962-8.

Cotrina ML, Lin JHC, Alves-Rodrigues A, Liu S, Li J, Azmi-Ghadimi H, Kang J, Naus CCG, Nedergaard M. 1998. Connexins regulate calcium signaling by controlling ATP release. Proceedings of the National Academy of Sciences 95(26):15735-15740.

D'Adamo AF, Jr., Gidez LI, Yatsu FM. 1968. Acetyl transport mechanisms. Involvement of N-acetyl aspartic acid in de novo fatty acid biosynthesis in the developing rat brain. Exp Brain Res 5(4):267-73.

D'Adamo AF, Jr., Yatsu FM. 1966. Acetate metabolism in the nervous system. N-acetyl-L-aspartic acid and the biosynthesis of brain lipids. J Neurochem 13(10):961-5.

Dahl G, Locovei S. 2006. Pannexin: to gap or not to gap, is that a question? IUBMB Life 58(7):409-19.

Dahl G, Nonner W, Werner R. 1994. Attempts to define functional domains of gap junction proteins with synthetic peptides. Biophys J 67(5):1816-22.

Davidson JS, Baumgarten IM. 1988. Glycyrrhetinic acid derivatives: a novel class of inhibitors of gap-junctional intercellular communication. Structure-activity relationships. J Pharmacol Exp Ther 246(3):1104-7.

Davies SE, Gotoh M, Richards DA, Obrenovitch TP. 1998. Hypoosmolarity induces an increase of extracellular N-acetylaspartate concentration in the rat striatum. Neurochem Res 23(8):1021-5.

Decleves X, Regina A, Laplanche JL, Roux F, Boval B, Launay JM, Scherrmann JM. 2000. Functional expression of P-glycoprotein and multidrug resistance-associated protein (Mrp1) in primary cultures of rat astrocytes. J Neurosci Res 60(5):594-601.

Deitmer JW, Verkhratsky AJ, Lohr C. 1998. Calcium signalling in glial cells. Cell Calcium 24(5-6):405-16.

Dermietzel R, Hertberg EL, Kessler JA, Spray DC. 1991. Gap junctions between cultured astrocytes: immunocytochemical, molecular, and electrophysiological analysis. J Neurosci 11(5):1421-32.

Dermietzel R, Hwang T, Buettner R, Hofer A, Dotzler E, Kremer M, Deutzmann R, thinnes FP, Fishman GI, Spray DC and others. 1994. Cloning and in situ Localization of a Brain-Derived Porin that Constitutes a Large-Conductance Anion Channel in Astrocytic Plasma Membranes. Proceedings of the National Academy of Sciences 91(2):499-503.

Derouiche A, Heimrich B, Frotscher M. 1993. Loss of layer-specific astrocytic glutamine synthetase immunoreactivity in slice cultures of hippocampus. Eur J Neurosci 5(2):122-7.

Dobrenis K, Chang HY, Pina-Benabou MH, Woodroffe A, Lee SC, Rozental R, Spray DC, Scemes E. 2005. Human and mouse microglia express connexin36, and functional gap junctions are formed between rodent microglia and neurons. J Neurosci Res 82(3):306-15.

Dringen R, Hamprecht B. 1998. Glutathione restoration as indicator for cellular metabolism of astroglial cells. Dev Neurosci 20(4-5):401-7.

Dringen R, Pfeiffer B, Hamprecht B. 1999. Synthesis of the antioxidant glutathione in neurons: supply by astrocytes of CysGly as precursor for neuronal glutathione. J Neurosci 19(2):562-9.

Duan S, Anderson CM, Keung EC, Chen Y, Chen Y, Swanson RA. 2003. P2X7 receptor-mediated release of excitatory amino acids from astrocytes. J Neurosci 23(4):1320-8.

Duffy HS, Ashton AW, O'Donnell P, Coombs W, Taffet SM, Delmar M, Spray DC. 2004. Regulation of Connexin43 Protein Complexes by Intracellular Acidification. Circ Res 94(2):215-222.

Dunwiddie TV. 1999. Adenosine and suppression of seizures. Adv Neurol 79:1001-10.

Ebihara L, Liu X, Pal JD. 2003. Effect of external magnesium and calcium on human connexin46 hemichannels. Biophys J 84(1):277-86.

Egelman DM, Montague PR. 1999. Calcium dynamics in the extracellular space of mammalian neural tissue. Biophys J 76(4):1856-67.

Eugenin EA, Branes MC, Berman JW, Saez JC. 2003. TNF-alpha plus IFN-gamma induce connexin43 expression and formation of gap junctions between human monocytes/macrophages that enhance physiological responses. J Immunol 170(3):1320-8.

Eugenin EA, Eckardt D, Theis M, Willecke K, Bennett MV, Saez JC. 2001. Microglia at brain stab wounds express connexin 43 and in vitro form functional gap junctions after treatment with interferon-gamma and tumor necrosis factor-alpha. Proc Natl Acad Sci U S A 98(7):4190-5.

Evans WH, Boitano S. 2001. Connexin mimetic peptides: specific inhibitors of gap-junctional intercellular communication. Biochem Soc Trans 29(Pt 4):606-12.

Faustmann PM, Haase CG, Romberg S, Hinkerohe D, Szlachta D, Smikalla D, Krause D, Dermietzel R. 2003. Microglia activation influences dye coupling and Cx43 expression of the astrocytic network. Glia 42(2):101-8.

Fedirko N, Avshalumov M, Rice ME, Chesler M. 2007. Regulation of postsynaptic Ca2+ influx in hippocampal CA1 pyramidal neurons via extracellular carbonic anhydrase. J Neurosci 27(5):1167-75.

Fedirko N, Svichar N, Chesler M. 2006. Fabrication and use of high-speed, concentric h+- and Ca2+-selective microelectrodes suitable for in vitro extracellular recording. J Neurophysiol 96(2):919-24.

Feustel PJ, Jin Y, Kimelberg HK. 2004. Volume-regulated anion channels are the predominant contributors to release of excitatory amino acids in the ischemic cortical penumbra. Stroke 35(5):1164-8.

Fischer G, Kettenmann H. 1985. Cultured astrocytes form a syncytium after maturation. Exp Cell Res 159(2):273-9.

Florian CL, Williams SR, Bhakoo KK, Noble MD. 1996. Regional and developmental variations in metabolite concentration in the rat brain and eye: a study using 1H NMR spectroscopy and high performance liquid chromatography. Neurochem Res 21(9):1065-1074.

Folbergrova J, Rehncrona S, Siesjo BK. 1979. Oxidized and reduced glutathione in the rat brain under normoxic and hypoxic conditions. J Neurochem 32(6):1621-7.

Fowler JC. 1990. Adenosine antagonists alter the synaptic response to in vitro ischemia in the rat hippocampus. Brain Research 509(2):331-334.

Fredholm BB, Ijzerman AP, Jacobson KA, Klotz K-N, Linden J. 2001. International Union of Pharmacology. XXV. Nomenclature and Classification of Adenosine Receptors. Pharmacol Rev 53(4):527-552.

Fremeau RT, Jr., Burman J, Qureshi T, Tran CH, Proctor J, Johnson J, Zhang H, Sulzer D, Copenhagen DR, Storm-Mathisen J and others. 2002. The identification of vesicular glutamate transporter 3 suggests novel modes of signaling by glutamate. Proceedings of the National Academy of Sciences 99(22):14488-14493.

Gabbita SP, Robinson KA, Stewart CA, Floyd RA, Hensley K. 2000. Redox Regulatory Mechanisms of Cellular Signal Transduction. Archives of Biochemistry and Biophysics 376(1):1-13.

Gahwiler BH, Capogna M, Debanne D, McKinney RA, Thompson SM. 1997. Organotypic slice cultures: a technique has come of age. Trends Neurosci 20(10):471-7.

Gegelashvili G, Schousboe A. 1998. Cellular Distribution and Kinetic Properties of High-Affinity Glutamate Transporters. Brain Research Bulletin 45(3):233-238.

Giaume C, McCarthy KD. 1996. Control of gap-junctional communication in astrocytic networks. Trends in Neurosciences 19(8):319-325.

Giaume C, Tabernero A, Medina JM. 1997. Metabolic trafficking through astrocytic gap junctions. Glia 21(1):114-23.

Goldberg GS, Moreno AP, Bechberger JF, Hearn SS, Shivers RR, MacPhee DJ, Zhang YC, Naus CC. 1996. Evidence that disruption of connexon particle arrangements in gap junction plaques is associated with inhibition of gap junctional communication by a glycyrrhetinic acid derivative. Exp Cell Res 222(1):48-53.

Gomes P, Srinivas SP, Van Driessche W, Vereecke J, Himpens B. 2005. ATP release through connexin hemichannels in corneal endothelial cells. Invest Ophthalmol Vis Sci 46(4):1208-18.

Goodenough DA, Goliger JA, Paul DL. 1996. Connexins, Connexons, and Intercellular Communication. Annual Review of Biochemistry 65(1):475-502.

Guillet B, Lortet S, Masmejean F, Samuel D, Nieoullon A, Pisano P. 2002. Developmental expression and activity of high affinity glutamate transporters in rat cortical primary cultures. Neurochemistry International 40(7):661-671.

Guo N, McIntosh C, Shaw C. 1992. Glutathione: New candidate neuropeptide in the central nervous system. Neuroscience 51(4):835-842.

Guo N, Shaw C. 1992. Characterization and localization of glutathione binding sites on cultured astrocytes. Brain Res Mol Brain Res 15(3-4):207-15.

Hagberg H, Andersson P, Lacarewicz J, Jacobson I, Butcher S, Sandberg M. 1987. Extracellular adenosine, inosine, hypoxanthine, and xanthine in relation to tissue nucleotides and purines in rat striatum during transient ischemia. J Neurochem 49(1):227-31.

Hagberg H, Lehmann A, Sandberg M, Nystrom B, Jacobson I, Hamberger A. 1985. Ischemia-induced shift of inhibitory and excitatory amino acids from intra- to extracellular compartments. J Cereb Blood Flow Metab 5(3):413-419.

Hansen AJ. 1985. Effect of anoxia on ion distribution in the brain. Physiol Rev 65(1):101-48.

Hansen AJ, Zeuthen T. 1981. Extracellular ion concentrations during spreading depression and ischemia in the rat brain cortex. Acta Physiol Scand 113(4):437-45.

Hansson E, Ronnback L, Persson LI, Lowenthal A, Noppe M, Alling C, Karlsson B. 1984. Cellular composition of primary cultures from cerebral cortex, striatum, hippocampus, brainstem and cerebellum. Brain Res 300(1):9-18.

Hegemann L, van Rooijen LA, Traber J, Schmidt BH. 1991. Polymyxin B is a selective and potent antagonist of calmodulin. Eur J Pharmacol 207(1):17-22.

Henke W, Hagen T, Jung K, Loening SA. 1998. Periodate-oxidized ATP stimulates the permeability transition of rat liver mitochondria. Biochim Biophys Acta 1363(3):209-16.

Heurteaux C, Lauritzen I, Widmann C, Lazdunski M. 1995. Essential Role of Adenosine, Adenosine A1 Receptors, and ATP-Sensitive K+ Channels in Cerebral Ischemic Preconditioning. Proceedings of the National Academy of Sciences 92(10):4666-4670.

Hirrlinger J, Konig J, Dringen R. 2002a. Expression of mRNAs of multidrug resistance proteins (Mrps) in cultured rat astrocytes, oligodendrocytes, microglial cells and neurones. J Neurochem 82(3):716-9.

Hirrlinger J, Konig J, Keppler D, Lindenau J, Schulz JB, Dringen R. 2001. The multidrug resistance protein MRP1 mediates the release of glutathione disulfide from rat astrocytes during oxidative stress. J Neurochem 76(2):627-36.

Hirrlinger J, Schulz JB, Dringen R. 2002b. Glutathione release from cultured brain cells: multidrug resistance protein 1 mediates the release of GSH from rat astroglial cells. J Neurosci Res 69(3):318-26.

Hirst-Jensen BJ, Sahoo P, Kieken F, Delmar M, Sorgen PL. 2007. Characterization of the pH-dependent Interaction between the Gap Junction Protein Connexin43 Carboxyl Terminus and Cytoplasmic Loop Domains. J Biol Chem 282(8):5801-5813.

Homolya L, Varadi A, Sarkadi B. 2003. Multidrug resistance-associated proteins: Export pumps for conjugates with glutathione, glucuronate or sulfate. Biofactors 17(1-4):103-14.

Hopkins FG. 1921. On an Autoxidisable Constituent of the Cell. Biochem J 15(2):286-305.

Huang Y, Grinspan JB, Abrams CK, Scherer SS. 2007. Pannexin1 is expressed by neurons and glia but does not form functional gap junctions. Glia 55(1):46-56.

Hur KC, Shim JE, Johnson RG. 2003. A potential role for cx43-hemichannels in staurosporin-induced apoptosis. Cell Commun Adhes 10(4-6):271-7.

Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M. 1999. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev 13(1):76-86.

Jagatha B, Mythri RB, Vali S, Bharath MM. 2008. Curcumin treatment alleviates the effects of glutathione depletion in vitro and in vivo: Therapeutic implications for Parkinson's disease explained via in silico studies. Free Radic Biol Med 44(5):907-17.

Janaky R, Ogita K, Pasqualotto BA, Bains JS, Oja SS, Yoneda Y, Shaw CA. 1999. Glutathione and Signal Transduction in the Mammalian CNS. Journal of Neurochemistry 73(3):889-902.

Janáky R, Varga V, Hermann A, Saransaari P, Oja SS. 2000. Mechanisms of L-Cysteine Neurotoxicity. Neurochemical Research 25(9):1397-1405.

Jentsch TJ, Stein V, Weinreich F, Zdebik AA. 2002. Molecular structure and physiological function of chloride channels. Physiol Rev 82(2):503-68.

John SA, Kondo R, Wang SY, Goldhaber JI, Weiss JN. 1999. Connexin-43 hemichannels opened by metabolic inhibition. J Biol Chem 274(1):236-40.

Johnson JW, Ascher P. 1987. Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature 325(6104):529-531.

Kamermans M, Fahrenfort I, Schultz K, Janssen-Bienhold U, Sjoerdsma T, Weiler R. 2001. Hemichannel-mediated inhibition in the outer retina. Science 292(5519):1178-80.

Kardami E, Dang X, Iacobas DA, Nickel BE, Jeyaraman M, Srisakuldee W, Makazan J, Tanguy S, Spray DC. 2007. The role of connexins in controlling cell growth and gene expression. Prog Biophys Mol Biol 94(1-2):245-64.

Kimelberg HK. 1995. Current concepts of brain edema. Review of laboratory investigations. J Neurosurg 83(6):1051-9.

Kimelberg HK, Goderie SK, Higman S, Pang S, Waniewski RA. 1990. Swelling-induced release of glutamate, aspartate, and taurine from astrocyte cultures. J Neurosci 10(5):1583-1591.

Kobayashi M, Itoh K, Suzuki T, Osanai H, Nishikawa K, Katoh Y, Takagi Y, Yamamoto M. 2002. Identification of the interactive interface and phylogenic conservation of the Nrf2-Keap1 system. Genes Cells 7(8):807-20.

Kohling R, Gladwell SJ, Bracci E, Vreugdenhil M, Jefferys JG. 2001. Prolonged epileptiform bursting induced by 0-Mg(2+) in rat hippocampal slices depends on gap junctional coupling. Neuroscience 105(3):579-87.

Koller KJ, Coyle JT. 1984. Ontogenesis of N-acetyl-aspartate and N-acetyl-aspartyl-glutamate in rat brain. Brain Res 317(1):137-140.

Koller KJ, Zaczek R, Coyle JT. 1984. N-acetyl-aspartyl-glutamate: regional levels in rat brain and the effects of brain lesions as determined by a new HPLC method. J Neurochem 43(4):1136-1142.

Kondo RP, Wang SY, John SA, Weiss JN, Goldhaber JI. 2000. Metabolic inhibition activates a non-selective current through connexin hemichannels in isolated ventricular myocytes. J Mol Cell Cardiol 32(10):1859-72.

Konig J, Nies AT, Cui Y, Leier I, Keppler D. 1999. Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. Biochim Biophys Acta 1461(2):377-94.

Korzeniewski C, Callewaert DM. 1983. An enzyme-release assay for natural cytotoxicity. J Immunol Methods 64(3):313-20.

Kraft AD, Johnson DA, Johnson JA. 2004. Nuclear Factor E2-Related Factor 2-Dependent Antioxidant Response Element Activation by tert-Butylhydroquinone and Sulforaphane Occurring Preferentially in Astrocytes Conditions Neurons against Oxidative Insult. J Neurosci 24(5):1101-1112.

Kranich O, Dringen R, Sandberg M, Hamprecht B. 1998. Utilization of cysteine and cysteine precursors for the synthesis of glutathione in astroglial cultures: preference for cystine. Glia 22(1):11-8.

Kranich O, Hamprecht B, Dringen R. 1996. Different preferences in the utilization of amino acids for glutathione synthesis in cultured neurons and astroglial cells derived from rat brain. Neuroscience Letters 219(3):211-214.

Kreutzberg GW. 1996. Microglia: a sensor for pathological events in the CNS. Trends Neurosci 19(8):312-8.

Kruh GD, Belinsky MG. 2003. The MRP family of drug efflux pumps. Oncogene 22(47):7537-52.

Kukley M, Barden JA, Steinhauser C, Jabs R. 2001. Distribution of P2X receptors on astrocytes in juvenile rat hippocampus. Glia 36(1):11-21.

Lanius RA, Shaw CA, Wagey R, Krieger C. 1994. Characterization, distribution, and protein kinase C-mediated regulation of [35S]glutathione binding sites in mouse and human spinal cord. J Neurochem 63(1):155-60.

Lehmann A. 1987. Alterations in hippocampal extracellular amino acids and purine catabolites during limbic seizures induced by folate injections into the rabbit amygdala. Neuroscience 22(2):573-8.

Leybaert L, Braet K, Vandamme W, Cabooter L, Martin PE, Evans WH. 2003. Connexin channels, connexin mimetic peptides and ATP release. Cell Commun Adhes 10(4-6):251-7.

Lin JH, Lou N, Kang N, Takano T, Hu F, Han X, Xu Q, Lovatt D, Torres A, Willecke K and others. 2008. A central role of connexin 43 in hypoxic preconditioning. J Neurosci 28(3):681-95.

Lindroth P, Mopper K. 1979. High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with o-phthaldialdehyde. Anal Chem 51:1667 - 1674.

Linsdell P, Hanrahan JW. 1998. Glutathione permeability of CFTR. Am J Physiol 275(1 Pt 1):C323-6.

Locovei S, Scemes E, Qiu F, Spray DC, Dahl G. 2007. Pannexin1 is part of the pore forming unit of the P2X(7) receptor death complex. FEBS Lett 581(3):483-8.

Markwardt F, Lohn M, Bohm T, Klapperstuck M. 1997. Purinoceptor-operated cationic channels in human B lymphocytes. J Physiol 498(Pt_1):143-151.

Martin ED, Fernandez M, Perea G, Pascual O, Haydon PG, Araque A, Cena V. 2007. Adenosine released by astrocytes contributes to hypoxia-induced modulation of synaptic transmission. Glia 55(1):36-45.

Martin ME, Munoz FM, Dickinson DA, Forman HJ, Martin del Rio R, Salinas M, Fando JL. 2005. Protective effect of L-trans-pyrrolidine-2,4-dicarboxilic acid preload against cell

death induced by oxygen/glucose deprivation in differentiated PC12 cells. J Neurosci Res 82(1):93-102.
Martinez-Hernandez A, Bell KP, Norenberg MD. 1977. Glutamine synthetase: glial localization in brain. Science 195(4284):1356-8.
Matthias K, Kirchhoff F, Seifert G, Huttmann K, Matyash M, Kettenmann H, Steinhauser C. 2003. Segregated expression of AMPA-type glutamate receptors and glutamate transporters defines distinct astrocyte populations in the mouse hippocampus. J Neurosci 23(5):1750-8.
Mehta V, Namboodiri MA. 1995. N-acetylaspartate as an acetyl source in the nervous system. Brain Res Mol Brain Res 31(1-2):151-7.
Meister A, Tate SS, Griffith OW. 1981. Gamma-glutamyl transpeptidase. Methods Enzymol 77:237-53.
Miller RF. 2004. D-Serine as a glial modulator of nerve cells. Glia 47(3):275-83.
Minich T, Riemer J, Schulz JB, Wielinga P, Wijnholds J, Dringen R. 2006. The multidrug resistance protein 1 (Mrp1), but not Mrp5, mediates export of glutathione and glutathione disulfide from brain astrocytes. J Neurochem 97(2):373-84.
Miyake M, Kakimoto Y. 1981. Developmental changes of N-acetyl-L-aspartic acid, N-acetyl-alpha-aspartylglutamic acid and beta-citryl-L-glutamic acid in different brain regions and spinal cords of rat and guinea pig. J Neurochem 37(4):1064-1067.
Moffett JR, Namboodiri MA, Cangro CB, Neale JH. 1991. Immunohistochemical localization of N-acetylaspartate in rat brain. Neuroreport 2(3):131-134.
Moi P, Chan K, Asunis I, Cao A, Kan YW. 1994. Isolation of NF-E2-Related Factor 2 (Nrf2), a NF-E2-Like Basic Leucine Zipper Transcriptional Activator that Binds to the Tandem NF-E2/AP1 Repeat of the {beta}-Globin Locus Control Region. Proceedings of the National Academy of Sciences 91(21):9926-9930.
Molchanova SM, Oja SS, Saransaari P. 2005. Mechanisms of enhanced taurine release under Ca2+ depletion. Neurochem Int 47(5):343-9.
Morgello S, Uson RR, Schwartz EJ, Haber RS. 1995. The human blood-brain barrier glucose transporter (GLUT1) is a glucose transporter of gray matter astrocytes. Glia 14(1):43-54.
Mulcahy RT, Wartman MA, Bailey HH, Gipp JJ. 1997. Constitutive and beta -Naphthoflavone-induced Expression of the Human gamma -Glutamylcysteine Synthetase Heavy Subunit Gene Is Regulated by a Distal Antioxidant Response Element/TRE Sequence. J Biol Chem 272(11):7445-7454.
Mulligan SJ, MacVicar BA. 2004. Calcium transients in astrocyte endfeet cause cerebrovascular constrictions. Nature 431(7005):195-9.
Nadler JV, Cooper JR. 1972. N-acetyl-L-aspartic acid content of human neural tumours and bovine peripheral nervous tissues. J Neurochem 19(2):313-9.
Nagelhus EA, Mathiisen TM, Ottersen OP. 2004. Aquaporin-4 in the central nervous system: Cellular and subcellular distribution and coexpression with KIR4.1. Neuroscience 129(4):905-913.
Nagy JI, Rash JE. 2000. Connexins and gap junctions of astrocytes and oligodendrocytes in the CNS. Brain Res Brain Res Rev 32(1):29-44.
Nakamura Y. 2002. Regulating factors for microglial activation. Biol Pharm Bull 25(8):945-53.
Nicolet BH. 1930. The Structure of Glutathione. Science 71(1849):589-590.
Nilius B, Droogmans G. 2003. Amazing chloride channels: an overview. Acta Physiologica Scandinavica 177(2):119-147.

Nodin C, Nilsson M, Blomstrand F. 2005. Gap junction blockage limits intercellular spreading of astrocytic apoptosis induced by metabolic depression. Journal of Neurochemistry 94(4):1111-1123.

Noraberg J, Kristensen BW, Zimmer J. 1999. Markers for neuronal degeneration in organotypic slice cultures. Brain Res Brain Res Protoc 3(3):278-90.

North RA, Surprenant A. 2000. Pharmacology of cloned P2X receptors. Annu Rev Pharmacol Toxicol 40:563-80.

Ogita K, Enomoto R, Nakahara F, Ishitsubo N, Yoneda Y. 1995. A possible role of glutathione as an endogenous agonist at the N-methyl-D-aspartate recognition domain in rat brain. J Neurochem 64(3):1088-96.

Okada Y. 1997. Volume expansion-sensing outward-rectifier Cl- channel: fresh start to the molecular identity and volume sensor. Am J Physiol 273(3 Pt 1):C755-89.

Okada Y. 2006. Cell volume-sensitive chloride channels: phenotypic properties and molecular identity. Contrib Nephrol 152:9-24.

Orkand RK, Nicholls JG, Kuffler SW. 1966. Effect of nerve impulses on the membrane potential of glial cells in the central nervous system of amphibia. J Neurophysiol 29(4):788-806.

Panchin Y, Kelmanson I, Matz M, Lukyanov K, Usman N, Lukyanov S. 2000. A ubiquitous family of putative gap junction molecules. Curr Biol 10(13):R473-4.

Pangrsic T, Potokar M, Stenovec M, Kreft M, Fabbretti E, Nistri A, Pryazhnikov E, Khiroug L, Giniatullin R, Zorec R. 2007. Exocytotic Release of ATP from Cultured Astrocytes. J Biol Chem 282(39):28749-28758.

Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG. 1994. Glutamate-mediated astrocyte-neuron signalling. Nature 369(6483):744-747.

Parpura V, Fang Y, Basarsky T, Jahn R, Haydon PG. 1995. Expression of synaptobrevin II, cellubrevin and syntaxin but not SNAP-25 in cultured astrocytes. FEBS Letters 377(3):489-492.

Parri HR, Gould TM, Crunelli V. 2001. Spontaneous astrocytic Ca2+ oscillations in situ drive NMDAR-mediated neuronal excitation. Nat Neurosci 4(8):803-12.

Pelegrin P, Surprenant A. 2006. Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. Embo J 25(21):5071-82.

Pellerin L, Magistretti PJ. 1994. Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. Proc Natl Acad Sci U S A 91(22):10625-9.

Penuela S, Bhalla R, Gong X-Q, Cowan KN, Celetti SJ, Cowan BJ, Bai D, Shao Q, Laird DW. 2007. Pannexin 1 and pannexin 3 are glycoproteins that exhibit many distinct characteristics from the connexin family of gap junction proteins. J Cell Sci 120(21):3772-3783.

Plotkin LI, Bellido T. 2001. Bisphosphonate-induced, hemichannel-mediated, anti-apoptosis through the Src/ERK pathway: a gap junction-independent action of connexin43. Cell Commun Adhes 8(4-6):377-82.

Pomper JK, Graulich J, Kovacs R, Hoffmann U, Gabriel S, Heinemann U. 2001. High oxygen tension leads to acute cell death in organotypic hippocampal slice cultures. Brain Res Dev Brain Res 126(1):109-16.

Price DL, Ludwig JW, Mi H, Schwarz TL, Ellisman MH. 2002. Distribution of rSlo Ca2+-activated K+ channels in rat astrocyte perivascular endfeet. Brain Research 956(2):183-193.

Pumain R, Heinemann U. 1985. Stimulus- and amino acid-induced calcium and potassium changes in rat neocortex. J Neurophysiol 53(1):1-16.

Quist AP, Rhee SK, Lin H, Lal R. 2000. Physiological role of gap-junctional hemichannels. Extracellular calcium-dependent isosmotic volume regulation. J Cell Biol 148(5):1063-74.

Raivich G. 2005. Like cops on the beat: the active role of resting microglia. Trends Neurosci 28(11):571-3.

Rana S, Dringen R. 2007. Gap junction hemichannel-mediated release of glutathione from cultured rat astrocytes. Neurosci Lett 415(1):45-8.

Ray A, Zoidl G, Weickert S, Wahle P, Dermietzel R. 2005. Site-specific and developmental expression of pannexin1 in the mouse nervous system. Eur J Neurosci 21(12):3277-90.

Regan RF, Guo Y. 1999. Extracellular reduced glutathione increases neuronal vulnerability to combined chemical hypoxia and glucose deprivation. Brain Res 817(1-2):145-50.

Retamal MA, Cortes CJ, Reuss L, Bennett MV, Saez JC. 2006. S-nitrosylation and permeation through connexin 43 hemichannels in astrocytes: induction by oxidant stress and reversal by reducing agents. Proc Natl Acad Sci U S A 103(12):4475-80.

Retamal MA, Froger N, Palacios-Prado N, Ezan P, Saez PJ, Saez JC, Giaume C. 2007a. Cx43 Hemichannels and Gap Junction Channels in Astrocytes Are Regulated Oppositely by Proinflammatory Cytokines Released from Activated Microglia. J Neurosci 27(50):13781-13792.

Retamal MA, Schalper KA, Shoji KF, Bennett MV, Saez JC. 2007b. Opening of connexin 43 hemichannels is increased by lowering intracellular redox potential. Proc Natl Acad Sci U S A 104(20):8322-7.

Richman PG, Meister A. 1975. Regulation of gamma-glutamyl-cysteine synthetase by nonallosteric feedback inhibition by glutathione. J Biol Chem 250(4):1422-6.

Rose CR, Ransom BR. 1997. Gap junctions equalize intracellular Na+ concentration in astrocytes. Glia 20(4):299-307.

Rossi DJ, Oshima T, Attwell D. 2000. Glutamate release in severe brain ischaemia is mainly by reversed uptake. Nature 403(6767):316-21.

Rouach N, Avignone E, Meme W, Koulakoff A, Venance L, Blomstrand F, Giaume C. 2002a. Gap junctions and connexin expression in the normal and pathological central nervous system. Biol Cell 94(7-8):457-75.

Rouach N, Calvo CF, Glowinski J, Giaume C. 2002b. Brain macrophages inhibit gap junctional communication and downregulate connexin 43 expression in cultured astrocytes. Eur J Neurosci 15(2):403-7.

Rouach N, Segal M, Koulakoff A, Giaume C, Avignone E. 2003. Carbenoxolone blockade of neuronal network activity in culture is not mediated by an action on gap junctions. J Physiol 553(Pt 3):729-45.

Rudkouskaya A, Chernoguz A, Haskew-Layton RE, Mongin AA. 2008. Two conventional PKC isoforms, alpha and betaI, are involved in the ATP-induced regulation of VRAC and glutamate release in cultured astrocytes. J Neurochem.

Rudolphi KA, Schubert P, Parkinson FE, Fredholm BB. 1992. Neuroprotective role of adenosine in cerebral ischaemia. Trends Pharmacol Sci 13(12):439-45.

Rushmore TH, King RG, Paulson KE, Pickett CB. 1990. Regulation of Glutathione S-Transferase Ya Subunit Gene Expression: Identification of a Unique Xenobiotic-Responsive Element Controlling Inducible Expression by Planar Aromatic Compounds. Proceedings of the National Academy of Sciences 87(10):3826-3830.

Sabirov RZ, Dutta AK, Okada Y. 2001. Volume-dependent ATP-conductive Large-Conductance Anion Channel as a Pathway for Swelling-induced ATP Release. J Gen Physiol 118(3):251-266.

Sagara JI, Miura K, Bannai S. 1993. Maintenance of neuronal glutathione by glial cells. J Neurochem 61(5):1672-6.

Sager TN, Laursen H, Fink-Jensen A, Topp S, Stensgaard A, Hedehus M, Rosenbaum S, Valsborg JS, Hansen AJ. 1999. N-Acetylaspartate distribution in rat brain striatum during acute brain ischemia. J Cereb Blood Flow Metab 19(2):164-72.

Salvi M, Fiore C, Battaglia V, Palermo M, Armanini D, Toninello A. 2005. Carbenoxolone induces oxidative stress in liver mitochondria, which is responsible for transition pore opening. Endocrinology 146(5):2306-12.

Sandberg M, Butcher SP, Hagberg H. 1986a. Extracellular overflow of neuroactive amino acids during severe insulin-induced hypoglycemia: in vivo dialysis of the rat hippocampus. J Neurochem 47(1):178-84.

Sandberg M, Butcher SP, Hagberg H. 1986b. Extracellular overflow of neuroactive amino acids during severe insulin-induced hypoglycemia: in vivo dialysis of the rat hippocampus. J Neurochem 47(1):178-184.

Sanderson MJ, Charles AC, Boitano S, Dirksen ER. 1994. Mechanisms and function of intercellular calcium signaling. Molecular and Cellular Endocrinology 98(2):173-187.

Schousboe I, Tonder N, Zimmer J, Schousboe A. 1993. A developmental study of lactate dehydrogenase isozyme and aspartate aminotransferase activity in organotypic rat hippocampal slice cultures and primary cultures of mouse neocortical and cerebellar neurons. Int J Dev Neurosci 11(6):765-772.

Schubert P, Rudolphi KA, Fredholm BB, Nakamura Y. 1994. Modulation of nerve and glial function by adenosine--role in the development of ischemic damage. Int J Biochem 26(10-11):1227-36.

Schwiebert EM. 1999. ABC transporter-facilitated ATP conductive transport. Am J Physiol 276(1 Pt 1):C1-8.

Shaw CA, Pasqualotto BA, Curry K. 1996. Glutathione-induced sodium currents in neocortex. Neuroreport 7(6):1149-52.

Shih AY, Johnson DA, Wong G, Kraft AD, Jiang L, Erb H, Johnson JA, Murphy TH. 2003. Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. J Neurosci 23(8):3394-406.

Shintani-Ishida K, Uemura K, Yoshida K-i. 2007. Hemichannels in cardiomyocytes open transiently during ischemia and contribute to reperfusion injury following brief ischemia. Am J Physiol Heart Circ Physiol 293(3):H1714-1720.

Singh SP, Wishnok JS, Keshive M, Deen WM, Tannenbaum SR. 1996. The chemistry of the S-nitrosoglutathione/glutathione system. Proc Natl Acad Sci U S A 93(25):14428-33.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic acid. Analytical Biochemistry 150(1):76-85.

Soltoff SP, McMillian MK, Talamo BR. 1992. ATP activates a cation-permeable pathway in rat parotid acinar cells. Am J Physiol 262(4 Pt 1):C934-40.

Sonnhof U. 1987. Single voltage-dependent K+ and Cl- channels in cultured rat astrocytes. Can J Physiol Pharmacol 65(5):1043-50.

Steinhauser C, Berger T, Frotscher M, Kettenmann H. 1992. Heterogeneity in the Membrane Current Pattern of Identified Glial Cells in the Hippocampal Slice. Eur J Neurosci 4(6):472-484.

Steullet P, Neijt HC, Cuenod M, Do KQ. 2006. Synaptic plasticity impairment and hypofunction of NMDA receptors induced by glutathione deficit: relevance to schizophrenia. Neuroscience 137(3):807-19.

Stewart D, Killeen E, Naquin R, Alam S, Alam J. 2003. Degradation of Transcription Factor Nrf2 via the Ubiquitin-Proteasome Pathway and Stabilization by Cadmium. J Biol Chem 278(4):2396-2402.

Stoppini L, Buchs PA, Muller D. 1991. A simple method for organotypic cultures of nervous tissue. J Neurosci Methods 37(2):173-82.

Stout CE, Costantin JL, Naus CC, Charles AC. 2002. Intercellular calcium signaling in astrocytes via ATP release through connexin hemichannels. J Biol Chem 277(12):10482-8.

Strange K, Emma F, Jackson PS. 1996. Cellular and molecular physiology of volume-sensitive anion channels. Am J Physiol 270(3 Pt 1):C711-30.

Suadicani SO, Brosnan CF, Scemes E. 2006. P2X7 receptors mediate ATP release and amplification of astrocytic intercellular Ca2+ signaling. J Neurosci 26(5):1378-85.

Sun Z, Zhang S, Chan JY, Zhang DD. 2007. Keap1 Controls Postinduction Repression of the Nrf2-Mediated Antioxidant Response by Escorting Nuclear Export of Nrf2. Mol Cell Biol 27(18):6334-6349.

Surprenant A, Rassendren F, Kawashima E, North RA, Buell G. 1996. The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). Science 272(5262):735-8.

Szatkowski M, Barbour B, Attwell D. 1990. Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. Nature 348(6300):443-446.

Tabernero A, Giaume C, Medina JM. 1996. Endothelin-1 regulates glucose utilization in cultured astrocytes by controlling intercellular communication through gap junctions. Glia 16(3):187-95.

Tallan HH. 1957. Studies on the distribution of N-acetyl-L-aspartic acid in brain. J Biol Chem 224(1):41-45.

Tallan HH, Moore S, Stein WH. 1956. N-acetyl-L-aspartic acid in brain. J Biol Chem(219):257-264.

Tate SS, Meister A. 1974. Interaction of ggr-Glutamyl Transpeptidase with Amino Acids, Dipeptides, and Derivatives and Analogs of Glutathione. J Biol Chem 249(23):7593-7602.

Taylor DL, Davies SE, Obrenovitch TP, Urenjak J, Richards DA, Clark JB, Symon L. 1994. Extracellular N-acetylaspartate in the rat brain: in vivo determination of basal levels and changes evoked by high K+. J Neurochem 62(6):2349-2355.

Theis M, Sohl G, Eiberger J, Willecke K. 2005. Emerging complexities in identity and function of glial connexins. Trends Neurosci 28(4):188-95.

Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, Biswal S. 2002. Identification of Nrf2-regulated Genes Induced by the Chemopreventive Agent Sulforaphane by Oligonucleotide Microarray. Cancer Res 62(18):5196-5203.

Thompson RJ, Zhou N, MacVicar BA. 2006. Ischemia opens neuronal gap junction hemichannels. Science 312(5775):924-7.

Tower DB, Young OM. 1973. The activities of butyrylcholinesterase and carbonic anhydrase, the rate of anaerobic glycolysis, and the question of a constant density of glial cells in cerebral cortices of various mammalian species from mouse to whale. J Neurochem 20(2):269-78.

Tranberg M, Stridh MH, Guy Y, Jilderos B, Wigstrom H, Weber SG, Sandberg M. 2004. NMDA-receptor mediated efflux of N-acetylaspartate: physiological and/or pathological importance? Neurochem Int 45(8):1195-204.

Tranberg M, Stridh MH, Jilderos B, Weber SG, Sandberg M. 2005. Reversed-phase HPLC with UV detection for the determination of N-acetylaspartate and creatine. Anal Biochem 343(1):179-82.

Trexler EB, Bukauskas FF, Bennett MV, Bargiello TA, Verselis VK. 1999. Rapid and direct effects of pH on connexins revealed by the connexin46 hemichannel preparation. J Gen Physiol 113(5):721-42.

Tsai G, Coyle JT. 1995. N-acetylaspartate in neuropsychiatric disorders. Prog Neurobiol 46(5):531-540.

Waagepetersen HS, Shimamoto K, Schousboe A. 2001. Comparison of effects of DL-threo-beta-benzyloxyaspartate (DL-TBOA) and L-trans-pyrrolidine-2,4-dicarboxylate (t-2,4-PDC) on uptake and release of [3h]D-aspartate in astrocytes and glutamatergic neurons. Neurochem Res 26(6):661-6.

Valiunas V, Weingart R. 2000. Electrical properties of gap junction hemichannels identified in transfected HeLa cells. Pflügers Archiv European Journal of Physiology 440(3):366-379.

Wallin C, Abbas AK, Tranberg M, Weber SG, Wigstrom H, Sandberg M. 2003. Searching for mechanisms of N-methyl-D-aspartate-induced glutathione efflux in organotypic hippocampal cultures. Neurochem Res 28(2):281-91.

Wallin C, Weber SG, Sandberg M. 1999. Glutathione efflux induced by NMDA and kainate: implications in neurotoxicity? J Neurochem 73(4):1566-72.

Wallraff A, Kohling R, Heinemann U, Theis M, Willecke K, Steinhauser C. 2006. The Impact of Astrocytic Gap Junctional Coupling on Potassium Buffering in the Hippocampus. J Neurosci 26(20):5438-5447.

Wallraff A, Odermatt B, Willecke K, Steinhauser C. 2004. Distinct types of astroglial cells in the hippocampus differ in gap junction coupling. Glia 48(1):36-43.

Walters JD, Johnson JD. 1988. Inhibition of cyclic nucleotide phosphodiesterase and calcineurin by spermine, a calcium-independent calmodulin antagonist. Biochim Biophys Acta 957(1):138-42.

Walz W. 2000. Role of astrocytes in the clearance of excess extracellular potassium. Neurochemistry International 36(4-5):291-300.

Vanden Abeele F, Bidaux G, Gordienko D, Beck B, Panchin YV, Baranova AV, Ivanov DV, Skryma R, Prevarskaya N. 2006. Functional implications of calcium permeability of the channel formed by pannexin 1. J Cell Biol 174(4):535-546.

Wang J, Ma M, Locovei S, Keane RW, Dahl G. 2007. Modulation of membrane channel currents by gap junction protein mimetic peptides: size matters. Am J Physiol Cell Physiol 293(3):C1112-9.

Wang Q, Sun AY, Simonyi A, Jensen MD, Shelat PB, Rottinghaus GE, MacDonald RS, Miller DK, Lubahn DE, Weisman GA and others. 2005. Neuroprotective mechanisms of curcumin against cerebral ischemia-induced neuronal apoptosis and behavioral deficits. J Neurosci Res 82(1):138-48.

Vanharreveld A, Crowell J, Malhotra SK. 1965. A Study of Extracellular Space in Central Nervous Tissue by Freeze-Substitution. J Cell Biol 25:117-37.

Varecka L, Peterajova E, Pogady J. 1987. Polymyxin B, a novel inhibitor of red cell Ca2+-activated K+ channel. FEBS Lett 225(1-2):173-7.

Weik R, Lonnendonker U. 1990. Polymyxin B as a highly effective gating modifier of high-conductance Ca2(+)-activated K+ channels in mouse skeletal muscle. Pflugers Arch 415(6):671-7.

Venance L, Stella N, Glowinski J, Giaume C. 1997. Mechanism involved in initiation and propagation of receptor-induced intercellular calcium signaling in cultured rat astrocytes. J Neurosci 17(6):1981-92.

Vesce S, Bezzi P, Volterra A. 2001. Synaptic Transmission with the Glia. News Physiol Sci 16(4):178-184.

Vessey JP, Lalonde MR, Mizan HA, Welch NC, Kelly ME, Barnes S. 2004. Carbenoxolone inhibition of voltage-gated Ca channels and synaptic transmission in the retina. J Neurophysiol 92(2):1252-6.

Whitaker JR, Granum PE. 1980. An absolute method for protein determination based on difference in absorbance at 235 and 280 nm. Anal Biochem 109(1):156-159.

Wilhelmsson U, Li L, Pekna M, Berthold C-H, Blom S, Eliasson C, Renner O, Bushong E, Ellisman M, Morgan TE and others. 2004. Absence of Glial Fibrillary Acidic Protein and Vimentin Prevents Hypertrophy of Astrocytic Processes and Improves Post-Traumatic Regeneration. J Neurosci 24(21):5016-5021.

Willecke K, Eiberger J, Degen J, Eckardt D, Romualdi A, Guldenagel M, Deutsch U, Sohl G. 2002. Structural and functional diversity of connexin genes in the mouse and human genome. Biol Chem 383(5):725-37.

Winterbourn CC, Metodiewa D. 1994. The Reaction of Superoxide with Reduced Glutathione. Archives of Biochemistry and Biophysics 314(2):284-290.

Volterra A, Steinhauser C. 2004. Glial modulation of synaptic transmission in the hippocampus. Glia 47(3):249-57.

Vornov JJ, Park J, Thomas AG. 1998. Regional vulnerability to endogenous and exogenous oxidative stress in organotypic hippocampal culture. Exp Neurol 149(1):109-22.

Vornov JJ, Tasker RC, Coyle JT. 1991. Direct observation of the agonist-specific regional vulnerability to glutamate, NMDA, and kainate neurotoxicity in organotypic hippocampal cultures. Exp Neurol 114(1):11-22.

Xu HL, Mao L, Ye S, Paisansathan C, Vetri F, Pelligrino DA. 2008. Astrocytes are a key conduit for upstream signaling of vasodilation during cerebral cortical neuronal activation in vivo. Am J Physiol Heart Circ Physiol 294(2):H622-32.

Yang CS, Chou ST, Lin NN, Liu L, Tsai PJ, Kuo JS, Lai JS. 1994. Determination of extracellular glutathione in rat brain by microdialysis and high-performance liquid chromatography with fluorescence detection. J Chromatogr B Biomed Appl 661(2):231-5.

Ye ZC, Wyeth MS, Baltan-Tekkok S, Ransom BR. 2003. Functional hemichannels in astrocytes: a novel mechanism of glutamate release. J Neurosci 23(9):3588-96.

Zhao J, Kobori N, Aronowski J, Dash PK. 2006. Sulforaphane reduces infarct volume following focal cerebral ischemia in rodents. Neurosci Lett 393(2-3):108-12.

Zoidl G, Petrasch-Parwez E, Ray A, Meier C, Bunse S, Habbes HW, Dahl G, Dermietzel R. 2007. Localization of the pannexin1 protein at postsynaptic sites in the cerebral cortex and hippocampus. Neuroscience 146(1):9-16.

Zonta M, Angulo MC, Gobbo S, Rosengarten B, Hossmann KA, Pozzan T, Carmignoto G. 2003. Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. Nat Neurosci 6(1):43-50.

Zundorf G, Kahlert S, Reiser G. 2007. Gap-junction blocker carbenoxolone differentially enhances NMDA-induced cell death in hippocampal neurons and astrocytes in co-culture. J Neurochem 102(2):508-21.

Zur Nieden R, Deitmer JW. 2006. The role of metabotropic glutamate receptors for the generation of calcium oscillations in rat hippocampal astrocytes in situ. Cereb Cortex 16(5):676-87.