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Accumulation of Iron Oxide and Silver Nanoparticles in Cultured Glial Cells

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

Bremen, März 2013

(Eva M. Luther)

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I Acknowledgements

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II STRUCTURE OF THE THESIS

This thesis is structured into the main sections Introduction, Results and Summarizing discussion. The Introduction gives insights into the basic knowledge on brain astrocytes and microglial cells, on iron oxide and silver nanoparticles (IONPs and AgNPs) and the consequences of an exposure of glial cells to metal-containing nanoparticles. It also contains a recently published review article on handling of IONPs and AgNPs by astrocytes.

The second part (Results) presents the results obtained during the work for this thesis. The results chapter is divided into three sub-chapters which contain two articles that describe the uptake of AgNPs by astrocytes and the biocompatibility of these particles. In addition a manuscript on the uptake of IONPs by microglial cells and on the intracellular localization of these particles is included which has been submitted for publication and is now in revision. The already published articles (chapter 1.5; chapter 2.1 and chapter 2.2) are embedded into this thesis as portable document format (pdf) in the style defined by the respective journal. The manuscript included as chapter 2.3 has very recently been reviewed and the suggestions made by the reviewers will be addressed in a revised version of this manuscript. As some additional experiments are required which, due to time constrains, cannot be performed before submission of this thesis, the initially submitted version of the manuscript has been included. The manuscript has been adapted to the general style of this thesis, but the text and the figures have not been altered. In this chapter the figures and tables are positioned with their legends between the results and the discussion part.

The third part of the thesis presents the Summarizing discussion of the key experimental findings presented in this thesis. Furthermore it gives an outlook on future studies that are based on the work presented in this thesis.

Figures and tables that are not included in the publications or manuscripts are labelled with the number of the respective main chapter followed by a running number according to their appearance in the text (for example, figure 1.2 refers to the second figure in the first chapter).

III SUMMARY

Iron oxide nanoparticles (IONPs) and silver nanoparticles (AgNPs) are frequently used in everyday products as well as for biomedical applications. As nanoparticles (NPs) are known to cross the intact or damaged blood brain barrier, brain cells have to deal with NPs and NP-derived metal ions. Astrocytes and microglia are the first brain cells that are discussed to encounter NPs which reach the brain, but at the start of this thesis only little was known about the effects of AgNPs or IONPs on those cell types.

The consequences of an exposure of astrocytes to AgNPs were studied on astrocyte-rich primary cultures as model systems. These cells efficiently took up AgNPs by endocytotic mechanisms and were neither acutely impaired in their viability nor during prolonged presence of accumulated AgNPs. Neither silver nor AgNPs was exported from the cells but presence of AgNPs in the cells was accompanied by an upregulation of metallothioneins that may safely store AgNP-derived silver ions, thereby protecting astrocytes against the potential toxicity of silver ions. These results are in line with the view that in brain astrocytes efficiently accumulate potentially toxic metals and metal-containing NPs and thereby provide protection for other brain cells.

Primary microglial cultures were established and characterized as cell culture model of microglial cells and used to study the effects of an exposure of microglia to IONPs. Fluorescently labelled IONPs were applied to visualize the uptake and intracellular localization of IONPs. These NPs were rapidly taken up by microglia into lysosomal compartments via endocytotic mechanisms. Viable microglia appeared not to suffer from oxidative stress as the cellular glutathione levels remained stable, however, in contrast to astrocytes that had been treated with comparable IONPs, microglia only tolerated moderate concentrations of accumulated IONPs for a few hours before their viability was impaired which may be a consequence of a liberation of iron ions from the accumulated IONPs.

The data presented in this thesis support the described differences regarding toxicity and uptake of NPs in astrocytes and microglia. This allows the assumption that astrocytes, due to their high capacity to take up NPs without impairment in their viability, may provide protection for microglial cells which efficiently accumulate NPs but are already damaged by the accumulation of relatively low concentrations.

IV ZUSAMMENFASSUNG

Eisenoxid- und Silber-Nanopartikel (IONPs und AgNPs) finden in Haushaltsprodukten und im medizinischen Bereich eine breite Anwendung. Nanopartikel (NPs) können die intakte oder geschädigte Blut-Hirn-Schranke überwinden und so mit Zellen des Gehirns in Kontakt kommen. Astrozyten und Mikroglia sind vermutlich die ersten Zelltypen, die auf ein NP-Vorkommen im Gehirn reagieren. Über Effekte von NPs auf diese Zelltypen war am Beginn dieser Arbeit jedoch nur wenig bekannt.

Die Konsequenzen einer Exposition von Astrozyten gegenüber AgNPs wurden anhand von astrozytenreichen Primärkulturen untersucht. Diese Zellen akkumulierten große Mengen an AgNPs ohne akut oder durch anhaltende Präsenz von AgNPs in ihrer Vitalität beeinträchtigt zu sein. Das akkumulierte Silber wurde nicht exportiert, es konnte jedoch eine Hochregulation von Metallothioneinen beobachtet werden. Die Hochregulation dieser Speicherproteine gilt als Schutzmechanismus um das Gehirn vor möglichen Metall-induzierten Schäden zu bewahren, was die These unterstützt, dass Astrozyten potentiell toxische Metalle akkumulieren um andere Zelltypen des Gehirns vor Schäden zu bewahren.

Primäre Mikrogliakulturen wurden etabliert und charakterisiert um die Effekte einer Exposition von Mikroglia gegenüber IONPs zu untersuchen. Fluoreszierende IONPs ermöglichten die visuelle Darstellung akkumulierter NPs in den Zellen. Nach einer schnellen Aufnahme der IONPs über endozytotische Pfade wurden diese dem Abbau in Lysosomen zugeführt. Unter subtoxischen Bedingungen schienen die Zellen keinen oxidativen Stress zu erleiden, da keine Veränderung des zellulären Glutathiongehaltes festgestellt wurde. Im Gegensatz zu Astrozyten, die mit vergleichbaren IONPs inkubiert worden waren, konnten Mikroglia jedoch nur eine begrenzte Menge an akkumulierten IONPs tolerieren, bevor sie Schaden nahmen, was in der Freisetzung von Eisenionen aus den NPs begründet sein könnte.

Die Daten dieser Doktorarbeit bestätigen die beschriebenen Unterschiede in Aufnahme und Toxizität von NPs in Mikroglia und Astrozyten. Dies lässt die Vermutung zu, dass Astrozyten durch ihre hohe Kapazität für die Aufnahme von NPs, ohne in ihrer Vitalität geschädigt zu werden, eine Schutzfunktion für Mikroglia ausüben könnten, die NPs zwar sehr effizient aufnehmen, aber bereits durch vergleichsweise geringe Konzentrationen geschädigt werden.

V ABBREVIATIONS

°C	degree Celsius
%	percent
µ	micro
ζ	zeta
Ag^+	silver ions
AgNPs	silver nanoparticles
AlNPs	alumina nanoparticles
ANOVA	analysis of variance
ATP	adenosine triphosphate
AuNPs	gold nanoparticles
BBB	blood brain barrier
BODIPY	boron-dipyrromethene
BP	BODIPY, boron-dipyrromethene
BSA	bovine serum albumin
c	centi
C	control
CD68	cluster of differentiation 68
CLC	Chang liver cells
CME	clathrin-mediated endocytosis
CNTs	carbon nanotubes
CNS	central nervous system
CTAB	cetyl trimethylammonium bromide
CvME	caveolin-mediated endocytosis
d	day/days
Da	dalton
DAPI	4',6-diamidino-2-phenylindole
DLS	dynamic light scattering
DMEM	Dulbecco´s modifies Eagle´s medium
DMSA	dimercapto succinic acid
DMT1	divalent metal transporter 1
EDX	energy-dispersive X-ray spectroscopy
e	electron
ed/eds	editor/editors
e.g.	*exempli gratia*, Latin for “for example”
EIPA	5-(N-ethyl-N-isopropyl) amiloride
ER	endoplasmatic reticulum
et al.	*et alii*, Latin for "and others"
FCS	fetal calf serum
FDA	United States Food and Drug Administration
FPN	ferroportin
Ft	ferritin
g	gram/grams
GABA	gamma-aminobutyric acid
GCM	glia-conditioned medium

GFAP	glial fibrillary acidic protein
GSH	glutathione
GSSG	glutathione disulfide
GSx	total glutathione
H33342	Hoechst 33342
h	hour/hours
HEPES	N-(2-hydroxyethyl)piperazine-N´-(2-ethanesulfonic acid)
HIV	human immunodeficiency virus
hMSC	human mesenchymal cells
ho-1	haeme oxigenase-1 gene
HO-1	haeme oxigenase-1
IB	incubation buffer
i.e.	*id est* Latin for "that is"
IL-1β	interleukin 1 beta
IONPs	iron oxide nanoparticles
IRE	iron response element
IRP	iron regulatory protein
k	kilo
l	litre
LDH	lactate dehydrogenase
Lf	lactoferrin
LPS	lipopolysaccharide
m	milli/metre
M	molar (moles/l)
min	minute/minutes
mol	moles
MP	macropinocytosis
MRI	magnetic resonance imaging
mRNA	messenger RNA
MS	multiple sclerosis
MTs	metallothioneins
Mtf	melanotransferrin
MTF-1	metal regulatory transcription factor 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n	number of experiments/ nano
n/a	not available
NADH	nicotinamide adenine dinucleotide
NFκB	nuclear factor kappa B
NP	nanoparticle
ox. Stress	oxidative stress
PBS	phosphate buffered saline
pdf	portable document format
PEG	polyethylene glycol
pH	*pondus hydrogenii* Latin for "potential hydrogen" or "Amount of hydrogen"
PI	propidium iodide
ppm	parts per million

PtdSer	phosphatidylserine
PVP	polyvinyl pyrrolidone
QDs	quantum dots
RNS	reactive nitrogen species
ROS	reactive oxygen species
rtPCR	reverse transcriptase polymerase chain reaction
SiO_2NPs	silicium dioxide nanoparticles
TEM	transmission electron microscopy
Tf	transferrin
TfR	transferrin receptor
TGF-β	transforming growth factor beta
TiO_2NPs	titanium dioxide nanoparticles
TLR	toll-like receptor
TMHF	3,5,5-trimethyl hexanoyl ferrocene
TNF-α	tumour necrosis factor alpha
V	volt

1

INTRODUCTION

1.1

GLIAL CELLS

1.1 GLIAL CELLS

The central nervous system (CNS) consists of different types of cells which can be classified into two major groups – excitable neurons and non-excitable glia cells. Neurons account for approximately 10% of the total cell number in the brain (Verkhratsky and Butt 2007). The remaining 90% are glial cells which are crucial for brain development and maintenance (Araque and Navarrete 2010, Verkhratsky 2010, Kettenmann *et al.* 2011, Pellerin and Magistretti 2012) and fulfil a number of important tasks, including structural and metabolic support, insulation of neurons, immune surveillance and guidance of development (Araque and Navarrete 2010, Sofroniew and Vinters 2010, Verkhratsky 2010, Kettenmann *et al.* 2011, Pellerin and Magistretti 2012). Glial cells can be sub-divided into microglia and macroglia.

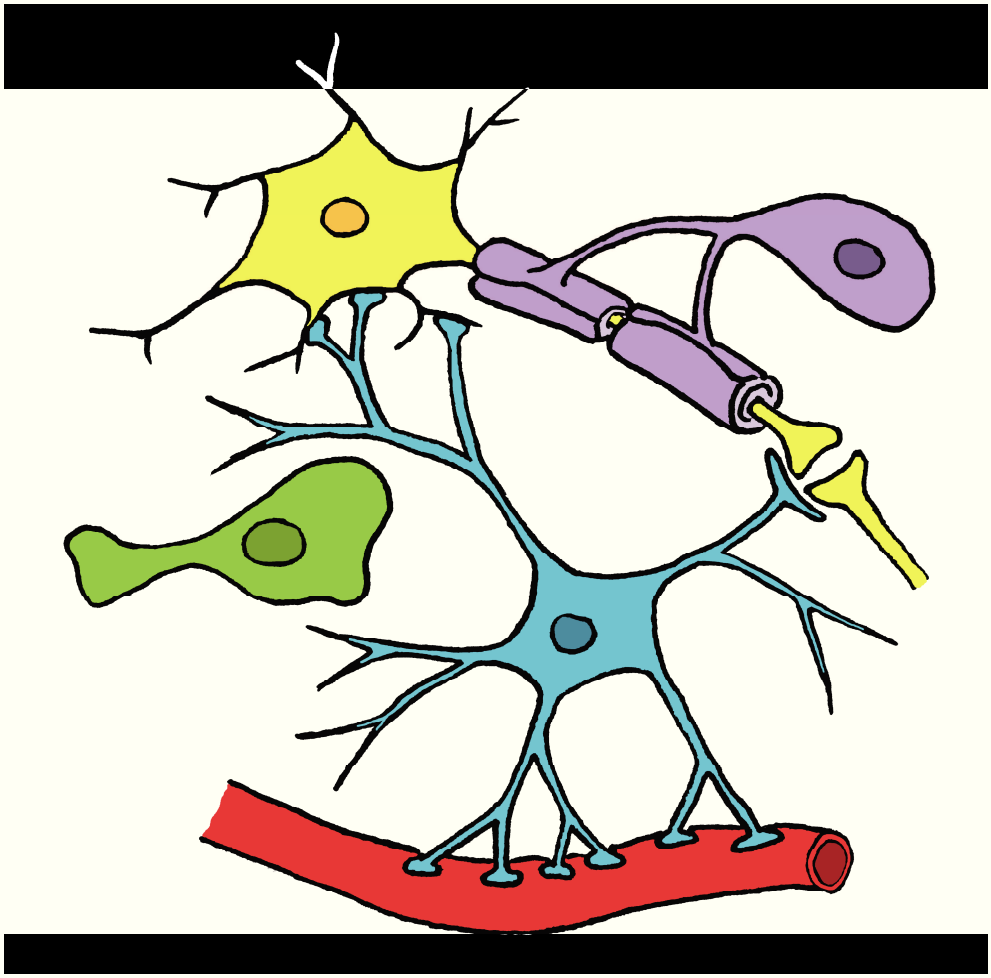

Figure 1.1: Schematic picture of the different brain cell types. Yellow: neuron; purple: oligodendrocyte; blue: astrocyte; green: microglia; red: blood capillary.

Microglia are the immune competent cells of the brain (Kettenmann *et al.* 2011) while macroglia can be further divided into oligodendrocytes, astrocytes and ependymal cells. Oligodendrocytes build the myelin sheaths around neuronal axons to enable saltatory signal conduction along the axons (Miron *et al.* 2011), ependymal cells form the walls of brain ventricles (Verkhratsky and Butt 2007, Kishimoto and Sawamoto 2012) and astrocytes have a variety of structural and metabolic functions. This thesis particularly focuses on astrocytes and microglial cells that are introduced in detail below.

1.1.1 Astrocytes

Astrocytes are the most abundant cells type in brain (Araque and Navarrete 2010) and outnumber neurons by five-fold (Sofroniew and Vinters 2010). Astrocytes were first described already in 1858 by Rudolf Virchow as tissue that binds together nervous elements (cited in Parpura *et al.* 2012), but these cells received their name "astrocytes" much later by Michael von Lenhossek in 1891 (cited in Parpura *et al.* 2012). Although known for more than 100 years, astrocytes were deemed for a long time to be only supporting cells for neurons and intensive studies on glial cells emerged only in the last decades (Verkhratsky 2010, Parpura *et al.* 2012). Nowadays it is generally accepted that astroglial cells are much more than neuronal glue.

1.1.1.1 Astrocytes during development

Astrocytes are of ectodermal origin (Sofroniew and Vinters 2010), their differentiation occurs during the late embryonic and early postnatal period (Lee *et al.* 2009). This is in temporal coincidence with the formation of the blood brain barrier (BBB) where astrocytes are described to participate (Lee *et al.* 2009). In addition, astrocytes play a crucial role in brain architecture by shaping dendritic morphology (Korn *et al.* 2011) and forming structures which guide migrating neurons (Powell and Geller 1999, Verkhratsky and Butt 2007). They are also essential for the formation (Faissner *et al.* 2010, Pfrieger 2010), maintenance and elimination of synapses (Stevens 2008). However since synaptogenesis of GABAergic (GABA: gamma-aminobutyric acid) neurons takes place before astrocytes differentiate (Huang and Scheiffele 2008), only the development of glutamatergic connections is considered to depend on astrocytes (Pfrieger 2010).

1.1.1.2 Astrocytes and brain architecture

Astrocytes in the vertebrate brain form ten-thousands to millions of processes (Oberheim *et al.* 2009, Verkhratsky 2010). They play a crucial role in sustaining brain architecture as there is no region in the brain that is devoid of astrocytes (Sofroniew and Vinters 2010). Astrocytes divide the brain tissue into relatively independent structural units according to their functional territories which is known as "tiling" (Sofroniew and Vinters 2010, Verkhratsky 2010). These territories only overlap in the very distal tips of processes. Here gap junctions are formed between the cells by connexins and several astrocytes build a functional syncytium (Verkhratsky 2010, Giaume and Liu 2012) that allows the intercellular exchange of ions like Ca^{2+}, Na^{+} or K^{+} and of small molecules with a molecular mass less than 1.2 kDa like adenosine triphosphate (ATP), glucose, glutathione (GSH) or messenger molecules (Theis and Giaume 2012). This astrocytic syncytium supports, for example, long distance energy substrate trafficking towards activated neurons (Parpura *et al.* 2012, Theis and Giaume 2012).

1.1.1.3 Astrocytes and synaptic transmission

Astrocytes have also been described to take part in synaptogenesis and synapse maintenance in the adult brain (Pfrieger 2010). A single astrocyte contacts several hundred dendrites and covers a large number of synapses of numerous neurons (Sofroniew and Vinters 2010, Parpura *et al.* 2012). Astrocytes are able to respond to neuronal changes in activity by the release of gliotransmitters like ATP, glutamate or GABA (Sofroniew and Vinters 2010) and they ensure undisturbed synaptic transmission by maintaining pH, ion and neurotransmitter homeostasis in the interstitial fluid (Sofroniew and Vinters 2010). Neuronal activity can also result in the mobilization of intracellular Ca^{2+} stores from the endoplasmatic reticulum (ER) (Nimmerjahn 2009) and generation of a Ca^{2+}-wave that is transmitted intra- and intercellularly (Cornell-Bell *et al.* 1990, Fiacco and McCarthy 2006, Agulhon *et al.* 2012). The released Ca^{2+} may reach even distant cells and serves as an intercellular signal (Perea and Araque 2005, Goldberg *et al.* 2010).

1.1.1.4 Metabolic support by astrocytes

Astrocytes and neurons are metabolically coupled as described for several metabolic pathways. The theory of the astrocyte-neuron lactate shuttle is known for 16 years now (Magistretti and Pellerin 1996, Pellerin and Magistretti 2012) and still under debate (Dienel 2012). According to this theory astrocytes metabolize glucose to lactate which they release. The lactate is then taken up by neurons where it serves as an energy substrate (Barros and Deitmer 2010, Kimelberg and Nedergaard 2010, Pellerin and Magistretti 2012). Additionally, astrocytes supply amino acid precursors for the neuronal synthesis of the antioxidant GSH (Dringen *et al.* 2000, Dringen and Hirrlinger 2003, Fernandez-Fernandez *et al.* 2012, Schmidt and Dringen 2012, Valdovinos-Flores and Gonsebatt 2012) and take part in the modulation of synaptic transmission by the uptake and release of neurotransmitters like glutamate or GABA (Sofroniew and Vinters 2010; Öz *et al.* 2012; Yudkoff *et al.* 2012). With their endfeet astrocytes cover virtually all vascular surfaces (Parpura *et al.* 2012). This enables bidirectional interactions including regulation of the blood flow by release of various mediators that increase or decrease blood vessel diameter (Takano *et al.* 2006, Iadecola and Nedergaard 2007, Sofroniew and Vinters 2010) and uptake of nutrients from the blood via the BBB (Sofroniew and Vinters 2010). Astrocytes are also the first cells that encounter all other substances that have crossed the BBB like drugs or toxins. They play an important role in the detoxification of xenobiotics (Schmidt and Dringen 2009, Schmidt and Dringen 2010) or reactive oxygen species (Dringen *et al.* 2005, Hirrlinger and Dringen 2010) and are involved in metal homeostasis in the brain (Tiffany-Castiglioni and Qian 2001, Dringen *et al.* 2007, Scheiber and Dringen 2013).

1.1.1.5 Astrocytes and metals

Astrocytes are described to play an important role in metal metabolism in the brain as they have a strategically important position between blood capillaries and other brain cells (Dringen *et al.* 2007). The brain contains potentially toxic levels of essential metals like iron (Fe), copper (Cu), manganese (Mn) or zinc (Zn) (Tiffany-Castiglioni *et al.* 2001) which have important functions in the normal brain metabolism, for example as co-factors for enzymatic reactions, in protein and lipid synthesis or in cell proliferation (Dringen *et al.* 2007, Lee and Koh 2010, Filipov and Dodd 2012, Scheiber and Dringen 2013). Astrocytes have been shown to be able to accumulate and store

such metals to prevent other brain cells from metal derived toxicity (Tiffany-Castiglioni and Qian 2001, Hoepken *et al.* 2004, Scheiber *et al.* 2010). The handling of Fe, Cu or Mn by astrocytes has been reviewed earlier (Tiffany-Castiglioni and Qian 2001, Dringen *et al.* 2007, Moos *et al.* 2007, Tiffany-Castiglioni *et al.* 2011, Scheiber and Dringen 2013), but also other metals, that have no functions in normal brain metabolism like lead (Pb) or silver (Ag) can be taken up efficiently by astrocytes (Tiffany-Castiglioni and Qian 2001, White *et al.* 2007).

Astrocytes possess machineries to increase their capacity to store potentially toxic metals by upregulation of metal storage proteins like ferritin (Ft) or metallothioneins (MTs) (Hidalgo *et al.* 1994, Kramer *et al.* 1996, Aschner *et al.* 1998, Hoepken *et al.* 2004, Geppert *et al.* 2012). Such an upregulation allows these cells to withstand high metal excess without suffering from metal-induced toxicity.

1.1.2 MICROGLIA

Microglial cells are the immune competent cells in the brain which were first described by Pio del Rio-Hortega in 1932 (cited in Kettenmann *et al.* 2011). These cells possess various properties of peripheral macrophages (Vilhardt 2005). Microglia release neurotrophic factors and anti-inflammatory cytokines and promote synaptic plasticity (Czeh *et al.* 2011, Kettenmann *et al.* 2011, Schafer *et al.* 2012), but upon injury of the brain or immunological stimuli microglia become activated resulting in the production of pro-inflammatory proteins and reactive oxygen species (ROS) that may result in neuronal damage (Ransohoff and Perry 2009, Lull and Block 2010, Smith *et al.* 2012).

1.1.2.1 Microglia in developing brain

Microglia are of myeloid origin and migrate into the brain from bone marrow during development (Chan *et al.* 2007, Lull and Block 2010, Kettenmann *et al.* 2011). First microglial progenitors occur in the head region of rat embryos already around embryonic day 11 – 14 (Sorokin *et al.* 1992, Pont-Lezica *et al.* 2011). With proceeding brain development the number of early microglial cells increases. This is described to be linked to the occurrence of apoptotic cells (Pont-Lezica *et al.* 2011, Harry and Kraft 2012). In the developing brain, neurons form numerous synaptic connections from which only a subset is

maintained and strengthened with proceeding development (Stevens *et al.* 2007, Pfrieger 2010, Schafer *et al.* 2012). Additionally, many neurons undergo apoptosis around the time of birth (Wakselman *et al.* 2008, Schlegelmilch *et al.* 2011) and are shown to be phagocytosed by microglia which participate in the modulation of synapses (Schafer *et al.* 2012). In this stage microglial cells have an amoeboid morphology that facilitates their phagocytotic properties (Lull and Block 2010). After birth microglia become more ramified as they fully differentiate. This process is hypothesized to coincide with the maturation of neurons and glia cells (Pont-Lezica *et al.* 2011, Harry and Kraft 2012).

1.1.2.2 Activation of microglia

In healthy brain microglia are reported to be in a "resting" state with a ramified morphology. However, "resting" microglia are not inactive, but are constantly surveying their immediate surroundings (Kettenmann *et al.* 2011). Their membranes are equipped with a variety of receptors (Kettenmann *et al.* 2011) which enable detection and classification of substances or particles in the extracellular space. Depending on the type of receptor activated, microglia respond differently in their downstream signalling. The response of microglia to such stimuli is referred to as "activation" which includes numerous states from moderate response to neurotoxic over-activation (Lull and Block 2010, Kettenmann *et al.* 2011). The cells undergo morphological changes from ramified to amoeboid which is believed to favour phagocytosis and motility (Lull and Block 2010). The presence of apoptotic cells or cell debris is recognized by the phosphatidylserine receptor leading to phagocytosis and simultaneous release of anti-inflammatory cytokines like transforming growth factor β without any inflammation (Napoli and Neumann 2009). This process can be classified as mainly beneficial moderate activation. Many pathogens on the other hand express patterns that are recognized via toll-like receptors triggering the release of pro-inflammatory signals like tumour necrosis factor α (TNF-α), interleukin 1β (IL-1β), nitric oxide (NO) or ROS (Napoli and Neumann 2009, Lull and Block 2010). A third way described to regulate microglial response is the absence of signal. Constitutively expressed calming signals from surrounding cells carry information about normal neuronal activity. The release of neurotransmitters or the binding of proteins to respective receptors on microglial cells have an inhibitory effect on microglial activation (Bessis *et al.* 2007, Hanisch and Kettenmann 2007, Lull and Block 2010, Saijo and Glass 2011). Any impairment of neuronal integrity may lead to the disruption

of these signals, alerting microglia and subsequently resulting in their activation (Hanisch and Kettenmann 2007). This enables microglial cells to react to any unknown disturbance without activation of a specific receptor (Hanisch and Kettenmann 2007, Kettenmann *et al.* 2011).

1.1.2.3 Microglia in disease

Upon exposure to stress- or damage-signals from other cells, microglia become acutely activated to proliferate, migrate to these sites and work in a neuroprotective mode by releasing anti-inflammatory cyto- and chemokines (Graeber and Streit 2010). If the support provided by microglia is not sufficient and the damaged cells cannot be saved, microglia will clear up the debris by phagocytosis (Graeber and Streit 2010). If the activation signals from damaged cells are continuously released, microglia become chronically activated. In this state of over-activation, also termed "reactive microgliosis", microglia continuously produce and release pro-inflammatory substances like TNF-α and IL-1β as well as ROS and NO which can damage surrounding neurons (Graeber and Streit 2010, Lull and Block 2010). Prolonged over-activation is described to lead to ageing and senescent microglia and subsequently to microglial degeneration (Graeber and Streit 2010). Therefore microglia seem to play both causative and amplifying roles in many neurodegenerative disorders (Lull and Block 2010) like Parkinson´s or Alzheimer´s disease (Saijo and Glass 2011, Phani *et al.* 2012, Rubio-Perez and Morillas-Ruiz 2012), multiple sclerosis (MS) or HIV-associated neurocognitive disorder (Ransohoff and Perry 2009, Saijo and Glass 2011) as well as in conditions like stroke (Thiel and Heiss 2011).

1.1.2.4 Microglia and metals

Disturbance of metal homoeostasis is often described to be associated with diseases like Alzheimer's disease (Batista-Nascimento *et al.* 2012), MS (Williams *et al.* 2012) or Parkinson´s disease (Sian-Hülsmann *et al.* 2011, Mounsey and Teismann 2012) in which microglial activation is a common observation (Saijo and Glass 2011, Phani *et al.* 2012, Rubio-Perez and Morillas-Ruiz 2012). Therefore the effects of metals on brain cells have often been studied in the context of microglial activation (Zheng *et al.* 2010, Higashi *et al.* 2011, Rathnasamy *et al.* 2011, Filipov and Dodd 2012). Especially iron is essential for basic cellular processes like energy production or cell metabolism (Fretham *et al.* 2011). Iron excess, however, is dangerous and

can lead to cellular impairment and radical production via Fenton chemistry (Jomova *et al.* 2010). Iron deficiency as well as an excess of iron are connected with many diseases and mental impairments (Jomova *et al.* 2010, Fretham *et al.* 2011, Rathnasamy *et al.* 2011, Batista-Nascimento *et al.* 2012). Therefore a strict regulation of the iron metabolism in brain is necessary.

In the developing and early postnatal brain most iron is present in microglia (Connor *et al.* 1995, Schonberg and McTigue 2009) and activated microglia are described to contain Ft (Cheepsunthorn *et al.* 1998, Hoepken 2005) and the transferrin receptor (TfR) (Kaur and Ling 1995, Hoepken 2005). With maturation of the other glial cells and differentiation of microglia, the brain iron store is shifted from microglia towards oligodendrocytes which become the major iron-containing cells in brain (Connor *et al.* 1995) as many iron-dependent enzymes are required for myelination of axons (Connor and Menzies 1996). In their "resting" state microglia do contain only low amounts of iron and iron-related proteins (Moos 1995, Hoepken 2005, Moos *et al.* 2007).

However, upon extracellular iron excess or activation, differentiated microglial can revive their ability to transport and store iron and are described to actively regulate iron homeostasis (Rathnasamy *et al.* 2011). In such conditions microglia contain proteins for iron uptake like the divalent metal transporter 1 (DMT1) (Rathore *et al.* 2012), melanotransferrin (Mtf) (Jefferies *et al.* 1996) or TfR (Kaur and Ling 1995, Rathnasamy *et al.* 2011) as well as iron binding and storing proteins like Ft (Cheepsunthorn *et al.* 1998, Cheepsunthorn *et al.* 2001, Zhang *et al.* 2006) or lactoferrin (Lf) (Fillebeen *et al.* 2001, An *et al.* 2009) which the cells may release to supply surrounding cells with a redox-inactive form of iron (Fillebeen *et al.* 2001, Zhang *et al.* 2006). Whether they also contain the iron exporter ferroportin (FPN) is still controversially discussed (Moos *et al.* 2007, Rathore *et al.* 2012).

Figure 1.2 compiles the knowledge on pathways that are described to be involved in the iron metabolism in microglia during activation or in connection with diseases. However, it has to be stated, that some pathways are described for a single disease only. Mtf, for example, was detected in microglia in senile plaques in Alzheimer´s disease, while in other pathological tissues it was

not expressed (Jefferies *et al.* 1996). It also should be kept in mind that in the state of disease only a subset of microglia might be involved in iron-processing or -storing (Kaur and Ling 1995, Lopes *et al.* 2008). In addition to the pathways depicted in figure 1.2, cytokines or ROS produced during activation may influence the expression of iron influx and storage proteins like DMT1 and Ft (Rathore *et al.* 2012).

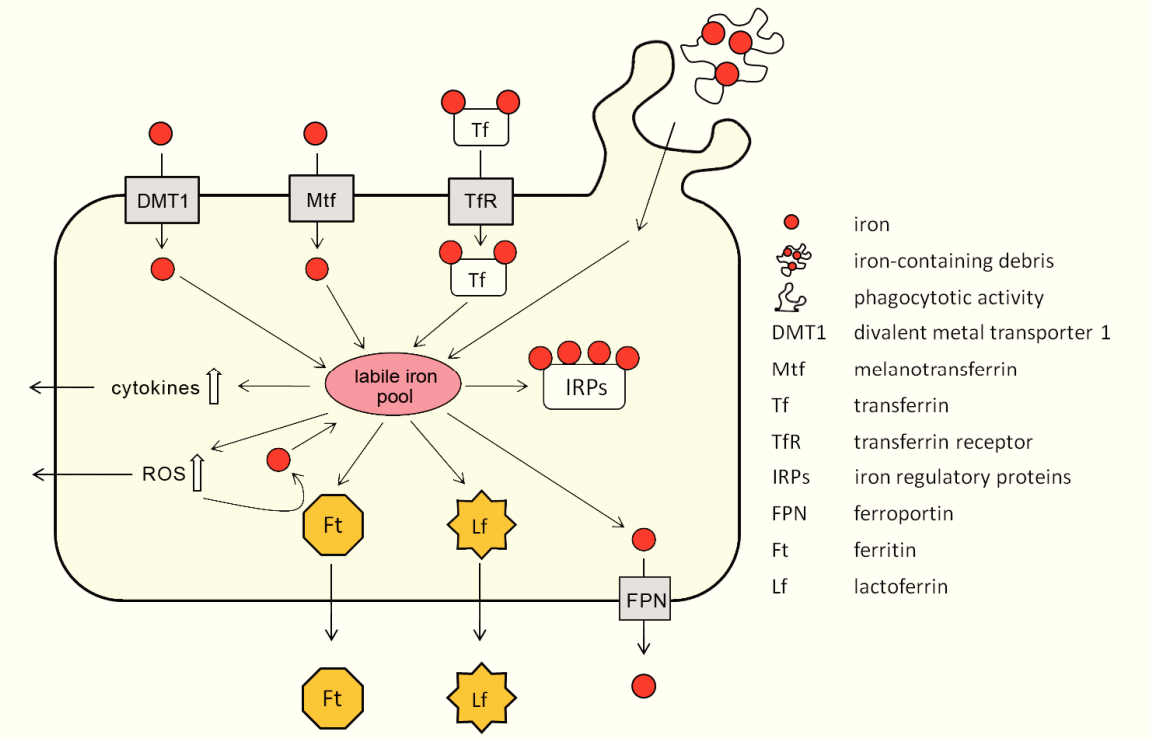

Figure 1.2: Pathways described to be involved in the iron metabolism in microglia upon extracellular iron excess. The depicted pathways have been described for one or more pathological conditions that are connected with iron excess. Activated microglia increase their cellular labile iron pool by taking up iron by transferrin-independent mechanisms via DMT1 or Mtf or bound to Tf via the transferrin receptor (TfR). Due to their phagocytotic properties microglia may also take up iron-containing debris from their surroundings. Excess of intracellular iron leads to the upregulation of iron binding proteins Ft and Lf, which help to safely store iron in a redox-inactive form, but also elevates cytokine and ROS production. Intracellular ROS may release iron from Ft. Whether ferroportin is expressed in microglia is still under debate, but they are described to release of Ft and Lf to supply other cells with a safe, redox-inactive form of iron.

1.2

NANOPARTICLES

1.2 NANOPARTICLES

Nanoparticles (NPs) have been defined as particles that have a size of 1 to 100 nm in two or three dimensions (Auffan *et al.* 2009). They can be divided into two main groups – naturally occurring and manufactured NPs. Even though naturally occurring nanoparticles like carbon black, clay minerals or silica have always been part of this world and nanoparticle with anthropogenic origin are almost as old as mankind itself (Peterson 2004, Oberdorster *et al.* 2005, Suh *et al.* 2009, Wiesenthal *et al.* 2011), nanotechnology as a separate field of research is rather young (Suh *et al.* 2009). Since the late 1960s NPs are considered for medical applications in drug delivery or as vaccines, but NPs were initially considered to be "pharmaceutical curiosities with no or only extremely limited application" (Kreuter 2007). Towards the end of the 20th century this view changed completely and nanotechnology is now considered as the emerging technology of the 21st century (Mangematin and Walsh 2012) and is expected to become a US$ 2.5 trillion market by 2015 (Invernizzi 2011).

NPs can consist of various core materials like carbon, organic polymers, metals or metal oxides (Suh *et al.* 2009, Ai *et al.* 2011, Peralta-Videa *et al.* 2011) and are often covered with a coat to stabilize them in the environment they are designed for (Fatisson *et al.* 2012). Frequently used coating materials are inorganic and organic molecules, polymers or proteins (table 1.2; chapter 1.5, table 2).

The small size and huge surface to volume ratio give NPs physical and chemical properties that differ from the respective bulk material (Auffan *et al.* 2009, Behari 2010), which opens new fields of research and application for already known materials. For example, carbon nanotubes (CNTs) display special properties regarding elasticity and strength as well as electrical conductivity, what makes them an interesting tool for development of ultra strong fibers or biosensors (Lu *et al.* 2012). Quantum dots (QDs) are frequently used for visualization of cell tissue (table 1.2) due to their optical properties. Polymer NPs serve as vehicles for drug delivery (Elsabahy and Wooley 2012).

Among the different types of NPs, silver nanoparticles (AgNPs) and iron oxide nanoparticles (IONPs) are of special interest, because AgNPs have antimicrobial properties and are already widely used in consumer products

(Wijnhoven *et al.* 2009, Ahamed *et al.* 2010, Dastjerdi and Montazer 2010, Tolaymat *et al.* 2010, Schluesener and Schluesener 2013) and IONPs are considered for a variety of medical applications (Weinstein *et al.* 2010, Kievit and Zhang 2011, Maier-Hauff *et al.* 2011). Therefore these two types of particles are introduced further in the following sections.

1.2.1 Silver nanoparticles

On the world markets the number of products containing nanomaterials has risen immensely during the last years. The "Project on Emerging Nanotechnologies", a data base established by the Woodrow Wilson International Center for Scholars and the Pew Charitable Trusts in 2005 (http://www.nanotechproject.org/), registered an increase in manufacturer-identified nano-consumer products within 5 years by over 500% from 212 to 1317 products in March 2011. Silver is by far the most frequently used material in nano-consumer products due to the potent antimicrobial properties and a relatively low toxic potential of AgNPs towards mammalian cells (Chen and Schluesener 2008, Dastjerdi and Montazer 2010).

In March 2011, 313 consumer products containing AgNPs were registered in the projects database of which some examples are shown in figure 1.3. The large number of products containing AgNPs makes human exposure to AgNPs in everyday life very likely. As AgNPs are reported to enter the brain via the olfactory epithelium (Aschner 2009) or by causing inflammation or disruption of the BBB (Tang *et al.* 2008, Sharma *et al.* 2010a, Sharma *et al.* 2010b, Tang *et al.* 2010), it is highly likely, that the frequent exposure to AgNPs from consumer products leads to an accumulation of AgNPs in the body and subsequently in the brain. The consequences of an exposure of brain cells to AgNPs are a main topic of this thesis.

The antimicrobial properties of AgNPs arise from a slow release of silver ions (Ag^+) (Xiu *et al.* 2012, Schluesener and Schluesener 2013), which makes AgNPs much more effective than low molecular weight silver. Silver applied as silver salt dissolves quickly in the surrounding media and is bound by various substances including phosphate, chloride, sulphide or organic acids (Choi *et al.* 2009, Xiu *et al.* 2011) which reduce the bioavailability of Ag^+ before reaching the intended site of action (Xiu *et al.* 2012).

Figure 1.3: Consumer products containing silver nanoparticles. Depicted are examples for products that are described to contain nano-silver. Picture credits can be found in table 1.3 at the end of the reference section.

In AgNPs the rate of Ag^+ released from the particles is influenced by many factors (Chernousova and Epple 2013) like the particle size (Morones *et al.* 2005, Panáček *et al.* 2006, Carlson *et al.* 2008, Sotiriou and Pratsinis 2010), surface charge (El Badawy *et al.* 2010), shape (Pal *et al.* 2007) or coating (Yang *et al.* 2011). Therefore, AgNP-toxicity can be indirectly influenced by varying these parameters. AgNPs display a potent antimicrobial tool, whose properties can be individually designed to meet specific requirements in the intended products.

1.2.2 Iron oxide nanoparticles

IONPs are used in a much lower scale in consumer products compared to AgNPs, but IONPs are qualitatively important as potent tools in medicine (Weinstein *et al.* 2010, Kievit and Zhang 2011, Maier-Hauff *et al.* 2011). Due to their high relaxivity IONPs are in clinical use as contrast agents for magnetic resonance imaging (MRI). Several products containing IONPs like Resovist® or

Endorem®/Feridex® have been approved by the United States Food and Drug Administration (FDA) for clinical application (Soenen and De Cuyper 2010). Those products are used as contrast agent for MRI in liver or spleen (Reimer and Vosshenrich 2004, Matuszewski *et al.* 2007, Soenen and De Cuyper 2010) but IONPs are also considered for an application in brain (Weinstein *et al.* 2010, Xie *et al.* 2011). In cancer treatment IONPs can be utilized to outline tumours for precise surgery (Trehin *et al.* 2006) or for hyperthermia to destroy tumour tissue in an alternating magnetic field (Maier-Hauff *et al.* 2011). Due to their magnetic properties they are also considered as a tool for targeted drug delivery (Veiseh *et al.* 2010, Weinstein *et al.* 2010). These applications of IONPs are also interesting for a use in the brain. In hyperthermia, IONPs could be administered directly to the brain by injection into the tumour tissue (Giustini *et al.* 2011), but mostly IONPs are injected in the periphery and have to cross the BBB to reach the brain. In various animal studies the presence of IONPs in brain after peripheral administration of the particles has been shown (table 1.1) indicating that IONPs cross the intact BBB. More information on the synthesis and the properties of IONPs is given in chapter 1.5.

1.2.3 Effects of metal-containing nanoparticles on glial cells

Metal-containing NPs have been detected in brain upon peripheral application via different routes of exopsure by many research groups (table 1.1). They were shown to enter the brain by crossing or disrupting the BBB (Tysiak *et al.* 2009, Sharma *et al.* 2010a, Tang *et al.* 2010) or via the olfactory epithelium (Aschner 2009) and will therefore come in contact with glial cells. Uptake of different NPs has been shown for all glial cell types (Calvo *et al.* 2001, Pickard and Chari 2010, Geppert *et al.* 2011, Jenkins *et al.* 2011), but the following sections will focus only on the effects of NPs on astrocytes and microglial cells as those cell types are main subjects of this thesis.

A detailed description on the handling of NPs by astrocytes will not be given in this paragraph, since chapter 1.5 of this thesis represents a very recent and comprehensive review article on this topic. Therefore, the reader is referred to this article for an up to date overview on the consequences of a treatment of astrocytes with NPs as well as on the uptake and the metabolism of NPs in astrocytes.

Table 1.1: Selected studies in which NPs were detected in brain after application via different routes of exposure

Nanoparticle	Route of exposure	References
IONPs	Injection into the brain	Wang *et al.* (2011a)
	Intravenous injection	Trehin *et al.* (2006), Rausch *et al.* (2002), Xie *et al.* (2011), Beckmann *et al.* (2011)
	Intraperitoneal injection	Alison *et al.* (2010)
	Subcutaneous injection	Oude Engberink *et al.* (2010)
	Intragastric injection	Wang *et al.* (2010)
	Intranasal	Wang *et al.* (2011b), Wu *et al.* (2013)
	?	Fleige *et al.* (2001)
AgNPs	Intravenous injection	Dziendzikowska *et al.* (2012), Sharma *et al.* (2010a), Tysiak *et al.* (2009)
	Subcutaneous injection	Tang *et al.* (2008), Tang *et al.* (2009)
	Oral	Loeschner *et al.* (2011), van der Zande *et al.* (2012), Hadrup *et al.* (2012)
	Intranasal	Genter *et al.* (2012), Liu *et al.* (2012)
AuNPs	Intraperitoneal injection	Lasagna-Reeves *et al.* (2010)
AlNPs	Intravenous injection	Sharma *et al.* (2010a)
CuNPs	Intravenous injection	Sharma *et al.* (2010a)
CuONPs	Intraperitoneal injection	An *et al.* (2012)

AuNPs: gold nanoparticles; AlNPs: alumina nanoaprticles; CuNPs: copper nanoaprticles; CuONPs: copper oxide nanoaprticle; ?: no information given

Microglia have been frequently reported to be affected by metal-containing NPs *in vivo* and *in vitro* (table 1.2). Depending on the core and coating material and the test system used, the consequences of an exposure of microglial cells to those NPs differ strongly. Microglial exposure to IONPs and QDs has been studied by several groups, but little is currently known on the effects of an exposure of microglial cells to other types of metal-containing NPs. Only a few studies have described the effects of alumina-, gold- or titanium dioxide-NPs (AlNPs, AuNPs and TiO_2NPs, respectively). A list of articles describing the effects of metal-containing NPs on microglia is given in table 1.2.

Table 1.2: Literature describing the current knowledge on effects of metal-containing NPs on microglial cells.

NP core	NP coating	Microglial cells	Consequences on microglia	References
Iron oxide	Dextran	*In vivo*	Co-localization with activated cells, labelling of microglia	Rausch *et al.* (2002)
Iron oxide	Dextran-Cy5.5	*In vivo*	Uptake in activated cells, labelling of microglia, outlining of brain tumours	Trehin *et al.* (2006)
Iron oxide	Dextran-goat anti mouse IgG	*In vivo*	No enhanced activation compared to control	Raju *et al.* (2011)
Iron oxide	Citrate	*In vivo*	Uptake into vesicular structures, labelling of microglia	Tysiak *et al.* (2009)
Iron oxide	Polysaccharide with lipophilic green fluorescent dye	Mixed primary culture	Effective uptake into vesicular structures	Pinkernelle *et al.* (2012)
Iron oxide	n/a	*In vivo*, BV-2	Phagocytotic uptake into vesicles, proliferation, activation, release of ROS and NO	Wang *et al.* (2011b)
Iron oxide	n/a	BV-2	Uptake into endosomes	Rosenberg *et al.* (2012)
Iron oxide	Dextran-fluorescein	*In vivo*, secondary culture	Time- and concentration-dependent uptake into vesicular structures, labelling of microglia, outlining of brain tumours, toxic effects in high concentrations	Fleige *et al.* (2001)
Iron oxide	Dextran, aminoPVA +/- Cy3.5	N9, N11	Time- and concentration-dependent uptake, enhanced in activated cells	Cengelli *et al.* (2006)
Polystyrene -iron oxide	Carboxyl-modified with fluorophor Nile Red	Secondary culture	Time-, concentration- and temperature-dependent uptake, toxic effects in high concentrations	Pickard and Chari (2010)

N9, N11, BV-2: microglial cell lines; IgG: immune globulin G; n/a: not available; PVA: polyvinyl alcohol.

Table 1.2 continued: Literature describing the current knowledge on effects of metal-containing NPs on microglial cells.

NP core	NP coating	Microglial cells	Consequences on microglia	References
QD	Amino/PEG	*In vivo*	Uptake in activated cells, labelling of microglia, outlining of brain tumours	Jackson *et al.* (2007)
QD	ZnS with LPS surface	*In vivo*, N9	Activation, formation of lipid droplets, enhanced expression of TLR2 and CD68, NO release	Lalancette-Hebert *et al.* (2010)
QD	None or BSA	N9	Uptake, size-dependent cellular localization and toxicity	Lovric *et al.* (2005)
QD	Streptavidin	*In vivo*, primary culture	Uptake via receptor-binding and clathrin-mediated endocytosis, no release of cytokines	Minami *et al.* (2012)
QD	Bound to amiloride-β peptide	Primary culture	Time-dependent uptake into lysosomes	Tokuraku *et al.* (2009)
TiO_2	None	*In vivo*, BV-2	Activation, increased expression of TNFα and NF-κB in activated microglia	Shin *et al.* (2010)
TiO_2	None	BV-2	ROS production	Long (2006)
AlNP	n/a	*In vivo*	Activation	Li *et al.* (2009)
AuNP	PEG or CTAB	*In vivo*, N9	Shape-dependent uptake and activation pattern, co-localization with lysosomal compartments, enhanced expression of TLR2, cytokine release	Hutter *et al.* (2010)

N9, BV-2: microglial cell lines, PEG: Poly(ethylene glycol), LPS: Lipopolysaccharide, TLR2: Toll-Like receptor 2, CD68: cluster of differentiation 68 (lysosomal membrane protein), BSA: Bovine serum albumin, TNFα: Tumour necrosis factor α, NF-κB: Nuclear factor κB, CTAB: cetyl trimethylammonium bromide; n/a: not available.

Most of the applied types of NPs seem to trigger activation processes in microglia. The peripheral administration of AlNPs led to an increase in the number of activated microglial cells 60 days after the treatment *in vivo* (Li *et al.* 2009). Upon treatment with AuNPs, a transient activation of microglia *in vivo* and in the cell line N9 was shown, which strongly depended on shape and surface structure of the NPs (Hutter *et al.* 2010). Upon microglial exposure to TiO_2NPs activation of the cells, increased expression of TNF-α and the nuclear factor κB (NFκB) as well as ROS production have been reported (Long 2006, Shin *et al.* 2010). For QDs and IONPs contradictory results were found regarding the activation of cells. Raju *et al.* (2011) and Minami *et al.* (2012) report no enhanced activation and no release of cytokines upon IONP and QD exposure, respectively. In contrast, Wang and co-workers showed activation and release of ROS and NO after IONP treatment (Wang *et al.* 2011b) and Lalancette-Herbert *et al.* (2010) described activation and NO release upon QD exposure.

Uptake of NPs into microglia has been shown for AuNPs (Hutter *et al.* 2010), QDs (Lovric *et al.* 2005, Jackson *et al.* 2007, Tokuraku *et al.* 2009, Lalancette-Herbert *et al.* 2010, Minami *et al.* 2012) and IONPs (Fleige *et al.* 2001, Trehin *et al.* 2006, Tysiak *et al.* 2009, Pickard and Chari 2010, Wang *et al.* 2011b, Pinkernelle *et al.* 2012, Rosenberg *et al.* 2012). In addition, detailed time- and concentration-dependent uptake of IONPs and QDs into vesicular or lysosomal compartments has been described in several reports (Fleige *et al.* 2001, Tokuraku *et al.* 2009, Tysiak *et al.* 2009, Wang *et al.* 2011b, Minami *et al.* 2012, Pinkernelle *et al.* 2012, Rosenberg *et al.* 2012). This uptake involves most likely endocytotic and/or phagocytotic processes (Wang *et al.* 2011b, Minami *et al.* 2012) which are also described for the uptake of IONPs into peripheral macrophages or macrophage cell lines (Gu *et al.* 2011, Lunov *et al.* 2011). As especially activated microglial cells are described to efficiently take up QDs and IONPs (Rausch *et al.* 2002, Trehin *et al.* 2006, Jackson *et al.* 2007), these cells can be selectively labelled by IONPs and QDs. Thus, such NPs display potent tools for drug delivery into brain tumours or for marking tumour outlines by using fluorescence-labelled particles or MRI techniques (Fleige *et al.* 2001, Rausch *et al.* 2002, Trehin *et al.* 2006, Jackson *et al.* 2007).

1.3

Aim of the thesis

1.3 Aim of the thesis

This thesis investigates the uptake and metabolism of AgNPs and IONPs into cultured brain astrocytes and microglia. Due to their location and key functions in brain (Sofroniew and Vinters 2010, Kettenmann *et al.* 2011), these glial cells are especially prone to nanoparticle contact (Pickard and Chari 2010, Geppert *et al.* 2011) and are considered as first lines of defence against metal stress in the brain (Oshiro *et al.* 2008) that may originate from NPs.

This thesis will describe the consequences of an exposure of cultured astrocytes to AgNPs. The molecular mechanisms involved in AgNP uptake as well as potential alterations of metabolism after exposure to AgNPs will be studied. Furthermore, the consequences of a long term presence of AgNPs in astrocytes will be investigated.

A second experimental part of this thesis will deal with cultured microglial cells and how they respond to an exposure to fluorescent IONPs. Primary microglial cultures will be established and characterized. These cultures will be exposed to fluorescent IONPs and the accumulation of iron in the cells and the involved uptake mechanisms will be studied. The fluorescence of the IONPs will be used as tool to localize the particles within the cells.

1.4

References

1.4 References

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Table 1.3: Picture credits for figure 1.3. All web references were last accessed on 2013/02/04.

Product class	Web reference
Medical products	• http://www.bmbf.de/pub/nanoparticles_small_things_big_effects.pdf • http://www.modernmedicine.com/news/acs-silver-delivery-system-promotes-wound-healing
Spray	• http://www.nanogist.com/English/products/spray.htm
Personal hygiene	• http://lktrading.en.ec21.com/Toothbrush--2298648.html • http://lktrading.en.ec21.com/Oragan_Nano_SIlver_Toothpaste--2553324_2298958.html
Cosmetics	• http://www.healthcare-exp.com/b2b/pharmaceutical_chemicals/1/
Food packaging	• http://www.alwaysfreshstoragecontainers.com
Textiles	• http://www.nanotechproject.org/inventories/consumer/browse/products/5121/ • http://bjsocks.e.tradeee.com/product_view/51177103/Nano-Silver Socks with Foot Support and Half Cushion.html
Electrical equipment	• shop.12v-kuehlgeraete.de/ • http://www.washingmachineproblems.advicev.com • http://www.nanotechproject.org/inventories/consumer/browse/products/7218/
Paint	• http://www.scientificamerican.com/article.cfm?id=silver-coating-fights-microbes Picture by Luis Carlos, iStockphoto • http://www.remmers.de/Schimmel-Protect.1718.0.html#bot

1.5

PUBLICATION 1

HANDLING OF IRON OXIDE AND SILVER NANOPARTICLES BY ASTROCYTES

MICHAELA C. HOHNHOLT, MARK GEPPERT, EVA M. LUTHER, CHARLOTTE PETTERS, FELIX BULKE AND RALF DRINGEN

NEUROCHEMICAL RESEARCH 38 (2013) 227-239

Contributions of Eva M. Luther:

- First draft of the parts on silver nanoparticles

The other parts of the manuscript as well as the Figures were prepared by the co-authors.

Doi: 10.1007/s11064-012-0930-y

2

RESULTS

2.1

PUBLICATION 2

ACCUMULATION OF SILVER NANOPARTICLES BY CULTURED PRIMARY BRAIN ASTROCYTES

EVA M. LUTHER, YVONNE KOEHLER, JOERG DIENDORF,
MATTHIAS EPPLE AND RALF DRINGEN
NANOTECHNOLOGY 22 (2011) 375101

Contributions of Eva M. Luther:

- Performance of experiments for Figures 3, 4, 5, 6, 7 and Table 1
- Preparation of the first draft of the manuscript

Yvonne Koehler performed the experiments for Figure 1, Joerg Diendorf synthesized and characterized the nanoparticles and performed experiments for Figure 2.

Doi:10.1088/0957-4484/22/37/375101

2.2

PUBLICATION 3

UPREGULATION OF METALLOTHIONEINS AFTER EXPOSURE OF CULTURED PRIMARY ASTROCYTES TO SILVER NANOPARTICLES

EVA M. LUTHER, MAIKE M. SCHMIDT, JOERG DIENDORF,
MATTHIAS EPPLE AND RALF DRINGEN
NEUROCHEMICAL RESEARCH 37 (2012) 1639-1648.

Contributions of Eva M. Luther:

- Performance of experiments for Figures 1 - 3 and Table 1
- Preparation of the first draft of the manuscript

Maike M. Schmidt performed experiments for Figure 4, Joerg Diendorf and Matthias Epple kindly provided the silver nanoparticles.

Doi: 10.1007/s11064-012-0767-4

2.3

PUBLICATION/MANUSCRIPT 4

ENDOCYTOTIC UPTAKE OF IRON OXIDE NANOPARTICLES BY CULTURED BRAIN MICROGLIAL CELLS

EVA M. LUTHER, CHARLOTTE PETTERS, FELIX BULKE, ACHIM KALTZ, KARSTEN THIEL, ULF BICKMEYER AND RALF DRINGEN

SUBMITTED TO ACTA BIOMATERIALIA, *IN REVISION*

Contributions of Eva M. Luther:

- Performance of experiments for Figures 2 - 7 and Tables 2 and 3
- Preparation of the first draft of the manuscript

Charlotte Petters prepared the BP-IONPs and performed experiments for Figure 1 and Table 1. Felix Bulke established the initial fluorescent coating which was further improved and characterized by Achim Kaltz. Karsten Thiel provided the TEM image and the EDX analysis shown in Figure 1A,B.

Manuscript for Acta Biomaterialia

Endocytotic uptake of iron oxide nanoparticles by cultured brain microglial cells

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Running title: Accumulation of nanoparticles by microglia

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Abstract

Microglia are the phagocytotic cells of the brain that rapidly respond to alterations in brain homeostasis. Since iron oxide nanoparticles (IONPs) are used for diagnostic and therapeutic applications in the brain, the consequences of an exposure of microglial cells to IONPs are of particular interest. To address this topic we have synthesized and characterized fluorescent BODIPY®-labelled IONPs (BP-IONPs). The average hydrodynamic diameter and the ζ-potential of BP-IONPs in water were about 65 nm and -49 mV, respectively. Both values increased after dispersion of the particles in serum containing incubation medium to around 130 nm and -8 mV. Exposure of cultured rat microglial cells with BP-IONPs caused a time-, concentration- and temperature-dependent uptake of the particles as demonstrated by strong increases in cellular iron contents and cellular fluorescence. Incubation for 3 h with 150 µM and 450 µM iron as BP-IONPs increased the cellular iron content from a low basal level of around 50 nmol iron/mg to 219 ± 52 and 481 ± 28 nmol iron/mg protein, respectively. These conditions did not affect cell viability, but exposure to higher concentrations of BP-IONPs or for longer incubation periods severely compromised cell viability. The BP-IONP fluorescence in viable microglial cells was co-localized with lysosomes. In addition, BP-IONP accumulation was lowered by 60% in the presence of the endocytosis inhibitors 5-(N-ethyl-N-isopropyl)amiloride, tyrphostin 23 and chlorpromazin. These results suggest that the rapid accumulation of BP-IONPs by microglial cells is predominantly mediated by macropinocytosis and clathrin-mediated endocytosis which direct the accumulated particles into the lysosomal compartment.

Key words: endocytosis; iron; lysosomes; microglia; nanoparticles; transport

1. Introduction

Iron oxide nanoparticles (IONPs) are used for neurobiological applications including cancer treatment by hypothermia, as contrast agents for magnetic resonance imaging (MRI) as well as for targeted drug delivery and cell transfection [1, 2]. Direct access of IONPs to brain tissue is achieved by injection into the affected brain area for treatment of brain tumours [3]. However, also IONPs that are administered peripherally by oral application, intravenous injection or by inhalation have been reported to enter the brain by crossing the blood brain barrier or via the olfactory system [1, 2, 4-6].

Microglial cells are the immune competent cells of the brain. Depending on the situation, microglial cells can act beneficial or harmful to their neighbouring cells. In the healthy adult brain, so-called “resting” microglia survey their microenvironment for nutrients or debris, release neurotrophic factors and anti-inflammatory cytokines and promote synaptic plasticity [7, 8]. However, upon activation by brain injury or infections, microglial cells migrate to the site of the impact and secrete inflammatory proteins and reactive oxygen species (ROS) that may damage neighbouring cells [7, 9]. Microglial cells will encounter nanoparticles that have entered the brain, since these cells are known to literally scan their surroundings for debris and particles which are subsequently taken up [7-9]. Indeed, exposure of animals with IONPs as contrast agents for MRI revealed that in brain especially the microglial cells are strongly labelled [10-13].

Metal containing nanoparticles (NP) such as IONPs, titan dioxide NPs, gold NPs, alumina NPs or quantum dots have been reported to affect microglial functions *in vivo* and have been connected with cell toxicity, microglial activation, production of ROS and cytokine release [10-18]. However, so far little information is available on the mechanisms involved in the uptake of IONPs by microglial cells. A few studies have used microglial cell lines as model systems to gain information on the consequences of an exposure of microglial cells with IONPs [12, 19-21]. However, it has to be considered that the advantageous feature of immortality of cell lines may be accompanied by properties and behaviours that differ to those of primary cells [8, 22].

Cells in secondary microglial cultures have been shown to accumulate fluorescent IONPs by analysis of their cellular fluorescence [13, 23]. However, a detailed quantitative analysis of IONP uptake into microglial cells as well as

an identification of mechanisms involved in particle uptake have to our knowledge not been reported so far. To address such questions we have synthesized and characterized fluorescent BODIPY®-labelled IONPs (BP-IONPs) that were subsequently used to investigate IONP uptake into cultured primary microglial cells. These cells efficiently accumulated BP-IONPs in a time-, concentration- and temperature-dependent manner by endocytotic processes which direct the accumulated particles into the lysosomal compartment.

2. Materials and Methods

2.1. Materials

Fetal calf serum (FCS), trypsin solution and penicillin/streptomycin solution were obtained from Biochrom (Berlin, Germany). Dulbecco´s modified Eagle´s medium (DMEM) was from Gibco (Karlsruhe, Germany) and 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) from Roth (Karlsruhe, Germany). Bovine serum albumin and NADH were from Applichem (Darmstadt, Germany). BODIPY® FL C_1-IA [N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-yl)methyl) iodoacetamide] and lysotracker Red DND-99 were purchased from Invitrogen (Darmstadt, Germany). 5-(N-ethyl-N-isopropyl)amiloride (EIPA), tyrphostin 23, ferrozine, dimercaptosuccinic acid (DMSA), 4´,6-diamidino-2-phenylindol hydrochloride (DAPI) and paraformaldehyde were purchased from Sigma-Aldrich (Steinheim, Germany). Mouse anti-rat CD11b (Ox-42) antibody was purchased from Serotec (Düsseldorf, Germany) and the Cy3-conjugated anti-mouse immunoglobulin from Dianova (Hamburg, Germany). Other chemicals of the highest purity available were purchased from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany). 96-well microtiter plates and 6-well cell culture plates were from Nunc (Wiesbaden, Germany) and 24-well cell culture plates from Sarstedt (Nümbrecht, Germany).

2.2. Synthesis and characterization of BP-IONP

IONPs were synthesized by chemical co-precipitation of ferrous and ferric iron salts as described previously [24]. The nanoparticles were coated with BODIPY® (BP)-labelled DMSA according to a modification [25] of a published method [26]. BP-DMSA was synthesized by thoroughly mixing 712.5 µM BODIPY® FL C_1-IA and 4.75 mM DMSA in 47.5 mM glycine/NaOH buffer (pH 10). A 30 min incubation at room temperature (RT) led to the complete derivatization of BODIPY® FL C_1-IA with thiol groups of DMSA (data not

shown). Electrospray ionization mass spectrometry revealed the expected signals at 470 m/z and 759 m/z for DMSA labelled with BP on one or both thiol groups, respectively (data not shown). IONPs were added to the BP-DMSA reaction mixture to a final concentration of 21.4 mM and the mixture was acidified to pH 3 by concentrated HNO_3. After mixing for 30 min at RT, the particles were separated from the solution by magnetic force, resuspended in H_2O and redispersed by increasing the pH value with NaOH to pH 9-10. Finally, the pH was lowered to 7.4 by adding HCl. This dispersion was diluted with water to a final iron concentration of 40 mM and stored at 4°C. The concentrations of BP-IONPs used in the individual experiments are given here as concentration of the iron present in the nanoparticle dispersion and do not represent the concentration of particles.

Samples for transmission electron microscopy (TEM) were prepared by dropping 5 µL of 1 mM BP-IONP dispersion in water onto carbon-coated copper grids and subsequent air drying at RT. Images were taken by a FEI Tecnai F20 S-TWIN (Hillsboro, Oregon, USA) operated at 200 kV equipped with a GATAN GIF2001 SSC-CCD camera. Energy dispersive X-ray analysis (EDX) was used for elemental analysis in the scanning mode of the microscope (STEM) with an EDAX r-TEM-EDX-detector with an energy resolution of 136 eV. The hydrodynamic diameters and the ζ-potentials of 1 mM BP-IONPs dispersed in different media were determined at 25°C by dynamic and electrophoretic light scattering in a Beckman Coulter (Krefeld, Germany) Delsa™ Nano C Particle analyzer at scattering angles of 165° and 15°, respectively. The fluorescence spectra of diluted BP-IONP solutions (50 µM in water) were recorded using a Cary Eclipse fluorimeter (Varian, Darmstadt, Germany).

The hydrodynamic diameter, ζ-potential and the fluorescence intensity of the dispersed BP-IONPs did not change during storage for at least up to one month, nor was release of any low molecular weight iron from the particles detectable during storage (data not shown).

2.3. Cell cultures

Primary microglial cultures were prepared from astroglia-rich primary cultures by tryptic removal of the astrocyte layer using a modification of a published method [27]. Astroglia-rich primary cultures were prepared from the whole brains of neonatal Wistar rats [28] and three hundred thousand viable cells

were seeded per well of a 24-well plate with or without coverslips in 1 mL culture medium (90% DMEM, 10% FCS, 20 U/mL of penicillin G and 20 µg/mL of streptomycin sulphate) or 1.5 million cells per well of a 6-well plate in 2.5 mL medium. The cultures were grown in a cell incubator (Sanyo, Osaka, Japan) that contained a humidified atmosphere of 10% CO_2/90% air and the culture medium was renewed every seventh day. To obtain microglial cultures, confluent 14- to 23-day-old astroglia-rich cultures were incubated for 30 min with 0.5% (w/v) trypsin in serum-free DMEM. This treatment resulted in the detachment of an intact top layer of cells that contain virtually all the astrocytes and left a population of firmly attached microglial cells in the wells. The microglial cells were washed with 2 mL culture medium and cultured in 1.5 mL glia-conditioned medium (GCM; 0.2 µm filtered glia-conditioned culture medium harvested after 1 d incubation of astroglia-rich primary cultures) for additional sixteen to twenty hours before experiments were performed. The cultures obtained by this method are highly enriched in microglial cells, as more than 98% of the cells in these cultures are positive for the microglial marker protein CD11b.

2.4. Experimental incubations

If not stated otherwise, microglial cultures on 6-well dishes were incubated at 37°C with 1 mL of GCM containing BP-IONPs and other compounds in the concentrations indicated in the legends of the figures and tables. To test for the temperature dependence of BP-IONP uptake, microglial cells were incubated at the given temperature with 1 mL GCM containing 20 mM HEPES (adjusted to pH 7.4 by addition of 5 M NaOH). After the desired incubation period, the media were collected for measurement of extracellular lactate dehydrogenase (LDH) activity and the cells were washed once with 1 mL of ice-cold phosphate-buffered saline (PBS: 10 mM potassium phosphate buffer pH 7.4, containing 150 mM NaCl). Cells were either lysed in 1 mL 1% (w/v) Triton X-100 in serum-free DMEM for analysis of the LDH activity or in 1 mL 1% (w/v) sulfosalicylic acid for determination of the glutathione content, fixed in 3.5% (w/v) paraformaldehyde in PBS for microscopy or stored dry at -20°C until quantification of their iron and protein contents.

2.5. Determination of cell viability, protein content and glutathione content

The viability of microglial cultures was determined by quantification of cellular and extracellular activity of the cytosolic enzyme lactate dehydrogenase (LDH) or by investigating the membrane permeability for propidium iodide (PI). LDH activity was determined as previously described [29] with the modification that 180 µL of lysates or media were used in the assay. For analysis of the membrane permeability the cells were incubated with PI as described previously [30]. To visualize all cell nuclei present, the cells were counterstained with the membrane-permeable Hoechst-dye H33342. An increase in the number of PI-positive cells or in the amount of extracellular LDH reflects a loss in cell viability. The protein content of the cultures was determined according to the Lowry method (Lowry *et al.* 1951) after solubilisation of the cells in 700 µL of 50 mM NaOH using bovine serum albumin as a standard. The contents of total glutathione (GSx, amount of GSH plus two times amount of glutathione disulfide (GSSG)) and GSSG in cell lysates were determined by a modified colorimetric Tietze assay [31]. Control experiments revealed that none of these colorimetric assays was affected by the presence of IONPs (data not shown).

2.6. Iron quantification and histochemical Perls´ staining for iron

The total iron content of cells, media or particle dispersions was determined by a colorimetric ferrozine method as previously described [25]. Iron was also cytochemically visualized by a modification of the histochemical Perls' staining as previously described [24]. For the co-localization of cellular BP-IONP fluorescence with Perls´ staining, the fluorescence pictures were taken before the iron staining was completed by diaminobenzidine-nickel enhancement.

2.7. Activation of microglial NADPH oxidase

Microglial cells were incubated with 0.5 mL of 1 mM nitroblue tetrazolium chloride in GCM with or without 150 µM BP-IONPs for 3 h. To some wells 50 nM phorbol 12-myristate 13-acetate was added after 2 h of incubation as positive control for activation of NADPH oxidase [32]. The formation of blue formazan crystals was monitored with an Eclipse TE-2000U fluorescence microscope (Nikon, Düsseldorf, Germany).

2.8. Immunocytochemical staining

Cells grown on coverslips in wells of 24-well dishes were washed once with 1 mL ice-cold PBS and fixed with 3.5% (w/v) paraformaldehyde in PBS for 10 min at 4°C. If not stated otherwise, the cells were washed thrice (5 min each) with PBS between the different steps of the staining procedure. After incubating the fixed cells with 0.1% (w/v) glycine in PBS for 5 min at RT, the membranes of the cells were permeabilized with 0.3% Triton X-100 in PBS for 10 min at RT. Incubation of the cells with mouse anti-CD11b (1:100 diluted in PBS) was carried out for 2 h at RT in a humidified atmosphere, followed by an incubation with the secondary Cy3-coupled goat anti-mouse antibody (1:200 diluted in PBS) for 30 min at RT. For visualization of the nuclei, the cells were treated with DAPI (1 mg/mL in PBS) for 5 min at RT. Prior to mounting the coverslips in Mowiol mounting media, an ethanol gradient of 70%, 90% and 100% in 1 min intervals was applied. Fluorescence images were taken by using the Eclipse TE-2000U fluorescence microscope.

For co-localization of accumulated BP-IONPs with lysotracker the cells were pre-incubated for 3 h with 150 µM iron as BP-IONPs, gently washed with 1 mL GCM and incubated for 0 or 90 min in GCM before 75 nM lysotracker was applied. After 60 min incubation with lysotracker, the cells were washed once with 1 mL of PBS and fixed with 3.5% (w/v) paraformaldehyde for 10 min. The images of cellular fluorescence for BP-IONPs (excitation: 488 nm, emission: 500–520 nm) and lysotracker (excitation: 561 nm, emission: 590–620 nm) were taken by a Leica SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany).

2.9. Presentation of data

If not stated otherwise, quantitative data are presented as means ± SD of values from at least three experiments that were performed on independently prepared cultures. Analysis of significance of the differences between groups of data was performed by ANOVA followed by the Dunnetts´ *post-hoc* test. The significance of difference between two sets of data was analysed by the t-test. $p>0.05$ was considered as not significant.

3. Results

3.1. Characterization of BP-IONPs

BP-IONPs were synthesized as described in the methods section. TEM analysis revealed that the synthesized BP-IONPs displayed a spherical morphology with a particle diameter of 5 to 20 nm (Fig. 1A). EDX analysis confirmed the presence of iron and sulphur, demonstrating successful coating of IONPs with the sulphur-containing BP-DMSA (Fig. 1B). Presence of the fluorescent dye BP in BP-IONPs was confirmed by fluorescence spectroscopy of the particles that showed maxima of 490 nm and 510 nm in the excitation and emission spectra, respectively (Fig. 1C). The average hydrodynamic diameter and the ζ-potential of BP-IONPs dispersed in water were 65 ± 4 nm and -49 ± 2 mV, respectively (Fig. 1D; Tab. 1).

For cell experiments, IONPs have to be dispersed in physiological media or buffers to maintain the cell viability. Since the composition of the medium can strongly affect the properties of engineered NPs [33], we investigated the effects of different media on the size and the ζ-potential of BP-IONPs. Dispersion of BP-IONPs in plain DMEM culture medium caused rapid precipitation of the particles as demonstrated by the strong increase in the average hydrodynamic diameter of the particles (Tab. 1). This precipitation was not observed for DMEM containing 10% FCS or for GCM that also contained 10% FCS. For these conditions, the diameter of the particles was doubled (to around 130 nm) compared to that determined for the water dispersion (65 nm), while the ζ-potential became more positive to around -9 mV (Tab. 1).

3.2. Characterization of microglial cell cultures

The primary microglial cultures prepared by the trypsinization method [27] contained cells with the typical amoeboid or bipolar morphologies (Fig. 2A) that have previously been described for cultured microglia [8, 9, 27]. Immunocytochemical staining with the antibody Ox-42 for the microglial marker protein CD11b [8, 23] demonstrated that almost all cells in the cultures were positive for this microglial marker (Fig. 2B).

3.3. Viability of microglial cells after exposure to BP-IONPs

To test for the consequences of an exposure of cultured microglial cells to BP-IONPs, the cells were incubated for up to 6 h in GCM with BP-IONPs. While the incubation of microglial cells without BP-IONPs for 6 h (Fig. 3A,B) or with up to 150 µM BP-IONPs for 3 h (Fig. 3D,E) did at best marginally affect cell

viability (Fig. 3A,D) and the cellular protein content (Fig. 3B,E), the incubation of microglial cells for more than 3 h with 450 µM or 1500 µM iron as BP-IONPs severely compromised the cell viability as shown by the significant loss in cellular LDH activity and by the accompanying increase in extracellular LDH activity (Fig. 3A,D). The compromised viability of microglial cells that had been treated with higher concentrations of BP-IONPs (Fig. 3D) was confirmed by PI staining (Fig. 4). After a 3 h exposure to 1500 µM iron as BP-IONPs the membranes of the majority of cells were permeable for the dye (Fig. 4K). For longer incubations, even BP-IONPs applied in the low concentration of 150 µM caused severe permeability of the cells for PI (data not shown).

Accumulated IONPs have been reported to induce oxidative stress in cultured cells [34, 35]. To test whether cultured microglial cells may suffer from oxidative stress during exposure to BP-IONPs, we tested for potential alterations in the cellular GSH redox state. However, microglial cells that had been exposed for 3 h to 150 µM or 450 µM iron as BP-IONPs had almost identical cellular GSx values as control cells and did not show any increase in cellular GSSG values (Tab. 2) that would indicate a severe oxidative stress.

3.4. Accumulation of iron from BP-IONPs by cultured microglial cells

Exposure of microglial cells to BP-IONPs caused a time- and concentration-dependent increase in the cellular iron content (Fig. 3C,F, Fig. 5), while the iron content of microglial cells incubated without BP-IONPs was not altered (Fig. 3C,F). After a 3 h incubation with 150 µM and 450 µM iron supplied as BP-IONPs, the cellular iron content was increased 4- and 10-fold to 219 ± 52 and 481 ± 28 nmol iron/mg protein, respectively, compared to the initial iron content of 49 ± 65 nmol iron/mg protein (Fig. 3C,F). Visualization of the cellular iron content by cytochemical staining for iron by the Perls´ method confirmed for BP-IONPs the concentration dependent increase in cell-associated iron (Fig. 5). While microglial cells that had been incubated without BP-IONPs hardly contain Perls´-detectable iron (Fig. 5A), the dark staining of precipitates formed in BP-IONP exposed cells became more intense with increasing concentration of applied BP-IONPs (Fig. 5C,E,G). For all concentrations of BP-IONPs applied, the iron visualized by the Perls´ method was almost perfectly co-localized with the BP-fluorescence of the cells (Fig. 5). While microglial cells incubated without BP-IONPs did hardly show any fluorescence (Fig. 5B), the fluorescence intensity of the cells increased with the

concentration of BP-IONPs applied (Fig. 5D,F,H). After incubation of the cultures with 450 µM BP-IONPs, all cells in the microglial cultures were Perls´ positive for iron (Fig. 5G) and showed a strong BP fluorescence (Fig. 5H).

To test for the influence of the incubation temperature on the accumulation of BP-IONPs by microglial cells, the cells were incubated with BP-IONPs in GCM that was pH stabilized at 4°C or 37°C by addition of HEPES. Incubation of microglial cells in HEPES-stabilized GCM at 4°C did neither alter the cellular protein content (Fig. 6B) nor compromise cell viability (Fig. 6C), but almost completely prevented the accumulation of BP-IONPs by microglial cells (Fig. 6A). Treatment of microglial cells with the respective 37°C condition led to a substantial accumulation of BP-IONPs but the cell viability was also compromised as indicated by the increased extracellular LDH activity (Fig. 6C) compared to an incubation in HEPES-free GCM. This temperature dependence of BP-IONP accumulation in microglial cells was confirmed by Perls´ staining and fluorescence microscopy which revealed that hardly any cell in microglial cultures that had been exposed to BP-IONPs at 4°C was Perls´ positive or showed BP fluorescence (data not shown).

3.5. Endocytosis and intracellular localization of BP-IONPs

Microglial cells contain a large number of lysosomal vesicles that can be stained with lysotracker (Fig. 7B). In the fluorescence channel used to detect BP-fluorescence, these cells showed only a weak autofluorescence (Fig. 7A) which was co-localized with the lysotracker-stained lysosomes (Fig. 7B). After exposure of the cells to 150 µM BP-IONPs for 3 h, microglial cells revealed a strong punctuated fluorescence staining (Fig. 7D), suggesting that most of the cellular BP-IONP fluorescence was associated with vesicular structures. Lysotracker co-staining revealed that only a part of these BP-IONP positive structures were lysosomes (Fig. 7E). However, after a further 90 min incubation period of BP-IONP treated microglial cells in NP-free GCM the number of large BP-IONP positive vesicle had increased (Fig. 7G) and the majority of these vesicles were now positively stained with lysotracker (Fig. 7H).

Inhibitors of endocytotic pathways were used to investigate which pathways may be involved in the observed accumulation of BP-IONPs by microglial cells. EIPA, a known inhibitor of macropinocytosis [36, 37] as well as tyrphostin 23 and chlorpromazin, inhibitors of clathrin-dependent uptake [36-38], lowered

the iron accumulation in BP-IONP treated microglial cells by around 30% compared to control conditions (Tab. 3), while combination of these three inhibitors reduced the cellular iron contents by almost 60% (Tab. 3). The presence of these inhibitors did not lower cell viability as demonstrated by the absence of any significant increase in the extracellular LDH activity (Tab. 3).

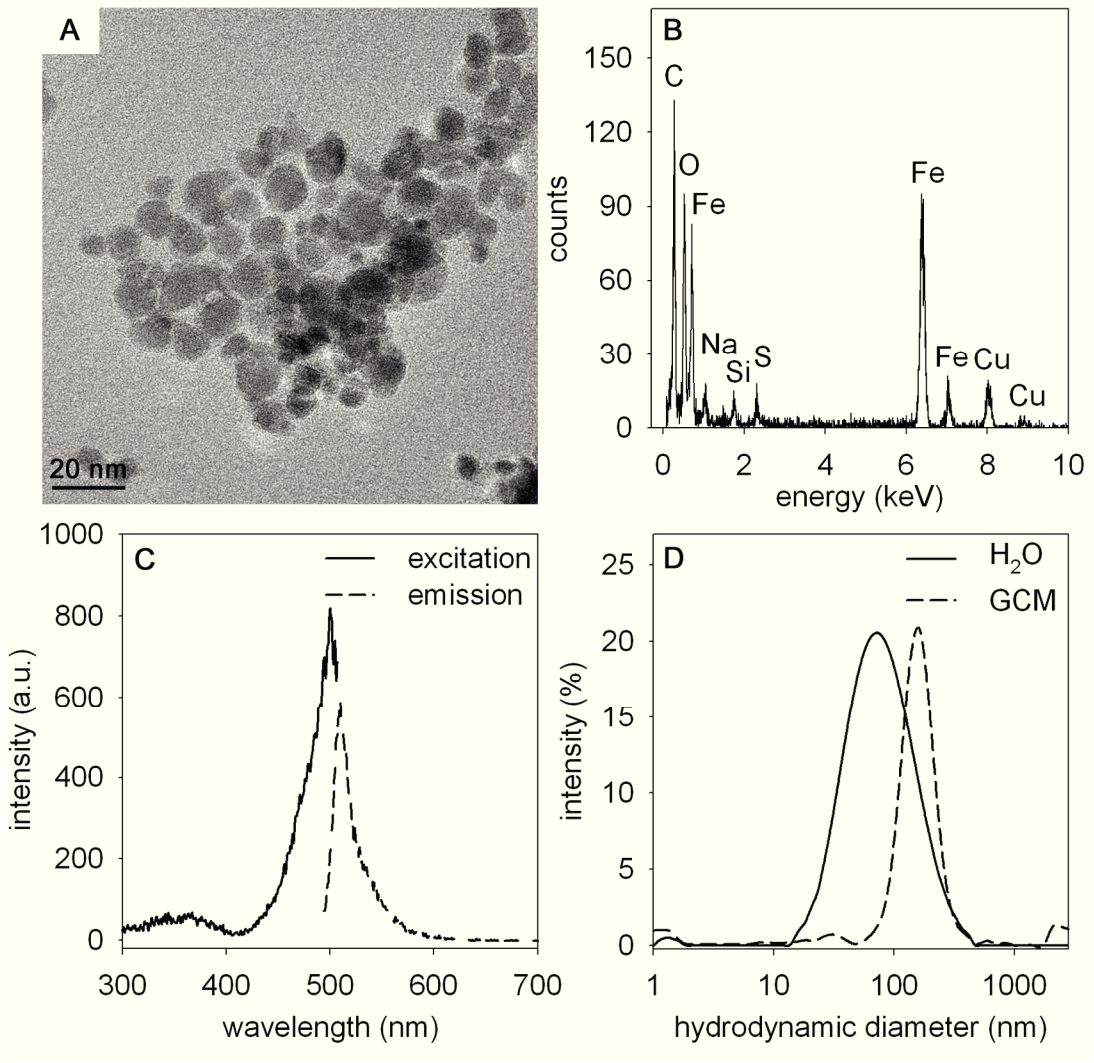

Fig. 1: Characterization of BP-IONPs. A: Transmission electron microscopy picture of BP-IONPs. **B:** Energy dispersive X-ray spectrum of BP-IONPs. **C:** Emission (excitation at 490 nm) and excitation (emission at 510 nm) fluorescence spectra of a 50 µM dispersion of BP-IONPs in water. **D:** Intensity distribution of the hydrodynamic diameter of BP-IONPs (1 mM) dispersed in water or GCM as determined by dynamic light scattering.

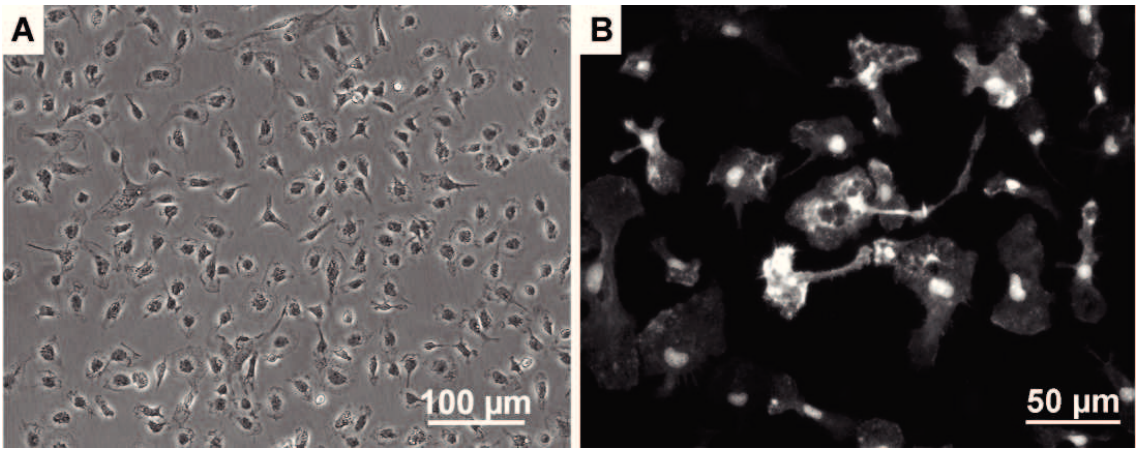

Fig. 2: Characterization of primary microglial cultures. A: Light microscopical image of a microglia-rich culture**. B:** Immunocytochemical staining of the cultures for the microglial marker protein CD11b. Nuclei were counter stained with DAPI.

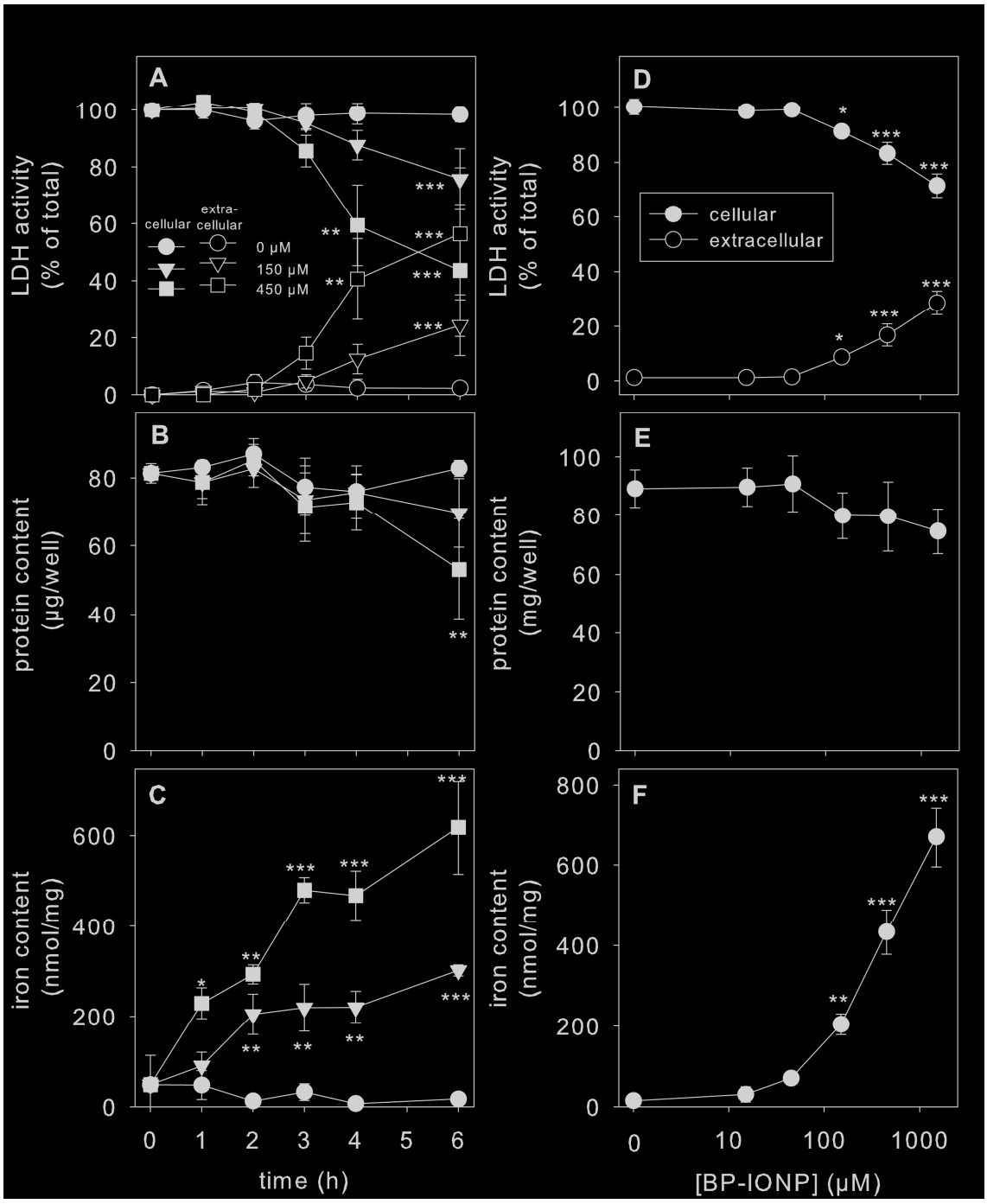

Fig. 3: Consequences of an exposure of microglial cultures to BP-IONPs. The cells were incubated without (0 µM) or with 150 µM or 450 µM BP-IONPs for up to 6 h (**A-C**) or for 3 h with the indicated concentrations of BP-IONPs (**D-F**) and the cellular and extracellular LDH activities (**A,D**), the protein content (**B,E**) as well as the specific iron content of the cells (**C,F**) were determined. Indicated is the significance of differences between the values obtained for BP-IONP-treated cells compared with controls (absence of BP-IONPs) (*p<0.05; **p<0.01; ***p<0.001).

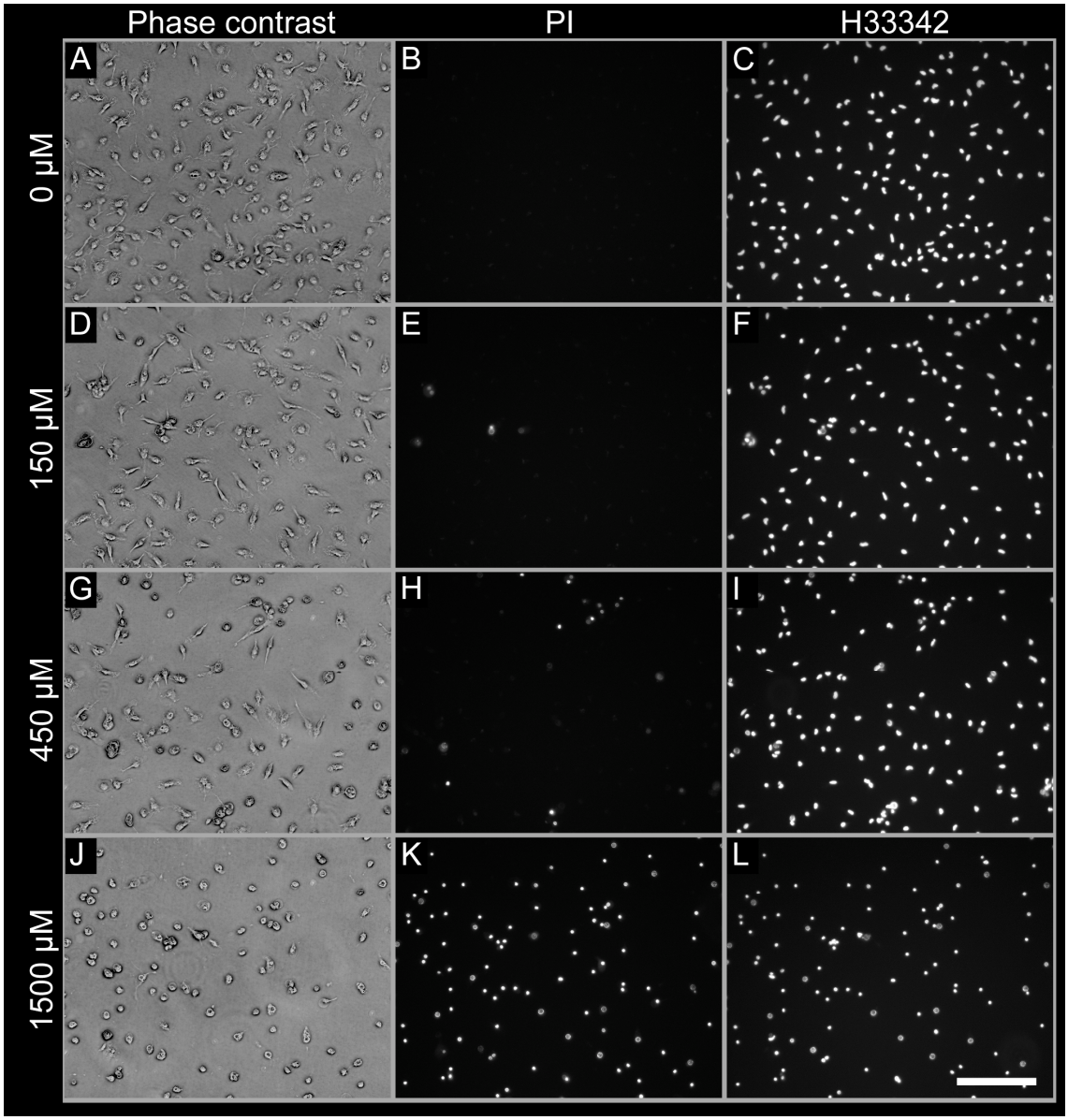

Fig. 4: Effects of BP-IONPs on the membrane integrity of microglial cells. The cells were incubated without (**A-C**) or with 150 µM (**D-F**), 450 µM (**G-I**) or 1500 µM (**J-L**) iron as BP-IONPs for 3 h. Shown are the phase contrast images of the cells (**A,D,G,J**), the PI staining which indicates nuclei of cells with permeabilized membranes (**B,E,H,K**) and the Hoechst 33342 (H33342) staining which identifies the nuclei of all cells present (**C,F,I,L**). The scale bar in **L** represents 100 µm and applies to all panels.

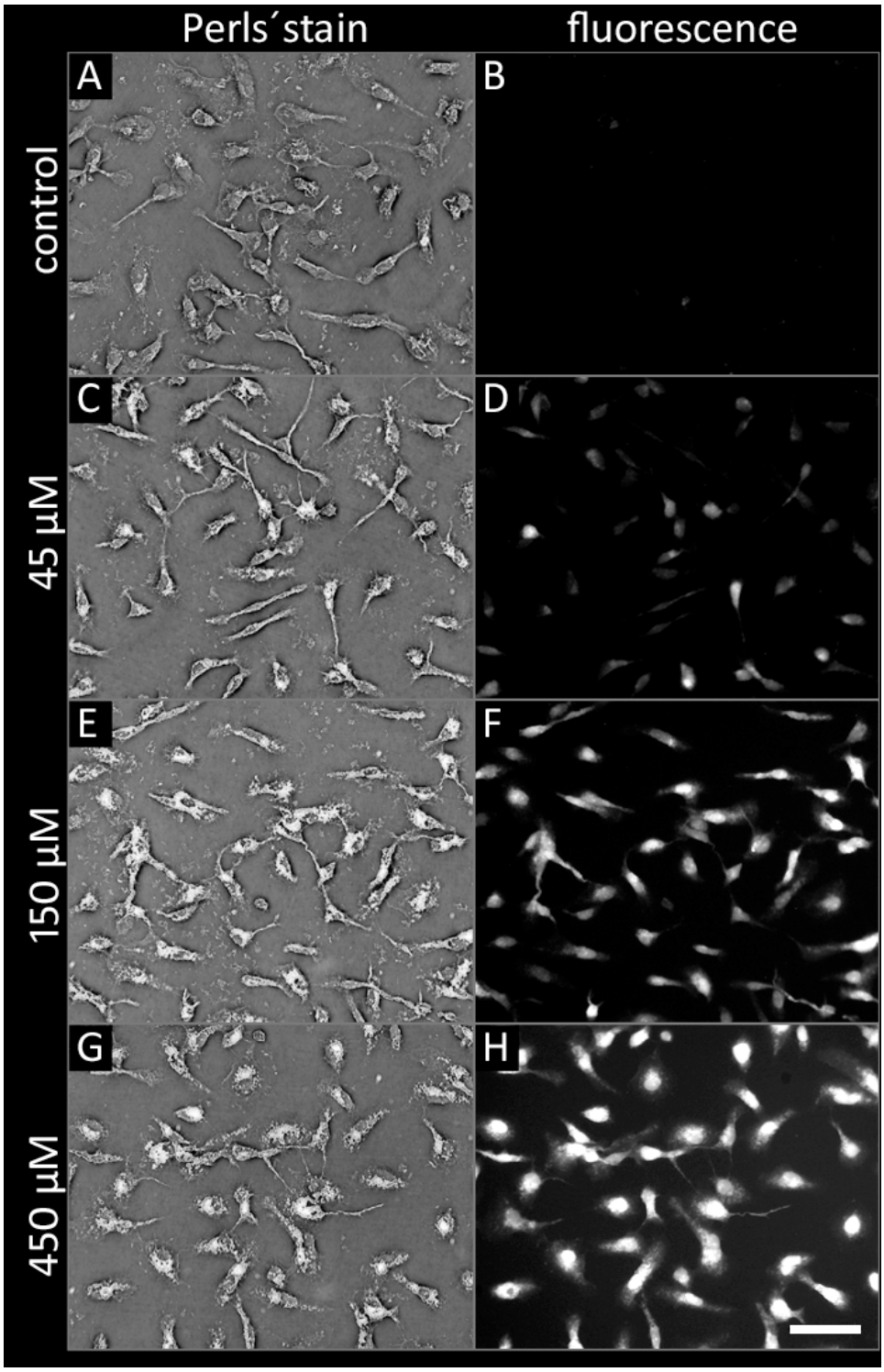

Fig. 5: Perls´ iron staining and BP-fluorescence of cultured microglial cells after exposure to BP-IONPs. The cells were incubated without (control, **A,B**) or with 45 µM (**C,D**), 150 µM (**E,F**) or 450 µM (**G,H**) of iron as BP-IONPs. Shown are overlays of the phase contrast image of the cells with the transmission light images of the Perls´ staining for cellular iron (**A,C,E,G**) and the BP fluorescence images (**B,D,F,H**) The size bar in panel **H** represents 50 µm and applies to all panels.

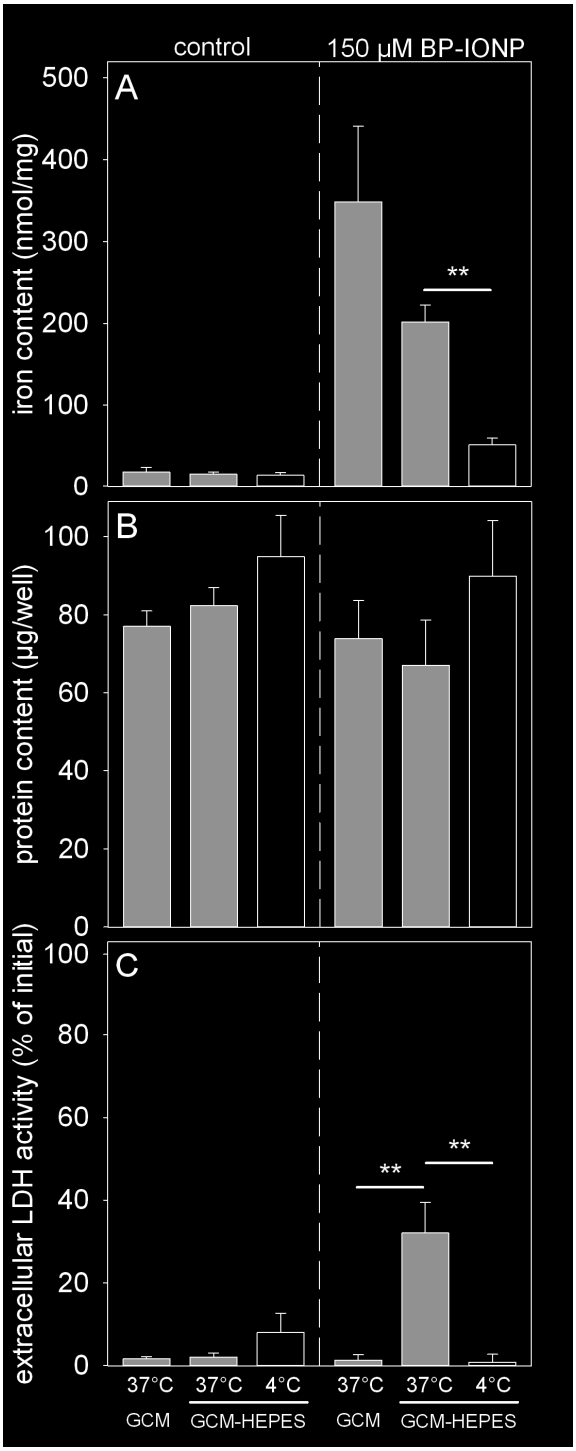

Fig. 6: Temperature dependent uptake of iron from BP-IONPs by cultured microglial cells. The cells were incubated for 3 h without (control) or with 150 µM of iron as BP-IONPs in GCM at 37°C or in GCM-HEPES at 37°C or 4°C. The specific iron content (**A**), the protein content (**B**) and the extracellular LDH activity (**C**) were measured. Indicated is the significance of differences compared to the values obtained for cells that had been incubated at 37°C in GCM-HEPES (**p<0.01).

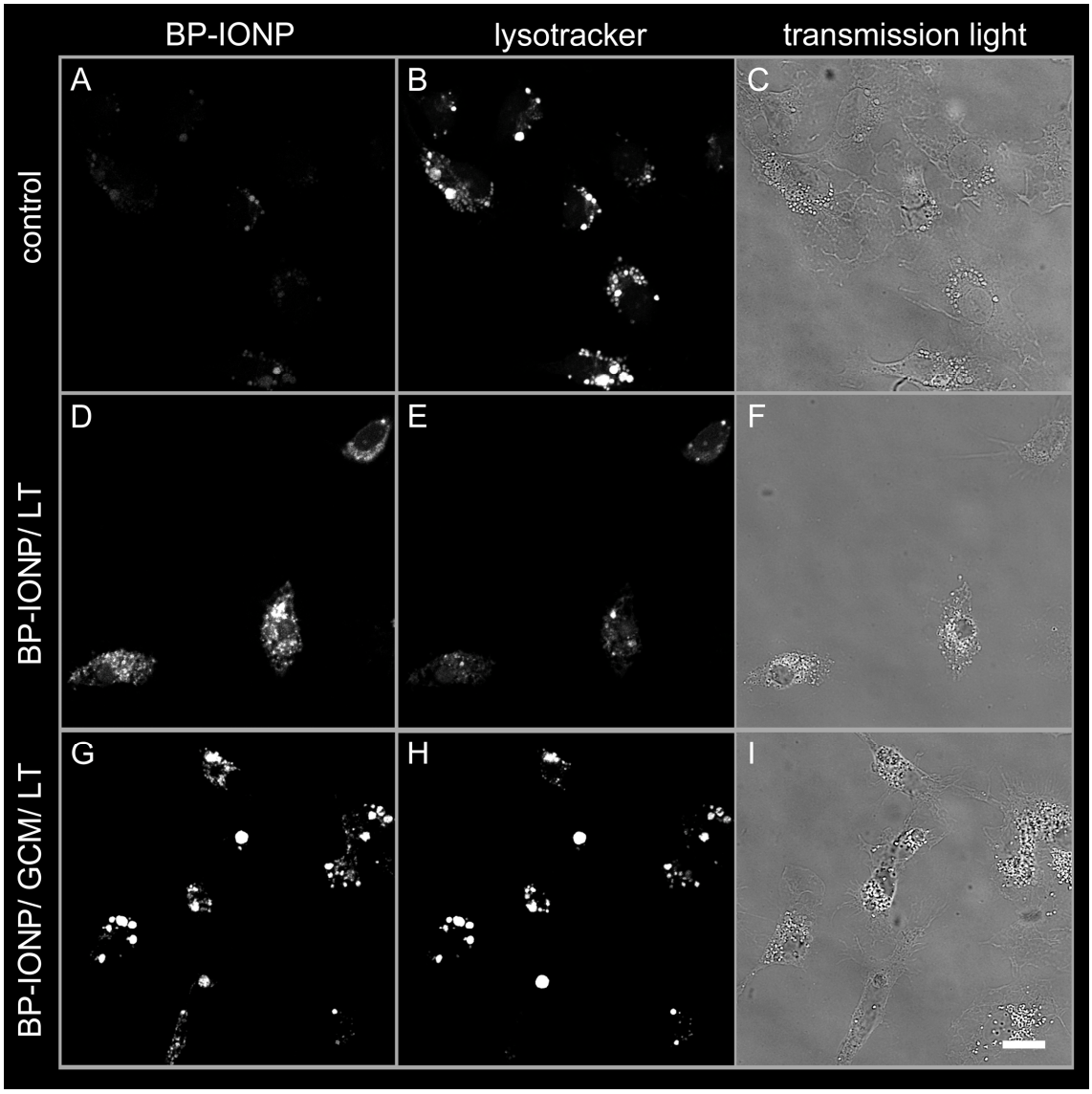

Fig. 7: Confocal co-localization of BP-IONPs and lysosomes in microglial cells. Microglial cells were incubation with lysotracker (LT) for 1 h either without prior incubation with BP-IONPs (control, **A-C**) or directly (**D-E**) or 90 min (**G-I**) after a 3 h loading of the cells with 150 µM BP-IONPs. The scale bar in **I** represents 10 µm and applies to all panels.

Table 1: Hydrodynamic diameter and ζ-potential of BP-IONPs in different media.

Medium	Hydrodynamic diameter (nm)	n	ζ-potential (mV)	n
H_2O	65 ± 4	5	-49 ± 2	4
DMEM	2221 ± 332	4	-18 ± 0	4
DMEM + FCS	128 ± 54	4	-9 ± 1	4
GCM	139 ± 9	3	-8 ± 3	3

The hydrodynamic diameter and the ζ-potential were determined for dispersions of BP-IONPs in the given media that contained 1 mM iron as BP-IONPs. The data represent mean values ± SD of n independently performed experiments.

Table 2: Effects of BP-IONPs on the glutathione content of cultured microglial cells.

BP-IONP (µM)	GSx content (nmol/mg)	GSSG content (nmol GSx/mg)	Protein content (µg/well)
initial	15.3 ± 3.2	0.2 ± 0.2	68 ± 14
0	10.3 ± 1.9	0.0 ± 0.0	84 ± 12
150	10.7 ± 1.7	0.2 ± 0.2	80 ± 7
450	10.2 ± 2.5	0.2 ± 0.2	85 ± 4

Cultured microglial cells were incubated for 3 h without (0 µM) or with 150 µM or 450 µM of iron as BP-IONPs and the specific contents of total glutathione (GSx) and GSSG as well as the protein content were measured. The data represent mean values ± SD of 3 experiments performed on independently prepared cultures.

Table 3: Effects of endocytosis inhibitors on the cell viability and the accumulation of BP-IONPs by cultured microglial cells.

Inhibitor/ Treatment	Concentration	Cellular LDH activity	Extracellular LDH activity	Protein content	Specific iron content		n
	(µM)	(% of total)	(% of total)	(µg/well)	(nmol/mg)	(% of control)	
Control (1xDMSO)		90 ± 11	10.4 ± 10.7	55 ± 12	257 ± 33	100 ± 0	3
EIPA	25	98 ± 1	1.5 ± 1.2	66 ± 22	186 ± 16 **	73 ± 10 *	3
Tyrphostin 23	100	100 ± 1	0.1 ± 0.1	63 ± 18	191 ± 25 **	74 ± 4 *	3
Chlorpromazine	10	99 ± 2	1.3 ± 1.5	70 ± 13	189 ± 9 **	74 ± 7 *	3
Control (3xDMSO)		98 ± 2	2.5 ± 2.3	76 ± 23	220 ± 83	100 ± 0	5
EIPA +	25 +						
Tyrphostin 23 +	100 +						
Chlorpromazin	10	98 ± 3	2.0 ± 2.1	67 ± 20 *	87 ± 26 ***	42 ± 15 ***	5

The cells were incubated for 3 h with 150 µM iron applied as BP-IONPs in the absence or the presence of the indicated endocytosis inhibitors. Since all inhibitors were applied from concentrated stock solutions in dimethyl sulfoxide (DMSO), the effect of the respective DMSO control was also investigated. The data represent mean values ± SD of n experiments performed on independently prepared cultures. Indicated are the significances between values obtained for cells treated with the indicated inhibitor(s) and the respective control (*p<0.05; **p<0.01; ***p<0.001).

4. Discussion

4.1. Characterization of BP-IONPs

Fluorescent BP-IONPs were successfully synthesized and coated with BP-labelled DMSA as previously shown for unlabelled DMSA [25, 39, 40]. DMSA has been reported to form a cage like structure around the IONPs by binding of the carboxyl groups of DMSA to the nanoparticle surface and by formation of disulfide bridges between adsorbed DMSA molecules [26, 41]. Complete derivatization of the BP applied labelled only 7.5% of the thiol groups of the available DMSA, thereby leaving sufficient DMSA thiol groups for the formation of a disulfide-linked cage around the core of the IONPs. The characterization of BP-IONPs by TEM and dynamic light scattering revealed the presence of individual particles of around 10 nm diameter which had formed small aggregates in dispersion, as previously also reported for DMSA-coated IONPs [25]. Fluorescence spectroscopy confirmed the presence of the fluorescent dye BP in the particles, as BP-IONPs showed the fluorescence maxima around 490 nm and 510 nm in the excitation and emission spectra, respectively, that were also recorded for BP by us (data not shown) and others [42, 43].

BP-IONPs were not stably dispersed in plain DMEM culture medium. Reason for this observation may be the high ionic strength of this medium and/or the presence of phosphate which caused precipitation of DMSA-coated IONPs (data not shown). However, presence of serum prevented the precipitation of BP-IONPs, most likely by forming a protein corona around the particles as previously described for AgNPs [44, 45] and IONPs [46, 47]. This hypothesis is supported by the increase in hydrodynamic diameter as well as by the positivation of the ζ-potential of BP-IONPs dispersed in serum-containing media.

4.2. Microglial cell cultures

Highly purified microglial cultures were generated by tryptic removal of the astrocyte layer from astroglia-rich primary cultures. Cell morphology and immunocytochemical staining confirmed literature data [8, 27] demonstrating that the cultures obtained were highly enriched for microglial cells. Although microglial cells in culture are described to be in an activated mode due to the artificial environment [7], the microglial cultures used in our study could be further activated by incubation with a phorbol ester (data not shown) which

induces superoxide generation by NADPH oxidase [32, 48]. However, no enhanced superoxide production was observed for microglial cultures after exposure to BP-IONPs (data not shown) which confirms literature data that cultured microglial cells are not activated by an exposure to IONPs [23].

4.3. Accumulation of BP-IONPs by viable microglial cells

Exposure of microglial cells to moderate concentrations of BP-IONPs (up to 450 µM) for up to 3 h did not compromise cell viability, whereas higher concentrations of IONPs or elongated incubation periods led to a severe loss in cell viability. This is consistent with literature data described for IONP-treated secondary microglial cultures [13, 23]. Quantification of cellular iron contents of BP-IONP treated microglial cells revealed a concentration dependence of BP-IONP accumulation, which was confirmed by fluorescence microscopy and cytochemical Perls´ staining for iron. The strict co-localization of fluorescence and iron deposits suggests that under the conditions used most of the accumulated particles remained intact after uptake.

Cultured viable microglial cells increased their cellular iron content after a 3 h incubation with 450 µM BP-IONPs by 10-fold. This cellular amount of accumulated IONPs of around 500 nmol/mg protein, which represented less than 10% of the iron applied as BP-IONPs, appears to be the maximal amounts of accumulated IONPs that can be tolerated by viable microglial cells, since higher concentrations of IONPs or longer incubations that led to higher specific cellular iron contents compromised the cell viability. This observation is consistent with the view that microglial cells take up particles until they die [13]. The loss in cell viability upon exposure to high concentrations of IONPs may be a consequence of a rapid liberation of iron ions from the accumulated particles, as low molecular weight iron has been described to be toxic for microglial cells [49, 50] and to cause detachment of cells [51]. This hypothesis is consistent with the observation that cellular BP-IONP fluorescence in microglial cells was co-localized with lysosomes where the low pH has been described to facilitate IONP-degradation by liberation of iron ions [52].

To test to which extend the determined cellular iron of IONP-treated microglial cells represented internalised iron or IONPs that were bound extracellularly to the cell membrane, the cells were exposed to BP-IONPs at 4°C, since this low temperature is known to slow down transport processes across the membrane and the internalisation of IONPs [25, 53, 54]. After incubation at 4°C, only

20% of the cellular iron contents of the respective 37°C incubation were observed, suggesting that only around 20% of the cellular iron determined for BP-IONP treated microglial cells can be considered as externally attached. This is consistent with data obtained for the uptake of other types of fluorescent IONPs by microglial cells [23]. In contrast, membrane-attached IONPs represented around 50% of the total cellular iron determined for IONP-treated astrocytes [25]. Thus, cultured microglial cells appear to be more efficient to internalise membrane-bound IONPs or are less efficient to bind IONPs than cultured astrocytes, although the specific capacity of cultured astrocytes to accumulate IONPs is much higher [25, 53] than that of cultured microglial cells.

4.4. Mechanisms of BP-IONP uptake into microglial cells

The uptake of BP-IONPs into vesicles and the subsequent co-localization of BP-IONPs in microglial cells with lysosomes suggest that endocytotic processes are involved in the accumulation of BP-IONPs into microglial cells. Although microglial cells have the capacity for phagocytosis [8], this process appears not to contribute substantially to the observed BP-IONP accumulation by cultured microglial cells. At least the phagocytosis inhibitor cytochalasin D which is described to inhibit α-synuclein-aggregate uptake in microglia [55] did not affect the BP-IONP accumulation by microglial cells (data not shown). Since the aggregates of BP-IONPs formed in GCM had an average size of 140 nm, they may be too small to be taken up by phagocytosis, since this process has been discussed to be predominantly involved in uptake of particles larger than 200 nm [56], while smaller particles are taken up by endocytotic pathways [57]. Indeed, tyrphostin 23, chlorpromazin and EIPA, inhibitors of macropinocytosis and clathrin-dependent endocytosis [38, 58, 59] significantly lowered the accumulation of BP-IONPs into microglial cells by around 60% in an additive fashion, suggesting that these two endocytotic pathways contribute to the uptake of BP-IONPs by microglial cells. This is in line with a recent report that demonstrates also a clathrin-dependent uptake of quantum dots into microglial cells [18]. However, it should be stressed here that the specificity of inhibitors that are commonly used to identify endocytotic pathways is still under debate [36, 37].

4.5. Conclusions

In summary, fluorescent BP-IONPs were synthesized and characterized as tools to investigate IONP uptake into cultured microglial cells. These cells efficiently accumulated BP-IONPs as demonstrated by quantification of cellular iron contents as well as the co-localization of cellular iron and cellular fluorescence. The localization of BP-IONPs in lysosomes as well as the inhibition of BP-IONP uptake by endocytosis inhibitors demonstrates that the BP-IONPs are taken up into microglial cells by endocytosis and enter the lysosomal pathway. Although the low pH of the lysosomes may liberate some iron from the accumulated BP-IONPs, the cells remained viable after exposure to moderate concentrations of the particles and did not show any indications for enhanced activation or oxidative stress. Thus, microglial cells may act as a first defence line in brain that fast and efficiently takes up IONPs, thereby helping to protect the brain against damage by IONPs and IONP-derived iron.

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3

SUMMARIZING DISCUSSION

3 SUMMARIZING DISCUSSION

This thesis investigated the effects of metal-containing NPs on cultured brain astrocytes and microglia, as those cells can be considered as first lines of defense against potential NP-derived effects in brain that protect other brain cells from damage. Due to the high prevalence rate of AgNPs in consumer products and the medical importance of IONPs those two types of NPs are of high relevance. Therefore this thesis focused on the investigation of the consequences of an exposure of brain cells to AgNPs and IONPs.

3.1 UPTAKE AND METABOLISM OF SILVER NANOPARTICLES BY ASTROCYTES

After peripheral application, a distribution of AgNPs throughout the whole body, including the brain, has frequently been reported (table 1.1). In addition, increased silver levels in brain cells upon treatment with AgNPs cause neurotoxicity and cognitive impairment in fish and rats (Kashiwada 2006, Asharani *et al.* 2008, Hadrup *et al.* 2012, Lee *et al.* 2012, Liu *et al.* 2012a). However, only little is known on the mechanisms behind these observed effects. The consequences of an exposure to AgNPs have been studied in CA1 neurons in brain slices, where the excitability and the signal transduction were altered by the depression of K^+- and Na^+-channels (Liu *et al.* 2009, Liu *et al.* 2011, Liu *et al.* 2012b), while in primary neural cells, mainly consisting of neurons and astrocytes, toxicity, oxidative stress and calcium signals have been observed (Haase *et al.* 2012). This thesis provides new insights into the biocompatibility and metabolism of PVP-coated AgNPs in astrocyte-rich primary cultures.

Astrocytes efficiently accumulated silver from AgNPs in a time- and concentration-dependent manner (chapter 2.1) as also described for the uptake of other NPs like IONPs (chapter 1.5, Geppert *et al.* 2009, Geppert *et al.* 2011, Lamkowsky *et al.* 2012) or polymer NPs (Chang *et al.* 2012). The capacity of astrocytes to accumulate silver from AgNPs was substantially higher compared to that of other cells types. For example, it exceeded that of cultured A549 lung cancer cells (Foldbjerg *et al.* 2011) by almost 20-fold.

The uptake of NPs by astrocytes is an energy-dependent process that involves endocytotic processes (chapter 1.5, Hohnholt *et al.* 2010b, Pickard

et al. 2011, Lamkowsky *et al.* 2012, Geppert *et al.* 2013). Vesicular structures filled with NPs including AgNPs have frequently been shown by TEM in astrocytes (Geppert *et al.* 2011, Pickard *et al.* 2011, Haase *et al.* 2012, Jenkins *et al.* 2013). However, the mechanisms involved in the uptake of NPs have been demonstrated only for IONPs so far (Pickard *et al.* 2011, Lamkowsky *et al.* 2012, Geppert *et al.* 2013). The uptake of AgNPs into astrocytes has been investigated in detail for the first time in this thesis (chapter 2.1). Lowering of the incubation temperature from 37°C to 4°C and thereby inhibiting active and energy-dependent uptake mechanisms decreased the cellular silver content by 80%. The active uptake of AgNPs into astrocytes was in part mediated by macropinocytosis (MP) and via the endosomal pathway as inhibition of those pathways significantly lowered the AgNP-uptake.

Comparing the endocytotic uptake of IONPs and AgNPs into astrocytes reveals, that the effects of the individual endocytosis inhibitors seem to be dependent on the incubation conditions, e.g. presence of serum, and the particles used (table 3.1). Therefore, general statements can hardly be made which pinpoint an individual pathway for NP uptake by astrocytes. However, at least the caveolin-mediated endocytosis (CvME) seems not to participate in the uptake of NPs into astrocytes as inhibitors of this pathway (Filipin III and Methyl-β-cyclodextrin) were not effective in preventing NP uptake in any of the described studies (table 3.1). In addition, the inhibitory potential of endocytosis inhibitors appears to be rather cell-type specific, as compounds like wortmannin and chlorpromazine, that were ineffective in inhibiting the AgNP uptake in astrocytes (chapter 2.1) partially prevented AgNP uptake into IMR-90 human lung fibroblasts (AshaRani *et al.* 2009), human mesenchymal stem cells (Greulich *et al.* 2011b), U251 human glioblastoma cells (AshaRani *et al.* 2009) and the mouse macrophage cell line J774A.1 (Wang *et al.* 2012). The NP-size also seems to play an important role for the inhibitory potential of endocytosis inhibitors, as upon application of very small AgNPs with a diameter of 5 nm to U937 macrophages, endocytotic uptake could neither be prevented by inhibition of CME nor by blocking of MP or CvME. The majority of these particles was discussed to be taken up via passive membrane penetration (Kim and Choi 2012). However, it should be considered, that the specificity of the described inhibitors for an individual endocytotic pathway is questionable (Ivanov 2008).

Table 3.1: Studies reporting the contribution of endocytotic pathways in NP uptake into astrocytes.

NP type	Size (nm)	Inhibitors affecting NP uptake	Inhibitors not affecting NP uptake	Reference
Serum present during the incubation				
PVP-AgNP	75	Chloroquine (1 mM) Amiloride (1 mM)	Methyl-β-cyclodextrin (2.5 mM) 3-Methyladenine (2.5 mM) Wortmannin (100 nM) EIPA (25 µM) Chlorpromazine (20 µM)	chapter 2.1
Carboxyl-modified Polystyrene-IONP	360	Tyrphostin 23 (350 µM) Dynasore (80 µM) Amiloride (1 mM) EIPA (100 µM)	Filipin III (5 µg/ml)	Pickard and Chari (2010a)
DMSA-IONP	70	Chlorpromazine (20 µM) Wortmannin (100 nM)	EIPA (25 µM) 3-Methyladenine (2.5 mM)	Geppert *et al.* (2013)
Serum absent during the incubation				
DMSA-IONP	70	--	Chlorpromazine (20 µM) EIPA (25 µM) Wortmannin (100 nM) 3-Methyladenine (2.5 mM)	Geppert *et al.*(2013)
DMSA-IONP	60	--	Chlorpromazine (20 µM) EIPA (25 µM)	Lamkowsky *et al.* (2012)

EIPA: 5-(N-ethyl-N-isopropyl)amiloride

In the studies described within this thesis, astrocytes were shown to accumulate PVP-coated AgNPs very efficiently without any acute or delayed loss in cell viability. This is in contrast to data on peptide-coated AgNPs that induced toxicity in astrocytes (Haase *et al.* 2012). In addition, PVP-coated AgNPs induced toxicity in other cell types, like in human mesenchymal stem cells (hMSCs) (Greulich *et al.* 2009). Therefore, also the toxic potential of AgNPs appears to strongly depend on the NP-composition and the individual cell-types investigated.

The reported cell toxicity of AgNPs is likely to originate from silver ions (Ag^+) that were released from AgNPs (Johnston *et al.* 2010, Kittler *et al.* 2010). Ag^+ have been shown to be much more toxic than AgNPs for many cells types, like A549 lung cancer cells, THP-1 monocytes, hMSCs, HepG2 cells, Chang liver cells (CLC), PC12 cells or trout gill cells (Foldbjerg *et al.* 2009, Greulich *et al.* 2009, Kim *et al.* 2009, Kittler *et al.* 2009, Foldbjerg *et al.* 2011, Powers *et al.* 2010b, Farkas *et al.* 2011, Piao *et al.* 2011) and also for astrocytes (chapter 2.1). As evident from figure 3.1, the resistance towards Ag^+ was similar in astrocytes and other cell types, but astrocytes appeared to have a much higher capacity to survive a treatment with AgNPs and tolerated AgNP concentrations that outranged toxic Ag^+ concentrations by at least 10-fold, whereas in other cell types already 1- to 6-times higher concentrations of AgNPs compared to Ag^+ led to a significant loss in viability (figure 3.1).

Differences in the release of Ag^+ may also explain the observed discrepancies in toxicity of PVP- and peptide-coated AgNPs in astrocytes (chapter 2.1, Haase *et al.* 2012) as it could be possible that the peptide coat is degraded faster or is less dense than the PVP-coat supporting a faster liberation of Ag^+ and subsequently a higher toxicity of peptide-coated AgNPs.

Oxidative stress has frequently been described as a consequence of an incubation of various cell lines from human, rat or mouse origin with AgNPs (table 3.2). Interestingly, oxidative stress is described for AgNPs with various coatings regardless of the size of the particles (table 3.2). Therefore the potential to induce oxidative stress in cell lines appears to be a general property of AgNPs which is likely to originate from the Ag^+ released from the particles as silver in low molecular form is known to induce oxidative stress (Powers *et al.* 2010b).

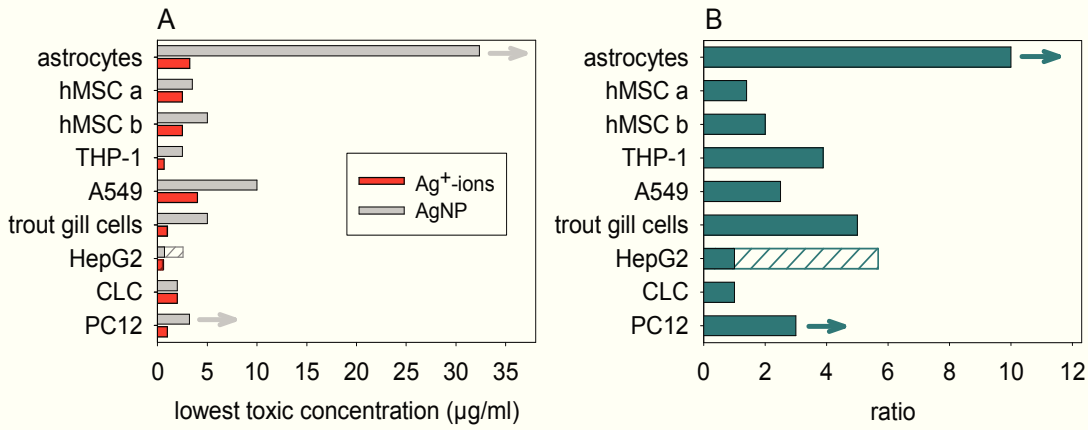

Figure 3.1: **Difference in the toxicity observed in various cell types after application of AgNPs and Ag^+.** Panel A shows the lowest concentrations of AgNPs and Ag^+ applied, that resulted in a significant loss in viability. Panel B shows the ratio of the lowest reported toxic concentration of AgNPs to Ag^+. Data are compiled from chapter 2.1 of this thesis (astrocytes), Greulich *et al.* 2009 (hMSC a), Kittler *et al.* 2009 (hMSC b), Foldbjerg *et al.* 2009 (THP-1), Foldbjerg *et al.* 2011 (A549), Farkas *et al.* 2011 (trout gill cells), Kim *et al.* 2009 (HepG2), Miura and Shinohara 2009 (HeLa), Piao *et al.* 2011 (CLC) and Powers *et al.* 2010b (PC12) and represent results obtained by different toxicity assays. However, it should be noted that the values for the onset of toxicity in each cell type depends on the assay used, as shown for HepG2 cells, where filled bars represent values from the alamar blue test, whereas the dashed bars show data from the MTT assay. In the studies on astrocytes and PC12 cells the arrows indicate underestimation of the values as no toxicity was observed in the highest concentrations of AgNPs tested.

Besides this thesis only few studies describe the effects of AgNPs on primary cells (Arora *et al.* 2009, Greulich *et al.* 2011a, Haase *et al.* 2012). The cellular antioxidative machinery has been reported to be altered in all of those studies upon application of AgNP concentrations between 10 and 30 µg/ml. In primary mouse fibroblasts and primary liver cells oxidative stress due to a 24 h-incubation with 30 µg/ml AgNPs has been described to elevate cellular GSH levels (Arora *et al.* 2009), whereas in peripheral blood mononuclear cells (PBMCs) and mixed neural cultures ROS has been detected already upon an exposure to 10 µg/ml AgNPs for 24 and 3 h, respectively (Greulich *et al.* 2011a, Haase *et al.* 2012). This is in contrast to the data obtained in this thesis, where neither shift in the cellular GSH to GSSG ratio nor a cellular depletion of GSH or ROS formation were detectable in astrocytes upon treatment with 10.8 µg/ml (100 µM) AgNPs for 4 h or after a subsequent recovery phase of up to 1 week. This again reveals that the effects observed upon treatment of cells with AgNPs dependent strongly on the NP-composition and cell-type used.

Table 3.2: Exposure of different cell lines to AgNPs induces oxidative stress.

Cell type	NP size (nm)	NP coat	Time#	Concentration§	Toxicity	Effects	References
A431	7-20	n/a	24 h	6.25 µg/ml	yes	GSH ↓; lipid peroxidation ↑	Arora *et al.* (2008)
BEAS-2B	43-260	Pt/Pd	24 h	0.01 µg/ml	no	ROS ↑	Kim *et al.* (2011)
BRL3A	15	n/a	24 h	25 µg/ml	yes	GSH ↓; ROS ↑	Hussain *et al.* (2005)
	100	n/a	24 h	25 µg/ml	yes	GSH ↓; ROS ↑	
HaCaTT	68.5	n/a	0.5 h	7.81 µg/ml	no	ROS ↑	Mukherjee *et al.* (2012)
		n/a	24 h	200 µg/ml	yes	GSH ↓	
hCLS	28-35	n/a	0.5 h	n/a	n/a	GSH ↓, ROS ↑	Piao *et al.* (2011)
HCT 116	n/a	n/a	12 h	n/a	n/a	ROS ↑	Hsin *et al.* (2008)
HeLa	5-10	n/a	4 h	2.5 µg/ml	no	ho-1 gene ↑	Miura and Shinohara (2009)
HeLa	65.5	n/a	1 h	0.625 µg/ml	no	ROS ↑	Mukherjee *et al.* (2012)
			24 h	25 µg/ml	n/a	GSH ↓	
HepG2	10	none	confluency of control	0.12 µg/ml	n/a	GSH ↓	Nowrouzi *et al.* (2010)
				0.24 µg/ml	n/a	NO ↑	
HepG2	5-10	n/a	1 h	0.2 µg/ml	n/a	ROS ↑	Kim *et al.* (2009)
HepG2	5	PVP	2 h	1 µg/ml	yes	ROS ↑	Liu *et al.* (2010)
	20	PVP	2 h	10 µg/ml	yes	ROS ↑	
	50	PVP	2 h	10 µg/ml	yes	ROS ↑	

#: shortest incubation time after which oxidative Stress was detected; §: lowest concentration at which a sigificant change in oxidative stress or antioxidative parameters was observed; n/a: not availabe; GSH: glutathione; ROS: reactive oxygen species; ↑: increased; ↓: decreased.

Table 3.2 continued: Exposure of different cell lines to AgNPs induces oxidative stress.

Cell type	NP size (nm)	NP coat	Time[#]	Concentration [§]	Toxicity	Effects	References
HepG2	69 ± 5	none	5 h	10% v/v	n/a	ROS ↑	Stevanovic *et al.* (2011)
	45 ± 5	PGA	5 h	10% v/v	n/a	ROS ↑	
HT-1080	7-20	n/a	24 h	6.25 µg/ml	yes	GSH ↓; lipid peroxidation ↑	Arora *et al.* (2008)
NIH3T3	n/a	n/a	6 h	n/a	n/a	ROS ↑	Hsin *et al.* (2008)
PC12	10	citrate	4 d	3.24 µg/ml	yes	ROS ↑	Powers *et al.* (2010a)
	10	PVP	4 d	3.24 µg/ml	n/a	ROS ↑	
	50	PVP	4 d	1.08 µg/ml	n/a	ROS ↑	
RAW 264.7	69 ± 30	FBS	24 h	0.4 ppm	no	GSH ↓; NO ↑	Park *et al.* (2010)
THP-1	69 (TEM), 100-200 (DLS)	none	6 h	5 µg/ml	yes	ROS ↑	Foldbjerg *et al.* (2009)

#: shortest incubation time after which oxidative stress was detected; §: lowest concentration at which a sigificant change in oxidative stress or antioxidative parameters was observed; n/a: not availabe; GSH: glutathione; ROS: reactive oxigen species; ↑: increased; ↓: decreased.

Upon exposure to an excess of metals, including application of metal containing NPs, astrocytes have frequently been shown to upregulate proteins that allow metal storage like Ft or metallothioneins (MTs) (chapter 1.5, chapter 2.2, Tiffany-Castiglioni *et al.* 2001, Tiffany-Castiglioni and Qian 2001, Dringen *et al.* 2007, Jones 2012, Scheiber and Dringen 2013) and/or proteins like heme oxigenase-1 (HO-1) that provide protection from the adverse effects that may arise from those metals (Ni *et al.* 2011).

The strong resistance of astrocytes against AgNP-derived toxicity is likely to be due to their high metal storing capacity which is increased by upregulation of metal storage proteins like MTs. The molecular mechanisms which induce this upregulation are not known so far. An involvement of the metal regulatory transcription factor 1 (MTF-1) seems likely which could be activated by zinc ions that were replaced by silver ions in MTs, since silver has a much higher affinity to MTs than zinc (Floriańczyk 2007). Activated MTF-1 would bind to metal response elements in the MT gene and trigger its synthesis (Vašák and Meloni 2011). Another possible way of MT upregulation involves the binding of the transcription factor Nrf-1 to antioxidative response elements within the MT-gene which can be triggered by oxidative stress (Ohtsuji *et al.* 2008, Reisman *et al.* 2009). A similar pathway could also result in an upregulation of HO-1 as described for neural cells (Haase *et al.* 2012), although HO-1 is not regulated via Nrf-1, but by Nrf-2, a member of the same family of transcription factors, which also binds to antioxidative response elements in the genes of target proteins (Syapin 2008).

In mixed neural cell cultures which contain mainly neurons and astrocytes an induction of the cytoprotective HO-1 was first seen after 8 h and increased further after 12 and 24 h (Haase *et al.* 2012). A significant induction of MTs in astrocyte cultures was visible after 24 h and increased strongly after 72 h and 168 h (chapter 2.2). The fast regulation of HO-1 and slightly delayed increase in MTs may provide optimal protection of the cells against Ag^+-derived toxicity or ROS.

Figure 3.2 summarizes the current knowledge on the handling of AgNPs by astrocytes.

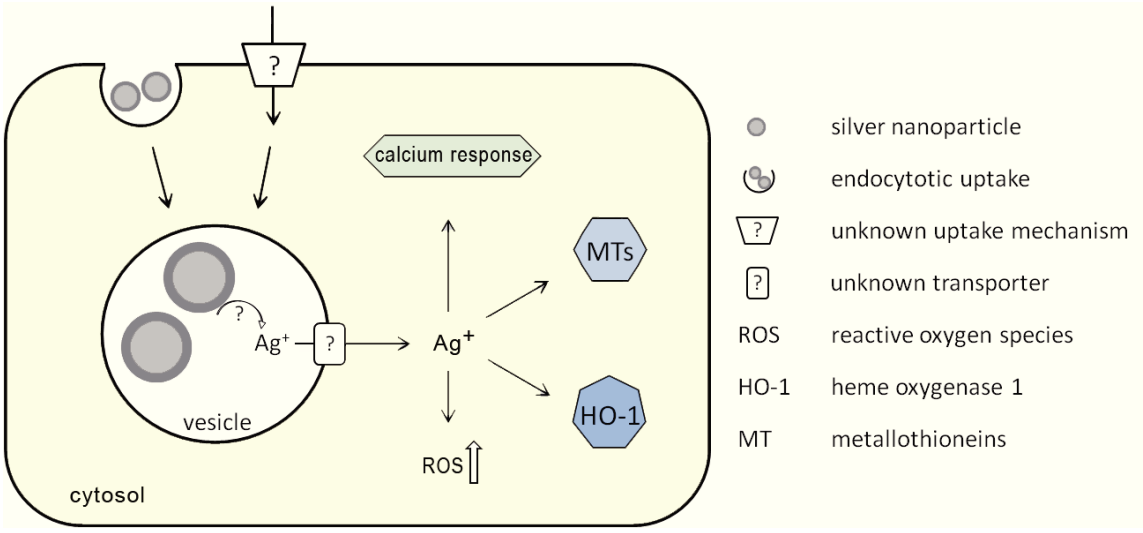

Figure 3.2: Handling of silver nanoparticles by cultures astrocytes. Astrocytes efficiently accumulate silver applied as AgNPs by endocytotic mechanisms but other uptake mechanisms appear to be involved in the AgNP uptake as well. The intracellular presence of AgNPs coincides with the upregulation of MTs and HO-1 and induces the release of a calcium reponse in the cells. This is likely to be due to the liberation of Ag^+ from the particles. The cellular presence of AgNPs may also lead to the formation of ROS.

3.2 Consequences of an accumulation of iron oxide nanoparticles by micoglial cells

The uptake of IONPs by microglia has frequently been shown *in vivo* and in cell lines (table 1.1), while only limited data are available on the effects of IONPs on primary or secondary microglial cells (chapter 2.3, Fleige *et al.* 2001, Pickard and Chari 2010b). This thesis for the first time gives quantative data on the uptake of IONPs by primary microglial cells of rat brain and provides new information on the localization of fluorescent IONPs in the cells.

Microglia efficiently took up BP-DMSA-coated IONPs in a time-, concentration- and temperature-dependent manner, confirming literature data (Fleige *et al.* 2001, Pickard and Chari 2010b). Within an incubation for 3 h with 450 µM iron as BP-DMSA-coated IONPs viable microglia increased their celluar iron content by 10-fold to around 500 nm/mg protein. This seems to be the maximal amount of intracellular iron which could be tolerated by the cells as a further increase led to a loss in viability. This supports literature data, where microglia only survived short

incubations of 30 or 120 min with high concentrations of 18 mM or 3 mM iron as Texas red-labelled IONPs, respectively, but higher concentrations or longer exposure times led to cell death (Fleige *et al.* 2001). Incubations with 20 µg/ml carboxyl-modified Nile red-fluorescent polystyrene-IONPs, which contain only 15-20% iron, however, were tolerable for microglial cells for even 24 h (Pickard and Chari 2010b). Due to the different time frames investigated, a direct comparison of the toxic effects of the different IONP-types is not possible, but it seems that the ability of microglial cells to tolerate internalized IONPs over time depends on the iron content the cells are exposed to.

The observed toxic effects of IONPs are likely to originate from metal ions released from the particles, as described for other NPs (Johnston *et al.* 2010, Kittler *et al.* 2010). The release of ions from NPs due to degradation of the particles in microglia can be expected to appear rather fast, as already after 90 min the majority of accumulated BP-DMSA-coated IONPs were co-localized with lysosomes (chapter 2.3), where the degradation of IONPs is facilitated by the low pH (Levy *et al.* 2010). A degradation of polystyrene-IONPs in microglial lysosomes has also been shown by TEM imaging (Jenkins *et al.* 2013). Therefore the amount of low molecular iron in the cells is likely to increase quickly to levels that are known to induce cytotoxicity in microglial cells (Zhang *et al.* 2006, Oshiro *et al.* 2008, Rathore *et al.* 2012). Thus, microglia appear to accumulate IONPs very efficiently, but due to their ability to quickly degrade those particles, the cells are liklely to generate high cellular levels of low molecular iron which they cannot tolerate over time.

Other cell types in brain who are not as specialized on degradation of debris should not, to the same extent, be affected by IONPs and IONP-derived low molecular iron. Astrocytes, which are described to be the metal storing cells in the brain (Tiffany-Castiglioni and Qian 2001, Dringen *et al.* 2007, Tiffany-Castiglioni *et al.* 2011, Scheiber and Dringen 2013), should tolerate iron and IONPs (Geppert *et al.* 2011, Pickard *et al.* 2011, Lamkowsky *et al.* 2012, Geppert *et al.* 2013) much better than microglia (chapter 2.3). Indeed, comparable studies on astrocytes (Geppert *et al.* 2011, Pickard *et al.* 2011, Lamkowsky *et al.* 2012, Geppert *et al.* 2013) and microglia (chapter 2.3, Pickard and Chari 2010b) utilizing either carboxyl-modified Nile red-fluorescent polystyrene-IONPs (Pickard and Chari 2010b,

Pickard *et al.* 2011) or DMSA-coated IONPs (chapter 2.3, Geppert *et al.* 2011, Lamkowsky *et al.* 2012, Geppert *et al.* 2013) revealed that a concentrations of IONPs that resulted in a loss in viability in microglia did not induce toxicity in astrocytes. For DMSA-coated IONPs even an application of 10-times higher iron concentrations than that toxic for microglia (450 µM, chapter 2.3), did not result in cell death of astrocytes (Geppert *et al.* 2011, Lamkowsky *et al.* 2012, Geppert *et al.* 2013).

The observed enhanced toxicity of microglia compared to astrocytes might be due to the observation, that microglia accumulate IONPs much faster than astrocytes and therewith earlier reach a maximal IONP-load which can be tolerated without a loss in viability. Astrocytes were reported to accumulate DMSA-coated IONPs with a rate of 24 ± 4 nmol/(h*mg) upon incubation with 1 mM IONPs (Geppert *et al.* 2013), whereas microglia accumulated similar particles at an almost doubled rate of 45 ± 4 nmol/(h*mg) from only 450 µM IONPs applied (chapter 2.3). This observation is consistent with studies on low molecular iron. Within 6 h microglia accumulated about 10-times more low molecular iron compared to astrocytes (Bishop et al. 2010) and concentrations of iron that were toxic for microglia did to not impair the viability of astrocytes (Rathore *et al.* 2012). Similar effects have also been shown for peripheral cells. Phagocytes were shown to be much more sensitive to NPs than non-phagocytotic cells due to their better ability to efficiently accumulate and internalize NPs (Herd *et al.* 2011, Mohamed *et al.* 2011, Rabolli *et al.* 2011, Fedeli *et al.* 2013).

The mechanisms behind the observed toxicity of IONPs in mircoglia, however, remain to be elucidated. Even though upon uptake of IONPs in microglia enhanced levels of ROS have been detected in the cell line BV-2 (Wang *et al.* 2011b), oxidative stress seems not to be the cause for the toxicity of IONPs observed in primary microglia. At least neither a shift in the cellular GSH to GSSG ratio nor a depletion of cellular GSH were detected in those cells upon treatment with sub-toxic concentrations of IONPs.

The uptake of IONPs into microglia is described to be dependent on clathrin-mediated endocytosis (CME) and macropinocytosis (MP) (chapter 2.3) as well as phagocytotic uptake (Wang *et al.* 2011b). However, the

involvement of phagocytotic pathways was concluded from TEM pictures where NP-filled vesicles were shown that could also originate from endocytotic uptake. At least in our studies inhibition of phagocytosis did not result in a reduced IONP uptake in microglia (data not shown). For astrocytes clathrin-mediated endocytosis (CME) and macropinocytosis (MP) have also been identified to be responsible for the uptake of similar particles compared to those discussed in chapter 2.3 from serum containing media (Geppert *et al.* 2013). However, a part of the uptake of IONPs seem to be mediated by an unknown mechanism as only 60% of the uptake into microglia and 50% into astrocytes could be inhibited by blocking CME and MP. Therefore, in both cell types about half of the IONP uptake appears to be mediated by the same pathways. The mechanisms resposible for the remaining part of the uptake remain to be identified in further studies.

3.3 Consequences of an exposure of the brain to iron oxide and silver nanoparticles

IONPs and AgNPs were described to reach the brain via various exposure routes (table 1.1). However, the impact that NPs have on the brain strongly depends on the type of NPs and the state, i.e. healthy or diseased, the brain is in. NPs that cross the BBB without eliciting any inflammation hardly have an influence on healthy brain regions. IONPs, for example, pass the intact BBB after peripheral application (Wang *et al.* 2010) and do not lead to an activation of brain cells *in vitro* (chapter 2.3, Raju *et al.* 2011) or *in vivo* (Alison *et al.* 2010, Raju *et al.* 2011). The efficient accumulation of IONPs by astrocytes *in vitro* (chapter 1.5, table 2) suggests that IONPs are also taken up by astrocytes *in vivo*. As this cell type covers the brain vasculature almost completely (Mathiisen *et al.* 2010, Parpura *et al.* 2012), astrocytes would be the first cells to encounter the NPs that have passed the BBB before microglia come in contact to those NPs. However, data from *in vivo* studies indicate, that in healthy brain regions almost no accumulation of IONPs is found (Alison *et al.* 2010).

In state of disease or inflammation however, where an immune reaction is triggered, microglia are activated and accumulate high amounts of NPs (table 1.1) which have passed the astrocytes. In case of IONPs this phenomenon has

been used for outlining tumour margins or visualization of inflammed brain regions (Fleige *et al.* 2001, Rausch *et al.* 2002, Trehin *et al.* 2006, Alison *et al.* 2010, Oude Engberink *et al.* 2010, Wang *et al.* 2011a, Wang *et al.* 2011b). Other NPs such as AgNPs enter the brain by causing inflammation or disruption of the BBB themselves, which has been shown *in vivo* (Tang *et al.* 2008, Tang *et al.* 2009, Sharma *et al.* 2010a, Sharma and Sharma 2012) and *in vitro* (Tang *et al.* 2010) and an accumulation of those NPs in brain can be expected as well.

The effects of such an NP invasion into the brain on the cellular metabolism *in vivo* only recently became a matter of research and are controversially discussed. Increased levels of H_2O_2 as well as reduced GSH contents have been detected after 7 days of intranasal application of IONPs in rats indicating that the brains suffered from oxidative stress, although no acute toxicity was observed (Wu *et al.* 2013). Upon oral administration of AgNPs to rats over 28 days the concentrations of neurotransmitters like dopamine were described to be altered in brain homogenates (Hadrup *et al.* 2012). In addition, 14 days of nasal administration of AgNPs led to an impaired synaptic plasticity as well as enhanced levels of ROS in rat hippocampus (Liu *et al.* 2012a) hinting towards NP-mediated oxidative stress. Dziendzikowska and co-workers, however, could not detect any adverse effects in rat brain *in vivo* after a bolus injection of AgNPs, but suggested impairments on the cellular level (Dziendzikowska *et al.* 2012). In a study by Genter *et al.* (2012), the levels of GSH in mouse brain were not altered upon a sinlge intranasal instillation of AgNPs compared to controls. Thus, for both types of NPs adverse effects have been described in rats after repeated administration of NPs with oxidative stress as the most prominent endpoint. A single application of NPs, however, appears to be not sufficient to trigger effects that are connected with oxidative stress.

The involvement of the different brain cell-types in the observed effects has not been shown *in vivo* so far. It can only be assumed based on the known functions of the individual cells-types and the *in vitro* data available. The first cells that encounter NP which invade diseased or inflamed brain regions are believed to be astrocytes and microglia (see chapter 1). Astrocytes efficiently accumlated large amounts of NPs *in vitro* and increased their capacity for storage of potentially toxic NP-derived metal ions (chapter 1.5, chapter 2.1, chapter 2.2). Therefore, this cell-type is considered to protect the brain from

adverse effects of NPs or NP-derived metal ions. Microglia are known to accumulate NPs *in vitro* even faster than astrocytes, but their capacity to tolerate high levels of NPs is rather small compared to that of astrocytes as mentioned above. Usually, the big advantage of microglia is their ability to accumulate and degrade debris or material that is not supposed to be in the brain like pathogens or xenobiotics rather fast and efficient and therewith clear potentially harmful compounds from the brain (Aloisi 2001). Regarding NPs, this ability may become a disadvantage, as the degradation products of IONPs or AgNPs are low molecular metal ions that display a severe risk for the cells as they are likely to be much more toxic than the original particles (figure 3.1).

The highly efficient uptake and degradation of NP by microglia may therefore increase the possible threat to the brain due to a faster release of metal ions. The major part of the protection of the brain from NP-derived damage seems to be provided by astrocytes which trigger protein regulation and increase their storage capacities to safely store NPs or, in case of metal-containing NPs, also NP-derived ions.

3.4 Future perspectives

In this thesis the effects of AgNPs on cultured astrocytes and the effects of fluorescently labelled IONPs on cultured microglia have been investigated *in vitro.* However, some important aspects have not been addressed so far.

AgNPs mostly are utilized due to their antimicrobial properties (Chen and Schluesener 2008, Dastjerdi and Montazer 2010) which are believed to depend on the release of Ag^+ (Xiu *et al.* 2012). This is as much an advantage as it bears a risk to consumers who are exposed to those NPs. Unintended release of Ag^+ from AgNPs might cause severe problems, as Ag^+ is toxic to many cells types already in low concentrations (figure 3.1). Purposed release of Ag^+ only in locally defined regions, however, would allow for example a delivery of a potent antimicrobial agent, i.e. Ag^+, to the intended sites of action.

Designing such tailor made particles for an application with minimized risks to consumers requires not only understanding the influence of the particle properties like size or coating on the release rates of Ag^+ (Chernousova and Epple 2013) but also on the accumulation of those particles in cells or

organisms (Lankveld *et al.* 2010, Powers *et al.* 2010a). Additionally, knowledge on toxicity of AgNPs themselves and the ability and capacity of cells to degrade and metabolize AgNPs is essential to design AgNPs that display a minimal risk together with maximal antimicrobial properties. As AgNPs were shown to damage the BBB (Tang *et al.* 2008, Sharma *et al.* 2010a, Sharma *et al.* 2010b, Tang *et al.* 2010), a special focus should be put to the effects of AgNPs on brain cells where more basic research is required to understand the effects of AgNPs on the brain.

This thesis provides some new insights in the handling of AgNPs by astrocytes describing the uptake and potential storage of AgNPs as well as their influence on the cell metabolism. Endocytotic uptake of AgNPs into astrocytes has been shown by the means of inhibition of the individual endocytotic pathways and a lysosomal degradation was proposed. The localization of AgNPs in vesicular structures was also shown by others using TEM imaging (Haase *et al.* 2012). To confirm the proposed lysosomal degradation, fluorescently labelled AgNPs could be used to follow their intracellular localization together with the application of lysotracker (Neun and Stern 2011). This would show whether the particles are indeed taken up into vesicles that undergo lysosomal degradation. Due to the low pH in lysosomes a slow release of low molecular silver from AgNPs in brain cells has been considered to lead to upregulation of storage and stress proteins like MTs (chapter 2.2) and HO-1 (Haase *et al.* 2012), which protect the cells from Ag^+-derived damage.

To visualize the release of Ag^+ in the cells and to confirm the causative role of the released ions in the induction of protein regulation, fluorescent indicators based on boron-dipyrromethene (BODIPY) could be applied, whose fluorescence is described to be enhanced or shifted upon binding to low molecular silver (Coskun and Akkaya 2005, Kim *et al.* 2008, Boens *et al.* 2012). BODIPY is a molecule frequently used for imaging in live cells (Beatty *et al.* 2011, Lee *et al.* 2011, Qu *et al.* 2012, Kim *et al.* 2013), therefore it seems suitable to test the described silver-chelation capacity of BODIPY 63 and 98, respectively (Coskun and Akkaya 2005, Kim *et al.* 2008) in brain cell cultures.

To elucidate the impact of a safe storage of AgNP-derived Ag^+ in proteins like MTs on survival and metabolism of astrocytes, the effects of AgNPs could be investigated in astrocyte cultures derived from MT-deficient animals in comparison to wild-type animals (Yao *et al.* 2000). MTs and HO-1, which both

have been reported to be upregulated as reaction of brain cells to the presence of AgNPs in different studies (chapter 2.2, Haase *et al.* 2012), should be investigated in the same samples of AgNP-treated cells. The time frame after which the individual proteins are upregulated upon AgNP-treatment appears to differ, as HO-1 was already upregulated after 8 to 12 h (Haase *et al.* 2012), whereas MT-upregulation increased from 1 to 7 days (chapter 2.2). The monitoring of those proteins in the same setup would allow an investigation of a possible interplay of MT and HO-1 during the protective response on NP exposure in the brain.

In addition, similar studies on AgNP uptake as those described for astrocytes should be performed on the other brain cell types. So far only for neurons in culture or in brain slices (Liu *et al.* 2009, Liu *et al.* 2011, Haase *et al.* 2012, Liu *et al.* 2012b) as well as for cell lines (Powers *et al.* 2010a, AshaRani *et al.* 2012, Hadrup *et al.* 2012) some information is available on the effects of AgNPs. Also *in vivo* the current data is insufficient and the published results are even contradictory (Genter *et al.* 2012, Hadrup *et al.* 2012, Liu *et al.* 2012a, Dziendzikowska *et al.* 2012). To investigate, which type of brain cell is involved in the response to AgNPs, *in vitro* and *in vivo* studies should be carried out with the same particle type. To enable visualization of the particles within the cells or in the tissue, fluorescent AgNPs could be applied. This would also allow a co-localization of AgNPs with markers for storage proteins like MTs, for cell organelles like lysosomes or for the different cell types in brain.

IONPs are already licensed as diagnostic tool in medicine (Soenen and De Cuyper 2010). They serve as contrast agent in MRI for the use in liver and spleen (Reimer and Vosshenrich 2004, Matuszewski *et al.* 2007) but are also considered for a use in brain (Weinstein *et al.* 2010, Xie *et al.* 2011). Many studies describe the effects of an application of IONPs to the brain *in vivo* focussing only on their use as MRI contrast agent for labelling of cells (reviewed in Weinstein *et al.* 2010, Deddens *et al.* 2012). Upon exposure to IONPs, a decrease in the number of intact neurons as well as induced microglial activation have been described (Wang *et al.* 2011b). Data on the effects of an exposure of the brain to IONPs on the cell metabolism *in vivo* are very limited. Wu *et al.* (2013) describe increased H_2O_2 levels and a reduced

GSH content in brain homogenates 7 days after intranasal application but the contribution of the single cell-types to the observed alterations remain to be elucidated. Several studies investigated the effects of an IONP exposure to brain cells *in vitro* (table 1.1, chapter 1.5, table 2). As microglial cells were shown to accumulate IONPs very efficiently *in vivo*, a special focus needs to be put on those cells also in *in vitro* studies. Most of the information available results from studies on microglial cell lines (table 1.1). Only little is known on the handling of IONPs by primary or secondary microglial cultures (Fleige *et al.* 2001, Pickard and Chari 2010b).

This thesis provides new insights into the effects of an exposure of primary microglial cells to fluorescent IONPs. As microglia are known to efficiently degrade iron-containing magnetic particles (Jenkins *et al.* 2013), the fate of the internalized BP-DMSA coated IONPs should be investigated in more detail. Such studies would require elongation of the incubation times as for example changes in cytokine expression are only described in microglial cells several hours after the loading of the cells with NPs (Hutter *et al.* 2010, Shin *et al.* 2010). However, the incubation of microglia with fluorescent IONPs utilized in this thesis was shown to result in a loss of cell viability already after 3 to 6 hours. To overcome the described experimental limitations regarding incubation times and IONP concentrations, the cause of the observed loss in cell viability should be investigated in more detail. Microglia have been described to accumulate NPs to an extent that cannot be tolerated by the cells and that leads to swelling of the cells and subsequent cell death (Fleige *et al.* 2001). But whether this is triggered by the quantity of internalized NPs or the release of high amounts of low molecular iron due to the fast and efficient degradation of the particles remains to be elucidated.

If the released iron is causative for the observed cell loss after longer incubation periods, the use of membrane permeable iron chelators such as 1,10-phenanthroline or 2,2´-dipyridyl (Hohnholt *et al.* 2010a) could allow a monitoring of viable cells and an intracellular metabolization of NPs over time as no low molecular iron-related toxicity due to degradation of the particles should occur. TEM images could give more information on the electron dense material within the lysosomes, comparable to studies with 10-times larger iron containing magnetic particles where the high capacity of microglial cells to degrade magnetic particles and a condensation of iron in vesicular structures have recently been shown (Pickard and Chari 2010b, Jenkins *et al.* 2013). If

the number of NPs taken up by the cells is the limiting factor, alternative experimental conditions have to be found to sufficiently load the cells with NPs without the described over-accumulation. This could be approached by lowering the concentrations of NPs and applying these only for a short loading phase with a subsequent removal of NP excess in the incubation medium. Culturing IONP-loaded microglial cells over several hours would allow monitoring of a possible increase in mRNA and upregulation of iron transport and storage proteins as depicted in figure 1.2 by reverse transcription PCR and Western blot analysis. IONP-related effects on the production and release of cytokines, ROS and NO should also be studied in such a setup as this has been described in microglial cells for other NPs (Long 2006, Hutter *et al.* 2010, Lalancette-Hébert *et al.* 2010, Shin *et al.* 2010) as well as for IONPs (Wang *et al.* 2011b).

Irrespective of the type of nanoparticle addressed, it is essential to study the effects of NPs in brain and to combine *in vitro* and *in vivo* approaches utilizing the same type of NPs to understand the consequences that those NPs have on the individual cell types as well as on the interplay of NP-exposed brain cells. This knowledge may help to develop new approaches for the design of safe NP-based consumer and medical products.

3.5 References

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4

APPENDIX

4.1 CURRICULUM VITAE

Personal facts:

Date of birth	26.08.1981
Place of birth	Frankfurt/M, Germany

Education:

01/2009 - 04/2013	**Doctoral thesis in neurobiochemistry** "Accumulation of iron oxide and silver nanoparticles in cultured glial cells", University of Bremen, Germany
01/2009 - 01/2012	Recipient of a scholarship of the Hans-Böckler-Foundation
04/2008 - 12/2008	Office Management Assistant, Frankfurt/M, Germany
01/2007 - 10/2007	**Diploma thesis** „Untersuchungen zur Migration endokrin wirksamer Substanzen aus Kunststoffen", Goethe University, Frankfurt/M, Germany
10/2002 - 10/2007	**Studies of biology** major subjects: Aquatic ecotoxicology, neurobiology, animal physiology, Goethe University, Frankfurt/M, Germany,
2001	**Abitur** (general qualification for university entrance) *Gymnasium* „Musterschule", Frankfurt/M, Germany

Study experience abroad:

09/2005 - 10/2005	**Volunteer work** Peron National Park and Monkey Mia Dolphin resort, Shark Bay, Australia
07/2005 - 09/2005	**Practical course in animal physiology** University of Western Australia, Perth, Australia
03/2005 - 04/2005	**Excursion "Marine Habitats"** HYDRA Institute for Marine Sciences, Fetovaia, Elba, Italy

Miscellaneous:

since 08/2008	Member of the European Society for Neurochemistry
since 2009	Trainer for ballroom dancing College sports, University of Bremen, Germany
2005 – 2007	Trainer for ballroom dancing College sports, Goethe University, Frankfurt/M, Germany

4.2 List of Publications

Luther EM, Petters C, Bulcke F, Kaltz A, Thiel K, Bickmeyer U and Dringen R (2013) Endocytotic uptake of iron oxide nanoparticles by cultured brain microglial cells. Acta Biomater, *in revision*

Filser J, Arndt D, Baumann J, Geppert M, Hackmann S, **Luther EM**, Pade C, Prenzel K, Wigger H Arning J, Hohnholt MC, Köser J, Kück A, Lesnikov E, Neumann J, Schütrumpf S, Warrelmann J, Bäumer M, Dringen R, von Gleich A, Swiderek P and Thöming J (2013) Intrinsically green iron oxide nanoparticles? From synthesis via (eco-)toxicology to scenario modelling. Nanoscale 5, 1034-1046

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