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Effects of Xenobiotics on the Glutathione and Glucose Metabolism of Cultured Brain Astrocytes

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This thesis is dedicated to my beloved family.

Content

I Acknowledgements

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II Information on the structure of the thesis

This thesis contains two introduction chapters, five result chapters and a summarising discussion. The introduction chapter 1.1 introduces the reader to functions of brain astrocytes, while the introduction chapter 1.2 represents a review article on glutathione synthesis and metabolism in the brain that has been accepted for publication. The first result chapter (2.1) describes the charaterisation of astroglia-rich primary cultures and contains basal biochemical parameters of these cultures as well as first results on the effects of xenobiotics on the glutathione content of the cells. The other result chapters describe in detail the effects of xenobiotics and of 2-deoxyribose on cultured astrocytes.

These chapters represent two accepted articles (chapters 2.2 & 2.3) and two manuscripts that have been submitted (chapter 2.4 & 2.5). The publications representing the chapters 2.2 and 2.3 are included as portable document format. The running text of the review article (chapter 1.2) and of the submitted manuscripts (chapter 2.4 and 2.5) have been adapted to the layout chosen for this thesis, but text, figures and tables are otherwise identical to the versions that have been submitted. To comfort the reader, the figures and tables with their legends were included directly in the text of the review article (chapter 1.2), while the figures and tables of the publications/manuscripts 4 and 5 (chapter 2.4 & 2.5) with their legends have been placed between the results part and the discussion of the respective manuscript. It should be noted that the numbering of Figures and Tables in one chapter addresses exclusively the respective chapter.

For the multi-author publications and manuscripts, the contributions of the first author is listed on the first page of the respective chapter.

III Summary

In the mammalian brain, astrocytes possess a large variety of important functions. These cells modulate synaptic transmission and are involved in the regulation of brain pH and ion homeostasis. In addition, astrocytes provide essential metabolic support for neighbouring neurons for example by supplying amino acids as precursor for neuronal glutathione (GSH) synthesis. In astrocytes, the tripeptide GSH has a key function in antioxidative and detoxification processes. This thesis investigated the effects of xenobiotics such as halogenated acetates, iodoacetamide and fumaric acid esters as well as cell derived compounds such as 2-deoxyribose on the GSH and glucose metabolism of brain astrocytes. For these studies, astroglia-rich primary cultures were used and characterised as model system. The compounds investigated differed strongly in their potential to affect cellular GSH contents and lactate production. While micromolar concentrations of iodoacetate, iodoacetamide and fumaric acid dimethyl or diethyl esters were sufficient to deprive viable astrocytes within minutes of their GSH, monochloroacetate and 2-deoxyribose had to be applied in millimolar concentration and for hours to severely lower the cellular GSH content. In contrast, neither fumaric acid monoesters nor poly-chlorinated acetates affected the cellular GSH metabolism. The depletion of cellular GSH observed for the GSH-depriving compounds is most likely the consequence of a reaction of the compounds itself or of its metabolites with GSH to form GSH-conjugates. In addition to their strong effect on the cellular GSH content, exposure of cultured astrocytes to halogenated acetates or iodoacetamide altered the glucose metabolism of the cells as indicated by a lowered cellular lactate production. For iodoacetate, iodoacetamide and monochloroacetate, inhibition of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase was identified as underlying mechanism for the compromised glycolysis, whereas poly-chlorinated acetates are likely to lower lactate production by increasing pyruvate dehydrogenase activity and oxidative metabolism. Since astrocytes play important roles in detoxifying processes and in supplying neighbouring neurons with precursors for GSH synthesis and with metabolic fuel molecules such as lactate, disturbances in astrocytic GSH and/or glucose metabolism by xenobiotics are likely to indirectly affect also the metabolism and the functions of neurons.

IV Zusammenfassung

Astrozyten erfüllen im Säugetiergehirn eine Vielzahl von wichtigen Funktionen. So sind Astrozyten an der Modellierung der synaptischen Weiterleitung, an der Regulation des pH-Wertes im Gehirn sowie an der Ionenhomöostase beteiligt. Zudem versorgen sie benachbarte Neurone mit Vorläufern der Aminosäuresubstrate für deren Glutathion-(GSH)-Synthese. Das Tripeptid GSH spielt in Astrozyten eine entscheidende Rolle in antioxidativen und Entgiftungsprozessen. Im Rahmen dieser Arbeit wurden die Effekte von Fremdstoffen, zum Beispiel von halogenierten Acetaten und Fumarsäurestern, sowie von endogenen Substanzen wie 2-Desoxyribose auf den GSH- und Glukosestoffwechsel von Astrozyten untersucht. Für diese Studien wurden Astroglia-reiche Primärkulturen als Modellsystem verwendet und charakterisiert. Die untersuchten Substanzen unterschieden sich stark in ihrem Potential den zellulären GSH-Gehalt und/oder die zelluläre Laktatbildung zu beeinträchtigen. Eine Inkubation mit mikromolaren Konzentrationen von Jodacetat, Jodacetamid oder Fumarsäuredialkylestern war ausreichend, um den zellulären GSH-Gehalt von Astrozyten innerhalb weniger Minuten zu verarmen, während Monochloroacetat oder 2-Desoxyribose in millimolaren Konzentrationen über mehrere Stunden verabreicht werden mussten, um ähnliche Effekte zu erzielen. Die Abnahme des zellulären GSH-Gehaltes nach Zugabe der untersuchten GSH-verarmenden Substanzen ist vermutlich auf Konjugation der Substanzen und/oder ihrer Metabolite mit GSH zurück zu führen. Die Exposition von Astrogliakulturen mit halogenierten Acetaten und Jodacetamid wirkte sich auch auf den Glukosestoffwechsel der Zellen aus, was in einer verringerten Laktatbildung resultierte. Für Jodacetat, Jodacetamid und Monochloroacetat konnte die Inhibierung des glykolytischen Enzyms Glycerinaldehyd-3-phosphat-Dehydrogenase als mechanistische Ursache für die verringerte Laktatbildung identifiziert werden. Dichloroacetat und Trichloroacetat hingegen verringerten die zelluläre Laktatbildung vermutlich durch die beschriebene Steigerung der Aktivität der Pyruvatdehydrogenase und des oxidativen Stoffwechsels. Da Astrozyten eine entscheidende Rolle in Entgiftungsprozessen und bei der Versorgung benachbarter Neurone mit Aminosäuren und Energiesubstraten wie Laktat spielen, würden sich Beeinträchtigungen des astroglialen Stoffwechsels durch Fremdstoffe indirekt auch auf den Stoffwechsel und die Funktionen von Neuronen auswirken.

V Abbreviations

°C	degree celsius
2dRib	2-deoxyribose
2VP	2-vinylpyridine
4HNE	4-hydroxy-2-nonenal
Abbrev.	abbreviation
Ac-CoA	acetyl-CoA
Ace	acetate
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
ApN	aminopeptidase N
ARE	antioxidative response elements
ATP	adenosintriphosphate
BBB	blood-brain barrier
BSO	buthionine sulfoximine
CDNB	1-chloro-2,4-dinitrobenzene
CNS	central nervous system
conc.	concentration
DAPI	4´-6-diamidino-2-phenylindole dihydrochloride
DCA	dichloroacetate
DEF	diethyl fumarate
DMEM	Dulbecco´s modified Eagle´s medium
DMF	dimethyl fumarate
DTNB	5,5´-dithiobis-(2-nitrobenzoic acid)
EAAC	excitatory amino acid carrier
ed.	editor
EDTA	ethylenediamine tetraacetate
eds.	editors
et al.	Latin: *et alii*, and others
FA	fumaric acid
FAE	fumaric acid esters
Fig.	figure
Figs.	figures
g	acceleration of gravity
GABA	γ-aminobutyric acid
G6PDH	glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCL	glutamate cysteine ligase
GFAP	glial fibrillary acidic protein
GPx	glutathione peroxidase(s)
GR	glutathione reductase
GS-2VP	glutathion conjugate of 2-vinylpyridine
GSH	glutathione
GSSG	glutathione disulfide
GST	glutathione-S-transferase

GSx	total glutathione
GS-bimane	glutathione conjugate of monochlorobimane
GSy	GSH synthetase
γGT	γ-glutamyltranspeptidase
HEPES	N-(2-hydroxyethyl)piperazine-N´-(2-ethanesulfonic acid)
HO	hemeo xygenase
IA	iodoacetate
IAA	iodoacetamide
IB	incubation buffer
K_{cat}	catalytic constant
K_i	inhibition constant
Km	Michaelis-Menten constant
KPi	potassium phosphate buffer
L	liter
LDH	lactate dehydrogenase
M	molar (mol/L)
MCA	monochloroacetate
MCB	monochlorobimane
MEF	monoethyl fumarate
min	minute(s)
MMF	monomethyl fumarate
Mrp1	multidrug resistance protein 1
n	number of individual experiments
$NADH/NAD^+$	nicotinamide adenine dinucleotide, reduced/oxidized
$NADPH/NADP^+$	nicotinamide adenine dinucleotide phosphate, reduced/oxidized
nm	nanometer
p.	page
PBS	phosphate buffered saline
PDH	pyruvate dehydrogenase
pp.	pages
RT	room temperature
SD	standard deviation
TCA	trichloroacetate
TNB	5-thio-2-nitrobenzoate
TNFα	tumor necrosis factor alpha
Tris	tris(hydroxymethyl)aminomethane
v/v	volume per volume
Vol.	volume
w/v	weight per volume

1

Introduction

1.1 Properties and functions of astrocytes

1.2 **Publication/Manuscript1:**

Schmidt, M. M. & Dringen, R.: Glutathione synthesis and metabolism. In: *Advances in Neurobiology, Volume: Neural Metabolism in vivo* (In-Young Choi & Rolf Gruetter eds.). Springer Science, New York. In press.

1.1

Properties and functions of astrocytes.

Maike M. Schmidt

1.1.1 The brain and its cell types

The mammalian brain is a highly complex organ based on the non-assessable dimensions of homocellular and heterocellular networks and on the functional as well as morphological interactions between different brain cell types. In general, the brain consists of two major classes of cells: glial cells and neurons. The idea of a coexistence of excitable and non-excitable cells in brain was first proposed by Gabriel Gustav Valentin in 1836 (Valentin 1836; cited in Verkhratsky & Butt 2007). From the very beginning of neurosciences until today, neurons attract the highest attention among the different types of brain cells. These excitable cells respond to external stimulation by the generation of an action potential which propagates through the neuronal network. Neurons can be grouped by their excitatory, inhibitory or modulatory effects, their motor, sensor or secretory function and/or their repertoire of neurotransmitters (Siegel *et al.* 2006).

Glial cells account for more than 90% of all cells in the human brain, although representing only 50% of cell mass (Verkhratsky & Butt 2007). In contrast to neurons, glial cells possess no conventional synaptic contacts (Siegel *et al.* 2006). Glial cells can be divided into three broad groups of cells: macroglial, microglial and ependymal cells (Fig. 1). Macroglial cells, also termed as “true glial cells”, are of ectodermal origin (Verkhratsky & Butt 2007), such as astrocytes (see 1.1.2) and the myelin-producing oligodendrocytes (Bradl & Lassmann 2010).

Microglial cells are of mesodermal origin and invade the central nervous system (CNS) during very early embryonic development (Cuadros & Navascues 1998). They represent the major immuno-competent cells in the brain and possess many features of monocytes (Färber & Kettenmann 2005, Graeber & Streit 2010). The third group of glial cells are formed by ependymal cells that line the brain ventricles and the spinal cord central canal. These cells show distinct morphological features such as a covering with microvilli and a central cluster of long cilia on their cell surface (Del Bigio 2010). However, the morphological and functional characteristics within one group of brain cells can differ strongly, depending on their anatomical localisation in the brain.

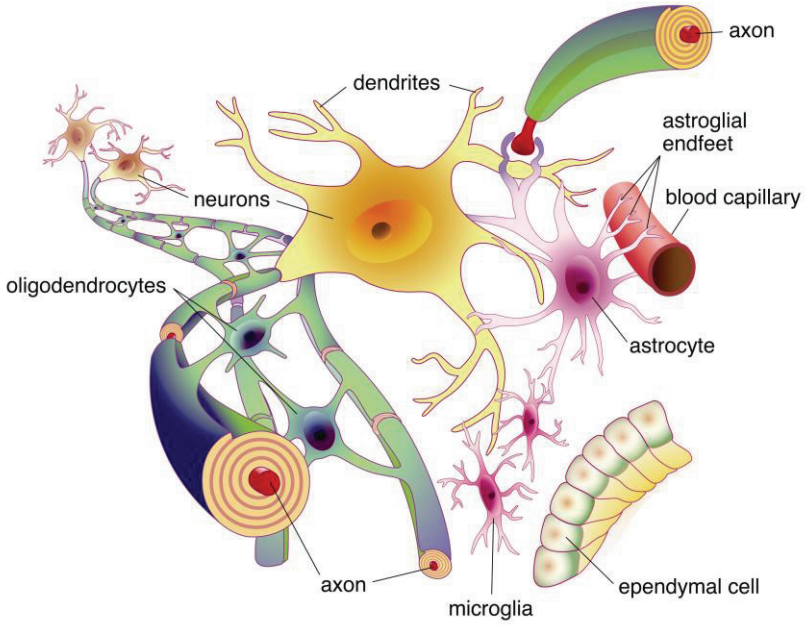

Figure 1: Schematic representation of the diverse cell types in the mammalian brain. This figure was kindly provided by Dr. Johannes Hirrlinger (University of Leipzig, Germany).

1.1.2 Astrocytes

1.1.2.1 General introduction

Already in 1858, Rudolf Ludwig Karl Virchow formed the term "glia" but contempted these cells as connective tissue, devoid of any other cellular elements (Virchow 1858; cited in Verkhratsky & Butt 2007). However, the use of first staining techniques and microscopical analysis by Camillo Golgi and Santiago Ramón y Cajal allowed the description of fine structures of diverse parts of the central nervous system and revealed a huge diversity of glial cells (De Carlos & Borrell 2007, Grant 2007). Michael von Lenhossek was the first to define the term astrocyte for star-like appearing glial cells in 1895 (Lenhossek 1895; cited in Verkhratsky & Butt 2007). At the end of the 19th century, many different opinions on the functions of glial cells were prominent, ranging from structural elements to the idea of active neuron-glial interactions (Verkhratsky & Butt 2007). Today, we know that astrocytes are specialised glial cells with heterogenous morphology and physiology that serve an amazingly large and diverse variety of functions (Table 1; Barres 2008,

Kimelberg 2010, Matyash & Kettenmann 2010, Seth & Koul 2008, Sofroniew & Vinters 2010, Volterra & Meldolesi 2005, Wang & Bordey 2008).

Table 1: Selected references summarising functions of brain astrocytes

Function	Selected References
Synaptogenesis	
Formation, maintenance and elimination	Barker & Ullian 2008, Fellin 2009, Klein 2009, Pfrieger 2010, Stevens *et al.* 2007, Theodosis *et al.* 2008, Ullian *et al.* 2004
Brain microarchitecture	
Astrocytic domains	Bushong *et al.* 2002, Bushong *et al.* 2004, Halassa *et al.* 2007, Halassa & Haydon 2010, Nedergaard *et al.* 2003
Glial syncytium	Giaume *et al.* 2010, Giaume & Theis 2010, Orellana *et al.* 2009, Rouach *et al.* 2008
Blood-brain barrier	Abbott 2002, Abbott *et al.* 2006, Bernacki *et al.* 2008, Haseloff *et al.* 2005, Lee *et al.* 2009
Brain microenvironment	
pH homeostasis	Deitmer & Rose 2010, Obara *et al.* 2008
Cerebrovascular regulation	Carmignoto & Gómez-Gonzalo 2010, Iadecola & Nedergaard 2007, Koehler *et al.* 2006, Koehler *et al.* 2009, Paulson *et al.* 2010
Astrocyte-neuron interaction	
Glio-transmitters	Beattie *et al.* 2002, Bergersen & Gundersen 2009, Bezzi *et al.* 2004, Chen *et al.* 2006, Hamilton & Attwell 2010, Pangrsic *et al.* 2007, Pascual *et al.* 2005
Gliotransmission & signalling	Araque & Perea 2004, Deitmer & Rose 2010, Parpura & Zorec 2010, Perea & Araque 2010, Perea *et al.* 2009, Reichenbach *et al.* 2010, Santello & Volterra 2009, Schipke & Kettenmann 2004, Schousboe & Waagepetersen 2006, Todd *et al.* 2006
Calcium signalling	Agulhon *et al.* 2008, Carmignoto 2000, Deitmer *et al.* 2006, Fiacco & McCarthy 2006, Fiacco *et al.* 2009, Perea & Araque 2005, Scemes & Giaume 2006
Metabolic coupling	Barros & Deitmer 2010, Benarroch 2010, Castro *et al.* 2009, Hertz *et al.* 2007, Hirrlinger & Dringen 2010, Maciejewski & Rothman 2008, Magistretti 2006, Magistretti 2009, Pellerin *et al.* 2007
Myelin biogenesis	Simons & Trajkovic 2006

In general, astrocytes are divided into two main subtypes, based on their differences in anatomical localisation and cellular morphology (Matyash & Kettenmann 2010, Sofroniew & Vinters 2010). Fibrous astrocytes are located in the white matter of the brain and are morphologically characterised by many long fiber-like processes,

whereas protoplasmic astrocytes are mainly found in the grey matter and are endowed with many fine processes in a uniform globoid distribution (Oberheim *et al.* 2006, Sofroniew & Vinters 2010). Although these two subtypes differ in localisation and morphology, both have been shown to intensively contact blood vessels thereby integrating the glia-vascular interface with neurons (Abbott *et al.* 2006). The blood-brain barrier (BBB) between intercerebral blood vessels and the brain parenchyma is formed by adjacent endothelial cells lining the blood vessel wall that are closely enwrapped by astrocytic end-feet (Abbott *et al.* 2006, Kacem *et al.* 1998, Lee *et al.* 2009). The primary function of the BBB as impermeable physical barrier is the protection of the brain microenvironment. Therefore, the BBB is characterised by tight, adherence and gap junctions and specialised transporter systems (Abbott 2002, Bernacki *et al.* 2008, Lee *et al.* 2009). Although astrocytes are not part of the BBB itself, they modulate the BBB permeability by the release of a variety of regulatory factors (Abbott 2002, Verkhratsky & Butt 2007).

Beside their function in the formation of the BBB (Lee *et al.* 2009), astrocytes play an important role in the functional architecture of the brain. These cells tile the entire CNS, possessing their own three-dimensional non-overlapping territory in which only the very distal tips of processes interdigitate one cell with another (Nedergaard *et al.* 2003, Sofroniew & Vinters 2010, Volterra & Meldolesi 2005). Especially in the grey matter, astrocytes divide brain regions into functional domains (Grosche *et al.* 1999, Grosche *et al.* 2002), in which theoretically hundreds of dendrites (Halassa *et al.* 2007) and more than 100,000 synapses are associated with a single astrocyte (Bushong *et al.* 2002).

Astrocytes form a functional syncytium by intercellular contacts known as gap junctions that are formed by the docking of two hemichannels or connexons of neighbouring cells (Giaume *et al.* 2010). Gap junctions allow the intercellular exchange of small molecules including ions involved in cellular excitability, metabolites such as ATP, glucose or glutathione (GSH), second messengers and other molecules (Giaume *et al.* 2010, Giaume & Theis 2010, Orellana *et al.* 2009).

The extend of the astroglial syncytium is diversly modulated for example by microglia activation (Faustmann *et al.* 2003) or by neuronal activity (Koulakoff *et al.* 2008). Impairment of the astroglial network has been shown to be neurotoxic (Blanc *et al.* 1998, Ozog *et al.* 2002). Dye-coupling experiments demonstrated that gap junctions are not limited to astrocyte-astrocyte communication but may also be formed between different brain cell types such as between astrocytes and oligodendrocytes (Nagy & Rash 2000) or neurons (Nagy *et al.* 2004).

1.1.2.2 Functions of astrocytes in the developing brain

In the developing brain, astrocytes play a crucial role in the guidance of neural precursor migration (Verkhratsky & Butt 2007) as well as in the formation, maintenance (Barker & Ullian 2010, Faissner *et al.* 2010, Pfrieger 2010) and elimination of synapses (Stevens *et al.* 2007). Neuronal guidance is a cooperation of fetal radial glial cells, since channels formed by astrocytes provide mechanical and guidance substrate for axon growth (Verkhratsky & Butt 2007). The regulation of these processes is very complex and depends on a variety of membrane bound as well as diffusible molecules (Barker & Ullian 2010, Pfrieger 2010). The requirement of glia during synaptogenesis varies with the neuronal cell type and its state of differentiation (Pfrieger 2010). However, gene ablation of astrocytes induced dramatic neuronal damage, demonstrating their relevance in synaptogenesis (Pfrieger 2010).

1.1.2.3 Astrocytes in modulation of synaptic transmission and signalling

Anatomical studies and three-dimensional illustrations demonstrated the extensive contacts between astrocytes and synaptic sites (Grosche *et al.* 2002, Grosche *et al.* 1999, Ventura & Harris 1999, Witcher *et al.* 2007) which allowed to extrapolate that a single astrocyte might envelope more than 100,000 synapses (Bushong *et al.* 2002). The term of the tri-partite synapse was coined in 1999 (Araque *et al.* 1999) and describes the bidirectional communication of astrocytes together with the neuronal presynaptic and postsynaptic elements at the synaptic site (Halassa *et al.* 2009, Perea *et al.* 2009). Glial cells are capable to express the same extended variety of ionotropic and metabotropic receptors for neurotransmitters and

neuromodulators and ionchannels as neurons do (Porter & McCarthy 1997, Verkhratsky & Steinhauser 2000, Verkhratsky & Butt 2007). In addition, astrocytes possess efficient neurotransmitter transport systems (Eulenburg & Gomeza 2010, Marcaggi & Attwell 2004) which enable these cells to be active partners in synaptic transmission. Besides the clearance of neurotransmitters such as glutamate, γ-aminobutyric acid (GABA) or glycine from the synaptic cleft, signals derived from neuronal activity can induce the release of glio-transmitters such as glutamate, ATP or D-serine (Araque & Perea 2004) by non-vesicular (Cotrina *et al.* 1998, Takano *et al.* 2005) or exocytotic mechanisms (Bezzi *et al.* 2001, Bezzi *et al.* 2004, Parpura & Haydon 2000, Zhang *et al.* 2004). On the other hand, neuronal activity can result in an increase of the cellular calcium ion concentration in astrocytes (Cornell-Bell *et al.* 1990, Verkhratsky & Kettenmann 1996). Calcium ions are released from cellular stores leading to calcium oscillations or the propagation of calcium waves via the astrocytic syncytium (Carmignoto 2000, Deitmer *et al.* 2006, Deitmer & Rose 2010, Scemes & Giaume 2006). The numerous abilities of astrocytes to modulate neuronal excitability and synaptic transmission have been reviewed recently (Hamilton & Attwell 2010, Parpura & Zorec 2010, Perea & Araque 2010, Theodosis *et al.* 2008).

The modulation of synaptic plasticity is based on the complex and irregular arborisation and ramification of astrocytic processes as the very distal lamellipodia- and filopodia-like processes are very motile structures that stabilise synapses (Reichenbach *et al.* 2010). Such interactions of astrocytic processes and dendritic spines have recently been demonstrated by dynamic imaging (Benediktsson *et al.* 2005, Haber *et al.* 2006, Hirrlinger *et al.* 2004, Nestor *et al.* 2007, Nishida & Okabe 2007).

1.1.2.4 Astrocyte functions in regulating the brain microenvironment

The maintenance of ion, pH and water homeostasis is crucial for brain functions. To maintain the ion homeostasis, astrocytes possess a variety of ion channels such as voltage-gated potassium channels that enable these cells to prevent extracellular potassium accumulation rather by local uptake than by gap junction mediated spatial potassium buffering (Meeks & Mennerick 2007, Schools *et al.* 2006, Wallraff *et al.* 2006). In addition, astrocytes express sodium and calcium channels, as well as

calcium-dependent potassium channels, chloride channels (Verkhratsky & Butt 2007, Verkhratsky & Steinhauser 2000) and aquaporins for water transport (Yool 2007). Since the uptake of neurotransmitters depends strongly on ion gradients and on transmembrane ion fluxes, small fluctuations in extracellular ion concentrations and/or pH can affect neural excitability and synaptic transmission (Deitmer & Rose 2010).

Since neurons are metabolically highly active cells that produce large amounts of CO_2 (Attwell & Laughlin 2001), pH regulation in the brain depends primarily on the carbonate/bicarbonate buffer system (Deitmer & Rose 2010). Both, glial cells and neurons posses several acid/base transport system such as the Na^+/H^+ exchanger for acid extrusion or the Cl^-/HCO_3^- exchanger to extrude base equivalents (Deitmer & Rose 2010, Obara *et al.* 2008). The enzymatic conversion of CO_2 to H_2CO_3 by the catalytic action of carbonic anhydrases fuels other transport systems such as the bidirectional sodium/bicarbonate transporter that is exclusively expressed in most macroglial cells and the monocarboxylate transporter which use bicarbonate and protons as cosubstrates, respectively (Deitmer 2002). The sodium/bicarbonate transporter primarily counterbalances neuronal activity-induced pH transients (Deitmer & Rose 2010), whereas the monocarboxylate transporters export metabolically generated acids such as lactic acid from the cells (Pierre & Pellerin 2005). There is growing evidence that altered pH homeostasis is connected to hypoxia/anoxia and epilepsy as well as to cancer and human immunodeficiency virus related CNS dysfunctions (Obara *et al.* 2008).

1.1.2.5 Metabolic cooperation between astrocytes and neurons

Metabolic cooperations between astrocytes and neurons appear to be a common principle for the metabolism of the brain. Experimental evidence for such cooperations has been reported for the basic glucose metabolism ("astrocyte-neuron-lactate shuttle hypothesis"; Barros & Deitmer 2010, Magistretti 2006, Pellerin *et al.* 2007) and the glutamate metabolism which is described in more detail below. In addition, metabolic cooperations between astrocytes and neurons have been shown for amino acids such as serine (Dringen *et al.* 1998) and branched chain amino acids (Bixel & Hamprecht 1995, Bixel *et al.* 1997, Yudkoff *et al.* 1994), the

metabolism of ketone bodies (Auestad *et al.* 1991, Bixel & Hamprecht 1995) and acetate (Brand *et al.* 1997), the synthesis of creatine (Dringen *et al.* 1998) and taurine (Brand *et al.* 1998) as well as for the GSH metabolism (chapter 1.2).

Astrocytes play a crucial role in the maintenance of glutamate homeostasis to ensure synaptic transmission and to prevent glutamate mediated excitotoxicity (Hertz & Zielke 2004). For these cells, glutamate serves as precursor amino acid for GSH synthesis (Dringen 2009) but also as metabolic fuel that can be oxidatively degraded (Zielke *et al.* 1998, Zielke *et al.* 2007). During synaptic activity, the glutamate released from neurons into the synapic cleft is rapidly taken up by perisynaptic astrocytes (Danbolt 2001) and converted to glutamine by the catalytic action of glutamine synthetase which in the brain is almost exclusively expressed in astrocytes (Norenberg & Martinez-Hernandez 1979). The non-neuroactive glutamine released from astrocytes is taken up by neurons and hydrolysed to glutamate by the activity of phosphate-activated glutaminase (Albrecht *et al.* 2007, Kvamme *et al.* 2000). This glutamate can then serve as precursor for neuronal GSH (Aoyama *et al.* 2008, Dringen 2009), for GABA synthesis (Leke *et al.* 2008, Sonnewald *et al.* 1993) or can be transported into synaptic vesicles to be used as neurotransmitter to maintain neural activity. Although this glutamate-glutamine cycle between neurons and astrocytes was initially suggested on the basis of cell culture experiments, more recent studies give experimental support for the existence of the glutamate-glutamine cycle in brain (Gruetter *et al.* 1998, Lebon *et al.* 2002, Serres *et al.* 2008, Sibson *et al.* 1998).

The uptake of glutamate in astrocytes is coupled to sodium uptake (Danbolt 2001). Subsequently, the elevated cellular sodium concentration after glutamate uptake activates the Na^+/K^+-ATPase which stimulates aerobic glycolysis in astrocytes (Pellerin & Magistretti 1994). The increased aerobic glycolysis leads to increased lactate production and release into the extracellular space which can serve neighbouring neurons as metabolic fuel. This metabolic interaction couples neuronal activity to astrocytic glucose consumption (Chatton *et al.* 2000, Magistretti 2009).

1.1.2.6 Astrocytes in pathology

In the last decades the ancient view of astrocytes as structural components and/or metabolic support for neuronal cells has been redefined. The growing insights into the vast variety of functions of brain astrocytes (Table 1) demonstrates that these cells are active participants in the maintenance of brain functions (Sofroniew & Vinters 2010), in the modulation of neural transmission and in signalling processes (Deitmer & Rose 2010, Parpura & Zorec 2010, Perea & Araque 2010). Emerging evidence suggests also a prominent role of astrocytes in the onset and/or the progression of neurological disorders (Table 2; Heneka *et al.* 2010).

Table 2: Selected references summarising functions and dysfunctions of brain astrocytes

Disease	Selected References
Several	Barres 2008, De Keyser *et al.* 2008, Duncan & Heales 2005, Heales *et al.* 2004, Heneka *et al.* 2010, Maragakis & Rothstein 2006, Miller 2005, Mrak & Griffin 2005, Ricci *et al.* 2009, Rossi & Volterra 2009, Seifert *et al.* 2006, Sofroniew & Vinters 2010
ALS	Barbeito *et al.* 2004, Blackburn *et al.* 2009, Holden 2007, Yamanaka *et al.* 2008
Astrogliosis	Fuller *et al.* 2009a, Ortinski *et al.* 2010, Sofroniew 2005, Sofroniew 2009
Brain ischemia	Rossi *et al.* 2007, Takano *et al.* 2009, Tuttolomondo *et al.* 2008
Epilepsy	Binder & Steinhauser 2006, Cloix & Hevor 2009, D'Ambrosio 2004, Eid *et al.* 2008, Friedman *et al.* 2009, Jabs *et al.* 2008, Seifert *et al.* 2010, Wetherington *et al.* 2008
Parkinson´s disease	Chen *et al.* 2005, Gates & Dunnett 2001, McGeer & McGeer 2008, Teismann & Schulz 2004
Schizophrenia	Bernstein *et al.* 2009, Kondziella *et al.* 2007

The only primary disease of astrocytes that has so far been associated with intrinsic astrocytic malfunctions appears to be Alexander´s Disease which is characterised by mutations in the gene encoding the glial fibrillary acidic protein (GFAP) (Mignot *et al.* 2004). In other neurodegenerative disorders such as Alzheimer´s disease (Fuller *et al.* 2009a, Fuller *et al.* 2009b), Huntington´s disease, epilepsy (Wetherington *et al.* 2008), brain ischemia (Rossi *et al.* 2007), amyotrophic lateral sclerosis (ALS; Yamanaka *et al.* 2008) and viral brain infections (Kramer-Hammerle *et al.* 2005), the implication of altered astrocyte functions has been shown to range from altered neurotransmitter clearance and/or ion homeastasis (especially

glutamate and potassium) to disturbances in intercellular communication, the increase of pro-inflammatory chemokine production, reactive astrogliosis (Ortinski *et al.* 2010) and glial scar formation (Sofroniew 2009).

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GSH synthesis and metabolism.

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Chapter 6.5:
GSH Synthesis and Metabolism

by
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Chapter content :

1 Abstract

The tripeptide glutathione (GSH) is present in brain cells in millimolar concentrations. GSH has many important cellular and extracellular functions, of which the detoxification of reactive oxygen species and of xenobiotics are especially important. In the brain, astrocytes play a central role in the GSH metabolism. Due to their broad metabolic potential astrocytes can utilise various extracellular precursors to produce the amino acids glutamate, cysteine and glycine that are required as cellular substrates for GSH synthesis. In contrast, neurons rely for their GSH synthesis on the availability of extracellular cysteine that is provided by astrocytes. The mechanism involved in this metabolic supply of cysteine includes export of GSH from astrocytes, extracellular processing of the exported GSH by ectoenzymes, and uptake into neurons of the amino acid precursors for GSH synthesis. Disturbances of this metabolic interaction between astrocytes and neurons will affect GSH homeostasis in brain and could contribute to a compromised antioxidative defense in neurological diseases. In this article we will review the current knowledge on the metabolism of GSH in brain cells with a special focus on the mechanisms that are involved in the metabolic supply of cysteine by astrocytes for the synthesis of GSH in neurons.

Key words: astrocytes, conjugation, cysteine, export, glutamate, glutamate cysteine ligase, γ-glutamyl transpeptidase, glutathione, GSH, GSSG, glycine, Mrp1, multidrug resistance proteins, neurons, neuron-glia interaction, PepT2, peptidases, peptide transporter, peroxidase, oxidative stress, metabolic cooperation, transferase, transport

2 Introduction

The tripeptide glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine) is present in cells in millimolar concentrations (Cooper and Kristal, 1997). GSH has essential cellular functions as important antioxidant. In addition, GSH serves as cofactor for some enzymes, is a storage and transport form of the amino acid cysteine and keeps the cellular thiol reduction potential in a strongly reduced form to protect essential thiol groups in enzymes against oxidation (Meister and Anderson, 1983; Cooper and Kristal, 1997; Janáky et al, 2007; Aoyama et al, 2008; Dringen, 2009; Forman et al, 2009; Lu, 2009). Recently, GSH has been demonstrated to be involved in cellular regulation, since the activity of many proteins can be modulated by glutathionylation (Dalle-Donne et al, 2007; Ghezzi and Di Simplicio, 2007; Mieyal et al, 2008). Moreover, GSH is involved in processes such as cell proliferation (Pallardó et al, 2009), apoptosis (Circu and Aw, 2008; Franco and Cidlowski, 2009), autoimmunity (Perricone et al, 2009), and aging (Maher, 2005; Rebrin and Sohal, 2008). For all these cellular processes and functions, the availability of a sufficiently high concentration of GSH is essential. Thus, it is not surprising that a dysregulation of GSH metabolism has been connected with various human diseases (Franco et al, 2007; Ristoff and Larsson, 2007; Ballatori et al, 2009b; Lu, 2009).

The antioxidative function of GSH appears to be especially important for the brain, since the cells of this organ continuously generate reactive oxygen species (ROS) in substantial amounts as side product of an extensive oxidative metabolism (Clarke and Sokoloff, 1999). This ROS generation has to be counter balanced by an appropriate antioxidative defense to prevent cell damage. Alterations in the homeostasis and the metabolism of the antioxidant GSH in brain have been connected with oxidative stress and oxidative damage. Evidence is growing that a compromised GSH metabolism contributes to the progression of neurological disorders such as Parkinson's disease (Chinta et al, 2007; Zeevalk et al, 2008; Cuadrado et al, 2009; Martin and Teismann, 2009), Alzheimer's disease (Liu et al, 2004; Ballatori et al, 2009b; Fuller et al, 2009), as well as schizophrenia and bipolar disorders (Gysin et al, 2007; Matsuzawa et al, 2008; Andreazza et al, 2009; Behrens and Sejnowski, 2009; Dean et al, 2009; Do et al, 2009; Wood et al, 2009).

Here we summarise the current knowledge on the cellular and extracellular metabolism of GSH in brain cells with the special focus on the mechanisms that are involved in the metabolic cooperation between astrocytes and neurons in the GSH metabolism of the brain.

3 GSH content in brain and neural cells

The specific GSH content of the brain depends on the species investigated and differs between brain regions. Forebrain and cortex seem to have the highest GSH content, followed by cerebellum, hippocampus, striatum and substantia nigra (Kang et al, 1999; Calabrese et al 2002), whereas brain stem, spinal cord, and the sciatic nerve have very low GSH contents (Cooper, 1998; Janáky et al, 2007). Since the cellular GSH content is determined by the rates of GSH synthesis and GSH consumption, the observed differences in specific GSH contents of different brain regions may reflect regional variation in the availability of GSH precursors and/or in the demand of GSH for various cellular and extracellular functions. In addition, regional differences in the ratio of glial cells to neurons, are likely to contribute to the variation in GSH contents between different brain regions (Rice and Russo-Menna, 1998, Srinivasan et al 2010). For example, white matter contains significantly less GSH than gray matter (Srinivasan et al 2010).

The most important functions of GSH in cells and in the extracellular space are listed in Table 1. It is important to consider that the concentrations of GSH in cells and in the extracellular space differ by three orders of magnitude. While GSH is present in the cytosol of cells in millimolar concentrations (Cooper and Kristal, 1997; Dringen and Hamprecht, 1998), the extracellular GSH in brain, as quantified by microdialysis, appears to reach just concentrations of up to 2 μM (Orwar et al, 1994; Yang et al, 1994; Lada and Kennedy, 1997; Han et al, 1999). These differences in GSH concentrations have to be considered for the potential functions of GSH in brain cells and in the extracellular space (Table 1).

Brain cell cultures are frequently used to investigate the metabolism of GSH of the different types of brain cells. Substantial information is available on the GSH metabolism of cultured astrocytes and neurons, while less is known on the GSH

metabolism of oligodendroglial and microglial cells (Dringen, 2000; Dringen, 2005; Dringen et al, 2005; Dringen, 2009; Dringen and Hirrlinger, 2009). Cultures that are enriched in astrocytes, neurons, oligodendroglial cells or microglial cells contain normally specific GSH contents between 20 and 40 nmol/mg protein (Dringen et al, 2005), although such values can differ strongly depending on the culturing conditions. Among the different types of cultured brain cells, microglial cells have the highest specific cellular GSH content, which is likely to contribute to the selfdefense of microglial cells against the ROS that they produce and release (Dringen, 2005; Dringen and Hirrlinger, 2009).

Table 1: Putative functions of GSH in brain

Intracellular functions (mM concentration range)	**Extracellular functions (µM concentration range)**
Radical scavenger	Substrate of enzymes (γGT)
Electron donor for non-enzymatic reduction	Radical scavenger
Substrate of enzymes (GPx, GST, isomerases)	Modulator of glutamate receptors
Leucotriene metabolism	Neurohormone
Detoxification of xenobiotics	Transport form of cysteine
Redox buffer	
Covalent modulator of protein functions via S-glutathionylation	
Storage form of cysteine	

4 Synthesis of GSH

GSH synthesis in brain cells follows the pathway that is well known for other tissues (Meister, 1974). GSH is synthesised by the consecutive reactions of the two ATP-consuming enzymes glutamate cysteine ligase (GCL; γ-glutamylcysteine synthetase) and GSH synthetase. GCL generates the dipeptide γGluCys from glutamate and cysteine. GSH synthetase catalyses the reaction of this dipeptide with glycine to GSH (Fig. 1). GCL is the rate limiting enzyme for GSH synthesis (Griffith, 1999; Griffith and Mulcahy, 1999) and is strongly regulated by GSH feedback inhibition (Richman and Meister, 1975; Misra and Griffith, 1998). Detailed information on structure, function and post-translational regulation of GCL have recently been summarised (Franklin et al, 2009). GCL is a heterodimeric protein that consists of a catalytic

(GCLc) and a modulatory (GCLm) subunit. The catalytical subunit GCLc possesses γGluCys synthesis activity, while the modifier subunit GCLm modulates the activity of GCLc by decreasing the K_m value for the substrates ATP and glutamate, by increasing the K_{cat} value for γGluCys synthesis and by increasing the K_i value for the feedback inhibition of GCL by GSH (Chen et al, 2005). In the absence of the modifier subunit, the catalytic subunit is efficiently inhibited already by low concentrations of GSH, as indicated by the high sensitivity of the GCL for GSH inhibition in GCLm knockout mice (Yang et al, 2002). A competition by GSH with glutamate on the substrate binding site and the reduction of an intersubunit disulfide bond are considered as molecular mechanisms of the GCL feedback inhibition by GSH (Huang et al, 1993; Fraser et al, 2002; Fraser et al, 2003; Franklin et al, 2009). GCL activity is known to be modulated by a variety of chemical and physical agents (Soltaninassab et al, 2000). Recent data confirmed that GCL activity is increased by prostaglandins (Saito et al, 2007), thyroid hormones (Dasgupta et al, 2007), polyphenols, flavonoids and substances that activate the nuclear erythroid 2-related factor 2 (Nrf2) pathway (Lavoie et al, 2009).

The consequences of an impaired GSH synthesis become evident by the relative rare inborn errors of metabolism for the GSH synthesis enzymes GCL or GSH synthetase that can lead to dramatic phenotypes including neurological symptoms such as mental retardation and motor functional disturbances. GCL deficiency in humans has only been reported for nine patients that all suffered from rather mild haemolytic anemia. Only in four cases CNS dysfunction such as spinocerebellar degeneration and neuropathies belonged to the disease pattern (Ristoff and Larsson, 2007). GSH synthetase deficiency leads to lower levels of cellular GSH and an accumulation of the dipeptide γGluCys in the cells. The patients suffer from haemolytic anemia, jaundice, metabolic acidosis and progressive CNS disorders (Dalton et al, 2004) with lesions in cortex and thalamus, cerebral necrosis and cerebral atrophy (Njalsson, 2005).

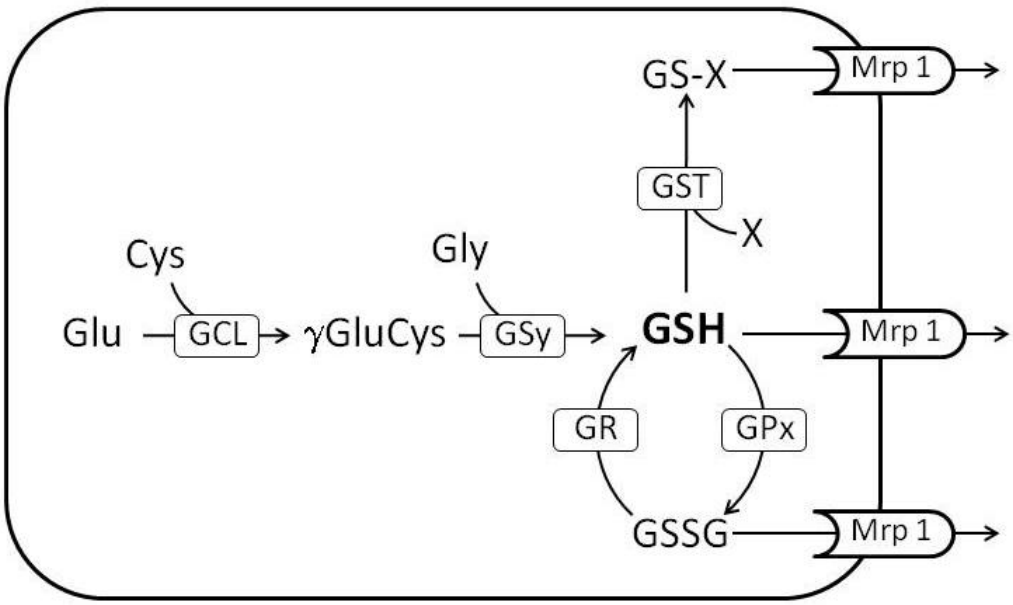

Figure 1: Synthesis and metabolism of GSH. GSH is synthesised from the amino acid substrates glutamate, cysteine and glycine by the consecutive reactions of glutamate cysteine ligase (GCL) and GSH synthetase (GSy). GSH is oxidised during peroxide disposal by glutathione peroxidases (GPx) to GSSG and is regenerated by glutathione reductase (GR) from GSSG. GSH can also react with xenobiotics and endogenous compounds (X) in reactions that are catalysed by glutathione-S-transferases (GST). GSH, GSSG and GSH conjugates can be exported from cells by multidrug resistance proteins such as Mrp1.

Several mouse models have been developed that were used to study the consequences of alterations in the activity of the enzymes involved in GSH synthesis (Dalton et al, 2004; Botta et al, 2008; Ballatori et al, 2009b). The deletion of the catalytical subunit of GCL leads to an embryonic lethal phenotype, whereas heterozygous mice are viable and fertile but show a lowered tissue content of GSH (Dalton et al, 2000). In addition, the knockdown of GCLc using an RNAi technique resulted in mild oxidative stress in neurons (Diaz-Hernandez et al, 2007). GCLm knockout mice are viable and develop normally, but have tissue GSH levels that are below 20% of that of wildtype mice (Yang et al, 2002). Brain cells of GCLm deficient mice are more vulnerable to xenobiotics such as domoic acid (Giordano et al, 2006; Giordano et al, 2007; Giordano et al, 2008b; Giordano et al, 2009) or environmental pollutants like flame retardants (Giordano et al, 2008a).

The transcription rate of the enzymes involved in GSH synthesis is controlled by the antioxidative reponse element (ARE) promotor, that is regulated by Nrf2. Both subunits of the GCL and the GSH synthetase are regulated by Nrf2 and are preferentially activated in astrocytes (Lee et al, 2003; Shih et al, 2003). Nrf2 activation or overexpression protects brain cells from oxidative or mitochondrial stress (Shih et al, 2003; Kraft et al, 2004; Shih et al, 2005) and leads to elevated levels of cellular GSH in cultured brain cells (Shih et al, 2003; Sun et al, 2005; Vargas et al, 2006; Vargas et al, 2008). In contrast, Nrf2 deficient mice are more vulnerable to insults that are applied in animal models of neurodegeneration (Johnson et al, 2008; Nguyen et al, 2009; Vargas and Johnson, 2009).

In addition to the enzymes responsible for GSH synthesis, the cellular GSH content depends strongly on the intracellular availability of the substrates glutamate, cysteine and glycine (Fig. 1). Since glutamate and glycine are neurotransmitters and cysteine in high concentrations is toxic to neurons, the extracellular availability of these amino acids in the brain is limited (Dringen, 2009). Thus, GSH synthesis in brain cells depends on their ability to take up suitable extracellular GSH precursors and/or to produce sufficient amounts of the substrates for GSH synthesis from such precursors. Cell culture experiments have demonstrated that uptake and metabolism of GSH precursors vary between different types of brain cells. GSH synthesis in neurons depends strongly on the availability of extracellular cysteine (Kranich et al, 1996; Dringen et al, 1999b), while astrocytes can use a wide range of amino acids and peptides that are taken up by the respective transporters and are subsequently converted to the substrate amino acids of GSH synthesis (Dringen and Hamprecht, 1998). Cultured neurons do not use extracellular cystine as source for the GSH substrate cysteine and prefer glutamine as best extracellular precursor for the glutamate moiety of GSH (Kranich et al, 1996). In contrast, astrocytes prefer glutamate and cystine as extracellular GSH precursors (Kranich et al, 1998). Cultured oligodendrocytes rely on the presence of cysteine or cystine in the culture media. Absence of these amino acids leads to a decline in cellular GSH levels of cultured oligodendrocytes that is followed by cell death (Yonezawa et al, 1996; Back et al, 1998). These differential preferences for extracellular GSH precursors lower the competition between different types of brain cells for GSH precursors.

The availability of the sulfur-containing amino acids cystine and/or cysteine appears to be crucial for the GSH synthesis in brain cells. A variety of transporters have been described to mediate the cellular uptake of cysteine or cystine. Both astrocytes and neurons have been reported to take up cystine by the Xc^- transport system, an electron neutral, sodium-independent cystine-glutamate exchanger (McBean 2002). In brain, the expression of this transporter varies between brain cell types and different brain regions (Shanker & Aschner 2001; Burdo et al, 2006). In addition, at least for astrocytes a contribution of the sodium-dependent X_{AG}- transport system (Bender et al 2000) and of the γ-glutamyl transpeptidase (γGT) system (Shanker & Aschner 2001) have been described to contribute to cellular cystine uptake. Transport of cysteine into astrocytes and neurons involves sodium-independent as well as sodium-dependent transport systems (Shanker & Aschner 2001). For neurons, members of the sodium-dependent X_{AG}- transporter family represent the predominat mechanism for cysteine uptake (Shanker & Aschner 2001). Absence of the X_{AG}- transporter family member excitatory amino acid carrier 1 (EAAC1) causes neuronal GSH deficiency, oxidative stress and age-dependent neurodegeneration (Aoyama et al, 2006; Aoyama et al, 2008). Differences in the expression levels of cysteine and cystine transporters are likely to contribute to the observed preferences of brain cells for sulfur-containing amino acids as extracellular precursors for cellular GSH synthesis.

5 Redox metabolism of GSH

GSH is an essential antioxidant for the cellular detoxification of ROS, since it directly reacts with radicals and is electron donor for the peroxide reduction by glutathione peroxidases (GPx). GSH is oxidised to GSSG during detoxification of peroxides (Fig. 1). For mammals eight GPx isoenzymes have been identified that differ in their tissue expression, substrate specificity and subcellular distribution (Ursini et al, 1995; Margis et al, 2008; Toppo et al, 2009). The cytosolic isoform GPx1 appears to be especially important for hydrogen peroxide reduction in brain cells (Dringen et al, 2005). The involvement of GPx1 in protecting brain cells against peroxide toxicity has been shown for GPx1-deficient neurons (de Haan et al, 1998; Taylor et al, 2005) and astrocytes (Liddell et al, 2006a; Liddell et al, 2006b). In addition, for several *in*

vivo stress paradigms it was demonstrated that brains of GPx1-deficient mice are more seriously damaged than those of wild type mice (de Haan et al, 1998; Crack et al, 2001; Flentjar et al, 2002; Crack et al, 2006; Wong et al, 2008).

GSH is regenerated from GSSG (Fig. 1) by cellular glutathione reductase (GR) (Schirmer et al, 1989; Lopez-Barea et al, 1990). This enzyme uses NADPH as electron donor and has been shown to be present in all types of brain cells (Dringen et al, 2005). Reduction of GSSG by GR is highly efficient due to the low K_m-values of GR for GSSG and NADPH (Dringen and Gutterer, 2002). Therefore, under unstressed conditions GSSG is hardly detectable in cells. However, during oxidative stress GR becomes rate limiting for GSH redox cycling as indicated by the cellular accumulation of GSSG (Dringen and Hamprecht, 1997) and a corresponding shift in the thiol reduction potential of the stressed cells (Hirrlinger and Dringen, 2010). For efficient GR-dependent reduction of GSSG to GSH, a suitable supply of reduction equivalents in the form of NADPH is required. Brain cells contain multiple pathways in cytosol and mitochondria that reduce $NADP^+$ to provide NADPH for the GR reaction (Dringen et al, 2005; Dringen et al, 2007; Hirrlinger and Dringen, 2010).

All types of brain cells contain GPx and GR (Dringen et al, 2005) and are therby able to oxidise GSH to GSSG and to regenerate GSH. Among the different types of brain cells in culture, oligodendrocytes contain the highest specific activities of the enzymes GPx and GR and are highy efficient in peroxide detoxification (Hirrlinger et al, 2002a; Baud et al, 2004). Exposure of cultured brain cells to hydrogen peroxide caused transient accumulation of cellular GSSG (Dringen et al, 1999a; Hirrlinger et al, 2000; Hirrlinger et al, 2001; Hirrlinger et al, 2002a; Liddell et al, 2006a), demonstrating that the activity of GR is not sufficient to immediately reduce the GSSG that is formed by GPx. However, after removal of the peroxide all types of brain cells were able to rapidly restore their initial high GSH to GSSG ratio by GR (Dringen et al, 1998b; Dringen et al, 1999a; Kussmaul et al, 1999; Hirrlinger et al, 2000; Liddell et al, 2006a; Liddell et al, 2006b). The GSH/GSSG redox cycling by GPx and GR does not deprive brain cells of their total glutathione (GSH plus GSSG). In contrast, GSH conjugation and export of GSH or GSSG lower the cellular GSH concentration and *de novo* synthesis of GSH is required to compensate for these types of GSH consumption.

6 Conjugation of GSH

Due to its reactive thiol group, GSH can rapidly react with various compounds (Fig. 1) in reactions that are either enzyme independent or are accelerated by glutathione-S-transferases (GSTs) (Commandeur et al, 1995; Salinas and Wong, 1999; Hayes et al, 2005; Blair, 2006). Four major groups of enzymes with GST activity have been identified, of which the large group of canonical soluble GSTs is best characterised. This group is subdivided in 7 classes, of which the α, μ, and π classes are most prominent in mammals (Mannervik et al, 2005; Zimniak and Singh, 2007). Although GSTs are generally considered as transferases that connect GSH to endogenous or xenobiotic acceptor substances, some of theses enzymes act also as isomerases or show glutathione peroxidase activity (Zimniak, 2007). GSTs are multifunctional enzymes with an impressive list of substrates (Sharma et al, 2007). GST isoenzymes are expressed in a tissue-dependent manner. In the brain, expression of GST isoenzymes of the α, μ, and π classes have been described (Dhanani and Awasthi, 2007) and the expression of different GSTs depends strongly on the cell type as well as on the brain region (Johnson et al, 1993; Shang et al, 2008).

The physiological functions of individual GSTs in brain and in the different types of brain cells have not been elucidated in detail. Since compounds such as monochlorobimane, iodoacetate or iodoacetamide, and fumaric acid diesters rapidly lower the cellular GSH contents of cultured astrocytes or neurons (DeCory et al, 2001; Waak and Dringen, 2006; Schmidt and Dringen, 2009; Schmidt and Dringen, 2010), a contribution of GSTs in the metabolism of such compounds has been postulated. In addition, GST-dependent detoxification in neurons has also been considered for various substances that cause selective necrosis to granule cells in the cerebellum (Fonnum and Lock, 2004).

7 Export of GSH, GSSG and glutathione conjugates

Export of GSH, GSSG and GSH conjugates has been reported for various cell types and tissues including the brain (Borst and Elferink, 2002; Dallas et al, 2006; Deeley and Cole, 2006; Toyoda et al, 2008; Ballatori et al, 2009a). Such processes consume

cellular GSH. Therefore, GSH synthesis is required to compensate for the cellular loss of GSH (Fig. 1).

Members of the family of multidrug resistance proteins (Mrps) are predominately responsible for the cellular export of GSH and its conjugates. Mrps are ATP-driven export pumps of organic anions and belong to the family of ATP-binding cassette transporters (Toyoda et al, 2008; Zhou et al, 2008; Ballatori et al, 2009a). Nine mammalian Mrps have been characterised that differ in their tissue distribution and in their substrate specificity. In addition to Mrps, also organic anion transporters (OATPs) or the cystic fibrosis transductance regulator (CFTR) have been reported to contribute to the transport of GSH (Ballatori et al, 2005). The current knowledge on the expression and functions of the different types of transporters that are involved in the export of GSH, GSSG and GSH conjugates in brain and neural cells has recently been summarised (Dallas et al, 2006; Ballatori et al, 2009a).

In addition to Mrps, the Ral-interacting protein RLIP76 has been described as novel GSH conjugate and multidrug transporter (Singhal et al, 2009). Although functional properties of RLIP76 have been predominantly obtained for cancer cells, at least the expression of RLIP76 in human brain tissue has been shown (Awasthi et al, 2007; Singhal et al, 2009). In epileptic brain, RLIP76 co-localises exclusively with endothelial cells but not with glial cells or neurons (Awasthi et al, 2007). Whether this transporter is involved in the export of GSH conjugates from the brain remains to be elucidated.

Among cultured brain cells, only cultured astrocytes release substantial amounts of GSH (Hirrlinger et al, 2002b). GSH export from astrocytes depends strongly on the cellular content of GSH (Sagara et al, 1996) and is accelerated by glutamate (Frade et al, 2008). Under unstressed conditions these cells release around 10% of their cellular GSH per hour (Dringen et al, 1997), and even protect the GSH exported against oxidation (Stewart et al, 2002; Pope et al, 2008). Mrp1 has been shown to mediate about 60% of the GSH export from astrocytes (Hirrlinger et al, 2002b; Hirrlinger and Dringen, 2005; Minich et al, 2006; Dringen and Hirrlinger, 2009). The transporter(s) responsible for the residual 40% of GSH export from astrocytes has not been identified so far. However, pharmacological studies on cultured Mrp1-

deficient astrocytes make a contribution of Mrps 4 and 5, OATPs 1 and 2 or CFTR in the GSH export from astrocytes unlikely (Minich et al, 2006). In addition to Mrp1, GAP junction hemichannels have been reported to mediate GSH release from astrocytes in culture (Rana and Dringen, 2007) and brain slices (Stridh et al, 2008) under certain conditions.

Under unstressed conditions, GSSG is hardly detectable in brain cells (Hirrlinger and Dringen, 2010). Thus, under such conditions astrocytes release exclusively GSH and only minute amounts of GSSG are detected in the media (Hirrlinger et al, 2002b; Minich et al, 2006). In contrast, during sustained peroxide stress, GSSG accumulates quickly in astrocytes and is efficiently exported (Hirrlinger et al, 2001; Hirrlinger et al, 2002c; Minich et al, 2006). The complete absence of any extracellular GSSG accumulation after exposure of Mrp1-deficient astrocytes to sustained peroxide stress (Minich et al, 2006) demonstrates that Mrp1 is exclusively responsible for GSSG export from astrocytes. Export of GSSG during oxidative stress has been discussed as protective mechanism that helps to at least partially prevent a shift in the thiol reduction potential of the cells (Akerboom and Sies, 1990; Keppler, 1999; Hirrlinger et al, 2001). However, it has to be considered that exported GSSG cannot serve anymore as substrate for cytosolic GSH regeneration by GR. Thus, ATP-dependent GSH *de novo* synthesis is required to compensate for the loss of cellular GSH due to GSSG export during oxidative stress.

8 Extracellular metabolism of GSH

Extracellular GSH and GSH conjugates are substrates of the ectoenzyme γ-glutamyl transpeptidase (γGT). This enzyme catalyses the transfer of the γ-glutamyl moiety onto a suitable acceptor and the hydrolysis of the γ-glutamyl bond (Ikeda and Taniguchi, 2005). In brain, γGT is strongly expressed in the endothelial cells of capillaries and has been suggested to be involved in the ventilatory response to hypoxia (Lipton et al, 2001). However, γGT is also expressed in astrocytes. Presence of γGT activity (Dringen et al, 1997; Garcion et al, 1999; Gegg et al, 2003) and γGT immunoreactivity (Shine and Haber, 1981; Philbert et al, 1995; Zhang et al, 1997) have been reported for astrocytes *in vivo* and in culture. γGT activity has also been reported for cultured oligodendrocytes, whereas hardly any activity of this enzyme

was detectable for cultured microglial cells and neurons (Ruedig and Dringen, 2004). The activity of γGT in cultured astrocytes is elevated after treatment of the cells with lipopolysaccharide (Garcion et al, 1999), NO donors (Gegg et al, 2003), tumor necrosis factor α (TNFα) (Ruedig and Dringen, 2004), thyroid hormone (Dasgupta et al, 2005), 6-hydroxydopamine (Zhang et al, 2005) or ethanol (Rathinam et al, 2006). Such increases in γGT activity improve the ability of the cells to process the GSH that was released from astrocytes (Ruedig and Dringen, 2004).

The product of the reaction of γGT with GSH is the dipeptide CysGly. This dipeptide is produced by γGT in equimolar concentrations to the GSH consumed. CysGly can be taken up into cells by peptide transporters or can be hydrolysed by ectopeptidases (Fig. 2). Both pathways appear to be realised in brain. The high affinity proton-coupled oligopeptide transporter PepT2 is widely expressed in brain (Fujita et al, 2004; Groneberg et al, 2004; Shen et al, 2004). The highest level of expression was found in the choroid plexus (Shen et al, 2004). In addition to ependymal and choroid plexus cells, astrocytes and neurons have been reported to express PepT2 in brain (Berger and Hediger, 1999; Shen et al, 2004). PepT2 expression has also been shown for cultured astrocytes (Dringen et al, 1998a; Xiang et al, 2006), whereas mRNA of PepT2 was not found in cultured neurons (Dieck et al, 1999). In cultured astrocytes, PeptT2 is responsible for the uptake of CysGly that is subsequently cleaved by a cellular peptidase to obtain cysteine and glycine for GSH synthesis (Dringen et al, 1998a). In contrast, cultured neurons appear not to take up intact CysGly. These cells express aminopeptidase N (ApN) (Dringen et al, 2001; Rathinam et al, 2006). Since the inactivation of this enzyme prevents the utilization of CysGly for GSH synthesis in neurons, CysGly is rather extracellularly hydrolysed by neurons to deliver substrates for GSH synthesis than taken up as intact dipeptide (Dringen et al, 2001). ApN is widely expressed in various brain regions (Noble et al, 2001) and could therefore be responsible for the extracellular cleavage of the γGT-product CysGly in brain that is demonstrated by the observation that inhibition of γGT lowers the extracellular concentration of cysteine in brain (Han et al, 1999).

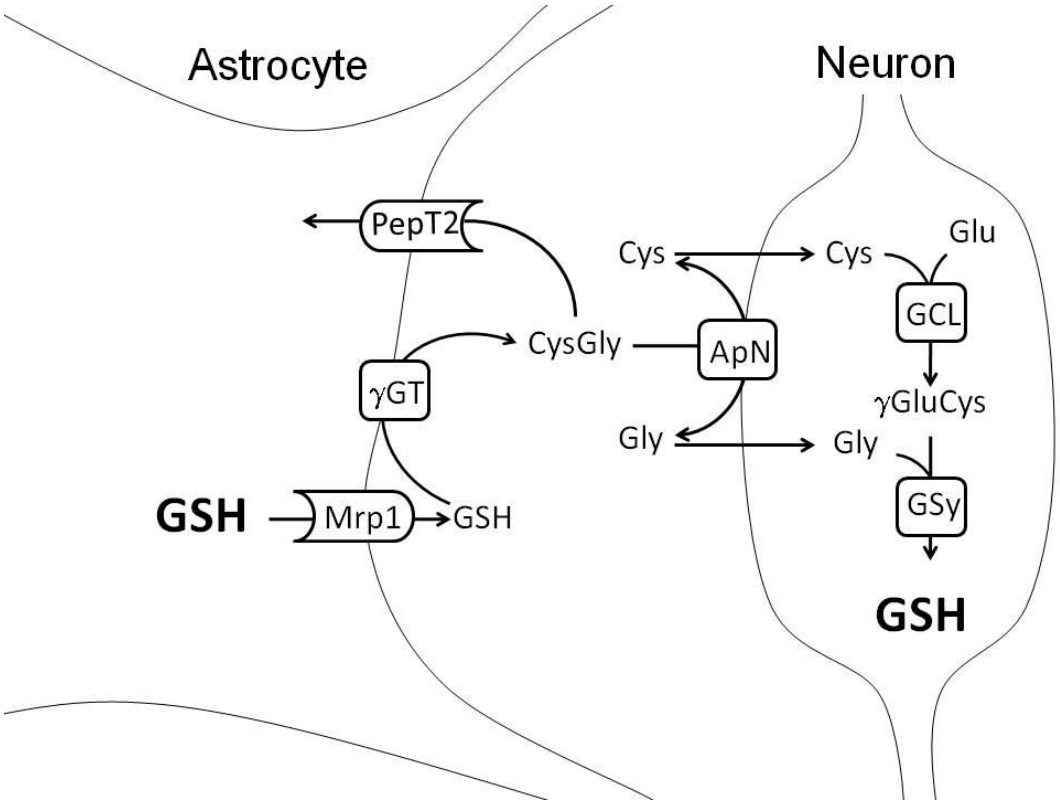

Figure 2: Metabolic interaction between astrocytes and neurons in the GSH metabolism. The extracellular cysteine that is required for neuronal GSH synthesis is provided by astrocytes. This metabolic supply is initiated by GSH export from astrocytes via Mrp1 or other transporters. Extracellular GSH is used as substrate of the astrocytic ectoenzyme γ-glutamyl transpeptidase (γGT) that produces the dipeptide CysGly. This dipeptide can be hydrolysed by neuronal aminopeptidase N (ApN) and the generated amino acids cysteine and glycine are taken up into neurons. Alternatively, CysGly can be taken up into astrocytes by the peptide transporter PepT2.

The uptake of cysteine and glycine into neurons is likely to be mediated by sodium dependent transporters. The sodium-dependent excitatory amino acid carrier 1 (EAAC1) is most likely responsible for the majority of cysteine uptake into neurons (Aoyama et al, 2008). Absence of EAAC1 causes neuronal GSH deficiency, oxidative stress and age-dependent neurodegeneration (Aoyama et al, 2006; Aoyama et al, 2008). The glutamate transporter-associated protein (GTRAP3-18) modulates the EAAC1-mediated cysteine uptake into neurons by interacting with EAAC1 in the plasma membrane. A decline of the GTRAP3-18 protein leads to an increase of the GSH content in neurons (Watabe et al, 2008).

The uptake of the amino acid glycine can be mediated by glycine transporter such as GlyT1 and GlyT2. These transporters differ in their expression patterns, stoichiometries, reversed uptake capacities and pre-steady-state kinetics (Roux and Supplisson, 2000; Supplisson and Roux, 2002; Eulenburg et al, 2005; Betz et al, 2006). GlyT1 appears to be expressed throughout the brain (Adams et al, 1995; Zafra et al, 1995; Jursky and Nelson, 1996) and GlyT1 immunoreactivity has been shown for astrocytes and some subpopulations of neurons (Zafra et al, 1995; Cubelos et al, 2005), whereas GlyT2 immunoreactivity was found only for glycinergic neurons (Jursky and Nelson, 1995). Studies on GlyT1 or GlyT2 knockout mice revealed that GlyT1 has an important function in the clearing of glycine from the synaptic cleft, whereas GlyT2 provides cytosolic glycine for vesicular release (Gomeza et al, 2003; Eulenburg et al, 2005; Betz et al, 2006). Whether the known glycine transporters or other transporters are involved in the uptake of the glycine that is required for GSH synthesis in neurons remains to be elucidated.

9 Metabolic cooperation between astrocytes and neurons in GSH metabolism

In brain, especially neurons appear to be disadvantaged regarding GSH metabolism. Since neurons cannot use extracellular cystine as GSH precursor (Kranich et al, 1996), these cells rely on the supply of cysteine from neighbouring cells (Dringen and Hirrlinger, 2003; Dringen, 2009). Indeed, presence of astrocytes maintains or even increases GSH levels in co-cultured neurons (Bolaños et al, 1996; Dringen et al, 1999b; Chen et al, 2001; Shih et al, 2003; de Bernardo et al, 2004; Gegg et al, 2005; Griffin et al, 2005; Watts et al, 2005; Rathinam et al, 2006; Vargas et al, 2006; Woehrling et al, 2007; Vargas et al, 2008; Sandhu et al, 2009). The pathway involved in the supply of GSH precursors from astrocytes to neurons (Fig. 2) is initiated by the Mrp1-dependent export of GSH from astrocytes (Minich et al, 2006). Since neurons do not have the ability to take up intact GSH (Sagara et al, 1996), the extracellular breakdown of the GSH exported from astrocytes by the astroglial ectoenzyme γGT (Dringen et al, 1997; Dringen et al, 1999b) and by a neuronal ectopeptidase (Dringen et al, 2001) are inevitable for the supply of the GSH precursor cysteine for neuronal GSH synthesis (Fig. 2). This scenario is strongly supported by the observation that inhibition of γGT in astrocyte-neuron co-cultures

totally prevented the astrocyte induced increase of the neuronal GSH content (Dringen et al, 1999b). In addition, also the coordinated neuroprotective upregulation of GSH efflux from astrocytes, γGT activity in astrocytes, and ApN activity in neurons by ethanol (Rathinam et al, 2006) supports the view that the connection of these three metabolic steps are important for the cysteine supply from astrocytes for neuronal GSH synthesis. With the supply by astrocytes of glutamine and CysGly, precursors of all three amino acids that are needed for GSH synthesis are provided from astrocytes to neurons. Most evidences for the interaction of astrocytes and neurons in GSH metabolism of the brain have been deduced from data obtained on cultures and co-cultures of brain cells. However, γGT-dependent generation of cysteine from GSH by brain cells has been confirmed for brain slices (Li et al, 1996; Li et al, 1999) and for the brain (Han et al, 1999), strongly suggesting that also *in vivo* astrocytes supply neurons with cysteine for GSH synthesis.

10 Conclusions

Metabolic cooperation between astrocytes and neurons is important for the homeostasis of GSH in brain. Neurons rely on the supply of GSH precursors, especially of cysteine, from neighbouring astrocytes. The metabolic pathway that provides astrocyte-derived cysteine to neurons is initiated by the export of GSH from astrocytes. The exported GSH is subsequently processed by ectoenzymes to neuronal GSH precursors. Alterations of this metabolic cooperation between astrocytes and neurons will substantially disturb the availability of the GSH precursor cysteine for neurons. Such processes may contribute to a lowered antioxidative defense in neurons, to an increased susceptibility to oxidative stress and to neuronal death in neurodegenerative diseases. On the other hand, improvement of the ability of astrocytes to synthesise, export and/or process extracellular GSH is likely to enhance the supply of GSH precursors to neurons and could be beneficial to improve the antioxidative potential of neurons in brain.

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2

Results

2.1 Astroglia-rich primary cultures as model system to study metabolism and glutathione-mediated detoxification of xenobiotics in brain astrocytes.

2.2 **Publication 2:**
Schmidt, M. M. & Dringen, R. (2009): Differential effects of iodoacetamide and iodoacetate on glycolysis and glutathione metabolism of cultured astrocytes. *Front Neuroenergetics,* **1,** 1-10.

2.3 **Publication 3:**
Schmidt, M. M. & Dringen, R. (2010): Fumaric acid diesters deprive cultured primary astrocytes rapidly of glutathione. *Neurochem Int*. In press.

2.4 **Publication/Manuscript 4:**
Schmidt, M. M. *et al.*: Effects of chlorinated acetates on the glutathione metabolism and on glycolysis of cultured astrocytes. *Neurotox Res*. In revision.

2.5 **Publication/Manuscript 5:**
Schmidt, M. M. *et al.*: 2-Deoxyribose deprives cultured astrocytes of their glutathione. *Neurochem Res.* Submitted.

2.1

Astroglia-rich primary cultures as model system to study metabolism and glutathione-mediated detoxification of xenobiotics in brain astrocytes.

Maike. M. Schmidt

2.1.1 Abstract

Astroglia-rich primary cultures were used in this thesis to study metabolism and glutathione-mediated detoxification of xenobiotics in brain astrocytes. Immunocyto-chemical characterisation showed that more than 95% of the total number of cells in these cultures were positively stained for the astrocytic marker glial fibrillary acidic protein. At a culture age between 15 and 21 days, the cultures contained a total protein content of 101 ± 23 µg per well and a specific total glutathione content of 36.1 ± 8.4 nmol/mg protein. In addition, specific activities of several enzymes involved in glutathione or glucose metabolism of cultured astrocytes were comparable to those published previously. The specific glutathione content did not significantly differ for cultures of an age between 15 and 21 days. Therefore, astroglia-rich primary cultures in the third week of culture age were considered as stable *in vitro* model system to investigate glutathione metabolism and functions of brain astrocytes. Using these cultures, the differential effects of xenobiotics such as halogenated acetates, iodoacetamide or fumaric acid esters and of cell derived compounds such as 2-deoxyribose on the cellular glutathione content were investigated by a highly sensitive quantitative determination method for glutathione as well as by a qualitative fluorescence method to visualise cellular glutathione.

2.1.2 Introduction

The mammalian brain is a highly complex organ that contains an extensive neuronal and glial network (Bushong *et al.* 2002, Giaume *et al.* 2010, Halassa *et al.* 2007) and is characterised by various functional interactions between different types of brain cells (Dringen 2009, Hertz *et al.* 2007, Magistretti 2006). Within the human brain, glial cells are the most abundant cells that outnumber neurons by over five fold (Sofroniew & Vinters 2010). A vast variety of functions of astrocytes have been described that were explored by *in vitro* and *in vivo* experimental paradigms (Seth & Koul 2008, Sofroniew & Vinters 2010). Especially astrocytes have been difficult to study *in vivo* since the glial syncytium formed via gap junctions between astrocytes (Giaume *et al.* 2010, Giaume & McCarthy 1996) and other brain cell types (Nagy *et al.* 2004, Nagy & Rash 2000) complicates controlled experimental manipulations. Astrocyte-rich cell cultures have been extensively used as model system for brain astrocytes to investigate astrocytic functions in amino acid, energy and glutathione (GSH) metabolism, metal homeostasis, oxidative defence and signalling (Table 1).

This chapter describes basic metabolic parameters for astroglia-rich primary cultures such as specific total glutathione (GSx) contents and specific enzyme activities. Knowledge of these parameters is important for defining astroglia-rich cultures as suitable model system to investigate the disturbances of cellular GSH and glucose metabolism by xenobiotics, which are described in much more detail in the next four chapters of this thesis. In addition, this chapter describes a method to visualise cellular GSH by fluorescence microscopy and compares the sensitivity of the qualitative fluorescence method with a quantitative enzymatic cycling method for GSH determination.

Table 1: Selected references for metabolic pathways that have been investigated by using astrocyte cell cultures

Metabolic pathways	Selected References
Amino acids	
Arginine	Schmidlin & Wiesinger 1994, Schmidlin & Wiesinger 1998
Carnosin	Schulz *et al.* 1987
Citrulline	Schmidlin *et al.* 2000
Cysteine	Kranich *et al.* 1996
Glycine	Dringen *et al.* 1998d, Verleysdonk *et al.* 1999
Isoleucine	Murin *et al.* 2009a
Leucine	Bixel & Hamprecht 1995, Bixel *et al.* 2004, Yudkoff *et al.* 1994
Serine	Verleysdonk & Hamprecht 2000
Valine	Murin *et al.* 2009b
Carbohydrates and energy metabolism	
Creatine	Möller & Hamprecht 1989
Fructose	Bergbauer *et al.* 1996, Schmoll *et al.* 1995a
Glucose & glycogen	Dello Russo *et al.* 2003, Dringen & Hamprecht 1992, Dringen & Hamprecht 1993a, Dringen & Hamprecht 1993b, Dringen *et al.* 1993, Lavado *et al.* 1997, Pfeiffer-Guglielmi *et al.* 2000, Psarra *et al.* 1998, Rahman *et al.* 2000, Schmoll *et al.* 1995b, Wiesinger *et al.* 1997
Isocitrate	Minich *et al.* 2003
Malic enzyme	Vogel *et al.* 1998
Mannose	Dringen *et al.* 1994
Sorbitol	Wiesinger *et al.* 1990
Glutathione metabolism	Diaz-Hernandez *et al.* 2005, Dringen & Hamprecht 1996, Dringen & Hamprecht 1998, Dringen *et al.* 1997, Dringen *et al.* 1998a, Drukarch *et al.* 1997, Hirrlinger *et al.* 2001, Hirrlinger *et al.* 2002b, Hirrlinger *et al.* 2002c, Kranich *et al.* 1996, Langeveld *et al.* 1996, Minich *et al.* 2006, Rana & Dringen 2007, Ruedig & Dringen 2004, Stewart *et al.* 2002, Vasquez *et al.* 2001, Waak & Dringen 2006
Metabolic coupling	
Gap junctions	Bolaños & Medina 1996, Lavado *et al.* 1997, Vera *et al.* 1996
Glutamine	Broer *et al.* 1999, Deitmer *et al.* 2003
Ketone bodies	Auestad *et al.* 1991, Bixel & Hamprecht 1995, Blazquez *et al.* 1998, Blazquez *et al.* 1999, Daikhin & Yudkoff 1998, Leite *et al.* 2004, Suzuki *et al.* 2009, Yudkoff *et al.* 1997
others	Broer *et al.* 1997, Dringen *et al.* 1995, Frade *et al.* 2008, Knorpp *et al.* 2006

Table 1 (continued): Selected references for metabolic pathways that have been investigated by using astrocyte cell cultures

Metabolic pathways	Selected References
Metal homeostasis	
Copper	Brown 2004, Hidalgo *et al.* 1994, Pope *et al.* 2008, Scheiber *et al.* 2010
Iron	Bishop *et al.* 2010a, Geppert *et al.* 2009, Hoepken *et al.* 2004, Keenan *et al.* 2010, Qian *et al.* 1999, Qian *et al.* 2000, Riemer *et al.* 2004, Swaiman & Machen 1985, Swaiman & Machen 1986, Swaiman & Machen 1991, Takeda *et al.* 1998, Tulpule *et al.* 2010
Manganese	Aschner *et al.* 1992, Erikson & Aschner 2002, Erikson & Aschner 2006, Hazell *et al.* 1999a, Hazell *et al.* 1999b, Milatovic *et al.* 2007, Sidoryk-Wegrzynowicz *et al.* 2009, Tjalkens *et al.* 2006
Zinc	Aschner *et al.* 1998a, Bishop *et al.* 2007, Bishop *et al.* 2010b, Hidalgo *et al.* 1994, Huszti *et al.* 2001, Suh *et al.* 2007
Nitric oxide	Almeida *et al.* 2001, Almeida *et al.* 2002, Almeida *et al.* 2004, Barker *et al.* 1998, Bolaños & Medina 1996, Bolaños *et al.* 1995, Bolaños *et al.* 1994, Brown *et al.* 1995, Cidad *et al.* 2001, Garcia-Nogales *et al.* 1999, Garcia-Nogales *et al.* 2003, Gegg *et al.* 2003, Gegg *et al.* 2005, Jacobson *et al.* 2005, Stewart *et al.* 1997, Vega-Agapito *et al.* 1999
Peroxides	Dringen & Hamprecht 1997, Dringen *et al.* 1998b, Dringen *et al.* 1998c, Dringen *et al.* 1999, Knorpp *et al.* 2006, Kussmaul *et al.* 1999, Liddell *et al.* 2004, Liddell *et al.* 2006a, Liddell *et al.* 2006b, Liddell *et al.* 2009, Sokolova *et al.* 2001
Osmolytes	
Myoinositol	Aschner *et al.* 1998b, Hijab *et al.* 2010, Isaacks *et al.* 1999, Wiesinger 1991
Taurine	Aschner *et al.* 1998b, Brand *et al.* 1998, Cruz-Rangel *et al.* 2008, Junyent *et al.* 2009
Signalling	Baltrons *et al.* 2002, Baltrons *et al.* 2008, Boran & Garcia 2007, Friedl *et al.* 1985, Friedl *et al.* 1989, Hasselblatt *et al.* 2003, Löffler *et al.* 1985, Navarra *et al.* 2004, Pedraza *et al.* 2003, Sardon *et al.* 2004, Schulz *et al.* 1989

2.1.3 Materials and Methods

2.1.3.1 Materials

Glutathione disulfide (GSSG), glutathione reductase (GR), glucose-6-phosphate, glyceraldehyde-3-phosphate, maleimide and potassium arsenate were purchased from Roche Diagnostics (Mannheim, Germany). 1-chloro-2,4-dinitrobenzene (CDNB), 2-deoxy-D-ribose (2dRib), 2-vinylpyridine (2VP), 4´-6-diamidino-2-phenylindole dihydrochloride (DAPI), 5,5´-dithiobis-(2-nitrobenzoic acid) (DTNB), buthionine sulfoximine (BSO), dichloroacetate (DCA), diethyl fumarate (DEF), dimethyl fumarate (DMF), iodoacetamide (IAA), iodoacetate (IA), monochloroacetate (MCA), monochlorobimane (MCB), monomethyl fumarate (MMF) and trichloroacetate (TCA) were obtained from Sigma-Aldrich (Steinheim, Germany). Bovine serum albumin, NAD^+, NADH, $NADP^+$, NADPH and sulfosalicylic acid were purchased from Applichem (Darmstadt, Germany). Fetal calf serum, streptomycin sulfate and penicillin G were from Biochrom (Berlin, Germany). Dulbecco´s modified Eagle´s medium (DMEM) was purchased from Invitrogen (Karlsruhe, Germany). Goat serum and the primary polyclonal antibody against glial fibrillary acidic protein (GFAP) were obtained from DakoCytomation (Glostrup, Denmark). The secondary anti-rabbit cyanine-labelled antibody was obtained from Dianova (Hamburg, Germany). All other chemicals were obtained from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany) at analytical grade. Sterile cell culture material and unsterile 96-well plates were from Nunc (Roskilde, Denmark) and Sarstedt (Karlsruhe, Germany).

2.1.3.2 Cell cultures

Astroglia-rich primary cultures derived from the whole brains of neonatal Wistar rats were prepared according to a previously described method (Hamprecht & Löffler 1985). Three hundred thousand viable cells were seeded in 1 mL culture medium (90% DMEM, 10% fetal calf serum, 20 units/mL of penicillin G, 20 µg/mL of streptomycin sulfate) per well of a 24-well cell culture dish without or with glass coverslips (10 mm diameter; Roth, Karlsruhe, Germany) and cultured in a cell

incubator (Sanyo, Osaka, Japan) that contained a humidified atmosphere of 10% CO_2/90% air. The culture medium was renewed every seventh day.

2.1.3.3 Immunocytochemical characterisation of the cultures

The immunocytochemical staining procedure was performed at room temperature (RT). Before and after addition of the antibodies, the cells were washed thrice with 1 mL phosphate-buffered saline (PBS; 10 mM potassium phosphate buffer, 150 mM NaCl, pH 7.4) for 5 min periods. Primary and secondary antibodies were diluted by a factor of 200 in PBS containing 10% (v/v) goat serum. Cells were washed with 1 mL ice-cold PBS and fixed with 0.4 mL 3.5% (w/v) paraformaldehyde in PBS for 10 min. After washing the cells thrice with 1 mL PBS for 5 min each washing period, the cells were incubated with 0.4 mL 0.1% (w/v) glycine in PBS for 5 min to derivatise free aldehyde groups in order to prevent crosslinking of proteins. Cells were subsequently permeabilised with 0.4 mL 0.3% (w/v) Triton-X100/0.1% (w/v) glycine in PBS for 10 min. The fixed and permeabilised cells were incubated for 2 h with 30 µL of the polyclonal antibody against GFAP followed by 30 min incubation with 30 µL of the cyanine-labeled secondary antibody in the dark. To counterstain the nuclei, the cells were incubated for additional 5 min with 30 µL DAPI (1 µg/mL in pure water) in the dark. The cover slips were mounted with DPX mounting media (Fluka), before analysing the fluorescence by using a fluorescence microscope (TS-2000u, NIKON, Düsseldorf, Germany). Further processing of the image data was performed using the software NIS Elements Basic Research (NIKON).

2.1.3.4 Experimental incubation of the cells

To study the consequences of treatment of astrocytes with various compounds, the cells were washed with 1 mL of prewarmed (37°C) incubation buffer (IB: 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.4 mM KCl, 145 mM NaCl, 0.8 mM Na_2HPO_4, 20 mM HEPES, 5 mM D-glucose, pH 7.4) and incubated at 37°C with 0.5 mL IB containing chlorinated acetates, iodoacetate, iodoacetamide, 2-deoxyribose or fumaric acid esters in the concentrations given in the legends of the figures or in the tables. For the determination of total glutathione (GSx) contents (GSx = amount of GSH plus

twice the amount of GSSG), protein contents or enzyme activities, the cells were washed with 1 mL ice-cold PBS after the incubation periods indicated in the legends of the figures or tables, followed by the treatment as described below for the individual methods. For visualisation of GSH using MCB, the medium was removed and replaced by 0.5 mL IB containing 100 µM MCB and the staining procedure and analysis of the data was followed as described below (2.1.3.6).

2.1.3.5 Determination of glutathione

The contents of GSx and GSSG (Fig. 1) in cell lysates were determined as described previously (Dringen & Hamprecht 1996, Dringen *et al.* 1997) in microtiter plates according to the colorimetric method originally described by Tietze (Tietze 1969).

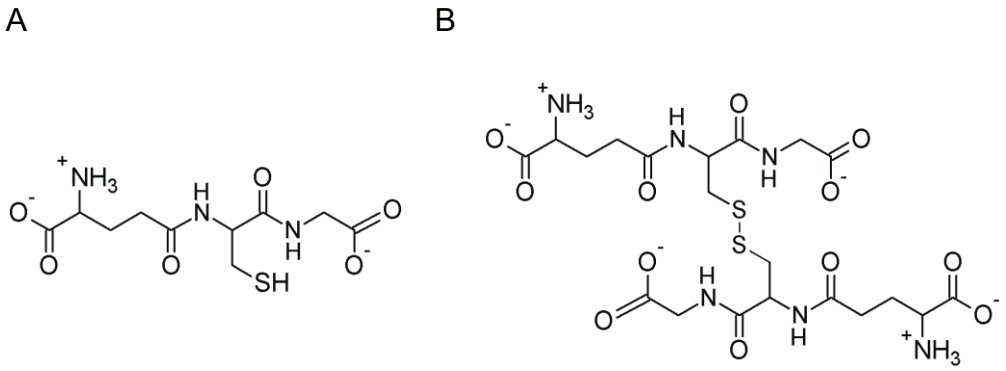


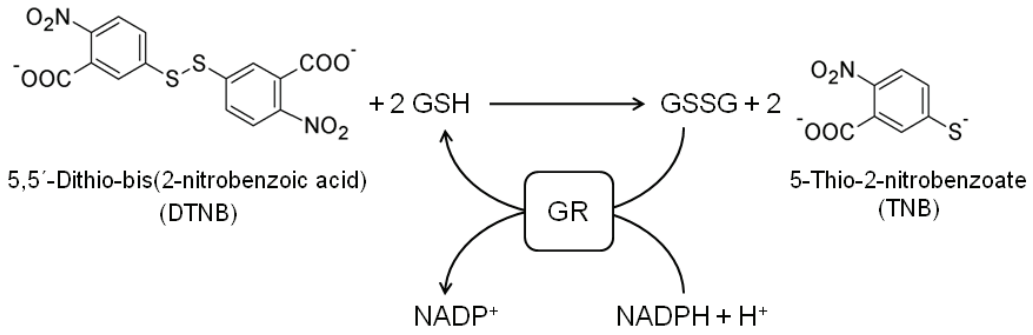
Figure 1: Structural formulas of GSH (A) and GSSG (B).

The underlying mechanism of this assay is the reduction of DTNB by GSH to 5-thio-2-nitrobenzoate (TNB) that can be detected at 405 nm (Fig. 2A). Glutathione reductase (GR) and its cosubstrate NADPH are added to generate a highly sensitive enzymatic cycling system (Fig. 2A), which ensures by GR specificity that the assay detects only GSH and GSSG. The conditions of the assay are chosen in a way that exclusively the amount of GSx limits the rate of TNB formation. Therefore, the increase in absorbance per unit of time correlates with the GSx content in the sample, which is quantified by comparison to the increase in absorbance determined for GSx standards (Fig. 3).

To discriminate between GSH and GSSG, 2VP is added to the sample to derivatise GSH by the formation of a GS-2VP conjugate that is not accepted as substrate by GR (Griffith 1980). As GSSG does not react with 2VP (Fig. 2B), the remaining GSx content detected for a sample after 2VP treatment represents exclusively GSSG.

For quantification of the GSx and GSSG contents, cells were washed with 1 mL ice-cold PBS and lysed with 0.5 mL 1% (w/v) sulfosalicylic acid in water. The cell lysates were centrifuged for 1 min at 12,000 g at RT and 10 µL of the supernatant was diluted with 90 µL of pure water in a well of a microtiter plate. The cycling reaction was started by adding 100 µL reaction mixture (0.3 mM DTNB, 0.4 mM NADPH, 1 mM EDTA in 0.1 M sodium phosphate buffer, pH 7.5) and the increase in absorbance due to the formation of TNB was followed at 405 nm in a Sunrise microtiter plate reader (TECAN, Groning, Austria).

A: GSx assay principle



B: Derivatisation of GSH by 2VP

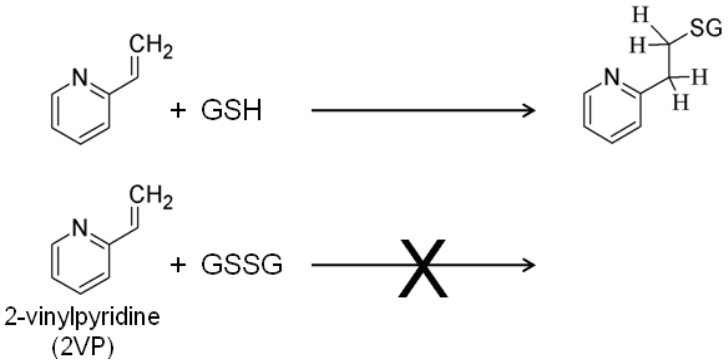


Figure 2: Schematic representation of the principle of the GSx assay (A). The derivatisation of GSH by 2VP is used to discriminate between GSH and GSSG (B). For details see text.

For determination of GSSG, 5 µL of 2VP was added to 130 µL of the supernatant of the cell lysates. Subsequently the pH was adjusted to pH 6 by addition of 0.2 M Tris solution. After incubation for 60 min at RT, 10 µL of the derivatised sample was used to determine the GSSG content, using the GSx assay as described above. To quantify the amounts of GSx or GSSG in the samples, the slope of the increase of absorbance was compared to those of GSx standards (GSSG in concentrations of 0 to 5 µM; treated identically as cell lysates; Fig. 3). The detection limit of this assay was about 0.2 nmol GSx per 500 µL lysate or medium.

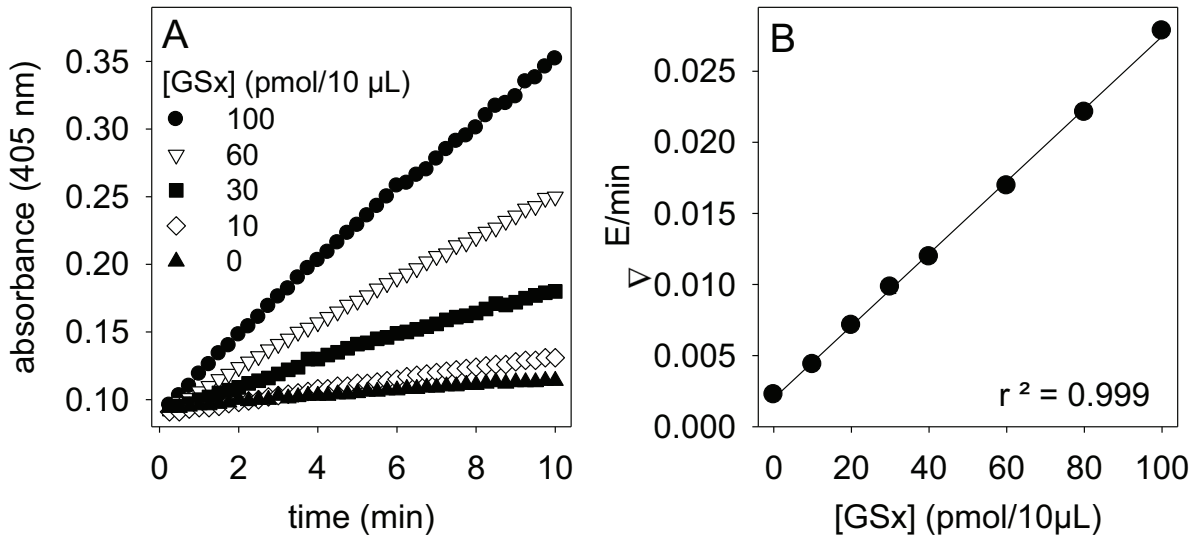


Figure 3: Detection of GSx by the enzymatic cycling assay. A: Increase of absorbance over time after application of 10 µL of the indicated concentrations of GSx standards. B: Slope of the increase in absorbance presented as function of the GSx standards applied.

2.1.3.6 Visualisation of cellular GSH using monochlorobimane

MCB is frequently used to determine the cellular GSH content of cells, including brain cells (Bragin *et al.* 2010, Keelan *et al.* 2001, Sun *et al.* 2006). The underlying mechanism is the glutathione-S-transferase (GST) catalysed reaction of the non-fluorescent MCB with GSH to form a fluorescent GS-bimane adduct (Fig. 4; Eklund *et al.* 2002).

To visualise the cellular GSH content in astrocytes by the MCB method, the cells were washed with 1 mL prewarmed (37°C) IB and subsequently incubated with

0.5 mL 100 µM MCB in IB for 2 min. The medium was removed and the cells were washed twice with 1 mL ice-cold PBS and the fluorescence signal of the GSH conjugate with MCB (GS-bimane) was immediately analysed using a fluorescence microscope (TS-2000u, NIKON). If not stated otherwise, the images presented in this chapter were taken with identical exposure time and were equally digital processed using the software NIS Elements Basic Research (NIKON). Consequently, the intensity of the GS-bimane signals for the different conditions used are directly comparable.

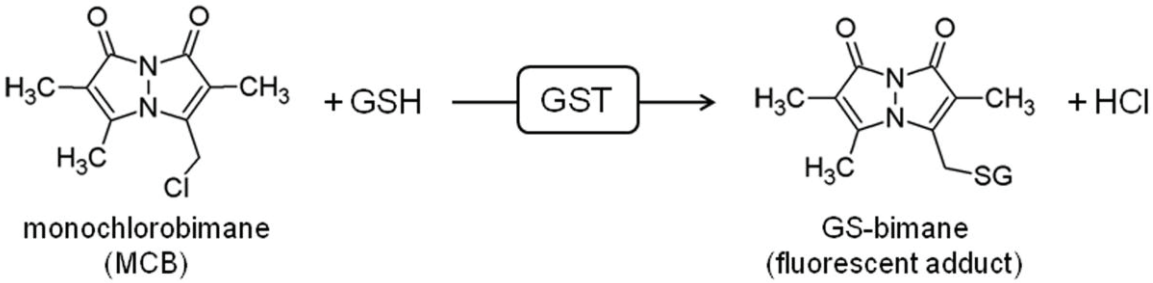


Figure 4: GST-catalysed reaction of monochlorobimane with GSH to form the fluorescent GS-bimane adduct.

2.1.3.7 Determination of protein content

The protein content of astroglia-rich primary cultures in wells of 24-well cell culture dishes was quantified according to the method originally described by Lowry (Lowry *et al.* 1951) using bovine serum albumin as a standard. For determination of the total protein content, the cells were solubilised in 200 µL of 0.5 M NaOH for 2 h. Lysis of the cells in 200 µL 20 mM potassium phosphate buffer (KPi) pH 7.0 for 10 min on ice and subsequent centrifugation (12,000 g; 1 min; RT) was performed to determine the soluble protein content in the supernatant. Total protein content per well and soluble protein content in the supernatants of cell lysates were used to calculate specific GSx or GSSG contents and specific enzyme activities, respectively.

2.1.3.8 Determination of enzyme activities

The cells were washed with 1 mL ice-cold PBS and subsequently lysed in 200 µL 20 mM KPi, pH 7.0 for 10 min on ice. The cell lysates were scrapped from the wells, transferred into Eppendorf cups and centrifuged (12,000 g; 1 min; RT). If not stated otherwise, 20 µL of the supernatant was used for determination of enzyme activities at RT in wells of microtiter plates.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was determined using a method described by Bisswanger (Bisswanger 2004), modified for the use of microtiter plates. The assay takes advantage of a side reaction of GAPDH. The physiological substrate phosphate is replaced by arsenate leading to the formation of 1-arseno-3-phosphoglycerate instead of 1,3-bisphosphoglycerate. The former is not stable and immediately hydrolyses to 3-phosphoglycerate. This hydrolysis shifts the equilibration of the GAPDH-catalysed reaction to the product side. The reaction mixture contained in a total volume of 360 µL 0.9 mM glyceraldehyde-3-phosphate, 3 mM potassium dihydrogen arsenate, 2 mM NAD^+ and 93 mM triethylamine hydrochloride/NaOH buffer, pH 7.6. The increase of absorbance due to the reduction of NAD^+ to NADH was followed at 340 nm over 5 min.

Glucose-6-phosphate dehydrogenase (G6PDH) activity was determined according to Deutsch (Deutsch 1984). This enzyme catalyses the $NADP^+$ dependent oxidation of glucose-6-phosphate to 6-phosphogluconolactone. The product 6-phosphoglucono-lactone is hydrolysed spontaneously. Maleimide is present in the assay to prevent additional NADPH production by 6-phosphogluconate dehydrogenase activity that is also present in the cell lysates. The reaction mixture contained in a total volume of 200 µL 6.3 mM $MgCl_2$, 5 mM maleimide, 3.3 mM glucose-6-phosphate, 0.4 mM $NADP^+$ and 75 mM Tris/HCl buffer, pH 7.5. The increase of absorbance at 340 nm due to the reduction of $NADP^+$ to NADPH was followed over 5 min.

Glutathione reductase (GR) activity was determined using a method described previously (Gutterer *et al.* 1999) following the formation of $NADP^+$ due to the reduction of GSSG to 2 GSH. The reaction mixture contained in a total volume of 300 µL 1 mM GSSG, 0.2 mM NADPH and 1 mM EDTA in 100 mM KPi, pH 7.0. The

decrease of absorbance at 340 nm due to the oxidation of NADPH was followed over 5 min.

Glutathione-S-transferase (GST) activity was determined according to a method originally described by Habig and colleagues (Habig *et al.* 1974). The assay is based on the GST-catalysed nucleophilic aromatic substitution of GSH to 1-chloro-2,4-dinitrobenzene (CDNB). Briefly, after lysis of the cells in 200 µL 20 mM KPi, pH 7.0 for 10 min on ice, 100 µL 260 mM KPi, pH 6.5 was added to adjust the pH of this solution. 50 µL of this mixture was used per well of a microtiter plate to determine GST activity. The reaction mixture contained in a total volume of 200 µL 1 mM CDNB and 10 mM GSH in 100 mM KPi, pH 6.5. The increase of absorbance at 340 nm due to the GST-catalysed formation of 2,4-dinitrophenyl-glutathione was followed over 5 min.

Lactate dehydrogenase (LDH) activity was determined using the method described previously (Dringen *et al.* 1998b) by monitoring the reduction of pyruvate to lactate with NADH as cosubstrate. The reaction mixture contained in a total volume of 360 µL 1.8 mM pyruvate, 0.2 mM NADH, 200 mM NaCl and 80 mM Tris/HCl buffer, pH 7.2. The decrease of absorbance at 340 nm due to the oxidation of NADH to NAD^+ was followed over 5 min.

2.1.3.9 Presentation of the data

If not stated otherwise, the data are presented as means ± standard deviation (SD) of values obtained in experiments that had been performed on at least three independently prepared cultures. Statistical analysis of the significance of differences between multiple sets of data was performed by ANOVA followed by Bonferroni post hoc test. $p>0.05$ was considered as not significant.

2.1.4 Results

2.1.4.1 Immunocytochemical characterisation

For immunocytochemical characterisation of astroglia-rich primary cultures, the cells were fixed, permeabilised and stained for the astrocytic marker GFAP and for the nuclei using DAPI. Figure 5A and B show phase contrast pictures of an untreated 18 day old confluent astroglia-rich primary culture in 200-fold (Fig. 5A) and 400-fold (Fig. 5B) magnification. Figure 5C shows the phase contrast of cells after fixation with paraformaldehyde. Immunocytochemical staining demonstrated that GFAP positive cells dominated quantitatively the number of cells in the culture (Fig. 5D). Correlating the number of cells by comparing the DAPI signal to that of GFAP revealed that the number of astrocytes exceeded 95% of all cells in the culture (Fig. 5D), confirming that the cultures used are indeed astroglia-rich. In the cultures, at least two types of astrocytic morphology could be distinguished (Fig. 5E,F). Astrocytes with a distinctive GFAP network consisting of rather fine processes that appeared in a globoid distribution dominated quantitatively in the culture (Fig. 5E), whereas some cells showed a more star-like appearance with a low number of prominent fiber-like main processes (Fig. 5F).

2.1.4.2 Protein and glutathione contents and enzyme activities

To investigate whether cellular protein and GSx contents vary with the age of the cultures, these parameters were quantified for astroglial cultures that were in the third week in culture (Fig. 6). At an age between 15 and 21 days after seeding, the cultures contained a protein content of 101 ± 23 µg per well and a specific GSx content of 36.1 ± 8.4 nmol/mg protein (n = 94). Compared to 15 day old cultures the protein content of 17, 19 and 20 day old cultures was slightly but significantly elevated (Fig. 6A). In addition, the GSx content per well of 15 day old cultures was significantly lower than that obtained for 18, 20 and 21 day old cultures ($p>0.05$; Fig. 6B). However, the specific GSx content of the cultures at the age between 15 and 21 days was almost identical (Fig. 6C). Thus, since the specific GSx content was not altered in astroglia-rich primary cultures between 15 and 21 days of culture age,

this period of culture age was identified as reliably stable to study the effects of xenobiotics on the GSx contents of cultured astrocytes.

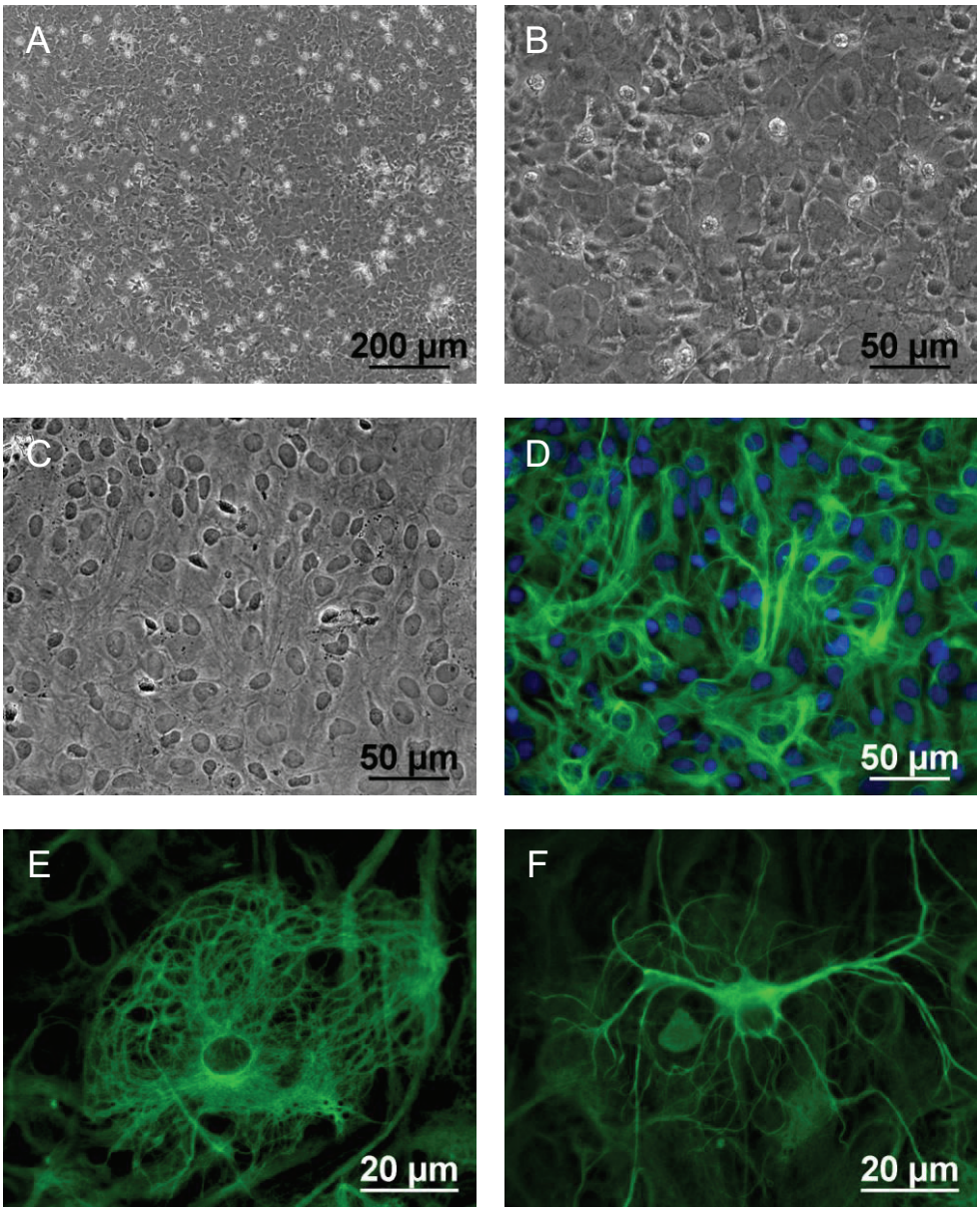


Figure 5: Immunocytochemical characterisation of astroglia-rich primary cultures. The immunocytochemical staining was performed on a confluent 18 day old culture. Phase contrast images represent cultures before (A,B) and after fixation (C). For identification of astrocytes, the cells were stained for GFAP (D-F, green signal) and counterstained with DAPI to visualise the nuclei (D, blue signal). Different morphologies of GFAP positive cells are shown in E and F.

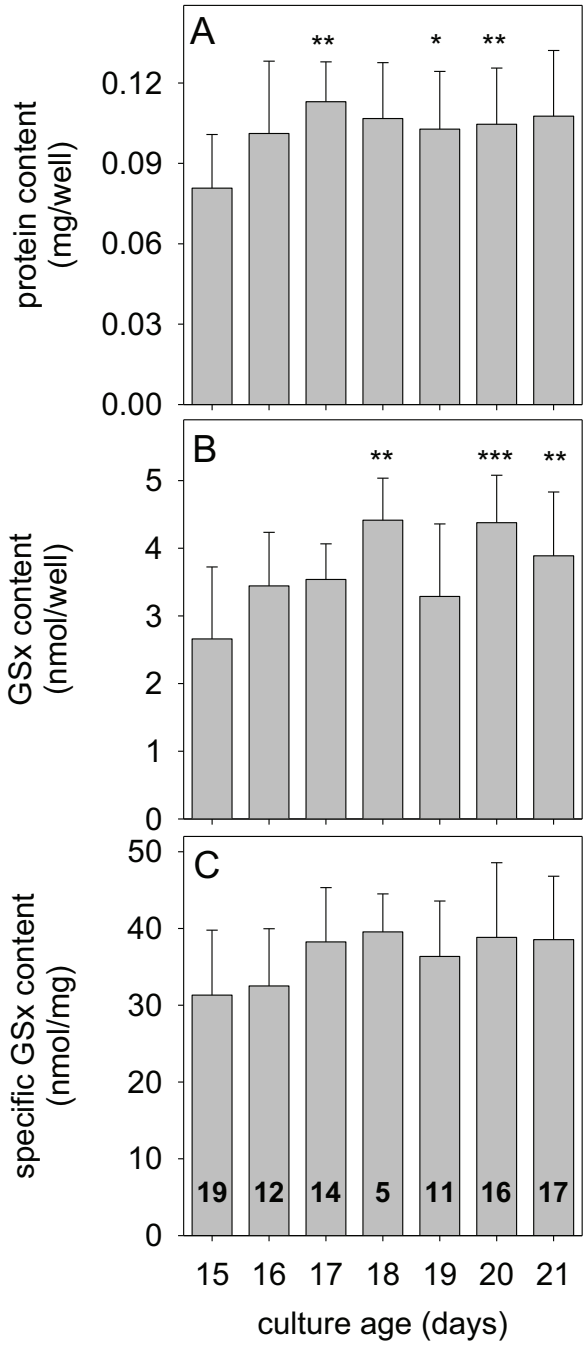


Figure 6: Protein and GSx contents of astroglia-rich primary cultures. Protein and GSx contents were determined for 15 to 21 day old cultures. The results represent means ± SD of data that were obtained on n (bold numbers in columns in C) independently prepared cell cultures. The means ± SD of total protein per well and specific GSx content for all cultures investigated (n = 94) was 101 ± 23 µg and 36.1 ± 8.4 nmol/mg protein, respectively. Significance of differences compared to the values obtained for 15 day old cultures are indicated as *p<0.05, **p<0.01 and ***p<0.001.

The cellular ratio of GSH to GSSG can be used as indicator of disturbances of the cellular thiol reduction potential (Hirrlinger & Dringen 2010). Therefore, the cellular GSSG content of untreated astroglial cultures was quantified and compared with that of GSx. In untreated cells, the specific GSSG content was 2.8 ± 1.6 nmol/mg protein, which represented around 7% of the GSx content of these cells (Table 2).

Table 2: Cellular glutathione and protein contents of untreated astroglia-rich primary cultures

Compound	Unit	Value
GSx content	nmol/well	3.8 ± 0.7
GSSG content	nmol GSx/well	0.2 ± 0.1
	% of GSx	7 ± 3
Protein content	µg/well	90 ± 15
Specific GSx content	nmol/mg	42.6 ± 8.9
Specific GSSG content	nmol GSx/mg	2.8 ± 1.6

The data represent means ± SD of values obtained on 12 independently prepared cultures.

Table 3: Specific enzyme activities of astroglia-rich primary cultures.

Enzymes	Abbrev.	Specific activity nmol/(min x mg protein)	n
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	475 ± 152	12
Glucose-6-phosphate dehydrogenase	G6PDH	70.4 ± 27.3	12
Glutathione reductase	GR	30.8 ± 16.0	12
Glutathione-S-transferase	GST	298 ± 32	3
Lactate dehydrogenase	LDH	1720 ± 348	12

Enzyme activities were determined for untreated astroglia-rich primary cultures of an age between 15 and 21 days. The results represent means ± SD of data that were obtained on n independently prepared cultures that contained 28 ± 4 µg (n = 3) or 48 ± 13 µg (n = 12) soluble protein and 97 ± 5 µg (n = 3) or 90 ± 15 µg (n = 12) total protein per well.

Enzyme activities of key enzymes for various metabolic pathways have been determined for astroglial cultures that were exposed to various xenobiotics (chapters 2.2 to 2.5). Table 3 lists the specific enzyme activities of untreated astroglial cultures. These cultures contained in nmol/(min x mg) a specific GR activity of 30.8 ± 16.0, a specific GST activity of 298 ± 32 and a specific G6PDH activity of 70.4 ± 27.3 (Table 3). In addition, the specific activities of GAPDH and LDH in untreated cultures were 475 ± 152 nmol/(min x mg) and 1720 ± 348 nmol/(min x mg), respectively (Table 3).

2.1.4.3 Comparison of the effects of xenobiotics on the cellular GSH content

The chapters 2.2 to 2.5 of this thesis deal in detail with the effects of various compounds on the cellular GSH content of astroglia-rich primary cultures. For direct comparison of the effects of halogenated acetates, IAA, fumaric acid esters (FAE) or 2dRib on the cellular GSH content, the cells were exposed to these compounds for the incubation times and the concentrations indicated in Table 4. The remaining cellular GSx content was quantified using an enzymatic cycling assay. In addition, MCB staining was performed to compare qualitatively GS-bimane staining with the data from the quantitative GSx assay (Table 4; Figs. 7 to 12). None of the conditions used led to compromised cell viability as indicated by the absence of any increase in extracellular LDH activity (data not shown).

The initial GSx content of untreated astroglial cultures accounted for 32.7 ± 3.9 nmol/mg protein. Incubation of the cells without xenobiotics for 30 min or 1 hour did not lower the cellular GSx content, whereas after 4 hours incubation the GSx content was significantly ($p<0.001$) lowered to 66% of the initial value (Table 4). The application of fumaric acid esters to astroglial cells revealed differential effects on the cellular GSx contents of the cells. While exposure of the cells to 100 µM MMF did not significantly alter the cellular GSx content within 30 min, the fumaric acid diesters DMF (100 µM) and DEF (100 µM) significantly ($p<0.001$) lowered the cellular GSx content to 17.2 ± 4.0% and 28.2 ± 6.9% of the initial content, respectively, in this time frame (Table 4).

Table 4: Cellular GSx content and cellular GS-bimane fluorescence of astroglia-rich primary cultures after exposure to xenobiotics or 2-deoxyribose

Compound	Abbrev.	Conc. (mM)	Time of incubation (h)	Cellular GSx content (nmol/mg)	Cellular GSx content (% of initial)	GS-bimane signal intensity
None (initial value)		–	0	32.7 ± 3.9	100.0 ± 6.0	++++
None		0	0.5	33.6 ± 0.9	103.9 ± 11.5	+++
Monomethyl fumarate	MMF	0.1	0.5	30.0 ± 3.0	92.2 ± 8.0	+++
Dimethyl fumarate	DMF	0.1	0.5	5.5 ± 0.6***,###	17.2 ± 4.0***,###	no signal
Diethyl fumarate	DEF	0.1	0.5	9.0 ± 1.0***,###	28.2 ± 6.9***,###	no signal
None		0	1	32.8 ± 1.7	101.1 ± 7.7	+++
Iodoacetate	IA	1	1	4.6 ± 0.5***,###	14.3 ± 3.3***,###	no signal
Iodoacetamide	IAA	1	1	2.5 ± 0.1***,###	7.9 ± 1.4***,###	no signal
None		0	4	21.4 ± 0.9***	66.4 ± 11.0***	++
Monochloroacetate	MCA	1	4	10.1 ± 0.1***,###	31.1 ± 3.5***,###	+
Dichloroacetate	DCA	1	4	23.0 ± 0.5**	71.0 ± 7.8**	++
Trichloroacetate	TCA	1	4	19.3 ± 2.6***	59.2 ± 6.0***	++
2-Deoxyribose	2dRib	30	4	4.7 ± 0.3***,###	14.3 ± 1.0***,###	no signal

The cells were incubated in the absence (none) or the presence of various compounds in the concentrations and for the incubation times listed. The results represent means ± SD of data obtained on three independently prepared cultures. The cultures contained initial protein contents of 88 ± 3 µg protein per well. Significance of differences, as calculated using ANOVA followed by Bonferroni post hoc test, compared to the initial GSx content, are indicated as **p<0.01 or ***p<0.001 and compared to the values for the corresponding control (none) are indicated as $^{###}$p<0.001. The intensity of the fluorescence of the GS-bimane signal is indicated as very strong (++++), strong (+++), weak (++) and very weak (+). Abbrev.: abbreviation; Conc.: concentration.

Halogenated acetates and IAA are known to diversely affect the metabolism of cells (Fonnum *et al.* 1997, Hayes *et al.* 1973, Liu *et al.* 1996, Stacpoole *et al.* 1998, Whitehouse *et al.* 1974). The glycolysis inhibitors IA and IAA (Sabri & Ochs 1971, Williamson 1967) deprived the cells efficiently of their cellular GSx (Table 4). The remaining GSx content after 60 min incubation represented 14.3 ± 3.3% and 7.9 ± 1.4% of the initial GSx content for IA and IAA, respectively, and differed significantly to the GSx contents obtained for control cells ($p<0.001$). In addition, amongst the chlorinated acetates applied for 4 hours in a concentration of 1 mM, only MCA but not DCA or TCA lowered the cellular GSx content significantly ($p<0.001$) compared to controls (absence of chlorinated acetates) to 10.1 ± 0.1 nmol/mg protein, which represents about 30% of the initial GSx content (Table 4). However, also for control conditions (absence of chlorinated acetates) and after exposure to DCA or TCA the cellular GSx content was significantly lowered within 4 hours of incubation to about 60% to 70% of the initial GSx content (Table 4).

To investigate the effects of reducing sugars, astroglial cultures were treated with 30 mM 2dRib for 4 hours. In the presence of 2dRib the cellular GSx content was significantly lowered ($p<0.001$) compared to the initial GSx content and to the GSx values obtained for control cells (Table 4). The remaining GSx content after 4 hours treatment with 2dRib was 4.7 ± 0.3 nmol/mg protein, representing 14.3 ± 1.0% of the initial GSx content (Table 4).

MCB staining has frequently been used to demonstrate the presence of GSH in cultured brain cells (Chatterjee *et al.* 1999, Chatterjee *et al.* 2000, Kaur *et al.* 2006, Kaur *et al.* 2008, Legare *et al.* 1993, Tauskela *et al.* 2000, Waak & Dringen 2006). To visualise the presence of cellular GSH before or after treatment with various compounds that may affect GSH metabolism of cultured astrocytes, the cells were incubated with MCB for 2 min and the signal of the formed GS-bimane adduct was subsequently analysed by fluorescence microscopy. Untreated cells showed a strong staining that was well distributed throughout the well (Fig. 7A,C). Depriving the cells by 80% of their cellular GSH by preincubation with the γ-glutamylcysteinyl ligase inhibitor (BSO; 0.1 mM) for 24 hours, lowered the cellular GSx content to 5.5 ± 0.1 nmol/mg and resulted in an almost complete loss of any detectable GS-bimane fluorescence after incubation with MCB for 2 min (Fig. 7B,D). Increasing

the exposure time showed that the remaining GS-bimane signal (Fig. 7F) was limited to small cells of the top layer of the cultures (Fig. 7E).

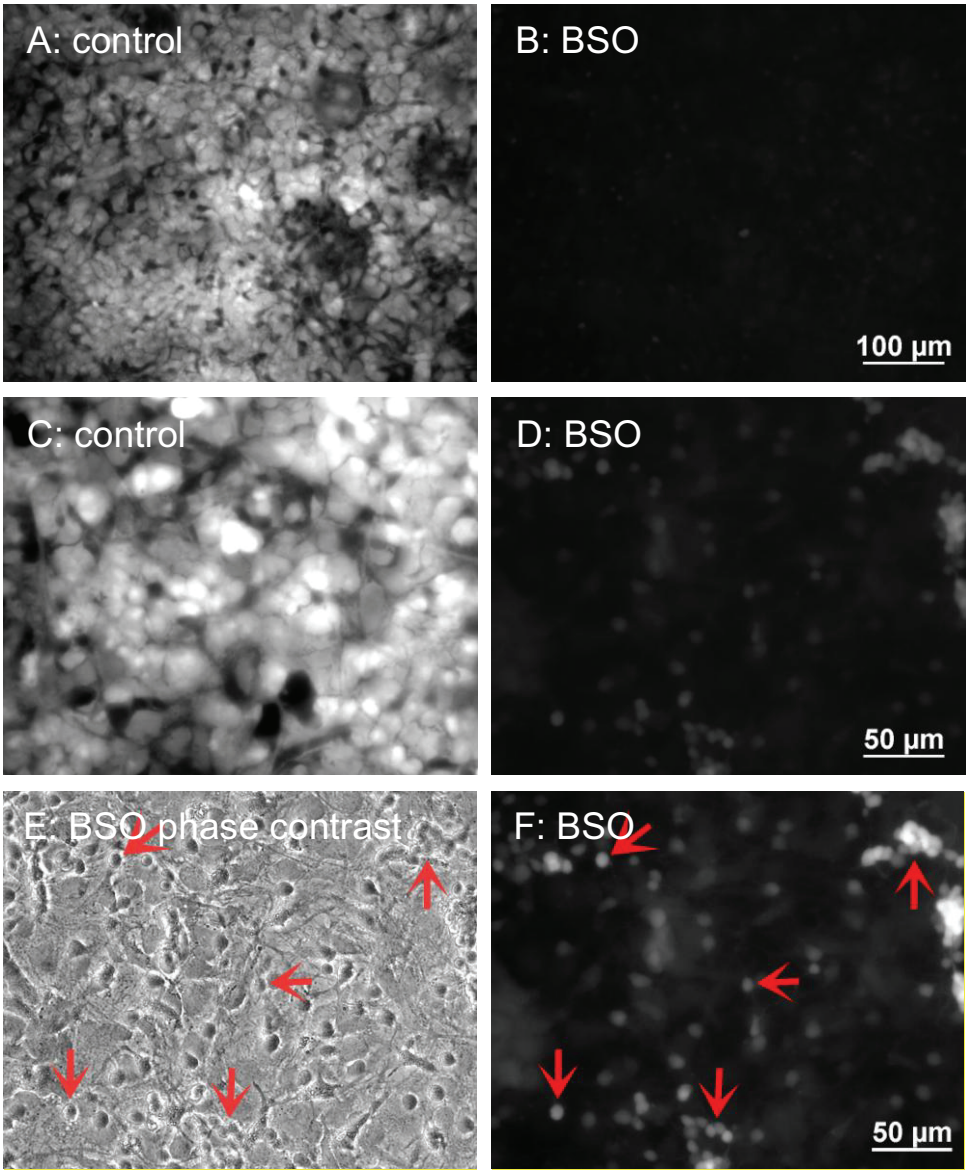


Figure 7: Visualisation of GSH in astroglia-rich primary cultures by MCB. The cells were preincubated without (A,C) or with 0.1 mM BSO (B,D,E,F) for 24 hours before MCB was applied. The pictures were taken from an experiment on a 18 day old culture that contained 89 ± 5 µg protein per well. The GSx content of the cells before MCB application was 34.9 ± 2.4 nmol/mg protein (A,C) and 5.5 ± 0.1 nmol/mg protein (B,D,E,F). For better visualisation of the remaining GS-bimane signal after BSO treatment, the exposure time in panel F was increased compared to panels A to D. The scale bar in B applies also to A, the bar in D also to C and the bar in F applies also to E.

Cultured astroglial cells release, in contrast to other types of cultured neural cells, substantial amounts of GSH (Hirrlinger *et al.* 2002c) with a rate of about 10% of their cellular GSH per hour (Dringen *et al.* 1997). Incubation of the cultures in the absence of amino acids for 4 hours led to a significant ($p<0.001$) decline in the cellular GSH content by 40% compared to the initial GSx content (Table 4). This loss in cellular GSx was confirmed by a time dependent decrease of the GS-bimane signal (Fig. 8). Interestingly, the loss of GS-bimane signal was heterogeneous, leading to the appearance of cell clusters that remained GS-bimane positive (Fig. 8).

After incubation of the astroglial cultures with xenobiotics or cell-derived compounds such as 2dRib, also the GS-bimane fluorescence intensity reflected the lowering effects of these compounds on the cellular GSH content of astrocytes as determined by the enzymatic quantification method (Table 4). The intensity of the GS-bimane signal after preincubation of the cultures with MMF (Fig. 9B) did not obviously differ to that of controls (Fig. 9A), whereas after incubation with the fumaric acid diesters DMF or DEF, no GS-bimane signal was detectable for the cultures (Fig. 9C,D). In addition, there was no detectable GS-bimane signal after incubation of cultured astrocytes with IA or IAA (Fig. 10B,C). However, incubation of the cultures with IA or IAA and subsequent derivatisation of the remaining GSH by MCB application led to changes in morphology that were characterised by a condensed appearance of the cells (data not shown).

After incubation of the cultures with chlorinated acetates, the GS-bimane signal after treatment with DCA (Fig. 11C) or TCA (Fig. 11D) was not very different to that of controls (Fig. 11A), whereas after incubation of the cells with MCA, only a weak GS bimane signal was detectable (Fig. 11B) that was substantially lower than that of control cells (Fig. 11A). After exposure of the cultures for 4 hours to 30 mM 2dRib no GS-bimane signal was detectable (Fig. 12B).

In conclusion, the lowering effect of the compounds investigated on the cellular GSH content of cultured astrocytes that was quantified by the enzymatic cycling assay could be confirmed by visualising cellular GSH by the MCB method (Table 4).

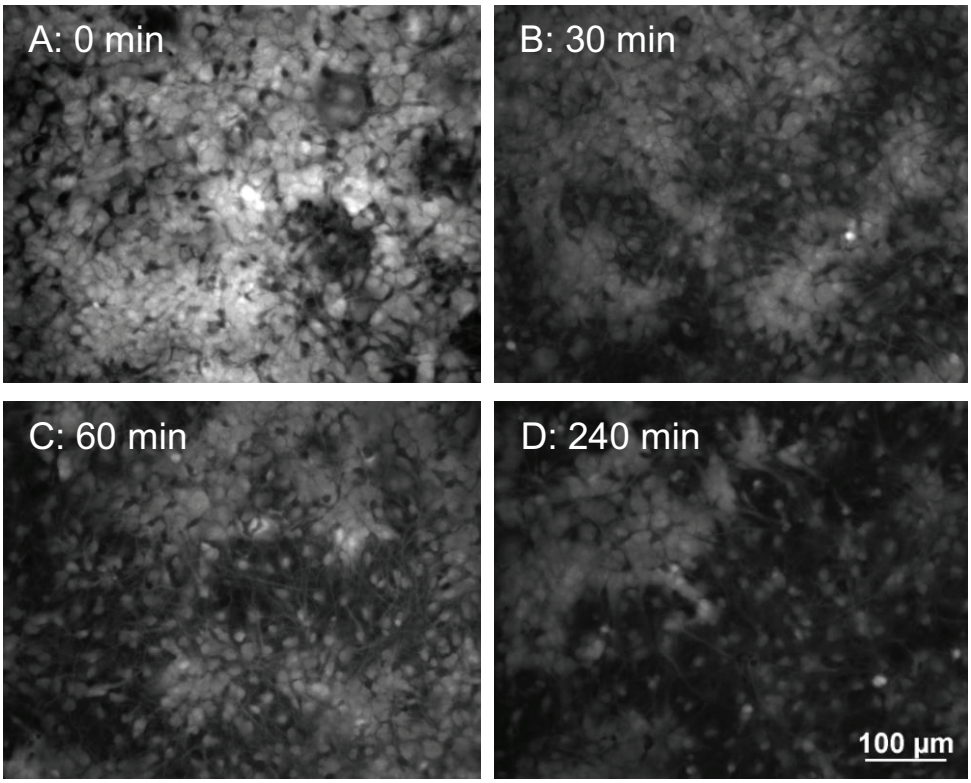


Figure 8: Effects of an incubation of cultured astrocytes in the absence of amino acids on the MCB detectable GSH content. Cells were incubated in the absence of amino acids for 0 min (A), 30 min (B), 60 min (C) or 240 min (D) before GSH was derivatised by MCB application. The pictures were taken from an experiment on a 18 day old culture that contained 89 ± 5 µg protein per well. The GSx content of the cells before MCB application was 33.2 ± 0.5 nmol/mg protein (0 min), 32.5 ± 0.6 nmol/mg protein (30 min), 33.3 ± 0.3 nmol/mg protein (60 min) and 20.5 ± 0.6 nmol/mg protein (240 min). The scale bar in D applies to all panels.

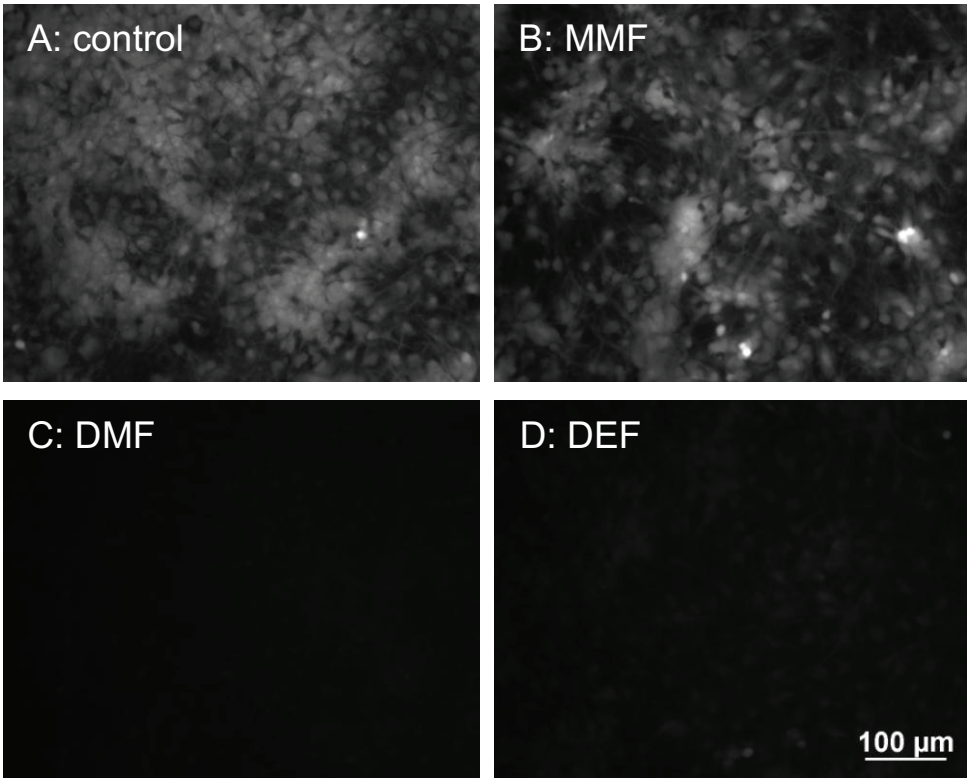


Figure 9: Effects of fumaric acid esters on the MCB detectable GSH content of cultured astrocytes. The cells were incubated in the absence (A) or presence of 100 µM MMF (B), 100 µM DMF (C) or 100 µM DEF (D) for 30 min before GSH was derivatised by MCB application. The pictures were taken from an experiment on a 18 day old culture that contained 89 ± 5 µg protein per well. The GSx content of the cells before MCB application was 33.3 ± 0.3 nmol/mg protein (A), 29.0 ± 0.3 nmol/mg protein (B), 5.4 ± 0.7 nmol/mg protein (C) and 8.9 ± 0.2 nmol/mg protein (D). The scale bar in D applies to all panels.

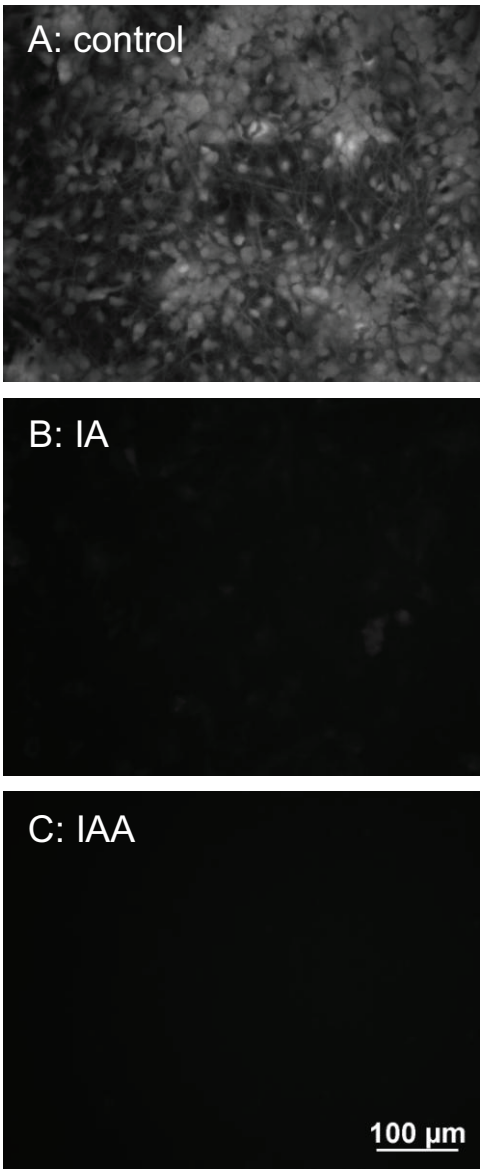


Figure 10: Effects of IA or IAA on the MCB detectable GSH content of cultured astrocytes. Cells were incubated in the absence (A) or presence of 1 mM IA (B) or IAA (C) for 60 min before GSH was derivatised by MCB application. The pictures were taken from an experiment on a 18 day old culture that contained 89 ± 5 µg protein per well. The GSx content of the cells before MCB application was 33.3 ± 0.3 nmol/mg protein (A), 4.7 ± 0.2 nmol/mg protein (B) and 2.4 ± 0.1 nmol/mg protein (C). The scale bar in C applies to all panels.

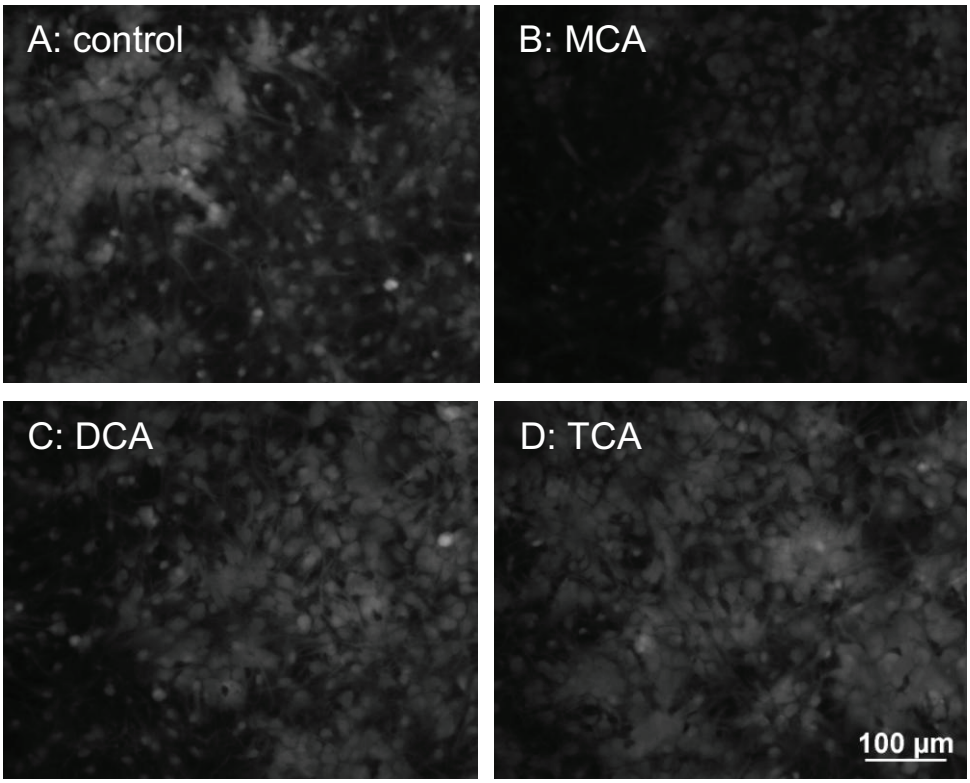


Figure 11: Effects of chlorinated acetates on the MCB detectable GSH content of cultured astrocytes. Cells were incubated in the absence (A) or presence of 1 mM MCA (B), DCA (C) or TCA (D) for 4 hours before GSH was derivatised by MCB application. The pictures were taken from an experiment on a 18 day old culture that contained 89 ± 5 µg protein per well. The GSx content of the cells before MCB application was 20.5 ± 0.5 nmol/mg protein (A), 10.1 ± 0.3 nmol/mg protein (B), 23.0 ± 0.6 nmol/mg protein (C) and 18.3 ± 0.2 nmol/mg protein (D). The scale bar in D applies to all panels.

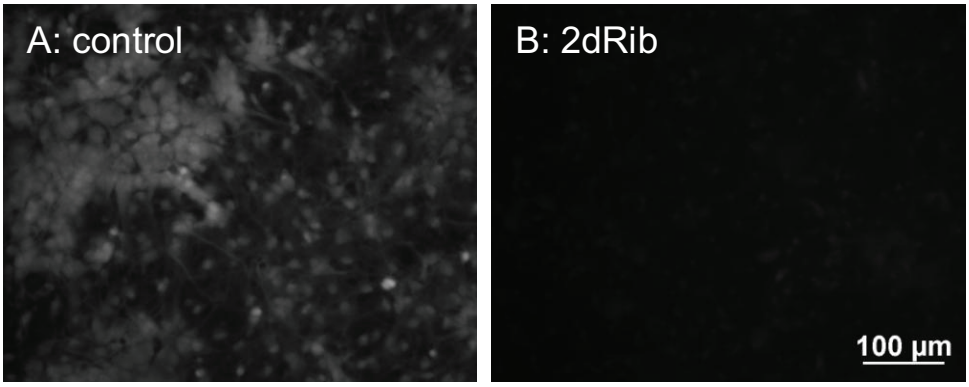


Figure 12: Effects of 2dRib on the MCB detectable GSH content of cultured astrocytes. Cells were incubated in the absence (A) or presence of 30 mM 2dRib (B) for 4 hours before GSH was derivatised by MCB application. The pictures were taken from an experiment on a 18 day old culture that contained 89 ± 5 µg protein per well. The GSx content of the cells before MCB application was 20.5 ± 0.5 nmol/mg protein (A) and 4.9 ± 0.2 nmol/mg protein (B). The scale bar in B applies also to A.

2.1.5 Discussion

Astroglia-rich primary cultures have frequently been used as model system to study astrocytic functions (Table 1) and were used in this thesis to study the disturbances of cellular GSH and glucose metabolism by xenobiotics or by cell-derived compounds. This chapter describes the characterisation of the cultures and gives basic metabolic parameters to define these cultures as suitable model system.

2.1.5.1 Immunocytochemical characterisation

GFAP, a member of the cytoskeletal protein family that is important for the modulation of motility and shape in mature astrocytes (Eng *et al.* 2000), was used as astrocytic target protein for immunocytochemical characterisation of astroglia-rich primary cultures. In the astroglial cultures used for this thesis, the GFAP staining was very prominent and appeared in most of the cells. Counterstaining with DAPI for visualisation of the nuclei revealed that more than 95% of the total number of cells in culture were GFAP-positive. Amongst the GFAP-positive cells, at least two distinct morphologies could be discriminated that suggest the presence of protoplasmic and fibrous astrocytes (Sofroniew & Vinters 2010) in these cultures. Although, GFAP staining is commonly used for characterisation of astrocytes in culture and in brain sections, it has its limitations. GFAP expression depends on the activation state of the cells and/or on the anatomical localisation (Eng *et al.* 2000) that may lead to heterogenous staining and underestimation of astroglial cell numbers. In addition, immunocytochemical labelling of GFAP will not give a complete representation of astrocyte morphology, since many of the very fine processes are GFAP-negative (Bushong *et al.* 2002, Ogata & Kosaka 2002). Therefore, in addition to GFAP other cell type specific proteins such as the calcium-binding protein S100β (Goncalves *et al.* 2008, Himeda *et al.* 2006, Ouyang *et al.* 2007, Pfeiffer *et al.* 1992, Yasuda *et al.* 2004) or the enzyme glutamine synthetase (Fages *et al.* 1988, Hallermayer & Hamprecht 1984, Ong *et al.* 1995, Ong *et al.* 1993) have been used for the identification of astrocytes in culture or in brain sections.

Astroglia-rich primary cultures prepared from whole perinatal brain have been reported to contain predominantly GFAP-positive cells, minor contamination of

other cell types such as progenitor cells, oligodendroglial cells, ependymal cells and microglia, but no neurons (Cesar & Hamprecht 1995, Dang *et al.* 2010, Gutterer *et al.* 1999, Kurz *et al.* 1993, Pawlowski & Dringen 2003, Reinhart *et al.* 1990). Using transmitting light microscopy, the presence of ependymal cells in the astroglial cultures used was obvious by the pulsation (data not shown) of their central cluster of long cilia (Gabrion *et al.* 1998, Prothmann *et al.* 2001, Verleysdonk *et al.* 2005). However, due to the high proportion of GFAP-positive cells in astroglia-rich primary cultures no further detailed analysis have been performed to verify the presence of other brain cell types in these cultures, for example by immunostaining for cell-type specific markers such as myelin-basic protein or galactocerebrosid for oligodendroglial cells (Baumann & Pham-Dinh 2001, Cahoy *et al.* 2008, Gutterer *et al.* 1999, Hirrlinger *et al.* 2000, Hirrlinger *et al.* 2002a, Pfeiffer *et al.* 1993, Ranscht *et al.* 1982, Satoh *et al.* 1996) and by using the antibody Ox42 to identify microglial cells (Chatterjee *et al.* 1999, Chatterjee *et al.* 2000, Dalmau *et al.* 1996, Hirrlinger *et al.* 2000).

Due to the high enrichment of GFAP-positive cells in the astroglia-rich primary cultures, these cultures were considered as suitable model system to study properties and functions of astrocytes.

2.1.5.2 Cellular contents of protein, GSH and GSSG

The cellular GSx content and its modulation by xenobiotics is the key topic of this thesis. To study whether contents of cellular protein, GSH or GSSG are altered with culture age, we determined these parameters within the third week of culture age that has been preferentially used previously to investigate GSH metabolism (Dringen & Hamprecht 1998).

The cultures in wells of 24-well cell culture dishes contained an average protein content of 101 ± 23 µg protein per well which remained constant at a culture age between 16 and 21 days. The specific GSx content of the cultures was 36.1 ± 8.4 nmol/mg protein which is comparable to a previous report of an average GSx content of 32.8 ± 3.2 nmol/mg protein (Dringen & Hamprecht 1998) for cultures between 14 and 21 days of age. However, the SD of the data obtained in this thesis

accounted for 23% of the mean value. Such variations are not unexpected for primary cultures. Reasons for that may be changes in cell culture material and media, inconsistent handling in the procedure of culture preparation and differences in the age of the pups (between 5 and 24 hours after birth) that were used to prepare the cultures. Nevertheless, despite of the significant but small differences in the protein content and in the GSx content per well, the specific GSx content remained constant and was independent of the culture age within the third week of culture age. In addition, cellular GSSG contents in untreated cells accounted for 7% of the total GSx, which is in range of the detection limit of the assay used. This indicates that the basic culture conditions did not lead to any oxidative stress that would have been detectable by an alteration of the cellular GSH to GSSG ratio.

2.1.5.3 Enzyme activities

Since xenobiotics such as IAA, IA or MCA inactivate enzymes and affect at least glycolysis (Bakken *et al.* 1998, Bickler & Kelleher 1992, Gemba *et al.* 1994, Loreck *et al.* 1987, Ogata *et al.* 1995, Sakai *et al.* 2005), the specific activities of some key enzymes of several metabolic pathways were quantified for untreated astroglia-rich primary cultures. As enzymes related to GSH metabolism, the activities of GR, GST and G6PDH were determined. While GR activity is essential for the reduction of GSSG (Gutterer *et al.* 1999), GST mediate the formation of conjugates of GSH with various nucleophilic substances (Hayes *et al.* 2005). G6PDH strongly affects cellular GSH metabolism as this enzyme plays a crucial role in the regeneration of NADPH in the pentose phosphate pathway that is required for the GR reaction (Dringen *et al.* 2007, Hirrlinger & Dringen 2010). The specific GR activity of 30.8 ± 16.0 nmol/(min x mg protein) obtained here for astroglia-rich primary cultures is comparable to the activities reported previously for cultured astrocytes which are in the range of 8 to 106 nmol/(min x mg protein) (Bishop *et al.* 2007, Gutterer *et al.* 1999, Hirrlinger *et al.* 2000, Huang & Philbert 1995, Huang & Philbert 1996, Lopez *et al.* 2007, Manganaro *et al.* 1995).

The determined specific activity of cytosolic GST of 298 ± 32 nmol/(min x mg protein) in astroglia-rich primary cultures is comparable to published GST activities of 67 to 119 nmol/(min x mg protein) for cultured astrocytes (Ahmed *et al.* 2002,

Huang & Philbert 1995, Huang & Philbert 1996, Waak 2005). Several isoforms of GST have been reported to be expressed in astrocytes in culture and *in vivo* (Ahmed *et al.* 2002, Dhanani & Awasthi 2007, Martinez-Lara *et al.* 2003, Sagara & Sugita 2001, Waak 2005). Which of these isoforms contributed most to the determined GST activity in astroglial cultures remains to be elucidated.

Also the specific G6PDH activity of 70.4 ± 27.3 nmol/(min x mg protein) determined for the cultures used here is in the range of 55 to 110 nmol/(min x mg protein) (Garcia-Nogales *et al.* 2003, Garcia-Nogales *et al.* 1999, Hagedorn 2008, Kußmaul 1999, Liddell *et al.* 2009) that has previously been reported for cultured astrocytes.

Since astrocytes play an important role in supplying neighbouring neurons with metabolic fuel molecules such as lactate (Barros & Deitmer 2010), we investigated the specific activities of GAPDH and LDH as enzymes involved in glycolytic lactate production. The specific activity for GAPDH of 475 ± 152 nmol/(min x mg protein) obtained for astroglia-rich primary cultures is in the range of activities of 350 to 2000 nmol/(min x mg protein) that have been published for cultured astrocytes (Buchczyk *et al.* 2000, Hazell *et al.* 1999b, Huang & Philbert 1996, Liddell *et al.* 2009). Also the specific LDH activity of 1720 ± 348 nmol/(min x mg protein) obtained here is comparable to published activities for cultured astrocytes that range from 1110 to 1900 nmol/(min x mg protein) (Hirrlinger *et al.* 1999, Liddell *et al.* 2009, Minich *et al.* 2003, O'Brien *et al.* 2007).

In conclusion, specific activities for the five enzymes investigated for untreated astroglia-rich primary cultures are similar to specific activities that have been published previously for astroglial cultures.

2.1.5.4 Visualisation of cellular GSH by MCB

To study the potential of brain astrocytes for GSH mediated detoxification processes, cultured astrocytes were exposed to various xenobiotics or endogenously derived substances. The quantification of the cellular GSx content after treatment of cultured astrocytes with these compounds using an enzymatic cycling assay for quantification (Dringen & Hamprecht 1996, Dringen *et al.* 1997) revealed that astrocytic GSH metabolism is compromised by many of the compounds. In addition

to the quantitative GSx determination, the GSH sensitive dye MCB was used to visualise GSH by fluorescence microscopy. MCB reacts predominantly with GSH in a GST-catalysed reaction forming a fluorescent GS-bimane adduct (Chatterjee *et al.* 1999, Cook *et al.* 1991, Eklund *et al.* 2002, Ublacker *et al.* 1991). MCB staining is considered as a fast and reliable method that has frequently been used to visualise GSH of brain cells in culture (Chatterjee *et al.* 1999, Chatterjee *et al.* 2000, Kaur *et al.* 2006, Kaur *et al.* 2008, Keelan *et al.* 2001, Legare *et al.* 1993, Tauskela *et al.* 2000, Waak & Dringen 2006), in brain slices (Bragin *et al.* 2010) and *in vivo* (Sun *et al.* 2006). Also in this thesis, MCB staining was successful established for visualisation of the GSH content in cultured astrocytes.

The cells of astroglia-rich primary cultures showed a very prominent staining after treatment with MCB that was substantially lowered after preincubation of the cells with GSH-depleting substances. The obtained intensity of the GS-bimane signal correlated well to the GSx contents quantified enzymatically for identical conditions. However, the GS-bimane signal intensity was not homogenously distributed over the whole well as became evident from the patches of cells in the cultures that were considerably stronger stained than neighbouring cells. This pattern of heterogenous staining was more prominent in conditions where the cellular GSH content was altered. The occurrence of heterogenous staining in astroglia-rich primary cultures has also been shown after immunolabelling of enzymes such as glutamine synthetase (Fages *et al.* 1988, Hallermayer & Hamprecht 1984), glycogen phosphorylase (Pfeiffer *et al.* 1992, Reinhart *et al.* 1990), glycogen phosphorylase kinase (Psarra *et al.* 1998) or glutathione reductase (Gutterer *et al.* 1999) as well as after cytochemical staining of glycogen (Dringen & Hamprecht 1993a) or iron (Bishop *et al.* 2010a, Dang *et al.* 2010). These observations were discussed to result either from the presence of different brain cell types in astroglia-rich primary cultures, from the origin of one cell type from different brain regions or as consequences of different stages of astroglial differentiation. For example, the GSH content has been shown to vary between different brain regions (Calabrese *et al.* 2002, Cooper 1998, Janáky *et al*. 2007, Kang *et al.* 1999). This may be a consequence of regional variation in the availability of GSH precursors, in the demand of GSH for various cellular and extracellular functions and/or may be due to differences in the

glial to neuron ratio (Rice & Russo-Menna, 1998, Srinivasan *et al.* 2010). In addition, astrocyte cultures prepared from various brain regions have been shown to differ in their specific GSx content (Langeveld *et al.* 1996) which could also explain the heterogenous GS-bimane signal observed in cultures prepared from total brain.

The intensity of the GS-bimane signal in astroglia-rich cultures appeared to some degree also to be cell type dependent which was most obvious after GSH deprivation with the inhibitor BSO. The remaining GS-bimane staining was limited to small cells of the top layer, which is consistent to earlier data published on glial cultures (Chatterjee *et al.* 1999, Chatterjee *et al.* 2000). Immunocytochemical labeling after MCB treatment identified these cells to be predominantly microglia (Chatterjee *et al.* 1999, Chatterjee *et al.* 2000). Since microglia have been shown to posses the highest specific GSx content in cultures (Hirrlinger *et al.* 2000) and a very low rate of GSx efflux (Hirrlinger *et al.* 2002c), incubation with BSO possibly led to a higher remaining cellular GSH level in these contaminating cells compared to astrocytes, which consequently would have resulted in a stronger remaining GS-bimane signal.

The GS-bimane conjugate formed upon MCB application is efficiently exported from astroglial cells in a process that is predominantly mediated by multidrug resistance protein (Mrp) 1 (Waak & Dringen 2006). Despite of Km values of Mrp1 for GSH conjugates such as leucotriene C4, S-dinitrophenyl-glutathione and GS-bimane in the nanomolar to low micromolar range (Homma *et al.* 1999, Jedlitschky *et al.* 1996, Leier *et al.* 1994, Loe *et al.* 1996, Müller *et al.* 1994), substantial loss of cellular GS-bimane signal from cultured astrocytes occurred only after incubation times exceeding 5 min (Waak & Dringen 2006), which was confirmed for the experimental conditions used here (data not shown). Consequently, GS-bimane export from the cells during the chosen incubation time of 2 min can most likely be excluded to contribute to the observed of the MCB staining.

Compared to the GSH quantification by the enzymatic cycling assay, the MCB method reached its limits when the remaining cellular GSH content was lower than 15 to 20% of the initial GSx content as indicated by the absence of any GS-bimane fluorescence for such conditions, confirming literature data that MCB appears to visualise only part of the cellular GSH content as the cellular GS-bimane content did

not exceed 43% of the initial GSx content (Waak & Dringen 2006). Since GST are involved in the formation of the GS-bimane conjugate (Chatterjee *et al.* 1999, Cook *et al.* 1991, Eklund *et al.* 2002, Ublacker *et al.* 1991), GSH deprivation of the cell may affect the activities of GST. The Km values of GST for GSH in conjugation reactions with compounds such as 4-hydroxynonenal, CDNB and MCB have been reported to range from 3 to 100 µM (Hubatsch *et al.* 1998, Young *et al.* 1994). Since cultured astrocytes contain a cellular GSx concentration of 8 mM (Dringen & Hamprecht 1998), 20% of the initial cellular GSx content still account for about 2 mM cytosolic GSH. Thus, insufficient GST catalysed GS-bimane formation as reason for the limited detection of lower concentration of cellular GSH can most likely be excluded. Furthermore, insufficient exposure of the cells to MCB to allow sufficient GS-bimane formation can be excluded, since the maximum the GS-bimane signal intensity was already reached within the first minute of incubation (data not shown).

A further disadvantage of the MCB method to investigate the GSH metabolism of cultured cells is that GSSG is not detected by MCB, since the fluorescence develops only after conjugation to GSH. Consequently, the MCB method cannot distinguish whether a lowered cellular GS-bimane signal results from stimulated GSH export and/or oxidation of GSH to GSSG. In addition, the fixation of the cells after MCB application using paraformaldehyde for the storage of the samples was limited to untreated cells, since the fixation procedure of cells that were pretreated with GSx lowering compounds led to an almost complete detachment of the cells.

Despite some disadvantages, MCB staining is a suitable method as an additional tool to qualitatively visualise the cellular GSH content of cells in astroglia-rich primary cultures and to study the GS-bimane export in living cells. Since the exposure time to document the GS-bimane signal of untreated cells was used as a reference for all the other conditions applied here, adaptation of the exposure time and the graphical processing could enhance the sensitivity of the MCB method. However, this staining procedure cannot replace the highly sensitive and extremely reliable enzymatic cycling assay for the quantification of GSH and GSSG that is necessary to investigate the consequences of a treatment with xenobiotics on the GSH metabolism of cultured astrocytes.

2.1.6 References

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Publication 2

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Differential effects of iodoacetamide and iodoacetate on glycolysis and glutathione metabolism of cultured astrocytes

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Iodoacetamide (IAA) and iodoacetate (IA) have frequently been used to inhibit glycolysis, since these compounds are known for their ability to irreversibly inhibit the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). However, the consequences of a treatment with such thiol reagents on the glutathione (GSH) metabolism of brain cells have not been explored. Exposure of astroglia-rich primary cultures to IAA or IA in concentrations of up to 1 mM deprived the cells of GSH, inhibited cellular GAPDH activity, lowered cellular lactate production and caused a delayed cell death that was detectable after 90 min of incubation. However, the two thiol reagents differed substantially in their potential to deprive cellular GSH and to inhibit astrocytic glycolysis. IAA depleted the cellular GSH content more efficiently than IA as demonstrated by half-maximal effects for IAA and IA that were observed at concentrations of about 10 and 100 μM, respectively. In contrast, IA was highly efficient in inactivating GAPDH and lactate production with half-maximal effects observed already at a concentration below 100 μM, whereas IAA had to be applied in 10 times higher concentration to inhibit lactate production by 50%. These substantial differences of IAA and IA to affect GSH content and glycolysis of cultured astrocytes suggest that in order to inhibit astrocytic glycolysis without substantially compromising the cellular GSH metabolism, IA – and not IAA – should be used in low concentrations and/or for short incubation periods.

Keywords: alkylation, astrocytes, carboxymethylation, GAPDH, glycolysis, GSH, lactate, thiol reagents

INTRODUCTION

The thiol reagents iodoacetamide (IAA) and iodoacetate (IA) (**Figure 1**) are frequently used as alkylating reagents to modify thiol groups in proteins by S-carboxyamidomethylation and S-carboxymethylation, respectively. IAA and IA have been used to study the importance of cysteine residues in catalytic reactions of enzymes and in transport processes of brain cells (Albrecht et al., 1993; Aschner et al., 1994; Gali and Board, 1997) and to derivatise cysteine residues in proteins for proteomic approaches (Adachi et al., 2005; Sun et al., 2008; Williams et al., 2008). The inactivation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by IAA and IA (Sabri and Ochs, 1971; Williamson, 1967) is often used as text book example for an irreversible enzyme inhibition. The essential cysteine residue in the active center of GAPDH forms a thioether bond with IAA or IA and can therefore not react anymore with the physiological substrate glyceraldehyde-3-phosphate. As a consequence, GAPDH is inactivated after exposure to IAA or IA and glycolysis is inhibited.

On cultured astrocytes and glial cell lines, IAA and IA have been used to inhibit glycolysis (Bakken et al., 1998; Bickler and Kelleher, 1992; Gemba et al., 1994; Loreck et al., 1987; Ogata et al., 1995) and to study the consequences of ATP depletion and hypoglycemia (Kauppinen et al., 1988; Nodin et al., 2005; Parkinson et al., 2002; Sun et al., 1993; Taylor et al., 1996). However, during treatment of cells or tissue with IAA or IA, side effects that are caused by the unspecific reaction of IAA and IA as thiol reagents cannot be excluded. In this context, especially the reactions of IAA and IA with low molecular weight cellular thiols such as GSH (Chen and Stevens, 1991; Liu et al., 1996) have to be considered.

The tripeptide GSH (γ-L-glutamyl-L-cysteinylglycine) is a cellular thiol that is present in millimolar concentrations in mammalian cells (Cooper and Kristal, 1997). GSH has many important functions in cells. Among those, the antioxidative and detoxifying functions of GSH are most likely the most important ones for many cell types and tissues. GSH serves as electron donor for the reduction of peroxides by glutathione peroxidases (Margis et al., 2008) and is substrate for the detoxification of xenobiotics in the reactions that are catalyzed by glutathione-*S*-transferases (Hayes et al., 2005).

In the brain, astrocytes play a very important role in the antioxidative defense and in the detoxification of xenobiotics (Aoyama et al., 2008; Ballatori et al., 2008; Cooper and Kristal, 1997; Dringen, 2000, 2009). Cultured astrocytes contain GSH in a cytosolic concentration of 8 mM (Dringen and Hamprecht, 1998). These cells rely on a high GSH concentration for the rapid clearance of peroxides (Dringen et al., 2005; Liddell et al., 2006a,b) for the resistance against oxidative stress (Bi et al., 2008; Bishop et al., 2007; Gegg et al., 2003, 2005; Giordano et al., 2008) and for the GSH-dependent detoxification of xenobiotics (Kubatova et al., 2006; Sagara and Sugita, 2001; Waak and Dringen, 2006).

Although IAA and IA have often been used to modulate the glucose metabolism of cultured astrocytes, the consequences of a

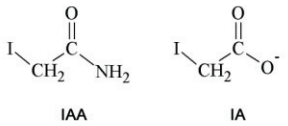

FIGURE 1 | Structural formulas of iodoacetamide (IAA) and iodoacetate (IA).

treatment of astrocytes with such thiol reagents on the GSH content of the cells have not been reported. Here we describe that exposure of cultured astrocytes to IAA or IA lowers the cellular GSH content and inhibits glycolysis, but that the two thiol reagents differ strongly in their potential to do so. IA is highly efficient in low concentrations to inhibit GAPDH activity and to lower cellular lactate production of cultured astrocytes, whereas IAA is much more potent to deprive cells of GSH than to inactivate glycolysis. These results suggest that for experiments that require inhibition of glycolysis in astrocytes IA – but not IAA – should be used in micromolar concentrations or only for short incubation periods to avoid a severe compromise of cellular GSH metabolism.

MATERIALS AND METHODS

MATERIALS

IAA, IA and 5,5′-dithio-bis(2-nitrobenzoic acid) were obtained from Sigma-Aldrich (Steinheim, Germany). Glucose-6-phosphate, glutamate pyruvate transaminase, GSH, glutathione disulfide (GSSG), glutathione reductase (GR), glyceraldehyde-3-phosphate, lactate dehydrogenase (LDH), maleimide and potassium arsenate were from Roche Diagnostics (Mannheim, Germany). Bovine serum albumin, NAD^+, NADH, $NADP^+$, NADPH and sulfosalicylic acid were purchased from Applichem (Darmstadt, Germany). Fetal calf serum, streptomycin sulfate and penicillin G were from Biochrom (Berlin, Germany). Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen (Karlsruhe, Germany). All other chemicals were obtained from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany) at analytical grade. Sterile cell culture material and unsterile 96-well plates were from Nunc (Roskilde, Denmark) and Sarstedt (Karlsruhe, Germany).

CELL CULTURES

Astroglia-rich primary cultures derived from the whole brains of neonatal Wistar rats were prepared as previously described (Hamprecht and Löffler, 1985). Three hundred thousand viable cells were seeded per well of a 24-well dish in 1 mL culture medium (90% DMEM, 10% fetal calf serum, 20 U/mL of penicillin G and 20 µg/mL of streptomycin sulfate) and cultured in a cell incubator (Sanyo, Osaka, Japan) that contained a humidified atmosphere of 10% CO_2/90% air. The culture medium was renewed every seventh day. The results described here were obtained on 14- to 23-day-old cultures.

EXPERIMENTAL INCUBATION OF CELLS

To study the consequences of a treatment of astrocytes with IAA or IA, the cells were washed with 1 mL of prewarmed (37°C) incubation buffer (IB; 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.4 mM KCl, 145 mM NaCl, 0.8 mM Na_2HPO_4, 20 mM HEPES, 5 mM D-Glucose, pH 7.4) and incubated in the cell incubator with 0.5 mL IB containing IAA or IA in the concentrations given in the figures. For analysis of the total cellular glutathione content (GSx = amount of GSH plus twice the amount of GSSG), the cells were washed with 1 mL phosphate-buffered saline (PBS; 10 mM potassium phosphate buffer, 150 mM NaCl, pH 7.4) and lysed with 0.5 mL 1% (w/v) sulfosalicylic acid. Ten microliters of aliquote parts of the lysates were used to quantify the cellular GSx content.

CHEMICAL REACTION OF GSH WITH IAA OR IA

GSH (10 µM) was incubated with the indicated concentrations of IAA or IA in a total volume of 1 mL at room temperature in IB for up to 60 min. At the time points indicated, 10 µL sample of the reaction mixture were mixed with 10 µL 1% sulfosalicylic acid before the GSx content was determined as described below.

DETERMINATION OF GLUTATHIONE

The contents of GSx and GSSG in cell lysates and incubation media were determined as described previously (Dringen and Hamprecht, 1996; Dringen et al., 1997) in microtiter plates according to the colorimetric method originally described by Tietze (Tietze, 1969). The detection limit of this assay was about 0.2 nmol GSx per 500 µL lysate or medium.

DETERMINATION OF LACTATE IN CULTURE MEDIUM

Extracellular lactate concentration in culture media was determined using a modification of an established assay (Dringen et al., 1993). Briefly, 20 µL media sample were diluted with 160 µL purified water in wells of a microtiter plate and mixed with 180 µL reaction mixture (5.6 mM NAD^+, 19.9 U/mL LDH, 1.94 U/mL glutamate pyruvate transaminase in 250 mM glutamate/NaOH buffer, pH 8.9). After incubation for 90 min in a humidified atmosphere at 37°C the absorbance of the NADH generated from lactate was determined at 340 nm using a Sunrise microtiter plate photometer (Tecan, Austria). Media samples containing no lactate were used as blanks.

DETERMINATION OF ENZYME ACTIVITIES

The cells were washed with 1 mL ice-cold PBS and subsequently lysed by incubation with 200 µL 20 mM potassium phosphate buffer (pH 7.0) for 10 min on ice. The lysates were scrapped of the wells and transferred into Eppendorf cups. After centrifugation (1 min, 12,000*g*, room temperature) 20 µL volumes of the supernatant were used to determine enzyme activities in wells of microtiter plates at room temperature. GAPDH activity was determined using the method described by Bisswanger (2004). The reaction mixture contained in a total volume of 360 µL 0.9 mM glyceraldehyde-3-phosphate, 3 mM potassium dihydrogen arsenate, 2 mM NAD^+ and 93 mM triethylamine hydrochloride/NaOH buffer, pH 7.6. The increase of absorbance at 340 nm due to the reduction of NAD^+ to NADH was followed over 10 min time. Glucose-6-phosphate dehydrogenase (G6PDH) activity was determined according to Deutsch (1984). The reaction mixture contained in a total volume of 200 µL 6.3 mM $MgCl_2$, 5 mM maleimide, 3.3 mM glucose-6-phosphate, 0.4 mM $NADP^+$ and 75 mM Tris/HCl buffer, pH 7.5. The increase of absorbance at 340 nm due to the reduction of $NADP^+$ to NADPH was followed over 5 min. LDH activity was

determined using the method described previously (Dringen et al., 1998). The reaction mixture contained in a total volume of 360 µL 1.8 mM pyruvate, 0.2 mM NADH and 80 mM Tris/HCl buffer containing 200 mM NaCl, pH 7.2. The decrease of absorbance at 340 nm due to the oxidation of NADH to NAD^+ was followed over 5 min. GR activity was determined using a method described previously (Gutterer et al., 1999). The reaction mixture contained in a total volume of 300 µL 1 mM GSSG, 0.2 mM NADPH and 1 mM EDTA in 100 mM potassium phosphate buffer, pH 7.0. The decrease of absorbance at 340 nm due to the oxidation of NADPH was followed over 5 min.

DETERMINATION OF CELL VIABILITY AND PROTEIN CONTENT

Loss of cell viability was analyzed by comparing the activity of LDH in the incubation medium with that of the cells using the microtiter plate assay described previously (Dringen et al., 1998). The presence of IAA or IA in a concentration of 1 mM did not affect LDH activity in lysates of cultured astrocytes (data not shown). The protein content per well of a 24-well dish was quantified after solubilization of the cells in 200 µL of 0.5 M NaOH according to the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard. Total protein content per well and cytosolic protein in the supernatants of cell lysates (cytosolic protein) were used to calculate specific GSx or GSSG contents and specific enzyme activities, respectively.

PRESENTATION OF THE DATA

If not stated otherwise, the data are presented as mean ± SD of values obtained in experiments on three independently prepared cultures. In the figures the bars have been omitted if they were smaller than the symbols representing the mean values. Statistical analysis of the significance of differences between multiple sets of data were performed by ANOVA followed by Bonferroni *post hoc* test. If not stated otherwise, statistical analysis of the significance of differences between two sets of data was performed using the paired *t*-test. The data shown in **Figure 2** for IAA and IA were obtained separately and were therefore analyzed for significance by the unpaired *t*-test. $p > 0.05$ was considered as not significant.

RESULTS

CHEMICAL REACTION OF IAA OR IA WITH GSH

To test whether IAA and IA react chemically with GSH, IAA or IA were mixed with GSH and the amount of detectable GSx was determined during an incubation period of up to 60 min. In the presence of IAA (**Figure 2A**) or IA (**Figure 2B**) the amount of detectable GSx was lowered in a time- and concentration-dependent manner. For all concentrations of IAA or IA applied, the amount of GSx determined after 60 min of incubation was significantly lower after treatment with IAA than after exposure to IA (**Figure 2C**). Half-maximal effects on the GSx content after 60 min of incubation with IAA and IA were observed for concentrations of about 0.2 and 1 mM, respectively (**Figure 2C**), demonstrating that IAA was more potent to react with GSH than IA.

INACTIVATION OF ASTROCYTIC GAPDH BY IAA OR IA

IAA and IA are well known to inhibit GAPDH activity (Sabri and Ochs, 1971; Williamson, 1967). To test for the potential of these compounds to inactivate astrocytic GAPDH, cultured astrocytes were lysed and the GAPDH activity in the lysates was determined in the absence or presence of IAA or IA. In the absence of IAA and IA, the increase in absorbance that was caused by the GAPDH-dependent formation of NADH was almost linear for up to 30 min

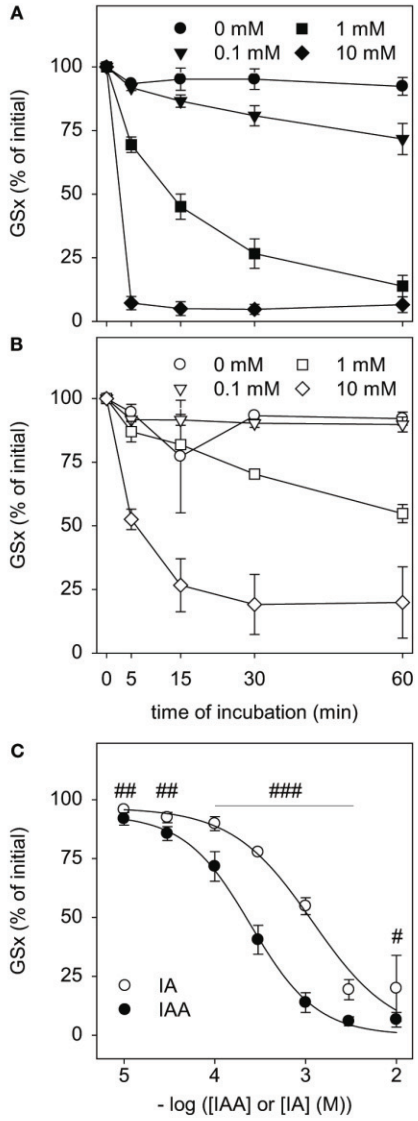

FIGURE 2 | Disappearance of GSH after exposure to IAA or IA. GSH in a concentration of 10 µM was incubated with the indicated concentrations of IAA **(A)** or IA **(B)** for up to 60 min. **(C)** shows the GSx content that was detected after 60 min incubation with the given concentrations of IAA or IA. The data shown represent mean ± SD of 6 values that were obtained in two independent experiments, each performed in triplicates with individually prepared solutions. The significance of the differences between the values obtained for IAA and IA was calculated by the unpaired *t*-test and is indicated by $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ or $^{\#\#\#}p < 0.001$).

(**Figure 3**). If the GAPDH reaction was monitored in the presence of IAA, the increase in NADH absorbance during the first 20 min of incubation was not significantly different ($p > 0.05$) from that of the control condition (absence of inhibitor) and it took about 60 min before the enzyme was completely inhibited (**Figure 3**). In contrast, in the presence of IA the increase in NADH absorbance was completely prevented within a few minutes (**Figure 3**).

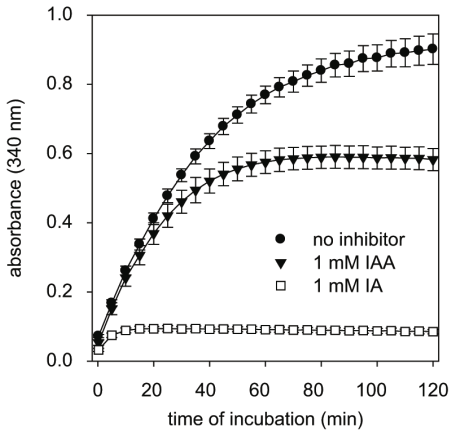

FIGURE 3 | GAPDH activity in cell lysates of astrocyte cultures in the absence or the presence of IAA or IA. The absorbance at 340 nm that indicates the formation of NADH by GAPDH was monitored. The results are presented as mean ± SD of data that were obtained on cell lysates derived from three independently prepared cultures.

CONSEQUENCES OF A TREATMENT OF ASTROCYTE CULTURES WITH IAA OR IA

To test for the consequences of a treatment of cultured astrocytes with IAA or IA, the cells were incubated with various concentrations of these compounds for up to 2 h. After the indicated incubation times the extracellular activity of LDH as indicator for the loss of cell viability, the specific cellular GSx content as indicator for cellular GSH, and the lactate concentration in the medium as indicator for glycolytic activity were monitored.

During the first 60 min of incubation with IAA or IA, the cells remained viable as indicated by the lack of any increase ($p > 0.05$) in extracellular LDH activity compared to control (absence of IAA and IA) (**Figures 4A,B**). However, incubation of astrocytes with IAA or IA for longer than 60 min caused a significant increase in extracellular LDH activity that was moderate for 0.1 mM IAA or IA but more severe for higher concentrations of both compounds (**Figures 4A,B**).

Exposure of cultured astrocytes to IAA caused a rapid decline of the cellular specific GSx content ($p < 0.001$ already after 5 min) that resulted after 30 min incubation in cellular GSx levels below 20% of control (absence of IAA) (**Figure 4C**). Also IA caused a decline

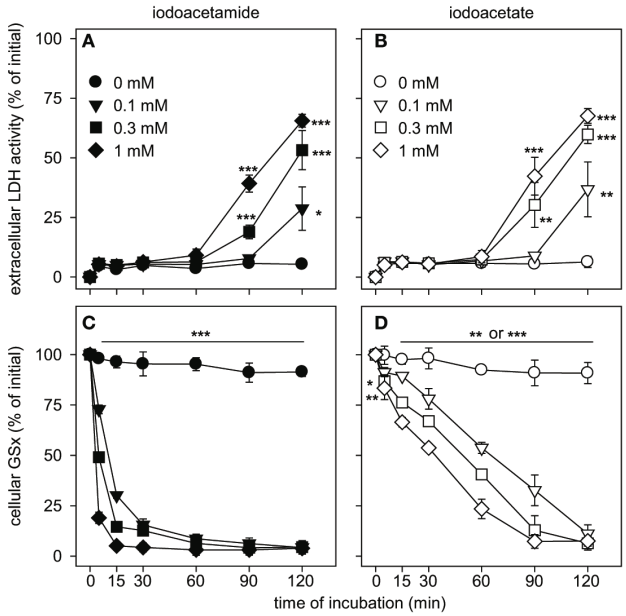

FIGURE 4 | Consequences of an application of IAA (A,C) or IA (B,D) on cellular viability (A,B) and specific cellular GSx content (C,D) in astrocyte cultures. The cells were incubated for up to 120 min without or with IAA or IA in the concentrations indicated in **(A)** and **(B)**. The results represent mean ± SD of data that were obtained on three independently prepared cultures. The cultures contained initial protein contents of 80 ± 8 µg protein per well and initial specific GSx contents (100%) of 46.2 ± 10.2 nmol/mg protein. The significance of differences to the data obtained for the control condition (absence of inhibitor) are indicated by $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$.

in cellular GSx content that depended on the concentration of IA applied (**Figure 4D**). However, this decline in cellular GSx after IA application (**Figure 4D**) was much slower than that observed for identical concentrations of IAA (**Figure 4C**) ($p < 0.001$ after 5 min for all concentrations of IAA and IA applied). The concentrations causing half-maximal deprivation of cellular GSx after exposure of cultured astrocytes for 60 min to IAA and IA were about 10 and 100 μM, respectively, and differed by one order of magnitude (**Figure 5**).

The decline in cellular GSx that was observed after exposure of cultured astrocytes to IAA or IA (**Figures 4C,D and 5**) was not accompanied by an increase in cellular GSSG nor by an increase in the extracellular concentrations of GSx or GSSG (**Table 1**). In contrast, the extracellular contents of GSx and GSSG were lowered after incubation of the cells with IAA or IA (**Table 1**).

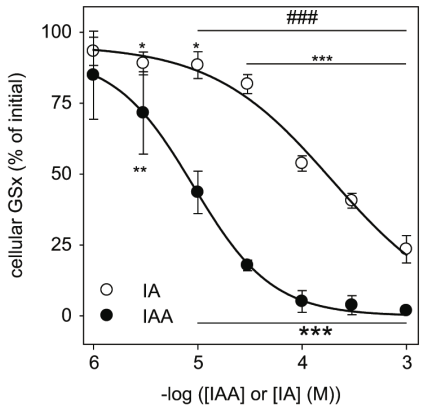

FIGURE 5 | Concentration dependency of the GSH depletion by IAA or IA in cultured astrocytes. The cells were incubated for 60 min with IAA or IA in concentrations of up to 1 mM. The results represent mean ± SD of data that were obtained on three independently prepared cultures that contained 80 ± 8 μg protein per well. The initial specific GSx content (100%) corresponded to 46.2 ± 10.2 nmol/mg protein. The significance of differences to the data obtained for the control condition (absence of inhibitor) are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$. The significance of differences between the data observed after treatment with identical concentrations of IAA and IA was calculated by the paired *t*-test and is indicated by $^{\#\#\#}p < 0.001$.

Exposure of astrocytes to IAA hardly affected the extracellular accumulation of lactate during the first 60 min of incubation compared to control (**Figure 6A**), whereas a further increase of extracellular lactate was slowed during longer incubations, at least for IAA concentrations of 0.3 and 1 mM (**Figure 6A**). The extracellular lactate concentration determined for cells that were treated with IAA for 60 min did not differ significantly to that of control cells (**Figure 7A**). In contrast, incubation of astrocytes with IA in a concentration of 1 mM inhibited extracellular lactate accumulation much quicker than IAA (**Figure 6B**). In concentrations of 0.1 or 0.3 mM IA almost completely prevented extracellular lactate accumulation within 30 min of incubation (**Figure 6B**). After 60 min of incubation the extracellular lactate concentrations of astrocyte cultures that were treated with 0.1, 0.3 and 1 mM IA were significantly lowered to 40 ± 7, 24 ± 16 and 6 ± 8%, respectively, of the concentration determined for cells that were incubated without inhibitor (**Figure 7A**).

To demonstrate that indeed the inhibition of GAPDH by IAA or IA was responsible for the observed decline in the rate of glycolytic lactate production in astrocyte cultures, the specific activity of GAPDH was determined for cells that were treated for 60 min with various concentrations of IAA or IA. When the cells were exposed to 0.1 mM IAA the specific GAPDH activity was significantly lowered to half of that of controls (**Figure 7B**). In contrast, already after treatment of the cells with 0.1 mM IA for 60 min GAPDH activity was not detectable anymore (**Figure 7B**). In concentrations of 0.3 and 1 mM, both IAA and IA completely inhibited GAPDH activity of astrocyte cultures within 60 min of incubation (**Figure 7B**).

To analyze how quickly the cellular GAPDH activity of cultured astrocytes was inactivated after exposure to IAA or IA, the cells were incubated with various concentrations of IAA or IA for time periods in the minute range (**Figure 8**). Half-maximal inhibition of astrocytic GAPDH by IAA that was applied in concentrations of 0.1, 0.3 and 1 mM was observed after about 60, 30 and 5 min of incubation, respectively (**Figure 8A**). In contrast, inactivation of GAPDH by the presence of IA was much faster that that by IAA. GAPDH activity was not detectable anymore after exposure of the cells for 5, 15 and 30 min to IA in concentrations of 1, 0.3 and 0.1 mM, respectively (**Figure 8B**).

To test whether IAA or IA are also able to inactivate other metabolic enzymes in cultured astrocytes, the cells were exposed for 60 min to IAA or IA in concentrations of 1 mM. Although these

Table 1 | Cellular and extracellular GSx and GSSG contents and extracellular LDH activity of primary astrocyte cultures after exposure to IAA or IA.

	Control	IAA	IA	*n*
Cellular GSx (nmol/mg)	41.9 ± 7.6	3.1 ± 3.6***	8.9 ± 5.6***	4
Cellular GSSG (nmol/mg)	5.1 ± 0.6	2.0 ± 2.0	2.9 ± 1.1	3
Extracellular GSx (nmol/mg)	4.7 ± 0.5	1.4 ± 1.1*	2.4 ± 1.4	3
Extracellular GSSG (nmol/mg)	3.8 ± 0.5	1.6 ± 0.9*	2.6 ± 0.9	3
Extracellular LDH (%)	6.4 ± 1.8	12.2 ± 6.5	8.9 ± 2.2	4

Primary astrocyte cultures were incubated in the absence (control) or the presence of 1 mM IAA or IA. The cellular and extracellular contents of GSx and GSSG and extracellular LDH activity (as % of initial) were determined after 60 min of incubation. The results represent mean ± SD of data that were obtained on n independently prepared cultures that contained 87 ± 16 μg protein per well. The significance of differences to the data obtained for the control condition (absence of inhibitor) are indicated by $^{*}p < 0.05$ or $^{***}p < 0.001$.

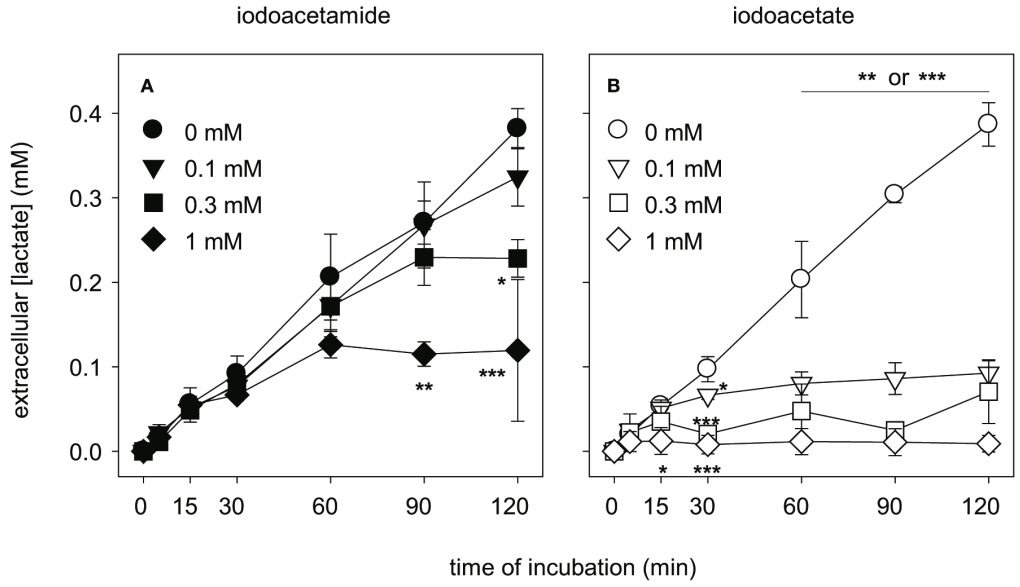

FIGURE 6 | Consequences of an application of IAA (A) or IA (B) on the extracellular lactate concentration in astrocyte cultures. The cells were incubated for up to 120 min without or with IAA or IA in the concentrations indicated in **(A)** and **(B)**. The results represent mean ± SD of data that were obtained on three independently prepared cultures. The cultures contained initial protein contents of 80 ± 8 µg protein per well. The significance of differences to the data obtained for the control condition (absence of inhibitor) are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$.

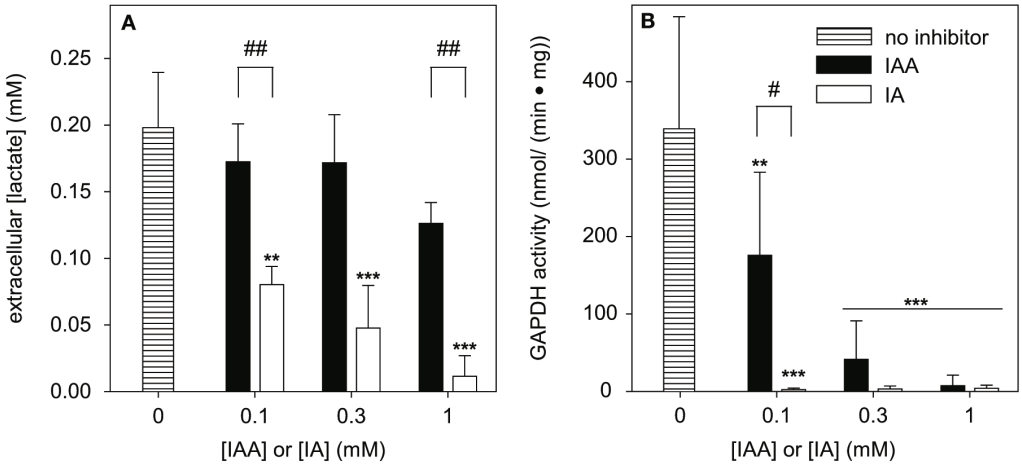

FIGURE 7 | Consequences of a treatment of cultured astrocytes with IAA or IA on the extracellular lactate concentration (A) and on the specific cellular GAPDH activity (B). The cells were incubated for 60 min with IAA or IA in concentrations of up to 1 mM. The results represent mean ± SD of data that were obtained on three **(A)** or six **(B)** independently prepared cultures that contained 80 ± 8 µg total protein per well **(A)** or 37 ± 11 µg cytosolic protein per well **(B)**. The significance of differences to the data obtained for the control condition (absence of inhibitor) are indicated by $^{**}p < 0.01$ or $^{***}p < 0.001$. The significance of differences between the data observed after treatment with identical concentrations of IAA and IA was calculated by the paired *t*-test and is indicated by $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$.

high concentration of IAA or IA completely inactivated GAPDH (**Figure 8**; **Table 2**), they had no significant effect on the specific activities of G6PDH or LDH. However, 60 min incubation of cultured astrocytes with 1 mM of IAA significantly lowered the specific activity of GR to 45% of controls (**Table 2**), whereas treatment of astrocytes with 1 mM of IA caused only some decline

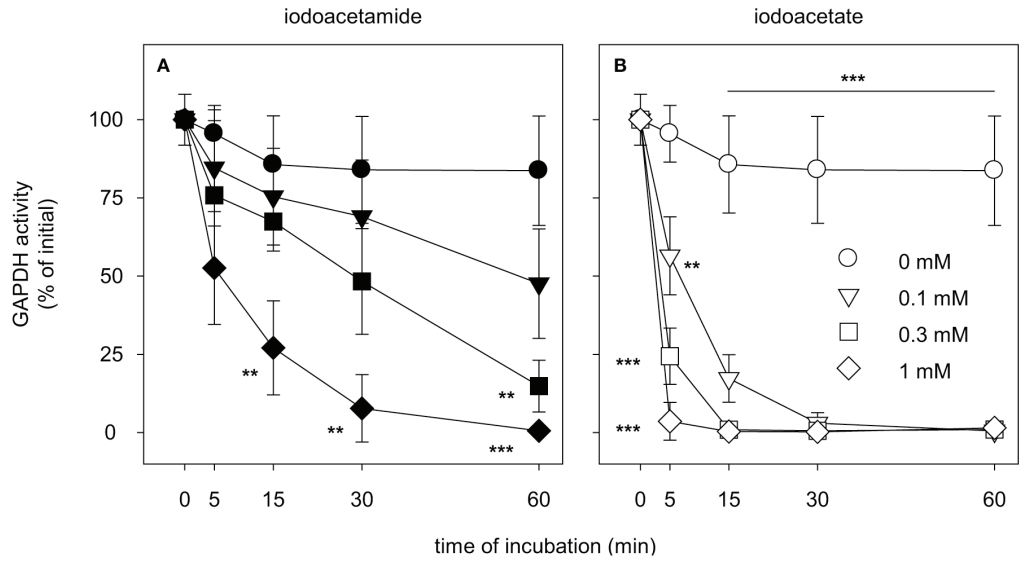

FIGURE 8 | Time dependency of the inhibition of GAPDH by IAA or IA in cultured astrocytes. The cells were incubated for up to 60 min without (circles) or with IAA **(A)** or IA **(B)** in concentrations of 0.1 mM (triangles), 0.3 mM (squares) or 1 mM (diamonds). The results represent mean ± SD of data that were obtained on three independently prepared cultures that contained 45 ± 5 μg cytosolic protein per well. The initial specific GAPDH activity (100%) corresponded to 492 ± 45 nmol/(min × mg) cytosolic protein. The significance of differences to the data obtained for the control condition (absence of inhibitor) are indicated by $^{**}p < 0.01$ or $^{***}p < 0.001$.

Table 2 | Specific activities of enzymes in cultured astrocytes after exposure to IAA or IA.

Enzyme	Inhibitor	Enzyme activity (nmol/(min × mg))	*n*
GAPDH	None	339 ± 145	6
	IAA	8 ± 13***	6
	IA	4 ± 4***	6
G6PDH	None	108 ± 12	3
	IAA	96 ± 8	3
	IA	104 ± 10	3
GR	None	56 ± 3	3
	IAA	25 ± 12*	3
	IA	40 ± 6	3
LDH	None	3278 ± 374	3
	IAA	3798 ± 379	3
	IA	3832 ± 331	3

Primary astrocyte cultures were incubated in the absence or the presence of 1 mM of IAA or IA. After 60 min incubation, the cells were lysed and the specific activities of the enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glucose-6-phosphate dehydrogenase (G6PDH), lactate dehydrogenase (LDH) and glutathione reductase (GR) were determined. The results represent mean ± SD of data that were obtained on n independently prepared cultures that contained 45 ± 5 μg (n = 3) or 37 ± 11 μg (n = 6) cytosolic protein per well. The significance of differences to the data obtained for the control condition (none) is indicated by $^{*}p < 0.05$ or $^{***}p < 0.001$.

in GR activity that did not reach the level of significance for the conditions investigated (**Table 2**).

DISCUSSION

The thiol reagents IAA and IA have frequently been used to inhibit astrocytic glycolysis (Bakken et al., 1998; Bickler and Kelleher, 1992; Gemba et al., 1994; Loreck et al., 1987; Ogata et al., 1995), but no information has been reported whether such treatments also affect the cellular GSH metabolism of astrocytes. To test for the consequences of a treatment of cultured astrocytes with IAA or IA on the GSH metabolism, the cells were exposed to various concentrations of the thiol reagents for up to 120 min. During the first 60 min incubation with IAA or IA in concentrations of up to 1 mM the cells remained viable as indicated by the lack of extracellular LDH activity, although glycolysis was inhibited and the cells were deprived of GSH. This lack of acute toxicity of such thiol reagents on cultured astrocytes confirms literature data (Gemba et al., 1994; Kahlert and Reiser, 2004; Nodin et al., 2005).

Comparison of the potential of IAA and IA to deprive cells of GSH and to inhibit glycolysis revealed substantial differences that have to our knowledge not been described so far. IAA was highly efficient to deprive the cells of GSH, whereas IA quickly inactivated GAPDH activity and slowed glycolysis. These differential effects are best demonstrated by the concentrations of the thiol reagents that had to be applied to obtain half-maximal effects for IAA and IA on GSH deprivation and lactate production which differed in both cases by one order of magnitude.

The GSH content of cultured astrocytes was lowered after exposure to IAA or IA. Such a decline of cellular non protein thiols has also been described for the kidney proximal tubule cell line LLC-PK1 after treatment with IAA (Chen and Stevens, 1991; Liu

et al., 1996). The deprivation of cellular GSH after exposure to IAA or IA is most likely the direct consequence of the alkylation of GSH in viable cells, since both IAA and IA reacted also efficiently with GSH in a cell-free system in a time- and concentration-dependent manner. Loss of cellular GSx that is caused by damage of the cell membrane after exposure to IAA or IA or by an elevated export of GSH or GSSG from cultured astrocytes (Hirrlinger et al., 2001, 2002; Minich et al., 2006) can be excluded for the conditions used here, since an increased extracellular accumulation of GSx or GSSG was not observed.

The cellular GSH concentration of cultured astrocytes was quicker deprived by exposure to IAA than by treatment with IA. This difference is likely to reflect different chemical reactivity of IAA and IA with GSH, since IAA was also in cell-independent experiments more potent than IA to react with GSH. The reaction of a thiol with IAA or IA is initiated by a nucleophilic attack of the sulfhydryl group to the α-carbon of IAA or IA. Since the α-carbon of IA is less positive than that of IAA due to the neighboring negative carboxylate in IA compared to the uncharged carboxyamido group in IAA, IA will alkylate the thiol group of GSH less efficiently than IAA. Also differences in cellular uptake of IAA and IA could contribute at least in part to the observed differences in the potential of IAA and IA to deprive cultured astrocytes of their GSH. Different mechanisms of uptake of the uncharged IAA and the negatively charged IA could result in a higher cellular concentration of IAA, thereby further accelerating the chemical reaction of cellular GSH with IAA. However, the quick inhibition of GAPDH that was observed after exposure of cultured astrocytes to IA clearly demonstrates that the charged IA does enter the cells and that cellular IA concentrations are quickly reached that are sufficient to efficiently inhibit GAPDH.

Despite of its stronger reactivity toward GSH, IAA appears to be a less potent inhibitor of astrocytic glycolysis than IA. Already low concentrations of IA were highly efficient to inactivate GAPDH and to prevent lactate production by cultured astrocytes. Different rates of transport of IAA and IA can be excluded as explanation of this observation, since IA was also much more potent than IAA to inactivate GAPDH in cell lysates and since the quick deprivation of cellular GSH clearly demonstrates that IAA is efficiently taken up by the cells.

Reasons for this difference of IA and IAA to inactivate GAPDH could be that the active center of the enzyme binds the negatively charged IA better than the neutral IAA and/or that for sterical reasons IA fits better than IAA into the active side of GAPDH in an orientation that facilitates the alkylation of the thiol group in the active center. Alternatively, the alkylation of the astrocytic GAPDH by IAA and IA could be inversely modulated by the GAPDH cosubstrate NAD^+. At least for yeast GAPDH was reported that the presence of NAD^+ supports alkylation by IA but inhibits alkylation by IAA (Byers and Koshland Jr., 1975). Such a behavior would explain the different potential of IAA and IA to inhibit GAPDH in astrocytic lysates, since the NAD^+ concentration in the GAPDH activity assay used was 2 mM, a concentration that has been reported to support alkylation of yeast GAPDH by IA and to suppress alkylation by IAA (Byers and Koshland Jr., 1975). With a specific NAD^+ content of about 1.5 nmol/mg protein (X. Chen and R. Dringen, unpublished results) and a cytosolic volume of 4.1 μL/mg protein (Dringen and Hamprecht, 1998), the cytosolic NAD^+ concentration of cultured astrocytes is about 0.4 mM. This concentration of NAD^+ may already be sufficient to support alkylation of GAPDH from IA in living astrocytes, whereas the alkylation from IAA is slowed down.

Regarding the inhibition of enzymes in viable astrocytes, IAA and IA appear to be remarkable specific for GAPDH. The highly reactive thiol group in the active center of GAPDH appears to be an excellent target to become alkylated by these thiol reagents. In contrast, other dehydrogenases such as G6PDH or LDH were not inactivated by exposure of astrocytes to 1 mM of IAA or IA. Only the cellular GR activity was partly inactivated by IAA. This slow but significant inactivation of GR confirms literature data that demonstrate that at least purified GR is sensitive to inactivation by IAA (Arscott et al., 1981; Pai and Schulz, 1983; Untucht-Grau et al., 1981). Thus, the quick depletion of cellular GSH appears to be the strongest side effect of a treatment of cultured astrocytes with 1 mM of IAA or IA.

Incubation of cultured astrocytes with IAA or IA caused severe cell death after incubation of the cells for more than 60 min with the thiol reagents. Both the inhibition of glycolysis and the decline in cellular GSH could contribute to the observed toxicity of IAA and IA after longer exposure to astrocytes. Since cultured astrocytes are able to survive depletion of their cellular GSH for several hours (Waak and Dringen, 2006), the cellular GSH deprivation appear not to be the primary cause of the delayed cell death observed after treatment with the thiol reagents. It is more likely that the depletion of cellular ATP that has been reported for cultured brain cells at least for the treatment with IA (Hernandez-Fonseca et al., 2008; Nodin et al., 2005; Ogata et al., 1995) is responsible for the observed cell death after exposure to IAA or IA.

IAA and IA are frequently used to slow glycolysis by inhibiting the GAPDH in cultured astrocytes. For such studies millimolar concentrations of IAA or IA have been used for incubation periods above 5 min [10 mM IAA for 2.5 h (Loreck et al., 1987), 3.5 mM IA for 15 min (Bickler and Kelleher, 1992), 1 mM IA for 30 min (Gemba et al., 1994), 5 mM IA for 30 min (Ogata et al., 1995)]. In our hands, such conditions deprive cultured astrocytes quickly of GSH. Thus, a contribution of a compromised cellular GSH metabolism to the reported effects observed after treatment of cultured astrocytes with millimolar concentrations of IAA or IA has to be considered. Since IAA lowers quickly the cellular GSH content of cultured astrocytes, we recommend not to use this compound for inactivation of GAPDH and glycolysis. In contrast to IAA, IA lead to a quick inactivation of GAPDH activity and slows down lactate production, without affecting the cellular GSH content substantially. Therefore, we suggest to use a low concentration of IA (0.1 mM) for 30 min or a high concentration of IA (1 mM) for 5 min to inhibit glycolysis in cultured astrocytes, since these conditions do not substantially compromise the GSH metabolism of the cells.

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2.3

Publication 3

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Fumaric acid diesters deprive cultured primary astrocytes rapidly of glutathione

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ABSTRACT

Fumaric acid esters (FAE) are used for the systemic therapy of psoriasis and are now considered for the treatment of autoimmune-based neurological disorders such as multiple sclerosis. Currently, the cellular metabolism of FAE as well as the mechanisms of their therapeutic action are poorly understood. Since cellular glutathione (GSH) is involved in the detoxification of xenobiotics, we analysed the consequences of an application of FAE on the content of GSH in brain cells using astroglia-rich primary cultures as model system. Micromolar concentrations of dimethyl fumarate (DMF) or diethyl fumarate (DEF) lowered the cellular GSH content in a time- and concentration-dependent manner. Halfmaximal effects after 60 min of incubation were observed for 10 μM DMF or DEF. In contrast to the diesters, monomethyl fumarate (MMF), monoethyl fumarate (MEF) or fumarate had to be applied in concentrations of 10 mM for 60 min to significantly lower the cellular GSH content. During 60 min exposure, DMF or DEF did not significantly affect the cell viability, increase the cellular content of glutathione disulfide, nor altered the specific activities of glucose-6-phosphate dehydrogenase, glutathione reductase, or lactate dehydrogenase. After removal of DMF or DEF, cultured astrocytes restored their cellular GSH content completely within 4 h. These data demonstrate that acute exposure to fumaric acid diesters deprives astrocytes of their GSH, most likely by the reaction of the reactive α,β-unsaturated diesters with GSH.

1. Introduction

Fumaric acid esters (FAE) are successfully used to treat psoriasis, a wide spread chronic inflammatory skin disorder, and are now considered as drugs to treat autoimmune-based neurological disorders such as multiple sclerosis (Linker et al., 2008a; Yazdi and Mrowietz, 2008; Moharregh-Khiabani et al., 2009). However, the underlying mechanisms of the biological action of FAE are not fully understood. An involvement of a modulation of T-cell numbers, the initiation of apoptosis, changes in cytokine production, the inhibition of NFκB activity and the interference with cellular redox systems have been suggested as molecular basis of FAE functions (Mrowietz and Asadullah, 2005; Moharregh-Khiabani et al., 2009). For brain cells, dimethyl fumarate (DMF) gained interest as inducer of antioxidative/electrophilic response elements which modulate the expression of various enzymes including glutamate cysteine ligase, glutathione-S-transferases and NAD(P)H:quinone reductase (Murphy et al., 2001; Wierinckx et al., 2005).

The tripeptide GSH (γ-L-glutamyl-L-cysteinylglycine) is the most abundant cellular thiol that is present in millimolar concentrations in mammalian cells (Cooper and Kristal, 1997). GSH possess a variety of functions in the cells of which the antioxidative and detoxifying functions are most likely the most important ones. During the reduction of peroxides by glutathione peroxidases, GSH serves as electron donor (Margis et al., 2008). In addition, GSH is substrate for the detoxification of xenobiotics in glutathione-S-transferase catalysed reactions (Sies and Packer, 2005; Awasthi, 2007).

In the brain, astrocytes play a key role in the antioxidative defence and in the detoxification of xenobiotics (Cooper and Kristal, 1997; Cooper, 1998; Dringen, 2000, 2009; Aoyama et al., 2008; Ballatori et al., 2009). Primary cultures of astrocytes contain GSH in a cytosolic concentration of 8 mM (Dringen and Hamprecht, 1998). This high GSH concentration is crucial for the rapid clearance of peroxides (Dringen et al., 2005; Liddell et al., 2006a,b), for the resistance against oxidative stress (Gegg et al., 2003, 2005; Bishop et al., 2007; Bi et al., 2008; Giordano et al., 2008), and for the GSH-dependent detoxification of xenobiotics (Sagara and Sugita, 2001; Kubatova et al., 2006; Waak and Dringen, 2006). In addition, the cellular concentration of GSH determines the release rate of GSH (Sagara et al., 1996). Since export of GSH from astrocytes is the essential first step in the supply of GSH precursors from astrocytes

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to neurons (Dringen, 2009), any modulation of the cytosolic GSH concentration of astrocytes will also affect the GSH synthesis of neurons.

Due to their structural properties as α,β-unsaturated electrophilic systems, FAE react with nucleophiles such as GSH (Boyland and Chasseaud, 1967; Kubal et al., 1995; Schmidt et al., 2007). Thus, a spontaneous or enzyme catalysed reaction of FAE with GSH is likely to occur in cells that encounter such compounds. Indeed, for several cell types including cultured lipopolysaccharide (LPS)-activated mixed glial cultures (Dethlefsen et al., 1988; Held et al., 1988; Nelson et al., 1999; Wierinckx et al., 2005), a decline in cellular GSH content after exposure to DMF has been reported. Since the consequences of a treatment with different types of FAE on the GSH metabolism of brain cells have not been reported, we have investigated how an acute exposure of cultured astrocytes to fumaric acid monoesters and diesters affects the GSH content of these cells. Here we report that fumaric acid diesters, but not the monoesters, are highly potent to deprive viable astrocytes in culture of their GSH.

2. Materials and methods

2.1. Material

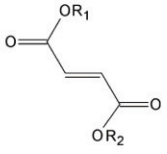
Buthionine sulfoximine (BSO), fumaric acid (FA), monomethyl fumarate (MMF), monoethyl fumarate (MEF), dimethyl fumarate (DMF) and diethyl fumarate (DEF) were obtained from Sigma–Aldrich (Steinheim, Germany). Glucose-6-phosphate, GSH, glutathione disulfide (GSSG), glutathione reductase, glyceraldehyde-3-phosphate, maleimide and potassium arsenate were from Roche Diagnostics (Mannheim, Germany). Bovine serum albumin, NAD^+, NADH, $NADP^+$, NADPH and sulfosalicylic acid were purchased from Applichem (Darmstadt, Germany). Fetal calf serum, streptomycin sulfate and penicillin G were from Biochrom (Berlin, Germany). Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen (Karlsruhe, Germany). All other chemicals were obtained from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany) at analytical grade. Sterile cell culture material and unsterile 96-well plates were from Nunc (Roskilde, Denmark) and Sarstedt (Karlsruhe, Germany).

2.2. Cell cultures

Astroglia-rich primary cultures derived from the whole brains of neonatal Wistar rats were prepared according to a published method (Hamprecht and Löffler, 1985). Three hundred thousand viable cells were seeded in 1 mL culture medium (90% DMEM, 10% fetal calf serum, 20 units/mL of penicillin G, 20 μg/mL of streptomycin sulfate) per well of a 24-well dish and cultured in a cell incubator (Sanyo, Osaka, Japan) that contained a humidified atmosphere of 10% CO_2/90% air. The culture medium was renewed every seventh day. The results described here were obtained on 15–21-day-old cultures. In this range, the investigated cellular parameters of the cultures did not significantly differ as a function of the culture age. The astrocyte-rich cultures used comprise more than 90% astrocytes, and contain small fractions of oligodendroglial, ependymal, and microglial cells but no neurons (Löffler et al., 1986; Reinhart et al., 1990; Gutterer et al., 1999; Dang et al., 2010).

2.3. Experimental incubation of cells

To study the consequences of a treatment of astrocytes with FAE, the cells were washed with 1 mL of prewarmed (37 °C) incubation buffer (IB; 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.4 mM KCl, 145 mM NaCl, 0.8 mM Na_2HPO_4, 20 mM HEPES, 5 mM D-glucose, pH 7.4) and incubated at 37 °C with 0.5 mL IB containing fumaric acid or its esters (Fig. 1) in the concentrations given in the figures or tables. For analysis of the total cellular glutathione content (GSx = amount of GSH plus twice the amount of GSSG), the cells were washed with 1 mL phosphate-buffered saline (PBS; 10 mM potassium phosphate buffer, 150 mM NaCl, pH 7.4) and lysed with 0.5 mL 1% (w/v) sulfosalicylic acid. Ten microliter aliquot parts of the lysates were used to quantify the cellular GSx content.



R_1	R_2	
- H	- H	fumaric acid
- CH_3	- H	monomethyl fumaric acid
- CH_2CH_3	- H	monoethyl fumaric acid
- CH_3	- CH_3	dimethyl fumaric acid
- CH_2CH_3	- CH_2CH_3	diethyl fumaric acid

Fig. 1. Structural formulas of fumaric acid and fumaric acid esters.

2.4. Cell-independent reaction of GSH with DMF

GSH (10 μM) was incubated with the indicated concentrations of DMF in a total volume of 1 mL IB at room temperature for up to 60 min. At the time points indicated, 10 μL sample of the reaction mixture was mixed with 10 μL 1% sulfosalicylic acid before the GSx content was determined.

2.5. Determination of glutathione

The contents of GSx and GSSG in cell lysates and incubation media were determined as described previously (Dringen and Hamprecht, 1996; Dringen et al., 1997) in microtiter plates according to the colorimetric method originally described by Tietze (1969). The detection limit of this assay was about 0.2 nmol GSx per 500 μL lysate or medium.

2.6. Determination of enzyme activities

The activities of the enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glucose-6-phosphate dehydrogenase (G6PDH), glutathione reductase (GR) and lactate dehydrogenase (LDH) were determined in lysates of astrocyte cultures as described previously (Schmidt and Dringen, 2009). Specific activities were calculated by normalizing the activities determined for cell lysates to the protein contents of the respective lysates.

2.7. Determination of cell viability and protein content

Loss of cell viability was analysed by comparing the activity of LDH in the incubation medium with that of the cells using the microtiter plate assay described previously (Dringen et al., 1998). The presence of fumarate and its esters even in the highest concentration used did not affect the determination of LDH activity in cell lysates of cultured astrocytes (data not shown), excluding that the presence of FAE may prevent the detection of extracellular LDH. The total protein content per well of a 24-well dish was quantified according to the Lowry method (Lowry et al., 1951) after solubilisation of the cells in 200 μL of 0.5 M NaOH, using bovine serum albumin as a standard. Total protein content per well and protein in the supernatants of cell lysates were used to calculate specific GSx or GSSG contents and specific enzyme activities, respectively.

2.8. Presentation of the data

If not stated otherwise, the data are presented as means ± standard deviation (SD) of values obtained in experiments on at least three independently prepared cultures. In the figures, the bars have been omitted, if they were smaller than the symbols representing the mean values. Statistical analysis of the significance of differences between multiple sets of data was performed by ANOVA followed by Bonferroni's post hoc test ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). $p > 0.05$ was considered as not significant.

3. Results

3.1. Consequences of a treatment of cultured astrocytes with DMF

To test for the consequences of a treatment of cultured astrocytes with DMF, the cells were incubated with various concentrations of this compound for up to 4 h. During the first 2 h of incubation with DMF in concentrations of up to 100 μM, the cells remained viable as indicated by the lack of any increase in extracellular LDH activity ($p > 0.05$) compared to control (absence of DMF) (Fig. 2A) and by the absence of any obvious alteration in cell morphology (data not shown). However, incubation of astrocytes with DMF in concentrations higher than 10 μM for longer than 2 h caused a significant increase in extracellular LDH activity, that was strongest for the highest concentration of DMF applied. In contrast to the delayed cell death observed after exposure of astrocytes to DMF (Fig. 2A), the presence of DMF caused a rapid and highly significant decline in cellular GSx content already during the first 60 min of incubation (Fig. 2B). While exposure with 10 μM DMF for 60 min deprived the cells of half of the cellular GSx content, higher concentrations of DMF caused an almost complete disappearance of GSx from the cells within 60 min (Fig. 2B).

3.2. Cell-independent reaction of DMF with GSH

To test whether DMF reacts with GSH in a cell-independent reaction, 10 μM of GSH was mixed with different concentrations of

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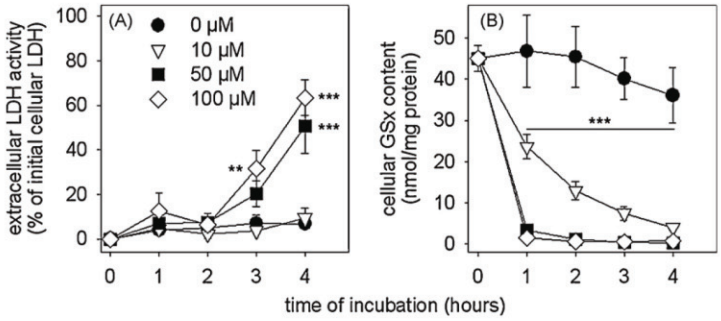

Fig. 2. Effects of DMF on cell viability (A) and cellular GSx content (B) of cultured astrocytes. The cells were incubated without or with DMF in the concentrations indicated in (A) for up to 4 h. The results represent means ± SD of data obtained on three independently prepared cell cultures that contained 82 ± 4 µg total protein per well and an initial specific cellular LDH activity of 723 ± 17 nmol/(min mg total protein). The significance of differences to the data obtained for the control condition (absence of DMF) are indicated as $^{**}p < 0.01$ or $^{***}p < 0.001$.

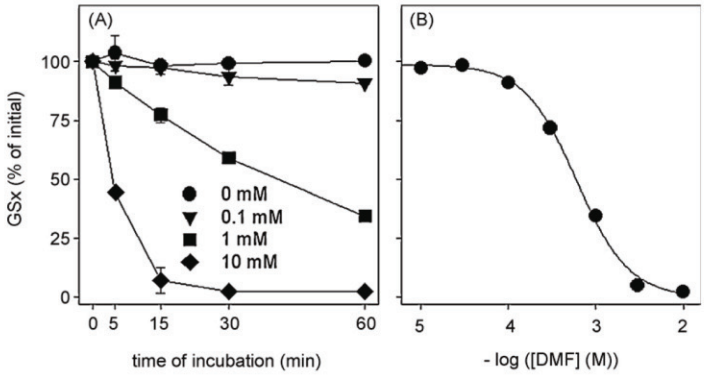

Fig. 3. Cell-independent disappearance of GSH after exposure to DMF. GSH in a concentration of 10 µM (100%) was incubated with the indicated concentrations of DMF for up to 60 min. (A) The amount of GSx that was detectable at the indicated time of incubation. (B) The GSx content that was detectable after 60 min incubation with the given concentrations of DMF. The data shown represent means ± SD of six values that were obtained in two independent experiments each performed in triplicates with individually prepared solutions.

DMF and the amount of detectable GSx was determined during an incubation period of up to 60 min. Presence of DMF lowered the amount of detectable GSx in a time- and concentration-dependent manner (Fig. 3). While presence of 100 µM caused only a small (10%) but significant ($p < 0.01$) loss in detectable GSx during 60 min incubation, 1 mM DMF lowered the amounts of detectable GSx by about 70% within 60 min, whereas hardly any GSx was detectable already after about 15 min of incubation with 10 mM DMF (Fig. 3A). The halfmaximal GSx-lowering effect after 60 min of incubation was observed for a concentration of about 600 µM DMF (Fig. 3B). Incubation of GSH with DEF caused a similar decline of the detectable GSx content, whereas presence of FA, MMF or MEF in concentrations of up to 10 mM did not lower the amount of detectable GSx (data not shown).

3.3. Consequences of a treatment of astrocytes with various fumaric acid esters

To directly compare the effects of different FAE (Fig. 1) on cultured astrocytes, the cells were exposed to the compounds for up to 60 min. In a concentration of 100 µM, none of the investigated compounds caused any significant increase in extracellular LDH activity during 60 min of incubation compared to controls (Table 1) and none of these conditions altered the morphology of the cells (data not shown). In contrast, the presence of the fumaric acid diesters DMF or DEF caused within 60 min an almost complete disappearance of cellular GSx to less than 5% of that of controls (absence of FAE), whereas fumaric acid and the monoesters MMF or MEF in concentrations of 100 µM did not affect cellular GSx content (Table 1). None of the compounds investigated elevated the cellular GSSG content significantly compared to the values obtained for the control condition (absence

Table 1
Cellular and extracellular GSx and GSSG contents and extracellular LDH activity of cultured astrocytes after exposure to fumarate or fumaric acid esters.

Compound	GSx (nmol/mg protein)		GSSG (nmol/mg protein)		LDH (% of intial)
	Cells	Media	Cells	Media	
none	33.0 ± 5.3	5.0 ± 2.5	2.3 ± 1.3	2.2 ± 1.3	6 ± 3
FA	32.1 ± 5.7	5.0 ± 2.0	2.3 ± 1.1	1.7 ± 1.0	8 ± 2
MMF	32.3 ± 5.7	4.8 ± 1.9	2.4 ± 1.2	1.8 ± 1.1	6 ± 4
MEF	32.1 ± 7.8	5.1 ± 2.4	2.3 ± 1.3	2.0 ± 1.2	6 ± 4
DMF	2.0 ± 1.4***	2.7 ± 1.8	1.8 ± 1.2	1.8 ± 1.2	9 ± 6
DEF	2.4 ± 1.7***	3.1 ± 1.7	2.5 ± 1.6	1.9 ± 1.2	5 ± 2

The cells were incubated in the absence (none) or the presence of 100 µM of FA or of the indicated FAE. The cellular and extracellular contents of GSx and GSSG and the extracellular LDH acitivity were determined after 60 min of incubation. The results represent means ± SD of data that were obtained on five independently prepared cultures that contained 87 ± 21 µg protein per well, an initial GSx content of 34.5 ± 8.0 nmol/mg protein, and an initial specific cellular LDH activity of 741 ± 38 nmol/(min mg total protein). The significance of differences to the data obtained for the control (none) are indicated as $^{***}p < 0.001$.

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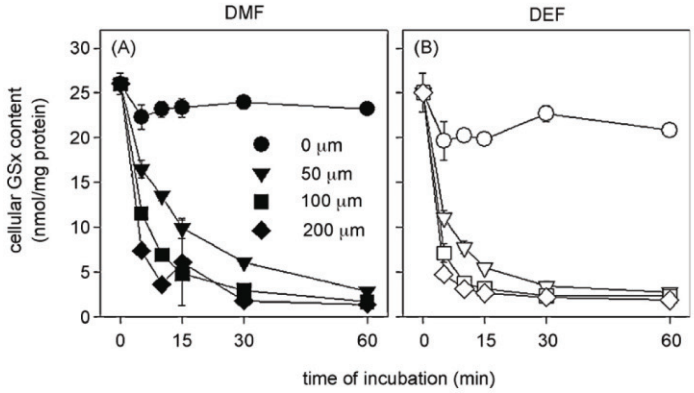

Fig. 4. Consequences of an application of DMF or DEF on the specific cellular GSx content of astrocytes. The cells were incubated for up to 60 min without or with DMF (A) or DEF (B) in the concentrations indicated in (A). The results represent data (means ± SD of triplicates) of representative experiments that were reproduced on at least two independently prepared cultures with comparable results. The cultures contained initial total protein contents of 145 ± 6 µg per well (A) and 120 ± 5 µg per well (B).

of FAE) (Table 1). The extracellular GSx content after 60 min of incubation, a part of which represented GSSG, was less than 20% of the initial cellular GSx content for all conditions applied (Table 1).

The application of DMF or DEF to cultured astrocytes caused a rapid concentration-dependent decline in the specific cellular GSx content that resulted after 10 min of incubation with the highest concentration applied (200 µM) in cellular GSx levels lower than 20% of controls (absence of DMF and DEF) (Fig. 4A and B). 60 min of incubation with concentrations of DMF or DEF between 50 and 200 µM caused almost complete disappearance of cellular GSx (Fig. 4). The concentrations causing halfmaximal deprivation of the specific cellular GSx content after exposure of cultured astrocytes for 60 min to DMF or DEF were about 10 µM for both compounds (Fig. 5A). In contrast, cellular GSx contents remained unaltered for 60 min incubation in the presence of FA, MMF, or MEF in concentrations of up to 1 mM (Fig. 5B), and were only significantly lowered after exposure to one of these compounds in a concentration of 10 mM (Fig. 5B). None of the conditions applied caused any significant increase in the extracellular LDH activity compared to control conditions (no FAE) (Fig. 5C and D). Application of DMF or DEF in concentrations of 100 µM for 60 min did not cause any significant alteration in the specific

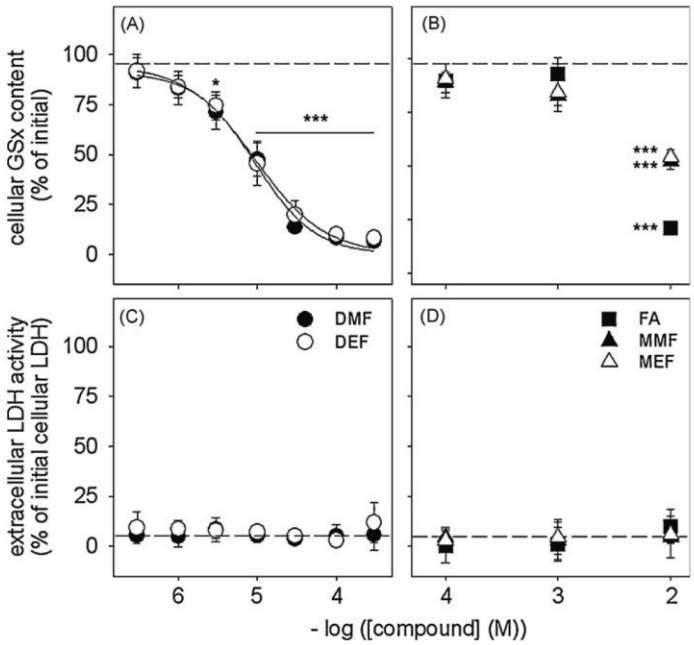

Fig. 5. Consequences of an application of DMF or DEF (A and C) and fumarate (FA), MMF or MEF (B and D) on the specific cellular GSx content (A and B) and the viability (C and D) of cultured astrocytes. The cells were incubated for 60 min with the indicated concentrations of FAE or FA. The results represent means ± SD of data that were obtained on three independently prepared cultures that contained 73 ± 17 µg total protein per well. The initial GSx content (100%) corresponded to 33.8 ± 5.6 nmol/mg protein and the initial specific cellular LDH activity to 659 ± 119 nmol/(min mg total protein). Dashed lines represent the control values (no FAE) and correspond to 93 ± 6% (A and B) and 5 ± 1% (C and D). The significance of differences to the data obtained for the control condition (absence of fumarate and FAE) are indicated as $^{*}p < 0.05$ and $^{***}p < 0.001$.

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Table 2
Specific activities of enzymes in cultured astrocytes after exposure to DMF or DEF.

Enzyme	Compound	Enzyme activity (nmol/(min × mg))
G6PDH	None	37.3 ± 6.5
	DMF	32.8 ± 17.5
	DEF	27.5 ± 6.1
GR	None	27.4 ± 9.6
	DMF	18.3 ± 5.3
	DEF	28.1 ± 3.4
LDH	None	1569 ± 74
	DMF	1397 ± 113
	DEF	1330 ± 66
GAPDH	None	324 ± 10
	DMF	263 ± 33*
	DEF	252 ± 20*

The cells were incubated in the absence (none) or presence of 100 μM DMF or DEF. After 60 min of incubation, the cells were lysed and the specific activities of the enzymes glucose-6-phosphate dehydrogenase (G6PDH), glutathione reductase (GR), lactate dehydrogenase (LDH), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined. The results represent means ± SD of data that were obtained on three independently prepared cell cultures that contained 59 ± 3 μg soluble protein per well. The significance of differences to the data obtained for the control (none) are indicated as $^{*}p < 0.05$.

activities of the cellular enzymes G6PDH, GR and LDH (Table 2). However, the specific GAPDH activity was significantly decreased after 60 min incubation with 100 μM DMF or DEF to about 80% of controls (Table 2).

3.4. Restoration of cellular GSH after removal of fumaric acid diesters

To investigate whether GSH synthesis is affected by DMF or DEF, cultured astrocytes were preincubated with 100 μM DMF or DEF for 30 min, the cells were washed with incubation medium and subsequently incubated for a recovery period of up to 8 h in serum-free DMEM without DMF or DEF. The preincubation for 30 min with 100 μM DMF or DEF significantly lowered the cellular GSx content by more than 80% ($p < 0.001$, Fig. 6A and B), as shown earlier (Fig. 4A and B). After removal of DMF or DEF, the cells restored their initial GSx content completely within 4 h of incubation (Fig. 6A). In contrast, if the glutamate cysteine ligase inhibitor BSO was present during the restoration period after removal of fumaric acid diesters, the restoration of cellular GSx was prevented (Fig. 6B). In addition, the cellular content of cells that were preincubated without FAE declined in the presence of BSO significantly to 37% and 21% of the initial content during incubation for 4 and 8 h, respectively (Fig. 6B). None of the conditions applied caused any significant increase in extracellular LDH activity (data not shown), demonstrating that the cells remained viable during these incubations.

Treatment of cultured astrocytes with 100 or 200 μM of DMF for 60 min became cell toxic after removal of DMF and subsequent incubation in medium for 24 h as indicated by a significant increase in extracellular LDH activity (data not shown). However, if the cells were exposed to 50 μM DMF for 60 min, the cell viability was not compromised during a subsequent incubation in DMEM (data not shown) and the specific cellular GSx content increased within 24 h significantly from 28.6 ± 2.6 nmol GSx/mg to 36.8 ± 4.0 nmol GSx/mg ($p < 0.05$; $n = 3$ individual experiments).

4. Discussion

FAE have been reported to react with thiols such as GSH in cell-free systems (Kubal et al., 1995; Frycak et al., 2005; Schmidt et al., 2007). Since FAE are discussed as promising drug for the treatment of autoimmune-based neurological disorders such as multiple sclerosis (Kappos et al., 2008; Linker et al., 2008b; Moharregh-Khiabani et al., 2009) and since the interference of these compounds with the cellular thiol reduction systems has been discussed as potential therapeutical mode of action (Held et al., 1988; Pellmar et al., 1992; Barchowsky et al., 1996; Nelson et al., 1999; Wierinckx et al., 2005), we have investigated the acute consequences of an exposure of cultured astrocytes to FAE. The dialkyl esters DMF and DEF in micromolar concentrations deprived the cells quickly of GSH. This observation confirms literature data for DMF on various types of cells including LPS-treated mixed glial cultures (Dethlefsen et al., 1988; Held et al., 1988; Nelson et al., 1999; Wierinckx et al., 2005).

The observed GSH deprivation in cultured astrocytes by DMF or DEF is unlikely to be the consequence of a loss in membrane integrity, at least for incubation periods of up to 2 h, since no significant increase in extracellular LDH activity or in extracellular GSx content was observed after exposure to DMF or DEF. In addition, since the amounts of extracellular GSx and GSSG were not significantly increased in the presence of DMF or DEF, and since 100 μM of the diesters were unable to mask low concentrations of GSH by chemical reactions in a cell-free system, a stimulation of GSH export by DMF or DEF via multidrug resistance protein 1 (Hirrlinger et al., 2001; Minich et al., 2006) can be excluded to explain the loss of cellular GSx. Moreover, since no elevation in the

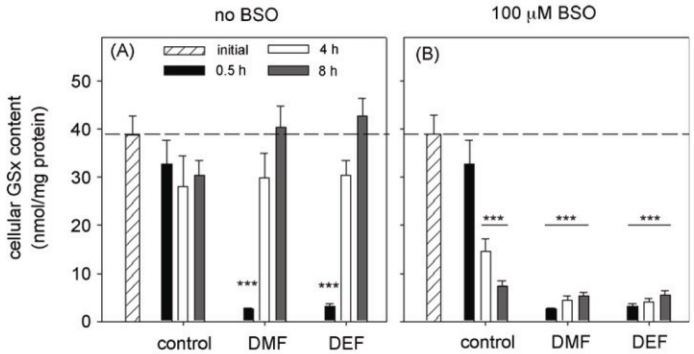

Fig. 6. Consequences of a pretreatment of cultured astrocytes with fumaric acid diesters on GSH synthesis. The cells were preincubated (0.5 h) in the absence (control) or the presence of 100 μM DMF or DEF for 30 min. The media were replaced by DMEM without (A) or with (B) 100 μM of the GSH synthesis inhibitor BSO and the cellular GSx content was monitored after 4 or 8 h. The results represent means ± SD of data that were obtained on three independently prepared cultures that contained 108 ± 8 μg total protein per well. The significance of differences to the data obtained for the initial value are indicated as $^{***}p < 0.001$.

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contents of cellular or extracellular GSSG was found for diester-treated cells, oxidative stress and accelerated GSSG export can also be excluded as a consequence of FAE-treatment.

The decline in cellular GSH content appears to be quite specific for the diesters of fumaric acid, since neither MMF nor MEF or fumarate in micromolar concentrations affected the cellular GSx content. One reason for this difference to the uncharged diesters is most likely the low efficiency of transport of the charged monoesters and of fumarate over the plasma membrane of astrocytes. This hypothesis is supported by the observation that the cellular GSH content of astrocytes was lowered at least to some extent after exposure of the cells to very high concentrations (10 mM) of the monoesters.

The glutamate cysteine ligase inhibitor BSO lowered the cellular GSx content with a halftime of around 4 h as previously reported (Devesa et al., 1993). The velocity of GSx deprivation by fumaric acid diesters was much faster than that observed for BSO, demonstrating that a simple inhibition of GSH synthesis by the diesters cannot be responsible for the disappearance of GSH from the cells. In addition, since astrocytes were able to restore their cellular GSx content after removal of the diesters, the synthesis of GSH from its substrate amino acids appears not to be irreversibly affected by the presence of such compounds. This interpretation is strongly supported by literature data demonstrating that after an initial decline in cellular GSx content after incubation of LPS-treated mixed glial cells with 30 μM DMF the cellular GSx content increased within 24 h to about 150% of the initial content (Wierinckx et al., 2005). This elevation of cellular GSx content was confirmed for astrocytes that were incubated for 24 h in DMEM after exposure for 60 min to 50 μM DMF.

GSH disappeared in a cell-free system in a time- and concentration-dependent manner in the presence of fumaric acid diesters. Since FAE are α,β-unsaturated carbonyl systems, nucleophiles such as GSH react enzyme-independently with the double bond of the FAE in a Michael-type of addition. Such reactions with GSH to the corresponding thioethers have been reported in cell-independent studies for fumaric acid diesters at physiological pH (Kubal et al., 1995; Schmidt et al., 2007). The lack of any GSH reaction with fumaric acid monoesters under conditions used is consistent with the much slower reaction of such compounds with GSH compared to the reaction of the diesters (Frycak et al., 2005; Schmidt et al., 2007). The higher reactivity of the diesters compared to monesters or fumaric acid towards GSH has been discussed as a consequence of the stronger electron withdrawing mesomeric effect (−M effect) of the methyl ester group compared to the carboxyl group (Frycak et al., 2005). These data suggest that also the quick deprivation of cellular GSx after exposure to DMF or DEF is most likely the consequence of a conjugation process with GSH. Since the deprivation of cellular GSH was much quicker than that observed in a cell-independent reaction, the disappearance of cellular GSH is likely to be accelerated by glutathione-S-transferases, since such enzymes are known to catalyse the reaction of GSH with α,β-unsaturated carbonyls (Mahajan and Atkins, 2005).

Exposure of cultured astrocytes to DMF in concentrations that quickly deprived the cells completely of GSH was toxic for the cells as indicated by the strong increase of extracellular LDH activity that was observed after a few hours of incubation. Thus, although acute GSH depletion does not immediately compromise cell viability, prolonged depletion of GSH by DMF becomes toxic. This situation is similar to that described for cultured astrocytes that had been deprived of GSH by incubation with monochlorobimane (Waak and Dringen, 2006). Since astrocytes are able to survive complete GSH deprivation for some hours, the cellular GSH deprivation itself appears not to be the primary cause of the delayed cell death observed after treatment with DMF. Potential reasons for this delay could be the impaired antioxidative potential of the GSH-deprived cells or the reaction of DMF with other cellular thiols that will subsequently impair cellular functions. At least in *in vitro* studies the interaction of DMF with thiol-containing peptides has been shown (Frycak et al., 2005). Such reactions may impair enzyme functions that would compromise cellular functions and could subsequently lead to the delayed toxicity observed. One cellular enzyme that was affected in its activity by treatment with DMF or DEF is GAPDH. The inactivation of GAPDH by fumaric acid diesters might be a direct consequence of the Michael-like addition of DMF or DEF to the cysteine residue in the active center of the enzyme as it has been demonstrated for the succination of GAPDH by fumarate in diabetes (Blatnik et al., 2008a,b). An inactivation of GAPDH activity of astrocytes by 20%, as found after 60 min incubation of astrocytes with DMF or DEF, does not affect lactate production in the cells (Schmidt and Dringen, 2009). However, a further inactivation of GAPDH activity by prolonged exposure of cells to dialkyl fumarates could compromise glycolytic flux as it has been shown for other inhibitors of GAPDH (Schmidt and Dringen, 2009) and chronic peroxide stress (Liddell et al., 2009). DMF and DEF appear to be quite specific to react with highly reactive thiol groups such as those of GSH or GAPDH. At least the activity of several other cellular enzymes such as LDH, G6PDH or GR was not affected by the presence of DMF or DEF in micromolar concentrations.

One important function of astrocytes is the supply of neurons with precursors for GSH synthesis. This metabolic cooperation involves export of GSH by multidrug resistance protein 1 and extracellular processing of the released GSH to cysteine by the ectoenzymes γ-glutamyl transpeptidase and aminopeptidase N (Dringen, 2009). Since the export of GSH from astrocytes depends strongly on the cellular GSH concentration (Sagara et al., 1996), all compounds and conditions that modulate the astrocytic GSH concentration will severly affect GSH release and subsequently also the supply of GSH precursors to neurons. Alterations of cellular GSH concentrations and lowered GSH release have been observed for astrocytes that were exposed to dopamine (Hirrlinger et al., 2002), chronic oxidative stress (Minich et al., 2006), monochlorobimane (Waak and Dringen, 2006) or alkylating reagents such as iodoacetamide and iodoacetate (Schmidt and Dringen, 2009). Thus, also deprivation of astrocytic GSH content by DMF or DEF will lower GSH export and subsequently the supply of extracellular GSH precursors to neurons. In contrast, an increase in cellular GSH would cause an accelerated GSH export from astrocytes. This situation is likely to occur after longer exposure to subtoxic concentrations of DMF (Wierinckx et al., 2005). An elevated cellular GSH content of astrocytes will accelerate GSH export (Sagara et al., 1996) and thereby increase the availability of GSH precursors for neurons. This increased supply of GSH precursors could contribute – in addition to the induction of antioxidative enzymes (Wierinckx et al., 2005) – to the observed neuroprotective function of fumarate diesters (Lukashev et al., 2008).

In conclusion, fumaric acid diesters deprive cultured astrocytes quickly of their cellular GSH, most likely due to the reaction of the nucleophile GSH with the double bond of the diesters. Since astrocytes have key functions in the detoxification of reactive oxygen species and xenobiotics in brain (Cooper, 1998; Benarroch, 2005; Dringen, 2009), the exposure of such cells to fumaric acid diesters could become critical for such GSH-dependent processes. These complications should be considered for situations when brain cells may encounter fumaric acid diesters. It can be assumed that fumaric acid diesters as uncharged hydrophobic compounds can pass the blood brain barrier, if these compounds could reach the brain in sufficiently high concentrations. However, at least for oral application of DMF this situation is unlikely to occur, since DMF is quickly hydrolysed in the periphery to MMF. After

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application of a single tablet dose of 120 mg DMF (plus 95 mg MEF), MMF was transiently detectable in the serum of test subjects in concentrations of less than 10 μM, while DMF was not detectable in the serum (Litjens et al., 2004). Whether the charged MMF would be able to pass the blood brain barrier remains to be elucidated. Nevertheless, cultured astrocytes appear to be a good model system to investigate the consequences of a treatment of brain cells with FAE. The results presented here demonstrate that at least MMF, which has been considered to be the most effective immunomodulating FAE (Nibbering et al., 1993), does not acutely affect the GSH metabolism of cultured astrocytes.

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2.4

Publication/Manuscript 4

Effects of chlorinated acetates
on the glutathione metabolism and on glycolysis
of cultured astrocytes.

Maike M. Schmidt, Astrid Rohwedder & Ralf Dringen

Neurotoxicity Research, revised manuscript submitted

Contributions of Maike M. Schmidt:

- Establishment of all experimental paradigms and conditions
- Data shown in Figures 3, 4 & 5 and Tables 2 & 3
- Preparation of the first draft of the manuscript

The data shown in Figures 1 & 2 and Table 1 were obtained by A. Rohwedder during a lab rotation that was supervised by M. M. Schmidt.

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Effects of chlorinated acetates on the glutathione metabolism and on glycolysis of cultured astrocytes

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Abstract

The chlorinated acetates monochloroacetate (MCA), dichloroacetate (DCA) and trichloroacetate (TCA) are generated in water disinfection processes and are formed during metabolic detoxification of industrial solvents such as trichloroethylene. To test for consequences of an exposure of brain cells to the different chlorinated acetates, glutathione levels and lactate production of primary astrocyte cultures were investigated as indicators for the potential of chlorinated acetates to disturb cellular detoxification processes and glucose metabolism, respectively. Application of MCA to cultured astrocytes caused a time and concentration dependent deprivation of cellular glutathione, inactivation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity, and loss in cell viability with halfmaximal effects observed for MCA concentrations between 0.3 mM and 3 mM. In contrast, presence of acetate, DCA, or TCA in a concentration of 10 mM did not compromise cell viability nor affect cellular glutathione content or GAPDH activity. However, presence of DCA and TCA significantly lowered the rate of cellular lactate production in viable astrocytes. These data demonstrate that the extent of chlorination strongly determines the potential of chlorinated acetates to disturb glutathione and/or glucose metabolism of astrocytes.

Keywords

monochloroacetate, dichloroacetate, trichloroacetate, glyceraldeyde-3-phosphate dehydrogenase, detoxification

1 Introduction

Monochloroacetate (MCA), dichloroacetate (DCA) and trichloroacetate (TCA) are environmental toxins that are produced during water disinfection processes in reaction of chlorine with humic and fulvic acids [1]. In addition, DCA and TCA are metabolites that are generated during detoxification of industrial solvents such as perchloroethylene or trichloroethylene [2, 3]. The adverse potential of the different chlorinated acetates to affect mammals is already known for many decades [4-8]. However, on the cellular level, predominantly the toxic potential of DCA and TCA has been extensively studied, while little information is available on the effects of MCA on cells. DCA and TCA have been investigated for peripheral cells in respect to hepatocarcenogenity [9-13], the generation of oxidative stress [2, 14-16], and an alteration in enzyme activities [17-22] or gene expression [23]. In addition, over the last two decades, DCA gained interest as investigational drug for the treatment of metabolic acidosis, myocardial or cerebral ischemia, and cancer as well as for modulating exercise tolerance [24-26].

So far little is known on the consequences of a treatment of neural cells with chlorinated acetates. Exposure to DCA causes an increase in the oxidative metabolism in astrocytes *in vitro* and *in vivo* which is due to a DCA-induced modulation of pyruvate dehydrogenase (PDH) activity [27, 28]. In addition, application of DCA led to peripheral neuropathy *in vivo* [29] and to reversible inhibition of the expression of myelin-related proteins in Schwann cells that were cocultured with dorsal root ganglia neurons [30]. In contrast to DCA, consequences of a treatment of brain cells with MCA or TCA as well as a direct comparison of the effects of different chlorinated acetates on metabolic properties and the viability of brain cells have to our knowledge not been reported so far.

The tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine; GSH) is essential for antioxidative processes and for the detoxification of xenobiotics in mammalian cells [31, 32]. In the brain, astrocytes play a key role in antioxidative defence [33-35] and in the clearance of xenobiotics [36-38]. In addition, the astrocytic GSH metabolism is essential for the supply of GSH precursors to neurons. This process involves release of GSH from astrocytes and subsequent extracellular processing of the exported GSH

[36]. Since the release rate of GSH from astrocytes depends on their cellular GSH concentration [39], any modulation of the cellular GSH concentration of astrocytes will also have consequences for neuronal GSH synthesis.

In the brain, astrocytes are localised between capillary endothelial cells and neurons [40]. Thus, astrocytes are likely to encounter first any potential toxin that is crossing the blood-brain barrier. Since the toxic and/or beneficial consequences of a treatment of brain cells with different chlorinated acetates have not been reported so far, we have investigated how cultured astrocytes respond to an exposure to MCA, DCA or TCA. These compounds differ strongly in their effects on cultured astrocytes. While MCA compromised cell viability, depleted cells of GSH and inactivated GAPDH activity, cultured astrocytes remained viable during exposure to DCA and TCA and lowered their production of lactate.

2 Materials and Methods

2.1 Material

MCA (sodium salt), DCA (sodium salt), TCA (sodium salt), 5,5'-dithio-bis(2-nitrobenzoic acid) and 1-chloro-2,4-dinitrobenzol (CDNB) were obtained from Sigma-Aldrich (Steinheim, Germany). GSH, glutathione disulfide (GSSG), glutathione reductase (GR), glutamate pyruvate transaminase and lactate dehydrogenase (LDH) were from Roche Diagnostics (Mannheim, Germany). Bovine serum albumin, NAD^+, NADH, $NADP^+$, NADPH, sulfosalicylic acid and sodium acetate were purchased from Applichem (Darmstadt, Germany). Fetal calf serum, streptomycin sulfate and penicillin G were from Biochrom (Berlin, Germany). Dulbecco´s modified Eagle´s medium (DMEM) was purchased from Invitrogen (Karlsruhe, Germany). All other chemicals were obtained from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany) at analytical grade. Sterile cell culture material and unsterile 96-well plates were from Nunc (Roskilde, Denmark) and Sarstedt (Karlsruhe, Germany).

2.2 Cell cultures

Astroglia-rich primary cultures derived from the brains of neonatal Wistar rats were prepared according to a published method [41]. Three hundred thousand viable cells were seeded in 1 mL culture medium (90% DMEM, 10% fetal calf serum, 20 units/mL of penicillin G, 20 µg/mL of streptomycin sulfate) per well of a 24-well dish and cultured in a cell incubator (Sanyo, Osaka, Japan) that contained a humidified atmosphere of 10% CO_2/90% air. The culture medium was renewed every seventh day. The results described here were obtained on 15 to 22 day-old cultures.

2.3 Experimental incubation of cells

To study the consequences of a treatment of astrocytes with chlorinated acetates, the cells were washed with 1 mL of prewarmed (37°C) incubation buffer (IB: 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.4 mM KCl, 145 mM NaCl, 0.8 mM Na_2HPO_4, 20 mM HEPES, 5 mM D-glucose, pH 7.4) and incubated at 37°C with 0.5 mL IB containing chlorinated acetates or acetate in concentrations between 0 and 10 mM as given in the legends

of the figures or in the tables. After the indicated incubation periods, the cells were washed with 1 mL ice-cold phosphate-buffered saline (PBS; 10 mM potassium phosphate buffer, 150 mM NaCl, pH 7.4). For determination of enzyme activities the cells were lysed with 200 µL 20 mM potassium phosphate buffer (KPi) pH 7.0. For analysis of the total cellular glutathione content (GSx = amount of GSH plus twice the amount of glutathione disulfide (GSSG)) the cells were lysed with 500 µL 1% (w/v) sulfosalicylic acid.

2.4 Determination of glutathione

The contents of GSx and GSSG in cell lysates and incubation media were determined as described previously [42, 43] in microtiter plates according to the colorimetric Tietze method [44]. The detection limit of this assay was about 0.2 nmol GSx per 500 µL lysate or medium.

2.5 Specific enzyme activities

The activities of the enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glucose-6-phosphate dehydrogenase (G6PDH), glutathione reductase (GR) and lactate dehydrogenase (LDH) were determined in lysates of astrocyte cultures as described previously [45]. The activity of glutathione-S-transferases (GST) was determined according to a method originally described by Habig et al [46]. Briefly, after lysis of the cells in 200 µL 20 mM KPi pH 7.0 for 10 min on ice, 100 µL 260 mM KPi 6.5 were added to adjust pH. Fifty microliters of the diluted cell lysate were mixed with 50 µL water and 100 µL reaction mixture (2 mM CDNB, 20 mM GSH in 100 mM KPi pH 6.5) in wells of a microtiter plate. The increase of absorbance at 340 nm due to the GST-catalysed formation of 2,4-dinitrophenylglutathione was followed using a Sunrise microtiter plate photometer (Tecan, Grödig, Austria) at room temperature for 5 min. Specific activities were calculated by normalizing the activities determined for cell lysates to the protein contents of the respective lysates.

2.6 Determination of lactate in culture medium

Extracellular lactate concentration in culture media was determined using a modification of an established assay [47, 48]. Briefly, 20 µL media sample were diluted with 160 µL purified water in wells of a microtiter plate and mixed with 180 µL reaction mixture (5.6 mM NAD^+, 19.9 U/mL LDH, 1.94 U/mL glutamate pyruvate transaminase in 250 mM glutamate/NaOH buffer pH 8.9). After incubation for 90 min in a humidified atmosphere at 37°C the absorbance of the NADH generated from lactate was determined at 340 nm.

2.7 Determination of cell viability and protein content

Loss of cell viability was analyzed by comparing the activity of LDH in the incubation medium with that of the cells using the microtiter plate assay described previously [49]. The presence of chlorinated acetates even in the highest concentration used did not affect the determination of LDH activity in cell lysates of cultured astrocytes (data not shown), excluding that the presence of chlorinated acetates may prevent the detection of extracellular LDH. The protein content per well of a 24-well dish was quantified according to the Lowry method [50] after solubilisation of the cells in 200 µL of 0.5 M NaOH, using bovine serum albumin as a standard. The cultures used contained between 76 and 105 µg total protein per well and between 23 and 31 µg soluble protein in the cell lysate of one well. Total protein content per well and protein in the supernatants of cell lysates were used to calculate specific GSx or GSSG contents and specific enzyme activities, respectively.

2.8 Presentation of the data

If not stated otherwise, the data are presented as means ± standard deviation (SD) of values obtained in experiments on three or four independently prepared cultures. In the figures the bars have been omitted, if they were smaller than the symbols representing the mean values. Statistical analysis of the significance of differences between multiple sets of data was performed by ANOVA followed by Bonferroni post hoc test and are indicated as *$p<0.05$, **$p<0.01$ or ***$p<0.001$. $p>0.05$ was considered as not significant.

3 Results

3.1 Effects of MCA on the viability and the glutathione metabolism of astrocytes

To test for the consequences of a treatment of cultured astrocytes with MCA, the cells were incubated with various concentrations of this compound for up to 8 hours (Fig. 1). During the first two hours of incubation with MCA in concentrations of up to 10 mM the cells remained viable as indicated by the lack of any increase in extracellular LDH activity ($p>0.05$, Fig. 1a). However, while exposure of cells for up to 8 h with 0.1 mM MCA did not cause any loss in cell viability, incubation with MCA in concentrations of 10 and 1 mM significantly increased the extracellular LDH activity within 4 h and 8 h of incubation, respectively (Fig. 1a).

In the absence of MCA the cellular GSx content declined by 60% within 8 hours of incubation (Fig. 1b). This loss in cellular GSx was accelerated by MCA in a time and concentration dependent manner. Presence of 1 mM and 10 mM MCA lowered already within 4 h and 2 h, respectively, the cellular GSx content significantly (Fig. 1b), demonstrating that in the presence of MCA the loss in cellular GSx content (Fig. 1b) preceded the loss in cell viability (Fig. 1a). A more detailed concentration dependency confirmed these results. MCA had to be present in concentrations above 3 mM to cause a significant loss in cell viability (Fig. 2a) within 4 hours of incubation, while the cellular GSx content was already significantly lowered in the presence of 0.3 mM MCA (Fig. 2b). Halfmaximal effects (EC-50 values) of MCA for the deprivation of cellular GSx and for compromised cell viability within 4 h were about 0.3 mM and 10 mM, respectively (Fig. 2).

To test whether the loss in cellular GSx after exposure of astrocytes to MCA was caused by export of GSH or GSSG, the cellular and extracellular contents of GSx and GSSG were determined. After 4 h incubation with MCA in concentrations of 0.1 and 1 mM the cellular GSx contents were significantly lowered by 20% and 82% and the extracellular contents were lowered by 30% and 38% of the values determined for controls (absence of MCA), respectively (Table 1). In contrast, the specific contents of cellular and extracellular GSSG were not altered in MCA-treated cultures compared to controls (absence of MCA) and the cellular GSSG values remained at

around 2 nmol GSx/mg protein (Table 1). The conditions used did not cause any increase in extracellular LDH activity (Table 1), confirming that treatment of astrocytes with up to 1 mM MCA for 4 h is not toxic for the cells.

3.2 Inactivation of astrocytic GAPDH activity by MCA

Since iodoacetate is known to strongly inactivate GAPDH activity in cultured astrocytes [45], we investigated the potential of MCA to inactivate GAPDH and to affect glycolysis. Exposure of cultured astrocytes with MCA in concentrations of up to 10 mM did not induce any significant increase in extracellular LDH activity during 3 h of incubation (Fig. 3a), but lowered the cellular GAPDH activity already within 1 h and 2 h to 19% and 3% of the initial value, respectively (Fig. 3b). In addition, presence of 10 mM MCA did not affect the linear extracellular lactate accumulation during the first 2 h of incubation, but almost completely prevented the further lactate accumulation as indicated by the significant lowered lactate concentrations in the media of MCA-treated cells compared to controls (Fig. 3c). After 4 h exposure of astrocytes to 10 mM MCA about 60% of the initial LDH were detected in the medium of cells (Fig. 3a), cellular GAPDH activity was not detectable anymore (Fig. 3b) and the extracellular lactate accumulation was prevented (Fig. 3c). In contrast, presence of MCA in a concentration of 1 mM for 4 h did not affect cell viability (Fig. 3a) nor lower extracellular lactate accumulation (Fig. 3c), but significantly inactivated GAPDH activity by about 40% (Fig. 3b).

To test in more detail the inactivation of astrocytic GAPDH by MCA, the cells were exposed for 2 h to various concentrations of MCA. Under these conditions, MCA in concentrations of up to 10 mM did not cause any loss in cell viability as indicated by the absence of any increase in extracellular LDH activity (Fig. 4a). However, MCA concentrations of above 0.7 mM caused within 2 h a significant inhibition of GAPDH activity (Fig. 4b). The EC-50 value for the inhibition by MCA of GAPDH activity after incubation of the cells for 2 h was about 3 mM (Fig. 4b). In contrast to GAPDH activity, exposure of astrocytes for 2 h with 10 mM MCA did not alter the specific activities of G6PDH, GR, LDH or GST (Table 2).

3.3 Comparison of different chlorinated acetates regarding their effects on cell viability, cellular GSx content and on enzyme activities

To test whether in addition to MCA, also the other chlorinated acetates or acetate compromise the viability and the cellular GSH metabolism, cultured astrocytes were exposed to 10 mM of the different chlorinated acetates or of acetate for 8 h. Exclusively MCA, but not DCA, TCA or acetate, affected cell viability as indicated by the significant increase of extracellular LDH activity of MCA-treated cells compared to the control condition (none) or to the other acetates (Fig. 5a), lowered the specific cellular GSx content (Fig. 5b) and the extracellular GSx content (Fig. 5c). In addition, exclusively MCA, but not the other compounds investigated, lowered the specific GAPDH activity of astrocytes (Table 2). None of the chlorinated acetates nor acetate affected significantly the specific activities of G6PDH, GR, LDH or GST under the conditions used (Table 2).

3.4 Effects of chlorinated acetates on lactate release rates

DCA has been reported to lower cellular lactate production due to a modulation of PDH kinase activity [51, 52]. Since inhibition of this enzyme activates PDH and accelerates oxidative metabolism, we have investigated the effects of the different chlorinated acetates on the lactate release rate of cultured astrocytes as indicator for glucose metabolism by glycolysis. No effects on lactate release rates were detectable when the cells were exposed to acetate in concentrations of up to 10 mM or to MCA in non-toxic concentrations (up to 1 mM) for 4 h (Table 3). In contrast, presence of DCA (1 mM and 10 mM) or TCA (1 mM) lowered the extracellular lactate accumulation significantly by about 20%, whereas TCA in a concentration of 10 mM lowered lactate production even stronger by about 40% (Table 3).

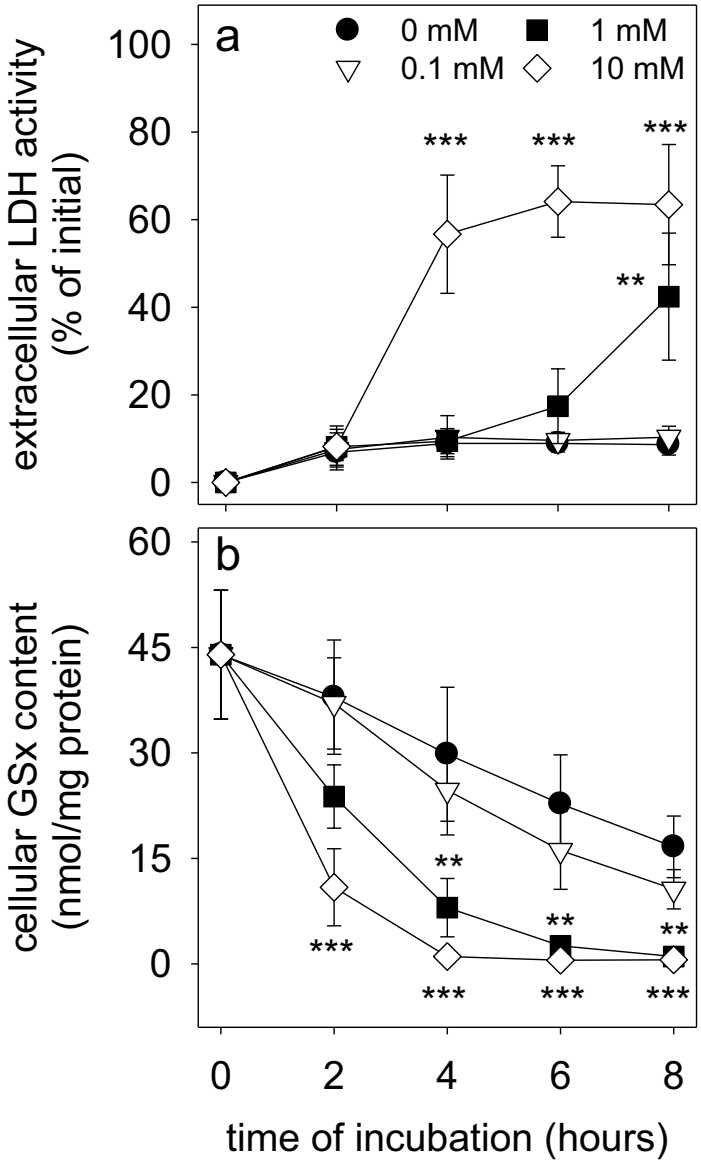

Figure 1: Time dependend effects of an application of MCA on the cell viability (a) and the specific cellular GSx content (b) of cultured astrocytes. The cells were incubated for up to 8 hours without or with MCA in concentrations of up to 10 mM. The significance of differences to the data obtained for the control (absence of MCA) are indicated.

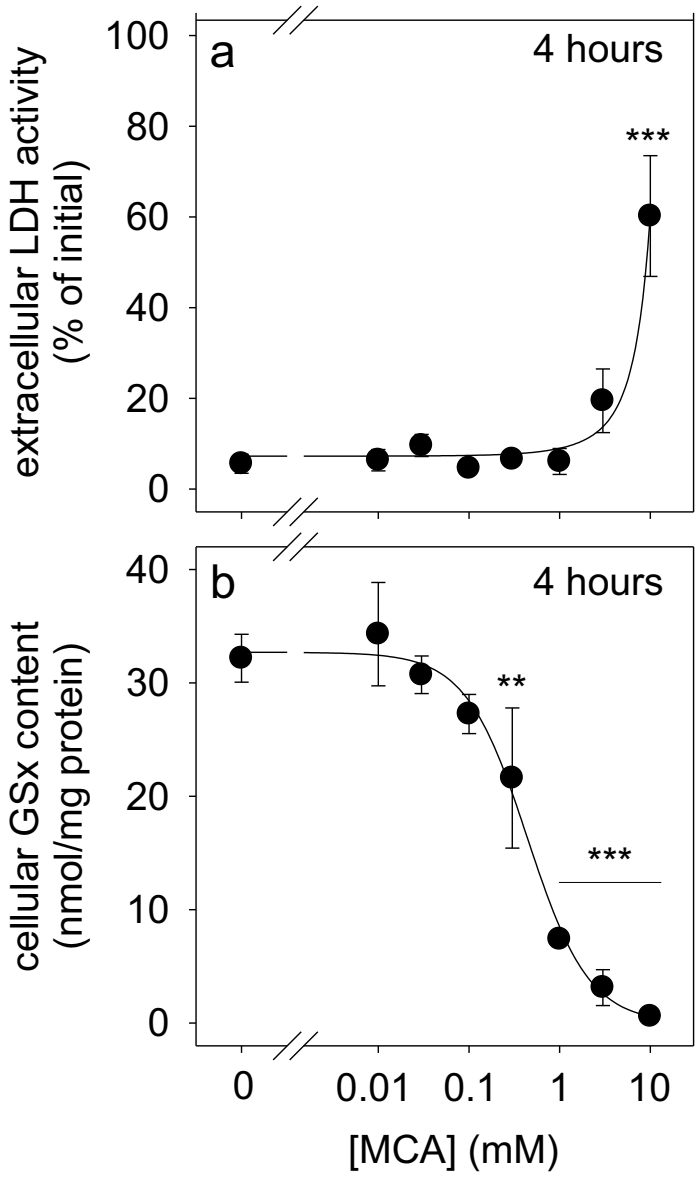

Figure 2: Concncentration dependent effects of MCA on the cell viability (a) and the cellular GSx content (b) of cultured astrocytes. The cells were incubated for 4 hours with MCA in concentrations of up to 10 mM. The cultures contained an initial specific GSx content of 46.6 ± 3.3 nmol/mg protein. The significance of differences to the data obtained for the control (absence of MCA) are indicated.

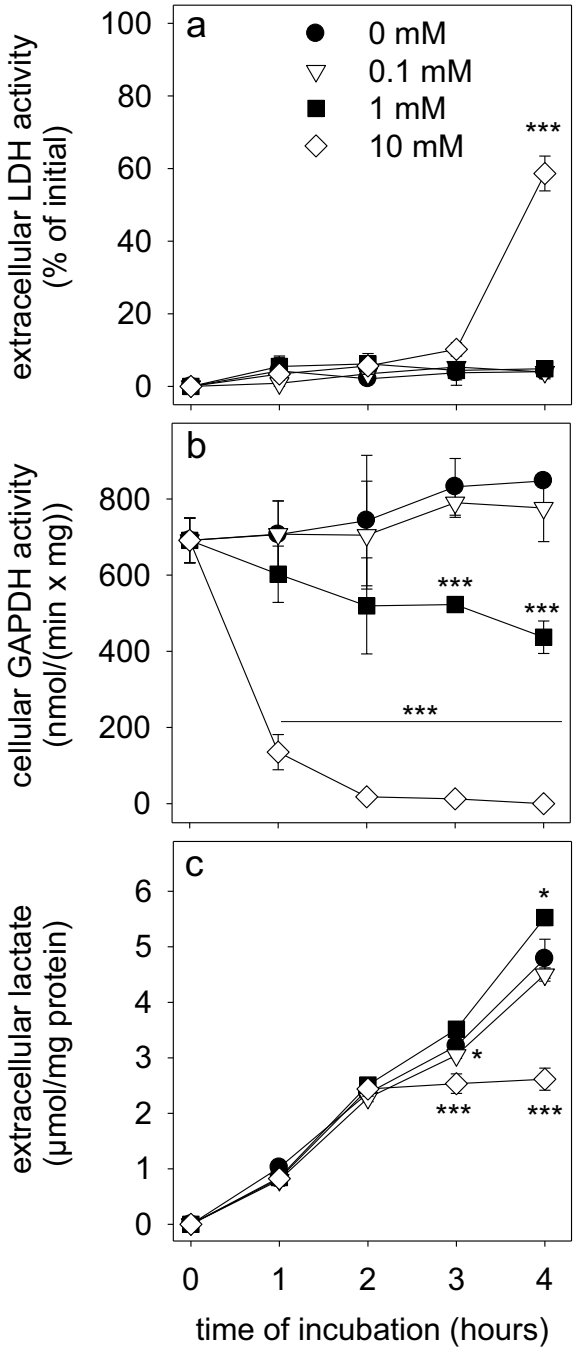

Figure 3: Consequences of a treatment of cultured astrocytes with MCA on the cell viability (a), the specific cellular GAPDH activity (b) and the extracellular lactate accumulation (c). The cells were incubated for up to 4 hours in the absence or presence of MCA in the concentration indicated in panel a. The significance of differences of data compared to those of control conditions (absence of MCA) are indicated.

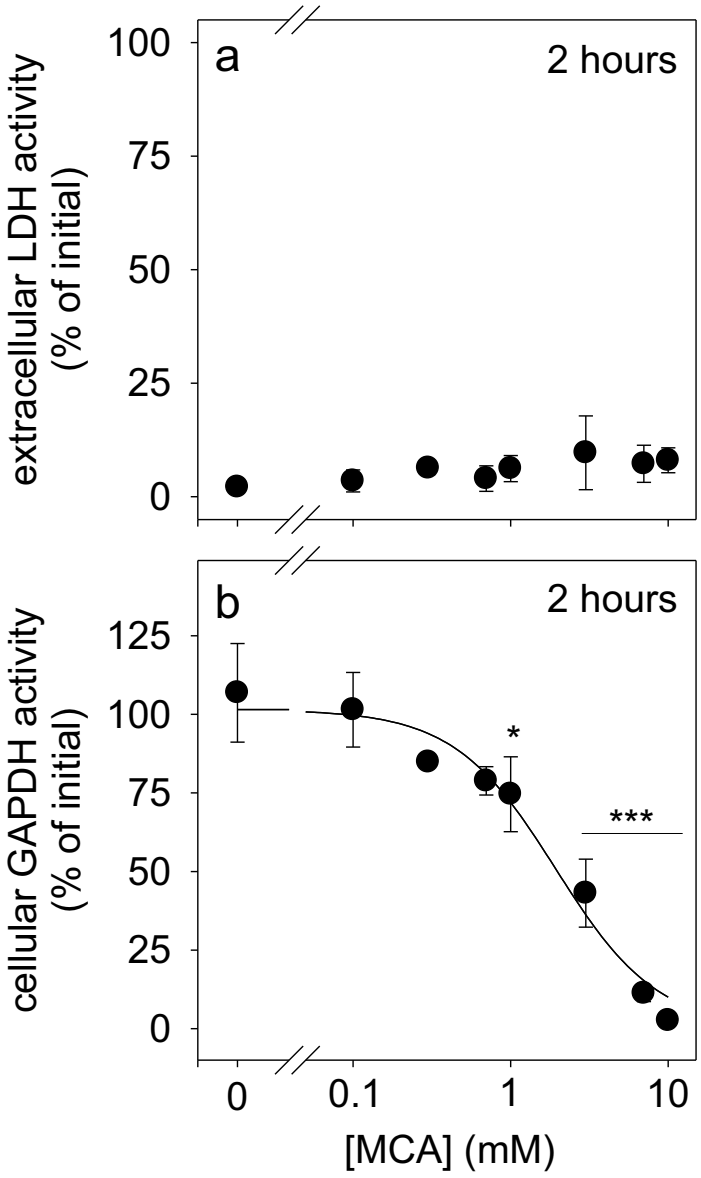

Figure 4: Concentration dependent effects of MCA on cell viability (a) and specific GAPDH activity (b) in cultured astrocytes. The cells were incubated for 2 hours with MCA in concentrations of up to 10 mM. . The initial GAPDH activity (100%) corresponded to 691 ± 59 nmol/(min x mg soluble protein). The significance of differences of data compared to those of the control condition (absence of MCA) are indicated.

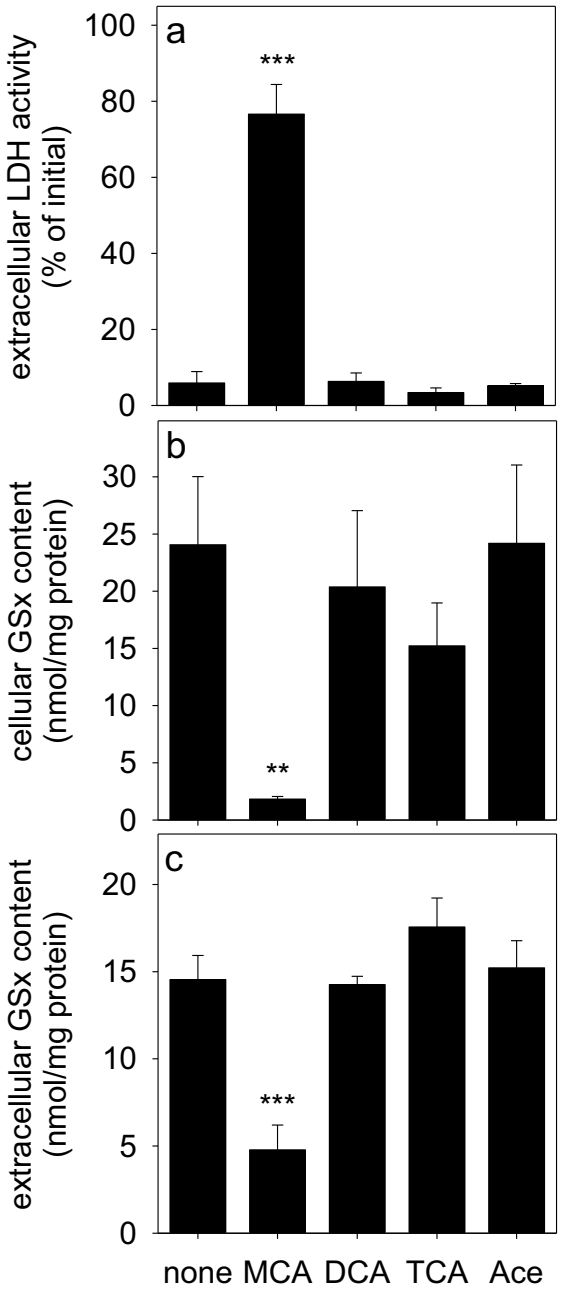

Figure 5: Effects of various chlorinated acetates on cell viability (a), cellular (b) and extracellular (c) GSx contents of cultured astrocytes. The cells were incubated for 8 hours in the absence (none) or presence of 10 mM MCA, DCA, TCA, or acetate (Ace). The cultures contained an initial specific GSx content of 43.7 ± 7.6 nmol/mg protein. The significance of differences to the data obtained for the control (none) are indicated.

Table 1: Cellular and extracellular GSx and GSSG contents and extracellular LDH activity of astrocyte cultures after exposure to MCA

			0 mM MCA	0.1 mM MCA	1 mM MCA
Cells	GSx	(nmol/mg)	35.4 ± 2.4	28.1 ± 2.2 *	9.9 ± 2.4 ***
	GSSG	(nmol/mg)	2.3 ± 0.4	2.2 ± 0.5	1.6 ± 0.4
Media	GSx	(nmol/mg)	16.8 ± 0.7	11.7 ± 1.6 **	10.4 ± 0.7 ***
	GSSG	(nmol/mg)	0.4 ± 0.5	0.4 ± 0.3	0.1 ± 0.3
	LDH activity	(% of initial)	8.7 ± 0.3	11.1 ± 0.2	11.9 ± 2.9

Primary astrocyte cultures were incubated in the absence or the presence of MCA. The cellular and extracellular contents of GSx and GSSG and extracellular LDH activity (as % of initial cellular LDH activity) were determined after 4 hours of incubation. The cultures contained an initial specific cellular GSx content of 48.0 ± 1.9 nmol/mg protein and an initial specific cellular GSSG content of 2.7 ± 1.3 nmol GSx/mg protein. The significance of differences to the data obtained for the control condition (absence of MCA) are indicated.

Table 2: Specific activities of enzymes in cultured astrocytes after exposure to chlorinated acetates.

Enzyme	Compound	Enzyme activity (nmol/(min x mg))
GAPDH	none	609 ± 22
	MCA	19 ± 9***
	DCA	611 ± 32
	TCA	607 ± 48
	Acetate	617 ± 36
G6PDH	none	82.7 ± 4.9
	MCA	86.2 ± 5.8
	DCA	82.1 ± 10.3
	TCA	85.9 ± 5.8
	Acetate	88.2 ± 4.3
GR	none	15.3 ± 3.7
	MCA	11.2 ± 5.0
	DCA	15.8 ± 3.9
	TCA	18.2 ± 0.9
	Acetate	11.6 ± 1.7
LDH	none	1367 ± 113
	MCA	1512 ± 115
	DCA	1473 ± 91
	TCA	1536 ± 22
	Acetate	1493 ± 79
GST	none	280 ± 11
	MCA	253 ± 6
	DCA	286 ± 11
	TCA	271 ± 13
	Acetate	297 ± 11

The cells were incubated in the absence (none) or presence of 10 mM MCA, DCA, TCA or acetate. After 2 hours of incubation, the cells were lysed and the specific activities of the enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glucose-6-phosphate dehydrogenase (G6PDH), glutathione reductase (GR), lactate dehydrogenase (LDH) and glutathione-S-transferase (GST) were determined. The initial specific enzyme activities (in nmol/(min x mg)) of the cells were 617 ± 27 (GAPDH), 72.1 ± 8.8 (G6PDH), 18.9 ± 1.2 (GR), 1363 ± 95 (LDH) and 297 ± 32 (GST). The significance of differences to the data obtained for the controls (none) are indicated.

Table 3: Effect of chlorinated acetates on lactate release rates and viability of astrocytes.

compound	(mM)	Lactate release rates (µmol/(h x mg))	Lactate release rates (% of control)	LDH activity (% of initial)
none	0	1.15 ± 0.31	100 ± 6.0	3.7 ± 1.2
Acetate	0.1	1.07 ± 0.25	93.4 ± 5.6	6.9 ± 2.8
	1	1.17 ± 0.34	101.2 ± 2.2	4.9 ± 1.1
	10	1.08 ± 0.21	94.4 ± 7.1	5.1 ± 2.2
MCA	0.1	1.23 ± 0.07	98.4 ± 5.3	4.0 ± 1.4
	1	1.56 ± 0.03*	124.5 ± 2.7*	4.9 ± 1.4
	10	---	---	58.6 ± 4.8***
DCA	0.1	1.05 ± 0.27	91.4 ± 1.6	0.9 ± 1.2
	1	0.94 ± 0.26	81.7 ± 4.7*	5.4 ± 0.5
	10	0.91 ± 0.18	79.5 ± 7.7*	4.3 ± 4.4
TCA	0.1	1.08 ± 0.30	93.5 ± 5.9	7.6 ± 8.4
	1	0.94 ± 0.21	82.1 ± 3.8*	5.4 ± 0.8
	10	0.68 ± 0.22	58.4 ± 5.3***	6.7 ± 1.4

The cells were incubated in the absence (none) or presence of the indicated compounds in the concentrations given. The lactate release rates were calculated from the linear increases in extracellular lactate concentration during the incubation for up to 4 h. The significance of differences to the data obtained for the control condition (none) are indicated.

4 Discussion

Monohalogenated acetates are known for their potential to severly affect the metabolism of astrocytes. Iodoacetate strongly inhibits GAPDH activity and quickly deprives astrocytes of GSH [45], whereas fluoroacetate is used by astrocytes as substrate for citrate synthase to produce fluorocitrate which strongly inactivates aconitase and blocks citric acid cycle [53]. In contrast, the consequences of a treatment of astrocytes with MCA or a direct comparison of the different chlorinated acetates on metabolic properties and the viability of brain cells have not been reported so far. Here we demonstrate that MCA, but not DCA, TCA or acetate, deprives cultured astrocytes of GSH, inactivates GAPDH activity and compromises cell viability.

In MCA-treated astrocytes, the increase in extracellular LDH activity was delayed compared to the deprivation of cellular GSH and the inactivation of GAPDH. A similar order of events was observed for iodoacetate-treated astrocytes where GSH deprivation and GAPDH inactivation also proceded a delayed cell death [45]. Both the depletion of the antioxidant GSH as well as inhibition of glycolytic ATP production by GAPDH inhibition are likely to be involved in the cell death observed for MCA-treated astrocytes. The observations that MCA deprives astrocytes of GSH and compromises cell viability are in accord with published data for hepatocytes that show a decline in cellular GSH content and a loss in viability after exposure to MCA in concentrations of up to 5 mM for up to 120 min [54].

The mechanism involved in the MCA-induced deprivation of cellular GSH has not been identified so far. For astrocytes, impaired membrane integrity can be excluded as reason for the cellular loss in GSx content after MCA-treatment, since no elevation of extracellular GSx was found for MCA-treated cells and since the loss in cellular GSH always preceded by hours the occurance of extracellular LDH. In addition, exposure of astrocytes to MCA induced a decline in the extracellular GSx content, excluding a MCA-induced stimulation of an export of cellular GSH or GSSG by multidrug resistance proteins [55, 56] as reasons for the observed loss in cellular GSH. Furthermore, an irreversible inhibition of GSH synthesis enzymes by MCA is unlikely to contribute to the observed MCA-induced GSx depletion, since cultured

astrocytes were able to fully restore their cellular GSx content within 8 h after removal of subtoxic (preincubation with 1 mM MCA for 2 h) concentrations of MCA (data not shown).

The deprivation of cellular GSx after exposure to MCA is most likely the consequence of a direct reaction of MCA with GSH in viable cells to S-carboxymethyl-glutathione. A similar mechanism has been postulated for astrocytes that are deprived of GSH by iodoacetate [45]. However, compared to iodoacetate (incubation with 1 mM iodoacetate deprived in 90 min the cellular GSx content by more than 90%; [45]), MCA had to be applied in 10fold higher concentrations and for 4 h to deprive viable astrocytes almost completely of their GSH. The higher reactivity of iodoacetate compared with MCA reflects the different reactivities of halogenated alkanes for substitution reactions with thiols [5].

In cell-free conditions, MCA and the other chlorinated acetates did not react with GSH, as demonstrated by the lack of any detectable loss in GSH content after incubation of 10 mM MCA with 10 µM GSH for 4 h (data not shown), contrasting the situation described for iodoacetate [45]. Thus, the disappearance of cellular GSH in MCA-treated astrocytes is most likely the consequence of a reaction that is catalysed by one or several of the GST isoforms which have been reported to be expressed in astrocytes in culture and *in vivo* [57-59], rather than the consequence of an enzyme-independent reaction.

In addition to the cellular GSH deprivation, MCA inactivated GAPDH activity and affected glycolysis in cultured astrocytes. Such a susceptibility of GAPDH towards MCA has already been reported for isolated liver GAPDH and is considered to be mediated by an alkylation of the reactive thiol group of a cysteine moiety in the active center of GAPDH [21], similar to the inactivation of GAPDH by iodoacetate (see [45] and references cited therein). The reaction of MCA with GAPDH appears to be specific for the highly reactive thiol group in the active center of GAPDH, since other dehydrogenases such as G6PDH, GR or LDH as well as GSTs were not inactivated by exposure of astrocytes to 10 mM of MCA.

Almost complete inhibition of GAPDH by MCA prevented extracellular lactate accumulation, while the residual 20% GAPDH activity detected after 1 h of

incubation with 10 mM MCA did not affect extracellular lactate accumulation. However, it should be considered that GAPDH is present in cultured astrocytes in a specific activity of above 600 nmol/(min x mg). Thus, the residual 20% of specific GAPDH activity will still be substantially higher than that of hexokinase (30 nmol/(min x mg); [60]). GAPDH will only become limiting for glycolytic flux, if this enzyme is almost completely inhibited, as observed after more than 2 h of incubation of astrocytes with 10 mM MCA.

In contrast to MCA, DCA and TCA even in concentrations of 10 mM did not affect the cellular GSH content, the GAPDH activity nor compromise the viability of cultured astrocytes. Differences in the uptake of the various chlorinated acetates, which is most likely mediated by monocarboxylate carriers [61], into astrocytes as well as different reactivities of astrocytic GSTs towards these compounds are likely to contribute to the observed differences of chlorinated acetates on the cellular GSH content. However, since both DCA and TCA lower the lactate productions by astrocytes, these compounds have to be present in the cells at least in concentrations sufficient to mediate the observed effects on lactate production.

Reason for the lowered extracellular lactate production by DCA and TCA is most likely the activation of PDH by noncompetitive inhibition of the PDH kinase [51, 52]. This leads to accelerated pyruvate oxidation by PDH and to a decreased extracellular lactate concentration *in vivo* and in cultured astrocytes [27, 62-66]. Our results confirm a previous report for cultured astrocytes [27] that DCA lowered extracellular lactate accumulation significantly but only to a low extent. In contrast to DCA, TCA has so far not been reported for its potential to lower lactate production. In our hands, TCA was even more potent than DCA. Since long time exposure of rats, mice or cultured cells to DCA and TCA did not reveal a greater toxic or cancerogenic potential of TCA compared with DCA [15, 54, 67, 68], also TCA could be considered to inhibit lactate production in astrocytes during metabolic acidosis.

Since DCA and TCA in concentrations of up to 10 mM did not show any effect on GSH metabolism or the cell viability of cultured astrocytes, these cells appear not to be able to dechlorinate DCA or TCA to MCA with reaction rates that generate toxic concentrations of MCA. This information is important for considerations to use DCA

or even TCA to decrease astrocytic lactate production to prevent metabolic acidosis in brain [27, 28].

5 Conclusion

Chlorinated acetates are environmental toxins that are generated during water disinfection processes [1] and are metabolites in the detoxification of industrial chlorinated solvents [2, 3]. Among these compounds only MCA was identified to strongly compromise metabolism and viability of astrocytes. MCA had to be applied in millimolar concentrations to induce acute toxicity to cultured astrocytes. However, since concentrations of chlorinated acetates in drinking water have been shown to sporadically reach concentration ranges of 0.1 to 0.5 mM [1], sustained exposure to chlorinated water could provide a situation where critical amounts of chlorinated acetates could reach the brain and affect the metabolism of astrocytes.

Intraveneous application of DCA has been shown to lower metabolic acidosis in brain [27, 28], suggesting that this compound is able to enter the brain. Indeed, presence of DCA in brain and in cerebral spinal fluid has been confirmed after oral application of DCA [63]. For liver cells was demonstrated that chlorinated acetates are substrates of monocarboxylate transporters (MCT) [61]. Since MCT are expressed in capillary endothelial cells [69] and in cultured astrocytes [70, 71], these transporters could be involved in the uptake of chlorinated acetates into the brain and astrocytes. Due to the localisation of astrocytes in brain, these cells are likely to encounter as first parenchymal cells of the brain chlorinated acetates after environmental or clinical exposure to such compounds. Since astrocytes have been suggested to supply energy substrates such as lactate [72] as well as GSH precursors [73] to neighbouring neurons, alterations in the GSH metabolism and glycolytic flux of astrocytes by chlorinated acetates would indirectly also affect neurons.

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2.5

Publication/Manuscript 5

2-Deoxyribose deprives cultured astrocytes of their glutathione.

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Neurochemical Research, manuscript submitted

Contributions of Maike M. Schmidt:

- Establishment of the experimental paradigms and conditions
- Data shown in Table I & II
- Data shown in Table IV with the exception of the sugar quantification
- Preparation of the first draft of the manuscript

The data shown in the figures and Table III were obtained by Helena Greb during a lab rotation that was supervised by M. M. Schmidt.

Quantification of glucose and 2-deoxyribose (Table IV) was performed by Hendrik Koliwer-Brandl.

Manuscript for *Neurochemical Research*

2-Deoxyribose deprives cultured astrocytes of their glutathione

by

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Running head: 2-deoxyribose and astroglial GSH

Abstract

High concentrations of 2-deoxy-D-ribose (2dRib) have been reported to cause oxidative stress and to disturb the glutathione (GSH) metabolism of various cell types. Exposure of astrocyte-rich primary cultures to millimolar concentrations of 2dRib or its stereoisomer 2-deoxy-L-ribose, but not the incubation with ribose, 2-deoxyglucose, glucose, fructose or saccharose, lowered the cellular GSH content in a time and concentration dependent manner. After exposure for 4 h to 30 mM 2dRib the cells contained 2dRib in a concentration of about 24 mM. Under these conditions 2dRib did not compromise cell viability and the ability of the cells to synthesise GSH, nor were the cellular ratio of glutathione disulfide (GSSG) to GSH and the extracellular concentrations of GSH or GSSG increased. These data demonstrate that 2dRib deprives viable cultured astrocytes of GSH and suggest that a cellular reaction of GSH with 2dRib or its metabolites is involved in the deprivation of astrocytic GSH.

Key words: astrocytes; 2-deoxyribose; GSH; oxidative stress; reducing sugars

1 Introduction

2-deoxy-D-ribose (2dRib) is produced in cells as product of the enzyme thymidine phosphorylase [1] and is generated during the degradation of DNA [2]. In addition, 2dRib has frequently been applied in high concentrations of up to 100 mM to investigate the mechanisms involved in sugar-induced cell damage, for example in the context of diabetes [3-8]. 2dRib reacts enzyme-independently in Maillard reactions with amino groups of proteins to form Amadori or Heynes products [9, 10]. Further reactions of such glycoadducts lead to the formation of highly reactive dicarbonyl compounds [11] that induce irreversible molecular damage by forming heterocyclic protein adducts and intra- or intermolecular crosslinks, the so called advanced glycation endproducts [12-14]. Such processes are likely to contribute to the potential of 2dRib to inhibit proliferation [15], to be cytotoxic [4, 16, 17], to cause oxidative stress [3, 6, 8] and to induce apoptosis [3, 18-20].

The tripeptide glutathione (GSH; γ-L-glutamyl-L-cysteinylglycine) is the most abundant cellular thiol in mammalian cells and has a variety of important functions, of which the detoxification and antioxidative functions are most relevant [21, 22]. GSH is substrate for the detoxification of endogenously derived compounds such as products of lipidperoxidation and/or DNA oxidation [23, 24] as well as for the detoxification of xenobiotics in glutathione-S-transferase catalysed reactions [25, 26]. In addition, GSH serves as electron donor for the reduction of peroxides in glutathione peroxidase-mediated reactions [27]. Thus, a compromised GSH metabolism will affect several metabolic cellular pathways. In the brain, astrocytes play a key role both in detoxification and antioxidative processes. In addition, astrocytes supply precursors for GSH synthesis to neighbouring neurons [21, 22, 28]. Therefore, any modulation of the cellular GSH content of astrocytes is likely to also affect neuronal GSH metabolism.

So far little information is available on the consequences of a treatment of brain cells with 2dRib. In long time exposure experiments on cultured astroglial cells, 2dRib has been shown to induce apoptosis and/or oxidative stress [16, 18]. Since GSH is one of the important antioxidants in astrocytes and since 2dRib has been reported to lower the cellular GSH content of peripheral cells [17, 20, 29, 30], we have

investigated the consequences of a treatment with 2dRib on the GSH metabolism of astrocyte-rich primary cultures. Here we report that millimolar concentration of 2dRib, but not of other sugars, deprive viable astrocytes in culture of their GSH in a time and concentration dependent manner. Since a stimulated export of GSH and GSSG by 2dRib can be excluded, the deprivation of cellular GSH is most likely a consequence of a derivatisation of GSH with 2dRib or its metabolites.

2 Experimental Procedures

2.1 Materials

Buthionine sulfoximine (BSO), 2-deoxy-D-glucose, 2-deoxy-D-ribose, D-glucose, D-fructose and D-ribose were obtained from Sigma-Aldrich (Steinheim, Germany), saccharose from Janssen Chimica (Geel, Belgium) and 2-deoxy-L-ribose from Acros Organics (Geel, Belgium). GSH, glutathione disulfide (GSSG) and glutathione reductase were from Roche Diagnostics (Mannheim, Germany). Bovine serum albumin, NAD^+, NADH, $NADP^+$, NADPH and sulfosalicylic acid were purchased from Applichem (Darmstadt, Germany). Fetal calf serum, streptomycin sulfate and penicillin G were from Biochrom (Berlin, Germany). Dulbecco´s modified Eagle´s medium (DMEM) was purchased from Invitrogen (Karlsruhe, Germany). All other chemicals were obtained from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany) at analytical grade. Sterile cell culture material and unsterile 96-well plates were from Nunc (Roskilde, Denmark) and Sarstedt (Karlsruhe, Germany).

2.2 Cell cultures

Astrocyte-rich primary cultures derived from the whole brains of neonatal Wistar rats were prepared as described [31]. Three hundred thousand viable cells were seeded in 1 mL culture medium (90% DMEM, 10% fetal calf serum, 20 units/mL of penicillin G, 20 μg/mL of streptomycin sulfate) per well of a 24-well dish and cultured in a cell incubator (Sanyo, Osaka, Japan) that contained a humidified atmosphere of 10% CO_2/90% air. The culture medium was renewed every seventh day. The results described here were obtained on 15 to 21 day-old cultures.

2.3 Experimental incubation of cells

To study the consequences of a treatment of astrocytes with sugars, the cells were washed with 1 mL of prewarmed (37°C) incubation buffer (IB: 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.4 mM KCl, 145 mM NaCl, 0.8 mM Na_2HPO_4, 20 mM HEPES, 5 mM D-glucose, pH 7.4) and incubated at 37°C with 0.5 mL IB containing 2dRib and/or other compounds in the concentrations given in the legends of the figures and tables. For analysis of the total cellular glutathione content (GSx = amount of GSH plus twice the

amount of GSSG), the cells were washed with 1 mL ice-cold phosphate-buffered saline (PBS; 10 mM potassium phosphate buffer, 150 mM NaCl, pH 7.4) and lysed with 0.5 mL 1% (w/v) sulfosalicylic acid. Ten microliter aliqotes of the lysates were used to quantify the cellular GSx content.

2.4 Determination of glutathione

The contents of GSx and GSSG in cell lysates and incubation media were determined as described previously [32, 33] by a modification of the colorimetric Tietze method [34]. The detection limit of this assay was about 0.2 nmol GSx per 500 µL lysate or medium.

2.5 Determination of enzyme activities

The activities of the cellular enzymes glucose-6-phosphate dehydrogenase (G6PDH), glutathione reductase (GR), lactate dehydrogenase (LDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined in cell lysates of cultured astrocytes as described previously [35]. The specific enzyme activities were calculated by normalizing the activities to the content of soluble protein determined for the respective lysates.

2.6 Determination of cell viability and protein content

Cell viability was analysed by comparing the activity of extracellular LDH with that of the cells using the microtiter plate assay described previously [36]. The presence of 2dRib even in the highest concentration used did not affect the LDH activity in cell lysates of cultured astrocytes (data not shown), excluding that the presence of 2dRib may prevent the detection of extracellular LDH. The protein content per well of a 24-well dish was quantified according to the Lowry method [37] after solubilisation of the cells in 200 µL of 0.5 M NaOH, using bovine serum albumin as a standard.

2.7 Detection of cellular 2-deoxyribose

Cellular contents of 2dRib and glucose were determined using high pH anion exchange chromatography with pulsed amperiometric detection (HPAE-PAD)

analysis. The cells were lysed in 200 µL pure H_2O for 10 min on ice. For protein precipitation, the lysate was mixed with 800 µL ice-cold acetone containing 50 µM lactose as internal standard and incubated over night at -20°C. The sample was centrifuged at 20,000 g at 4°C for 15 minutes to remove protein precipitates and cell debris. The dried supernatant was dissolved in 125 µL pure H_2O for HPAE-PAD which was carried out by using a DX600 system (Dionex, Sunnyvale, CA, USA) with an electrochemical detector (ED50), a gradient pump (GP50) and a autosampler (AS50). Carbohydrates were separated by HPAE on a CarboPAC PA1 (4 x 250 mm) analytical column (Dionex) together with a guard column (4 x 50 mm) using a constant flow rate of 1 mL/min. Sample volumes of 25 µL were injected and the chromatography was performed using an isocratic flow of 100 mM NaOH for 15 min. For PAD of the carbohydrates the typical quadruple waveform was used as described previously [38]. The column was regenerated by washing for 10 min with 100 mM sodium acetate in 200 mM NaOH followed by 10 min with 100 mM NaOH. The Dionex software Chromeleon 6.40 SP8 was used for data acquisition and data evaluation.

2.8 Presentation of the data

The data are presented as means ± standard deviation (SD) of values obtained in experiments performed on three independently prepared cultures. In the figures the bars have been omitted, if they were smaller than the symbols representing the mean values. Statistical analysis of the significance of differences between multiple sets of data was performed by ANOVA followed by Bonferroni post hoc test, comparison of two sets of data was performed by t-test analysis. $p>0.05$ was considered as not significant.

3 Results

3.1 Consequences of a treatment of cultured astrocytes with 2dRib

After a 4 hour incubation of cultured astrocytes without or with 30 mM 2dRib, the cells remained viable as indicated by the lack of any increase in extracellular LDH activity compared to the corresponding control (absence of 2dRib) (Table I). In the absence of 2dRib the cellular GSx content was significantly lowered within 4 hours to 63 ± 5% of the initial GSx content (Fig. 1, $p<0.001$). However, incubation with 30 mM 2dRib caused a highly significant acceleration of this decline, depriving the cells by 50% of their GSx already within 2 hours and leading to an almost complete disappearance of GSx (13 ± 6 % of initial content) within 4 hours of incubation (Fig. 1).

The application of 2dRib to cultured astrocytes caused a concentration dependent decline of the specific cellular GSx content. A 4 hour incubation with 2dRib in concentrations higher than 3 mM lowered the cellular GSx content significantly compared to controls (absence of 2dRib) (Fig. 2A). The 2dRib concentration causing halfmaximal deprivation of the cellular GSx content under these conditions was about 20 mM (Fig. 2A). After 4 hours of incubation, only the highest concentration of 2dRib applied (100 mM) caused a small but significant increase of the extracellular LDH activity (Fig. 2B).

The decline of cellular GSx that was observed after exposure of cultured astrocytes to 2dRib was not accompanied by an increase of cellular GSSG nor by an accumulation of GSx or GSSG in the culture media (Table I). In contrast, the extracellular levels of GSx were even lowered after incubation of the cells with 2dRib and still represented predominately GSH, since the content of extracellular GSSG was not increased during incubation with 2dRib (Table I).

3.2 Consequences of a treatment of astrocytes with 2dRib and other sugars

To test for the specificity for 2dRib to cause cellular GSH deprivation, the cells were incubated for 4 hours with various sugars in concentrations of 30 mM. None of these sugars impaired cell viability as indicated by the lack of any increase in extracellular

LDH activity compared to controls (Table II). Of the sugars applied, exclusively 2dRib and its stereoisomer 2-deoxy-L-ribose, but not ribose, 2-deoxyglucose, glucose, fructose or saccharose, lowered the cellular GSx content of astrocytes within 4 hours by more than 80% of the initial content (Table II). 2dRib and 2-deoxy-L-ribose did not differ in their potential to deprive the cells of GSx, as indicated by the almost identical GSx contents determined after a 4 hour treatment of the cell with each of the stereoisomers in concentrations of 3 mM, 10 mM or 30 mM (Fig. 3; Table II). In addition, 2dRib, 2-deoxy-L-ribose and glucose were tested for their potential to inactivate cellular enzymes. However, in contrast to the cellular GSx content, application of glucose, 2dRib or 2-deoxy-L-ribose in a concentration of 30 mM did not cause any significant alteration in the specific acitivities of the cellular enzymes G6PDH, GR, LDH or GAPDH (Table III).

3.3 Effects of 2dRib on GSH synthesis in cultured astrocytes

To test whether GSH synthesis is affected by 2dRib, cultured astrocytes were treated with or without 30 mM 2dRib in the absence or presence of extracellular precursors for GSH synthesis (1 mM glutamate, 0.2 mM glycine, 0.2 mM cystine) to prevent or allow GSH synthesis, respectively. None of these conditions caused any significant increase in the extracellular LDH activity compared to the corresponding controls (Fig. 4B). Presence of amino acids raised the specific cellular GSx content in the absence of 2dRib significantly to 138 ± 17% of the initial GSx content (36.9 ± 1.5 nmol/mg) and completely prevented the loss of cellular GSx observed after exposure of the cells to 30 mM 2dRib. However, also the increase of the cellular GSx content found after application of amino acids was abolished in the presence of 2dRib (Fig. 4A).

As second approach to investigate whether 2dRib affects GSH synthesis, cultured astrocytes were incubated with glutamate, glycine and cystine in the absence or the presence of 10 mM 2dRib and/or 1 mM of the GSH synthesis inhibitor buthionine sulfoximine (BSO) for 4 hours (Fig. 5). The lack of any increase in extracellular LDH activity revealed that none of these conditions compromised cell viability (Fig. 5B). The cellular GSx content in the control condition (absence of 2dRib and BSO) increased significantly to 133 ± 10% compared to the initial content, whereas

exposure of cells to BSO and 2dRib lowered the cellular GSx content significantly to 69 ± 13% and 86 ± 7% of the initial content, respectively (Fig. 5A). However, application of both 2dRib plus BSO lowered the cellular GSx level further to 29 ± 2% of the initial content, which was significantly lower than the values determined for cell treated with 2dRib alone (Fig. 5A).

3.4 Determination of the cellular content of 2dRib

To determine the content of 2dRib in viable astrocytes, the cells were incubated in the absence or presence of 30 mM 2dRib. The cells remained viable under these incubation conditions, since no increase of LDH activity was detectable (Table IV). After 4 hours of incubation, the cells contained about 8 nmol/mg glucose both after incubation in the absence or presence of 2dRib. In contrast, 2dRib was only detectable in cells that had been exposed to 30 mM 2dRib. These cells contained a specific 2dRib content of 104 ± 14 nmol/mg protein (Table IV).

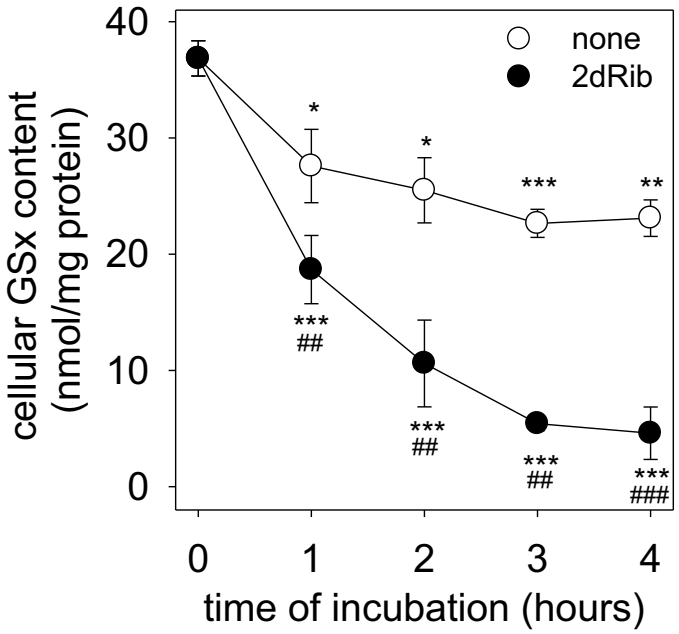

Figure 1: Time dependent effects of 2dRib on the specific cellular GSx content of astrocyte cultures. The cells were incubated for up to 4 hours without (none) or with 30 mM 2dRib. The cultures contained an initial protein content of 100 ± 7 µg protein per well. The significance of differences to the initial GSx content are indicated as *p<0.05, **p<0.01 and ***p<0.001. Significances of differences of data obtained for cells treated without or with 2dRib are indicated by $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001.

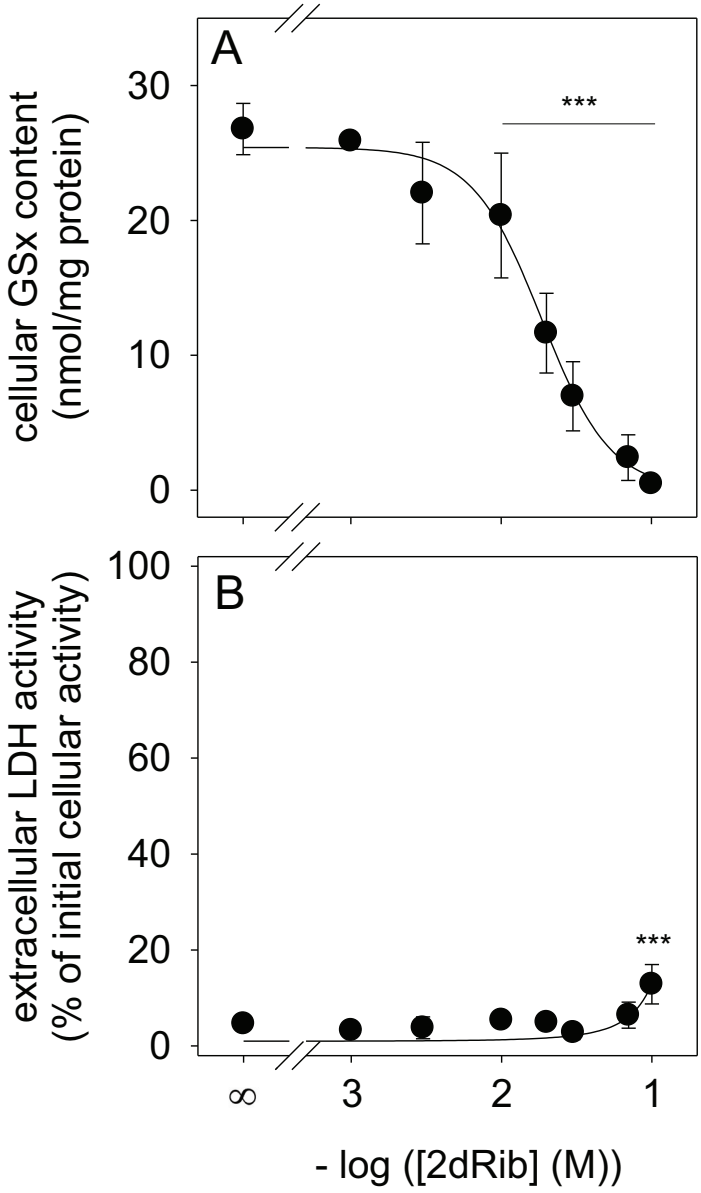

Figure 2: Concentration dependent effects of 2dRib on the GSx content (A) and the cell viability (B) of cultured astrocytes. The cells were incubated for 4 hours with 2dRib in the indicated concentrations. The cultures contained an initial protein content of 97 ± 18 µg protein per well and an initial specific GSx content of 41.0 ± 2.2 nmol/mg protein. The significance of differences to the data obtained for the control (absence of 2dRib) are indicated as ***p<0.001.

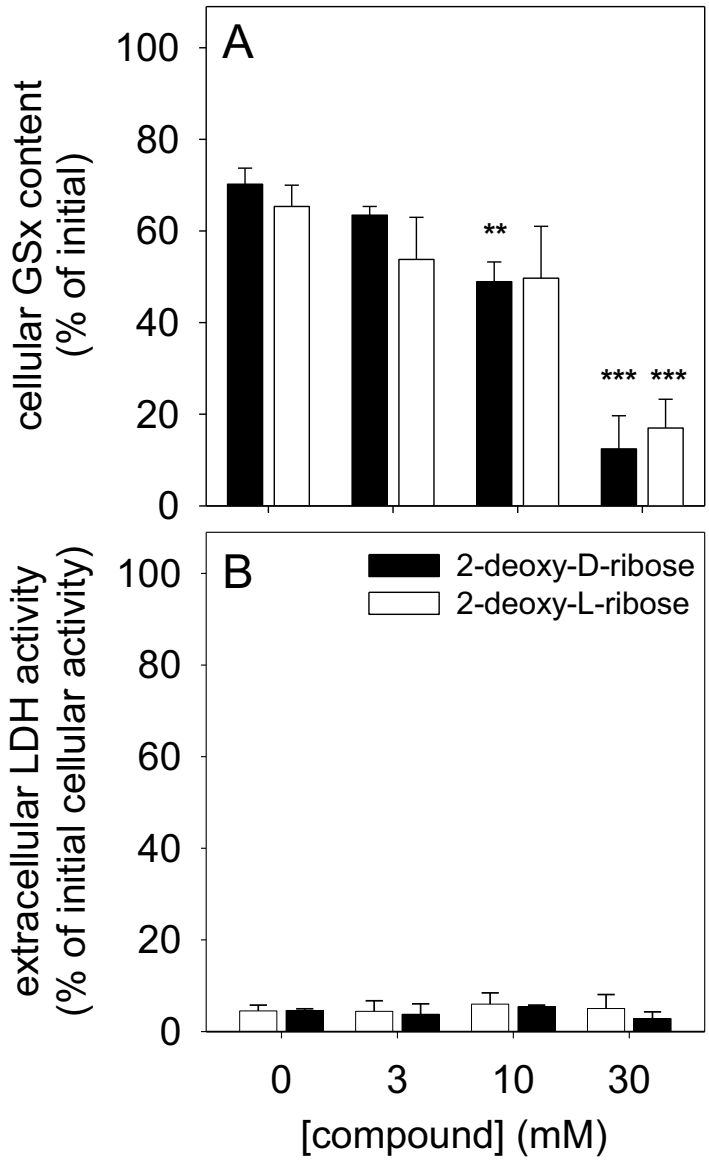

Figure 3: Effects of 2dRib and 2-deoxy-L-ribose on the GSx content (A) and the cell viability (B) of cultured astrocytes. The cells were incubated for 4 hours in the absence or presence of the indicated concentration of 2dRib or 2-deoxy-L-ribose. The cultures contained an initial protein content of 91 ± 24 µg protein per well and an initial GSx content of 41.7 ± 1.4 nmol/mg protein. The significance of differences to the data obtained for the control (absence of 2-deoxyriboses) are indicated as **p<0.01 and ***p<0.001.

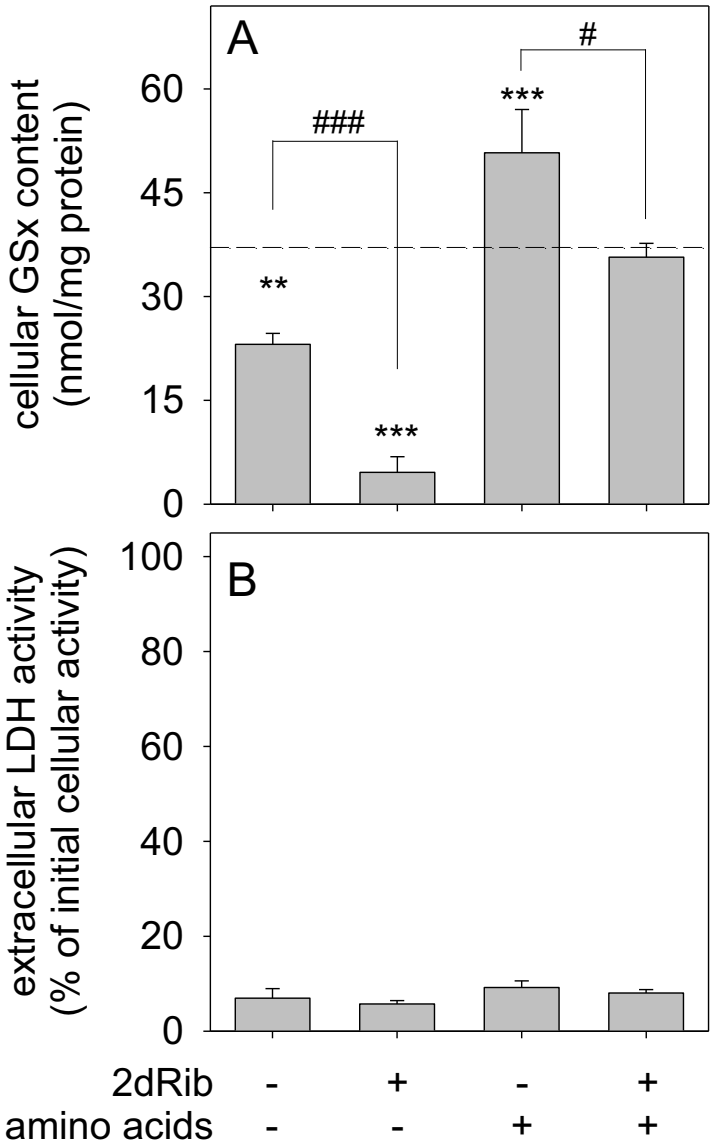

Figure 4: Effects of 2dRib and extracellular GSH precursors on the specific cellular GSx content (A) and the cell viability (B) of astrocyte cultures. The cells were incubated for 4 hours without or with 30 mM 2dRib in the absence or presence of extracellular amino acids (1 mM glutamate, 0.2 mM cystine, 0.2 mM glycine). The cultures contained an initial protein content of 100 ± 7 µg protein per well. The dashed line in A represents the initial GSx content of 36.9 ± 12.5 nmol/mg. The significance of differences to the data obtained for the initial GSx content are indicated as **$p<0.01$ and ***$p<0.001$, significance of differences between the indicated data sets as $^{\#}p<0.05$ and $^{\#\#\#}p<0.001$.

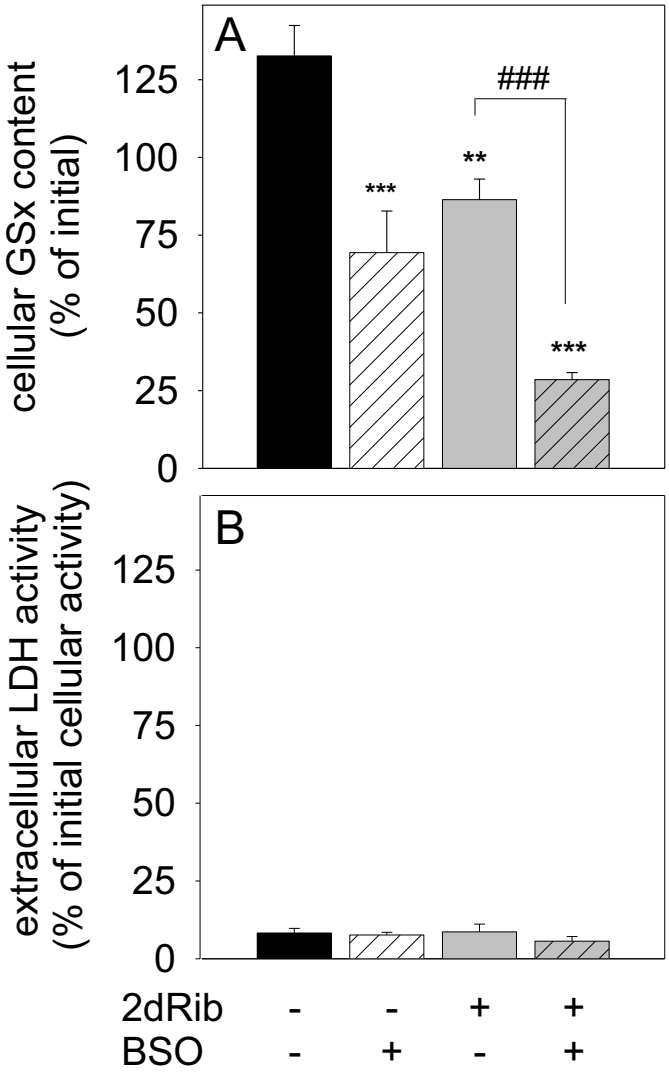

Figure 5: Effects of 2dRib and BSO on the specific GSx content (A) and the cell viability (B) of cultured astrocytes. The cells were incubated with 1 mM glutamate, 0.2 mM cystine and 0.2 mM glycine for 4 hours in the absence or presence of 10 mM 2dRib and/or 1 mM of the GSH synthesis inhibitor BSO. The cultures contained an initial protein content of 115 ± 8 µg protein per well and an initial specific GSx content of 31.8 ± 8.3 nmol/mg protein. The significance of differences to the data obtained for the control (absence of 2dRib and BSO) are indicated as **p<0.01 and ***p<0.001, that for comparison of the indicated data set as $^{###}$p<0.001.

Table I: Cellular and extracellular GSx and GSSG contents and extracellular LDH activity of astrocyte cultures after exposure to 2-deoxyribose

		none	2-deoxy-D-ribose	2-deoxy-L-ribose
cells	GSx (nmol/mg)	34.0 ± 1.0	8.7 ± 0.5***	9.5 ± 1.5***
	GSSG (nmol GSx/mg)	2.1 ± 0.2	1.8 ± 0.1	2.1 ± 0.3
media	GSx (nmol/mg)	9.0 ± 1.5	4.4 ± 0.1*	5.1 ± 1.3
	GSSG (nmol GSx/mg)	1.6 ± 0.6	1.5 ± 0.1	1.3 ± 0.1
	LDH activity (% of initial cellular activity)	5 ± 3	2 ± 1	3 ± 2

Astrocyte cultures were incubated in the absence (none) or the presence of 30 mM of 2-deoxy-D-ribose or 2-deoxy-L-ribose for 4 hours. The cultures contained 102 ± 6 µg protein per well and an initial specific GSx content of 44.7 ± 2.1 nmol/mg protein. The significance of differences to the data obtained for the control condition (none) are indicated as *p<0.05 and ***p<0.001.

Table II: Cellular GSx contents and extracellular LDH activity of astrocyte cultures after exposure to various sugars

Sugar	Cellular GSx content (% of initial)	Extracellular LDH activity (% of initial cellular activity)
none	66 ± 1	5 ± 0
2-deoxy-D-ribose	12 ± 1***	5 ± 2
2-deoxy-L-ribose	11 ± 1***	6 ± 2
D-ribose	59 ± 5	5 ± 0
2-deoxy-D-glucose	55 ± 8	6 ± 3
D-glucose	66 ± 3	5 ± 1
D-fructose	67 ± 7	4 ± 1
saccharose	66 ± 3	6 ± 5

Astrocyte cultures were incubated for 4 hours in the absence (none) or the presence of the indicated sugars (30 mM). The cultures contained 112 ± 4 µg protein per well and an initial specific GSx content of 36.7 ± 6.3 nmol/mg protein. The significance of differences to the data obtained for the control condition (none) is indicated as ***$p<0.001$.

Table III: Specific activities of enzymes in cultured astrocytes after exposure to reducing sugars

Enzyme	Compound	Enzyme activity (nmol/(min x mg))
G6PDH	none	64.5 ± 8.9
	glucose	50.5 ± 13.1
	2-deoxy-D-ribose	62.8 ± 6.9
	2-deoxy-L-ribose	54.5 ± 5.9
GR	none	18.5 ± 1.5
	glucose	16.4 ± 7.0
	2-deoxy-D-ribose	13.1 ± 6.2
	2-deoxy-L-ribose	19.6 ± 4.8
LDH	none	1764 ± 172
	glucose	1828 ± 259
	2-deoxy-D-ribose	1960 ± 163
	2-deoxy-L-ribose	1654 ± 94
GAPDH	none	441 ± 27
	glucose	434 ± 38
	2-deoxy-D-ribose	420 ± 27
	2-deoxy-L-ribose	455 ± 43

The cells were incubated in the absence (none) or the presence of 30 mM of the indicated sugars. After 4 hours of incubation, the cells were lysed and the specific activities of the enzymes glucose-6-phosphate dehydrogenase (G6PDH), glutathione reductase (GR), lactate dehydrogenase (LDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined. The cultures contained 41 ± 6 µg soluble protein per well.

Table IV: Cellular contents of 2-deoxyribose and glucose in cultured astrocytes

	Glucose content (nmol/mg protein)	2dRib content (nmol/mg protein)	GSx content (nmol/mg protein)	Extracellular LDH activity (% of initial cellular activity)
none	8.1 ± 3.1	not detectable	34.0 ± 1.0	5 ± 3
2dRib	8.1 ± 4.3	104 ± 14	8.7 ± 0.5	2 ± 1

Primary astrocyte cultures were incubated in the absence (none) or the presence of 30 mM 2dRib for 4 hours. The cultures contained 102 ± 6 µg protein per well and a specific initial GSx content of 44.7 ± 2.1 nmol/mg protein.

4 Discussion

The reducing sugar 2dRib has been reported to be cytotoxic for various cell types by disrupting the cellular GSH and thiol homeostasis [3, 17, 20, 29, 30] and by inducing apoptosis [3, 18-20]. Since little information is available on the effects of a treatment of brain cells with 2dRib, we have investigated the acute consequences of an exposure of cultured primary astrocytes to 2dRib. Here we demonstrate that both 2dRib and its stereoisomer 2-deoxy-L-ribose lower in millimolar concentrations the cellular GSH content of viable astrocytes.

2dRib strongly accelerated the normal decline of astrocytic GSH, which is predominately due to cellular export of GSH [33] via multidrug resistance protein (Mrp) 1 [39, 40]. For astrocytes, GSH depletion was only observerd after application of 2dRib in a concentration of at least 10 mM, while 30 mM 2dRib almost completely deprived astrocytes of GSH within 4 hours. Compared with other cell types, astrocytes responded much stronger to the presence of 2dRib. Human fibroblast, HL-60 cells and embryonic stem cells had to be exposed to 50 mM 2dRib for 24 h [17], to 15 mM for 96 h [30] and to 70 mM 2dRib for 4 h [20], respectively, to almost completely deprive these cells of GSH. Only erythrocytes lost already after 1 h incubation with 5 mM 2dRib their GSH almost completely [29].

The cellular GSH content was lowered in astrocytes by millimolar concentrations of 2dRib with halfmaximal effects observed at a 2dRib concentration of about 20 mM. Thus, 2dRib had to be applied in much higher concentrations and for longer times than compounds such as iodoacetamide or fumaric acid diesters which lower already in micromolar concentrations astrocytic GSH significantly within minutes [35, 41]. One reason for the need of such high concentrations could be a slow uptake of 2dRib into the cells. However, sugar analysis revealed a cellular 2dRib content of 104 nmol/mg after incubation of the cells with 30 mM 2dRib for 4 hours, which corresponds to a cytosolic 2dRib concentration of 24 mM (calculated by using a specific volume of 4.1 µL/mg astrocytic protein [42]). Thus, within 4 hours of incubation intracellular and extracellular concentrations of 2dRib were almost identical, clearly demonstrating that 2dRib is able to enter the cells with a

substantial rate. However, the mechanism(s) contributing to the uptake of 2dRib into astrocytes remains to be elucidated.

Potential reasons for the observed GSH deprivation by 2dRib in cultured astrocytes could be a loss of cell viability as it has been shown after exposure to 2dRib for various cell types [3, 4, 17, 19, 20], including cultured astroglial cells that had been exposed to 2dRib for up to 72 h [16, 18]. However, for the conditions used here, a loss of cell viability can be excluded as reason for the decline of cellular GSH after exposure to 30 mM 2dRib. Only application of 100 mM 2dRib affected significantly cell viability, most likely by osmotic damage.

For various cell types and cultures, evidence of oxidative stress has been reported as a consequence of an exposure to 2dRib [16, 17, 19, 20]. During oxidative stress astrocytes oxidise GSH to GSSG which is subsequently exported by Mrp1 [39, 43]. However, since the cellular and extracellular GSSG contents of astrocytes were not significantly elevated after exposure to 2dRib, consistent with data reported for embryonic stem cells [20], oxidative stress and accelerated GSSG export cannot explain the 2dRib-induced deprivation of astrocytic GSH. In addition, the deprivation of cellular GSH by a 2dRib-stimulated GSH export, which has recently been reported for embryonic stem cells [20], can be excluded for astrocytes, since presence of 2dRib did not cause an increase but rather a significant decrease in extracellular GSH levels.

Treatment of embryonic stem cells with 2dRib lowered the activity of γ-glutamyl cysteine ligase (GCL), the first enzyme involved in GSH synthesis [20]. However, presence of amino acid substrates prevented the 2dRib-induced decline in astrocytic GSH, demonstrating that GSH synthesis takes place in presence of 2dRib. In addition, application of the GCL inhibitor BSO caused a further decline in the GSH content of 2dRib-treated astrocytes, suggesting independent additive modes of action of BSO and 2dRib to lower cellular GSH content. Thus, an impairment of GSH synthesis can most likely be excluded as molecular mechanism of the 2dRib-induced loss in astrocytic GSH.

Since the deprivation of cellular GSH in astrocytes during exposure to 2dRib appears not to be a consequence of a loss of cell viability, of a stimulated export of GSH or

GSSG or of a compromised GSH synthesis, a direct interaction of GSH with 2dRib or products that are derived from the cellular metabolism of 2dRib are likely to be responsible for the observed decline in astrocytic GSH. 2dRib has been shown to react with proteins by the Maillard reaction, forming Amadori or Heynes products [9]. However, despite of its potential to interact with proteins, 2dRib appeared to predominantly affect the GSH content of cultured astrocytes. At least the activities of the cellular enzymes GAPDH, G6PDH, GR and LDH were not altered, although 2dRib was present in the cells in a concentration of up to 24 mM.

For sugars such as glucose or fructose a direct reaction with GSH to form pyroglutamic acid related Amadori compounds and N-2-deoxy-glucosyl glutathione, respectively, have been reported [44, 45]. However, a direct chemical reaction of 2dRib with GSH was not observed under the conditions used here, since even a 1000fold excess of 2dRib over GSH did not lower the amount of detectable GSH in cell-free conditions (data not shown). In addition, the deprivation of cellular GSH was highly specific for 2dRib and was not observed for ribose, glucose or fructose, nor for the deoxysugar 2-deoxyglucose. Thus, a product of the cellular metabolism of 2dRib is likely to react with cellular GSH, rather than 2dRib itself. Although cell-independent autoxidation of 2dRib does not take place at a rate sufficient to explain the cellular effects of 2dRib found in the hours range [29], the 2dRib moiety in cellular DNA is continuously oxidised in living cells, leading to the formation of various α,β-unsaturated carbonyl species [2]. This structural element is also present in fumaric acid dialkyl esters which rapidly deprive astrocytes of GSH [41]. Thus, an oxidation of free 2dRib in astrocytes to α,β-unsaturated carbonyls which efficiently react with GSH may be involved in the GSH deprivation of astrocytes by 2dRib. Such an enzyme-independent oxidation of 2dRib is not stereospecific and would explain why GSH deprivation of astrocytes was observed for both 2dRib and its stereoisomer 2-deoxy-L-ribose.

In conclusion, 2dRib lowered in millimolar concentrations the cellular GSH content of viable astrocytes. Since 2dRib does not affect GSH synthesis nor the export of GSH or GSSG, the mechanism underlying the GSH deprivation appears rather to be a reaction of GSH with a product of 2dRib metabolism. The high concentration of intracellular 2dRib that is necessary to acutely lower cellular GSH suggest that

2dRib-induced consumption of cellular GSH occurs at best slowly under physiological conditions, when 2dRib or its oxidation products are generated during normal DNA repair processes. However, elevated DNA damage during oxidative stress may provide additional 2dRib and/or its oxidation products that will deprive the cells of GSH, thereby further compromising the antioxidative defence of the cells. In addition, for experimental paradigms that use 2dRib to study the effects of high concentrations of reducing sugares on astrocytes, 2dRib-mediated decline in cellular GSH has to be considered.

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3

Summarising Discussion

The tripeptide GSH is the most abundant low molecular weight thiol in mammalian cells (Cooper & Kristal 1997) and has vast variety of functions (chapter 1.2). In the brain, astrocytes play a key role in the antioxidative defence (Dringen *et al.* 2005, Liddell *et al.* 2006a, Liddell *et al.* 2006b) and in the GSH-mediated detoxification of endogenous derived compounds (Blair 2006, Hirrlinger *et al.* 2002b, Kubatova *et al.* 2006) or xenobiotics (Sagara & Sugita 2001, Waak & Dringen 2006). In addition, astrocytes are essential for providing GSH precursor amino acids (Aoyama *et al.* 2008, Dringen 2009, Dringen *et al.* 1999) as well as metabolic fuel molecules such as lactate to neighbouring neurons (Pellerin *et al.* 2007). Consequently, alterations of the astrocytic metabolism are likely to indirectly affect also the cellular functions of neurons (Fig. 1).

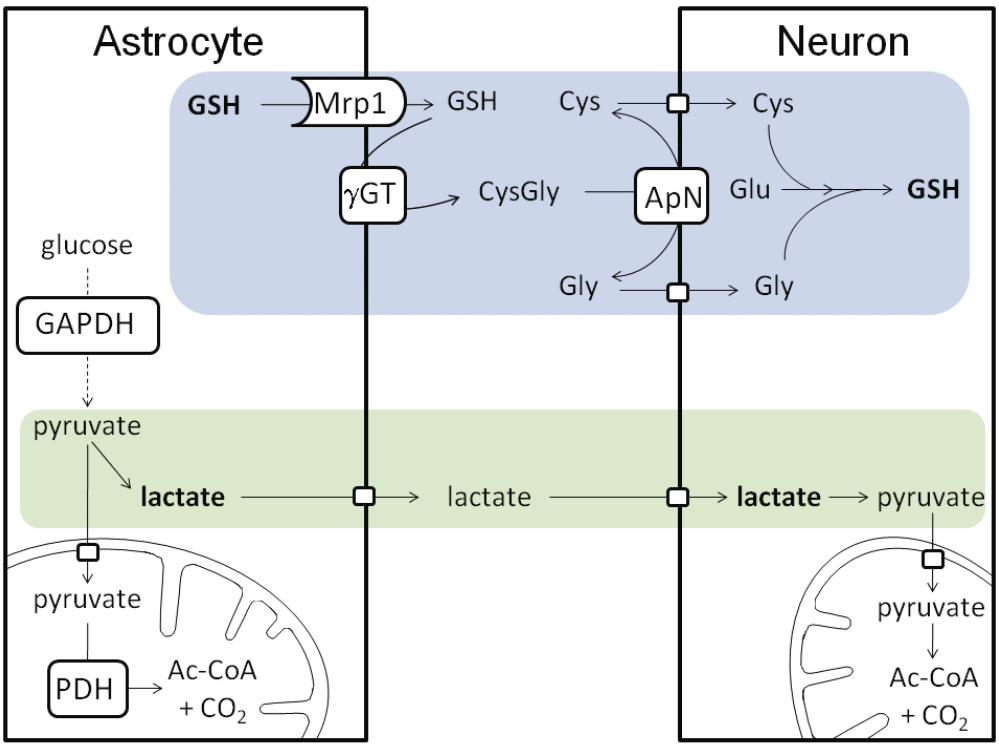

Figure 1: Metabolic coupling between astrocytes and neurons in GSH (blue box) and glucose (green box) metabolism. Ac-CoA: acetyl-CoA; ApN: aminopeptidase-N; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; γGT: γ-glutamyl transpeptidase; Mrp1: multidrug resistance protein 1; PDH: pyruvate dehydrogenase.

In the brain, astrocytes are anatomically located between the brain capillaries and neurons (Sofroniew & Vinters 2010) and are most likely the first cells to encounter xenobiotics that have crossed the blood-brain barrier. Depending on their chemical properties, xenobiotics can diversely affect cellular metabolism. For example xenobiotics can alter enzyme activities or decrease the cellular GSH content of the cells due to conjugation with GSH and subsequent export of these conjugates from the cells (Fig. 2).

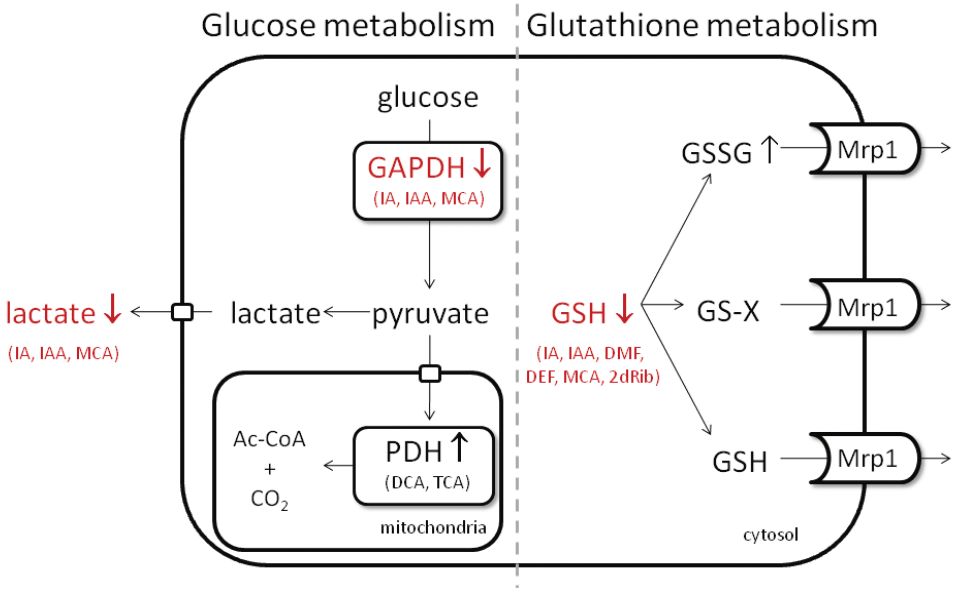

Figure 2: Effects of xenobiotics on glucose and GSH metabolism of brain astrocytes. Processes that play a predominant role in the reactions of astrocytes to the xenobiotics investigated here are indicated in red, others in black. Ac-CoA: acetyl-CoA; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GS-X: glutathione conjugate; Mrp1: multidrug resistance protein 1; PDH: pyruvate dehydrogenase.

The aim of this thesis was to study the GSH-mediated detoxification of xenobiotics by brain astrocytes, using astroglia-rich primary cultures as model system. Immunocytochemical characterisation of these cultures used in this thesis demonstrated that more than 95% of the cells were stained positive for the astrocytic marker GFAP (Eng *et al.* 2000), and that the cultures contain at least two distinct morphologies that most likely represent the presence of protoplasmic and fibrous astrocytes (chapter 2.1; Sofronview & Vinters 2010). In addition, basal biochemical parameters such as the specific GSx content and the activities of

enzymes involved in GSH or glucose metabolism were determined for astroglia-rich primary cultures. The values obtained were comparable to those previously reported for cultured astrocytes (chapter 2.1). Thus, astroglia-rich primary cultures can be considered as reliable and suitable model to investigate the consequences of an exposure of astrocytes to xenobiotics.

In addition to the quantification of cellular GSx contents by a highly sensitive enzymatic cycling assay (Dringen & Hamprecht 1996, Dringen *et al.* 1997), the GSH sensitive dye MCB was used to visualise the cellular GSH content of the cells after treatment with xenobiotics. This staining method is based on the GST-catalysed reaction of MCB with GSH forming a fluorescent GS-bimane conjugate (Eklund *et al.* 2002). The qualitative results obtained with this staining method for various experimental conditions confirmed the results obtained by the quantification of cellular GSx (chapter 2.1).

3.1 Effects of xenobiotics on GSH contents

GSH-mediated detoxification of xenobiotics and endogenous compounds by cultured astrocytes was investigated for halogenated acetates, iodoacetamide (IAA), fumaric acid esters (FAE) and 2-deoxyribose (2dRib) (Table 1). IAA and iodoacetate (IA) are frequently used to inhibit glycolysis (Bakken *et al.* 1998, Bickler & Kelleher 1992, Gemba *et al.* 1994, Loreck *et al.* 1987, Ogata *et al.* 1995), whereas chlorinated acetates are toxins produced during water disinfection processes (Boorman 1999) and/or metabolites generated during detoxification of industrial solvents such as perchloroethylene (Larson & Bull 1992, Lash *et al.* 2000). FAE are successfully used for the systemic therapy of psoriasis (Rostami & Mrowietz 2008) and gained interest as a putative drug for the treatment of autoimmune-based neurological disorders such as multiple sclerosis (Linker *et al.* 2008, Moharregh-Khiabani *et al.* 2009). On the other hand, the reducing sugar 2dRib represents a physiological occurring substance that is endogenously produced as a consequence of DNA damage (Dedon 2008), but has also been frequently applied to investigate sugar-induced cell damage in peripheral cells in the context of diabetes (Ardestani *et al.* 2008, Koh *et al.* 2005, Koh *et al.* 2010, Lee & Choi 2008, Lee *et al.* 2010, Suh *et al.* 2009).

The various compounds investigated differed strongly in their potential to affect the cellular GSH metabolism of astrocytes. The halfmaximal effects on the cellular GSx content (Table 1) demonstrate that IAA, IA and fumaric acid dialkyl esters were highly potent in micromolar concentration to deprive cultured astrocytes of GSH within minutes, whereas monochloroacetate (MCA) and 2dRib had to be applied in 3 to 2000 times higher concentration and for longer incubation periods to severely affect the cellular GSx content.

Small chemical modifications of the compounds caused already severe alterations of their potential to react with GSH, as evident for example from the fact that the reactivity of IAA towards GSH was one order of magnitude higher than that of IA. Also, the fumaric monalkylesters had to be applied in 100 fold higher concentrations compared to the diesters to significantly lower the cellular GSx content in viable cells (Table 1). The differential effects of fumaric mono- and dialkylesters on the cellular GSx content of brain cells have recently been confirmed for oligodendroglial cells (Thiessen *et al.* 2010). Furthermore, in contrast to MCA, dichloroacetate (DCA) and trichloroacetate (TCA) showed no reactivity towards GSH at all. The potential reasons for the different reactivities towards GSH of one compound within its group of compounds have been discussed in detail in the chapters 2.2 to 2.5 of this thesis.

A decrease of cellular GSx content can be a consequence of either unspecific GSx release due to a loss of membrane integrity, of stimulated GSH and/or GSSG export or of enzyme-independent or GST-catalysed conjugation with GSH. All GSx-depriving compounds applied here led to a loss of cell viability, as indicated by the increase of extracellular LDH activity that depended on the concentration of the compound and also on the duration of the treatment (Table 2). However, the deprivation of cellular GSx always preceded the loss of cell viability for all the GSx-depriving compounds applied. Therefore, unspecific GSx release due to compromised membrane integrity can be excluded as the reason for the loss of cellular GSx, after exposure to the compounds investigated here.

Table 1: GSx deprivation of cultured astrocytes by xenobiotics or 2dRib

Compound		Halfmaximal GSx deprivation Time (min)	Halfmaximal GSx deprivation [compound] (mM)
Inhibitors of glycolysis	IA	60	0.1
	IAA	60	0.01
Fumaric acid esters	MMF	60	10
	MEF	60	10
	DMF	60	0.01
	DEF	60	0.01
Chlorinated acetates	MCA	>240	0.3
	DCA	>240	>10*
	TCA	>240	>10*
Reducing sugars	2dRib	240	20

*) no detectable decrease in GSx content found.

Compound		Halfmaximal GSx deprivation [compound] (µM)	Halfmaximal GSx deprivation Time (min)
Inhibitors of glycolysis	IA	100	10
	IAA	100	60
Fumaric acid esters	MMF	100	No effect (60 min)
	MEF	100	No effect (60 min)
	DMF	100	5
	DEF	100	5
Chlorinated acetates	MCA	100	No effect (240 min)
	DCA	100	No effect (240 min)
	TCA	100	No effect (240 min)
Reducing sugars	2dRib	100	No effect (240 min)

The data shown were taken from the results chapters of this thesis.

Table 2: Effect of xenobiotics and 2dRib on the viability of cultured astrocytes

Compound		Onset of detectable toxicity	
		[compound] (mM)	Time (min)
Inhibitors of glycolysis	IA	0.3	90
	IAA	0.3	90
Fumaric acid esters	DMF	0.1	180
Chlorinated acetates	MCA	10	240
Reducing sugars	2dRib	100	240

The data shown were taken from the results chapters of this thesis and represent the lowest concentration of a compound and the shortest incubation time that led to a significant increase in the extracellular activity of LDH compared to controls.

Possible reason for the deprivation of cellular GSx on treatment of cultured astrocytes with xenobiotics might be a stimulated export of GSH and/or GSSG from the cells. Astrocytes are the only brain cell type in culture that has been shown to release substantial amounts of GSH with a rate of 1.8 to 3.8 nmol/(hour x mg) (Dringen *et al.* 1997, Hirrlinger *et al.* 2002c, Sagara *et al.* 1996, Stridh *et al.* 2010). Stimulated GSH export has been reported as a consequence of exposure to elevated glutamate concentrations (Frade *et al.* 2008), ethanol (Rathinam *et al.* 2006, Watts *et al.* 2005), human immunodeficiency virus-1 envelope glycoprotein gp120 (Ronaldson & Bendayan 2008) and compounds such as MK571 that modulate the affinity of Mrp1 for GSH (Hirrlinger *et al.* 2002c, Loe *et al.* 2000). However, since none of the GSx-depriving substances investigated in this thesis led to an elevated extracellular accumulation of GSx, a stimulation of GSH export by these compounds can be excluded. Oxidising compounds such as peroxides (Hirrlinger *et al.* 1999, Hirrlinger *et al.* 2001, Minich *et al.* 2006) or dopamine (Hirrlinger *et al.* 2002b) cause cellular GSx deprivation by inducing oxidation of GSH to GSSG which is subsequently exported by Mrp1 from astrocytes (Hirrlinger *et al.* 2001, Hirrlinger *et al.* 2002c, Minich *et al.* 2006). As none of the substances applied caused any detectable rise in cellular GSSG nor a stimulated GSSG export from the cells, oxidative stress and accelerated GSSG export can also be excluded as a consequence of a treatment of cultured astrocytes with halogenated acetates, IAA, FAE or 2dRib.

Since a loss of membrane integrity and stimulated GSH and/or GSSG export could be excluded as reason for the observed GSx deprivation by various compounds, a direct interaction of the compounds investigated here with GSH appears to be the most likely explanation for their potential to deprive astrocytes of their GSx. For IAA, IA and fumaric acid diesters this hypothesis is strongly supported by the observation that these compounds induced the rapid disappearance of detectable GSH in cell-free systems (chapters 2.2 & 2.3). This ability to react in an enzyme-independent manner with GSH was consistent with effects obtained on cultured astrocytes, as IAA, IA and fumaric acid diesters possessed the highest potential of the investigated compounds to deprive the cells of GSH (Table 1). Whether the reactions of IAA, IA or fumaric acid diesters with GSH in cells is enzyme-independent or requires GST-activity remains to be elucidated.

In order to identify and characterise the possible conjugates of dimethyl fumarate (DMF) with GSH, an analytical method using gas chromatography/tandem mass spectrometry has been established (data not shown). However, despite successful detection of the conjugate in standard solutions derived from the pure chemical reaction of DMF with GSH, the signals obtained for incubation media and cell lysates were not reliable to identify and quantify the expected low concentration of conjugates (data not shown).

In contrast to IAA, IA and fumaric acid dialkylesters, neither chlorinated acetates, fumaric acid monoesters, nor 2dRib reacted with GSH in a cell-free system, suggesting that the deprivation of cellular GSH in viable astrocytes after exposure of the cells to these compounds is likely to be a consequence of reactions that are catalysed by GST. Several isoforms of GST have been shown to be expressed in astrocytes in culture and *in vivo* (Dhanani & Awasthi 2007, Johnson *et al.* 1993, Martinez-Lara *et al.* 2003, Sagara & Sugita 2001). These enzymes possess a highly conserved GSH binding site and an electrophilic substrate binding site that varies significantly between different isoforms (Mahajan & Atkins 2005). Halogenated compounds and α,β-unsaturated carbonyls are known substrates of cytosolic GST (Hayes *et al.* 2005). Thus, GST are likely to play a role in the conjugation with GSH of at least some of the compounds investigated here. To study a possible involvement of GST in the deprivation of cellular GSH by halogenated acetates, IAA, fumaric acid

diesters and 2dRib, we attempted to inhibit the cytosolic GST by the inhibitor sulfasalazine (Cao *et al.* 2003, Li *et al.* 2005). Already micromolar concentrations of this inhibitor decreased the specific activities of cytosolic GST in cell lysates by about 60%, but even 1 mM of the inhibitor did not prevent the cellular GSx deprivation of cultured astrocytes after treatment with some of the compounds investigated (data not shown). Thus, a contribution of GST in the deprivation of cellular GSH by the xenobiotics investigated here remains to be elucidated.

Besides the differential chemical reactivities of the compounds towards GSH and their ability to potentially serve as GST substrates, also the cellular presence of the compounds in sufficient concentrations is a prerequisite for their ability to react with cellular GSH. Uncharged molecules such as IAA and fumaric acid diesters are likely to penetrate the membrane by simple diffusion, whereas the membrane permeability of IA and fumaric acid monosters is likely to be limited due to their charged nature. However, the strong inhibitory effect of IA on GAPDH activity (chapter 2.2) reveals that this compound was present at least in sufficiently high concentration in the cells to inhibit this enzyme. The negatively charged fumaric monoesters did not affect the cellular GSH content in micromolar concentrations, but extracellular concentration in the millimolar range led to a significant decrease in cellular GSx, suggesting that limited transport efficiency of these compounds can at least in part be overcome by increasing extracellular concentrations.

Chlorinated acetates have been described as substrates for monocarboxylate transporters (MCT) in rat liver cells (Jackson & Halestrap 1996). Since MCT have been demonstrated to be expressed in astroglial cells (Broer *et al.* 1997, Debernardi *et al.* 2003), these transporter could be involved in the uptake of chlorinated acetates into cultured astrocytes. However, MCT are unlikely to be responsible at least for MCA uptake, since a 10 fold excess of the physiological MCT substrate pyruvate did not lower the observed cellular GSx deprivation by MCA (data not shown). In contrast to the xenobiotics, cultured astrocytes had to be exposed to millimolar concentrations of 2dRib to significantly affect the cellular GSH content (chapter 2.5), while application of other sugars in this concentration range did not affect the cellular GSH content. Since 2dRib was present in astrocytes in a concentration of 24 mM, a limited transport efficiency as reason for the high

concentration of 2dRib that had to be applied to lower the astroglial GSx content can be excluded.

3.2 Effects of xenobiotics on glucose metabolism

Astrocytes play a key role in brain glucose metabolism due to their extensive contacts to brain capillaries (Abbott 2002) and their metabolic coupling with neurons (Benarroch 2005, Benarroch 2010, Castro *et al.* 2009). The astrocyte-neuron lactate-shuttle hypothesis postulates that lactate released from astrocytes is provided as an essential metabolic fuel molecule to neighbouring neurons (Pellerin *et al.* 2007). Consequently, altered glucose metabolism of astrocytes by xenobiotics would also affect neuronal activity. To study whether the xenobiotics investigated in this thesis would alter the glucose metabolism of cultured astrocytes, their influence on lactate production and release was investigated. Amongst the compounds used, IAA, IA and chlorinated acetates lowered lactate release from cultured astrocytes, but differed strongly in their potential to do so. Activation of the oxidative part of the pentose phosphate pathway, inhibition of enzymes involved in glycolysis and enhanced mitochondrial oxidation of pyruvate have been discussed to lower lactate release from astrocytes (Liddell *et al.* 2009). For IAA, IA and MCA GAPDH inhibition was identified as the underlying mechanism for the lowered lactate release observed after treatment of cultured astrocytes with these compounds. The inhibition of GAPDH by IAA, IA and MCA results most likely from a covalent modification of the highly reactive thiol group in the active center of GAPDH (Byers & Koshland 1975, Sakai *et al.* 2005). Amongst the compounds investigated, IA appears to be the most potent inhibitor of GAPDH. In a concentration of 1 mM this compound led to a complete inhibition within 5 min of treatment of the cells, whereas MCA had to be applied in 10 times higher concentrations for several hours to inhibit GAPDH activity (chapters 2.2 and 2.4). This inhibition by IA appears to be specific for GAPDH as the activities of other dehydrogenases such as G6PDH or LDH were not and the activity of GR was only partially altered (chapter 2.2).

The mechanism involved in lowering lactate release after exposure of cultured astrocytes to DCA or TCA is most likely the activation of the pyruvate dehydrogenase (PDH) complex by noncompetitive inhibition of the PDH kinase

(Whitehouse *et al.* 1974, Whitehouse & Randle 1973). At least for DCA the activation of the PDH complex has been shown for astrocytes in culture and *in vivo* (Chang *et al.* 1992, Colohan *et al.* 1986, Corbett *et al.* 1998, Itoh *et al.* 2003, Kuroda *et al.* 1984, Miller *et al.* 1990), whereas the mechanism of TCA to lower lactate release in cultured astrocytes remains to be elucidated.

3.3 Future perspectives

Although several attempts were made in collaboration to identify and quantify the conjugates that were postulated to be formed during exposure of cultured astrocytes with xenobiotics and 2dRib, this aspect is still pending. Suitable assays for the suggested GSH conjugates would allow to study i) the involvement of GST in the formation of the conjugates, ii) the clearance of these conjugates from cells and the role of transport proteins in their export from the cells, and iii) secondary effects of the conjugates on the metabolism of brain cells. A variety of methods, including liquid and gas-chromatography coupled with mass spectrometric analysis and nuclear magnetic resonance spectroscopy techniques, have been reported for the detection of conjugates of GSH with 4-hydroxynonenal (Falletti & Douki 2008, Kuiper *et al.* 2010, Long *et al.* 2010, Warnke *et al.* 2008), dopamine (Zhou & Lim 2009) or acetaminophen (Thatcher & Murray 2001) in tissues or in cell cultures. In addition, these methods were used in cell-free conditions for structural analysis of GSH interaction with fumaric acid esters (Kubal *et al.* 1995, Schmidt *et al.* 2007) or for glucose-triggered modifications of GSH (Jeric & Horvat 2009). The published methods differ strongly in complexity but appear to provide a basis for the development of analytical methods to detect GSH conjugates in cultured astrocytes and their media after exposure to xenobiotics. A successful detection and quantification of the conjugates formed is crucial to address the detoxification of xenobiotics via conjugation to GSH and the export of the conjugates from the cells. Those assays would also allow to approach mechanistical aspects, such as to study the involvement of GST in the formation of the conjugates and the putative export mechanisms.

GST describe a family of phase II-detoxifying enzymes that catalyse the conjugation with GSH of a broad spectrum of substrates, including endogenously derived

substances, therapeuticals and toxins (Hayes *et al.* 2005, Mahajan & Atkins 2005). A great variety of GST inhibitors are known and have recently been summarised (Mahajan & Atkins 2005, Zhao & Wang 2006). However, some of the frequently used inhibitors such as ethacrynic acid lower GST activity by depleting the substrate GSH (Huang & Philbert 1996) and are therefore not suitable to study the GST catalysed formation of GSH conjugates after treatment of the cells with xenobiotics. Assuming the availability of functional assays for conjugates, inhibitors such as sulfasalazine, but also GSH analogs with S-alkylated cysteine moieties could be used to study the GST-dependent conjugate formation, at least in cell lysates. In addition, GST knockout mice (Henderson & Wolf 2005) and gene-silencing techniques (Smeyne *et al.* 2007) could help to specify the GST isoforms involved in conjugate formation in cultured astrocytes.

Several Mrp isoforms have been shown to be expressed in brain (Hirrlinger *et al.* 2002a, Nies *et al.* 2004) that are discussed to be involved in the export of GSH conjugates from the cells (Kruh & Belinsky 2003). In astroglia-rich primary cultures, Mrp1 has been identified to predominantly mediate the export of GSH or GSSG (Hirrlinger *et al.* 2001, Hirrlinger *et al.* 2002c, Minich *et al.* 2006) and GS-MCB conjugates (Waak & Dringen 2006). Inhibitors such as MK571 (Leier *et al.* 1994, Leier *et al.* 1996) or Mrp knockout models (Minich *et al.* 2006) would be suitable to identify transporters of this family that are potentially involved in the export of GSH conjugates of the xenobiotics investigated here. Future work on conjugate export from astrocytes should also include the transporter RLIP76 (Singhal *et al.* 2009), a novel non-ABC transporter which appears to dominate the export of xenobiotics and GSH-conjugates in peripheral cells (Awasthi *et al.* 2009). The putative expression of RLIP76 in neural cells has been controversially discussed (Awasthi *et al.* 2005, Soranzo *et al.* 2007) and its expression in astrocytes still remains to be elucidated.

Conjugation of xenobiotics to GSH and the prevention of cellular accumulation of the conjugates by efficient export mechanisms serve to minimise toxicity (Awasthi *et al.* 2009), which has been nicely illustrated for 4-hydroxy-2-nonenal (4HNE), a product derived from lipidperoxidation (Niki 2009). Since 4HNE modulates gene expression and stress signalling (Awasthi *et al.* 2003, Forman *et al.* 2003, Yang *et al.* 2003), the export of its GSH conjugate is one important regulator of 4HNE function. However,

for the xenobiotics investigated here and their GSH conjugates, little is known about their potential to modulate cellular functions of brain cells. Application of micromolar concentrations of fumaric acid dialkylesters that deprived cultured astrocytes within minutes of their GSH (chapter 2.3) led to a strong hemeoxygenase 1 (HO-1) induction both in cultured astrocytes (Schmidt & Dringen 2009) and oligodendroglial OLN-93 cells (Thiessen *et al.* 2010). These data demonstrate at least for fumaric acid dialkylesters a correlation of GSH-mediated detoxification and stress signalling, which should be addressed in further studies. In this context it should be discriminated whether GSH depletion itself, the kinetic of GSH depletion, the presence of the conjugate formed or the presence of the xenobiotic modulate cellular functions, such as the HO-1 expression, in addition to the potential of a compound to modulate the cellular GSH content.

Cell cultures are used as model system to simplify the cellular complexity of the brain. However, cell culture data obtained on metabolic pathways and their alterations might not extrapolate to the *in vivo* situation. For this purpose, detoxification studies of xenobiotics could be extended to other brain cell types in culture (e.g. neurons, microglial or oligodendroglial cells) and to systems with an increased level of complexity such as astrocyte-neuron co-culture, brain slices and *in vivo* animal models. This would help to better understand the metabolic interactions of brain cells during exposure to xenobiotics and would verify that the data observed for cultured astroglial cells have *in vivo* relevance.

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4

Appendix

4.1 Curriculum vitae

Personal Information

Date of birth: 28th of June 1979
Place of birth: Freiburg im Breisgau, Germany

Education

03/07 – 06/10	**Doctoral thesis in Neurochemistry**, *University of Bremen*, Germany "Effects of Xenobiotics on the Glutathione and Glucose Metabolism of Cultured Brain Astrocytes" Supervisor: Prof. Dr. Ralf Dringen, *University of Bremen*, Germany
12/06	**Diploma in Biology**, *University of Bremen*, Germany Diploma thesis: "Age dependent changes of the physiological stress response of the queen scallop *Aequipecten opercularis*" Supervisor: PD Dr. Doris Abele, *Alfred-Wegener Institute for Marine and Polar Research*, Bremerhaven, Germany
10/01-12/06	**Studies of Biology**, *University of Bremen*, Germany
09/01	**Pre-Diploma in Biology**, *Albert-Ludwigs-University of Freiburg*, Germany
10/98-09/01	**Studies of Biology**, *Albert-Ludwigs-University of Freiburg*, Germany
06/98	**Abitur (general qualification for university entrance)** *Marie-Curie-Gymnasium Kirchzarten*, Germany

Teaching Experience

03/07 – 06/10	**Supervisor in biochemical courses** *University of Bremen*, Germany
07/07 – 08/07	**Course instructor**, *Deutsche SchülerAkademie*, Rostock, Germany

Course –related Activities

03/02 – 09/05	**Student research assistant**, *Alfred-Wegener Institute for Marine and Polar Research*, Bremerhaven, Germany
01/04 – 03/04	**Expedition participant as student research assistant** European Iron Fertilization Experiment EIFEX ANT XXI/3, Polarstern *Alfred-Wegener Institute for Marine and Polar Research*, Bremerhaven, Germany

4.2 List of publications

Philipp, E., **Schmidt, M.**, Gsottbauer, C., Sänger, A M. & Abele, D. (2008). Size- and age-dependent changes in adductor muscle swimming physiology of the scallop *Aequipecten opercularis. J Exp Biol,* **211,** 2492-2501.

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Schmidt, M. M., Rohwedder, A. & Dringen, R. Effects of chlorinated acetates on the glutathione metabolism and on glycolysis of cultured astrocytes. *Neurotox Res*, revised manuscript.

Scheiber, I. F., **Schmidt, M. M.** & Dringen, R. Zinc prevents the copper-induced damage of cultured astrocytes. *Neurochem Int,* in revision.

Schmidt, M. M., Greb, H., Koliwer-Brandl, H., Kelm, S. & Dringen, R. 2-Deoxyribose deprives cultured astrocytes of their glutathione. *Neurochem Res,* Submitted.