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Effects of Antiretroviral Drugs on the Glutathione and Glucose Metabolism of Cultured Brain Cells

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Maria Brandmann

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Dekan: Prof. Dr. T. Hoffmeister

Erster Gutachter: Prof. Dr. R. Dringen

Zweiter Gutachter: Prof. Dr. J. Hirrlinger

Hiermit erkläre ich, die vorliegende Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt zu haben. Diese Arbeit wurde zuvor nicht an anderer Stelle eingereicht.

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(Maria Brandmann)

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II Summary

The highly active antiretroviral therapy (HAART) has been successfully used for 30 years to treat the aquired immuno deficiency syndrome (AIDS) and the human immuno deficiency virus (HIV)-associated dementia. However, as minor neurocognitive deficits persist in treated HIV patients potential adverse consequences of antiretroviral drugs on brain cells are currently intensively discussed, but lack sufficient experimental proof. To address such questions, this thesis investigated the acute effects of various antiretroviral drugs from the classes of reverse transcriptase (RT) inhibitors and protease inhibitors on viability, glutathione (GSH) metabolism and glycolytic flux of brain cells, using primary cultures of astrocytes and neurons as model systems.

A treatment with protease and RT inhibitors did not acutely damage cultured brain cells. However, the incubation of viable cultured astrocytes or neurons with protease inhibitors, but not with RT inhibitors, strongly stimulated cellular GSH release. The protease inhibitor-induced acceleration of GSH export was completely blocked by an inhibitor of the multidrug resistance protein 1 (Mrp1), suggesting that this exporter mediates the protease inhibitor-induced GSH depletion of brain cells. Protease inhibitors or RT inhibitors did not modulate the glycolytic flux of cultured astrocytes. In contrast, 8-hydroxy efavirenz (8-OH-efv), the primary metabolite of the frequently used RT inhibitor efavirenz, accelerated the glycolysis-derived lactate release from viable cultured astrocytes. However, in contrast to respiratory chain inhibitors, a direct inhibition of mitochondrial respiration by 8-OH-efv appears not to be the mechanism underlying the 8-OH-efv-mediated stimulation of glycolytic flux in astrocytes.

As the lifelong treatment of HIV patients with antiretroviral drugs establishes a chronic exposure of brain cells to such compounds or their metabolites, alterations in basic metabolism of brain cells, such as those reported in this thesis, should be considered to contribute to the reported mild neurocognitive impairments of treated HIV patients.

III Zusammenfassung

Die hochaktive antiretrovirale Therapie (HAART) wird seit 30 Jahren mit großem Erfolg eingesetzt, um das erworbene Immunschwächesyndrom (AIDS, **a**quired **i**mmuno **d**eficiency **s**yndrome) und die humane Immunschwächevirus (HIV)-assoziierte Demenz in HIV-Patienten zu behandeln. Da in behandelten HIV-Patienten kognitive Beeinträchtigungen zu beobachten sind, werden potentielle Nebenwirkungen von antiretroviralen Therapeutika auf Gehirnzellen als mögliche Ursache intensiv diskutiert, auch wenn experimentelle Beweise dafür noch nicht erbracht sind. Die vorliegende Arbeit untersucht daher an Primärkulturen von Astrozyten und Neuronen als Modellsystemen die Effekte von antiretroviralen Substanzen aus den Klassen der Protease-Inhibitoren sowie der Reverse-Transkriptase (RT)-Inhibitoren auf die Vitalität, den Glutathion (GSH)-Stoffwechsel und den glykolytischen Fluss von Gehirnzellen.

Weder die Behandlung mit Protease- noch mit RT-Inhibitoren führte zu einer akuten Schädigung von Gehirnzellen. Die Inkubation von vitalen Astrozyten und Neuronen mit Protease-Inhibitoren, jedoch nicht mit RT-Inhibitoren, resultierte dagegen in einer starken Stimulation der Freisetzung von zellulärem GSH. Die durch Protease-Inhibitoren induzierte Beschleunigung des GSH-Exports wurde durch einen Inhibitor des Multidrug-Resistance-Protein 1 (Mrp1) vollständig blockiert. Dies weist darauf hin, dass dieser Transporter die Protease-Inhibitor-stimulierte GSH-Verarmung von Gehirnzellen vermittelt. Weder Protease- noch RT-Inhibitoren veränderten den glykolytischen Fluss in Astrozytenkulturen. Im Gegensatz dazu stimulierte 8-Hydroxy-Efavirenz (8-OH-efv), der Primärmetabolit des vielfach eingesetzten RT-Inhibitors Efavirenz, den Export des Glykolyseproduktes Laktat aus vitalen Astrozyten. Die Ursache dieser Wirkung scheint keine direkte Inhibition der Atmungskette durch 8-OH-efv zu sein, ganz im Gegensatz zu der analogen Wirkung von Inhibitoren der mitochondrialen Atmungskette.

Da die lebenslange Behandlung von HIV-Patienten mit antiretroviralen Therapeutika eine chronische Exposition von Gehirnzellen mit solchen Substanzen oder deren Metaboliten hervorruft, sollten Veränderungen des basalen Metabolismus von Gehirnzellen, wie sie in dieser Arbeit beschrieben wurden, als eine der möglichen Ursachen für die beobachteten leichten neurokognitiven Schäden in HIV-Patienten berücksichtigt werden.

IV Abbreviations

°C	degree Celsius
%	percent
µ	micro
γ	gamma
ABC	ATP-binding cassette
ADP	adenosine diphosphate
AIDS	aquired immunodeficiency syndrome
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANLS	astrocyte-neuron lactate shuttle
ANOVA	analysis of variance
ApN	aminopeptidase N
ATP	adenosine triphosphate
BBB	blood-brain barrier
BSA	bovine serum albumin
CD4	cluster of differentiation 4
CNS	central nervous system
CSF	cerebrospinal fluid
CYP	cytochrome P450
Cys	cysteine
CysGly	cysteine-glycine dipeptide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
8-OH-efv	8-hydroxy efavirenz
e.g.	*exempli gratia*, Latin for "for example"
ed(s).	editor(s)
et al.	*et alii*, Latin for "and others"
FAC	ferric ammonium citrate
Fig.	figure
g	gram(s) or acceleration of gravity
GABA	γ-aminobutyric acid
γ-GluCys	γ-glutamylcysteine

γ-GT	γ–glutamyl transpeptidase
GCL	glutamate cysteine ligase
Gln	glutamine
GlnT	glutamine transporter
Glu	glutamate
GLUT(1/4)	glucose transporter 1/4
Gly	glycine
gp	glycoprotein
GR	glutathione reductase
GS	glutathione synthetase
GSH	glutathione
GSSG	glutathione disulfide
GSx	total glutathione
h	hour(s)
H33342	Hoechst 33342
HAART	highly active antiretroviral terapy
HAD	HIV-associated dementia
HAND	HIV-associated neurocognitive disorders
HEPES	4-(2-hydroxyl)-1-piperazine ethanesulfonic acid
HIV	human immunodeficiency virus
IB	incubation buffer
Idv/IDV	indinavir
K_m	Michaelis-Menten constant
L	liter(s)
LDH	lactate dehydrogenase
LPV	lopinavir
m	milli or meter(s)
M	molar (mol/L)
MCT	monocarboxylate transporter
mol	mole(s)
MRP	human multidrug resistance transporter
Mrp	multidrug resistance transporter
n	nano or number of experiments
NADH	nicotinamide adenine dinucleotide

NADPH	nicotinamide adenine dinucleotide phosphate
Nfv/NFV	nelfinavir
NMDA	N-methyl-D-aspartate
NNRTI	non-nucleoside RT inhibitor
N(t)RTI	nucleoside/nucleotide RT inhibitor
n.s.	not significant
PBS	phosphate-buffered saline
PDH	pyruvate dehydrogenase complex
PDK	PDH kinase
PFK	phosphofructo kinase
P-gp	phospho-glycoprotein
pH	*pondus hydrogenii*, Latin for "potential hydrogen"
PI	propidium iodide
Pr-I	protease inhibitor
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	reverse transcriptase
RTV	ritonavir
SD	standard deviation
SDT	sodium dependent transporter
SOD	superoxide dismutase
US	United States
V_{max}	maximum velocity
w/v	weight per volume

1 Introduction

1.1 Brain cells

The brain is the primary control unit of our body which operates all life-maintaining body functions. It receives and processes signals coming from the rest of the body and sends back signals to the periphery. But in the beginning of science in ancient Greece, as proposed by Aristotle, the heart, not the brain was initially considered the "center of perception" (Agnati *et al.* 2007; Bennett 1999). It was the Greek researcher Galen (129-201) in the second century, who directed the focus to brain and therewith provided the cornerstone for the developing field of neuroscience (Agnati *et al.* 2007; Bennett 1999). Since then anatomy, structure and the diverse functions of the brain in health and disease have been extensively investigated.

The brain consists of four distinct main cell types. The human brain is the most complex brain within all mammals with a total cell number of more than 10^{12} (Azevedo *et al.* 2009). The brain consists of different cell types. The most acknowledged brain cell type are the neurons. Neurons are responsible for signal transduction and will be further introduced in chapter 1.1.1. The remaining cell types, are microglia, oligodendrocytes and astrocytes and are commonly summarized as glia (Corty and Freeman 2013). Microglia, as the major immune competent cell type of the brain, are crucial for protection of the brain against pathogens and injury (Benarroch 2013; Biber *et al.* 2014; Cronk and Kipnis 2013). Oligodendrocytes conduct the essential function of the generation of myelin sheaths surrounding the neuronal axons to enhance signal transduction (White and Krämer-Albers 2014; Zoupi *et al.* 2011). Astrocytes have many important functions in the brain which will be addressed in more detail in chapter 1.1.2.

1.1.1 Neurons

Neurons are the most pleomorphic cell type in the body. They do not only vary in size and shape. Nerve cells can also be distinguished by their modulatory, excitatory or inhibitory effects, as well as by their function as motor, secretory or sensory neurons (Raine 2006). In the early times of neuroscience L. Galvani (1737-1798) was the first to show that electrical signals are sent from the brain to the skeletal muscles along nerves. These signals were further described as transient, spatial electrical changes conducted along the nerves by L. Nobili (1784-1835), C. Matteucci (1811-1865) and H. von Helmholtz (1821-1894), which are today called action potentials (Agnati *et al.* 2007; Bennett 1999). This transduction along the membrane of a single nerve cell in form of action potentials is a complex and energy demanding process including release of K^+, uptake of Na^+ or Ca^{2+} and restoration of K^+ and Na^+ gradients by the Na^+- K^+-ATPase (Hille and Catterall 2006).

The cornerstone of modern, molecular neuroscience was provided in 1888 by R. Y Cajal and his postulated "neuron doctrine" (López-Muñoz and Alamo 2009), in which he specifies the nervous system as an association of single, independent nerve cells, communicating with each other via special regions, now known as synapses (López-Muñoz and Alamo 2009). Those cells were named neurons in 1891 (López-Muñoz and Alamo 2009), based on the Ancient Greek word *neuron* (nerve). And indeed, the postulated interneuronal transduction of signals via chemical neurotransmitters at synapses was discovered and extensively investigated in the last century (Agnati *et al.* 2007; López-Muñoz and Alamo 2009). The release of those neurotransmitters, stored in vesicles, from the pre-synaptic neuron into the synaptic cleft is triggered by action potentials. Released neurotransmitters subsequently bind to post-synaptic receptors, which directly or indirectly regulate ion channels via signaling cascades (Holz and Fisher 2006).

1.1.2 Astrocytes

Over ten years after the first proposal of neuroglia as the "nervenkitt" (nerve glue) by R. Virchow in 1858 (Virchow 1858), C. Golgi visualized astrocytes for the first time and further established the description of glia as connective tissue (Golgi 1871). Yet, the term *astrocyte* was firstly introduced 20 years later in 1891 by M. von Lenhossek (Lenhossek 1891). It is derived from a combination of the Latin word *astra* (star) and the ancient Greek word *kytos* (cell or vessel). Although described and accepted as star-like cells, astrocytes display very heterogeneous morphologies and functions (Oberheim *et al.* 2012). Their forms range from the very branchy, protoplasmic astrocytes with a rather complex morphology and numerous processes which are mostly found in gray matter and fibrous astrocytes, rather simple in morphology which possess less branches and are located in the white matter (Andriezen 1893; Kölliker 1889). Although astrocytes have been identified very early and are considered to be the major brain cell type, the importance of astrocyte function for neuronal function has been underestimated for long and has gradually been recognized and investigated only in the past 25 years (Sofroniew and Vinters 2010).

In the brain astrocytes cover around 99 % of blood capillaries as well as precapillary arterioles with their endfeet (Kacem *et al.* 1998; Mathiisen *et al.* 2010), making them the first parenchymal brain cell type to encounter energy substrates, pathogens, toxins or any other compound transported to the brain by the blood. Not only the close proximity of astrocytes to the brain vasculature is important but also the simultaneous close connection to neurons, especially synapses, through numerous astrocytic processes (Parpura *et al.* 2012). This strategically important positioning enables astrocytes to conduct various important functions, some of which are summarized in Table 1.1, to maintain proper neuronal functioning.

Table 1.1: important functions of astrocytes in the brain

function	**mechanism**	**reference**
synapse formation and remodeling	release of signal molecules (e.g. thrombospondins)	Risher and Eroglu 2012 Pfrieger 2010
modulation of synaptic activity	release of gliotransmitters (e.g. GSH, glutamate, GABA, ATP, D-serine)	Kettenmann and Zorec 2013 Janáky *et al.* 2007
regulation of cerebral blood flow	synthesis and release of vasoactive compounds (e.g. arachidonic acid, epoxyeicosatrienoic acids, prostaglandin E2)	Howarth *et al.* 2012 Kowiański *et al.* 2013
control of extracellular pH	transport of H^+ and HCO_3^-	Deitmer and Rose 2010
regulation of ion homeostasis	uptake of K^+	Kimelberg and Nedergaard 2010
neurotransmitter clearance	uptake of glutamate and GABA	Schousboe *et al.* 2013
regulation of water homeostasis	expression of aquaporin 4	Mack and Wolburg 2013
energy supply to neurons	hypothesized supply of energy substrate lactate to neurons	Hirrlinger and Dringen 2010 Pellerin and Magistretti 2012
storage of energy	Synthesis and degradation of glycogen	DiNuzzo *et al.* 2012
defense against oxidative stress	high levels of antioxidants (e.g. GSH, ascorbate, vitamin E), high activities of ROS-detoxifying enzymes, proposed supply of GSH-precursors to neurons	Schmidt and Dringen 2012 Aoyama and Nakaki 2013

e.g., for example; GSH, glutathione; GABA, γ-aminobutyric acid; ATP, adenosine triphosphate; ROS, reactive oxygen species

Due to the metabolic coupling of astrocytes and neurons, metabolic changes in astrocytes may not only alter or impair astrocyte functions alone but are likely to at least add to neuronal dysfunction (Allaman *et al.* 2011). Indeed various studies suggest that astrocytic malfunctions are involved in the pathogenesis of neurological diseases, such as multiple sclerosis, amyotrophic lateral sclerosis, Alexander disease, Alzheimer's disease, depression, epilepsy, Huntington's disease or leukodystrophy (Armato *et al.* 2013; Brosnan and Raine 2013; Clabough 2013; Devinsky *et al.* 2013; Lanciotti *et al.* 2013; Sanacora and Banasr 2013; Valori *et al.* 2014; Yoshida and Nakagawa 2012).

1.2 Brain energy metabolism

To maintain the full function as control unit of the body, the brain has very high energy demands. As there is only minute energy reservoirs in the brain, a continuous supply with energy substrates is crucial for normal brain function (Schousboe *et al.* 2007). The high energy demand of the brain, mainly to restore and maintain ion gradients and clear synapses of neurotransmitters for proper signal transduction, is strongly underlined by the high oxygen and glucose consumption of the brain in respect to the entirety of the body. Around 20 % of the total oxygen and 25 % of the total glucose are used exclusively by the brain, while it only constitutes around 2 % of the total body mass (Attwell and Laughlin 2001; Clarke and Sokoloff 1999).

Glucose represents the most important energy substrate in the brain (Dienel 2012), although fuels like lactate or ketone bodies, transported in the blood, have been shown to be transiently utilized as energy sources in the brain, especially early in development or during intense exercise (Prins 2012; Quistorff *et al.* 2008).

In the last two decades lactate has also been strongly propagated to be a regular, alternative energy substrate for neurons provided by astrocytes (Bélanger *et al.* 2011; Pellerin 2010; Pellerin and Magistretti 1994, 2012). According to the astrocyte-neuron lactate shuttle (ANLS) hypothesis (Figure 1.1) glucose is taken up into astrocytes by the glucose transporter 1 (GLUT1) and is not completely oxidized but rather metabolized to lactate. Lactate is exported by astrocytes and subsequently taken up by neurons via monocarboxylate transporters (MCT) and further oxidized in neurons to fulfill their energy demands.

Whether lactate is indeed a regular energy fuel for neurons to meet their energy demand is, however, highly debated (Dienel 2012).

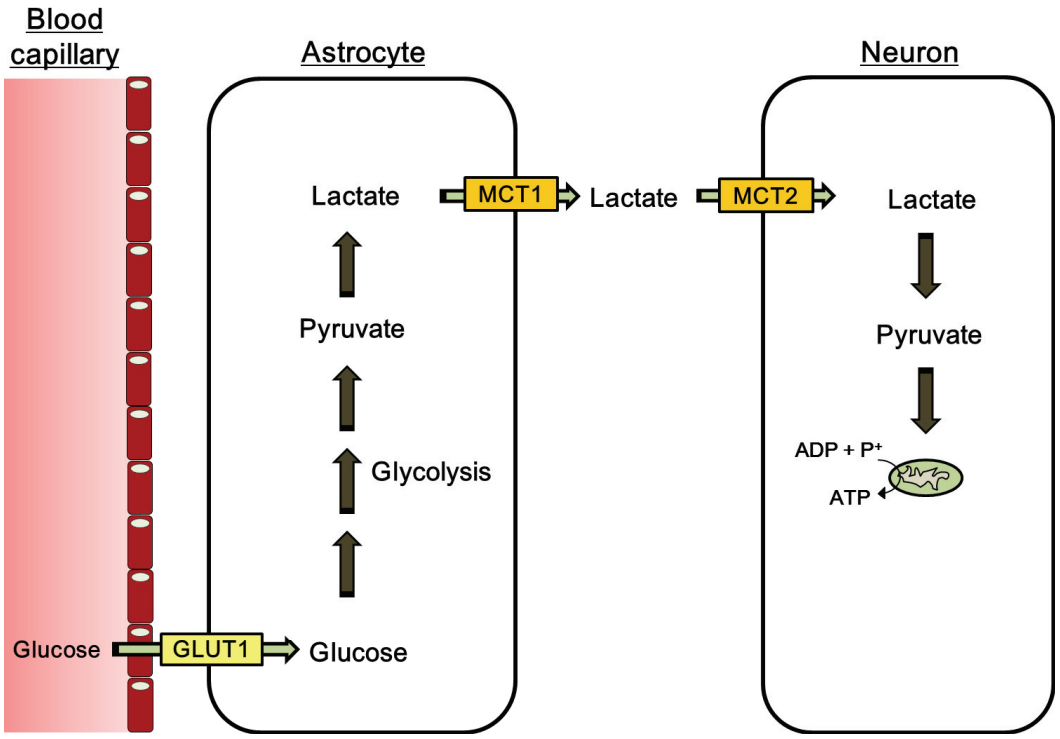


Figure 1.1: Astrocyte-neuron lactate shuttle (ANLS) hypothesis. Astrocytes take up glucose from the blood by the glucose transporter 1 (GLUT1) and metabolize the intracellular glucose to lactate. The generated lactate is exported from astrocytes by the monocarboxylate transporter 1 (MCT1). Extracellular lactate is taken up by the neuronal monocarboxylate transporter 2 (MCT2) and further metabolized in neurons to produce ATP via oxidative phosphorylation.

1.3 Glutathione metabolism in the brain

The tripeptide Glutathione (GSH) is an important thiol in the brain and is a key player for healthy brain functions as it is crucial for the defense against xenobiotics and oxidants and for the maintenance of a reduced thiol reduction state (Aoyama and Nakaki 2013; Hirrlinger and Dringen 2010; Johnson *et al.* 2012; Lu 2013; Schmidt and Dringen 2012). Its concentration in cells is in the millimolar range but can vary in different brain regions and between different neural cell types

(Aoyama and Nakaki 2013; Cooper 1997; Dringen and Hamprecht 1998; Schmidt and Dringen 2012). GSH was firstly identified in a yeast extract by J. de Rey-Pailhade in 1888 and named "philothione" originating from the ancient Greek words *philia* (love) and *theion* (sulfur) due to its potential to reduce sulfur to hydrogen sulfide (Aoyama and Nakaki 2013; De Rey-Pailhade 1888; Meister 1988). In 1921 F. G. Hopkins further characterized this compound as a dipeptide containing glutamate and cysteine and proposed the new name "glutathione" (Hopkins 1921), but later corrected his description of GSH to be a tripeptide also containing glycine (Hopkins 1929). However after these discoveries extensive investigations concerning the importance and functions of GSH, particularly in the central nervous system (CNS), were started after a gap of around forty years in the 1970s (Aoyama and Nakaki 2013).

1.3.1 Functions of glutathione

Due to the high energy demand and the continuously high oxygen consumption of the brain, there is a constant production of reactive oxygen species (ROS) in brain mitochondria. Especially superoxide (O_2^-) is generated as side product of oxidative phosphorylation (Venditti *et al.* 2013). O_2^- can undergo dismutation to hydrogen peroxide (H_2O_2) in a reaction catalyzed by superoxide dismutase (SOD) or reacts with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$) (Venditti *et al.* 2013). Furthermore H_2O_2 can form hydroxyl radicals (OH^-) by reacting with ferrous iron in the Fenton reaction (Halliwell and Gutteridge 1992; Halliwell and Gutteridge 1999). ROS can severely damage cells by modifying proteins, inducing lipid peroxidation or causing DNA strand breaks (Murphy *et al.* 2011; Venditti *et al.* 2013). Thus, to prevent severe oxidative damage of brain cells by ROS an effective antioxidative defense is crucial. As the brain, compared to the kidney or liver, has lower specific activities of antioxidative enzymes like SOD and catalase, GSH

represents an especially important antioxidant in the defense against ROS (Cooper 1997; Dringen 2000; Ho *et al.* 1997; Schmidt and Dringen 2012). GSH is involved in two antioxidative mechanisms. Firstly, GSH can react non-enzymatically with O_2^-, $ONOO^-$ and OH^- (Saez *et al.* 1990; Winterbourn and Metodiewa 1994). Secondly, GSH can function as electron donor for the glutathione peroxidase catalyzed reduction of peroxides like H_2O_2 (Dringen *et al.* 2005; Schmidt and Dringen 2012). In this reaction GSH is oxidized and forms glutathione disulfide (GSSG) which is subsequently reduced to GSH by glutathione reductase (GR) (Dringen *et al.* 2005; Schmidt and Dringen 2012). In cells under unstressed conditions GSH is mostly present in its reduced form (Cooper 1998) as demonstrated by a high GSH:GSSG ratio. However, during oxidative stress this ratio can transiently drop, due to the limiting activity of the GSH regenerating enzyme GR (Dringen and Hamprecht 1997). Thus, the ratio of GSH:GSSG is a good indicator for the presence of oxidative stress. A lowering in the GSH:GSSG can result in reversible conjugation of proteins to GSH (S-glutathionylation) catalyzed by glutathione-S-transferases to protect or alter their function (Chatterjee 2013; Cooper *et al.* 2011; Gould *et al.* 2013). Furthermore the thiol redox state in form of the GSH:GSSG ratio has been indicated to be involved in the regulation of gene expression, cell proliferation and apoptosis (Aoyama and Nakaki 2013; Chatterjee 2013; Cooper *et al.* 2011). S-glutathionylation of xenobiotics and therapeutic drugs is also very important for an efficient detoxification of such compounds (Cooper *et al.* 2011; Schmidt and Dringen 2012).

In addition to many intracellular functions of GSH in the brain, extracellular GSH has been reported to function as neuromodulator by binding to N-methyl-D-aspartate (NMDA) glutamate receptors (Janáky *et al.* 2007).

Considering the important functions of GSH in the brain, it is not surprising that alterations of the GSH homeostasis in brain may be detrimental for proper brain functioning. A dysregulation in brain GSH homeostasis has been discussed for many neurological diseases, including multiple sclerosis, Alzheimer's disease and

Parkinson's disease (Ferreira *et al.* 2013; Johnson *et al.* 2012; Pocernich and Butterfield 2012; Smeyne and Smeyne 2013).

1.3.2 Synthesis of glutathione

GSH is a hydrophilic compound. However, literature information whether and to which extent GSH can passively cross the blood-brain barrier (BBB) or can be actively transported is currently not consistent and under debate (Cornford *et al.* 1978; Peterson *et al.* 2013). As GSH plasma concentrations in humans have been reported to be much lower than brain concentrations (Flagg *et al.* 1993; Jones *et al.* 1998), brain GSH is considered to be mainly synthesized in brain cells from substrate amino acids. Synthesis of the tripeptide GSH in the cytosol is a two-step, ATP-consuming reaction catalyzed by glutamate cysteine ligase (GCL) and GSH synthetase (GS) (Meister 1974) (Figure 1.2). In the first reaction glutamate and cysteine form the dipeptide γ-glutamylcysteine (γ–GluCys) catalyzed by the GCL and in the second reaction glycine is added to the γ–GluCys to form GSH, catalyzed by the GS. Besides the activity of GCL (Griffith 1999; Griffith and Mulcahy 1999), the limiting step of GSH synthesis is the availability of the amino acid precursors, especially cysteine. Its disulfide form cystine is efficiently used at least by cultured astrocytes for GSH synthesis after intracellular reduction to cysteine as GSH precursor (Kranich *et al.* 1998). In contrast, neurons prefer cysteine over cystine (Kranich *et al.* 1996) and therefore are considered to rely on sufficient supply of cysteine from astrocytes.

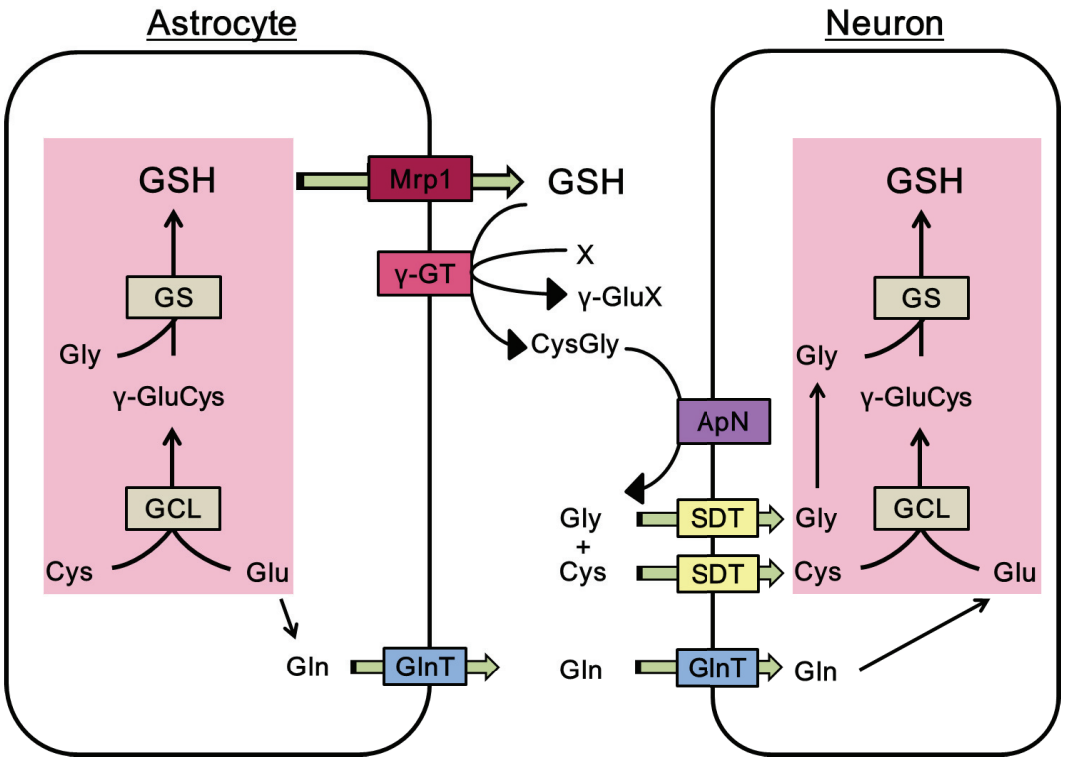


Figure 1.2: **Glutathione (GSH) synthesis and supply of precursors for GSH synthesis from astrocytes to neurons**. In brain cells GSH is synthesized (pink boxes) in two ATP-consuming reactions. In the first reaction glutamate cysteine ligase (GCL) uses the substrates glutamate (Glu) and cysteine (Cys) to generate γ-glutamylcysteine (γ–GluCys). In the second reaction, catalyzed by GSH synthetase (GS), glycine (Gly) is added to γ–GluCys to form GSH. Astrocytic GSH is mainly exported by the multidrug resistance protein 1 (Mrp1). Subsequently the γ–glutamyl moiety is transferred from extracellular GSH to an acceptor (X) by the astrocytic ecto-enzyme γ–glutamyl transpeptidase (γ-GT) generating γ–GluX and the dipeptide CysGly. CysGly is cleaved to Cys and Gly by the neuronal aminopeptidase N (ApN). After uptake via different sodium dependent transporters (SDT) Cys and Gly are utilized as substrates for neuronal GSH synthesis. Glutamate for GSH synthesis is provided to neurons in form of glutamine (Gln). Export of Gln from astrocytes and uptake of Gln into neurons takes place by different Gln transporters (GlnT). Modified from Dringen (2009).

1.3.3 Export of astrocytic glutathione and supply of glutathione precursors to neurons

Cultured astrocytes contain with around 8 mM very high intracellular GSH levels (Dringen and Hamprecht 1998), and can export approximately 10 % of their GSH per hour via multidrug resistance proteins (Mrp) (Dringen *et al.* 1997).

This export provides neurons with the necessary precursor cysteine for GSH synthesis (Dringen 2009; Dringen *et al.* 1999) (Figure 1.2). Exported GSH is substrate of the astrocytic ecto-enzyme γ–glutamyl transpeptidase (γ-GT) which transfers the γ–glutamyl moiety to an acceptor, for example amino acids, peptides or water (Tate and Meister 1974) and generates the dipeptide CysGly (Dringen *et al.* 1999). Subsequently CysGly is further cleaved by the neuronal aminopeptidase N (ApN) to generate cysteine and glycine which are taken up into neurons as substrates for GSH synthesis (Dringen *et al.* 2001). In addition, astrocytes provide neurons with glutamate in form of glutamine (Dringen 2009).

1.4 Human immunodeficiency virus

The infection with the human immunodeficiency virus (HIV) and its deleterious consequences on the human immune system firstly caught the world's eye in 1981 (Centers for Disease Control and Prevention 1981). However at that time only the fatal immune deficiency had been observed in sexually active homosexual patients which was named acquired immunodeficiency syndrome (AIDS). The virus itself as cause of this disease was isolated 2 years later in 1983 by Luc Montagnier and colleagues (Barré-Sinoussi *et al.* 1983) and the first initial evidence of the connection between the virus and AIDS was obtained around one year later (Gallo *et al.* 1984). In 1986, after several years of research, the virus was finally named HIV (Coffin *et al.* 1986). The introduction of a combinatorial treatment, successfully turned HIV infection from a fatal into a chronic, but manageable disease (Deeks *et al.* 2013).

Today, more than thirty years after discovery, there are 35 million patients living with HIV infection worldwide. Although the number of new infections is decreasing, still around 2.3 mio people were infected with HIV in 2012 (UNAIDS 2013). These numbers show that HIV infection is still an important issue for research and medicine (Ippolito *et al.* 2013; Mastro and Abdool Karim 2009).

1.4.1 Virus structure and course of infection

HIV is a retrovirus belonging to the sub-class of lentiviruses (Rambaut *et al.* 2004). This virus mainly targets cells of the human immune system like lymphocytes, monocytes and thymocytes (Fang *et al.* 2008; Klimas *et al.* 2008; Nilsson *et al.* 2007; Okoye and Picker 2013) but also cells of the CNS, especially microglia and astrocytes (Ghafouri *et al.* 2006; Gray *et al.* 2014; Lamers *et al.* 2014; Spudich 2013; Ton and Xiong 2013). There are two genetically distinct types of HIV, called HIV-1 and HIV 2 (Klimas *et al.* 2008). HIV-1 is the major type of virus, which is found in most countries in the world, whereas HIV-2 is the predominant virus type in a small number of Western African countries (Klimas *et al.* 2008; Usach *et al.* 2013).

The HIV genome consists of two copies of RNA which are, together with the three essential viral enzymes reverse transcriptase (RT), integrase and HIV aspartic protease enclosed by the viral p24 capsid proteins (Figure 1.3). This nucleo-capsid is further surrounded by the p17 matrix proteins and a lipid bilayer. Imbedded into the lipid bilayer are the viral glycoproteins (gp) 120 and 41 proteins. Gp 120 recognizes and attaches mainly to cluster of differentiation 4 (CD4) host cell receptors and the essential CC chemokine receptor 5 or CXC chemokine receptor 4 co-receptors (Barbaro *et al.* 2005; Levy 2009; von Recum and Pokorski 2013). The gp 41 induces fusion with the host cell membrane which results in the entry of the viral core into the host cell (Barbaro *et al.* 2005).

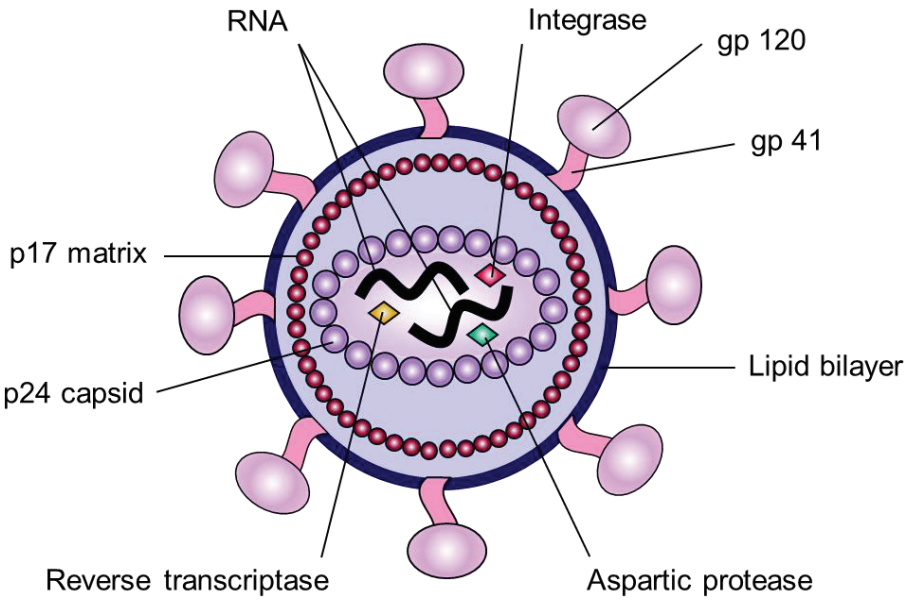


Figure 1.3: Structure of the human immuno deficiency virus (HIV). Two copies of RNA and the viral enzymes reverse transcriptase, integrase and aspartyl protease are enclosed by the p24 capsid proteins. This nucleo-capsid is further coated by p17 matrix proteins and a lipid bilayer. The lipid bilayer contains the two glycoproteins (gp) 41 and 120. Modified from Turner and Summers (1999).

HIV is predominantly transmitted by body fluids, like genital liquids and contaminated blood but a mother to child transmission has also been reported (Klein 2000; Klimas *et al.* 2008; Levy 2009; Royce 1997). During the first weeks after infection many patients show strong immune responses similar to flew-like symptoms (Klimas *et al.* 2008; Levy 2006). This initial stage, also called acute HIV-1 infection syndrome, is followed by an asymptomatic period, lasting for approximately 10 years, in which continuous antiviral immune responses are accompanied by continuous slow virus replication (Levy 2009). After the entry into the symptomatic phase increased viral replication continuously destroys immune competent cells resulting in the occurrence of minor, but not life-threatening

opportunistic infections, such as herpes simplex and fungal infections (Klimas *et al.* 2008). The progress of intensive viral replication accompanied by the complete breakdown of the immune activities finally causes severe and lethal opportunistic infections and cancers, which are designated as predominant features of AIDS (Centers for Disease Control and Prevention 1993; Klimas *et al.* 2008).

1.4.2 Infection of the brain

Besides the development of cancers and the occurrence of opportunistic infections many AIDS patients also show the distinct symptoms of HIV-associated neurocognitive disorders (HAND) (Clifford and Ances 2013; Lamers *et al.* 2014; Mirza and Rathore 2012). These deficits can range from minor impairments to severe dementia, also known as HIV-associated dementia (HAD) and are a result of the infection of the brain with HIV (Ghafouri *et al.* 2006; Lamers *et al.* 2014; Mirza and Rathore 2012).

Although it is still not completely understood how HIV crosses the BBB, the virus is widely believed to enter the brain mainly via infected monocytes early in the course of infection (Trojan horse hypothesis) as is shown in Figure 1.4 (Spudich and Gonzalez-Scarano 2012; Zhou and Saksena 2013).

Theoretically the infection of all brain cell types by HIV is possible, as all types express the necessary receptors and/or co-receptors to enable virus entry (Gonzalez-Scarano and Martin-Garcia 2005; Mirza and Rathore 2012). However, besides macrophages, mostly microglia and astrocytes, are infected with HIV (Gray *et al.* 2014; Mirza and Rathore 2012; Spudich 2013; Ton and Xiong 2013).

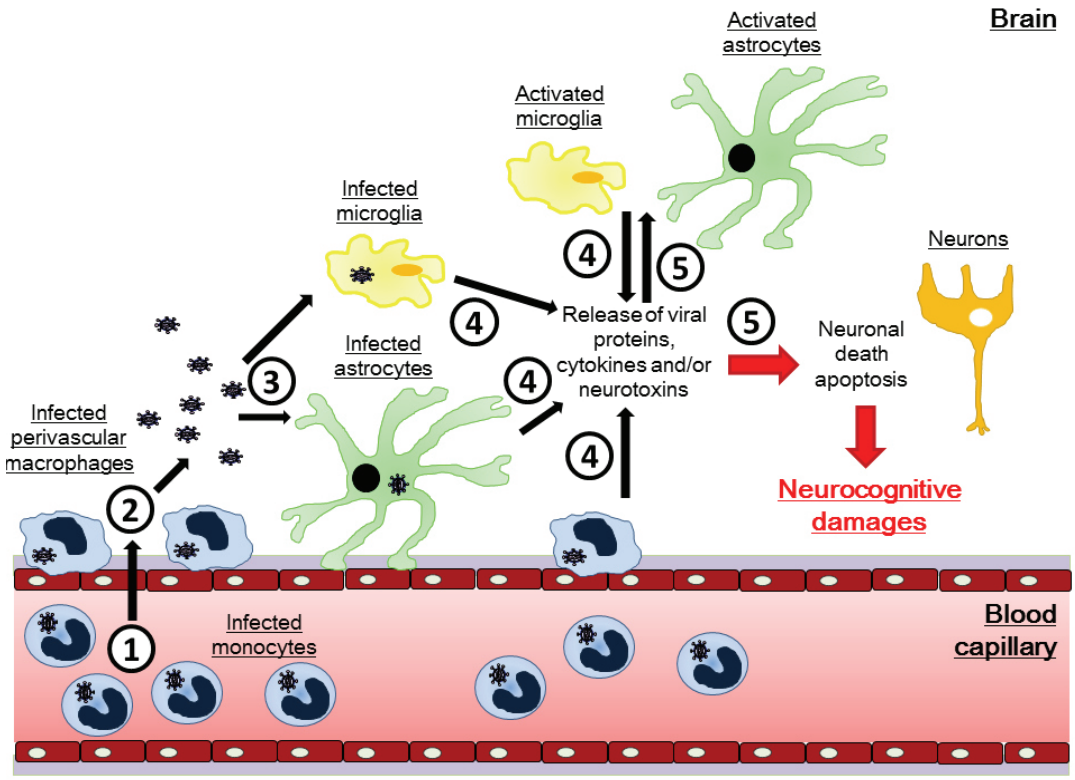


Figure 1.4: Model of HIV entry into the brain and mechanisms of neuronal damage. HIV is considered to enter the brain via infected monocytes (**1**). These monocytes differentiate into perivascular macrophages and produce infectious viral particles (**2**), which subsequently infect astrocytes and microglia (**3**). Infected microglia, macrophages and astrocytes will release viral proteins and/or cytokines/neurotoxins (**4**), which will directly damage neurons and activate non-infected microglia and astrocytes (**5**), which in turn release additional cytokines and neurotoxins. Modified from Spudich and Gonzalez-Scarano (2012).

As neuronal death or damage due to direct infection with HIV has until today not been proven, neuronal damage is rather considered a result of indirect mechanisms, which are depicted in (Figure 1.4) (Spudich 2013). On one hand viral proteins, secreted by infected macrophages or microglia, might directly damage neurons and have been shown to disrupt the BBB (Louboutin and Strayer 2012; Xu

et al. 2012) resulting in the entry of inflammatory cells into the brain. On the other hand infected macrophages or microglia may release various cytokines and neurotoxins, which can directly induce apoptosis in neurons but may also further stimulate their production by activation of non-infected microglia and astrocytes. This cascade of events is believed to interfere with normal neuronal functions, to modulate synaptic transmission and to finally induce neuronal death which results in neurocognitive deficits in HIV infected patients (Ghafouri *et al.* 2006).

1.5 Highly active antiretroviral therapy

Despite intense research for more than thirty years it is still not possible to cure patients suffering from HIV infection (Dieffenbach and Fauci 2011). However HIV infection has turned from a lethal into a chronic but manageable disease (Deeks *et al.* 2013). This has been achieved by the introduction of the highly active antiretroviral therapy (HAART) in the 1990s (Arts and Hazuda 2012), a combinatorial therapy closely connected to the viral life cycle (Figure 1.5) which combines different classes of antiretroviral drugs to simultaneously block viral replication at different stages (Barbaro *et al.* 2005; Bazzoli *et al.* 2010). Unfortunately, such a combination of several classes of antiretroviral drugs is not able to remove the virus from already infected cells but will prevent the occurrence of new waves of infection within the body. This results in an at least partial restoration of a functional immune system and will slow down the progress of the disease.

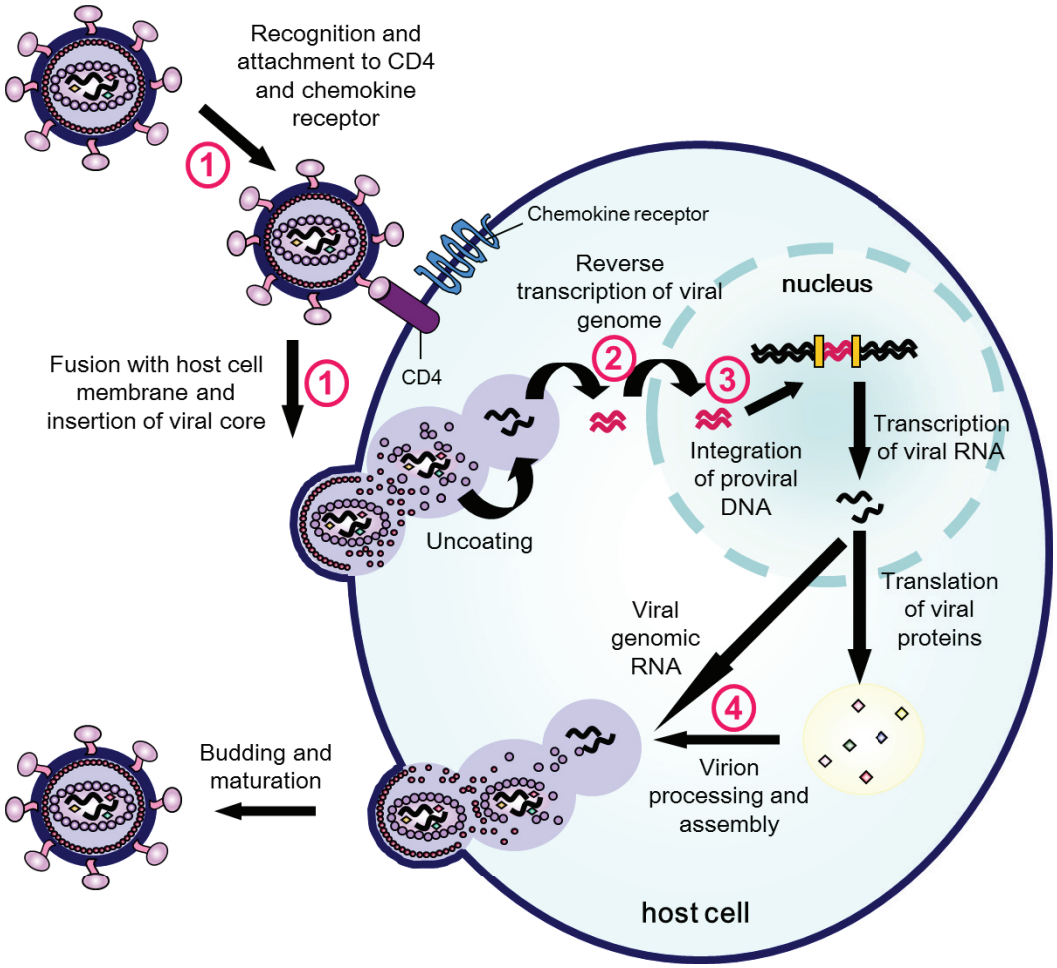


Figure 1.5: Targets of antiviral therapy in the HIV life cycle. The attachment and fusion of HIV is targeted by entry-/fusion inhibitors (**1**). After the entry of HIV the reverse transcription of the viral RNA can be inhibited by non-nucleoside and/or nucleoside/nucleotide reverse transcriptase inhibitors (**2**). The subsequent integration of the proviral DNA is prohibited by integrase inhibitors (**3**). The processing of the viral precursor poly-proteins is blocked by protease inhibitors (**4**). Modified from Rambaut *et al*. (2004).

The main targets of HAART in the life cycle of HIV are depicted in Figure 1.5. The entry of HIV as initial step of the viral life cycle can be blocked by entry inhibitors which prevent virus attachment to host cell receptors/co-receptors or fusion with

the host cell membrane (Bazzoli *et al.* 2010; De Clercq 2009; von Recum and Pokorski 2013). Several subclasses of RT inhibitors, non-nucleoside RT inhibitors (NNRTI) and nucleoside/nucleotide RT inhibitors (N(t)RTI), prevent reverse transcription of the viral RNA genome into DNA, the second stage of the HIV life cycle, by inhibiting the essential viral enzyme RT (Bazzoli *et al.* 2010; Cihlar and Ray 2010; De Clercq 2009; Usach *et al.* 2013). The crucial step of viral DNA integration is prohibited by the rather recently developed class of integrase inhibitors (Bazzoli *et al.* 2010; De Clercq 2013; Stock 2013). Finally, the class of HIV protease inhibitors impedes the cleavage of viral precursor poly-proteins into mature and functional viral proteins by the HIV protease, the fourth essential step of the viral life cycle (Bazzoli *et al.* 2010; De Clercq 2013; Wensing *et al.* 2010).

The next sections will give a more detailed introduction on HIV protease inhibitors and the NNRTI efavirenz, as the effects of those drugs on the glutathione- and glucose-metabolism of brain cells were extensively investigated in this thesis.

1.5.1 Protease inhibitors

The discovery that the HIV protease is a crucial enzyme needed to produce mature, infectious viral particles (Kohl *et al.* 1988; McQuade *et al.* 1990) made it a well-investigated prime target for therapy (Arts and Hazuda 2012; Wensing *et al.* 2010; Wlodawer and Vondrasek 1998; Zha *et al*. 2012). HIV protease is a homodimer with an active center that is very similar to those of pepsin-like proteases (Navia *et al.* 1989; Wlodawer and Vondrasek 1998). Unlike human aspartic proteases, HIV protease has initially been described to be able to cleave tyrosine-proline or phenylalanine-proline bonds within the viral precursor poly-proteins (Wlodawer and Vondrasek 1998). However, more recently HIV protease has been shown to recognize the asymmetric shape of the precursor proteins rather

than specific amino acid sequences, thus enabling the cleavage at sites which share no sequence homology with other substrates of aspartic proteases (Wensing *et al.* 2010).

For the HIV protease selective inhibitors containing a hydroxyethylene core have been synthesized, mimicking the typical structure of the substrate cleavage sites. These peptidomimetics efficiently bind to the active center of the enzyme but take more space than the natural substrate. They are not cleaved but instead occupy the enzyme and inhibit hydrolysis of the HIV precursor poly-proteins (Flexner 1998; Wensing *et al.* 2010). Currently there are 10 different protease inhibitors approved for therapy by the US Food and Drug Administration (De Clercq 2013; US Food and Drug Administration 2013). Figure 1.6 shows the structures of the protease inhibitors indinavir, nelfinavir, ritonavir and lopinavir, which were investigated for their effects on cultured brain cells in this thesis.

All four protease inhibitors are very lipophilic, in the order of nelfinavir>lopinavir>ritonavir>indinavir, indicating that those drugs may enter cells by passive diffusion (Ford *et al.* 2004). However active uptake via organic anion transporters can also not be excluded (Griffin *et al.* 2011). Although the uptake of protease inhibitors is not fully understood and might vary for the different protease inhibitors, intracellular accumulation of the therapeutically active drugs is limited by effective export systems such as P-glycoprotein (P-gp) and Mrps (Griffin *et al.* 2011). Furthermore, the high affinity of protease inhibitors to bind plasma proteins might influence cellular accumulation, as it is not known whether only the very low non-protein-bound fraction of the drugs is available for cellular uptake or if also the high percentage of protein-bound fraction is available (Ford *et al.* 2004; Gimenez *et al.* 2004). In addition to those factors, metabolism, mostly in the liver by cytochrome P450 (CYP) systems, limits the availability of the protease inhibitors indinavir, nelfinavir and lopinavir resulting in relatively short blood plasma half-lives (Bazzoli *et al.* 2010; Griffin *et al.* 2011). Only ritonavir has

been shown to inhibit CYP 3A and is therefore currently used in a low, non-therapeutic dose together with other protease inhibitors, except nelfinavir, to slow down their metabolism and increase their half-life in the body (Cooper *et al.* 2003; von Hentig and Haberl 2012).

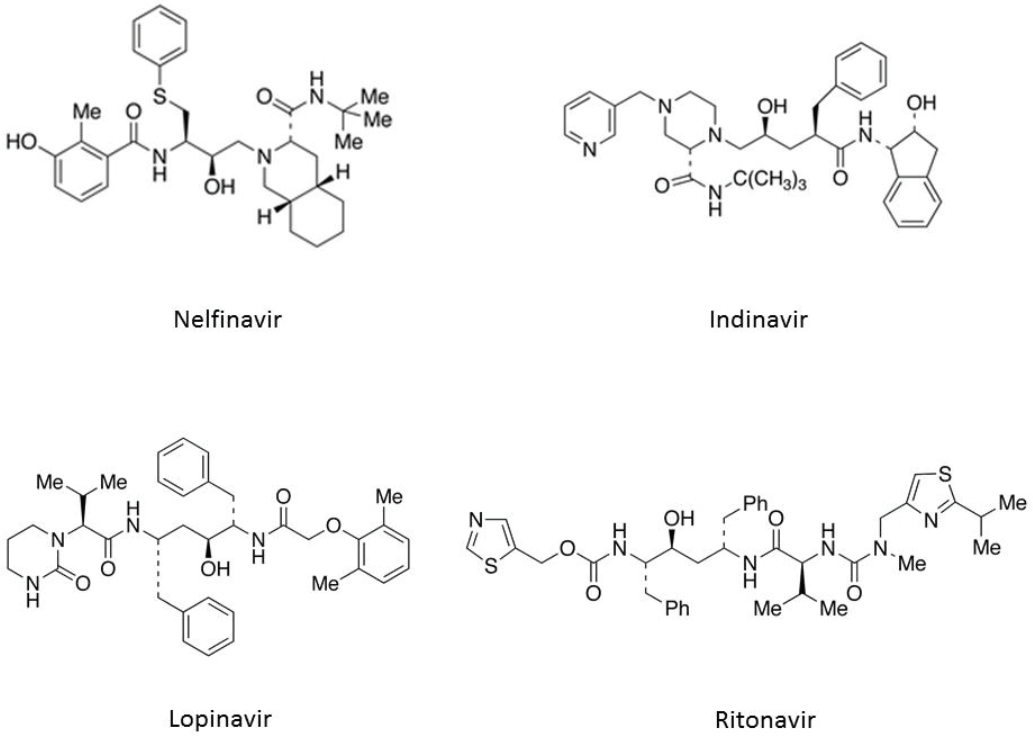


Figure 1.6: Structures of the HIV protease inhibitors nelfinavir, indinavir, lopinavir and ritonavir. The structures are taken from the homepage of the supplier Toronto Research Chemicals Inc. (*www.trc.com*_(2014/02/21)).

Even though the physicochemical properties of protease inhibitors limit the availability and half-life in the body to some extent, severe clinical manifestations in HIV patients, such as insulin resistance, disturbances in lipid metabolism,

including lipodystrophy, as well as cardiovascular disease have been linked to protease inhibitor-containing regimen (Bavinger *et al.* 2013; Hruz 2011; Souza *et al.* 2013). Studies on hepatocytes, adipocytes, peripheral blood mononuclear cells and endothelial cells have revealed potential explanations for those adverse effects of protease inhibitor treatment and are summarized in Table 1.2.

Table 1.2: Proposed mechanisms involved in clinical side effects of protease inhibitor-containing regimen

clinical manifestation	**proposed mechanism**	**investigated cell type**	**Reference**
insulin resistance	- inhibition of insulin-dependent GLUT4 transporter - ROS production - decrease in adiponectin levels	adipocytes	Parker *et al.* 2005 Ben-Romano *et al.* 2006 Lagathu *et al.* 2007
lipid disturbances	- endoplasmic reticulum stress - inhibition of the degradation of transcription factors controlling lipid pathways - cell type specific induction or inhibition of triglyceride and/or cholesterol synthesis - ROS production - decrease in adiponectin levels	adipocytes, hepatocytes, peripheral blood mononuclear cells	Taura *et al.* 2013 Williams *et al.* 2004 Zhou *et al.* 2006 Lenhard *et al.* 2000 Lagathu *et al.* 2007
cardiovascular disease	- inhibited expression of NO-synthase - ROS production	coronary arteries, endothelial cells	Chai *et al.* 2005 Mondal *et al.* 2004

1.5.2 Non-nucleoside reverse transcriptase inhibitors

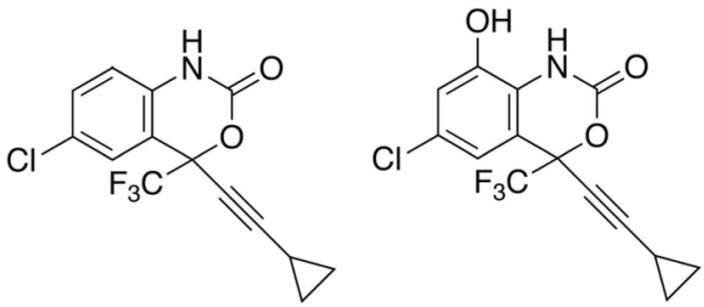
RT is the first viral enzyme in the HIV life cycle and catalyzes the reverse transcription of the viral RNA into DNA to clear the way of DNA integration into the host genome. RT displays three distinct functions to generate a viral double stranded DNA molecule. It first functions as an RNA-dependent DNA-polymerase, which translates the viral RNA into DNA and produces an RNA-DNA double strand. Secondly it exerts an RNase H activity, hydrolyzing the RNA template strand and producing a DNA single strand. Lastly through its function as DNA-dependent DNA-polymerase it converts the HIV single stranded DNA into a double stranded molecule by synthesizing the second DNA strand (Barbaro *et al.* 2005). This is a crucial step for the successful viral life cycle making the RT the first, but also still today, a very important target for the development and use of antiretroviral therapeutic drugs (Arts & Hazuda 2012; Barbaro *et al.* 2005; Cihlar and Ray 2010; Mitsuya *et al.* 1985; Usach *et al.* 2013).

RT is targeted by several subclasses of antiretroviral drugs. N(t)RTIs are nucleoside/nucleotide analogues, which are incorporated into the nascent DNA molecule but terminate the elongation of the strand due to the absence of 3' hydroxyl group (Cihlar and Ray 2010; De Clercq 2009). In contrast, NNRTIs exclusively inhibit HIV-1 RT (Usach *et al.* 2013) by binding to an allosteric pocket, which is functionally and spatially associated to the substrate binding site (De Clercq 2009).

There are currently 5 approved NNRTIs which are regular components of HAART (US Food and Drug Administration 2013). Efavirenz, one of the most commonly used NNRTI, is a benzoxazinone derivative (Figure 1.7) which is very lipophilic and highly bound to plasma proteins (Usach *et al.* 2013). Organic anion transporters are involved in the cellular accumulation of efavirenz but these transporters do not appear to be exclusively responsible for cellular efavirenz

uptake (Janneh *et al.* 2009). In contrast to protease inhibitors, efavirenz is not considered to be efficiently exported by export systems like P-gp or Mrps (Janneh *et al.* 2009; Störmer *et al.* 2002).

The therapeutic actions of efavirenz have been shown to be limited by its metabolism. Efavirenz is primarily metabolized in the liver by mostly the monooxygenases CYP 2B6 and CYP 3A4 to its therapeutically non-active primary metabolite 8-hydroxy-efavirenz (8-OH-efv) (Fig. 1.7) (Desta *et al.* 2007; Usach *et al.* 2013). As efavirenz has been shown to additionally induce its own metabolism (Usach *et al.* 2013) 8-OH-efv strongly accumulates, which is indicated by high blood plasma concentrations of up to 7 µM in treated HIV patients (Avery *et al.* 2013; Habtewold *et al.* 2011; Ngaimisi *et al.* 2013; Ngaimisi *et al.* 2010). Despite the knowledge of the rapid oxidation of efavirenz and the accumulation of its primary metabolite, there is hardly any information available on potential effects of 8-OH-efv on different cell types.



Efavirenz

8-hydroxy-efavirenz
(8-OH-efv)

Figure 1.7: Structures of the non-nucleoside reverse transcriptase inhibitor efavirenz and its primary metabolite 8-hydroxy-efavirenz (8-OH-efv). The structures are taken from the homepage of the supplier Toronto Research Chemicals Inc. (*www.trc.com*_(2014/02/21)).

Table 1.3 summarizes various side effects of a treatment of HIV patients with efavirenz. Despite the long list of adverse effects, the cellular and/or molecular mechanisms underlying the reported side effects remain to be elucidated in more detail. Exposure of hepatic cell culture models or human peripheral blood mononuclear cells to efavirenz, has been reported to induce apoptosis, endoplasmic reticulum stress, mitochondrial dysfunction due to inhibition of respiration and increase in mitochondrial ROS production, and decrease in cellular GSH levels (Apostolova *et al.* 2013; Apostolova *et al.* 2010; Blas-Garcia *et al.* 2010). Such mechanisms may contribute to the development of the described clinical manifestations during treatment with efavirenz-containing regimen.

Table 3: Clinical adverse effects of a treatment with efavirenz-containing regimen

clinical manifestation	**reference**
hepatotoxicity	Brück *et al.* 2008; Fink and Bloch 2013; [a]
skin rashes	Ananworanich *et al.* 2005; [a]
increase in cholesterol and triglycerides	Negredo *et al.* 2004; Mankhatitham *et al.* 2012; [a]
lipodystrophy/lipoatrophy	Haubrich *et al.* 2009; [a]
gastrointestinal effects	- [a]
joint pain	- [a]
muscle pain	- [a]
myopathy	- [a]
asthenia	- [a]
breathlessness	- [a]
gynecomastia	- [a]
tinnitus	- [a]

[a] information taken from prescribing information of Sustiva (brand name of efavirenz) from Bristol-Myers Squibb (*packageinserts.**bms**.com/pi/pi_**sustiva**.pdf*_(2014/04/02))

1.6 Effects of antiretroviral drugs on brain cells

HAART successfully lowers viral replication in the body. However, although the incidence of HAD, the most severe form of HAND, has been reduced since the introduction of HAART, milder forms of neurocognitive deficits are still common (Lamers *et al.* 2014; Manji *et al.* 2013; Mothobi and Brew 2012). This persistence of HAND is intensively discussed and might be a consequence of multiple factors, including damages caused by HIV before treatment, immune restoration disorders caused by antiretroviral drugs, co-morbidities like drug abuse and psychological illnesses, insufficient viral suppression due to restricted drug penetration, ageing and neurotoxicity of HAART drugs (Mothobi and Brew 2012). A contribution of the applied antiretroviral drugs or their metabolites in the formation of HAND, although being taken into consideration, currently lacks sufficient experimental proof on the cellular level and needs to be vastly extended (Mothobi and Brew 2012; Tan and McArthur 2012).

Despite various information about adverse effects on the periphery induced by HIV protease inhibitors or the NNRTI efavirenz, studies on brain cells remain scarce. *In vivo* it was shown that lopinavir boosted with a low dose of ritonavir affected memory performance and caused brain injury in mice (Gupta *et al.* 2012; Pepping *et al.* 2014; Pistell *et al.* 2010). In an *in vivo* rat model ritonavir-containing regimen has been described to induce synaptic damage and neuronal loss (Akay *et al.* 2014). Furthermore ritonavir showed moderate toxicity after longterm-treatment of cultured neurons (Akay *et al.* 2014; Liner *et al.* 2010; Robertson *et al.* 2012). In addition it was shown that ritonavir, lopinavir and nelfinavir inhibited amyloid-ß-peptide production in neuron cultures, which however was not seen *in vivo* (Lan *et al.* 2012). During treatment with efavirenz-containing regimen 40-70 % of patients suffer from neurological adverse effects, including suicidal tendencies, hallucinations, impaired concentration, dizziness, anxiety, insomnia, abnormal dreams, memory disorders and depression (Cavalcante *et al.* 2010; Cespedes and

Aberg 2006; Fumaz *et al.* 2005; Jena *et al.* 2009; Lochet *et al.* 2003). The cellular mechanisms leading to such effects yet remain largely unknown. *In vitro*, efavirenz has been shown to be neurotoxic (Liner *et al.* 2010; Robertson *et al.* 2012; Tovar-y-Romo *et al.* 2012). Exposure of mice to efavirenz caused an inhibition of creatine kinase and complex IV of the respiratory chain (Streck *et al.* 2011; Streck *et al.* 2008), suggesting that alterations in brain energy metabolism may possibly contribute to observed CNS side effects observed during efavirenz-treatment. However, considering the rapid metabolism of efavirenz it remains to be elucidated whether efavirenz or its accumulating primary metabolite is responsible for these effects. There is currently only one study describing 8-OH-efv as a potent neurotoxin and inducer of calcium influx into neurons (Tovar-y-Romo *et al.* 2012).

1.7 Aim of the thesis

HAART has proven to efficiently slow down HIV replication in patients and has thus saved the lives of millions of HIV patients for over 30 years. However, the persistent development of neurocognitive damages in the HAART-era concerns many physicians and scientists, and cannot be fully explained. Whether antiretroviral drugs contribute to those neurological adverse effects has still not been supported by sufficient experimental evidence despite intensive discussion (Mothobi and Brew 2012; Tan and McArthur 2012).

This thesis aims to investigate on cell culture models of astrocytes and neurons (Contestabile 2002; Lange *et al.* 2012; Tulpule *et al.* 2014) whether commonly used antiretroviral drugs have the potential to acutely damage brain cells or alter important metabolic pathways, such as GSH or glucose metabolism.

Due to their frequent use in therapy of HIV patients, especially HIV protease inhibitors and the RT inhibitor efavirenz will be applied to cultured primary rat astrocytes or neurons.

It will be investigated, whether antiretroviral drugs compromise cell viability, induce oxidative stress, alter GSH metabolism or GSH export or modulate glucose consumption or lactate production.

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2 Results

2.1 Publication 1

Brandmann M., Tulpule K., Schmidt M. M. and Dringen R. (2012)

The antiretroviral protease inhibitors indinavir and nelfinavir stimulate Mrp1-mediated GSH export from cultured brain astrocytes.

J Neurochem. **120**:78-92

Contributions of Maria Brandmann:
Data shown in Figures 1-5 and 7
Data shown in Table 1
Preparation of the first draft of the manuscript

DOI: 10.1111/j.1471-4159.2011.07544.x

2.2 Publication 2

Arend C., Brandmann M. and Dringen R.
(2013)

The antiretroviral protease inhibitor ritonavir accelerates glutathione export from cultured primary astrocytes.

Neurochem Res. **38**:732-741

Contributions of Maria Brandmann:
Supervision of Christian Arend during his Bachelor Thesis
Data shown in Figures 4 and 5
Improvement of the manuscript

DOI: 10.1007/s11064-013-0971-x

2.3 Publication 3

Brandmann M., Hohnholt M. C., Petters C. and Dringen R. (2014)

Antiretroviral protease inhibitors accelerate the multidrug resistance protein 1-mediated glutathione export from cultured neurons.

Neurochem Res. **39,** 883-892

Contributions of Maria Brandmann:
Data shown in Figures 1-5
Data shown in Table 1
Preparation of the first draft of the manuscript

DOI: 10.1007/s11064-014-1284-4

2.4 Publication 4

Brandmann M., Nehls U. and Dringen R. (2013)

8-Hydroxy-efavirenz, the primary metabolite of the antiretroviral drug efavirenz, stimulates the glycolytic flux in cultured rat astrocytes.

Neurochem Res. **38**:2524-2534

Contributions of Maria Brandmann:
Data shown in Figures 1-5
Data shown in Table 1
Preparation of the first draft of the manuscript

DOI: 10.1007/s11064-013-1165-2

3 Summarizing Discussion

Treatment of HIV patients with antiretroviral drugs is a successful means to improve the life expectancy of patients with HIV infection (Harrison *et al.* 2010; Nakagawa *et al.* 2012; Nakagawa *et al.* 2013). However, it is still rarely investigated whether the approved drugs have the potential to damage and/or alter important metabolic pathways in brain cells. In this thesis primary cultures of rat astrocytes and cerebellar granule neurons were used as established and frequently used model systems for astrocytes and neurons (Contestabile 2002; Lange *et al.* 2012; Tulpule *et al.* 2014) to investigate potential acute effects of antiretroviral drugs from the classes of RT and protease inhibitors on cell viability, glycolysis and GSH metabolism.

3.1 Effects of protease inhibitors on cultured brain cells

Among the various antiretroviral drugs from different classes investigated in this thesis, only the four HIV protease inhibitors indinavir, nelfinavir, ritonavir and lopinavir were found to acutely stimulate GSH export from both, cultured astrocytes and neurons, without compromising cell viability.

3.1.1 Cell viability

Low micromolar concentrations of indinavir, nelfinavir, ritonavir and/or lopinavir did not acutely compromise cell viability of cultured astrocytes or neurons in the hour range (chapters 2.1- 2.3). This indicates that both cell types are not highly susceptible to damages induced by these protease inhibitors. Similarly, in a very recent study bovine endothelial cells were reported not to be damaged after exposure for several hours to low micromolar concentrations of ritonavir (Chen

and Mak 2014). Apart from this study, investigations of the acute cell toxicity of protease inhibitors after short-term exposure are scarce.

24 h was the longest exposure time of astrocytes and neurons to indinavir, nelfinavir, ritonavir and/or lopinavir studied in this thesis. For this condition only nelfinavir showed some toxic potential on astrocytes (chapter 2.1), which was not observed for neurons (chapter 2.3). However, a direct comparison of the data obtained for astrocytes and neurons is impossible, as nelfinavir was applied in a three times higher concentration to astrocytes than to neurons. All the other investigated protease inhibitors did not compromise viability of astrocytes or neurons within a 24 h incubation (Arend 2012; chapters 2.1, 2.3).

Due to the necessary lifelong treatment of HIV patients with HAART (Deeks *et al.* 2013) investigations of the toxic potential of protease inhibitors have rather focused on longer incubation periods to study damage by chronic exposure to various cell types. For example, exposure of neuron and cortical neuroglial cultures to micromolar concentrations of ritonavir for up to 8 days resulted in a time- and concentration dependent loss of cell viability (Akay *et al.* 2014; Robertson *et al.* 2012). Cultured neurons exposed to low micromolar concentrations of nelfinavir, ritonavir or lopinavir for 48 h were only modestly damaged by nelfinavir, while ritonavir and lopinavir were not toxic (Lan *et al.* 2012). In other cell types, like adipocytes, macrophages, auditory cells and endothelial cells which were treated for at least one day with protease inhibitors, the extend of toxicity seems to be cell type and/or species specific (Chandler *et al.* 2003; Chen and Mak 2014; Lagathu *et al.* 2007; Manda *et al.* 2011; Thein *et al.* 2014).

The reported loss of cell viability after longterm-treatment of cultures neuroglial and peripheral cells with protease inhibitors is considered to be linked to oxidative stress (Akay *et al.* 2014; Chen and Mak 2014; Manda *et al.* 2011). However severe oxidative stress was not observed after treatment of cultured astrocytes or neurons

with the protease inhibitors indinavir, nelfinavir, ritonavir and/or lopinavir as almost all the glutathione determined inside and outside of the cells represented GSH. Only little amounts, in the range of the detection limit (Hohnholt and Dringen 2014), have been identified as oxidized GSSG (chapters 2.1-2.3). The good antioxidative potential of astrocytes (Dringen *et al.* 2005) and neurons (Hohnholt and Dringen 2014) could be responsible for preventing oxidative stress during short-term treatments of cultured astrocytes and neurons with protease inhibitors.

Interestingly, a 24 h exposure of astrocytes to indinavir resulted in an increase in ROS production although the cells remained viable (chapter 2.1). In contrast, treatment with nelfinavir for 24 h compromised the viability of astrocytes to some extent, without accelerating ROS production (chapter 2.1). Thus, increased formation of ROS after exposure and the toxic potential of protease inhibitors appear not to be directly linked in astrocytes. For nelfinavir, the induction of apoptosis, as already shown in various other cell types, including cancer cells (Braga-Neto *et al.* 2012; Gills *et al.* 2007; Kraus *et al.* 2013; Kushchayeva *et al.* 2014) might explain its delayed toxic potential in astrocytes.

3.1.2 Glutathione export

Cultured astrocytes and neurons show a basal release of GSH. Specific GSH export rates from astrocytes, however, are higher than those from neurons (Table 3.1) which is in line with previous reports (Hirrlinger *et al.* 2002; Tulpule 2012). Similarly, the basal loss of cellular GSH/h from astrocytes is higher than from neurons (Table 3.1). Comparison of the loss of cellular GSH for astrocytes to literature data reveals that the here reported loss (3.5 %/h) is lower than the previously described 10 % for cultured astrocytes (Dringen *et al.* 1997). Possible reasons for this discrepancy include differing seeding densities, as well as different

culture and experimental conditions. Treatment of both cell types with indinavir, nelfinavir and lopinavir caused a 3-fold stimulation GSH export rates while ritonavir increased GSH export rates by approximately 6-fold (Table 3.1). Interestingly, the loss of cellular GSH from astrocytes after treatment with protease inhibitors was doubled compared to those from treated neurons, whereas basal loss of GSH from astrocytes was only increased 1.6-fold. This shows that protease inhibitors deplete astrocytes more rapidly of their GSH than neurons. Reasons for this cell type specific difference may include lower uptake and/or higher metabolism or export of protease inhibitors from neurons. This scenario is similar to that reported for an exposure of brain cell to formaldehyde. GSH export rates of formaldehyde-treated astrocytes were higher compared to GSH export rates from formaldehyde-treated neurons (Tulpule and Dringen 2011; Tulpule *et al.* 2013; Tulpule *et al.* 2012).

Table 3.1: GSH export rates of cultured astrocytes and neurons in the absence or presence of protease inhibitors.

	Astrocytes		**Neurons**	
Protease inhibitor	**GSH export rate (nmol/(mg x h)**	**Loss of cellular GSH (%/h)**	**GSH export rate (nmol/(mg x h)**	**Loss of cellular GSH (%/h)**
none	1.5 ± 0.3	3.5 ± 0.3	0.5 ± 0.1	2.2 ± 0.4
indinavir	4.4 ± 1.3	10.1 ± 1.8	1.6 ± 0.3	6.4 ± 0.4
nelfinavir	4.6 ± 1.2	10.6 ± 2.1	1.4 ± 0.2	5.6 ± 1.0
lopinavir	4.6 ± 1.1	10.7 ± 2.5	1.4 ± 0.4	5.6 ± 0.8
ritonavir	8.5 ± 2.3	19.6 ± 3.6	2.6 ± 0.5	10.5 ± 1.6

Cultured astrocytes or neurons were incubated in the absence (none) or presence of 10 µM indinavir, nelfinavir, lopinavir or ritonavir for 3 h or 4 h, respectively. Shown are means ± SD of data determined in three (astrocytes) or four (neurons) experiments on independently prepared cultures. Initial cellular GSH contents were 43.2 ± 8.9 nmol/mg protein (astrocytes) or 24.9 ± 6.5 (neurons) and initial protein contents were 174 ± 19 µg/well (astrocytes) or 49 ± 10 µg/well. The data are derived from results shown in chapters 2.2 and 2.4 with the exception of data for astrocytes treated with lopinavir which have not been published so far.

All investigated protease inhibitors have the potential to stimulate the export of GSH from viable cultured neurons and astrocytes (chapters 2.1-2.3). This thesis is the first to describe GSH export as new cellular target of protease inhibitors. Previously indinavir, nelfinavir and ritonavir have been shown to decrease cellular GSH or reduced thiol levels in endothelial cells, adipocytes and malaria parasites (Chen and Mak 2014; He *et al.* 2009; Manda *et al.* 2011; Weakley *et al.* 2011). Yet, extracellular GSH concentrations have not been determined in these studies and a potential stimulation of GSH export was not considered. Very recently a stimulation of GSH export from lung cells into the alveolar fluid of HIV patients treated with HAART was described (Cribbs *et al.* 2014). However, as the authors do not provide the information which antiretroviral drugs were components of the investigated HAART treatment, a comparison of this new study with the data provided here is unfortunately not possible.

After a treatment of astrocytes and neurons with protease inhibitors the levels of GSSG in cells and media were very low (chapters 2.1-2.3). This not only indicates that neither cultured astrocytes nor neurons suffered from acute, severe oxidative stress, but also shows that protease inhibitors predominantly stimulate GSH export from cultured brain cells. Similar acceleration of GSH export has been shown after exposure of astrocytes and/or neurons to formaldehyde or arsenics (Meyer *et al.* 2013; Tadepalle 2014; Tulpule and Dringen 2011; Tulpule *et al.* 2013). The potential formation and export of protease inhibitor-GSH conjugates seems unlikely under the conditions used as the sums of cellular and extracellular GSH were not affected by the presence of protease inhibitors (chapters 2.1-2.3). A conjugation of GSH to protease inhibitors would have decreased the level of detectable GSH as shown for astrocytes exposed to fumaric acid diesters, iodoacetate, iodoacetamide, deoxyribose, chlorinated acetates or monochlorobimane (Schmidt and Dringen 2009, 2010; Schmidt *et al.* 2010; Schmidt *et al.* 2011; Waak and Dringen 2006) or for

reactive metabolites of the drugs diclophenac, acetaminophen and clozapine under cell-free conditions (Dragovic *et al.* 2014).

The potential of protease inhibitors to stimulate GSH export differed in a similar way for both, neurons and astrocytes. Ritonavir treatment resulted in a stronger stimulation of GSH export in both cell types than indinavir, nelfinavir or lopinavir (chapters 2.2, 2.3). For both, astrocytes and neurons, GSH export was maximally accelerated by ritonavir, nelfinavir or lopinavir in a concentration of 10 μM, whereas indinavir further increased GSH export when applied in concentrations higher than 10 μM. The obtained data do unfortunately not give an explanation for those differences. Several factors, including uptake and export rates of the inhibitors, their potential to bind to protein intracellularly and their metabolism by CYP systems, may be responsible for the differences observed for the investigated protease inhibitors.

The superfamily of ATP-binding cassette (ABC) transporters plays a major role in the cellular detoxification of xenobiotics and (therapeutic) drugs by efficiently exporting them. For these transport processes the ABC transporters P-gp and Mrp1 are particularly important (Cole 2014; Sharom 2008, 2011). The activity of especially P-gp, but also of Mrps, have been linked to the observed limited accumulation of protease inhibitors in the brain and to low cellular accumulation (Bachmeier *et al.* 2005; Janneh *et al.* 2007; Jones *et al.* 2001; Marzolini *et al.* 2013; van der Sandt *et al.* 2001). As Mrp1 has also been shown to be involved in the export of GSH, GSH conjugates and GSSG from rodent astrocytes (Hirrlinger *et al.* 2001; Hirrlinger *et al.* 2002; Minich *et al.* 2006; Waak and Dringen 2006) and as protease inhibitors have been shown to interact with Mrp1 either as substrates or as inhibitors (Bachmeier *et al.* 2005; Janneh *et al.* 2007; Jones *et al.* 2001; Olson *et al.* 2002; van der Sandt *et al.* 2001), it was investigated whether the protease inhibitor-mediated acceleration of GSH export might be linked to Mrp1. Indeed, the observed stimulation of GSH export by protease inhibitors was diminished in the

presence of the known Mrp1 inhibitor MK571 (Hirrlinger *et al.* 2002; Minich *et al.* 2006), indicating that Mrp1 is involved in the protease inhibitor-mediated acceleration of GSH export from neural cells. A similar involvement of Mrp1 in the stimulated GSH export from cultured astrocytes has been described for formaldehyde, arsenate and arsenite (Meyer *et al.* 2013; Tadepalle 2014; Tulpule and Dringen 2011; Tulpule *et al.* 2012) and for H_2O_2 exposed neurons (Hohnholt and Dringen 2014). However, the Mrp1 inhibitor MK571 has so far only been shown to exclusively inhibit Mrp1-mediated GSH export in cultured mouse astrocytes (Minich *et al.* 2006). Therefore, it can currently not be completely excluded, that in rat brain cells other GSH transporters, such as organic anion transporting polypeptides, cystic fibrosis transmembrane conductance regulators (Ballatori *et al.* 2009) or GSH export via gap-junction hemichannels (Stridh *et al.* 2010) may also be affected by MK571 and may be involved in the protease inhibitor-stimulated GSH export.

The mechanism by which protease inhibitors stimulate the basal Mrp1-mediated GSH export (Figure 3.1a) is not clear. A recruitment of Mrp1-containing vesicles to the plasma membrane during protease inhibitor treatment provides one possible mechanism (Figure 3.1b). Such a recruitment of Mrp1 transporters has been shown for cultured astrocytes after exposure of astrocytes to bilirubin (Gennuso *et al.* 2004). Furthermore, the strong increase in V_{max}-value but not of the K_m-value for GSH export, after treatment of cultured astrocytes with formaldehyde (Tulpule *et al.* 2012) indicates translocation of Mrp1 transporters into the plasma membrane. It is also possible that protease inhibitors stimulate GSH export directly by binding to Mrp1 (Figure 3.1c). Such a direct binding has been shown for the Mrp1 substrates vincristine, etoposide, daunorubicin or mitoxantrone (Loe *et al.* 1998; Morrow *et al.* 2006; Rappa *et al.* 1997; Renes *et al.* 1999). Alternatively, protease inhibitors may also indirectly stimulate Mrp1-mediated GSH export by triggering the binding of

an endogenous protein to Mrp1 which modulates its transport properties (Figure 3.1d).

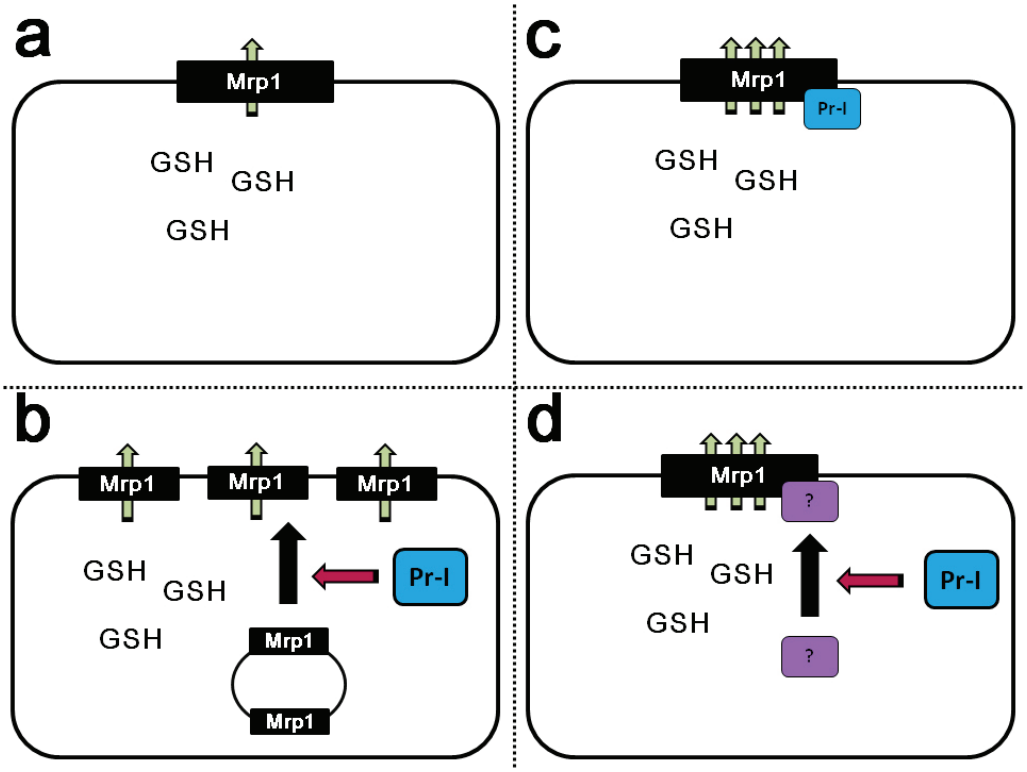

Figure 3.1: Potential mechanisms of protease inhibitor-mediated stimulation of glutathione (GSH) export. Basal GSH export by the multidrug resistance protein 1 (Mrp1) is shown in (**a**). Protease inhibitors (Pr-I) could accelerate GSH export by recruitment of intracellular vesicular Mrp1 to the plasma membrane (**b**). Alternatively, protease inhibitors could directly stimulate GSH export by binding to Mrp1 (**c**) or by triggering the binding of a transport-stimulating protein (purple square) to Mrp1 (**d**).

3.2 Stimulation of the glycolytic flux in cultured astrocytes by 8-hydroxy efavirenz

Inhibition of mitochondrial respiration in cultured astrocytes by known inhibitors like rotenone, antimycin A or sodium azide increases glycolytic flux, as demonstrated by accelerated glucose consumption and lactate release (Scheiber and Dringen 2011; Tulpule and Dringen 2012). Treatment of cultured astrocytes for several hours with low micromolar concentrations of various RT inhibitors or protease inhibitors neither compromised cell viability nor acutely affected glycolytic flux (chapter 2.4). This was unexpected at least for the NNRTI efavirenz, as treatment with efavirenz has been described to block complex I of the respiratory chain in cultured hepatocytes and to inhibit complex IV of the respiratory chain in brain after oral administration to mice (Blas-Garcia *et al.* 2010; Streck *et al.* 2011).

To evaluate potential effects of efavirenz, its extensive hepatic metabolism (Ogburn *et al.* 2010; Usach *et al.* 2013; Ward *et al.* 2003) and the accumulation of its primary metabolite 8-OH-efv in the blood of efavirenz-treated patients (Avery *et al.* 2013; Habtewold *et al.* 2011; Ngaimisi *et al.* 2013; Ngaimisi *et al.* 2010) should be taken into account. Thus 8-OH-efv, but not efavirenz itself, may cause the observed inhibition of respiration *in vitro* and *in vivo* (Blas-Garcia *et al.* 2010; Streck *et al.* 2011). However, this option was not considered as explanation for the reported effects of efavirenz exposure. Therefore, this thesis compared acute effects of efavirenz and its metabolite 8-OH-efv on the glycolytic flux of cultured astrocytes.

A number of studies describe the toxic effects of efavirenz on various cell types, including cancer cell lines, hepatocytes, auditory cells and neurons (Bumpus 2011; Hecht *et al.* 2013; Liner *et al.* 2010; Robertson *et al.* 2012; Thein *et al.* 2014; Tovar-y-Romo *et al.* 2012). Also treatment of cultured astrocytes for 3 h with 100 µM efavirenz strongly compromised cell viability (chapter 2.4). In contrast exposure of

astrocytes to low micromolar concentrations of 8-OH-efv for several hours did not acutely compromise cell viability. Cultured hepatocytes seem to be more vulnerable to 8-OH-efv than cultured astrocytes as a treatment with low micromolar concentrations of 8-OH-efv for 6 h severely damaged the cells (Bumpus 2011), whereas astrocytes remained viable under such conditions. Similarly, a treatment for 24 h with 8-OH-efv in concentrations higher than 10 nM severely damaged cultured neurons (Tovar-y-Romo *et al.* 2012), while astrocytes seem to be less vulnerable to 8-OH-efv, as concentrations higher than 3 µM were required to compromise cell viability within 24 h of incubation to some extent (data not shown).

The application of 8-OH-efv, but not efavirenz itself, caused a stimulation of glucose consumption and lactate release in viable astrocytes in a time and concentration dependent manner, with 10 µM 8-OH-efv displaying maximal effects (chapter 2.4). Thus this thesis identifies 8-OH-efv as a new compound that accelerates glycolytic flux in astrocytes. Similarly arsenite, arsenate and formaldehyde have previously been described to stimulate glycolytic flux in brain cells (Meyer *et al.* 2013; Tadepalle 2014; Tulpule and Dringen 2012; Tulpule *et al.* 2013). However, 10 µM 8-OH-efv did not significantly inhibit respiration of isolated astrocytic mitoplasts and a 6-times higher concentration of 8-OH-efv was necessary to at least partly affect respiration in mitoplasts (chapter 2.4). Thus the stimulation of glycolysis in astrocytes by 8-OH-efv appears not to be caused by an inhibition of mitochondrial respiration.

Accelerated glycogenolysis (Dringen *et al.* 1993) is unlikely to contribute to the increased lactate release as also uptake of extracellular glucose was increased in astrocytes by 8-OH-efv and the ratio of lactate release and glucose consumption was similar to that of control cells. In addition to this, the amount of glucose stored as glycogen in astrocytes (around 50 nmol/mg protein; Dringen *et al.* 1993) would even after complete conversion to lactate only account for roughly 10 % of even the

basal lactate generated (around 1 µmol/mg protein; chapter 2.4) from glucose within 1 h.

There are several potential mechanisms by which 8-OH-efv might accelerate glucose consumption and lactate release (Figure 3.2). Firstly, 8-OH-efv may stimulate glucose uptake by either activating GLUT1 which has been shown to be expressed in astrocytes (McEwen and Reagan 2004) or recruitment of GLUT1 to the plasma membrane which has for example been described in adipocytes and myoblasts (Andrisse *et al.* 2013; Liu *et al.* 2012). Secondly, 8-OH-efv could accelerate glycolysis by stimulating glycolytic key enzymes which may limit glycolytic flux, majorly phosphofructo kinase (PFK) (Hertz *et al.* 2007). The inhibition of the pyruvate dehydrogenase complex (PDH) during exposure of astrocytes to 8-OH-efv can so far also not be excluded as cause for acceleration of the glycolytic flux, although basal PDH activity in astrocytes has been reported to be already low compared to neurons (Halim *et al.* 2010).

Interestingly, the AMP-activated protein kinase (AMPK) has been shown to stimulate glucose uptake via GLUT1 (Abbud *et al.* 2000; Cura and Carruthers 2012; Wu and Wei 2012), to increase activity of glycolytic key enzymes (Holmes *et al.* 1999; Marsin *et al.* 2000; Marsin *et al.* 2002; Wu and Wei 2012) and to inactivate PDH (Wu *et al.* 2013). AMPK is an important component of the signaling cascade sustaining cellular energy balance (Carling *et al.* 2012) and is activated under hypoxic conditions, oxidative stress and nutrient deprivation (Mungai *et al.* 2011; Wang *et al.* 2011; Wu *et al.* 2013). In the brain, activation of AMPK has been connected to ischemic stroke and increased lactate levels (Li *et al.* 2010; Li *et al.* 2007). Especially the over-activation of AMPK in astrocytes was considered to deteriorate ischemic injury due to increases in glycolysis, resulting in lactic acidosis (Li *et al.* 2010). There is also a number of compounds activating AMPK, for example 5-aminoimidazole-4-carboxamide riboside, metformin or berberine, which all have been shown to increase glucose consumption and lactate release in

peripheral cells (Buzzai *et al.* 2007; Holmes *et al.* 1999; Kurth-Kraczek *et al.* 1999; Yin *et al.* 2008). Similarly, metformin has been recently shown to stimulate glycolytic flux in cultured astrocytes (E.-M. Blumrich, personal communication).

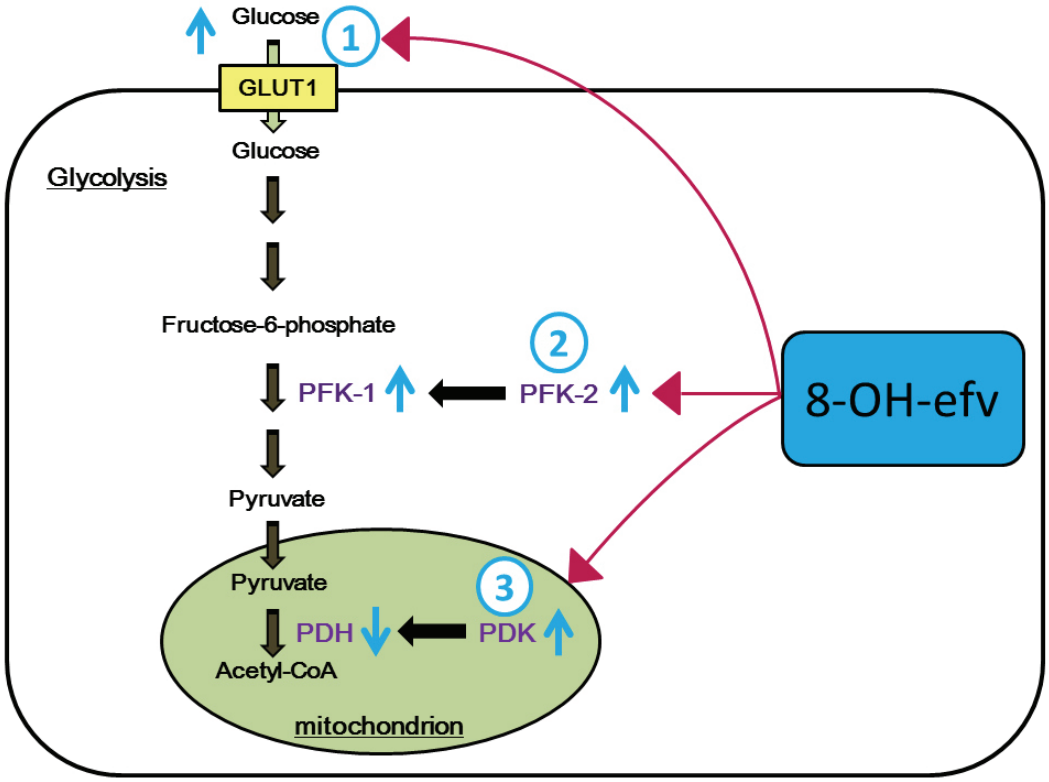

Figure 3.2: Potential mechanisms of 8-OH-efv-mediated stimulation of the glycolytic flux in astrocytes. 8-OH-efv might stimulate glucose uptake, by either increased recruitment of vesicular glucose transporter 1 (GLUT1) to the plasma membrane or by activation of GLUT1 (**1**). Alternatively, 8-OH-efv could activate phosphofructo kinase-2 (PFK-2) (**2**). PFK-2 will increase levels of fructose 2,6-bisphosphate which in turn activates the glycolysis key enzyme phosphofructo kinase-1 (PFK-1). The pyruvate dehydrogenase complex (PDH) could be inhibited via 8-OH-efv-induced activation of pyruvate dehydrogenase kinase (PDK) (**3**). ↑: activation/stimulation; ↓: inactivation/inhibition.

In cultured hepatocytes treated with efavirenz AMPK was activated (Blas-Garcia *et al.* 2010). As efavirenz is rapidly metabolized in hepatocytes (Desta *et al.* 2007; Usach 2013), it is likely that 8-OH-efv is the species activating AMPK. Thus, it is possible that 8-OH-efv stimulates glycolytic flux by increasing glucose consumption and lactate release in astrocytes via AMPK signaling.

3.3 Potential *in vivo* relevance of the findings of this thesis

Cultured brain cells are frequently used as model systems to investigate whether given drugs have the potential to damage brain cells or affect important metabolic pathways (Contestabile 2002; Lange *et al.* 2012; Tulpule *et al.* 2014). This thesis demonstrates that protease inhibitors acutely stimulate GSH export from astrocytes and neurons and the efavirenz metabolite 8-OH-efv accelerates the glycolytic flux in astrocytes, without compromising cell viability. Whether such alterations in the metabolism of brain cells are involved in persistent HAND in treated HIV patients is currently unknown. Sufficient accumulation of antiretroviral drugs or their metabolites in the brain would be a prerequisite for their ability to affect brain cell functions *in vivo* which in turn would highly depend on the penetration of the drugs into the brain and on their metabolism.

Even though reported plasma concentrations of the protease inhibitors (Baede-van Dijk *et al.* 2001; Burger *et al.* 2001; Lopez-Cortes *et al.* 2013; Marzolini *et al.* 2013) used in this thesis are in the micromolar concentration range, which stimulated GSH export from cultured brain cells, brain penetration of protease inhibitors is considered to be strongly limited by excessive export by ABC transporters at the BBB (Bachmeier *et al.* 2005; Marzolini *et al.* 2013; van der Sandt *et al.* 2001). Although in the cerebrospinal fluid (CSF) of protease inhibitor-treated HIV patients only very low protease inhibitor levels were detected (Best *et al.* 2009;

DiCenzo *et al.* 2009; Solas *et al.* 2003), it has been indicated in an animal model that real brain tissue concentrations might be higher than those estimated for CSF (Anderson *et al.* 2006; Anthonypillai *et al.* 2004; Kaddoumi *et al.* 2007). Sufficient accumulation of protease inhibitors in the brain is supported by studies, which report neurocognitive deficits in mice after treatment with ritonavir-boosted lopinavir doses (Gupta *et al.* 2012; Pepping *et al.* 2014; Pistell *et al.* 2010). A low accumulation of protease inhibitors in the brain *in vivo* is supported by a recent study, in which ritonavir, lopinavir and nelfinavir inhibited amyloid-ß-peptide production in neuron cultures but not *in vivo* (Lan *et al.* 2012). Nevertheless, it should be considered that the necessary life-long treatment of HIV patients with antiretroviral drugs may result in an accumulation of protease inhibitors in the brain which can alter brain functions.

Infection of the brain with HIV results in the release of viral proteins (see chapter 1.4.2). These viral proteins have at least *in vitro* been shown to disrupt the BBB (Louboutin and Strayer 2012; Xu *et al.* 2012). Such a disruption of the BBB prior to or during the initiation of HAART could foster the access of protease inhibitors to the brain and establish concentrations sufficiently high to alter GSH homeostasis in the brain. Under those conditions, protease inhibitor-induced GSH export may deprive brain cells of GSH *in vivo*, assuming that the loss of GSH is not compensated by synthesis.

Stimulated GSH export would be accompanied by an extracellular accumulation of GSH. Augmented extracellular concentrations of GSH might be detrimental to neurons. GSH can on one hand be increasingly hydrolyzed by the astrocytic ectoenzyme γ-GT (Dringen *et al.* 1997; Dringen *et al.* 1999) to generate higher levels of the neurotransmitter glutamate (Schmidt and Dringen 2012), which is considered to cause excitotoxicity by over-stimulating NMDA glutamate receptors (Rodriguez-Rodriguez *et al.* 2013). On the other hand elevated concentrations of extracellular GSH could also directly affect neurons, as it has been suggested to act

as neuromodulator and neurotransmitter on NMDA glutamate receptors (Aoyama *et al.* 2008; Janáky *et al.* 2007).

Due to the crucial role of GSH in the defense against ROS (Lu 2013; Schmidt and Dringen 2012), a GSH depletion of brain cells by protease inhibitors might also render brain cells more vulnerable to oxidative stress caused by other regularly co-administered antiretroviral drugs like RT nucleoside analogues, which have been shown to induce oxidative stress (Lagathu *et al.* 2007; Opii *et al.* 2007; Xue *et al.* 2013). Also HIV infection itself is linked to oxidative stress and GSH deficiency in patients (De Rosa *et al.* 2000; Ngondi *et al.* 2006; Suresh *et al.* 2009). Under this light chronic oxidative stress induced by HIV infection itself and/or co-applied antiretroviral drugs could further damage brain cells. In such a scenario it seems possible, that a lifelong treatment with protease inhibitor-containing regimen could persistently compromise the GSH homeostasis in the brain and could therewith contribute to the minor cognitive damages, observed in treated HIV patients (Lamers *et al.* 2014; Manji *et al.* 2013; Mothobi and Brew 2012).

Similar to protease inhibitors, also CSF concentrations of efavirenz and 8-OH-efv have been reported to be quite low in the low nanomolar range (Avery *et al.* 2013; Best *et al.* 2011; Tovar-y-Romo *et al.* 2012). However a sufficient penetration of efavirenz and/or 8-OH-efv into the brain is supported by studies showing that complex IV of the respiratory chain and creatine kinase in brain were inhibited after exposure of mice to efavirenz (Streck *et al.* 2011; Streck *et al.* 2008). Chronic exposure to efavirenz might therefore result in an accumulation of 8-OH-efv in the brain to concentrations, which could stimulate glycolysis and increase extracellular lactate levels which subsequently might result in acidosis. This chain of events may impair brain functions as acidosis causes astrocyte and neuron swelling *in vitro* (Ringel *et al.* 2006; Staub *et al.* 1990; Staub *et al.* 1993) and impairs synaptic transmission as well as neurocognitive functions *in vitro* and *in vivo* (Li *et al.* 2011; Tachibana *et al.* 2013; Zhao *et al.* 2011). As lactic acidosis has been linked to HIV patients treated with efavirenz-containing regimen (Chow *et al.* 2007), such a

scenario is likely to contribute to neurocognitive deficits in HAART-treated HIV patients. Especially in combination with protease inhibitors, an 8-OH-efv-induced lactic acidosis could be even more detrimental for brain function, as increased levels of lactate have been shown to prevent GSH synthesis in cultured primary astrocytes and neurons (Lewerenz *et al.* 2010).

Along these lines, a potential contribution of antiretroviral drugs to the development of cognitive deficits in treated patients should be carefully monitored in connection with the use of new approaches to increase cellular and brain drug concentrations. Such approaches include the inhibition of ABC transporters, as well as delivery of antiretroviral drugs by nanocarriers (Giacalone *et al.* 2013; Kaddoumi *et al.* 2007; Nair *et al.* 2013; Tshweu *et al.* 2013).

3.4 Future perspectives

This thesis provides the first experimental evidence that four members of the class of HIV protease inhibitors acutely accelerate GSH export from brain cells and that the primary efavirenz metabolite 8-OH-efv acutely increases glycolytic flux in astrocytes. These observations request that investigations concerning effects of antiretroviral drugs on brain cells should be more strongly considered and that the possibility of an altered brain metabolism may contribute to HAND in HAART-treated HIV patients.

GSH export from brain cells is a newly identified target of HIV protease inhibitors. Thus, it is important to deepen the knowledge about the consequences of a protease inhibitor treatment of brain cells on GSH export and to elucidate the involved mechanisms. Firstly, it is important to verify that indeed transport of GSH by Mrp1 is accelerated by protease inhibitors. As the inhibitor MK571 has only been shown to exclusively inhibit Mrp1 in mouse astrocytes (Minich *et al.*

2006), but not in rat astrocytes, it cannot so far not be excluded that also other transporters are blocked by MK571 and might be involved in the observed protease inhibitor-mediated stimulation of GSH export from rat brain cells. Thus, the specificity of MK571 to inhibit GSH export by Mrp1 in rat brain cells should be verified, for example by knockdown of Mrp1 by RNA interference technology (Siomi and Siomi 2009).

To investigate the mechanism of Mrp1-mediated stimulation of GSH export by protease inhibitors, immunostaining of Mrp1 (Gennuso *et al.* 2004) should be used to study a potential recruitment of Mrp1-containing vesicles to the plasma membrane after exposure to protease inhibitors. Potential direct interactions of protease inhibitors with Mrp1 and a co-export of such compounds with GSH could be tested by determination of transporter activity (Mao *et al.* 1999) or membrane vesicle transport studies (Loe *et al.* 1996).

Protease inhibitors stimulate GSH export from both, astrocytes and neurons. However, it is currently not clear how the close coupling of neuronal and astrocytic GSH metabolism (Dringen 2009) will be affected by protease inhibitors. Thus, co-culture experiments should be conducted to investigate, whether protease inhibitors would simultaneously deplete astrocytes and neurons of GSH and/or how such compounds would affect the cooperation of astrocytes and neurons in GSH metabolism.

Human astrocytes have been shown to predominantly express MRP4, but hardly MRP1 (Nies *et al.* 2004). This indicates that in human astrocytes MRP4 might be the predominant transporter for GSH (Nies *et al.* 2004). To address the human situation, which is relevant for HIV infection, it will be necessary to investigate the potential of protease inhibitors to also stimulate MRP4-mediated GSH export in cultures of human astrocytes. As protease inhibitors have been recently shown to

interact with Mrp4 (Fukuda *et al.* 2013) it is likely that the MRP4-mediated GSH export can also be accelerated by protease inhibitors.

The primary efavirenz metabolite 8-OH-efv acutely stimulates glycolysis in cultured astrocytes. However, the involved mechanism still remains to be elucidated. To investigate, whether glucose uptake is increased by recruitment of GLUT1 to the plasma membrane or by GLUT1 activation after exposure of astrocytes to 8-OH-efv, immunostaining of GLUT1 and uptake studies with labeled glucose should be performed (Freemerman *et al.* 2014; Loaiza *et al.* 2003; Maher 1995; Nijland *et al.* 2014).

To examine the possibility of a direct stimulation of glycolysis by 8-OH-efv, the activities of glycolytic key enzymes should be determined after treatment of astrocytes with 8-OH-efv, especially the activities of PFK-1 and PFK-2 should be measured (Ishikawa *et al.* 1990; Schaftingen and Hers 1981). To determine whether PDH inhibition during a treatment of astrocytes with 8-OH-efv is responsible for the stimulation of the glycolytic flux, the inactivation of PDH and the activation of PDK should be analyzed by Western blot of the phosphorylated PDK/PDH (Wu *et al.* 2013).

To investigate the potential involvement of an AMPK-activation by 8-OH-efv, a Western blot analysis of phosphorylated AMPK (Blas-Garcia *et al.* 2010) should be conducted. Furthermore it should be investigated, whether the inhibition of AMPK by the AMPK inhibitor Compound C (Zhou *et al.* 2001) might diminish the 8-OH-efv-mediated stimulation of the glycolytic flux.

During this thesis the effects of 8-OH-efv were only investigated on cultured astrocytes. Such studies should be extended to investigate whether 8-OH-efv has the potential to accelerate glycolytic flux in other brain cell types.

To learn more on the metabolism of antiretroviral drugs in brain cells it is crucial to determine intracellular concentrations of such compound and their metabolites. Cellular drug contents can be measured by liquid chromatography-tandem mass spectrometry (Fukuda *et al.* 2013; Koehn and Ho 2014; Rao *et al.* 2013; Srivastava *et al.* 2013). Such determinations might give first information which cellular concentrations of protease inhibitor or 8-OH-efv are needed to stimulate GSH export or glycolytic flux, respectively.

Furthermore determination of cellular protease inhibitor concentrations will identify inhibitor-specific differences. Such differences might provide an explanation for the distinct potential of the investigated protease inhibitors to stimulate GSH export or the possibility of co-export of GSH and protease inhibitors by Mrp1. The identification of protease inhibitor metabolites is also very important. The differentiation between the therapeutically active drugs and their metabolites will indicate, which species is most likely to stimulate GSH export, whether and to which extent astrocytes and neurons are able to metabolize protease inhibitors, and if there are any cell type-specific differences which may explain the different potential of protease inhibitors to deplete astrocytes or neurons of GSH export.

The treatment of astrocytes with efavirenz/8-OH-efv and the subsequent determination of intracellular levels of efavirenz, 8-OH-efv and/or other metabolites are important to investigate whether astrocytes are able to metabolize efavirenz and if 8-OH-efv can be further metabolized.

Considering that treatment with HAART is a life-long necessity, cultured brain cells should be chronically exposed to antiretroviral drugs. Although brain cells have been exposed to antiretroviral drugs for up to 8 days in some studies (Akay *et al.* 2014; Robertson *et al.* 2012), further studies addressing long-term effects of antiretroviral drugs on brain cells need to be conducted. Such experiments should

investigate exposure to single antiretroviral drugs but also combinations of drugs. Such studies would address the question whether a long-term exposure to single antiretroviral drugs damage brain cells, whether acute effects of virostatics, for example depletion of GSH by protease inhibitors, can be compensated by cellular adaption processes and whether a chronic exposure to drug combinations renders brain cells more vulnerable to damages compared to treatment with single drugs.

To assess whether protease inhibitors and efavirenz/8-OH-efavirenz show similar effects *in vivo*, studies in animal models should be conducted. In this context it will be important to conduct behavioral studies to assess potential CNS side effects and determine real brain tissue concentrations of the active drugs as well as metabolites to distinguish between effects of the applied drugs and their metabolites.

As the BBB has been shown to be disrupted by HIV proteins during HIV infection of the brain (Louboutin and Strayer 2012; Xu *et al.* 2012), the use of infected animal models, such as macaques infected with the simian immunodeficiency virus (Ambrose *et al.* 2007), should be considered to assess whether the penetration of antiretroviral drugs into the brain might be elevated and whether under such conditions higher levels of drugs would be present in the brain.

Cerebral microdialysis represents a very useful method in clinical research to monitor patients with neurological conditions (Revuelto-Rey *et al.* 2012). The analysis of extracellular low molecular weight compounds enables the assessment of the metabolic condition of the brain (Revuelto-Rey *et al.* 2012). This tool should be used in animal models to determine extracellular levels of compounds like lactate and GSH after exposure to antiretroviral drugs, such as protease inhibitors and efavirenz. This might help to further evaluate whether antiretroviral drugs have the potential to contribute to HAND in HIV patients.

3.5 References

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