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**NEUROINFLAMMATION AND IRON
HANDLING IN ANIMAL MODELS WITH
BRAIN IRON OVERLOAD**

**BY
LISA JUUL ROUTHE**

DISSERTATION SUBMITTED 2019



AALBORG UNIVERSITY
DENMARK

NEUROINFLAMMATION AND IRON HANDLING IN ANIMAL MODELS WITH BRAIN IRON OVERLOAD

PHD DISSERTATION

by

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Xiang J, **Routhe LJ**, Wilkenson D, Hua Y, Moos T, Xi G, Keep R: The choroid plexus as a site of damage in hemorrhagic and ischemic stroke and its role in responding to injury. *Fluids Barriers CNS*, 2017 Mar 28; 14(1):8.

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ENGLISH SUMMARY

Iron is essential for living organisms, and tight regulation of iron homeostasis is vital since defective iron homeostasis can have harmful effects. Throughout life, the brain takes up iron from the periphery through the blood-brain barrier (BBB) and the blood-cerebrospinal fluid (CSF) barrier. The turnover of iron is extremely low, which can explain why iron concentrations increase in the brain in healthy aging. In pathology, like hemorrhagic stroke and many neurodegenerative diseases, defective iron homeostasis can result in iron accumulation and consequently changes in the cellular distribution of iron and cell damage. Furthermore, an inflammatory reaction in the brain parenchyma is a common response in these diseases. The inflammatory process involves the activation of microglia and astrocytes and infiltration of circulating immune cells, which further perturbs iron homeostasis. Glia cells upregulate their iron uptake and increase numerous iron-related proteins, which include heme oxygenase-1 (HO-1) that releases free iron by degrading heme, and the iron storage protein, ferritin.

Several iron-related proteins, such as lipocalin 2 (LCN2) and Zrt-, Irt-like protein 14 (ZIP14) are regulated by inflammatory stimuli in the periphery. LCN2 functions to limit available iron for bacteria and participates in iron homeostasis, while ZIP14 imports divalent metal ions in the liver, heart, and pancreas. However, there is still lacking evidence of whether they are regulated during pathology in the brain. This dissertation focuses on iron handling in the brain in conditions with iron overload and neuroinflammation. In order to study this, animal models of hemorrhagic stroke, i.e. intracerebral hemorrhage (ICH) and intraventricular hemorrhage (IVH), and a model of chronic neurodegeneration were used.

In Study I, the role of the choroid plexus in handling hemoglobin was studied using *in vitro* and *in vivo* models of IVH. The regulation of the transferrin receptor, ferritin, HO-1, LCN2 and lipocalin receptor, 24p3R was investigated after hemoglobin exposure. An experimental model of ICH was established by administrating arterial autologous whole blood into the striatum of rats. Characterization of the model includes investigation of iron content, ferritin, and HO-1 expression. Study II examined the modulation of ZIP14 expression in the model of ICH, whereas Study III investigated the regulation of ZIP14 in an experimental model of chronic neurodegeneration in the substantia nigra pars reticulata (SNpr). Since astrocytes were shown to modulate ZIP14 expression in both models, it was examined whether ZIP14 is regulated by blood derivatives, iron overload, and inflammation in cultures of astrocytes.

Results from Study I demonstrate that the choroid plexus upregulates HO-1, acute-phase protein LCN2, and ferritin in response to hemoglobin exposure. While ferritin is probably upregulated as a cellular defense mechanism to store iron in a non-toxic

form thereby limiting iron-induced cell damage, upregulation of LCN2 may be detrimental to the choroid plexus. Study II and III show that reactive astrocytes increase their expression of the iron importer, ZIP14, in animal models with increased brain iron and neuroinflammation. Furthermore, astrocyte cultures increase their *Zip14* gene expression in response to hemoglobin, iron overload, and inflammation. Due to the dysregulation of iron homeostasis observed in ZIP14 knockdown astrocytes, we suggest that ZIP14 plays an essential role in astrocytic iron homeostasis in pathology. Additionally, ZIP14 could serve as a neuroprotective mechanism by limiting free extracellular iron and neuronal cell death.

Together, these studies show that LCN2 and ZIP14 may play significant roles in handling iron in the choroid plexus and astrocytes, respectively, in pathological conditions.

DANSK RESUME

Jern har essentielle funktioner for overlevelse af organismer, og regulering af jernhomøostasen er vigtig, da overskydende jern kan være toksisk. Hjernen optager jern fra cirkulationen gennem blod-hjerne barrieren (BBB) og blod-cerebrospinalvæske barrieren (blood-CSF-barrier) gennem hele livet. Da hjernen har en lille omsætning af jern, ses der er en øget jernkoncentration i hjernen ved aldring. Denne jernkoncentration er endnu højere ved patologiske tilstande såsom cerebrale hæmragier og neurodegenerative sygdomme og kan føre til jernakkumulering, ændringer i den cellulære distribuering af jern og cytotoxicitet, der kan være med til at forværre sygdomme. Derudover er sygdommene ofte akkompagneret af intraparenkymal inflammation, hvilket fører til aktivering af mikroglia og astrocytter samt invasion af immunceller fra cirkulationen, som er med til yderligere at forstyrre jernhomøostasen. Blandt andet er det tilfældet, at gliaceller opregulerer deres jernoptag og øger ekspresionen af forskellige jern-relaterede proteiner, såsom heme oxygenase 1 (HO-1), der frigiver ved nedbrydelse af hæg, samt jernlagringsproteinet, ferritin.

Mange jernrelaterede proteiner, herunder lipocalin 2 (LCN2) og Zrt-, Irt-like protein 14 (ZIP14) reguleres af inflammatoriske stimuli i periferien. LCN2 spiller en rolle i forsvaret mod bakterielle infektioner, hvor det mindsker tilgængeligheden af frit jern, mens ZIP14 fungerer som en importer af divalente metalioner i leveren, hjertet og bygspejtkirtlen. Der er dog stadig ingen evidens for, hvordan disse proteiner reguleres under patologiske tilstande i hjernen. Denne afhandling fokuserer på jernhåndteringen i hjernen i tilstande med jernophobning og neuroinflammation. For at undersøge dette er der anvendt forskellige dyremodeller, herunder modeller for intracerebral hæmoragi (ICH) og intraventrikulær hæmoragi (IVH), samt en model for kronisk neurodegeneration.

I Studie I undersøges håndteringen af hæmoglobin i plexus choroideus *in vitro* og *in vivo* i en model for IVH. Reguleringen af transferrin receptor, ferritin, HO-1, LCN2 og lipocalin receptor, 24p3R blev undersøgt efter hæmoglobinbehandling. Derudover blev en model for ICH etableret ved at injicere arterielt, autologt blod i striatum på rotter. Modellen blev karakteriseret for dens jernindhold samt ferritin og HO-1 ekspresion og blev senere brugt i Studie II. I Studie II og III blev ZIP14-ekspresionen undersøgt i modellen for intracerebral hæmoragi samt i en eksperimentel model for kronisk neurodegeneration i substantia nigra pars reticulata (SNpr). Ekspresion og regulering af ZIP14 blev endvidere undersøgt i astrocytter, da begge *in vivo* modeller viste ZIP14-opregulering i astrocytter. Astrocytkulturer blev behandlet med blod, jern og pro-inflammatoriske cytokiner, hvorefter ZIP14-ekspresionen blev undersøgt.

Resultater fra Studie I demonstrerer, at plexus choroideus opregulerer HO-1, LCN2 og ferritin i forbindelse med hæmoglobinbehandling. Mens ferritin formentlig opreguleres som en cellulær forsvarsmekanisme, der lagrer jern og dermed mindsker risikoen for jerninduceret cellulær skade, formodes det, at opreguleringen af LCN2 kan forårsage skade på plexus choroideus.

Resultater fra Studie II og III viser, at reaktive astrocytter øger deres ekspresion af jerntransporteren, ZIP14. Den øgede ekspresion af ZIP14 i astrocytter ses i begge dyremodeller, der blandt andet er kendt for at have inflammation samt jernophobning. Desuden viser studier med astrocytter, at ZIP14 reguleres efter behandling med hæmoglobin, øget jern og inflammation *in vitro*. Desuden viser vi, at mindsket ZIP14-ekspresion er associeret med en ubalance i den astrocytiske jernhomøostase, hvilket kan tyde på, at ZIP14 spiller en vigtig rolle for jernhomøostasen i astrocytter. Disse resultater indikerer, at opregulering af ZIP14 virker som en forsvarsmekanisme ved at mindske neuronal celledød ved sygdom med inflammation og jernakkumulering.

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LIST OF MANUSCRIPTS

*Manuscript I: **Routhe LJ**, Xiang J, Ye H, Hua Y, Moos T, Xi G, Keep RF. Regulation of iron-related proteins in the choroid plexus by hemoglobin. Submitted.*

*Manuscript II: **Routhe LJ**, Thomsen MS, Xi G, Moos T. Upregulated astrocytic expression of ZIP14 in an experimental model of intracerebral hemorrhage. In preparation.*

*Manuscript III: **Routhe LJ**, Andersen IK, Hauerslev LV, Issa II, Moos T, Thomsen MS. Altered expression of ZIP14 (SLC39A14) is part of the astrocytic reaction to chronic neurodegeneration with iron overload. Submitted.*

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Thomsen MS, **Routhe LJ**, Moos T. The vascular basement membrane in the healthy and pathological brain. *J Cereb Blood Flow Metab. 2017 Oct;37(10):3300-3317.*

LIST OF ABBREVIATIONS

BBB: blood-brain barrier

CNS: central nervous system

CSF: cerebrospinal fluid

Dcytb: duodenal cytochrome *b*

DMT1: divalent metal transporter 1

GFAP: glial fibrillary protein

HO-1: heme oxygenase-1

ICH: intracerebral hemorrhage

IL-1 β : interleukin-1 β

IL-6: interleukin-6

IRE: iron-responsive element

IRP: iron-regulatory protein

IVH: intraventricular hemorrhage

LCN2: lipocalin 2

LPS: lipopolysaccharide

NTBI: non-transferrin-bound iron

ROS: reactive oxygen species

SNpr: substantia nigra pars reticulata

STEAP: six-transmembrane epithelial antigen of the prostate

TLR: toll-like receptor

TNF- α : tumor necrosis factor- α

TRCP: transient receptor potential channels

UTR: untranslated region

ZIP8: Zrt-, Irt-like protein 8

ZIP14: Zrt-, Irt-like protein 14

CHAPTER 1. INTRODUCTION

Iron is fundamental for living organisms, as it is involved in numerous cellular processes such as DNA synthesis, oxygen transport, and the electron transport chain in the mitochondria. Although iron is necessary for cellular processes, unbound (free) iron promotes the production of reactive oxygen species through Fenton chemistry and can potentially cause oxidative stress, which in turn can lead to cellular damage and death (1). In the attempt to balance tissue iron concentrations and homeostasis, maintaining constant iron concentrations via regulation of import and export from the body is of importance. Iron homeostasis is primarily regulated at the site of uptake, and thus if iron accumulates in various organs, it hardly leaves (2).

Maintaining proper iron concentrations in the brain is also essential, since inappropriately high concentrations of iron, as in other organs, are toxic to the brain parenchyma. In the brain, iron is crucial for the synthesis of neurotransmitters and myelin production and thus plays a vital role in brain development and maintenance of normal brain functions. Developmental iron deficiency is thus thought to impair brain development by hampering mitotic activity, transmitter synthesis, and myelin production, while brain iron overload may propagate oxidative damage causing neuronal cell death (3–5).

Throughout life, the brain acquires iron from the circulation via transport of transferrin-bound iron through the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (blood-CSF barrier). This uptake is developmentally regulated, being particularly high early in life (6,7). The turnover of iron in the brain is very low, and as the uptake of iron into the brain occurs throughout life, an increased iron concentration is seen in the brain even in healthy aging (4). The concentration of iron differs among brain regions with the highest concentrations in, e.g. the basal ganglia and substantia nigra (8).

The following paragraphs cover systemic iron uptake and regulation, major routes of cellular iron uptake and storage mechanisms, iron transport through the brain barriers, and iron handling in neurons and glial cells. Moreover, iron handling is elucidated in hemorrhagic stroke, neurodegeneration, the inflammatory choroid plexus, and reactive glial cells.

1.1. SYSTEMIC IRON UPTAKE AND REGULATION

Iron is absorbed as heme or non-heme iron primarily by the enterocytes present in the proximal duodenum. Although non-heme iron constitutes most of the iron in diets, its absorption is less efficient compared to heme-iron, which, on the other hand, typically constitutes only a small part of dietary iron (9). The gastrointestinal uptake of non-heme iron is mediated by divalent metal transporter 1 (DMT1) expressed on the apical

membrane of enterocytes. DMT1, which belongs to the natural resistance-associated macrophage protein (NRAMP) family, is well-functioning at the low pH in the duodenum (10–12). Here it transports non-heme ferrous iron (Fe^{2+}) into the enterocytes. Upon its luminal uptake, ferric iron (Fe^{3+}) is reduced to Fe^{2+} through the ferrireductase duodenal cytochrome *b* (*Dcytb*) localized on the apical membrane of enterocytes (13,14).

Despite being an integral source of iron, little is known about the uptake of heme iron. It is suggested that it enters the enterocytes via heme carrier protein 1, although its function as a heme transporter is still debated (2). Subsequent degradation of heme by heme oxygenase 1 (HO-1) releases Fe^{2+} that enters the cytosolic pool of iron together with iron absorbed through DMT1 (2,15). Fe^{2+} is then either oxidized and incorporated in ferritin, the major iron storage protein capable of storing approximately 4500 Fe^{3+} atoms or exported into the circulation through the cellular iron-exporter ferroportin that transports Fe^{2+} (13,16). The export of Fe^{2+} through ferroportin is influenced by the membrane-bound ferroxidases, hephaestin and ceruloplasmin that oxidize Fe^{2+} to Fe^{3+} (2,17,18). In the circulation, Fe^{3+} rapidly binds to apo-transferrin, which has a high affinity for iron. Transferrin is mainly synthesized in the liver and can bind up to two Fe^{3+} , generally referred to as holo-transferrin (19). In pathological conditions with iron overload, the carrying capacity of transferrin can be exceeded leading to the presence of non-transferrin-bound iron (NTBI) in the blood plasma. NTBI is observed in the human disorder hemochromatosis, where iron is deposited throughout the body (20,21). An exception is the brain that shows only a modest increase in iron deposition in a mouse model of hemochromatosis. The modest increase was suggested to relate to the protective mechanisms of the BBB and blood-CSF barrier (22).

Systemic iron homeostasis is regulated by recycling of iron from dying red blood cells, mobilization of hepatic iron stores, and the gastrointestinal iron absorption, whereas excretion does not contribute to regulation of iron homeostasis (2). The gastrointestinal iron absorption is regulated by the liver-derived hormone, hepcidin, which is secreted in response to inflammation and systemic iron overload (23,24). It regulates the iron absorption by direct interaction with ferroportin at the basolateral side of enterocytes causing its proteolysis, thereby abolishing its capacity to export iron (25). Thus in conditions with increased hepcidin, the expression of ferroportin lowers, which results in decreased iron transport into the circulation (23).

1.2. CELLULAR IRON UPTAKE AND STORAGE

Transferrin carries iron from the site of absorption via the circulation to cells expressing transferrin receptors that subsequently facilitate receptor-mediated endocytosis of the holo-transferrin (6). Another mechanism for iron uptake relies on the uptake of NTBI although, in normal physiological conditions, NTBI is absent in the circulation. This means that NTBI-mediated uptake of iron is likely to occur only

in pathological conditions, where the iron-binding capacity of transferrin is exceeded (21).

1.2.1. TRANSFERRIN-MEDIATED IRON UPTAKE

The majority of cells in the body obtain iron via transferrin receptor-mediated endocytosis in clathrin-coated vesicles. The transferrin receptor is a transmembrane receptor that consists of two subunits, each capable of binding one holo-transferrin. Binding of holo-transferrin to the transferrin receptor triggers the formation of an endosome with a lower pH (Fig. 1.1) (26). Fe^{3+} dissociates from transferrin in the acidic environment of the endosome and is subsequently reduced to Fe^{2+} by a ferrireductase, like six-transmembrane epithelial antigen of the prostate 2 (STEAP2), STEAP3, or Dcytb (27,28). Fe^{2+} is then transported across the endosomal membrane via DMT1 and transferrin is recycled to the plasma membrane (6,12,29,30).

1.2.2. NON-TRANSFERRIN-BOUND IRON UPTAKE

For uptake of NTBI, Fe^{3+} is reduced to Fe^{2+} by a ferrireductase and then imported via an NTBI transporter (Fig. 1.1). Although cells are generally capable of acquiring NTBI (21,31), the uptake mechanisms are less described. It is widely accepted that NTBI uptake involves transport by DMT1, which is the most important transporter for intestinal iron uptake (32). Nevertheless, accumulative data suggest the involvement of other newly identified transporters in NTBI uptake. These transporters include the transient receptor potential channel (TRPC), L-type and T-type calcium channels, Zrt-, Irt-like protein 8 (ZIP8), and ZIP14 (21,33–36). The following section will focus on DMT1 and ZIP14 for uptake of NTBI.

DMT1 is a well-characterized iron transporter. It has multiple transmembrane domains and is located both in cellular membranes and endosomes, thus emphasizing the dual role of DMT1 in iron uptake (37,38). DMT1 transports divalent metal ions and is thought to transport Fe^{2+} predominantly (10). Four isoforms exist, of which two contain an iron-responsive element (IRE) in its 3' untranslated region (UTR) (30,39). The isoforms exhibit differential regulation and distribution patterns, for instance, the isoforms containing an IRE are located in the cell membrane and regulated by iron status, whereas the others are located in the endosomal compartment and not regulated by iron status (30,40).

In addition to DMT1, ZIP14 is also able to transport divalent metal ions. ZIP14 is an integral membrane protein and a member of the Zrt- and Irt-like protein family (41). It is detected in various non-neuronal tissues and cell types, such as liver, pancreas, heart, white adipose tissue, and hepatocytes (42–45). In nervous tissue, there is evidence for ZIP14 expression in the brain *in vivo* and cultured neurons and astrocytes (35,46,47). ZIP14 was initially characterized as a zinc transporter (48) but has later gained status as being an iron transporter (36,49).

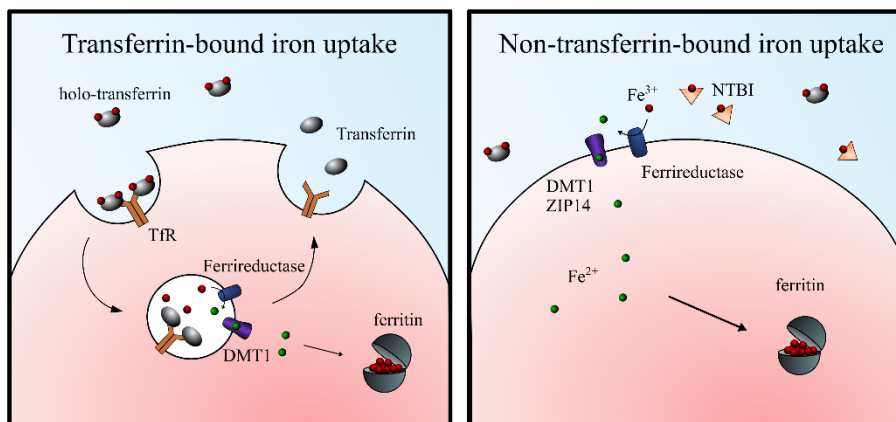


Figure 1.1. Two routes for cellular iron uptake. Left: Transferrin-bound iron uptake: The transferrin receptor (TfR) mediates uptake of holo-transferrin. Ferric iron (Fe^{3+}) is detached from transferrin in the endosome, reduced to ferrous iron (Fe^{2+}) by a ferrireductase and then pumped into the cytosol by divalent metal transporter 1 (DMT1) where iron can be stored in ferritin, while transferrin is recycled to the cell surface. Right: Non-transferrin-bound iron (NTBI) uptake: NTBI transporters, such as DMT1 or Zrt-, Irt-like protein 14 (ZIP14), facilitate uptake of Fe^{2+} . Upon uptake, Fe^{3+} is reduced to Fe^{2+} by a ferrireductase and Fe^{2+} is then transported into the cytosol through, e.g. DMT1 or ZIP14, where iron incorporates in ferritin.

1.2.3. STORAGE OF IRON

Once the uptake of iron has occurred, it can either be used in cellular processes, incorporated in ferritin, or exported through ferroportin to preserve iron homeostasis. Ferritin is important for binding and storage of iron. It consists of two isoforms, a heavy chain (H) and a light chain (L) with a molecular weight of 19 kDa and 21 kDa, respectively. The H chain exerts ferroxidase activity, while the L chain functions in mineralization and iron nucleation (50,51). Of note, lack of ferritin H is lethal in the early stages of development, whereas mutations in ferritin L can result in iron accumulation (52,53). Together, ferritin H and L form a spherical structure that stores iron in a non-toxic form, thereby neutralizing its deleterious effects.

1.2.4. REGULATION OF CELLULAR IRON HOMEOSTASIS

Regulation of iron homeostasis occurs at both the systemic and cellular level. Control of cellular iron homeostasis is complex and involves regulation of proteins implicated in iron uptake, storage, and export. It is partly controlled by post-transcriptional regulation via the IRE/iron-regulatory proteins (IRP) system. IREs are located in either the 3'-UTR of mRNA encoding iron uptake genes, DMT1 and transferrin receptor, or the 5'-UTR of mRNA encoding genes involved with iron storage or export, ferritin and ferroportin (54). IRPs that bind with IREs located at 3'-UTR increase mRNA stability, while binding to IREs in the 5'-UTR block their translation. During iron overload, IRPs lose their affinity to IREs, thus destabilizing DMT1 and

transferrin receptor, while the translation of ferritin and ferroportin increases (13,54,55).

1.3. IRON IN THE BRAIN

The brain is separated from the circulation by physical barriers, i.e. the blood-brain barrier (BBB) denoted by brain capillary endothelial cells and the blood-cerebrospinal fluid barrier (blood-CSF barrier) consisting of choroid plexus epithelial cells. The capillaries ensheathed by the choroid plexus epithelial cells are fenestrated, which contrasts with brain capillary endothelial cells and choroid plexus epithelial cells that are connected to adjacent cells by tight junctions, thereby forming the BBB and blood-CSF barrier. The barriers regulate the brain microenvironment by governing the entry of potentially toxic compounds, pathogens, and nutrients into the brain (56,57). Selective transporters exist to regulate the transport of vital components across the barriers, e.g. the transferrin receptor, which is responsible for iron uptake. Both barriers are major sites of iron exchange between the brain and periphery. The transferrin receptor is highly expressed on the luminal side of the BBB and has a broad distribution on the choroid plexus epithelial cells, where it facilitates iron transport from the circulation into the brain and CSF (6,58–60).

1.3.1. IRON TRANSPORT ACROSS THE BRAIN BARRIERS

It is generally acknowledged that the transferrin receptor constitutes the most central mechanism for iron uptake at the BBB. How iron is transported through the BBB is still a matter of debate, and different hypotheses exist. One route of iron transport into the brain is the transferrin receptor-mediated transcytosis, where endosomes containing holo-transferrin in complex with transferrin receptor traverse the endothelial cells of the BBB and release holo-transferrin into the brain interstitium (Fig. 1.2A) (61,62). Another route of transport involves transferrin receptor-mediated endocytosis. This is followed by separation of iron from the transferrin-transferrin receptor complex in the acidic environment of the endosome. Iron is then reduced by a ferrireductase, pumped through the endosome via DMT1 and reaches the abluminal surface of the endothelial cells, whereas the apo-transferrin and transferrin receptor are recycled to the luminal surface (Fig. 1.2A). At the abluminal surface, iron is exported into the brain interstitium via ferroportin and subsequently oxidized by hephaestin or ceruloplasmin (27,30,63). The latter observation combined with the fact that transferrin receptors were never detected at the abluminal side of the endothelium are observations that point against the idea of transport of transferrin across the BBB.

Much similar to the transport through the BBB, different pathways are described for iron uptake at the blood-CSF barrier (Fig. 1.2B). The choroid plexus epithelium expresses the transferrin receptor and is shown to transport iron via transcytosis of holo-transferrin (64). Additionally, several studies have proved evidence for expression of DMT1, ferrireductase, ferroportin, and ferroxidases in the choroid

plexus (57,65,66), indicative of transferrin receptor-mediated endocytosis and subsequent export by ferroportin. The choroid plexus epithelial cells have abundant expression of ferritin (57,67), suggesting they are also able to accumulate and store iron. Iron that is not stored in the choroid plexus is exported into the CSF (64). Due to the absence of a diffusional barrier between the CSF and the brain interstitium, components of the CSF may diffuse into the interstitium (57).

Once iron, transported through the BBB and blood-CSF barrier, enters the brain interstitium or CSF, it binds to apo-transferrin and enters transferrin-receptor expressing cells. Opposite to the plasma transferrin, transferrin within the CSF is believed to be saturated in non-pathological conditions, indicating that iron may bind to low molecular weight complexes like citrate, ascorbate, or ATP from where it can be taken up by neurons and glial cells by less characterized mechanisms (68–70).

1.3.2. IRON UPTAKE IN NEURONS AND GLIAL CELLS

Neurons

Neurons express transferrin receptors, suggesting that they are instrumental for neuronal iron uptake. Neuronal transferrin receptors are markedly upregulated during iron deficiency, which clearly advocates that neuronal iron uptake is regulated by the IRE/IRP system (6). Neurons in most regions of the brain contain iron. However, iron levels are higher later in life (71).

It is proposed that NTBI continuously occurs in the brain interstitium, where it might serve as the primary source of iron for glial cells, that in contrast to neurons, are devoid of detectable transferrin receptor (60,70,72). There is currently no solid evidence for NTBI uptake in neurons *in vivo*, but ZIP8 and ZIP14 are proposed to mediate uptake of iron in neuronal cultures. The expression of ferrireductase STEAP2 in the cellular membrane seemingly support this notion (35).

Microglia

Broadly distributed throughout the brain, microglia continuously survey the microenvironment and react to pathological changes in the brain. In non-pathological conditions, microglia exhibit an inactivated or ramified state, characterized by their thin, motile processes. In this state, they play pivotal roles in support of neurons, e.g. by releasing neurotrophic factors (73). Microglia take up NTBI *in vitro* and enhance their accumulation of iron in the absence of transferrin (31,74). More recently, primary microglia were reported to express *Dmt1* and the microglial cell line, IMG cells, exhibit *Dmt1*, *Zip8*, and *Zip14* gene expression (75).

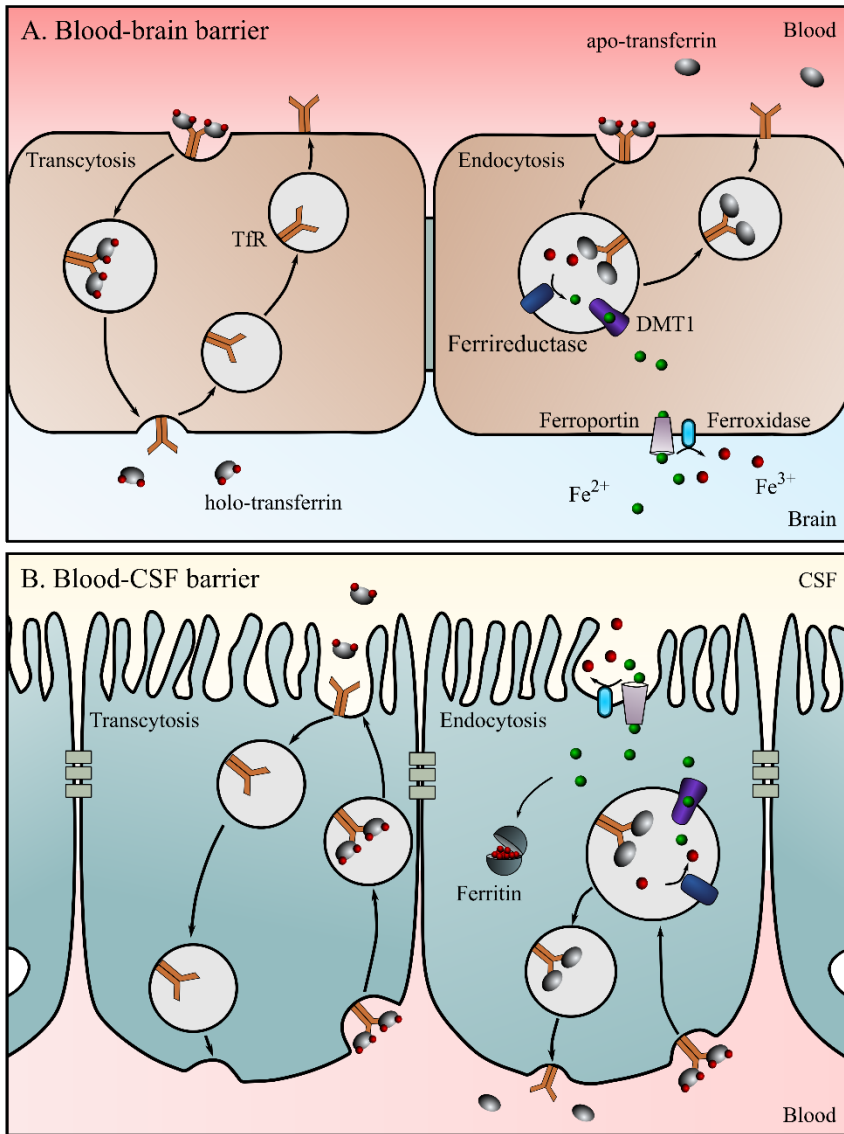


Figure 1.2. Iron transport across the blood-brain barrier (A) and blood-cerebrospinal fluid (CSF) barrier (B). A-B. Transcytosis: Holo-transferrin complexes with the transferrin receptor (TfR) on the BBB and blood-CSF barrier. The complex is internalized, and holo-transferrin is released in the brain or CSF, and the transferrin receptor is recycled. Endocytosis: Binding of holo-transferrin to the transferrin receptor triggers a cascade that results in internalization of the complex. Inside the endosome, iron is separated from transferrin, reduced by a ferrireductase and transported into the cytosol through divalent metal transporter 1 (DMT1). Iron is then either stored in ferritin or exported through ferroportin. Exported iron is oxidized by a ferrooxidase thus enabling binding of iron to transferrin.

Astrocytes

Astrocytes, the most abundant glial cell type in the brain, perform multiple functional and supportive roles for the brain microenvironment. Hence, astrocytes provide support for the BBB and neighboring neurons and maintain the extracellular ion homeostasis in the brain. In contrast to neurons and other glial cells, astrocytes of the normal brain are surprisingly vague in their expression of molecules related to iron homeostasis. For instance, they lack transferrin receptors *in vivo* (60). Therefore, their uptake of iron probably relies on NTBI uptake rather than transferrin receptor-mediated iron uptake. However, *in vitro* astrocytes were demonstrated to contain transferrin receptors and ferritin in cell cultures (76,77). NTBI uptake in cultured astrocytes has been studied comprehensively (33,76–80). *In vitro*, astrocytes can take up ferric iron from low molecular weight sources like ferric ammonium citrate (31,80). Dcytb or stromal cell-derived receptor 2 present in their cellular membranes are proposed to reduce Fe^{3+} after which it is imported via NTBI transporters (77,80). DMT1 probably mediates some NTBI uptake, but an expression of other transporters such as ZIP14 and TRPC were also recently reported in astrocytes *in vitro* (33,78).

1.4. IRON IN THE PATHOLOGICAL BRAIN

The pathological brain is supposedly challenged by inflammation and deposition of iron. There is a well-recognized link between inflammation and iron accumulation. Thus, chronic systemic inflammation causes iron retention in the reticuloendothelial system to limit the amount of available iron thereby preventing the growth and survival of bacterial microorganisms (81).

Neuroinflammation is a multifaceted response to many CNS injuries, such as hemorrhagic stroke and neurodegeneration, and includes barrier dysfunction, infiltration of immune cells from the periphery, and activation of glial cells that together release inflammatory mediators, such as IL-1 β and IL-6 (82–84). Furthermore, iron deposition occurs in the pathological brain and may be caused by iron-rich microglia and invading monocytes/macrophages that phagocytize damaged cells and subsequently undergo apoptosis, releasing iron into the brain (83). This iron deposition is supposedly even more exaggerated in cerebral hemorrhage, in which substantial amounts of iron are released from dying blood cells (85,86).

1.4.1. HEMORRHAGIC STROKE

Intracerebral hemorrhage (ICH) is a subtype of hemorrhagic stroke and accounts for approximately 10-15% of all strokes. In nearly 50% of patients with ICH, the bleeding spreads into the ventricular space (intraventricular hemorrhage, IVH), which is correlated to a worsened outcome (87). In ICH, rupture of a cerebral blood vessel releases erythrocytes into the brain parenchyma, thereby causing primary and secondary injury. The primary injury denotes the damage that occurs within minutes after the ictus because of the mass effect created by the hematoma, while secondary

injury refers to the long-term damage caused by the presence of blood in the brain and the hematoma resolution. Blood in the brain parenchyma and hematoma resolution are accompanied by inflammation, cytotoxicity of the blood, and oxidative stress (88,89).

Rupture of a vessel and subsequent bleeding into the brain parenchyma is associated with activation of microglia and astrocytes and infiltration of neutrophils and macrophages that secrete chemokines and cytokines, e.g. interleukin-1 (IL-1), IL-6, and tumor necrosis factor- α (TNF- α) (90–93). Together, the release of inflammatory mediators and formation of reactive oxygen species causes neuronal cell death (Fig. 1.3), modulates the basement membrane and increases the permeability of the BBB, resulting in brain edema (92,94,95). Evidence suggests that neutrophils participate in brain injury by activating microglia and astrocytes, amplifying inflammation, and compromising the integrity of the BBB. Thus, depletion of neutrophils is reported to attenuate these mechanisms, at least until 14 days after ICH (96).

While cytotoxicity of blood in the brain is attributable to many components, this section will focus on the toxicity of hemoglobin and iron, two main components of erythrocytes. Erythrocytes gradually start to lyse and release their content, i.e. hemoglobin, into the brain interstitium (Fig. 1.3) (85,97). The free hemoglobin complexes with haptoglobin, which has a high affinity for the CD163 receptor, located at the cell membrane of macrophages and microglia. CD163 is regulated by an increased extracellular content of hemoglobin-haptoglobin complexes as early as 24 hours after ICH (98–100) and is thought to play a distinct time-dependent role after ICH. For instance, CD163^{-/-} is correlated with smaller lesions and decreased iron content in early phases of ICH, while CD163^{-/-} results in larger lesions and increased iron in the long term, suggesting that CD163 mediates brain injury in the early phase of ICH and beneficial effects in the long term (101).

Hemoglobin degrades intracellularly into heme that is further degraded into free iron by HO-1. HO-1 is expressed by microglia, astrocytes, and neurons and is upregulated in the early phase after ICH (85). Increased HO-1 expression in astrocytes is suggested to be independent on the uptake of hemoglobin and heme, as astrocytes are proposed to take up small amounts hemoglobin (102). Comparable to CD163, HO-1 displays a distinct temporal role following ICH that has harmful effects in the early phase, while promoting recovery in later stages (103). The pathological increase in HO-1 expression may cause iron accumulation, which in microglia and macrophages coincide with increased ferritin levels (85).

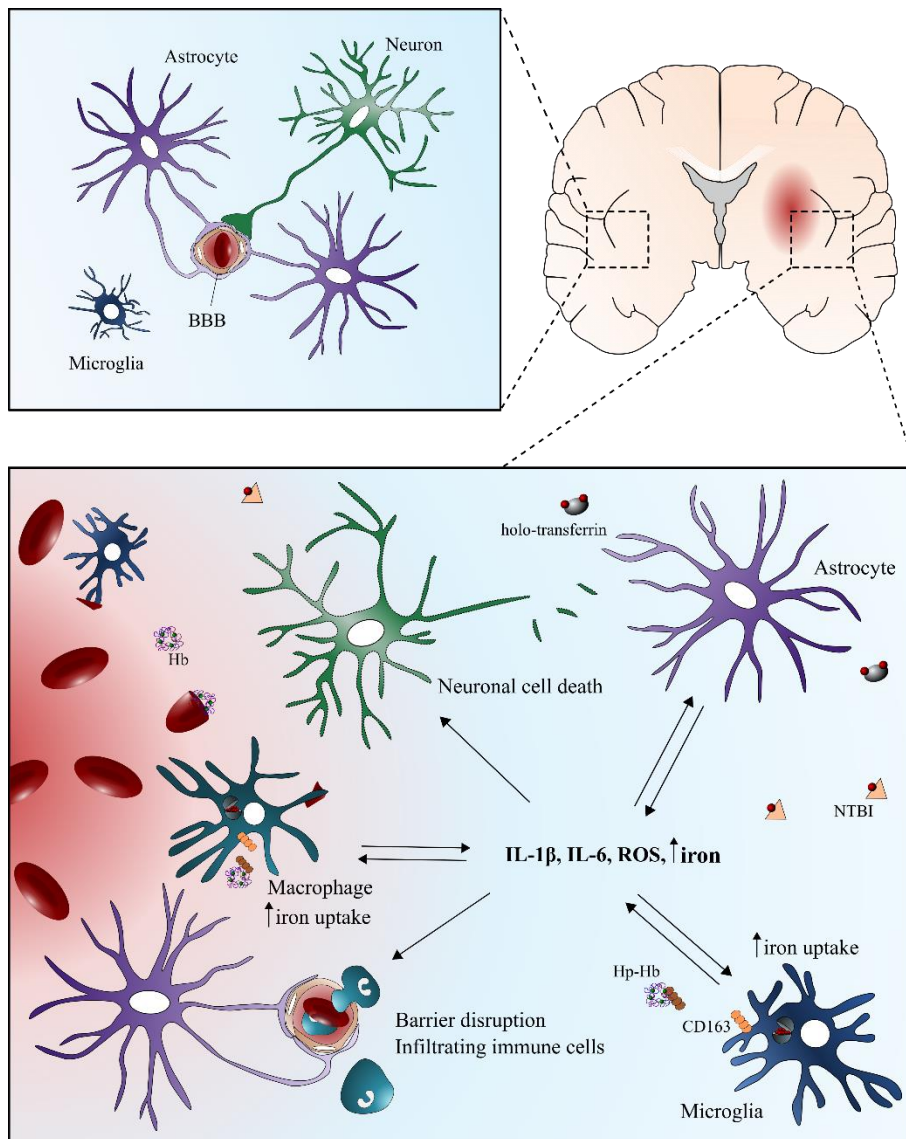


Figure 1.3. Cellular responses to intracerebral hemorrhage. Microglia and astrocytes activate, and inflammatory immune cells invade the brain parenchyma through, e.g. the blood-brain barrier (BBB) after rupture of a cerebral vessel. Microglia and macrophages phagocytose erythrocytes and contribute to the production of inflammatory mediators, such as interleukin-1 β (IL-1 β), IL-6, and reactive oxygen species (ROS), along with activated astrocytes. As erythrocytes lyse, hemoglobin (Hb) and iron are released into the brain. Hb is scavenged by haptoglobin (Hp) and taken up via CD163 expressed on macrophages and microglia. The increased iron uptake in microglia and macrophages results in an increased ferritin expression. Collectively, inflammation and iron dysregulation exert neuronal injury after ICH. NTBI, non-transferrin-bound iron.

Experimental Models of Intracerebral Hemorrhage

Many models have been established to study ICH, of which the most extensively used are intracerebral injection of either autologous whole blood or collagenase (104). The blood model has existed since the 1960s, where whole blood was injected into the brain of larger animals (105–107). Rats and mice models were later developed. In the rat model, arterial blood is sampled from the femoral artery into a syringe, and 100 μ l blood is stereotactically injected into the rat brain over 10 minutes by use of a microinfusion pump (108–110). This method only introduces blood and allows for control of hematoma size, although variations may occur as a consequence of bleeding into the ventricular space and backflow of injected blood along the needle injection site. Compared to the collagenase-induced model, studies have found milder neurological symptoms, less hematoma expansion, and shorter recovery (111). However, this method has been a useful tool in investigations of pathological changes that occur after ICH (85,112).

In the collagenase-induced model, developed by Rosenberg and colleagues (113), 0.01–0.5 U bacterial collagenase diluted in 2 μ l sterile saline is administered to the brain, causing dissolution of the endothelial basement membrane. Ultimately, this results in vascular disruption and hematoma formation that starts as early as 10 min after collagenase administration and continues for hours (90,113–116). Rosenberg et al. found a concentration-dependent increase in edema formation after collagenase injection and that rats injected with more than 0.5 U collagenase died within 24 hours (113). In contrast to the whole-blood induced model, injection of bacterial collagenase has been shown to induce greater BBB injury, more severe tissue destruction, persistent neurological deficits, and a greater inflammatory response, although the latter is not proved (116–120).

1.4.2. NEURODEGENERATION

Neurodegeneration is a progressive, irreversible process with damage of neuronal structures and loss of their essential functions, ultimately leading to cell death. Neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, and Parkinson's disease are heterogeneous in their clinical pathology, some affecting motor skills while others cause amnesia and cognitive deficits. Common to these irreversible diseases, chronic inflammation and progressive iron deposition occur in brain areas preferentially affected by the diseases (83,121–125). Accordingly, iron deposits in Lewy bodies in the substantia nigra in Parkinson's disease and in amyloid plaques and neurofibrillary tangles in Alzheimer's disease (126,127). The increased iron concentration found in the substantia nigra of patients with Parkinson's disease may be explained by increased DMT1 expression in dopaminergic neurons and reactive microglia in the substantia nigra pars compacta (SNpc) (128). Supporting this, mutations in DMT1 in animal models of Parkinson's disease (6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)) mitigates dopaminergic cell loss (128). In Alzheimer's disease, iron is also

thought to contribute to the formation of amyloid plaques through the modulation of amyloid precursor protein processing (4). The iron deposition may result in the generation of reactive oxygen species, which can exacerbate the neurodegeneration. However, the question arises whether iron is the initial cause of neurodegeneration or is due to deposition from dying neuronal cells and migrating inflammatory cells from the circulation.

Experimental Models of Chronic Neurodegeneration

Neurodegeneration can be induced by intracerebral microinjections of toxins, e.g. ibotenic acid, MPTP, and 6-OHDA (129–131). Intrastratial administration of ibotenic acid results in cytotoxicity of gamma-aminobutyric acid (GABA)ergic neurons in the striatum (129). Cell death among GABAergic striatal neurons projecting inhibitory stimuli to neurons in substantia nigra pars reticulata (SNpr) will eventually lead to excitotoxicity in SNpr. This excitotoxicity, exerted by glutamate, develops upon overstimulation of nigral neurons from the subthalamic nucleus and results in progressive loss of nigral neurons and shrinkage of SNpr (130,132). Shrinkage of SNpr is accompanied by iron deposition, long lasting gliosis, and infiltration of inflammatory cells from the periphery (129,132). The mechanisms by which iron increases in this model is not known however, it was recently proposed to be associated with infiltration of monocytes through a compromised BBB and an inflammatory response (83).

1.5. ALTERED IRON HOMEOSTASIS IN INFLAMMATION

Although, neuroinflammation is considered to play essential roles in tissue repair mechanisms within the CNS, persistent neuroinflammation can be detrimental (82,133). Additionally, many pathological conditions with inflammation are associated with altered iron metabolism (83,134).

1.5.1. IRON HANDLING IN THE INFLAMED CHOROID PLEXUS

The choroid plexus is in contact with many external factors and acts as an immune sensor. It undergoes morphological and physiological changes during aging and disease (87,135,136). For example, as a response to lipopolysaccharide (LPS)-induced inflammation, the choroid plexus induce the production of IL-1 β , IL-6, and TNF- α , which can regulate iron-related proteins (134,137). LPS is a major component of Gram-negative bacteria and is often used as a model of inflammation, where it activates immune cells through Toll-like receptor 4 (TLR4). Expression of TLR4 in the choroid plexus allows them to recognize LPS (134). Accordingly, LPS-induced inflammation results in a robust upregulation of lipocalin 2 (LCN2) in the choroid plexus (138).

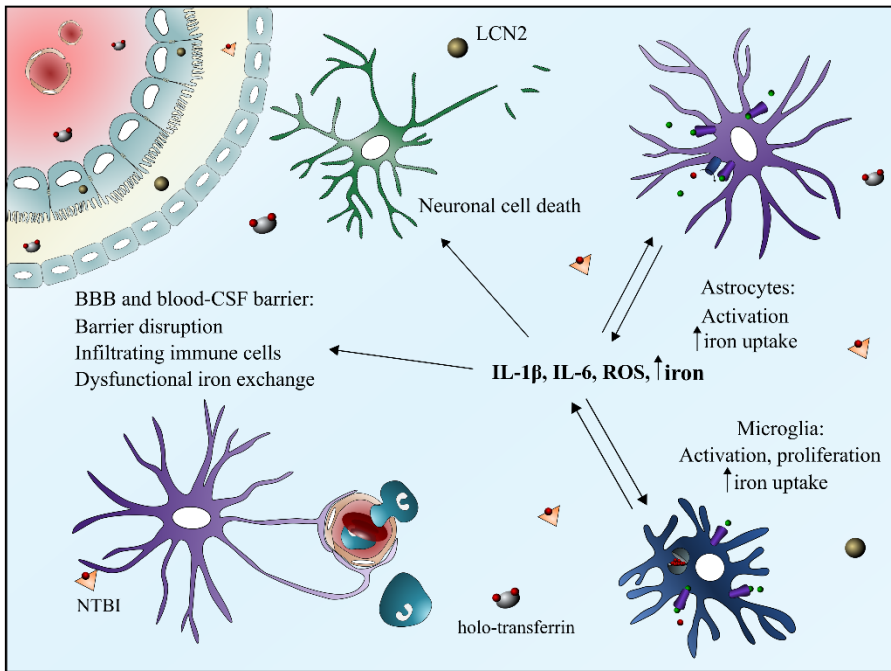


Figure 1.4. Neuroinflammation and iron overload contribute to neuronal cell death. Upon injury, microglia proliferate and activate, which leads to the release of pro-inflammatory mediators, such as interleukin-1 β (IL-1 β), IL-6 and reactive oxygen species (ROS). The release of pro-inflammatory mediators causes astrocyte activation, an inflammatory response in the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (blood-CSF barrier), and monocyte/macrophage invasion into the brain parenchyma through the brain barriers. As a response to injury, the blood-CSF barrier modulates the expression of iron-related proteins and produce lipocalin 2 (LCN2). Excess iron is taken up by microglia and astrocytes that both increase their expression of non-transferrin-bound iron (NTBI) transporters and their iron uptake. In microglia, this results in increased ferritin expression.

LCN2 is an acute-phase protein that acts in the antimicrobial defense, as it chelates the bacterial iron-containing siderophores, thereby limiting iron availability for the bacteria (139). Additionally, a growing body of literature indicates that LCN2 participates in iron homeostasis. Thus, LCN2 interacts with cell membrane receptor 24p3R to increase or decrease intracellular iron levels (140). Based on the evidence that LCN2^{-/-} mice have less BBB disruption compared to wild type mice in hemorrhagic stroke, LCN2 also influences barrier properties (141).

Barrier dysfunctions are suggested to cause an imbalance in the iron exchange between the circulation and the brain (134). Supporting this notion, the choroid plexus responds rapidly to inflammation by increasing gene expression of ferritin H and *Hamp* (the gene encoding hepcidin) (136). The authors propose that these changes in

the choroid plexus can lead to changes in the composition of the CSF, which may affect cells within the brain, like microglia and astrocytes (136).

1.5.2. IRON HANDLING IN REACTIVE MICROGLIA

Microglia are the earliest responders to CNS injury and increase in number in the early phase of inflammation (92). During activation, microglia change into amoeboid shape with hypertrophied processes (132). Their activation can be elicited by a myriad of stimuli, such as infection, trauma, pro-inflammatory cytokines, and neuronal injury (142). For instance, microglia express TLR4 (143) that enable them to detect LPS. Interestingly, LPS induces a pro-inflammatory response that activates microglia and accentuates their cellular iron uptake and DMT1 expression (Fig. 1.4) (75,144,145). Furthermore, hepcidin increases in inflammatory conditions and is shown to decrease the expression of ferroportin while increasing DMT1 expression in microglia (145), supporting that microglia increase their iron content during inflammation. In addition to changes in iron homeostasis, microglia secrete a variety of cytokines, IL-1 α , IL-1 β , IL-6, TNF- α , chemokines, and reactive oxygen species that engage the activation of astrocytes (146–150).

1.5.3. IRON HANDLING IN REACTIVE ASTROCYTES

Increasing evidence suggests that astrocytes, upon activation, lose their neuroprotective role while gaining neurotoxic functions that exacerbate neuroinflammation (150,151). It is suggested that they undergo major morphological and molecular changes during activation. Regarding morphological changes, the conversion to a reactive state induces hypertrophy of astrocyte processes (152). In contrast to microglia, astrocytes do not proliferate in inflammation-induced CNS insults. However, an increase in the number of astrocytes is seen in traumas, where they contribute to scar formation (150,153,154).

Similar to microglia, astrocytes modify their iron homeostasis during inflammation. For instance, they induce DMT1 expression and ferritin expression *in vitro* when stimulated with pro-inflammatory cytokines or LPS (33,145,155). While it is well documented that astrocytes take up more iron during inflammation and iron overload (Fig. 1.4) (31,33), their iron uptake mechanisms are still debated. DMT1 has received much attention and support in its function as a significant NTBI transporter during inflammation (33). However, DMT1 expression *in vivo* is restricted to astrocyte endfeet (65). Based on this, others claim that DMT1 only contributes to iron uptake in non-pathological conditions, while new NTBI transporters are important during pathology (80). ZIP14 is regulated by iron overload and inflammation, suggesting it plays a role in pathological conditions (43,44,156,157).

Together, this implies an essential role for the choroid plexus and activated glial cells in modifying the inflammatory response and iron homeostasis, which may be relevant in neurological conditions where basal levels of iron and pro-inflammatory mediators are elevated (4,82).

CHAPTER 2. THESIS OBJECTIVES

The objective of this dissertation was to understand how the brain handles iron in conditions with inflammation and iron overload, using *in vitro* cultures and *in vivo* models of intraventricular hemorrhage, intracerebral hemorrhage and neurodegeneration.

The hypotheses were:

- The choroid plexus responds to hemoglobin exposure by changing the expression of iron-handling proteins (Study I)
- The expression of the iron-transporter ZIP14 increases in conditions with inflammation and iron overload, caused by cerebral hemorrhage (Study II)
- The expression of ZIP14 increases in an experimental model of chronic neurodegeneration with inflammation and iron accumulation as a mechanism to limit deposition of available free iron (Study III)

In order to address the overall objective, three separate studies were conducted. The aims of the studies were:

Study I: To investigate the regulation of iron-related proteins, i.e. HO-1, ferritin, and LCN2, in the choroid plexus *in vitro* and *in vivo* following exposure of hemoglobin.

Study II: To establish an experimental model of ICH that would enable analysis of iron deposition and correlative increases in HO-1 and ferritin expression. The model was additionally employed to examine the regulation of ZIP14 during ICH *in vivo* and astrocyte cultures in experimental conditions mimicking the molecular events occurring during ICH.

Study III: To investigate the expression and regulation of ZIP14 in an experimental model of chronic neurodegeneration with inflammation and progressive iron deposition. Results were supplemented with *in vitro* studies of ZIP14 expression and regulation in astrocytes.

CHAPTER 3. METHODS AND RESULTS

3.1. STUDY I

Regulation of iron-related proteins in the choroid plexus by hemoglobin

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Manuscript submitted

Abstract

Introduction: Following intraventricular hemorrhage (IVH), lysis of red blood cells within the ventricular system can release hemoglobin and heme into CSF. Heme may be further degraded by heme oxygenase (HO) hence increasing the concentration of iron in CSF. The response of the choroid plexus (CP), site of the blood-CSF barrier, within the ventricles to IVH has not been well studied. It may be a site of injury or iron clearance.

Aims: In this study, we aimed to characterize the hemoglobin-induced regulation of iron-handling proteins in the choroid plexus *in vitro* and *in vivo*.

Methods: *In vitro*, freshly isolated CP, primary cultures of CP epithelial cells, and immortalized rat choroid plexus epithelial Z310 cells were treated with different concentrations of hemoglobin, after which RT-qPCR was performed to evaluate regulation of ferritin, HO-1, transferrin receptor (TfR), lipocalin (LCN2), and lipocalin receptor (24p3R). *In vivo*, mice had an intraventricular injection of hemoglobin or saline and CP ferritin, HO-1, and LCN2 expression was examined by immunohistochemistry.

Results: While there were some differences in the baseline of mRNA levels between the different *in vitro* preparations (notably in the Z310 cells), each of the preparations showed similar increases in the mRNA levels for ferritin, HO-1, and LCN2, but not TfR and 24p3R after 6 hours of hemoglobin exposure. *In vivo*, immunohistochemistry confirmed hemoglobin-induced upregulation of ferritin, HO-1, and LCN2.

Discussion: The impact of IVH on the CP has received little attention. The current study shows increased ferritin expression, which may protect the CP from hemoglobin-derived iron. The impact of the increased HO-1 expression is less certain, as it both degrades potentially toxic heme and releases iron that can enhance free radical production. Similarly, while LCN2 may be involved in clearing iron from CSF, it may also participate in CP iron overload, neuroinflammation and ICH-induced brain injury.

3.2. STUDY II

To mimic ICH in Study II, the experimental model with injection of autologous blood into the brain parenchyma was established. The impact of the intraparenchymal deposition and degradation of blood on iron deposition and changes in protein expression was examined by Perls' staining for histological iron, and immunohistochemistry for detection of HO-1 and ferritin in rats sacrificed 1, 3, 7, 14, and 28 days post-surgery. Subsequently, the role of ZIP14 was investigated.

3.2.1. PERLS' STAINING

Perls' staining is a method used to visualize non-heme iron present when iron is bound to ferritin or transferrin. The staining reaction depends on the conversion of ferrocyanide into crystal precipitates, Prussian blue, in an acidic environment and detects both ferrous and ferric iron with main sensitivity for detection of ferric iron (158). The acidic environment initiates iron release from ferritin and transferrin and thus enables the formation of precipitates with deep blue color (158). This method is extensively used to detect iron in tissue sections either as Perls' staining alone or in combination with DAB-enhancement that increases the sensitivity of the staining reaction (159).

In the ICH model, pathological iron deposition was not detected in the area of hemorrhage in rats sacrificed 1 day post-surgery (Fig. 3.1). However, iron-positive cells became apparent in the perihematomal zone 3 days after ICH. The staining was observed in cells with a morphology corresponding to amoeboid macrophages or neutrophils. After 7 days, a blue rim was observed between the hematoma and the adjacent tissue. Here iron-positive cells showed round, as seen after 3 days, or ramified morphology (microglia or macrophages). The blue rim was still apparent after 14 and 28 days (Fig. 3.1) and stainings were similar to that observed after 7 days, thus suggesting that iron is present in inflammatory cells after ICH in rats.

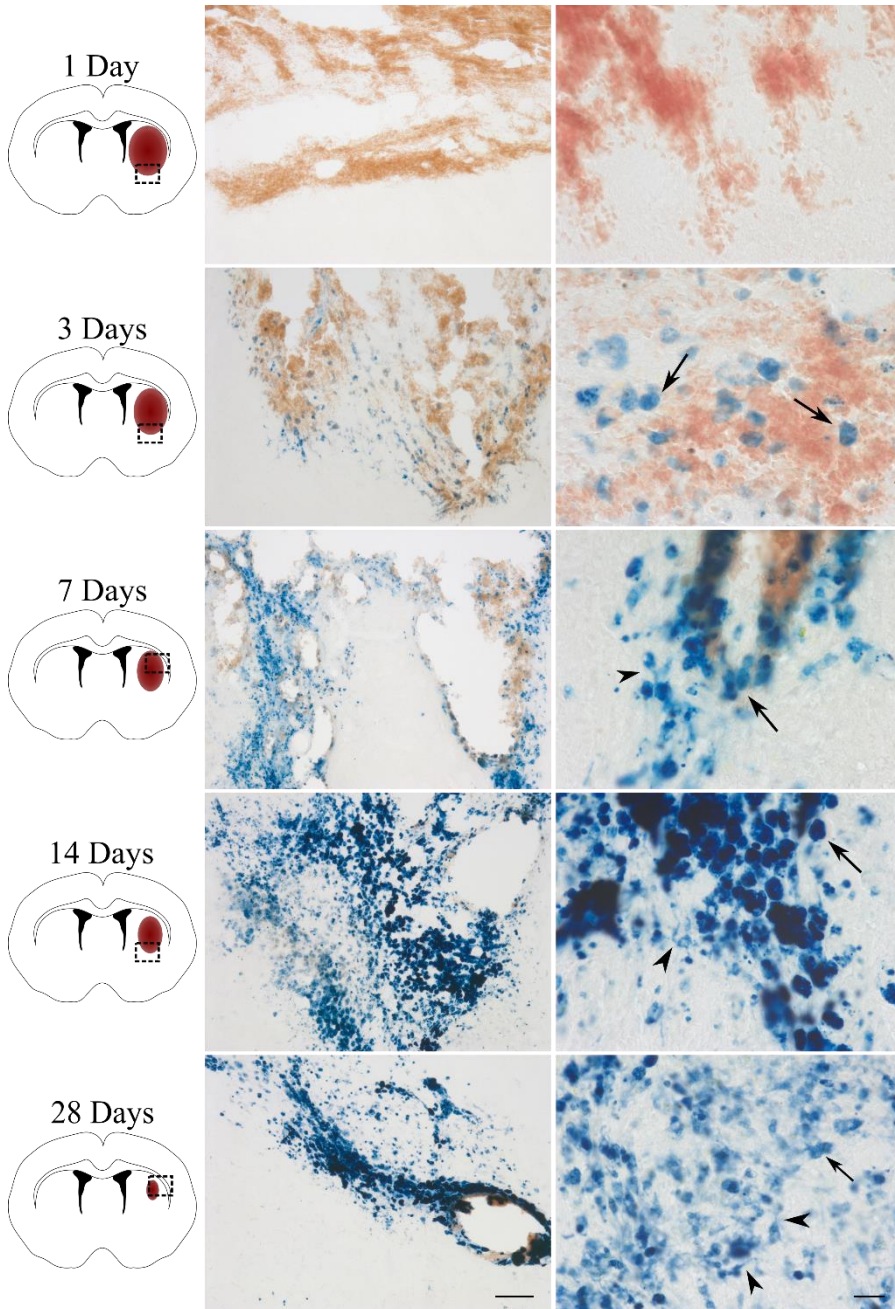


Figure 3.1. Perls' stain after induction of ICH. Non-heme iron was detected in the tissue after 3 days and onwards. Based on the morphology of the cells, iron staining was observed in amoeboid (arrow) and ramified (arrowhead) immune cells. Scale bar = 100 μm and 20 μm .

3.2.2. IMMUNOCYTOCHEMISTRY

Resolution of the hematoma in ICH includes degradation of the red blood cells with release of intracellular proteins into the extracellular space, intracellular conversion of hemoglobin to heme and iron-mediated by HO-1, and storage of unbound iron by ferritin (85). Immunohistochemical analysis showed a rapid upregulation of HO-1 in the ipsilateral side as early as 1 day after ICH, while the contralateral side remained unlabeled (Fig. 3.2). This expression was still pronounced after 28 days, which indicate a prolonged HO-1 expression after ICH. A vast majority of HO-1 was observed in cells with a morphology corresponding to microglial cells (1, 3, 7, 14, and 28 days), although some astrocyte-like and weakly stained neuronal-like cells were also observed.

Cells exhibiting ferritin immunoreactivity revealed a temporal upregulation from 1 day to 28 days after ICH (Fig. 3.3). Based on cell morphology, ferritin staining was induced in macrophage and microglia-like cells adjacent to the site of the hematoma. In contrast, the contralateral side showed few ferritin-labeled cells. These results were in agreement with results published by other investigators, which used the same model of ICH (85). In further studies, we used rats sacrificed after 1, 7, 14, and 28 days.

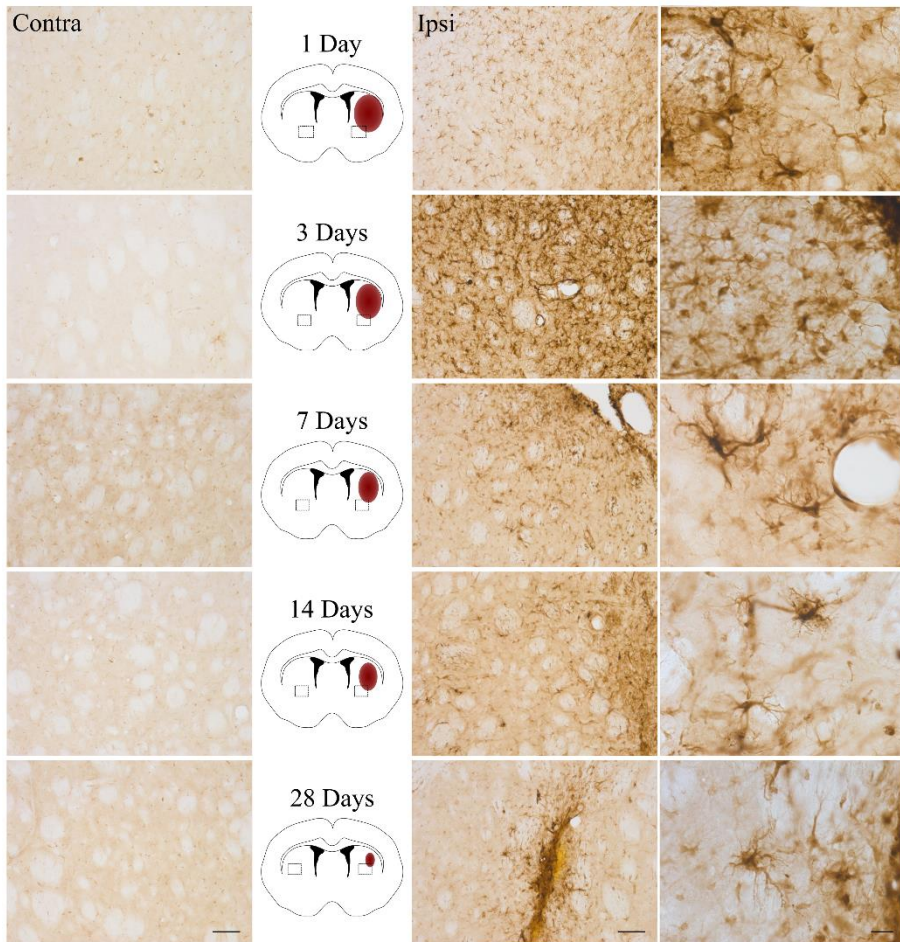


Figure 3.2. HO-1 in the experimental model of intracerebral hemorrhage (ICH). Representative images of HO-1 in the contralateral (Contra) and ipsilateral side (Ipsi) of rats euthanized 1, 3, 7, 14 and 28 days after ICH. HO-1-positive cells were found in the ipsilateral side with most cells observed during the early phase of ICH (1 and 3 days). A majority of HO-1 immunoreactive cells had a morphology corresponding to microglia/macrophages after ICH. However, astrocyte-like cells were also observed. In contrast, the contralateral side remained unlabeled. Scale bars = 100 μm and 20 μm .

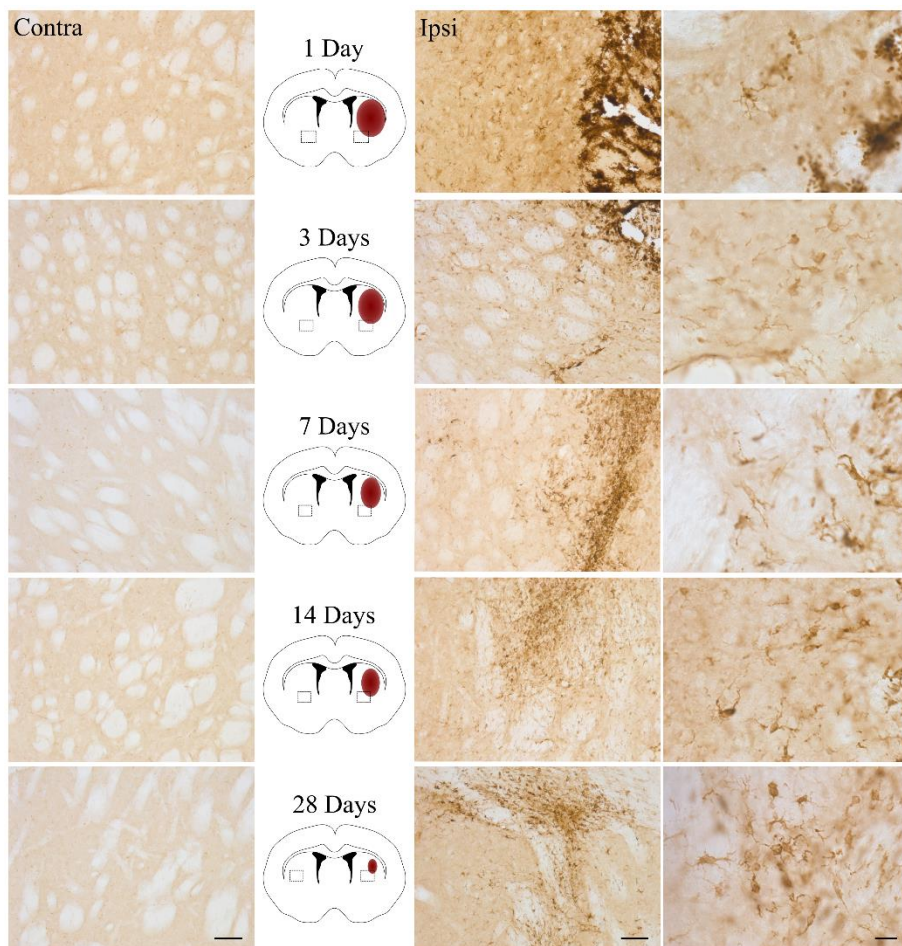


Figure 3.3. Ferritin in the experimental model of intracerebral hemorrhage (ICH). Representative images of ferritin in the contralateral (Contra) and ipsilateral side (Ipsi) of rats euthanized 1, 3, 7, 14 and 28 days after ICH. In the early phase of ICH, a few ferritin-positive cells were observed (1-7 days). In contrast, more ferritin-positive cells appeared in the perihematomal zone after 14 and 28 days. Ferritin expression is confined to cells with microglia/macrophage morphology. Scale bars = 100 μ m and 20 μ m.

Upregulated astrocytic ZIP14 expression in an experimental model of intracerebral hemorrhage

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Manuscript in preparation

Abstract

Introduction: Intracerebral hemorrhage (ICH) is associated with an increased level of non-transferrin-bound iron (NTBI) causing an overall increase in the concentration of iron in the brain. NTBI may represent a toxic form of iron that promotes oxidative stress through the Fenton reaction chemistry. The transporter Zrt-, Irt-like protein 14 (ZIP14) is suggested to import NTBI through the cellular membrane. Although all cells in the brain probably take up NTBI, regulated uptake of NTBI in astrocytes may be neuroprotective. In the present study, the occurrence of ZIP14 was characterized after hemorrhagic stroke *in vivo* and *in vitro*.

Methods: Male Sprague-Dawley rats were stereotactically injected with autologous whole blood (ICH) and sacrificed after 1, 7, 14, and 28 days. ZIP14 expression was analyzed with immunohistochemistry, Western blotting, and RT-qPCR. Primary rat astrocyte cultures were treated with whole blood or hemoglobin and analyzed for protein and mRNA expression of ZIP14 after 1 or 7 days.

Results: After ICH, upregulation of ZIP14 was observed together with an increase in the number of ZIP14-positive cells in the ipsilateral side 7, 14, and 28 days after ICH compared to the contralateral side. There was no difference in the gene expression of either *Zip14* or *Il-6* between the ipsilateral and contralateral side or between the ipsilateral sides of ICH and sham-operated animals. ZIP14 co-localized with GFAP in the experimental model of ICH, suggestive of an astrocytic expression. This was further supported *in vitro*, where gene expression analysis revealed increased *Zip14* expression in astrocytes treated with hemoglobin or 5% whole blood.

Conclusion: The increased expression of ZIP14 is indicative of the brain's attempt to recover following ICH via uptake of iron by astrocytes.

3.3. STUDY III

Altered expression of ZIP14 (SLC39A14) is part of the astrocytic reaction to chronic neurodegeneration with iron overload

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Abstract

Introduction: Neurodegeneration is associated with inflammation and a mismanaged iron homeostasis, leading to increased concentration of non-transferrin-bound iron (NTBI) in the brain. NTBI can be taken up by cells expressing Zrt-, Irt-like protein-14 (ZIP14), which is regulated by iron overload and inflammatory cytokines like interleukin-1 β (IL-1 β) and IL-6. Thus, this study focuses on the involvement and regulation of Zip14 in chronic neurodegeneration with iron overload and inflammation.

Methods: Male Wistar rats were unilaterally injected with the glutamate receptor agonist, ibotenic acid, in the striatum, which eventually leads to excitotoxicity and neuronal loss in substantia nigra pars reticulata (SNpr). ZIP14 expression was measured in the SNpr using immunohistochemistry, Western blotting, and RT-qPCR. Furthermore, primary astrocyte cultures were examined for *Zip14* mRNA expression after stimulation with ferric ammonium citrate (FAC), IL-6, or IL-1 β . Iron uptake was investigated after treatment with IL-1 β and siRNA-mediated ZIP14 knockdown.

Results: ZIP14 was widely expressed in the normal rat brain. In the lesioned SNpr, reactive astrocytes revealed altered ZIP14 expression with a main confinement to cell bodies and cellular processes. *In vitro*, FAC and IL-1 β stimulation resulted in increased *Zip14* expression and increased uptake of ^{59}Fe . Similarly, increased ^{59}Fe uptake was observed after siRNA-mediated ZIP14 knockdown.

Conclusion: In conclusion, these data imply a vital role of ZIP14 for astrocytic iron homeostasis, and provides evidence for astrocytes as being important for management of iron in the pathological brain.

CHAPTER 4. GENERAL DISCUSSION

Brain iron increases with age, but the observations that iron accumulation is even higher in neurodegeneration has led to the speculations that dysregulated iron homeostasis may contribute to the brain pathology (4,160,161). While there has been a development in the understanding of systemic iron homeostasis, mechanisms of iron handling in the brain are still poorly understood. Thus, studies of iron handling in the brain often draw parallels to known mechanisms in the periphery. The aim of this dissertation was to study iron handling in the pathological brain. The current study was inspired by findings from models with systemic iron overload, e.g. hemochromatosis, in which NTBI increase systemically, thus triggering the induction of iron-handling proteins (21).

This chapter is dedicated to a general discussion that focuses on the expression of iron-related proteins in the choroid plexus, with particular emphasis on LCN2, and the importance of astrocytic ZIP14, which are both proteins that are regulated by iron accumulation and inflammation (43,44,138,141,156). Lastly, model limitations are discussed.

4.1. THE CHOROID PLEXUS AS A RESPONDER TO HEMOGLOBIN

In contrast to many other organs, the brain shows a surprisingly small increase in iron accumulation during iron overload pathology, which, as mentioned, may be due to the protective effects of the BBB and blood-CSF barrier (22). There has been considerable interest in examining the BBB in stroke and only a few studies investigating the role of the choroid plexus epithelial cells (87). The choroid plexus is responsible for secretion and regulation of CSF components and suggested to play a key role in brain iron homeostasis (57). Existing work has confirmed a modulation of the expression of iron-handling proteins in the choroid plexus in response to inflammation (134). The modifications of the choroid plexus transport function may cause altered brain iron homeostasis (57,136). As there is little evidence for uptake of NTBI at the brain barriers (22), we decided not to look at NTBI transporters in Study I but investigated the regulation of many other iron-handling proteins after hemoglobin exposure. We found that the choroid plexus responded by increasing gene expression of *Lcn2*, *HO-1*, *Ftl*, and *Fth*.

The function of LCN2 has gained much attention in the last decades since it is upregulated in the CNS in models of Alzheimer's, Parkinson's disease, and hemorrhagic stroke (131,136,141,162). A growing body of evidence reports that LCN2 is regulated by blood derivatives, pro-inflammatory cytokines, and LPS (138,141,163–166). Supporting this notion, we found that LCN2 is upregulated in the

choroid plexus after hemoglobin exposure (Study I). The involvement of LCN2 in pathological processes in the CNS is controversial and point towards both harmful and protective mechanisms (167). For instance, LCN2^{-/-} accentuates inflammation in an animal model of multiple sclerosis (168) although, in many other disease models, LCN2^{-/-} alleviates CNS inflammation and iron accumulation (141,166,169), suggesting that effects of LCN2 depend on the disease. Regarding iron homeostasis, LCN2^{-/-} mice had unchanged homeostasis in multiple sclerosis, whereas decreased ferritin expression and decreased number of ferritin-positive cells were observed in LCN2^{-/-} mice after ICH (141,168). Based on these observations, it was postulated that LCN2 mediates iron-induced toxicity after ICH, and possibly contribute to iron overload in cells expressing the lipocalin receptor, 24p3R (136,141), which was found to be expressed in the choroid plexus in Study I.

In Study I, hemoglobin exposure induced HO-1 expression in the choroid plexus, thus potentially releasing free iron. A prominent expression of ferritin found in the epithelial cells of the choroid plexus suggests that they play a role in iron storage (57,67). Following hemoglobin exposure, the increased ferritin expression, suggests that the choroid plexus has increased iron accumulation (Study I). Ferritin H, in particular, seems crucial for normal function of the choroid plexus and deletion of ferritin H in the choroid plexus of the lateral ventricles caused hydrocephalus in mice (170). Ferritin probably plays a vital role in detoxifying iron. However, whether iron accumulation in the choroid plexus serves to protect the brain from iron overload or contributes to dysfunction of the choroid plexus and the blood-CSF barrier after IVH, remains a central question. In a model of aceruloplasminemia with knockout of the ferroxidase, ceruloplasmin, the choroid plexus is the first brain region to show severe iron deposition (171). The authors propose that this iron accumulation may cause leakiness of the blood-CSF barrier as the choroid plexus is vulnerable to increased iron (171). Similarly, dysregulated iron homeostasis and upregulation of markers of oxidative stress are suggested to contribute to the morphological and functional changes of the choroid plexus in Alzheimer's disease (135,136,172).

In general, it can be speculated that changes to the brain barriers lead to a dysfunctional iron exchange between the circulation and brain that in turn result in brain iron overload, which then must be handled by neurons and glial cells in the brain parenchyma. This will be the topic of the following paragraph.

4.2. UNCOVERING THE ROLE OF ASTROCYTIC ZIP14 ALTERATIONS

In the brain, microglia and macrophages are shown to handle iron after experimental ICH (Fig. 3.1 and 3.3) and experimental chronic neurodegeneration (132). However, we show that astrocytes also participates in the handling of iron during disease in Study II and III.

To date, reactive astrocytes have been observed in numerous animal models with CNS injury (150,152). Reactive astrocytes are essential in the repair of the BBB, regulation of leukocyte trafficking, and limiting neuronal injury. Accordingly, inhibiting reactive astrocytes in a transgenic mouse model with the eradication of dividing GFAP positive cells after stab injury resulted in prolonged BBB dysfunction and increased infiltration of leukocytes (173). While reactive astrocytes that limit neuronal injury and BBB dysfunction are beneficial, other reactive astrocytes are proposed to exert neurotoxic and inflammatory effects. In neurodegeneration, the presence of reactive astrocytes is confirmed in brain regions affected by Alzheimer's disease, Huntington's disease, and Parkinson's disease (150,154). Findings from this dissertation are consistent with previous findings in that reactive astrocytes are found in rat models of ICH and chronic neurodegeneration (Study II and III).

As earlier mentioned, characteristic features of hemorrhagic stroke and neurodegenerative diseases are excess iron accumulation and inflammation, both critical regulators of ZIP14 (43,44,83). Accordingly, iron status regulates ZIP14 and an increase in protein levels are observed in iron-loaded hepatoma HepG2 cells, whereas the levels decrease in iron-depleted cells (174). In line with these findings, Study II showed that astrocytes increase their expression of ZIP14 7-28 days after ICH and after stimulation with whole blood and hemoglobin in astrocyte cultures. *In vivo*, the ZIP14 upregulation coincided with iron deposition. The induction of HO-1 in the early phase of ICH initializes release and accumulation of free iron from 3-28 days after surgery assessed by Perls' staining. Measuring iron with spectrophotometry, Wu et al. found similarly that non-heme iron concentrations were increased during this time course (85). Ferritin that incorporates free iron was also upregulated during this period, possibly as a neuroprotective mechanism (85). Since upregulation of ZIP14 occurs in the recovery phase of ICH, it is likely that it plays a role in handling excess iron occurring after the breakdown of heme.

Besides iron overload, chronic inflammation and pro-inflammatory cytokines such as IL-1 β and IL-6, augment hepatic ZIP14 expression (44,156). Treating astrocytes with LPS, as a model of inflammation, resulted in increased *Zip14* expression in astrocytes in Study II. Whether astrocytes express TLR4 and thus respond to LPS stimulation is still a matter of debate. One group was not able to detect an astrocytic reaction to LPS and argue that the lack of response is attributable to the absence of TLR4 in astrocytes (150). The astrocyte cultures used in Study II may have few microglia that could theoretically interact with LPS and create a response. However, astrocytic TLR4 expression was confirmed by other groups (175–177) and LPS induced *Zip14* expression (Study II), pro-inflammation, and iron homeostatic changes in astrocytes (178), indicating that astrocytes can interact with LPS.

In Study III, stimulating astrocyte cultures with IL-1 β and iron-induced *Zip14* mRNA expression and revealed an increased iron uptake in activated astrocytes. Inflammation has been implicated in early stages of neurodegeneration, which led to

the investigation of regulation and involvement of ZIP14 in an excitotoxicity-induced model of neurodegeneration that is associated with accumulation of iron in SNpr and presence of inflammatory cells (129,132). Here, we found ZIP14 expression in hypertrophied astrocytic processes in the SNpr of the lesioned side, whereas the non-lesioned side showed ZIP14 expression around the nuclei.

Returning to the question, “does astrocytic ZIP14 expression reflect a protective response or contribute to disease mechanisms after CNS trauma?” Although there is no difference in the iron accumulation between ZIP14^{-/-} mice and wild type mice, *Dmt1* mRNA increases in ZIP14^{-/-} mice, and ZIP14 knockout is associated with neuroinflammation and neurodegeneration (46,179,180). In ZIP14^{-/-} mice, systemic inflammation is augmented, and IL-6 production increased compared to wild type mice, suggesting that ZIP14 may mediate protective effects by reducing chronic systemic inflammation (179). Knockdown of ZIP14 in astrocytes (in Study III) induced iron accumulation, proposing that ZIP14 plays an essential role in maintaining iron homeostasis. Thus during pathology, increases in astrocytic ZIP14 may protect neurons from iron overload.

4.3. MODEL LIMITATIONS

Throughout history, animal models have contributed immensely in obtaining knowledge about human biology and pathology. Rats and mice are extensively used as models of human neurological disorders. However, it should be kept in mind that translation of results obtained from rodent animal models is still far from perfect and the models cover only aspects of human disease. For instance, in clinical ICH, bleeding results from an arterial source whereas experimental models with an injection of collagenase results in more diffuse bleeding that may originate from small vessels near the injection site (116–119). Using the blood model neither mimics clinical ICH due to the lack of vasculature pathology and disruption, which is thought to be the primary etiology of human ICH (89,117). Choosing the right animal model thus largely depends on the objectives of the experimental study. Accordingly, the blood model is useful when investigating mechanistic effects linked with the presence of blood in the brain (181), i.e., iron handling and inflammation, which was assessed in Study II.

Similarly, toxin-induced neurodegeneration causes immediate onset of symptoms that do not resemble the insidious, progressive nature of human neurodegeneration. In the model of chronic neurodegeneration, the injection of ibotenic acid causes cell death of striatal neurons (129). The finding that N-methyl-D-aspartic acid (NMDA) antagonists protect against neuronal cell death in striatum suggests that ibotenic acid acts via the NMDA-receptor (182). The relevance of using the excitotoxicity-induced model of neurodegeneration is based on the role of excitotoxicity in the pathogenesis of neurodegeneration, where a continuous stimulation of NMDA-receptors can lead to neuronal injury by elevated intracellular calcium content and increased enzyme

activity (183). In Huntington's disease, neurons with a high expression of NMDA-receptors in the striatum are lost early in disease (184).

A possible limitation of this study is the choice to use young animals when assessing iron handling in brain iron overload. However, this choice was made to mimic disease models of ICH and neurodegeneration in published papers (112,132,141). Since age is the predominant cause of neurodegeneration and ICH often affects the elderly, more attention should be given to the use of aged animals. Comparing young and aged rats, ICH is shown to cause decreased lesion resolution, and a delayed and more pronounced microglia activation in aged rats (185). Similarly, the inflammatory response is delayed and increased in aged rats (91,186). Despite these changes in aged rats, Wasserman et al. did not detect any difference in neuronal cell death compared to young rats (185). In contrast to microglia, more dispute prevails in the existing work on reactive astrocytes in young and aged rats after ICH. While one study reports an increased number of astrocytes in the edge of the lesion in aged rats, another study attributes the more severe tissue damage observed in aged rats to repression of astrocyte activation (185,186), thus again emphasizing the importance of the choice of animal model.

Indeed, care should be taken when deciding on a suitable animal model and age of the animal in experiments that seek to unravel mechanisms and treatment of human disease.

CHAPTER 5. CONCLUSIONS AND FUTURE PERSPECTIVES

Although a dysregulated iron homeostasis is implicated in many CNS diseases, relatively little is known about the underlying mechanisms. However, results obtained from the studies described in this dissertation have provided novel insights into the handling of iron during brain pathology. They demonstrate that iron accumulation and inflammation, two characteristic features of CNS disease, cause regulation of iron-handling proteins, namely LCN2 and ZIP14. Furthermore, it is demonstrated that the choroid plexus and astrocytes participate in the iron handling during ICH and neurodegeneration.

In response to hemoglobin exposure, the choroid plexus alters the expression of ferritin, LCN2, and HO-1. While increased ferritin expression in the choroid plexus may play a protective role by storing iron in a non-toxic form, upregulation of HO-1 and LCN2 can lead to iron accumulation. Altered iron metabolism in the choroid plexus can interrupt the blood-CSF barrier, which, in turn, may enhance neuronal cell death. Thus from a clinical point of view, uncovering the influence of LCN2 in the CNS could add significantly to pharmacologically targeting iron overload and inflammation.

Using animal models of ICH and excitotoxicity-induced chronic neurodegeneration, we show that reactive astrocytes increased their expression of ZIP14 as a response to iron accumulation and inflammation. Reactive astrocytes exert neuroprotective as well as deleterious effects. They are less susceptible to iron toxicity than neurons and thus upregulation of ZIP14 in reactive astrocytes in ICH and chronic neurodegeneration may serve a protective role for neurons by limiting available iron. However, research focusing on the communication between glial cells and neurons may further outline the role of ZIP14 in iron homeostasis in CNS health and disease. Unraveling the role of ZIP14 in brain pathology and understanding of whether upregulation may be neuroprotective or neurotoxic, can be key to target these diseases therapeutically.

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