Synthesis and characterization of copper oxide nanoparticles and investigation of their effects on the vitality and metabolism of astrocytes

Dissertation

Zur Erlangung eines Doktors in den Naturwissenschaften (Dr. rer. nat.)

Fachbereich 2 (Biologie/Chemie)

Universität Bremen

Felix Bulcke

2015

Erster Gutachter:	Prof. Dr. Andrea Hartwig Karlsruher Institut für Technologie, Deutschland
Zweiter Gutachter:	Assoc. Prof. Dr. Anthony White University of Melbourne, Australia

Hiermit erkläre ich, Felix Bulcke, dass die vorliegende Doktorarbeit selbstständig und nur unter Verwendung der angegebenen Quellen angefertigt wurde. Diese Arbeit wurde zuvor nicht an anderer Stelle eingereicht.

Bremen, Januar 2016

Felix Bulcke

1.1 Table of content

I. Acknowledgements ... 3
II. Structure of the thesis ... 5
III. Summary ... 7
IV. Zusammenfassung ... 9
V. Abbreviations and symbols ... 11

1. **Introduction** ... 13
1.1 Nanoparticles ... 15
1.1.1 Basic concepts ... 15
1.1.2 Nanoparticles in industry, environment and human health ... 18
1.1.3 Copper oxide nanoparticles ... 22
1.2 Astrocytes ... 24
1.2.1 Functions at the synapse ... 24
1.2.2 Metabolic interactions with neurons ... 25
1.2.3 Detoxification ... 26
1.2.4 Functions in the metal metabolism of the brain ... 26
1.2.5 Astrocytes and nanoparticles ... 27
1.3 Publication 1: Handling of copper and copper oxide nanoparticles by astrocytes ... 29
1.4 Aim of the thesis ... 41
1.5 References ... 43

2. **Results** ... 55
2.1 Publication 2: Uptake and toxicity of copper oxide nanoparticles in cultured primary brain astrocytes ... 57
2.2 Publication 3: Copper oxide nanoparticles simulate glycolytic flux and increase the cellular contents of glutathione and metallothioneins in cultured astrocytes ... 69
2.3 Publication 4: Modulation of copper accumulation and copper-induced toxicity by antioxidants and copper chelators in cultured primary brain astrocytes ... 83

2.4 Endocytotic uptake of CuO-NPs by cultured primary astrocytes 93
2.4.1 Introduction 94
2.4.2 Materials and methods..94
2.4.3 Results 97
2.4.4 Discussion...105
2.4.5 References 107

3. **Summarizing discussion**..111
3.1 Synthesis and characterization of copper oxide nanoparticles 113
3.2 Uptake of copper oxide nanoparticles by astrocytes 117
3.3 Toxicity of copper and copper oxide nanoparticles on astrocytes 119
3.4 Stimulation of the astrocytic metabolism by copper oxide nanoparticles 122
3.5 Reflections on the *in vivo* relevance of the data obtained 124
3.6 Future perspectives..126
3.7 References 131

I. Acknowledgements

At first, I want to thank the supervisor of my project Prof. Dr. Ralf Dringen. Throughout my thesis you constantly supported me with valuable advice and helped me to improve my work and work on myself. Your devotion to science is remarkable and you never run out of new ideas. I admire your broad knowledge and that you prioritize all your students equally.

Secondly, I want to cordially thank Prof. Dr. Andrea Hartwig and Assoc. Prof. Dr. Anthony White for being the reviewers of my thesis.

I also want to thank the co-authors of my publications for contributing valuable information, data and discussion.

Thanks to all recent and former members of the neurobiochemistry working group. No matter if scientific or non-scientific topics were discussed, I really enjoyed the time having you around. Even in stressful times you all made my day. I want to thank Michaela, Maike, Eva and Mark for taking care of me in my early years. Further, I want to thank Ivo for all the work on copper he has conducted, which was the fundament of my experimental work. Especially I want to thank Charlotte, Ketki and Maria, who made up a big part of my life during this time. We were not only colleagues but good friends who supported each other on good and bad days. I also want to thank Yvonne, who always gave good advice in the lab and had an open ear and mind for any problem. After the years passed and oneself found to be the "oldest one" I also had the pleasure to pass knowledge ahead to the "younger generation" of PhD-students. Eva, Chris, Wiebke and recently Erik also were more friends than colleagues. We had a lot of fun on various occasions. My message to you is that you should keep up the spirit and the good mood of the group as it is now. I'm very glad that I was part of such an enjoyable group.

Additionally, I want to thank my family for all the support throughout this time. And last but not least, I want to thank Anni. The life with you made me a better person and helped me trough this time.

II. Structure of the thesis

This thesis consists of the three chapters introduction (Chaper 1), results (Chapter 2) and summarizing discussion (Chapter 3). The introduction part (Chapter 1) is composed of 3 subchapters. The first part describes the properties, applications and impact of nanomaterials with a special focus on copper oxide nanoparticles (Chapter 1.1). The second part describes the role of astrocytes as a central key player in the brain homeostasis (Chapter 1.2). The last part of the introduction is a publication reviewing the handling of copper and copper oxide nanoparticles by astrocytes (Chapter 1.3).

The results part of this thesis consists of three publications (Capter 2.1-2.3) and the description and analysis of an unpublished dataset (Chapter 2.4), describing the effects of copper and copper oxide nanoparticles on astrocytes. In the summarizing discussion (Chapter 3), which is the third and last part of this thesis, the most important results of this thesis will be discussed and future perspectives will be elucidated.

The contributions of all authors to publications are listed on the first page of the corresponding chapter.

III. Summary

Copper oxide nanoparticles (CuO-NPs) recently raised the industry's interest due to their interesting chemical and physical properties. The continuous increase of products containing CuO-NPs and the unintentional generation of CuO-NPs by technical processes establish an increased risk of human exposure. Since nanoparticles can reach the brain upon exposure, it is of high interest to evaluate the uptake and potential adverse effects on brain cells. In this context astrocytes are of special interest due to their central role in the brain homeostasis and in defence processes.

In the presented thesis, a method for the synthesis of CuO-NPs was established. After a detailed analysis of the physico-chemical properties of the synthesized CuO-NPs, primary astrocytes cultures were exposed to these CuO-NPs. The accumulation and uptake mechanism of CuO-NPs by cultured astrocytes as well as the resulting effects on the cell vitality and metabolism were investigated. It was shown that cultured astrocytes strongly accumulated CuO-NPs in a time-, concentration-, temperature- and media-dependent manner. Results from experiments with pharmacological inhibitors of different endocytotic pathways suggest that clathrin-mediated endocytosis, macropinocytosis and the recycling of membranes are involved in the uptake of CuO-NPs by astrocytes. Accumulated CuO-NPs exerted a strong toxicity when the specific cellular copper contents reached values above 10 nmol copper per mg protein. The mechanism of toxicity was assigned to a strong increase in reactive oxygen species in the treated cells. Cultured astrocytes treated with subtoxic concentrations of CuO-NPs over a time period of 24 h strongly increased their gycolytic flux, their glutathione content as well as the levels of the copper storage protein metallothioneine. The observed increase in glycolytic flux and metallothioneine levels was prevented in presence of the cell permeable copper chelator tetrathiomolybdate. This chelator was also capable of preventing the strong toxicity and the increased generation of reactive oxygen species in acute exposure scenarios where high concentrations of CuO-NPs were applied to cultured astrocytes. Similarly, toxicity observed after the exposure of cultured astrocytes with $CuCl_2$ was prevented by copper chelators while copper reducing antioxidants increased copper uptake and copper derived toxicity.

The data presented in this thesis reveal that CuO-NPs can have severe deleterious effects on astrocytes, which otherwise are very robust against several toxins. The

vulnerability of astrocytes to CuO-NPs suggests that brain cells may be severely damaged if they encounter such nanoparticles. Hence, the exposure of humans to CuO-NPs should be minimized and carefully evaluated to prevent potential health hazards.

IV. Zusammenfassung

Kupferoxid Nanopartikel (CuO-NPs) haben Aufgrund ihrer interessanten physikochemischen Eigenschaften unlängst das Interesse der Industrie geweckt. Die stetige Zunahme CuO-NP beinhaltender Produkte und die unkontrollierte Freisetzung von CuO-NPs steigern das Risiko einer menschlichen Exposition. Da Nanopartikel durch eine Exposition in das Gehirn gelangen können, ist es von großem Interesse die Aufnahme und potentielle negative Effekte auf Gehirnzellen zu evaluieren. In diesem Kontext sind Astrozyten von besonderem Interesse, da ihnen eine zentrale Rolle in der Homöostase des Gehirns und in detoxifizierenden Prozessen zugeschrieben wird.

In der vorliegenden Arbeit wurde eine Methode zur Synthese von CuO-NPs etabliert. Nach einer detaillierten Analyse der physikochemischen Eigenschaften der synthetisierten CuO-NPs wurden primäre Astrozytenkulturen mit diesen Partikeln behandelt. Die Akkumulation von CuO-NPs durch Astrozyten und der Aufnahmeweg, sowie die Effekte von CuO-NPs auf die Vitalität und den Metabolismus der behandelten Zellen wurden untersucht. Astrozyten nahmen CuO-NPs in Abhängigkeit von Zeit, Konzentration, Temperatur und Inkubationsmedium auf. Unter Zuhilfenahme pharmakologischer Inhibitoren verschiedener Endozytosewege wurde eine Beteiligung von Clathrin-vermittelter Endozytose, Makropinozytose und Membran Recycling an der Aufnahme von CuO-NPs festgestellt. Aufgenommene CuO-NPs übten eine starke Toxizität aus, wenn der spezifische zelluläre Kupfergehalt der behandelten Zellen einen Wert von 10 nmol Kupfer pro mg Protein überstiegen hat. Der Mechanismus der Zellschädigung wurde der gestiegenen Produktion reaktiver Sauerstoffspezies zugeschrieben. Wurden kultivierte Astrozyzen über einen Zeitraum von 24 Stunden mit sub-toxischen Konzentrationen von CuO-NPs behandelt, so steigerten sich deren glykolytischer Fluss, deren Glutathiongehalt und deren Menge an den Kupferspeicherproteinen Metallothionein. Die gezeigte Steigerung im glykolytischem Fluss und in der Menge von Metallothioneinen konnte mittels des membrangängigen Kupferchelators Tetrathiomolybdat unterdrückt werden. Dieser Chelator konnte ebenfalls die strake Toxizität und gesteigerte Produktion reaktiver Sauerstoffspezies in akuten Expositionszenarien, in denen die Zellen mit hohen Konzentrationen von CuO-NPs behandelt wurde, mindern. In vergleichbarer Weise konnte die toxische Wirkung von $CuCl_2$ auf Astrozytenkulturen mittels Kupferchelatoren verringert werden,

wohingegen Kupfer-reduzierende Antioxidantien die Kupferaufnahme und Kupfer vermittelte Toxizität steigerten.

Die Daten aus der vorliegenden Arbeit zeigen, dass CuO-NPs bei einer Exposition von Astrozyten, welche andernfalls sehr robust gegen Toxine sind, schwerwiegende schädliche Effekte hervorrufen können. Die schädigende Wirkung von CuO-NPs auf Astrozyten spricht dafür, dass ebenfalls andere Gehirnzelltypen von diesen Nanopartikeln beeinträchtigt werden könnten. Demzufolge sollte eine Exposition von Menschen mit CuO-NPs minimiert und das Expositionsrisiko mit Sorgfalt abgeschätzt werden um mögliche Gesundheitsrisiken zu vermeiden.

V. Abbreviations and symbols

Ami	Amiloride
ARE	Antioxdative response elements
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CuO-NPs	Copper oxide nanoparticles
pCuO-NPs	BSA coated CuO-NPs
CPZ	Chlorpromazine
CQ	Chloroquine
Ctr1	Copper transport protein 1
DMSA	Dimercaptosuccinic acid
DMSO	Dimethyl sulfoxide
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
DMEM-FCS	Dulbecco's modified Eagle's medium containing 10% FCS
EU	European Union
FCS	Fetal calf serum
GFAP	Glial fibrillary acidic protein
H33342	Bisbenzimide Hoechst 33342
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
IB	Incubation buffer
IB-BSA	Incubation buffer containing 0.5 mg BSA per mL
LDH	Lactate dehydrogenase
LC3	Microtubule-associated protein 1 light chain 3
MAP	Mitogen-activated protein
MRE	Metal response elements

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Nrf2	Nuclear factor erythroid 2-related factor 2
PI	Propidium iodide
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
ROS	Reactive oxygen species
siRNA	Small interfering RNA
TMP	Tetramethylpyratine
US	United States
UV	Ultraviolet
Wort	Wortmannin

1. Introduction

1.1 Nanoparticles

1.2 Astrocytes

1.3 Publication 1: Handling of copper and copper oxide nanoparticles by astrocytes.

1.4 Aim of the thesis

1.5 References

1. Introduction

1.1 Nanoparticles

1.1.1 Basic concepts

The history of nanomaterials reaches back thousands of years. Archeologists found that already 3000 years ago in Egypt and China gold, silver, platinum and palladium nanoparticles were used in paint, even without the knowledge of the existence and the properties of these materials (Daniel and Astruc 2004, Heiligtag and Niederberger 2013). Since the medieval times colloidal gold and silver were used in European churches for coloring glass as well as in the glaze of porcelain and other pottery (Horikoshi and Serpone 2013). As until the last century no analytical method existed to study materials of a size smaller than 1 µm, the knowledge on those materials was limited. Nanotechnology is a science that emerged in the beginning of the last century by the development of the high resolution microscopy techniques, transmission electron microscopy (1932) and scanning electron microscopy (1937), revealing the scale of 1 billionth of a meter (1 nm = 10^{-9} m) which is the size range of molecules and atoms (Knoll and Ruska 1932, von Ardenne 1938). In 1959 Richard Feynman proposed in his speech “There’s plenty of room at the bottom” the possibility of the synthesis of materials by direct manipulation of atoms (Feynman 1960). Although the term nanotechnology was first used by Norio Taniguchi in 1974, it was not until 1982 when the scanning tunneling microscope, which was invented by Gerd Binnig and Heinrich Rohrer who received a noble prize in 1986, was able to resolve molecular structures and atomic bonds (Taniguchi 1974, Binnig and Rohrer 1986). In the year 1989, Donald Mark Eigler used the scanning tunneling microscope technique to manipulate individual atoms validating the theory of Feynman (Eigler and Schweizer 1990).

Several new nanomaterials with interesting physical properties were discovered along the last 50 years, for example carbon nanofibers (Koyama and Endo 1973), fullerenes (Kroto et al. 1985), carbon nanotubes (Iijima 1991) and graphene (Novoselov et al. 2004). Some of these discoveries were awarded with noble prizes. There are several definitions available from different international organizations for the terms “nanomaterials” and “nanoparticles” (Table 1.1). Conclusively, it can be said that nanoparticles are defined as objects with at least 2 dimensions in the nanoscale (1-100 nm) and that nanomaterials have an external or internal structures in the nanoscale.

Table 1.1: Definitions of the terms "nanomaterials" and" nanoparticle" by international organizations

Organization	Nanomaterial	Nanoparticle	Standard access code (Reference)
International Organization for Standardization	"material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale"	"particle with a nominal diameter smaller than about 100 nm"	ISO/TS 80004-1 ISO/TR 27628 (https://www.iso.org/obp/ui/#iso:std:iso:ts:80004:-2:ed-1:v1:en)
European Union	"natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm"	"defined physical boundaries in the dimension between 1-100 nm"	L 275/38 (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:275:0038:0040:en:PDF)
British Standards Institution	"material having one or more external dimensions in the nanoscale or which is nanostructured"	"particle with all three external dimensions in the nanoscale"	PAS 136:2007 PAS 71:2011 (http://shop.bsigroup.com/upload/Shop/Download/Nano/PAS136.pdf) (http://shop.bsigroup.com/upload/Shop/Download/Nano/PAS71.pdf)
American Society of Testing and Materials	"physically or chemically distinguishable components, at least one of which is nanoscale in one or more dimensions"	"particle with lengths in two or three dimensions greater than 1 nm and smaller than about 0.1 µm"	E2456 (http://webstore.ansi.org/ansidocstore/find.asp?find_spec=ASTM+E2456-06)

Besides the size of nanoparticles, there are many other aspects that have to be considered to define nanoparticles (Figure 1.1) (Borm et al. 2006). The chemical and physical properties of nanoparticles do not only depend on their size, but also on their composition which can be organic (polymers, lipids) as well as inorganic (metal, metal oxides, metalloid, metalloid oxides) or a mixture of both (Akhtar et al. 2013, Allouche 2013, Cupaioli et al. 2014). Also the shape affects the physical properties of nanoparticles (Jo et al. 2015). The size of nanoparticles and their shape define their surface area (Kettler et al. 2014). In comparison to bigger particles, the surface area of smaller particles is higher in relation to their volume. For example, a cube with a side length of 1 cm has a surface area of 6 cm^2. The volume of this cube (1 cm^3) is equal to the volume of 1000 cubes with a side length of 1 mm, whereas the surface area of these cubes is in total 60 cm^2. If the same volume is filled up with cubes of 1 nm side length, the total surface area increases to $6 \cdot 10^7$ cm^2. Therefore, the big advantage of nanomaterials is that only a low amount of material is required to create a very large surface.

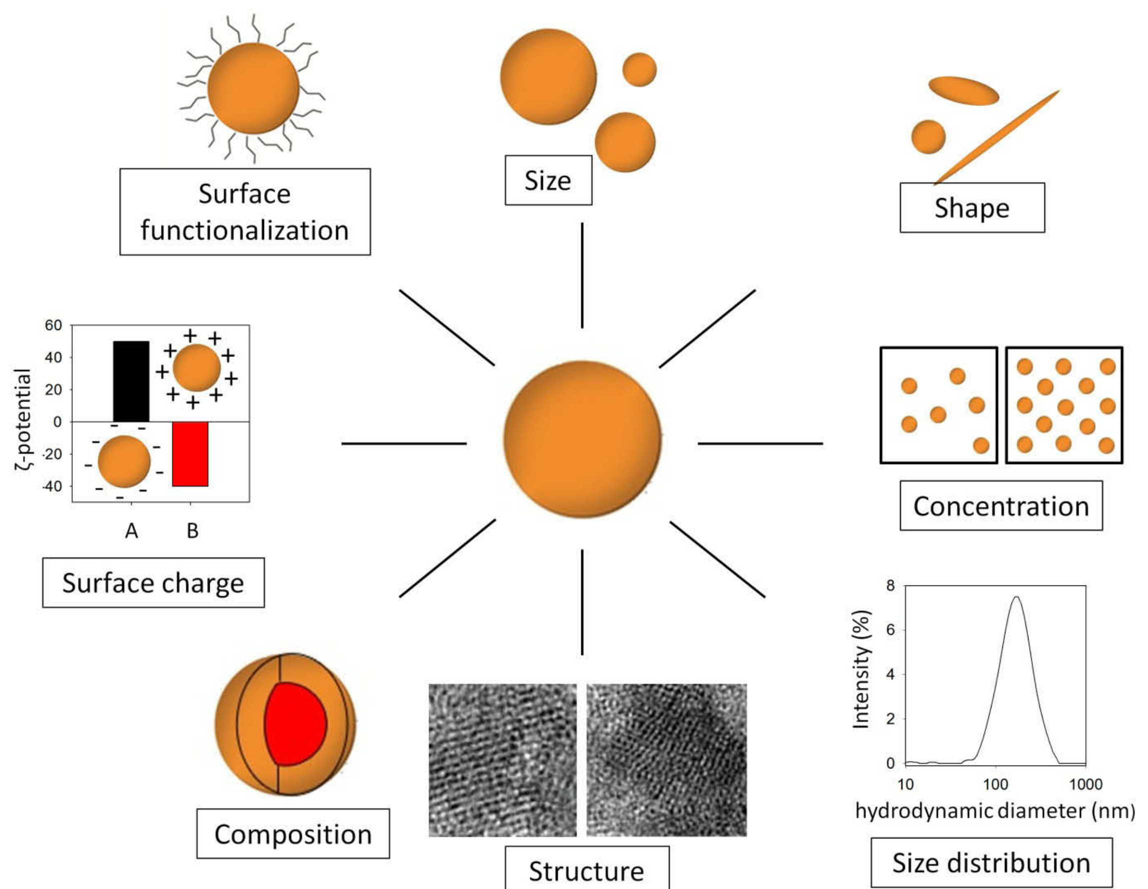

Figure 1.1: Chemical and physical properties of nanoparticles.

The surface of the nanoparticles is an important aspect, which determines the properties of the material by defining its catalytic activity/inactivity, the resistance to environmental factors, the binding ability to molecules and other surfaces as well as interactions with cells (Kettler et al. 2014, Jo et al. 2015). Due to the importance of the nanoparticle surface, it is of high interest to improve this surface by optimized synthesis or further modification to obtain the desired material properties. The native surface of nanoparticles is either charged or uncharged, depending on the used material and the synthesis method. Modifications of the surface can be performed by the addition of a so called "coating" material. These substances are mostly polar, polymeric or oligomeric compounds such as polyethylene glycol, polyvinylpyrrolidone, polyacrylic acid, polysorbate or dextran (Sperling and Parak 2010, Amin et al. 2015). These are used to give high aqueous dispersity, prevent agglomeration and increase the stability of the particles in biological media (Sperling and Parak 2010, Amin et al. 2015). The coating of the nanoparticles also allows the introduction of additional compounds to the particle surface (Sperling and Parak 2010, Amin et al. 2015). Such modifications could increase the catalytic activity of the material or add further features to the nanoparticles (Figure 1.2), including antibodies, proteins or glycans for cell targeting, or drugs, fluorescent dyes, enzymes, DNA and many other (Sperling and Parak 2010, Amin et al. 2015, Kang et al. 2015a, Pérez-Herrero and Fernández-Medarde 2015). The possibility to add several modifications to the nanoparticle surface makes it even more interesting to build tailor-made tools for specific applications. Especially nanomedicine seems to be a promising technology for individual and highly specific treatment of patients (Pautler and Brenner 2010, Chang et al. 2015).

Due to the wide variety of different possible combinations of those materials, it is unfortunately very difficult for the scientific community and the regulatory organizations to unify and simplify regulations, common standards and safety issues of nanomaterials (Borm et al. 2006, Cupaioli et al. 2014).

1.1.2 Nanoparticles in industry, environment and human health

Over the last decade the amount of nanoparticle-containing consumer products strongly increased to a number of above 1,800 products, as listed in the databases "Project on emerging nanotechnologies" (Consumer Products Inventory 2015) and "The Nanodatabase" (Danish Consumer Council 2015). Approximately the half of the registered products was listed into the categories cosmetics and clothing. Although not

all registered nanoparticle containing products were analyzed to inform the consumer about the ingredients, the most commonly used material for applied nanoparticles was silver. Depending on the used material, nanoparticles are used for various technical and biomedical applications and can appear in certain products (Table 1.2).

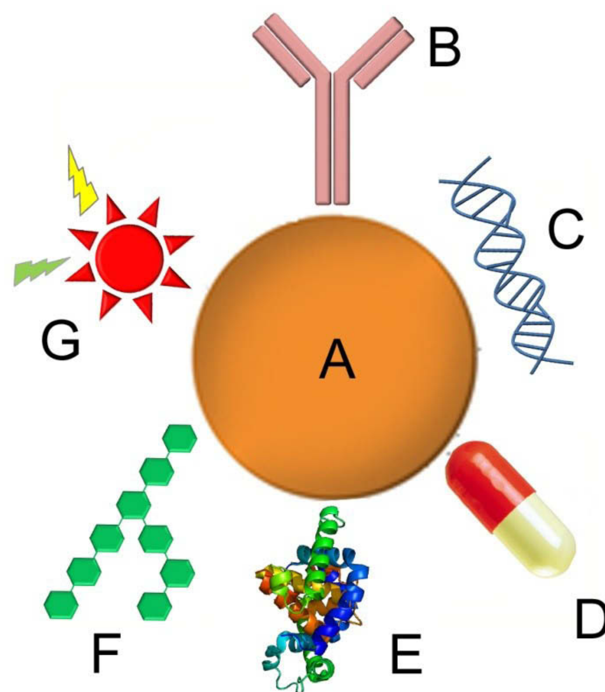

Figure 1.2: Surface modifications of nanoparticles. Additional features can be introduced to nanoparticles by binding of different substances to the surface of nanoparticles. Nanoparticles (A) can be modified by introducing antibodies (B), DNA (C), drugs (D), proteins or enzymes (E), glycans (F) or fluorescent dyes (G).

The annual production of nanomaterials increases each year and in 2012 reached a value of >10,000 tons and is predicted to further increase to an amounts of up to 100,000 tons till the year 2020 (Piccinno et al. 2012, Zhang et al. 2015). Therefore, it can be assumed that the environment is also increasingly polluted with nanomaterials. In the beginning of this century almost nothing was known about the environmental impact of nanomaterials (Krug 2014). Before the year 2000 less than 200 papers were published on nanotoxicology (Krug 2014). Since then an exponential increase of publications on this topic was recorded to a total number greater than 10000 papers (in 2013) (Krug 2014).

Table 1.2: Reported applications for some nanomaterials and nanomaterial-containing products listed by their core material.

Material	**Applications**	**References**
Aluminium nanoparticles/ alumina nanoparticles	Biosensors, bone substitutes, paper additive, screening tool for microorganisms, tissue engineering, ultra high capacity batteries	Schmid and Riediker 2008, Wang et al. 2011, Ingham et al. 2012, Ogihara et al. 2012, Li et al. 2015
Carbon nanoparticles	Bioimaging tool, cosmetics, protective coatings, rubber additives, sensors, toner	Amaratunga et al. 1996, Athanassiou et al. 2006, Schmid and Riediker 2008, Chandra et al. 2011, Gminski et al. 2011, Zhang et al. 2015
Carbon nanotubes	Biosensors, energy storages, light and hard composite matarials, microelectronics, solar cells, water filters	Gethard et al. 2011, De Volder et al. 2013
Copper oxide nanoparticles	Biosensor, microelectronics, additive in paints, lubricants, heat-transfer fluids, plastics, textiles and propellants	**See chapter 1.1.3**
Iron oxide nanoparticles	Additive in plastics, contrast agents, drug carrier, gene therapy, hyperthermia therapy	Dobson 2006, Schmid and Riediker 2008, Tseng et al. 2015
Gold nanoparticles	Biosensors, drug delivery, gene therapy, surface coatings, textiles, water treatment	Paciotti et al. 2006, Qian et al. 2008, Schmid and Riediker 2008, Conde et al. 2010, Zhang et al. 2015
Platinum nanopaticles	Biosensors, catalysts	(Rioux et al. 2006, Khoshfetrat et al. 2015)
Quantum Dots	Bioimaging, biosensors, laser and light emitting devices, molecular computation, photocatalysts, solar cells	Klimov et al. 2000, Zhao et al. 2013, He et al. 2014, Concina et al. 2015, Kang et al. 2015b, Wang et al. 2015
Silica nanoparticles	Biosensors, cosmetics, drug delivery, microelectronics, paints, paper additives, pharmaceutical products, UV-protection	Trewyn et al. 2007, Schmid and Riediker 2008
Silver nanoparticles	Antimicrobial coatings, catalysts, electronics, wound healing	Abou El-Nour et al. 2010, Kwan et al. 2011, Prabhu and Poulose 2012
Titanium dioxide nanoparticles	Additive in plastics, cleaning products, food packaging, microelectronics, photocatalysts, solar cells, sunscreens	Schmid and Riediker 2008, Paz 2011
Zink oxide nanoparticles	Catalysts, medical products, microelectronics, paints, UV-protection	Schmid and Riediker 2008, Zhang et al. 2015

The gain of applications of these new products and the increasing production output raised awareness of the governments and the scientific community towards the potential adverse environmental consequences of this technology (Krug 2014). Unfortunately, although a lot of ecotoxicological studies on nanomaterials were performed, the high variety of nanomaterials and the strong variation between different test systems makes it difficult to give clear statements on the toxicity of these materials (Juganson et al. 2015). Nevertheless, solid databases might help to predict potential and evaluate the ecological impact of nanomaterials (Juganson et al. 2015). Fortunately, several research frameworks/clusters were formed to evaluate the potential environmental dangers of nanomaterials, for example the EU "NanoSafetyCluster" or the US "National Nanotechnology Initiative".

Silver nanoparticles are a good example for the impact of nanomaterials on the environment. These nanoparticles are increasingly used for functional clothing and can strongly leach out of their support material into the environment, polluting sewage plants and rivers (Schlich et al. 2013, Zhang et al. 2015). Also copper oxide nanoparticles (CuO-NPs), which are used on a big scale in anti fouling paint of ships and in wood preservatives, have close contact to the environment and a high chance to deposit there (Almeida et al. 2007, Perreault et al. 2012b). Those nanoparticles can become extremely toxic to soil and aquatic organisms if the corresponding ions leach out of the particles (Bondarenko et al. 2013). Especially for heavy metal containing nanomaterials, it is of high interest to understand the interaction of the materials with the environment and evaluate the potential risk of an environmental contamination and accumulation during the life cycle of these products (Glover et al. 2011, Zhang et al. 2015). The understanding of the underlying mechanisms of nanomaterial life cycles could assist in the creation of sustainable and non-toxic nanomaterials.

Beside the environmental impact of nanomaterials, substantial amounts of research are focused on the question how those materials could directly affect the human health (Paur et al. 2011). Due to the widespread applications of nanoparticles (Table 2) the risk of an exposure of man to those materials is high. Not only the release of nanoparticles into the environment but also the direct contact to nanoparticle-containing products and the occupational exposure to such materials could create a risk for the human health that has to be assessed. To understand the potential impact of nanoparticles on the human health, it is necessary to consider several aspects such as nanoparticle generation and

occurrence, nanoparticle characteristics, possible exposure routes, targeted organs and effects of those particles on cells and whole organisms. For humans, the primary exposure routes for nanoparticles are ingestion and inhalation, whereas the skin is hardly penetrated (Borm et al. 2006, Kimura et al. 2012). The majority of airborne nanoparticles are incidentally created by the burning of fuels, mechanical abrasion or generation of fumes in the metal industry (Borm et al. 2006, Szymczak et al. 2007, Balkhyour and Goknil 2010).

To investigate the toxicity of nanoparticles several test systems and model organisms can be used (Frohlich and Salar-Behzadi 2014). Many studies on the toxicity of different nanoparticles were carried out in vitro on cultured lung cells (Paur et al. 2011). Nevertheless, cell studies have their limitations and cannot mimic the entire organism. Therefore, to assess the whole picture of nanoparticle toxicity also *in vivo* inhalation and injection studies are necessary. Considering many comparative *in vitro* and *in vivo* studies on the potential toxicity of nanoparticles, it was shown that CuO-NPs were among the most toxic nanoparticles (Fahmy and Cormier 2009, Cronholm et al. 2013, Ivask et al. 2015, Katsnelson et al. 2015).

1.1.3 Copper oxide nanoparticles

The cheap price and the special features of CuO-NPs led to an increased interest from the industry towards this material (Yurderi et al. 2015). Due to their physical properties CuO-NPs are excellent electric conductors, making them interesting for microelectronic products such as conductive ink or sensor elements (Dharmadasa et al. 2013, Yang et al. 2015). Another physical property of CuO-NPs is their effective heat conductivity, making them efficient additives for heat transfer fluids and lubricants (Ettefaghi et al. 2013, Manimaran et al. 2014, Jatti and Singh 2015). The cheap price, the high surface reactivity and the nanoparticle specific high surface area of this material qualifies CuO-NPs as cost-effective catalyst for various chemical reactions (White et al. 2006, Yurderi et al. 2015). However, despite their high application potential there are various disadvantages of this material. The most double edged feature of CuO-NPs is their biocidal activity. On the one hand, CuO-NPs are effectively used in anti-fouling paint, wood preservatives, sterile surface coatings, water filters or in textiles and bandages (Almeida et al. 2007, Evans et al. 2008, Ahmad et al. 2012, Perreault et al. 2012b, Ben-Sasson et al. 2014, Dankovich and Smith 2014). On the other hand, the biocidal activity of CuO-NPs could be unintentionally harmful to the environment and human health

(Karlsson et al. 2008). Therefore, close monitoring of the toxic potential of those particles is necessary to evaluate their risk.

To understand the toxic mechanisms of CuO-NPs it is important to elucidate the uptake and distribution of such particles in the body. In this context it is important to consider, especially for the occupational exposure scenario, that CuO-NPs are unintentionally released from electric motors or during welding (Szymczak et al. 2007, Balkhyour and Goknil 2010). Airborne CuO-NPs are easily inhaled and can thereby enter the body. *In vitro* studies showed the high toxic potential of CuO-NPs to several lung cell lines (Karlsson et al. 2008, Cronholm et al. 2013, Kim et al. 2013, Semisch et al. 2014, Ahamed et al. 2015, Ivask et al. 2015, Jing et al. 2015). *In vivo* inhalation and injection studies on mice and rats confirmed this high toxicity (Chen et al. 2006, Liao and Liu 2012, Privalova et al. 2014, Jing et al. 2015). Interestingly, one of these inhalation studies reported that CuO-NPs were more toxic than µm sized copper oxide particles (Yokohira et al. 2008). It was also reported that inhaled nanoparticles can enter into the blood stream and are also able to cross the blood brain barrier (Kreyling et al. 2002, Oberdorster et al. 2004, Sharma and Sharma 2012, Yim et al. 2012). *In vivo* studies of CuO-NPs showed that those particles can accumulate in the brain and have a high capacity to alter brain functionality (An et al. 2012, Privalova et al. 2014). So far, only very few studies were performed to evaluate the toxicity of CuO-NPs on brain cells (Li et al. 2007, Chen et al. 2008, Xu et al. 2009, Prabhu et al. 2010, Liu et al. 2011, Perreault et al. 2012a). However, all those studies focused on neuronal cell lines, whereas nothing was known about the effect of those particles on astrocytes.

1.2 Astrocytes

Brain cells can be classified in general into two types: the signal conducting and integrating neuronas and the supportive glial cells. The human brain consists of approximately 86 billion neurons and 85 billion glial cells (Herculano-Houzel 2014). The neuron to glia ratio differs strongly between certain brain regions (Herculano-Houzel 2014). In the brain, the glial cell types are divided into microglia, oligodendrocytes and astrocytes (Figure 1.3) (Sofroniew and Vinters 2010). Between these very differing group of cells, astrocytes are the most abundant cell type (Herculano-Houzel 2014). Astrocytes are located between the endothelia cells of the brain blood vessels and neurons (Mathiisen et al. 2010, Sofroniew and Vinters 2010). Various important functions of the brain homeostasis are attributed to astrocytes, of which a few will be described in the following subchapters. After elucidating the key role of astrocytes in the brain, it is no surprise that several neurological and psychiatric disorders are connected to disturbances in the astrocytic functions (Allaman et al. 2011, Rossi 2015, Verkhratsky et al. 2015, Verkhratsky and Parpura 2015, Yamamuro et al. 2015).

1.2.1 Functions at the synapse

Astrocytes are responsible to maintain the neural plasticity in the developing and mature brain (Parpura et al. 2012, Haydon and Nedergaard 2015). They are important interacting partners with neurons and are contributing strongly to the genesis, maturation and maintenance of the synaptic connectivity (Verkhratsky et al. 2015, Verkhratsky and Parpura 2015). Moreover, astrocytes are responsible for the homeostasis of neurotransmitters and ions in the synaptic cleft (Verkhratsky et al. 2015, Verkhratsky and Parpura 2015). Astrocytes are also able to modulate the synaptic transmission by the release of so called gliotransmitters such a glutamate, ɣ-aminobutyric acid, D-serine, ATP and lactate (Volterra and Meldolesi 2005, Gundersen et al. 2015, Verkhratsky et al. 2015, Verkhratsky and Parpura 2015). Due to their connection with neurons and their involvement in signal modulation, astrocytes seem to be strongly involved in cognition, emotion as well as motoric and sensory processing (Oliveira et al. 2015).

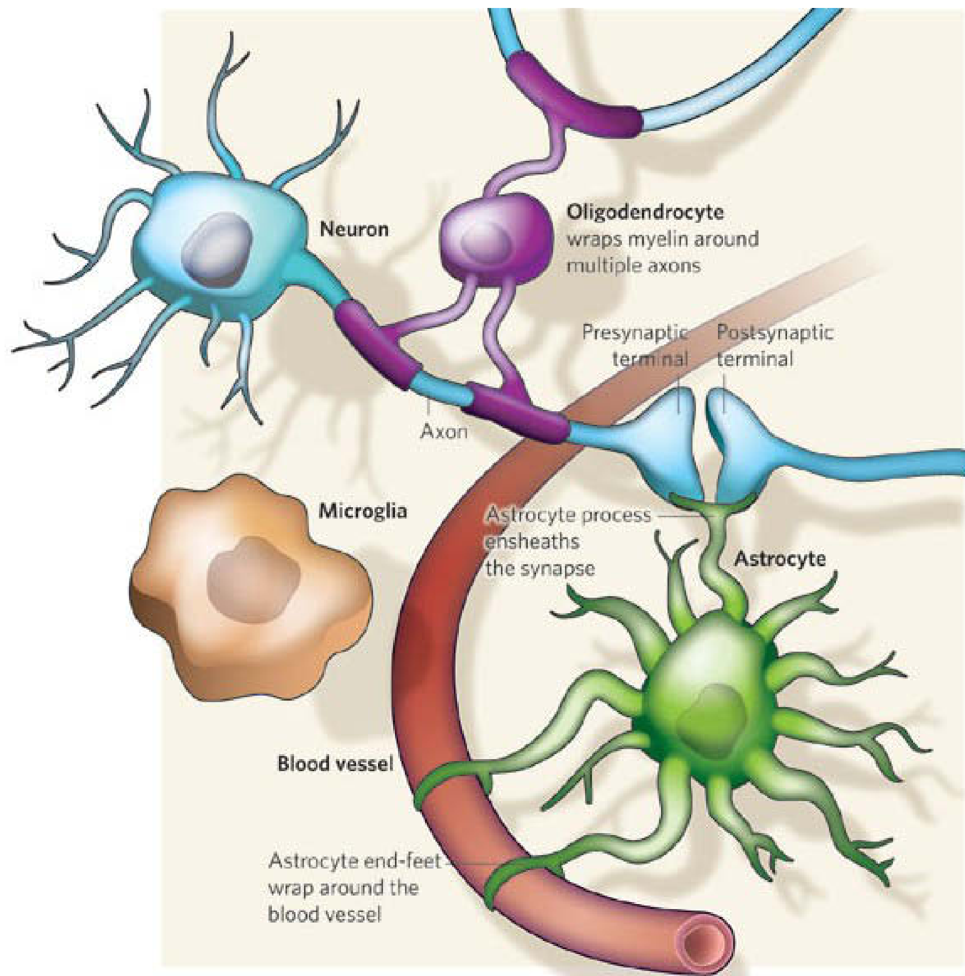

Figure 1.3: Schematic illustration of the cellular organization in the brain. The signal transducing neurons (blue) and their interaction with the major glial cell types such as astrocytes (green), oligodendrocytes (purple) and microglial cells (brown) is shown (Allen and Barres 2009). Adapted with permission from Macmillan Publishers Ltd: Nature, Allen and Barres 2009, "Neuroscience: Glia - more than just brain glue", copyright 2009.

1.2.2 Metabolic interactions with neurons

Neurons also have a tight metabolic interconnection with astrocytes (Pellerin and Magistretti 1994, Magistretti 2006, Allaman et al. 2011, Brekke et al. 2015). Astrocytes are highly glycolytic and are able to export large amounts of lactate (Pellerin and Magistretti 1994, Itoh et al. 2003). Neurons rely on oxidative metabolism and are able to use lactate as an oxidative substrate to produce high amounts of ATP (Bélanger et al. 2011). However, it is still under discussion whether astrocytes export lactate as primary energy fuel for neurons (Mangia et al. 2011, Pellerin and Magistretti 2012, Brekke et al. 2015, Lundgaard et al. 2015). The so called glutamate-glutamine cycle is another metabolic pathway which describes the tight metabolic interaction of astrocytes and

neurons in the synthesis of neurotransmitters (Brekke et al. 2015). Astrocytes export large amounts of glutamine to supply neurons with a precursor for glutamate and ɣ-aminobutyric acid synthesis, since neurons are unable to synthesize sufficient amounts of the neurotrasmitting glutamate and ɣ-aminobutyric acid from glucose (Brekke et al. 2015). Further, astrocytes supply neurons with glutathione precursors to support neuronal glutathione synthesis (Hirrlinger and Dringen 2010, Dringen et al. 2015a).

1.2.3 Detoxification

Due to the tight coverage of the endothelia cells of the blood capillaries by the astrocytic endfeet, astrocytes are the first parenchymal cells of the brain that encounter harmful substances that are crossing the blood brain barrier, such as toxins, heavy metals or drugs (Mathiisen et al. 2010, Sofroniew and Vinters 2010). Therefore it is of high importance that astrocytes are able to handle such compounds. Indeed, astrocytes are capable to tolerate and detoxify various toxins, heavy metals and drugs (Scheiber et al. 2010, Brandmann et al. 2012, Arend et al. 2013, Tulpule and Dringen 2013, Dringen et al. 2015a, Dringen et al. 2015b). Astrocytes have high glutathione contents and a high capability to perform glutathione-dependent detoxification processes (Dringen et al. 2015a). The tripeptide glutathione, which is the major low molecular weight antioxidant of cells, is involved in the detoxification of exogenous toxic substances but is also able to mitigate endogenously generated oxidants (Dringen et al. 2015a). For example, redox active metals like copper can accelerate reactive oxygen species production by effectively catalyzing the formation of reactive oxygen species in presence of H_2O_2 or superoxide in Fenton-like reactions (Pham et al. 2013). However, due to the fact that astrocytes are able to increase their glutathione contents upon exposure to copper and various other potential toxic substances (Dringen et al. 2015a), these cells are considered as the central element for detoxification in the brain (Hirrlinger and Dringen 2010, Schreiner et al. 2015). However, impairment of the astrocytic glutathione homeostasis can lead to a severe loss in the tolerance against oxidative stress (Liddell et al. 2006, Lee et al. 2010).

1.2.4 Functions in the metal metabolism of the brain

Metals such as iron and copper are essential for several cellular functions (Liu et al. 2014). Iron is essential component of iron-sulfur clusters and heme groups in proteins (Liu et al. 2014). These groups are required for the electron transport of the respiratory

chain and are involved in various other enzymatic processes (Liu et al. 2014). Copper is a component of various enzymes which are required for energy metabolism, antioxidative defence, iron metabolism and neurotransmitter synthesis (Liu et al. 2014, Scheiber et al. 2014).

The strategic localization of astrocytes at the endothelia cells of the blood capillaries and the effective uptake and export capabilities of those cells for iron and copper strongly suggests that astrocytes have a central role in the trafficking of those metals to other brain cells (Dringen et al. 2007, Scheiber et al. 2014). Despite the essential function of both metals, iron and copper can become toxic if they are accumulated at higher levels by causing severe generation of reactive oxygen species (Jomova and Valko 2011). Therefore, the uptake, storage and export of those metals by astrocytes are tightly regulated (Tiffany-Castiglioni and Qian 2001, Dringen et al. 2007, Dringen et al. 2013, Scheiber et al. 2014). Further, these cells are able to tolerate and sequester excess amount of toxic metals and therefore are considered as metal sink of the brain (Tiffany-Castiglioni and Qian 2001). The role of astrocytes in the copper metabolism of the brain was recently reviewed in detail (Dringen et al. 2013, Scheiber et al. 2014).

1.2.5 Astrocytes and nanoparticles

Several studies were conducted on cultured astrocytes to test for their resistance against iron oxide and silver nanoparticles (Geppert et al. 2011, Luther et al. 2011, Hohnholt and Dringen 2013, Petters et al. 2014). An overview about these studies was published earlier (Hohnholt et al. 2013). Throughout these studies it has been shown that cultured astrocytes are not very sensitive to those nanomaterials (Geppert et al. 2011, Luther et al. 2011, Hohnholt and Dringen 2013, Petters et al. 2014). These studies also analyzed the uptake and storage mechanism with the help of electron microscopy and fluorescent molecules which were attached to those nanoparticles (Geppert et al. 2011, Petters et al. 2014, Petters et al. 2015). In contrast to iron oxide and silver nanoparticles, recent studies have shown that cultured astrocytes are sensitive against zinc oxide and titanium dioxide nanoparticles and the observed toxicity was correlated to increased oxidative stress (Wang et al. 2014, Wilson et al. 2015). Concerning the ability of astrocytes to deal with CuO-NPs, no literature data were available at the start of the experimental work of the presented thesis. An up to date description how cultured astrocytes deal with CuO-NPs and copper which includes most of the data shown in the presented thesis is included as chapter 1.3 below.

1.3 Publication 1

Handling of copper and copper oxide nanoparticles by Astrocytes

Felix Bulcke and Ralf Dringen

Neurochemical Research 2015, in press

DOI 10.1007/s11064-015-1688-9

Contributions of Felix Bulcke:

- First draft of the chapters “Uptake of Copper Oxide Nanoparticles” and “Conclusions and Perspectives”
- Preparation of all Figures and Tables
- Improvement of the manuscript

The pdf-document of this publication is not displayed due to copyright reasons.

The publication can be accessed at:

http://link.springer.com/article/10.1007%2Fs11064-015-1688-9

DOI: 10.1007/s11064-015-1688-9

1.4 Aim of the thesis

The aim of this thesis is to investigate the uptake and toxicity of CuO-NPs on cultured primary astrocytes. For this purpose, a synthesis method of CuO-NPs will be established and the physicochemical properties of those nanoparticles will be characterized. Subsequently, CuO-NPs will be applied to cultured primary astrocytes in acute and chronic exposure scenarios. The accumulation of CuO-NPs will be evaluated by quantification of cellular copper content and the data obtained for CuO-NPs will be compared to those for copper ions. In this context, experiments will be performed to elucidate the mechanism of nanoparticle uptake. Potential deleterious effects of CuO-NPs and ionic copper on astrocytes will be investigated and it will be tested if compounds like copper chelators and antioxidants can prevent copper mediated toxicity in astrocytes. Finally, it will be investigated whether cultured astrocytes respond to a treatment with sub-toxic concentrations of CuO-NPs with a stimulation of protective mechanisms and with alterations in the cell metabolism.

1.5 References

Abou El-Nour KMM, Eftaiha Aa, Al-Warthan A, Ammar RAA (2010) Synthesis and applications of silver nanoparticles. Arabian J Chem 3:135-140

Ahamed M, Akhtar MJ, Alhadlaq HA, Alrokayan SA (2015) Assessment of the lung toxicity of copper oxide nanoparticles: Current status. Nanomedicine (Lond) 10:2365-2377

Ahmad Z, Vargas-Reus MA, Bakhshi R, Ryan F, Ren GG, Oktar F, Allaker RP (2012) Antimicrobial properties of electrically formed elastomeric polyurethane-copper oxide nanocomposites for medical and dental applications. Methods Enzymol 509:87-99

Akhtar MS, Panwar J, Yun Y-S (2013) Biogenic synthesis of metallic nanoparticles by plant extracts. ACS Sustain Chem Eng 1:591-602

Allaman I, Bélanger M, Magistretti PJ (2011) Astrocyte–neuron metabolic relationships: For better and for worse. Trends Neurosci 34:76-87

Allen NJ and Barres BA (2009) Neuroscience: Glia - more than just brain glue. Nature 457:675-677

Allouche J (2013). Synthesis of organic and bioorganic nanoparticles: An overview of the preparation methods. In: Nanomaterials: A danger or a promise? Eds: R Brayner, F Fiévet and T Coradin, Springer, London, United Kingdom: 27-74

Almeida E, Diamantino TC, de Sousa O (2007) Marine paints: The particular case of antifouling paints. Prog Org Coat 59:2-20

Amaratunga GAJ, Chhowalla M, Kiely CJ, Alexandrou I, Aharonov R, Devenish RM (1996) Hard elastic carbon thin films from linking of carbon nanoparticles. Nature 383:321-323

Amin ML, Joo JY, Yi DK, An SSA (2015) Surface modification and local orientations of surface molecules in nanotherapeutics. J Control Release 207:131-142

An L, Liu S, Yang Z, Zhang T (2012) Cognitive impairment in rats induced by nano-CuO and its possible mechanisms. Toxicol Lett 213:220-227

Arend C, Brandmann M, Dringen R (2013) The antiretroviral protease inhibitor ritonavir accelerates glutathione export from cultured primary astrocytes. Neurochem Res 38:732-741

Athanassiou EK, Grass RN, Stark WJ (2006) Large-scale production of carbon-coated copper nanoparticles for sensor applications. Nanotechnology 17:1668

Balkhyour MA and Goknil MK (2010) Total fume and metal concentrations during welding in selected factories in Jeddah, Saudi Arabia. Int J Environ Res Public Health 7:2978-2987

Bélanger M, Allaman I, Magistretti Pierre J (2011) Brain energy metabolism: Focus on astrocyte-neuron metabolic cooperation. Cell Metab 14:724-738

Ben-Sasson M, Zodrow KR, Genggeng Q, Kang Y, Giannelis EP, Elimelech M (2014) Surface functionalization of thin-film composite membranes with copper nanoparticles for antimicrobial surface properties. Environ Sci Technol 48:384-393

Binnig G and Rohrer H (1986) Scanning tunneling microscopy. IBM J Res Dev 30:4

Bondarenko O, Juganson K, Ivask A, Kasemets K, Mortimer M, Kahru A (2013) Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: A critical review. Arch Toxicol 87:1181-1200

Borm PJ, Robbins D, Haubold S, Kuhlbusch T, Fissan H, Donaldson K, Schins R, Stone V, Kreyling W, Lademann J, Krutmann J, Warheit D, Oberdorster E (2006) The potential risks of nanomaterials: a review carried out for ECETOC. Part Fibre Toxicol 3:11

Brandmann M, Tulpule K, Schmidt MM, Dringen R (2012) The antiretroviral protease inhibitors indinavir and nelfinavir stimulate Mrp1-mediated GSH export from cultured brain astrocytes. J Neurochem 120:78-92

Brekke E, Morken TS, Sonnewald U (2015) Glucose metabolism and astrocyte–neuron interactions in the neonatal brain. Neurochem Int 82:33-41

Chandra S, Das P, Bag S, Laha D, Pramanik P (2011) Synthesis, functionalization and bioimaging applications of highly fluorescent carbon nanoparticles. Nanoscale 3:1533-1540

Chang EH, Harford JB, Eaton MAW, Boisseau PM, Dube A, Hayeshi R, Swai H, Lee DS (2015) Nanomedicine: past, present and future – a global perspective. Biochem Biophys Res Commun 468:511-517

Chen J, Zhu J, Cho H-H, Cui K, Li F, Zhou X, Rogers JT, Wong STC, Huang X (2008) Differential cytotoxicity of metal oxide nanoparticles. J Exp Nanosci 3:321-328

Chen Z, Meng H, Xing G, Chen C, Zhao Y, Jia G, Wang T, Yuan H, Ye C, Zhao F, Chai Z, Zhu C, Fang X, Ma B, Wan L (2006) Acute toxicological effects of copper nanoparticles in vivo. Toxicol Lett 163:109-120

Concina I, Manzoni C, Grancini G, Celikin M, Soudi A, Rosei F, Zavelani-Rossi M, Cerullo G, Vomiero A (2015) Modulating exciton dynamics in composite nanocrystals for excitonic solar cells. J Phys Chem Lett 6:2489-2495

Conde J, de la Fuente JM, Baptista PV (2010) In vitro transcription and translation inhibition via DNA functionalized gold nanoparticles. Nanotechnology 21:505101

Consumer Products Inventory (2015) Project on emerging nanotechnologies. Retrieved 09.01.2016, from nanotechproject.org/cpi

Cronholm P, Karlsson HL, Hedberg J, Lowe TA, Winnberg L, Elihn K, Wallinder IO, Moller L (2013) Intracellular uptake and toxicity of Ag and CuO nanoparticles: a comparison between nanoparticles and their corresponding metal ions. Small 9:970-982

Cupaioli FA, Zucca FA, Boraschi D, Zecca L (2014) Engineered nanoparticles. How brain friendly is this new guest? Prog Neurobiol 119–120:20-38

Daniel MC and Astruc D (2004) Gold nanoparticles: assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology. Chem Rev 104:293-346

Danish Consumer Council (2015) The Nanodatabase. Retrieved 09.01.2016, from nanodb.dk

Dankovich TA and Smith JA (2014) Incorporation of copper nanoparticles into paper for point-of-use water purification. Water Res 63:245-251

De Volder MFL, Tawfick SH, Baughman RH, Hart AJ (2013) Carbon nanotubes: Present and future commercial applications. Science 339:535-539

Dharmadasa R, Jha M, Amos DA, Druffel T (2013) Room temperature synthesis of a copper ink for the Intense pulsed light sintering of conductive copper films. ACS Appl Mater Interfaces 5:13227-13234

Dobson J (2006) Gene therapy progress and prospects: magnetic nanoparticle-based gene delivery. Gene Ther 13:283-287

Dringen R, Bishop G, Koeppe M, Dang T, Robinson S (2007) The pivotal role of astrocytes in the metabolism of iron in the brain. Neurochem Res 32:1884-1890

Dringen R, Brandmann M, Hohnholt M, Blumrich E-M (2015a) Glutathione-dependent detoxification processes in astrocytes. Neurochem Res 40:2570-2582

Dringen R, Scheiber IF, Mercer JFB (2013) Copper metabolism of astrocytes. Front Aging Neurosci 5:9

Dringen R, Spiller S, Neumann S, Koehler Y (2015b) Uptake, metabolic effects and toxicity of arsenate and arsenite in astrocytes. Neurochem Res, in press DOI: 10.1007/s11064-11015-11570-11069

Eigler DM and Schweizer EK (1990) Positioning single atoms with a scanning tunnelling microscope. Nature 344:524-526

Ettefaghi E-o-I, Ahmadi H, Rashidi A, Mohtasebi SS, Alaei M (2013) Experimental evaluation of engine oil properties containing copper oxide nanoparticles as a nanoadditive. Int J Ind Chem 4:1-6

Evans P, Matsunaga H, Kiguchi M (2008) Large-scale application of nanotechnology for wood protection. Nat Nanotechnol 3:577

Fahmy B and Cormier SA (2009) Copper oxide nanoparticles induce oxidative stress and cytotoxicity in airway epithelial cells. Toxicol in Vitro 23:1365-1371

Feynman R (1960) There's plenty of room at the bottom. Eng Sci 23:22-36

Frohlich E and Salar-Behzadi S (2014) Toxicological assessment of inhaled nanoparticles: role of in vivo, ex vivo, in vitro, and in silico studies. Int J Mol Sci 15:4795-4822

Geppert M, Hohnholt M, Thiel K, Nürnberger S, Grunwald I, Dringen R (2011) Uptake of dimercaptosuccinate-coated magnetic iron oxide nanoparticles by cultured brain astrocytes. Nanotechnology 22:145101

Gethard K, Sae-Khow O, Mitra S (2011) Water desalination using carbon-nanotube-enhanced membrane distillation. ACS Appl Mater Interfaces 3:110-114

Glover RD, Miller JM, Hutchison JE (2011) Generation of metal nanoparticles from slver and copper objects: nanoparticle dynamics on surfaces and potential sources of nanoparticles in the environment. ACS Nano 5:8950-8957

Gminski R, Decker K, Heinz C, Seidel A, Könczöl M, Goldenberg E, Grobéty B, Ebner W, Gieré R, Mersch-Sundermann V (2011) Genotoxic effects of three selected black toner powders and their dimethyl sulfoxide extracts in cultured human epithelial A549 lung cells in vitro. Environ Mol Mutagen 52:296-309

Gundersen V, Storm-Mathisen J, Bergersen LH (2015) Neuroglial transmission. Physiol Rev 95:695-726

Haydon PG and Nedergaard M (2015) How do astrocytes participate in neural plasticity? Cold Spring Harb Perspect Biol 7:a020438

He X, Li Z, Chen M, Ma N (2014) DNA-pogrammed dynamic assembly of quantum dots for molecular computation. Angew Chem Int Ed 53:14447-14450

Heiligtag FJ and Niederberger M (2013) The fascinating world of nanoparticle research. Mater Today 16:262-271

Herculano-Houzel S (2014) The glia/neuron ratio: How it varies uniformly across brain structures and species and what that means for brain physiology and evolution. Glia 62:1377-1391

Hirrlinger J and Dringen R (2010) The cytosolic redox state of astrocytes: Maintenance, regulation and functional implications for metabolite trafficking. Brain Res Rev 63:177-188

Hohnholt M, Geppert M, Luther E, Petters C, Bulcke F, Dringen R (2013) Handling of iron oxide and silver nanoparticles by astrocytes. Neurochem Res 38:227-239

Hohnholt Michaela C and Dringen R (2013) Uptake and metabolism of iron and iron oxide nanoparticles in brain astrocytes. Biochem Soc Trans 41:1588-1592

Horikoshi S and Serpone N (2013). Introduction to Nanoparticles. In: Microwaves in nanoparticle synthesis: Fundamentals and applications. Eds: S Horikoshi and N Serpone, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany: 1-24

Iijima S (1991) Helical microtubules of graphitic carbon. Nature 354:56-58

Ingham CJ, ter Maat J, de Vos WM (2012) Where bio meets nano: The many uses for nanoporous aluminum oxide in biotechnology. Biotechnol Adv 30:1089-1099

Itoh Y, Esaki T, Shimoji K, Cook M, Law MJ, Kaufman E, Sokoloff L (2003) Dichloroacetate effects on glucose and lactate oxidation by neurons and astroglia in vitro and on glucose utilization by brain in vivo. Proc Natl Acad Sci 100:4879-4884

Ivask A, Titma T, Visnapuu M, Vija H, Kakinen A, Sihtmae M, Pokhrel S, Madler L, Heinlaan M, Kisand V, Shimmo R, Kahru A (2015) Toxicity of 11 metal oxide nanoparticles to three mammalian cell types in vitro. Curr Top in Med Chem 15:1914-1929

Jatti V and Singh TP (2015) Copper oxide nano-particles as friction-reduction and anti-wear additives in lubricating oil. J Mech Sci Technol 29:793-798

Jing X, Park JH, Peters TM, Thorne PS (2015) Toxicity of copper oxide nanoparticles in lung epithelial cells exposed at the air–liquid interface compared with in vivo assessment. Toxicol in Vitro 29:502-511

Jo DH, Kim JH, Lee TG, Kim JH (2015) Size, surface charge, and shape determine therapeutic effects of nanoparticles on brain and retinal diseases. Nanomedicine 11:1603-1611

Jomova K and Valko M (2011) Advances in metal-induced oxidative stress and human disease. Toxicology 283:65-87

Juganson K, Ivask A, Blinova I, Mortimer M, Kahru A (2015) NanoE-Tox: New and in-depth database concerning ecotoxicity of nanomaterials. Beilstein J Nanotechnol 6:1788-1804

Kang L, Gao Z, Huang W, Jin M, Wang Q (2015a) Nanocarrier-mediated co-delivery of chemotherapeutic drugs and gene agents for cancer treatment. Acta Pharm Sin B 5:169-175

Kang YF, Li YH, Fang YW, Xu Y, Wei XM, Yin XB (2015b) Carbon quantum dots for zebrafish fluorescence imaging. Sci Rep 5:11835

Karlsson HL, Cronholm P, Gustafsson J, Möller L (2008) Copper oxide nanoparticles are highly toxic: A comparison between metal oxide nanoparticles and carbon nanotubes. Chem Res Toxicol 21:1726-1732

Katsnelson BA, Privalova LI, Sutunkova MP, Gurvich VB, Loginova NV, Minigalieva IA, Kireyeva EP, Shur VY, Shishkina EV, Beikin YB, Makeyev OH, Valamina IE

(2015) Some inferences from in vivo experiments with metal and metal oxide nanoparticles: the pulmonary phagocytosis response, subchronic systemic toxicity and genotoxicity, regulatory proposals, searching for bioprotectors (a self-overview). Int J Nanomedicine 10:3013-3029

Kettler K, Veltman K, van de Meent D, van Wezel A, Hendriks AJ (2014) Cellular uptake of nanoparticles as determined by particle properties, experimental conditions, and cell type. Environ Toxicol Chem 33:481-492

Khoshfetrat SM, Ranjbari M, Shayan M, Mehrgardi MA, Kiani A (2015) Wireless electrochemiluminescence bipolar electrode array for visualized genotyping of single nucleotide polymorphism. Anal Chem 87:8123-8131

Kim JS, Peters TM, O'Shaughnessy PT, Adamcakova-Dodd A, Thorne PS (2013) Validation of an in vitro exposure system for toxicity assessment of air-delivered nanomaterials. Toxicol in Vitro 27:164-173

Kimura E, Kawano Y, Todo H, Ikarashi Y, Sugibayashi K (2012) Measurement of skin permeation/penetration of nanoparticles for their safety evaluation. Biol Pharm Bull 35:1476-1486

Klimov VI, Mikhailovsky AA, Xu S, Malko A, Hollingsworth JA, Leatherdale CA, Eisler H-J, Bawendi MG (2000) Optical gain and stimulated emission in nanocrystal quantum dots. Science 290:314-317

Knoll M and Ruska E (1932) Das Elektronenmikroskop. Z Physik 78:318-339

Koyama T and Endo M (1973) Structure and growth processes of vapor-grown carbon fibers. Oyo Buturi 42:690-696

Kreyling WG, Semmler M, Erbe F, Mayer P, Takenaka S, Schulz H, Oberdorster G, Ziesenis A (2002) Translocation of ultrafine insoluble iridium particles from lung epithelium to extrapulmonary organs is size dependent but very low. J Toxicol Environ Health A 65:1513-1530

Kroto HW, Heath JR, O'Brien SC, Curl RF, Smalley RE (1985) C60: Buckminsterfullerene. Nature 318:162-163

Krug HF (2014) Nanosafety research—Are we on the right track? Angew Chem Int Ed 53:12304-12319

Kwan KHL, Liu X, To MKT, Yeung KWK, Ho C-m, Wong KKY (2011) Modulation of collagen alignment by silver nanoparticles results in better mechanical properties in wound healing. Nanomedicine 7:497-504

Lee M, Cho T, Jantaratnotai N, Wang YT, McGeer E, McGeer PL (2010) Depletion of GSH in glial cells induces neurotoxicity: relevance to aging and degenerative neurological diseases. FASEB J 24:2533-2545

Li F, Zhou X, Zhu J, Ma J, Huang X, Wong S (2007) High content image analysis for human H4 neuroglioma cells exposed to CuO nanoparticles. BMC Biotech 7:1-11

Li S, Niu J, Zhao YC, So KP, Wang C, Wang CA, Li J (2015) High-rate aluminium yolk-shell nanoparticle anode for Li-ion battery with long cycle life and ultrahigh capacity. Nat Commun 6:7872

Liao M and Liu H (2012) Gene expression profiling of nephrotoxicity from copper nanoparticles in rats after repeated oral administration. Environ Toxicol Pharmacol 34:67-80

Liddell JR, Dringen R, Crack PJ, Robinson SR (2006) Glutathione peroxidase 1 and a high cellular glutathione concentration are essential for effective organic hydroperoxide detoxification in astrocytes. Glia 54:873-879

Liu J, Chakraborty S, Hosseinzadeh P, Yu Y, Tian S, Petrik I, Bhagi A, Lu Y (2014) Metalloproteins containing cytochrome, iron–sulfur, or copper redox centers. Chem Rev 114:4366-4469

Liu Z, Liu S, Ren G, Zhang T, Yang Z (2011) Nano-CuO inhibited voltage-gated sodium current of hippocampal CA1 neurons via reactive oxygen species but independent from G-proteins pathway. J Appl Toxicol 31:439-445

Lundgaard I, Li B, Xie L, Kang H, Sanggaard S, Haswell JDR, Sun W, Goldman S, Blekot S, Nielsen M, Takano T, Deane R, Nedergaard M (2015) Direct neuronal glucose uptake heralds activity-dependent increases in cerebral metabolism. Nat Commun 6:6807

Luther E, Koehler Y, Diendorf J, Epple M, Dringen R (2011) Accumulation of silver nanoparticles by cultured primary brain astrocytes. Nanotechnology 22:375101

Magistretti PJ (2006) Neuron–glia metabolic coupling and plasticity. J Exp Biol 209:2304-2311

Mangia S, DiNuzzo M, Giove F, Carruthers A, Simpson IA, Vannucci SJ (2011) Response to "comment on recent modeling studies of astrocyte-neuron metabolic interactions": Much ado about nothing. J Cereb Blood Flow Metab 31:1346-1353

Manimaran R, Palaniradja K, Alagumurthi N, Sendhilnathan S, Hussain J (2014) Preparation and characterization of copper oxide nanofluid for heat transfer applications. Appl Nanosci 4:163-167

Mathiisen TM, Lehre KP, Danbolt NC, Ottersen OP (2010) The perivascular astroglial sheath provides a complete covering of the brain microvessels: An electron microscopic 3D reconstruction. Glia 58:1094-1103

Novoselov KS, Geim AK, Morozov SV, Jiang D, Zhang Y, Dubonos SV, Grigorieva IV, Firsov AA (2004) Electric field effect in atomically thin carbon films. Science 306:666-669

Oberdorster G, Sharp Z, Atudorei V, Elder A, Gelein R, Kreyling W, Cox C (2004) Translocation of inhaled ultrafine particles to the brain. Inhal Toxicol 16:437-445

Ogihara N, Usui Y, Aoki K, Shimizu M, Narita N, Hara K, Nakamura K, Ishigaki N, Takanashi S, Okamoto M, Kato H, Haniu H, Ogiwara N, Nakayama N, Taruta S, Saito N (2012) Biocompatibility and bone tissue compatibility of alumina ceramics reinforced with carbon nanotubes. Nanomedicine (Lond) 7:981-993

Oliveira JF, Sardinha VM, Guerra-Gomes S, Araque A, Sousa N (2015) Do stars govern our actions? Astrocyte involvement in rodent behavior. Trends Neurosci 38:535-549

Paciotti GF, Kingston DGI, Tamarkin L (2006) Colloidal gold nanoparticles: a novel nanoparticle platform for developing multifunctional tumor-targeted drug delivery vectors. Drug Dev Res 67:47-54

Parpura V, Heneka MT, Montana V, Oliet SH, Schousboe A, Haydon PG, Stout RF, Jr., Spray DC, Reichenbach A, Pannicke T, Pekny M, Pekna M, Zorec R, Verkhratsky A (2012) Glial cells in (patho)physiology. J Neurochem 121:4-27

Paur H-R, Cassee FR, Teeguarden J, Fissan H, Diabate S, Aufderheide M, Kreyling WG, Hänninen O, Kasper G, Riediker M, Rothen-Rutishauser B, Schmid O (2011) In-vitro cell exposure studies for the assessment of nanoparticle toxicity in the lung—A dialog between aerosol science and biology. J Aerosol Sci 42:668-692

Pautler M and Brenner S (2010) Nanomedicine: Promises and challenges for the future of public health. Int J Nanomed 5:803-809

Paz Y (2011) Self-assembled monolayers and titanium dioxide: From surface patterning to potential applications. Beilstein J Nanotechnol 2:845-861

Pellerin L and Magistretti PJ (1994) Glutamate uptake into astrocytes stimulates aerobic glycolysis: A mechanism coupling neuronal activity to glucose utilization. Proc Natl Acad Sci 91:10625-10629

Pellerin L and Magistretti PJ (2012) Sweet sixteen for ANLS. J Cereb Blood Flow Metab 32:1152-1166

Pérez-Herrero E and Fernández-Medarde A (2015) Advanced targeted therapies in cancer: Drug nanocarriers, the future of chemotherapy. Eur J Pharm Biopharm 93:52-79

Perreault F, Melegari SP, da Costa CH, de Oliveira Franco Rossetto AL, Popovic R, Matias WG (2012a) Genotoxic effects of copper oxide nanoparticles in Neuro 2A cell cultures. Sci Total Environ 441:117-124

Perreault F, Oukarroum A, Melegari SP, Matias WG, Popovic R (2012b) Polymer coating of copper oxide nanoparticles increases nanoparticles uptake and toxicity in the green alga Chlamydomonas reinhardtii. Chemosphere 87:1388-1394

Petters C, Irrsack E, Koch M, Dringen R (2014) Uptake and metabolism of iron oxide nanoparticles in brain cells. Neurochem Res 39:1648-1660

Petters C, Thiel K, Dringen R (2015) Lysosomal iron liberation is responsible for the vulnerability of brain microglial cells to iron oxide nanoparticles: comparison with neurons and astrocytes. Nanotoxicology, in press DOI:10.3109/17435390.2015.1071445

Pham AN, Xing G, Miller CJ, Waite TD (2013) Fenton-like copper redox chemistry revisited: Hydrogen peroxide and superoxide mediation of copper-catalyzed oxidant production. J Catal 301:54-64

Piccinno F, Gottschalk F, Seeger S, Nowack B (2012) Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. J Nanopart Res 14:1-11

Prabhu BM, Ali SF, Murdock RC, Hussain SM, Srivatsan M (2010) Copper nanoparticles exert size and concentration dependent toxicity on somatosensory neurons of rat. Nanotoxicology 4:150-160

Prabhu S and Poulose E (2012) Silver nanoparticles: mechanism of antimicrobial action, synthesis, medical applications, and toxicity effects. Int Nano Lett 2:1-10

Privalova LI, Katsnelson BA, Loginova NV, Gurvich VB, Shur VY, Valamina IE, Makeyev OH, Sutunkova MP, Minigalieva IA, Kireyeva EP, Rusakov VO, Tyurnina AE, Kozin RV, Meshtcheryakova EY, Korotkov AV, Shuman EA, Zvereva AE, Kostykova SV (2014) Subchronic toxicity of copper oxide nanoparticles and its attenuation with the help of a combination of bioprotectors. Int J Mol Sci 15:12379-12406

Qian X, Peng X-H, Ansari DO, Yin-Goen Q, Chen GZ, Shin DM, Yang L, Young AN, Wang MD, Nie S (2008) In vivo tumor targeting and spectroscopic detection with surface-enhanced Raman nanoparticle tags. Nat Biotechnol 26:83-90

Rioux RM, Song H, Grass M, Habas S, Niesz K, Hoefelmeyer JD, Yang P, Somorjai GA (2006) Monodisperse platinum nanoparticles of well-defined shape: Synthesis, characterization, catalytic properties and future prospects. Top Catal 39:167-174

Rossi D (2015) Astrocyte physiopathology: At the crossroads of intercellular networking, inflammation and cell death. Prog Neurobiol 130:86-120

Scheiber IF, Mercer JFB, Dringen R (2010) Copper accumulation by cultured astrocytes. Neurochem Int 56:451-460

Scheiber IF, Mercer JFB, Dringen R (2014) Metabolism and functions of copper in brain. Prog Neurobiol 116:33-57

Schlich K, Klawonn T, Terytze K, Hund-Rinke K (2013) Hazard assessment of a silver nanoparticle in soil applied via sewage sludge. Environ Sci Eur 25:1-14

Schmid K and Riediker M (2008) Use of nanoparticles in Swiss industry: A targeted survey. Environ Sci Technol 42:2253-2260

Schreiner B, Romanelli E, Liberski P, Ingold-Heppner B, Sobottka-Brillout B, Hartwig T, Chandrasekar V, Johannssen H, Zeilhofer Hanns U, Aguzzi A, Heppner F, Kerschensteiner M, Becher B (2015) Astrocyte depletion impairs redox homeostasis and triggers neuronal loss in the adult CNS. Cell Rep 12:1377-1384

Semisch A, Ohle J, Witt B, Hartwig A (2014) Cytotoxicity and genotoxicity of nano - and microparticulate copper oxide: role of solubility and intracellular bioavailability. Part Fibre Toxicol 11:10

Sharma HS and Sharma A (2012) Neurotoxicity of engineered nanoparticles from metals. CNS Neurol Disord Drug Targets 11:65-80

Sofroniew M and Vinters H (2010) Astrocytes: Biology and pathology. Acta Neuropathol 119:7-35

Sperling RA and Parak WJ (2010) Surface modification, functionalization and bioconjugation of colloidal inorganic nanoparticles. Philos Trans A Math Phys Eng Sci 368:1333-1383

Szymczak W, Menzel N, Keck L (2007) Emission of ultrafine copper particles by universal motors controlled by phase angle modulation. J Aerosol Sci 38:520-531

Taniguchi N (1974) On the basic concept of 'Nano-technology'. Proc Intl Conf Prod Eng Tokyo, Part II, Japan Society of Precision Engineering 18-23

Tiffany-Castiglioni E and Qian Y (2001) Astroglia as metal depots: Molecular mechanisms for metal accumulation, storage and release. Neurotoxicology 22:577-592

Trewyn BG, Giri S, Slowing II, Lin VSY (2007) Mesoporous silica nanoparticle based controlled release, drug delivery, and biosensor systems. Chem Comm 3236-3245

Tseng SH, Chou MY, Chu IM (2015) Cetuximab-conjugated iron oxide nanoparticles for cancer imaging and therapy. Int J Nanomed 10:3663-3685

Tulpule K and Dringen R (2013) Formaldehyde in brain: An overlooked player in neurodegeneration? J Neurochem 127:7-21

Verkhratsky A, Nedergaard M, Hertz L (2015) Why are astrocytes important? Neurochem Res 40:389-401

Verkhratsky A and Parpura V (2015) Astrogliopathology in neurological, neurodevelopmental and psychiatric disorders. Neurobiol Dis 85:254–261

Volterra A and Meldolesi J (2005) Astrocytes, from brain glue to communication elements: the revolution continues. Nat Rev Neurosci 6:626-640

von Ardenne M (1938) Das Elektronen-Rastermikroskop. Z Physik 109:553-572

Wang J, Deng X, Zhang F, Chen D, Ding W (2014) ZnO nanoparticle-induced oxidative stress triggers apoptosis by activating JNK signaling pathway in cultured primary astrocytes. Nanoscale Res Lett 9:117

Wang L, Zheng J, Yang S, Wu C, Liu C, Xiao Y, Li Y, Qing Z, Yang R (2015) Two-photon sensing and imaging of endogenous biological cyanide in plant tissues using graphene quantum dot/gold nanoparticle conjugate. ACS Appl Mater Interfaces 7:19509-19515

Wang M, Meng G, Huang Q, Li M, Li Z, Tang C (2011) Fluorescence detection of trace PCB101 based on PITC immobilized on porous AAO membrane. Analyst 136:278-281

White B, Yin M, Hall A, Le D, Stolbov S, Rahman T, Turro N, O'Brien S (2006) Complete CO oxidation over Cu2O nanoparticles supported on silica gel. Nano Lett 6:2095-2098

Wilson CL, Natarajan V, Hayward SL, Khalimonchuk O, Kidambi S (2015) Mitochondrial dysfunction and loss of glutamate uptake in primary astrocytes exposed to titanium dioxide nanoparticles. Nanoscale 7:18477-18488

Xu LJ, Zhao JX, Zhang T, Ren GG, Yang Z (2009) In vitro study on influence of nano particles of CuO on CA1 pyramidal neurons of rat hippocampus potassium currents. Environ Toxicol 24:211-217

Yamamuro K, Kimoto S, Rosen KM, Kishimoto T, Makinodan M (2015) Potential primary roles of glial cells in the mechanisms of psychiatric disorders. Front Cell Neurosci 9:154

Yang M, He J, Hu M, Hu X, Yan C, Cheng Z (2015) Synthesis of copper oxide nanoparticles and their sensing property to hydrogen cyanide under varied humidity conditions. Sens Actuators B 213:59-64

Yim YS, Choi JS, Kim GT, Kim CH, Shin TH, Kim DG, Cheon J (2012) A facile approach for the delivery of inorganic nanoparticles into the brain by passing through the blood-brain barrier (BBB). Chem Comm 48:61-63

Yokohira M, Kuno T, Yamakawa K, Hosokawa K, Matsuda Y, Hashimoto N, Suzuki S, Saoo K, Imaida K (2008) Lung toxicity of 16 fine particles on intratracheal instillation in a bioassay model using F344 male rats. Toxicol Pathol 36:620-631

Yurderi M, Bulut A, Ertas İE, Zahmakiran M, Kaya M (2015) Supported copper–copper oxide nanoparticles as active, stable and low-cost catalyst in the methanolysis of ammonia–borane for chemical hydrogen storage. Appl Catal B 165:169-175

Zhang Y, Leu Y-R, Aitken R, Riediker M (2015) Inventory of engineered nanoparticle-containing consumer products available in the Singapore retail market and likelihood of release into the aquatic environment. Int J Environ Res Public Health 12:8717

Zhao J, Holmes MA, Osterloh FE (2013) Quantum confinement controls photocatalysis: A free energy analysis for photocatalytic proton reduction at CdSe nanocrystals. ACS Nano 7:4316-4325

2. Results

2.1 Publication 2: Uptake and toxicity of copper oxide nanoparticles in cultured primary brain astrocytes

2.2 Publication 3: Copper oxide nanoparticles simulate glycolytic flux and increase the cellular contents of glutathione and metallothioneins in cultured astrocytes

2.3 Publication 4: Modulation of copper accumulation and copper-induced toxicity by antioxidants and copper chelators in cultured primary brain astrocytes

2.4 Endocytotic uptake of CuO-NPs by cultured primary astrocytes

2.1 Publication 2

Uptake and toxicity of copper oxide nanoparticles in cultured primary brain astrocytes

Felix Bulcke, Karsten Thiel and Ralf Dringen

Nanotoxicology. 2014, 8:775-85
DOI: 10.3109/17435390.2013.829591

Contributions of Felix Bulcke:

- Experimental work except Figure 1A-C
- Preparation of the first draft of the manuscript
- Preparation of Figures and Tables
- Improvement of the manuscript

Karsten Thiel performed transmission electron microscopy and the elemental analysis of CuO-NPs as seen in Figure 1A-C.

The pdf-document of this publication is not displayed due to copyright reasons.

The publication can be accessed at:

http://www.tandfonline.com/doi/abs/10.3109/17435390.2013.829591

DOI: 10.3109/17435390.2013.829591

2.2 Publication 3

Copper oxide nanoparticles simulate glycolytic flux and increase the cellular contents of glutathione and metallothioneins in cultured astrocytes

Felix Bulcke and Ralf Dringen

Neurochemical Research 2015, 40:15-26
DOI: 10.1007/s11064-014-1458-0

Contributions of Felix Bulcke:

- Experimental work
- Preparation of the first draft of the manuscript
- Preparation of Figures and Tables
- Improvement of the manuscript

The pdf-document of this publication is not displayed due to copyright reasons.

The publication can be accessed at:

http://link.springer.com/article/10.1007%2Fs11064-014-1458-0

DOI: 10.1007/s11064-014-1458-0

2.3 Publication 4

Modulation of copper accumulation and copper-induced toxicity by antioxidants and copper chelators in cultured primary brain astrocytes

Felix Bulcke, Patricia Santofimia-Castaño, Antonio Gonzalez-Mateos and Ralf Dringen

Journal of Trace Elements in Medicine and Biology 2015, 32:168-76
DOI: 10.1016/j.jtemb.2015.07.001.

Contributions of Felix Bulcke:

- Experimental work for Figure 2, 3 and 5
- Half of the experimental work for Table 1
- Preparation of the first draft of the manuscript in collaboration with Patricia Santofimia-Castaño
- Praparation of all Figures and Tables

Patricia Santofimia-Castaño obtained the experimental data shown in Figure 1, 4 and half of the date for Table 1.

The pdf-document of this publication is not displayed due to copyright reasons.

The publication can be accessed at:

http://www.sciencedirect.com/science/article/pii/S0946672X15300158

DOI: 10.1016/j.jtemb.2015.07.001.

2.4 Endocytotic uptake of CuO-NPs by cultured primary astrocytes

2.4.1 Introduction

2.4.2 Materials and methods

2.4.3 Results

2.4.4 Discussion

2.4.5 Refecences

2.4 Endocytotic uptake of CuO-NPs by cultured primary astrocytes

2.4.1 Introduction

The cellular uptake of nanoparticles can be mediated by different endocytotic pathways (Zhang et al. 2009, Iversen et al. 2011). Primary cultured astrocytes are able to accumulate iron oxide nanoparticles and silver nanoparticles by macropinocytosis and clathrin-mediated endocytosis (Luther et al. 2011, Geppert et al. 2012). In contrast, the uptake mechanism for copper oxide nanoparticles (CuO-NPs) into these cells has not been reported so far. The experimental data presented in this chapter will elucidate a potential contribution of different endocytotic pathways in the uptake of CuO-NPs into cultured primary astrocytes by applying inhibitors of different endocytotic pathways.

2.4.2 Materials and methods

All materials and chemical used, were purchased from the suppliers given in the previous chapters (Chapter 2.1-2.3) or recent publications (Luther et al. 2011, Geppert et al. 2012, Petters and Dringen 2015).

2.4.2.1 Synthesis and characterization of protein coated CuO-NPs

CuO-NPs were synthesized according to a previously published method (Bulcke et al 2014 - Chapter 2.1). These particles were coated with bovine serum albumin (BSA). For this purpose, a solution of 20 mg per mL BSA was prepared in water and 72.5 µl of this solution was mixed with 500 µL CuO-NPs (30-40 mM) for 30 s. The resulting solution was mixed with 1 mL of a high salt mixture (200 mM HEPES, 50 mM glucose, 18 mM $CaCl_2$, 10 mM $MgCl_2$, 54 mM KCl, 1.45 M NaCl, pH 7.4 at room temperature) for 30 s and subsequently centrifuged for 5 min at 12000 g. The resulting supernatant was discarded and the remaining pellet containing BSA coated CuO-NPs (pCuO-NPs) was redispersed in 500 µL H_2O and sonified for 30 s as described previously (Bulcke et al 2014 - Chapter 2.1). The copper concentration of the resulting pCuO-NP solution was determined by atomic absorption spectroscopy as described previously (Bulcke et al 2014 - Chapter 2.1). Concentrations of pCuO-NPs were given as concentration of the total copper present in the pCuO-NP-containing dispersions used and not as particle concentrations. The synthesized pCuO-NPs were characterized by dynamic and electrophoretic light scattering as described previously (Bulcke et al 2014 - Chapter 2.1).

The hydrodynamic diameter and the ζ-potential of the synthesized pCuO-NP was in water 152 ± 9 nm and -35 ± 3 mV (n=3, measurements were kindly performed by Arundhati Joshi). When dispersed in in incubation buffer (20 mM HEPES, 5 mM glucose, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.4 mM KCl, 145 mM NaCl, adjusted to pH 7.4 at 37°C) containing 0.5 mg per mL BSA (IB-BSA), the hydrodynamic diameter pCuO-NPs slightly increased and the ζ-potential was less negative (165 ± 12 nm; -14 ± 4 mV). The synthesized pCuO-NPs had a slightly higher hydrodynamic diameter and less negative ζ-potential compared to previously obtained data on non protein coated particles (Bulcke et al. 2014 - Chapter 2.1). However, in IB-BSA the pCuO-NPs had similar properties as compared to CuO-NPs dispersed in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS; DMEM-FCS) (Bulcke et al. 2014 - Chapter 2.1).

2.4.2.2 Cell culture experiments

Primary astrocytes cultures were obtained as described previously and seeded in 24-well plates (Tulpule et al. 2014). Presence of astrocytes was determined by immunocytochemical staining for the astrocytes specific marker glial fibrillary acidic protein (GFAP) as described previously (Petters and Dringen 2014). Confocal images were taken using a Zeiss LSM 510 META laser scanning microscope equipped with argon and helium-neon lasers (Zeiss, Oberkochen, Germany). The immunocyotochemical staining for GFAP in the prepared primary cultures confirmed the presence of GFAP-positive astrocytes (Figure 2.1). For incubations, confluent primary astrocyte cultures of an age between 14 and 28 days were washed twice with 1 mL prewarmed (37°C) phosphate buffered saline (PBS; 10 mM potassium phosphate buffer, containing 150 mM sodium chloride, adjusted to pH 7.4 at 37°C) and subsequently incubated with pCuO-NPs in 1 mL IB-BSA for up to 4 h at 37°C. To test for the participation of different endocytotic pathways, the cells were incubated in IB-BSA with 50 µM pCuO-NPs for 1 h in absence or presence of different endocytosis inhibitors. For this purpose, concentrated stock solutions of 200 mM chlorpromazine, 1 mM wortmannin and 100 mM amiloride were prepared in DMSO and a 100 mM stock solution of chloroquine was prepared in water. These concentrated stock solutions were diluted in IB-BSA to prepare incubation solutions containing the indicated final concentrations of the inhibitors. DMSO in the indicated final concentrations was used as solvent control. The endocytosis inhibitors applied have different cellular targets. Chloroquin prevents the acidification of vesicles, vesicle fusion and the recycling of

membranes (Steinman et al. 1983). Chlorpromazine induces a loss of clathrin from the cell membrane and is therefore considered as inhibitor of the clathrin mediated endocytosis, whereas wortmannin and amiloride block the cellular macropinocytosis (Huth et al. 2006).

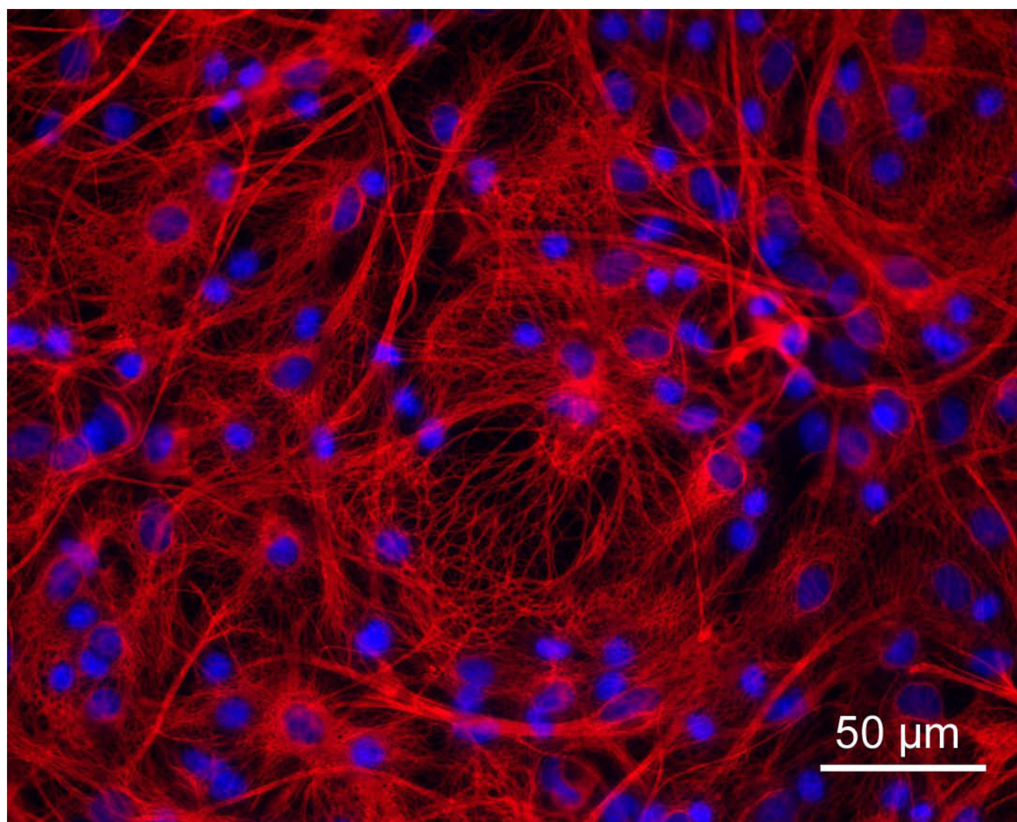

Figure 2.1: Confocal microscopy image of primary astrocyte cultures stained for the astrocyte specific glial fibrillary acidic protein (GFAP). The cell nuclei were stained with DAPI (blue), whereas GFAP was immunocytochemical detected with an α-GFAP antibody from rabbit and a Cy3 conjugated α-rabbit secondary antibody (red).

Several cellular parameters were investigated after the treatment of the cells with pCuO-NPs in presence or absence of the inhibitors. The cellular copper and protein content were obtained from cell lysates as previously described (Scheiber et al. 2010, Bulcke et al. 2014, Tulpule et al. 2014). Briefly, after the treatment, the cells were washed twice with PBS and lysed with 400 µL 50 mM NaOH. 100 µL of the generated lysate was used to determine the protein content of the cells, whereas another 100 µL of the lysates was used for analysis of the cellular copper content (Scheiber et al. 2010, Bulcke et al. 2014, Tulpule et al. 2014).

The viability of the cells was assessed by determination of the activity of cellular reductases, the activity of the enzyme lactate dehydrogenase (LDH) and by testing the membrane permeability. The reduction capacity of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used as indicator for the activity of cellular reductases (Mosmann 1983). For this purpose, a modification of a previously published method was used (Scheiber et al. 2010, Bulcke et al. 2014). Briefly, after the indicated treatments, the cells were incubated with 1 mL MTT in IB-BSA for 90 min at 37°C. Subsequently, the media was removed and the cells as well as the generated cellular formazan were dissolved in 500 µL DMSO. 20 µL of the DMSO-lysate was mixed with 180 µL of a mixture of 90% DMSO and 10% 50 mM NaOH in wells of a 96-well plate and the absorbance was determined at 540 nm as described previously (Scheiber et al. 2010, Bulcke et al. 2014). Staining for membrane integrity of the treated cells was performed with propidium iodide as previously described (Bulcke et al. 2014, Tulpule et al. 2014). The extra- and intracellular activites of the cytosolic enzyme LDH were determined as previously described (Bulcke et al. 2014, Tulpule et al. 2014) for 20 µL of the incubation media or 20 µL of cell lysates. For obtaining the lysates, the cells were washed twice with 1 mL ice cold PBS and subsequently lysed in 1 mL IB-BSA containing 1% Triton X-100 for 15 min at 37°C.

2.4.3 Results

To test for the accumulation of pCuO-NPs by primary astrocyte cultures, these cells were incubated with up to 250 µM pCuO-NPs in IB-BSA for up to 4 h and their MTT reduction capacity, their LDH activity, protein content and copper content were determined (Figure 2.2). Incubations of primary astrocytes with pCuO-NPs did not increase the extracellular LDH activity (Figure 2.2 A). The cellular LDH activity was determined as additional indicator for cell viability (Figure 2.2 B). The presence of pCuO-NPs led to a time- and concentration-dependent decrease in the cellular LDH activity. After 1 h of treatment only the highest concentration of pCuO-NPs applied (250 µM) significantly decreased the cellular LDH activity (Figure 2.2 B). Longer incubation periods led to a significant decrease of the LDH activity in concentrations above 100 µM pCuO-NPs. After 4 h almost no cellular LDH activity was detectable if pCuO-NPs had applied in concentrations above 100 µM (Figure 2.2 B). The loss in cell vitality was also mirrored in the MTT reduction capacity of the cells but with an earlier onset of imparired reduction capacity (Figure 2.2 D).

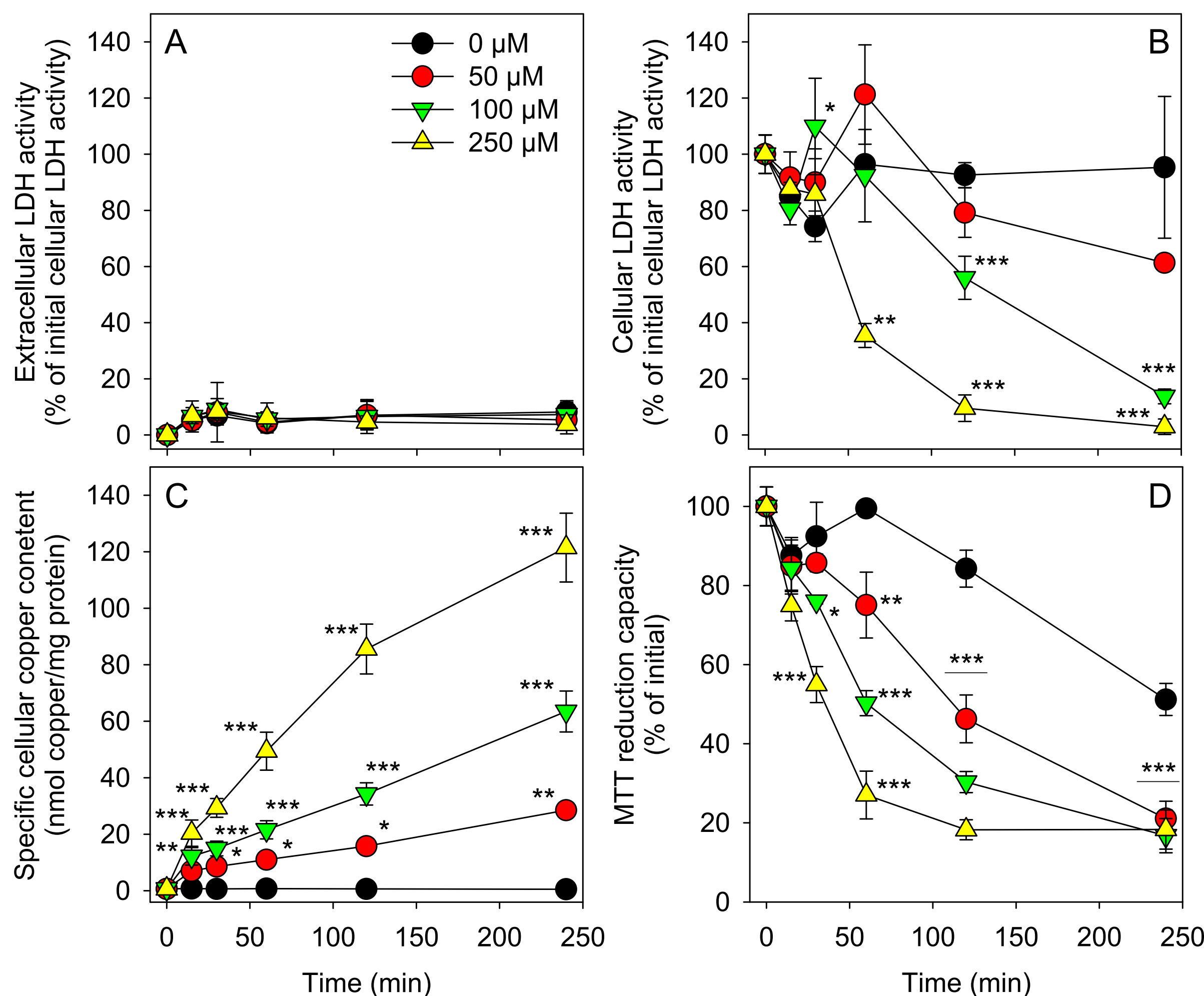

Figure 2.2: Time dependent accumulation of CuO-NPs. Primary astrocytes were incubated with up to 250 µM CuO-NPs in IB-BSA for up to 4 h. Extracellular (A) and cellular LDH activity (B), the specific cellular copper contents (C) and MTT reduction capacity (D) were determined. Significance of differences was analyzed between the absence or presence of CuO-NPs in the indicated concentrations (n=3; ANOVA; *p<0.05; **p<0.01; ***p<0.001).

However, even in the absence of pCuO-NPs (control condition) a loss in the MTT reduction capacity of 45% was detected in the after an incubation time of 4 h in comparison to the initially obtained values (Figure 2.2 D). Already after 30 min of treatment with 100 µM pCuO-NPs the MTT-reduction capacity of the treated cells was significantly reduced by 25% (Figure 2.2.D). Increasing incubation time or concentration of pCuO-NPs led to an increased loss in MTT reduction capacity (Figure 2.2 D). The Specific cellular copper content of the treated cells increased in a time- and concentration-dependent manner (Figure 2.2 C). However, all treatments which resulted

in specific cellular copper contents higher than 10 nmol copper per mg protein (Figure 2.2 C) led to a decreased MTT reduction capacity (Figure 2.2 D).

To analyze the endocytotic uptake mechanisms of pCuO-NPs in cultured primary astrocytes, sub-toxic conditions had to be defined in which a sufficient amount of copper was accumulated by the treated cells. After 1 h of treatment the cells accumulated 10.9 ± 1.2 nmol copper per mg protein when treated with 50 µM pCuO-NPs, which is approximately a 10-fold higher copper content in comparison to cells which had been treated in the absence of pCuO-NPs (0.7 ± 0.1 nmol copper per mg protein) (Figure 2.2 C). This amount of accumulated copper appears sufficient to manipulate the pCuO-NP uptake with inhibitors. For this incubation condition the cell vitality was not compromised. At least no loss in cellular LDH activity was determined for incubations of astrocytes for 1 h with 50 µM pCuO-NPs (Figure 2.2 B). Previously it was reported, that the extracellular activity of the enzyme LDH might be severely inhibited in presence of excess amount of copper (Lai and Blass 1984). To verify if 50 µM pCuO-NPs can interfere with the determination of the LDH activity cell lysates were incubated for 1 h with 50 µM pCuO-NPs. The determined activity of LDH was not different in comparison to the initially obtained values (data not shown). Therefore, the extracellular LDH activity can also be used in presence of 50 µM pCuO-NPs as reliable indicator for cell viability. However, it was not tested if higher concentrations of pCuO-NPs affect the activity of LDH. The treatment of astrocytes with 50 µM pCuO-NPs did not increase the extracellular LDH activity (Figure 2.2 A). A significant decrease in the MTT-reducticon capacity of the incubated cells by 25 ± 8% was found after the treatment with 50 µM pCuO-NPs compared to incubations performed in the absence of pCuO-NPs. As the MTT reduction capacity of the cells is a very early indicator for impaired cell vitality and the cellular LDH activity is a very late indicator (Hohnholt et al 2015), an additional method was applied to verify vitality of the astrocytes treated with 50 µM pCuO-NPs for 1 h. For this purpose a staining for membrane integrity was performed (Figure 2.3). The number of cells per well, as determined by the positive signal for the membrane permeable DNA-stain H33342 was similar for all treatments (Figure 2.3 E-H). The PI staining for membrane integrity of cultured astrocytes revealed that no impairment of the astrocytic membranes was observed in the absence of pCuO-NPs (Figure 2.3 A), while treatment of the cells with 100 µM silver nitrate, which was used as positive control for toxicity (Luther et al 2011), showed that each cell was PI-positive (Figure 2.3 B). The strong signal for PI under these conditions was

accompanied by a strong background (Figure 2.3 B). PI-positive cells were not observed when the cells were treated with 50 µM pCuO-NPs (Figure 2.3 C). In contrast, an increased number of PI-positive cells could be determined if the cells were treated with 100 µM pCuO-NPs (Figure 2.3 D).

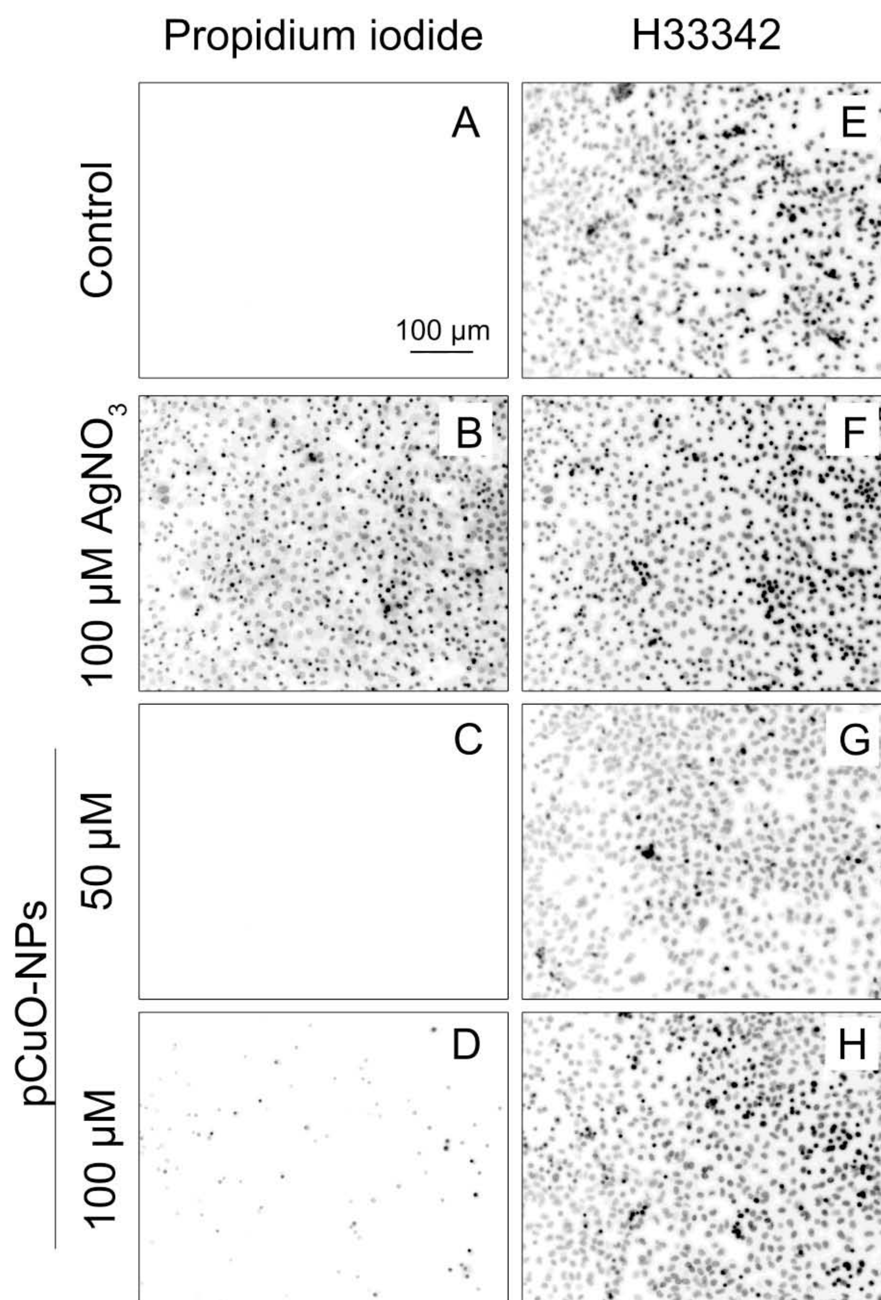

Figure 2.3: Staining for membrane integrity of pCuO-NP-treated astrocytes. Primary astrocyte cultures were incubated for 1 h in the absence or presence of 50 µM or 100 µM pCuO-NPs in IB-BSA. Subsequently, the membrane integrity of the treated cells was determined by propidium iodide staining (A-D). Cell nuclei were stained with H33343 (E-H). 100 µM $AgNO_3$ was used as positive control for impaired membranes (B) (Luther et al. 2011). The scale bar in panel A applies to all panels.

These data verifiy that astrocytes that had been treated for 1 h with 50 µM pCuO-NPs accumulated substantial amounts of copper (Figure 2.3 C) whereas the vitality of those cells was not compromised (Figure 2.2; Figure 2.3 A,B,D).

These conditions were chosen to test for the inhibition of endocytotic pathways by different endocytosis inhibitors (Table 2.1). Cultured astrocytes were treated in presence of 50 µM pCuO-NPs for 1 h in the absence or presence of either 1 mM chloroquine, 20 µM chlorpromazine, 100 nM wortmannine, 1 mM amiloride or a combination of those inhibitors. The extracellular LDH activity was not impaired in any of the chosen conditions. The MTT reduction capacity of some conditions was reduced to some extend in comparison to the control condition (Table 2.1). However, already treatment with DMSO alone led to a decrease of the MTT reduction capacity by 10%. For example, in comparison to the vehicle control (water) chloroquine showed a decreased MTT reduction capacity of 82 ± 7%. Stronger decreases in the MTT-reduction capacity were observed in the combinations of chlorpromazine and wortmannin as well as chloroquine and amiloride (Tavle 2.1). Staining for membrane integrity with propidium iodide was performed to confirm the viability of the cells treated in presence of 50 µM pCuO-NPs and in presence of the inhibitors chloroquin, chlorpromazine and amiloride (Figure 2.4). After these treatments, the number of cell nuclei, as determined by H33342 staining, was not altered compared to control conditions (Figure 2.4 F-J). As shown previously (Figure 2.3 B), the application of silver nitrate severely increased the number of PI-potive cells (Figure 2.5 B). The membrane integrity of all cells treated in presence of pCuO-NPs and of one of the applied endocytosis inhibitors was not affected by the treatment, as demonstrated by the absence of PI positive cells (Figure 2.4 C-E). Also the combined application of chloroquine and amiloride in presence of pCuO-NPs only led to the occurance of a few PI-positive cells (data not shown). The endocytosis inhibitors alone did not lead to the occurrence of PI-positive cells during incubation in the absence of pCuO-NPs (data not shown). After exposure to 50 µM pCuO-NPs, astrocytes had accumulated 10.7 ± 1.2 nmol copper per mg protein at 37°C (Table 2.1). Strong inhibition of the active copper accumulation was determined for incubations performed at 4°C (5.9 nmol copper per mg protein). To calculate the internalized copper (Figure 2.5), the extracellular attached copper, which was assumed from the copper values obtained at incubations performed at 4°C (Table 2.1) was substracted from the cellular copper content obtained at incubations performed at 37°C (Table 2.1).

Table 2.1: Inhibition of CuO-NP uptake by endocytosis inhibitors

Treatment	Specific copper content (nmol/mg protein)		extracellular LDH activity (% of initial cellular LDH activity)		MTT reduction capacity (% of control)		n
	Control	CuO-NPs	Control	CuO-NPs	Control	CuO-NPs	
None	0.7 ± 0.3	10.7 ± 1.2$^{\#\#\#}$	5.6 ± 2.2	3.1 ± 1.6	100.0 ± 3.4	89.4 ± 4.8$^{\#\#}$	5
4°C	0.6 ± 0.1	5.9 ± 0.5***$^{/\#\#\#}$	2.6 ± 1.4	2.7 ± 2.3	91.8 ± 6.1	83.9 ± 6.4$^{\#}$	5
CQ	0.5 ± 0.2	8.3 ± 1.5***$^{/\#\#\#}$	3.9 ± 1.1	2.7 ± 1.2	81.5 ± 6.6***	73.4 ± 11.2*	5
0.1% DMSO	0.7 ± 0.3	10.7 ± 1.6$^{\#\#\#}$	4.6 ± 2.2	2.4 ± 2.2	91.9 ± 3.7	85.0 ± 6.1	5
CPZ	0.6 ± 0.2	8.7 ± 0.6**$^{/\#\#\#}$	5.7 ± 2.2	1.5 ± 1.6	82.4 ± 2.8	74.5 ± 5.8	5
Wort	0.6 ± 0.3	9.5 ± 1.4$^{\#\#\#}$	5.3 ± 1.6	2.6 ± 2.2	87.0 ± 2.7	80.8 ± 5.1$^{\#}$	5
0.2% DMSO	0.8 ± 0.2	9.9 ± 0.8$^{\#\#\#}$	4.1 ± 2.9	1.7 ± 1.9	90.1 ± 3.6	81.7 ± 3.3$^{\#\#}$	4
CPZ+Wort	0.6 ± 0.3	9.1 ± 0.9$^{\#\#\#}$	3.5 ± 0.7	2.8 ± 2.2	74.9 ± 2.1**	64.4 ± 4.1*$^{/\#\#}$	4
1% DMSO	0.7 ± 0.3	10.4 ± 1.1$^{\#\#\#}$	4.3 ± 2.9	0.8 ± 1.6	90.4 ± 5.1	82.9 ± 5.7	5
Ami	0.6 ± 0.2	7.4 ± 1.4***$^{/\#\#\#}$	3.8 ± 1.7	1.8 ± 1.8	80.5 ± 6.5	71.0 ± 4.9	5
CQ+AMI	0.5 ± 0.3	7.1 ± 0.3***$^{/\#\#\#}$	2.0 ± 2.0	2.9 ± 2.7	66.7 ± 2.3***	58.0 ± 8.8***	4

Cultured astrocytes were incubated for 1 h in IB-BSA in the absence (Control) or the presence of 50 µM pCuO-NPs without (None) or with 1 mM chloroquine (CQ), 20 µM chlorpromazine (CPZ), 100 nM wortmannin (Wort), 1 mM amiloride (Ami) or in the indicated combinations of those. The specific cellular copper content, the extracellular LDH activity and the MTT reduction capacity were determined. The data were obtained in experiments performed on n independently prepared cultures. Asterisks indicate the significance of differences of data obtained from cells treated with the given endocytosis inhibitors or the respective solvent control (ANOVA; $^*p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$), whereas hashes indicate the significane of differences of data obtained from cells treated in the absence or presence of pCuO-NPs (paired t-test; $^{\#}p<0.05$; $^{\#\#}p<0.01$).

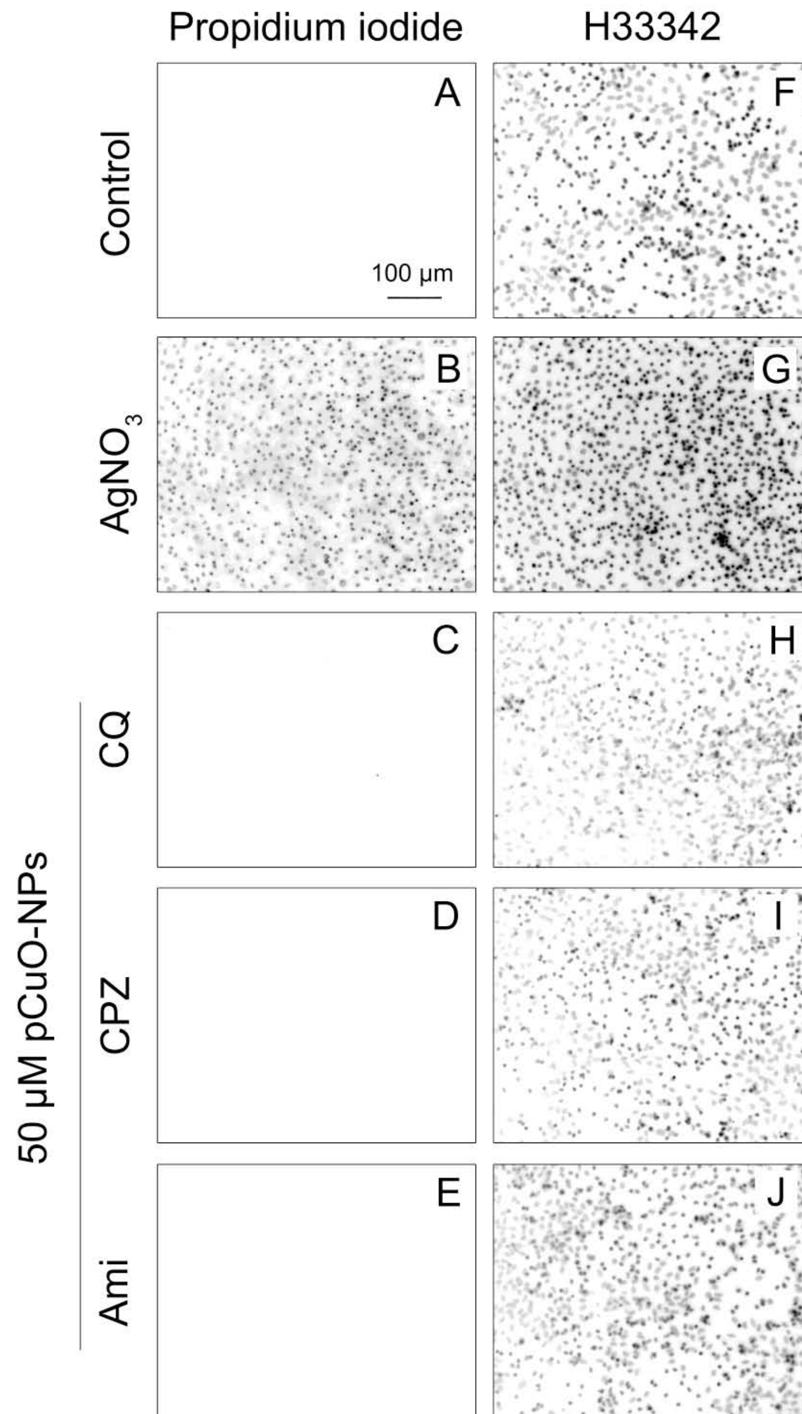

Figure 2.4: Staining for membrane integrity of astrocytes treated with pCuO-NP and endocytosis inhibitors. Primary astrocyte cultures were incubated for 1 h with 50 µM pCuO-NPs in IB-BSA in absence (A,F) or presence of 100 µM $AgNO_3$, 1 mM chloroquine (CQ), 20 µM chlorpromazine (CPZ), or 1 mM amiloride (Ami). Subsequently, the membrane integrity of the treated cells was determined by propidium iodide staining (A-E). Cell nuclei were stained with H33343 (F-J). The $AgNO_3$ treatment was used as positive control to impair cell membranes (B) (Luther et al. 2011). The scale bar in panel A applies to all panels.

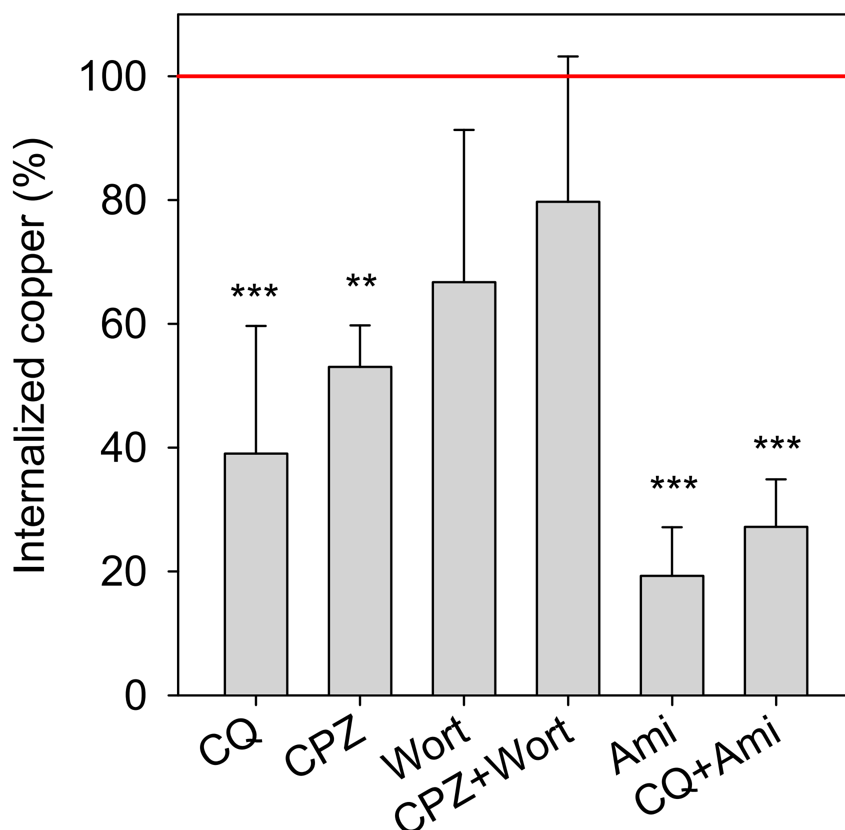

Figure 2.5: Inhibition of endocytotic CuO-NP uptake. Cultured astrocytes were incubated for 1 h in the presence of 50 µM CuO-NPs without (100%; solid line; 10.7 ± 1.2 nmol copper per mg protein) or with 1 mM chloroquine (CQ), 20 µM chlorpromazine (CPZ), 100 nM wortmannin (Wort), 1 mM amiloride (Ami) or the indicated combinations of inhibitors. Subsequently, the specific cellular copper contents were determined (Table 2.1). Copper values derived form incubations performed at 4°C (0%; 5.9 ± 0.5 nmol copper per mg protein) were substracted from the copper values obtained at 37°C to calculate the internalized copper. The relative inhibitory effect on the internalization of pCuO-NP by the indicated compounds was calculated in comparison to the respective control. Asterisks indicate the significance of differences of data obtained from cells treated with the given endocytosis inhibitors and the respective solvent control (ANOVA; **$p<0.01$; ***$p<0.001$).

Under the chosen conditions the inhibitors chloroquin and amiloride showed the highest effect and decreased the amount of actively accumulated copper to 39 ± 21% and 19 ± 8% without severe decreases in cell viability (Table 1; Figure 2.4). Chlorpromazine also lowered the copper accumulation to 53 ± 7%. The inhibitors wortmannin and the different combinations of endocytosis inhibitors did not effectively lowered the copper accumulation or severely decreased the MTT-reduction capacity.

2.4.4 Discussion

The presented data shows that pCuO-NPs are taken up in a time-, concentration-, and temperature-dependent manner. These results confirm previously obtained data which reported the uptake of CuO-NPs (Bulcke et al. 2014 - Chapter 2.1). However, in contrast to the non -protein coated CuO-NPs the pCuO-NPs seem to be more efficiently taken up and show higher toxicity. For example incubations with 100 µM CuO-NPs for 6 h in DMEM-FCS were necessary to obtain copper values around 10 nmol copper per mg protein (Bulcke et al. 2014 - Chapter 2.1), whereas in this study the same amount of copper was obtained after 1 h treatment with 50 µM pCuO-NPs in IB-BSA. This might relate to the protein content of the media, which contained 10% FCS in the case of non protein coated CuO-NPs, in contrast to the 0.5 mg BSA per mL used in the presented results. Previous experiments with iron oxide nanoparticles revealed that those particles are taken up by cultured astrocytes depending on the protein content of the media (Geppert et al. 2012). Hereby it was reported, that increasing concentrations of protein in the incubation media decreased the cellular nanoparticle accumulation (Geppert et al. 2012). The time- and concentration-dependent accumulation of pCuO-NPs by astrocytes corresponded to a decrease in cell vitality, which was determined by a loss of cellular LDH activity and MTT-reduction capacity as well as increased membrane permeability. If the specific cellular copper contents exceeded a threshold of 10 nmol copper per mg protein a decrease in the cell viability was observed, confirming previously obtained data (Bulcke and Dringen 2015 - Chapter 1.3).

Different pharmacological inhibitors of endocytosis were tested for their potential to inhibit the accumulation of CuO-NPs by astrocytes. Several vitality assays were applied to test for the viability of the cells treated in presence of pCuO-NPs and the inhibitors. Throughout the different incubation conditions no increase in the extracellular LDH activity was observable. Altough the extracellular LDH activity was not affected under those conditions, a significant drop in the MTT reduction capacity of 27% was determined in cells treated with chloroquine or chlorpromazine in the presence of pCuO-NPs. Nevertheless, both substances were already shown to decrease MTT reduction capacity in astrocytes whereas other vitality assays, such as WST-1 reduction, were not affected by chloroquin and chlorpromazine (Berridge and Tan 1993, Isobe et al. 1999). Endocytosis was reported to be involved in the cellular uptake of MTT (Liu et al. 1997). Therefore, an impaired uptake of MTT can result in a loss of signal in MTT-reduction capacity of cells treated with endocytosis inhibitors. To rule out that the drop

in MTT-reduction capacity originated from non-viable cells, an additional vitality assay was performed. The staining for membrane integrity with propidium iodide confirmed the viability of the treated cells. To sum it up, the treatment of astrocytes with 50 µM pCuO-NPs for 1 h in presence of either chloroquin, chlorpromazine or amiloride did not affected the viability of the treated cells. Therefore, a decreased copper content of the cells treated under those condtions can be attributed the inhibition of the respective endocytotic pathway.

The copper accumulation of pCuO-NP treated astrocytes was effectively prevented by amiloride, chloroquin and chlorpromazine (Figure 2.5). Figure 2.6 summarizes the proposed endocytotic mechanisms for the uptake of pCuO-NPs by cultured astrocytes, which were determined by the respectice inhibitors.

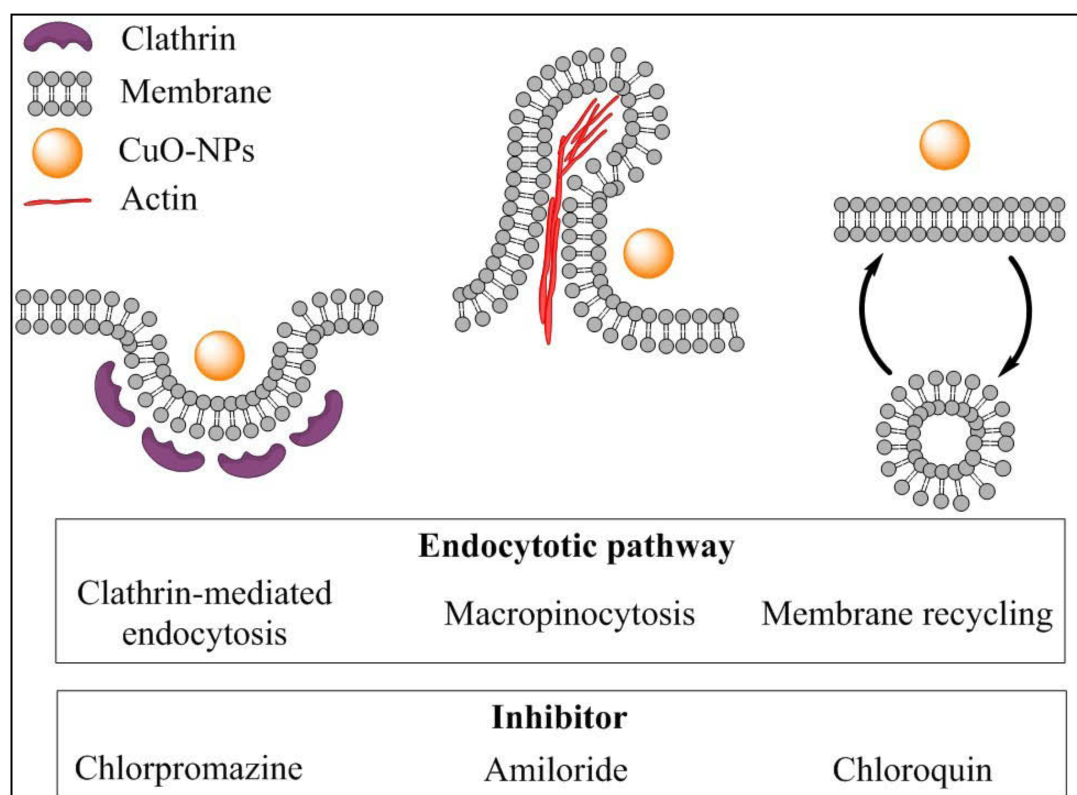

Figure 2.6: Proposed endocytotic uptake mechanisms of CuO-NPs in cultured astrocytes. Pharmacological inhibition of clathrin-mediated endocytosis with chlorpromazine, macropinocytosis with amiloride and membrane recycling with chloroquine effectively lowered the internalization of CuO-NPs in cultured astrocytes.

Amiloride was among the tested inhibitors the most potent inhibitor of pCuO-NP internalization by astrocytes, reducing the internalized copper by 80%. Amiloride is an effective inhibitor of macropinocytosis which also strongly inhibited the accumulation of silver nanoparticles in cultured astrocytes (Luther et al. 2011). The pharmacological inhibition of macropinocytosis with amiloride revealed that macropinocytosis is the predominant pathway for the uptake of TiO_2-Nanoparticles in U373 human glial cells (Huerta-García et al 2015). The inhibitor of vesicle fusion and membrane cycling chloroquine (Steinman et al. 1983) effectively lowered the internalization of pCuO-NPs into astrocytes. It was reported that the accumulation of silver nanoparticles could be strongly prevented with chloroquine (Luther et al 2011). The inhibition of the clathrin-mediated endocytosis with chlorpromazine (Huth et al 2006, Vercauteren et al. 2010) lowered the internalization of pCuO-NPs. It was reported that the accumulation of iron oxide nanoparticles and silver nanoparticles by astrocytes could be effectively prevented by chlorpromazine (Luther et al 2011, Geppert et al 2012).

The applied pCuO-NPs seem to be accumulated into primary astrocyte cultures by similar endocytotic pathways as iron oxide nanoparticles and silver nanoparticles (Luther et al. 2011, Geppert et al. 2012). Nevertheless, due to the effective inhibition of pCuO-NP uptake with inhibitors of different endocytosis pathways, it seems that not an individual pathway is responsible for uptake of pCuO-NPs. In addition, if one uptake route is inhibited other mechanisms might participate stronger in the uptake of pCuO-NPs (Huth et al. 2006, Vercauteren et al. 2010). However, results obtained with pharmacological inhibitors of endocytosis have to be evaluated carefully, as the specificity of those is poor (Vercauteren et al. 2010, Ivanov 2014). Other methods of inhibition, such as RNA interference should be considered for further approaches to elucidate the role of individual endocytotic pathways in the uptake of pCuO-NPs.

2.4.5 References

Berridge MV and Tan AS (1993) Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): Subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophys 303:474-482

Bulcke F and Dringen R (2015) Handling of Copper and Copper Oxide Nanoparticles by Astrocytes. Neurochem Res, in press DOI: 10.1007/s11064-015-1688

Bulcke F, Thiel K, Dringen R (2014) Uptake and toxicity of copper oxide nanoparticles in cultured primary brain astrocytes. Nanotoxicology 8:775-785

Geppert M, Petters C, Thiel K, Dringen R (2012) The presence of serum alters the properties of iron oxide nanoparticles and lowers their accumulation by cultured brain astrocytes. J Nanopar Res 15:1-15

Huerta-García E, Márquez-Ramírez SG, Ramos-Godinez MP, López-Saavedra A, Herrera LA, Parra A, Alfaro-Moreno E, Gómez EO, López-Marure R (2015) Internalization of titanium dioxide nanoparticles by glial cells is given at short times and is mainly mediated by actin reorganization-dependent endocytosis. Neurotoxicology, 51:27-37

Huth US, Schubert R, Peschka-Süss R (2006) Investigating the uptake and intracellular fate of pH-sensitive liposomes by flow cytometry and spectral bio-imaging. J Control Release 110:490-504

Hohnholt MC, Blumrich EM, Dringen R (2015) Multiassay analysis of the toxic potential of hydrogen peroxide on cultured neurons. J Neurosci Res 93:1127-1137

Isobe I, Michikawa M, Yanagisawa K (1999) Enhancement of MTT, a tetrazolium salt, exocytosis by amyloid β-protein and chloroquine in cultured rat astrocytes. Neurosci Lett 266:129-132

Ivanov AI (2014) Pharmacological inhibitors of exocytosis and endocytosis: novel bullets for old targets. Methods Mol Biol 1174:3-18

Iversen TG, Skotland T, Sandvig K (2011) Endocytosis and intracellular transport of nanoparticles: Present knowledge and need for future studies. Nano Today 6:176-185

Lai JC and Blass JP (1984) Neurotoxic effects of copper: inhibition of glycolysis and glycolytic enzymes. Neurochem Res 9:1699-1710

Liu Y, Peterson DA, Kimura H, Schubert D (1997) Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. J Neurochem 69:581-593

Luther E, Koehler Y, Diendorf J, Epple M, Dringen R (2011) Accumulation of silver nanoparticles by cultured primary brain astrocytes. Nanotechnology 22:375101

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65:55-63

Petters C and Dringen R (2014) Comparison of primary and secondary rat astrocyte cultures regarding glucose and glutathione metabolism and the accumulation of iron oxide nanoparticles. Neurochem Res 39:46-58

Petters C and Dringen R (2015) Accumulation of iron oxide nanoparticles by cultured primary neurons. Neurochem Int 81:1-9

Scheiber IF, Mercer JFB, Dringen R (2010) Copper accumulation by cultured astrocytes. Neurochem Int 56:451-460

Steinman R, Mellman I, Muller W, Cohn Z (1983) Endocytosis and the recycling of plasma membrane. J Cell Biol 96:1-27

Tulpule K, Hohnholt MC, Hirrlinger J, Dringen R (2014). Primary cultures of rat astrocytes and neurons as model systems to study metabolism and metabolite export from brain cells. In: Neuromethods 90: Brain Energy Metabolism. Eds: J Hirrlinger and H Waagepetersen. New York, Springer. 90: 45-72

Vercauteren D, Vandenbroucke RE, Jones AT, Rejman J, Demeester J, De Smedt SC, Sanders NN, Braeckmans K (2010) The use of inhibitors to study endocytic pathways of gene carriers: Optimization and pitfalls. Mol Ther 18:561-569

Zhang S, Li J, Lykotrafitis G, Bao G, Suresh S (2009) Size-dependent endocytosis of nanoparticles. Adv Mater 21:419-424

3. Summarizing discussion

3.1 Synthesis and characterization of copper oxide nanoparticles

3.2 Uptake of copper oxide nanoparticles by astrocytes

3.3 Toxicity of copper and copper oxide nanoparticles on astrocytes

3.4 Stimulation of the astrocytic metabolism by copper oxide nanoparticles

3.5 Reflection on the *in vivo* relevance of the data obtained

3.6 Future perspectives

3.7 References

3. Summarizing Discussion

3.1 Synthesis and characterization of copper oxide nanoparticles

The CuO-NPs used in this study were synthesized by an alkaline precipitation method, which is a modification of a published method (Kobayashi et al. 2011). In this preparation procedure copper nitrate was precipitated at 75°C by the addition of sodium hydroxide under the formation of copper hydroxide (Reaction 1). In a further reaction (Reaction 2) copper hydroxide eliminates water at 75°C and generates copper oxide in a nanosized form (Chapter 2.1).

Reaction 1: $Cu(NO_3)_2 + 2\ NaOH \xrightarrow{75°C} Cu(OH)_2 + 2\ Na^+ + 2\ NO_3^-$

Reaction 2: $Cu(OH)_2 \xrightarrow{75°C} CuO + H_2O$

Subsequently, the coating material dimercaptosuccinic acid (DMSA) was added to the nanoparticles to facilitate the colloidal dispersion of those. The negatively charged carboxyl groups of DMSA can bind to the surface of the CuO-NPs. Further, oxidation of the thiol groups of the DMSA results in the formation of disulfide bridges between individual DMSA molecules, stabilizing the coat formation by generating a cage-like structure (Fauconnier et al. 1997). DMSA is an ideal coating material due to the strong binding to the surface of the particles and the intermolecular linking of DMSA molecules (Fauconnier et al. 1997). DMSA also has the advantage to be highly biocompatible and is frequently used in heavy metal chelation therapy (Brandt et al. 2015). Additionally, DMSA was already used as coating material for iron oxide nanoparticles, which were thoroughly investigated for their effect on brain cells (Geppert et al. 2011, Hohnholt et al. 2011, Petters and Dringen 2014, Petters et al. 2014, Petters and Dringen 2015, Petters et al. 2015). Those iron oxide nanoparticles were not toxic to astrocytes (Geppert et al. 2011, Petters et al. 2014). The DMSA coating material can be easily modified by the addition of fluorescent dyes to the thiol groups, which allows the subcellular localization of those particles (Luther et al. 2013, Petters and Dringen 2014). Thus, DMSA has several advantages as coating material for nanoparticles. Other substances such as antibodies, glycans or drugs can be easily introduced to the DMSA coat to add further functionality to the particles.

Various other methods are available to synthesize CuO-NPs, which might harvest smaller, more uniform or more stable particles (Lignier et al. 2012). The advantage of the synthesis method established in the presented thesis is the easy procedure that requires no special equipment and no organic solvents, while highly reproducibile results were generated (Chapter 2.1).

For a detailed analysis of nanoparticle properties it is necessary to use several methods to get an overview about the particle composition, size, shape, state of agglomeration, stability and surface charge (Kettler et al. 2014). Knowing the properties of nanoparticles will also help to predict the potential behavior of these nanoparticles under certain environmental conditions (Nel et al. 2006, Nel et al. 2009). Effects of nanoparticles on organisms, for example toxicity or bioavailability, can already strongly differ if only one property of the material is altered. This was clearly shown for differently sized CuO-NPs (Karlsson et al. 2009), for differently charged polystyrene nanoparticles (Walczak et al. 2015), for nanoparticles with different core materials but otherwise same properties (Kim et al. 2014) and for CuO-NPs with different shapes (Di Bucchianico et al. 2013).

The synthesized CuO-NPs were characterized with different analysis methods (Chapter 2.1). Electron microscopy revealed that CuO-NPs are present in agglomerates in which the individual spherical primary particles had a crystalline structure and a size between 5-10 nm. Electron dispersive X-ray spectroscopy was used to obtain the elemental composition of the synthesized CuO-NPs. The elemental analysis revealed that the atomic composition of the particles was 54% for copper and 46% for oxygen. Dynamic light scattering reavealed an average hydrodynamic diameter of 136 ± 4 nm for CuO-NP-agglomerates determined in water. The size distribution of the particles in water was moderately polydisperse with a polydispersity index of 0.15 ± 0.01. In water, the ζ-potential of the DMSA-coated CuO-NPs was -49.0 ± 7.2 mV, indicating that the negatively charged carboxyl groups of the DMSA are exposed on the nanoparticle surface. However, uncoated nanoparticles had a positive surface charge of 35 ± 1.3 mV, suggesting that the negatively charged DMSA binds via electrostatic interactions to the surface of the positively charged bare CuO-NPs during the coating procedure.

The physico-chemical properties of CuO-NPs synthesized for this work, delivered a concise collection of information on the particles. This dataset allows the comparison of the results obtained from this work to others. However, due to different methods used

for CuO-NPs synthesis and differing particle properties throughout scientific publications, such comparisons have to be conducted carefully.

The stability of CuO-NPs, in regard to the alteration of physico-chemical properties of those particles, is an important factor to consider for the application of such particles in biological systems. CuO-NPs can dissolve over time, which might cause problems as the liberation of copper ions could interfere with the interpretation of experimental data obtained from biological test systems (Studer et al. 2010, Semisch et al. 2014). The CuO-NPs used in the presented thesis were stable in aqueous dispersion for up to two weeks after the preparation (Table 3.1; Chapter 2.1). Also other studies have reported that the colloidal stability of CuO-NPs is not very high in several media (Fedele et al. 2011, Son et al. 2015). In this context, especially protein free media with high ionic strengths causes accelerated formation of agglomerates ultimately resulting in particle precipitation (Fedele et al. 2011, Son et al. 2015).

Table 3.1: Characterization of the synthesized CuO-NPs in different dispersants.

	Dispersant	Hydrodynamic diameter (nm)	Zeta potential (mV)	Reference
CuO-NPs	Water	136 ± 4	-49 ± 7	Chapter 2.1
	IB	957 ± 246	-27 ± 9	Chapter 2.1
	DMEM-FCS	178 ± 12	-10 ± 3	Chapter 2.1
pCuO-NPs	Water	152 ± 9	-35 ± 3	Chapter 2.4
	IB-BSA	165 ± 12	-14 ± 4	Chapter 2.4

CuO-NPs and protein coated CuO-NPs (pCuO-NPs) were dispersed in the indicated media and their hydrodynamic diameter and zeta potential were determined (n=3). The dispersants were water, incubation buffer (IB), incubation buffer containing 0.5 mg BSA per mL (IB-BSA) or Dulbecco's Modified Eagle Medium containing 10% fetal calf serum (DMEM-FCS). The detailed formulation of the media is given in Chapter 2.1 and 2.4.

Derjaguin, Landau, Vervey, and Overbeek (DLVO) developed in the beginning of the 1940s a theory on the stability of colloidal solutions (Derjaguin and Landau 1941, Verwey and Overbeek 1948). The DLVO theory describes the force between charged surfaces by the combination of the attractive van der Waals force and the electrostatic repulsion. Colloidal stability is given, when the electrostatic repulsion of the nanoparticle surfaces is higher than the attraction by the van der Waals forces. However, if high salt concentrations are present in such dispersions, the electrostatic repulsion decreases to such an extent that the sum of the repulsive and attractive forces benefits the attraction of the particles (Derjaguin and Landau 1941, Verwey and

Overbeek 1948). The particles agglomerate under such circumstances, and if a certain agglomeration state is reached, the stability of the colloidal dispersion is impaired and subsequently the particles precipitate (Derjaguin and Landau 1941, Verwey and Overbeek 1948). The DLVO theory explains the observed agglomeration of CuO-NPs in high ionic strength media, as seen in the dispersion of CuO-NPs in IB (Table 3.1).

Interestingly, in the case that proteins were present in the dispersion media no precipitation of the CuO-NPs was observed (Table 3.1), as also reported by others (Xu et al. 2012, Miao et al. 2015). Proteins seem to stabilize CuO-NPs (Xu et al. 2012, Miao et al. 2015), most likely by an interaction with the charged surface of nanoparticles and binding via electrostatic interactions to form a so called "corona" (Nel et al. 2009, Treuel et al. 2015). The formation of protein coronas around nanoparticles will of course alter the surface properties of those particles (Nel et al. 2009). For example, the hydrodynamic diameter of CuO-NPs used for this work increased in presence of 10% fetal calf serum by 30%, while the strong negative surface charge of those particles was reduced from -49 mV to -10 mV (Table 3.1). Similarly, the ζ-potential of the BSA coated pCuO-NPs, which were dispersed in BSA containing incubation buffer, was less negative than pCuO-NPs which were dispersed in water, whereas the hydrodynamic diameters were comparable in both conditions (Table 3.1). Because the surface properties of nanoparticles determine characteristics like bioavailability and toxicity in biological systems, it is of high importance to keep track of such phenomena (Lynch et al. 2009, Tenzer et al. 2013, Treuel et al. 2015). The formation of protein coronas on nanoparticles is inevitable in biological systems in which proteins are always present (Treuel et al. 2015). The dynamic process of the protein corona formation and the corona itself can be highly variable in presence of multiple proteins (Monopoli et al. 2012). To circumvent potential problems of this highly variable condition it might be beneficial to coat nanoparticles with defined proteins like serum albumins (Röcker et al. 2009), as done for this work (Chapter 2.4). However, even after the formation of the protein corona it can be dynamically altered by an exchange of proteins (Röcker et al. 2009, Walczyk et al. 2010). This implies for CuO-NPs, that a rapid alteration of the surface characteristics occurs if those particles encounter biological environments in which their will imidiately interact with surrounding substances. This is of special interest regarding the uptake of nanoparticles by for example humans.

3.2 Uptake of copper oxide nanoparticles by astrocytes

Several studies on cultured primary astrocytes reported the ability of astrocytes to accumulate different types of nanoparticles (Geppert et al. 2011, Luther et al. 2011, Petters et al. 2014, Wang et al. 2014, Wilson et al. 2015). A detailed overview of different types of nanoparticles, which were tested for their interaction with astrocyte cultures, was recently published (Hohnholt et al. 2013). However, at the start of this PhD project no no literature data was available on how cultured astrocytes are affected by an exposure to CuO-NPs. To investigate the uptake and potential toxic action of CuO-NPs, which were synthesized and characterized as described above, we applied those CuO-NPs on primary astrocytes cultures (Chapter 2.1; Chapter 2.2; Chapter 2.4). Primary astrocyte cultures accumulated those particles in a time-, concentration- and temperature-dependent manner (Chapter 2.1; Chapter 2.2; Chapter 2.4). However, strong differences in the amount of copper accumulated by astrocytes were observed for protein free CuO-NPs and CuO-NPs that had been coated with BSA prior to the incubation. Additionally the incubation media strongly affected the accumulation of CuO-NP (Chapter 2.1, Chapter 2.4). The specific copper contents of astrocytes treated with 50 µM BSA coated CuO-NPs was 10.7 ± 1.2 nmol copper per mg protein when the incubation was performed for 1 h in incubation buffer containing 0.5 mg BSA per mL (Chapter 2.4). In a different incubation scenario with with non protein coated CuO-NPs and a treatment of the cells DMEM containing 10% FCS, astrocytes accumulated 9.4 ± 0.7 nmol copper per mg protein after a treatment with 100 µM CuO-NPs for 4 h (Chapter 2.1; Chapter 2.2). As described in the previous chapter, the formation of a protein corona around the nanoparticles strongly affects the properties of those particles. CuO-NPs seem to be taken up differently by astrocytes depending on the concentration and composition of proteins present in the incubation media, as it was already shown for the accumulation of iron oxide nanoparticles by astrocytes (Geppert et al. 2012). The altered ζ-potential of the particles (Table 3.1) might contribute to the observed facilitated accumulation of CuO-NPs in BSA containing incubation media. As discussed before, the composition of the protein corona can also substantially contribute to differences in the uptake of nanoparticles (Nel et al. 2009).

The specific cellular copper contents of astrocytes after exposure to CuO-NPs for 3 h, were significantly higher in comparison to the specific cellular copper contents of astrocytes exposed to similar concentrations of the copper salts $CuCl_2$ and $Cu(NO_3)_2$

(Chapter 2.1). However, the internalized copper content was similar for CuO-NPs and copper salts (Chapter 2.1). This hints to the different mechanisms which seem to be involved in the uptake of nanosized and ionic copper. Chapter 1.3 describes in detail the proposed uptake mechanisms of CuO-NPs and copper ions by cultured astrocytes (for an overview see Figure 3.1).

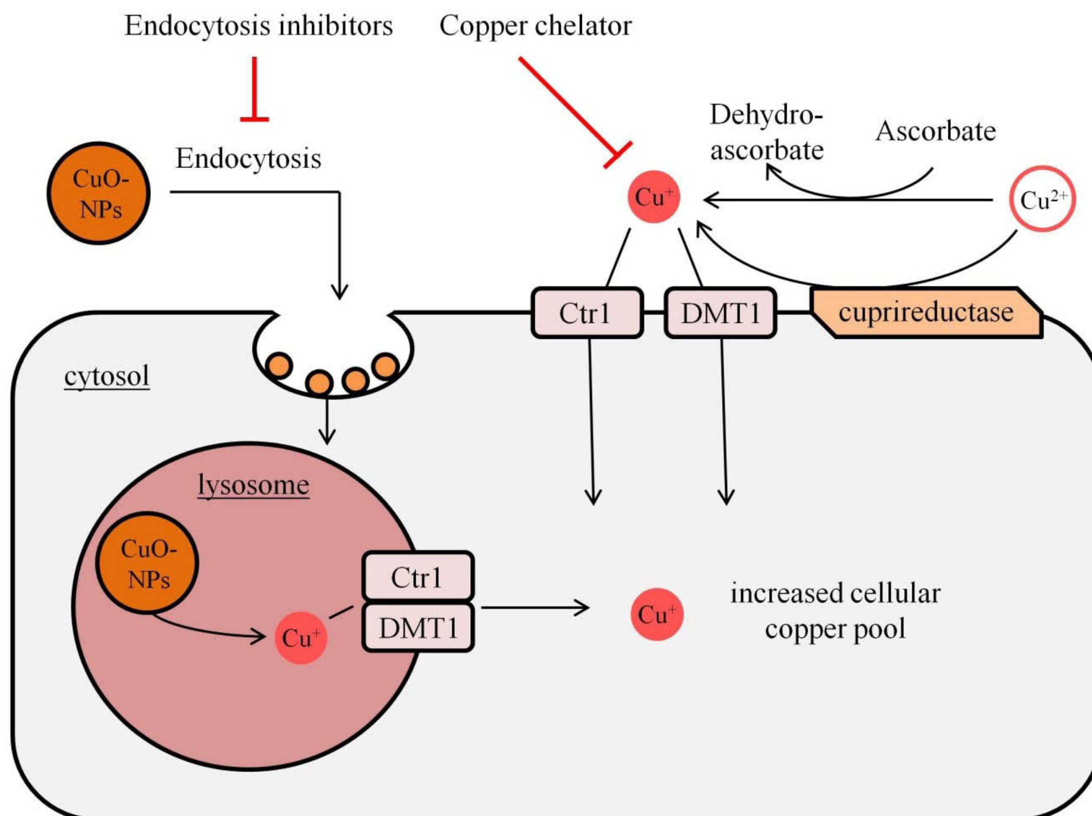

Figure 3.1: Uptake of CuO-NPs and ionic copper by astrocytes. The uptake of CuO-NPs is mediated by endocytosis, whereas ionic copper is accumulated copper transporter Ctr1 and the divalent metal transporter 1 (DMT1). The endocytotic mechanisms responsible for the uptake of CuO-NPs were identified with the help of pharmacological inhibitors. Vesicles containing internalised CuO-NPs will undergo the lysosomal pathway, where copper ions are liberated and exported via Ctr1 or DMT1 into the cytosol. The uptake of ionic copper by Ctr1 and DMT1 is facilitated by an extracellular reduction of Cu^{2+} to Cu^{+}, which is catalysed by ectocuprireductases or presence of reducing substances such as ascorbate. Application of extracellular copper chelators effectively prevented the accumulation of ionic copper. Adapted with kind permission from Springer Science+Business Media: Neurochemical Research, Bulcke and Dringen 2015, “Handling of Copper and Copper Oxide Nanoparticles by Astrocytes”, Figure 1, copyright 2015.

The inhibition of different endocytotic pathways suggests that CuO-NPs are taken up by cultured astrocytes via endocytotic mechanisms, which involve the acidification, fusion and recycling of vesicles, as well as clathrin-mediated endocytosis and macropinocytosis (Chapter 2.4, Steinman et al. 1983, Huth et al. 2006). The experimental inhibition of the vesicle acidification and membrane recycling with chloroquin as well as the inhibition of macropinicytosis with amiloride reduced the amount of actively accumulated copper by 61 ± 21% and 81 ± 7%, respectively. A less but also significant reduction in the active copper accumulation by approximately 47% was obtained by the inhibition of clathrin mediated endocytosis with the inhibitor chlorpromazine (Chapter 2.4). However, complete inhibition of active copper accumulation, as seen in incubations performed at 4°C, could not be achieved, hinting towards a contribution of multiple endocytotic processes in the uptake of CuO-NPs by astrocytes (Figure 3.1). Comparable results for the inhibition of nanoparticles accumulation were reported previously for silver nanoparticles and ironoxide nanoparticles (Luther et al. 2011, Geppert et al. 2012), as discussed previously (Chapter 2.4).

In contrast to CuO-NPs, ionic copper can be transported into astrocytes by the copper transporter Ctr1 and by the divalent metal transporter 1 (Scheiber et al. 2010, 2014). The transport of copper was strongly accelerated when the applied Cu^{2+} was reduced to Cu^{+} in presence of reducing agents such as ascorbate or other antioxidants, demonstrating the preference of those transporters for Cu^{+} (Chapter 2.3). The application of extracellular copper chelators prevented the uptake of ionic copper even in the reduced form (Chapter 2.3). Despite the different mechanisms involved in the uptake of CuO-NPs or copper ions (Figure 3.1), the accumulated copper dramatically affected the viability of the exposed cells, as described in the next chapters.

3.3 Toxicity of copper and copper oxide nanoparticles on astrocytes

Several cell parameters were investigated to obtain a concise knowledge on the toxic effects of CuO-NPs. Hereby it was shown that the different methods used provide readings at different stages of toxicity (Chapter 2.3). The reduction capacity of the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by copper-exposed astrocytes appears to be the earliest indicator of toxicity (Chapter 2.3). This method provides an insight into the activity of cellular reductases which are responsible

for the reduction of MTT, although the detailed mechanisms involved in the cellular reduction of MTT are so far not elucidated (Stockert et al. 2012). Nevertheless, the determination of the cellular MTT reduction capacity gives an idea about the metabolic state of the cell which does not necessarily account for a loss in cell viability (Riss et al. 2013). Methods which determine later stages of toxicity test for the integrity of the cellular membranes by investigating the permeability of those for dyes or enzymes (Hohnholt et al. 2015). In the presented thesis it is shown, that staining cells with propidium iodide and determining the activity of the cytosolic enzyme lactate dehydrogenase are reliable indicators for cell death (Chapter 2.1-2.4). However, determining the activity of enzymes can strongly be affected by the presence of copper, as copper is a potent inhibitor of several enzymes including lactate dehydrogenase (Lai and Blass 1984). Recently it was reported that several biological assays can be affected by different nanoparticles (Kroll et al 2012, Ong et al 2014). Conclusively, it is necessary to apply a combination of several assay systems to get robust information about the vitality of cells and to test the reliability of those assays in presence of ionic copper and CuO-NPs.

Astrocytes exposed to CuO-NPs strongly accumulated copper in a time-, concentration-, temperature- and media-dependent manner (Chapter 2.1; Chapter 2.2; Chapter 2.4). In these performed studies was demonstrated, that an increase in the cellular copper contents is mirrored by a decrease in cellular vitality (Chapter 2.1-2.4). If in this case the cellular copper content of the exposed cells exceeded a threshold value of 10 nmol copper per mg protein, the vitality of the cells started to decrease (Chapter 1.3). To our knowledge, this is the first report of such a threshold value for the intracellular copper content of astrocytes regarding toxicity. Accordingly, increasing the cellular copper contents by facilitating the copper uptake resulted hereby in even stronger toxicity (Chapter 2.3).

The presented work demonstrates, that copper can severely damage astrocytes if it is accumulated by those cells in high quantities (Chapter 2.1-2.4, for an overview see Figure 3.2). The primary source of the observed toxicity is most likely copper facilitates the increased generation of reactive oxygen species (Chapter 2.1; 2.3). Indeed, exposure of astrocytes with CuO-NPs as well as copper ions led to an increased generation of reactive oxygen species (Chapter 2.1; 2.3). The occurrence of reactive oxygen species and the loss in cell viability after exposure to CuO-NPs was drastically lowered by the

application of an intracellular copper ion chelator (Chapter 2.1; 2.3), indicating an intracellular liberation of copper ions from the internalized CuO-NPs (Chapter 1.3).

The ability of astrocytes to store nanoparticles in vesicles was shown for iron oxide nanoparticles (Geppert et al. 2011, Petters et al. 2015). The endocytotic uptake of CuO-NPs by astrocytes hints towards their vesicular storage (Figure 3.1; Chapter 1.3; Chapter 2.4). As endocytotic vesicles containing CuO-NPs might rapidly enter the lysosomal pathway, those particles will encounter an reducing and acidic environment (de Duve 1983, Chiang and Maric 2011, Wang et al. 2013) The stability of CuO-NPs under these conditions might be affected, as these conditions were already reported to strongly facilitate the dissolution of CuO-NPs (Studer et al. 2010, Odzak et al. 2014, Semisch et al. 2014, Son et al. 2015). So far it can not be excluded that the toxicity observed in treatments of astrocytes with CuO-NPs exclusively resulted from the liberation of copper ions. The lysosomal degradation of accumulated CuO-NPs and the liberation of copper ions were also discussed for other cell types like HeLa cells and A549 cells (Limbach et al. 2007, Karlsson et al. 2008, Studer et al. 2010, Semisch et al. 2014), hinting towards a similar mechanism in astrocytes. Conclusively, astrocytes are most likely not able to cope with this dramatic increase in the intracellular pool of copper ions and the associated increase in reactive oxygen species, resulting in the observed decrease in the vitality of CuO-NP-treated astrocytes (Figure 3.2).

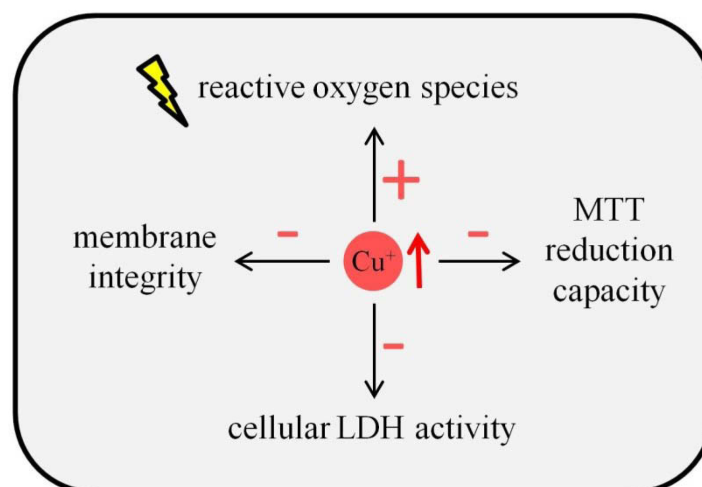

Figure 3.2: Toxicity indicators in astrocytes exposed to copper. Astrocytes treated with ionic copper or CuO-NPs increased their intracellular copper pool (see Figure 3.1). Cellular copper contents exceeding threshold values of 10 nmol copper per mg protein result in increased generation of reactive oxygen species as well as decreased membrane integrity, MTT reduction capacity and cellular LDH activity.

3.4 Stimulation of the astrocytic metabolism by copper oxide nanoparticles

Beside the strong toxicity, as discussed above, a copper treatment of astrocytes can results in a variety of different consequences (Chapter 1.3). After a chronic treatment of astrocytes with sub-toxic concentrations of CuO-NPs the cells were able to adapt to increased cellular copper levels (Chapter 2.2). Astrocytes which were incubated with 100 µM CuO-NPs for 24 h showed an accelerated glycolytic flux, increased glutathione and metallothioneine levels (Figure 3.3; Chapter 2.2). Interestingly, the observed effects were almost completely abolished in presence of an cell permeable chelator of copper ions (Chapter 2.2). This demonstrates the contribution of low molecular weight copper in the observed alterations, which might be liberated from internalized CuO-NPs as discussed above. Indeed, similar stimulatory effects on glycolysis, glutathione synthesis and metallothioneine content and were reported for astrocytes which were exposed to $CuCl_2$ (Scheiber and Dringen 2011a, Scheiber and Dringen 2011b, Scheiber and Dringen 2013).

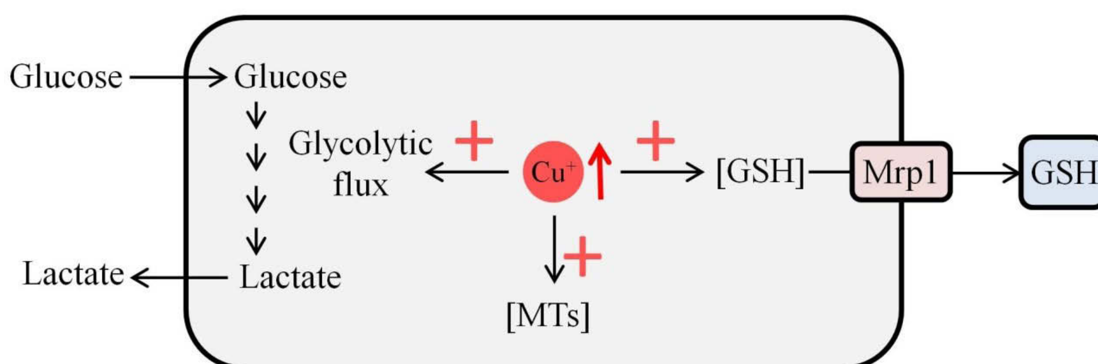

Figure 3.3: Stimulation of the astrocytic metabolism by increased cellular copper levels. Increased cellular copper levels strongly affected cellular levels of metallothioneins (MTs) and glutathione (GSH), accelerated the glutathione export by the multidrug resistance protein 1 and increased the glycolytic flux. Adapted with kind permission from Springer Science+Business Media: Neurochemical Research, Bulcke and Dringen 2015, "Handling of Copper and Copper Oxide Nanoparticles by Astrocytes", Figure 3, copyright 2015.

At least the stimulation of the glycolytic flux by copper seems to depend on protein synthesis (Scheiber and Dringen 2011b). Therefore, a potential explanation for the observed effects caused by a treatment of astrocytes with copper could be the activation

of transcription factors (Grubman and White 2014). For instance, stabilization of the hypoxia inducible factor (HIF) 1α was reported to stimulate the glycolytic flux in cultured astrocytes (Schubert et al. 2009). Further, it was shown that at least in human umbilical vein endothelial cells the expression of HIF-1α regulated genes is partially copper dependent (Zhang et al. 2014). Nevertheless, in cultured astrocytes the stabilization of HIF-1α was excluded as reason for the increased glycolcytic flux in presence of copper (Scheiber and Dringen 2011b).

An alternative transcriptional activator, the nuclear factor erythroid 2-related factor 2 (Nrf2), was stimulated in A172 glioblastoma cells upon exposure to copper and other heavy metals (Simmons et al. 2011). Nrf2 has a central role in the activation of the transcription of metal response elements and antioxidant response elements (Figure 3.4, Song et al. 2014).

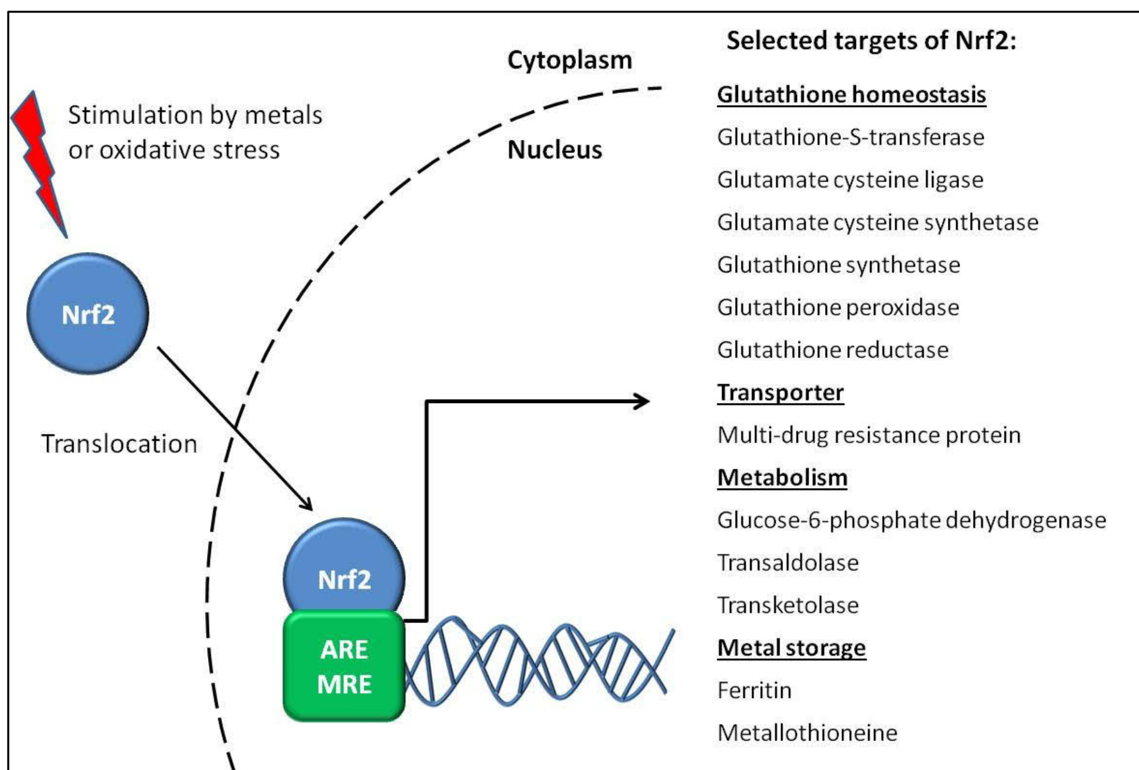

Figure 3.4: Scheme of the activation of nuclear factor erythroid 2-related factor 2 (Nrf2). The transcription factor Nrf2 is activated by metals or oxidative stress. Activated Nrf2 is translocated to the nucleus and binds to antioxdative response elements (ARE) or metal response elements (MRE) and activates the transcription of the indicated selected targets (Tufekci et al. 2011, Song et al. 2014).

The stimulation of those gene elements results in increased expression of glycolytic enzymes, enzymes involved in the glutathione homeostasis and metallothioneines (Tufekci et al. 2011, Song et al. 2014). Therefore, the activation of Nrf2 in astrocytes upon exposure to $CuCl_2$ or CuO-NPs could explain the stimulated expression of proteins which mediate for the stimulated glycolysis, increased glutathione synthesis and metallothioneine levels (Figure 3.4).

The effective stimulation of various protective pathways under copper mediated stress underlines the role of astrocytes in the brain as metal sink, central element of detoxification and metabolic support of neurons (Tiffany-Castiglioni and Qian 2001, Hirrlinger and Dringen 2010, Brekke et al. 2015).

3.5 Reflections on the *in vivo* relevance of the data obtained

The results of this thesis clearly show that CuO-NPs in high concentrations have adverse effects on cultured primary astrocytes. This data in combination with the results of others shows that CuO-NPs are strongly toxic and exert severe deleterious effects on different cell types (Karlsson et al. 2009, Ahamed et al. 2015, Ivask et al. 2015). However, to perform a complete risk assessment for CuO-NPs, additional information has to be obtained (Figure 3.5).

CuO-NPs can be fabricated or unintentionally released into the environment (Figure 3.5). Several studies reported the strong ecotoxicity of CuO-NPs, as lately reviewed (Bondarenko et al. 2013). However, also humans could be exposed to CuO-NPs. The most endangered persons are those constantly exposed to CuO-NP containing exhausts, smoke and fumes (Szymczak et al. 2007, Balkhyour and Goknil 2010). In an occupational exposure scenario especially welders are most probable exposed to CuO-NPs (Balkhyour and Goknil 2010, DFG 2012, Lehnert et al. 2012, DFG 2015). Workers in this branch of industry are strongly exposed to nanoparticles and are known to suffer from severe health impairments such as pulmonary inflammation, chronic kidney and liver damage (Balkhyour and Goknil 2010, DFG 2012, Lehnert et al. 2012, Andujar et al. 2014). The threshold limit value for copper fumes in a daily exposure scenario without adverse health effects is at 0.1 mg per m^3 ambient air at the workplace (DFG 2012, 2015).

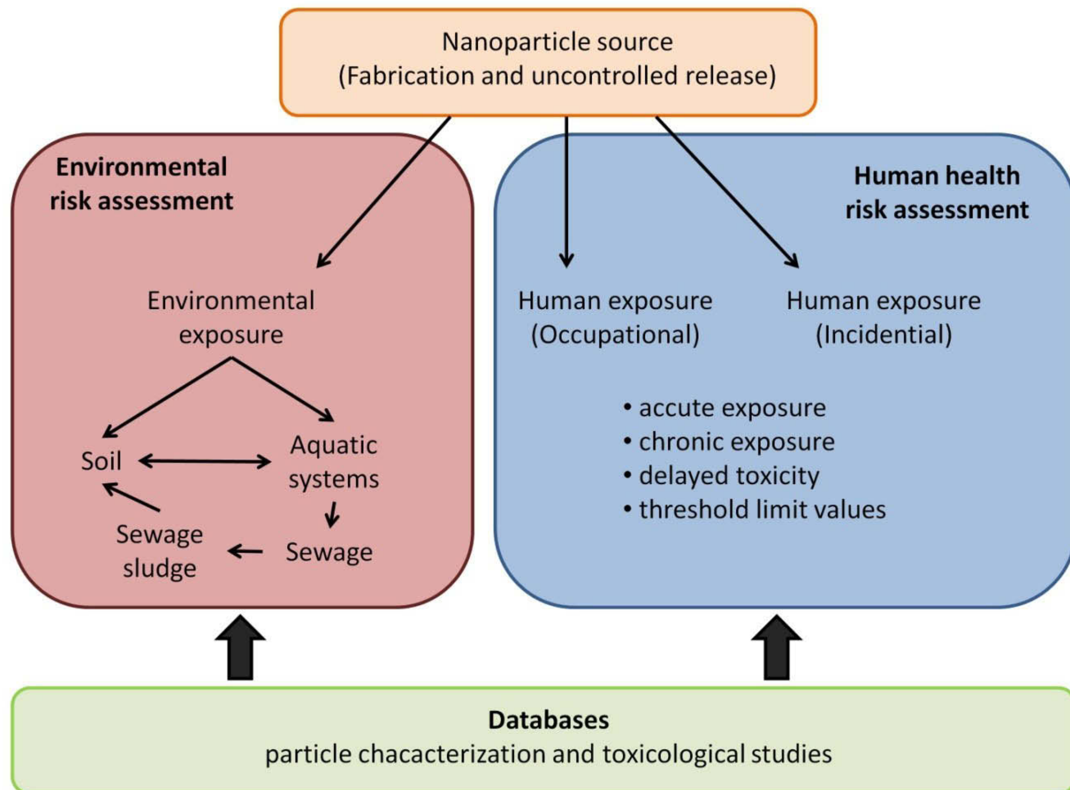

Figure 3.5: Schematic overview of the risk assessment of nanoparticles. Databases with increasing amounts of studies will help in the risk assessment of nanoparticles for the environment and human health.

Several studies report that nanoparticles are able to enter the body by different routes, but inhalation is the most probable uptake route for nanoparticles (Kreyling et al. 2002, Hoet et al. 2004, Oberdörster et al. 2004, Oberdörster et al. 2005). Nanoparticles are able to enter the brain upon inhalation either directly by translocation over the nerve endings of the olfactory bulb or indirectly after uptake into the blood stream (Kreyling et al. 2002, Oberdörster et al. 2004, Sharma and Sharma 2012). However, the amount of nanoparticles which might reach the brain through the bloodstream and subsequently enter the brain by transcytosis through the endothelia cells of the brain capillaries might be very low for various types of nanoparticles (Kreyling et al. 2002). This view may be not correct for CuO-NPs, as CuO-NPs which were applied into the bloodstream of rats are reported to disrupt the integrity of the blood brain barrier and thereby facilitate this entering into the brain (Sharma and Sharma 2012).

In the presented work CuO-NPs were applied in toxic and sub-toxic concentrations over a period of maximal 24 h, which demonstrates already the limitation of the performed *in vitro* experiments. However, the obtained results on cultured astrocytes, which demonstrated the strong uptake and toxicity of CuO-NPs and ionic copper (Chapter 2.1-2.4) might help to explain the deleterious effects of CuO-NPs, which were observed in the subsequent *in vivo* experiments (Migliore et al. 2015). *In vivo* injection and inhalation experiments with CuO-NPs on rats showed that the treated animals suffered severe cognitive impairments and damage of the blood brain barrier (Sharma and Sharma 2007, An et al. 2012, Sharma and Sharma 2012). However, the role of astrocytes in this process of neurodegeneration was so far not elucidated. Taking into account that astrocytes play a key role in the brain homeostasis and that those cells are able to accumulate CuO-NPs over time and strongly react to this treatment, it is necessary to perform further *in vivo* inhalation or injection studies with a focus on astrocytes in the brain, to evaluate the potential role of these cells in the neurotoxicity of CuO-NPs.

3.6 Future perspectives

CuO-NPs synthesized for this work were a good tool to investigate the accumulation and toxicity of CuO-NPs in cultured astrocytes. However, the hydrodynamic size of the particles used in dispersion is relatively large and the stability of the particles is poor in comparison to other types of nanoparticles (Geppert et al. 2011, Luther et al. 2011, Petters et al. 2014). Several improvements of the particle synthesis are possible to obtain smaller and more stable particles. The current method describes the alkaline precipitation of CuO-NPs with a subsequent coating (Chapter 2.1). An improvement of this method is the alteration of the synthesis method by alkaline precipitation of CuO-NPs in presence of the coating material (Lüken 2014). This improved synthesis method harvested particles with a hydrodynamic diameter of 59 nm in water, which is half of the hydrodynamic diameter of the CuO-NPs used in the presented work. To improve the stability of DMSA-coated CuO-NPs against agglomeration in media containing low amounts of protein, an additional coating of CuO-NPs with proteins, as described in chapter 2.4 for BSA, could be used for future studies. As so far no data on the long term stability of protein coated CuO-NPs were obtained, such experiments need to be conducted. The particle dissolution of CuO-NPs should be reduced by modification of the nanoparticle surface, for example with carbon or natural organic matter (Odzak et al.

2014, Son et al. 2015). In any case, altering the synthesis method of CuO-NPs involves a detailed characterization of the particles to ensure stability and reproducibility. As described previously, small changes in the particle properties could lead to differences in the bioavailability and toxicity (Chapter 3.1).

Fluorescent nanoparticles were reported to be a good tool for the intracellular visualization and for tracking the fate of the nanoparticles in cells (Kim et al. 2008, Wang et al. 2015). DMSA as coating material allows the introduction of fluorescent dyes as it was already done for iron oxide nanoparticles (Luther et al. 2013, Petters et al. 2014). In these reports, the thiol groups of the DMSA were functionalized with a fluorescent molecule. All thiol reactive dyes could possibly be introduced to DMSA. Fluorescent CuO-NPs should be synthesized and characterized to analyze the fate of those nanoparticles in cells.

The intracellular localization of nanoparticles can be analyzed by electron microscopy (Geppert et al. 2011). However, the limitation of this technique is that it just gives insight if nanoparticles are present or stored in vesicular structures. Several fluorescent markers and antibodies for the visualization of vesicles, different stages of endosomes and lysosomes are commercially available. These markers in combination with the fluorescently labeled CuO-NPs could visualize the uptake pathway and fate of those nanoparticles in cells. Moreover, those studies should be performed in cultured astrocytes, to verify the hypothesis that CuO-NPs are present in lysosomes after the accumulation, as already confirmed for fluorescent labeled iron oxide nanoparticles (Petters et al. 2015).

In the presented work, endocytosis inhibitors were used to investigate the uptake mechanism of CuO-NPs by astrocytes. However, the low specificity of the used inhibitors is the limitation of this method (Vercauteren et al. 2010). For a detailed analysis of the uptake mechanism, the small interfering RNA (siRNA) technique could be used to specifically suppress targeted endocytotic pathways (Chang et al. 2014). Nevertheless, it should be taken into account that endocytosis is a very dynamic process in which alternative pathways for the uptake of material could be used if one pathway is shut down (Huth et al. 2006, Vercauteren et al. 2010). Despite this disadvantage, the siRNA technique should be applied to identify the endocytotic pathways involved in the uptake of CuO-NPs.

CuO-NPs severely reduced the vitality of astrocytes if they were accumulated in high amounts. This toxicity was attributed to the extended generation of reactive oxygen species (Chapter 2.1). *In vivo* experiments with mice and *in vitro* experiments with podocytes showed, that a treatment with CuO-NPs results in oxidative DNA damage and the formation of micronuclei (Perreault et al. 2012, Song et al. 2012). The verification of oxidative DNA damage and the formation of micronuclei was so far not elucidated for astrocytes and therefore should be performed. Additionally, the pathway of cell death in astrocytes exposed to CuO-NPs was so far not revealed. Several cellular signaling pathways can be affected by the presence of copper (Grubman and White 2014). Recently it was shown, that the CuO-NPs induced toxicity is regulated at least in fibroblasts and keartinocytes by the mitogen-activated protein (MAP) kinase and p53 (Luo et al. 2014). Further it was reported, that at least in A549 lung epithelia cells CuO-NPs induced autophagy, as shown by reduced levels of caspase-3 and microtubule-associated protein 1 light chain 3 (LC3) (Sun et al. 2012). Westen blot experiments with CuO-NP treated astrocytes should be performed to elucidate the participation of the MAP kinase, p53, caspase-3 and LC3 in the cell death.

Astrocytes treated with sub-toxic concentrations of CuO-NPs showed a stimulated glycolytic flux and increased their glutathione and metallothioneine levels (Chapter 2.2). However, the mechanism behind these protective measures is so far not known and should be elucidated. As discussed above, a potential target involved in the upregulation of these processes could be the transcription factor Nrf2 (Chapter 3.4). Recently it was shown that tetramethylpyratine (TMP), an enhancer of Nrf2 activity, could protect PC-12 cells from $CoCl_2$ derived toxicity (Guan et al. 2015). TMP enhanced the activity of Nrf2 and γ-glutamylcysteine ligase, whereas the hypoxia-inducible factor 1α and the NADPH oxidase 2 mediated generation of reactive oxygen species was suppressed (Guan et al. 2015). The involvement of Nrf 2 was determined in this study by Western blot analysis and the transcriptional activity was determined by a luciferase reporter gene assay (Guan et al. 2015). Similar studies should be performed to investigate wheather TMP can protect astrocytes from CuO-NP derived toxicity and wheather Nrf2 is involved in the upregulation of the observed protective mechanism.

The tissue copper content strongly differs between certain brain regions (Davies et al. 2013). For example, with 11.4 µg copper per g wet weight the substantia nigra contains almost double the amount of copper compared to the cerebellum or the visual cortex

(Davies et al. 2013). Certain cell types like astrocytes are more efficient to accumulate copper that other cell types, for example neuronal cells (Scheiber et al. 2010, Liu et al. 2013). Different cell types can also have different capabilities to take up nanoparticles, as recently shown for the uptake of iron oxide nanoparticles in different brain cells (Petters et al. 2015). Therefore it is of high interest to study the uptake and also the toxicity of CuO-NPs in different cell types to investigate whether certain types of cells are more susceptible to the CuO-NP derived toxicity. Those *in vitro* experiments can be used to clarify the mechanisms behind the toxicity. It will be necessary to conduct *in vivo* experiments to transform the results obtained from such *in vitro* experiments to the occupational exposure scenario. *In vivo* inhalation studies should be performed to clarify if harmful amounts of CuO-NPs can even reach the brain trough inhalation and how the distribution of those particles will take place between different organs and cell types. As recently reviewed, only few *in vivo* inhalation studies were conducted with CuO-NPs which mostly focused on the lung toxicity of this material (Ahamed et al. 2015). Only one *in vivo* study focused on the neurotransmitter changes of the brain after intranasal instillation of CuO-NPs (Zhang et al. 2012). Additionally, some *in vivo* injection studies with CuO-NPs were performed that showed the disruption of the blood brain barrier, neuronal damage and behavioral changes of the test animals (An et al. 2012, Sharma and Sharma 2012).

Several adverse effects of CuO-NPs were discussed up to this point, but so far not much was told about the potential benefits of CuO-NPs. Among their broad range of applications (Chapter 1.1), CuO-NPs could potentially be used as biomedical agent. As mentioned in the previous chapters it was reported, that CuO-NPs can be accumulated by several cell types including astrocytes. Additionally it was shown, that CuO-NPs are prone to dissolve over time. If the properties of CuO-NPs could be altered in such a way that the release of low molecular weight copper ions is defined in a slow manner, those particles could be used to serve as copper depot. Astrocytes, which are suggested as a fulcrum of the copper homeostasis in brain (Scheiber and Dringen 2013), can be seen as a copper storing and distributing cell type (Tiffany-Castiglioni et al. 2011). Several neurodegenerative disorders are linked to disturbances in the copper metabolism of the brain (Grubman et al. 2014a, Scheiber et al. 2014). Menkes disease is characterized by decreased copper levels in the body due to a mutation of the copper transport protein ATP7A, which results in a decreased copper absorption from the intestine (Barnes et al. 2005, Ahuja et al. 2015). In contrast to Menkes disease in which a general copper

deficiency is a hallmark, Alzheimer's disease and Parkinson's disease are characterized by a local copper deficiency in which either the proteins amyloide-β or α-synuclein aggregate and strongly bind copper (Ahuja et al. 2015, Dusek et al. 2015). Subsequently, the copper bound to those aggregates is unavailable for neurons (Ahuja et al. 2015, Dusek et al. 2015). Amyotrophic lateral sclerosis is another neurodegenerative disorder in which a defective superoxide dismutase 1 has a decreased metal binding capacity connected with a toxic gain of function (Roberts et al. 2014). Copper delivering therapies are currently under investigation for the treatment of the mentioned neurodegenerative disorders (Iakovidis et al. 2011, Bica et al. 2014, Grubman et al. 2014b, Roberts et al. 2014). CuO-NPs should be utilized in these deficiency scenarios to supply neurons and other cells of the brain with the essential copper. Several animal model systems for different neurodegenerative disorders are commercially available (Ghorayeb et al. 2011, Bezard et al. 2013, Ribeiro et al. 2013), in which CuO-NPs should be tested for their treatment capability of copper deficiency.

In contrast to the copper depot approach, CuO-NPs could also be utilized as anti-cancer tool. In this scenario, CuO-NPs would be selectively directed to cancer cells and cause death of these cells. It was already shown that cancer cells seem to be more sensitive to CuO-NPs in comparison to normal cells (Wang et al. 2012). The CuO-NPs of the here present work were tested in astrocytes, therefore a direct comparison to glioma cell lines like C6-cells should be conducted to investigate weather CuO-NPs affect cancer cells stronger than normal cells. In case this hypothesis can be verified, approaches to target and direct those nanoparticles specifically to selected cancer cells by surface modifications, for examples with glycans, peptides or antibodies, should be performed. In case the targeting of the CuO-NPs to cancer cells is effective in cancer cell containing mixed cell cultures, these CuO-NPs should be tested in vivo for their ability to selectively reach and kill cancer cells. The focus of research on nanoparticles for cancer therapy and diagnostic over the last years was predominantly performed for iron oxide, gold, silica and polymeric nanoparticles (Ediriwickrema and Saltzman 2015, Sharma et al. 2015). Those nanoparticles were mainly used as carriers for drugs or siRNA delivery, with the advantage of a low toxicity of the nanoparticle core material (Ediriwickrema and Saltzman 2015, Sharma et al. 2015). Several nanoparticles based drugs for cancer therapy are currently in clinical trials, whereas only few are approved for usage (Wicki et al. 2015). However, CuO-NPs might be a promising alternative to the currently investigated nanoparticles in cancer therapy.

3.7 References

Ahamed M, Akhtar MJ, Alhadlaq HA, Alrokayan SA (2015) Assessment of the lung toxicity of copper oxide nanoparticles: current status. Nanomedicine (Lond) 10:2365-2377

Ahuja A, Dev K, Tanwar RS, Selwal KK, Tyagi PK (2015) Copper mediated neurological disorder: Visions into amyotrophic lateral sclerosis, Alzheimer and Menkes disease. J Trace Elem Med Biol 29:11-23

An L, Liu S, Yang Z, Zhang T (2012) Cognitive impairment in rats induced by nano-CuO and its possible mechanisms. Toxicol Lett 213:220-227

Andujar P, Simon-Deckers A, Galateau-Sallé F, Fayard B, Beaune G, Clin B, Billon-Galland M-A, Durupthy O, Pairon J-C, Doucet J, Boczkowski J, Lanone S (2014) Role of metal oxide nanoparticles in histopathological changes observed in the lung of welders. Part Fibre Toxicol 11:23-23

Balkhyour MA and Goknil MK (2010) Total fume and metal concentrations during welding in selected factories in Jeddah, Saudi Arabia. Int J Environ Res Public Health 7:2978-2987

Barnes N, Tsivkovskii R, Tsivkovskaia N, Lutsenko S (2005) The copper-transporting ATPases, Menkes and Wilson disease proteins, have distinct roles in adult and developing cerebellum. J Biol Chem 280:9640-9645

Bezard E, Yue Z, Kirik D, Spillantini MG (2013) Animal models of Parkinson's disease: limits and relevance to neuroprotection studies. Mov Dis 28:61-70.

Bica L, Liddell JR, Donnelly PS, Duncan C, Caragounis A, Volitakis I, Paterson BM, Cappai R, Grubman A, Camakaris J, Crouch PJ, White AR (2014) Neuroprotective copper bis(thiosemicarbazonato) complexes promote neurite elongation. PLoS ONE 9:e90070

Bondarenko O, Juganson K, Ivask A, Kasemets K, Mortimer M, Kahru A (2013) Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: a critical review. Arch Toxicol 87:1181-1200

Brandt U, Deters A, Steinbuchel A (2015) A jack-of-all-trades: 2-mercaptosuccinic acid. Appl Microbiol Biotechnol 99:4545-4557

Brekke E, Morken TS, Sonnewald U (2015) Glucose metabolism and astrocyte–neuron interactions in the neonatal brain. Neurochem Int 82:33-41

Chang C-C, Wu M, Yuan F (2014) Role of specific endocytic pathways in electrotransfection of cells. Mol Ther Methods Clin Dev 1:14058

Chiang H-S and Maric M (2011) Lysosomal thiol reductase negatively regulates autophagy by altering glutathione synthesis and oxidation. Free Radic Biol Med 51:688-699

Davies KM, Hare DJ, Cottam V, Chen N, Hilgers L, Halliday G, Mercer JFB, Double KL (2013) Localization of copper and copper transporters in the human brain. Metallomics 5:43-51

de Duve C (1983) Lysosomes revisited. Europ J Biochem 137:391-397

Derjaguin B and Landau L (1941) Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solutions of electrolytes. Acta Phys Chim 14:633-662

DFG (2012). Kupfer und seine anorganischen Verbindungen [MAK Value Documentation in German language, 2007]. In: The MAK-Collection for Occupational Health and Safety. Eds: DFG. Weinheim, Germany, Wiley-VCH Verlag GmbH & Co. KGaA

DFG (2015). Stoffliste. In: MAK- und BAT-Werte-Liste 2015. Eds: DFG. Weinheim, Germany, Wiley-VCH Verlag GmbH & Co. KGaA

Di Bucchianico S, Fabbrizi MR, Misra SK, Valsami-Jones E, Berhanu D, Reip P, Bergamaschi E, Migliore L (2013) Multiple cytotoxic and genotoxic effects induced in vitro by differently shaped copper oxide nanomaterials. Mutagenesis 28:287-299

Dusek P, Roos PM, Litwin T, Schneider SA, Flaten TP, Aaseth J (2015) The neurotoxicity of iron, copper and manganese in Parkinson's and Wilson's diseases. J Trace Elem Med Biol 31:193-203

Ediriwickrema A and Saltzman WM (2015) Nanotherapy for cancer: Targeting and multifunctionality in the future of cancer therapies. ACS Biomat Sci Eng 1:64-78

Fauconnier N, Pons JN, Roger J, Bee A (1997) Thiolation of maghemite nanoparticles by dimercaptosuccinic acid. J Colloid Interface Sci 194:427-433

Fedele L, Colla L, Bobbo S, Barison S, Agresti F (2011) Experimental stability analysis of different water-based nanofluids. Nanoscale Res Lett 6:300

Geppert M, Hohnholt MC, Thiel K, Nurnberger S, Grunwald I, Rezwan K, Dringen R (2011) Uptake of dimercaptosuccinate-coated magnetic iron oxide nanoparticles by cultured brain astrocytes. Nanotechnology 22:145101

Geppert M, Petters C, Thiel K, Dringen R (2012) The presence of serum alters the properties of iron oxide nanoparticles and lowers their accumulation by cultured brain astrocytes. J Nanopart Res 15:1-15

Ghorayeb I, Page G, Gaillard A, Jaber M (2011). Neurochemical mechanisms in disease: Animal models of neurodegenerative diseases. In: Advances in neurobiology. Eds: Blass JP. New York, Springer: 49-101

Grubman A, James SA, James J, Duncan C, Volitakis I, Hickey JL, Crouch PJ, Donnelly PS, Kanninen KM, Liddell JR, Cotman SL, de J, White AR (2014a) X-ray fluorescence imaging reveals subcellular biometal disturbances in a childhood neurodegenerative disorder. Chem Sci 5:2503-2516

Grubman A, White AR, Liddell JR (2014b) Mitochondrial metals as a potential therapeutic target in neurodegeneration. Br J Pharmacol 171:2159-2173

Grubman A and White AR (2014) Copper as a key regulator of cell signalling pathways. Expert Rev Mol Med 16:e11

Guan D, Su Y, Li Y, Wu C, Meng Y, Peng X, Cui Y (2015) Tetramethylpyrazine inhibits CoCl2-induced neurotoxicity through enhancement of Nrf2/GCLc/GSH and suppression of HIF1α/NOX2/ROS pathways. J Neurochem 134:551-565

Hirrlinger J and Dringen R (2010) The cytosolic redox state of astrocytes: Maintenance, regulation and functional implications for metabolite trafficking. Brain Res Rev 63:177-188

Hoet P, Bruske-Hohlfeld I, Salata O (2004) Nanoparticles - known and unknown health risks. J Nanobiotechnology 2:12

Hohnholt M, Geppert M, Luther E, Petters C, Bulcke F, Dringen R (2013) Handling of iron oxide and silver nanoparticles by astrocytes. Neurochem Res 38:227-239

Hohnholt MC, Blumrich EM, Dringen R (2015) Multiassay analysis of the toxic potential of hydrogen peroxide on cultured neurons. J Neurosci Res 93:1127-1137

Hohnholt MC, Geppert M, Dringen R (2011) Treatment with iron oxide nanoparticles induces ferritin synthesis but not oxidative stress in oligodendroglial cells. Acta Biomat 7:3946-3954

Huth US, Schubert R, Peschka-Süss R (2006) Investigating the uptake and intracellular fate of pH-sensitive liposomes by flow cytometry and spectral bio-imaging. J Control Release 110:490-504

Iakovidis I, Delimaris I, Piperakis SM (2011) Copper and its complexes in medicine: A biochemical approach. Mol Biol Int 2011:594529

Ivask A, Titma T, Visnapuu M, Vija H, Kakinen A, Sihtmae M, Pokhrel S, Madler L, Heinlaan M, Kisand V, Shimmo R, Kahru A (2015) Toxicity of 11 metal oxide nanoparticles to three mammalian cell types in vitro. Curr Top Med Chem 15:1914-1929

Karlsson HL, Cronholm P, Gustafsson J, Möller L (2008) Copper oxide nanoparticles are highly toxic: A comparison between metal oxide nanoparticles and carbon nanotubes. Chem Res Toxicol 21:1726-1732

Karlsson HL, Gustafsson J, Cronholm P, Möller L (2009) Size-dependent toxicity of metal oxide particles—A comparison between nano- and micrometer size. Toxicol Lett 188:112-118

Kettler K, Veltman K, van de Meent D, van Wezel A, Hendriks AJ (2014) Cellular uptake of nanoparticles as determined by particle properties, experimental conditions, and cell type. Environ Toxicol Chem 33:481-492

Kim BYS, Jiang W, Oreopoulos J, Yip CM, Rutka JT, Chan WCW (2008) Biodegradable quantum dot nanocomposites enable live cell labeling and imaging of cytoplasmic targets. Nano Letters 8:3887-3892

Kim JE, Kim H, An SS, Maeng EH, Kim MK, Song YJ (2014) In vitro cytotoxicity of SiO2 or ZnO nanoparticles with different sizes and surface charges on U373MG human glioblastoma cells. Int J Nanomedicine 9 Suppl 2:235-241

Kobayashi Y, Maeda T, Watanabe K, Ihara K, Yasuda Y, Morita T (2011) Preparation of CuO nanoparticles by metal salt-base reaction in aqueous solution and their metallic bonding property. J Nanopart Res 13:5365-5372

Kreyling WG, Semmler M, Erbe F, Mayer P, Takenaka S, Schulz H, Oberdorster G, Ziesenis A (2002) Translocation of ultrafine insoluble iridium particles from lung epithelium to extrapulmonary organs is size dependent but very low. J Toxicol Environ Health A 65:1513-1530

Kroll A, Pillukat MH, Hahn D, Schneckenburger J (2012) Interference of engineered nanoparticles with in vitro toxicity assays. Arch Toxiclo 86:1123-1136

Lai JC and Blass JP (1984) Neurotoxic effects of copper: inhibition of glycolysis and glycolytic enzymes. Neurochem Res 9:1699-1710

Lehnert M, Pesch B, Lotz A, Pelzer J, Kendzia B, Gawrych K, Heinze E, Van Gelder R, Punkenburg E, Weiss T, Mattenklott M, Hahn J-U, Möhlmann C, Berges M, Hartwig A, Brüning T, The Weldox Study G (2012) Exposure to inhalable, respirable, and ultrafine particles in welding fume. Ann Occup Hyg 56:557-567

Lignier P, Bellabarba R, Tooze RP (2012) Scalable strategies for the synthesis of well-defined copper metal and oxide nanocrystals. Chem Soc Rev 41:1708-1720

Limbach LK, Wick P, Manser P, Grass RN, Bruinink A, Stark WJ (2007) Exposure of engineered nanoparticles to human lung epithelial cells: Influence of chemical composition and catalytic activity on oxidative stress. Environ Sci Technol 41:4158-4163

Liu JJ, Kim Y, Yan F, Ding Q, Ip V, Jong NN, Mercer JFB, McKeage MJ (2013) Contributions of rat Ctr1 to the uptake and toxicity of copper and platinum anticancer drugs in dorsal root ganglion neurons. Biochem Pharmacol 85:207-215

Lüken KP (2014) Synthese und Charakterisierung fluoreszierender Kupfer(II)oxid-Nanopartikel. Bachelor thesis in chemistry, University of Bremen, Germany

Luo C, Li Y, Yang L, Zheng Y, Long J, Jia J, Xiao S, Liu J (2014) Activation of Erk and p53 regulates copper oxide nanoparticle-induced cytotoxicity in keratinocytes and fibroblasts. Int J Nanomedicine 9:4763-4772

Luther E, Koehler Y, Diendorf J, Epple M, Dringen R (2011) Accumulation of silver nanoparticles by cultured primary brain astrocytes. Nanotechnology 22:375101

Luther EM, Petters C, Bulcke F, Kaltz A, Thiel K, Bickmeyer U, Dringen R (2013) Endocytotic uptake of iron oxide nanoparticles by cultured brain microglial cells. Acta Biomat 9:8454-8465

Lynch I, Salvati A, Dawson KA (2009) Protein-nanoparticle interactions: What does the cell see? Nat Nano 4:546-547

Miao L, Wang C, Hou J, Wang P, Ao Y, Li Y, Lv B, Yang Y, You G, Xu Y (2015) Enhanced stability and dissolution of CuO nanoparticles by extracellular polymeric substances in aqueous environment. J Nanopart Res 17:1-12

Migliore L, Uboldi C, Di Bucchianico S, Coppedè F (2015) Nanomaterials and neurodegeneration. Environ Mol Mutagen 56:149-170

Monopoli MP, Aberg C, Salvati A, Dawson KA (2012) Biomolecular coronas provide the biological identity of nanosized materials. Nat Nano 7:779-786

Nel A, Xia T, Mädler L, Li N (2006) Toxic potential of materials at the nanolevel. Science 311:622-627

Nel AE, Madler L, Velegol D, Xia T, Hoek EMV, Somasundaran P, Klaessig F, Castranova V, Thompson M (2009) Understanding biophysicochemical interactions at the nano-bio interface. Nat Mater 8:543-557

Oberdörster G, Oberdörster E, Oberdörster J (2005) Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect 113:823-839

Oberdörster G, Sharp Z, Atudorei V, Elder A, Gelein R, Kreyling W, Cox C (2004) Translocation of inhaled ultrafine particles to the brain. Inhal Toxicol 16:437-445

Odzak N, Kistler D, Behra R, Sigg L (2014) Dissolution of metal and metal oxide nanoparticles in aqueous media. Environ Pollut 191:132-138

Ong KJ, MacCormack TJ, Clark RJ, Ede JD, Ortega VA, Felix LC, Dang MKM, Ma G, Fenniri H, Veinot JCG, Goss GG (2014) Widespread nanoparticle-assay interference: implications for nanotoxicity testing. PLoS ONE 9:e90650

Perreault F, Melegari SP, da Costa CH, de Oliveira Franco Rossetto AL, Popovic R, Matias WG (2012) Genotoxic effects of copper oxide nanoparticles in Neuro 2A cell cultures. Sci Total Environ 441:117-124

Petters C and Dringen R (2014) Comparison of primary and secondary rat astrocyte cultures regarding glucose and glutathione metabolism and the accumulation of iron oxide nanoparticles. Neurochem Res 39:46-58

Petters C and Dringen R (2015) Accumulation of iron oxide nanoparticles by cultured primary neurons. Neurochem Int 81:1-9

Petters C, Irrsack E, Koch M, Dringen R (2014) Uptake and metabolism of iron oxide nanoparticles in brain cells. Neurochem Res 39:1648-1660

Petters C, Thiel K, Dringen R (2015) Lysosomal iron liberation is responsible for the vulnerability of brain microglial cells to iron oxide nanoparticles: comparison with neurons and astrocytes. Nanotoxicology, in press DOI: 10.3109/17435390.17432015.11071445

Ribeiro FM, Camargos ERS, Souza LCD, Teixeria AL (2013) Animal models of neurodegenerative diseases. Rev Bras Psiquiatr 35:S82-91

Riss TL, Moravec RA, Niles AL, Benink HA, Worzella TJ, Minor L, Storts D, Reid Y (2013). Cell Viability Assays. In: Assay Guidance Manual. Eds: GS Sittampalam, NP Coussens, H Nelson et al. Bethesda (MD): Available from: ncbi.nlm.nih.gov/books/NBK144065/

Roberts BR, Lim NK, McAllum EJ, Donnelly PS, Hare DJ, Doble PA, Turner BJ, Price KA, Lim SC, Paterson BM, Hickey JL, Rhoads TW, Williams JR, Kanninen KM, Hung LW, Liddell JR, Grubman A, Monty JF, Llanos RM, Kramer DR, Mercer JF, Bush AI, Masters CL, Duce JA, Li QX, Beckman JS, Barnham KJ, White AR, Crouch PJ (2014) Oral treatment with Cu(II)(atsm) increases mutant SOD1 in vivo but protects motor neurons and improves the phenotype of a transgenic mouse model of amyotrophic lateral sclerosis. J Neurosci 34:8021-8031

Röcker C, Potzl M, Zhang F, Parak WJ, Nienhaus GU (2009) A quantitative fluorescence study of protein monolayer formation on colloidal nanoparticles. Nat Nano 4:577-580

Scheiber IF and Dringen R (2011a) Copper-treatment increases the cellular GSH content and accelerates GSH export from cultured rat astrocytes. Neurosci Lett 498:42-46

Scheiber IF and Dringen R (2011b) Copper accelerates glycolytic flux in cultured astrocytes. Neurochem Res 36:894-903

Scheiber IF and Dringen R (2013) Astrocyte functions in the copper homeostasis of the brain. Neurochem Int 62:556-565

Scheiber IF, Mercer JFB, Dringen R (2010) Copper accumulation by cultured astrocytes. Neurochem Int 56:451-460

Scheiber IF, Mercer JFB, Dringen R (2014) Metabolism and functions of copper in brain. Prog Neurobiol 116:33-57

Schubert D, Soucek T, Blouw B (2009) The induction of HIF-1 reduces astrocyte activation by amyloid beta peptide. Eur J Neurosci 29:1323-1334

Semisch A, Ohle J, Witt B, Hartwig A (2014) Cytotoxicity and genotoxicity of nano - and microparticulate copper oxide: role of solubility and intracellular bioavailability. Part Fibre Toxicol 11:10

Sharma H, Mishra PK, Talegaonkar S, Vaidya B (2015) Metal nanoparticles: a theranostic nanotool against cancer. Drug Discov Today 20:1143-1151

Sharma HS and Sharma A (2007) Nanoparticles aggravate heat stress induced cognitive deficits, blood–brain barrier disruption, edema formation and brain pathology. Prog Brain Res 162:245-273

Sharma HS and Sharma A (2012) Neurotoxicity of engineered nanoparticles from metals. CNS Neurol Disord Drug Targets 11:65-80

Simmons SO, Fan C-Y, Yeoman K, Wakefield J, Ramabhadran R (2011) NRF2 oxidative stress induced by heavy metals is cell type dependent. Curr Chem Genomics 5:1-12

Son J, Vavra J, Forbes VE (2015) Effects of water quality parameters on agglomeration and dissolution of copper oxide nanoparticles (CuO-NPs) using a central composite circumscribed design. Sci Total Environ 521–522:183-190

Song M-F, Li Y-S, Kasai H, Kawai K (2012) Metal nanoparticle-induced micronuclei and oxidative DNA damage in mice. J Clin Biochem Nutr 50:211-216

Song MO, Mattie MD, Lee C-H, Freedman JH (2014) The role of Nrf1 and Nrf2 in the regulation of copper-responsive transcription. Exp Cell Res 322:39-50

Steinman R, Mellman I, Muller W, Cohn Z (1983) Endocytosis and the recycling of plasma membrane. J Cell Biol 96:1-27

Stockert JC, Blázquez-Castro A, Cañete M, Horobin RW, Villanueva Á (2012) MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. Acta Histochem 114:785-796

Studer AM, Limbach LK, Van Duc L, Krumeich F, Athanassiou EK, Gerber LC, Moch H, Stark WJ (2010) Nanoparticle cytotoxicity depends on intracellular solubility: Comparison of stabilized copper metal and degradable copper oxide nanoparticles. Toxicol Lett 197:169-174

Sun T, Yan Y, Zhao Y, Guo F, Jiang C (2012) Copper oxide nanoparticles induce autophagic cell death in A549 cells. PLoS ONE 7:e43442

Szymczak W, Menzel N, Keck L (2007) Emission of ultrafine copper particles by universal motors controlled by phase angle modulation. J Aerosol Sci 38:520-531

Tenzer S, Docter D, Kuharev J, Musyanovych A, Fetz V, Hecht R, Schlenk F, Fischer D, Kiouptsi K, Reinhardt C, Landfester K, Schild H, Maskos M, Knauer SK, Stauber RH (2013) Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology. Nat Nano 8:772-781

Tiffany-Castiglioni E, Hong S, Qian Y (2011) Copper handling by astrocytes: Insights into neurodegenerative diseases. Int J Dev Neurosci 29:811-818

Tiffany-Castiglioni E and Qian Y (2001) Astroglia as metal depots: Molecular mechanisms for metal accumulation, storage and release. Neurotoxicology 22:577-592

Treuel L, Docter D, Maskos M, Stauber RH (2015) Protein corona – from molecular adsorption to physiological complexity. Beilstein J Nanotechnol 6:857-873

Tufekci KU, Civi Bayin E, Genc S, Genc K (2011) The Nrf2/ARE pathway: A promising target to counteract mitochondrial dysfunction in Parkinson's disease. Parkinsons Dis 2011:314082

Vercauteren D, Vandenbroucke RE, Jones AT, Rejman J, Demeester J, De Smedt SC, Sanders NN, Braeckmans K (2010) The use of inhibitors to study endocytic pathways of gene carriers: Optimization and pitfalls. Mol Ther 18:561-569

Verwey E and Overbeek J (1948). The interaction of sol particles having an electric double layer. In: Theory of the stability of lyophobic colloids. Eds: E Verwey and J Overbeek. Amsterdam, Elsevier Pub. Co

Walczak AP, Hendriksen PJ, Woutersen RA, van der Zande M, Undas AK, Helsdingen R, van den Berg HH, Rietjens IM, Bouwmeester H (2015) Bioavailability and biodistribution of differently charged polystyrene nanoparticles upon oral exposure in rats. J Nanopart Res 17:231

Walczyk D, Bombelli FB, Monopoli MP, Lynch I, Dawson KA (2010) What the cell "sees" in bionanoscience. J Am Chem Soc 132:5761-5768

Wang C, Wang Y, Li Y, Bodemann B, Zhao T, Ma X, Huang G, Hu Z, DeBerardinis RJ, White MA, Gao J (2015) A nanobuffer reporter library for fine-scale imaging and perturbation of endocytic organelles. Nat Commun 6:8524

Wang J, Deng X, Zhang F, Chen D, Ding W (2014) ZnO nanoparticle-induced oxidative stress triggers apoptosis by activating JNK signaling pathway in cultured primary astrocytes. Nanoscale Res Lett 9:117

Wang Y, Zi X-Y, Su J, Zhang H-X, Zhang X-R, Zhu H-Y, Li J-X, Yin M, Yang F, Hu Y-P (2012) Cuprous oxide nanoparticles selectively induce apoptosis of tumor cells. Int J Nanomedicine 7:2641-2652

Wang Z, von dem Bussche A, Kabadi PK, Kane AB, Hurt RH (2013) Biological and environmental transformations of copper-based nanomaterials. ACS Nano 7:8715-8727

Wicki A, Witzigmann D, Balasubramanian V, Huwyler J (2015) Nanomedicine in cancer therapy: Challenges, opportunities, and clinical applications. J Control Release 200:138-157

Wilson CL, Natarajan V, Hayward SL, Khalimonchuk O, Kidambi S (2015) Mitochondrial dysfunction and loss of glutamate uptake in primary astrocytes exposed to titanium dioxide nanoparticles. Nanoscale 7:18477-18488

Xu M, Li J, Iwai H, Mei Q, Fujita D, Su H, Chen H, Hanagata N (2012) Formation of nano-bio-complex as nanomaterials dispersed in a biological solution for understanding nanobiological interactions. Sci Rep 2:406

Zhang L, Bai R, Liu Y, Meng L, Li B, Wang L, Xu L, Le Guyader L, Chen C (2012) The dose-dependent toxicological effects and potential perturbation on the neurotransmitter secretion in brain following intranasal instillation of copper nanoparticles. Nanotoxicology 6:562-575

Zhang Z, Qiu L, Lin C, Yang H, Fu H, Li R, Kang YJ (2014) Copper-dependent and -independent hypoxia-inducible factor-1 regulation of gene expression. Metallomics 6:1889-1893