



**Formaldehyde Metabolism and
Formaldehyde-induced Alterations in Glucose and
Glutathione Metabolism of Cultured Brain Cells**

Dissertation

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Herewith, I, Ketki Tulpule, declare that this dissertation is my own research work and that the thesis was written by me. Any contributions by others have been duly acknowledged.

Bremen, November 2012

(Ketki Tulpule)

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II. Structure of the thesis

This thesis consists of introduction (Chapter 1), methods (Chapter 2), results (Chapter 3) and summarizing discussion (Chapter 4). Chapter 2 contains a manuscript on methods to generate neural cultures as well as use of these cultures for metabolic studies that has been submitted for an invited book chapter in 'Neuromethods: Brain Energy Metabolism'. Chapter 3 comprises of four parts with three publications (3.1, 3.2 and 3.3) and a submitted manuscript (3.4).

The publications in chapters 3.1 to 3.3 are included as portable document formats. The submitted manuscripts (Chapters 2 and 3.4) have been formatted to maintain uniformity in the layout of this thesis, without altering the content. For ease of reading the manuscripts, tables and figures with their legends have been placed in the text body (Chapter 2) or before the discussion section (Chapter 3.4). The figures and tables in chapters 1 and 4 are numbered with a different style than that in manuscripts/publications. At the end of each chapter the references are listed pertaining to the citations in that chapter. For publications and manuscripts, the experimental contributions of all authors are listed on the first page of each chapter.

III. Summary

Formaldehyde is an environmental pollutant that is also generated in the body during normal metabolic processes. Interestingly, several pathological conditions are associated with an increase in formaldehyde-generating enzymes in the body. The level of formaldehyde in the brain is elevated with increasing age and in neurodegenerative conditions which may contribute to lowered cognitive functions. Although the neurotoxic potential of formaldehyde is well established, the molecular mechanisms involved remain, to a great extent, obscure. Also, the ability of the different types of brain cells to metabolize formaldehyde has not been reported so far.

This thesis investigated the capacity of cultured brain cells to metabolize formaldehyde and studied the effects of a formaldehyde exposure on the glucose and the glutathione metabolism by using primary cultures of cerebellar granule neurons or astrocytes as well as the oligodendroglial cell-line OLN-93 as model systems. These cultured cells were remarkably resistant towards acute toxicity of formaldehyde and expressed the mRNAs for enzymes that are known to be involved in formaldehyde generation and disposal, suggesting that brain cells are able to metabolize this aldehyde. Furthermore, all three types of cultures cleared exogenously applied formaldehyde with almost identical rates, but differed in the extent of the formation of the formaldehyde oxidation product, formate.

Since formate is a known inhibitor of the cytochrome c oxidase of the mitochondrial respiratory chain and since the metabolism of formaldehyde involves the important antioxidant glutathione, the effect of an exposure of cultured brain cells to formaldehyde on their glucose and glutathione metabolism was also investigated. Formaldehyde application accelerated the export of glycolysis-derived lactate and induced a rapid multidrug-resistance protein 1-mediated export of glutathione from cultured brain cells. These formaldehyde-induced alterations in metabolic pathways of brain cells may contribute to the known impairments in memory and learning that have been reported for neurodegenerative conditions and for formaldehyde-exposed animals.

IV. Zusammenfassung

Formaldehyd ist ein Umwelttoxin, das jedoch auch als normales Stoffwechselintermediat im Körper gebildet wird. Interessanterweise ist der Formaldehydgehalt in Gehirn bei zunehmendem Alter und bei neurodegenerativen Erkrankungen erhöht, was zu verringerten kognitiven Leistungen beitragen könnte. Obwohl das neurotoxische Potential von Formaldehyd gut bekannt ist, sind die beteiligten molekularen Mechanismen weitgehend unerforscht. Ebenso ist das Potential der verschiedenen Typen von Gehirnzellen zur Metabolisierung von Formaldehyd bis dato nicht beschrieben.

Die vorliegende Dissertation untersuchte die Fähigkeit von Gehirnzellen in Kultur zur Metabolisierung von Formaldehyd sowie Wirkung einer Formaldehydexposition auf den Glukose- und Glutathionmetabolismus an Primärkulturen von cerebellaren Körnerzellen oder Astrozyten sowie an der oligodendroglialen Zelllinie OLN-93 als Modellsystemen. Diese Zellkulturen waren außerordentlich widerstandsfähig gegenüber akuter Formaldehydtoxizität und exprimierten die mRNAs für Enzyme, die an der Bildung und Beseitigung von Formaldehyd beteiligt sind. Dies weist darauf hin, dass diese Gehirnzellen Formaldehyd metabolisieren können. Weiterhin beseitigten die drei untersuchten Zelltypen exogen appliziertes Formaldehyd mit nahezu identischer Geschwindigkeit, unterschieden sich aber im Ausmaß der Bildung des Formaldehyd-Oxidationsproduktes Formiat.

Da Formiat ein Hemmstoff der Cytochrom-c-Oxidase der mitochondrialen Atmungskette ist, und bei der Formaldehydoxidation das wichtige Antioxidans Glutathion beteiligt ist, wurden die Effekte von Formaldehyd auf den Glukose- und Glutathionstoffwechsel in Gehirnzellkulturen untersucht. Die Gabe von Formaldehyd steigerte den Export des Glycolyseproduktes Laktat und führte zu einem schnellen, *Multidrug resistance protein-1*-vermittelten Export von Glutathion aus Gehirnzellen. Die Formaldehyd-induzierten Veränderungen in diesen wichtigen Stoffwechselwegen von Gehirnzellen könnten zu den Beeinträchtigungen von Gedächtnis und Lernen beitragen, welche für neurodegenerative Erkrankungen sowie eine Formaldehydexposition von Tieren beschrieben wurden.

V. Abbreviations

°C	degree
%	percent
ϵ	molar extinction co-efficient
μ	micro
γ	gamma
λ_{em}	emission wavelength
λ_{ex}	excitation wavelength
2VP	2-vinylpyridine
Aal	acetaldehyde
A β	beta-amyloid
AD	Alzheimer's disease
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
ANOVA	analysis of variance
APC(s)	astroglia-rich primary culture(s)
ApN	aminopeptidase N
Ara-C	cytosine β -D-arabinofuranoside
ATP	adenosine triphosphate
BBB	blood-brain barrier
bp	base pairs
BSA	bovine serum albumin
BSO	buthionine sulfoximine
c	concentration
CAS	Chemical Abstracts Service
cDNA	complementary deoxyribonucleic acid
CGNC(s)	cerebellar granule neuron culture(s)
CNP	2',3'-cyclic nucleotide 3'-phosphodiesterase
CNS	central nervous system
d	path length
DAPI	4'-6-diamidino-2-phenylindole dihydrochloride
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
EBSS	Earle's balanced salt solution
Ed(s).	editor(s)
EDTA	ethylenediamine tetraacetate
et al.	Latin: <i>et alii</i> , and others
Fa	formate
Fal	formaldehyde
FCS	fetal calf serum

Abbreviations

Fig.	figure(s)
g	acceleration of gravity
GABA	γ -aminobutyric acid
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
G6P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
GR	glutathione reductase
GPT	glutamate pyruvate transaminase
GSH	glutathione
GSSG	glutathione disulfide
GSx	total glutathione
γ -GT	γ -glutamyl transpeptidase
h	hour(s)
H33342	Hoechst 33342
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HK	hexokinase
IARC	International Agency for Research on Cancer
IB	incubation buffer
IL	interleukin
JHDM	JmjC domain-containing histone demethylases
K _m	Michaelis-Menten constant
L	liter
LDH	lactate dehydrogenase
LSD	lysine-specific demethylase
m	milli/meter
M	molar (mol per L)
MAG	myelin-associated glycoprotein
MAP	microtubule-associated protein
MCT	monocarboxylate transporter
MeOH	methanol
mEBSS	modified Earle's balanced salt solution
min	minute(s)
mol	mole
mRNA	messenger ribonucleic acid
Mrp	multidrug resistance protein
MS	multiple sclerosis
MTHFD	methylene tetrahydrofolate dehydrogenase
n	nano
n.a	not applicable
n.d	not detectable/not determined
NAD ⁺ /NADH	nicotinamide adenine dinucleotide, oxidized/reduced
NAD ⁺ /NADH	nicotinamide adenine dinucleotide phosphate, oxidized/reduced
NMDA	N-methyl-D-aspartate

p	probability
PBS	phosphate-buffered saline
PCR(s)	polymerase chain reaction(s)
PD	Parkinson's disease
PDL	poly-D-lysine
pH	potenz Hydrogen (negative decadic logarithm of proton concentration)
PI	propidium iodide
ppm	parts per million
r ²	linearity coefficient
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR(s)	reverse transcription polymerase chain reaction(s)
s	second(s)
sd	standard deviation
SSA	sulfosalicylic acid
SSAO	semicarbazide-sensitive amine oxidase
TCA	tricarboxylic acid
THF	tetrahydrofolate
TNB	5-thio-2-nitrobenzoate
Tris	tris (hydroxymethyl) aminomethane
U	unit (enzyme)
VAP	vascular adhesion protein
V _{max}	maximum velocity
v/v	volume per volume
WHO	World Health Organisation
w/v	weight per volume

1.

Introduction

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1.1. Formaldehyde

Formaldehyde, also known as methanal, is the simplest aldehyde and was first described in 1855 by a Russian scientist named Alexander Michailowitsch Butlerow (Salthammer et al., 2010). The chemical synthesis of formaldehyde by methanol dehydration was first achieved in 1867 by a German chemist, August Wilhelm von Hofmann (Salthammer et al., 2010). In the years to follow, the properties of formaldehyde were extensively studied and this compound was one of the earliest to obtain a Chemical Abstracts Service (CAS) registry number (50-00-0). Formaldehyde is highly reactive and can undergo hydration or form hemiacetals with alcohols, thiohemiacetals with thiols and Schiff bases with amines (Vollhardt and Schore, 2005) which makes this aldehyde an important compound in industries (Tang et al., 2009). The following section highlights the different modes of formaldehyde exposure in humans, its cellular metabolism and also implications of increased formaldehyde levels in diseases.

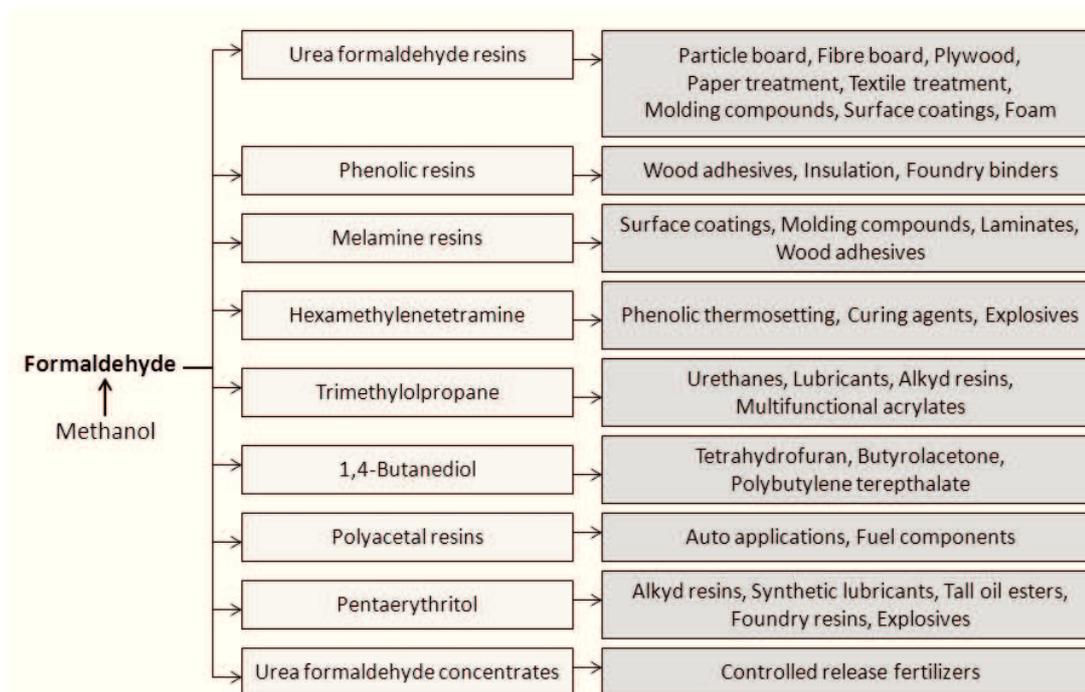


Figure 1.1: Industrial use of formaldehyde as a chemical feedstock.

Modified from Salthammer et al. (2010).

1.1.1. Exogenous sources of formaldehyde

Formaldehyde is commercially synthesized by silver- or ferric molybdate-catalyzed oxidation of methanol (Kralj, 2010; Qian et al., 2003). In 2006 the annual global production of formaldehyde was 32 million tons, with China (34%), United States (14%) and Germany (8%) being the highest contributors (Tang et al., 2009). Such a high production of formaldehyde can be attributed to the vast industrial applications of this compound (Fig. 1.1).

Owing to the extensive industrial use of formaldehyde, a number of commonly used products contain either this compound or formaldehyde-releasing substances (de Groot et al., 2009; Sasseville, 2004). Some examples of such products are construction materials, agricultural fertilizers, fumigants, paints, cosmetics, antiperspirants, polish, cleaning agents and toiletries (de Groot et al., 2010; de Groot et al., 2009; Sasseville, 2004). In addition, formaldehyde can be produced and released from burning of wood, coal, tobacco, natural gas and kerosene (de Groot et al., 2009; Laitinen et al., 2010). Such a broad spectrum of products and processes that contain and/or release formaldehyde makes this aldehyde a ubiquitous compound. Hence, it is not surprising that formaldehyde can also be detected in residential indoor air across the globe (Table 1.1).

Table 1.1: Some studies reporting the presence of formaldehyde in residential indoor air.

Country	References
Canada	Gilbert et al., 2005; Heroux et al., 2010
Japan	Azuma et al., 2007; Ohura et al., 2006; Park and Ikeda, 2006; Sakai et al., 2004
China	Guo et al., 2009; Li et al., 2008; Wang et al., 2007; Zhao et al., 2004
USA	Hun et al., 2010; Kinney et al., 2002; Offermann et al., 2009
France	Clarisse et al., 2003; Marchand et al., 2006; Marchand et al., 2008
Germany	de Bruin et al., 2008; Ullrich, 2002
Sweden	Sakai et al., 2004; Gustafson et al., 2005; Gustafson et al., 2007;
Mexico	Baez et al., 2003; Serrano-Trespalcios et al., 2004

The World Health Organization (WHO) reviewed literature on formaldehyde levels in the air and its consequences on health in 2010 to set a short-term (30 min) guideline for maximal formaldehyde concentration in the air of 0.1 mg/m³ (WHO, 2010) which corresponds to around 0.08 ppm. Guidelines for formaldehyde concentration in indoor air are also set by health authorities in different countries. For example, in Germany an indoor air guideline value of 0.1 ppm was established in 1977 and has remained unchanged even after the recent reassessment in 2006 (Salthammer et al., 2010).

Inhalation and contact with skin are not the only modes of formaldehyde exposure in humans. Formaldehyde can also enter the body by consumption of coffee, codfish, meat, poultry and maple syrup which naturally contain formaldehyde (de Groot et al., 2009; Dhareshwar and Stella, 2008). Moreover, the illicit use of Rongalite®, a food preservative synthesized from formaldehyde, increases the potential risk of exposure to this aldehyde (Tang et al., 2009). Furthermore, aspartame, an artificial sweetener, and some prodrugs also release formaldehyde on their metabolism in the body (Dhareshwar and Stella, 2008; Monte, 2010).

1.1.2. Endogenous formaldehyde generation

A number of metabolic processes in the body generate formaldehyde. Some of these important endogenous reactions contributing to formaldehyde production are described in this section.

1.1.2.1. Methanol oxidation

Methanol can be produced within the body by hydrolysis of protein carboxymethyl esters either non-enzymatically or catalyzed by methylesterases (Lee et al., 2008). Additionally, accidental or intentional intake of methanol can further expose the body to this alcohol. In the body, methanol oxidation to formaldehyde can occur by three different pathways (Fig. 1.2). The first pathway involves the cytosolic enzyme alcohol dehydrogenase (ADH) 1 which oxidizes

methanol to formaldehyde along with the reduction of NAD^+ to NADH (Friedenson, 2011; Harris et al., 2003; MacAllister et al., 2011). Alternatively, hydrogen peroxide, most likely generated by NADPH-dependent electron transfer, contributes to the direct oxidation of methanol by catalase (Fig. 1.2). The hydrogen peroxide can also undergo Fenton chemistry to form hydroxyl radical which oxidizes methanol, thereby generating formaldehyde (Fig. 1.2). Although in all animals the principle site of methanol oxidation is the liver, the metabolic pathways involved in this process depend on the species (Skrzydowska, 2003; Sweeting et al., 2011; Tephly, 1991). While humans and monkeys oxidize methanol with the aid of ADH1, this step is mediated predominantly by catalase in rats and mice (Skrzydowska, 2003; Sweeting et al., 2011; Tephly, 1991).

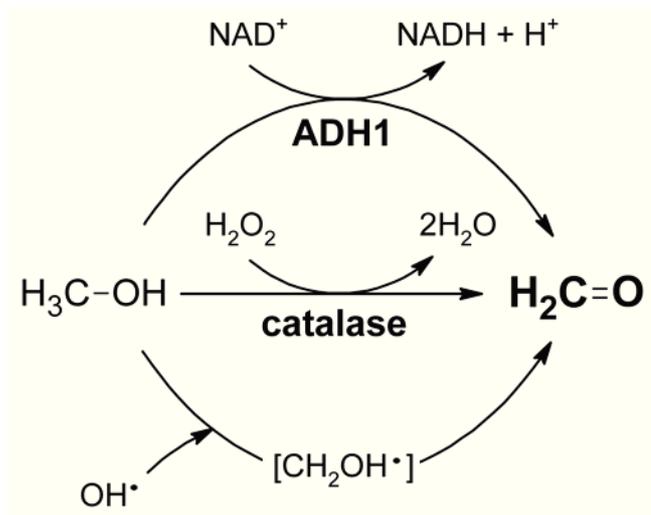


Figure 1.2: Formaldehyde generation by methanol oxidation. Methanol can be oxidized to formaldehyde in an enzymatic reaction catalyzed by alcohol dehydrogenase (ADH) 1 or catalase and non-enzymatically by the reaction with hydroxyl radicals. Modified from MacAllister et al. (2011).

1.1.2.2. Deamination of methylamine

Semicarbazide-sensitive amine oxidase (SSAO) is a common name for a group of copper-containing amine oxidases which are inhibited by semicarbazide and most of them contain topa-quinone at their catalytic centre (Jalkanen and Salmi, 2001; Yu et al., 2003). The oxidative deamination of primary amines by SSAO yields the corresponding aldehyde, ammonia and hydrogen peroxide (Jalkanen and Salmi, 2008; O'Sullivan et al., 2004). Thus, when methylamine, an endogenous amine (Li et al., 2004; Nunes et al., 2011) is utilized as a substrate in SSAO-catalyzed reaction formaldehyde is generated (Fig. 1.3) (O'Sullivan et al., 2004; Yu et al., 2003). Vascular adhesion protein (VAP) 1 is an enzyme which belongs to SSAO (Jalkanen and Salmi, 2008; Smith and Vainio, 2007) and is one of the most extensively studied members of this group. SSAO is found in a wide range of organisms from microbes to plants and mammals (Brazeau et al., 2004; MacPherson and Murphy, 2007) and plays an important role in the detoxification of xenobiotic amines, glucose uptake and cell-adhesion in leukocyte trafficking (Jalkanen and Salmi, 2001, 2008; Yu et al., 2003). In mammals, SSAO is either membrane-associated or in a soluble form circulating in the vascular system (Jalkanen and Salmi, 2001).



Figure 1.3: Deamination of methylamine generates formaldehyde.

Semicarbazide-sensitive amine oxidase (SSAO) catalyzes the oxidative deamination of methylamine to yield formaldehyde, hydrogen peroxide and ammonia.

1.1.2.3. Histone demethylation

Lysine-specific demethylase (LSD) 1 and JmjC domain-containing histone demethylases (JHDM) are enzymes that remove methyl groups from methylated lysine residues in histones, thereby altering the chromatin structure and playing an important role in regulation of gene expression (Cheng and Zhang,

2007; Cloos et al., 2008; Hou and Yu, 2010; Izzo and Schneider, 2010). Removal of a methyl group by either LSD1 or JHDM yields formaldehyde as a by-product (Fig. 1.4) (Cloos et al., 2008; Hou and Yu, 2010). LSD1 is a flavin-containing enzyme that selectively demethylates the mono- or dimethylated lysine residue in position 4 of histone H3 (Forneris et al., 2009; Hou and Yu, 2010; Shi, 2007). On the other hand, JHDM require Fe^{2+} and α -ketoglutarate as cofactors and can remove methyl groups from mono-, di- or trimethylated lysine residues (Cheng and Zhang, 2007; Cloos et al., 2008; Hou and Yu, 2010). LSD1 and JHDM homologues are present in a number of organisms like yeast, plants and mammals (Shi, 2007; Sun and Zhou, 2008).

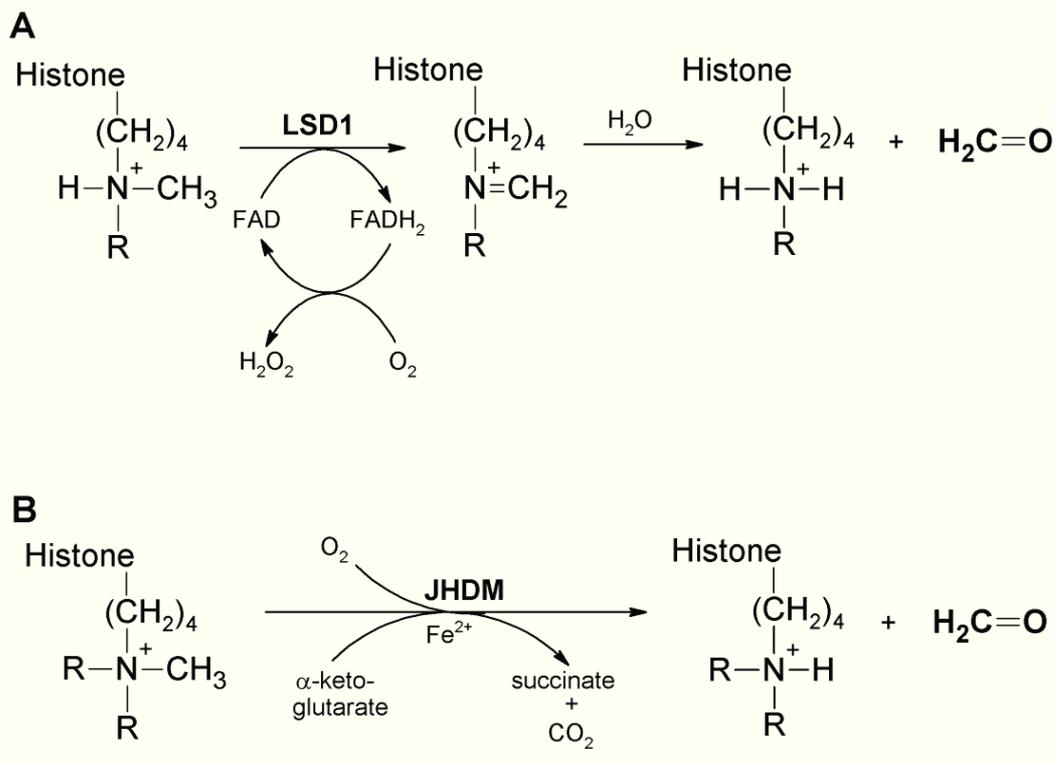


Figure 1.4: Formaldehyde generation by demethylation of histones.

Lysine-specific demethylase (LSD) 1 (A) or JmjC domain-containing histone demethylases (JDHM) (B) remove methyl groups from lysine residues in histones by two different mechanisms leading to formaldehyde generation. R represents either a hydrogen atom or a methyl group.

1.1.3. Formaldehyde metabolism

In order to maintain a low physiological level of formaldehyde, cells are equipped with the enzymatic machinery necessary for its disposal. Cellular metabolism of formaldehyde may yield methanol or formate by the reduction or oxidation of formaldehyde, respectively (Fig. 1.5). The reduction of formaldehyde to methanol can be catalyzed by ADH1 while formate can be generated by two independent pathways mediated by either the cytosolic ADH3 or the mitochondrial aldehyde dehydrogenase (ALDH) 2 (Friedenson, 2011; MacAllister et al., 2011; Teng et al., 2001).

ADH3, also known as formaldehyde dehydrogenase can oxidize formaldehyde to formate in a two-step process that requires glutathione (GSH) (Harris et al., 2003; MacAllister et al., 2011; Staab et al., 2009; Thompson et al., 2010). In the first step, GSH reacts with formaldehyde in an enzyme-independent manner to form S-hydroxymethyl GSH that is subsequently used as a substrate by ADH3 to generate S-formyl GSH, along with the reduction of NAD⁺ to NADH (Harris et al., 2003; MacAllister et al., 2011; Staab et al., 2009; Thompson et al., 2010). The formation of S-hydroxymethyl GSH strongly depends on the concentrations of formaldehyde and GSH present as this hemithioacetal is in equilibrium with the reactants (Ahmed and Anders, 1978). The conjugate S-formyl GSH is hydrolyzed by a thiolase to generate formate and GSH (Harris et al., 2003; MacAllister et al., 2011; Teng et al., 2001). On the other hand, the reaction catalyzed by ALDH2 is GSH-independent wherein NADH is generated along with formaldehyde oxidation to formate (MacAllister et al., 2011; Teng et al., 2001). The enzymes ADH3 and ALDH2 differ in their K_m-values for their respective substrates, S-hydroxymethyl GSH and free formaldehyde (Casanova-Schmitz et al., 1984; Heck et al., 1990). While ADH3 has a very low K_m-value for S-hydroxymethyl GSH (less than 10 μM), that of ALDH2 for formaldehyde is around 0.2-0.5 mM indicating that in the presence of GSH, ADH3 is a more important catalyst for formaldehyde oxidation (Casanova-Schmitz et al., 1984; Heck et al., 1990).

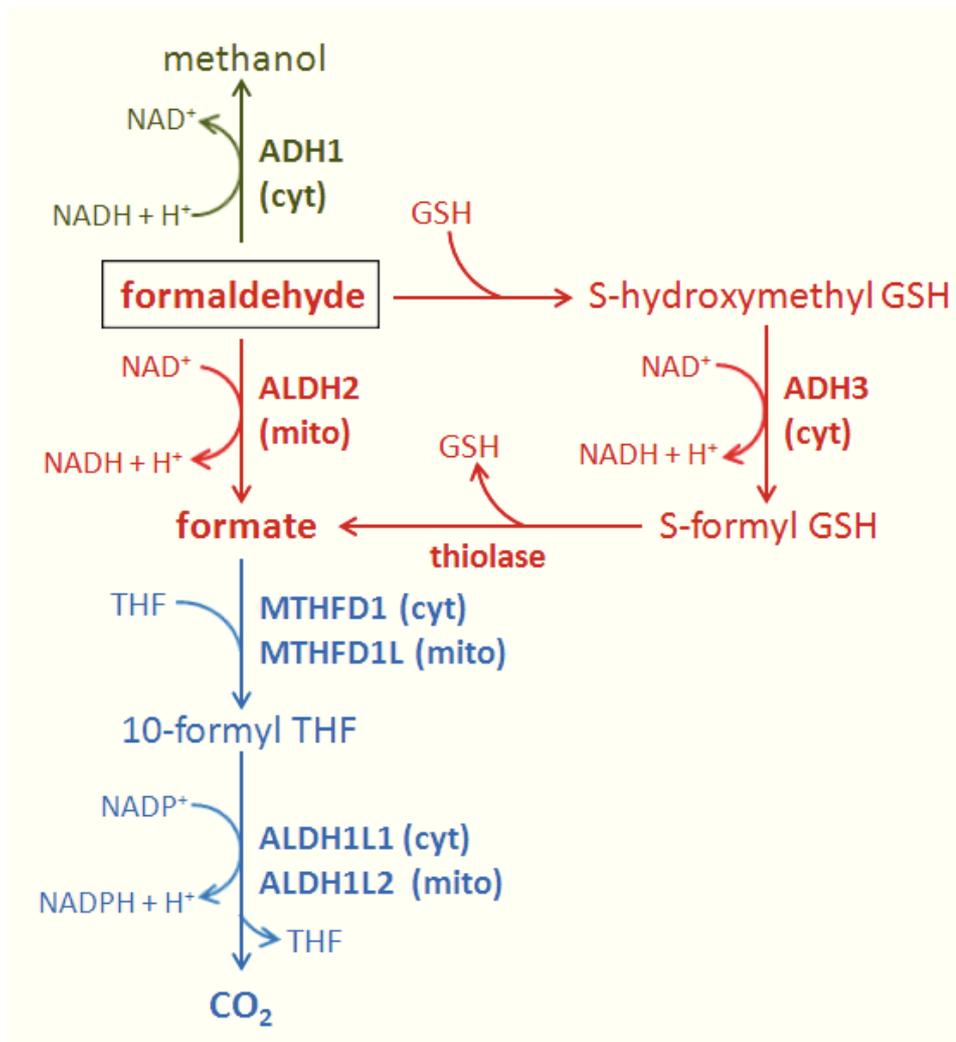


Figure 1.5: Cellular metabolism of formaldehyde. Formaldehyde can be oxidized to formate in a reaction catalyzed by cytosolic (cyt) glutathione (GSH)-dependent alcohol dehydrogenase (ADH) 3 or by the mitochondrial (mito) aldehyde dehydrogenase (ALDH) 2. Reduction of formaldehyde by ADH1 can result in generation of methanol. Formate can undergo further oxidation to CO_2 via a pathway involving tetrahydrofolate (THF) where 10-formyl THF is synthesized in the first step catalysed by methylene tetrahydrofolate dehydrogenase (MTHFD) 1 or MTHFD1L in an ATP-dependent reaction. In the next step, 10-formyl THF dehydrogenase ALDH1L1 or ALDH1L2 acts on 10-formyl THF to produce CO_2 and regenerate THF.

The formate generated by formaldehyde oxidation can undergo further oxidization to carbon dioxide by a pathway dependent on tetrahydrofolate (THF) wherein formate is first converted to 10-formyl THF (Fig. 1.5) in an ATP-dependent reaction (Krupenko et al., 2010; Krupenko, 2009; Skrzydlewska, 2003). The cytosolic form of the enzyme catalyzing this reaction is called methylene tetrahydrofolate dehydrogenase (MTHFD) 1 while the mitochondrial form is MTHFD1L (Krupenko, 2009). In the next step the enzyme 10-formyl THF dehydrogenase, also known as ALDH1L1 (cytosolic) or its mitochondrial isoform ALDH1L2 oxidizes 10-formyl THF to carbon dioxide using NADP⁺ as a co-factor and regenerates THF (Krupenko et al., 2010; Krupenko, 2009; Skrzydlewska, 2003). Although formate oxidation takes place predominantly by the THF-dependent pathway, catalase-mediated oxidation can also occur (Cook et al., 2001; Skrzydlewska, 2003).

1.1.4. Formaldehyde in diseases

The concentration of formaldehyde in the blood has been reported to be around 0.1 mM (Heck and Casanova, 2004) while that in the brain is about 0.2 mM (hippocampus) and 0.4 mM (cortex) (Tong et al., 2012) which can be attributed to the balance between formaldehyde-generating and -disposing processes described in previous sections. Exposure to exogenous formaldehyde or an increase in the activity of formaldehyde-generating enzymes without an increase in the capacity to clear this formaldehyde may elevate formaldehyde level in the body. Indeed an increased expression/activity of the formaldehyde-generating enzymes SSAO, LSD1 and JHDM has been reported in pathology (Table 1.2). While elevated levels of SSAO are associated with a broad spectrum of pathological conditions, an increase in the expression of histone demethylases, LSD1 and JHDM, has been observed in different types of cancer (Table 1.2). In line with the increase in formaldehyde-generating enzymes in pathology, increased formaldehyde levels have been reported in patients suffering from neurodegenerative diseases like Alzheimer's disease (AD) or multiple sclerosis (MS) (Khokhlov et al., 1989 cited in Miao and He, 2012; Tong et al., 2012; Tong et al., 2011), in diabetic rats (Tong et al., 2012) and in cancer tissue (Spanel et

al., 1999; Tong et al., 2010). Moreover, some human cancer cell lines have also been reported to have elevated formaldehyde levels (Kato et al., 2001; Tong et al., 2010).

Table 1.2: Elevation of formaldehyde-generating enzymes in disease.

Enzyme	Disease	References
SSAO/ VAP1	Alzheimer's disease	del Mar Hernandez et al., 2005; Ferrer et al., 2002; Unzeta et al., 2007
	Multiple sclerosis	Airas et al., 2006
	Heart disease	Boomsma et al., 2000; Boomsma et al., 2005
	Diabetes mellitus and diabetic complications	Boomsma et al., 2005; Gronvall-Nordquist et al., 2001; Karadi et al., 2002; Meszaros et al., 1999; Obata, 2006
	Chronic liver disease	Kurkijarvi et al., 2000
LSD1/ JHDM	Sarcoma	Bennani-Baiti et al., 2012; Schildhaus et al., 2011
	Peripheral nerve sheath tumor	Schildhaus et al., 2011
	Neuroblastoma	Schulte et al., 2009
	Bladder cancer	Hayami et al., 2011; Hayami et al., 2010
	Breast cancer	Lim et al., 2010
	Prostate cancer	Kahl et al., 2006; Xiang et al., 2007

1.1.5. Formaldehyde toxicity

Several *in vitro* as well as *in vivo* studies have demonstrated formaldehyde-mediated cross-linking of proteins, DNA and DNA-proteins (Barker et al., 2005; Casanova et al., 1989; Lu et al., 2008a; Metz et al., 2004; Moeller et al., 2011; Shaham et al., 2003; Sutherland et al., 2008; Toews et al., 2008) which could have deleterious effects on cellular functions. The formation of DNA adducts

following formaldehyde exposure are considered to represent important events in carcinogenesis and mutagenesis (Lu et al., 2011; Swenberg et al., 2011). Based on the extensive literature available on the occurrence of cancer on formaldehyde exposure, the International Agency for Research on Cancer (IARC) classified this compound as a carcinogenic agent in humans (IARC, 2006). Besides its carcinogenic potential, animal studies have revealed that exposure to formaldehyde can also cause reproductive and developmental deficits (Duong et al., 2011; Köse et al., 2011; Wang et al., 2012). Of particular interest for the current work is formaldehyde-induced neurotoxicity (Songur et al., 2010) which is described in detail in Section 1.3.3.

1.2. Brain cells

The brain is the most complex organ in the body consisting of more than 100 billion cells (Azevedo et al., 2009). Brain cells interact extensively with each other to ensure proper functioning of this vital organ. This section describes the different cells that constitute the brain (Fig. 1.6) and the intimate association between them in health and disease.

1.2.1. Neurons

Amongst all the constituent cells of the brain, neurons have been in the spotlight for the longest time. The first descriptions of nerve cells were done in the period from 1833 to 1837 almost simultaneously by two scientists, Christian Gottfried Ehrenberg and Johann Evangelista Purkinje (Ehrenberg, 1836; Lopez-Munoz et al., 2006; Purkinje, 1837). The first microscopic image of a nerve cell was published in 1836 by Gabriel Gustav Valentin, a student of Purkinje (Lopez-Munoz et al., 2006; Valentin, 1836). However, a major breakthrough in the field of neuroscience happened in 1888-89 when the Spanish neurobiologist, Santiago Ramón y Cajal, postulated the neuron doctrine (De Carlos and Borrell, 2007; Lopez-Munoz et al., 2006; Serrano-Castro and Garcia-Torrecillas, 2012). Using cerebellum and retina of birds, Cajal described,

in detail, the morphology and arrangement of neurons which is widely accepted till date with few modifications (De Carlos and Borrell, 2007; Lopez-Munoz et al., 2006; Serrano-Castro and Garcia-Torrecillas, 2012).

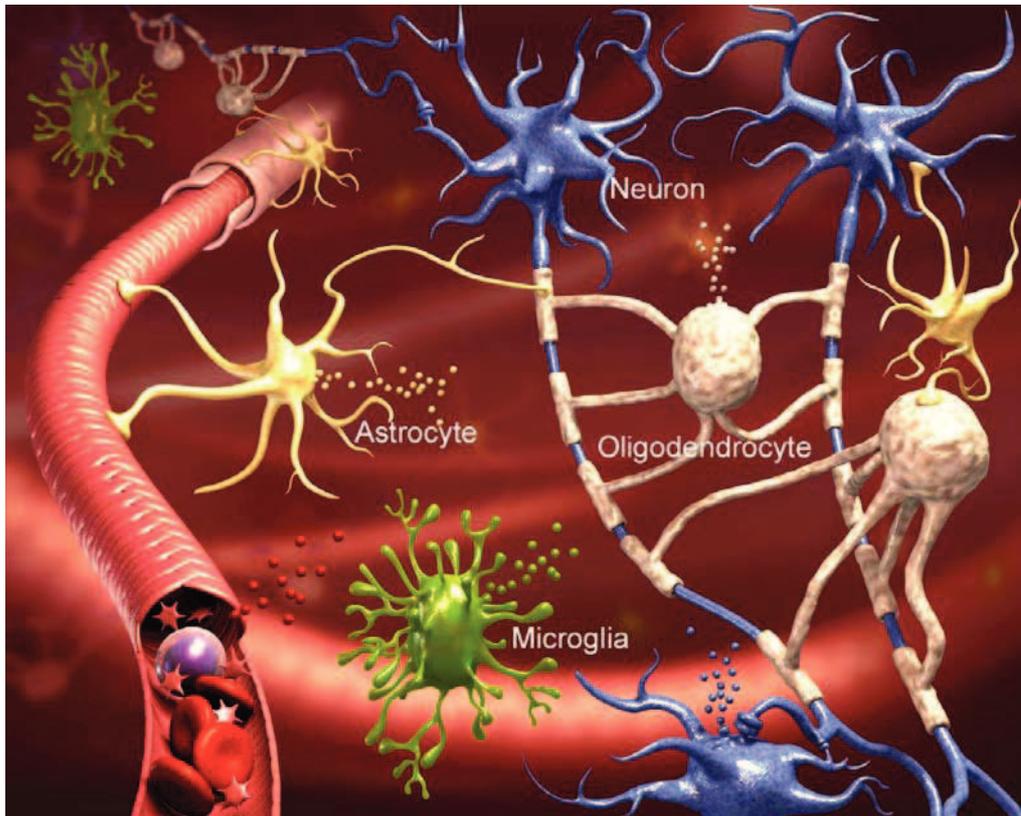


Figure 1.6: Cellular constitution of the brain. The brain is mainly composed of neurons and glial cells (astrocytes, oligodendrocytes and microglia). Modified from Colombo et al. (2012).

Neurons are characterized by a polarized morphology consisting of a cell-body, axon and several highly branched dendrites (Nicholls et al., 2011; Raine, 2007). Neurons are excitatory, inhibitory or modulatory in their effects and motor, sensory or secretory in their functions (Raine, 2007). These cells communicate with each other by means of chemical transmission at synapses (Holz and Fisher, 2007). Synaptic transmission involves a series of complex processes like depolarization of the presynaptic nerve terminal leading to influx of Ca^{2+} which

in turn triggers exocytosis of small synaptic vesicles that store neurotransmitters (Holz and Fisher, 2007; Nicholls et al., 2011). The released neurotransmitters interact with receptors on the postsynaptic neuron that are either coupled directly with ion channels or act through secondary messengers (Holz and Fisher, 2007; Nicholls et al., 2011). The inactivation of neurotransmitters occurs either by reuptake at the nerve terminal, degradation or uptake and metabolism by glial cells (Holz and Fisher, 2007; Nicholls et al., 2011). Some examples of neurotransmitters are glutamate and acetylcholine that belong to the class of excitatory neurotransmitters (Lester et al., 2010; Yang et al., 2011) while γ -amino butyric acid (GABA) and glycine are inhibitory neurotransmitters (Bowery and Smart, 2006).

For efficient functioning of the nervous system, a close co-operation between neurons and glial cells is required which is described in detail in the following section. Of particular interest in the context of this thesis is the reliance of neurons on astrocytic supply of lactate, an energy metabolite, and also GSH precursors which is described in Section 1.2.2.1.

1.2.2. Glial cells

Prior to 1856 when Rudolf Virchow coined the term 'neuroglia' (glia means glue in Greek) to describe the 'connecting material' surrounding nerve cells (Kettenmann and Verkhratsky, 2008), the brain was thought to comprise merely of nerve cells. In 1858, Virchow wrote in a book titled 'Cellular Pathology'- *'Hitherto, gentlemen, in considering the nervous system, I have only spoken of the really nervous parts of it. But if we would study the nervous system in its real relations in the body, it is extremely important to have a knowledge of that substance also which lies between the proper nervous parts, holds them together and gives the whole its form in a greater or less degree'* (Kettenmann and Verkhratsky, 2008; Virchow, 1858). Thereafter the concept of neuroglia spread in the world and in years to follow astrocytes, oligodendrocytes and microglia were described as the different components of the neuroglia (Fig. 1.6).

During evolution, the ratio of glia to neurons in the frontal cortex has increased from about 0.3 in rodents to 1.65 in humans (Kettenmann and Verkhratsky, 2008; Sherwood et al., 2006), suggesting an important role of the former cell types in higher organisms. Glial cells constitute more than 90% of all cells in the human brain (He and Sun, 2007) and have several specialized functions. The functions of different brain glial cell types are described below with special emphasis on astrocytes.

1.2.2.1. Astrocytes

Astrocytes, the major type of neuroglia in the brain, were named by Michael von Lenhossek (Lenhossek, 1891; Parpura et al., 2012) due to their star-shaped appearance. These cells can be divided into two sub-types on the basis of their morphology and location in the brain - fibrous astrocytes residing in the white matter and protoplasmic astrocytes found in the grey matter (Molofsky et al., 2012; Sofroniew and Vinters, 2010). Fibrous astrocytes possess elongated fiber-like processes while protoplasmic astrocytes have fine branched processes in a uniform globoid pattern (Molofsky et al., 2012; Sofroniew and Vinters, 2010). During evolution, the complexity and size of astrocytes increased disproportionately to neurons, which is proposed to reflect the increased importance of astrocytic functions in higher organisms (Oberheim et al., 2006). In humans, every astrocyte contacts and enwraps about 2 million synapses compared to about 0.1 million synapses covered by rodent astrocytes (Kettenmann and Verkhratsky, 2008; Oberheim et al., 2006). Astrocytes are involved in many important processes including development and maintenance of the blood-brain barrier (BBB), guidance of neuronal migration, synaptic transmission and neuronal excitability, synaptic generation, regulation of the brain microenvironment, in particular regarding neurotransmitter and ionic homeostasis, regulation of energy metabolism, scavenging of reactive oxygen species, detoxification, metal sequestration and providing metabolic support to neurons (Fernandez-Fernandez et al., 2012; Nedergaard and Verkhratsky, 2012; Parpura et al., 2012; Schmidt and Dringen, 2012; Tiffany-Castiglioni et al., 2011).

The BBB is a diffusion barrier that regulates the exchange of molecules between the blood and the brain and protects the central nervous system (CNS). The brain capillaries are about 50-100 times tighter compared to the peripheral microvessels owing to the presence of this barrier (Li et al., 2012). Although the primary seal of the BBB is formed by the endothelial tight junctions, majority of the abluminal surface of cerebral microvascular endothelium is ensheathed by astrocytic endfeet (Correale and Villa, 2009; Mathiisen et al., 2010). Several factors released by astrocytes such as transforming growth factor- β 1, glial cell line-derived neurotrophic factor (GDNF), basic fibroblast growth factor, interleukin (IL) 6, and angiopoietin 1 are considered to be important for the induction and maintenance of the BBB (Correale and Villa, 2009). Besides, the astrocytic endfeet express purinergic P2Y receptors, potassium channel Kir4.1 and water-channel protein aquaporin-4, indicating a pivotal role of these cells in gliovascular signaling and in regulation of brain water and electrolyte metabolism (Correale and Villa, 2009; Liebner et al., 2011).

Astrocytes express a wide array of neurotrophic factors that are essential for the proliferation, survival and maturation of multipotent neural stem/progenitor cells (Barkho et al., 2006). Astrocytes, like neurons, also release a wide array of factors that are necessary for the growth and maturation of oligodendrocytes (Moore et al., 2011). Furthermore, during adult neurogenesis, neuroblasts migrate in the brain by sliding over astrocyte-lined tunnels (Mamber et al., 2010; Ming and Song, 2011). Synaptogenesis is a process that occurs during brain development as well as in the adult brain (Kelsch et al., 2010; Pfrieger, 2010) where astrocytes contribute immensely to the formation, maturation and maintenance of synapses (Faissner et al., 2010; Pfrieger, 2010). According to the concept of a tripartite synapse, astrocytic processes are in close vicinity of the neuronal pre- and post-synapse which facilitate efficient modulation of synaptic transmission and plasticity by communication between the two cell types via neurotransmitters and gliotransmitters (Faissner et al., 2010; Nedergaard and Verkhratsky, 2012; Verkhratsky and Parpura, 2010). On the other hand, rapid clearance of neurotransmitters released from nerve terminals is important for the survival and functioning of neurons. A number of transporters expressed by astrocytes function in clearance of the

neurotransmitters from the synaptic cleft (Coulter and Eid, 2012; Eulenburg and Gomez, 2010).

Astrocytes play a central role in regulation of extracellular K^+ (Verkhratsky and Parpura, 2010) and express voltage-gated potassium channels as well as passive potassium channels (Verkhratsky and Steinhauser, 2000). These cells communicate with each other via gap junctions that also allow spatial K^+ buffering required for maintaining potassium homeostasis in the brain (Benfenati and Ferroni, 2010). Furthermore, astrocytes express calcium-activated potassium channels and also sodium, calcium and chloride channels (Deitmer and Rose, 2010; Oka et al., 2004; Verkhratsky and Steinhauser, 2000) that regulate the levels of these ions in the brain. The intracellular pH of brain cells is actively maintained in a range from 7.0 to 7.4 and is similar to the extracellular pH (7.1 to 7.3) in nervous tissue (Deitmer and Rose, 2010). This pH regulation in the brain is mediated by neurons as well as glial cells which possess several transporters that carry acid or base equivalents across the plasma membrane (Deitmer and Rose, 2010).

Proper functioning of neurons requires sufficient production of ATP, around 90% of which occurs via oxidative phosphorylation in the mitochondria (Cai et al., 2011). According to the astrocyte-neuron lactate shuttle hypothesis, astrocytes convert glucose to lactate and subsequently export this metabolite which is taken up by neurons (Fig. 1.7) (Fernandez-Fernandez et al., 2012; Genc et al., 2011; Parpura et al., 2012; Pellerin and Magistretti, 2012). Neurons oxidize lactate to pyruvate which undergoes further oxidation via the tricarboxylic acid (TCA) cycle in the mitochondrion along with the generation of ATP (Fig. 1.7) (Fernandez-Fernandez et al., 2012; Genc et al., 2011; Parpura et al., 2012; Pellerin and Magistretti, 2012). Besides glucose, another energy source in the brain is glycogen which is predominantly stored in astrocytes and can undergo glycogenolysis in these cells to ultimately produce and release lactate (Dringen et al., 1993; Obel et al., 2012). The metabolic coupling between astrocytes and neurons with regard to lactate is in agreement with the neuronal requirement of astrocytic glycogen-derived lactate for the process of memory formation in the hippocampus (Newman et al., 2011; Suzuki et al., 2011).

However, the existence of a coupling between astrocytes and neurons with respect to lactate is complicated by the observation that ATP generation in the brain during neuronal activity occurs by oxidative phosphorylation (Hall et al., 2012) and that neurons take up glucose following brain stimulation (Dienel, 2012; DiNuzzo et al., 2010; Mangia et al., 2011).

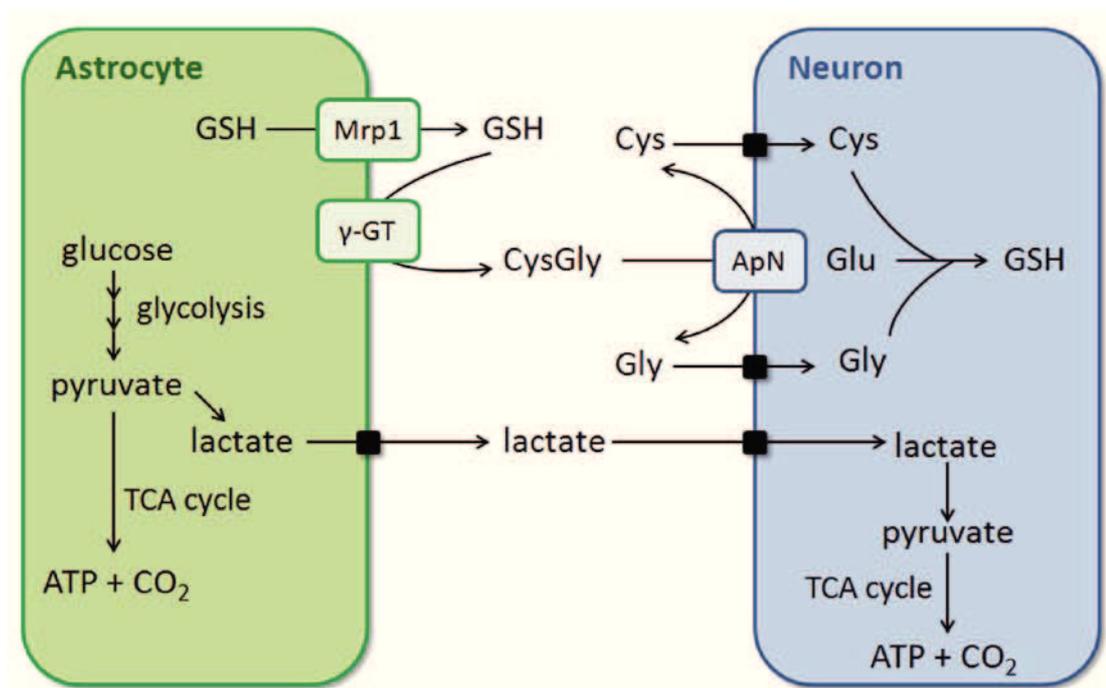


Figure 1.7: Schematic representation of the metabolic coupling between astrocytes and neurons. Astrocytes provide neurons with lactate and precursors for glutathione (GSH) synthesis. ApN: aminopeptidase N; γ -GT: γ -glutamyl transpeptidase; Mrp1: multidrug resistance protein, TCA: tricarboxylic acid. For details please see text.

Another example of metabolic coupling between astrocytes and neurons is the glutamate–glutamine cycle where the astrocytic enzyme glutamine synthetase converts glutamate to glutamine which is subsequently released and taken up by neurons (Rothman et al., 2011). This glutamine can be used by neurons for synthesis of the neurotransmitters, glutamate and GABA (Coulter and Eid, 2012), or for neuronal protein and GSH synthesis (Hirrlinger and Dringen,

2010; Schmidt and Dringen, 2012). Furthermore, the other two precursors for GSH synthesis, glycine and cysteine are also provided by astrocytes (Fig. 1.7) in a process that involves the multidrug resistance protein (Mrp) 1-mediated export of GSH and its cleavage by the astrocytic ectoenzyme γ -glutamyl transpeptidase (γ -GT) to form a cysteinyl-glycine dipeptide (Dringen et al., 1999; Fernandez-Fernandez et al., 2012; Hirrlinger and Dringen, 2010; Schmidt and Dringen, 2012). This dipeptide is cleaved extracellularly by the neuronal enzyme aminopeptidase N (ApN) to generate the two constituent amino acids which are taken up by neurons (Dringen et al., 2001; Fernandez-Fernandez et al., 2012; Hirrlinger and Dringen, 2010; Schmidt and Dringen, 2012). The synthesis of GSH takes place in a two-step ATP-requiring process where, at first, γ -glutamylcysteine ligase generates the dipeptide γ -glutamyl-cysteine followed by GSH synthetase-catalyzed addition of a glycyl moiety to the dipeptide to form GSH (Lu, 2012; Schmidt and Dringen, 2012).

1.2.2.2. Oligodendrocytes

In the CNS oligodendrocytes are specialized cells that myelinate the axons of neurons (Emery, 2010; Nave, 2010). Myelin extends from the plasma membrane of oligodendrocytes and spirally enwraps an axonal segment in the form of a multilamellar compact sheath (Nave and Trapp, 2008). In the brain one oligodendrocyte can produce up to 40 myelin segments on multiple axons with a capacity of myelinating oligodendrocytes to produce as much as $5\text{-}50 \times 10^3 \mu\text{m}^2$ of membrane each day (Miron et al., 2011). Proper myelination is necessary for neuronal propagation of action potentials, since abnormal development and/or maintenance of myelin sheaths leads to nerve degeneration associated with disorders like MS and leukodystrophies (Lassmann et al., 2007; Nave, 2010; Sriram, 2011). Oligodendrocytes and neurons also communicate with each other through certain factors. For example, production of GDNF by oligodendrocytes provides trophic support to neurons (Du and Dreyfus, 2002; Wilkins et al., 2003) while several factors released by neurons aid the process of myelination (Nave and Trapp, 2008). Moreover, there is also evidence that oligodendrocytes play a role in maintaining the nodal sodium

channel clusters on neurons which are necessary for saltatory conduction (Dupree et al., 2004; Ochab-Marcinek et al., 2009).

The development and maturation of oligodendrocytes from oligodendrocyte precursor cells is a complex process regulated by extracellular signals and a number of intrinsic factors (Barca-Mayo and Lu, 2012; Miron et al., 2011). This process initiates during embryonic development and continues into young adult life (Barca-Mayo and Lu, 2012). Each developmental stage of oligodendrocytes is characterized by expression of different molecular markers which help in their identification (Bradl and Lassmann, 2010). Briefly, oligodendrocytes precursor cells differentiate into pre-myelinating oligodendrocytes with an intermediate pro-oligodendrocytic stage and the pre-myelinating oligodendrocytes finally differentiate into myelinating oligodendrocytes (de Castro and Bribian, 2005; Emery, 2010). During this process the axons release signal molecules that regulate the development of oligodendrocytes (Bozzali and Wrabetz, 2004).

Amongst all types of brain cells, oligodendrocytes contain the highest amount of iron (Benkovic and Connor, 1993) which might be due to their requirement of iron for proliferation and differentiation (Todorich et al., 2009). In this regard, a recent study has shown that knocking out the iron efflux protein, ferroportin, in astrocytes negatively affects remyelination in the brain which has been discussed to be a consequence of decreased proliferation and/or differentiation of oligodendrocytes progenitor cells (Schulz et al., 2012). Oligodendrocytes can also use lactate as a metabolic fuel molecule or as a precursor for lipids with a six-fold higher rate of lipid synthesis on supply of lactate compared to astrocytes and neurons (Sanchez-Abarca et al., 2001). In line with this finding, lowering glucose availability has been shown to reduce myelination which can be rescued by supplying lactate (Rinholm et al., 2011). On the other hand, oligodendrocyte-derived products of aerobic glycolysis (pyruvate and lactate) have been suggested to be utilized by myelinated axons under certain conditions (Fünfschilling et al., 2012; Lee et al., 2012).

1.2.2.3. Microglia

The term “microglia” was introduced by Pio del Rio-Hortega in 1932 (Kettenmann et al., 2011). Microglia are the resident macrophages in the brain and play an important role in the innate immune response (Czeh et al., 2011; Kettenmann et al., 2011). In the healthy CNS ‘resting’ microglia have a ramified morphology consisting of a small cell body with fine cellular processes (Czeh et al., 2011; Kettenmann et al., 2011). The resting microglia carry out surveillance by constantly scanning their environment for signals that may pose a threat to the brain homeostasis (Czeh et al., 2011; Kettenmann et al., 2011). As a consequence of any disturbance in homeostasis that indicates a potential threat to the CNS, a phenomenon called “microglial activation” occurs which is characterized by changes in the microglial cell shape, gene expression and functions (Czeh et al., 2011; Kettenmann et al., 2011).

Under pathological conditions, microglial cells are activated and migrate to the site of injury (Black and Waxman, 2012; Ohsawa and Kohsaka, 2011). Both activation and migration is mediated by a wide repertoire of complex signals which are perceived by microglia owing to the presence of numerous receptors on their cell surface (Kettenmann et al., 2011; Saijo et al., 2012). For example, CX3CL1 is a chemokine which is either membrane-bound or soluble that is expressed by neurons and modulates microglial activity by interacting with the CX3CL1 receptor present on the latter cell type (Marchesi et al., 2010; Pabon et al., 2011). Activated microglia initiate immune responses such as phagocytosis, antigen processing and presentation and the production of a number of factors (Kettenmann et al., 2011; Smith et al., 2012; Sosa and Forsthuber, 2011). Microglia produce cytotoxic factors (e.g. nitric oxide, reactive oxygen species), neurotrophic factors (e.g. brain-derived neurotrophic factor, nerve growth factor) as well as pro- (IL-1, tumor necrosis factor- α) and anti-inflammatory (IL-4 and IL-10) cytokines (Czeh et al., 2011; Kettenmann et al., 2011; Smith et al., 2012). During brain development, these cells also regulate apoptosis, cell proliferation, angiogenesis and neuronal differentiation (Czeh et al., 2011; Harry and Kraft, 2012; Pont-Lezica et al., 2011). Finally, microglia play a role in

restructuring of neuronal circuits by synaptic modification and elimination (Tremblay and Majewska, 2011; Tremblay et al., 2011).

1.2.3. Brain cells in diseases

Under normal physiological conditions, an efficient cross-talk between different brain cells is essential for optimal brain functions (Garden and La Spada, 2012). A dysfunction in such an intercellular communication in pathology may play a role in initiation and progression of diseases. A few examples of diseases of the CNS and the interplay between different brain cell types in their pathology are briefly described in this section.

AD patients suffer from a progressive memory loss and dysfunction of higher cognitive domains (Stopford et al., 2012). This neurodegenerative disease is characterized by aggregation and deposition of β -amyloid ($A\beta$) peptides and formation of neurofibrillary tangles that ultimately lead to neuronal death (Benilova et al., 2012; Wesson et al., 2011). Besides these hallmarks of the disease, inflammation is also seen in AD brain (Broussard et al., 2012; Wyss-Coray, 2006; Wyss-Coray and Rogers, 2012). Although the precise mechanism behind the pathology of AD is still obscure, according to one view $A\beta$ results in a cascade of hemichannel activation wherein microglial and astroglial release of glutamate as well as ATP promotes neuronal death (Orellana et al., 2011a; Orellana et al., 2011b). On the other hand, $A\beta$ has been discussed to activate astrocytes and microglia which in turn release pro- and anti-inflammatory mediators resulting in neuroinflammation (Broussard et al., 2012; Wyss-Coray and Rogers, 2012). Although oligodendrocytes have not been extensively studied with respect to AD, myelin abnormalities in the presence of $A\beta$ deposits have been reported *in vitro* as well as *in vivo* (Mitew et al., 2010; Roth et al., 2005).

Parkinson's disease (PD) is a neurodegenerative disorder that affects motor functions (Fasano et al., 2012). In PD, dopaminergic neurons in the substantia nigra degenerate along with the occurrence of α -synuclein inclusions in neuronal cell bodies and axons (Halliday and Stevens, 2011; Steiner et al., 2011). However, during the progression of the disease, these inclusions are also seen

in protoplasmic astrocytes which in turn activate microglia by the release of cytokines and chemokines (Halliday and Stevens, 2011). These activated phagocytotic microglial cells target neurons and also cause inflammation in PD brains (Halliday and Stevens, 2011; Politis et al., 2012). During later stage of the disease, the non-myelinating oligodendrocytes also accumulate α -synuclein (Halliday and Stevens, 2011) and could further contribute to neuronal death.

MS is an autoimmune inflammatory demyelinating disorder of the CNS (Lassmann et al., 2007; Sriram, 2011). In MS astrocytes and microglia are considered to elicit an inflammatory response and majorly target oligodendrocytes and neurons (Chastain et al., 2011; Miljkovic et al., 2011). Moreover, a disruption in the BBB as well as the blood-cerebrospinal fluid barrier in MS facilitates infiltration of leukocytes into the brain which further damage oligodendrocytes and affect myelin (Alvarez et al., 2011). An extensive loss in myelin and the underlying axons observed in MS (Bradl and Lassmann, 2010; Sriram, 2011) is detrimental as demyelination of neurons can affect memory and cognitive functions (Fields, 2008; Huang et al., 2009).

1.3. Formaldehyde and brain

The BBB is a physical obstruction that monitors the entry of compounds into the brain tissue. However, methanol and formaldehyde from blood can easily cross this barrier owing to their small size and uncharged nature. The concentration of formaldehyde in the healthy brain has been reported to be 0.2-0.4 mM (Tong et al., 2012) which may reflect the balance between formaldehyde-generating and -disposing processes in the brain. This section deals with the production and metabolism of formaldehyde in the brain, involvement of formaldehyde in pathology of some neurodegenerative diseases and the neurotoxicity of formaldehyde.

1.3.1. Presence of formaldehyde-generating and -disposing enzymes

Several reports have demonstrated the presence of enzymes that produce and metabolize formaldehyde in the brain on the mRNA or protein level (Table 1.3). These reports suggest that local formaldehyde generation can occur in the brain and that the brain cells have the potential to dispose this formaldehyde. However, unlike other enzymes, the expression of ADH1 in the brain has been controversial since this dehydrogenase was not detected in some investigations (Galter et al., 2003; Julia et al., 1987).

1.3.2. Formaldehyde in pathology of neurodegenerative diseases

The observation that certain neurodegenerative diseases like AD and MS are associated with an increase in formaldehyde-generating enzymes (Section 1.1.4) has led to postulation of hypotheses wherein elevated formaldehyde levels are linked to pathology of these diseases. Monte (2010) suggested that formaldehyde methylates proteins such as tau protein (in AD) or myelin basic protein (in MS) which in turn elicits an immune response by the body (Monte, 2010). In line with this hypothesis, inhibition of SSAO in a murine mouse model of MS has been shown to reduce the incidence and severity of this disease (Wang et al., 2006) which may, at least in part, be due to lowering of formaldehyde generation. Also, some human subjects who suffered from methanol poisoning developed symptoms of MS which has been discussed to be an effect of methanol oxidation to formaldehyde (Henzi, 1984; Schwyzer and Henzi, 1983) and the subsequent modification of proteins as described above. Furthermore, formaldehyde exposure has been indicated to be a risk factor for development of amyotrophic lateral sclerosis (Weisskopf et al., 2009), another neurodegenerative motor neuron disease (Kiernan et al., 2011).

Table 1.3: Formaldehyde-producing and -metabolizing enzymes in the brain.

Enzymes	Brain region	References
Formaldehyde generation		
ADH1	Cerebellum, hippocampus, cerebral cortex	Martinez et al., 2001
Catalase	Cerebellum, olfactory bulb, hippocampus,	Schad et al., 2003; Zimatkin and Lindros, 1996
SSAO/ VAP1	Meningeal and parenchymal blood vessels	Ferrer et al., 2002; Unzeta et al., 2007; Valente et al., 2012
LSD1	Hippocampus, cerebral cortex	Sun et al., 2010; Zhang et al., 2010; Zibetti et al., 2010
JHDM	Total brain	Fukuda et al., 2011; Wolf et al., 2007
Formaldehyde oxidation		
ADH3	Cerebral cortex, cerebellum, hippocampus	Galter et al., 2003; Iborra et al., 1992; Julia et al., 1987
ALDH2	Total brain	Alnouti and Klaassen, 2008; Stewart et al., 1996
Formate oxidation		
MTHFD1	Total brain	Anthony and Heintz, 2007; MacFarlane et al., 2009
MTHFD1L	Total brain	Prasannan et al., 2003
ALDH1L1	Cerebral cortex, basal ganglia, cerebellum	Cahoy et al., 2008; Neymeyer et al., 1997
ALDH1L2	Total brain	Krupenko et al., 2010

Yu (2001) proposed that in AD, SSAO-mediated formaldehyde generation can induce A β cross-linkage, deposition and subsequently result in plaque formation adjacent to the cerebrovessels. Furthermore, it is suggested that in AD formaldehyde may cause cytotoxicity, inducing inflammation and release of more SSAO, thereby producing a cascade of toxic cycles (Yu, 2001). An indirect indication for a putative role of formaldehyde in AD pathology is found in two

studies where formaldehyde enhanced A β aggregation *in vitro* (Chen et al., 2007) and SSAO was shown to co-localize with A β deposits in AD brain (Jiang et al., 2008). On the other hand, increased formaldehyde levels in the brain and cerebrospinal fluid of patients suffering from AD and MS (Khokhlov et al., 1989 cited in Miao and He, 2012; Tong et al., 2012; Tong et al., 2011) are indicative of the involvement of formaldehyde in the pathology of these diseases.

Besides neurodegenerative diseases, aging and diabetes are also characterized by elevated concentration of formaldehyde in brain (Tong et al., 2012). In aging an increase in cerebral protein carboxymethylation occurs (Sellinger et al., 1988) which can serve as a source of methanol and hence of formaldehyde. On the other hand, elevated SSAO activity in diabetes (Boomsma et al., 2005; Gronvall-Nordquist et al., 2001; Karadi et al., 2002; Meszaros et al., 1999; Obata, 2006) may contribute to the observed increase in brain formaldehyde levels in diabetes mouse models (Tong et al., 2012).

1.3.3. Formaldehyde neurotoxicity

The neurotoxic potential of formaldehyde is well-known (Songur et al., 2010). Alterations in brain morphology following formaldehyde exposure in rats include a decrease in the number of neurons and a reduction in the volume of brain hemispheres (Aslan et al., 2006; Gurel et al., 2005; Sarsilmaz et al., 2007; Songur et al., 2010). Formaldehyde exposure also compromises the antioxidative defense of the brain by decreasing the level of GSH and also activities of the antioxidative enzymes superoxide dismutase and catalase (Gurel et al., 2005; Lu et al., 2008b; Songur et al., 2008; Zararsiz et al., 2006; Zararsiz et al., 2007; Zararsiz et al., 2011). This compromised antioxidative capacity is accompanied by an increase in nitric oxide levels and also in the amounts of markers of oxidative stress such as malondialdehyde and protein carbonyls (Gurel et al., 2005; Lu et al., 2008b; Songur et al., 2008; Zararsiz et al., 2006; Zararsiz et al., 2007). Also, PC12 cells treated with formaldehyde exhibit endoplasmic reticulum stress, decrease in levels of the antioxidant proteins thioredoxin and paraoxonase 1, reduction in tyrosine hydroxylase

expression and apoptosis (Lee et al., 2008; Luo et al., 2012; Tang et al., 2011). An increase in apoptotic events has also been observed, *in vivo* in brains of formaldehyde-treated rats (Zararsiz et al., 2006; Zararsiz et al., 2007). Formaldehyde application blocks the N-methyl-D-aspartate (NMDA) receptor (Tong et al., 2012) which is considered to be crucial for memory formation (Mayford et al., 2012; Timofeeva and Levin, 2011). In line with this finding, exposure to formaldehyde has been shown to affect behavior, memory and learning (Lu et al., 2008b; Malek et al., 2003; Pitten et al., 2000; Sorg et al., 2004; Tong et al., 2012; Tong et al., 2011; Turkoglu et al., 2008; Usanmaz et al., 2002). Furthermore, formaldehyde alters the expression of glutamate receptor and decreases glutamate uptake in cultured astrocytes (Song et al., 2010). Although the above mentioned toxicity of formaldehyde treatment, both *in vivo* and *in vitro*, could be due to formaldehyde, *per se*, there is a likelihood that formate, the oxidation product of formaldehyde, may contribute, at least in part, to the observed toxicity. Formic acid is known to inhibit complex IV of the respiratory chain (Nicholls, 1975) which would diminish ATP production by oxidative phosphorylation (Wallace et al., 1997). Besides, formic acid has been shown to cause neuronal death in brain slices (Kapur et al., 2007) as well as in primary neural cultures (Dorman et al., 1993).

1.4. Aim of the thesis

Formaldehyde is implied to play a role in the pathology of several neurodegenerative diseases, yet little is known about the metabolism of formaldehyde in brain cells and on the effects of formaldehyde exposure on brain cells. Therefore, this thesis aims to focus on the following consequences of formaldehyde application on cell culture model systems for neural cells, namely, astrocyte-rich primary cultures (Lange et al., 2012), primary cultures of cerebellar granule neurons (Drejer et al., 1985) and the oligodendroglial cell-line OLN-93 (Richter-Landsberg and Heinrich, 1996):

- Assessment of the vulnerability of different brain cell-types to formaldehyde-induced toxicity.

- Investigation of the capacity of brain cells to metabolize exogenously applied formaldehyde by measuring formaldehyde, formate and methanol.
- Alterations in glucose metabolism of cultured brain cells as accumulation of formate within cells may inhibit complex IV (Nicholls, 1975) of the respiratory chain and consequently enhance glycolysis.
- Effect of formaldehyde on GSH homeostasis in brain cells, since formaldehyde metabolism by ADH3 is GSH-dependent (Harris et al., 2003; MacAllister et al., 2011; Staab et al., 2009; Thompson et al., 2010) and exposure to this aldehyde results in oxidative stress in the brain (Gurel et al., 2005; Lu et al., 2008b; Songur et al., 2008; Zararsiz et al., 2006; Zararsiz et al., 2007).

These studies will give an insight into the metabolic consequences of a formaldehyde exposure *in vitro* which is likely to also have physiological relevance *in vivo*.

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2.

Methods

Publication/Manuscript 1

Primary cultures of astrocytes and neurons as model systems to study the metabolism and metabolite export from brain cells

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Invited book chapter submitted for publication in
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- Contribution of Ketki Tulpule:
 - Data generation for Fig. 4, 5 and 6
 - Preparation of first draft of the manuscript
- Michaela C. Hohnholt obtained data for Fig. 1, 2 and 3

**Manuscript for the book
“Neuromethods: Brain Energy Metabolism”**

**Primary cultures of astrocytes and neurons as model systems to
study the metabolism and metabolite export from brain cells**

by

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Abstract

Primary cultures of astrocytes and neurons are frequently used to investigate metabolic properties of these two important brain cell types. Here we describe methods to generate primary cultures that are highly enriched in astrocytes or cerebellar granule neurons. These cultures are good model systems to investigate the basal metabolism of astrocytes and neurons as well as metabolite export from these cells. As examples for such studies, we describe here in detail the robust practical procedures that we use to investigate cellular glucose consumption and lactate production as well as the cellular contents and the export of glutathione from cultured brain cells. In this context we also describe some viability assays that are useful to confirm that a given experimental treatment is not toxic for cultured neural cells.

Key words: astrocytes, metabolite export, glucose, glycolytic flux, GSH, neurons, lactate, viability

Running head: Metabolism of cultured brain cells

1. Introduction

The brain is a complex organ that consists of various cell types which all have important and essential functions. While neurons are mainly responsible for the transmission and processing of information, astrocytes have many supporting, protective and modulatory functions **(1-3)**. The complexity of the brain makes it rather difficult to gain information on the metabolic potential of one brain cell type by direct quantification of metabolites from brain tissue. In most cases such analysis identifies neither the cellular origin of the metabolite of interest nor whether the metabolite was located in a given cell type or extracellularly. In order to investigate the metabolic potential of the different types of brain cells, primary cell cultures are frequently used that are enriched for only one type of brain cells. Such cultures allow metabolic studies of one brain cell type in the absence of the other cell types that constitute the brain. For example, primary cultures of astrocytes have been used extensively to investigate metabolic properties of this cell type in order to understand the functions of astrocytes **(4)**. However, it should be considered that highly enriched cell cultures have the disadvantage that the potential modulation of metabolic properties of one type of brain cells by another is prevented. In addition, cultured cells do not necessarily present the same metabolic properties as brain cells *in vivo*. Therefore, while studies in cell cultures can provide valuable insights in the metabolic potential of brain cells, the extrapolation to the *in vivo* situation has to be done carefully and should, if possible, be verified in more complex or even intact neural systems (see chapters_____ of this book).

Many protocols have been used to prepare neural cell cultures that are strongly enriched in either astrocytes or neurons. We describe here in detail the methods we use to prepare astrocyte-rich primary cultures (APCs) and cerebellar granule neuron cultures (CGNCs) that are based on the protocols described by Hamprecht and Löffler **(5)** and by Anggono and colleagues **(6)**. APCs are highly enriched for astrocytes as demonstrated by immunocytochemical staining for the astrocyte marker protein, glial fibrillary acidic protein (GFAP) (Fig. 1C), while hardly any cell in these cultures is positive for the neuron-specific marker protein microtubule-associated protein 2 (MAP-

2; Fig. 1C). In contrast, most cells in CGNCs express MAP-2 and only a few cells in these cultures are positive for GFAP (Fig. 1D). The detailed methods to prepare and maintain APCs and CGNCs are described below in chapter 2.

To confirm that data from experiments designed to investigate metabolic properties of cultured cells reflect the metabolism of viable cells, it is essential to test for a potential compromised cell viability of a given experimental paradigm. In addition to frequent microscopic inspection of the cell morphology and determination of the protein content of the adherent cultured cells, we investigate routinely for each experimental condition whether the cell membrane integrity is compromised. This is done by determining the extracellular activity of the cytosolic enzyme lactate dehydrogenase (LDH) **(7)** and by investigating the permeability of the cell membranes for the fluorescent dye propidium iodide (PI) **(8)**. The detailed methods for these two viability assays are presented in chapter 4.

We frequently use cell cultures as models to investigate the metabolism of brain cells. As examples for such studies we describe here the determination of glucose consumption and lactate release by APCs and CGNCs as well as the quantification of the cellular glutathione (GSH) content and of the GSH export from these cells. The incubation conditions recommended for such metabolic studies as well as the microtiter plate-based colorimetric assays that we use for the quantification of glucose, lactate and GSH are described in detail in chapters 3 and 5-7.

2. Preparation of primary cultures from rat brain

Infrastructure: Special infrastructure is required for the generation and maintenance of primary brain cell cultures. An animal breeding facility or a room for housing company-delivered animals (either pregnant or mother with pups) is a pre-requisite for the preparation of primary cell cultures. Animal handling has to be conducted in accordance with the valid guidelines for care and use of laboratory animals and has to be licensed by the appropriate authorities.

Special cell culturing infrastructure is required for the preparation and the maintenance of the primary cell cultures. This includes a laminar air flow bench for sterile work, an incubator with CO₂ gas supply for culturing of cells in carbonate-buffered media, a centrifuge for spinning down cell suspensions, fridge and freezer for storage of media and solutions as well as a water bath for pre-warming media to 37°C. Since the cells are kept in culture for several weeks, it is mandatory to perform all cell culturing steps under sterile conditions. For a regular monitoring of cell growth and morphology during culturing and experimental incubations a microscope is required, preferably in close vicinity of the cell incubator to avoid transport-induced fluctuations in temperature or pH of the culture medium. Used cell culture solutions, media and cell culture waste should be disposed off after autoclaving. Animal cadavers should be disposed according to the appropriate regulations of the country and the scientific institution.

2.1. Astrocyte-rich primary cultures (APCs) from rat brain

APCs are prepared from the brains of newborn Wistar rats that have an age of maximal 24 h.

2.1.1. Materials and solutions

Materials: Dulbecco's modified Eagle's medium (DMEM), ethanol (70%), fetal calf serum (FCS), D-glucose, NaCl, KCl, KH₂PO₄, Na₂HPO₄, penicillin G, streptomycin sulphate, sucrose, sterile disposable filter units (0.2 µm), surgical scissors (length: 15 cm), two bent forceps with blunt tips (length: 15 cm), sterile (autoclaved) nylon mesh filter bags (size of around 1.5 x 4 cm) prepared by heat-induced sealing of nylon meshes (4 x 4 cm) of pore diameters of 210 µm or 132 µm (Sefar, Heiden, Switzerland), Neubauer counting chamber, sterile 10 mL pipettes, sterile 50 mL centrifugation tubes, sterile 5 cm, 10 cm and 24-well cell culture plates.

APC preparation solution: The solution contains 137 mM NaCl, 5.4 mM KCl, 0.22 mM KH_2PO_4 , 0.17 mM Na_2HPO_4 , 5 mM glucose, 58.4 mM sucrose, 200 U/mL penicillin G and 200 $\mu\text{g}/\text{mL}$ streptomycin sulphate. This solution should be filtered sterile through a 0.2 μm sterile filter and can be stored at 4°C for a few months.

APC medium: DMEM containing 25 mM D-glucose, 44 mM sodium bicarbonate, 1 mM pyruvate, 200 U/L penicillin G and 200 $\mu\text{g}/\text{L}$ streptomycin sulfate should be sterile filtered (0.2 μm filter) and supplemented with 10% (v/v) of sterile FCS. This sterile culture medium can be stored at 4°C for a month.

2.1.2. Method

1. Clean the laminar air flow bench with 70% ethanol and sterilize it with UV light for 30 min prior to any use for cell culture work.
2. Place three 10 cm cell culture dishes (A-C) containing cold APC preparation solution (dish A: 20 mL, dish B: 10 mL, dish C: 10 mL) on a tray filled with ice under the bench. In dishes B and C put one nylon mesh filter bag of pore diameters of 210 μm and 132 μm , respectively. Stack 5 cm dishes in the bench, one for each brain. Fill a glass dish (4-5 cm high) (dish D) with pre-warmed preparation solution. Sterilize forceps and surgical scissors by rinsing with 70% ethanol and a careful flame treatment. Place the scissors and forceps on a metal stand on the bench, avoiding contact of the sterile parts with the surface of the bench.
3. Take one new-born rat with a pair of forceps and briefly immerse the animal in pre-warmed preparation solution (dish D). Decapitate the animal with scissors and collect the head in a 5 cm cell culture dish. Remove the entire brain of the animal by carefully squeezing its head by using two forceps from mouth to neck, collect the brain and transfer it into the ice-cold preparation solution in dish A. Repeat these steps until all rat brains (use at least 3 brains and up to 15 brains) are collected in dish A.
4. Transfer 3-5 rat brains into the 210 μm nylon mesh filter bag in dish B and pass the brains through the net into dish B by gentle squeezing the brains

through the nylon mesh using bent forceps with blunt tips. Repeat this until all brains have been passed through the 210 μm nylon mesh filter bag. Finally, filter the remaining preparation solution from dish A through the 210 μm nylon mesh bag into dish B using a 10 mL pipette.

5. Pass the total 30 mL cell suspension from dish B through the 132 μm nylon mesh bag into dish C using a 10 mL pipette. Ensure that all material has passed through the mesh.
6. Transfer the total 40 mL cell suspension into one 50 mL centrifugation tube and spin down cells with 400 g for 5 min at 4°C. Carefully remove the supernatant and resuspend the cell pellet in pre-warmed (37°C) culture medium (2 mL per brain).
7. One neonatal rat brain will yield approximately 30 million viable cells. Count the harvested cells in a Neubauer counting chamber and add culture medium to the cell suspension to obtain a seeding density to 150,000 cells per cm^2 . Plate the cell suspension in cell culture dishes (24-well dish: 300,000 viable cells in 1 mL medium; 5 cm dish: 3 million viable cells in 5 mL medium).
8. Maintain the cultured cells in a cell incubator at 37°C with an atmosphere that contains 90% air, 10% CO_2 and 100% relative humidity. The APC medium should be renewed every seventh day of cultivation. APCs reach confluency after about 10 d and the cultures can be used for experiments between 14-28 d after seeding.

2.1.3. Results and Notes

1. It is essential to routinely characterize the cultures by microscopic inspection and by immunocytochemical staining using antibodies for cell-type specific markers. Fig. 1A shows a phase contrast image of an APC. Immunocytochemical staining of APCs revealed that around 95% of the cells in these cultures are positive for the astrocyte marker protein GFAP (Fig. 1C). However, APCs also contain low numbers of other types of glial cells (microglia, oligodendrocytes and ependymal cells) **(5,9,10)**, but hardly any cells that express the neuronal marker MAP-2 (Fig. 1C).

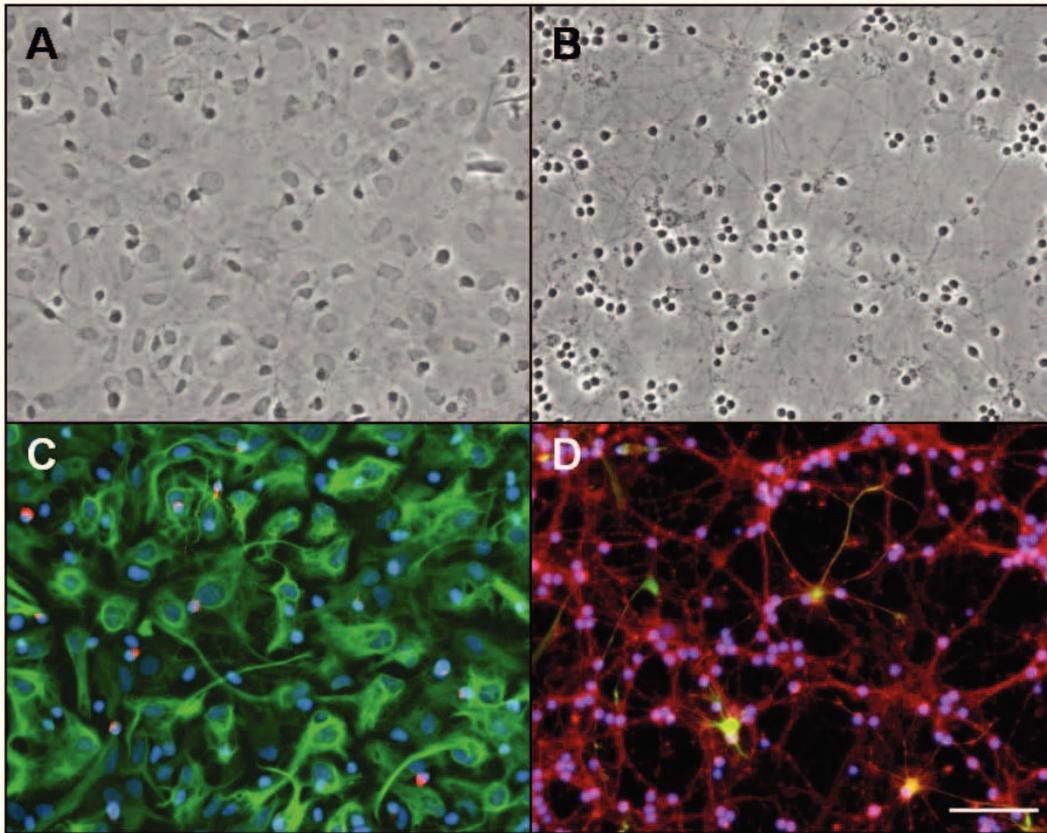


Figure 1: Phase contrast images (A,B) and immunocytochemical staining (C,D) of APCs (A,C) or CGNCs (B,D). Immunocytochemistry (C,D) was performed for the presence of the astrocytic marker protein GFAP (green) and the neuronal marker protein MAP-2 (red). The cell nuclei are co-stained with DAPI (blue). Immunocytochemistry was performed as previously described **(32)** by using rabbit anti-GFAP (1:500), Cy-2-coupled anti-rabbit IgG (1:200), mouse anti-MAP-2 (1:250) and Cy3-coupled anti-mouse IgG (1:200). The scale bar in D represents 50 μm and applies to all panels.

2. The protocol described here can also be used to prepare APCs from the brains of newborn mice. However, substantially lower numbers of cells will be obtained from one mouse brain compared to a rat brain.
3. This protocol can also be used to prepare astrocytes from a specific brain region like e.g. the cortex. In this case, the brain has to be carefully dissected out of the skull and the brain region of interest should be

harvested. After collection of the desired brain region from several brains, the preparation can be done as described above.

4. In case a cell incubator has to be used that supplies less than 10% CO₂, the APC medium can be adjusted by lowering the sodium bicarbonate concentration to establish the desired pH. The concentration of NaCl should be increased accordingly to maintain a constant osmolarity.
5. Depending on the type of metabolic experiment to be performed it should be considered to feed APCs with fresh culture medium (with or without serum) one day prior to the experiment to replenish the cells with nutrients.
6. For immunocytochemical staining the use of sub-confluent APCs should be considered, since individual astrocytes are easier to distinguish from each other in such cultures.
7. Metabolite contents and enzyme activities of cultured brain cells are frequently normalized on the protein contents of the cells. To determine the protein content per well of APCs or CGNCs in wells of 24-well dishes, we solubilize the cells for 2 h in 200 µL of 0.5 M NaOH and determine the protein content in such lysates by the Lowry method **(11)**.

2.2. Cerebellar granule neuron primary cultures (CGNCs) from rat brain

CGNCs are prepared from the brains of 7 d old Wistar rats **(6)**. Up to 7 cerebella can be harvested for one procedure. If more cells are needed, it is recommended to perform the preparation twice. In contrast to many other types of cultured neurons, CGNCs require a high concentration of K⁺ for maintenance. Therefore, culture media and buffers for CGNCs are supplemented with additional KCl.

2.2.1. Materials and solutions

Materials: Bovine serum albumin (BSA), cytosine β-D-arabinofuranoside (Ara-C), deoxyribonuclease, D-glucose, Earle's balanced salt solution (EBSS), ethanol (70%), L-glutamine, FCS, KCl, KH₂PO₄, K₂HPO₄, minimal essential medium (MEM), MgSO₄, NaCl, penicillin G, poly-D-lysine (PDL), soybean trypsin

inhibitor, streptomycin sulphate, trypsin, sterile (autoclaved) glass pipettes, a pair each of bent (length: 15 cm) and straight surgical scissors (length: 14.5 cm), bent (length: 11 cm) and straight (length: 13 cm) forceps, Neubauer counting chamber, sterile scalpel, sterile 5 cm, 10 cm and 24-well cell culture dishes.

Coating of cell culture dishes with poly-D-lysine (PDL): Cerebellar granule neurons will attach very well to cell culture dishes that are covered with positive charges due to a coating with PDL. Prepare a sterile 15 µg/mL PDL hydrobromide (molecular weight: 30,000 - 70,000) solution in pure water. The solution can be stored at 4°C for a few weeks. Cell culture dishes are coated with PDL by 2 h incubation with an appropriate volume of the sterile PDL solution at room temperature under sterile conditions. Subsequently, the PDL solution is removed, the wells are washed once with sterile water and the cell cultures dishes are dried under sterile conditions. Coated sterile cell cultures dishes can be stored at 4°C for a few days.

Modified Earle's balanced salt solution (mEBSS): Dissolve 4% (w/v) BSA and 3 mM MgSO₄ in EBSS and filter the solution sterile. Aliquots of this solution can be stored in sterile tubes at -20°C for several months.

Phosphate-buffered saline (PBS): Prepare a sterile 10 mM potassium phosphate buffer pH 7.4 containing 150 mM NaCl. This solution can be stored for months at 4°C.

CGNC preparation buffer: Prepare for each CGNC preparation 100 mL of a fresh sterile solution of 14 mM D-glucose, 60 mM MgSO₄ and 0.3% (w/v) BSA in PBS.

Trypsin solution: Prepare 20 mL of a sterile solution of 0.25% (w/v) trypsin in CGNC preparation buffer shortly before each CGNC preparation.

Trypsin inhibitor solution: Dissolve 600 U deoxyribonuclease, 0.5 mg soybean trypsin inhibitor and 1.5 mM MgSO₄ in 10 mL of CGNC preparation buffer shortly before each CGNC preparation. Before use dilute 3.2 mL of this solution with 16.8 mL CGNC preparation buffer to obtain 20 mL of the sterile diluted trypsin inhibitor solution.

CGNC medium: Prepare MEM containing 26 mM sodium bicarbonate, 30 mM D-glucose, 25 mM KCl, 2 mM L-glutamine, 200 U/L penicillin G, 200 µg/L streptomycin sulfate. Filter it sterile (0.2 µm filter) and add 10% (v/v) heat-inactivated FCS. Heat-inactivated FCS is prepared by incubation of a bottle of FCS in a water bath at 55°C for 1 h (aliquots of 50 mL can be stored frozen at -20°C until use). The CGNC medium can be stored at 4°C for up to one month. In addition, prepare culture medium which is supplemented with 10 µM Ara-C.

2.2.2. Method

1. Clean the laminar air flow bench with 70% ethanol and sterilize it with UV light for 30 min prior to any use for cell culture work.
2. Put 5 mL CGNC preparation buffer in one 5 cm cell culture dish to collect the cerebella. Sterilize forceps and surgical scissors by rinsing with 70% ethanol and careful flame treatment.
3. Warm all required solutions to room temperature and perform all preparations steps at this temperature.
4. Decapitate the animals with scissors and collect the heads in 10 cm cell cultures dishes. Open the skull with surgical scissors and dissect the cerebellum with the bent forceps. Place the extracted cerebella in the 5 cm cell culture dish containing CGNC preparation buffer. Repeat this step until up to 7 cerebella are collected in preparation buffer.
5. Transfer all the cerebella in a new 5 cm dish and cut the tissue into small pieces with a scalpel.
6. Transfer the minced cerebella to 20 mL trypsin solution and incubate at 37°C until the tissue is digested (approximately 20 min). Regularly shake the solution gently to improve the access of the trypsin to the tissue.

7. Flame polish 3 sterile glass pipettes using the Bunsen burner flame under the bench. Narrow the necks of two pipettes using the flame to obtain one fine and one medium bore. Flame-polish the third pipette-neck only to remove all sharp edges of the glass.
8. After the trypsinization is completed add 20 mL of diluted trypsin inhibitor solution to the digest and centrifuge at 800 g for 1 min at room temperature.
9. Remove the supernatant and resuspend the pellet in about 2 mL of the trypsin inhibitor solution using the wide-bore glass pipette. Thereafter, consecutively use the medium and fine narrow bore pipettes to triturate the cells thoroughly for generation of a single cell suspension.
10. Layer the cell suspension carefully above 12 mL of the mEBSS solution and centrifuge for 5 min at 1300 g and room temperature. Gently remove the supernatant and thoroughly resuspend the pellet in 2 to 4 mL CGNC medium.
11. One rat cerebellum will yield approximately 30 million viable cells. Count the harvested cells in a Neubauer cell counting chamber and adjust the cell suspension with CGNC medium to the desired cell number (375,000 cells per cm²) and plate the cell suspension in cell culture dishes (24-well dish: 0.75 million viable cells in 1 mL medium).
12. Incubate the cultured cells in a cell incubator at 37°C with an atmosphere that contains 95% air, 5% CO₂ and 100% relative humidity. The culture medium should be changed after 24 h of incubation to CGNC medium containing 10 μM Ara-C to inhibit the proliferation of contaminating cells (mainly astrocytes). The cultures can be used in the range between 7- 12 days after seeding.

2.2.3. Results and Notes

1. It is essential to routinely characterize the cultures by microscopic inspection and by immunocytochemical characterization for cell type specific marker proteins. Fig. 1B shows a phase contrast image of a CGNC. Immunocytochemical staining of these cultures revealed that around 99% of

the cells in these cultures are MAP-2 positive and only 1% of the cells express GFAP (Fig. 1D).

2. CGNCs are more delicate to handle compared to APCs. Therefore, it is recommended to be very careful when changing the medium especially after experimental incubations, since the treatment might already have stressed the cells and they may detach easily.

3. Incubation of cultured brain cells for short-term metabolic studies

Metabolic studies on cultured brain cells can be performed in the complex cell culture media used to maintain the cultures. However, due to the large number of compounds present in complex culture media, especially if undefined protein-containing serum is added, an interference of media components with uptake, metabolism and/or export of substrates and metabolites is difficult to exclude. For example the high glucose content of many commercial culture media (25 mM) prevents a reliable determination of the cellular glucose consumption in the hour range, while the cystine present in such media reacts chemically with GSH exported from cells to form mixed cysteine-GSH disulfide. A good alternative to culture media, at least for short time metabolic studies, are defined isotonic incubation buffers that can be used to incubate APCs and CGNCs for hours without compromising cell viability. Here we describe the buffers and treatments that we use regularly to investigate glucose and GSH metabolism of cultured brain cells in the hour range.

3.1. Materials and solutions

Materials: CaCl₂, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), D-Glucose, KCl, MgCl₂, NaCl, Na₂HPO₄, NaOH.

Incubation buffer (IB): Prepare a solution containing 1.8 mM CaCl₂, 1 mM MgCl₂, 5.4 mM KCl, 145 mM NaCl, 0.8 mM Na₂HPO₄, 20 mM HEPES and 5 mM glucose, pH 7.4 at 37°C. For experiments on CGNCs it is necessary to

supplement the IB with additional KCl to a final concentration of 30 mM. If the incubation is to be performed at 4°C, adjust the pH of IB to 7.4 at this temperature. IB should not be stored for longer than 1 week at 4°C, since microbial contamination as well as formation of salt precipitates cannot be excluded for longer storage periods.

3.2. Method

1. Dissolve all compounds that should be applied to the cultured cells in IB to establish the appropriate final concentrations of test substances in the incubation medium. If test compounds cannot be directly dissolved in IB but have to be taken from a concentrated stock solution in an organic solvent (e.g. dimethyl sulfoxide or ethanol), always include the solvent in the appropriate final concentration as an additional control. Before starting the incubation, ensure that all incubation media are warmed up to 37°C, preferably by using a water bath.
2. Place a 24-well culture plate containing cells onto a metal grid that is warmed to 37°C in a water bath (the water level should be a few millimeters above the grid) and aspirate the culture medium. Wash the cells twice with 1 mL pre-warmed (37°C) IB and then add a defined volume of pre-warmed incubation medium containing the test compounds of interest. The volume of incubation medium can be varied between 200 µL and 1 mL per well, depending on the design of the experiment, the costs of the test compounds and the sensitivity of the assay systems that will be applied. Use triplicate wells for each condition to confirm reproducibility. For experimental incubation of cultured neurons use only one washing step to lower the risk of cell detachment from the culture plate.
3. Transfer the plate to an incubator set at 37°C with CO₂-free humidified atmosphere and incubate the plate for the desired incubation period.
4. To terminate the incubation, harvest the incubation media and store them at the appropriate storage temperature until further analysis. For determination of cellular metabolites (e.g., glutathione) or cellular enzyme activities (e.g., LDH), wash the cells twice with 1 mL unsterile ice-cold PBS

to stop transport processes and cell metabolism before applying an appropriate volume of lysis solution to the washed cells.

3.3. Notes

The procedure described above can be used for an incubation of cultured cells for up to 6 h, unless the viability of cells is compromised. For long incubation periods, all washing and incubation steps should be performed under sterile conditions to avoid interference by microbial contamination.

1. Careful treatment of the cells during all washing and incubation steps is essential to avoid detachment of cells and damage of the cell layer. Ensure that temperature and pH of all solutions are properly adjusted to the desired values.
2. For incubations at a given temperature always use IB the pH of which was adjusted to the desired temperature for washing and for incubations.
3. Depending on the type of experiment and the type of metabolite measured, it is important to feed the cells with fresh culture medium 1 d prior to performance of the experiment to replenish the cells with nutrients.
4. In the case that serum components may affect a metabolic pathway of interest or disturb the analysis of a metabolite, the cultures should be washed twice and subsequently incubated with serum-free culture medium for one day before the experiment is started.

4. Viability assays

To confirm that a metabolic study was performed on viable cells it is important to assess the biocompatibility of a given treatment or test substance. Two methods for testing cell viability following an experimental incubation are described here in detail, the quantification of extracellular lactate dehydrogenase (LDH) **(7)** and the staining of cell nuclei with propidium iodide (PI) **(8)**. Both assays determine a compromised integrity of the cell membrane. Extracellular LDH activity and PI-positive cells are observed only if the cell

membrane is sufficiently permeabilised to allow release of the cytosolic enzyme LDH from the cells and permeation of the fluorescent dye PI into the cells, respectively.

4.1. Release of lactate dehydrogenase (LDH) from cells

4.1.1. Principle

LDH catalyzes the reduction of pyruvate to lactate using NADH as an electron donor (Fig. 2A). LDH is localized in high activity in the cytosol of cultured cells. Severe damage of the cell membrane during an experimental incubation causes a release of this enzyme into the incubation medium. Thus, the extracellular activity of LDH can be used as an indicator of the loss in cell membrane integrity, while the cellular LDH activity is an indicator for viable cells that remained attached to the cell culture dish. LDH activity in incubation media and cells is determined by a microtiter plate-based method (7). Pyruvate and NADH are added to the LDH-containing sample and the decline in the absorbance of NADH at 340 nm is recorded (Fig. 2A). Comparison of the NADH generation from media samples with those of completely lysed cells allows quantification of the relative activity of released LDH which serves an indicator for a loss in cell viability.

4.1.2 Materials and solutions

Materials and equipment: HCl, NaCl, NADH, sodium pyruvate, Triton X-100, Tris (tris (hydroxymethyl) aminomethane), 96-well microtiter plates, microtiter plate reader with a filter for 340 nm and a kinetic measurement mode.

LDH buffer: Prepare 80 mM Tris-HCl buffer containing 200 mM NaCl (pH 7.2). This buffer can be stored at 4°C for a few months.

Triton X-100 solution: Prepare a solution containing 1% (w/v) of Triton X-100 in the incubation medium used for the experiments. This solution can be used for a week when stored at 4°C.

LDH reaction mixture: Sufficient amounts of a fresh reaction mixture containing 3.6 mM sodium pyruvate and 0.44 mM NADH in LDH buffer should be prepared shortly before starting the measurement.

4.1.3. Method

1. For quantification of extracellular or cellular LDH activities following an experimental incubation, the initial cellular LDH activity of untreated cells is commonly used as a reference. To obtain samples for initial cellular LDH activity, untreated cells are washed twice with ice-cold PBS and lysed in an appropriate volume of Triton X-100 solution for 30 min at 37°C. The lysis volume should be adjusted to the amount of cells to be lysed. For example, for APCs and CGNCs in wells of 24-well plates a lysis volume of 200 μ L is appropriate.
2. Collect the incubation media after incubation and store them at 4°C until determination of LDH activity. If the cellular LDH activity is to be determined after incubation, wash the cells twice with ice-cold PBS and lyse them in an appropriate volume of Triton X-100 solution for 30 min at 37°C. Collect the lysates and store them at 4°C. Do not freeze LDH-containing samples as that is likely to damage the enzyme. It is recommended to measure the LDH activity of media and cell lysates within 24 h.
3. Mix 10 μ L of samples (media or lysates) with 170 μ L LDH buffer in wells of a 96-well microtiter plate. As controls use 10 μ L of cell lysate of untreated cells (100% LDH release) and 10 μ L of incubation medium that never came in contact with cells (0% LDH release). Depending on the LDH activity present in lysates or media, the sample volume can be increased to up to 180 μ L at the expense of LDH buffer.
4. Add 180 μ L of the freshly prepared LDH reaction mixture to each well of the microtiter plate. Ideally, initiation of the LDH reaction is done by a

multichannel dispensing pipette that allows addition of reaction mixture into all wells within seconds.

5. Measure the LDH-dependent decrease in absorbance of NADH at 340 nm (Fig. 2B). Calculate the slope from the linear part of the decline in absorbance and compare the LDH activity obtained for all samples with those obtained for lysates of untreated cells (100% LDH) and medium blanks (0% LDH). The extracellular LDH activity determined is proportional to the amount of dead cells, while absence of extracellular LDH suggests that the cells remained viable during the treatment.
6. The extracellular LDH activity can be expressed as percent of initial cellular LDH activity ($= \text{LDH}_{\text{medium}} \times 100 / \text{LDH}_{\text{untreated cells}}$) or as percent of the total LDH activity for a given time point ($= \text{LDH}_{\text{medium}} \times 100 / (\text{LDH}_{\text{lysate of treated cells}} + \text{LDH}_{\text{medium of treated cells}})$).

4.1.4. Results and Notes

1. Fig. 2B shows the time-dependent decline in absorbance at 340 nm for 10 μL of lysis buffer (IB with 1% Triton X-100) that was never in contact with cells or for 10 μL of cell lysates prepared by adding lysis buffer to APCs or CGNCs. Only the linear part of the decrease in absorbance (between 1 and 6 min) should be used to calculate LDH activity in the lysates.
2. Under certain conditions the extracellular LDH activity cannot be measured reliably due to interference of the test compound with the assay and/or inhibition of the released LDH by the test compound. To test for such interferences, the respective test compound should be added to the Triton X-100 lysate of untreated cells. Unaltered decline of NADH compared to the control (absence of test compound) is a good indication that the test compound does not give false negative results when the extracellular LDH activity is used as a marker for loss in cell viability.

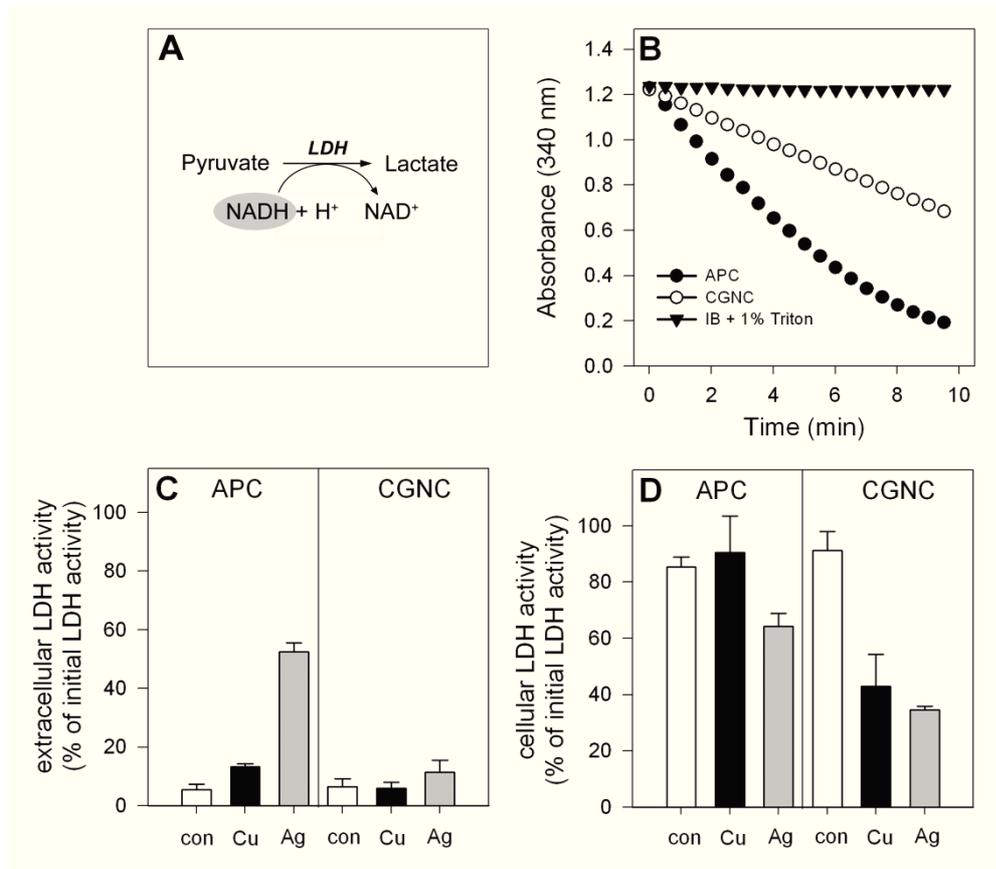


Figure 2: Measurement of lactate dehydrogenase (LDH) activity as a parameter for cell viability. **A:** LDH catalyzes the reduction of pyruvate to lactate by using NADH as an electron donor. **B:** Decline in absorbance at 340 nm of a reaction mixture containing 10 μ L lysis buffer (1% Triton X-100 in IB) or 10 μ L lysates of APCs or CGNCs. **C,D:** Cultured APCs or CGNCs were incubated in IB without (con) or with 30 μ M CuCl_2 (Cu) or 30 μ M AgNO_3 (Ag) for 3 h and the extracellular (C) and the cellular (D) LDH activities were determined as percent of the initial cellular LDH activity.

- To test for the potential toxicity of copper and silver ions to culture brain cells, APCs and CGNCs were treated with IB alone (control) or with IB containing 30 μ M CuCl_2 (Cu) or 30 μ M AgNO_3 (Ag) for 3 h and the cellular and extracellular LDH activities were determined. Control cells that had been incubated without metal ions showed only a very low extracellular LDH activity of less than 10% of the initial cellular LDH activity (Fig. 2C) and the cellular LDH activity was only lowered by up to 15% (Fig. 2D), indicating

that the incubation conditions hardly harmed the cells. Presence of copper or silver ions during the incubation of APCs increased the extracellular LDH activity to 13% and 52% of the initial cellular LDH activity, respectively (Fig. 2C) with a comparable lowering of the cellular LDH activity (Fig. 2D). For CGNCs that had been treated with copper or silver ions no increase in extracellular LDH activity was observed (Fig. 2C), while the cellular LDH activity was lowered by 60% and 70%, respectively (Fig. 2D). The discrepancy between the initial cellular LDH activity and the sum of extracellular plus cellular LDH activities after treatment may be caused by detachment of cells with intact cell membranes, leading to a decrease in cellular LDH activities without corresponding increases in extracellular LDH activities. To verify this possibility, Triton X-100 can be added to a final concentration of 1% to the harvested incubation medium which will lyse detached intact cells that are present in the medium and makes their LDH detectable. An alternative explanation for the loss in total LDH activity could be that LDH released from damaged cells may have become inactivated by the metal ions as described before **(12,13)**.

4. As detection limit for extracellular LDH activity we consider an absorbance difference of 0.02 absorbance units obtained within 10 min measurement. This value corresponds to 1.29 nmol/min LDH activity in a well of a microtitre plate. The detection of LDH in cell lysates depends on the investigated cell type. If the cellular LDH activity or the cell density per well is very low, the volume of cell lysates applied as sample can be increased. In addition, the measurement time of 10 min could be prolonged to increase the detection limit. However, for such altered assay conditions the stability of the LDH would have to be tested.

4.2. Cell permeability for propidium iodide (PI)

4.2.1. Principle

The PI staining method visually identifies cells with damaged cell membranes that became permeable to PI **(8)**. The fluorescent dye PI (λ_{ex} : 535 nm; λ_{em} : 612 nm) intercalates into DNA **(14)**. Since PI cannot penetrate through an intact cell membrane, intact cells are PI-negative while cells with permeabilized membranes contain PI-positive nuclei. The membrane permeable fluorescent dye Hoechst 33342 (H33342; λ_{ex} : 341 nm; λ_{em} : 461 nm) is applied together with PI to visualize all cell nuclei.

4.2.2. Materials and solutions

Materials and equipment: H33342, KH_2PO_4 , K_2HPO_4 , NaCl, PI, paraformaldehyde, fluorescence microscope with appropriate excitation and emission filters.

Solutions of PI and H33342: Prepare a 1.5 mM PI stock solution and a 1.78 mM stock solution of H33342 in pure water. These solutions can be stored in the dark for several weeks at 4°C. Dilute the stock solutions shortly before application to cells in pre-warmed IB to obtain the PI-H33342 solution with final concentrations of 5 μM PI and 10 μM H33342 that will be applied to the cells.

Paraformaldehyde solution: If the cells are fixed after PI-H33342 staining, a solution of 3.5% (w/v) paraformaldehyde in PBS has to be prepared. This solution can be stored at -20°C for a month.

4.2.3. Method

1. Wash the cells after the experimental incubation twice with pre-warmed (37°C) IB.
2. Incubate the cells with the PI-H33342 solution (200-500 μ L per well of a 24-well cell culture dish, depending on the cell density) at 37°C for 15 min in the dark.
3. Wash the cells twice with pre-warmed IB and analyse the cells immediately for fluorescence under a fluorescence microscope.
4. If many experimental incubation conditions have to be analysed, it is recommended to fix the cells with 3.5% paraformaldehyde in PBS (400 μ L per well of a 24-well cell culture dish) for 10 min at room temperature.
5. After paraformaldehyde fixation, wash the cells twice with PBS. The cells can then be stored for several hours in the dark at 4°C until analysis of fluorescence.

4.2.4. Results and Notes

1. Fig. 3 shows PI-H33342-stainings of APCs and CGNCs that were incubated for 3 h without or with 30 μ M CuCl_2 (Cu) or 30 μ M AgNO_3 (Ag) in 200 μ L IB. After 3 h of incubation, the membrane integrity of APCs and CGNCs that had been treated without metal ions were not compromised as indicated by the absence of any PI-positive cells (Fig. 3A,C), while a high number of cells was present in the dishes as indicated by the H33342-staining of all cell nuclei (Fig. 3B,D). After treatment with CuCl_2 , APCs (Fig. 3E), but not CGNCs (Fig. 3G) contained a large number of PI-positive cells. In contrast, at best, a low number of PI-positive cells were observed for APCs after exposure to AgNO_3 (Fig. 3I), while CGNCs contained an increased number of PI-positive cells after such a treatment (Fig. 3K). Thus, the PI-staining suggests that under the conditions used the cells in APCs are more vulnerable to Cu^{2+} treatment, whereas cells in CGNCs are preferentially damaged by exposure to Ag^+ ions.

- It is recommended to include a positive control for each PI/H33342 staining, especially if no loss of cell viability is expected for the experimental incubations. For APCs and CGNCs, Cu^{2+} and Ag^+ treatments, respectively, work well as positive controls. The concentrations of toxic metal ions can be increased or lowered depending on the incubation medium and the incubation time chosen. As alternative to metal ions, compounds that directly interfere with the cell membrane (e.g. treatment with 0.1% Triton-X 100 for 10 min) can be used to compromise the integrity of the cell membrane.
- All storage and handling as well as microscopic examinations and documentations should be done in the dark to avoid loss of the fluorescent signals.

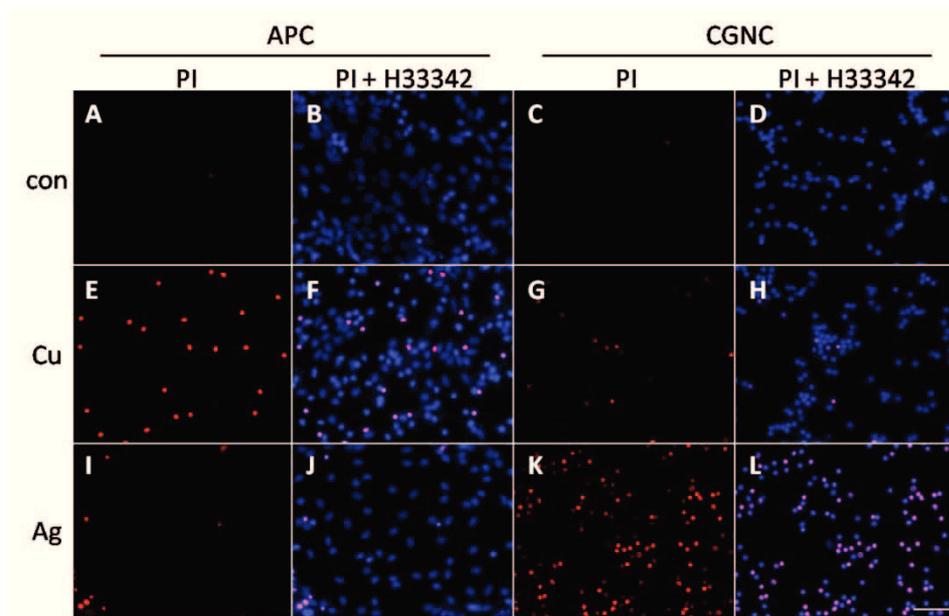


Figure 3: Staining of cell nuclei with propidium iodide (PI) and Hoechst 33342 (H33342) to assess cell viability. APCs or CGNCs were incubated in IB without (con) or with 30 μM CuCl_2 (Cu) or 30 μM AgNO_3 (Ag) for 3 h. PI-stained nuclei identify cells that have a permeabilized cell membrane, while staining with the membrane permeable dye H33342 identifies the nuclei of all cells present. The scale bar in panel L corresponds to 50 μm and applies to all panels.

4. It is recommended to document the positive and negative controls first to adjust the settings for illumination of PI-positive and H33342-positive cell nuclei. These settings should be kept constant for the documentation of the stainings of all conditions used in one experiment.
5. It should be noted that results obtained for cell viability by PI staining (Fig. 3) may not always be consistent with data obtained by determining extracellular LDH activity (Fig. 2).

5. Glucose assay

5.1. Principle

The microtiter plate-based glucose assay uses the coupled enzymatic reactions of hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PDH) to quantify glucose in incubation media **(15,16)**. The glucose in the sample solution is phosphorylated by HK to glucose-6-phosphate (G6P) in an ATP and Mg^{2+} -dependent reaction. The G6P generated by HK is subsequently oxidized by G6PDH to 6-phosphogluconate along with the reduction of $NADP^+$ to NADPH (Fig. 4A). The amount of NADPH generated is equimolar to the amount of glucose consumed and can be monitored by the increase in absorbance at 340 nm.

5.2. Materials and solutions

Materials and equipment: ATP, glucose, G6PDH, HK, KOH, $MgCl_2$, $NADP^+$, triethanolamine hydrochloride (TEA), 96-well microtiter plates, microtiter plate reader with a filter for 340 nm.

Glucose assay buffer: Prepare 0.3 M TEA/KOH buffer (pH 7.6). This buffer can be stored at 4°C for up to a few months. Warm the buffer to room temperature before use.

Glucose reaction mixture: Sufficient amounts of reaction mixture consisting of 2.44 U/mL G6PDH, 1.7 U/mL HK, 1.5 mM NADP⁺, 2 mM ATP and 2 mM MgCl₂ in glucose assay buffer should be prepared fresh shortly before analyzing the glucose contents.

5.3. Method

1. After an experimental incubation, the harvested media samples for glucose measurement can be stored at 4°C for a few hours or at -20°C for weeks. Dilute 10 µL of media samples in 170 µL pure water in wells of a microtiter plate. If the media should contain glucose concentrations higher than 7.5 mM, dilute the samples appropriately. Use as a negative control (blank) 10 µL of the incubation medium without glucose (or use pure water if this medium is not available) and as a positive control add 10 µL of the glucose-containing incubation medium which had no contact to cells.
2. To each well add 180 µL of a freshly prepared glucose reaction mixture and incubate the plate at room temperature for 10 min.
3. Measure the absorbance at 340 nm using a microtiter plate reader and convert the absorbance after correction for the blank values to the concentration of NADPH generated (in mM) using the Lambert-Beer law with a path length of $d = 0.9$ cm (see note below) and the extinction coefficient of NADPH at 340 nm ($\epsilon = 6.2 \text{ mM}^{-1}\text{cm}^{-1}$). Alternatively, standards with known glucose concentrations may be used to obtain a calibration curve. The value calculated should be multiplied by the appropriate dilution factor (the dilution factor is 36, if 10 µL of sample was used) to get the glucose concentration in the sample. The concentration of glucose consumed by cells is calculated by subtracting the medium glucose concentration after incubation of cells from the value determined for the medium that was applied to the cells.

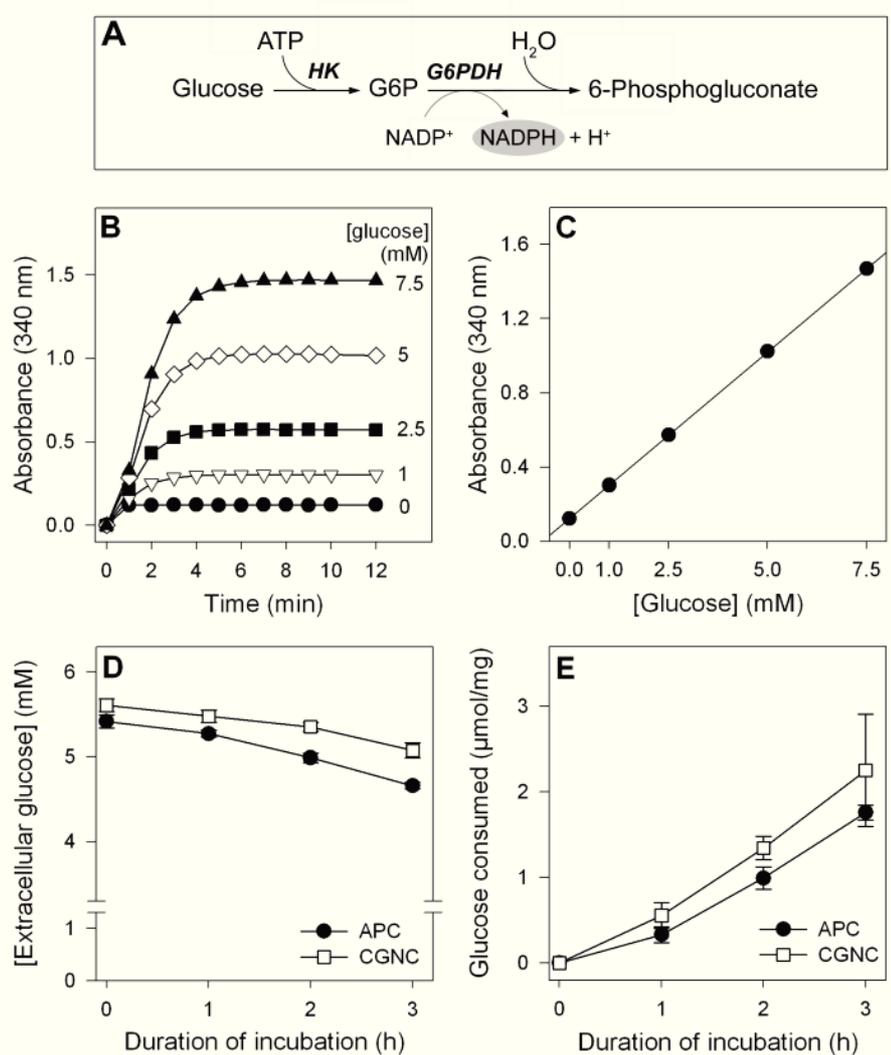


Figure 4: Determination of glucose and glucose consumption. **A:** Glucose is quantified by a coupled enzymatic assay. Hexokinase (HK) phosphorylates glucose to glucose-6-phosphate (G6P) which is subsequently oxidized by the $NADP^+$ -dependent glucose-6-phosphate dehydrogenase (G6PDH) to 6-phosphogluconate. **B:** For 10 μL sample volumes of the indicated glucose concentrations in IB the absorbance of NADPH at 340 nm increased with time to maximal values within a few minutes. **C:** The absorbance recorded at 340 nm after 10 min increased proportionally to the concentration of glucose present. **D,E:** APCs or CGNCs were incubated with 0.2 mL IB (which contains 5 mM glucose). Panels **D** shows the glucose concentration determined for the indicated incubation periods and panel **E** the specific amount of glucose consumed by the cells as calculated as protein-normalized difference between the glucose applied at the onset of the experiment and the glucose determined at the indicated incubation time.

5.4. Results and Notes

1. Fig. 4 shows the increase in absorbance of NADPH at 340 nm as a function of time and concentration of glucose (Fig. 4B,C). The absorbance of NADPH generated by the coupled HK and G6PDH reactions increased within a few minutes to maximal values (Fig. 4B). After 10 min of incubation a linear relationship was obtained between the concentration of glucose applied and the absorbance at 340 nm (Fig. 4C).
2. Incubation of APCs and CGNCs in wells of 24-well plates with 200 μ L IB revealed that the concentration of glucose was lowered during an incubation for up to 3 h (Fig. 4D). APCs and CGNCs did not differ substantially in their specific glucose consumption (Fig. 4E).
3. The assay has a detection limit of 0.13 mM glucose in the medium (when 10 μ L of the sample is used). However, by applying higher volumes (up to 180 μ L) media at the expense of the diluting water, the sensitivity of the assay can be increased by a factor of up to 18.
4. The path length of $d = 0.9$ cm used for calculation of the sample concentration by the Lambert-Beer law depends on the geometry of the 96-well plate used for the measurement as well as on the reaction volume within each well. Therefore, the d -value has to be determined for each type of 96-well plate, for example by measuring the absorbance of a defined volume of a solution with a known concentration of a chromophore (e.g., NADH at 340 nm) and subsequent calculation of the d -value by the Lambert-Beer law.
5. It is important to test for the potential interference of a test component with the glucose assay. For this purpose, record the NADPH formation in a reaction that contains 10 μ L of a 5 mM glucose solution in IB as well as the test compound in the final concentrations used for the cell experiment and compare the results obtained without and with the test component.

6. Lactate assay

6.1. Principle

The lactate assay described here has been used to measure the lactate concentration in various incubation media **(16-18)**. The enzyme lactate dehydrogenase (LDH) catalyzes the oxidation of lactate to pyruvate using NAD⁺ as a co-factor, thereby generating NADH which can be quantified at 340 nm (Fig. 5A). Since the equilibrium of the LDH-catalyzed reaction strongly favors lactate formation, the lactate oxidation by LDH has to be coupled with another reaction to guarantee complete lactate oxidation. The pyruvate generated by LDH from lactate is removed by a transamination reaction with glutamate catalyzed by the enzyme glutamate pyruvate transaminase (GPT) (Fig. 5A). The high concentration of glutamate in the glutamate/NaOH buffer together with the alkaline pH of the reaction solution that buffers the protons generated in the LDH reaction ensures that the formation of NADH by lactate oxidation is maximal (Fig. 5A). Due to the 1:1 stoichiometry between lactate oxidation and NADH formation the lactate concentration can be calculated directly by the Lambert-Beer law from the absorbance of NADH at 340 nm as described for the glucose assay.

6.2. Materials and solutions

Materials and equipment: Glutamic acid, GPT, L-(+)-lactate, LDH, NAD⁺, NaOH, 96-well microtiter plates, microtiter plate reader with a filter for 340 nm.

Lactate assay buffer: Prepare a 0.5 M glutamate/NaOH buffer (pH 8.9). The solubility of glutamic acid will improve during adjustment of pH towards the alkaline range. This buffer can be stored at 4°C for up to a few months.

Lactate reaction mixture: Prepare sufficient amounts of a fresh reaction mixture consisting of 3.89 U/mL GPT, 39.7 U/mL LDH and 5.6 mM NAD⁺ in lactate assay buffer.

6.3. Method

1. After an experimental incubation, the harvested samples for lactate measurement can be stored at 4°C for a few hours or at -20°C for weeks. Dilute 10 µL of media samples in 170 µL pure water in a well of a microtiter plate. The volume of media samples can be adjusted according to the concentration of lactate in the medium. As a negative control (blank) use 10 µL of the incubation medium that had no contact to cells, whereas for a positive control for complete oxidation of lactate use 10 µL of a 5 mM lactate solution in IB.
2. To each well add 180 µL of a freshly prepared lactate reaction mixture and incubate the plate in a humidified atmosphere of a CO₂-free cell incubator at 37°C for 90 min.
3. Measure the absorbance at 340 nm using a microtiter plate reader and convert the absorbance after correction for the blank values to the concentration of NADH as described above for the glucose assay. Alternatively, standards with known lactate concentrations may be used to obtain a calibration curve. Use the appropriate dilution factors to calculate the concentration of lactate in the samples.

6.4. Results and Notes

1. Fig. 5B shows the increase in absorbance of NADH at 340 nm as a function of time and concentration of lactate. The absorbance of NADH generated by the LDH reaction increased within 90 min to maximal values (Fig. 5B). After 90 min of incubation a linear relationship was obtained between the concentration of lactate applied and the absorbance at 340 nm (Fig. 5C).
2. Incubation of APCs and CGNCs in wells of 24-well plates in 200 µL IB (which contains 5 mM glucose) for up to 3 h caused a strong increase in the extracellular lactate concentrations (Fig. 5D). The specific lactate release of APCs was almost doubled compared to that determined for CGNCs (Fig. 5E).

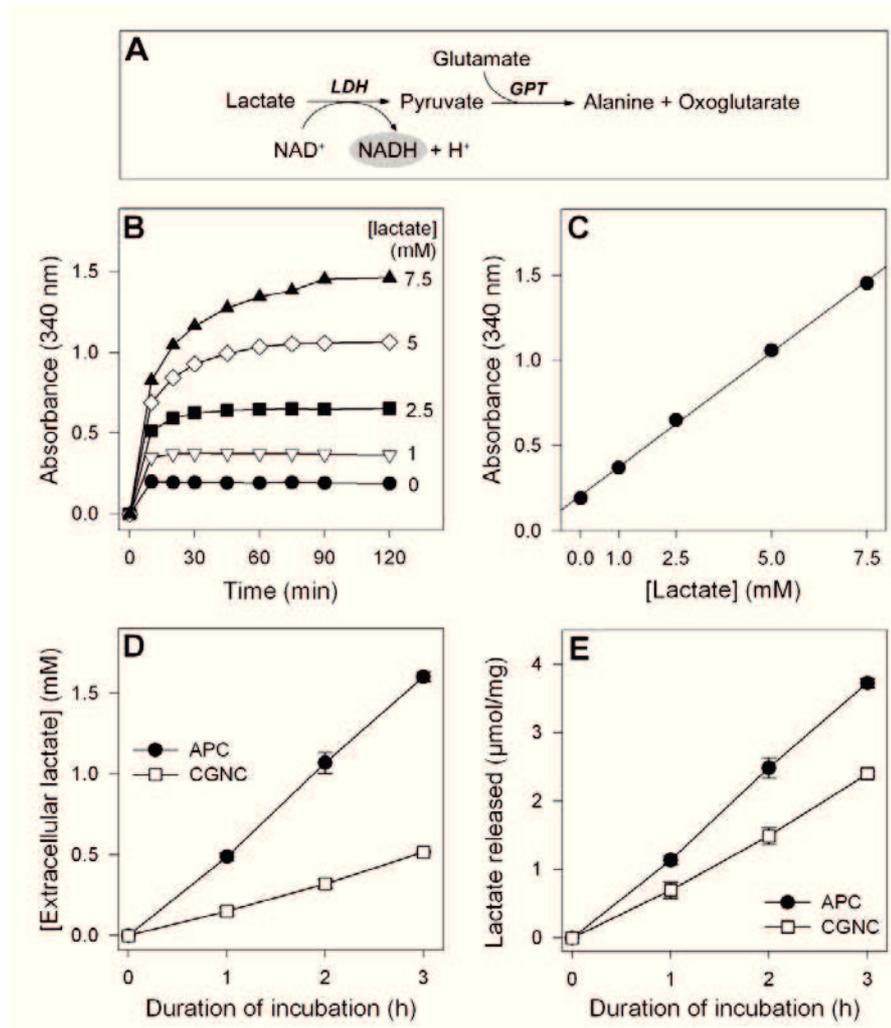


Figure 5: Determination of lactate and lactate production. **A:** Lactate is quantified by a coupled enzymatic assay. Lactate dehydrogenase (LDH) oxidizes lactate to pyruvate, thereby reducing NAD^+ to $NADH$. To establish complete oxidation of lactate, the pyruvate formed in the LDH reaction is transaminated by glutamate pyruvate transaminase (GPT) to alanine in presence of a large excess of glutamate. The high pH of the reaction buffer (pH 8.9) buffers the proton generated during lactate oxidation, thereby also supporting the complete oxidation of lactate. **B:** For 10 μL sample volumes of the indicated lactate stock solutions in IB the absorbance of $NADH$ at 340 nm increased with time to a maximal value after 90 min. **C:** The absorbance recorded after 90 min at 340 nm increased proportionally to the concentration of lactate present. **D,E:** APCs or CGNCs were incubated with 0.2 mL IB (which contains 5 mM glucose). Panel **D** shows the extracellular lactate concentration determined for the indicated incubation periods and panel **E** the specific amount of lactate produced by the cells.

3. For 10 μL of medium sample the assay has a detection limit for lactate of about 0.13 mM. However, by applying higher volumes (up to 180 μL) media at the expense of the water, the sensitivity of the assay can be increased by a factor of 18.
4. Alternatively to the photometric quantification of the NADH generated, the amount of NADH produced by the assay reaction can also be measured by fluorescence (excitation 340 nm, emission 460 nm) which allows an even higher sensitivity and a lower detection limit for lactate. For this fluorescence method, the use of a lactate calibration curve is required.
5. It is important to test for the potential interference of a test component with the lactate assay. For this purpose, record the NADH formation in a reaction that contains 10 μL of a 5 mM lactate solution in IB as well as the test compound in the final concentrations used for the cell experiment and compare the results obtained for the conditions without and with the test component.

7. Glutathione (GSH) assay

7.1. Principle

The enzymatic cycling method for quantification of glutathione (GSH) has originally been described by Tietze (**19**) and was modified for the use of microtiter plates (**20-22**). The underlying mechanism of this assay is the reduction of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) by GSH to form 5-thio-2-nitrobenzoate (TNB) that can be monitored at 405 nm (Fig. 6A). The glutathione disulfide (GSSG) formed during this reaction is reduced to GSH by the NADPH-dependent glutathione reductase (GR). Thus, the GSH and the GSSG present in a sample are continuously cycled by DTNB-mediated oxidation and GR-catalysed reduction, thereby leading to a signal amplification by the continuous formation of TNB. The rate of TNB formation depends exclusively on the total amounts of GSH and GSSG in the sample. Thus, this method determines in its basal version the amount of total glutathione (GSx = amount of GSH plus twice the amount of GSSG) in a sample. The assay is highly

specific for the detection of GSH and GSSG due to the high substrate specificity of GR for GSSG. The slope of the increase in absorbance at 405 nm per minute correlates to the GSx content in the sample and can be quantified by comparison to that of GSx standards. To discriminate between GSH and GSSG, 2-vinylpyridine (2VP) can be added to the sample which masks GSH by the formation of a GSH-2VP conjugate that cannot be detected by this assay. As GSSG does not react with 2VP (**23**), all remaining GSx that is detectable after derivatization of a sample with 2VP represents GSSG.

7.2. Materials and solutions

Materials and equipment: DTNB, ethylenediamine tetraacetic acid (EDTA), GR, GSSG, NADPH, NaH₂PO₄, Na₂HPO₄, sulfosalicylic acid (SSA), Tris base (Tris), 2VP, 96-well microtiter plates, microtiter plate reader with a filter for 405 nm and a kinetic measurement mode.

GSx assay buffer: Prepare a 0.1 M sodium phosphate buffer containing 1 mM EDTA, pH 7.5. This solution can be stored at 4°C for a few months.

SSA solution: Prepare a 1% (w/v) SSA solution in pure water. This solution is stable for a few months when stored at 4°C.

GSx standards: Prepare standard concentrations of GSSG in 1% SSA solution in duplicates ranging from 0 to 50 pmol/10 µL. These standards correspond to GSx concentrations of 0 to 100 pmol/10 µL (0-10 µM). The GSx standards can be used for weeks, if stored at 4°C.

Tris solution: Prepare a solution of 0.2 M Tris in pure water. This solution can be stored at 4°C for a few months.

GSx reaction mixture: Directly before application to the GSx-containing samples in wells of a microtiter plate, prepare sufficient amounts of a fresh reaction mixture containing 0.6 U/mL GR, 400 µM NADPH and 400 µM DTNB

in GSx assay buffer. It is important to prepare this mixture just prior to addition to the samples in the 96-well plate as a slow chemical reaction will result in the formation of TNB within this reaction mixture, even in the absence of GSx.

7.3. Method for GSx quantification

1. For analysis of GSx content in media samples, collect media samples and immediately mix the samples with an identical volume of ice-cold 1% SSA. Store the sample at 4°C till measurement. For analysis of cellular GSx contents, wash the cells with ice-cold PBS as previously described (chapter 3), remove the residual washing fluid and immediately add 200 μ L of ice-cold 1% SSA to each well of a 24-well plate. Lyse the cells at 4°C for 10 min. Transfer the lysates to cups, spin them at 12,000 g for 1 min and store the supernatant at 4°C until GSx quantification.
2. Dilute 20 μ L of the SSA-diluted media samples or 10 μ L of the cell lysates with water in wells of a microtiter plate to a total volume of 100 μ L. Similarly, dilute 10 μ L of GSx standards with 90 μ L of water in wells of the microtiter plate.
3. Prepare a fresh GSx reaction mixture and add 100 μ L of this reaction mixture to each well of the microtiter plate. This should be done by a multichannel dispensing pipettor that allows adding of reaction mixture into all wells within seconds.
4. Monitor the increase in absorbance at 405 nm over 10 min at 30 s intervals. Compare the slopes in absorbance recorded for the samples with those of the standards and calculate the amount of GSx in the samples.

7.4. Method for GSSG quantification

1. For analysis of the GSSG content of cell lysates or media samples, it is necessary to conjugate the GSH with 2VP. For quantification of GSSG in cell lysates, transfer 130 μ L of the centrifuged cell lysates or 130 μ L GSx

standards into fresh sample cups. For GSSG quantification in media, transfer 130 μL of SSA-diluted media samples or 130 μL GSx standards that had been diluted 1:1 with the incubation medium into fresh cups.

2. Add 5 μL of 2VP to each cup under a proper safety hood as 2VP is highly toxic. Adjust the pH with an appropriate volume of 0.2 M Tris solution to around pH 6. Mix the samples well directly after addition of the Tris solution and incubate at room temperature for 1 h.
3. Dilute 10 μL of each derivatization reaction for cell lysates (or their standards) and 20 μL of derivatized media samples (or their standards) with water in wells of a microtiter plate to a total volume of 100 μL .
4. Prepare a fresh GSx reaction mixture and add 100 μL of this reaction mixture to each well of the microtiter plate. This should be done by a multichannel dispenser pipette that allows adding of reaction mixture into all wells within seconds.
5. Monitor the increase in absorbance at 405 nm over 10 min at 30 s intervals. Compare the slopes in absorbance recorded for the samples with those of the standards and calculate the amount of GSSG (given as GSx) in the samples.

7.5. Results and Notes

1. Fig. 6B shows the time-dependent increase in absorbance at 405 nm for 8 different concentrations of GSx standards. The slopes calculated from the linear increases in absorbance with time are proportional to the concentration of GSx applied to the cycling reactions (Fig. 6C).
2. Incubation of cultured brain cells in wells of 24-well plates in 200 μL IB caused a substantial decrease in cellular GSx content of APCs within 3 h, but only a mild loss of cellular GSx from CGNCs (Fig. 6D). The cellular loss of GSx in APCs was accompanied by the appearance of substantial amounts of GSx in the incubation medium, while only low amounts of GSx were detected in the media of CGNCs (Fig. 6E).
3. Use a fresh tip for each pipetting of 1% SSA to guarantee the application of identical volumes of the SSA solution.

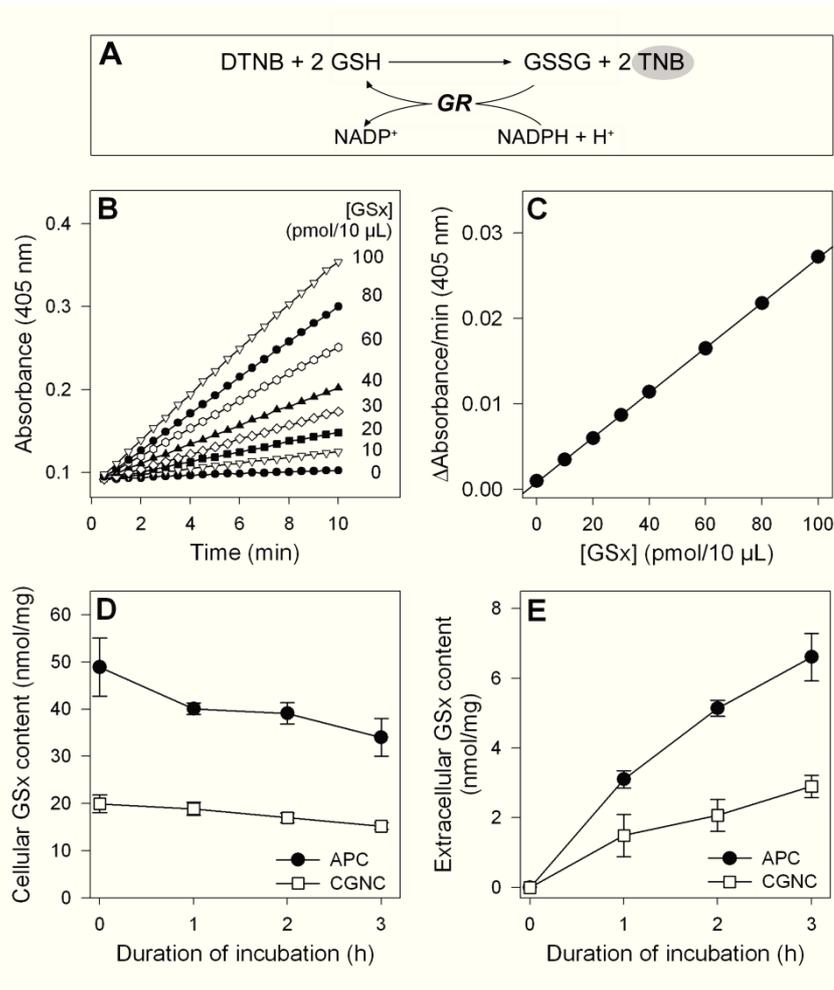


Figure 6: Determination of total glutathione (GSx = amount of GSH plus twice the amount GSSG). **A:** GSx is quantified by a highly sensitive enzymatic cycling assay. Two molecules of GSH reduce the disulfide dithionitrobenzoate (DTNB) to 2 molecules of thionitrobenzoate (TNB). The GSSG generated from GSH in this reaction as well as GSSG that was already present in the sample is subsequently reduced to GSH by glutathione reductase (GR) which uses NADPH as electron donor. This GSH starts a new cycle of DTNB reduction and enzymatic GSH regeneration. The increase in absorbance of TNB over time at 405 nm is proportional to the amount of GSx present in the reaction. **B:** Increase in absorbance at 405 nm for 10 μL samples of the indicated concentrations of GSx in the reactions. **C:** The slope of the linear increase in absorbance of TNB with time (calculated from the data presented in B) is proportional to the concentration of GSx cycling within the reactions. **D,E:** APCs or CGNCs were incubated with 0.2 mL IB. Under those conditions, the cell viability was not compromised (data not shown). Panel **D** shows the specific cellular GSx contents and panel **E** the specific amount of extracellular GSx determined for the indicated incubation periods.

4. The contents of GSx and GSSG in media or cell lysates should be measured within hours after collecting the media samples and preparing the cell lysates as the amount of detectable GSx is already lowered within 1 d of storage even at temperatures of -80°C or -20°C.
5. The detection limit for GSx in the assay described here is 0.2 nmol in 0.2 mL lysate or media samples.
6. The 2VP used for derivatization of GSH is highly toxic and has a repulsive odor. Handle all samples under a fume hood and dispose all liquids as well as the microtiter plate used into toxic waste.
7. For each combination of Tris solution and 1% SSA solution (or SSA solution diluted with media) test in advance how much of the Tris solution has to be added to adjust the pH to around pH 6 (tolerable range 5.5 to 6.5).
8. Specific values for GSx contents of cultured cells strongly depend on the culturing conditions, the age of the cultures, the availability of GSH precursors and the consumption of cellular GSH for export and detoxification reactions. In addition, even for cultures that had been prepared following the same protocol, the specific values for the GSx contents of untreated cultures may strongly differ, in extreme cases by a factor of up to 2.

8. Concluding remarks

In this chapter, we present methods to prepare cultures that are highly enriched in astrocytes or cerebellar granule neurons as well as several methods that have been successfully used to determine the glucose and GSH metabolism of these cells. However, astrocyte- and neuron-rich cultures have also been used to investigate other metabolic pathways of brain cells, for example the glycogen metabolism (**17,24,25**), the glutamate metabolism (**26**), the NAD⁺/NADH redox state (**27,28**), the formation of nitric oxide (**29**) and many more. In addition, such cultures have also been used to study the activity of cell type-specific enzymes involved in brain energy metabolism (**9,30**). The methods described here in detail are useful for studies of the metabolism of cultured astrocytes and neurons. However, by small adaptations especially concerning

incubation and sample volumes, the methods described here can also be used for the investigation of the metabolism of other types of cultured cells, for example that of cultured oligodendrocytes or microglial cells **(31)**.

9. Acknowledgements

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3.

Results

3.1. Publication 2

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3.2. Publication 3

Tulpule, K., Dringen, R., 2011. Formaldehyde stimulates Mrp1-mediated glutathione deprivation of cultured astrocytes. *J Neurochem* 116, 626-635. 109

3.3. Publication 4

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3.4. Publication/Manuscript 5

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3.1.

Publication 2

Formate generated by cellular oxidation of formaldehyde accelerates the glycolytic flux in cultured astrocytes

Ketki Tulpule & Ralf Dringen

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- Contribution of Ketki Tulpule:
 - Experimental work
 - Preparation of first draft of the manuscript



Formate Generated by Cellular Oxidation of Formaldehyde Accelerates the Glycolytic Flux in Cultured Astrocytes

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KEY WORDS

glucose; formate; lactate; metabolism; respiration

ABSTRACT

Formaldehyde is a neurotoxic compound that can be endogenously generated in the brain. Because astrocytes play a key role in metabolism and detoxification processes in brain, we have investigated the capacity of these cells to metabolize formaldehyde using primary astrocyte-rich cultures as a model system. Application of formaldehyde to these cultures resulted in the appearance of formate in cells and in a time-, concentration- and temperature-dependent disappearance of formaldehyde from the medium that was accompanied by a matching extracellular accumulation of formate. This formaldehyde-oxidizing capacity of astrocyte cultures is likely to be catalyzed by alcohol dehydrogenase 3 and aldehyde dehydrogenase 2, because the cells of the cultures contain the mRNAs of these formaldehyde-oxidizing enzymes. In addition, exposure to formaldehyde increased both glucose consumption and lactate production by the cells. Both the strong increase in the cellular formate content and the increase in glycolytic flux were only observed after application of formaldehyde to the cells, but not after treatment with exogenous methanol or formate. The accelerated lactate production was not additive to that obtained for azide, a known inhibitor of complex IV of the respiratory chain, and persisted after removal of formaldehyde after a formaldehyde exposure for 1.5 h. These data demonstrate that cultured astrocytes efficiently oxidize formaldehyde to formate, which subsequently enhances glycolytic flux, most likely by inhibition of mitochondrial respiration. © 2012 Wiley Periodicals, Inc.

INTRODUCTION

Formaldehyde is a small volatile compound that easily penetrates cell membranes, has neurotoxic potential (Songur et al., 2010), and causes deficits in memory and learning in mice (Lu et al., 2008; Malek et al., 2003; Tong et al., 2011). As a common environmental pollutant (Flyvholm and Andersen, 1993; Logue et al., 2011; Sofuoglu et al., 2011), exogenous formaldehyde can harm animals and man (IARC, 2006; NTP, 2010). However, formaldehyde is also produced endogenously by enzymatic processes. The semicarbazide-sensitive amine oxidase (SSAO), also known as vascular adhesion protein-1 (Salmi and Jalkanen, 2001), produces formalde-

hyde by deamination of methylamine (O'Sullivan et al., 2004; Yu et al., 2003), whereas the lysine-specific demethylase 1 (LSD1) demethylates lysine residues in histones (Shi et al., 2004; Wang et al., 2009) to generate formaldehyde. An additional endogenous source of formaldehyde is the carboxymethyl esters of proteins, which are generated by post-translational modification by carboxymethyltransferases (Lee et al., 2008). Hydrolysis of such carboxymethyl esters generates methanol that can be oxidized to formaldehyde by alcohol dehydrogenases, catalase, or microsomal mixed function oxidase systems (Lee et al., 2008; Liesivuori and Savolainen, 1991; Macallister et al., 2011; Tephly, 1991).

In cells, formaldehyde is metabolized either by enzymatic reduction to methanol or by oxidation to formate. The oxidation of formaldehyde to formate can be catalyzed either by the cytosolic formaldehyde dehydrogenase (ADH3) or by the mitochondrial aldehyde dehydrogenase 2 (ALDH2; Harris et al., 2003; Koivusalo et al., 1989; Macallister et al., 2011; Teng et al., 2001). For ADH3-dependent formaldehyde oxidation, the aldehyde has to first react with cellular glutathione (GSH) in an enzyme-independent process to form the ADH3 substrate *S*-hydroxymethyl-GSH (Staab et al., 2009; Thompson et al., 2009). The oxidation of *S*-hydroxymethyl-GSH by ADH3 generates *S*-formyl-GSH, which is subsequently hydrolyzed by *S*-formyl-GSH hydrolase to formate and GSH (Macallister et al., 2011; Teng et al., 2001). Formaldehyde-derived formate can be further oxidized to carbon dioxide predominantly by a folate-dependent pathway (Skrzydłowska, 2003; Tibbetts and Appling, 2010).

The brain is capable of producing formaldehyde, as the enzymes involved in formaldehyde generation are expressed in this organ (Diliberto and Axelrod, 1976; Obata, 2002; Zhang et al., 2010; Zuo and Yu, 1994). The increase of SSAO activity in Alzheimer's disease (AD) (del Mar Hernandez et al., 2005; Ferrer et al., 2002;

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Unzeta et al., 2007) as well as the elevation in protein carboxymethylation with age (Sellinger et al., 1988) suggest that formaldehyde production may be altered during neurodegeneration and aging. This view is further strengthened by the recent demonstration of increased levels of formaldehyde in the hippocampus of AD brain and in brains of mouse models of AD and dementia (Tong et al., 2011).

Little is known about the metabolism of formaldehyde and its oxidation product formate in brain cells. Both formaldehyde-oxidizing enzymes ADH3 and ALDH2 are expressed in astrocytes in culture (Iborra et al., 1992; Yang et al., 2005), suggesting that these cells have the potential to oxidize formaldehyde to formate. In addition, astrocytes have been implicated to play an important role in formate oxidation to carbon dioxide as the rate-limiting enzyme of this pathway, 10-formyltetrahydrofolate dehydrogenase, is predominantly localized in these cells in the brain (Neymeyer et al., 1997). Cultured astrocytes are remarkably resistant against acute formaldehyde toxicity. Application of formaldehyde in concentrations of up to 1 mM does not compromise cell viability (Tulpule and Dringen, 2011). However, exposure of astrocytes to formaldehyde affects important metabolic processes of these cells. Recently, it has been shown that formaldehyde treatment inhibits glutamate uptake in astrocytes by decreasing glutamate transporter expression (Song et al., 2010) and stimulates the export of the antioxidative tripeptide GSH from these cells (Tulpule and Dringen, 2011).

Despite the reported expression of the formaldehyde-oxidizing enzymes ADH3 and ALDH2 in astrocyte cultures (Iborra et al., 1992; Yang et al., 2005), it was not known whether astrocytes are able to metabolize formaldehyde. Therefore, we have investigated the potential of astrocytes to metabolize formaldehyde and to generate formate by using astrocyte-rich primary cultures as model system. Here, we report that although under the conditions used cultured astrocytes do not appear to produce endogenous formaldehyde, these cells are highly efficient in oxidizing exogenously applied formaldehyde to formate in a time-, concentration-, and temperature-dependent manner. In addition, the exposure of the cells to formaldehyde caused a delayed accelerated glucose consumption and lactate production, suggesting that the formate generated by formaldehyde oxidation inhibits mitochondrial respiration in astrocytes.

MATERIALS AND METHODS

Materials

Fetal calf serum (FCS) and penicillin/streptomycin solution were obtained from Biochrom (Berlin, Germany), and Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Karlsruhe, Germany). Formaldehyde, Folin-Ciocalteu phenol reagent, sodium potassium tartrate, sodium azide, sodium dihydrogen phosphate, sodium pyruvate, and triethanolamine hydrochloride were obtained from Merck (Darmstadt,

Germany), and acetaldehyde, sodium formate, methanol, and trizma base were purchased from Sigma-Aldrich (Steinheim, Germany). Agarose, bovine serum albumin, NADP⁺, NAD⁺, and NADH were purchased from AppliChem (Darmstadt, Germany). Formaldehyde dehydrogenase and formate dehydrogenase were obtained from Sigma-Aldrich (Steinheim, Germany), and glucose-6-phosphate dehydrogenase, glutamate pyruvate transaminase, hexokinase, and lactate dehydrogenase (LDH) were purchased from Roche Diagnostics (Mannheim, Germany). Deoxyribonucleoside triphosphate (dNTP) mix, DNA loading dye, GeneRuler™, 50 bp DNA ladder, RevertAid™ H Minus First Strand cDNA Synthesis Kit, magnesium chloride, Taq buffer, and Taq polymerase were obtained from Fermentas (St. Leon-Rot, Germany). The RNeasy® Mini Kit was from Qiagen (Hilden, Germany). Primers were obtained from MWG Biotech (Ebersberg, Germany) and RedSafe™ was from HiSS Diagnostics (Freiburg, Germany). All other chemicals of the highest purity available were obtained from Fluka (Buchs, Switzerland), Riedel-deHaen (Seelze, Germany), or Roth (Karlsruhe, Germany). The 96-well microtiter plates and sterile 24-well plates were obtained from Nunc (Wiesbaden, Germany).

Cell Culture

Astrocyte-rich primary cultures were prepared from the brains of newborn Wistar rats using a previously described method (Hamprecht and Löffler, 1985). The cells were suspended in culture medium (90% DMEM, 10% FCS, 1 mM pyruvate, 20 U/mL penicillin G, and 20 µg/mL streptomycin sulfate) and seeded in wells of 24-well plates (3×10^5 cells in 1 mL) or in 5-cm dishes (3×10^6 cells in 5 mL). The cultures were incubated in a Sanyo (Osaka, Japan) incubator at 37°C with 10% CO₂ and a humidified atmosphere. The culture medium was renewed every seventh day. For experiments, cultures at an age between 15 and 21 days were used. Cultures in 5-cm dishes were used for extraction of mRNA, whereas all other experiments were performed on cultures in wells of 24-well dishes.

Reverse Transcription Polymerase Chain Reaction Analysis

Total RNA was extracted from the astrocyte-rich cultures seeded in 5-cm dishes or from whole neonatal rat brain using the RNeasy® Mini Kit following the instructions provided by the supplier. The RevertAid™ H Minus First Strand cDNA Synthesis Kit was used for reverse transcription of the mRNA in the extracted RNA according to the instructions provided by the supplier. PCRs were performed as previously described (Tulpule et al., 2010). The primers sequences, annealing temperatures, and expected sizes of the amplification products are listed in Table 1.

Each cycle of PCR consisted of an initial 3-min denaturing at 94°C followed by 0.5-min denaturing at 94°C,

TABLE 1. Nucleotide Sequences and Annealing Temperatures of Primers Used for RT-PCR Analysis

Amplified mRNA	Forward primer	Reverse primer	Annealing temperature (°C)	Expected band size (bp)
SSAO	5'-AGTGTCCCAAGATGGACAG-3'	5'-CTCCCAAGCAGGAAGCTCAG-3'	59	229
LSD1	5'-CGAATGACCTCTCAGGAAGC-3'	5'-AGACCGTGGCGCTTAAGTA-3'	59	224
ADH3	5'-CAGCACGTTTCCGAGTACA-3'	5'-GCCACTTACAGCCCATGAT-3'	60	213
ALDH2	5'-GACCTGGACAAGCCAATTA-3'	5'-TCTTCTGTGGCACTTTGACG-3'	60	193
β -Actin	5'-GGGTCAAGGACTCTCTACG-3'	5'-GTCTCAAACATGATCTGGG-3'	59	248

0.5-min annealing at the desired annealing temperature (Table 1), and 0.5-min synthesis at 72°C. A total of 35 cycles were performed, followed by a final 10-min elongation at 72°C. All PCR products were analyzed on a 2.5% agarose gel in TAE-buffer (40 mM Tris/acetate buffer and 1 mM EDTA, pH 8.0) containing Redsafe™. Bands were visualized in UV light using a DeVision G imaging system, and images were taken by a DECON DC camera (DC Science, Hohengandern, Germany).

Experimental Incubation of Cells

The medium of the cultures was changed to serum-free DMEM 1 day before the experiment. Before treatment with formaldehyde or other compounds, the cells were washed twice with 1 mL prewarmed (37°C) or ice-cold (4°C) incubation buffer (IB; 145 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 0.8 mM Na₂HPO₄, 20 mM HEPES, and 5 mM glucose, pH 7.4). Thereafter, 0.2 mL IB containing formaldehyde and/or the other compounds investigated were applied in the concentrations indicated in the figures and tables and the cells were incubated at 37 or 4°C. After incubation of cells for the indicated time, the medium was collected and analyzed for LDH activity and for the concentrations of formaldehyde, formate, lactate, or glucose as described below. For analysis of cellular formate levels, cells were washed twice with 1 mL of ice-cold phosphate-buffered saline (10 mM potassium phosphate buffer, pH 7.4, containing 150 mM NaCl) and lysed as described below. For experiments where formaldehyde was removed after preincubation, the preincubation medium was aspirated, cells were washed twice with 1 mL of prewarmed (37°C) IB, and incubated further with 0.2 mL of IB containing the indicated compounds. At intervals of either 30 min or 1 h, 5 μ L media samples were harvested and directly added to wells of microtiter plates for determining lactate contents.

Determination of Extracellular Formaldehyde and Formate

The concentration of formaldehyde in the medium was determined using the NAD⁺-dependent oxidation of formaldehyde to formate by the GSH-independent formaldehyde dehydrogenase from *Pseudomonas putida* (Ho and Richards, 1990; Ho and Samanifar, 1988). During this reaction, the generation of NADH was monitored by the increase in absorbance at 340 nm. Briefly, 10 μ L media samples were mixed with 170 μ L pure water in

wells of a microtiter plate and 180 μ L reaction mixture (0.1 M potassium phosphate buffer, pH 7.4, at 37°C containing 18 mU formaldehyde dehydrogenase and 2.4 mM NAD⁺) was added. The reaction was incubated in a humidified atmosphere at 37°C for 30 min after which the absorbance was measured at 340 nm using a Sunrise microtiter plate reader (Tecan, Austria). Samples containing only IB were used as blank. This assay is highly specific for formaldehyde and does not detect formate or methanol (data not shown).

For determination of formate in the media samples, an assay based on formate dehydrogenase from *Candida bodinii* was used (Ogata and Iwamoto, 1990; Popov and Lamzin, 1994). The principle of this assay is the enzymatic oxidation of formate to carbon dioxide with reduction of NAD⁺ to NADH, which can be spectrophotometrically quantified at 340 nm. For quantitation of formate, the procedure was identical to that described above for formaldehyde detection with the exception that the reaction mixture consisted of 0.1 M potassium phosphate buffer, pH 7.4, at 37°C containing 126 mU formate dehydrogenase and 14 mM NAD⁺. This method is highly specific for formate and does not detect formaldehyde (data not shown).

Determination of Cellular Formate

A fluorescence method was used to quantify cellular formate levels, because the sensitivity of the photometric NADH detection method for quantification of formate in media was not sufficiently high to reliably measure cellular formate. Cultured astrocytes were washed twice with ice-cold phosphate-buffered saline and lysed with 100 μ L of 20 mM potassium phosphate buffer, pH 7.0, at room temperature for 1 h on a shaker. Thereafter, cell lysates from three wells were pooled and centrifuged for 1 min at 12,000g. In wells of a black microtiter plate, 250 μ L of the supernatant was mixed with 50 μ L of reaction mixture (0.1 M potassium phosphate buffer, pH 7.4, at 37°C containing 105 mU formate dehydrogenase and 42 mM NAD⁺) and incubated for 30 min at 37°C. The cellular formate content was quantified by comparing the fluorescence intensity of the NADH generated in the samples to that of NADH standards (0–15 nmol). Excitation and emission wavelengths of 355 and 460 nm, respectively, were used to determine the fluorescence in the microtiter plate fluorimeter Fluoroscan Ascent FL (Thermo, Waltham, MA). The amount of cellular formate was normalized to the protein content in the respective lysate supernatants.

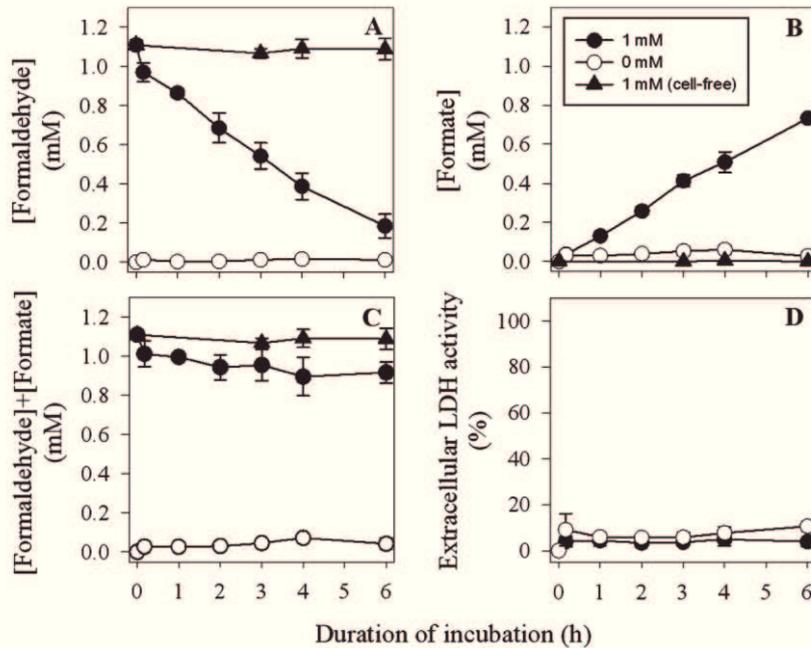


Fig. 1. Formaldehyde oxidation by cultured astrocytes. Cells were incubated without (0 mM, open circles) or with 1 mM formaldehyde (closed circles) for up to 6 h, and the extracellular concentrations of formaldehyde (A) and formate (B) as well as the extracellular LDH activity (D), given as percent of the initial cellular LDH activity, were

determined. The filled triangles represent control incubations of 1 mM formaldehyde in the absence of cells to monitor the cell-independent disappearance of formaldehyde or generation of formate. Panel C represents the sum of the extracellular concentrations of formaldehyde plus formate. The cultures used contained $124 \pm 20 \mu\text{g}$ protein per well.

Determination of Extracellular Lactate and Glucose

For quantitation of extracellular lactate and glucose contents, 5 or 20 μL media samples were used and the amounts were determined as described previously (Dringen et al., 1993; Liddell et al., 2009; Schmidt and Dringen, 2009).

Determination of Cell Viability and Protein Content

For assessment of cell viability, the activity of LDH in the media was determined using the microtiter plate assay described previously (Dringen et al., 1998) with the modification that 20 μL of lysates and media were used. None of the compounds investigated affected the determination of LDH activity in media or cell lysates of cultured astrocytes (data not shown). The protein content of the cultures was determined, after lysis of cells in well of a 24-well plate with 200 μL of 0.5 M NaOH, according to the Lowry's method (Lowry et al., 1951), using bovine serum albumin as a standard.

Presentation of Data

Data are presented as means \pm SD of values that were obtained in at least three experiments performed

on independently prepared cultures. The analysis of significance between groups of data was performed by ANOVA followed by Bonferroni *post hoc* tests, whereas that between two sets of data was analyzed by the *t*-test with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. $P > 0.05$ was considered as not significant.

RESULTS

Formaldehyde Oxidation by Cultured Astrocytes

To investigate the formaldehyde-oxidizing and formate-generating potential of cultured astrocytes, the cells were treated without or with 1 mM formaldehyde for up to 6 h (Fig. 1). During the incubation of the cells with formaldehyde, the viability was not compromised as indicated by the low extracellular LDH activity (Fig. 1D). For cells exposed to formaldehyde, the formaldehyde concentration in the medium decreased almost linearly during the incubation (Fig. 1A). This decrease in formaldehyde concentration was accompanied by a matching accumulation of formate in the medium (Fig. 1B), whereas the sum of extracellular concentrations of formaldehyde plus formate remained almost constant at 1 mM (Fig. 1C). A small drop in extracellular formaldehyde concentration was observed during the first 10 min of incubation that was not fully matched by a rise in the

TABLE 2. Rates of Formaldehyde and Glucose Metabolism in Cultured Astrocytes

	0 mM formaldehyde	1 mM formaldehyde
Formaldehyde clearance rate [$\mu\text{mol}/(\text{h} \times \text{mg})$]	n.d.	0.23 ± 0.02
Formate release rate [$\mu\text{mol}/(\text{h} \times \text{mg})$]	n.d.	0.20 ± 0.03
Ratio of formate release to formaldehyde clearance	–	0.88 ± 0.08
Lactate release rate [$\mu\text{mol}/(\text{h} \times \text{mg})$]	1.34 ± 0.09	$1.97 \pm 0.20^{**}$
Glucose consumption rate [$\mu\text{mol}/(\text{h} \times \text{mg})$]	0.73 ± 0.01	$1.07 \pm 0.05^{***}$
Ratio of lactate release to glucose consumption	1.84 ± 0.16	1.85 ± 0.13

Cultured astrocytes were incubated without or with 1 mM formaldehyde for up to 4 h. The rates were determined from the slope of the linear decrease in extracellular formaldehyde or glucose contents and of the linear increase in the contents of extracellular formate or lactate. The results were obtained on cultures that contained 124 ± 20 (formaldehyde metabolism) or 91 ± 11 (glucose metabolism) μg protein per well. The significance of differences to the data obtained for cells incubated without formaldehyde is indicated. n.d., not detectable.

extracellular formate concentration (Fig. 1A,B). In the absence of exogenous formaldehyde, no extracellular accumulation of formaldehyde or formate was observed (Fig. 1A,B), indicating that under the conditions used cells are not able to endogenously produce formaldehyde and oxidize it to formate. Thus, the formate released into the medium was formed exclusively by oxidation of the applied formaldehyde.

Control incubations with formaldehyde in the absence of cells but otherwise under identical conditions were performed to test for potential disappearance of the volatile formaldehyde during the incubation in wells of 24-well dishes at 37°C . Incubation of 1 mM formaldehyde for up to 6 h in the absence of cells neither lowered the concentration of detectable formaldehyde (Fig. 1A) nor caused any appearance of formate (Fig. 1B), excluding that evaporation of formaldehyde or cell-independent oxidation to formate occurred under the incubation conditions used.

The linear decrease and increase in extracellular formaldehyde and formate concentrations, respectively, were used to calculate formaldehyde clearance and formate release rates (Table 2). The rate of formaldehyde clearance was almost identical to that of formate release, and the ratio of these two rates was 0.88 ± 0.08 (Table 2), indicating that most of the formaldehyde that had disappeared from the medium was oxidized to formate and subsequently released from the cells.

The concentration dependence of formaldehyde oxidation was investigated by incubating cells for 3 h with formaldehyde in concentrations of up to 1 mM (Fig. 2). None of the concentrations of formaldehyde applied compromised the viability of cells (data not shown). Concentrations of formaldehyde higher than 1 mM were not used, because of the known toxicity to astrocytes of formaldehyde in concentrations above 1 mM (Tulpule and Dringen, 2011). For all concentrations of formaldehyde applied, formaldehyde disappeared during the 3-h incubation (Fig. 2A) and formate accumulated in the medium (Fig. 2B) and in the cells (Fig. 2C). The cellular formate amounted, for all concentrations of formalde-

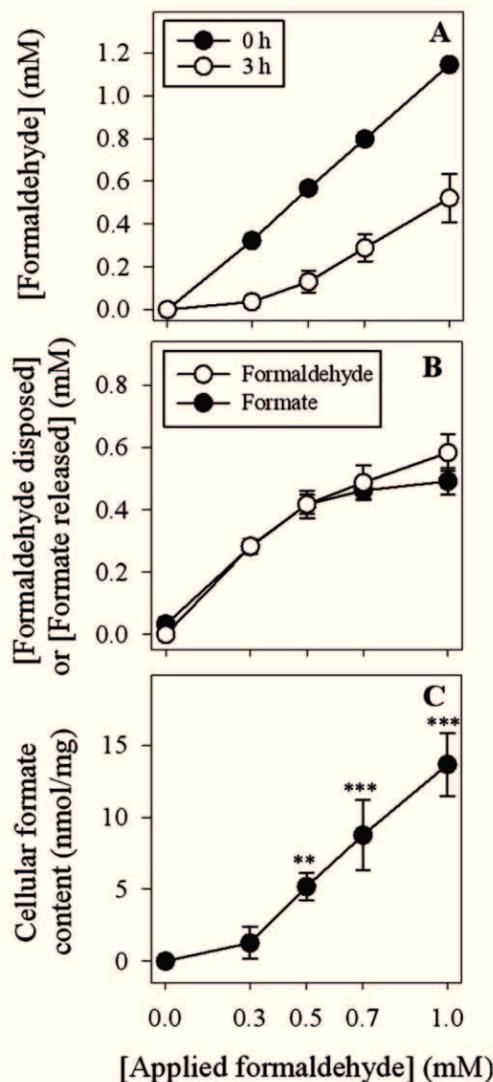


Fig. 2. Concentration-dependent formaldehyde oxidation in cultured astrocytes. Cells were treated with the indicated concentrations of formaldehyde for 3 h, and the concentrations of formaldehyde before and after the incubation (A) as well as of formate after 3 h (B) were determined. Panel B also gives the concentration of disposed formaldehyde as calculated from the difference between the formaldehyde concentrations determined for 0 and 3 h shown in panel A. Panel C represents the cellular formate contents after the incubation. In panel C, the significance of differences to data obtained for the control condition (absence of formaldehyde) is indicated. The results were obtained on cultures that contained 93 ± 3 (panels A and B) or 98 ± 19 (panel C) μg protein per well.

hyde applied, to less than 1% of the extracellular formate. The cellular content of formate determined after 3 h of incubation (Fig. 2C), but not the amount of extracellular formate (Fig. 2B), increased proportionally to the

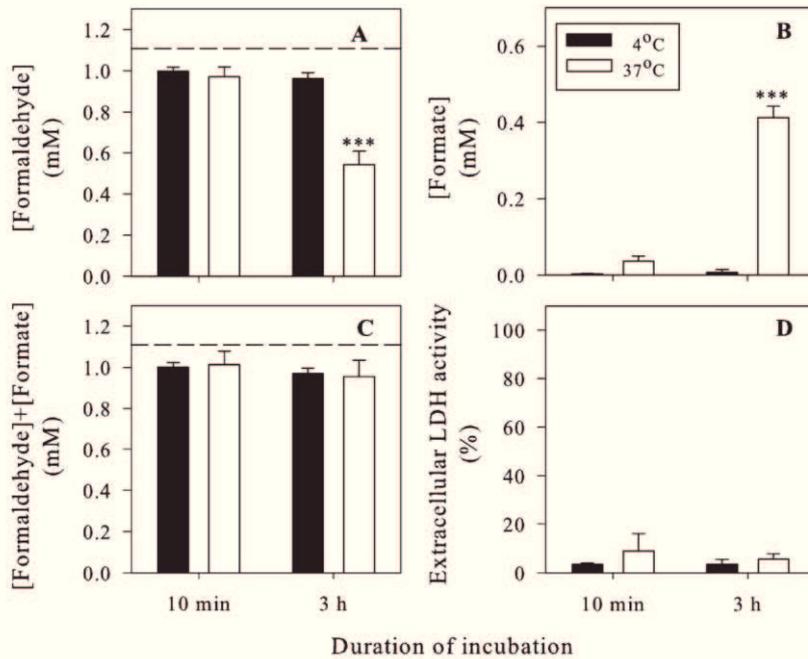


Fig. 3. Temperature dependence of formaldehyde oxidation by cultured astrocytes. After incubation of cells with formaldehyde for 10 min or 3 h at 4 or 37°C, the concentrations of formaldehyde (A) and formate (B) as well as the LDH activity (D) in the medium, given as percent of the initial cellular LDH activity, were determined. Panel C represents the sum of extracellular concentrations of formaldehyde plus formate.

The dashed lines in panel A and C indicate the concentration of formaldehyde applied to the cells (1.11 ± 0.03 mM). The results were obtained on cultures that contained 123 ± 20 μ g protein per well. The significance of differences between the data obtained for cells that had been incubated at 4 and 37°C is indicated.

concentration of formaldehyde applied. Although the cells almost completely oxidized 0.3 mM formaldehyde to formate within 3 h, only half of the applied 1 mM formaldehyde was detected as extracellular formate (Fig. 2B). The hyperbolic curves obtained for the concentration dependencies of formaldehyde disposal and extracellular formate accumulation were fitted using the Michaelis–Menten equation. Linearization of the data from three independent experiments by the Hanes–Woolf plot revealed K_M and V_{max} values of 0.19 ± 0.04 mM and 0.43 ± 0.07 μ mol/(h \times mg), respectively, for formaldehyde disposal and 0.14 ± 0.03 mM and 0.40 ± 0.06 μ mol/(h \times mg), respectively, for formate release.

Effect of Temperature on Formaldehyde Oxidation

To ascertain that the decline in extracellular formaldehyde and the accumulation of formate in the medium was due to metabolism of formaldehyde by astrocytes, cells were incubated with 1 mM formaldehyde for 10 min or for 3 h at 4 or 37°C (Fig. 3). Although there was a slight drop in the extracellular formaldehyde concentration during the first 10 min of incubation at both incubation temperatures, the formaldehyde concentration remained constant during further incubation at 4°C

for up to 3 h (Fig. 3A) and the formate released into the medium was negligible (Fig. 3B). In contrast, at 37°C about half of the formaldehyde applied was removed by the cells (Fig. 3A) and a matching amount of formate was generated and released into the medium (Fig. 3B). For both temperatures, the sum of extracellular concentrations of formaldehyde plus formate remained almost constant and was only slightly lower than the concentration of formaldehyde applied (Fig. 3C). None of the conditions used compromised the viability of the cells, as indicated by the absence of any significant increase in extracellular LDH activity compared with controls (Fig. 3D).

Expression of Formaldehyde-Generating and -Oxidizing Enzymes

To test whether the primary astrocyte-rich cultures used in this study and neonatal rat brain express the mRNAs for formaldehyde-generating and formaldehyde-oxidizing enzymes, qualitative RT-PCRs were performed (Fig. 4). Specific amplification products of the expected sizes (Fig. 4, Table 1) were obtained for SSAO, LSD1, ADH3, and ALDH2, confirming the presence of mRNAs of these enzymes in neonatal rat brain and in the astrocyte-rich primary cultures used (Fig. 4).

Consequences of Formaldehyde Treatment on Glycolysis in Cultured Astrocytes

Formate is a known inhibitor of complex IV of the respiratory chain (Nicholls, 1975; Petersen, 1977). Because inhibition of the respiratory chain stimulates glycolytic flux in astrocytes (Pauwels et al., 1985; Scheiber and Dringen, 2011; Walz and Mukerji, 1988), we investigated the consequences of formaldehyde oxidation on astrocytic glucose consumption and lactate production. Compared with control cells, the treatment of astrocytes with 1 mM formaldehyde resulted in a time-dependent increase in both lactate release and glucose consumption that became significant after 1 and 3 h of incubation, respectively (Fig. 5A,C). The rates of lactate release and

glucose consumption, calculated from the slopes of the linear increase in the contents of extracellular lactate and consumed glucose, were significantly elevated by about 50% in cells exposed to 1 mM formaldehyde compared with controls (Table 2), whereas the ratio of the lactate release rate to the glucose consumption rate remained unaltered for both control and formaldehyde-treated cells (Table 2). The stimulation of glycolytic flux after exposure of astrocytes to formaldehyde depended on the concentration of formaldehyde applied (Fig. 5B,D). A significantly elevated lactate release was only observed after exposure of the cells to at least 0.7 mM formaldehyde (Fig. 5B), whereas 1 mM formaldehyde had to be applied to significantly increase cellular glucose consumption of the cells (Fig. 5D). Thus, despite the substantial increases in cellular and extracellular formate levels observed for formaldehyde concentrations below 0.7 mM (Fig. 2B,C), a significant increase in glycolytic flux was only observed after exposure of cells to at least 0.7 mM formaldehyde (Fig. 5).

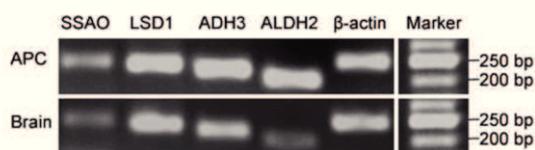


Fig. 4. RT-PCR analysis of astrocyte-rich primary cultures and neonatal rat brain for the presence of mRNAs of the formaldehyde-generating enzymes, SSAO and LSD1, and the formaldehyde-oxidizing enzymes, ADH3 and ALDH2. β -Actin was used as an internal positive control.

Effects of Treatment with Formaldehyde or Its Metabolites on Glycolytic Flux in Astrocytes

To study whether the formaldehyde-stimulated glycolytic flux was due to formaldehyde itself or its

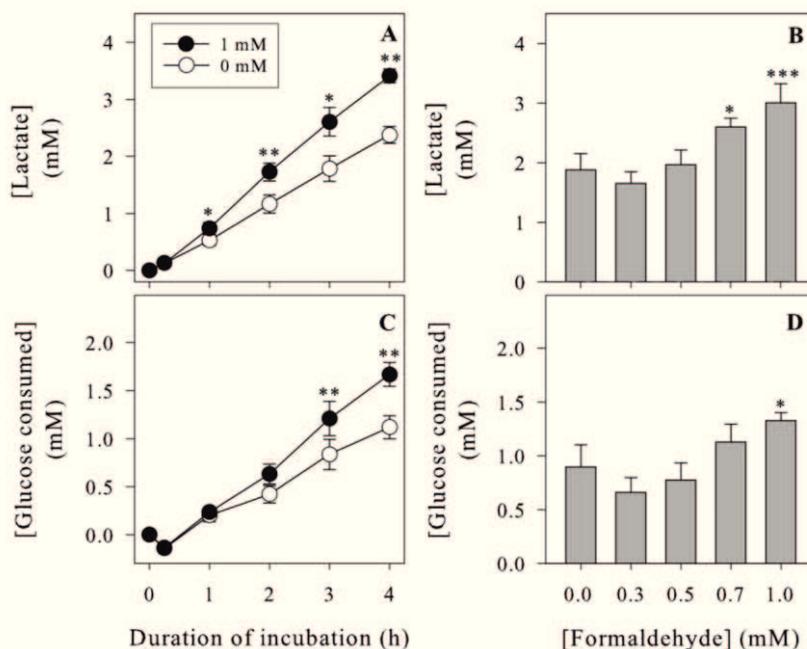


Fig. 5. Effects of formaldehyde on the glucose metabolism of cultured astrocytes. Cells were incubated without or with 1 mM formaldehyde for up to 4 h (A, C) or with the indicated concentrations of formaldehyde for 3 h (B, D) and the media concentrations of lactate (A, B) and the concen-

tration of glucose consumed (C, D) were determined. The results were obtained on cultures that contained 91 ± 11 (A, C) and 93 ± 3 (B, D) μg protein per well. The significance of differences to the data obtained for the control condition (absence of formaldehyde) is indicated.

TABLE 3. Effects of Formaldehyde and Its Metabolites on the Glucose Metabolism of Cultured Astrocytes

Compound	Lactate released (mM)	Glucose consumed (mM)	Extracellular LDH activity (% of initial)	n	Cellular formate content (nmol/mg)	Extracellular LDH activity (% of initial)	n
None	1.98 ± 0.17	0.92 ± 0.08	4 ± 2	9	0.1 ± 0.1	3 ± 1	4
Formaldehyde	2.84 ± 0.28***	1.35 ± 0.04**	8 ± 3**	9	13.6 ± 1.8***	7 ± 1**	4
Methanol	1.91 ± 0.29	0.97 ± 0.23	4 ± 2	6	0.1 ± 0.2	4 ± 2	3
Formate	1.71 ± 0.23	1.05 ± 0.17	2 ± 2	4	4.3 ± 1.4**	3 ± 1	3

Cells were incubated with 1 mM of the indicated compounds for 3 h. The significance of differences to the values of controls (none) is indicated.

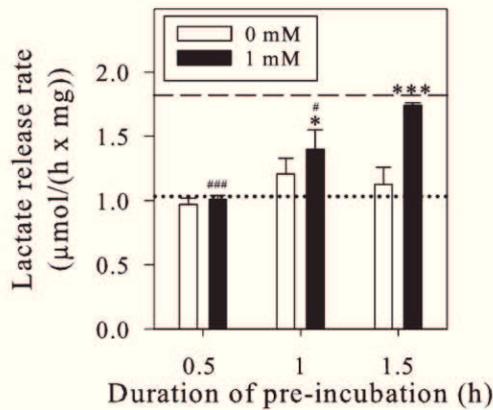


Fig. 6. Consequence of removal of formaldehyde on the lactate release rate of cultured astrocytes. Cells were preincubated without or with 1 mM formaldehyde for 0.5, 1, or 1.5 h after which the medium was removed and the main incubation was performed in the absence of formaldehyde. The lactate release rates were calculated from the linear increase in the extracellular lactate content during the 2.5-h main incubation. The dotted and dashed lines indicate the lactate release rates of control cells on prolonged incubation without and with formaldehyde and correspond to $1.09 \pm 0.03 \mu\text{mol}/(\text{h} \times \text{mg})$ and $1.82 \pm 0.33 \mu\text{mol}/(\text{h} \times \text{mg})$, respectively. The results were obtained on cultures that contained $114 \pm 12 \mu\text{g}$ protein per well. The significance of differences between cells that had been preincubated without or with formaldehyde is indicated by stars. Hashes ($^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$) indicate the significance of differences between specific lactate release rates of cells that were continuously exposed to formaldehyde (dashed line) and those exposed only for the preincubation to formaldehyde.

metabolites, cells were treated with 1 mM formaldehyde, methanol, or sodium formate for 3 h and the concentrations of lactate released and glucose consumed as well as the cellular levels of formate were determined (Table 3). The cell viability was not compromised for cells treated with methanol or formate (Table 3), whereas exposure of astrocytes to 1 mM formaldehyde caused a small (less than 10% of initial cellular LDH activity) but significant increase in extracellular LDH activity (Table 3). Exposure of cells to methanol and formate did not have any influence on lactate release and glucose consumption, whereas formaldehyde treatment significantly increased glycolysis compared with control cells (Table 3). In addition, the specific level of cellular formate determined for formaldehyde-treated cells was significantly ($P < 0.001$) higher (about 14 nmol/mg) than that found for cells incubated with exogenous formate (about 4 nmol/mg), whereas no cellular formate was observed for methanol-treated cells (Table 3).

Effect of Removal of Formaldehyde on Formaldehyde-Stimulated Glycolytic Flux

To investigate whether continuous presence of formaldehyde throughout the incubation is required to accelerate glycolytic flux in cultured astrocytes, cells were preincubated without or with 1 mM formaldehyde for 0.5, 1, or 1.5 h and subsequently incubated in formaldehyde-free medium (main incubation; Fig. 6). The lactate release rate was calculated from the linear increase in the extracellular lactate concentration during a 2.5 h main incubation that followed the preincubation. As controls, cells were incubated in formaldehyde-free or formaldehyde-containing (1 mM) medium throughout preincubation and main incubation without changing the medium (Fig. 6). For these control conditions, the lactate release rate was significantly higher ($P < 0.001$; $n = 3$) on treatment with 1 mM formaldehyde [$1.82 \pm 0.33 \mu\text{mol}/(\text{h} \times \text{mg})$] compared with the formaldehyde-free controls [$1.09 \pm 0.03 \mu\text{mol}/(\text{h} \times \text{mg})$]. For formaldehyde-free incubations, these rates were not altered by renewing the formaldehyde-free media for the main incubation (Fig. 6). However, replacement of formaldehyde-containing preincubation medium by formaldehyde-free medium for the main incubation altered the lactate production rate depending on the duration of the preincubation with formaldehyde. Although cells exposed for only 30 min to formaldehyde did not show any accelerated lactate production after removal of formaldehyde, the formaldehyde-induced accelerated lactate production rate was partially and fully maintained in cultures that had been preincubated with formaldehyde for 1 and 1.5 h, respectively, before removing formaldehyde for the main incubation (Fig. 6).

Effect of Azide on Formaldehyde-Stimulated Lactate Release

Formaldehyde oxidation by cultured astrocytes produces formate (Fig. 1), which is a known inhibitor of complex IV of the respiratory chain (Nicholls, 1975; Wallace and Starkov, 2000). Besides formate, azide is also an inhibitor of this complex (Zamboni et al., 2010; Ziabreva et al., 2010). To investigate whether inhibition of complex IV is involved in the observed stimulation of glycolytic flux in formaldehyde-treated astrocytes, the cells were preincubated with 1 mM formaldehyde for 1.5 h and subsequently incubated without or with 10 mM azide for further 3 h (Table 4). Indeed, application of azide significantly increased the lactate release rate from

TABLE 4. Effect of Complex IV Inhibition by Azide on the Formaldehyde-Stimulated Lactate Release

	Lactate release rate [$\mu\text{mol}/(\text{h} \times \text{mg})$]		LDH activity (% of initial)		<i>n</i>
	Control	Formaldehyde	Control	Formaldehyde	
None	0.99 ± 0.10	$1.48 \pm 0.16^{***}$	8 ± 2	10 ± 3	6
Azide	$1.99 \pm 0.13^{***}$	$1.53 \pm 0.08^{\#}$	9 ± 4	12 ± 1	3

Cells were preincubated without (control) or with 1 mM formaldehyde for 1.5 h, and the lactate release was measured in the subsequent main incubation for up to 3 h in the absence (none) or presence of 10 mM azide. The lactate release rates were calculated from the linear increase in extracellular lactate content in the main incubation. The results were obtained on *n* cultures that contained 105 ± 24 (none) or 87 ± 12 (azide) μg protein per well, respectively. The significance of differences to the values obtained for cells that had been incubated without azide (none) is indicated by stars, whereas that between cells pretreated in the presence or absence of formaldehyde is indicated by $^{\#}P < 0.05$ or $^{***}P < 0.001$.

astrocytes compared with control (Table 4). However, the treatment of cells with formaldehyde and subsequently with azide did not further increase the lactate production rate compared with the values obtained for cells that were treated with formaldehyde alone (Table 4).

DISCUSSION

Cultured astrocytes were used as a model system to investigate the metabolism of formaldehyde as well as potential consequences of a formaldehyde treatment on astrocytes. Astrocyte cultures were remarkably resistant against formaldehyde-induced toxicity. At least a treatment with 1 mM formaldehyde did not cause any substantial cell damage, confirming our previous observations (Tulpule and Dringen, 2011). The capacity of cultured astrocytes to quickly metabolize formaldehyde could be responsible for this resistance of astrocytes, because its metabolites, methanol and formate, have been shown to be less toxic than formaldehyde (Lee et al., 2008; Oyama et al., 2002; Tulpule and Dringen, 2011). Indeed, astrocytes efficiently and almost quantitatively oxidized formaldehyde to formate in a time- and concentration-dependent manner, while reduction of formaldehyde to methanol was not observed for cultured astrocytes (data not shown). Formaldehyde oxidation was completely prevented by lowering the incubation temperature to 4°C, demonstrating that temperature-sensitive processes such as uptake of formaldehyde, cellular oxidation, and/or formate export are involved in formaldehyde metabolism by astrocytes. The initial small drop in the medium formaldehyde level after exposure of cells with formaldehyde is likely to reflect at least in part the chemical reaction of formaldehyde with cellular macromolecules (Bolt, 1987; Gubisne-Haberle et al., 2004; Heck and Casanova, 1999; O'Connor and Fox, 1989; Saito et al., 2005), because this disappearance of formaldehyde was not entirely matched by formate generation and was observed even for the 4°C condition, which completely prevented formate formation.

As an uncharged small molecule, formaldehyde is considered to efficiently penetrate cell membranes (Gurel et al., 2005; Tulpule and Dringen, 2011). Because cultured astrocytes express at least the mRNAs for ADH3 and ALDH2, which confirms previous reports (Iborra

et al., 1992; Yang et al., 2005), these two enzymes are likely to be involved in formaldehyde oxidation to formate, as also described for other cells and tissues (Harris et al., 2003; Koivusalo et al., 1989; Macallister et al., 2011; Teng et al., 2001). The formaldehyde oxidation rate of intact astrocytes after exposure to 1 mM formaldehyde was found to be about $0.2 \mu\text{mol}/(\text{h} \times \text{mg})$. This value is similar to the reported specific formaldehyde-oxidizing capacity of brain homogenate ($0.16 \mu\text{mol}/(\text{h} \times \text{mg})$; Iborra et al., 1992), but is lower than the respective value reported for rat liver cells (about $1.1 \mu\text{mol}/(\text{h} \times \text{mg})$; Dicker and Cederbaum, 1984). Most of the formate generated by the oxidation of formaldehyde in astrocytes is exported from the cells, as demonstrated by the low amounts of cellular formate detected and by almost identical rates of formaldehyde disposal and extracellular formate accumulation. Potential transporters that could be involved in formate export from astrocytes are gamma-amino butyric acid receptors, which have been shown to be permeable to formate (Kaila, 1994; Mason et al., 1990) and are expressed in astrocytes (Lee et al., 2011; Velez-Fort et al., 2011). In addition, the membrane protein pendrin that has been described as a formate transporter (Soleimani et al., 2001) could contribute to formate export from astrocytes, because this transporter is expressed in the brain (Ramachandran et al., 2006). In contrast to formate export, the oxidation of formate to carbon dioxide, which is considered as the predominant end product of methanol oxidation in the rat liver after methanol poisoning (Skrzydowska, 2003; Tephly, 1991), does not appear to contribute substantially to the formaldehyde metabolism in cultured rat astrocytes.

The hyperbolic concentration dependence of formaldehyde disposal and formate release suggest that a saturable process is involved in formaldehyde oxidation in astrocytes. The calculated V_{max} value for this process in astrocytes ($0.4 \mu\text{mol}/(\text{h} \times \text{mg})$) was similar to such V_{max} values reported for rat hepatocytes ($0.25 \mu\text{mol}/(\text{h} \times \text{mg})$) and human bronchial epithelial cells ($0.36 \mu\text{mol}/(\text{h} \times \text{mg})$) (Ovrebø et al., 2002). In astrocytes, both the cytosolic ADH3 and mitochondrial ALDH2 are likely to contribute to the observed formate generation. Prerequisite for ADH3-mediated formaldehyde oxidation is the formation of *S*-hydroxymethyl-GSH by the rapid reaction of formaldehyde with cellular GSH, which is present in astrocytes in a cytosolic concentration of 8 mM (Dringen and Hamprecht, 1998). ADH3 has a K_M value below 10 μM for *S*-hydroxymethyl-GSH (Casanova-Schmitz et al., 1984; Ovrebø et al., 2002; Uotila and Koivusalo, 1974) and will therefore catalyze its reaction efficiently, even if substantial amounts of GSH have been exported by the formaldehyde-mediated accelerated GSH export (Tulpule and Dringen, 2011). To which extent the formaldehyde that has penetrated the cell membrane will escape under the conditions used the high affinity cytosolic ADH3-dependent oxidation and will reach the mitochondrial ALDH2 remains to be elucidated.

Cultured astrocytes are known to be rather glycolytic and to possess basal rates of lactate release that are

almost twice that of their glucose consumption rates (Fonseca et al., 2005; Scheiber and Dringen, 2011; Schmidt and Dringen, 2009). The reason for this extensive glycolytic metabolism is most likely the strong inhibition of the pyruvate dehydrogenase complex via phosphorylation of its α -subunit (Halim et al., 2010). Treatment of astrocytes with formaldehyde, but not with methanol or acetaldehyde (data not shown), accelerated the rates of both glucose consumption and extracellular lactate accumulation without altering the ratio of these rates. Because formate is a known complex IV inhibitor (Nicholls, 1975; Petersen, 1977), the formate generated by cellular oxidation of formaldehyde is likely to serve as an endogenously generated inhibitor of complex IV. Thus, formaldehyde-derived formate should also accelerate glycolytic flux, as previously described for other respiratory chain inhibitors (Pauwels et al., 1985; Scheiber and Dringen, 2011; Walz and Mukerji, 1988). This view is supported by the observation that the formaldehyde-induced stimulation of glycolytic flux was not additive to the enhancement observed for the known complex IV inhibitor azide (Zamboni et al., 2010; Ziabreva et al., 2010). Application of extracellular formate did not accelerate glycolytic flux, whereas formaldehyde-derived formate appears to efficiently inhibit astrocytic respiration. Likely reason for this discrepancy is a low permeation or a low rate of uptake of the charged formate through the cell membrane compared with the rapid penetration of the uncharged formaldehyde into the cells. Indeed, only low levels of cellular formate were determined for cells treated with 1 mM of exogenous formate. The specific cellular formate content for formate-treated cells was lower than that found for cells exposed to 0.5 mM formaldehyde, which did not show accelerated glycolytic flux. Thus, inefficient accumulation of exogenous formate by astrocytes is likely to prevent the inhibition of respiration and the acceleration of glycolysis.

Further evidence in favor of formate-mediated, and against a direct formaldehyde-mediated, stimulation of glycolysis is provided by the delay observed for the formaldehyde-induced stimulation of glycolytic flux. At least 1 h of incubation with formaldehyde was required before the increase in extracellular lactate concentration became significant. In addition, also the observation that formaldehyde-induced stimulation of glycolytic flux became persistent after incubation with formaldehyde for at least 1 h supports the hypothesis that formaldehyde itself does not affect glycolytic flux. Both observations are consistent with the intracellular generation of a formaldehyde metabolite such as formate that subsequently acts as inhibitor of complex IV. Because of the efficient export of formate from astrocytes, the cellular accumulation of sufficiently high amounts of formate to inhibit complex IV is likely to take some time and to cause a delay in the onset of the stimulation of glycolytic flux in astrocytes after application of formaldehyde. This delay contrasts strongly with the rapid consequences of a formaldehyde treatment on the GSH metabolism of astrocytes. Formaldehyde stimulates already within minutes the multidrug resistance protein 1 (Mrp1)-medi-

ated GSH export from astrocytes and the removal of formaldehyde immediately terminates this effect (Tulpule and Dringen, 2011).

Formate inactivates complex IV by forming a complex with the heme iron of the cytochrome a_3 (Nicholls, 1975). Whether in viable cells formate enters complex IV from the intermembrane and/or the matrix site of mitochondria to inactivate the enzyme remains to be elucidated. However, because cultured astrocytes express both cytosolic ADH3 and mitochondrial ALDH2, formate is likely to be generated in these cells by formaldehyde oxidation in both cytosol and mitochondria. In addition, at least for isolated rat liver mitochondria formate has been shown to permeate mitochondria (Cybulski and Fisher, 1977; Tibbetts and Appling, 2010). Thus, formate generated in the cytosol is likely to enter astrocytic mitochondria, whereas formate produced in mitochondria is likely to be released into the cytosol and subsequently exported by the cells. Further studies are required to elucidate the contributions of cytosolic and mitochondrial formaldehyde oxidation and the potential of the formate generated in these compartments to inhibit respiration and/or to be released from the cells.

In brain, astrocytes are likely to encounter formaldehyde that is generated within the brain (Diliberto and Axelrod, 1976; Obata, 2002; Zhang et al., 2010; Zuo and Yu, 1994). The presence of mRNAs of the formaldehyde-generating enzymes SSAO and LSD1 in astrocyte cultures suggests that even astrocytes themselves could generate formaldehyde. However, at least under the conditions used, cultured astrocytes do not appear to generate substantial amounts of endogenous formaldehyde or formate. Thus, a more likely reason for the surprisingly high capacity of astrocytes to oxidize formaldehyde is that astrocytes are prepared to encounter formaldehyde produced by other cells. Because astrocytes cover brain capillaries with their endfeet (Mathiisen et al., 2010), astrocytes will be exposed to formaldehyde generated by cells of the cerebrovascular tissue, which contains substantial activity of SSAO (Castillo et al., 1998) and to formaldehyde coming from the periphery. Under normal conditions, the formaldehyde concentration in blood is around 0.1 mM (Heck and Casanova, 2004; Heck et al., 1985). Because of its uncharged nature, formaldehyde can easily penetrate the blood-brain barrier to attain an equilibrium between the concentration in blood and brain. Thus, even under physiological conditions, astrocytes are likely to continuously generate some formate because of their potential to oxidize blood-derived formaldehyde. However, after exposure to environmental formaldehyde and in the case of elevated formaldehyde levels in brain under pathological conditions such as AD (Tong et al., 2011), astrocytic formate generation may substantially increase. This could contribute to the reported lowered mitochondrial complex IV activity in brain (Castellani et al., 2002; Mancuso et al., 2003; Sullivan and Brown, 2005) and to an increased lactate concentration observed for the cerebrospinal fluid of AD patients (Mancuso et al., 2003; Parnetti et al., 2000).

In conclusion, the efficient formate generation by formaldehyde oxidation strongly affects astrocytic glucose metabolism as indicated by the observed increase in glycolytic flux. *In vivo*, the export of formaldehyde-derived formate as well as the formate-induced accelerated lactate export from astrocytes could have severe consequences for the neighboring cells. Enhanced export of these acids is likely to induce a metabolic acidosis (Rachoin et al., 2010; Skrzydlewska, 2003). Although accelerated lactate release from astrocytes could be considered to improve the supply of the energy substrate lactate to neurons (Barros and Deitmer, 2010; Pellerin et al., 2007), presence of an excess of extracellular formate can harm neurons, as demonstrated by the formate-induced neuronal death reported for brain slices (Kapur et al., 2007). Thus, both the consequences of formaldehyde oxidation on the glucose metabolism in brain cells as well as formate-induced neurotoxicity may be involved in the development of cognitive defects associated with formaldehyde exposure (Lu et al., 2008; Malek et al., 2003; Tong et al., 2011).

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3.2.

Publication 3

Formaldehyde stimulates Mrp1-mediated glutathione deprivation of cultured astrocytes

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- Contribution of Ketki Tulpule:
 - Experimental work
 - Preparation of first draft of the manuscript

Formaldehyde stimulates Mrp1-mediated glutathione deprivation of cultured astrocytes

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Abstract

Formaldehyde (Fal) is an environmental neurotoxin that is also endogenously produced in brain. Since the tripeptide glutathione (GSH) plays an important role in detoxification processes in brain cells, we have investigated the consequences of a Fal exposure on the GSH metabolism of brain cells, using astrocyte-rich primary cultures as model system. Treatment of these cultures with Fal resulted in a rapid time- and concentration-dependent depletion of cellular GSH and a matching increase in the extracellular GSH content. Exposure of astrocytes to 1 mM Fal for 3 h did not compromise cell viability but almost completely deprived the cells of GSH. Half-maximal

deprivation of cellular GSH was observed after application of 0.3 mM Fal. This effect was rather specific for Fal, since methanol, formate or acetaldehyde did not affect cellular GSH levels. The Fal-stimulated GSH loss from viable astrocytes was completely prevented by semicarbazide-mediated chemical removal of Fal or by the application of MK571, an inhibitor of the multidrug resistance protein 1. These data demonstrate that Fal deprives astrocytes of cellular GSH by a multidrug resistance protein 1-mediated process.

Keywords: astrocytes, detoxification, formaldehyde, glutathione, multidrug resistance protein 1, transport.

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Formaldehyde (Fal) is an environmental pollutant (Flyvholm and Andersen 1993; Bono *et al.* 2010; Sofuoglu *et al.* 2010) that has been classified as a potential class 3 carcinogenic agent in humans (IARC 2006). However, Fal is also endogenously produced by semicarbazide-sensitive amine oxidase (SSAO)-mediated deamination of methylamine (Yu *et al.* 2003; O'Sullivan *et al.* 2004). A further endogenous source of Fal is the oxidation of methanol (MeOH) that is hydrolysed from carboxy methyl esters of proteins which have been formed during post-translational modification by carboxymethyl transferases (Lee *et al.* 2008). In addition, the enzyme lysine-specific demethylase 1, a nuclear homologue of amine oxidase has been shown to generate Fal by demethylation of a lysine residue in histones (Shi *et al.* 2004; Wang *et al.* 2009). The presence of SSAO, lysine-specific demethylase 1 and carboxymethyl transferase in brain (Diliberto and Axelrod 1976; Zuo & Yu 1994; Obata 2002; Zhang *et al.* 2010) suggest that this organ continuously produces Fal. Interestingly, increased activity of SSAO has been demonstrated for pathological conditions like Alzheimer's disease (AD) (Ferrer *et al.* 2002; Unzeta *et al.* 2007), while protein carboxymethylation has been shown to increase with age (Sellinger *et al.* 1988). Accordingly,

elevated levels of Fal have been reported to be present in the hippocampus of AD brain and in the brains of mouse models of AD and dementia (Tong *et al.* 2011). Formaldehyde is a highly reactive compound that can interact with macromolecules like DNA and proteins (Bolt 1987; O'Connor and Fox 1989; Heck and Casanova 1999; Gubisne-Haberle *et al.* 2004; Saito *et al.* 2005). Such reactions of Fal in brain may contribute to the Fal-induced disturbances in learning and memory (Malek *et al.* 2003; Lu *et al.* 2008; Tong *et al.* 2011).

The tripeptide glutathione (GSH) (γ -L-glutamyl-L-cysteinyl-glycine) is the most abundant thiol present in mammalian

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Abbreviations used: Aal, acetaldehyde; AD, Alzheimer's disease; Fa, formate; Fal, formaldehyde; GSH, glutathione; GSSG, glutathione disulphide; GSx, total glutathione; IB, incubation buffer; LDH, lactate dehydrogenase; MeOH, methanol; Mrp1, multidrug resistance protein 1; PI, propidium iodide; SSAO, semicarbazide-sensitive amine oxidase.

cells with intracellular concentrations in the millimolar range (Cooper and Kristal 1997). GSH has essential functions in the detoxification of reactive oxygen species and of xenobiotics (Hirrlinger and Dringen 2010; Schmidt and Dringen in press). In the brain, astrocytes play an important role in GSH metabolism and homeostasis (Aoyama *et al.* 2008; Dringen 2009; Schmidt and Dringen in press). These cells supply neurons with precursors of GSH synthesis in a process that involves release of GSH from astrocytes via multidrug resistance protein 1 (Mrp1) and subsequent processing of the extracellular GSH to amino acids which serve as substrates for neuronal GSH synthesis (Dringen *et al.* 1999; Minich *et al.* 2006; Hirrlinger and Dringen 2010). Thus, an alteration in GSH homeostasis in astrocytes is likely to also have severe consequences for neuronal GSH synthesis.

Glutathione is involved in the cellular detoxification of Fal. The first step of this process is the enzyme-independent conjugation of Fal with GSH to form *S*-hydroxymethyl-GSH (Koivusalo *et al.* 1989; Harris *et al.* 2003). This conjugate is oxidised by Fal dehydrogenase to *S*-formyl-GSH which is subsequently hydrolysed by *S*-formyl-GSH hydrolase to formate (Fa), thereby regenerating the substrate GSH (Koivusalo *et al.* 1989; Harris *et al.* 2003). Of these enzymes, at least Fal dehydrogenase has been demonstrated to be present in astrocytes (Iborra *et al.* 1992), suggesting that astrocytes have the potential to metabolise Fal.

Considering the neurotoxic potential of Fal (Songur *et al.* 2010) and the known interconnection of Fal and GSH metabolism (Koivusalo *et al.* 1989; Harris *et al.* 2003), it is surprising that consequences of Fal on the GSH metabolism of brain cells have not been reported so far. Here, we demonstrate that Fal strongly affects the GSH homeostasis of cultured astrocytes by stimulating a Mrp1-mediated process which rapidly deprives astrocytes of their GSH.

Materials and methods

Materials

Acetaldehyde (Aal), ammonium Fa, MeOH and trizma base were purchased from Sigma-Aldrich (Steinheim, Germany). Formaldehyde was from Merck (Darmstadt, Germany). Foetal calf serum and penicillin/streptomycin solution were obtained from Biochrom (Berlin, Germany) while Dulbecco's modified Eagle's medium was purchased from Gibco (Karlsruhe, Germany). Bovine serum albumin, NADH and NADPH were purchased from Applichem (Darmstadt, Germany). All other chemicals of the highest purity available were from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Riedel-deHaen (Seelze, Germany) or Roth (Karlsruhe, Germany); 96-well microtitre plates and 24-well plates were from Nunc (Wiesbaden, Germany).

Cell cultures

Astrocyte-rich primary cultures were prepared from the brains of newborn Wistar rats (Hamprecht and Löffler 1985). The cells were seeded in culture medium (90% Dulbecco's modified Eagle's

medium, 10% foetal calf serum, 1 mM pyruvate, 20 U/mL penicillin G and 20 µg/mL streptomycin sulphate) in wells of 24-well plates (300 000 cells in 1 mL) and incubated in the humidified atmosphere of a Sanyo (Osaka, Japan) incubator with 10% CO₂. The cultures were maintained by renewing the culture medium every seventh day. The cultures were used for experiments at an age between 15 and 21 days.

Experimental incubation of cells

Cells were washed twice with 1 mL pre-warmed (37°C) incubation buffer (IB; 145 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 0.8 mM Na₂HPO₄, 20 mM HEPES, 5 mM D-glucose, pH 7.4) and incubated at 37°C with 0.2 mL IB containing Fal and/or the other compounds investigated in the concentrations indicated in the figures and the table. For experiments that tested for the time-dependent effects of semicarbazide and MK571, 10 µL of concentrated stock solutions of these compounds were applied to obtain a final concentration of 10 mM (semicarbazide) or 50 µM (MK571) 30 or 60 min after application of Fal. After incubation of cells for the indicated time, the medium was collected and the cells were washed twice with 1 mL ice-cold phosphate-buffered saline (10 mM potassium phosphate buffer, pH 7.4, containing 150 mM NaCl). For analysis of the total glutathione content [GSx = amount of GSH plus twice the amount of glutathione disulphide (GSSG)], the cells were lysed with 0.5 mL 1% (w/v) sulfosalicylic acid and 10 µL of the lysate was used for quantification. Ten microlitre of the medium was mixed with 10 µL 1% sulfosalicylic acid before quantification of the medium GSx content.

Determination of GSx and GSSG

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 microtitre plates according to the colorimetric Tietze method (Tietze 1969). Under all conditions used here, the amounts of GSSG were in the range of the detection limit of the assay used.

Determination of cell viability

Cell viability was assessed by determining the activity of lactate dehydrogenase (LDH) in the media using the microtitre plate assay described previously (Dringen *et al.* 1998) with the modification that 20 µL volumes of lysates and media were used. None of the compounds investigated affected the determination of LDH activity in media or cell lysates of cultured astrocytes (data not shown). Propidium iodide (PI) staining was used to test for membrane integrity of astrocytes as described previously (Scheiber *et al.* 2010). Briefly, following the experimental incubation, the cells were washed twice with 1 mL of pre-warmed (37°C) IB and then incubated for 15 min at 37°C in 0.5 mL IB containing 5 µM PI and 10 µM of the membrane permeable dye Hoechst 33342 (Fluka, Buchs, Switzerland) (to visualise all nuclei). Cells were then washed twice with 1 mL of phosphate-buffered saline and analysed for fluorescence using a Nikon (Düsseldorf, Germany) Eclipse TS2000U microscope.

Determination of protein

The protein content was determined after lysis of the cells in 200 µL 0.5 M NaOH according to the Lowry method (Lowry *et al.* 1951), using bovine serum albumin as a standard.

Cell-independent reaction of GSH with Fal

Glutathione (15 μM) or GSSG (7.5 μM) was incubated in IB with the indicated concentrations of Fal in a total volume of 1 mL at 37°C for 3 h. Thereafter, a 10 μL sample was mixed with 10 μL 1% (w/v) sulfosalicylic acid and the GSx content was measured.

Presentation of data

Data are presented as means \pm SD of values that were obtained in three independent experiments performed on independently prepared cultures, if not stated otherwise. The analysis of significance between groups of data was performed by ANOVA followed by Bonferroni *post hoc* tests with $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. $p > 0.05$ was considered as not significant.

Results

Effects of Fal on the cell viability of cultured astrocytes

To investigate the consequences of a treatment of astrocytes with Fal, astrocyte-rich primary cultures were incubated with Fal in various concentrations (Fig. 1). Formaldehyde in concentrations of up to 1 mM did not compromise cell viability within 3 h of incubation as indicated by the lack of any increase in extracellular LDH activity compared with control (Fig. 1a and e) and by the absence of PI-positive cells (Fig. 2a, b, e and f). In contrast, exposure of cells to higher concentrations of Fal damaged the cells. Exposure of astrocytes for 3 h to 2 or 3 mM Fal resulted in a significant elevation in the extracellular LDH activity (Fig. 1e) and in the appearance of PI-positive cells (Fig. 2c, d, g and h).

Effects of Fal on the GSH and GSSG contents of cultured astrocytes

Incubation of astrocytes with Fal resulted in a rapid decline in the cellular GSx content which depended on the concentration of Fal applied and on the duration of exposure (Fig. 1b and f). The disappearance of cellular GSx was rapid in the first hour of treatment with Fal and slowed down during longer incubation (Fig. 1b). Exposure to 1 mM Fal almost completely deprived the cells of GSx within 3 h (Fig. 1b). Half-maximal deprivation of cellular GSx was observed after exposure of the cells to about 0.3 mM Fal for 3 h (Fig. 1f). The decrease in cellular GSx content after exposure to Fal (Fig. 1b and f) was accompanied by a corresponding increase in the extracellular GSx content (Fig. 1c and g). As a consequence, the sum of cellular plus extracellular GSx remained almost constant, at least for Fal concentrations of up to 1 mM (Fig. 1d and h). Maximal extracellular GSx contents were observed for cells that had been exposed to 1 mM Fal, while less GSx was detectable in media of cells that had been exposed to 2 or 3 mM Fal (Fig. 1g). Compared to controls (absence of Fal) the sum of extra- and cellular GSx contents was slightly lower after exposure of cells to 1 mM Fal (Fig. 1d and h), while treatment with 2 or 3 mM

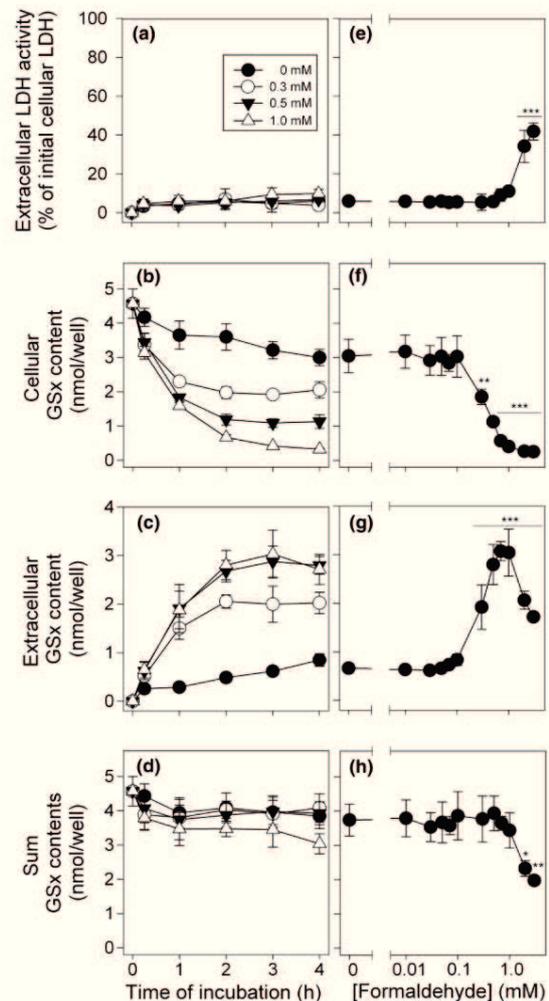


Fig. 1 Effects of formaldehyde on the viability and the glutathione content of cultured astrocytes. The cells were incubated without or with 0.3, 0.5 or 1 mM formaldehyde for up to 4 h (a–d) or with the indicated concentrations of formaldehyde for 3 h (e–h). (a, e) Extracellular LDH activity; (b, f) cellular GSx content; (c, g) extracellular GSx content; (d, h) sum of cellular plus extracellular GSx content. The results show means \pm SD of data obtained on three independently prepared cultures that contained $91 \pm 11 \mu\text{g}$ (a–d) and $95 \pm 11 \mu\text{g}$ (e–h) protein per well. In panels (e–h), the significance of differences to the data obtained for the control condition (absence of formaldehyde) is indicated by $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$.

Fal significantly lowered the sum of GSx contents by about 50% (Fig. 1h).

Analysis of the oxidation state of GSx in cells and media for control cells and Fal-treated astrocytes revealed that GSSG accounted only for minute amounts of GSx that were in the range of the detection limit of the assay used (Table 1).

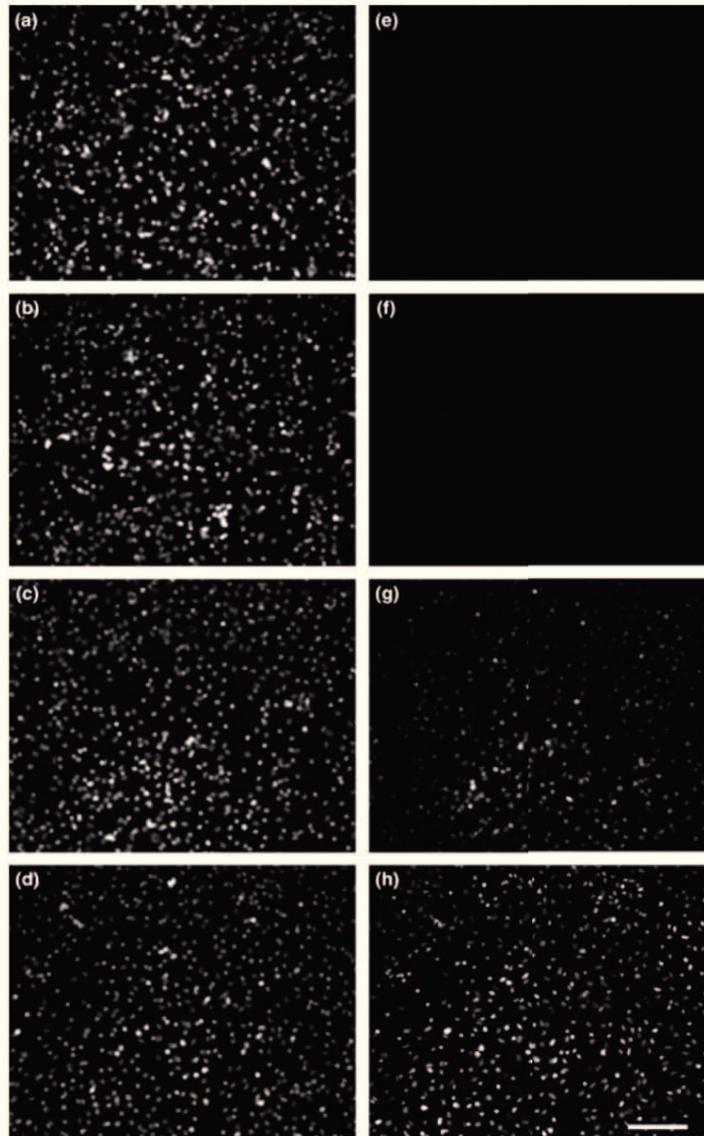


Fig. 2 Effect of formaldehyde on the membrane integrity of cultured astrocytes. The cells were incubated without (a, e) or with formaldehyde in concentrations of 1 mM (b, f), 2 mM (c, g) and 3 mM (d, h) for 3 h. The cells were then co-incubated with Hoechst 33342 and propidium iodide (PI) to visualise all cell nuclei (a–d) and to identify by PI-staining (e–h) cells with impaired membrane integrity. Shown are data from a representative experiment that was performed on a 16-day-old culture. The scale bar in panel (h) represents 100 μm and applies to all panels.

Cell-independent reaction of Fal with GSH

Formaldehyde in concentrations above 1 mM caused significant disappearance of GSx from astrocyte cultures, which may be a consequence of the reported formation of Fal adducts with GSH (Naylor *et al.* 1988; Hopkinson *et al.* 2010). To investigate the extent to which such reactions occur under the incubation conditions used, Fal in different concentrations was incubated with GSH or GSSG in the absence of cells (Fig. 3). While no loss in the amount of detectable GSSG was observed after incubation with Fal in concentrations of up to 3 mM for 3 h, the presence of Fal in

concentrations above 0.3 mM caused a significant ($p < 0.01$) concentration-dependent decline in the detectable GSH. Presence of 3 mM Fal lowered the amount of detectable GSH by about 40% (Fig. 3).

Consequences of a treatment of cultured astrocytes with Fal, its metabolites MeOH and Fa, or Aal

To investigate the specificity of the Fal-induced effects on the GSx content of astrocytes, cells were incubated with Fal, its metabolites MeOH and Fa, or with ethanol or Aal in concentrations of 1 or 10 mM for 3 h (Fig. 4). With the

Table 1 Effects of formaldehyde and MK571 on the GSx and GSSG contents as well as on the viability of astrocyte cultures

	Control	Formaldehyde	MK571	Formaldehyde plus MK571
Cellular GSx (nmol/well)	3.01 ± 0.44	0.32 ± 0.08***	3.69 ± 0.83	2.88 ± 0.34
Extracellular GSx (nmol/well)	0.75 ± 0.25	2.67 ± 0.60***	0.30 ± 0.14	0.66 ± 0.24
Sum GSx (nmol/well)	3.76 ± 0.62	2.98 ± 0.65	3.99 ± 0.90	3.54 ± 0.57
Cellular GSSG (nmol/well)	0.17 ± 0.06	0.16 ± 0.07	0.22 ± 0.05	0.18 ± 0.07
Extracellular GSSG (nmol/well)	0.12 ± 0.03	0.27 ± 0.05**	0.07 ± 0.01	0.10 ± 0.03
Extracellular LDH activity (% of initial cellular LDH)	5 ± 2	12 ± 2	9 ± 5	15 ± 5

The cells were incubated without or with 1 mM formaldehyde in the presence or absence of 50 μ M MK571 for 3 h. The results represent means \pm SD of data obtained on three independently prepared cultures that contained 109 \pm 14 μ g protein per well. The significance of differences to the data obtained for the control condition (absence of both formaldehyde and MK571) is indicated by ** p < 0.01 or *** p < 0.001.

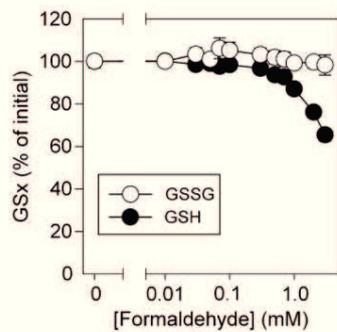


Fig. 3 Cell-independent disappearance of glutathione (GSH) after exposure to formaldehyde. GSH or glutathione disulphide (GSSG) was incubated with the indicated concentrations of formaldehyde for 3 h and the GSx content of the solution was measured. The 100% values represent 15 μ M GSH and 7.5 μ M GSSG. The results indicate means \pm SD of data obtained on three independent experiments.

exception of 10 mM Fal, none of these conditions resulted in a loss of cell viability as indicated by the absence of any significant increase in the extracellular LDH activity compared with controls (Fig. 4a). Of the different compounds investigated, exclusively Fal lowered the cellular GSx content in both concentrations applied (Fig. 4b), while the significant increase in the extracellular GSx content was only detectable for cells that were treated with 1 mM Fal (Fig. 4c). Presence of 10 mM Fal, but not of 1 mM Fal caused a significant decline in the sum of cellular plus extracellular GSx (Fig. 4d).

Effects of a removal of Fal on the stimulated GSH deprivation of astrocytes

To investigate whether Fal has to be present in the medium to maintain the rapid export of GSH from astrocytes, cells were incubated without and with 1 mM Fal. Semicarbazide (10 mM final concentration) was added after 30 or 60 min incubation to remove the Fal from the medium (Fig. 5) by

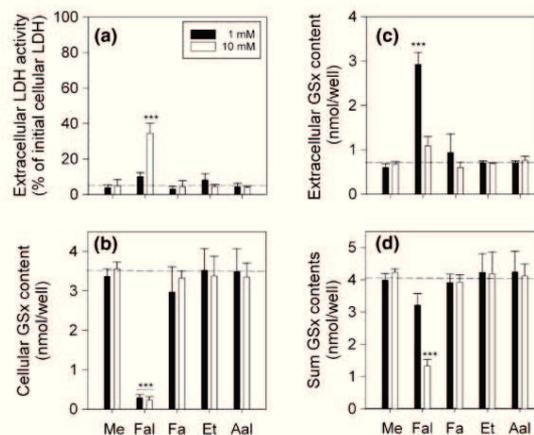


Fig. 4 Consequences of a treatment of cultured astrocytes with formaldehyde (Fal), methanol (Me), formate (FA), ethanol (Et) or acetaldehyde (Aal) on the viability (a), the cellular GSx content (b), the extracellular GSx content (c) and the sum of cellular plus extracellular GSx content (d). The cells were incubated with the compounds in the indicated concentrations for 3 h. The dashed lines represent mean values of the control condition (absence of any of the indicated compounds) for the different parameters investigated. The results represent means \pm SD of data obtained on three independently prepared cultures that contained 91 \pm 12 μ g protein per well. The significance of differences to the values of controls is indicated by *** p < 0.001.

the chemical reaction to its semicarbazone (Savenije-Chapel and Noordhoek 1980). Application of semicarbazide did not compromise the viability of astrocytes that were treated in the absence (Figure S1) or the presence of Fal (Fig. 5a). Also, the cellular or extracellular contents of GSx or the sum of cellular plus extracellular GSx were unaffected in the absence of Fal (data not shown). In contrast, the Fal-induced loss in cellular GSx (Fig. 5b, filled circles) and the accelerated accumulation of extracellular GSx (Fig. 5c, filled circles) were completely prevented after addition of semicarbazide (Fig 5b and c, triangles and squares), while the sum

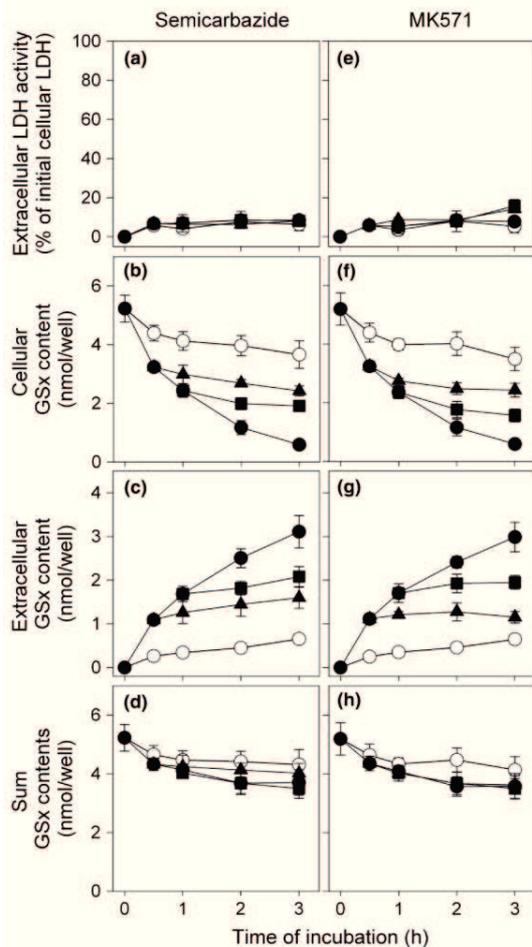


Fig. 5 Effects of semicarbazide and MK571 on the formaldehyde-induced alteration in the glutathione content of cultured astrocytes. The cells were incubated without (open circles) or with (filled symbols) 1 mM formaldehyde for up to 3 h. Semicarbazide (a–d) or MK571 (e–h) was added to a final concentration of 10 mM and 50 μ M, respectively, after 30 min (triangles) or 60 min (squares) of incubation. (a, e) Extracellular LDH activity; (b, f) cellular GSx content; (c, g) extracellular GSx content; (d, h) sum of cellular plus extracellular GSx content. The results represent means \pm SD of data obtained on four (a–d) or three (e–h) independently prepared cultures that contained 118 ± 2 (a–d) or 118 ± 3 (e–h) μ g protein per well.

of cellular plus extracellular GSx remained almost constant (Fig. 5d).

Effect of Mrp1 inhibition on the Fal-induced loss of GSH from astrocytes

Mrp1 has been reported to mediate the majority of GSH export from cultured astrocytes (Hirrlinger *et al.* 2002; Minich *et al.* 2006). To investigate whether Mrp1 is involved

in the rapid loss of cellular GSH and in the accumulation of extracellular GSx in Fal-treated astrocyte cultures, the cells were incubated without or with 1 mM Fal in the presence of 50 μ M of the Mrp1 inhibitor MK571 (Hirrlinger *et al.* 2002) for up to 3 h. Presence of MK571 did not compromise the viability of astrocytes under the conditions used (Table 1, Fig. 5e). However, MK571 completely prevented the Fal-induced loss in cellular GSx and the accelerated accumulation of extracellular GSx when it was applied to the cells together with Fal (Table 1) or 30 or 60 min after Fal application (Fig. 5f and g, triangles and squares), while the sum of cellular plus extracellular GSx and the ratio of GSSG to GSx were not significantly altered by the presence of MK571 (Table 1, Fig. 5h).

Discussion

Astrocyte-rich primary cultures were used to investigate the consequences of a treatment with Fal on the viability and the GSH metabolism of astrocytes. Formaldehyde in concentrations of up to 1 mM did not compromise cell viability or membrane integrity, while higher concentrations damaged astrocytes, as also reported for PC12 cells (Lee *et al.* 2008). However, treatment of astrocytes with 1 mM Fal during longer exposure than 4 h resulted in toxicity (data not shown), which is in accordance with a recent report (Song *et al.* 2010). The well-known reactivity of Fal with macromolecules like DNA and proteins (Bolt 1987; O'Connor and Fox 1989; Heck and Casanova 1999; Gubisne-Haberle *et al.* 2004; Saito *et al.* 2005) is likely to be responsible for the observed cell death of astrocytes that had been exposed to high concentrations of Fal. Since Fal reacts with thiols such as GSH (Naylor *et al.* 1988), also a reaction of Fal with reactive thiol groups in enzymes has to be considered. Such reactions may inactivate enzymes, thereby contributing to a compromised metabolism and a delayed cell death, as it has been shown for the inhibition of glyceraldehyde-3-phosphate dehydrogenase by thiol reagents such as iodoacetate, iodoacetamide or fumaric acid diesters (Schmidt and Dringen 2009, 2010). In contrast to Fal, MeOH, Fa, ethanol and Aal failed to compromise cell viability, even if applied in concentrations of 10 mM for up to 3 h. This is consistent with literature data which demonstrate that Fal is more toxic to cells than its metabolites (Oyama *et al.* 2002; Lee *et al.* 2008).

Formaldehyde caused a time- and concentration-dependent decrease in the cellular GSx content of cultured astrocytes that was accompanied by a corresponding increase in extracellular GSx. Since the sum of cellular plus extracellular GSx was hardly affected by Fal in concentrations of up to 1 mM, this compound appears to predominantly alter the distribution of GSx between the cellular and extracellular compartment of the cultures rather than to react with GSH under such conditions. Disappearance of low amounts of detectable GSH from viable cells after applica-

tion of 1 mM Fal could reflect the formation of *S*-hydroxymethyl-GSH and/or the potential of the cells to synthesise formyl-GSH by oxidation of *S*-hydroxymethyl-GSH. However, large amounts of formyl-GSH are unlikely to accumulate in cells, since this compound is hydrolysed to liberate GSH (Koivusalo *et al.* 1989). The detectability of GSH in medium was only lowered after application of Fal in higher concentrations, which could be a consequence of a reaction of GSH with Fal to a conjugate that is not detectable by the GSx assay used. Indeed, a variety of such reaction products have been identified (Naylor *et al.* 1988; Hopkinson *et al.* 2010). Under the conditions used, the reaction between millimolar concentrations of Fal with GSH is likely to involve the thiol group of GSH, since the detectability of GSSG was not lowered in the presence of Fal.

Formaldehyde in concentrations of up to 1 mM did not induce LDH release or PI membrane permeability. Thus, a leakage of GSH through damaged cell membranes can be excluded as reason for the Fal-induced extracellular GSx accumulation. The loss of cellular GSx from astrocytes after Fal treatment appears to be rather mediated by stimulation of an export process. This stimulation seems to be specific for Fal, since neither Aal nor MeOH and Fa, the products of cellular Fal metabolism by catalase, alcohol dehydrogenases or aldehyde dehydrogenases (Liesivuori and Savolainen 1991; Teng *et al.* 2001), altered the distribution of GSx between cellular and extracellular compartment.

In other experimental paradigms, Fal exposure has been demonstrated to lead to oxidative stress and impairment of antioxidative mechanisms (Gurel *et al.* 2005; Zararsiz *et al.* 2007; Songur *et al.* 2008, 2010). However, since no alteration in the cellular ratio of GSSG to GSH was observed after exposure to Fal, astrocytes do under the conditions used not suffer from a substantial oxidative stress, contrasting the situation described for astrocytes that were exposed to peroxide stress (Hirrlinger *et al.* 2001; Minich *et al.* 2006; Liddell *et al.* 2009). Under such stress conditions, astrocytes release substantial amounts of GSSG by Mrp1 (Hirrlinger *et al.* 2001; Minich *et al.* 2006). However, since GSSG accounted for only minute parts of the GSx contents of cells and media, a Fal-induced oxidative stress that would cause GSH oxidation and subsequent GSSG export from astrocytes can be excluded as reason for the observed loss of cellular GSx.

The data obtained suggest that the cellular loss of GSx is caused by a transporter-mediated export of GSH. A similar Fal-stimulated GSH efflux has been reported for cultured rat hepatocytes (Ku and Billings 1984) and for perfused rat liver (Krieter *et al.* 1985), although the transporters involved in these processes have not been identified in these studies. For both rat and mouse astrocyte cultures, GSH is predominantly exported by Mrp1 (Hirrlinger *et al.* 2002; Minich *et al.* 2006). Application of the specific Mrp1 inhibitor MK571 (Minich *et al.* 2006), completely prevented the Fal-stimu-

lated loss of GSH, demonstrating clearly the involvement of a Mrp1-dependent mechanism. To investigate whether Fal stimulates in addition to the cellular GSH loss also other Mrp1-mediated transport processes, the export of the Mrp1-substrate GSSG was investigated. In astrocytes, GSSG export is exclusively mediated by Mrp1 (Hirrlinger *et al.* 2001; Minich *et al.* 2006). However, since Fal did not accelerate astrocytic GSSG export during oxidative stress (data not shown), a general stimulation by Fal of Mrp1-mediated export processes can be excluded.

Formaldehyde is well known to covalently modify proteins (Metz *et al.* 2004, 2006; Toews *et al.* 2008). Such a modification of Mrp1 could be the molecular mechanism underlying a Fal-stimulated GSH export by Mrp1 in astrocytes. However, the observation that the Fal-induced stimulation of GSH release required continuous presence of Fal and was immediately prevented after removal of Fal by either washing the cells (data not shown) or by inactivation of Fal by semicarbazide (Saito *et al.* 2005), suggests that such a modification of Mrp1 by Fal would be rather labile and quickly reversible. Alternatively, Fal itself or its GSH conjugate could act as a stimulator of Mrp1-mediated GSH efflux, as it has previously been described for other compounds (Rappa *et al.* 1997; Loe *et al.* 2000; Hirrlinger *et al.* 2002; Kruh and Belinsky 2003).

The data obtained here on Fal-stimulated Mrp1-mediated GSH deprivation of astrocytes would also be consistent with an alternative scenario. Cellular loss of GSH and extracellular accumulation of GSH could also be a consequence of a Mrp1-mediated export of *S*-hydroxymethyl-GSH. The formation, stability and disintegration of *S*-hydroxymethyl-GSH depends strongly on the concentrations of Fal and GSH, since this hemimercaptal is in equilibrium with Fal and GSH (Ahmed and Anders 1978). In cultured astrocytes, the high cytosolic GSH concentration of 8 mM (Dringen and Hamprucht 1998) is likely to favour the formation of *S*-hydroxymethyl-GSH in the presence of Fal. Considering that GSH conjugates are good substrates for Mrp1-mediated export (Cole and Deeley 2006; Minich *et al.* 2006; Waak and Dringen 2006) also the *S*-hydroxymethyl-GSH formed in cells is likely to be efficiently exported by Mrp1. In the cell medium that contains at best micromolar concentration of GSH, break down of *S*-hydroxymethyl-GSH is likely to occur (Krieter *et al.* 1985). As consequence, the exported *S*-hydroxymethyl-GSH would be determined as extracellular GSH. This alternative scenario for the molecular process underlying the Fal-induced stimulation of astrocytic GSH loss would also be compatible with the observed effects of semicarbazide or MK571. Thus, further studies are required to discriminate whether Fal directly stimulates GSH export or whether presence of Fal generates a labile GSH conjugate that is efficiently exported by Mrp1.

Formaldehyde will, as small uncharged molecule, easily penetrate the blood–brain barrier. Thus, similar extracellular

concentrations of Fal can be expected for blood and brain. Endogenously produced Fal levels in human blood have been reported to be approximately 0.1 mM (Heck *et al.* 1985; Heck and Casanova 2004). Such a normal concentration of Fal in brain is unlikely to substantially affect the GSH homeostasis. At least for cultured astrocytes 0.1 mM Fal did not significantly stimulate GSH loss. In contrast, for conditions of accelerated endogenous Fal production or after excessive environmental Fal exposure, GSH export from astrocytes is likely to be stimulated in brain. The levels of Fal are almost doubled in the hippocampus of AD patients and in brains of senescence accelerated SAMP8 mice (Tong *et al.* 2011), suggesting that Fal-induced stimulation of Mrp1-mediated GSH deprivation could occur in astrocytes under these conditions. Consequence of such a process would be a disturbed GSH homeostasis in brain which is likely to contribute to oxidative stress. Indeed, the elevated Fal levels in the hippocampus of AD patients (Tong *et al.* 2011) correlate well with the elevated oxidative stress of this brain area compared with other parts of the AD brain (Hensley *et al.* 1995; Sultana *et al.* 2009). Thus, Fal-stimulated GSH loss from astrocytes could compromise astrocytic functions which in turn could lead to the occurrence of oxidative stress in pathological conditions such as AD.

In conclusion, Fal stimulates Mrp1-mediated GSH loss from viable cultured astrocytes and increases the extracellular GSH concentration. Further studies are now required to elucidate the *in vivo* relevance of the observed effects of Fal on the GSH metabolism of astrocytes. In addition to the consequences of a Fal-induced loss of GSH from astrocytes in brain, which could compromise detoxification functions of these cells, the appearance of elevated extracellular GSH levels could modulate brain functions. Extracellular GSH is substrate of astrocytic γ -glutamyl transpeptidase which in concert with neuronal aminopeptidase processes GSH to the extracellular amino acids which are required for neuronal GSH synthesis (Hirrlinger and Dringen 2010). Thus, Fal-stimulated increase in astrocyte-derived extracellular GSH could be beneficial by increasing the supply of GSH precursors to neurons. However, since extracellular GSH has also been considered as endogenous modulator of glutamate receptors and to act as neurohormone (Janáky *et al.* 2007), a Fal-induced increase in extracellular GSH concentration could alter synaptic neurotransmission. Such consequences of a Fal-stimulated alteration in GSH homeostasis in brain may contribute to the neurotoxicity (Songur *et al.* 2010) and to the cognitive deficiencies (Malek *et al.* 2003; Lu *et al.* 2008) that have been connected with Fal.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effects of semicarbazide on the glutathione content of cultured astrocytes.

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3.3.

Publication 4

Formaldehyde induces rapid glutathione export from viable oligodendroglial OLN-93 cells

Ketki Tulpule, Maike M. Schmidt, Karolin Boecker, Olaf Goldbaum, Christiane Richter-Landsberg & Ralf Dringen

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- Contribution of Ketki Tulpule:
 - Experimental work (except Fig. 1, 7B-E)
 - Preparation of first draft of the manuscript
- Maike M. Schmidt prepared Fig. 7B-E
- Karolin Boecker established incubation conditions for Fig. 2, 3, 6, 8
- Olaf Goldbaum prepared Fig. 1

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Formaldehyde induces rapid glutathione export from viable oligodendroglial OLN-93 cells

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ABSTRACT

Formaldehyde is a neurotoxic environmental pollutant that can also be produced in the body by certain enzymatic reactions. To test for the potential consequences of an exposure of oligodendrocytes to formaldehyde, we used OLN-93 cells as a model system. Treatment with formaldehyde altered the cellular glutathione (GSH) content of these cells by inducing a rapid time- and concentration-dependent export of GSH. Half-maximal effects were observed for a formaldehyde concentration of about 0.2 mM. While the basal GSH efflux from OLN-93 cells was negligible even when the cellular GSH content was doubled by pre-incubation of the cells with cadmium chloride, the formaldehyde-stimulated export increased almost proportionally to the cellular GSH content. In addition, the stimulated GSH export required the presence of formaldehyde and was almost completely abolished after removal of the aldehyde. Analysis of kinetic parameters of the formaldehyde-induced GSH export revealed similar K_m and V_{max} values of around 100 nmol/mg and 40 nmol/(h mg), respectively, for both OLN-93 cells and cultured astrocytes. The transporter responsible for the formaldehyde-induced GSH export from OLN-93 cells is most likely the multidrug resistance protein 1 (Mrp1), since this transporter is expressed in these cells and since the inhibitor MK571 completely prevented the formaldehyde-induced GSH export. The rapid export of GSH from formaldehyde-treated viable oligodendroglial cells is likely to compromise the cellular antioxidative and detoxification potential which may contribute to the known neurotoxicity of formaldehyde.

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1. Introduction

Formaldehyde is a common environmental pollutant (Logue et al., 2011; Sofuoglu et al., 2011) that has neurotoxic potential (Songur et al., 2010). However, formaldehyde is also generated endogenously as an oxidation product of methanol in liver (Macallister et al., 2011; Skrzydlewska, 2003) or as a product of lysine-specific demethylase 1 (LSD1) and semicarbazide-sensitive amine

oxidase (SSAO) in various organs including the brain (Hernandez-Guillamon et al., 2010; Obata, 2002; Zhang et al., 2010; Zibetti et al., 2010). Endogenously produced formaldehyde establishes a normal blood formaldehyde concentration of about 0.1 mM (Heck and Casanova, 2004; Heck et al., 1985). As a small uncharged molecule formaldehyde will easily penetrate from the blood into the brain. However, under certain conditions the local concentration of formaldehyde within some brain regions may even be higher than the blood concentration due to elevated endogenous formaldehyde generation. For example, the increased expression of SSAO in brains of patients who suffered from Alzheimer's disease (AD) and the elevated activity of serum SSAO observed for multiple sclerosis (MS) (Airas et al., 2006; Ferrer et al., 2002; Hernandez et al., 2005; Unzeta et al., 2007) are likely to increase brain formaldehyde levels. This has recently been confirmed at least in studies on post-mortem brains of AD patients (Tong et al., 2011).

Oligodendrocytes are the myelin forming cells in the brain (Emery, 2010; Nave, 2010). Since myelin sheaths in the brain show a prominent staining for formaldehyde dehydrogenase (Keller et al., 1990), oligodendrocytes may have the potential to oxidize

Abbreviations: AD, Alzheimer's disease; BSO, buthionine sulfoximine; CNP, 2', 3'-cyclic nucleotide 3'-phosphodiesterase; DMEM, Dulbecco's modified Eagle's medium; GFAP, glial fibrillary acidic protein; Fal, formaldehyde; FCS, fetal calf serum; GSH, glutathione; GSSG, glutathione disulfide; GSx, total glutathione; IB, incubation buffer; LDH, lactate dehydrogenase; LSD1, lysine-specific demethylase 1; MAG, myelin associated glycoprotein; Mrp, multidrug resistance protein; MS, multiple sclerosis; PBS, phosphate buffered saline; PI, propidium iodide; RT-PCR, reverse transcription polymerase chain reaction; SSAO, semicarbazide-sensitive amine oxidase.

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73 formaldehyde as recently reported for cultured astrocytes (Tulpule
74 and Dringen, 2012). Although, to our knowledge, direct conse-
75 quences of an exposure of oligodendrocytes to formaldehyde have
76 not been reported so far, a number of observations connect im-
77 paired oligodendrocyte functions to formaldehyde. For example
78 the demyelination of the optic nerve after methanol poisoning
79 (Gaul et al., 1995; Sharpe et al., 1982; Shin and Uhm, 2011) is con-
80 sidered to be the consequence of the adverse effects of the metha-
81 nol oxidation products formaldehyde and formate (Monte, 2010;
82 Skrzydlewska, 2003). In addition, some patients have been re-
83 ported to develop MS symptoms after methanol poisoning (Henzi,
84 1984), while inhibition of the formaldehyde-producing enzyme
85 SSAO reduces the incidence and the severity of MS in a mouse
86 model of this disease (Wang et al., 2006). Formaldehyde-modified
87 proteins which elicit an immune response have been considered to
88 be responsible for the development of MS symptoms (Monte,
89 2010; Schwyzer and Henzi, 1983).

90 The tripeptide glutathione (GSH) plays an important role in the
91 detoxification of toxins and reactive oxygen species in brain (Hir-
92 linger and Dringen, 2010; Schmidt and Dringen, 2012). The main-
93 tenance of a high cellular concentration of GSH is especially
94 important for oligodendrocytes, since a loss of GSH or insufficient
95 synthesis of GSH in these cells have been connected with oxidative
96 stress and cell damage (Back et al., 1998; Oka et al., 1993; Yonez-
97 awa et al., 1996). Since formaldehyde reacts rapidly with GSH in a
98 non-enzymatic process to S-hydroxymethyl-glutathione (Harris
99 et al., 2003; Macallister et al., 2011; Teng et al., 2001), an exposure
100 of oligodendroglial cells to formaldehyde may have severe conse-
101 quences on the GSH metabolism of these cells. To address this
102 hypothesis, we have used the oligodendroglial cell line OLN-93.
103 These cells morphologically resemble oligodendrocyte O-2A pro-
104 genitor cells and express various oligodendrocyte markers such
105 as galactocerobroside, myelin basic protein and myelin-associated
106 glycoprotein (MAG) (Richter-Landsberg and Heinrich, 1996). This
107 well established cell line has previously been used as a model system
108 to investigate the consequences of a treatment of oligodendro-
109 glial cells with drugs, metals or nanoparticles on the cell
110 viability, the GSH metabolism and the cellular response to oxida-
111 tive stress (Ernst et al., 2004; Hohnholt et al., 2010, 2011; Hohnholt
112 and Dringen, 2011; Stahnke et al., 2007; Steiner et al., 2011; Thies-
113 sen et al., 2010). OLN-93 cells allow studies on a homogeneous
114 population of cells with oligodendroglial properties and enable
115 the realization of biochemical experiments that require large num-
116 bers of cells and high cell densities, which would be difficult to per-
117 form on oligodendrocyte cultures due to the rather low cell yield
118 obtained for such cultures.

119 Here we report that formaldehyde induces a rapid GSH export
120 from viable OLN-93 cells by a process that involves the multidrug
121 resistance protein 1 (Mrp1), a member of the family of ATP-
122 dependent export pumps for anions such as glutathione and gluta-
123 thione-S-conjugates (Keppeler, 2011; Slot et al., 2011). Such a
124 formaldehyde-induced GSH loss is likely to compromise the cellular
125 antioxidative and detoxifying potential and may contribute to the
126 known neurotoxicity of formaldehyde (Songur et al., 2010) and to
127 the reported deficits in memory and learning after formaldehyde
128 exposure (Lu et al., 2008; Malek et al., 2003; Tong et al., 2011).

129 2. Materials and methods

130 2.1. Materials

131 Formaldehyde was purchased from Merck (Darmstadt, Ger-
132 many). Fetal calf serum (FCS) and penicillin/streptomycin solution
133 were purchased from Biochrom (Berlin, Germany) and Dulbecco's
134 modified Eagle's medium (DMEM) was from Gibco (Karlsruhe,

Germany). Bovine serum albumin, NADH and NADPH were pur-
135 chased from Applichem (Darmstadt, Germany). Deoxyribonucleo-
136 side triphosphate mix, DNA loading dye, GeneRuler™, 50 bp DNA
137 ladder, RevertAid™ H Minus First Strand cDNA Synthesis Kit, mag-
138 nesium chloride, Taq buffer and Taq polymerase were obtained
139 from Fermentas (St. Leon-Rot, Germany). The RNeasy® Mini Kit
140 was from Qiagen (Hilden, Germany). Primers were obtained from
141 MWG Biotech (Ebersberg, Germany) and RedSafe™ was from HISS
142 Diagnostics (Freiburg, Germany). The primary rat anti-MRPr1 anti-
143 body was purchased from Enzo (Lörrach, Germany), the secondary
144 donkey anti-rat IgG coupled to Cy3 and donkey serum were ob-
145 tained from Dianova (Hamburg, Germany). Rabbit polyclonal
146 anti- α -tubulin, mouse monoclonal anti- α -tubulin, mouse anti-gial
147 fibrillary acidic protein (anti-GFAP), mouse anti-2',3'-cyclic nucle-
148 otide 3'-phosphodiesterase (anti-CNP) and mouse anti-A2B5 were
149 from Sigma-Aldrich (Steinheim, Germany). Rabbit polyclonal
150 anti-MAG was a generous gift of Dr. T.V. Waehnel (Goettingen,
151 Germany). Texas Red-conjugated donkey anti-mouse and fluores-
152 cein isothiocyanate-conjugated donkey anti-rabbit secondary anti-
153 bodies were from Jackson ImmunoResearch (West Grove, PA, USA).
154 All other chemicals of the highest purity available were from Fluka
155 (Buchs, Switzerland), Merck (Darmstadt, Germany), Sigma-Aldrich
156 (Steinheim, Germany), Riedel-deHaen (Seelze, Germany) or Carl
157 Roth (Karlsruhe, Germany). 96-well microtitre plates, 5 cm dishes
158 and 24-well plates were from Nunc (Wiesbaden, Germany). Glass
159 coverslips were obtained from Omnilab (Bremen, Germany).
160

161 2.2. Cell cultures

162 OLN-93 is a permanent cell line derived from spontaneously
163 transformed cells in primary glial cultures of rat brain (Richter-
164 Landsberg and Heinrich, 1996). The cells (passage number
165 29–40) were cultured as previously described (Hohnholt et al.,
166 2010; Thiessen et al., 2010). If not stated otherwise, 100,000 cells
167 were seeded per well of 24-well plates in 1 mL culture medium
168 (90% DMEM, 10% FCS, 1 mM pyruvate, 20 units/ml penicillin G
169 and 20 μ g/mL streptomycin sulfate) and experiments were per-
170 formed 16–20 h after seeding. For experiments that required an
171 exposure to buthionine sulfoximine (BSO) or cadmium chloride
172 for up to 8 h, the culture medium was replaced with 1 mL medium
173 containing these compounds at an appropriate time-point such
174 that the treatment could be terminated exactly 20 h after seeding.
175 For experiments where cells were exposed to these compounds for
176 24 h, 50,000 cells in 1 mL culture medium were seeded per well of
177 24-well plates, the culture medium was replaced with 1 mL me-
178 dium containing BSO or cadmium chloride 16 h after seeding and
179 cells were incubated for additional 24 h. For RT-PCR experiments,
180 RNA was extracted from OLN-93 cells 16 h after seeding of
181 500,000 cells in 5 mL culture medium into 5 cm dishes.

182 Astrocyte-rich primary cultures were prepared from the brains
183 of newborn Wistar rats (Hamprrecht and Löffler, 1985). The cells
184 were seeded in culture medium in 24-well plates (300,000 cells
185 in 1 mL) or 5 cm dishes (3,000,000 cells in 5 mL) and incubated
186 in the humidified atmosphere of a Sanyo (Osaka, Japan) incubator
187 with 10% CO₂. The cultures were maintained by renewing the cul-
188 ture medium every seventh day and were used for experiments at
189 an age between 15 and 21 days. For pre-treatment of these cul-
190 tures, cells were pre-incubated in 0.5 mL of serum-free culture
191 medium without or with BSO for up to 24 h or with cadmium chlo-
192 ride, copper chloride or potassium arsenate for 24 h.

193 2.3. Experimental incubation of cells for GSH export studies

194 Cells were washed twice with 1 mL pre-warmed (37 °C) incuba-
195 tion buffer (IB: 1.8 mM CaCl₂, 1 mM MgCl₂, 5.4 mM KCl, 145 mM
196 NaCl, 0.8 mM Na₂HPO₄, 20 mM HEPES, 5 mM D-glucose, pH 7.4)

197 and then incubated at 37 °C with 0.2 mL IB containing formalde- 255
 198 hyde and/or other compounds in the concentrations indicated in 256
 199 the figures and the tables. For experiments involving removal of 257
 200 formaldehyde by washing, the formaldehyde-containing medium 258
 201 was aspirated after 30 min, the cells were washed twice with 259
 202 pre-warmed IB and subsequently incubated with 0.2 mL of IB con- 260
 203 taining 0 or 0.5 mM formaldehyde. For removal of formaldehyde 261
 204 by the chemical reaction to its semicarbazone, after 30 min of incu- 262
 205 bation 10 µL of 0.2 M semicarbazide in IB was added directly to the 263
 206 cultures incubated in 0.2 mL formaldehyde-containing IB. All incu- 264
 207 bations were terminated by collecting the medium and by washing 265
 208 the cells twice with 1 mL ice-cold phosphate-buffered saline (PBS; 266
 209 10 mM potassium phosphate buffer, pH 7.4, containing 150 mM 267
 210 NaCl).

211 2.4. Determination of cell viability

212 Cell viability was assessed by determining the activity of the en- 272
 213 zyme lactate dehydrogenase (LDH) in the media using the microti- 273
 214 tre plate assay described previously (Dringen et al., 1998) with the 274
 215 modification that 20 µL volumes of lysates and media were used. 275
 216 Membrane integrity of OLN-93 cells was investigated by propidi- 276
 217 um iodide (PI) staining as described recently (Hohnholt and Drin- 277
 218 gen, 2011; Scheiber et al., 2010). In this method, Hoechst 33342 278
 219 staining identifies the nuclei of all cells present, while PI staining 279
 220 identifies the cells which have damaged cell membranes. 280

221 2.5. Determination of glutathione and protein contents

222 Total glutathione contents (GSx = amount of GSH plus twice the 281
 223 amount of glutathione disulfide (GSSG)) and GSSG contents in cells 282
 224 and media were determined by the colorimetric Tietze assay as 283
 225 previously described (Dringen and Hamprecht, 1996; Dringen 284
 226 et al., 1997) in microtitre plates. Cellular protein contents were 285
 227 determined by the Lowry method (Lowry et al., 1951) using bovine 286
 228 serum albumin as a standard protein. 287

229 2.6. Reverse transcription polymerase chain reaction analysis (RT-PCR)

230 Total RNA was extracted from OLN-93 cells or astrocyte-rich 288
 231 primary cultures seeded in 5 cm dishes using the RNeasy® Mini 289
 232 Kit following the instructions provided by the supplier. Reverse 290
 233 transcription of mRNA in the extracted RNA was performed using 291
 234 the RevertAid™ H Minus First Strand cDNA Synthesis Kit. PCRs 292
 235 were performed as previously described (Tulpule et al., 2010) using 293
 236 the primers 5'-GCACCTGGCTTCTAACTATTGG-3' and 5'-TCTCATTG 294
 237 AAGTGTGAGTACAC-3' (for Mrp1) or 5'-GGGTCAGAAGGACTCT 295
 238 ACG-3' and 5'-GGTCTCAAACATGATCTGGG-3' (for β-actin) that 296
 239 have previously been used for the amplification of rat Mrp1 and 297
 240 β-actin (Hirrlinger et al., 2002a). Agarose gel electrophoresis 298
 241 showed amplification products of the expected sizes of 511 and 299
 242 248 bp for Mrp1 and β-actin, respectively. 300

243 2.7. Immunocytochemical stainings

244 For immunostaining of OLN-93 cells for cell type specific mark- 301
 245 ers, cells grown on coverslips were washed twice with PBS and 302
 246 fixed with 3% (w/v) paraformaldehyde for 15 min. Cells were 303
 247 washed thrice in 5 min intervals with PBS between the different 304
 248 steps of the staining procedure. Fixed cells were permeabilized 305
 249 with 0.1% Triton X-100 for 30 min and incubated overnight at 306
 250 4 °C in a humidified atmosphere with rabbit polyclonal anti-α- 307
 251 tubulin in combination with the mouse monoclonal antibodies 308
 252 anti-CNP, anti-A2B5 or anti-GFAP, or with mouse monoclonal 309
 253 anti-α-tubulin in combination with rabbit polyclonal anti-MAC 310
 254 (working dilutions for all antibodies: 1:200 in PBS). Cover slips 311
 312

were washed and incubated with a mixture of Texas Red- con- 255
 256 jugated donkey anti mouse (1:100 diluted in PBS) and fluorescein 257
 258 isothiocyanate-conjugated donkey anti rabbit (1:100 diluted in 259
 260 PBS) secondary antibodies for 1 h at room temperature, washed 261
 262 and mounted with Vectashield (Vector Laboratories, Burlingame, 263
 264 CA, USA) mounting medium. Fluorescence was studied using a 264
 265 Zeiss epifluorescence microscope (Oberkochen, Germany) 265
 266 equipped with a digital camera using a plan-neofluar objective 266
 267 (40×). OLN-93 cells express markers (De Vries and Boullerne, 267
 268 2010) for oligodendrocyte precursor cells (A2B5; Fig. 1G) and ma- 268
 269 ture oligodendrocytes (CNP; Fig. 1E and MAG; F) but not the 269
 270 astroglial protein GFAP (Fig. 1H).

271 For staining of presence of multidrug resistance protein 1, OLN- 271
 272 93 cells grown on coverslips were washed twice with ice-cold PBS 272
 273 and fixed with methanol for 10 min at -20 °C. If not stated other- 273
 274 wise, the cells were washed thrice in 5 min intervals with ice-cold 274
 275 PBS between the different steps of the staining procedure. Fixed 275
 276 cells were incubated with 0.3% Triton X-100 in PBS for 10 min at 276
 277 room temperature. Incubation of the cells with anti-MRP1 (1:50 277
 278 diluted in PBS) was carried out overnight at 4 °C in a humidified 278
 279 atmosphere. The secondary anti-rat IgG-Cy3 antibody (1:100 di- 279
 280 luted in PBS containing 10% donkey serum) was applied for 60 280
 281 min at room temperature. Prior to mounting the coverslips in 281
 282 Mowiol mounting medium (Arampatzidou et al., 2011), an ethanol 282
 283 gradient of 70%, 90% and 100% in 1 min intervals was applied. The 283
 284 fluorescent signal was documented using the Eclipse TS2000U 284
 285 microscope (Nikon, Düsseldorf, Germany) and the NIS-Elements 285
 286 Basic Research software (version 3.1, Nikon). 286

287 2.8. Presentation of data

288 Data are presented as means ± SD of values that were obtained 287
 289 in three independent experiments performed on three independ- 288
 290 ent passages of cells, if not stated otherwise. Pictures showing 289
 291 PI staining, immunocytochemical staining and RT-PCR were taken 290
 292 from representative experiments that were reproduced at least 291
 293 twice with comparable outcomes. The analysis of significance be- 292
 294 tween groups of data was performed by ANOVA followed by Bon- 293
 295 ferroni *post hoc* tests with **p* < 0.05, ***p* < 0.01 and ****p* < 0.001. 294
 296 Statistical comparisons of two data sets were performed by the 295
 297 paired *t*-test with **p* < 0.05, ##*p* < 0.01 and ###*p* < 0.001. *p* > 0.05 296
 297 was considered as not significant. 297

298 3. Results

299 3.1. Viability of oligodendroglial cells during formaldehyde exposure

300 To test for the potential toxicity of formaldehyde, OLN-93 cells 301
 302 were treated with formaldehyde in concentrations of up to 3 mM 302
 303 for up to 4 h. PI staining (Fig. 2A–L) as well as measurement of 303
 304 the extracellular LDH activity (Fig. 2M and N) revealed that a treat- 304
 305 ment with up to 0.5 mM formaldehyde for 4 h (Fig. 2E, K, M, and N) 305
 306 and a treatment with up to 3 mM formaldehyde for 2 h (Fig. 2F) did 306
 307 not compromise cell viability. In contrast, exposure of OLN-93 cells 307
 308 for 4 h to 3 mM formaldehyde was toxic for the cells as indicated 308
 309 by the presence of PI-positive cells (Fig. 2L) and by the appearance 309
 310 of extracellular LDH (data not shown). 310

311 3.2. Formaldehyde treatment alters the glutathione content of oligodendroglial cells

312 During incubation in IB for up to 4 h only minute amounts of 309
 310 extracellular total glutathione (GSx = amount of glutathione 310
 311 (GSH) plus twice the amount of glutathione disulfide (GSSG)) were 311
 312 observed for cultured OLN-93 cells (Fig. 3A). In contrast, applica- 312

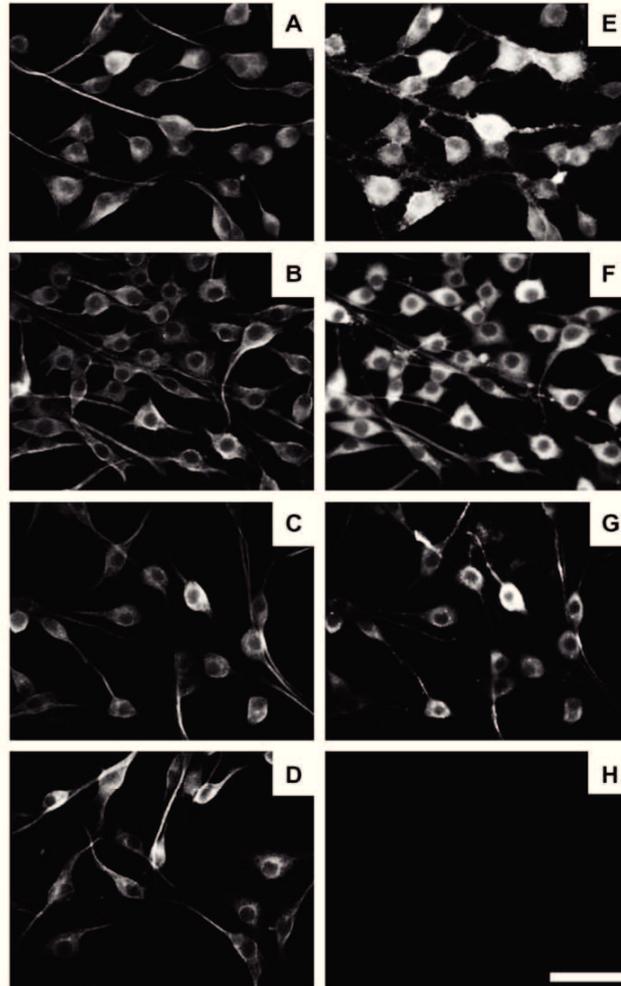


Fig. 1. Immunocytochemical staining of OLN93 cells for α -tubulin (A–D) in combination with antibodies against CNP (E), MAG (F), A2B5 (G) and GFAP (H). The scale bar in panel H corresponds to 50 μ m and applies to all panels.

313 tion of formaldehyde resulted in a strong concentration-dependent
314 increase in the extracellular accumulation of GSx from viable cells
315 (Fig. 3A). While presence of 0.1 mM formaldehyde caused only a
316 small increase in the extracellular GSx content, the accumulation
317 of GSx in the medium was much stronger for cells exposed to 0.3
318 or 0.5 mM formaldehyde (Fig. 3A). The formaldehyde-induced
319 increase in the extracellular GSx content was rapid in the initial
320 2 h of incubation and slowed down thereafter (Fig. 3A). The in-
321 crease in extracellular GSx contents in formaldehyde-treated cul-
322 tures was accompanied by a comparable decline in cellular GSx
323 contents after 4 h of incubation (data not shown). To investigate
324 the concentration-dependence of the formaldehyde-induced alter-
325 ations in the GSx contents of OLN-93 cells in more detail, the cells
326 were incubated with formaldehyde in concentrations of up to

327 1 mM for 2 h. The cellular and extracellular GSx contents remained
328 unaltered compared to that of control cells after exposure to form-
329 aldehyde in concentrations of up to 0.07 mM (Fig. 3B and C). How-
330 ever, treatment with higher concentrations of formaldehyde
331 significantly increased the amounts of extracellular GSx (Fig. 3B)
332 and lowered the cellular GSx levels (Fig. 3C). Application of form-
333 aldehyde in concentrations higher than 0.5 mM did not lead to a
334 further increase in the extracellular GSx content (Fig. 3B) or to
335 any further substantial decrease in cellular GSx contents
336 (Fig. 3C). Half-maximal effects on the cellular and extracellular
337 GSx contents were observed for a formaldehyde concentration of
338 about 0.2 mM. The sum of the cellular plus extracellular GSx con-
339 tents of OLN-93 cells after 2 h incubation with formaldehyde in
340 concentrations higher than 0.1 mM were significantly lowered by

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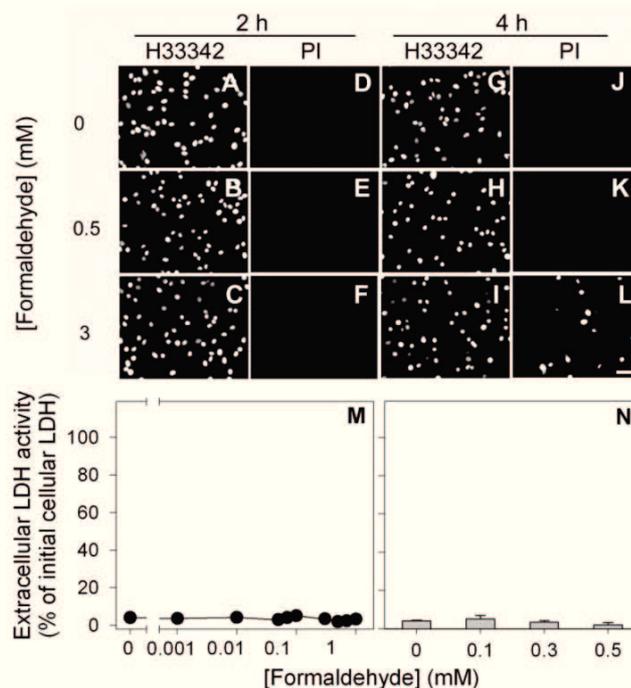


Fig. 2. Impact of formaldehyde exposure on the viability and membrane integrity of OLN-93 cells. The cells were treated without (A, D, G, and J) or with 0.5 mM (B, E, H, and K) or 3 mM formaldehyde (C, F, I, and L) for 2 h (A–F) or 4 h (G–L). The cells were stained with Hoechst 33342 (A–C, G–I) to visualize all nuclei and with propidium iodide (D–F, J–L) to identify cells with compromised membrane integrity. The scale bar in panel L corresponds to 50 μ m and applies to all panels. Panels M and N give the extracellular LDH activities of cells that had been exposed for 2 h (M) or 4 h (N) with the indicated concentrations of formaldehyde. The values shown in panels M and N are derived from three independent experiments on cells that had average protein contents of $77 \pm 3 \mu$ g (M) and $84 \pm 13 \mu$ g (N) per well.

341 up to 20% compared to control cells (Fig. 3D). None of the conditions
342 applied compromised the cell viability as indicated by the absence
343 of any significant ($p > 0.05$) increase in extracellular LDH
344 activity (Fig. 2M).

345 To test whether formaldehyde exposure alters the ratio of GSSG
346 to GSH in OLN-93 cells, the amount of GSSG contributing to the
347 GSx content was determined for cells and media of cultures that
348 had been treated without or with 0.5 mM formaldehyde for 2 h
349 (Table 1). For both conditions, the GSSG contents of cell lysates
350 and media were very low and remained in the range of the detection
351 limit of the method used for quantification (Table 1), demonstrating
352 that formaldehyde does not cause any substantial increase of the
353 GSSG levels in the cultures and that formaldehyde induces export
354 of GSH, not that of GSSG, from the cells.

355 3.3. Dependence of the formaldehyde-induced GSH export on the 356 cellular GSx content in OLN-93 cells and cultured astrocytes

357 GSH export from astroglial cultures has been reported to depend
358 on the cellular concentration of GSH (Sagara et al., 1996) and to be
359 strongly accelerated in the presence of formaldehyde (Tulpule and
360 Dringen, 2011). Therefore, the dependence of the formaldehyde-
361 stimulated GSH export rates on the initial cellular GSx content was
362 investigated for OLN-93 cells and was compared with that of cultured
363 astrocytes that were used as a positive control. To modulate cellular
364 GSx levels, the cultured cells were

365 pre-incubated with various compounds that either increased or
366 decreased the normal cellular GSx content of the cells before they
367 were exposed to formaldehyde.

368 OLN-93 cells were pre-incubated with the GSH synthesis inhibitor
369 buthionine sulfoximine (BSO) to lower the cellular GSx content
370 (Hohnholt and Dringen, 2011; Schmidt and Dringen, 2010; Thiesen
371 et al., 2010) or with cadmium chloride to increase cellular
372 GSx levels (Bannai et al., 1991; Sagara et al., 1996) before applica-
373 tion of formaldehyde. While the specific cellular GSx content of
374 control OLN-93 cells was not altered during the incubation in culture
375 medium, the presence of BSO lowered the cellular GSx content
376 within 8 h and 24 h by 50% and 80%, respectively (Fig. 4). On the
377 other hand, presence of cadmium chloride increased the cellular
378 GSx content of OLN-93 cells within 8 and 24 h by 60% and 100%,
379 respectively (Fig. 4). In the concentrations applied, neither BSO
380 nor cadmium chloride compromised the cell viability of OLN-93
381 cells during an incubation of up to 24 h (data not shown).

382 Pre-incubation of OLN-93 cells with BSO or cadmium chloride
383 for various time intervals was used to vary their specific cellular
384 GSx contents between 10 and 100 nmol/mg in order to investigate
385 how the cellular GSx content affects the GSH export rate in absence
386 or presence of formaldehyde. The almost negligible GSH export
387 rates (less than 1 nmol/(h mg)) observed for untreated OLN-93
388 cells (Fig. 3A and B) was not significantly altered by a modulation of
389 the cellular GSx content (Fig. 5A, open symbols). In contrast, the GSH
390 export rate of formaldehyde-treated OLN-93 cells depended

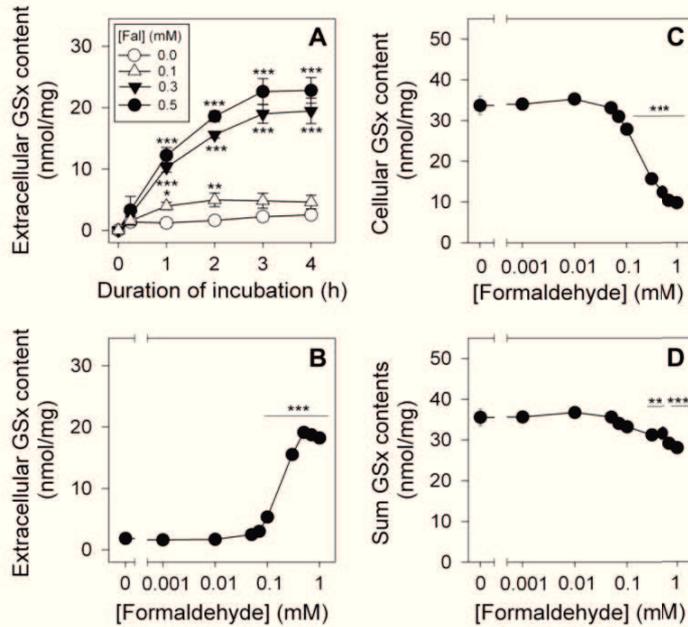


Fig. 3. Consequences of a formaldehyde treatment on the GSx content of OLN-93 cells. The cells were incubated for up to 4 h (A) or for 2 h (B-D) in the absence (0 mM) or the presence of the indicated concentrations of formaldehyde (Fal). Panel A shows the extracellular GSx contents for the indicated time points (A). Panel B gives the extracellular GSx content and panel C the cellular GSx content of OLN-93 cells after the 2 h incubation, while panel D gives the sum of the cellular plus extracellular GSx contents. The results represent means \pm SD of data obtained on three independent cultures. The cultures had average protein contents of $84 \pm 13 \mu\text{g}$ (A) and $77 \pm 3 \mu\text{g}$ (B-D) per well and initial specific cellular GSx contents of $46.9 \pm 4.2 \text{ nmol per mg}$ (A) and $42.4 \pm 0.9 \text{ nmol per mg}$ (B-D). The significance of differences (ANOVA) compared to the data obtained for the control condition (absence of formaldehyde) is indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Table 1
Effects of formaldehyde on the GSx and GSSG contents of OLN-93 cells.

		[Formaldehyde]	
		0 mM	0.5 mM
Medium	GSx content (nmol/mg)	1.85 ± 0.20	$19.14 \pm 0.88^{***}$
	GSSG content (nmol GSx/mg)	0.26 ± 0.45	$1.37 \pm 0.25^*$
	LDH activity (% of initial cellular LDH activity)	4 ± 0	2 ± 1
Cells	GSx content (nmol/mg)	33.70 ± 2.30	$12.40 \pm 0.64^{***}$
	GSSG content (nmol GSx/mg)	0.11 ± 0.17	0.07 ± 0.05

Cells were incubated without (0 mM) or with 0.5 mM formaldehyde for 2 h. The results represent means \pm SD of data obtained on three independent passages of cells that had an average protein content of $77 \pm 3 \mu\text{g}$ per well and an initial specific cellular GSx content of $42.4 \pm 0.9 \text{ nmol per mg}$. The significance of differences between data obtained for cells treated without or with formaldehyde (t-test) is indicated by * $p < 0.05$ and *** $p < 0.001$.

391 strongly on the cellular GSx content as indicated by the lowered
392 export rate found for BSO-treated cells and by the accelerated
393 GSx export from cadmium pre-treated OLN-93 cells (Fig. 5A, filled
394 symbols).

395 Besides OLN-93 cells, the dependence of GSH export on the cel-
396 lular GSH concentration in presence of formaldehyde was also
397 investigated for cultured astrocytes, since these cells are known
398 to export GSH with rates that strongly depend on the cellular
399 GSH concentration (Sagara et al., 1996). Elevated cellular GSx
400 contents were obtained by pre-incubation of cultured astrocytes

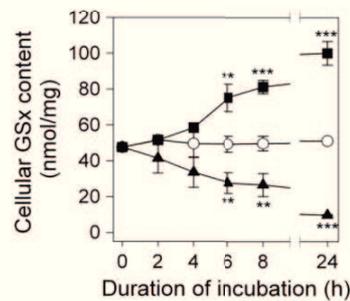


Fig. 4. Alteration of specific cellular GSx contents in OLN-93 cells after application of BSO or cadmium chloride. The cells were treated without (circles) or with $100 \mu\text{M}$ BSO (triangles) or $1 \mu\text{M}$ cadmium chloride (squares) for the indicated time and the specific cellular GSx content was determined. The results represent means \pm SD of data obtained from three independent experiments on cultures that had an average protein content of $65 \pm 8 \mu\text{g}$ per well. The significance of difference (ANOVA) to the values obtained at a given time point for the control condition (absence of BSO and CdCl₂, circles) is indicated by ** $p < 0.01$ and *** $p < 0.001$.

401 with cadmium chloride, copper chloride or arsenate, as reported
402 previously for different cell types (Eannai et al., 1991; Cheng
403 et al., 2008; Sagara et al. 1996; Scheiber and Dringen, 2011). In
404 contrast to astrocytes, exposure of OLN-93 cells to copper chloride

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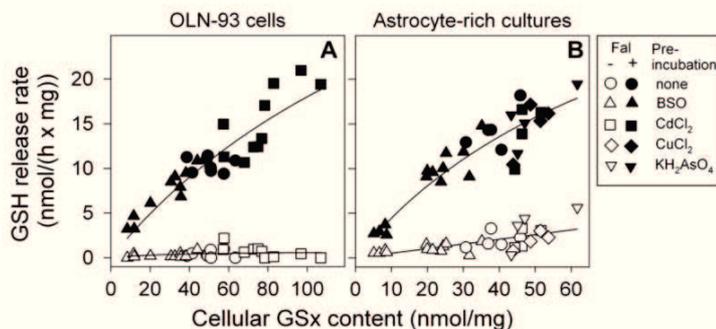


Fig. 5. Effects of an altered cellular GSx content on the GSH export from OLN-93 cells (A) and astrocyte-rich primary cultures (B). OLN-93 cells (A) were pre-incubated without (circles) or with 100 μ M BSO (triangles up) or 1 μ M cadmium chloride (squares) for up to 24 h to achieve various specific cellular GSx contents. Astrocyte cultures (B) were pre-incubated without (circles) or with 100 μ M BSO (triangles up) for up to 24 h or with 1 μ M cadmium chloride (squares), 20 μ M copper chloride (diamonds) or 5 μ M arsenate (triangles down) for 24 h. After the respective pre-incubations to modulate cellular GSx levels, cells were incubated in the absence (open symbols) or presence of formaldehyde (Fal; filled symbols; 0.5 mM for OLN-93 cells and 1 mM for cultured astrocytes) for up to 1 h. The specific GSH release rates were calculated from the linear increase in the extracellular GSx contents between 10 and 60 min and were plotted against the specific cellular GSx content.

405 or arsenate did not affect the GSx content of these cells (data not
406 shown).

407 Analysis of GSH export rates for astrocytes that had been pre-
408 treated with BSO, cadmium chloride, copper chloride or arsenate
409 to alter their cellular GSx contents revealed that the basal GSH ex-
410 port rate of astrocytes increased almost proportional to the cellular
411 GSx concentration (Fig. 5B, open circles) as previously reported
412 (Sagara et al., 1996; Scheiber and Dringen, 2011). Moreover, the
413 presence of formaldehyde strongly accelerated the GSH export
414 from cultured astrocytes for all cellular GSx contents investigated
415 (Fig. 5B, filled circles).

416 Hyperbolic fittings using the Michaelis–Menten equation gave
417 reasonable correlations of 0.78 (OLN-93 cells) and 0.83 (astrocytes)
418 for the dependence of the formaldehyde-induced GSH export rates
419 on the cellular GSx contents (Fig. 5). The calculated K_m and V_{max}
420 values of around 100 nmol/mg and 40 nmol/(h mg), respectively,
421 were almost identical for the GSH export from formaldehyde-treated
422 OLN-93 cells and astrocytes (Table 2). The kinetic parameters
423 for the basal GSH export in OLN-93 cells could not be determined,
424 but the calculated K_m and V_{max} values for the basal GSH export
425 from cultured astrocytes were 86 nmol/mg and 4 nmol/(h mg),
426 respectively (Table 2).

427 3.4. Formaldehyde removal terminates the accelerated GSH efflux from 428 OLN-93 cells

429 To investigate whether presence of formaldehyde is required to
430 maintain the observed accelerated GSH export from OLN-93 cells,

431 the cells were pre-incubated without or with 0.5 mM formalde-
432 hyde for 30 min, washed and subsequently incubated without or
433 with 0.5 mM formaldehyde for additional 1.5 h (Fig. 6A). The ac-
434 celerated GSH export observed during pre-incubation with formalde-
435 hyde was strongly reduced by removal of formaldehyde (Fig. 6A,
436 open triangles), but continued if formaldehyde was present during
437 the main incubation (Fig. 6A, filled circles). Accordingly, the low
438 extracellular GSx accumulation of OLN-93 cells that had been
439 pre-incubated without formaldehyde was maintained during a
440 main incubation in the absence of formaldehyde (Fig. 6A, open
441 circles), while application of formaldehyde during the main incu-
442 bation of these cells immediately accelerated GSH export (Fig. 6A,
443 filled triangles). None of these conditions compromised cell viabi-
444 lity as indicated by the absence of any significant increase in the
445 extracellular LDH activity compared with controls (data not
446 shown).

447 Alternatively to the washing approach, formaldehyde was re-
448 moved from the medium by chemical reaction with semicarbazide
449 to form a semicarbazone. While application of semicarbazide to
450 control cells after 30 min incubation in the absence of formalde-
451 hyde did not affect the, at best, very low extracellular accumula-
452 tion of GSx (Fig. 6B, open circles and triangles), semicarbazide
453 application (Fig. 6B, open diamonds) strongly reduced the accel-
454 erated extracellular GSx accumulation found for formaldehyde-tre-
455 ated cultures (Fig. 6B, filled circles). None of these conditions
456 compromised the viability of OLN-93 cells as indicated by the ab-
457 sence of any significant increase in extracellular LDH activity (data
458 not shown).

459 3.5. Expression of multidrug resistance protein 1 in OLN-93 cells

460 For cultured astrocytes, GSH export in absence or presence of
461 formaldehyde has been reported to be predominantly mediated
462 by Mrp1 (Hirrlinger et al., 2002b; Minich et al., 2006; Tulpule
463 and Dringen, 2011). To test whether OLN-93 cells express Mrp1,
464 the presence of mRNA for Mrp1 was investigated by RT-PCR and
465 immunocytochemical staining for Mrp1. A specific Mrp1 amplifica-
466 tion product of the expected size was obtained for OLN-93 cells as
467 well as for rat astrocyte-rich primary cultures (Fig. 7A) that are
468 known to express Mrp1 (Hirrlinger et al., 2002b), confirming the
469 presence of Mrp1 mRNA in OLN-93 cells. Immunocytochemical

Table 2

Kinetic parameters for GSH export from OLN-93 cells and cultured astrocytes.

Cells	Control		Formaldehyde	
	K_m (nmol/mg)	V_{max} (nmol/(h mg))	K_m (nmol/mg)	V_{max} (nmol/(h mg))
OLN-93 cells	n.d.	n.d.	105	35
Astrocyte cultures	86	4	101	47

The K_m and V_{max} values were calculated from the data shown in Fig. 4 using the Hanes–Woolf plot. n.d., not determined.

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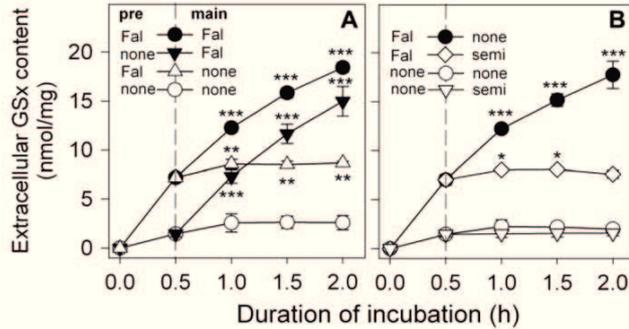


Fig. 6. Effect of formaldehyde-removal on the formaldehyde-induced GSH export from OLN-93 cells. (A) The cells were pre-incubated without (none) or with 0.5 mM formaldehyde (Fal) for 30 min (pre) and subsequently incubated (main) in the absence (open symbols) or presence (filled symbols) of 0.5 mM formaldehyde for further 1.5 h. (B) The cells were incubated without (none) or with 0.5 mM formaldehyde (Fal) for 30 min before 10 μ L IB (none) or 10 μ L semicarbazide (semi) in IB was added. During the further 1.5 h the GSx accumulation in the medium was determined. The results represent means \pm SD of data obtained on three independent passages of cell cultures that had an average protein content of 77 ± 11 μ g per well and an initial specific cellular GSx content of 46.2 ± 6.3 nmol per mg. For each condition, the significance of difference (ANOVA) between the values obtained for 30 min (onset of the main incubation) and those obtained for incubation times of 60, 90 or 120 min are indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

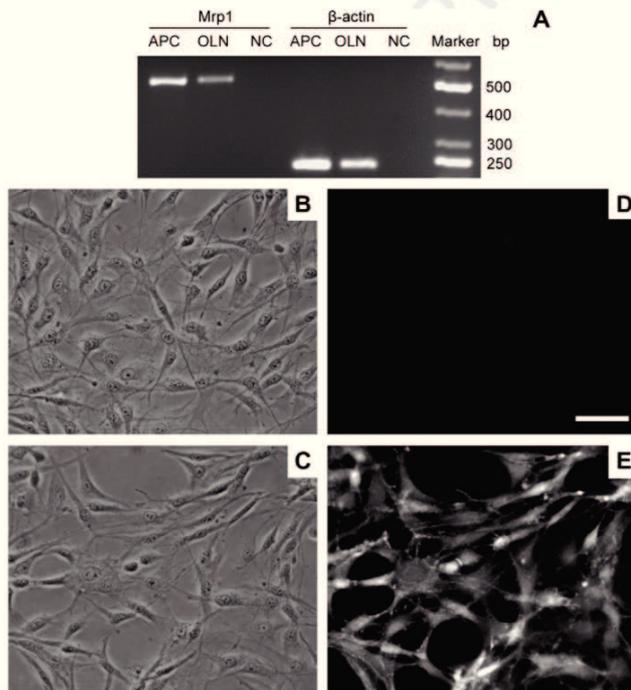


Fig. 7. Expression of Mrp1 in OLN-93 cells. (A) RT-PCR analysis of the presence of Mrp1 mRNA in OLN-93 cells (OLN) and astrocyte-rich primary cultures (APC). β -Actin was used as an internal positive control. Omission of cDNA was used as a negative control (NC). (B) Immunocytochemical detection of Mrp1 in OLN-93 cells. Representative phase contrast images (B and C) and the corresponding fluorescence images (D and E) are shown for cells that were treated without (B and D) or with (C and E) the primary Mrp1 antibody. The scale bar in panel C corresponds to 50 μ m and applies to all panels.

470
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staining of OLN-93 cells for Mrp1 revealed that these cells contain also Mrp1 protein (Fig. 7E), while no fluorescence signal for Mrp1

was observed when the primary anti-Mrp1 antibody was omitted (Fig. 7D).

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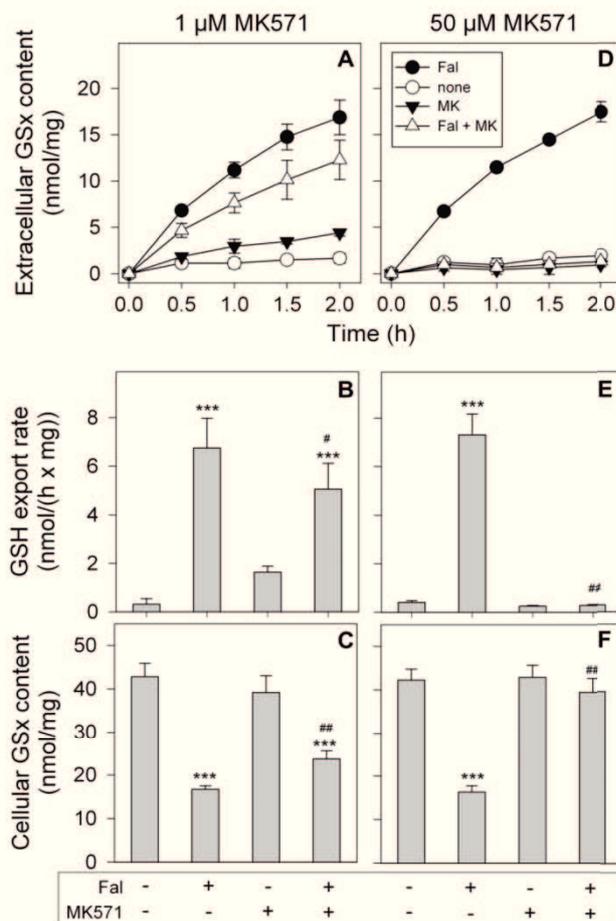


Fig. 8. Effects of MK571 on formaldehyde-stimulated GSH export from OLN-93 cells. The cells were incubated without (none) or with 0.5 mM formaldehyde (Fal) in the absence or presence of 1 μ M MK571 (A–C) or 50 μ M MK571 (D–F) for up to 2 h. (A and D) Time-dependent extracellular GSx accumulation. (B and E) Specific GSH export rates as calculated from the almost linear increase in extracellular GSx contents between 0.5 and 2.0 h. (C and F) Cellular GSx contents after 2 h of incubation. The results represent means \pm SD of data obtained on four (A–C) or three (D–F) independent passages of cells that had average protein contents of 75 \pm 9 (A–C) and 84 \pm 13 (D–F) μ g per well and initial specific cellular GSx contents of 42.8 \pm 5.9 (A–C) and 46.9 \pm 4.2 (D–F) nmol per mg. The significance of differences (ANOVA) of data compared with those of controls (absence of formaldehyde) is indicated by *** p < 0.001. The significance of differences between data obtained for cells treated with formaldehyde in the presence or absence of MK571 (t -test) is indicated by # p < 0.05 and ## p < 0.01.

474 3.6. Involvement of multidrug resistance protein 1 in formaldehyde-
475 stimulated GSH export

476 To investigate whether Mrp1 is involved in the formaldehyde-
477 induced GSH export from OLN-93 cells, the compound MK571
478 was applied which has been reported to stimulate Mrp1-mediated
479 GSH export at a concentration of 1 μ M, but to inhibit GSH export at
480 a concentration of 50 μ M (Hirrlinger et al., 2002b; Minich et al.,
481 2006). Application of 1 μ M MK571 to OLN-93 cultures in the absence
482 of formaldehyde resulted in a slight increase in the extracellular
483 GSx accumulation (Fig. 8A) and a significantly lowered
484 formaldehyde-induced GSH export by 25% (Fig. 8B), thereby partially
485 preventing the loss in cellular GSx (Fig. 8C). Treatment of

486 OLN-93 cells with 50 μ M MK571 did not affect the very low export
487 of GSH under control conditions, but almost completely prevented
488 the formaldehyde-induced extracellular GSx accumulation
489 (Fig. 8D), the increase in GSH export rate (Fig. 8E) and the loss in
490 cellular GSx content (Fig. 8F). None of these conditions affected
491 the viability of the cells as indicated by the absence of any significant
492 increase in extracellular LDH activity (data not shown).

4. Discussion

493 To investigate effects of formaldehyde on the GSH metabolism
494 of OLN-93 cells, we first compared basal parameters of the GSH
495 metabolism of these cells with those of cultured astrocytes. The
496

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high specific GSx content of untreated OLN-93 cells confirmed literature data (Hohnholt and Dringen, 2011; Thiessen et al., 2010) and was similar to values reported for cultured astrocytes (Brandmann et al., 2012; Tulpule and Dringen, 2011). The cellular GSx content of viable OLN-93 cells was strongly modulated by exposure of the cells to BSO or cadmium chloride. Presence of the GSH synthesis inhibitor BSO lowered GSH levels in OLN-93 cells as previously reported (Hohnholt and Dringen, 2011; Thiessen et al., 2010), while application of cadmium chloride for 24 h doubled the cellular GSx content. Potential reason for the latter observation could be a cadmium-induced increase in cystine uptake, as described for macrophages (Bannai et al., 1991), which subsequently would improve the availability of cellular cysteine as a substrate for GSH synthesis. Exposure of OLN-93 cells to copper chloride or arsenate did not elevate cellular GSx contents (data not shown), but these compounds increased the level of cellular GSx in cultured astrocytes. The underlying reason for this discrepancy could be that OLN-93 cells take up copper ions or arsenate less efficiently than astrocytes.

Oligodendroglial cells appear to release GSH with a, at best, very low basal rate, as shown for OLN-93 cells (Hohnholt and Dringen, 2011; present report) and cultured oligodendrocytes (Hirrlinger et al., 2002b). For OLN-93 cells, even a doubling of the cellular GSx content did not promote any substantial GSH export, contrasting the GSH export from cultured astrocytes which strongly depends on the cellular GSx content (Sagara et al., 1996; Scheiber and Dringen, 2011; present report).

For cultured astrocytes, Mrp1 has been reported to be predominantly responsible for basal and accelerated GSH export (Brandmann et al., 2012; Hirrlinger et al., 2002b; Minich et al., 2006; Tulpule and Dringen, 2011). However, despite the presence of Mrp1 mRNA and protein in OLN-93 cells, this transporter does not appear to mediate any substantial basal GSH export. Probable reasons for this difference between OLN-93 cells and astrocytes could be that the basal Mrp1-mediated GSH export requires a cell-type specific co-substrate, a regulatory protein and/or a modification of Mrp1 (Dringen and Hirrlinger, 2003), which is present in astrocytes but not in OLN-93 cells.

To test for consequences of a formaldehyde treatment on the GSH metabolism, OLN-93 cells were exposed to this aldehyde. OLN-93 cells were resistant towards acute toxicity of formaldehyde in concentrations of up to 1 mM. However, higher concentrations of formaldehyde or longer exposure time severely compromised the viability of OLN-93 cells as previously reported for astrocytes (Song et al., 2010; Tulpule and Dringen, 2011) and other cell types (Lovschall et al., 2002; Song et al., 2010; Tang et al., 2011). This toxicity is most likely due to the potential of formaldehyde to react with proteins and DNA (Cheng et al., 2003; Sutherland et al., 2008). In addition, formaldehyde is known to induce oxidative stress (Duong et al., 2011; Ozen et al., 2008; Songur et al., 2008; Zararsiz et al., 2007) which could contribute to the toxicity of higher concentrations of formaldehyde, since such conditions rapidly deprive OLN-93 cells of the important antioxidant GSH (data not shown).

Presence of moderate concentrations of formaldehyde induced a rapid time- and concentration-dependent export of GSH from viable OLN-93 cells. Since formaldehyde exposure did not cause any substantial increase in GSSG levels in cells or medium, a generation of cellular GSSG and subsequent export of GSSG can be excluded for formaldehyde-treated OLN-93 cells. The observed partial loss in the sum of cellular plus extracellular GSx during incubation of OLN-93 cells with formaldehyde is most likely a consequence of the formation of formaldehyde-GSH adducts (Bateman et al., 2007; Hopkinson et al., 2010) which are not detectable with the GSx assay used.

In the presence of formaldehyde, the GSH export rate increased almost proportionally to the cellular GSx content in both OLN-93

cells and cultured astrocytes. Almost identical K_m and V_{max} values of about 100 nmol/mg and 40 nmol/(h mg), respectively, were calculated for the formaldehyde-stimulated GSH export from OLN-93 cells and astrocytes, suggesting that the same transport mechanism is involved in these GSH export processes. Also, the K_m value for basal GSH export from cultured astrocytes was comparable to formaldehyde-stimulated GSH export, while the V_{max} value of the basal GSH export was only 10% of the value calculated for formaldehyde-treated cells. Considering that Mrp1 predominantly mediates the basal and stimulated GSH export from cultured astrocytes (Brandmann et al., 2012; Hirrlinger et al., 2002b; Minich et al., 2006; Tulpule and Dringen, 2011), that this protein is expressed in OLN-93 cells and that the Mrp1-inhibitor MK571 completely prevented the formaldehyde-stimulated GSH export from OLN-93 cells, it seems very likely that the formaldehyde-stimulated GSH export from OLN-93 cells is mediated by Mrp1.

The stimulation of GSH export from OLN-93 cells occurred rapidly after exposure of the cells to formaldehyde and was also terminated instantaneously after removing formaldehyde. These observations suggest that the formaldehyde-induced GSH export from OLN-93 cells is caused by an activation of the transporter which may involve a covalent reversible modification or by non-covalent binding of a formaldehyde-conjugate to the transporter. Alternatively, a formaldehyde-induced translocation of Mrp1 from intracellular vesicles to the plasma membrane, as previously reported for the bilirubin-treated astrocytes (Gennuso et al., 2004), could also be responsible for the formaldehyde-induced GSH export from OLN-93 cells.

Previously, we have hypothesized that a labile formaldehyde-GSH conjugate could be the substrate exported by Mrp1 rather than free GSH, thereby strongly accelerating GSH export in formaldehyde-treated astrocytes (Tulpule and Dringen, 2011). The reported K_m value of Mrp1 for GSH export is above 5 mM, while GSH conjugates are exported by this transporter with K_m values below 1 mM (Cole and Deeley, 2006; Deeley and Cole, 2006). The determined K_m values for GSH export for cultured astrocytes (100 nmol/mg) corresponds to a cytosolic GSH concentration of about 25 mM, as calculated by using a specific cytosolic volume of 4.1 μ L/mg protein (Dringen and Hamprecht, 1998). Thus, the remaining high K_m value observed for GSH export from formaldehyde-treated astrocytes suggests that an export of a labile formaldehyde-GSH conjugate is not involved in the formaldehyde-stimulated Mrp1-mediated GSH export from astrocytes. Due to the almost identical kinetic parameters for GSH export, these considerations for cultured astrocytes can also be applied to OLN-93 cells.

In conclusion, the presence of formaldehyde induces a rapid GSH export from viable OLN-93 cells which is most likely mediated by Mrp1. Further experiments should be performed on cultured oligodendrocytes and oligodendrocyte precursor cells to verify that the observed consequences of a formaldehyde treatment of OLN-93 cells are also valid for cultured oligodendrocytes. Assuming that presence of formaldehyde would also stimulate GSH export from oligodendrocytes in brain, a formaldehyde-induced loss of GSH would lower their antioxidative and detoxifying potential which could compromise their functions. Since oligodendrocytes play as myelinating cells an important role in learning and cognition (Fields, 2008; Huang et al., 2009), a formaldehyde-induced dysfunction of oligodendrocytes could contribute to the reported deficits in memory and learning after formaldehyde exposure (Lu et al., 2008; Malek et al., 2003; Tong et al., 2011).

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Formaldehyde metabolism and formaldehyde-induced alterations in glucose and glutathione metabolism of cultured neurons

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 - Design of the study
 - Experimental work
 - Preparation of first draft of the manuscript
- Michaela C. Hohnholt prepared the neuronal cultures

Formaldehyde metabolism and formaldehyde-induced alterations in glucose and glutathione metabolism of cultured neurons

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Running title: formaldehyde and neurons

Abbreviations: AD, Alzheimer's disease; ADH, alcohol dehydrogenase, ALDH, aldehyde dehydrogenase; APC, astrocyte-rich primary culture; BSA, bovine serum albumin; CGNC, cerebellar granule neuron culture; GSH, glutathione; GSSG, glutathione disulfide; GSx, total glutathione; IB, incubation buffer; LDH, lactate dehydrogenase; Mrp, multidrug resistance protein; MTHFD, methylene tetrahydrofolate dehydrogenase; PBS, phosphate buffered saline; PI, propidium iodide; SSAO, semicarbazide-sensitive amine oxidase, THF, tetrahydrofolate.

Abstract

Formaldehyde is endogenously produced in the human body and brain levels of this compound are elevated in neurodegenerative conditions. Although the toxic potential of an excess of formaldehyde has been studied, little is known on the molecular mechanisms underlying its neurotoxicity as well as on the ability of neurons to metabolize formaldehyde. To address these topics we have used cerebellar granule neuron cultures as model system. These cultures express mRNAs of various enzymes that are involved in formaldehyde metabolism and were remarkably resistant towards acute formaldehyde toxicity. Cerebellar granule neurons metabolized formaldehyde with a rate of around 200 nmol/(h x mg) which was accompanied by significant increases in the cellular and extracellular concentrations of formate. In addition, formaldehyde application almost doubled the rate of lactate release from viable neurons and strongly accelerated the export of the antioxidant glutathione. The latter process was completely prevented by inhibition of the known glutathione exporter multidrug resistance protein 1. These data indicate that cerebellar granule neurons are capable of metabolizing formaldehyde and that the neuronal glucose and glutathione metabolism is severely affected by the presence of formaldehyde.

Keywords: cerebellar granule neurons, formate, GSH, lactate, metabolism

Introduction

Formaldehyde is the simplest aldehyde that shows high reactivity towards cellular macromolecules like DNA and proteins (Lu *et al.* 2010, Ospina *et al.* 2011). Formaldehyde is not only ubiquitously present as an environment pollutant (de Groot *et al.* 2009, Dhahreshwar and Stella 2008), but is also generated in substantial amounts in the human body by enzyme-catalyzed reactions (Cloos *et al.* 2008, Hou and Yu 2010, O'Sullivan *et al.* 2004). However, owing to formaldehyde detoxification by cellular enzymes (MacAllister *et al.* 2011, Friedenson 2011), a steady state balance between formaldehyde-generating and -disposing processes is established that leads to a normal blood formaldehyde concentration of around 0.1 mM (Heck and Casanova 2004).

Formaldehyde-generating processes include methanol oxidation, methylamine deamination and histone demethylation (Cloos *et al.* 2008, Hou and Yu 2010, Lee *et al.* 2008, O'Sullivan *et al.* 2004), while formaldehyde disposal occurs either by reduction to methanol via the cytosolic alcohol dehydrogenase (ADH) 1 (MacAllister *et al.* 2011, Friedenson 2011) or by oxidation to formate (Fig. 1). Formaldehyde oxidation is catalyzed by cytosolic ADH3 or by mitochondrial aldehyde dehydrogenase (ALDH) 2 (Friedenson 2011, MacAllister *et al.* 2011). While ALDH2 acts directly on formaldehyde, the substrate for ADH3 is a formaldehyde-glutathione (GSH) adduct that is formed in an enzyme-independent reaction (MacAllister *et al.* 2011, Staab *et al.* 2009, Thompson *et al.* 2010). The formaldehyde-derived formate can either be exported from the cells via gamma-amino butyric acid receptors (Mason *et al.* 1990) and the monocarboxylate transporter 1 (Moschen *et al.* 2012) or can undergo further oxidation to CO₂ (Fig. 1). Formate oxidation is a tetrahydrofolate (THF)-dependent two-step process where formate first reacts with THF to form 10-formyl THF in an ATP-dependent enzymatic reaction catalyzed by the cytosolic methylene THF dehydrogenase (MTHFD) 1 or by the mitochondrial MTHFD1L (Krupenko *et al.* 2010, Krupenko 2009) (Fig. 1). Subsequently, the cytosolic ALDH1L1 or its mitochondrial isoform ALDH1L2 oxidize 10-formyl THF to generate carbon dioxide (Krupenko *et al.* 2010, Krupenko 2009) (Fig. 1). To a

lesser extent, catalase can also contribute to formate oxidation in a THF-independent manner (Skrzydłowska 2003).

The concentration of formaldehyde in the healthy human brain has been reported to be around 0.2 mM and 0.4 mM in the hippocampus and cortex, respectively (Tong *et al.* 2012). However, formaldehyde levels in the brain have been shown to increase even further with age and in Alzheimer's disease (AD) (Tong *et al.* 2012, Tong *et al.* 2011). The reason for these increases in formaldehyde concentrations could be the elevated expression of formaldehyde-generating enzymes, as reported for the methylamine deaminating semicarbazide-sensitive amine oxidase (SSAO) in AD (del Mar Hernandez *et al.* 2005, Ferrer *et al.* 2002, Unzeta *et al.* 2007). Moreover, during ageing increased activity of carboxymethyl esterase, an enzyme generating methanol by demethylation of post-translationally modified proteins, has been observed in the brain (Sellinger *et al.* 1988) which may foster elevated formation of formaldehyde by providing the substrate for methanol oxidation.

Animal studies have demonstrated that formaldehyde has toxic effects on the nervous system, causes oxidative damage to the brain and compromises memory and learning (Songur *et al.* 2010, Tong *et al.* 2012, Tong *et al.* 2011). However, there is limited information on the formaldehyde metabolism in brain cells and on the molecular mechanisms underlying its neurotoxicity. Recently we have shown that cultured astrocytes efficiently oxidize formaldehyde to formate and that formaldehyde exposure accelerates glycolytic flux and GSH export from glial cells (Tulpule and Dringen 2012, Tulpule and Dringen 2011, Tulpule *et al.* 2012). Here we show that also primary cerebellar granule neurons have the potential to oxidize formaldehyde and that presence of formaldehyde accelerates lactate production and multidrug resistance protein (Mrp) 1-mediated GSH export from viable neurons.

Materials and Methods

Materials

Fetal calf serum and penicillin/streptomycin solution were purchased from Biochrom (Berlin, Germany), minimal essential medium from Gibco Life Technologies (Darmstadt, Germany) and Earle's balanced salt solution from Life Technologies-Invitrogen (Darmstadt, Germany). Cytosine β -D-arabinofuranoside, poly-D-lysine and soybean trypsin inhibitor were obtained from Sigma-Aldrich (Steinheim, Germany). Formaldehyde was purchased from Merck (Darmstadt, Germany). Bovine serum albumin (BSA), NAD⁺, NADH and NADPH were from Applichem (Darmstadt, Germany). The enzymes glutamate pyruvate transaminase, glutathione reductase and lactate dehydrogenase (LDH) were from Roche (Mannheim, Germany), while formaldehyde dehydrogenase and formate dehydrogenase were from Sigma-Aldrich (Steinheim, Germany). Primers for RT-PCRs were purchased from MWG Biotech (Ebersberg, Germany), RedSafe™ from HiSS Diagnostics (Freiburg, Germany) and the RNeasy® Mini Kit from Qiagen (Hilden, Germany). Deoxyribonucleoside triphosphate (dNTP) mix, DNA loading dye, GeneRuler™, 50 bp DNA ladder, RevertAid™ H Minus First Strand cDNA Synthesis Kit, magnesium chloride, Taq buffer and Taq polymerase were purchased from Fermentas (St. Leon-Rot, Germany). Other chemicals of the highest purity available were from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany). 96-well microtitre plates (transparent and black) and 24-well plates were from Nunc (Wiesbaden, Germany).

Cell cultures

Cerebellar granule neuron-rich cultures were prepared from the brains of 7-8 day old Wistar rats according to a method described by Anggono *et al.* (2008). Briefly, the cerebellae were dissected and a single cell suspension was produced by tryptic digestion with 2.5% (w/v) trypsin in phosphate buffered saline (PBS; 10 mM potassium phosphate buffer, pH 7.4, containing 150 mM NaCl) containing 14 mM glucose, 60 mM MgSO₄ and 0.3% (w/v) BSA followed by manual trituration with glass pipettes. In wells of poly-D-lysine coated 24-well

dishes, 0.75 million cells were seeded in 1 mL of culture medium (90% minimal essential medium, 10% heat-inactivated fetal calf serum, 30 mM D-glucose, 25 mM KCl, 2 mM L-glutamine, 200 U/L penicillin G and 200 µg/L streptomycin sulfate). The cells were cultured at 37°C in a humidified atmosphere with 5% CO₂ in a cell incubator (Sanyo, Osaka, Japan). After one day in culture the medium was replaced by culture medium containing 10 µM cytosine β-D-arabinofuranoside. The cells were used for experiments at a culture age between 7 and 10 days.

Astrocyte-rich primary cultures were prepared from the brains of newborn Wistar rats (Hamprecht and Löffler 1985). Cells were seeded in culture medium in 24-well plates (0.3 million cells per 1 mL) and incubated in the humidified atmosphere of a Sanyo (Osaka, Japan) incubator with 10% CO₂. The culture medium was renewed every seventh day and the cultures were used for experiments at an age between 15 and 21 days.

Experimental incubation of cells

If not stated otherwise, cultured neurons were washed with 1 mL pre-warmed (37°C) incubation buffer (IB; 145 mM NaCl, 30.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 0.8 mM Na₂HPO₄, 20 mM HEPES, 5 mM glucose, pH 7.4) and exposed to formaldehyde in 200 µL IB containing formaldehyde and/or other compounds at 37°C. For incubations at 4°C, cells were washed with ice-cold (4°C) IB and incubated with 200 µL formaldehyde-containing IB at 4°C. The experimental incubations were terminated by collecting the incubation media and by washing the cells with 1 mL of ice-cold PBS. For experiments investigating the requirement of formaldehyde for the stimulated GSH export, the pre-incubation medium was aspirated, cells were washed once with 1 mL pre-warmed (37°C) IB and incubated further with 200 µL IB containing the indicated compounds. At 1 h intervals, 10 µL media samples were harvested to determine total glutathione (GSx) contents.

Determination of cell viability and protein content

Release of the cytosolic enzyme LDH and the membrane permeability for the fluorescent dye propidium iodide (PI) were assessed to determine compromised cell viability. LDH activity in cell lysates and media was measured using a microtitre plate-based photometric assay (Dringen *et al.* 1998). As a modification, some media samples were treated with Triton X-100 (final concentration of 0.5% (v/v)) to lyse potentially detached cells with intact cell membranes. PI staining of the cells was performed as previously described (Scheiber *et al.* 2010). The protein content of the cultured cells was determined after lysing of the cells with 200 μ L 0.5 M NaOH by the Lowry method (Lowry *et al.* 1951), using BSA as a standard protein. The neuron cultures contained an average protein content of 51 ± 10 μ g per well.

Determination of formaldehyde, formate, lactate and glutathione

The concentrations of formaldehyde, formate and lactate were determined by enzymatic assay systems as recently described (Liddell *et al.* 2009, Schmidt and Dringen 2009, Tulpule and Dringen 2012). Total glutathione contents (GSx = amount of GSH plus twice the amount of glutathione disulfide (GSSG)) and GSSG contents in cells and media were determined by the colorimetric Tietze cycling assay in microtitre plates (Hirrlinger and Dringen 2005).

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Cells seeded in 24-well plates were used for extraction of total RNA. The RNeasy[®] Mini Kit and the RevertAid[™] H Minus First Strand cDNA Synthesis Kit were used for extraction of RNA and its reverse transcription for synthesis of cDNA following the instructions provided by the supplier. PCRs were performed as previously described (Tulpule *et al.* 2010) using the primer sets and annealing temperatures listed in Table 1. Gel electrophoresis was performed in a 2.5% agarose gel containing 1x RedSafe[™].

Presentation of data

Data are presented as means \pm SD of values that were obtained in n experiments (given in the panels of the figures) performed on independently generated cultures. Pictures showing PI stainings or RT-PCRs were taken from representative experiments that were reproduced at least twice with comparable outcomes. The analysis of significance between groups of data was performed by ANOVA followed by the Bonferroni *post-hoc* tests with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Significance of differences between 2 data sets was analyzed with the paired t-test and is indicated by # $p < 0.05$, ## $p < 0.01$ or ### $p < 0.001$. $p > 0.05$ was considered as not significant.

Results

Viability of cultured neurons after exposure to formaldehyde

To test for potential neurotoxic effects of formaldehyde, cultured cerebellar granule neurons were incubated with formaldehyde in concentrations of up to 1 mM for up to 6 h (Fig. 2). Exposure of the cells to formaldehyde for 2 h did not significantly elevate the activity of LDH in the medium compared to controls, irrespective of whether the medium was treated with Triton X-100 or not (Fig. 2A). Accordingly, only a low number of PI-positive cells were found after a 2 h treatment without or with formaldehyde in concentrations of up to 1 mM (Fig. 2I-L). In contrast, all neurons in cultures that had been treated with 100 μ M silver nitrate for 2 h or 6 h (positive control for toxicity) were PI-positive (Fig. 2H,M,R,W). Also, an incubation of neurons for 6 h with 0.1 mM formaldehyde did not compromise cell viability (Fig. 2B,C,T), while exposure of the cells to 0.5 mM or 1 mM formaldehyde caused a small but significant increase in the LDH activity in Triton X-100-treated media (Fig. 2B) and a matching loss in cellular LDH activity (Fig. 2C). However, exposure of the cells to 0.5 mM or 1 mM formaldehyde did not severely increase the number of PI-positive neurons (Fig. 2U,V), suggesting that the membranes of the cells that remained attached to the well under those conditions were intact.

Formaldehyde clearance by neurons

The observed resistance of neurons to formaldehyde toxicity could be associated with their capacity to metabolize the applied formaldehyde. RT-PCRs were performed to test for expression in cerebellar granule neuron cultures (as well as in astrocyte primary cultures as control cells) of the enzymes involved in formaldehyde metabolism (Fig. 1). Indeed, bands of the expected sizes (Table 1) were observed for mRNAs of formaldehyde-metabolizing enzymes for both cell culture types (Fig. 3), suggesting that neurons may indeed have the capacity to reduce formaldehyde to methanol and/or to oxidize formaldehyde via formate to CO₂ (Fig. 1). Among the investigated enzymes, at best a very weak signal was

observed for the mRNA of ALDH1L1 in cultured neurons, while this band was clearly detectable for astrocyte cultures (Fig. 3).

To test whether neurons are indeed able to oxidize formaldehyde, cerebellar granule neurons were exposed to 0.5 mM formaldehyde for up to 3 h and formaldehyde and formate concentrations were determined in the media (Fig. 4A, circles). A control incubation without cells but otherwise under identical conditions revealed that hardly any formaldehyde was lost from the medium by evaporation or cell-independent oxidation to formate (Fig. 4A, triangles). In contrast, a decline in the extracellular formaldehyde concentration and an increase in the concentration of formate in the medium were observed on incubation of neurons with formaldehyde (Fig. 4A). The linear changes in the concentrations of formaldehyde and formate in the medium were used to calculate formaldehyde clearance and formate release rates of 210 ± 24 nmol/(h x mg) and 51 ± 10 nmol/(h x mg), respectively (Fig. 4B). Treatment of neurons with 0.5 mM formaldehyde for 2 h also increased the cellular level of formate significantly from a basal level of 4 ± 3 nmol/mg to 17 ± 3 nmol/mg (Fig. 4C). Under these conditions a reduction of formaldehyde is unlikely to contribute to the disappearance of formaldehyde, since no increase in the concentration of extracellular methanol was observed (data not shown).

Formaldehyde forms adducts with cellular macromolecules like proteins and DNA (Ospina *et al.* 2011, Lu *et al.* 2010). Such processes could contribute to the decline in detectable formaldehyde observed for cultured neurons. To differentiate between a chemical reaction of formaldehyde with macromolecules and metabolic formaldehyde oxidation, the cells were incubated with 0.5 mM formaldehyde for 2 h at 37°C or 4°C, as the low temperature is known to completely prevent cellular formaldehyde metabolism but not its chemical reactivity (Tulpule and Dringen 2012). Formate was not detectable in the medium of cells that had been exposed to formaldehyde at 4°C (Fig. 4D), while the disappearance of formaldehyde was lowered by around 50% compared to cells that had been exposed to the aldehyde at 37°C (Fig. 4D). The cell viability was not affected by incubation of cells with formaldehyde at these temperatures (data not shown).

Effect of formaldehyde exposure on lactate production by neurons

Formate accumulating within cells has the potential to affect ATP production as formate inhibits cytochrome c oxidase (Wallace *et al.* 1997, Nicholls 1975) which in turn may stimulate glycolysis (Walz and Mukerji 1988). To test whether exposure of neurons to formaldehyde affects cellular glucose metabolism, cerebellar granule neurons were treated with formaldehyde in a concentration of 0.5 mM. Already after 15 min of incubation a significantly elevated extracellular lactate concentration was determined for formaldehyde-treated cells compared to control neurons (Fig. 5A). Calculation of the lactate release rates (Fig. 5B) from the linear increase in extracellular lactate contents revealed that presence of formaldehyde almost doubled the lactate release rate compared to control cells from 0.89 ± 0.16 to 1.57 ± 0.27 $\mu\text{mol}/(\text{h} \times \text{mg})$ (Fig. 5B).

GSH export from formaldehyde-treated neurons

Formaldehyde treatment has been reported to stimulate GSH export from glial cells (Tulpule and Dringen 2011, Tulpule *et al.* 2012). Similarly, the application of formaldehyde to neurons caused a time- and concentration-dependent increase in extracellular GSx content (Fig. 6A,D) that was accompanied by a matching decline in cellular GSx contents (Fig. 6B,E), while the sum of cellular plus extracellular GSx remained almost constant for all the conditions used (Fig. 6C,F). In the absence of formaldehyde, cultured neurons exported, at best, low amounts of GSH while upon application of formaldehyde a rapid extracellular GSx accumulation was observed for the first 2 h of incubation which slowed down thereafter (Fig. 6A). Half-maximal effects on the cellular and extracellular GSx contents of neurons after incubation for 2 h were found for around 0.3 mM formaldehyde, while presence of 1 mM formaldehyde induced maximal effects (Fig. 6D,E).

Formaldehyde-accelerated GSH export from glial cells is mediated by Mrp1 (Tulpule and Dringen 2011, Tulpule *et al.* 2012). Mrp 1 transports anionic compounds such as GSH and conjugates of GSH in an ATP-dependent manner

(Keppler 2011). Since cultured neurons contain Mrp1 mRNA (data not shown), Mrp1 was considered as a potential transporter to mediate formaldehyde-induced GSH export from neurons. Indeed, presence of the Mrp1 inhibitor MK571 (Hirrlinger *et al.* 2002, Minich *et al.* 2006) almost completely prevented the formaldehyde-induced extracellular accumulation of GSx (Fig. 7A) and the corresponding cellular decline of GSx (Fig. 7B), while the sum of cellular plus extracellular GSx was not affected by the absence or presence of formaldehyde and/or MK571 (Fig. 7C). Quantification of GSSG content which contributes to the determined GSx amounts revealed that the very low GSSG levels in cerebellar granule neurons or their media were not increased after exposure of the cells to formaldehyde and/or MK571 (Fig. 7). None of these conditions compromised the cell viability as indicated by the absence of any significant increase in extracellular LDH activity (data not shown).

To investigate whether the formaldehyde-induced GSH export depends on the presence of formaldehyde, neurons were pre-incubated without or with 0.5 mM formaldehyde for 1 h after which the cells were exposed to fresh medium containing no or 0.5 mM formaldehyde (Fig. 8). The accelerated GSH export was only maintained by cells that were exposed to formaldehyde during the main incubation (Fig. 8A, filled triangles), while removal of formaldehyde (Fig. 8A, open triangles) slowed the further increase of extracellular GSx contents to values that were comparable to those of control cells that had no contact to formaldehyde (Fig. 8, circles). This is also reflected by the export rates calculated for the main incubation (Fig. 8B). The GSH export of cells that had been exposed to formaldehyde only during the pre-incubation did not significantly differ from the values determined for cells that had no contact with formaldehyde (about 1.5 nmol/(h x mg)), while the presence of 0.5 mM formaldehyde during the main incubation increased the GSH efflux rate by three-fold (Fig. 8B).

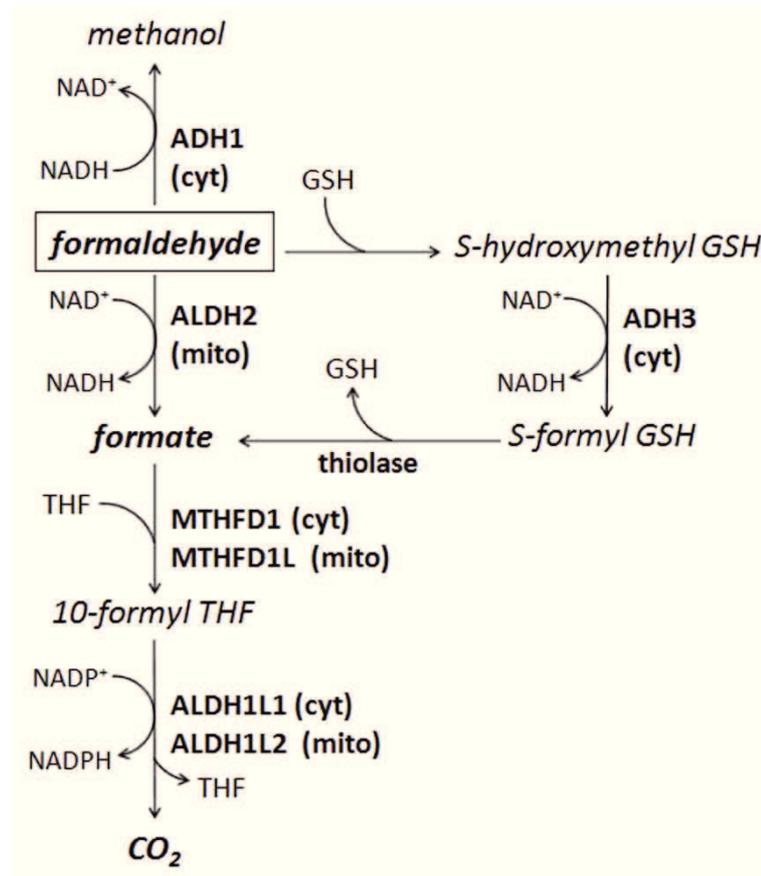


Figure 1: Formaldehyde metabolism. Formaldehyde can be reduced to methanol by alcohol dehydrogenase (ADH) 1 or oxidized to formate by cytosolic (cyt) glutathione (GSH)-dependent ADH 3 or by the mitochondrial (mito) aldehyde dehydrogenase (ALDH) 2. Further oxidation of formate to CO_2 involves the formation of 10-formyl tetrahydrofolate (THF) by the cytosolic methylene tetrahydrofolate dehydrogenase (MTHFD) 1 or the mitochondrial MTHFD1L and the subsequent oxidation of 10-formyl THF by the cytosolic 10-formyl THF dehydrogenase ALDH1L1 or the mitochondrial ALDH1L2 to CO_2 .

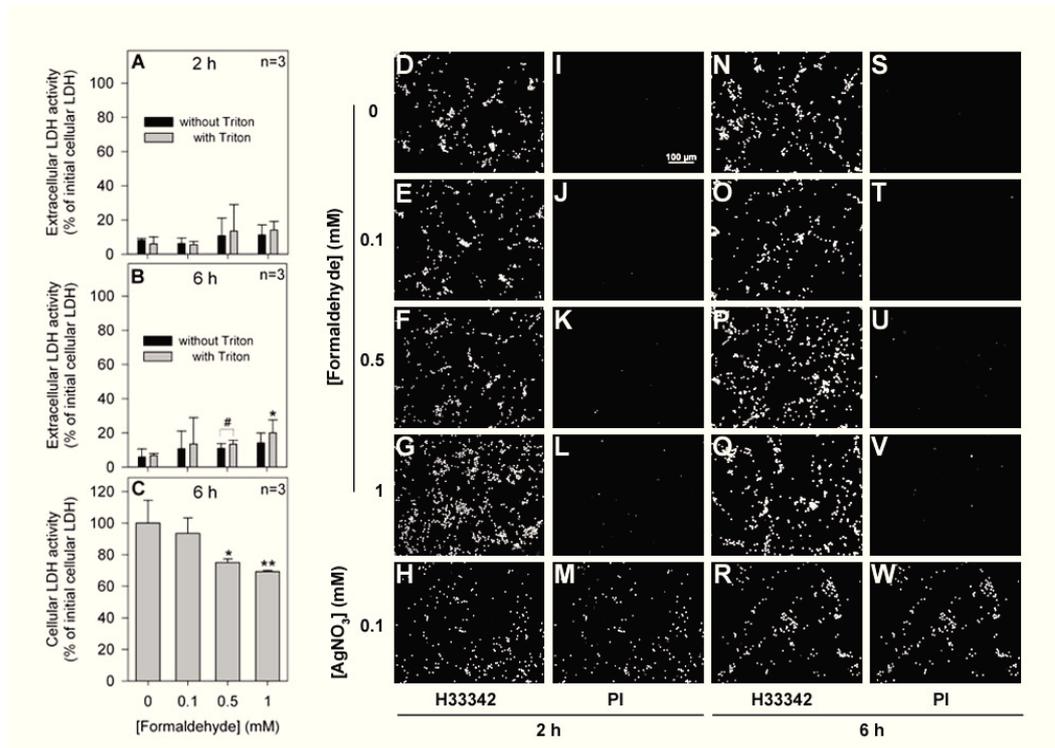


Figure 2: Formaldehyde toxicity in cultured neurons. Cells were incubated with the indicated concentrations of formaldehyde for up to 6 h. LDH activity in untreated or Triton X-100-treated media was determined after 2 h (A) or 6 h (B) while that in cells (C) was measured after 6 h of incubation. The nuclei of cells that had been treated for 2 h or 6 h with formaldehyde in the indicated concentrations (D-G, I-L, N-Q, S-V) or with 100 μ M AgNO₃ (positive control, H,M,R,W) were stained with Hoechst 33342 (D-H, N-R), while those of cells with impaired membrane integrity were positive for propidium iodide (PI) staining (I-M, S-W). The scale bar in panel I applies to the panels D-W. In A-C, the significance of differences compared to controls (ANOVA) is indicated by * $p < 0.05$ or ** $p < 0.01$ while that between treatment of media samples with or without Triton X-100 (paired t-test) is indicated by # $p < 0.05$.

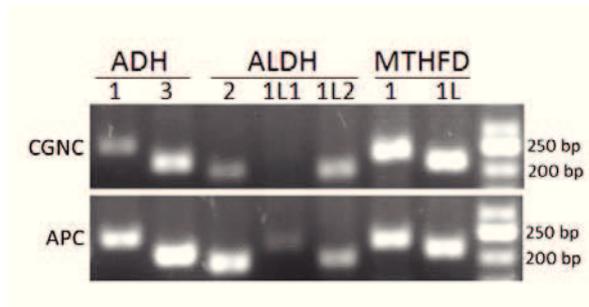


Figure 3: RT-PCR analysis of expression of enzymes involved in formaldehyde and formate metabolism. mRNAs harvested from cerebellar granule neurons culture (CGNC) or astrocyte-rich primary culture (APC) were used as source to generate specific amplification products that demonstrate the presence of mRNAs of the indicated alcohol dehydrogenases (ADH), aldehyde dehydrogenases (ALDH) or methylene tetrahydrofolate dehydrogenases (MTHFD).

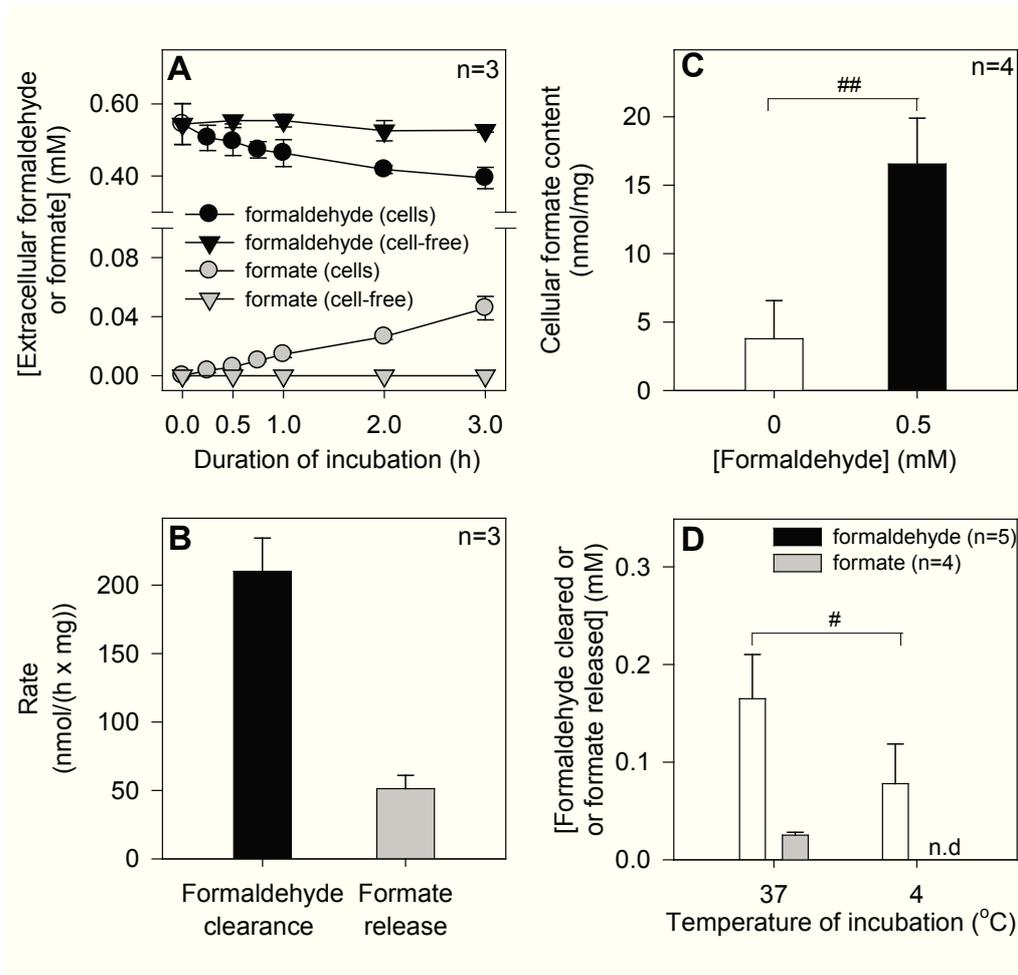


Figure 4: Formaldehyde metabolism in cultured neurons. A: Cells (circles) or cell-free wells (triangles) were incubated with 0.5 mM formaldehyde for up to 3 h and the concentration of formaldehyde and formate in the medium was determined. **B:** Rates of formaldehyde clearance and formate release as calculated from the slopes of the decrease and increase in extracellular formaldehyde and formate concentrations (A), respectively. **C:** Cellular content of formate in neurons that had been treated without or with 0.5 mM formaldehyde for 2 h. **D:** Concentrations of disposed formaldehyde and released formate after a 2 h incubation of cells with 0.5 mM formaldehyde at 37°C or 4°C. In C and D the significance of differences (paired t-test) between the indicated conditions is indicated by #p<0.05 or ##p<0.01. n.d, not detectable.

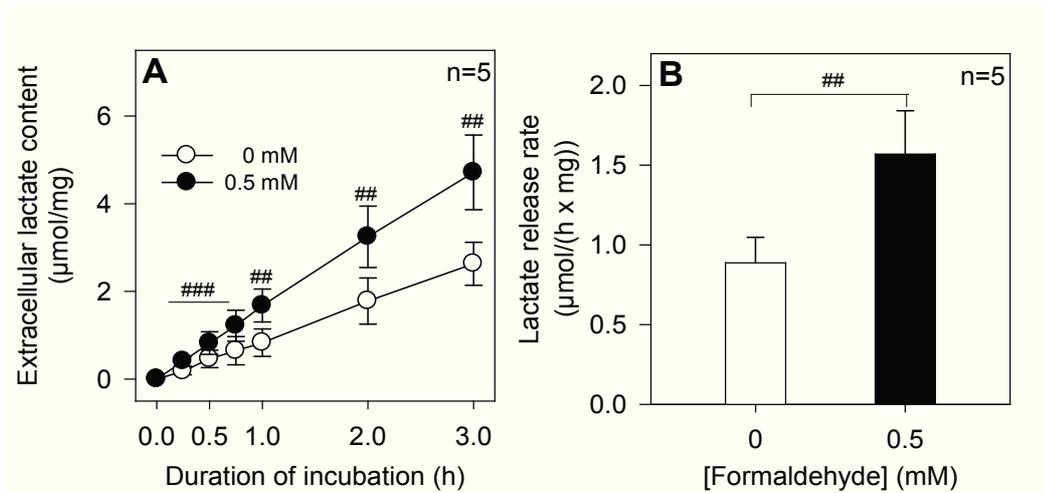


Figure 5: Formaldehyde treatment enhances lactate release from cultured neurons. The cells were incubated without or with 0.5 mM formaldehyde for up to 3 h and the lactate content in the medium was measured (A). The rates of lactate release (B) was calculated from the slopes of the linear increases in extracellular lactate contents shown in panel A. The significance of differences (paired t-test) between the data obtained for cells incubated in the absence or presence of formaldehyde is indicated as ##p<0.01 or ###p<0.001.

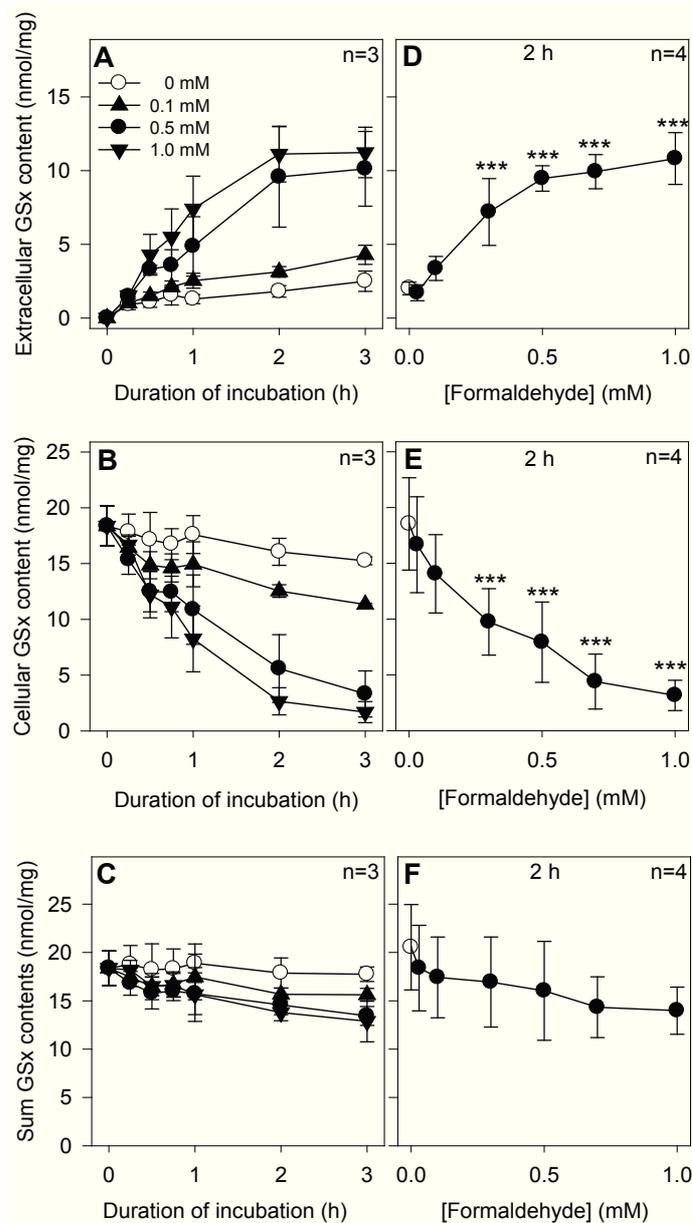


Figure 6: Formaldehyde exposure accelerates GSH export from cultured neurons. The cells were incubated for up to 3 h (A-C) without or with formaldehyde in the concentrations indicated in panel A or for 2 h (D-F) with formaldehyde in concentrations of up to 1 mM. The extracellular (A,D) and cellular (B,E) GSx contents were determined and the sum of GSx contents (C,F) was calculated for the respective conditions. The initial cellular GSx contents were 18.4 ± 1.8 (A-C) and 20.5 ± 4.4 (D-F) nmol per mg. The significance of differences (ANOVA) compared to control (absence of formaldehyde) is indicated by *** $p < 0.001$.

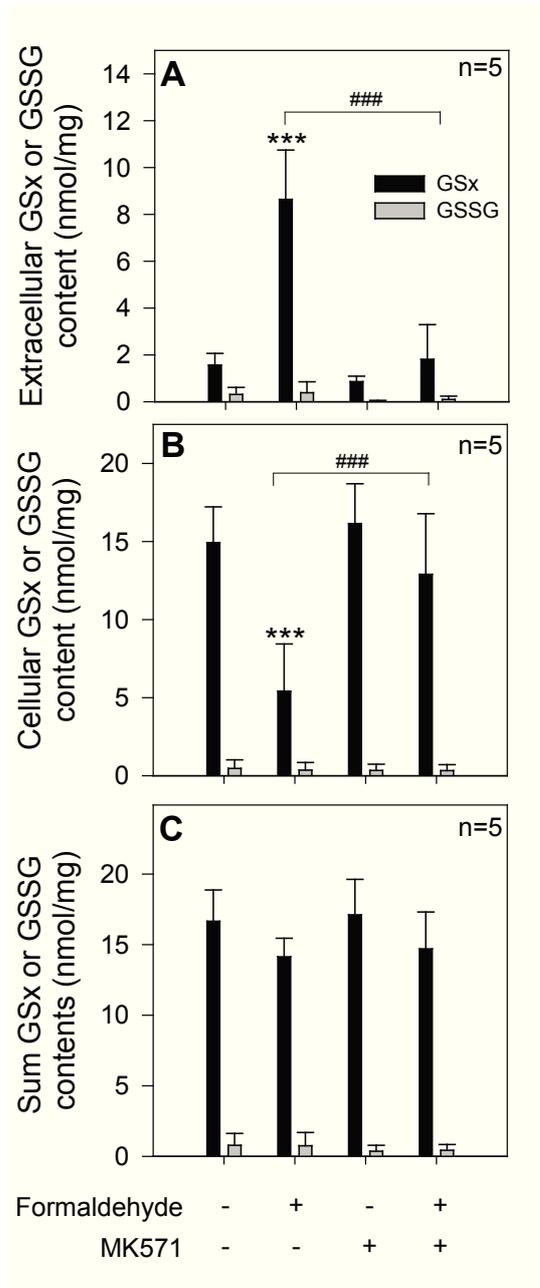


Figure 7: Prevention of formaldehyde-stimulated GSH efflux by Mrp1 inhibition.

Cultured neurons were treated without or with 0.5 mM formaldehyde in the absence or the presence of 50 μ M MK571 for 2 h. The extracellular (A) and cellular (B) GSx and GSSG contents were measured after the incubation and the sum of GSx (C) was calculated for each condition. The initial cellular GSx content was 19.2 ± 3.2 nmol per mg. The significance of differences (ANOVA) compared to control (absence of formaldehyde and MK571) is indicated by $***p < 0.001$, while that between formaldehyde treatment in the presence or absence of MK571 (paired t-test) is indicated by $###p < 0.001$.

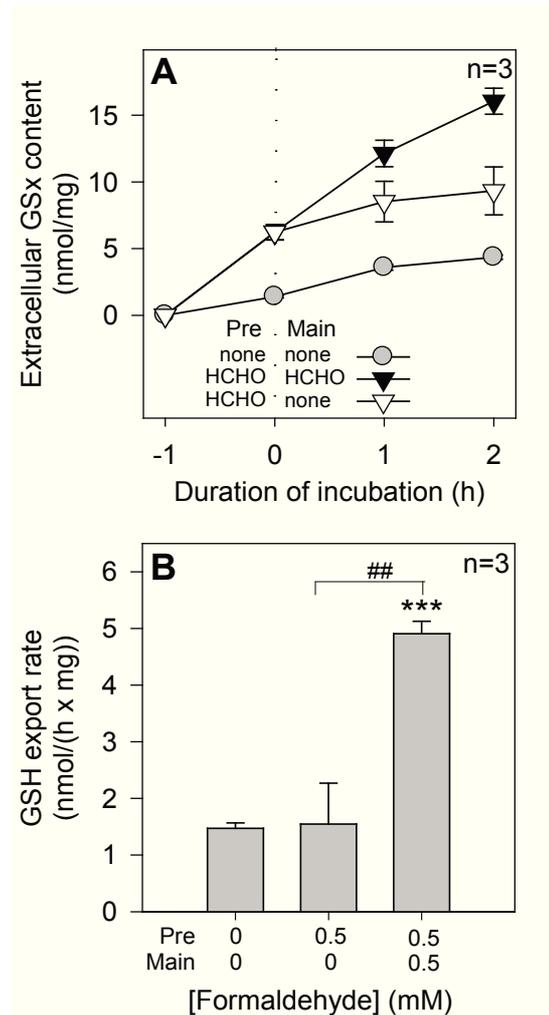


Figure 8: Removal of formaldehyde abolishes the formaldehyde-stimulated GSH efflux. Cultured neurons were pre-incubated for 1 h in the presence or absence of 0.5 mM formaldehyde. The pre-incubation medium was removed and cells were incubated with fresh medium containing no or 0.5 mM formaldehyde for further 2 h. Media samples were harvested at the indicated incubation periods and the extracellular GSx contents were determined. The GSH export rates (B) were calculated from the slopes of extracellular GSx accumulation during the main incubation (A). The initial cellular GSx content was 27.9 ± 2.9 nmol per mg. The significance of differences (ANOVA) compared to controls (absence of formaldehyde in pre- and main-incubation) is indicated by $***p < 0.001$ while that between main incubation in the presence or absence of formaldehyde (paired t-test) is indicated by $##p < 0.01$.

Table 1: Primer sets and annealing temperatures used for reverse transcription PCRs.

mRNA	Primers	Annealing temperature (°C)	Amplicon size (bp)
ADH1	5'-AGCCCATTCACCACTTCATC-3' 5'-GGCTGCTCCTGCTGTTTAC-3'	60	248
ADH3	5'-CAGCACGTTTTCCGAGTACA-3' 5'-GCCACTTTACAGCCCATGAT-3'	60	213
ALDH2	5'-GACCTGGACAAGGCCAATTA-3' 5'-TCTTCTGTGGCACTTTGACG-3'	60	193
ALDH1L1	5'-GTGCCGTGTTCAAGTTTCCT-3' 5'-CCATGAATGAGGGTCCAGTT-3'	60	226
ALDH1L2	5'-CTCAGGGGTCTTCACGAGAG-3' 5'-CGAGGGTCACTGTTTTGGTT-3'	60	197
MTHFD1	5'-CATGTGGTGGTTCTTGTTC-3' 5'-AGGTCCAGCTCAGCATCTGT-3'	58	230
MTHFD1L	5'-AATGTTTTGTTTCGCCCTTG-3' 5'-CACAATGTCTGCCTCTCGAA-3'	55	212

Discussion

Cultured cerebellar granule neurons were used as a model system to investigate the consequences of a treatment of neurons with formaldehyde. Exposure of these cells to formaldehyde in concentrations of up to 1 mM was not acutely toxic. This resistance of cultured cerebellar granule neurons against toxicity of low concentrations of formaldehyde for short time-frames is consistent with literature data for cortical neurons (Song *et al.* 2010), PC12 cells (Lee *et al.* 2008), astrocytes (Tulpule and Dringen 2011, Tulpule and Dringen 2012) and oligodendroglial OLN-93 cells (Tulpule *et al.* 2012). Cell viability of cerebellar granule neurons was only compromised during prolonged exposure of the cells to formaldehyde which may be caused by the observed alterations in cellular metabolism of formaldehyde-treated neurons and/or by the impairment of cellular functions due to its reactivity with cellular macromolecules (Lu *et al.* 2010, Ospina *et al.* 2011). Animal experiments have revealed that formaldehyde causes neuronal damage by oxidative stress (Gurel *et al.* 2005, Zararsiz *et al.* 2006, Zararsiz *et al.* 2007, Zararsiz *et al.* 2011). However, at least for viable neurons formaldehyde exposure did not increase the cellular or extracellular levels of GSSG, suggesting that cultured cerebellar granule neurons did not suffer from severe oxidative damage under the conditions used here.

Rapid metabolism of formaldehyde can be excluded as potential reason for the relative resistance of cerebellar granule neurons against formaldehyde toxicity, as even after 3 h exposure still around 70% of the applied formaldehyde was detectable in the incubation medium. Nevertheless, cultured cerebellar granule neurons have the potential to metabolize formaldehyde and also consistently express the mRNAs for enzymes known to contribute to formaldehyde reduction to methanol (ADH1) and for formaldehyde oxidation to formate (ADH3 and ALDH2), confirming literature data on the expression of these enzymes in neurons in brain sections (Galter *et al.* 2003, Martinez *et al.* 2001, Su *et al.* 2011). Also the mRNAs of the 10-formyl THF synthesizing enzymes, MTHFD1 and MTHFD1L, were found in cultures of cerebellar granule neurons. While MTHFD1 has been previously shown to be expressed in neurons (Anthony and Heintz 2007), the presence of MTHFD1L has been demonstrated at least for

adult brain tissue (Prasannan *et al.* 2003). mRNA of ALDH1L2 was detected for cultured cerebellar granule neuron and astrocytes, which is consistent with the presence of this enzyme in the brain (Krupenko *et al.* 2010). However, only a marginal expression of ALDH1L1 mRNA was found for the neuron cultures as expected from the reported highly specific expression of ALDH1L1 in astrocytes (Cahoy *et al.* 2008). The very weak signal for ALDH1L1 mRNA observed for cerebellar granule neuron cultures may result from a few contaminating astrocytes in these cultures.

Cultured cerebellar granule neurons are able to metabolize formaldehyde and cleared exogenous formaldehyde in a time- and temperature-dependent manner with a rate of about 200 nmol/(h x mg), which is similar to values recently reported for cultured astrocytes (Tulpule and Dringen 2012). Extracellular and cellular formate levels of formaldehyde-treated neurons accounted to 25% and 10%, respectively, of the formaldehyde that had disappeared within 2 h from the medium, while no methanol generation from formaldehyde was observed (data not shown). The rather low rate of formate generation by cerebellar granule neurons contrasts the situation reported for astrocytes where formaldehyde is almost quantitatively oxidized to formate (Tulpule and Dringen 2012). Evaporation of formaldehyde can be ruled out as the potential reason for the observed disappearance of formaldehyde as in the absence of neurons the decline in medium concentration of formaldehyde was only marginal. However, some interaction of formaldehyde with cellular macromolecules (Lu *et al.* 2010, Ospina *et al.* 2011) is likely to contribute to the observed loss of formaldehyde, since incubation of cells at 4°C prevented only half of the disappearance of formaldehyde, but completely prevented its oxidation to formate. In addition, since cultured neurons contain the mRNA for the enzymes responsible for formate oxidation, the complete enzymatic oxidation of some formaldehyde to CO₂ is likely to also contribute to the observed cell-dependent loss of formaldehyde.

Cultured cerebellar granule neurons produced lactate at a basal rate of around 0.8 μmol/(h x mg) which is similar to the glycolytic rates previous reported for cultured neurons (Itoh *et al.* 2003, Zwingmann and Leibfritz 2003), but lower

than that reported for cultured astrocytes (about 1.3 $\mu\text{mol}/(\text{h} \times \text{mg})$) (Tulpule and Dringen 2012, Scheiber and Dringen 2011). This was expected since astrocytes are reported to be more glycolytic than neurons (Itoh *et al.* 2003, Zwingmann and Leibfritz 2003) possibly due to an inhibited pyruvate dehydrogenase complex (Halim *et al.* 2010) and a lower capacity for NADH shuttling into the mitochondria (Berkich *et al.* 2007, Neves *et al.* 2012). Exposure of neurons to formaldehyde rapidly increased lactate release and doubled their lactate release rate. This accelerated lactate release is likely to be a consequence of the accumulation of formaldehyde-derived formate in the cells which is a known inhibitor of cytochrome c oxidase (Wallace *et al.* 1997, Nicholls 1975), as inhibition of mitochondrial respiration has been reported to accelerate glycolytic lactate production in cultured neurons (Walz and Mukerji 1988). A formaldehyde-induced stimulation of glycolytic flux has also been observed for cultured astrocytes (Tulpule and Dringen 2012). However, the onset of the increase in astrocytic lactate production was delayed by about one hour and the lactate release rate was only increased by up to 50% (Tulpule and Dringen 2012), while the extracellular lactate concentration in formaldehyde-treated neuron cultures was already significantly elevated after 15 min and the lactate release rate was doubled compared to control neurons. These differences between cultured astrocytes and neurons in the modulation of glycolytic flux after formaldehyde application may be a consequence of the more efficient formate export from astrocytes (Tulpule and Dringen 2012) and the higher basal glycolytic flux rate in astrocytes (Bolanos *et al.* 2010).

Cultured neurons release GSH only with a low specific rate, confirming literature data (Hirrlinger *et al.* 2002). However, application of formaldehyde strongly accelerated this basal GSH export in a concentration- and time-dependent manner in a process that was completely prevented by the Mrp1-inhibitor MK571, as recently also reported for cultured glial cells (Tulpule and Dringen 2011, Tulpule *et al.* 2012). Thus, formaldehyde-induced stimulation of Mrp1-mediated GSH export appears to be a common feature of neural cells. As formaldehyde application increases the V_{max} -value of the GSH export from astrocytes 10-fold without changing the K_{m} -value, a formaldehyde-induced

activation of Mrp1 and/or recruitment of additional transporters to the plasma membrane have been suggested as reasons for the accelerated GSH export (Tulpule *et al.* 2012). Such processes may also be involved in the accelerated Mrp1-mediated GSH export from formaldehyde-treated neurons. Furthermore, the observation that the formaldehyde-induced effect on Mrp1-mediated GSH transport is terminated by removing formaldehyde suggests that a rapidly reversible process is involved in the stimulated GSH export from neurons that requires the acute presence of formaldehyde.

The concentrations of formaldehyde that were effective in stimulating glycolytic lactate production and GSH export and to generate formate in cultured neurons (present report) or cultured glial cells (Tulpule and Dringen 2012, Tulpule and Dringen 2011, Tulpule *et al.* 2012) are in the range of formaldehyde concentration that have been reported for blood (0.1 mM), brain cortex (0.4 mM) and hippocampus (0.2 mM) (Tong *et al.* 2012, Tong *et al.* 2011, Heck and Casanova 2004). Thus, the observed metabolic consequences of an exposure of brain cells to formaldehyde are likely to be relevant for the *in vivo* situation, especially under conditions that have been connected with elevated brain formaldehyde levels such as ageing, AD or multiple sclerosis (Tong *et al.* 2012, Tong *et al.* 2011, Khokhlov *et al.* 1989 cited in Miao and He 2012, Ferrer *et al.* 2002, del Mar Hernandez *et al.* 2005, Unzeta *et al.* 2007, Airas *et al.* 2006).

The observed formaldehyde-induced alterations in the metabolism of brain cells may severely compromise brain functions. Excess of formate generation has adverse effects on brain cells as extracellular formate is known to be neurotoxic (Kapur *et al.* 2007) and elevated cellular levels of formate will lower mitochondrial ATP production (Wallace *et al.* 1997). In addition, accelerated proton-coupled export of lactate and formate will result in metabolic acidosis which is known to compromise cell viability (Rose 2010, Yao and Haddad 2004). Furthermore, accumulation of extracellular GSH and depletion of neuronal GSH has been shown to cause excitotoxicity (Regan and Guo 1999b, Lee *et al.* 2010) and a combination of extracellular GSH accumulation with energy depletion further potentiates neuronal death (Regan and Guo 1999a). Finally, acidosis may even impair neuronal restoration of GSH as a lowered pH has been

reported to inhibit GSH synthesis (Lewerenz *et al.* 2010). Thus, elevated formaldehyde generation, formaldehyde-derived formate generation as well as formaldehyde-induced alterations in glucose and GSH metabolism in brain are likely to trigger a number of events that will synergistically compromise viability and functions of brain cells. These processes may contribute to the reported formaldehyde-induced oxidative damage, neurotoxicity and impaired cognitive potential (Songur *et al.* 2010, Tong *et al.* 2012, Tong *et al.* 2011).

In summary, cerebellar granule neurons are capable of oxidizing formaldehyde and severely increase their lactate production and GSH release during exposure to formaldehyde. Such processes may contribute to the known neurotoxic potential of formaldehyde. Since ageing and disorders such as AD and multiple sclerosis have been connected both with elevated brain formaldehyde levels (Tong *et al.* 2012, Tong *et al.* 2011, Khokhlov *et al.* 1989 cited in Miao and He 2012), and with oxidative damage and impaired energy metabolism (Leuner *et al.* 2012, Correia *et al.* 2012, Haider *et al.* 2011, Soler-Lopez *et al.* 2012, Sohal and Orr 2012), further studies are now required to investigate whether elevated brain formaldehyde levels as well as a potentially accelerated formate generation contribute to the disturbances observed in brain metabolism for these conditions.

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Conflict of interest

The author have no conflict of interest to declare.

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4.

Summarizing Discussion

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4.1. Formaldehyde-induced neurotoxicity: comparison of exogenous and endogenous sources as risk factors

Human exposure to formaldehyde can occur by inhalation, ingestion or entry through the skin (de Groot et al., 2009; Dhareshwar and Stella, 2008; NTP, 2010). One pertinent question is whether exogenous formaldehyde can pose a big threat to the CNS by entering the blood and ultimately reaching the brain on crossing the BBB. Some studies have shown that formaldehyde inhalation does not substantially increase the level of formaldehyde in blood over the homeostatic value (Franks, 2005; Heck et al., 1985; Heck et al., 1982) and the brain (Heck et al., 1982). This is not surprising since the formaldehyde-oxidizing enzymes ADH3 and ALDH2 are ubiquitously expressed in all tissues (Alnouti and Klaassen, 2008; Nishimura and Naito, 2006). Also, since the homeostatic concentration of formaldehyde in the blood is about 0.1 mM (Heck and Casanova, 2004; Heck et al., 1985), the body would have to be exposed to high doses of formaldehyde in order to exert cerebral damage after escaping its metabolism, predominantly in the liver. Indeed, exposure to formaldehyde has been reported to cause neurotoxicity in humans as well as animals and depends on the dose and duration of formaldehyde exposure (Kilburn et al., 1985a; Kilburn et al., 1985b; Kilburn et al., 1989; Songur et al., 2010; Songur et al., 2008). Nevertheless, individuals carrying functional polymorphisms in the promoter of *ADH3* or genetic polymorphisms in *ALDH2* which are suggested to be associated with reduced formaldehyde-oxidizing capacity (Hedberg et al., 2001; Wang et al., 2002) may be more vulnerable to neural damage by exogenous formaldehyde even at low doses.

Under physiological conditions, the concentration of formaldehyde in brain (around 0.3 mM) (Tong et al., 2012) is higher than that in the blood (0.1 mM) (Heck and Casanova, 2004; Heck et al., 1985) which hints at a higher capacity of the brain to generate formaldehyde by endogenous processes. An elevation of such metabolic processes in diseases (Section 1.1.4) has the potential to expose this organ to considerably high levels of formaldehyde as observed in AD, as well as diabetes (Tong et al., 2012; Tong et al., 2011). Thus, the risk of neurotoxicity from an increase in formaldehyde generated within the brain

tissue seems higher compared to exposure to exogenous formaldehyde which may enter the brain after escaping peripheral metabolism.

4.2. Effects of formaldehyde application on brain cells

Since the brain encounters formaldehyde in physiological conditions and the level of exposure increases in pathological conditions (Tong et al., 2012; Tong et al., 2011), the ability of brain cells to metabolize formaldehyde was investigated in this thesis. Astrocyte-rich primary cultures, cerebellar granule neurons cultures as well as the oligodendroglial cell-line OLN-93 were employed as model systems for astrocytes, neurons and oligodendrocytes, respectively (Drejer et al., 2008; Lange et al., 2012; Richter-Landsberg and Heinrich, 1996). The advantage of these cultures is that they enable studies on a specific brain cell-type with minimal interference from other cells which constitute the brain. Formaldehyde oxidation generates formate, a mitochondrial respiration inhibitor (Nicholls, 1975; Wallace et al., 1997) while the pathway of formaldehyde oxidation involves GSH (Friedenson, 2011; MacAllister et al., 2011), an important antioxidant (Lushchak, 2012; Schmidt and Dringen, 2012). Therefore, it was interesting to test for the consequence of a formaldehyde treatment on the glucose and GSH metabolism of these cells.

The presence of mRNAs for the enzymes involved in formaldehyde generation and disposal (Sections 1.1.2 and 1.1.3) was analyzed qualitatively in these cultures using reverse transcription (RT)-PCR (Fig. 4.1). All cultures contained the mRNA of SSAO and LSD1 (formaldehyde-generating enzymes) as well as for ADH3 and ALDH2 (formaldehyde-oxidizing enzymes) which verifies previous reports on the expression of these enzymes in the brain (Table 1.3). Furthermore, the mRNA of ADH1 (formaldehyde-reducing enzyme) was also expressed in all the cultures which is in agreement with a previous study (Martinez et al., 2001). Amongst the enzymes involved in THF-dependent formate oxidation, MTHFD1 and MTHFD1L which have been shown to be expressed in the brain (Anthony and Heintz, 2007; MacFarlane et al., 2009; Prasannan et al., 2003) were also expressed in all the cultures. On the contrary,

mRNA of the 10-formyl THF-oxidizing enzyme, ALDH1L1 was hardly detectable in cultured neurons and OLN-93 cells which is not surprising since ALDH1L1 is considered to be an astrocyte-specific protein (Cahoy et al., 2008). Furthermore, the mRNA of ALDH1L2 which has been reported to be present in the total brain tissue (Krupenko et al., 2010), was present in all cultures except OLN-93 cells. Thus, all types of brain cell cultures used in this study may possess the capacity to generate and dispose formaldehyde.

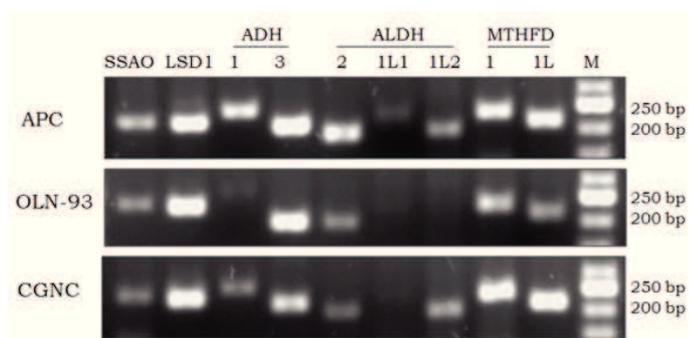


Figure 4.1: RT-PCR analysis of the formaldehyde-generating and -metabolizing enzymes in cultured brain cells.

4.2.1. Formaldehyde metabolism

The concentrations of formaldehyde used for exposure of cultures were in the (patho)physiologically relevant range (Heck and Casanova, 2004; Heck et al., 1985; Tong et al., 2012; Tong et al., 2011). Cultured astrocytes, OLN-93 cells as well as cultured cerebellar granule neurons withstood formaldehyde application in concentrations of up to 1 mM for up to 4 h (Chapters 3.1-3.4) which is in line with the reported resistance of cultured brain cells to formaldehyde toxicity for a time-frame of a few hours (Song et al., 2010; Tong et al., 2012). On the contrary, exposure of microglial cultures to formaldehyde resulted in an almost complete cell death, assessed by LDH release, within 30 min of incubation (data not shown). The resistance of cultured brain cells to formaldehyde toxicity could be due to their ability to metabolize the applied formaldehyde, since this compound is more cytotoxic than its metabolites, methanol and formate (Lee et al., 2008; Oyama et al., 2002).

Table 4.1: Metabolism of formaldehyde and consequences of formaldehyde exposure on the glucose and glutathione metabolism in cultured brain cells#.

Parameter	[HCHO] (mM)	APC		OLN-93		CGNC	
			n		n		n
Protein content ($\mu\text{g}/\text{well}$)		113 \pm 18	32	76 \pm 6***	22	51 \pm 10***	20
Formaldehyde metabolism							
Formaldehyde clearance rate (nmol/(h x mg))	0.5	299 \pm 27	3	n.d		321 \pm 120	8
	1	322 \pm 110	6	244 \pm 39	3	n.d	
Formate release rate (nmol/(h x mg))	0.5	299 \pm 34	3	n.d		51 \pm 7***	7
	1	276 \pm 89	6	188 \pm 8	3	n.d	
Formaldehyde clearance to formate release ratio	0.5	1.0 \pm 0.0	3	n.d		n.a	
	1	0.9 \pm 0.1	6	0.8 \pm 0.1	3	n.d	
Glucose metabolism							
Lactate release rate ($\mu\text{mol}/(\text{h} \times \text{mg})$)	0	1.4 \pm 0.2	18	1.0 \pm 0.1***	10	0.9 \pm 0.2***	5
	0.5	1.3 \pm 0.1	3	1.6 \pm 0.1 ^{sss}	3	1.6 \pm 0.3 ^{ss}	5
	1	2.0 \pm 0.2 ^{sss}	18	1.8 \pm 0.2 ^{sss}	10	n.d	
Glucose consumption rate ($\mu\text{mol}/(\text{h} \times \text{mg})$)	0	0.7 \pm 0.1	6	0.4 \pm 0.1***	10	n.d	
	0.5	0.6 \pm 0.1	6	0.6 \pm 0.0 ^s	3	n.d	
	1	1.0 \pm 0.1 ^{sss}	6	0.7 \pm 0.1***, ^{sss}	10	n.d	
Lactate release to glucose consumption ratio	0	1.8 \pm 0.4	6	2.5 \pm 0.6*	10	n.d	
	0.5	2.3 \pm 0.4	6	2.6 \pm 0.3	3	n.d	
	1	1.9 \pm 0.4	6	2.4 \pm 0.4*	10	n.d	
Glutathione export							
Cellular GSH content (nmol/mg)		51.5 \pm 12.4	19	43.8 \pm 4.4***	22	20.6 \pm 4.8***	15
GSH export rate (nmol/(h x mg))	0	1.5 \pm 0.3	13	0.4 \pm 0.3**	14	1.1 \pm 0.5*	6
	0.5	20.3 \pm 2.8 ^{sss}	3	10.7 \pm 1.2***, ^{sss}	14	4.7 \pm 1.3***, ^{sss}	6
	1	18.2 \pm 2.5 ^{sss}	13	11.8 \pm 2.6***, ^{sss}	3	5.2 \pm 0.9***, ^{sss}	3

#The data on formaldehyde metabolism and its impact on glucose metabolism in OLN-93 cells are from Tongaonkar (2012). The data on glutathione export rate from OLN-93 cells in the presence of 1 mM formaldehyde are from Boecker (2011). All other data sets are compiled from Chapters 3.1-3.4. Significance of differences compared to cultured astrocytes (ANOVA or unpaired t-test) is indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. For one culture type, significance of differences compared to the control condition (ANOVA or t-test) is indicated by ^s $p < 0.05$, ^{ss} $p < 0.05$ and ^{sss} $p < 0.001$. n.a, not applicable (unpaired data); n.d, not determined.

All types of cultures used in this study have the potential to clear the applied formaldehyde at a rate of about 0.3 $\mu\text{mol}/(\text{h} \times \text{mg})$ (Table 1.4) which is similar to the formaldehyde oxidation rate of brain cortex homogenate (around 0.2 $\mu\text{mol}/(\text{h} \times \text{mg})$) (Iborra et al., 1992), but is only a fifth of the oxidation rate found for liver cells (Dicker and Cederbaum, 1984). The K_m -value for formaldehyde clearance in cultured astrocytes was calculated to be around 0.19 mM (Chapter 3.1). Since the K_m -value of ADH3 (for S-hydroxymethyl GSH) and ALDH2 (for formaldehyde) is less than 10 μM and around 0.2-0.5 mM, respectively, (Casanova-Schmitz et al., 1984; Heck et al., 1990), under the conditions used, both the cytosolic ADH3 and mitochondrial ALDH2 are likely to contribute to formaldehyde oxidation.

The difference between the various brain cell cultures lies in the fate of the formaldehyde metabolized. While in astrocytes and OLN-93 cells, majority of the formate that was generated by formaldehyde oxidation was exported into the medium, in neurons only about 25% of the cleared formaldehyde was detectable as extracellular formate (Table 4.1). The reason behind these differences might be that cultured neurons export formate poorly and/or are better equipped with enzymes responsible for further oxidation of formate to carbon dioxide (Fig. 1.5). The putative exporters for formate are GABA receptors (Mason et al., 1990) and monocarboxylate transporter (MCT) 1 (Moschen et al., 2012), both of which are expressed in astrocytes (Debernardi et al., 2003; Lee et al., 2011; Velez-Fort et al., 2011), oligodendrocytes (Luyt et al., 2007) and neurons (Debernardi et al., 2003; Olsen and Sieghart, 2009; Rinholm et al., 2011). However, unlike other brain cells, the expression level of MCT1 in neurons has been reported to be very low (Debernardi et al., 2003). If poor export of formate would be the only reason behind the observation that in neurons only 25% of the disappearing formaldehyde was found as extracellular formate (Chapter 3.4), the remaining 75% of the formaldehyde-derived formate should be detected within cells. Indeed, the amount of formate that accumulated in neurons on treatment with 0.5 mM formaldehyde for 2 h was about three-fold higher than that in cultured astrocytes on application of the same concentration of formaldehyde for 3 h (Table 4.2). However, this formate accounted for only 10% of the formaldehyde

that had disappeared from the medium (Chapter 3.4). Thus, the lower extracellular accumulation of formate in cerebellar granule neurons compared to cultured astrocytes and OLN-93 cells may be due to a combination of poor export and further oxidation of the cellular formate to carbon dioxide.

Table 4.2: Cellular accumulation of formaldehyde-derived formate in cultured brain cells.

Culture	Incubation conditions		Cellular formate content (nmol/mg)	n	Reference
	[HCHO] (mM)	Time (h)			
APC	0.5	3	5.2 ± 0.9	3	Chapter 3.1
	1	3	13.6 ± 2.2	3	Chapter 3.1
OLN-93	1	5	22.2 ± 6.6	3	Tongaonkar, 2012
CGNC	0.5	2	14.7 ± 5.0	4	Chapter 3.4

4.2.2. Formaldehyde-stimulated lactate release

All the neural cell cultures used here oxidized the applied formaldehyde to formate leading to an elevated cellular level of formate (Chapters 3.1, 3.4; Tongaonkar, 2012). The formate present in the cells can potentially inhibit mitochondrial cytochrome c oxidase (Nicholls, 1975; Wallace et al., 1997) and alter the glucose metabolism of formaldehyde-treated cultures. Therefore, glucose consumption and lactate release of formaldehyde-treated cells was investigated. The basal rates of lactate release and glucose consumption in cultured astrocytes (Table 4.1) were similar to previous reports (Fonseca et al., 2005; Scheiber and Dringen, 2011) and the ratio of these rates was around 2 indicating that these cells are highly glycolytic. This feature of astrocytes has been attributed to the inhibition of pyruvate dehydrogenase complex (Halim et al., 2010) and a low NADH shuttling into the mitochondria (Berkich et al., 2007; Neves et al., 2012). Like astrocytes, OLN-93 cells are also glycolytic as evident from the high ratio of lactate release to glucose consumption, although

the respective rates are significantly lower compared to astrocytes (Table 4.1). While the glucose consumption in cultured neurons could not be measured reliably, the lactate release rate from these cultures is comparable to literature data (Itoh et al., 2003; Pauwels et al., 1985; Walz and Mukerji, 1988; Zwingmann and Leibfritz, 2003) and was significantly lower than that of cultured astrocytes (Table 4.1). This was expected, since astrocytes are reported to be more glycolytic compared to neurons (Walz and Mukerji, 1988; Zwingmann and Leibfritz, 2003). The release of lactate by astrocytes is considered highly important to support neuronal energy demand (Fernandez-Fernandez et al., 2012; Pellerin and Magistretti, 2012) and oligodendrocyte development and myelination (Rinholm et al., 2011), while lactate release from oligodendrocytes is suggested to provide energy substrate to myelinated neurons (Fünfschilling et al., 2012; Lee et al., 2012).

Formaldehyde exposure elevated lactate release from all types of brain cell cultures (Table 4.1). However, the extent of the increase in lactate release rate over basal rate differed between the culture types (Table 4.1). While at a formaldehyde concentration of 0.5 mM, the lactate release rate increased by about 60% and 100% in OLN-93 cells and cultured neurons, respectively, the rate was unaltered in cultured astrocytes. On the other hand, an application of 1 mM formaldehyde was required to increase glycolysis by about 50% in cultured astrocytes. Furthermore, in cultured astrocytes, the lactate release rate remained high compared to the basal rate even on removal of formaldehyde after 1 h of incubation and this persistent lactate release was not further enhanced by application of azide, another inhibitor of mitochondrial cytochrome c oxidase (Chapter 3.1). These observations strengthen the hypothesis that the effect of elevated glycolytic flux is not due to formaldehyde itself but due to formate. Thus, the differences in the extent of acceleration of lactate release on formaldehyde treatment of cultured astrocytes, OLN-93 cells and neurons may depend, at least partly, on the cellular concentrations of formate achieved to inhibit mitochondrial respiration. For example, in cultures the cell volume of human astrocytes is estimated to be ten times that of neurons (Zhang et al., 2002). Therefore, in astrocytes, substantially higher amounts of cellular formate

would be required to attain the same concentration of formate like that in neurons for mitochondrial inhibition.

4.2.3. Formaldehyde-accelerated glutathione deprivation

For all types of neuronal cultures, oxidized glutathione (GSSG) contributed only marginally to the amounts of total glutathione (GSx) measured both in cells and media (Chapters 3.2-3.4) indicating that most of the GSx was GSH. The initial cellular GSH content as well as the basal GSH export was the highest in astrocytes compared to other cultures which is in line with a previous report (Hirrlinger et al., 2002). Around 60% of the basal GSH export from astrocytes is mediated by Mrp1 (Minich et al., 2006), a member of ATP-binding cassette transporters (Keppler, 2011). Besides GSH, Mrp1 transports a wide array of substrates, including GSSG as well as GSH conjugates (Keppler, 2011). However, this transporter differs in its K_m -value for the different substrates. The K_m -value of Mrp1 for GSH is above 5 mM, while that for GSSG and GSH conjugates is about 100 μ M and less than 1 mM, respectively (Cole and Deeley, 2006; Deeley and Cole, 2006; Leier et al., 1996).

Table 4.3: Duration of incubation and concentration of formaldehyde required for half-maximal GSH export from cultured brain cells.

Culture	[HCHO] (mM)	Time (h)	Reference
APC	0.3	1	Chapter 3.2
OLN-93	0.2	0.75	Chapter 3.3
CGNC	0.3	1	Chapter 3.4

Formaldehyde treatment increased Mrp1-mediated GSH export from all types of cultured brain cells investigated (Chapters 3.2-3.4). The concentration of formaldehyde and the duration of incubation required for half-maximal GSH

export were similar for the different culture types (Table 4.3). The stimulated GSH efflux observed in the presence of formaldehyde was hypothesized to be due to the Mrp1-mediated export of either GSH or of the formaldehyde-GSH conjugate, S-hydroxymethyl GSH (Fig. 4.2). The latter is known to be labile (Ahmed and Anders, 1978) and is likely to disintegrate into formaldehyde and GSH immediately after its export due to the change in the equilibrium between the reactants and the product.

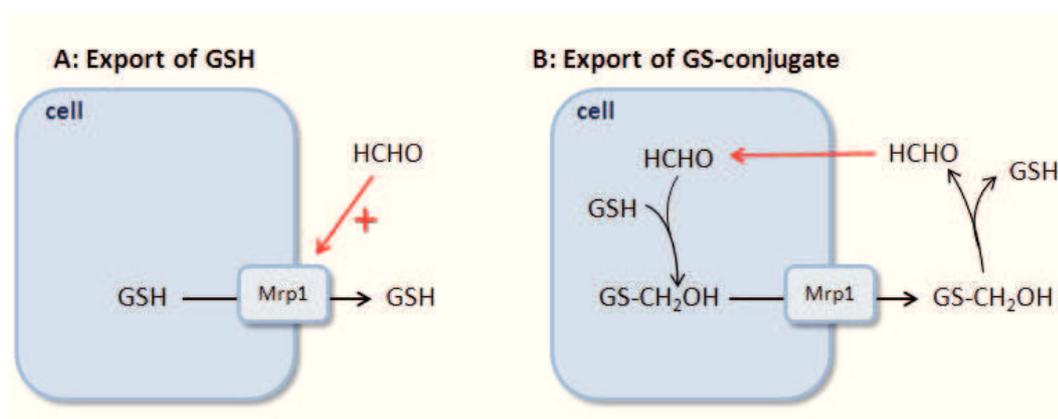


Figure 4.2: Hypothesized mechanism behind formaldehyde-stimulated GSH export. Formaldehyde may directly stimulate Mrp1-mediated GSH export (A). Alternatively, formaldehyde may react with GSH in cells to form S-hydroxymethyl GSH which is exported by Mrp1 and immediately disintegrates into formaldehyde and GSH on its release into the medium due to the labile nature of this conjugate (B).

To obtain a better insight into the identity of the GSH moiety exported in the presence of formaldehyde, the kinetic parameters for basal and formaldehyde-stimulated GSH export were determined, since Mrp1 has different K_m -values for GSH and its conjugates (Cole and Deeley, 2006; Deeley and Cole, 2006). The K_m - and V_{max} -value for the basal GSH export in cultured astrocytes was about 100 nmol/mg (about 25 mM) and 4 nmol/(h x mg), respectively (Chapter 3.3) which was comparable to literature data (Sagara et al., 1996). On application of formaldehyde to cultured astrocytes, the K_m -value for formaldehyde-stimulated GSH export was identical to that for the basal GSH export, while the V_{max} -

value increased by a factor of 10 (Chapter 3.3). This indicates that GSH rather than S-hydroxymethyl GSH is exported by Mrp1 in the presence of formaldehyde, since an export of S-hydroxymethyl GSH should lead to a lowering of the Km-value for GSH export, owing to the lower Km-value of Mrp1 for GSH conjugates (Cole and Deeley, 2006; Deeley and Cole, 2006). Although the kinetic parameters for the basal GSH export in OLN-93 cells and that for basal as well as stimulated GSH export in cultured neurons could not be determined, it is likely that in the presence of formaldehyde, GSH rather than the conjugate is exported from both types of cultures.

Since the stimulation of GSH export was observed within minutes after formaldehyde application (Chapters 3.2-3.4), *de novo* synthesis of Mrp1 seems unlikely to explain the stimulated GSH efflux. Furthermore, the observation that removal of formaldehyde instantly decelerates the stimulated GSH export (Chapters 3.2-3.4) indicates that the formaldehyde-accelerated GSH export is reversible. The molecular mechanism behind the accelerated GSH export in the presence of formaldehyde could be either recruitment of Mrp1 to the cell membrane and/or covalent activation of this transporter. Although no evidence for activation of Mrp1 is found in literature, a reversible translocation of Mrp1 from the Golgi apparatus to the cell surface has at least been reported for cultured astrocytes treated with bilirubin (Gennuso et al., 2004).

The amount of GSSG detected in cells and media on formaldehyde treatment for all the cultures used was marginal (Chapters 3.2-3.4). This indicates that the cells did not encounter severe formaldehyde-induced oxidative stress under the conditions used, although this aldehyde is known to induce oxidative stress in the brain (Songur et al., 2008; Zararsiz et al., 2006; Zararsiz et al., 2007; Zararsiz et al., 2011). The lack of severe oxidative stress observed in the cultures on exposure to formaldehyde could be due to a reinforced antioxidative capacity of brain cells in cultures compared to the respective cells *in vivo*. Nevertheless, studies on formaldehyde-induced oxidative damage *in vitro* used lower doses of formaldehyde for longer time-frames than those used in the current study (Song et al., 2010; Tang et al., 2011; Zhang et al., 2010). Hence it is likely that exposure to formaldehyde for a short duration does not induce

oxidative damage but that oxidative stress is rather a consequence of a chronic exposure.

Application of formaldehyde does not deprive the cells completely of their GSH and about 5% residual GSH still remains within cells (Chapters 3.2-3.4). The cellular concentration of the remaining GSH is sufficiently high (astrocytes have a cell volume of 4.1 $\mu\text{L}/\text{mg}$ (Dringen and Hamprecht, 1998)) to drive ADH3-catalyzed GSH-dependent formaldehyde oxidation, since the K_m -value of ADH3 for S-hydroxymethyl GSH is less than 10 μM (Casanova-Schmitz et al., 1984; Heck et al., 1990) and this reaction involves recycling of GSH (Fig. 4.3). Thus, the stimulated GSH export will not hamper the GSH-dependent formaldehyde oxidation.

Fig. 4.3 shows the current scheme on the metabolism of formaldehyde in brain cells and on consequences of such an exposure on the glucose and glutathione metabolism based on the data described in this thesis. Formaldehyde application to cultured brain cells results in oxidation of formaldehyde to generate formate which most likely inhibits complex IV of the mitochondrial respiratory chain (Nicholls, 1975; Wallace et al., 1997), thereby increasing the glycolytic flux. On the other hand, formaldehyde deprives cultured brain cells of GSH by stimulating Mrp1-mediated GSH export.

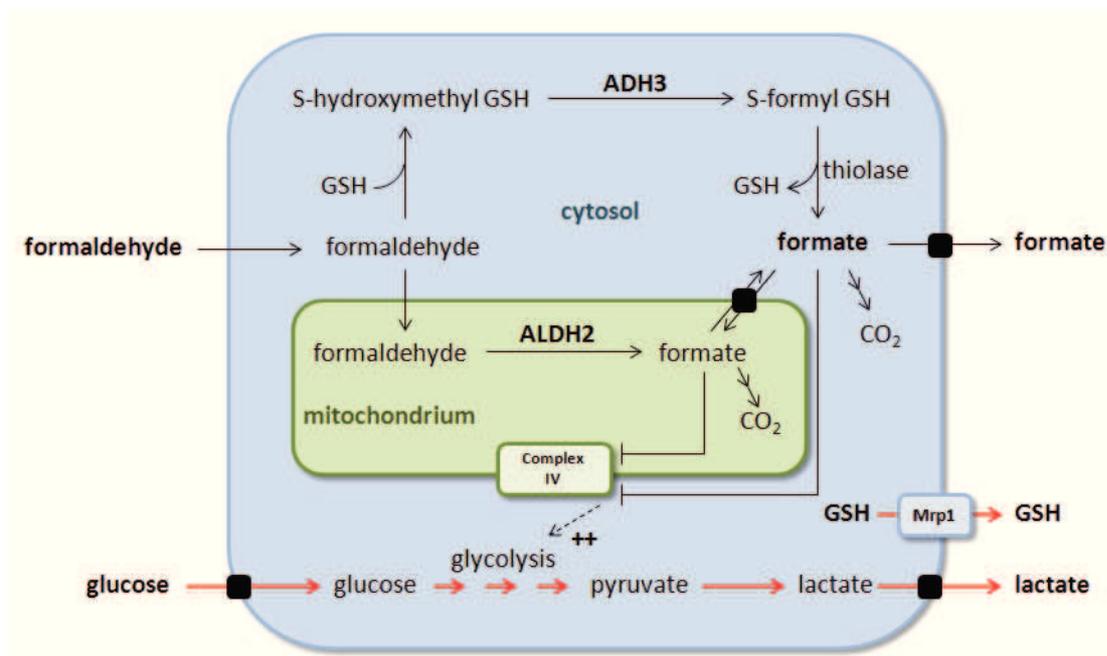


Figure 4.3: Formaldehyde oxidation by cultured brain cells and the acceleration of glycolytic flux and GSH export on formaldehyde exposure. Exogenously applied formaldehyde is oxidized to formate in a pathway mediated by cytosolic ADH3 in a GSH-dependent process or by the mitochondrial ALDH2. At least a part of the formate generated within cells is exported while a fraction may be further oxidized to carbon dioxide. Remaining cellular formate is likely to inhibit mitochondrial complex IV which in turn accelerates glycolysis resulting in elevated lactate release. Formaldehyde also induces a rapid GSH export in a process mediated by Mrp1. Red arrows indicate the metabolic processes stimulated on formaldehyde exposure.

4.3. (Patho)physiological implications

The present study gives a novel insight into the metabolism of formaldehyde by brain cells and on the alterations in the glucose and GSH metabolism on exposure of cells to formaldehyde using *in vitro* model systems. However, the extrapolation of the results obtained in this thesis to the situation *in vivo* when the brain encounters high levels of formaldehyde should be done with caution due to the limitations of these model systems (De Vries and Boullerne, 2010; Gibbons and Dragunow, 2010; Lange et al., 2012). Furthermore, it should also

be considered that despite the use of physiologically relevant concentrations in this study, the amount of formaldehyde that brain cells encounter *in vivo* would be far less than that *in vitro* due to the low (about 20%) volume fraction (ratio of volume of extracellular matrix to that of the tissue) *in vivo* (Nicholson, 2001; Sykova and Nicholson, 2008). This could also be an explanation for the increase in lactate and GSH efflux seen on treatment of cultured brain cells with concentrations of formaldehyde that are reported in the healthy brain (Tong et al., 2012), although such a situation *in vivo* may pose a severe threat to this organ. Nevertheless, when the formaldehyde concentration in the brain rises over the homeostatic value alterations in brain energy and GSH metabolism are likely to occur.

On exposure of brain to high concentrations of formaldehyde, astrocytes, oligodendrocytes and neurons may oxidize this aldehyde as has been reported for brain homogenates (Iborra et al., 1992). Subsequently, formate and lactate may be released from the cells (Fig. 4.3). Although the release of lactate by astrocytes and oligodendrocytes is considered to be neuroprotective (Fünfschilling et al., 2012; Lee et al., 2012; Pellerin and Magistretti, 2012), a formate-induced inhibition of mitochondrial cytochrome c oxidase in neurons would slow down oxidative ATP generation through the TCA cycle (Fig. 1.7). An up-regulation of glycolysis by brain cells may serve as a transient solution to meet their energy demands, however on prolonged exposure to formaldehyde the cells will suffer from an energy crisis that in turn will disrupt their functions. For example, oligodendrocytes have a high energy requirement for myelination (Harris and Attwell, 2012), hence disturbed energy metabolism of these cells on exposure to high levels of formaldehyde may affect the myelination of axons which is crucial for learning and cognition (Fields, 2008; Huang et al., 2009). Besides impairment of energy metabolism, formaldehyde-induced generation of both formic acid and lactic acid in the brain can lead to cerebral acidosis (Rose, 2010; Skrzydlewska, 2003; Yao and Haddad, 2004). Lactic acidosis results in swelling of astrocytes, impairment of signal transmission in neurons and neurological deficits (Li et al., 2011; Staub et al.,

1993; Zhao et al., 2011). In addition, formic acid has been shown to exert neurotoxicity in hippocampal brain slices (Kapur et al., 2007).

Exposure of brain cells to high levels of formaldehyde will cause a depletion of GSH in these cells and GSH accumulation in the extracellular space. As GSH has important cellular functions in the brain such as protection against reactive oxygen species and detoxification of xenobiotics (Schmidt and Dringen, 2012), GSH deprivation of cells by prolonged exposure to formaldehyde may cause severe oxidative stress as previously reported (Songur et al., 2008; Zararsiz et al., 2006; Zararsiz et al., 2007; Zararsiz et al., 2011), unless the capacity of cells to synthesize GSH is up-regulated sufficiently to compensate for this loss. However, acidosis, which is likely to be a manifestation of formaldehyde exposure due to production of formic acid and lactic acid (Rose, 2010; Skrzydlewska, 2003), has been shown to slow down GSH synthesis (Lewerenz et al., 2010). This may render the cells incapable of fully restoring their GSH levels.

The formaldehyde-induced accumulation of extracellular GSH in brain may also be detrimental since GSH has been suggested to act as a neurotransmitter and neuromodulator at glutamate receptors (Janáky et al., 2007) which play important roles in memory and learning (Davis et al., 2012; Mukherjee and Manahan-Vaughan, 2012). Likewise, GSH breakdown by the astrocytic ectoenzyme γ -GT (Fig. 1.7) generates the neurotransmitter, glutamate (Fernandez-Fernandez et al., 2012; Schmidt and Dringen, 2012). Thus, excessive accumulation of extracellular GSH, its oxidation product GSSG as well as GSH-derived glutamate can cause excitotoxicity which has at least been demonstrated *in vitro* (Regan and Guo, 1999a; Regan and Guo, 1999b).

Conditions such as ageing and diseases like MS and AD which are associated with increased levels of formaldehyde in brain (Khokhlov et al., 1989; Miao and He, 2012; Tong et al., 2012; Tong et al., 2011) have been connected to impaired mitochondrial function (Boumezbeur et al., 2010; Leuner et al., 2012; Mahad et al., 2008; Sullivan and Brown, 2005) along with an increase in brain lactate content (Paling et al., 2011; Parnetti et al., 2000; Ross et al., 2010). Also,

oxidative stress in the brain has been connected with ageing, MS and AD (Belkacemi and Ramassamy, 2012; Haider et al., 2011; Sohal and Orr, 2012; Steele and Robinson, 2012; van Horssen et al., 2011). These reports strengthen the view that formaldehyde may, at least to some extent, have a causal role in the initiation and/or progression of pathological symptoms of neurodegenerative conditions (Monte, 2010; Yu, 2001). An adequate supply of lactate to neurons has been shown to modulate memory formation (Suzuki et al., 2011) while GSH depletion in the brain has been demonstrated to result in behavioral changes (Steullet et al., 2010). Thus, the formaldehyde-induced alterations in glucose and GSH metabolism may contribute to the deficits in behavior, cognition and learning observed in animals after exposure to formaldehyde (Lu et al., 2008; Malek et al., 2003; Pitten et al., 2000; Tong et al., 2012; Tong et al., 2011) or in neurodegenerative conditions such as AD (Stopford et al., 2012).

4.4. Future perspectives

This thesis throws light on the capacity of cultured brain cells to clear exogenously applied formaldehyde and also the consequences of formaldehyde exposure on the energy and GSH metabolism of brain cells. Based on the experimental evidence presented in this thesis some aspects still need to be addressed to complete the scheme (Fig. 4.3) and to understand better the underlying mechanisms.

The expression of the enzymes involved in formaldehyde generation and metabolism should be ascertained on the protein level using Western blot analysis as well as immunocytochemistry. Also, the metabolism of formaldehyde to carbon dioxide, especially in neurons, remains to be verified. RNA interference technology (Siomi and Siomi, 2009) can be used to silence the genes of the enzymes involved in the metabolism of this aldehyde and formaldehyde oxidation can be assessed using the biochemical tools used in this thesis. As an alternative, these enzymes can also be inhibited using known chemical inhibitors. For example, ALDH2 can be inhibited by daidizin,

crotonaldehyde, chloral hydrate or disulfiram (MacAllister et al., 2011; Teng et al., 2001; Tong et al., 2012). Such studies would give a better insight into the contribution of different enzymes involved in the metabolism of formaldehyde in brain cells. Furthermore, a direct evidence for the inhibition of mitochondrial complex IV in cultured brain cells on formaldehyde treatment can be obtained by measuring the activity of this complex using a previously described method (Wharton and Tzagoloff, 1967).

The putative transporters responsible for the export of formate are GABA receptors and MCT1 (Mason et al., 1990; Moschen et al., 2012). However, the role of these transporters in the efflux of formaldehyde-derived formate from the cultures used in this study was not assessed. The involvement of GABA receptors can be investigated using zinc ions which inhibit the receptor (Hosie et al., 2003) while AR-C117977, a highly specific inhibitor of MCT1 (Ekberg et al., 2007) can be employed to test for its involvement. In order to evaluate the hypothesis that formaldehyde-stimulated GSH export involves trafficking of Mrp1 to the cell surface from the Golgi apparatus, immunofluorescence staining of this protein can be performed to investigate its localization before and after formaldehyde treatment by confocal microscopy.

Studies on formaldehyde exposure of microglial cultures could be performed to investigate the mechanism behind the unexpected vulnerability of these cells to formaldehyde. Such an investigation may provide an interesting insight into a potential selective vulnerability of microglia *in vivo* in conditions associated with elevated brain formaldehyde levels.

Formaldehyde has been hypothesized to be involved in the pathology of neurodegenerative diseases (Monte, 2010; Yu, 2001). However, the literature evidence for an increase in brain formaldehyde levels in neurodegeneration is limited. Also, such reports (Tong et al., 2011; Tong et al., 2012) are not verified by independent studies. Therefore, the level of formaldehyde in post-mortem brains of patients as well as animal models for the conditions associated with an increase in formaldehyde-generating enzymes should be determined. Besides brain, urine formaldehyde levels may also be of interest since cognitive deficits

post-operation or in senile dementia correlate with urine formaldehyde levels and have been suggested to be prognostic markers for these conditions (Tong et al., 2011; Wang et al., 2012). The radiometric Dimedone-¹⁴C method (Szarvas et al., 1986) or high performance liquid chromatography with a fluorescence detector (Luo et al., 2001) can be employed to determine formaldehyde levels in biological samples. Such studies may help in establishing a stronger link between formaldehyde and neurodegeneration.

Microdialysis is a widely used technique in neuroscience that facilitates sampling and collection of low molecular weight compounds from the extracellular space in the brain of humans as well as animals (Chefer et al., 2009). This valuable tool should be employed for determination of metabolites such as formate, lactate and GSH in healthy and the diseased brains for conditions associated with an increase in brain formaldehyde level. Comparison of the extracellular levels of these metabolites in the brain and correlating them to the levels of formaldehyde observed in (patho)physiology would help to validate the results obtained in cultured brain cells for the situation *in vivo*.

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5.

Appendix

5.1. Curriculum vitae

Personal information

Date of Birth: 4th May 1985
Place of Birth: Pune, India

Academic information

- 04/09 – 11/12 Doctoral thesis in Neurobiochemistry
University of Bremen, Germany
‘Formaldehyde metabolism and formaldehyde-induced alterations in glucose and glutathione metabolism of cultured brain cells’
Supervisor: Prof. Dr. Ralf Dringen, University of Bremen, Germany
- 10/08 M. Sc. in Biochemistry and Molecular Biology
University of Bremen, Germany
Master Thesis: *‘Uptake of ferrous iron by cultured astrocytes’*
Supervisor: Prof. Dr. Ralf Dringen, University of Bremen, Germany
- 10/06 – 10/08 Studies for master’s degree in Biochemistry and Molecular Biology
- 06/06 B. Sc. in Microbiology (vocational Industrial Microbiology)
Abasaheb Garware College, University of Pune, India
- 06/03 – 06/06 Studies for bachelor’s degree in Microbiology

Teaching Experience

04/09 – 11/12 Supervisor in biochemical courses

Course-related activities

- 02/07 – 10/08 Student research assistant
Max Plank Institute for Marine Microbiology, Bremen
- 06/05 Month long summer training at the Nicholas Piramal Research Centre, Mumbai, India

5.2. List of publications

Tulpule, K., Robinson, S.R., Bishop, G.M., Dringen, R., 2010. Uptake of ferrous iron by cultured astrocytes. *J Neurosci Res* 88, 563-571.

Tulpule, K., Dringen, R., 2011. Formaldehyde stimulates Mrp1-mediated glutathione deprivation of cultured astrocytes. *J Neurochem* 116, 626-635.

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Tulpule, K., Schmidt, M.M., Boecker, K., Goldbaum, O., Richter-Landsberg, C., Dringen, R. 2012. Formaldehyde induces rapid glutathione export from viable oligodendroglial OLN-93 cells. *Neurochem Int*, in press.

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