Copper metabolism and copper-mediated alterations in the metabolism of cultured astrocytes

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

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I Structure of the thesis

This thesis is composed of three main parts, introduction (1), results (2) and summarizing discussion (3). The first part introduces the reader to the biological functions of copper (1.1), to the molecular mechanisms of cellular copper transport (1.2), to the functions and metabolism of copper in the brain (1.3) and to the role of astrocytes in the copper metabolism of the brain (1.4). The second part (results) consists of five published articles that deal with copper transport (2.1 and 2.2), copper toxicity (2.3) and with copper-mediated alterations in the metabolism of cultured astrocytes (2.4 and 2.5). These publications are included as portable document format. For each article, the contributions of the first author are listed on the first page of the respective chapter. In the third part (summarizing discussion) the key-findings of this thesis are discussed and possible future directions for further research on the topics addressed by this thesis are proposed.

II Summary

Copper is an essential element that is required for a variety of important cellular functions. Since not only copper deficiency, but also excess of copper can seriously affect cellular functions, cellular copper metabolism is tightly regulated. Disturbances of copper homeostasis are the underlying defect of the inherited diseases Menkes and Wilson's disease and have also been linked to several neurodegenerative diseases including Alzheimer's disease and Parkinson's disease. Known astrocyte features strongly suggest a pivotal role of theses cells in the metal metabolism of the brain. Using astrocyte-rich primary cultures as model system, this thesis investigated the copper metabolism as well as copper-mediated alterations in the metabolism of astrocytes. Cultured astrocytes efficiently accumulated copper with saturable kinetics. The characteristics of the observed copper accumulation suggest that both copper transporter receptor 1 (Ctr1) and a Ctr1-independent mechanism are involved in astrocytic copper accumulation. Cultured astrocytes were also found to release copper in a time-, concentration- and temperature-dependent manner. Copper export from these cells most likely involves the copper-ATPase ATP7A. Thus, with being capable of both taking up and exporting copper, astrocytes possess the cellular machinery required to transport copper from the blood-brain barrier to the brain parenchyma. Cultured astrocytes were remarkably resistant against copper-induced toxicity. Nevertheless, prolonged copper treatment led to profound alterations in their metabolism. For example, copper accumulation by cultured astrocytes was accompanied by a stimulation of glycolytic flux, an increase in the cellular glutathione content and an acceleration of glutathione export. Such copper-mediated alterations in the metabolism of astrocytes may also occur *in vivo*, for example in copper overload conditions such in Wilson's disease and could either contribute to disease

progression or serve as compensatory response to protect the brain against the toxic effects of an excess of copper.

III Zusammenfassung

Kupfer ist ein essentielles Spurenelement, welches für zahlreiche wichtige zelluläre Funktionen benötigt wird. Da sowohl ein Mangel an Kupfer als auch dessen übermäßige Anreicherung zu schwerwiegenden Beeinträchtigungen zellulärer Funktionen führen kann, wird der Kupferstoffwechsel strengstens reguliert. Eine Störung des Kupferstoffwechels liegt den Erbkrankheiten Morbus Menkes und Morbus Wilson zugrunde und auch neurodegenerative Krankheiten wie Morbus Alzheimer und Morbus Parkinson werden mit einer beinträchtigten Kupferhomeostase in Verbindung gebracht. Astrozyten weisen eine Vielzahl an Eigenschaften auf, die auf eine bedeutende Rolle dieser Zellen im Metall-Stoffwechsel des Gehirns hindeuten. In der vorliegenden Arbeit wurden Astrozyten-reiche Primärkulturen als Modellsystem verwendet, um den Kupferstoffwechsel von Astrozyten sowie die Auswirkungen von Kupfer auf den Metabolismus dieser Zellen zu untersuchen. Astrozytenkulturen akkumulierten Kupfer mit sättigbarer Kinetik. Die Charakteristika der beobachteten Kupferakkumulation lassen sowohl auf eine Beteiligung vom Kupfer-Transporter-Rezeptor 1 (Ctr1) als auch auf eine Beteiligung eines Ctr1-unabhängigen Mechanismus' schließen. Es konnte zudem gezeigt werden, daß Astrozyten Kupfer in Zeit-, Konzentrations- und Temperatur-abhängiger Weise freisetzen, höchstwahrscheinlich unter Beteiligung der Kupfer-ATPase ATP7A. Mit den Fähigkeiten Kupfer aufzunehmen und zu exportieren, weisen Astrozyten die notwendigen Voraussetzungen auf, um Kupfer von der Blut-Hirn-Schranke in das Hirnparenchym zu transportieren. Astrozyten zeigten sich bemerkenswert resistent gegenüber Kupfer-bedingter Schädigung. Nichtsdestotrotz, führte eine längere Inkubation mit Kupfer zu ausgeprägten Änderungen ihres Stoffwechsels. So wurde die Kupferakkumulation von Astrozyten von einer Steigerung des glykolytischen Flusses, von einem Anstieg des zellulären Glutathiongehalts sowie von einem beschleunigten Glutathion-

Export begleitet. Solche durch Kupfer bewirkten Veränderungen im Stoffwechsel von Astrozyten treten möglicherweise auch *in vivo* auf, z.B. bei einer übermäßige Anreicherung von Kupfer wie beim Morbus Wilson, und tragen so möglicherweise zur Krankheitsprogression oder kompensatorisch zum Schutz des Gehirn gegen die toxischen Effekte eines Kupfer-Überschusses bei.

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IV Abbreviations

µg	microgram
µM	micromolar
AD	Alzheimer's disease
ADP	Adenosine diphosphate
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ApN	aminopeptidase N
Asp	aspartate
ATOX1	human analog of antioxidant protein 1
ATP	Adenosine triphosphate
ATX1	antioxidant protein 1
Aβ	amyloid β
BBB	blood-brain barrier
BCB	blood-cerebrospinal fluid barrier
BSE	bovine spongiform encephalopathy
C	cysteine
CCS	copper chaperone for superoxide dismutase
CHCH	coiled-coil-helix-coiled-coil-helix
CNS	central nervous system
Cp	ceruloplasmin
CSF	cerebrospinal fluid
Ctr	copper transporter receptor
Ctr1	copper transporter receptor 1
Ctr2	copper transporter receptor 2
Cu/Zn-SOD	copper/zinc superoxide dismutase
Cys	cystein
D	aspartate
d	day
Dcytb	duodenal cytochrome *b*
DMEM	Dulbecco's modified Eagle's medium
DMT1	divalent metal transporter 1
DβM	dopamine-β-monoxygenase
E	glutamate
ε	extinction coefficient
ECM	extracellular matrix
EC-SOD	extracellular superoxide dismutase
EPR	electron paramagnetic resonance
ER	endoplasmic reticulum
et al.	Latin: *et alii*, and others
F	phenylalanine

FGF-1	Fibroblast growth factor-1
FGF-2	Fibroblast growth factor-2
G	glycine
g	gram
GABA	γ-aminobutyric acid
GCL	glutamate cysteine ligase
GFAP	glial fibrillary acidic protein
Glu	glutamate
Gly	glycine
GPI	glycosylphosphatidylinositol
GS	glutathione synthase
GSH	glutathione
gtsm	glyoxalbis(N (4)- methyl-3-thiosemicarbazonato
H	Histidine
Hah1	human analog of Antioxidant protein 1
hCCS	human chaperone for superoxide dismutase
hCtr1	human copper transporter receptor 1
hCtr2	human copper transporter receptor 2
HD	Huntington's disease
HEK293	human embryonic kidney cell line 293
HeLa	Henrietta Lacks
HIF	hypoxia-inducible factor
HIF-1	hypoxia-inducible factor-1
HIF-1α	hypoxia-inducible factor-1α
HIF-1β	hypoxia-inducible factor-1β
His	histidine
IL-1α	interleukin-1α
IL-8	interleukin-8
IMS	intermembrane space
K	lysine
kDa	kilodalton
K_M	Michaelis-Menten constant
LA-ICP-MS	laser ablation inductive coupled plasma mass spectroscopy
L-DOPA	L-3,4-dihydroxyphenylalanin
LEC	Long-evans cinnamon
LMCT	ligand-to-metal charge transfer
LOX	lysyl oxidase
LPT	long-term potentiation
LTQ	lysyl tyrosine quinone
M	methionine
MAPK	mitogen-activated protein kinase
MBD	metal binding domain
Met	methioneine

mg	miligram
Mn-SOD	manganese superoxide dismutase
mRNA	messanger ribonucleic acid
Mrp1	multidrug resistance proteine 1
MT	metallothionein
MTF1	metal transcription factor 1
N	asparagine
NADPH	nicotineamide adenine dinucleotide phosphate, reduced
NF-κB	nuclear factor kappa B
NMDA	N-methyl-D-aspartate
nmol	nanomolar
PAL	peptidyl-α-hydroxyglycine α-amidating lyase
PAM	peptidylglycine α- amidating monoxygenase
PD	Parkinson's disease
PHM	peptidylglycine α-hydroxylating monoxygenase
PINA	pineal gland night-specific ATPase
PLC-PKC	phospholipase C-protein kinase C
PrP	prion protein
ROS	reactive oxygen species
S	serine
SOD	superoxide dismutase
SOD1	copper/zinc superoxide dismutase
SOD2	manganese superoxide dismutase
SOD3	extracellular superoxide dismutase
Steap	six transmembrane epithelial antigen of the prostate
T	threonine
TGN	*trans*-Golgi network
Thr	threonine
TM1	transmembrane domain 1
TM2	transmembrane domain 2
TM3	transmembrane domain 3
TM6	transmembrane domain 6
TM7	transmembrane domain 7
TM8	transmembrane domain 8
TPQ	trihydroxyphenylalanine quinone
Tyrp1	tyrosinase related protein 1
Tyrp2	tyrosinase related protein 2
US	United states of America
UV	ultra violet
VAP-1	vascular adhesion protein-1
VEGF	vascular endothelial growth factor
Vis	visible
$\mathbf{v_{max}}$	maximum reaction velocity

WHO	World Health Organization
x	any amino acid
XFM	x-ray fluorescence microprobe
Y	tyrosine
ZIP	ZRT-/IRT-like protein
γGT	γ-glutamyl transpeptidase

1

Introduction

1.1

Biology of copper

Copper is the lightest element of group 11 of the periodic table of the chemical elements. Although some compounds exist with copper in the oxidation states Cu^{3+} and Cu^{4+}, the copper chemistry is largely dominated by Cu^{+} and Cu^{2+} compounds (Holleman and Wiberg, 2007). Cu^{+} and Cu^{2+} form numerous complexes with both organic and inorganic ligands. The soft Cu^{+} ion prefers ligands that have large polarizable electron clouds, such as sulfur ligands or unsaturated nitrogen donor ligands usually exerting coordination numbers from two to four with linear, trigonal or tetrahedral coordination (Kaim and Rall, 1996; Crichton and Pierre, 2001; Wadas et al., 2007; Tisato et al., 2010). In contrast, the hard Cu^{2+} ion prefers hard sp^3 hybridized nitrogen and oxygen ligands (Kaim and Rall, 1996; Crichton and Pierre, 2001; Wadas et al., 2007; Tisato et al., 2010). Coordination numbers in Cu^{2+} complexes range from four to eight, allowing a large variety of coordination geometries (Kaim and Rall, 1996; Crichton and Pierre, 2001; Wadas et al., 2007; Tisato et al., 2010). The redox potential of the Cu^{2+}/Cu^{+} redox pair varies dramatically depending on the ligand environment and pH. Thus, the one electron oxidation of various Cu^{+}-complexes towards dioxygen has been reported to vary from -1.5 to + 1.3 V against the standard hydrogen electrode (Tisato et al., 2010).

Copper is a relatively modern bioelement that became bioavailable about 2-3 billion years ago with the advent of an oxygen atmosphere that allowed for the conversion of Cu^{+} to the more soluble Cu^{2+} ion (Kaim and Rall, 1996; Crichton and Pierre, 2001; MacPherson and Murphy, 2007). Since then copper has become an indispensable element for all organisms that have an oxidative metabolism. In humans, it represents the third most abundant essential transition metal (Lewinska-Preis et al., 2011). As a cofactor of several enzymes and/or as structural component, copper is involved in many physiological pathways. Furthermore, copper is associated with important biological processes including angiogenesis, response to hypoxia and neuromodulation.

1.1.1 Copper-dependent enzymes

Copper is an essential cofactor and/or a structural component in a number of important enzymes of plants and animals (Table 1). In general, these enzymes are involved in redox reactions (Kaim and Rall, 1996). The relatively high redox potential for the Cu^{2+}/Cu^{+} system found in copper enzymes is utilized by many of them for a direct oxidation of certain substrates that are easy to oxidize, such as superoxide by superoxide dismutase and catechols by tyrosinase (Tisato et al., 2010). Among others, copper-dependent enzymes participate in biological processes such as energy metabolism (e.g. cytochrome *c* oxidase), antioxidative defense (e.g. Zn,Cu-superoxide dismutase) and iron metabolism (e.g. ceruloplasmin).

Table 1: Mammalian copper-dependent enzymes

Enzyme	Function
Cytochrome *c* oxidase	Oxidative phosphorylation
Cu,Zn superoxide dismutase (SOD1)	Superoxide detoxification, signaling
Ceruloplasmin (Cp)	Ferroxidase
Lysyl oxidase (LOX)	Crosslinking of collagen and elastin
Tyrosinase	Melanin synthesis
Dopamin-β-monoxygenase (DβM)	Norepinephrine synthesis
Peptidylglycine α-amidating enzyme (PAM)	Activation of peptide hormones
Copper amine oxidase	Deamination of amines
Hephaestin	Ferroxidase
Coagulation factors V and VIII	Blood clotting

On the basis of their optical and electron paramagnetic resonance (EPR) features, copper-dependent enzymes are classified as type 1, 2 or 3 copper enzymes (Kaim and Rall, 1996; Rosenzweig and Sazinsky, 2006; MacPherson and Murphy, 2007). The distinct copper centers vary in their coordination

geometries, ligand environments surrounding the metal center and functions (Holm et al., 1996; Kaim and Rall, 1996; MacPherson and Murphy, 2007). Most copper enzymes contain only one type of copper center, but in some (e.g. ceruloplasmin, cytochrome *c* oxidase) more than one type can be found.

Type 1 copper sites, also known as blue copper sites, posses a very intense ligand-to-metal charge transfer (LMCT) absorption in the 600 nm region ($\varepsilon_{600\ nm} \cong 5000\ M^{-1}\ cm^{-1}$), due to an electron transfer from a cysteine thiolate ligand to Cu^{2+}. These centers exhibit an unusual ground-state EPR spectrum (Kaim and Rall, 1996; Gerdemann et al., 2002; Solomon, 2006; MacPherson and Murphy, 2007; Kosman, 2010a). Type 1 copper centers are mononuclear copper sites that contain copper typically coordinated by one cysteine and two histidines in an approximately trigonal-planar arrangement (Holm et al., 1996; Kaim and Rall, 1996; MacPherson and Murphy, 2007; Kosman, 2010a). Often an additional methionine coordinates axially, resulting in a strongly distorted tetrahedral geometry (Kaim and Rall, 1996; MacPherson and Murphy, 2007). This "transition state" between the favored coordination geometries of Cu^{+} (tetrahedral) and Cu^{2+} (square-planar) contributes to the unanimously high reduction-potential of type 1 copper sites compared to that of inorganic copper complexes in aqueous solution (Gray et al., 2000; Gerdemann et al., 2002). The binuclear Cu_A copper center in cytochrome *c* oxidase is an extension of the type 1 site (Holm et al., 1996; Kaim and Rall, 1996; Malmstrom and Leckner, 1998; MacPherson and Murphy, 2007). It is constituted of two copper ions that are connected via two bridging cysteine thiolates and a weak direct Cu-Cu bond (Blackburn et al., 1997; Solomon, 2006). Type 1 copper sites exclusively function in single electron transfer (Kaim and Rall, 1996; Solomon, 2006; MacPherson and Murphy, 2007; Kosman, 2010a).

Type 2 copper sites lack unique features in their UV/Vis and EPR spectra; accordingly the spectroscopic and magnetic characteristics of type 2 copper

centers resemble that of aqueous copper complexes (Kaim and Rall, 1996; Gerdemann et al., 2002; MacPherson and Murphy, 2007). Type 2 copper is typically square-planar or square-pyramidal coordinated with histidine representing one or more of the ligands (Kaim and Rall, 1996; MacPherson and Murphy, 2007). Type 2 sites often have a low reduction potential (Lancaster et al., 2009) and vacant coordination positions that allow binding of exogenous ligands such as dioxygen (Kaim and Rall, 1996; MacPherson and Murphy, 2007). Consequently, type 2 copper sites catalytically activate enzyme substrates by direct interaction rather than being involved in electron transfer (Kaim and Rall, 1996; MacPherson and Murphy, 2007).

In contrast to type 1 and 2 sites, type 3 copper sites are binuclear (Kaim and Rall, 1996; Rosenzweig and Sazinsky, 2006; MacPherson and Murphy, 2007). These copper sites are constituted of two closely spaced antiferromagnetically coupled copper ions, each of them coordinated by three histidines, which can be reversibly bridged by dioxygen (Kaim and Rall, 1996; Gerdemann et al., 2002; Rosenzweig and Sazinsky, 2006; MacPherson and Murphy, 2007; Solomon et al., 2011). Type 3 copper sites exhibit an intense LMCT absorption at 350 nm ($\varepsilon_{350\ nm} \cong 20000\ mM^{-1}\ cm^{-1}$) when molecular oxygen is bound (Kaim and Rall, 1996; Solomon et al., 2011) and are EPR silent due to the anti-parallel spin-spin coupling of the copper ions (Kaim and Rall, 1996; Gerdemann et al., 2002). The function of type 3 copper sites is the activation and transport of oxygen (Kaim and Rall, 1996; MacPherson and Murphy, 2007; Solomon et al., 2011).

1.1.1.1 Cytochrome c oxidase

Cytochrome *c* oxidase is a member of the super-family of heme-copper containing oxidases (Ferguson-Miller and Babcock, 1996; Stiburek et al., 2009; Popovic et al., 2010). It is embedded in the mitochondrial inner membrane where it catalyzes the electron transfer from reduced cytochrome *c* to dioxygen

in the final step of mitochondrial oxidative phosphorylation (Hatefi, 1985; Ferguson-Miller and Babcock, 1996; Tsukihara et al., 1996; Diaz, 2010). Since this four-electron reduction of molecular oxygen to water is coupled to a proton pumping process across the mitochondrial inner membrane, functional cytochrome *c* oxidase contributes to the maintenance of the mitochondrial membrane electrochemical gradient that provides the driving force for ATP synthase-dependent generation of ATP (Hatefi, 1985; Ferguson-Miller and Babcock, 1996; Hamza and Gitlin, 2002; Diaz, 2010).

Mammalian cytochrome *c* oxidase is a multimeric protein complex consisting of 13 subunits, encoded by both the mitochondrial and nuclear genome (Hatefi, 1985; Tsukihara et al., 1995, 1996; Leary et al., 2009b; Stiburek and Zeman, 2010). Biogenesis of the functional holoprotein is a complicated process that requires several specific proteins, so-called assembly factors, including Cox17, Sco1 and Sco2, and even a greater number of proteins with broader substrate specificities, such as mitochondrial ATP-dependent proteases (Shoubridge, 2001; Hamza and Gitlin, 2002; Leary et al., 2004; Diaz, 2010; Stiburek and Zeman, 2010). The mitochondria encoded subunits of cytochrome *c* oxidase, Cox1, Cox2 and Cox3, constitute the catalytic core at which the dioxygen reduction and proton translocation are carried out (Hatefi, 1985; Ferguson-Miller and Babcock, 1996; Hamza and Gitlin, 2002; Diaz, 2010). Cox1 contains two heme moieties, designated heme *a* and heme a_3, and one copper ion that is denoted Cu_B (Hatefi, 1985; Tsukihara et al., 1995, 1996). Cox2 contains a binuclear copper center, designated Cu_A, which serves as the initial electron acceptor from cytochrome *c* (Tsukihara et al., 1995, 1996). During dioxygen reduction electrons derived from cytochrome *c* are transferred from the Cu_A center first to heme *a* and then to the site of dioxygen binding and reduction, a binuclear center consisting of heme a_3 and Cu_B (Tsukihara et al., 1995; Ferguson-Miller and Babcock, 1996; Tsukihara et al., 1996). In contrast to Cox 1 and Cox 2, Cox3 does not contain any

prosthetic groups (Hatefi, 1985; Michel et al., 1998). The function of this subunit is not fully understood, but it appears to be essential for the assembly of the functional holoprotein (Michel et al., 1998; Hoffbuhr et al., 2000).

Cytochrome *c* oxidase deficiency is one of the most common causes of respiratory chain defects in humans (Borisov, 2002; Hamza and Gitlin, 2002; Diaz, 2010). Human cytochrome *c* oxidase deficiency comprehends a wide variety of disorders with distinct clinical phenotypes resulting from a number of unique genetic abnormalities (Borisov, 2002; Hamza and Gitlin, 2002; Diaz, 2010). Pathological features range from metabolic acidosis, weakness, cardiomyopathy to neurodegeneration (Borisov, 2002; Hamza and Gitlin, 2002; Diaz, 2010). Cytochrome *c* oxidase deficiency rarely arise from mutations located in mitochondrial or nuclear genes encoding the cytochrome *c* oxidases subunits, but is rather secondary to loss-of-function mutations in genes encoding for proteins required for the assembly of the functional holoprotein (Shoubridge, 2001; Diaz, 2010). Thus, mutations in genes encoding Sco1 and Sco2, both critical for the metallation of the Cu_A site, result in impaired cytochrome *c* oxidase function (Leary et al., 2004). Reduced insertion of copper might also be the reason for cytochrome *c* oxidase impairment in copper deficiency (Kodama et al., 1989; Milne and Nielsen, 1996).

1.1.1.2 Copper/zinc superoxide dismutase

The members of the ubiquitous family of superoxide dismutases (SODs) convert superoxide to dioxygen and hydrogen peroxide for further disposal by catalase and glutathione peroxidase (Dringen and Hirrlinger, 2010). Superoxide is produced during the reduction of dioxygen that occurs in respiration and during autoxidation of catecholamines as well as its metabolites (Halliwell and Gutteridge, 2007; Dringen and Hirrlinger, 2010). Superoxide is also generated by some enzymes, for example by the enzymatic activity of NADPH oxidases in

macrophages and microglial cells during the immune response (Morel et al., 1991; Dringen, 2005; Halliwell and Gutteridge, 2007). Since excess amounts of superoxide can lead to the formation of highly reactive oxygen species (ROS) that would damage cellular constituents and/or initiate lipid peroxidation (Halliwell and Gutteridge, 2007), SODs represent a first line of defense against the toxicity of the superoxide anion.

In eukaryotic cells three distinct isoforms of SOD that are encoded by three different genes have been identified: copper/zinc superoxide dismutase (Cu/Zn-SOD; SOD1), manganese superoxide dismutase (Mn-SOD; SOD2) and extracellular superoxide dismutase (EC-SOD; SOD3; Miao and St Clair, 2009; Perry et al., 2010). SOD1 is a homodimeric protein located largely in the cytosol with minor fractions being present in intracellular compartments including the nucleus, the intermembrane space of mitochondria, lysosomes and peroxisomes (Weisiger and Fridovich, 1973a; Thomas et al., 1974; Crapo et al., 1992; Okado-Matsumoto and Fridovich, 2001). However, some cell types also secrete SOD1 (Mondola et al., 1996; Mondola et al., 1998; Cimini et al., 2002). The homotetrameric SOD2 is a mitochondrial enzyme that resides within the matrix and is associated with the inner membrane of mitochondria (Weisiger and Fridovich, 1973a; Okado-Matsumoto and Fridovich, 2001; Miao and St Clair, 2009; Perry et al., 2010). The homotetrameric glycoprotein SOD3 is secreted by fibroblasts and glial cells and has been found in the extracellular matrix of tissues as well as in plasma, lymph and cerebrospinal fluid, where it protects cell membranes against oxidative stress (Petersen and Enghild, 2005; Antonyuk et al., 2009). While SOD2 contains manganese as metal cofactor (Weisiger and Fridovich, 1973b, a; Miao and St Clair, 2009), both SOD1 and SOD3 contain catalytic copper and structural zinc ions in their active sites (Carrico and Deutsch, 1970; Weisiger and Fridovich, 1973b; Tibell et al., 1987; Antonyuk et al., 2009; Miao and St Clair, 2009).

Crystallographic studies have revealed the detailed enzymatic mechanism for the catalysis of superoxide disproportionation by SOD1, during which the catalytic copper ion is cyclically reduced and oxidized (Hart et al., 1999; Perry et al., 2010). SOD1 activity can be regulated at the post-translational level by copper insertion and disulfide formation, which is facilitated by the copper chaperon for SOD1 (CCS; Leitch et al., 2009b; Miao and St Clair, 2009). Consequently, a decrease in the activity of SOD1 is observed in copper deficient subjects (Milne and Nielsen, 1996), which is often accompanied by an increase in SOD2, a well-characterized response to oxidative stress (Uriu-Adams and Keen, 2005).

Besides its function in the detoxification of superoxide, SOD1 has been connected with intracellular signaling (Mondola et al., 2004; Juarez et al., 2008). The activity of several growth factors, e.g. epidermal growth factor, platelet-derived growth factor and vascular endothelial growth factor, is redox regulated (Valko et al., 2007) and SOD1 has been demonstrated to play an essential role in mitogen-activated protein kinase (MAPK) signaling by mediating a hydrogen peroxide-dependent oxidation and inactivation of phosphatases in several tumor cell lines (Juarez et al., 2006; Juarez et al., 2008). Furthermore, secreted SOD1 has been shown to bind to SK-N-BE neuroblastoma cells, leading to an increase in intracellular calcium concentrations through a phospholipase C-protein kinase C (PLC-PKC)-dependent pathway and subsequently to an activation of the MAPK extracellular signal-regulated kinases 1 and 2 (Mondola et al., 2004). Interestingly the activation of the PLC-PKC pathway by SOD1 was independent of the enzymes superoxide dismutase activity (Mondola et al., 2004). Since SOD1 is also present in the neuronal microenvironment, a neuromodulatory role of SOD1 has been suggested (Mondola et al., 2004).

Mutations in SOD1 have been linked to amyotrophic lateral sclerosis (ALS) (Rosen, 1993). ALS is a progressive neurodegenerative disease preferentially but not exclusively affecting motor neurons in the spinal cord, brainstem and brain (Pasinelli and Brown, 2006; Bento-Abreu et al., 2010; Ticozzi et al., 2011). The gradual loss of motor-neurons results in weakness, muscle atrophy as well as spasticity and finally leads to death due to respiratory failure (Pasinelli and Brown, 2006; Zatta and Frank, 2007; Bento-Abreu et al., 2010; Ticozzi et al., 2011). The majority of cases of ALS are sporadic without family history. Only about 10% of all cases are familial ALS (Rosen, 1993; Pasinelli and Brown, 2006; Bento-Abreu et al., 2010; Ticozzi et al., 2011). Mutations in SOD1 are the most common cause (around 20%) of the familial form, but have also been found in roughly 3% of sporadic ALS patients (Rosen, 1993; Pasinelli and Brown, 2006; Bento-Abreu et al., 2010; Ticozzi et al., 2011). Currently there is no definite explanation how a mutant SOD1 provokes ALS, but it appears to be due to a yet unknown gain of toxic functions rather than to a loss of function (Hough et al., 2004; Pasinelli and Brown, 2006; Bento-Abreu et al., 2010; Ticozzi et al., 2011). The pathogenic capacity of mutant SOD1 has been ascribed to its potential to form harmful aggregates that result in endoplasmatic reticulum stress and malfunction of the proteasomal system, to its proapoptotic properties as well as to its abilities to damage mitochondria (Pasinelli and Brown, 2006; Kanekura et al., 2009; Bento-Abreu et al., 2010; Nassif et al., 2010; Shi et al., 2010). In addition to the toxic intracellular effects of mutant SOD1, extracellular mutant SOD1 induces motor neuron death by triggering microgliosis (Urushitani et al., 2006; Zhao et al., 2010).

1.1.1.3 Ceruloplasmin

Ceruloplasmin (Cp) belongs to the family of multicopper oxidases (Hellman and Gitlin, 2002; Kosman, 2010a). Members of this group of enzymes, which is present in all kinds of phyla, are characterized by the concomitant presence of

type 1, type 2 and type 3 copper sites (Hellman and Gitlin, 2002; Healy and Tipton, 2007; Quintanar et al., 2007; Dittmer and Kanost, 2010; Kosman, 2010a). Multicopper oxidases couple the one-electron oxidation of four substrate molecules with the four-electron reduction of dioxygen to water (Hellman and Gitlin, 2002; Healy and Tipton, 2007; Quintanar et al., 2007; Kosman, 2010a). The reducing substrates utilized in this reaction vary among the enzyme family members (Hellman and Gitlin, 2002). Known substrates include such diverse compounds as low valent transition metal ions (Fe^{2+}, Mn^{2+}, Cu^{+}), bilirubin, ascorbate, phenols and nitrate (Hellman and Gitlin, 2002; Quintanar et al., 2007; Kosman, 2010a).

Cp exhibits ferroxidase activity and has a critical role in iron homeostasis (Hellman and Gitlin, 2002; Healy and Tipton, 2007; Kosman, 2010b). It has been suggested to be an important contributor to the plasma antioxidative capacity, since it displays ferroxidase, cuprous oxidase and glutathione-peroxidase activities as well as the ability to scavenge ROS (Atanasiu et al., 1998; Stoj and Kosman, 2003; Healy and Tipton, 2007). Cp also possesses amine oxidase, catechol oxidase and ascorbate oxidase activities, although the physical importance of these enzymatic activities remains to be elucidated (Bielli and Calabrese, 2002; Healy and Tipton, 2007). Even though copper bound to Cp accounts for up to 95% of plasma copper, there is no evidence for a direct role of this plasma protein in copper transport to tissues (Meyer et al., 2001; Bielli and Calabrese, 2002; Healy and Tipton, 2007; Choi and Zheng, 2009).

Cp contains 6 copper atoms per molecule: three type 1 copper sites, a single type 2 copper ion and a binuclear type 3 copper site (Zaitseva et al., 1996; Bento et al., 2007). The LMCT absorption at 610 nm between the cysteine ligand sulfur and the type 1 copper ions confers the typical intense blue color to Cp (Bielli and Calabrese, 2002; Hellman and Gitlin, 2002; Healy and Tipton, 2007). The type 2 copper is located in close proximity to the type 3 copper center with

which it forms a trinuclear cluster that is the site of oxygen binding and reduction (Farver et al., 1999; Bielli and Calabrese, 2002; Bento et al., 2007; Healy and Tipton, 2007). During the catalytic cycle, electrons pass from the type 1 copper ions to this trinuclear copper cluster and subsequently to the oxygen molecule bound at this site (Farver et al., 1999; Bielli and Calabrese, 2002; Bento et al., 2007; Healy and Tipton, 2007).

The majority of Cp is synthesized by hepatocytes and secreted into circulation (Hellman and Gitlin, 2002; Healy and Tipton, 2007). Within the human central nervous system (CNS) and testes a glycosylphosphatidylinositol (GPI)-anchored form of Cp that is generated by alternative splicing has been identified for astrocytes and Sertoli cells respectively (Klomp et al., 1996; Patel and David, 1997; Fortna et al., 1999; Jeong and David, 2003; Mittal et al., 2003). During biosynthesis copper insertion into apo-Cp takes place late in the secretory pathway (Sato and Gitlin, 1991; Hellman and Gitlin, 2002; Hellman et al., 2002). In hepatocytes the copper transporting ATPase ATP7B and the Niemann-Pieck C1 protein are required for proper metallation of Cp (Terada et al., 1998; Meng et al., 2004; Yanagimoto et al., 2009; Yanagimoto et al., 2011).

Cp is an acute phase response protein whose synthesis and secretion can be strongly increased during pregnancy, inflammation, infection, and in diseases such as diabetes, cancer as well as cardiovascular diseases (Hughes, 1972; Louro et al., 2001; Bielli and Calabrese, 2002; Chiarla et al., 2008; Nowak et al., 2010). The elevated serum Cp levels in such conditions are mainly the result of increased Cp-biosynthesis in hepatocytes that can be induced in these cells by estrogen, proinflammatory cytokines and hypoxia-inducible factor (HIF)-1 (Limpongsanurak et al., 1981; Mukhopadhyay et al., 2000; Persichini et al., 2010; Sidhu et al., 2011). Copper deficiency does not affect the rates of biosynthesis and release of Cp by hepatocytes (Holtzman and Gaumnitz, 1970; Gitlin et al., 1992). However, impaired metallation causes an augmented release of apo-Cp

that lacks oxidase activity and possesses a shorter half-life than holo-Cp, thus leading to a lowering in Cp protein levels and oxidase activity in the serum of copper deficient rodents compared to control animals (Holtzman and Gaumnitz, 1970; Gitlin et al., 1992; Broderius et al., 2010).

Aceruloplasminemia is an autosomal recessive disorder resulting from a loss of function mutation in the Cp gene (Harris et al., 1995; Yoshida et al., 1995; Takahashi et al., 1996). Due to the importance of Cp in iron homeostasis, the lack of functional Cp in affected individuals is accompanied by excessive iron accumulation in most tissues (Harris et al., 1995; Yoshida et al., 1995; Miyajima et al., 1996; Gonzalez-Cuyar et al., 2008). Patients with aceruloplasminemia suffer from neurological symptoms such as retinal degeneration, mild dementia, dysarthria, dystonia as well as from diabetes mellitus (Harris et al., 1995; Yoshida et al., 1995; Miyajima et al., 1996; Takahashi et al., 1996; Gonzalez-Cuyar et al., 2008; McNeill et al., 2008). The neurological symptoms mirror the site of iron deposition in the brain (Miyajima, 2003) and iron-mediated oxidative stress is likely to contribute to the pathogenesis of aceruloplasminemia (Kaneko et al., 2002a; Kaneko et al., 2002b; Miyajima et al., 2002; Kono and Miyajima, 2006; Gonzalez-Cuyar et al., 2008).

1.1.1.4 Lysyl oxidase

Lysyl oxidase (LOX) is the eponym of the LOX-family of amine oxidases that currently consists of five members: LOX itself and the Lox-like proteins LOXL1-4 (Kagan and Li, 2003; Payne et al., 2007). LOX catalyzes the posttranslational oxidative deamination of certain peptidyl lysines in their target proteins to the peptidyl aldehyde α-aminoadipic-δ-semialdehyde and can be found in the extracellular matrix (ECM) where it is well-known for its crucial role in the formation, maturation and stabilization of connective tissue by catalyzing the cross-linking of tropoelastin and tropocollagen to insoluble fibers (Rucker et al.,

1998; Kagan and Li, 2003; Lucero and Kagan, 2006; Payne et al., 2007). LOX has also been demonstrated to be localized in the cytosol and/or nuclei in some cell types (Li et al., 1997; Nellaiappan et al., 2000; Li et al., 2004; Jansen and Csiszar, 2007; Saad et al., 2010) and to exhibit physiological functions that extend far beyond the oxidation of structural proteins of the ECM (Lucero and Kagan, 2006; Payne et al., 2007). Thus, LOX is likely to function in gene transcription and cell signaling, amongst others by deaminating specific lysyl residues in histone H1 and H2 as well as in basic fibroblast growth factor and transforming growth factor-1β (Kagan et al., 1983; Li et al., 1997; Giampuzzi et al., 2003; Li et al., 2003; Mello et al., 2011). Consequently, LOX has been demonstrated to influence tissue development, cell proliferation, intracellular signal responses and cell migration and to act as an antagonist or protagonist of malignant processes (Li et al., 2003; Maki et al., 2005; Erler and Giaccia, 2006; Payne et al., 2007; Polgar et al., 2007; Atsawasuwan et al., 2008; Saad et al., 2010).

LOX contains two cofactors essential for its catalytic function: a tightly bound copper ion and a lysyl tyrosine quinone (LTQ), a unique covalently integrated organic cofactor that is auto-catalytically derived from a specific tyrosine and a specific lysine residue within the nascent polypeptide chain (Gacheru et al., 1990; Wang et al., 1996; Rucker et al., 1998; Bollinger et al., 2005; Lucero and Kagan, 2006). Copper in LOX has long been considered to be involved in the transfer of electrons to and from oxygen in order to facilitate the oxidative deamination of targeted peptidyl lysyl groups. However, experimental evidence suggests that the copper is required for LTQ formation and enzyme integrity rather than being directly involved in the catalytic process of oxidative deamination (Tang and Klinman, 2001). According to the proposed mechanism for the formation of LTQ the enzyme-bound copper atom catalyzes in the first step the oxidation of the tyrosine residue to peptidyl dihydroxyphenylalanine quinone which is then followed by covalent addition of the ε-amino group of

the attacking lysine residue to the quinone ring (Wang et al., 1996; Rucker et al., 1998; Bollinger et al., 2005).

LOX is synthesized as a catalytically inactive propeptide that is processed further by glycosylation, addition of copper and autocatalytical formation of the LTQ cofactor (Kosonen et al., 1997; Rucker et al., 1998). The propeptide is activated by proteolytic cleavage by procollagen C-proteinase following secretion (Rucker et al., 1998; Kagan and Li, 2003; Lucero and Kagan, 2006). Copper incorporation takes place in the *trans*-Golgi network (TGN) where it is delivered to the propeptide by the copper transporting ATPase ATP7A (Kosonen et al., 1997; Tchaparian et al., 2000; Hardman et al., 2007). Accordingly, LOX activity is low in patients suffering from Menkes disease, which is caused by mutations in the ATP7A gene and is characterized by marked connective tissue dysfunctions (Royce et al., 1980; Royce and Steinmann, 1990; Tumer and Moller, 2010; Kodama et al., 2011). Dietary copper status also affects LOX activity, but does not alter tissue levels of the LOX protein (Rucker et al., 1996; Rucker et al., 1998). Interestingly, LOX activity is not only diminished when copper levels are low, but does also increase with rising copper levels which is hypothesized to be due to improved LTQ cofactor formation (Rucker et al., 1996; Rucker et al., 1998).

1.1.1.5 Tyrosinase

Tyrosinase is the key enzyme in the biogenesis of melanin pigments in all organisms. In mammals, tyrosinase is mainly expressed in melanocytes and retinal pigment epithelium cells where it is localized to specialized organelles known as melanosomes (Petris et al., 2000; Wang and Hebert, 2006; Ray et al., 2007; Simon et al., 2009). Mammalian melanins are assumed to function as photoprotectives that absorb UV radiation, as antioxidants that scavenge ROS and as sinks for heavy metals (Meredith and Sarna, 2006; Brenner and Hearing,

2008; Simon et al., 2008). Mutations in tyrosinase lead to the absence or reduced synthesis of melanins and thus are responsible for oculocutaneous albinism (Gronskov et al., 2007; Ray et al., 2007; Fistarol and Itin, 2010). Affected individuals are characterized by reduced or absent pigmentation of the skin, hair and eyes, defects of the visual system and enhanced sensitivity to skin and ocular cancers (Gronskov et al., 2007; Ray et al., 2007; Fistarol and Itin, 2010). While in lower organisms and plants tyrosinase is the only enzyme controlling melanin synthesis, in mammals, two additional enzymes that have originated by duplication of the ancestral tyrosinase gene, the tyrosinase-related proteins Tyrp1 and Tyrp2, participate in this biosynthetic pathway (Garcia-Borron and Solano, 2002; Wang and Hebert, 2006; Olivares and Solano, 2009).

Tyrosinase possesses cresolase activity, the ability to catalyze the oxidation of *o*-diphenols to *o*-quinones, as well as catecholase acticity, the capability to catalyze the hydroxylation of monophenols to *o*-diphenols (Solomon et al., 1996; Garcia-Borron and Solano, 2002; Gerdemann et al., 2002; Wang and Hebert, 2006; Olivares and Solano, 2009). Amongst others, tyrosinase catalyzes the hydroxylation of L-tyrosin to L-3,4-dihydroxyphenylalanin (L-DOPA), the rate-limiting step in the biosynthesis of melanins and dopamine, and its subsequent oxidation to DOPA quinone (Garcia-Borron and Solano, 2002; Wang and Hebert, 2006; Olivares and Solano, 2009). It contains two antiferromagnetically-coupled copper ions in their active centers, which serve as the site of substrate and oxygen binding (Solomon et al., 1996; Garcia-Borron and Solano, 2002; Gerdemann et al., 2002; Rosenzweig and Sazinsky, 2006; Decker et al., 2007). The active site of tyrosinase shares strong sequential, structural and mechanistic homology with that of catechol oxidases found in plants, insects and crustaceans, and that of hemocyanins found in the hemolymph of many mollusks and arthropods (Solomon et al., 1996; Jaenicke and Decker, 2004; Decker et al., 2007; Olivares and Solano, 2009; Solomon et al., 2011). Catechol

oxidases are capable of oxidizing catechols but lack cresolase activity, whereas hemocyanins are oxygen carrier proteins analogous to hemoglobins (Gerdemann et al., 2002; Jaenicke and Decker, 2004; Decker et al., 2007; Olivares and Solano, 2009). Together with these proteins, tyrosinases constitute the family of type 3 copper proteins (Gerdemann et al., 2002; Jaenicke and Decker, 2004; Decker et al., 2007; Olivares and Solano, 2009). The highest degree of conservation in members of this group of proteins is found in the copper binding regions, termed CuA and CuB for tyrosinases (Gerdemann et al., 2002; Jaenicke and Decker, 2004). Each CuA and CuB, as well as the respective regions in catechol oxidases and hemocyanins, contain three histidine residues that coordinate to the pair of copper ions and are almost perfectly conserved throughout the type 3 copper proteins (Gerdemann et al., 2002; Jaenicke and Decker, 2004; Decker et al., 2007). The functional differences between tyrosinases, catechol oxidases and hemocyanins are based on the different accessibilities of the dinuclear copper centers to potential substrates (Solomon et al., 1996; Gerdemann et al., 2002; Rosenzweig and Sazinsky, 2006; Solomon et al., 2011).

In mammalian cells the first DOPA oxidase-positive compartment is the TGN, so that presumably tyrosinase acquires copper in this organelle (Wang and Hebert, 2006; Ray et al., 2007; Setty et al., 2008). During trafficking from TGN to melanosomes tyrosinase looses its copper and must be reloaded within melanosomes to sustain its activity (Setty et al., 2008). For both compartments, copper loading depends on the copper transporting ATPase ATP7A (Petris et al., 2000; Wang and Hebert, 2006; Ray et al., 2007; Setty et al., 2008). Consequently, mutations in ATP7A are associated with diminished tyrosinase activity which manifests clinically in diffuse subcutaneous hypopigmentation (Fistarol and Itin, 2010). The presence of the typical copper-chaperone CxxC motif in both tyrosinase and ATP7A has led to the hypothesis that copper is

transferred from ATP7A first to the CxxC motif of tyrosinase and then to the final histidine-rich active sites (Garcia-Borron and Solano, 2002). However, the precise mechanism how ATP7A transfers copper to tyrosinase remains to be elucidated.

1.1.1.6 Dopamine-β-monoxygenase and peptidylglycine α-amidating monoxygenase

Dopamine-β-mooxygenase (DβM) and peptidylglycine α-amidating monoxygenase (PAM) belong to a small class of copper proteins found exclusively in animals (Klinman, 2006). Both enzymes, also referred to as noncoupled binuclear copper proteins, catalyze the dioxygen- and ascorbate-dependent hydroxylation of specific C-H-bonds in their target substrates (Klinman, 1996; Chen and Solomon, 2004; Klinman, 2006; Solomon et al., 2011).

Among others, DβM catalyzes the oxidative hydroxylation of dopamine to norepinephrine and thus plays an important role in the metabolism of these catecholamines (Stewart and Klinman, 1988; Klinman, 1996; Kim et al., 2002; Timmers et al., 2004; Klinman, 2006). DβM is a homotetrameric glycoprotein that localizes primarily within the chromaffin granules of the adrenal medulla and the large dense-core synaptic vesicles of noradrenergic neurons where it exists as both a soluble and a membrane-bound protein (Geffen et al., 1969; Stewart and Klinman, 1988; Kim et al., 2002). The stimulus-dependent secretion of the soluble enzyme accounts for the presence of DβM in blood and CSF (Stewart and Klinman, 1988; Kim et al., 2002).

Expression of PAM in adults is highest in the secretory vesicles of atrial myocytes, endocrine cells of the pituitary gland and in many neurons, but not limited to theses cell types (Rhodes et al., 1990; Prigge et al., 2000). PAM exclusively catalyzes the C-terminal α-amidation of various glycine-extended propeptides, a post-translational modification essential for the bioactivity of diverse physiological regulators including peptide hormones,

neurotransmitters and growth factors (Klinman, 1996; Prigge et al., 1997; Prigge et al., 2000; Bousquet-Moore et al., 2010a). Due to the physiological importance of PAM, lack of functional PAM in mice is embryonic lethal (Bousquet-Moore et al., 2010a). PAM is composed of two enzymatic domains that act sequentially on the target substrate (Klinman, 1996; Prigge et al., 1997; Prigge et al., 2000; Bousquet-Moore et al., 2010a). The amino-terminal domain, peptidylglycine α-hydroxylating monoxygenase (PHM) catalyzes the stereospecific hydroxylation of the glycine α-carbon of the peptidylglycine substrates whereas the second domain, peptidyl- α-hydroxyglycine α-amidating lyase (PAL), generates the α-amidated peptide product and glyoxylate (Prigge et al., 1997; Prigge et al., 2000; Bousquet-Moore et al., 2010a). The isolated domains that can be separated either through endoprotease cleavage or through independent expression retain their enzymatic activity (Prigge et al., 1997; Prigge et al., 2000). Tissue-specific and developmentally regulated alternative splicing gives rise to multiple isoforms of PAM, the most important being PAM-1, -2 and -3 (Klinman, 1996; Prigge et al., 2000; Bousquet-Moore et al., 2010a). While PAM-3 is a soluble protein, both PAM-1 and -2 are membranous proteins (Klinman, 1996; Bousquet-Moore et al., 2010a). However, PAM-1 can be post-translationally modified generating separated, soluble PHM and PAL, which are stored in large dense-core vesicles and secreted along with the neuropeptides or peptide hormones (Klinman, 1996; Prigge et al., 2000; Bousquet-Moore et al., 2010a).

Comparison of the primary sequence of the catalytic core of PHM with DβM indicates a central core of around 300 amino acids that is 27% identical and 40% homologues to the catalytic domain of PHM (Southan and Kruse, 1989; Prigge et al., 2000; Klinman, 2006; Kapoor et al., 2011). In particular, the copper binding sites of PHM and DβM show strong sequence similarity (Klinman, 1996, 2006). Both enzymes contain two type 2 coppers sites per subunit termed Cu_M and Cu_H, the former being coordinated by two histidine and one methionine, the

latter by three histidine residues (Solomon et al., 1996; Prigge et al., 1997; Prigge et al., 2004; Klinman, 2006; Hess et al., 2008; Chufan et al., 2010; Solomon et al., 2011). The nonequivalent copper centers are largely separated in space with no bridging ligand and no observable magnetic interaction (Klinman, 1996; Prigge et al., 1997; Gherman et al., 2006; Solomon et al., 2011). Cu_H solely functions as an electron transfer site, whereas Cu_M is the site at which dioxygen binding, activation and subsequent substrate hydroxylation take place (Prigge et al., 1997; Evans et al., 2003; Prigge et al., 2004; Gherman et al., 2006; Hess et al., 2008). In addition to its established catalytic function, the Cu_M site also plays a significant structural role (Siebert et al., 2005). During catalytic cycling one electron is transferred by each copper to molecular oxygen by a yet unclear mechanism (Prigge et al., 1997, 1999; Prigge et al., 2000; Evans et al., 2003; Chen and Solomon, 2004; Prigge et al., 2004; Klinman, 2006; Solomon et al., 2011). Due to the separation of the two copper ions by about 11 Å a direct electron transfer from Cu_H to the Cu_M-dioxygen complex is prohibited (Prigge et al., 1997, 1999; Prigge et al., 2000; Prigge et al., 2004). Several pathways for the electron transfer between the two copper sites have been proposed, but the exact mechanism remains an open question (Prigge et al., 1999; Prigge et al., 2000; Evans et al., 2003; Prigge et al., 2004; Klinman, 2006; Solomon et al., 2011).

Proper metallation of DβM and PHM is essential for their activity. In Menkes disease patients plasma catechol levels are altered and levels of amidated peptides are low, reflecting DβM and PHM deficiency, respectively (El Meskini et al., 2003; Steveson et al., 2003; Niciu et al., 2007; Kaler et al., 2008; Goldstein et al., 2009). Menkes disease is an inherited disorder caused by defects in the copper transporting ATPase ATP7A, which transports copper out of the cell as well as into the secretory pathway (Tumer and Moller, 2010). Thus, copper loading of both, DβM and PHM, is likely to depend on ATP7A. In support of this view, PAM activity is compromised in cells lacking functional ATP7A,

although expression levels of PAM are normal (El Meskini et al., 2003; Steveson et al., 2003; Niciu et al., 2007).

1.1.1.7 Copper amine oxidases

Amine oxidases catalyze the oxidative deamination of various amines and the subsequent reduction of oxygen to hydrogen peroxide (Klinman, 1996; Lyles, 1996; Toninello et al., 2006; Sebela et al., 2007; Boobis et al., 2009). Ubiquitously expressed in all forms of life, amine oxidases allow bacteria, yeast and fungi the use of amines as nitrogen source via the oxidative release of ammonium and play an important role in regulating the levels of various biogenic amines in higher eukaryotes (Klinman, 1996; Mure, 2004; O'Sullivan et al., 2004; Toninello et al., 2006; Boobis et al., 2009). Amine oxidases are a heterogeneous group of enzymes that can be divided according to their cofactor into copper amine oxidases and flavin-containing amine oxidases (Toninello et al., 2006; Sebela et al., 2007; Boobis et al., 2009).

Copper amine oxidases are almost always observed to be homodimers with the two subunit containing each a type 2 copper center (Klinman, 1996; Brazeau et al., 2004; MacPherson and Murphy, 2007). In addition to copper, trihydroxyphenylalanine quinone (TPQ) is utilized as cofactor (Klinman, 1996; Brazeau et al., 2004; Mure, 2004; MacPherson and Murphy, 2007; Sebela et al., 2007; Boobis et al., 2009). TPQ is auto-catalytically derived from a tyrosine residue within the nascent protein (Klinman, 1996; Brazeau et al., 2004; MacPherson and Murphy, 2007). The bound copper participates in this process, most likely by coordinating to the pre-TPQ residue and reaction intermediates (DuBois and Klinman, 2005; MacPherson and Murphy, 2007). Amine oxidation by copper amine oxidases follows a ping-pong mechanism during which TPQ is first reduced by the amine substrate and subsequently reoxidized by molecular oxygen (Klinman, 1996; Kishishita et al., 2003; Shepard et al., 2008). While the

reduction of TPQ is believed to proceed independent of copper (Kishishita et al., 2003), its role in the reoxidation step is still under debate. Copper may either be directly involved in the transfer of electrons from the reduced organic cofactor to dioxygen by activating molecular oxygen or may solely provide electrostatic stabilization of the superoxide anion intermediate (Mills et al., 2002; Kishishita et al., 2003; Brazeau et al., 2004; DuBois and Klinman, 2005; Mukherjee et al., 2008; Shepard et al., 2008).

In mammals two principal classes of copper amine oxidases exist: primary amine oxidases (also often referred to as semicarbazide-sensitive amine oxidase) and diamine oxidases (Schwelberger, 2007; Sebela et al., 2007; Boobis et al., 2009). Primary amine oxidases prefer primary monoamines as substrates and have no or little activity towards diamines (O'Sullivan et al., 2004; Boobis et al., 2009; Kaitaniemi et al., 2009). The physiological substrates are unspecified, but are believed to include methylamine, aminoacetone, dopamine and/or tyramine (Lizcano et al., 1991; O'Sullivan et al., 2004; Kaitaniemi et al., 2009). Mammalian primary amine oxidases comprise vascular adhesion protein-1 (VAP-1) and the homologous retinal-specific amine oxidase (Bono et al., 1998; Smith et al., 1998; Zhang et al., 2003; Schwelberger, 2007). Both enzymes are membrane proteins mainly located in the plasma membrane with large extracellular domains that harbor the catalytic site (Salmi and Jalkanen, 1992; Morris et al., 1997; Zhang et al., 2003). Proteolytic cleavage of VAP-1 from adipocytes and endothelial cells results in a soluble enzyme which accounts for the majority of primary amine oxidases activity in human and mouse plasma (Kurkijarvi et al., 1998; Kurkijarvi et al., 2000; O'Sullivan et al., 2004; Schwelberger, 2007). VAP-1 has been demonstrated to mediate leukocyte adhesion, to stimulate glucose uptake and to induce cell differentiation of adipocytes in a hydrogen peroxide-dependent manner (Salmi and Jalkanen, 2001; Salmi et al., 2001; Yu et al., 2003; O'Sullivan et al., 2004). Furthermore, the

products of the amine oxidase reaction have been postulated to have an important role in signaling, but may also contribute to the pathogenesis of various inflammation-associated diseases (Salmi and Jalkanen, 2001; O'Sullivan et al., 2004). Thus, plasma as well as membrane-bound primary amine oxidase activities are observed to be elevated in Alzheimer's disease, cancer and diabetes type 1 and 2 (Yu et al., 2003; O'Sullivan et al., 2004; Toninello et al., 2006). However, the physiological functions of primary amine oxidases are still not well understood (Salmi and Jalkanen, 2001; O'Sullivan et al., 2004; MacPherson and Murphy, 2007; Kaitaniemi et al., 2009).

Diamine oxidase preferentially oxidizes diamines (Sessa and Perin, 1994; Elmore et al., 2002; Boobis et al., 2009). Its physiological substrates include histamine, putrescine, cadaverine and the polyamine spermidine (Sessa and Perin, 1994; Elmore et al., 2002). Diamine oxidase is a soluble enzyme localized to intracellular vesicles with the highest expression levels found in kidney, placenta and intestine (Schwelberger et al., 1998; Elmore et al., 2002; Schwelberger, 2007). Plasma levels are usually very low, but increase in response to heparin and during pregnancy (Sessa and Perin, 1994; Elmore et al., 2002; Schwelberger, 2007; Boobis et al., 2009). Diamine oxidase is the main enzyme for metabolism of exogenous histamine and is postulated to play a role in the regulation of cell proliferation, inflammation and ischemia (Sessa and Perin, 1994; Elmore et al., 2002; Maintz and Novak, 2007; McGrath et al., 2009; Jones and Kearns, 2011).

1.1.2 Angiogenesis

Angiogenesis, the formation of new blood vessels from existing vasculature, is involved in many physiological and pathological conditions (Costa et al., 2004; Gupta and Zhang, 2005; D'Andrea et al., 2010; Carmeliet and Jain, 2011). Angiogenesis is a strictly regulated process that plays an essential role in

embryonic development, wound healing and ovulation (Costa et al., 2004; Gupta and Zhang, 2005; D'Andrea et al., 2010; Carmeliet and Jain, 2011). Insufficient angiogenesis is associated with cardiovascular diseases, stroke as well as a reduced capacity for tissue repair, while excessive angiogenesis is observed in cancer, retinopathies, arthritis and psoriasis (Gupta and Zhang, 2005; D'Andrea et al., 2010; Carmeliet and Jain, 2011). The crucial role of angiogenesis in the progression of tumor growth and metastasis has prompted extensive research into anti-angiogenic strategies as cancer-therapeutics (Gupta and Zhang, 2005; Finney et al., 2009; Tisato et al., 2010; Carmeliet and Jain, 2011). Several studies point to a pivotal role of copper in normal and pathological angiogenesis. However, in addition to the requirement of copper in the angiogenic process, copper itself has been identified to be angiogenic (Parke et al., 1988; Hu, 1998; Gerard et al., 2010).

The copper content of rabbit corneas has been shown to increase in response to an angiogenic stimulus (Gullino et al., 1990) and nutrition-induced copper depletion or treatment with copper chelators have been shown to inhibit neovascularization as well as endothelial cell proliferation and migration (Matsubara et al., 1989; Brem et al., 1990; Gullino et al., 1990; Juarez et al., 2006; Mamou et al., 2006; Finney et al., 2007; Hassouneh et al., 2007; Lowndes et al., 2009). In addition, the observed elevated tissue and serum levels of copper and Cp in patients suffering from various cancers with extensive angiogenesis further hint to an important role of copper in tumor angiogenesis (Coates et al., 1989; Gullino et al., 1990; Senra Varela et al., 1997; Nayak et al., 2004; Uriu-Adams and Keen, 2005; Doustjalali et al., 2006). The precise mechanisms by which bioavailable copper exerts its multiple effects on angiogenesis are not fully understood. However, numerous processes important for the regulation of angiogenesis have been found to be influenced by copper, either directly or indirectly (Finney et al., 2009; D'Andrea et al., 2010). Specifically copper, but not

other transition metals, has been shown to be required for the expression and/or secretion of several angiogenic mediators, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-1 , interleukin (IL)-1α and IL-8 (Prudovsky et al., 2003; Martin et al., 2005; Soldi et al., 2007; Di Serio et al., 2008; D'Andrea et al., 2010). X-ray fluorescence microprobe (XFM) approaches have shown copper to be highly spatially regulated during the angiogenic processes, as it is translocated from the cells perinuclear areas towards the tips of extending filopodia, followed by subsequent release into the extracellular space (Finney et al., 2007; Finney et al., 2009). Based on these result it has been postulated that copper activates an extracellular target essential for the angiogenic process (Finney et al., 2007; Finney et al., 2009). Consistent with this hypothesis copper influences the biological activity of proteins and protein-derived fragments that modulate several biological processes involved in angiogenesis, endothelial cell migration and proliferation. Copper has been shown to enhance the effects of VEGF and FGF-2 on angiogenesis (Gerard et al., 2010) and to increase specific binding of the potent angiogenic protein angiogenin to endothelial cells (Badet et al., 1989; Soncin et al., 1997; Hu, 1998). Furthermore, biomolecules such as heparin and the peptide glycyl-L-hystidyl-L-lysine have been found to induce the formation of new capillaries when bound to copper (Gullino et al., 1990).

Recognition of copper as critical factor in angiogenesis has encouraged research into the use of copper chelators in anti-angiogenic cancer therapy. Depletion of copper has been shown to inhibit angiogenesis in a wide variety of cancer cell and xenograft systems (Brem et al., 1990; Juarez et al., 2006; Hassouneh et al., 2007; Khan and Merajver, 2009; Kumar et al., 2010). Phase I and II clinical trials utilizing copper chelation as either an adjuvant or as primary therapy have shown promising results (Henry et al., 2006; Lowndes et al., 2008; Pass et al., 2008; Khan and Merajver, 2009; Lin et al., 2011).

1.1.3 Non-classical secretion

The transport of polypeptides across or into biological membranes is an important process in all living organisms. Proteins destined for translocation frequently possess specific signal sequences that target them to their particular destinations (van Vliet et al., 2003; Derby and Gleeson, 2007; Neupert and Herrmann, 2007; Wente and Rout, 2010). Most secretory proteins contain a hydrophobic N-terminal sequence for targeting into the endoplasmic reticulum (ER) from where they are exported from the cell through the classical ER-Golgi pathway (Prudovsky et al., 2003; van Vliet et al., 2003; Prudovsky et al., 2008). However, some proteins found in the extracellular milieu lack such sequences and are released via non-classical ER-Golgi-independent routes (Prudovsky et al., 2003; Prudovsky et al., 2008; Nickel, 2011). FGF-1 and IL-1α that belong to this group of proteins are secreted by a copper-dependent mechanism (Landriscina et al., 2001; Mandinova et al., 2003; Prudovsky et al., 2003; Sivaraja et al., 2006; Soldi et al., 2007; Di Serio et al., 2008). Both are exported as components of multiprotein complexes and a crucial role in the assembly of these complexes has been ascribed to copper (Landriscina et al., 2001; Mandinova et al., 2003; Sivaraja et al., 2006). In addition, copper might be required for the formation of FGF-1 homodimers that is critical for FGF-1 release (Landriscina et al., 2001; Prudovsky et al., 2008).

1.1.4 Hypoxia-inducible factor 1

The hypoxia-inducible factor (HIF)-1 is a key regulator of the transcriptional response to hypoxia in mammals (Martin et al., 2005; Ke and Costa, 2006; Majmundar et al., 2010). Among others, the corresponding gene products are involved in erythropoiesis, iron metabolism, angiogenesis, glucose uptake and glycolysis (Martin et al., 2005; Ke and Costa, 2006; Majmundar et al., 2010). HIF-1 is a $\alpha_1\beta_1$ heterodimer specifically recognizing HIF-1-binding sites within cis-

regulatory hypoxia response elements (Martin et al., 2005; Ke and Costa, 2006; Majmundar et al., 2010). Although constitutively synthesized, protein levels of the α-subunit (HIF-1α) are almost not detectable under normoxic conditions, since HIF-1α is subject of rapid ubiquitination by the van Hippel-Landau tumor suppressor protein and subsequent proteasomal degradation (Martin et al., 2005; Ke and Costa, 2006; Majmundar et al., 2010). Ubiquination by the van Hippel-Landau tumor suppressor protein requires the modification of HIF-1α by prolyl-4-hydroxylation, which is accomplished by a family of oxygen- and iron-dependent prolyl-4-hydroxylases (Martin et al., 2005; Ke and Costa, 2006; Majmundar et al., 2010). Hypoxic conditions diminish prolyl-4-hydroxylases activity and thus facilitate HIF-1α accumulation and translocation into the nucleus, where it dimerizes with HIF-1β and interacts with transcriptional cofactors to assemble the HIF-1 transcriptional complex (Martin et al., 2005; Ke and Costa, 2006; Majmundar et al., 2010). HIF-1 activity is further controlled by another iron-dependent dioxygenase, the factor inhibiting HIF-1, which can hydroxylate HIF-1α at a C-terminal asparagine residue, thereby preventing the recruitment of transcriptional cofactors (Martin et al., 2005; Ke and Costa, 2006; Majmundar et al., 2010).

Being required for HIF-1 binding to the hypoxia response elements of target genes as well as for the formation of the HIF-1 transcriptional complex, copper is essential for HIF-1 transcriptional activity (Feng et al., 2009). Consequently, copper deprivation suppresses the transcriptional activity of HIF-1 under conditions otherwise known to activate it (Jiang et al., 2007; Feng et al., 2009). Besides the need of copper for HIF-1 transcriptional activity, excess of copper stabilizes the HIF-1α protein, most likely by inhibition of prolyl-4-hydroxylases (Martin et al., 2005).

1.1.5 Essentiality and toxicity of copper

Copper is an essential element for all forms of life. According to the World Health Organization (WHO), a metal is categorized as essential when "absence or deficiency of the element from the diet produces either functional or structural abnormalities and that the abnormalities are related to, or a consequence of specific biochemical changes that can be reversed by the presence of the essential metal" (WHO, 1996). However, also excessive intake of copper can cause adverse health effects (Chambers et al., 2010). Beside inappropriate dietary copper supply a number of human disorders result in copper deficiency or copper toxicity (Huster, 2010; Tumer and Moller, 2010). Health effects in connection with states of low or elevated copper may range from sub-clinical effects via clinical effects to lethal effects (WHO, 2002; Zatta and Frank, 2007).

In the US the estimated mean copper intake from food is 1.2 – 1.6 mg/d for men and 1.0 – 1.1 mg/d for women (Food and Nutrition Board, 2001). Higher copper intakes have been reported for the European Union, where mean dietary copper intakes have been reported to range from 2.4 mg/d (United Kingdom) to 4.2 mg/d (Germany) in men and from 1.7 mg/d (United Kingdom) to 3.3 mg/d (Germany) in women (Flynn et al., 2009). The main dietary sources of copper are shellfish, organ meat, seeds, grains, nuts and beans (Hunt and Meacham, 2001; Tapiero et al., 2003; de Romana et al., 2011). Besides food, drinking water can significantly contribute to daily intake, although this strongly varies from country to country (de Romana et al., 2011). Dietary supplements containing copper are consumed by approximately 15% of adults in the US (Food and Nutrition Board, 2001; Flynn et al., 2009). For men and women the recommended dietary copper intake is 0.9 mg Cu/d in the US (Food and Nutrition Board, 2001; Trumbo et al., 2001) and 1.1 mg Cu/d in the European Union (Scientific Committee on Food, 2003). The upper safe limit of daily

copper intake was prescribed to 10 mg Cu/d and 5 mg Cu/d in the US (Food and Nutrition Board, 2001; Trumbo et al., 2001) and in the European Union (Scientific Committee on Food, 2003; Flynn et al., 2009), respectively. However, the upper safe limit was largely based on liver toxicity endpoints and does not take less severe but clinically important responses into consideration (Chambers et al., 2010).

1.1.5.1 Copper deficiency

Copper deficiency in humans can occur through multiple mechanisms (Uriu-Adams and Keen, 2005; Zatta and Frank, 2007; de Romana et al., 2011). It can be observed in premature and low-birth weight infants who can be born with low hepatic copper stores (Walravens, 1980; Nassi et al., 2009), in individuals receiving total parenteral nutrition without adequate copper supplementation (Angotti et al., 2008; Imataki et al., 2008; Shike, 2009), in malnourished infants (Cordano, 1998) and in persons with malabsorption syndromes (Jameson et al., 1985; Jaiser and Winston, 2010). Low copper intakes in the diet can result in marginal copper deficiency. Although the mean dietary copper intake in the US and European Union is higher then the recommended one, the diets of approximately 25% of individuals do not meet this recommendation (Stern, 2010; Klevay, 2011). Marginal copper deficiency is therefore assumed to be widespread among populations (Stern et al., 2007; Stern, 2010; Klevay, 2011). Secondary copper deficiency can occur as consequence of high dietary intake of zinc (Horvath et al., 2010), of pharmacological treatments with copper chelating agents such as D-penicillamine or tetrathiomolybdate and after gastrointestinal surgery (O'Donnell and Simmons, 2011). Furthermore, severe copper deficiency is a hallmark of the hereditary diseases Menkes disease and occipital horn syndrome, both originating from genetic defects in the copper transporting ATPase ATP7A (Tumer and Moller, 2010; Kaler, 2011; Kodama et al., 2011).

The clinical symptoms of copper deficiency in humans are numerous (Danks, 1988; Uauy et al., 1998). Early and common signs of acquired copper deficiency are hematological manifestations such as anemia, leucopenia, neutropenia and pancytopenia (Angotti et al., 2008; Halfdanarson et al., 2008; Imataki et al., 2008). Bone abnormalities including osteoporosis, bone fractures and bone malformation have often been observed in copper deficient low-birth-weight infants and young children (Sutton et al., 1985; Stern et al., 2007). Acquired copper deficiency may manifest with neurological symptoms, the clinical presentation resembling that of myeloneuropathy observed in vitamin B12 deficiency (Schleper and Stuerenburg, 2001; Jaiser and Winston, 2010). Moreover, acquired copper deficiency has been identified as a dietary factor that may increase the risk of colon cancer (Chambers et al., 2010). The clinical features of Menkes disease include severe progressive neurological degeneration, connective tissue abnormalities, muscular hypotonia and hypopigmentation of skin and hair (Tumer and Moller, 2010; Kaler, 2011; Kodama et al., 2011).

Many of the clinical symptoms of acquired copper deficiency and Menkes disease can be attributed to a decrease in the activities of copper-dependent enzymes. Thus, hypopigmentation of skin and hair results from reduced tyrosinase activity and abnormalities of bone and connective tissue are principally due to lowered LOX activity (Petris et al., 2000; Kaler, 2011; Kodama et al., 2011). While the exact mechanism of copper deficiency myelopathy is not known (Jaiser and Winston, 2010), neurological degeneration in Menkes disease is believed to be primarily caused by a decrease in the activity of neuronal cytochrome c oxidase (Rossi et al., 2004; Kodama et al., 2011). Oxidative stress as a consequence of a low copper status may contribute to some of the clinical effects observed in human copper deficiencies (Uriu-Adams and Keen, 2005).

1.1.5.2 Copper toxicity

Excess of copper compared to cellular needs is harmful. Acute copper toxicity has been described for individuals that accidentally or with suicidal intention ingested high doses of copper (Gunay et al., 2006; Franchitto et al., 2008; Sood and Verma, 2011). For copper doses up to 1 gram, gastrointestinal symptoms predominate (Gunay et al., 2006; Franchitto et al., 2008). Following ingestion of higher doses, nausea, vomiting, headache, diarrhea, hemolytic anemia, gastrointestinal hemorrhage, liver and kidney failure as well as death may occur (Gunay et al., 2006; Franchitto et al., 2008; Sood and Verma, 2011).

Chronic copper toxicity is a feature of Wilson's disease, Indian Childhood Cirrhosis and Idiopathic Chronic Toxicosis (Scheinberg and Sternlieb, 1996; Tanner, 1998; Huster, 2010). Wilson's disease originates from a genetic defect in the copper transporting ATPase ATP7B that results in excessive copper accumulation particular in liver and brain (Scheinberg and Sternlieb, 1996; Pfeiffer, 2007; Huster, 2010). Wilson's disease is connected with hepatic ophthalmologic, neurological and/or psychiatric symptoms (Scheinberg and Sternlieb, 1996; Pfeiffer, 2007; Huster, 2010). Indian Childhood Cirrhosis and Idiopathic Chronic Toxicosis are severe chronic liver diseases that are characterized by excessive hepatic copper accumulation (Scheinberg and Sternlieb, 1996; Tanner, 1998). The etiology of these rare diseases has been hypothesized to be a combination of an unknown genetic defect affecting the copper metabolism and high dietary copper intake (Scheinberg and Sternlieb, 1996; Tanner, 1998). However, the causative role of copper in Indian Childhood Cirrhosis has recently been doubted (Sriramachari and Nayak, 2008). Chronic copper toxicity in individuals with no genetic susceptibility is rare and not considered as a major human health concern (Uriu-Adams and Keen, 2005; de Romana et al., 2011).

Both acute and chronic copper toxicity are in part a consequence of the redox activity of copper. Similar to low-molecular weight iron, unbound or loosely bound copper can catalyze the production of hydroxyl radicals in a Fenton-like reaction, which may induce oxidative stress and subsequent damage of cellular components (Gunther et al., 1995; Britton, 1996; Burkitt, 2001; Uriu-Adams and Keen, 2005; Halliwell and Gutteridge, 2007). In addition, excess of copper has been discussed to manifests its toxicity by direct inhibition of protein functions (Boulard et al., 1972; Letelier et al., 2005; Pamp et al., 2005; Letelier et al., 2006; Schwerdtle et al., 2007).

1.2

Cellular copper homeostasis

Homeostasis denotes "the process through which a tightly controlled set of physiological mechanisms monitors and regulates the absorption, transport, distribution, tissue storage and excretion of essential nutritions in order to ensure a constant and sufficient supply under varying conditions of intake while simultaneously avoiding excessive body levels that may lead to toxicity" (Stern, 2010). Given the requirement for copper on one hand and the potential toxicity of copper on the other, cells have evolved mechanisms to regulate cellular copper concentrations. Many of the components involved in cellular copper homeostasis are well known at the molecular level. These include transporters that mediate the uptake and efflux of copper, biomolecules that sequester and store copper and specialized proteins called copper chaperones that guide copper to copper-dependent enzymes and to organelles (Table 2).

Table 2: Proteins involved in mammalian copper homeostasis

Protein	**Function**
Copper transporter receptor 1 (Ctr1)	Copper uptake
Copper transporter receptor 2 (Ctr2)	Copper uptake
Divalent metal transporter 1 (DMT1)	Copper uptake
Copper chaperone for superoxide dismutase (CCS)	Intracellular copper trafficking
ATOX1	Intracellular copper trafficking
Cox17	Intracellular copper trafficking
Glutathione (GSH)	Intracellular copper trafficking, storage and detoxification
Metallothionein (MT)	Storage and detoxification
ATP7A	Copper export
ATP7B	Copper export

1.2.1 Copper uptake

Members of the copper transporter receptor (Ctr) family that were first described for *Saccharomyces cerevisiae* (Dancis et al., 1994; Knight et al., 1996), play a key role in the uptake of copper in eukaryotic cells. In man, the two Ctr-members hCtr1 and hCtr2 have been identified (Zhou and Gitschier, 1997). Ctr1 is considered as the major contributor to high-affinity copper uptake in mammalian cells (Lee et al., 2001; Eisses et al., 2005; Maryon et al., 2007b; Kim et al., 2008). The human and mouse homologues, hCtr1 and mCtr1, were identified by functional complementation of a growth defect of a yeast copper uptake mutant (Zhou and Gitschier, 1997; Lee et al., 2000). Ctr1 mediates copper uptake with an apparent K_M-value of 1-8 μM (Eisses and Kaplan, 2002; Lee et al., 2002a; Lee et al., 2002b; Eisses and Kaplan, 2005). The transport of copper by hCtr1 does not depend on ATP hydrolysis or an ion gradient (Lee et al., 2002a), suggesting that hCtr1 is neither an ion pump nor a secondary active transporter. Since Ctr1-mediated copper uptake is effectively inhibited by Ag^+, which is isoelectric to Cu^+, and strongly stimulated by ascorbate, monovalent copper is thought to be the copper species transported by Ctr1 (Lee et al., 2002a; Lee et al., 2002b; Bertinato et al., 2010). In addition to hCtr1, hCtr2 has been shown to mediate the uptake of Cu^+ (van den Berghe et al., 2007; Bertinato et al., 2008). However, since hCtr2 transports copper with lower affinity compared to hCtr1 (Bertinato et al., 2008), does not complement the yeast copper uptake deficiency (Zhou and Gitschier, 1997) and is predominately located in intracellular compartments (van den Berghe et al., 2007; Bertinato et al., 2008), its exact role in copper transport in mammalian cells remains unclear (Gupta and Lutsenko, 2009).

hCtr1 is composed of 190 amino acids and is predicted to have a molecular mass of 23 kDa (Zhou and Gitschier, 1997). However, due to *N*- and *O*-glycosylation hCtr1 resolves as 30-35 kDa band in polyacrylamide gels (Eisses

and Kaplan, 2002; Klomp et al., 2002; Lee et al., 2002a). Human Ctr1 is substantially smaller than the yeast analogue (406 amino acids) and shares only 23% sequence homology with this protein (Dancis et al., 1994; Zhou and Gitschier, 1997). In contrast, the mouse and rat Ctr1-homologues share more than 90% sequence identity with hCtr1 (Zhou and Gitschier, 1997; Lee et al., 2000). Despite the poor sequence identity, mammalian Ctr1 and yeast Ctr1 have a similar overall architecture consisting of (1) an extracellular N-terminus, (2) an intracellular loop connecting the first and second transmembrane domain, (3) three membrane spanning α-helices and (4) an intracellular C-terminus.

The extracellular N-terminus of hCtr1 (Eisses and Kaplan, 2002; Puig et al., 2002; Klomp et al., 2003) contains two histidine-rich regions and two methionine motifs (Zhou and Gitschier, 1997; Eisses and Kaplan, 2002) and is *N*- and *O*-linked glycosylated at residues Asp^{15} and Thr^{27}, respectively (Eisses and Kaplan, 2002; Klomp et al., 2002; Maryon et al., 2007a). Mutation analysis revealed that deletion of the first methionine motif and/or of the His-rich regions has almost no effect on copper transport activity of hCtr1 (Puig et al., 2002; Eisses and Kaplan, 2005). However, these regions may be of importance under physiological conditions in accepting copper ions from copper binding proteins or copper complexes (Eisses and Kaplan, 2005; Maryon et al., 2007a). In contrast, mutation or deletion of methionine residues in the second methionine motif had a strong inhibitory effect on hCtr1-mediated copper uptake (Puig et al., 2002; Eisses and Kaplan, 2005; Larson et al., 2010). Glycosylation is not required for protein stability (Eisses and Kaplan, 2005) or plasma membrane delivery (Klomp et al., 2003) of hCtr1, but is essential for its copper transport activity and for endocytotic response to copper (Guo et al., 2004; Eisses and Kaplan, 2005). *N*-glycosylation has been suggested to stabilize or modify the structure of hCtr1 in a way that facilitates copper transport (Maryon et al., 2007a). *O*-glycosylation is required to protect the N-terminus of hCtr1 against

proteolytic cleavage (Maryon et al., 2007a; Maryon et al., 2009). In addition, the N-terminal domain has been shown to interact with itself and thus, may be involved in the constitution and/or stabilization of hCtr1-trimers (Klomp et al., 2003).

The function of the intracellular loop of Ctr proteins has not been reported so far. In hCtr1 a conserved dileucine motif (van den Berghe and Klomp, 2010), that is known to play a role in the internalization of membrane proteins in mammalian cells (Sandoval and Bakke, 1994), and a Yxx-(hydrophobic amino acid) internalization sequence (YNSM), suggests a possible function in the endocytosis of hCtr1 (van den Berghe and Klomp, 2010).

In contrast to most other transporters that are predicted to have 6-12 membrane-spanning domains, Ctr1 contains only three membrane spanning α-helices (Eisses and Kaplan, 2002; Klomp et al., 2003). Since three α-helices are insufficient to form a translocation pore, Ctr1 has to oligomerize to provide a pore that allows copper to pass through the plasma membrane (Eisses and Kaplan, 2005; De Feo et al., 2007). Indeed, biochemical, genetic and electron microscopy studies have demonstrated that Ctr1 forms homotrimers (Eisses and Kaplan, 2002; Lee et al., 2002a; Aller and Unger, 2006) and that oligomerization is essential for Ctr1 functionality (Aller et al., 2004). The primary sequences of the transmembrane regions are poorly conserved among Ctr proteins (De Feo et al., 2007). An exception is a MxxxM-X_{12}-GxxxG-motif, which links a MxxxM motif (MM4-motif) at the end of the second transmembrane domain (TM2) with an GxxxG-motif (GG4-motif) in the center of the third membrane spanning domain (TM3), and consequently was considered to represent a Ctr signature motif (De Feo et al., 2007). The strict conservation of the MM4- and GG4-motifs suggests that these two motifs and hence TM2 and TM3 contribute to the copper-permeable pore and/or to the mechanism of copper translocation (De Feo et al., 2007). Indeed, mutation

analysis have revealed the importance of the metal binding properties of the methionine residues in the MM4-motif for the copper transport activity of hCtr1 as well as of yeast Ctr1 (Puig et al., 2002; Eisses and Kaplan, 2005; De Feo et al., 2009) and the necessity of the GG4-motif for the oligorimerization of Ctr1 to functional homotrimers (Aller et al., 2004).

Truncation of the intracellular C-terminus of hCtr1 lowers the efficiency of hCtr1 mediated copper transport (Eisses and Kaplan, 2005). Since the carboxyterminus of yeast Ctr1 has been shown to be involved in protein-protein interactions with the copper chaperon Atox1 (Xiao and Wedd, 2002), it was speculated that truncation of the C-terminus impairs copper uptake at the copper release step (Eisses and Kaplan, 2005). Recently, a mechanistic model for Cu^{+} transport by hCtr1 has been proposed in which an important role is attributed to the HCH-sequence located at the C-terminus (De Feo et al., 2009). In addition, the C-terminus of yeast Ctr1 has been demonstrated to be required for the down-regulation of copper transport activity in the presence of excess copper (Wu et al., 2009).

Mammalian Ctr1 is ubiquitously expressed; however, expression levels are tissue specific, being highest in the liver, kidney and intestine (Zhou and Gitschier, 1997; Lee et al., 2002b; Kuo et al., 2006). In some tissues the expression level of Ctr1 depends on the copper status and is influenced by the physiological state, such as pregnancy and lactation (Kuo et al., 2006). Ctr1 plays an essential role in embryonic development as deletion of Ctr1 is embryonic lethal, most likely due to an insufficient supply of the developing embryo with copper (Kuo et al., 2001b; Lee et al., 2001). In brain and spleen of Ctr1-heterozygous knock-out mice copper levels are lowered to about 50% of that of wild type animals, but were normal in other organs, suggesting that alternative Ctr1-independent copper transport systems play an important role in the supply of these organs with copper (Lee et al., 2001). In addition, Ctr1-

homozygous knock-out cells, isolated from embryos obtained from the inter-cross of Ctr1-heterozygous mice, possess a residual copper transport activity (Lee et al., 2002b), further supporting the presence of alternative copper uptake pathways. Nevertheless, the importance of Ctr1 for a proper copper homeostasis is demonstrated by lower basal copper levels and lower activities of copper dependent enzymes in Ctr1-deficient cells compared to wild type cells (Lee et al., 2002b). Tissue-specific deletion of Ctr1 in mice intestine revealed an important role of this protein in dietary copper absorption (Nose et al., 2006). Intestinal epithelial cells generated from these mice hyperaccumulated copper, whereas all organs tested suffered from a severe copper deficit (Nose et al., 2006). Despite the up to 10 times higher copper levels in these cells compared to that of intestinal epithelial cells from control animals, activities of copper dependent enzymes were strongly reduced and levels of the copper chaperone for superoxide dismutase dramatically increased. These results and the absence of Ctr1 from the apical membrane (Zimnicka et al., 2007) indicate that Ctr1 is not responsible for transport of copper across the apical membrane, but is required to make copper absorbed from the lumen bioavailable (Nose et al., 2006).

In cultured cells, Ctr1 is typically observed at the plasma membrane and in cytoplasmic vesicles (Klomp et al., 2002; Gupta and Lutsenko, 2009), but the ratio of theses localizations depends on the cell type (Klomp et al., 2002). In polarized cells of the intestine, kidney and placenta Ctr1 was found both intracellularly and at the basolateral membrane (Hardman et al., 2006; Kuo et al., 2006; Nose et al., 2006; Zimnicka et al., 2007). The subcellular localization of hCtr1 in HEK293 and HeLa cells has been demonstrated to represent a steady-state localization which is the result of a dynamic process involving constitutive endocytosis and recycling of hCtr1 from and to the plasma membrane (Klomp et al., 2002; Petris et al., 2003). In some cell types the presence of copper in

concentrations close to the K_M-value for copper transport stimulates endocytosis of hCtr1 from the plasma membrane, thereby establishing a new steady-state (Petris et al., 2003; Guo et al., 2004; Molloy and Kaplan, 2009). Interestingly, Ag^+, which is isoelectric to Cu^+, is also capable to trigger endocytosis of hCtr1 with comparable efficiency as copper (Petris et al., 2003). Mutation analysis identified the second methionine motif in the amino-terminal region and the MM4-motif in TM2, which both are of great importance for the copper transport activity, to be also essential for the endocytotic response of hCtr1 to low micromolar copper concentrations (Guo et al., 2004). The fate of internalized hCtr1 remains uncertain, since two studies showed hCtr1 to be degraded (Petris et al., 2003; Guo et al., 2004), whereas another study found it to recycle back to the plasma membrane after removal of the copper stimulus (Molloy and Kaplan, 2009).

The stimulated endocytosis of hCtr1 in response to copper could represent a homeostatic control mechanism to prevent excessive copper uptake and potential copper toxicity (Petris et al., 2003). On the other hand, the observation that already copper concentrations close to the K_M-value for copper uptake by hCtr1 enhance endocytosis of hCtr1 has led to the proposal that copper uptake by Ctr1 is reminiscent to the uptake of transferrin-bound iron by the transferrin-receptor (Petris et al., 2003). In this model, binding of copper to Ctr1 is thought to trigger endocytosis of the copper-Ctr1 complex into an intracellular compartment from which copper is transported into the cytosol by hCtr1 (Petris et al., 2003).

A function of Ctr1 in the transport of copper from intracellular vesicles into the cytosol has also been assumed to explain the role of Ctr1 in dietary copper acquisition (Zimnicka et al., 2007). However, the apical copper entry into intestinal cells has clearly been demonstrated to be mediated by a copper transport system other than Ctr1 (Nose et al., 2006; Zimnicka et al., 2007). Also in other cell types alternative copper transport pathways are likely to contribute

to cellular copper uptake (Lee et al., 2002b). The identity of that system remains to be elucidated. One study provided evidence that the divalent metal transporter (DMT) 1 is involved in apical copper uptake by intestinal cells (Arredondo et al., 2003). DMT1 is constituted of 561 amino acids, contains 12 membrane spanning domains and is widely expressed in several tissues such as intestine, kidney and brain (Gunshin et al., 1997). It mediates ferrous iron transport across the plasma membrane and from intracellular compartments into the cytosol by a process that is coupled to the co-transport of H^+, hence, the transport of iron by DMT1 generates a positive inward current (Gunshin et al., 1997). In addition to iron, other transition metal ions including manganese, cadmium and copper provoked large inward currents in DMT1 expressing oocytes, suggesting that these metals might also be transported by DMT1 (Gunshin et al., 1997). Indeed, a decreased apical uptake of copper by CaCo2 cells was observed following partial knock-down of DMT1 and competition experiments revealed that copper inhibits apical iron uptake and vice versa (Arredondo et al., 2003). On the other hand it should also be pointed out that another study found no evidence for a role of DMT1 in apical copper transport by CaCo2 cells (Zimnicka et al., 2007).

Embryonic Ctr1-homozygous knock-out cells have been demonstrated to possess a residual copper transport activity of approximately 30% of that of wild type cells (Lee et al., 2002b). In contrast to Ctr1-mediated copper uptake, Ctr1-independent copper transport activity is of low affinity (K_M-value: 10 μM) and most likely represents the transport of divalent copper (Lee et al., 2002b). Even though the K_M-value of this transport pathway is close to that reported for hCtr2 (Bertinato et al., 2008) the lack of inhibition by Ag^+ makes Ctr2 unlikely to contribute to the copper uptake observed in Ctr1-deficient cells. Also, iron, manganese and cadmium, high-affinity substrates for DMT1 (Garrick et al., 2003), only slightly inhibited the residual copper uptake (Lee et al., 2002b). In

contrast, zinc strongly competed for Ctr1-independent copper transport, leading to the assumption that the alternative copper transport activity could be mediated by members of the ZIP (ZRT-/IRT-like protein) family of metal transporters (Lee et al., 2002b). In mammals, 14 ZIP transporters have been described (Kambe et al., 2004; Lichten and Cousins, 2009). These proteins have broad substrate specificity and were shown to transport zinc, manganese, cadmium and iron ions (Kambe et al., 2004; Eide, 2006). A contribution of ZIP members in copper transport has not been reported, but copper has been shown to compete for zinc and cadmium uptake by Zip1, Zip2 and Zip14 (Gaither and Eide, 2000, 2001; Girijashanker et al., 2008). However, no direct evidence for a contribution of Zip1 and Zip2 to Ctr1-independent copper uptake activity has been found (Lee et al., 2002b). Whether other members of this protein family may contribute to Ctr1-independent copper accumulation, remains to be elucidated.

Finally, Ctr1-independent copper uptake may not require a specific transporter, but may occur by endocytosis (Ferruzza et al., 2000; Zimnicka et al., 2007). In support of such a mechanism, apical copper uptake in CaCo2 cells has been shown to be not saturable (Ferruzza et al., 2000; Zimnicka et al., 2007). However, this lack of saturation might also simply result from the action of an unidentified transporter with a very high K_M-value for copper ions (Zimnicka et al., 2007).

1.2.2 Copper sequestration and storage

The accumulation of copper in the cytosol induces a risk for copper-mediated oxidative damage and binding of copper to essential biomolecules. However, under physiological conditions the concentration of free copper within the cell has been calculated to be around 10^{-18} M which amounts to less than one free copper ion per cell (Rae et al., 1999). Such low concentrations of free copper are

maintained by binding of copper to metallothioneins (MTs) and ligands of low molecular mass such as glutathione (GSH). MTs and GSH also represent the major molecules involved in the intracellular sequestering and storing of excess copper. In addition, mitochondria have been suggested to contribute to the cellular copper buffering capacity (Cobine et al., 2004; Maxfield et al., 2004; Leary et al., 2009b).

1.2.2.1 Glutathione

The tripeptide GSH is the most abundant low-molecular weight thiol in living cells, being present in millimolar concentrations (Hirrlinger and Dringen, 2010; Schmidt and Dringen, 2012). GSH is essential for the detoxification of reactive oxygen species, maintains the cellular thiol reduction potential in a strongly reduced state and is involved in redox regulation and signaling (Hirrlinger and Dringen, 2010; Schmidt and Dringen, 2012). In addition, GSH has been linked to the transport and the detoxification of metal ions including copper (Ballatori et al., 2009; Jomova et al., 2010).

GSH forms stable complexes with Cu^+ in cell-free systems even in the presence of oxygen (Ciriolo et al., 1990; Ascone et al., 1993; Kachur et al., 1998; Banci et al., 2010). Consistent with GSH functioning as an intracellular copper chelator, an elevation of the cellular GSH content has been shown to lower the bioavailable intracellular pool of copper (Chen et al., 2008a). Indeed, the majority of cytosolic copper is bound to GSH (Freedman et al., 1989) and copper in the form of a Cu(I)-GSH complex is believed to be a major contributor to the copper exchangeable pool in the cytosol (Miras et al., 2008; Banci et al., 2010). In accordance with this function, Cu(I)-GSH is capable of transferring copper to MTs (Ferreira et al., 1993) and SOD1 (Ciriolo et al., 1990; Ascone et al., 1993; Carroll et al., 2004) *in vitro*. *In vivo*, Cu(I)-GSH has been demonstrated to be required for the incorporation of copper into MTs and for the CCS-independent

activation of SOD1 (Freedman et al., 1989; Carroll et al., 2004; Jensen and Culotta, 2005; Leitch et al., 2009a; Huang et al., 2011). Cu(I)-GSH has further been suggested to represent the major source of copper for antioxidant protein 1 (Miras et al., 2008; Poger et al., 2008).

The susceptibility of cells to copper toxicity strongly correlates with their cellular GSH content (Freedman et al., 1989; Steinebach and Wolterbeek, 1994; White et al., 1999; Chen et al., 2008a; Du et al., 2008; Vidyashankar and Patki, 2010). Sequestering of copper by GSH protects cells from the toxic effects of free copper that arise from its ability to bind indiscriminately to essential proteins and/or to catalyze ROS generation (Hanna and Mason, 1992; Milne et al., 1993; Jimenez and Speisky, 2000; Burkitt, 2001). However, Cu(I)-GSH is redox-active and continuously reacts with molecular oxygen to produce superoxide (Speisky et al., 2008; Speisky et al., 2009; Aliaga et al., 2010; Aliaga et al., 2011). This feature accounts for the ability of Cu(I)-GSH to stimulate the release of Fe^{2+} from ferritin and to reduce Fe^{3+} (Aliaga et al., 2011), thus fostering iron-catalyzed production of hydroxyl radicals by the Fenton reaction in the presence of hydrogen peroxide. This pro-oxidative behavior of the Cu(I)–GSH complex has been postulated to underlie the oxidative stress and toxicity associated with the over-exposure of cells to copper (Aliaga et al., 2011)

GSH depletion has been found to lower the rates of copper uptake into liver cells and trophoblasts (Freedman et al., 1989; Tong and McArdle, 1995). Hence, the formation of the Cu(I)-GSH complex has been assumed to be involved in copper uptake (Freedman et al., 1989; Tong and McArdle, 1995). Alternatively, this lowered copper uptake might reflect the adaption of the cells to their intracellular bioavailable pool of copper. In accord with this view, down-regulation of hCtr1 was shown to account for lower rates of copper uptake in GSH depleted liver cells compared to control cells, whereas hCtr1 abundance and copper uptake rates were increased in cells with elevated GSH levels (Chen

et al., 2008a). Despite being slower in copper uptake, GSH depleted cells accumulate more copper than control cells, since copper export in these cells is impaired (Singleton et al., 2010).

In addition to its copper chelating and copper trafficking properties, GSH can participate in cellular copper homeostasis by regulating the activities of copper transport proteins via glutathionylation/deglutathionylation (Lim et al., 2006; Voronova et al., 2007a; Voronova et al., 2007b; Singleton et al., 2010). At least the activities of the copper transporting ATPases ATP7A and ATP7B have recently been demonstrated to be regulated by this post-translational mechanism (Singleton et al., 2010). Glutathionylation might also be involved in the regulation of the copper chaperone function of Cox17 as it has been shown to form mixed disulfides with GSH *in vitro* (Voronova et al., 2007a; Voronova et al., 2007b).

1.2.2.2 Metallothioneins

Metallothioneins (MTs) constitute a heterogeneous family of low-molecular weight, cysteine-rich proteins (Pulido et al., 1966; Buhler and Kagi, 1974; Kagi et al., 1974; Bremner and Davies, 1975; Palacios et al., 2011; Vasak and Meloni, 2011). Phylogenetically conserved members of this protein family are found in all eukaryotes, most fungi and certain prokaryotes (Capdevila and Atrian, 2011; Palacios et al., 2011). Several biological functions have been ascribed to MTs. In addition to a presumed role in zinc and copper homeostasis, MTs have been implicated in the detoxification of non-essential metals, protection against ROS, maintenance of the intracellular redox balance, regulation of cell proliferation and apoptosis, as well as in neuroprotection (Hidalgo et al., 2001; Cherian and Kang, 2006; Colvin et al., 2010; Maret, 2011; Thirumoorthy et al., 2011; Vasak and Meloni, 2011).

In mammals, four distinct MT isoforms exist that are denoted MT-1 to MT-4 (Kagi and Valee, 1960; Palmiter et al., 1992; Quaife et al., 1994; Thirumoorthy et al., 2011; Vasak and Meloni, 2011). The predominant isoforms MT-1 and MT-2 are ubiquitously expressed in almost all organs and tissues, being most abundant in liver and kidney (Thirumoorthy et al., 2011; Vasak and Meloni, 2011). The expression of MT-3 and MT-4 is confined mainly to the brain and stratified epithelium, respectively (Palmiter et al., 1992; Tsuji et al., 1992; Quaife et al., 1994; Garrett et al., 1999a; Garrett et al., 1999b; Hozumi et al., 2008; Thirumoorthy et al., 2011; Vasak and Meloni, 2011). MT-3 and MT-4 are constitutively expressed, while MT-1 and MT-2 are both basally expressed and inducible by various stressors, including heavy metals, oxidative stress and pro-inflammatory cytokines (Leone et al., 1985; Andrews, 2000; Kawai et al., 2000; Haq et al., 2003; Santon et al., 2008; Vasak and Meloni, 2011). Principally located in the cytosol, MTs have been observed to translocate into the nucleus under several physiological and pathological conditions (Nishimura et al., 1989; Kiningham et al., 1995; Nagano et al., 2000). In addition, despite the absence of a classical secretion sequence, MT-1, MT-2 and MT-3 have been demonstrated to be secreted by certain cell types (Moltedo et al., 2000; Trayhurn et al., 2000; Uchida et al., 2002; Chung et al., 2008; Miyazaki et al., 2011).

Mammalian MTs consist of a single polypeptide chain of 61-68 amino acids, 20 of which are strictly conserved cysteines that are arranged in a distinct pattern of CxC, CC and CxxC sequences (Kojima et al., 1976; Kissling and Kagi, 1977; Palmiter et al., 1992; Vasak and Meloni, 2011). They contain two metal binding domains, the N-terminal β-domain and the C-terminal α-domain, with different abilities for metal ion binding, the folding of each of these independent domains being induced by metal coordination to the apo-protein (Boulanger et al., 1982; Winge and Miklossy, 1982; Hunt et al., 1984; Salgado and Stillman, 2004; Duncan et al., 2006; Vasak and Meloni, 2011). Within cells MTs are present

in their apo-forms as well as in their metal bound forms (Yang et al., 2001; Petering et al., 2006). One MT can reversibly bind up to seven divalent metal ions in a three-metal-thiolate cluster (β-domain) and a four-metal-thiolate cluster (α-domain) or up to 12 monovalent ions in two six-metal-thiolate clusters (Boulanger et al., 1982; Hunt et al., 1984; Roschitzki and Vasak, 2002; Duncan et al., 2006). Although all mammalian MT isoforms can bind a variety of heavy metals, the physiological metals to bind to MTs are copper and zinc (Uchida et al., 1991; Quaife et al., 1994; Stillman, 1995; Bogumil et al., 1996; Orlowski and Piotrowski, 1998; Vasak and Meloni, 2011)

Multiple evidences suggest that MTs are involved in the maintenance of cellular copper homeostasis. MTs have been postulated to function as cytosolic copper storage in normal copper metabolism and to serve as metal reservoir in the event of copper deficit (Suzuki et al., 2002; Tapia et al., 2004; Ogra et al., 2006). Consistent with such functions, cells lacking MT-1 and MT-2 contain lower basal copper contents and are more sensitive to copper depletion than wild-type cells (Ogra et al., 2006; Miyayama et al., 2009). However, MT-1 and MT-2 appear not to play an obligatory role in normal copper homeostasis, since MT-1/2 knock-out mice do not show a copper-related phenotype (Michalska and Choo, 1993; Camakaris et al., 1999).

The expression of MTs is induced by an excess of copper (Wake and Mercer, 1985; Hidalgo et al., 1994; Kawai et al., 2000; Kuo et al., 2001a; Jiang et al., 2002). Elevated MT contents have been demonstrated in Wilson's disease patients and in some animal models (Klein et al., 1990; Mercer et al., 1992; Mulder et al., 1992; Sakurai et al., 1992; Suzuki-Kurasaki et al., 1997). Since MTs are capable of binding excess cellular copper an increase in the cellular MT content confers resistance against copper-induced toxicity (Freedman et al., 1986; Kawai et al., 2000; Jiang et al., 2002). Hence, the rise in MT levels reflects an adaption of cells to copper overload conditions. Despite the function of MTs in the cellular

detoxification of copper, cells lacking MT-1 and MT-2 are only slightly more sensitive to excess copper than wild type cells (Kelly and Palmiter, 1996; Park et al., 2001; Jiang et al., 2002; Tapia et al., 2004). This counterintuitive finding can be attributed to a lesser copper uptake by these cells (Tapia et al., 2004; Armendariz et al., 2006). Thus, in addition to their role in cytosolic copper storage and copper detoxification, MTs appear to be involved in the regulation of copper uptake by controlling the intracellular concentration of free copper.

1.2.3 Copper trafficking

The intracellular trafficking of copper is mediated by a group of proteins termed copper chaperones. These specialized proteins shuttle copper to specific cellular targets, thereby protecting it from being scavenged by MTs or GSH.

1.2.3.1 Antioxidant protein 1

Antioxidant protein 1 (Atx1) is a small copper-binding protein originally identified as a copper-dependent suppressor of oxygen toxicity in yeast strains lacking both SOD1 and SOD2 (Lin and Culotta, 1995). In yeast, Atx1 shuttles copper to the copper transporting P-type ATPase Ccc2 for subsequent transport into the secretory pathway and incorporation into copper-dependent enzymes (Klomp et al., 1997; Lin et al., 1997). For example Atx1-deficient yeast mutants are also deficient in iron uptake due to impaired copper incorporation into the multicopper oxidase Fet3, that is required to generate ferric iron as substrate for the high affinity iron importer Ftr1 (Lin et al., 1997; Robinson and Winge, 2010). Atx1 is phylogenetically conserved and homologous proteins have been found in many prokaryotic and eukaryotic species (Klomp et al., 1997; Boal and Rosenzweig, 2009).

The human homologue of Atx1, termed Hah1 or Atox1, is a 68 amino acid protein that shares 47% amino acid identity and 58% similarity with Atx1

(Klomp et al., 1997). Hah1 is abundant in all human tissues and distributed throughout the cytosol and nucleus of cells (Klomp et al., 1997; Hamza et al., 1999). Both expression level and intracellular localization of Hah1 do not depend on the intracellular copper content (Klomp et al., 1997; Hamza et al., 1999). Hah1 functionally complements mutant yeast strains deficient in Atx1 (Klomp et al., 1997; Hung et al., 1998) and has been demonstrated to bind and transfer Cu^{+} to the N-terminal metal-binding domains (MBDs) of the copper transporting P-type ATPases ATP7A and ATP7B (Hung et al., 1998; Hamza et al., 1999; Ralle et al., 2003; Tanchou et al., 2004; Wernimont et al., 2004; Banci et al., 2007a; Banci et al., 2008a; Banci et al., 2009b; Hussain et al., 2009; Rodriguez-Granillo et al., 2010; Benitez et al., 2011). In addition, Hah1 is involved in the regulation of the copper mediated intracellular trafficking and catalytic activity of both ATP7A and ATP7B (Tanchou et al., 2004; Banci et al., 2007a).

Hah1 adopts a βαββαβ ferredoxin fold regardless of the presence of copper ions (Hung et al., 1998; Wernimont et al., 2000; Anastassopoulou et al., 2004; Boal and Rosenzweig, 2009). The loop connecting the first β-strand with the first α-helix contains a copper-binding motif, MxCxxC, that is also present six times in the N-terminal MBDs of ATP7A and ATP7B (Klomp et al., 1997; Hung et al., 1998; Ralle et al., 2003; Tanchou et al., 2004). The cysteine residues within this motif are necessary and sufficient for copper binding by Hah1 (Klomp et al., 1997; Hung et al., 1998; Wernimont et al., 2000; Ralle et al., 2003; Anastassopoulou et al., 2004; Tanchou et al., 2004). Both monomeric (Ralle et al., 2003; Anastassopoulou et al., 2004; Wernimont et al., 2004) and dimeric (Wernimont et al., 2000; Tanchou et al., 2004) forms of copper-loaded Hah1 (Cu-Hah1) have been described. The copper induced homo-dimerization has been proposed to contribute to the sensing and/or regulation of the intracellular copper pool (Tanchou et al., 2004).

The transfer of copper from Cu-Hah1 to the N-terminal MBDs of ATP7A and ATP7B occurs most likely via specific protein-protein interactions (Hamza et al., 1999; Wernimont et al., 2000; Wernimont et al., 2004; Banci et al., 2007a; Banci et al., 2008a; Banci et al., 2009b). Accordingly, Hah1 has been demonstrated to form heterocomplexes with both copper transporting P-type ATPases (Hamza et al., 1999; Tanchou et al., 2004; Banci et al., 2007a; Banci et al., 2009b; Benitez et al., 2011). The interactions between Cu-Hah1 and its target proteins depend on the metal-binding cysteines in the MxCxxC motifs of the target domains, providing evidence for the presence of a bridging copper ion in the intermolecular adduct (Banci et al., 2007a; Banci et al., 2009a; Banci et al., 2009b; Benitez et al., 2011). In addition, electrostatic interactions between lysine residues on the surface of Hah1 with negatively charged residues on the surfaces of the target domains of ATP7A and ATP7B have been suggested to contribute to the stabilization of the heterocomplexes (Hamza et al., 1999; Wernimont et al., 2000; Ralle et al., 2003). Indeed lysine in position 60 of Hah1, has recently been demonstrated to facilitate adduct stability and copper transfer (Hussain et al., 2009). The copper transfer reaction from Cu-Hah1 to the MBDs of ATP7A and ATP7B has been proposed to proceed via intermediates in which the copper ion is coordinated by two cysteines of the MxCxxC motif of one protein and one cysteine of the MxCxxC motif of the other (Ralle et al., 2003; Wernimont et al., 2004; Banci et al., 2009b; Rodriguez-Granillo et al., 2010). Since the copper transfer reaction from Cu-Hah1 to the target domain is thermodynamically disadvantageous, it has been suggested to be coupled to subsequent Cu^+ translocation into the Golgi lumen and to ATP hydrolysis (Wernimont et al., 2004; Rodriguez-Granillo et al., 2010).

Mutations in ATP7B that lead to an impairment of protein-protein interaction with Hah1 have been identified in patients suffering from Wilson's disease (Hamza et al., 1999). This highlights the importance of specific protein-protein

interactions in the transfer of copper from Hah1 to the copper transporting P-type ATPases as well as the significance of Hah1 in cellular copper homeostasis.

1.2.3.2 Copper chaperone for superoxide dismutase

The copper chaperone for superoxide dismutase (CCS) is found widely distributed throughout eukaryotes (Casareno et al., 1998; Rae et al., 2001; Leitch et al., 2009b). CCS is involved in the maturation of SOD1 by inserting copper into SOD1 and facilitating the formation of the intramolecular disulfide between Cys^{57} and Cys^{146} that is critical for SOD1 function (Culotta et al., 1997; Rae et al., 2001; Furukawa et al., 2004; Leitch et al., 2009b). Although SOD1 from most species can be activated independently of CCS, maximal SOD1 activity in the majority of organisms relies on the presence of CCS (Rae et al., 2001; Leitch et al., 2009a; Leitch et al., 2009b). Primarily localized to the cytosol and nucleus of cells, CCS possesses a similar cellular distribution as its target protein (Culotta et al., 1997; Casareno et al., 1998). The expression level of CCS depends on the cellular copper content (Bertinato and L'Abbe, 2003; Prohaska et al., 2003; Caruano-Yzermans et al., 2006). Thus, copper deficiency has been demonstrated to result in an increase in CCS protein abundance due to a lowered rate of proteosomal degradation of CCS (Bertinato and L'Abbe, 2003; Caruano-Yzermans et al., 2006).

CCS consists of three structural domains (Casareno et al., 1998; Lamb et al., 1999; Leitch et al., 2009b). The N-terminal domain 1 is an Atx1-like region capable of binding Cu^{+} via the two cysteines of the MxCxxC motif (Lamb et al., 1999; Rae et al., 2001; Stasser et al., 2005; Stasser et al., 2007; Barry and Blackburn, 2008; Leitch et al., 2009b). The function of this domain in the CCS-mediated activation of SOD1 remains puzzling. For yeast CCS domain 1 has been shown to be functionally important only when cellular copper is limited, whereas it is essential for the activation of SOD1 by human CCS (hCCS;

Schmidt et al., 1999; Caruano-Yzermans et al., 2006; Stasser et al., 2007). A function of domain 1 in the acquisition of copper from an upstream source of copper (e.g. Ctr1) has been hypothesized (Rae et al., 2001; Leitch et al., 2009b; Wright et al., 2011). The central domain 2 of CCS strongly resembles its target protein, SOD1. Notably, the residues constituting the dimer interface of SOD1 are well conserved in CCS (Casareno et al., 1998; Lamb et al., 1999; Lamb et al., 2000; Lamb et al., 2001; Rae et al., 2001). Consequently, domain 2 is involved in the recognition and binding of SOD1 which is essential for the CCS-mediated activation of SOD1 (Lamb et al., 1999; Schmidt et al., 2000; Lamb et al., 2001; Caruano-Yzermans et al., 2006; Stasser et al., 2007; Barry and Blackburn, 2008; Leitch et al., 2009b; Robinson and Winge, 2010). The C-terminal domain 3 of CCS harbors a copper-binding CxC motif that is critical for its metal transfer and disulfide isomerase activity (Lamb et al., 2001; Rae et al., 2001; Stasser et al., 2005; Caruano-Yzermans et al., 2006; Stasser et al., 2007; Robinson and Winge, 2010). Further, the CxC motif has been shown to be required for the dimerization of CCS with SOD1 and the copper-dependent regulation of CCS expression (Schmidt et al., 2000; Caruano-Yzermans et al., 2006).

Regardless of its copper content, hCCS predominantly exists as a dimer (Casareno et al., 1998; Rae et al., 2001; Stasser et al., 2007; Leitch et al., 2009b; Wright et al., 2011). Apo-hCCS has been proposed to dimerize via the domain 2 SOD-like dimer interface (Stasser et al., 2007). Upon copper-loading, hCCS is believed to rearrange into a dimeric species in which the monomers are linked via a polynuclear copper cluster formed between the CxC motifs, thereby opening the domain 2 SOD-like dimer interface for association with SOD1 (Stasser et al., 2005; Stasser et al., 2007; Barry and Blackburn, 2008; Barry et al., 2008).

The activation of SOD1 by CCS is not a simple copper transfer reaction, but a oxygen-dependent redox process in which copper incorporation is

accompanied by the generation of the critical intramolecular disulfide between Cys^{57} and Cys^{146} (Brown et al., 2004; Furukawa et al., 2004; Stasser et al., 2007). Following association of the heterodimeric CCS-SOD1 complex, a disulfide bond forms between Cys^{57} of SOD1 and a cysteine of the CxC motif in domain 3 of CCS (Lamb et al., 2001; Stasser et al., 2007; Leitch et al., 2009b; Robinson and Winge, 2010). This intermolecular disulfide is believed to facilitate metal ion insertion by opening the SOD1 active site and to convert to the critical intramolecular disulfide in SOD1 (Lamb et al., 2001; Furukawa et al., 2004; Barry and Blackburn, 2008; Robinson and Winge, 2010).

1.2.3.3 Copper chaperones for cytochrome c oxidase

Biogenesis of cytochrome *c* oxidase requires the assembly of 13 subunits to a multimeric protein complex and the concomitant insertion of cofactors, including three copper ions, two heme *a* groups, one zinc ion and a magnesium ion (Hatefi, 1985; Diaz, 2010; Stiburek and Zeman, 2010). This complicated process is facilitated by several nuclear-encoded proteins, so-called assembly factors (Shoubridge, 2001; Hamza and Gitlin, 2002; Leary et al., 2004; Diaz, 2010; Stiburek and Zeman, 2010). Formation of the Cu_B and Cu_A site in the mitochondrial encoded subunits Cox1 and Cox2 takes place within the mitochondrial intermembrane space (IMS), and thus requires both the delivery of copper into this mitochondrial compartment as well as the insertion of copper into the two copper centers. Mitochondria have been shown to contain a significant matrix pool of non-proteinaceous copper which has been suggested to supply the IMS with copper (Cobine et al., 2004; Maxfield et al., 2004). However, the mechanism by which copper is shuttled into mitochondria is yet unknown (Kim et al., 2008; Leary et al., 2009b; Robinson and Winge, 2010). A number of proteins have been identified so far to be involved in the insertion of copper ions into mammalian cytochrome *c* oxidase (Heaton et al., 2000; Nobrega et al., 2002; Barros et al., 2004; Rigby et al., 2007; Banci et al., 2008c).

The initial event in the transfer of copper to Cox1 and Cox2 is the Cox17-mediated transfer of copper to the Sco-proteins Sco1 and Sco2 and Cox11 (Beers et al., 1997; Horng et al., 2004; Banting and Glerum, 2006; Banci et al., 2008b; Leary et al., 2009b; Robinson and Winge, 2010). This is followed by the subsequent insertion of copper into the nascent Cu_A and Cu_B sites (Beers et al., 1997; Banting and Glerum, 2006; Leary et al., 2009b; Robinson and Winge, 2010).

Cox17, initially identified in a yeast mutant displaying a respiratory defect, is essential for the metallation of eukaryotic cytochrome *c* oxidases (Glerum et al., 1996a; Heaton et al., 2000; Takahashi et al., 2002; Oswald et al., 2009). Cox17 is a small cysteine-rich, hydrophilic protein localized in the IMS and in the cytosol of cells (Glerum et al., 1996a; Beers et al., 1997; Maxfield et al., 2004; Oswald et al., 2009). Although this dual localization suggests a role of Cox17 in the shuttling of copper from the cytosol into the IMS, the primary function of Cox17 is the transfer of copper to Sco1, Sco2 and Cox11 within the IMS (Horng et al., 2004; Maxfield et al., 2004). Cox17 is composed of a coiled-coil-helix-coiled-coil-helix (CHCH) domain formed by a conserved twin Cx_9C motif and an unstructured N-terminal tail of about 15 amino acid residues (Banci et al., 2008c). A conserved KxCCxC motif, also present in MTs, is located between these two regions (Amaravadi et al., 1997; Banci et al., 2008c). The copper chaperone function of Cox17 depends essentially on the two adjacent cysteines in this motif (Heaton et al., 2000; Takahashi et al., 2002; Banci et al., 2008c), whereas the CHCH motif is important for the import of Cox17 into the IMS by the mitochondrial disulfide relay system (Mesecke et al., 2005; Banci et al., 2008c; Sideris et al., 2009; Banci et al., 2011). Mammalian Cox17 can exist in three different redox states (Palumaa et al., 2004; Voronova et al., 2007b). The fully reduced state ($Cox17_{SH}$) that contains no disulfide is thought to represent the prevalent oxidation state in the cytosol (Palumaa et al., 2004; Voronova et al., 2007b). $Cox17_{SH}$ binds four copper ions in a copper-thiolate cluster similar to

that observed in copper-substituted MTs, and a similar role of $Cox17^{SH}$ in storing and detoxification of potential toxic copper ions as known for MTs has been suggested (Palumaa et al., 2004; Voronova et al., 2007b). The partial reduced state ($Cox17^{2S\text{-}S}$) is considered to be the predominant redox species in the IMS (Voronova et al., 2007b; Banci et al., 2008c). The cysteines within the twin Cx_9C motif are present as two disulfides that are introduced during the import of $Cox17^{SH}$ into the IMS by the mitochondrial disulfide relay system (Mesecke et al., 2005; Banci et al., 2008c). $Cox17^{2S\text{-}S}$ binds one copper ion that is coordinated by the sulfurs of the two adjacent cysteines in the KxCCxC motif (Palumaa et al., 2004; Banci et al., 2008c). Metal-transfer experiments are consistent with $Cu^{1}Cox17^{2S\text{-}S}$ being the biologically active form, donating copper to its target proteins (Banci et al., 2008b). In the fully oxidized protein ($Cox17^{3S\text{-}S}$) also the two cysteines of the KxCCxC motif form a disulfide. Consequently, $Cox17^{3S\text{-}S}$ is not able to bind copper (Palumaa et al., 2004; Voronova et al., 2007b; Banci et al., 2008c). $Cox17^{3S\text{-}S}$ represents most likely only a transient intermediate in the course of copper transfer from $Cox17^{2S\text{-}S}$ to Sco1 (Voronova et al., 2007b; Banci et al., 2008b).

Sco proteins are required for the formation of the Cu_A site of cytochrome *c* oxidase (Mattatall et al., 2000; Leary et al., 2004; Horng et al., 2005; Stiburek et al., 2009). In humans, the two homologs hSco1 and hSco2 contribute to this process by transferring copper to the binuclear copper center and by acting as thiol-disulfide oxidoreductases (Leary et al., 2004; Horng et al., 2005; Leary et al., 2009a). Consistent with their critical role in the formation of the binuclear Cu_A center, mutations in Sco1 and Sco2 cause severe cytochrome *c* oxidase deficiencies (Horng et al., 2005; Leary et al., 2007; Stiburek et al., 2009; Diaz, 2010). In addition, hSco1 and hSco2 have been implicated in the maintenance of cellular copper homeostasis (Leary et al., 2007; Leary et al., 2009b; Stiburek et al., 2009; Stiburek and Zeman, 2010), especially in the regulation of copper

efflux (Leary et al., 2007). Sco proteins are tethered to the mitochondrial membrane by a single transmembrane domain and contain a large soluble domain, which protrudes into the IMS and adopts a thioredoxin-like fold (Glerum et al., 1996b; Rentzsch et al., 1999; Balatri et al., 2003; Abajian and Rosenzweig, 2006; Banci et al., 2006; Banci et al., 2007b). Sco-proteins can bind one copper ion by the cysteine residues of a CxxxC motif and a conserved histidine residue both located on the soluble domain (Glerum et al., 1996b; Rentzsch et al., 1999; Balatri et al., 2003; Horng et al., 2005; Banci et al., 2006; Banci et al., 2007b). Besides functioning in copper binding, the two cysteines of the CxxxC motif have been proposed to be the active site residues in the thiol-disulfide oxidoreductase function of Sco proteins (Banci et al., 2006; Leary et al., 2009a).

The Cu_B site of cytochrome *c* oxidase receives its copper ion from Cox11 (Hiser et al., 2000; Carr et al., 2002). Accordingly, cells lacking functional Cox11 have impaired cytochrome *c* oxidase activity (Hiser et al., 2000; Carr et al., 2002; Banting and Glerum, 2006; Thompson et al., 2010). Like the Sco proteins, Cox11 is tethered to the mitochondrial membrane and has a C-terminal domain protruding into the IMS (Carr et al., 2002; Carr et al., 2005). Cox11 forms a stable dimer that can bind two copper ions in a binuclear copper-thiolate cluster with each monomer contributing two cysteine residues from a CFCF motif strictly conserved among species (Carr et al., 2002; Banci et al., 2004; Horng et al., 2004; Banting and Glerum, 2006; Thompson et al., 2010). A third conserved cysteine, also absolutely required for function of Cox11, has been proposed to act as a transient copper ligand during copper transfer to Cox1 (Carr et al., 2002; Banting and Glerum, 2006; Thompson et al., 2010).

1.2.4 Copper export

Cellular copper export in mammals relies on the function of two proteins, ATP7A and ATP7B. These proteins belong to the protein family of P_{1B}-type ATPases, which have key functions in metal homeostasis in organisms of all kind of phyla (Vulpe et al., 1993; Arguello et al., 2007; Lutsenko et al., 2007a). P_{1B}-type ATPases are a subgroup of P-type ATPases that use the energy of ATP hydrolysis to transport heavy metals across cellular membranes (Arguello et al., 2007). Both ATP7A and ATP7B mediate Cu^{+} translocation with apparent K_M-values in the low micromolar range (Voskoboinik et al., 1998; Voskoboinik et al., 1999; Voskoboinik et al., 2001a; Voskoboinik et al., 2001b). In addition to their critical function in the efflux of excess cellular copper, ATP7A and ATP7B shuttle copper to the secretory pathway for incorporation into copper-dependent enzymes such as tyrosinase, PAM, DβM, LOX and Cp (Kosonen et al., 1997; Terada et al., 1998; Petris et al., 2000; Tchaparian et al., 2000; El Meskini et al., 2003; Steveson et al., 2003; Meng et al., 2004; Wang and Hebert, 2006; Hardman et al., 2007; Niciu et al., 2007; Ray et al., 2007; Setty et al., 2008). The importance of these proteins in the maintenance of copper homeostasis is dramatically illustrated by the human genetic disorders Menkes and Wilson's disease that are caused by mutations in ATP7A and ATP7B, respectively (Scheinberg and Sternlieb, 1996; Pfeiffer, 2007; Huster, 2010; Tumer and Moller, 2010; Kaler, 2011; Kodama et al., 2011).

Human ATP7A and ATP7B are large multispanning membrane proteins that share 50-60% amino acid sequence homology (Bull et al., 1993; Tanzi et al., 1993; Yamaguchi et al., 1996; Hung et al., 1997; Payne and Gitlin, 1998). Their overall structure consists of (1) a cytosolic amino-terminus, (2) eight transmembrane helices, (3) an ATP-binding domain (4) an actuator domain, and (5) a cytosolic carboxyl-terminus (Lutsenko et al., 2007a; Boal and Rosenzweig, 2009; Barry et al., 2010).

The N-terminal tail of human ATP7A and ATP7B harbors six MBDs (Bull et al., 1993; Chelly et al., 1993; Mercer et al., 1993; Tanzi et al., 1993; Vulpe et al., 1993; Petrukhin et al., 1994; Payne and Gitlin, 1998), each capable of binding one Cu^{+}-ion (Lutsenko et al., 1997; Jensen et al., 1999; DiDonato et al., 2000; Banci et al., 2007a; Banci et al., 2009b). The MBDs are similar in amino acid sequence and structure to Hah1, adopting a βαββαβ ferredoxin-like fold and containing a copper-binding MxCxxC sequence motif within the loop connecting the first β-strand with the first α-helix (Lutsenko et al., 2007a; Boal and Rosenzweig, 2009). Copper can be transferred by Cu-Hah1 to any of the six MBDs of ATP7A and ATP7B (Hung et al., 1998; Hamza et al., 1999; Ralle et al., 2003; Tanchou et al., 2004; Wernimont et al., 2004; Banci et al., 2007a; Banci et al., 2008a; Banci et al., 2009b; Hussain et al., 2009; Rodriguez-Granillo et al., 2010; Benitez et al., 2011). However, only the MBDs closest to the membrane, MBD5 and MBD6, are important for efficient copper transport (Forbes et al., 1999; Strausak et al., 1999; Mercer et al., 2003; Cater et al., 2004; Cater et al., 2007), while MBD1-4 primarily function in the regulation of the catalytic activity in response to copper (Lutsenko et al., 2007a; Barry et al., 2010).

The eight transmembrane helices of ATP7A and ATP7B are involved in the formation of the copper translocation pathway (Lutsenko et al., 2007a; Barry et al., 2010). Specific residues within TM6-TM8 are thought to contribute to the intramembrane copper coordination sites required for copper transmembrane transport (Arguello et al., 2007; Lutsenko et al., 2007a; Barry et al., 2010). Indeed, mutation of the cysteines in a CPC motif located in TM6, common for all P_{1B}-type ATPases (Arguello et al., 2007), and mutation of Met^{1393} in TM8 have been shown to result in an impaired catalytic activity of human ATP7B and murine ATP7A (Voskoboinik et al., 2001a; Cater et al., 2007).

The ATP-binding domain of both ATP7A and ATP7B, located between TM6 and TM7, comprises a nucleotide-binding domain (N-domain) and a

phosphorylation domain (P-domain) containing the DKTG, TGDN and GDGxND signature motifs of P-type ATPases (Bull et al., 1993; Tanzi et al., 1993; Vulpe et al., 1993; Petrukhin et al., 1994). The invariant Asp residue in the DKTG sequence motif of the P-domain is crucial for the catalytic cycle of P-type-ATPases (Voskoboinik et al., 2001b; Petris et al., 2002; Palmgren and Nissen, 2011). In the case of ATP7A and ATP7B, it accepts the γ-phosphate from the ATP upon binding of ATP to the N-domain and copper to the intramembrane copper sites (Voskoboinik et al., 2001b; de Bie et al., 2007; Lutsenko et al., 2007b). The formation of this phosphorylated intermediate induces conformational changes that allow the copper ion to be released on the other side of the membrane (Voskoboinik et al., 2001b; de Bie et al., 2007; Lutsenko et al., 2007b). The catalytic cycle is closed by the hydrolysis of the aspartyl phosphate bond and the return of the enzyme to its initial state (Voskoboinik et al., 2001a; de Bie et al., 2007; Lutsenko et al., 2007b). The dephosphorylation step is facilitated by the actuator domain (A-domain) linked to TM4 and TM5 (Voskoboinik et al., 2001b; de Bie et al., 2007; Lutsenko et al., 2007b). This domain harbors the TGE signature motif of the P-type ATPases that is strictly required for their phosphatase activity (Bull et al., 1993; Tanzi et al., 1993; Vulpe et al., 1993; Petrukhin et al., 1994; Palmgren and Nissen, 2011). Consequently, mutations of the TGE motif in ATP7A and ATP7B result in hyperphosphorylated and catalytic inactive proteins (Petris et al., 2002; Cater et al., 2007).

ATP7A continuously recycles between the TGN and the plasma membrane, whereas ATP7B traffics between the TGN and a cytosolic vesicular compartment (Petris et al., 1996; Petris and Mercer, 1999). When copper levels are normal both ATP7A and ATP7B have steady state localization at the TGN, where they transport copper from the cytosol to the TGN lumen for incorporation into copper-dependent enzymes (Petris et al., 1996; Yamaguchi et

al., 1996; Hung et al., 1997; La Fontaine et al., 1998; Petris and Mercer, 1999). A rise in cytosolic copper levels induces a shift in the steady state distribution from the TGN to the plasma membrane and/or to a distinct cytosolic vesicular compartment in close proximity to the plasma membrane (Petris et al., 1996; Hung et al., 1997; La Fontaine et al., 1998; Voskoboinik et al., 1998; Petris and Mercer, 1999; Voskoboinik et al., 1999; Forbes and Cox, 2000; Pase et al., 2004; Nyasae et al., 2007). Redistribution of ATP7A and ATP7B back to the TGN occurs when cellular copper levels return to normal (Petris et al., 1996; Pase et al., 2004; Nyasae et al., 2007). The ability of ATP7A and ATP7B to efflux copper is linked to their ability to undergo copper-induced redistribution (La Fontaine and Mercer, 2007). Consistently, mutations that impair the copper-dependent trafficking of these proteins have been associated with Menkes and Wilson's disease (Forbes and Cox, 2000; Kim et al., 2003).

ATP7A is expressed in most tissues, including intestine, skeletal muscle, placenta, brain heart and kidney, but its expression in liver is very low (Chelly et al., 1993; Vulpe et al., 1993; Paynter et al., 1994). In contrast, ATP7B is abundantly expressed in the liver and at lower levels in kidney, placenta, brain, lung and heart (Bull et al., 1993; Tanzi et al., 1993; Yamaguchi et al., 1996). This difference in expression patterns correlates well with the observed alterations in body copper homeostasis seen in Menkes and Wilson's disease. Inactivation of ATP7A in Menkes disease results in systemic copper deficiency due to diminished copper export from the intestine into the portal blood (Tumer and Moller, 2010; Kaler, 2011; Kodama et al., 2011), while failure of biliary copper excretion by ATP7B in Wilson's disease leads to copper overload in liver and other tissues (Scheinberg and Sternlieb, 1996; Pfeiffer, 2007; Huster, 2010).

1.3

Copper metabolism of the brain

The brain concentrates heavy metals including copper for metabolic use (Bush, 2000). Copper is of great importance for the normal development and function of the brain. Since not only copper deficiency, but also excess of copper can seriously affect brain functions, also the brain possesses mechanisms to regulate copper homeostasis. Impairments of these homeostatic mechanisms are associated with neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease or Huntington's disease.

1.3.1 Copper content and spatial distribution

The estimated copper content of the human brain ranges from 3.1 to 5.1 μg/g wet weight (Rahil-Khazen et al., 2002; Lech and Sadlik, 2007). Compared to human brains, mice appear to have a similar brain copper content (5.5 μg copper/g wet weight; Waggoner et al., 2000) whereas rat brains contain less copper (1 to 2.5 μg/g wet weight; Sugawara et al., 1992; Olusola et al., 2004). The cerebrospinal fluid contains copper in a concentration of 0.3 – 0.5 μM (Stuerenburg, 2000; Forte et al., 2004; Nischwitz et al., 2008; Strozyk et al., 2009). However, the extracellular copper concentration in brain tissue may be higher. At least for the synaptic cleft copper concentrations of up to 250 μM have been reported (Kardos et al., 1989; Hopt et al., 2003).

Several studies have demonstrated that copper is unevenly distributed in the brain (Warren et al., 1960; Smeyers-Verbeke et al., 1974; Bonilla et al., 1984; Faa et al., 2001; Becker et al., 2005; Dobrowolska et al., 2008; Serpa et al., 2008; Popescu et al., 2009; Wang et al., 2010). In general, copper contents are higher in the grey matter (1.6 to 6.5 μg/g wet weight) than in the white matter (0.9 to 2.5 μg/g wet weight; Warren et al., 1960; Smeyers-Verbeke et al., 1974; Bonilla et al., 1984; Becker et al., 2005; Dobrowolska et al., 2008). Quantification of copper in human brain sections by atomic absorption spectroscopy demonstrated that copper is enriched in the locus coeruleus and the substantia nigra, which both

are pigmented tissues and contain catecholaminergic cells (Warren et al., 1960; Goldberg and Allen, 1981; Popescu et al., 2009). Two-dimensional copper maps of human brain slices, obtained by laser ablation inductively coupled plasma mass spectroscopy (LA-ICP-MS), revealed that areas strongly enriched in copper are also present within the hippocampus (Dobrowolska et al., 2008). Compared to other grey matter regions the thalamus of human brain contains lower copper levels (Warren et al., 1960; Smeyers-Verbeke et al., 1974), whereas the copper content of the thalamus of rats is higher (Jackson et al., 2006), indicating that not only the copper content but also the copper distribution within the brain varies between species.

Histochemical and more recently LA-ICP-MS investigations showed that copper contents are higher in glial cells in brain than in neurons, under both pathological and normal conditions (Szerdahelyi and Kasa, 1986; Kodama et al., 1991; Becker and Salber, 2010). This finding indicates that copper is stored in glial cells, suggesting that glial cells play an important role in the copper metabolism of the brain. Furthermore, the presence of glial cells containing very high copper levels in regions near the ventricles suggests that glial cells also play a role in the uptake of copper from the cerebrospinal fluid (Szerdahelyi and Kasa, 1986). It is notable that in the locus coeruleus and the substantia nigra, the regions with the highest copper content in human brain, copper was not detectable in neurons but was exclusively observed in glial cells (Szerdahelyi and Kasa, 1986). However, another study found that most of the copper in the locus coeruleus was located on presynaptic membranes of a part of the afferent terminals contacting dendrites or somatic spines of neurons (Sato et al., 1994). The presence of substantial amounts of copper in nerve terminals was confirmed by subcellular fractionation of the rat cerebral cortex demonstrating that 23% of the total copper of the brain is contained in synaptosomes (Matsuba and Takahashi, 1970). The copper concentration in

synaptosomes was estimated to be about 15 μM, whereas the copper concentration in the synaptic vesicles was estimated to be about 291 μM, indicating an active transport of copper into the vesicles (Hopt et al., 2003).

Both brain copper content and distribution change during development, with age and in neurodegenerative diseases (Tarohda et al., 2004; Serpa et al., 2008; Wang et al., 2010). For example, the copper content in brains of Wilson's disease patients has been reported to be up to 125 μg/g dry weight, a strong increase compared to the reference value of 13 to 60 μg/g dry weight (Faa et al., 2001). In contrast, the brain copper contents of Menkes disease patients are strongly reduced (Nooijen et al., 1981; Willemse et al., 1982).

1.3.2 Importance of copper for the brain

Copper is utilized in the brain for general metabolic as well as for more brain specific functions (Lutsenko et al., 2010). In humans, the brain requirement for copper is best illustrated by Menkes disease. This fatal disease is characterized by a general copper deficit and the failure of copper delivery to several copper-dependent enzymes (Tumer and Moller, 2010; Kaler, 2011; Kodama et al., 2011). As a consequence of the reduced delivery of copper to the brain, Menkes disease patients exhibit severe mental and developmental impairment (Tumer and Moller, 2010; Kaler, 2011; Kodama et al., 2011).

1.3.2.1 Energy metabolism

The brain is one of the most energy-dependent tissues of the body (Rossi et al., 2004). Most of this energy requirement relates to active ion transport (Vergun et al., 2007). Since 95% of total ATP in the brain is estimated to be generated in mitochondria (Vergun et al., 2007), mitochondrial efficiency is essential for brain function, and consequently its impairment is associated with neurodegeneration (Rossi et al., 2004). Impaired mitochondrial function is also

thought to be a major factor in the devastating brain damage associated with Menkes disease (Kaler, 1998). Deficient cytochrome *c* oxidase activity has been observed in the brain of Menkes disease patients (Maehara et al., 1983) and animal models (Yoshimura et al., 1993) as well as in copper deficient rodents (Rusinko and Prohaska, 1985; Prohaska, 1991; Prohaska et al., 1995; Kunz et al., 1999; Gybina and Prohaska, 2006, 2008a, b). Elevated brain lactate levels and decreased levels of brain N-acetylaspartate, the synthesis of which is coupled to mitochondrial energy production in neurons (Moffett et al., 2007), are further indicators for a failure of oxidative phosphorylation under these conditions (Rusinko and Prohaska, 1985; Gybina and Prohaska, 2008a, 2009; Gybina et al., 2009). The alteration in ATP levels under copper deficiency in the brindled mouse model for Menkes disease appears to depend on age. A 50% decrease has been found in older mice (Rossi et al., 2001), whereas in younger mice, despite an impaired mitochondrial energy production, the levels of ATP, ADP and AMP did not differ to control animals (Rusinko and Prohaska, 1985). Also in copper deficient rats ATP, ADP and AMP levels were not altered compared to control animals, suggesting a compensatory mechanism (Gybina and Prohaska, 2008a). Indeed, AMP kinase has been shown to be activated in rat brain under copper deficiency conditions (Gybina and Prohaska, 2008b, a). However, despite this higher content of phosphorylated AMP kinase, lower concentrations of fructose-2,6-bisphosphate, most likely due to higher concentrations of citrate that may inhibit phosphofructokinase 2 and glycolytic inhibition due to the accumulation of lactate (Sotelo-Hitschfeld et al., 2012), were observed in cerebellum of copper-deficient rats (Gybina and Prohaska, 2008b).

1.3.2.2 Antioxidative defense

The brain is extremely prone to oxidative stress (Dringen, 2000; Rossi et al., 2004; Halliwell, 2006). Amongst others it has a high rate of oxidative

metabolism, shows low activities of enzymes involved in the antioxidant defense and is rich in polyunsaturated fatty acids (Dringen, 2000; Rossi et al., 2004; Halliwell, 2006). Copper deficit may render the brain even more susceptible to oxidative stress, since defective cytochrome *c* oxidase activity may result in increased superoxide production and/or impaired activity of SOD1 and SOD3 may weaken the antioxidant defense. Indeed, SOD1 immunoreactivity in brain of Menkes disease patients is strongly reduced, whereas the expression level of SOD2 was observed to be elevated (Shibata et al., 1995), a well-characterized response to oxidative stress (Uriu-Adams and Keen, 2005). However, while SOD1 activity under copper deficient conditions in rodents is strongly reduced in liver (Prohaska, 1991; Lai et al., 1994), it is maintained at almost normal levels in brain (Prohaska, 1991; Lai et al., 1994; Prohaska et al., 2003; Gybina and Prohaska, 2006). Moreover, although superoxide levels are enhanced in brain slices from SOD1-deficient mice compared to wild type mice (Sasaki et al., 2011), these mice show normal neurodevelopment (Reaume et al., 1996; Ho et al., 1998). Thus, the relative contribution of partial SOD deficiency to the neurodegeneration in Menkes disease is difficult to assign.

1.3.2.3 Iron metabolism

Iron is a necessary cofactor in many metabolic processes and the brain has a substantial requirement for this essential metal (Dringen et al., 2007; Crichton et al., 2011). However, just as iron deficiency can seriously affect brain function, an excess of iron can too (Dringen et al., 2007; Crichton et al., 2011). Cp has a critical role in iron-homeostasis (Hellman and Gitlin, 2002; Healy and Tipton, 2007; Kosman, 2010b) and thus represents a link between copper and iron metabolism. Aceruloplasminemia is an autosomal recessive disorder resulting from a loss of function mutation in the Cp gene (Harris et al., 1995; Yoshida et al., 1995; Takahashi et al., 1996) and is characterized by marked iron

accumulation in the brain and other tissues (Harris et al., 1995; Yoshida et al., 1995; Miyajima et al., 1996; Gonzalez-Cuyar et al., 2008). Amongst others, patients suffer from neurological symptoms such as dysarthria, dystonia and mild dementia (Harris et al., 1995; Yoshida et al., 1995; Miyajima et al., 1996; Takahashi et al., 1996; Gonzalez-Cuyar et al., 2008; McNeill et al., 2008). In Wilson's disease the transfer of copper to apo-Cp is impaired, at least in the liver (Terada et al., 1998; Meng et al., 2004), and the basal ganglia and the cortical grey matter of Wilson's disease patients contained more iron than the normal control (Cumings, 1948). Also in the brains of older Long-Evans Cinnamon (LEC) rats, an animal model for Wilson's disease, elevated iron contents have been reported (Sugawara et al., 1992; Kim et al., 2005). The lack of iron accumulation in the brains of younger LEC rats (Sugawara et al., 1992; Kim et al., 2005) is consistent with observations for Cp-deficient mice (Patel et al., 2002; Jeong and David, 2006). Copper deficient rodents also display a reduction in holo-Cp levels compared to control animals (Holtzman and Gaumnitz, 1970; Gitlin et al., 1992; Broderius et al., 2010). However, the consequences of copper deficiency on brain iron metabolism have not been studied so far.

1.3.2.4 Neurotransmitter and neuropeptide synthesis

Norepinephrine is the principal sympathetic neurotransmitter and an important modulator of mood, attention, arousal and cardiovascular function (Kim et al., 2002; Goldstein et al., 2003; Berridge, 2008; Goddard et al., 2010). The final step in norepinephrine synthesis, the oxidative hydroxylation of dopamine, is catalyzed by DβM located in granulated vesicles of sympathetic nerve terminals, adrenal medulla chromaffin cells, and noradrenergic and adrenergic neurons of the brain (Geffen et al., 1969; Stewart and Klinman, 1988; Klinman, 1996; Kim et al., 2002; Timmers et al., 2004; Klinman, 2006). An elevated dopamine to norepinephrine ratio has been observed in the plasma of Menkes disease patients (Prohaska and Smith, 1982; Kaler et al., 2008; Goldstein et al.,

2009) as well as in the brains of animal models for Menkes disease and copper deficient-rodents (Prohaska and Smith, 1982; Nelson and Prohaska, 2009), indicating a lack of DβM under severe copper deficiency conditions. Paradoxically, DβM activity measurements revealed an increased activity in the brain of copper deficient rodents (Prohaska and Smith, 1982; Prohaska et al., 1995; Nelson and Prohaska, 2009). However, the depletion of norepinephrine is known to induce apo-DβM synthesis, which is likely to become activated *in vitro* by trace amounts of copper during assaying the DβM activity (Prohaska and Smith, 1982; Nelson and Prohaska, 2009). Despite the fundamental role of norepinephrine in central nervous system function, the fact that patients with congenital DβM deficiency exhibit only subtle signs of central nervous system dysfunction (Robertson et al., 1991; Kim et al., 2002) makes a contribution of DβM deficiency to the neurodegeneration in Menkes disease difficult to assign.

Amidated neuropeptides are expressed in almost every neuron and are involved in a variety of function in the brain, including neuronal proliferation, energy metabolism and neuromodulation (Magistretti et al., 1998; Hansel et al., 2001; Bousquet-Moore et al., 2010b). The only enzyme known to catalyze the α-amidation of peptide precursors is PAM (Klinman, 1996; Prigge et al., 1997; Prigge et al., 2000; Bousquet-Moore et al., 2010a). Decreased PAM activity and low levels of several α-amidated peptides have been observed in the brains of animal models for Menkes disease and copper deficient rodents (Prohaska et al., 1995; Hansel et al., 2001; Steveson et al., 2003; Prohaska et al., 2005; Bousquet-Moore et al., 2010b). The lack of amidated peptides due to decreased PAM activity is though to contribute to neurodevelopmental delay and increased seizure frequency associated with Menkes disease (Steveson et al., 2003; Bousquet-Moore et al., 2010b; Kaler, 2011).

1.3.2.5 Neuromodulatory function

Synaptosomes and primary hippocampal neurons have been shown to release copper following depolarization, resulting in copper concentrations in the synaptic cleft that have been reported to range from 15 μM to 250 μM (Hartter and Barnea, 1988; Kardos et al., 1989; Hopt et al., 2003; Schlief et al., 2005). This suggests a potential neuromodulatory function of copper as has been demonstrated for zinc (Frederickson and Bush, 2001). Indeed, exogenous application of already low micromolar concentrations of copper produce an antagonistic effect on N-methyl-D-aspartate (NMDA)-, kainite-, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)- and γ-aminobutyric acid (GABA)-receptors as well as on the P2Y1, P2X4 and P2X7 purinoreceptors (Kardos et al., 1989; Li et al., 1996; Trombley and Shepherd, 1996; Vlachova et al., 1996; Weiser and Wienrich, 1996; Doreulee et al., 1997; Xiong et al., 1999; Sharonova et al., 2000; Horning and Trombley, 2001; Zhu et al., 2002; Coddou et al., 2003; Huidobro-Toro et al., 2008; Peters et al., 2011). Copper has further been shown to block glycine-mediated currents, when glycine receptors are activated by low, non-desensitizing concentrations of glycine (Trombley and Shepherd, 1996; Wang et al., 2002) and to potentate P2X2 receptor activity (Xiong et al., 1999; Huidobro-Toro et al., 2008). In contrast to its acute inhibitory effect on AMPA- and GABA-mediated currents, treatment of rat hippocampal neurons with copper for 3 h enhances synaptic activity (Peters et al., 2011). In addition to its effect on ligand-gated receptors, copper has been shown to inhibit voltage-gated Ca^{2+}- and K^{+}-channels (Horning and Trombley, 2001; Niu et al., 2005; Niu et al., 2006).

AMPA/kainite as well as NMDA receptors in cultured rat cortical neurons are noncompetitively blocked by copper (Vlachova et al., 1996; Weiser and Wienrich, 1996), whereas $GABA_A$ receptors and glycine receptors are blocked in a competitive manner (Sharonova et al., 2000; Wang et al., 2002; Zhu et al.,

2002). The inhibitory effect of copper on GABA-, NMDA- and glycine receptor-mediated currents is voltage-independent, in contrast to the effect of Mg^{2+}, suggesting that copper acts at a specific neuromodulatory site rather than as channel blocker (Trombley and Shepherd, 1996; Vlachova et al., 1996; Weiser and Wienrich, 1996; Wang et al., 2002). Experimental evidence suggests that copper catalyzes an S-nitrosylation of the NMDA receptor, which is a known mechanism of regulation of NMDA receptors (Schlief et al., 2006). An oxidation process has also been proposed to be involved in the inhibition of AMPA/kainate receptors by copper (Weiser and Wienrich, 1996). The increase in AMPAergic neurotransmission in response to prolonged exposure to copper has been ascribed to a copper-induced enrichment of the AMPA receptor at the plasma membrane (Peters et al., 2011).

The exact role of copper in synaptic physiology remains to be elucidated. However, synaptically released endogenous as well as exogenously applied copper protect primary hippocampal neurons against NMDA-mediated excitotoxic cell death, most likely by lowering the NMDA receptor-mediated intracellular Ca^{2+}-elevation following depolarization/activation (Schlief et al., 2006). Copper also appears to be involved in the modulation of synaptic plasticity. NMDA receptors participate in both the induction and the maintenance of long-term potentiation (LPT; Voglis and Tavernarakis, 2006). Consistent with its inhibitory function on NMDA receptor-mediated Ca^{2+}-currents hippocampus slices that had been exposed to exogenous copper (Doreulee et al., 1997; Salazar-Weber and Smith, 2011) as well as hippocampal slices of rats that had been fed a high-copper diet did not show any LTP (Goldschmith et al., 2005; Leiva et al., 2009). LTP has been associated with learning and memory in many organisms (Voglis and Tavernarakis, 2006). However, despite blocking LTP in the hippocampus, copper did not alter learning and memory in rats (Leiva et al., 2009).

1.3.3 Transport and storage

Currently little is known about the mechanism of copper transport and homeostasis in the brain. To enter the brain, copper has to be transported across the blood-brain barrier (BBB) and/or the blood-cerebrospinal fluid barrier (BCB), which separates the blood from the brain interstitial space and CSF, respectively (Zheng and Monnot, 2012). At both brain barrier systems copper is transported into the brain primarily as free copper (Choi and Zheng, 2009). Although the copper uptake into cerebral capillaries is much slower than into the choroid plexus, the copper acquired by cerebral capillaries appears to be more readily transported into the brain parenchyma than copper from the choroid plexus to the CSF (Choi and Zheng, 2009; Monnot et al., 2011). In fact, recent evidence indicates that the role of the BCB in brain copper homeostasis is rather to efflux copper from the CSF to the blood (Monnot et al., 2011). Based on theses findings, the following model has been postulated for the copper transport between the blood and the brain (Monnot et al., 2011; Zheng and Monnot, 2012). The BBB represents the major route for the transport of copper from the blood circulation into the brain parenchyma, where copper is utilized and subsequently released into the CSF via the brain interstitial fluid. The copper in the CSF can be taken up by choroid epithelial cells where it may be stored or effluxed to the blood. Thus, while the BBB determines the influx of copper into the brain, the BCB functions in the maintenance of the copper homeostasis in the brain extracellular fluids. However, the situation might be different in the developing brain for which the BCB has been suggested to be the primary route of copper entry (Donsante et al., 2010).

The knowledge of the copper handling machinery in different cell types of the central nervous system is rudimentary. Nevertheless, it is clear that all the key copper handling proteins mediating copper homeostasis in peripheral tissues are also present in the brain (Lutsenko et al., 2010; Tiffany-Castiglioni et al.,

2011). As for peripheral cells, Ctr1 is likely to be the major pathway for copper entry into brain cells. Ctr1 appears to be essential for delivery of copper into the brain, since the copper levels in brains of Ctr1-heterozygous knock-out mice are reduced to about 50% of that of wild type animals (Lee et al., 2001). In mammalian brain Ctr1 is ubiquitously distributed with its expression being most abundant in the choroid plexus and high in the endothelial cells of the capillaries (Nishihara et al., 1998; Gybina and Prohaska, 2006; Kuo et al., 2006; Zheng and Monnot, 2012). Ctr1 is enriched on the apical membrane of the choroid plexus where it may extract copper from the CSF, consistent with the proposed function of the choroid plexus in the maintenance of the copper homeostasis in the brain extracellular fluids (Kuo et al., 2006; Zheng and Monnot, 2012). Uptake of copper by the BCB could also be mediated by DMT1, which is also located towards the apical membrane of the choroid plexus (Wang et al., 2008). In brain capillary endothelial cells, Ctr1 has been proposed to be located at the luminal side where it may regulate the uptake of copper from the blood (Zheng and Monnot, 2012). Since DMT1 seems not to be expressed in brain capillary endothelial cells (Moos et al., 2006), Ctr1 is likely to be the predominant transporter involved in copper uptake at the BBB. In neural parenchymal tissue, expression of Ctr1 has only been studied in rat dorsal root ganglion tissue (Liu et al., 2009; Ip et al., 2010). In this tissue intense Ctr1-immunoreactivity has been observed in large-sized neurons, which are thought to have a strong need for Ctr1 to meet their high demands for copper delivery to cytochrome *c* oxidase (Liu et al., 2009; Ip et al., 2010).

Copper entering brain cells is likely to be sequestered by GSH and either stored as MT-copper complex or shuttled by copper chaperons to their specific cellular target sites. MTs exist in three isoforms in the brain: MT1, MT2 and MT3 and has been shown to be expressed in the BBB and BCB as well as in astrocytes and neurons, whereas microglial cells and oligodendrocytes are essentially devoid

of MTs (Uchida et al., 1991; Hidalgo et al., 1994; Anezaki et al., 1995; Hidalgo et al., 2001). Elevated brain copper levels have been observed to be accompanied by an increase in MT levels, most likely reflecting a compensatory response against copper-induced toxicity (Ono et al., 1997; Haywood et al., 2008; Zatta et al., 2008). Detailed studies of copper chaperones in the brain are scare. However, the copper chaperons Atox1, CCS and the copper chaperons for cytochrome *c* oxidase Cox17, Sco1 and Sco2 have been shown to be expressed in brain (Klomp et al., 1997; Nishihara et al., 1998; Naeve et al., 1999; Papadopoulou et al., 1999; Rothstein et al., 1999; Hamza et al., 2000; Punter et al., 2000; Takahashi et al., 2002; Gybina and Prohaska, 2006). Atox1 is expressed ubiquitously in the brain with the expression level being highest in the choroid plexus (Nishihara et al., 1998). CCS is expressed at a similar level in various human brain regions, with the exception of white matter from where it is largely absent (Rothstein et al., 1999). On a cellular level CCS was found to be more abundant in neurons than in astrocytes (Rothstein et al., 1999).

ATP7A is crucial for the supply of the brain with copper as demonstrated by the low brain copper levels observed in Menkes disease patients (Nooijen et al., 1981; Willemse et al., 1982) and animal models (Hunt, 1974; Kumode et al., 1993). ATP7A was shown to be expressed ubiquitously in brain with expression levels being most abundant in the choroid plexus (Chelly et al., 1993; Vulpe et al., 1993; Paynter et al., 1994; Nishihara et al., 1998; Niciu et al., 2006; Niciu et al., 2007). A dysfunction of ATP7A has been shown to result in copper accumulation in brain capillaries of macular and brindled mutant mice (Kodama, 1993; Yoshimura, 1994) indicating that ATP7A plays a role in copper transport across the BBB. The importance of ATP7A in copper export from brain capillary endothelial cells has also been demonstrated in a cell culture model for these cells (Qian et al., 1998b). In brain parenchyma, ATP7A has been shown to be expressed in both neurons and non-neuronal cells (Barnes et al.,

2005; El Meskini et al., 2005; Schlief et al., 2005; Niciu et al., 2006; Niciu et al., 2007). In cerebellum of adult mice, ATP7A is expressed in Bergmann glial cells but not in Purkinje cells (Barnes et al., 2005). In Bergmann cells ATP7A has been proposed to function in the regulation of the cytosolic copper concentration as well as in the export of copper for delivery to Purkinje neurons (Barnes et al., 2005). ATP7A has further been shown to be strongly expressed in small-sized neurons of the rat dorsal root ganglion that are the primarily site of substance P synthesis in this tissue (Ip et al., 2010). Since the biosynthesis of substance P requires PAM the strong expression of ATP7A in these neurons is thought to meet their high demands for copper delivery to PAM (Ip et al., 2010). In addition to its well known role in the export and delivery of copper to cuproenzymes in peripheral tissues, ATP7A is also required in the brain to release copper from hippocampal neurons upon NMDA-activation (Schlief et al., 2005). NMDA-receptor mediated Ca^{2+}-entry induces the identical trafficking of ATP7A to a cytoplasmic compartment adjacent to the plasma membrane as observed with copper (Schlief et al., 2005; Schlief et al., 2006) where ATP7A is thought to accumulate copper into a membrane bound compartment, forming and/or replenishing a pool of potentially releasable copper (Schlief et al., 2005; Schlief and Gitlin, 2006). Moreover, an ATP7A-mediated copper efflux has been suggested to play a role in neuronal process outgrowth and/or synaptogenesis of maturing olfactory receptor neurons (El Meskini et al., 2005). However, a secretion of copper into the extracellular space has not been observed during the formation of neuritic processes (Finney et al., 2007; Finney et al., 2009).

Compared to ATP7A, the function of ATP7B in the brain is less clear. ATP7B is ubiquitously expressed in all brain regions (Bull et al., 1993; Tanzi et al., 1993; Yamaguchi et al., 1996; Niciu et al., 2007) and has been observed in brain capillary endothelial cells, in choroidal epithelial cells, in ependymal cells as well as in Purkinje neurons of adult mice (Qian et al., 1998b; Barnes et al., 2005;

Niciu et al., 2006; Zheng and Monnot, 2012). The expression of ATP7B in Purkinje neurons is consistent with the expression of Cp being localized to these cells (Barnes et al., 2005). The pineal gland and adult retina express an alternative-spliced form of ATP7B, the pineal gland night-specific ATPase (PINA), which lacks the N-terminal MBDs and the first four membrane segments (Borjigin et al., 1999). Although representing only the C-terminal half of ATP7B, PINA has been shown to possess some copper transport activity (Borjigin et al., 1999). The pineal gland is a functional compartment of the circadian timing system and the expression of PINA is 100-fold higher at night than at daytime (Borjigin et al., 1999), suggesting a function of rhythmic copper metabolism in circadian rhythm. Interestingly, mice subjected to total darkness for up to 60 days showed a marked elevation in their brain copper content (Beltramini et al., 2004).

1.3.4 Neurodegenerative diseases

Impairment of copper homeostasis can lead to neurodegeneration, as exemplified by Menkes and Wilson's disease (Lorincz, 2010; Kodama et al., 2011). Alterations of copper homeostasis have also been associated with neurodegenerative diseases such as prion diseases, Alzheimer's disease, Parkinson's disease or Huntington's disease (Gaggelli et al., 2006; Rivera-Mancia et al., 2010). However, in contrast to Menkes and Wilson's disease the role of copper in these diseases is not fully understood.

1.3.4.1 Prion diseases

Refolding of the prion protein (PrP) into an abnormal conformation has been associated with transmissible neurodegenerative diseases, such as Creutzfeld-Jacob disease, Kuru and fatal familial insomnia in humans, bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep, which are summarized as prion diseases or transmissible spongiform encephalopathies (Aguzzi et al.,

2008; Cobb and Surewicz, 2009; Moore et al., 2009; Holman et al., 2010). Prion diseases are rare, but are generally fatal for anyone who gets infected (Moore et al., 2009; Holman et al., 2010). The most common prion disease in humans, with an annual incidence rate of about 1 case per 1.000.000, is the Creutzfeld-Jacob disease. It is characterized by a rapidly progressive dementia and neurological features including ataxia, tremor, bradykinesia and rigidity, with death occurring within 1 to 2 year of disease onset (Aguzzi et al., 2008; Cobb and Surewicz, 2009; Brown and Mastrianni, 2010). The PrP is ubiquitously expressed but most abundantly in neurons (Hu et al., 2007; Westergard et al., 2007; Linden et al., 2008). Being subject to constitutive endocytosis it continuously recycles between the plasma membrane and endosomes (Brown and Sassoon, 2002; Westergard et al., 2007; Linden et al., 2008). The physiological roles of the PrP are still under discussion. Among others it has been implicated in the protection against apoptosis and oxidative stress, in transmembrane signaling as well as in the formation and function of synapses (Brown and Sassoon, 2002; Rachidi et al., 2003; Hu et al., 2007; Westergard et al., 2007; Linden et al., 2008).

Cells expressing PrP are much more resistant to copper-treatment than PrP-deficient cells (Brown et al., 1998; Rachidi et al., 2003; Haigh and Brown, 2006). PrP is known to bind copper with low micromolar affinity (Brown et al., 1997; Westergard et al., 2007) and may protect against copper-induced toxicity by capturing extracellular copper and reducing copper-mediated ROS production (Brown et al., 1998; Rachidi et al., 2003; Haigh and Brown, 2006; Millhauser, 2007). Moreover, copper has been shown to stimulate endocytosis of PrP (Pauly and Harris, 1998; Brown and Harris, 2003). Based on this observation the PrP has been suggested to serve as a receptor for cellular uptake or efflux of copper (Pauly and Harris, 1998; Brown and Harris, 2003). Indeed some studies reported that the rate of cellular copper uptake depended on the expression level of PrP

(Brown, 2004; Urso et al., 2012). Further supporting a role of PrP in copper homeostasis, the copper contents of brain fractions of mice were shown to increase with increasing abundance of PrP (Brown et al., 1997). Also the copper contents of synaptosomes of PrP-deficient mice were found to be lower compared to that of wild type mice, which has led to the proposal that the PrP may play a role in regulating copper release at the synapse (Brown et al., 1997). Furthermore, the prion protein has been shown to be capable of reducing Cu^{2+} to Cu^{+} with maximal activity at pH 6.5 (Miura et al., 2005). Thus, it has been concluded that the PrP does not only transports extracellular copper into endosomes, but also reduces Cu^{2+} prior to the release into the cytosol (Miura et al., 2005). However, the potential role of PrP in cellular copper homeostasis is still under controversial debate (Westergard et al., 2007), since other studies did not found any alteration in the copper content of brain fractions of mice expressing different levels of prion protein (Waggoner et al., 2000; Giese et al., 2005) or failed to show a contribution of PrP to cellular copper uptake (Rachidi et al., 2003).

An alteration of copper homeostasis may also play a role in the development and progression of prion diseases (Leach et al., 2006; Millhauser, 2007; Rana et al., 2009). Brain copper contents of scrapie infected mice were found to be decreased by around 60%, pointing to a severe copper deficit in prion diseases (Wong et al., 2001; Thackray et al., 2002; Mitteregger et al., 2009). Consistently, copper supplementation of these mice reduced the progression of scrapie (Mitteregger et al., 2009). In contrast to copper, the manganese contents were shown to be elevated in prion-infected brain tissue (Wong et al., 2001; Thackray et al., 2002; Mitteregger et al., 2009) and manganese supplementation accelerated disease progression in scrapie infected mice (Mitteregger et al., 2009). Since PrP with manganese bound is able to induce nucleated-polymerization of PrP (Brazier et al., 2008), copper deficiency was hypothesized

to foster the development of prion diseases by favoring the incorporation of manganese into PrP instead of copper (Brown et al., 2000). However, whether the observed perturbation of copper and manganese in the brain are a cause or a consequence of prion diseases remains to be evaluated (Rana et al., 2009).

1.3.4.2 Alzheimer's disease

Alzheimer's disease (AD) is an irreversible and progressive disease that causes memory loss and psychiatric disturbances (Castellani et al., 2010; Salawu et al., 2011). It represents the most common neurodegenerative disease in humans, with currently an estimate of 18-25 million people worldwide suffering from this disease (Castellani et al., 2010; Ballard et al., 2011; Kenche and Barnham, 2011). The major risk factor in AD is advanced age and as a direct consequence of increased life span expectancies the number of people suffering from AD is projected to increase dramatically within the next decades (Castellani et al., 2010; Ballard et al., 2011; Kenche and Barnham, 2011; Salawu et al., 2011). Aside from age, other risk factors include genetic factors, gender and environmental factors (Castellani et al., 2010; Ballard et al., 2011). The pathological hallmarks of AD are the extracellular senile plaques and the intracellular neurofibrillary tangels in brain (Castellani et al., 2010; Ballard et al., 2011). The principal constituents of senile plaques are amyloid-β (Aβ) peptides of 40 and 42 residues, which are generated form the integral membrane amyloid precursor protein by the consecutive action of β- and γ-secretase (Borchardt et al., 1999; Castellani et al., 2010; Ballard et al., 2011; Budimir, 2011; Tougu et al., 2011).

Aβ peptides have been shown to bind copper with high affinity (Atwood et al., 2000) and senile plaques are strongly enriched in copper (Lovell et al., 1998). Furthermore, copper has been shown to precipitate Aβ peptides *in vitro* and it has been suggested that copper triggers the formation of senile plaques (Atwood et al., 2000; Kenche and Barnham, 2011; Tougu et al., 2011; Roberts et

al., 2012). In support of this view, Aβ deposition begins within the glutamatergic synapse (Cater et al., 2008), where both copper (Kardos et al., 1989; Hopt et al., 2003) and Aβ (Lesne et al., 2005; Tougu et al., 2011) are released during synaptic transmission. However, although accumulation of Aβ peptides in the form of senile plaques are the most prominent feature of AD, it is now widely accepted that soluble oligomeric Aβ species are the most toxic form of Aβ peptides (Karran et al., 2011; Roberts et al., 2012). Since Aβ can mediate the reduction of Cu^{2+} to Cu^{+}, copper may promote the toxicity of such Aβ oligomers through the formation of ROS (Robertson et al., 1991; Huang et al., 1999; Multhaup et al., 2002; Kenche and Barnham, 2011). In addition, copper has been reported to increase the inhibition of cytochrome *c* oxidase by Aβ (Crouch et al., 2005).

While the enhancement of Aβ-toxicity by copper *in vitro* suggest a detrimental role of copper in AD, the observed lower copper contents in the brain of AD patients (Deibel et al., 1996; Loeffler et al., 1996) and mouse models for AD (Maynard et al., 2002; Bayer et al., 2003) as compared to controls rather argue for a copper deficit contributing to the neurodegeneration in AD. In this line, copper supplementation of a mouse AD model improved the survival of these animals (Bayer et al., 2003). Improved cognitive functions were also observed in another mouse model of AD following administration of Cu(gtsm) as copper source (Crouch et al., 2009). However, intake of copper had no effect on cognition in patients with mild AD in a phase 2 clinical trial, even though a positive effect on a relevant AD biomarker was observed (Kessler et al., 2008a; Kessler et al., 2008b). Mechanistically, copper deficiency may exacerbate disease progression by influencing amyloid precursor protein-processing and Aβ-metabolism (Cater et al., 2008). Indeed, copper treatment has been shown to inhibit the amyloidogenic pathway in chinese hamster ovary cells, thereby reducing Aβ synthesis (Borchardt et al., 1999) and treatment of amyloid

precursor protein overexpressing cell lines with a clioquinol-copper complex has been associated with both an increase in copper levels and an up-regulation of Aβ degrading metalloproteinases (White et al., 2006). In addition, copper deficiency may also influence the activity of copper-dependent enzymes. In this regard low activities of cytochrome *c* oxidase (Mutisya et al., 1994; Parker et al., 1994; Maurer et al., 2000) and SOD1 (Chen et al., 1994; Marcus et al., 1998) have been reported for the AD brain.

In view of the high copper contents in senile plaques (Lovell et al., 1998) and the overall low copper content in AD brain, it is now widely acknowledged that in AD copper is abnormally redistributed to senile plaques, leaving the tissue and cells deficient in copper (Macreadie, 2008; Kaden et al., 2011; Roberts et al., 2012). Therapeutic strategies aiming to restore the normal copper distribution in AD brain are currently under investigation (Zatta et al., 2009; Kenche and Barnham, 2011). Thus, the copper chelator PBT-2, which has been shown to reduce senile plaques and to enhance brain copper levels by its copper ionophore function (Adlard et al., 2008), has recently been shown to improve AD biomarkers as well as cognition in AD patients in a phase 2 clinical trial (Lannfelt et al., 2008).

1.3.4.3 Parkinson's disease

With an overall prevalence ranging between 0.1% and 0.3%, Parkinson's disease (PD) is the second most common neurodegenerative disease in humans (Wirdefeldt et al., 2011). PD is characterized by a complex motor disorder known as Parkinsonism that manifests with resting tremor, bradykinesia, rigidity and postural instability, but clinical symptoms also include cognitive and psychiatric problems (Thomas and Beal, 2007; Breen and Barker, 2010; Ferrer et al., 2011). The pathological hallmarks of PD are the loss of neuromelanin containing dopaminergic neurons in the substantia nigra pars

compacta and the presence of intracellular inclusions, called Lewy bodies, in the remaining neurons (Thomas and Beal, 2007; Jomova et al., 2010; Ferrer et al., 2011; Sian-Hulsmann et al., 2011). The majority of cases are idiopathic with less than 10% of PD having a strict familial etiology (Thomas and Beal, 2007; Jomova et al., 2010; Sian-Hulsmann et al., 2011; Wirdefeldt et al., 2011). The underlying mechanisms of idiopathic PD are not fully understood. Among others mitochondrial dysfunction, oxidative stress and inflammation have been suggested in the pathogenesis of PD (Thomas and Beal, 2007; Jomova et al., 2010).

Parkinsonism is frequently present in patients with neurological Wilson's disease (Lorincz, 2010) and copper has been demonstrated to accelerate aggregation of α-synuclein into fibrillar plaques, the precursors to Lewy bodies (Uversky et al., 2001). However, while the total copper content in brains of PD patients does not differ strongly from healthy controls, copper levels are substantial lower in substantia nigra of PD patients (Dexter et al., 1989; Dexter et al., 1991; Loeffler et al., 1996; Popescu et al., 2009). This reduction in the copper content of the substantia nigra in PD has been discussed to result in an impairment of copper-dependent pathways, thereby contributing to the pathogenesis of PD (Double, 2012). In support of this view, copper supplementation has been shown to prevent the increase in lipid peroxidation, striatal dopamine depletion and the reduction in the activity of tyrosine hydroxylase in an animal model for PD (Alcaraz-Zubeldia et al., 2001; Alcaraz-Zubeldia et al., 2009) while copper chelation was reported not to be protective in PD animal models (Youdim et al., 2007).

1.3.4.4 Huntington's disease

Huntington's disease (HD) is a rare autosomal-dominant, progressive neurodegenerative disease that results in motor, cognitive and psychiatric

abnormalities (Anderson, 2011; Shannon, 2011). The genetic defect underlying HD is an expansion of the cytosine-adenine-guanine (CAG) repeat in exon 1 of the huntingtin gene that is translated into an expanded polyglutamine domain at the N-terminus of the huntingtin protein (McFarland and Cha, 2011). The length of this extension determines the onset as well as the velocity of the clinical progression of HD (Anderson, 2011; McFarland and Cha, 2011; Shannon, 2011). However, the exact pathogenic mechanism in HD remains to be elucidated. Among others, aggregation of the mutant huntingtin protein, oxidative stress, impaired energy metabolism, loss of neurotrophic support and transcriptional dysregulation have been discussed to contribute to the development and progression of HD (Gauthier et al., 2004; Chen, 2011; McFarland and Cha, 2011).

Accumulation of copper in the HD brain has been hypothesized to promote disease progression (Fox et al., 2007). The brain copper levels of HD patients (Dexter et al., 1991) and of a mouse model for HD (Fox et al., 2007) have been found to be elevated compared to controls and copper has been shown to strongly interact with the huntingtin protein and to promote its aggregation (Fox et al., 2007). In addition treatment with the copper chelators tetrathiomolybdate (Tallaksen-Greene et al., 2009) or clioquinol (Nguyen et al., 2005) delayed the decline in motor function in mouse models for HD, further supporting a potential role of copper in disease progression. Currently, the copper chelator PBT-2, an 8-hydroxyquinon derivate of clioquinol, is under clinical investigation for the treatment of HD (http://www.huntington-study-group.org/HSGResearch/ClinicalTrialsObservationalStudiesinProgress/Reach2HD/tabid/243/Default.aspx; date visited: 14/03/2012).

1.4

The role of astrocytes in brain copper homeostasis

Astrocytes, which constitute the main class of neuroglia, are the most abundant cells in the brain (Markiewicz and Lukomska, 2006; Sofroniew and Vinters, 2010). These cells are distributed throughout the entire brain and fulfill a range of important functions essential for brain physiology (Nedergaard et al., 2003; Sofroniew and Vinters, 2010; Parpura et al., 2012). Among other functions, astrocytes have been discussed to be involved in the control of brain energy metabolism, metabolic support of neurons, maintenance of the BBB as well as in the modulation of synaptic transmission, synaptic plasticity and extracellular ion homeostasis (Nedergaard et al., 2003; Pellerin et al., 2007; Barker and Ullian, 2010; Barros and Deitmer, 2010; Deitmer and Rose, 2010; Hirrlinger and Dringen, 2010; Perea and Araque, 2010; Dienel, 2011). Moreover astrocytes have been considered to play a key role in the metal metabolism of the brain (Tiffany-Castiglion and Qian, 2001; Dringen et al., 2007; Tiffany-Castiglioni et al., 2011).

Astrocytes possess several features that allow them to function as regulators for the uptake and distribution of essential metals to other types of brain cells and to serve as metal depots (Tiffany-Castiglioni et al., 2001; Dringen et al., 2007; Tiffany-Castiglioni et al., 2011). These cells have a strategically important location in the brain, being in close contact to neuronal cell bodies and to capillary endothelial cells via their cytoplasmic processes that terminate in so called end-feet (Demeuse et al., 2002; Nedergaard et al., 2003). Astrocytic end-feet cover most of the brain capillaries (Demeuse et al., 2002; Nedergaard et al., 2003; Mathiisen et al., 2010) and have been shown to express metal transport proteins such as DMT1 (Burdo et al., 2001) or ferroportin (Wu et al., 2004). Thus, astrocytes are the first brain parenchyma cells to encounter metals that cross the BBB. In addition, astrocytes can express ferritin in an iron-dependent manner (Hoepken et al., 2004) and contain high cellular contents of MTs (Aschner, 1997; Aschner et al., 1997; Dineley et al., 2000; Hidalgo et al., 2001) and GSH (Dringen

and Hamprecht, 1998; Hirrlinger and Dringen, 2010), endowing them with a high capacity to store and to prevent the toxicity of metals and of metal-induced oxidative stress.

At the start of the work on this thesis little was known on the copper metabolism of astrocytes. However, multiple evidence suggest that these cells are likely to play an important role in the copper homeostasis of the brain. Within the brain copper has been shown histochemically to concentrate in astrocytes and it has been suggested that astrocytes may regulate the copper supply to neurons (Szerdahelyi and Kasa, 1986; Kodama et al., 1991; Kodama, 1993). In the North Ronaldsay sheep elevated brain copper content was accompanied by copper accumulation in astrocytes and by a strong astrocytic immunoreactivity for MTs (Haywood et al., 2008). In cell culture, astrocytes have been reported to take up copper more efficiently than cultured neurons and to protect neurons from copper toxicity (Brown, 2004). This protection of neurons against copper toxicity could involve the removal of copper by uptake into astrocytes. In addition, astrocytes release compounds that prevent the copper-mediated inactivation/degradation of extracellular GSH (Pope et al., 2008). Since trafficking of GSH from astrocytes to neurons is essential to maintain neuronal GSH levels (Dringen et al., 1999; Hirrlinger and Dringen, 2010), this stabilization of extracellular GSH may also have neuroprotective functions (Pope et al., 2008).

Astrocytes have been demonstrated to accumulate copper *in vivo* (Haywood et al., 2008) and *in vitro* (Kodama et al., 1991; Brown, 2004). Copper accumulation by cultured astrocytes has been reported to follow Michaelis-Menten kinetic with apparent K_M and v_{max}-values of 2.9 nM and 12 pmol/(min x mg), respectively (Brown, 2004). Since Ctr1 mRNA has been shown to be expressed in cultured astrocytes (Qian and Tiffany-Castiglioni, 2003), this transporter is likely to participate in the copper uptake by astrocytes. DMT1, which has been

shown to be expressed in the astrocyte end-feet that contact capillary endothelial cells (Burdo et al., 2001) as well as in cultured astrocytes (Jeong and David, 2003; Erikson and Aschner, 2006; Tulpule et al., 2010), may also contribute to astrocytic copper uptake. Both Ctr1 and DMT1 have been reported to mediate the transport of Cu^{+} rather than of Cu^{2+} (Lee et al., 2002a; Lee et al., 2002b; Arredondo et al., 2003; Bertinato et al., 2010), necessitating a reduction of Cu^{2+} by an ecto-cuprireductase prior to transport. The reductase Dcyt*b*, which is known to reduce copper (Wyman et al., 2008) has been shown to be expressed in cultured astrocytes (Jeong and David, 2003; Tulpule et al., 2010) and could therefore provide Cu^{+} for cellular accumulation.

Despite the efficient accumulation of copper, astrocytes have been observed to be remarkably resistant against copper-induced toxicity (Chen et al., 2008b; Reddy et al., 2008). However, incubation of cultured astrocytes for hours or days with micromolar concentrations of copper has also been reported to severely compromise the integrity and function of these cells (Ferretti et al., 2003; Merker et al., 2005; Reddy et al., 2008). While elevated levels of peroxides and increased protein oxidation that have been observed for copper-treated human astrocytoma cells suggest an involvement of oxidative stress in copper-induced toxicity (Ferretti et al., 2003; Merker et al., 2005; Qian et al., 2005), cultured astrocytes did not show a significant increase in the levels of ROS after exposure to 100 μM copper sulfate (Gyulkhandanyan et al., 2003)

Neurons depend on sufficient amounts of copper which has been suggested to be supplied by neighboring astrocytes (Kodama et al., 1991; Kodama, 1993). For such a copper supply function astrocytes have to be able to release copper. C6 glioma cells, a commonly used model for astrocytes, have been shown to express ATP7A (Qian et al., 1997, 1998a) and to export copper with apparent K_M- and v_{max}-values of 0.15 μM and 65 pmol/(mg x h), respectively (Qian et al., 1995). Considering that astrocytes also express ATP7A (Barnes et al., 2005;

Niciu et al., 2006; Niciu et al., 2007) and that astrocytes in the brindled mouse model of Menkes disease excessively accumulate copper (Kodama et al., 1991), it is highly likely that these cells play an important regulatory role in the brain copper metabolism by providing the essential trace element copper to neurons and to other brain cells, as already postulated two decades ago (Kodama et al., 1991; Kodama, 1993).

1.5

Aim of the thesis

Copper is an essential element. Its cellular and tissue concentrations are strictly regulated by various complex mechanisms and disturbances of copper homeostasis have been linked to several neurodegenerative diseases. In brain, astrocytes are thought to be key players in the regulation of metal homeostasis. Since only little was known about the copper metabolism in astrocytes and on potential effects of copper on the metabolism of astrocytes, this thesis aims to experimentally address such questions by using astrocyte-rich primary cultures as model system for brain astrocytes.

This thesis will investigate:

- mechanism(s) that are involved in copper accumulation
- the presence of potential transporters involved in the copper transport by astrocytes
- the copper export after loading the cells with copper
- the trafficking of the copper-transporting ATPase ATP7A in response to extracellular copper
- the effects of copper accumulation on cellular integrity and functions
- copper-induced alterations of the glucose and GSH metabolism of astrocytes.

1.6

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2

Results

2.1

Publication 1:

Copper accumulation by cultured astrocytes

Ivo F. Scheiber
Ralf Dringen

2010
Neurochemistry International, **56**, 451-460.

Contributions of Ivo F. Scheiber:

- Design of the study
- Experimental work
- Preparation of the first draft of the manuscript

The article can be accessed via the following link:

http://dx.doi.org/10.1016/j.neuint.2009.12.002

2.2

Publication 2:

Copper export from cultured astrocytes

Ivo F. Scheiber
Maike M. Schmidt
Ralf Dringen

2012
Neurochemistry International, **60**, 292-300.

Contributions of Ivo F. Scheiber:

- Design of the study
- Performance of all experimental incubations
- Data shown in Figures 1, 2, 3, 4, 5 & 7 and Table 1
- Preparation of the first draft of the manuscript

The article can be accessed via the following link:

http://dx.doi.org/10.1016/j.neuint.2011.12.012

2.3

Publication 3:

Zinc prevents the copper induced-damage of cultured astrocytes

Ivo F. Scheiber
Maike M. Schmidt
Ralf Dringen

2010
Neurochemistry International, **57**, 314-322.

Contributions of Ivo F. Scheiber:

- Design of the study
- Performance of all experimental incubations
- Data shown in Figures 1 & 4 and Table 2
- Data shown in Figures 2 & 5 with the exception of the quantification of glutathione
- Preparation of the first draft of the manuscript

The article can be accessed via the following link:

http://dx.doi.org/10.1016/j.neuint.2010.06.010

2.4

Publication 4:

Copper accelerates glycolytic flux in cultured astrocytes

Ivo F. Scheiber
Ralf Dringen

2011
Neurochemical Research, **36**, 894-903.

Contributions of Ivo F. Scheiber:

- Design of the study
- Experimental work
- Preparation of the first draft of the manuscript

The article can be accessed via the following link:

http://dx.doi.org/10.1007/s11064-011-0419-0

2.5

Publication 5:

Copper-treatment increases the cellular GSH content and accelerates GSH export from cultured rat astrocytes

Ivo F. Scheiber
Ralf Dringen

2011
Neuroscience Letters, **498**, 42-46.

Contributions of Ivo F. Scheiber:

- Design of the study
- Experimental work
- Preparation of the first draft of the manuscript

3

Summarizing Discussion

Copper is an essential trace metal that is required for a variety of functions. Amongst the different type of brain cells, astrocytes are discussed to play a key role in metal homeostasis (Tiffany-Castiglion and Qian, 2001; Dringen et al., 2007; Tiffany-Castiglioni et al., 2011). In addition, astrocytes are metabolically coupled to neurons and provide them with GSH precursor amino acids (Hirrlinger and Dringen, 2010; Schmidt and Dringen, 2012) as well as substrates for energy production such as lactate (Pellerin et al., 2007; Barros and Deitmer, 2010; Dienel, 2011). Consequently, alterations of the astrocytic metabolism are likely to indirectly affect the functions of neurons.

The aim of this thesis was to study the copper homeostatic mechanisms of astrocytes and the effects of copper on the metabolism of these cells, using astrocyte-rich primary cultures as model system. Immunocytochemical characterization of the cultures used in this thesis had previously revealed that more than 90% of the cells were stained positive for the astrocytic marker glial fibrillary acidic protein (GFAP; (Reinhart et al., 1990; Gutterer et al., 1999; Schmidt, 2010). Basal biochemical parameters such as the specific GSH content, glucose metabolism as well as the activities of enzymes involved in GSH or glucose metabolism are very well known for these cultures (Schmidt, 2010). Thus, astrocyte-rich primary cultures were considered as suitable model system to investigate the transport and storage of copper in astrocytes as well as metabolic consequences of a copper exposure.

Copper metabolism as well as the effects of copper on the metabolism of cultured astrocytes was investigated for copper concentrations in the range from 0 to 30 μM which is likely to represent the physiological range of copper concentrations in the extracellular fluids in brain. Although the copper concentration in the CSF was estimated to be in the range of 0.3 μM – 0.5 μM (Stuerenburg, 2000; Forte et al., 2004; Nischwitz et al., 2008; Strozyk et al., 2009), the extracellular copper concentrations in brain tissue are likely to be much

higher, since at least for the synaptic cleft copper concentrations have been reported to range from 4 μM to up to 250 μM (Kardos et al., 1989; Hopt et al., 2003).

3.1 Copper metabolism in astrocytes

Astrocytes have long been considered to play a role in brain copper metabolism (Kodama et al., 1991; Tiffany-Castiglion and Qian, 2001; Tiffany-Castiglioni et al., 2011). However, the cellular copper metabolism in astrocytes or other brain cells has not been studied as extensively as that of peripheral cell types such as hepatocytes or intestinal cells. To gain more knowledge about the copper metabolism in astrocytes, this thesis investigated the copper metabolism of cultured astrocytes in detail. The results obtained and presented in chapters 2.1, 2.2 and 2.3 of this thesis extended the knowledge on functions of astrocytes in copper homeostasis. The updated current model of copper metabolism in astrocytes is depicted in Figure 1.

Untreated astrocyte-rich primary cultures contain a low basal copper content of about 1.5 nmol per mg protein. This copper level is most likely sufficient to meet the physiological requirement of astrocytes for copper. These cells accumulate copper efficiently *in vitro* (Brown, 2004; Qian et al., 2012) and *in vivo* (Haywood et al., 2008). Cultured astrocytes can take up copper by both Ctr1 and Ctr1-independent mechanisms (Chapter 2.1). These cells express Ctr1 protein, extending the previous observation that astrocytes contain mRNA for this high-affinity copper transporter (Qian and Tiffany-Castiglioni, 2003). The kinetic parameters observed for copper accumulation in astrocytes were also consistent with that reported for Ctr1-mediated copper transport in other cells (Eisses and Kaplan, 2002; Lee et al., 2002a; Eisses et al., 2005). However, results from zinc inhibition experiments suggest that astrocytes can take up copper by additional mechanisms. Such a Ctr1-independent transport has previously been

described for other cell types (Lee et al., 2002b; Arredondo et al., 2003; Eisses et al., 2005; Kidane et al., 2012), but the identity of the transporters involved in this process remains unknown. One likely candidate is DMT1, which has been demonstrated to account for at least 50% of copper uptake in the human

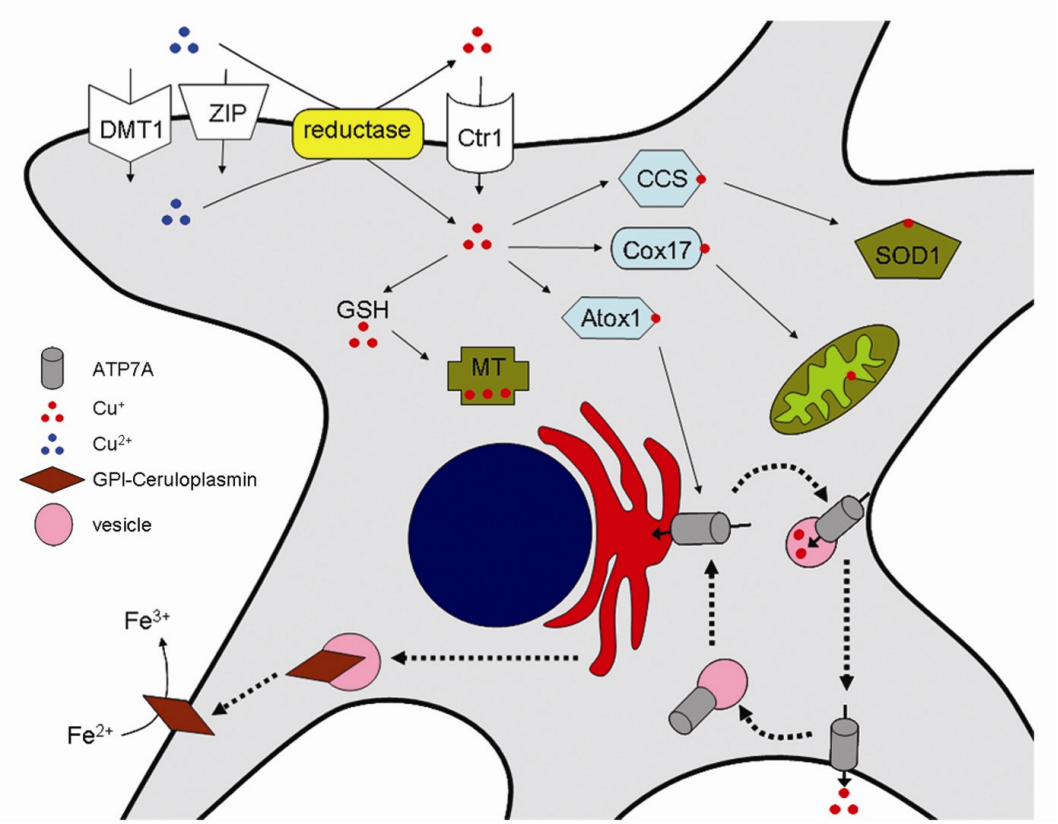

Figure 1: Copper metabolism in cultured astrocytes. Copper can be taken up into cultured astrocytes by the copper transporter receptor 1 (Ctr1) and probably by additional transporters such as the divalent metal transporter 1 (DMT1) or members of the ZIP family of metal transporters. Since Ctr1 transports Cu^{+} rather than Cu^{2+}, astrocytes are likely to possess an ecto-cuprireductase. Accumulated copper is sequestered by GSH, stored in MTs or shuttled to its specific cellular targets by copper chaperones. The copper chaperone for superoxide dismutase (CCS) delivers copper to superoxide dismutase 1 (SOD1). Cox17 delivers copper to Sco1, Sco2 and Cox11 for subsequent incorporation into cytochrome *c* oxidase. Antioxidant protein 1 (Atox1) delivers copper to ATP7A and ATP7B for translocation into the trans-Golgi network and subsequent incorporation into copper-dependent enzymes such as glycosyl phosphatidylinositol-anchored ceruloplasmin (GPI-Cp). When the cellular copper level rises above a certain threshold, ATP7A translocates via vesicles to the plasma membrane. ATP7A imports copper into these vesicles for release after fusion with the plasma membrane and/or exports copper directly from the astrocyte. Redistribution of ATP7A back to the trans-Golgi network occurs when cellular copper levels return to normal.

intestinal cell line CaCo2 (Arredondo et al., 2003). DMT1 has been shown to be expressed in cultured astrocytes (Jeong and David, 2003; Erikson and Aschner, 2006; Tulpule et al., 2010) and the increased copper accumulation at lower pH, the stimulation by ascorbate and the reduction of cellular copper accumulation by cadmium and manganese is consistent with a contribution of this transporter to the copper accumulation by astrocytes. The strong inhibition of copper accumulation by zinc hints to a possible involvement of members of the ZIP-family of metal transporters. ZIP transporters have broad substrate specificity and have been demonstrated to transport zinc, manganese, cadmium and iron (Kambe et al., 2004; Eide, 2006). Direct evidence of a contribution of ZIP transporters in copper uptake has not been reported so far, but the strong competition by copper of the uptake of zinc and cadmium by Zip1, Zip2 and Zip14 (Gaither and Eide, 2000, 2001; Girijashanker et al., 2008) suggests that these transporters may be capable of transporting copper. However, in addition to the functional characterization of the various members of the ZIP family proteins with respect to copper transport, the expression of these transporters in astrocytes needs further studies, since so far only mRNA for Zip1 (Belloni-Olivi et al., 2009) and Zip14 (Bishop et al., 2010) have been shown to be present in astrocytes.

Ctr1 and DMT1 have been reported to transport Cu^{+} rather than Cu^{2+} (Lee et al., 2002a; Arredondo et al., 2003), yet cultured astrocytes rapidly accumulated copper from Cu^{2+} when added to the culture medium. The copper accumulation was strongly stimulated by ascorbate, suggesting that reduction of Cu^{2+} to Cu^{+} is the rate-limiting step in copper uptake by astrocytes. This observation indicates the involvement of an ecto-cuprireductase in copper uptake that is located on the astrocyte plasma membrane in close proximity to Ctr1 and DMT1. All metal reductases known to reduce Cu^{2+} do also reduce Fe^{3+} and belong either to the cytochrome *b* (Knopfel and Solioz, 2002; Wyman et al.,

2008) or to the Steap family of proteins (Ohgami et al., 2006; Knutson, 2007). While the expression of members of the latter family of metal reductases has not been investigated for astrocytes, Dcyt*b*, the homologue of the cytochrome b family has been shown to be expressed in cultured astrocytes (Jeong and David, 2003; Tulpule et al., 2010). However, if a cupri-/ferri-reductase supports copper uptake into astrocytes, it would be expected that ferric iron would inhibit copper uptake from Cu^{2+}-salts, which was at least not the case for the copper accumulation investigated here (Chapter 2.1). Thus, astrocytes appear to possess mechanisms to transport both Cu^{+} and Cu^{2+}, and Cu^{2+}-transport in the absence of ascorbate appears to outweigh Cu^{+} transport.

Cultured astrocytes accumulated copper much more rapidly from amino acid-free buffers than from Dulbecco's modified Eagle's medium (DMEM; Chapter 2) or histidine-buffer (Brown, 2004). The reason for this difference is most likely the chelation of copper by components of the latter two media. The observed difference in the rates of copper accumulation from different media supports the view of the existence of multiple copper uptake processes in astrocytes: (1) a high-affinity and low uptake capacity process, which probably involves Ctr1-mediated transport, and (2) an additional process of high uptake capacity but low-affinity. The relative importance of these processes *in vivo* is difficult to assign, although Ctr1-mediated transport appears to prevail only when copper is bound to ligands such as histidine (Lee et al., 2002a). This observation, together with the almost complete inhibition of copper uptake by zinc, suggests that cultured astrocytes predominantly accumulate copper from amino acid-free buffers by a Ctr1-independent transport process. Thus, studying copper transport by cultured astrocytes under this condition may represent a suitable tool to further investigate Ctr1-independent transport.

In cells, copper is sequestered by GSH, stored in MTs or shuttled by copper chaperones to its specific targets, keeping the levels of potential toxic free

copper low (Freedman and Peisach, 1989; Tapia et al., 2004; Robinson and Winge, 2010). Astrocytes contain high cellular contents of GSH (Dringen and Hamprecht, 1998) and MTs (Aschner et al., 1997; Dineley et al., 2000), providing these cells with a high storage capacity for copper. When exposed to copper in DMEM for prolonged time cultured astrocytes elevate their contents of GSH (Chapter 2.5) and MTs (data not shown). These compensatory responses further increase their storage capacity for copper and are likely to explain the remarkably resistance of these cells against copper-induced toxicity (Brown et al., 1998; Chen et al., 2008b; Reddy et al., 2008). In contrast, when copper uptake is very rapid the protective mechanisms of astrocytes against copper-induced toxicity are overwhelmed. For example, exposure to 30 μM copper in amino acid-free buffer results in an 8-fold increase in cellular copper content within 1 h and led to severe toxicity within 2 h (Chapter 2.3). These observations indicate that the rate of copper accumulation determines the velocity and extent of copper-mediated cell damage in cultured astrocytes.

The expression and function of copper chaperones in astrocytes has not been addressed in this thesis. However, CCS which delivers copper to SOD1 has been demonstrated in astrocytes (Rothstein et al., 1999). In addition, the finding that cultured astrocytes express ATP7A (Chapter 2.2) provides indirect evidence for the presence of Atox1 in these cells, since the cellular functions of this copper-transporting ATPase relies on the presence of the copper chaperone Atox1 (Klomp et al., 1997; Hung et al., 1998).

Viable astrocytes have been shown for the first time in this thesis to export copper. This copper export involves most likely ATP7A, since this protein is expressed in astrocyte cultures (Chapter 2.2), confirming literature data (Barnes et al., 2005; Niciu et al., 2006; Niciu et al., 2007). However, a contribution of ATP7B in copper export from astrocytes can also not be excluded, since mRNA for ATP7B was found to be expressed in cultured astrocytes (data not shown).

As demonstrated for other cell types, ATP7A in astrocytes traffics to sites close to the plasma membrane when cellular copper levels exceed certain thresholds and returns back to its initial TGN localization after removal of copper (Chapter 2.2), further supporting a contribution of ATP7A in the observed copper export from astrocytes.

In conclusion, this thesis provides further evidence for a pivotal role of astrocytes in the copper metabolism of the brain. Astrocytes have been shown to efficiently accumulate copper by both Ctr1 and Ctr1-independent mechanisms. An increase in cellular copper levels was observed to cause an increase in cellular contents of GSH and MTs, providing astrocytes with a high storage capacity for copper and making them remarkably resistant against copper induced-toxicity. The ability to efficiently take up and store copper may be important to protect neurons from copper-induced toxicity and may contribute to the neuroprotective function of astrocytes against copper-mediated toxicity (Brown, 2004; Pope et al., 2008). Furthermore, astrocytes have been demonstrated in this thesis for the first time to be capable of exporting copper. With their strategically important localization between capillary endothelial cells and neuronal cell bodies (Nedergaard et al., 2003; Parpura et al., 2012) and with their potential to both taking up and exporting copper, astrocytes are ideal to provide copper to neurons. Disturbances of brain copper homeostasis have been connected with the development and progression of neurodegenerative diseases (Waggoner et al., 1999; Gaeta and Hider, 2005; Klevay, 2008; Macreadie, 2008; Hung et al., 2010; Rivera-Mancia et al., 2010). Assuming a pivotal role of astrocytes in brain copper homeostasis, astrocytic copper metabolism should be considered as a new therapeutic target in the treatment of such diseases.

3.2 Effects of copper on the metabolism of astrocytes

As discussed in the previous section, astrocytes may have a pivotal role in the regulation of brain copper homeostasis and are likely to encounter high copper concentrations when homeostatic mechanisms are impaired. Indeed, astrocytes have been shown to excessively accumulate copper in North Ronaldsay sheep, an animal model for copper toxicosis (Haywood et al., 2008). The consequences of pathological copper accumulation have been largely linked to copper-induced oxidative stress. However, the absence of any detectable ROS formation in astrocytes exposed to toxic concentrations of copper in DMEM (data not shown) argues against ROS-mediated damage as the major cause of copper-induced toxicity in these cells. This view is supported by the finding that at the early stage of copper accumulation in the liver of ATP7B deficient mice, strongly elevated copper concentrations did not correlate with liver damage (Huster and Lutsenko, 2007; Huster et al., 2007). In fact, in these animals elevated copper levels have been shown to affect specific cellular targets at the transcriptional and/or translational levels and to have distinct effects on metabolic functions of the liver (Huster et al., 2007; Sauer et al., 2011). Alterations of astrocytic metabolism by copper have been reported for the first time by articles that are part of this thesis (Chapters 2.4 and 2.5)

Astrocytes play a key role in brain glucose metabolism (Figure 2). They are in direct contact with brain capillary epithelial cells (Demeuse et al., 2002; Nedergaard et al., 2003; Parpura et al., 2012) and thus are likely to be the first cells to receive glucose from the blood. According to the astrocyte-neuron lactate shuttle hypothesis lactate released from astrocytes serves as an essential metabolic fuel for neighboring neurons (Pellerin et al., 2007; Barros and Deitmer, 2010; Dienel, 2011). Consequently, altered glucose metabolism of astrocytes by copper would also affect neurons. Treatment of cultured astrocytes with copper led to an increase in their glucose consumption and

lactate release in a time- and concentration-dependent manner (Chapter 2.4). The observed induction of glycolytic flux was not due to mitochondrial impairment, although opening of the mitochondrial transition pore was shown for copper-treated astrocytes (Reddy et al., 2008) and cerebella of ATP7B-deficient mice displayed lower activities of respiratory chain complexes (Sauer et al., 2011). The effect of copper on glucose metabolism of astrocytes depended

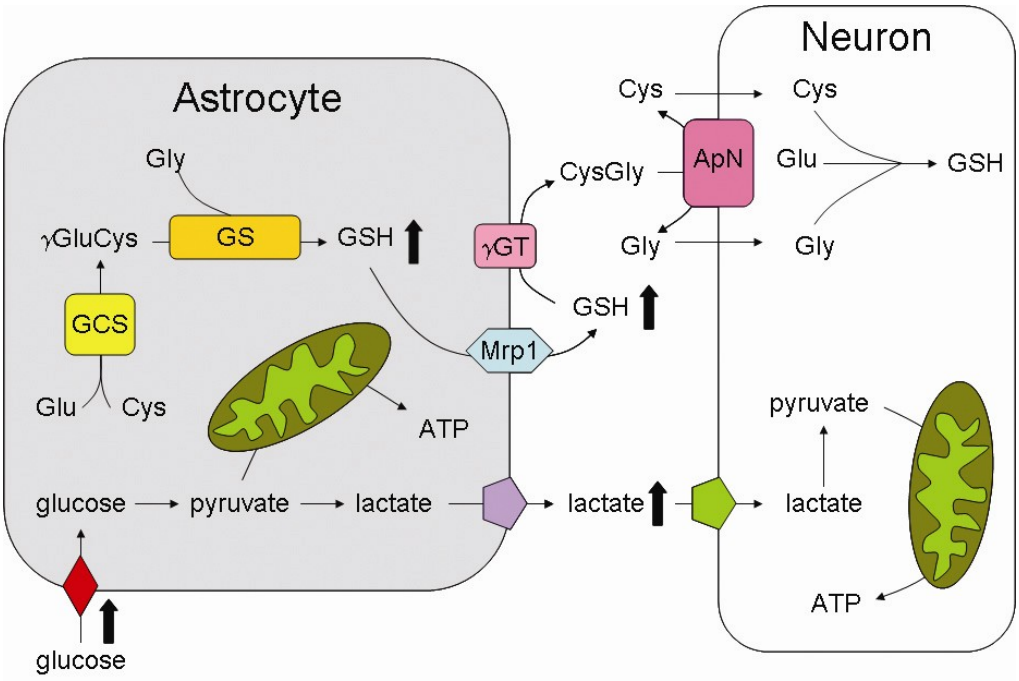

Figure 2: Potential consequences of copper-induced alterations in astrocyte metabolism and on the metabolic coupling between astrocytes and neurons. Astrocytes take up glucose entering the brain and metabolize it to pyruvate via glycolysis. Pyruvate is used for ATP production via mitochondrial respiration, but can also be reduced to lactate which is exported for uptake by neurons to serve as a substrate for oxidative phosphorylation. Exposure of cultured astrocytes to copper increased both glucose consumption and lactate release. Glutathione (GSH) is synthesized from the amino acid substrates glutamate (Glu), cysteine (Cys) and glycine (Gly) by the sequential action of glutamate cysteine ligase (GCL) and glutathione synthetase (GS). GSH can be exported by multidrug resistance protein 1 (Mrp1) from astrocytes. Extracellular GSH serves as substrate for astrocytic γ-glutamyl transpeptidase (γGT) to produce cysteinylglycine (CysGly). CysGly is hydrolyzed by neuronal aminopeptidase N (ApN) and the generated amino acids cysteine and glycine are taken up by neurons, where they can serve as precursors for GSH synthesis. Copper treatment increases the cellular GSH content and accelerates GSH export from cultured astrocytes.

on protein synthesis. Copper-induced changes in the transciptome have for example been observed in HepG2 cells exposed to copper (Song et al., 2009; Song and Freedman, 2011), in mice fed a copper-rich diet (Muller et al., 2007) and in mice deficient in ATP7B (Huster et al., 2007). Although the transcription factor Hif-1α is known to increase the glycolytic rate in cultured astrocytes (Schubert et al., 2009) and to be stabilized by copper (Martin et al., 2005), Hif-1α does not appear to be involved in the observed stimulation of glycolytic flux (Chapter 2.4). A possible role of other transcription factors in the copper-induced stimulation of glycolytic flux in astrocytes that are known to be activated by copper, such as the nuclear factor kappa B (NF-κB; McElwee et al., 2009) or the metal transcription factor 1 (MTF1; Mattie and Freedman, 2004), remains to be investigated. At least NF-κB has been shown to enhance glycolytic flux in mouse embryonic fibroblasts (Kawauchi et al., 2008). Interestingly, activation of NF-κB by copper was only observed for cells connected with the maintenance of copper homeostasis at the systemic level, whereas in other cell types copper had either no effect or an inhibitory effect on NF-κB-activation (McElwee et al., 2009).

Astrocytes play an important role in the brain GSH metabolism (Figure 2). These cells provide neighboring neurons with GSH precursors in a process that involves the release of GSH from astrocytes by multidrug resistance protein 1 (Mrp1) and subsequent processing of extracellular GSH to the amino acids required for neuronal GSH synthesis (Hirrlinger and Dringen, 2010; Schmidt and Dringen, 2012). GSH is synthesized in a two-step reaction by the consecutive action of glutamate cysteine ligase (GCL) and glutathione synthetase (Franklin et al., 2009; Lu, 2009; Schmidt and Dringen, 2012). The GCL-catalyzed reaction of glutamate and cysteine to γ-glutamylcysteine is the rate-limiting step of GSH synthesis (Franklin et al., 2009; Lu, 2009; Schmidt and Dringen, 2012). GCL is a heterodimeric protein that consists of a catalytic and a

modulator subunit (Franklin et al., 2009; Lu, 2009; Schmidt and Dringen, 2012). Both subunits, and thus GCL activity, are regulated on the transcriptional, post-transcriptional and post-translational level (Franklin et al., 2009; Lu, 2009). Formation of the GCL holoenzyme is required for efficient GCL activity and is thought to regulate GCL activity in a redox-dependent manner (Franklin et al., 2009; Lu, 2009).

Treatment of cultured astrocytes with copper strongly increased the cellular GSH content and led, most likely as a direct consequence of the elevated GSH content, to an accelerated Mrp1-dependent GSH release from these cells (Chapter 2.5). This strong increase of cellular GSH contrasts reports for other cell types that describe cellular depletion of GSH after exposure to copper (Hansen et al., 2006; Singleton et al., 2010; Vidyashankar and Patki, 2010). The mechanism responsible for the observed elevated GSH content in copper-treated astrocytes remains to be elucidated. However, it appears to be independent of protein synthesis, since an accelerated GSH release was also observed when protein synthesis was inhibited (data not shown). Consistently, nitric oxide, which has been reported to elevate GCL activity in astrocytes by inducing synthesis of GCL mRNA (Gegg et al., 2003), is unlikely to be involved in the elevation of the cellular GSH content of copper-treated astrocytes. More likely, elevated copper levels may enhance GCL activity by a post-translational mechanism. One possibility is that copper may facilitate the formation of the disulfide bond linking the catalytic with the regulatory subunit, thereby enhancing the levels of GCL holoenzyme (Franklin et al., 2009). Although an enhanced ROS production was not detected in copper-treated astrocytes, slight transient increases in ROS production induced by copper could also have caused an increase in GCL activity. Alternatively, an increased uptake of the GSH precursors cysteine or cysteine into astrocytes (Kranich et al., 1998) may contribute to the increased specific GSH content as reported for astrocytes

exposed to ammonia, cadmium or arsenite (Sagara et al., 1996; Wegrzynowicz et al., 2007).

In conclusion, this thesis described for the first time effects of copper on the metabolism of cultured astrocytes. Copper was shown to alter both glucose metabolism and GSH metabolism of cultured astrocytes. The increase in glycolytic flux appeared not to be required for the survival of astrocytes in the presence of high copper concentrations. *In vivo* an increased export of lactate from copper-loaded astrocytes could be deleterious, since it could result in a lactic acidosis (Rehncrona, 1985; Paschen et al., 1987). Alternatively, moderately increased lactate availability could be neuroprotective, since lactate is considered as substrate for the energy metabolism of neurons (Pellerin et al., 2007; Barros and Deitmer, 2010; Dienel, 2011). Whether such copper-induced alterations in astrocytic glucose metabolism occur *in vivo* is unclear. While one study found no evidence for an increased lactate concentration in the brain of Wilson's disease patients, indicating that brain energy metabolism was normal (Kraft et al., 1999), another study showed strong lactate accumulation in the basal ganglia (Juan et al., 2005). The elevation of the cellular GSH content is likely to confer copper-treated astrocytes with increased resistance against copper-mediated toxicity. In addition, the accelerated release of GSH may also provide protection to neurons against the toxic effects of copper, since extracellular GSH may sequester extracellular copper and could supply neurons with GSH precursors. However, whether such metabolic changes occur in copper overload condition *in vivo* is questionable. At least in the brain of ATP7B deficient mice, increased copper levels were not associated with a significant change of GSH levels (Sauer et al., 2011) and the GSH content in brains of copper intoxicated rats was decreased (Alexandrova et al., 2008; Ozcelik and Uzun, 2009).

3.3 Future perspectives

Astrocytes have been shown to efficiently accumulate copper. The mechanism contributing to copper uptake by astrocytes has been shown to strongly depend on the medium in which copper is provided. While in amino acid-free media Ctr1-independent copper uptake appears to dominate, copper uptake is more likely to involve Ctr1 in DMEM. To proof this hypothesis and to study the relative contribution of the individual transporters to copper uptake by astrocytes under the different incubation conditions, knock-down approaches by RNA interference (McManus and Sharp, 2002) for the different candidate transporters could be used. Alternatively, astrocyte cultures derived from animals deficient in these transporters (e.g. Belgrade rat for studying possible contribution of DMT1; Fleming et al., 1998)) could be generated and investigated regarding their copper accumulation. If the hypothesis that copper accumulation from amino acid-free buffers by astrocytes primarily represents Ctr1-independent transport is validated, this system would provide a valuable tool to study the molecular mechanisms of Ctr1-independent transport in detail.

Ctr1-independent transport has not been studied extensively. For CaCo2 cells DMT1 has been shown to contribute to copper accumulation (Arredondo et al., 2003) and could be a likely candidate for Ctr1-independent copper uptake in astrocytes. Preliminary experiments have shown that extracellular copper specifically induces the translocation of DMT1 from the plasma membrane to cytosolic compartments (data not shown). Members of the ZIP family of metal transporters are additional candidates for Ctr1-independent copper uptake by astrocytes and other cell types. Currently 14 different ZIP transporters are known in mammals, but copper transport has not been studied for any of them. Thus, before investigating the contribution of ZIP transporters to copper transport by astrocytes, ZIP transporters should first be characterized with respect to their copper transport properties.

Astrocytes have been shown to be capable of exporting copper. This process is likely to be mediated by ATP7A. However, also mRNA of ATP7B has been found in astrocyte cultures (data not shown). Presence of ATP7B in these cultures should be investigated on protein level. If expression of ATP7B protein could be confirmed, altered copper export rates in cultured astrocytes derived from animals either deficient in ATP7A (e.g. mottled mice; Hunt, 1974) or ATP7B (e.g. LEC rats; Yamaguchi et al., 1994) could reveal the relative contribution of both ATPases in astrocytic copper export. In addition, such cultures could be used to study whether ATP7A and ATP7B can functionally substitute for each other in astrocytes. This could be tested by studying the activity of tyrosinase, LOX and/or PAM in ATP7A deficient astrocyte cultures and the Cp activity in ATP7B deficient cells. Of special interest would also be the investigation of the iron metabolism in astrocytes deficient in ATP7B, since those astrocytes may be unable to release iron as reported for astrocytes derived from Cp-deficient animals (Jeong and David, 2003)

Astrocytes are remarkably resistant against copper-toxicity, most likely due to the strong elevation of cellular GSH contents in response to copper treatment. Although MT expression was found up-regulated in astrocytes when exposed to copper, it appears not to be essential for the protection of astrocytes to copper toxicity (data not shown). MTs have been reported to be secreted by astrocytes in response to brain injury. These secreted MTs are protective to neurons, which can take up extracellular MTs in a process that involves interaction with the megalin receptor (Chung et al., 2008). Given that copper induces MT expression and that copper is involved in non-classical secretion (Prudovsky et al., 2008), a release of MTs by astrocytes may also take place in response to copper. To test for the contribution of MT-release in the neuroprotective function of astrocytes against copper-mediated toxicity, the survival of neurons in co-cultures with

either wild type astrocytes or MT-deficient astrocytes (Yao et al., 2000) could be compared.

While some information is now available on the uptake, storage and export of copper in astrocytes, almost nothing is known about the intracellular copper transport in these cells. To complete the model of copper metabolism in astrocytes, the expression and cellular localization of the known copper chaperones should be investigated.

The hypothesis that astrocytes serve as a copper depots for the brain, distribute copper to brain parenchyma and protect other types of brain cells against copper toxicity is well-supported by literature data (Tiffany-Castiglioni et al., 2011). However, this hypothesis has not been studied directly so far. Generation of mice with astrocyte specific knock-out of copper transporters (ATP7A, Ctr1) or chaperons/storage proteins (Atox1, MTs) by an inducible Cre/lox system (Sauer, 1998; Metzger and Chambon, 2001; Sun et al., 2007) should be considered to confirm the important role of astrocytes in brain copper metabolism *in vivo*.

Copper has been shown to alter the glucose and GSH metabolism of cultured astrocytes. Mechanistically, these alterations have not been elucidated so far and should be further studied. A possible function of NF-κB in the stimulation of glycolytic flux by copper would be worth to investigate. In addition, the alteration of other metabolic pathways (e.g. lipid metabolism) in astrocytes by copper should be tested. Also, the consequence of a copper deficit on specific metabolic pathways in astrocytes has not been addressed so far. Such investigations may aid in the understanding of the role of astrocytes in neurodegenerative diseases associated with impaired copper homeostasis such as neurological Wilson's disease, Alzheimer's disease or Parkinson's disease (Gaggelli et al., 2006; Lorincz, 2010; Rivera-Mancia et al., 2010).

Finally, since copper is essential for every type of brain cell the experiments and methods described here for investigating the metabolism of copper in and its metabolic effects on cultured astrocytes should be applied to study copper metabolism of other types of brain cells such as neurons, oligodendrocytes and microglial cells.

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Appendix

4.1 Curriculum vitae

Personal information

Date of birth: 29th of February 1980

Place of birth: Herdecke, Germany

Education

07/07 – 06/12 **Doctoral thesis in Neurochemistry**, University of Bremen, Germany

"Copper metabolism and copper-mediated alterations in the metabolism of cultured astrocytes", Supervisor: Prof. Dr. Ralf Dringen, *University of Bremen*, Germany

05/07 **Diploma in Chemistry**, *University of Bremen*, Germany

Diploma thesis: "Untersuchungen zum Zinktransport in Astrozyten", Supervisor: Prof. Dr. Ralf Dringen, *University of Bremen*, Germany

10/01 – 05/07 **Studies in Chemistry**, *University of Bremen*, Germany

10/00 – 09/01 **Studies in Philosophy**, *Ruhr-Universität Bochum*, Germany

06/99 **Abitur (general qualification for university entrance),** *Gymnasium im Schulzentrum Holthausen*, Hattingen, Germany

Teaching Experience

03/06 – 02/12 **Supervisor in biochemical courses for students of chemistry and biology**, *University of Bremen*, Germany

04/04 – 07/05 **Supervisor in chemical courses for students of chemistry**, *University of Bremen*, Germany

04/04 – 07/05 **Supervisor in chemical courses for students of physics**, *University of Bremen*, Germany

Course-related activities

03/03 – 03/04 **Student research assistant,** *Max Planck Institute for Marine Microbiology*, Bremen, Germany

4.2 List of publications

Bishop, G.M., **Scheiber, I.F.**, Dringen, R., Robinson, S.R., 2010. Synergistic accumulation of iron and zinc by cultured astrocytes. *J Neural Transm* **117**, 809-817.

Scheiber, I.F., Mercer, J.F., Dringen, R., 2010. Copper accumulation by cultured astrocytes. *Neurochem Int* **56**, 451-460.

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