

Changed iron metabolism and iron toxicity in scrapie-infected neuroblastoma cells

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ABSTRACT

Reactions and interactions of iron and oxygen can be both beneficial and detrimental to cells and tissues. Iron is mainly found in our blood where it functions as a mediator of the transport of oxygen to the cells and is further vital for the cellular respiration reducing the oxygen to water. The flexible redox state of iron makes it ideal to contribute in single electron transfers, but may also catalyze reactions with oxygen resulting in cell damaging reactive oxygen species (ROS). Normally the cells are protected against iron toxicity by controlling iron uptake and storage. When the intracellular demand for iron increases; the iron uptake is promoted by increasing the expression of transferrin receptor (TfR) and by decreasing the expression of the iron storage protein ferritin. Ferritin has a central role in the cellular iron detoxification by keeping it in a non reactive but still bioavailable form. However, in neurodegenerative diseases like in Alzheimer's and Parkinson's disease the iron storage capacity is disturbed and iron induced oxidative stress adds to the pathology of the diseases. The role of iron and its possible contribution to the pathology of prion diseases, like Creutzfeldt-Jakob disease, is less explored. In the first three studies of this thesis, the iron metabolism and the mutual relation between iron and oxygen are studied in scrapie-infected mouse neuroblastoma cells (ScN2a) as compared to control cells (N2a). In the fourth study we have analyzed the expression of ferritin and TfR in response to inflammation by treating the cells with the bacterial endotoxin lipopolysaccharide (LPS). LPS promotes the expression of inducible nitric oxide synthase (iNOS), a producer of nitric oxide (NO), a well known regulator of the iron metabolism.

In the first study, the scrapie infection was found to reduce the iron levels, mRNA and protein levels of ferritin and the TfR. In addition, reduced levels and activities of the iron regulatory proteins 1 and 2 were observed as compared to the uninfected N2a cells.

In the second study, the addition of iron to the cell medium strongly increased the level of ROS and decreased the cell viability of the ScN2a cells, whereas the N2a cells were unaffected. The ferritin expression in N2a cells in response to the iron treatment was strongly increased and the concomitant measurement of the labile iron pool (LIP) revealed the LIP to be normalized within four hours. In the ScN2a cells the induction of ferritin expression was lower resulting in elevations in LIP that lasted up to 16 h, indicating that the increased ROS levels were iron catalyzed.

In the third study, the cells were challenged with hydrogen peroxide (H₂O₂) to elevate the oxidative stress and to analyze the effects on the LIP and cell viability. The ScN2a cells were sensitive to the increased oxidative stress according to the cell viability test, and responded to the treatment with marked increase in the LIP levels, probably derived from an intra-cellular source. The cell viability could be reset by the co-addition of an iron chelator to the cell media. The N2a cells did not elevate the LIP and resisted higher concentrations of H₂O₂ than the ScN2a cells, according to the cell viability assay.

In the fourth study, the LPS treatment resulted in increased mRNA levels of the heavy chain of ferritin, increased the protein levels of ferritin light chain and decreased the protein levels of the TfR in N2a cells, whereas no effects were observed in the ScN2a cells. Co-treatment with LPS and the iNOS inhibitor aminoguanidine did not affect the LPS induced decrease of TfR in N2a cells, whereas the free radical scavenger N-acetyl-L-cysteine reversed the effect of LPS on TfR expression, indicating that the changes were mediated by an oxidative rather than a nitric oxide mechanism in the N2a cells.

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IV. S. Fernaeus, K. Reis, J. Hålldin, K. Bedecs and T. Land.

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Stimulation of G-proteins in human control and Alzheimer's disease brain by FADmutants of APP(714-723): implication of oxidative mechanisms.
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ABBREVIATIONS

Aβ amyloid beta	LBP LPS binding protein
AD Alzheimer's disease	L-Ft ferritin light chain
A adenine	LIP labile iron pool
APP amyloid precursor protein	LPS lipopolysaccharide
BBB blood brain barrier	2-ME 2-mercaptoethanol
BIP 2,2'-bipyridyl	Mn SOD manganese SOD
BSE bovine spongiform encephalopathy	MtF mitochondrial ferritin
C cytosine	NAC N-acetyl-L-cysteine
CJD Creutzfeldt-Jakob disease	NADH reduced nicotinamide adenine dinucleotide
CM-H₂DCFDA Calcein-AM and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate	N2a mouse neuroblastoma cell line
CNS central nervous system	NBIA neurodegeneration with brain iron accumulation
CSF cerebrospinal fluid	NF-κB nuclear factor κ B
Cu²⁺ cupric ion	NO[•] nitric oxide radical
Cu/Zn SOD copper/zink SOD	NO⁺ nitrosonium ion
Dcytb duodenal cytochrome b	nNOS neuronal NOS
DFO desferrioxamine	NOS nitric oxide synthase
DMT1 divalent metal transporter 1	O₂⁻ superoxide anion
eNOS endothelial NOS	OH[•] hydroxyl radical
ER endoplasmic reticulum	ONOO⁻ peroxynitrite
FAC ferric ammonium citrate	PC12 pheochromocytoma cells
FADH₂ reduced flavin adenine dinucleotide	PD Parkinson's disease
FFI fatal familial insomnia	PIPLC phosphatidylinositol-specific phospholipase C
Fe²⁺ ferrous iron	PK proteinase K
Fe³⁺ ferric iron	PKAN pantothenate kinase-associated neurodegeneration
[4Fe-4S] iron sulfur cluster	Prnp prion protein gene
Ft ferritin	PrP prion protein
GABA gamma aminobutyric acid	PrP^C cellular prion protein
GPI glycosylphosphatidylinositol	PrP^{Sc} scrapie prion protein
GPX glutathione peroxidase	PrP^{Ctm} N-terminal transmembrane isoform of PrP
GR glutathione reductase	PrP^{Ntm} C-terminal transmembrane isoform of PrP
GSH reduced glutathione	PUFA poly unsaturated fatty acids
GSS Gerstmann-Sträussler-Sheinker syndrome	ROO[•] peroxy radical
GSSG oxidized glutathione	ROS reactive oxygen species
GT1 mouse hypothalamic cell line	ScGT1 scrapie-infected GT1 cells
H-Ft ferritin heavy chain	ScN2a scrapie-infected N2a cells
H₂O₂ hydrogen peroxide	SIH salicylaldehyde
IκB inhibitory κ B	SOD superoxide dismutase
IKK I κ B kinase	Tf transferrin
IL-1α interleukin α	TfR transferrin receptor
IFN-γ interferon γ	TLR toll like receptor
iNOS inducible NOS	TNF-α tumor necrosis factor α
IRAK interleukin-1 receptor-associated kinase	TSE transmissible spongiform encephalopathy
IRE iron responsive element	U uracil
IRP iron regulatory protein	UTR untranslated region
IscS cysteine desulfurase	UQH₂ ubiquinol
JNK c-jun n-terminal kinase	UQ ubiquinone

INTRODUCTION

Iron, a vital metal for living organisms from bacteria to man, is utilized for oxygen transport in the red blood cells, electron transfer in the respiratory chain of the mitochondria, facilitation of red-ox reactions and sometimes its presence in proteins is essential for structure and functionality. However, when iron is mediating single electron transfers in the vicinity of dioxygen (O_2), reactive free radicals are formed. These are able to damage biomolecules vital for normal cellular function and structure. If the cellular control of iron levels and the cellular antioxidative defence machinery are functional, the leakage of electrons which frequently occurs, will not damage the cells. However, in several diseases, including neurodegenerative diseases like Alzheimer's and Parkinson's disease, the iron balance and the antioxidative defence is disturbed and the toxicity of free radicals formed will add to the pathology of the diseases. The involvement of iron and its possible contribution to the pathology of prion diseases, like Creutzfeldt-Jakob disease, is less explored. In this thesis the iron metabolism and the mutual relation between iron and oxygen is studied using neuronal cell line cultures from mouse, infected with the disease associated pathogen, the prion.

Proteins of the iron metabolism and regulation of iron uptake

A grown up human contains 3-5 g of iron. About 60-70% of the body iron resides in red blood cells where it transports oxygen, 20-30% is stored in the liver, and the rest locates to muscles and other tissues. There is no physiologically active transporter to export excess iron from the body. Iron leaves the body by non-specific routes like shedding of epithelial cells of skin and cells of the intestine, and through loss of blood. A healthy individual absorbs 1-2 mg iron/day to compensate for the daily loss of iron. The daily erythropoiesis requires about 30 mg iron, so the iron within the body is recycled by mononuclear phagocytes which phagocytose senescent red blood cells and release iron for reutilization [Papanikolaou and Pantopoulos, 2005]. The uptake of iron is highly regulated, first on the level of dietary iron absorption and second on a cellular level. Here follows the description of proteins involved in the mammalian iron metabolism and how the proteins interact to maintain body and cellular iron homeostasis.

Iron regulatory proteins

Iron regulatory proteins (IRPs) are post-transcriptional regulators recognizing and binding iron responsive elements (IREs) located in untranslated regions (UTRs) of the mRNA transcripts of target genes. The IRE consists of about 30 nucleotides and forms a stem-loop structure on the transcript. The centre of the sequence contains six nucleotides in a loop having a conserved sequence CAGUGX, where X is most often a uracil (U) or cytosine (C) and sometimes adenine (A), and the flanking sequences base-pair and form the stem interrupted in a few places by unpaired nucleotide bulges. The IREs are evolutionary conserved in vertebrates, in some bacteria and insects [Aziz and Munro, 1987, Theil and Eisenstein, 2000]. The effect of the IRP/IRE binding is dependent upon where on the transcript the IRE is located. When IRPs bind to IREs located at the 3' UTR of the mRNA, the transcript is stabilized and translation is promoted. If IRPs bind to IREs at the 5' UTR of the mRNA they will inhibit translation resulting in decreased steady state levels of the proteins.

Iron regulatory protein 1

The cytosolic iron regulatory protein 1 (IRP1) was purified and identified as a protein of about 90 kDa able to bind with high affinity to the IRE structure [Rouault *et al*, 1989]. Next the cDNA of the protein was cloned and the mRNA was found to be expressed by a single gene [Rouault *et al*, 1990]. Later several lines of evidence supported that the IRP1 was identical to the cytosolic aconitase [Hentze and Argos, 1991, Kaptain *et al*, 1991, Kennedy *et al*, 1992]. The homologous mitochondrial aconitase is a well characterized iron-sulfur [4Fe-4S] cluster containing enzyme functioning in the Krebs cycle converting citrate into isocitrate. The cytosolic aconitase also needs a [4Fe-4S] cluster for its enzymatic activity, however, the metabolic relevance of a cytosolic aconitase is still poorly understood. The presence or not of the [4Fe-4S] cluster determines the function of the aconitase/IRP1 and consequently, mechanisms of cluster synthesis and disassembly is in focus in the field of iron-research and is recently reviewed by Rouault and Tong [Rouault and Tong, 2005]. The [4Fe-4S] cluster is an unstable structure and during cellular iron deficiency the cluster disassembles and IRP1 gains high affinity for mRNA containing IRE(s) and conversely,

when the cellular iron levels increase, the cluster reassembles and IRP1 loses its IRE binding activity regaining its aconitase activity [Haile *et al*, 1992, Klausner and Rouault, 1993].

Iron regulatory protein 2

A second iron regulatory protein (IRP2) cloned by Rouault *et al* in 1990, was also found to bind IRE structures with similar affinity as IRP1. The amino acid sequence of IRP2 was found to be 57% identical and 75% similar to IRP1, having a molecular mass of 105 kDa [Rouault *et al*, 1990, Samaniego *et al*, 1994]. IRP2 contains an inserted stretch, consisting of 73 amino acids, able to sense the intracellular iron levels. When cells are iron replete, specific amino acids are oxidized in an iron dependent manner, followed by ubiquitination and degradation by the proteasome [Guo *et al*, 1995, Iwai *et al*, 1998 Samaniego *et al*, 1994].

Both IRPs are broadly expressed, but their relative levels vary in a tissue and cell specific manner. Meyron-Holtz *et al* analyzed the expression pattern of IRP1 and IRP2 in mice by *in situ* hybridization and Western blotting. The IRP1 protein was strongly expressed in brown fat, liver and kidney, and mRNA levels were also found at high levels in the small intestine. IRP2 protein was mostly expressed in forebrain, cerebellum, skeletal muscle and heart, and IRP2 mRNA levels were also strongly expressed in the dorsal root ganglia, thymus and retina [Meyron-Holtz *et al*, 2004].

Transferrin receptors

Transferrin Receptor 1

The transferrin receptor 1, often referred to as just the transferrin receptor (TfR), is a homodimer consisting of two 90 kDa transmembrane glycoprotein subunits linked by disulfide bonds. It is located on the cell surface within lipid rafts and its polypeptide chain traverses the cell membrane once resulting in a small cytoplasmic domain and a large extracellular domain. TfR binds two molecules of transferrin (Tf) with highest affinity to diferric transferrin and lowest affinity towards apo-transferrin. After binding, the complex is internalized into the cell by receptor mediated endocytosis in clathrin-coated pits. The acidic pH of the endosomes, besides mediation of the iron release from Tf (see below), also increases the affinity of the TfR for the apo-form of Tf promoting recycling of Tf to the cell surface by exocytosis. Iron uptake through the Tf/TfR complex is well conserved

among mammals and constitutes the major iron uptake pathway of inorganic iron in the cells. The expression of the TfR is regulated by IRPs, depending on the iron status of the cell. The mRNA of TfR has five IREs located at the 3' UTR, consequently, when the iron levels are decreased and IRPs become active the binding of IRPs to the IREs will increase the stability of the transcript and promote the protein expression, and in opposite, when iron levels are high the lack of binding of IRPs to the TfR mRNA will promote its degradation and decrease the numbers of TfR on the cell surface (Fig. 1) [Casey *et al*, 1988, Rouault *et al*, 1990].

Transferrin receptor 2

Transferrin receptor 2 is homologous to TfR, sharing 45% sequence identity, but its transcript does not contain any IREs and its expression appears to not be regulated by iron [Kawabata *et al*, 1999]. Two transcripts of the gene have been identified, one encoding TfR2- α having a molecular mass of 95-105 kDa dependent upon glycosylations, and the other encodes only the intracellular part of the receptor denoted TfR2- β . TfR2- α is a transmembrane homodimer highly expressed in the liver, erythroid precursor cells, erythroleukaemic cell lines, and bone marrow samples from patients with erythroleukemia and myeloid leukaemia. TfR2- α is also expressed in several other tissues, but to a much lesser extent as compared to TfR which is found to be expressed by all types of cells except mature erythroid cells. TfR2- α binds Tf, but with 20-30-fold lower affinity as compared to TfR, and also undergoes receptor mediated endocytosis. The role of TfR2- α is at present unclear, but may be involved in the regulation of iron absorption and sensing of the body iron levels [Trinder *et al*, 2003].

Transferrin

Transferrin is a soluble glycoprotein of 80 kDa, consisting of two ferric binding domains and is synthesized in the liver, brain, testes, and mammary glands. It circulates in the plasma and in interstitial fluids where it binds and delivers iron to the cells by receptor-mediated endocytosis after its binding to the TfR. At the slight alkaline pH 7.4 of the extracellular fluids, Tf binds two ferric (Fe^{3+}) ions with high affinity coordinated by one histidine, two tyrosines and one asparagine. The release of iron occurs after protonation of

the histidine and/or tyrosine ligands as a result of the drop in pH within the endosomes [Aisen *et al*, 1978, Cheng *et al* 2004]. In the circulation of healthy individuals the Tf molecules are not fully saturated but constitute a mix of diferric, monoferric and apo-transferrin, thus providing an iron-buffering capacity to avoid free toxic iron in the circulation and interstitial space [Moos and Morgan, 2000].

The ferritins

Cytosolic ferritin

Ferritin (Ft) is highly conserved in its three-dimensional structure among plants and animals. It is a multimeric protein consisting of 24 polypeptides of heavy ferritin (H-Ft) and light ferritin (L-Ft) chains forming a large cavity in its centre where it can store up to about 4000 iron atoms in a non-toxic and bioavailable manner. Ft has a molecular weight of about 450 kDa containing varying ratio of H and L chain polypeptides (21 and 19 kDa, respectively). The H and L chain share sequence homology but are derived from two separate genes [Chou *et al*, 1986, Jain *et al*, 1985]. The ratio of the polypeptides affects the functional properties of Ft. The H-chain has ferroxidase activity and H-rich ferritins are found in tissues like heart and brain, and within cells typically having high metabolic activity and utilization of iron e.g. for the respiratory chain of the mitochondria [Connor *et al*, 1994]. The L-rich ferritins seem to dominate in tissues like liver and spleen, characterized by cells having high iron storage capacity [Cairo *et al*, 1991]. The ferritin structure further contains eight exit or entry pores on the surface and a number of ferroxidase centres on the H-subunits. When ferrous (Fe^{2+}) iron enters through the pore and localizes to the ferroxidase centre it is oxidized by O_2 into a short lived diferric peroxo intermediate ($\text{Fe}^{3+}\text{-2O-Fe}^{3+}$) before it is further transformed into a diferric-oxo ($\text{Fe}^{3+}\text{-O-Fe}^{3+}$) mineral precursor. The mineral precursor is subject for two additional hydrolytic reactions during its path into the cavity where it is finally stored as ferrihydrite. Fe^{2+} iron can leave through the pores after rehydration and reduction [Biemond *et al*, 1984, Chasteen and Harrison, 1999, Theil, 2003].

The translation of Ft is regulated by the intracellular iron levels and demands for iron [Rittling and Woodworth, 1984]. Both the H-Ft and L-Ft mRNA contain an IRE located in the 5'-UTR and when the cellular demand for iron is increased activated IRPs bind to the

IRE, inhibiting the translation resulting in less cellular storage capacity of iron (Fig. 1)[Aziz and Munro, 1987, Leibold and Munro, 1988, Rouault *et al*, 1990].

The H-Ft can also be regulated on a transcriptional level, by the cytokines tumour necrosis factor α (TNF- α), interferon γ (IFN- γ) and interleukin 1 α (IL-1 α) as have been showed in various mesenchymal cells of mouse and human origin [Fahmy *et al*, 1993, Miller *et al*, 1991, Torti *et al*, 1988, Wei *et al*, 1990]. Kwak *et al* found TNF- α signaling to initiate the binding of the transcription factor nuclear factor κ B (NF- κ B) and activate the transcription of ferritin. They found in the murine ferritin promoter, two independent *cis*-acting elements approximately 4.8 kb upstream of the transcriptional start site, one very close in similarity to the consensus sequence of NF- κ B binding element and one novel sequence with similarities to the NF- κ B sequence [Kwak *et al*, 1995].

The function of Ft besides iron storage is also to supply cells with iron for usage in heme and iron-sulfur (Fe-S) cluster synthesis. Ferritin has also a role as a cytoprotector against oxidative stress, a situation of increased levels of reactive oxidative species (ROS), a role accomplished by controlling the free intracellular iron levels. Early work by Balla *et al*, demonstrated a synergistic effect of heme and hydrogen peroxide treatment on cytotoxicity in porcine aortic endothelial cells, but when first pretreating the cells with only heme, they found the cells to resist the cytotoxicity of heme and hydrogen peroxide, an effect conferred by increased ferritin expression [Balla *et al*, 1992]. Orino *et al* analyzed HeLa cells stably transfected with L-Ft or H-Ft, under control by an inducible promotor, and when cells were challenged with hydrogen peroxide (H₂O₂) both ferritin subunits had protective effects when their expression was induced [Orino *et al*, 2001].

In addition, ferritin mRNA and protein expression have been found to be induced by pro-oxidants [Cairo *et al*, 1995, Orino *et al*, 2001]. The mechanism by how this is accomplished is not fully understood, but ROS may increase the free labile iron pool (LIP, see definition below) by both heme destruction and ferritin degradation. Thus, increased LIP will decrease IRP activities and ferritin synthesis will be stimulated on a post-transcriptional level. In addition, ROS is known as a mediator in signal transduction pathways and is implicated in up-stream activation of NF- κ B and H-Ft gene expression [Cairo *et al*, 1995, Pham *et al*, 2004]. Furthermore, TNF- α induced apoptosis was found to be inhibited by the

antioxidative effect of H-Ft simultaneously expressed in a NF- κ B dependent manner [Pham *et al*, 2004].

Mitochondrial ferritin

Levi *et al* recently reported of a mitochondrial ferritin (MtF) having ferroxidase activity and sharing high similarities with the H-chain of cytosolic ferritin. An intron less gene encodes a precursor protein of 242 amino acids, with a molecular mass of approximately 30 kDa, containing a 60 amino acids long mitochondrial targeting sequence. The mature protein has a molecular mass of 22 kDa [Levi *et al*, 2001]. The human MtF protein has 80 % sequence identity to the human H-Ft. Recombinant human MtF, expressed in *E. coli*, assembles into a 24-mer binding 200-300 iron atoms per subunit similar to cytosolic Ft [Langlois d'Estaintot *et al*, 2004]. Drysdale *et al* found MtF to have a limited expression in human tissues, and strong expression of the transcript was only found in the testis. In the other tissues examined the expression of MtF was very low according to Northern blotting. Immunostaining of bone marrow derived erythrocytes with MtF antibodies revealed low expression of MtF in normal cells, but very high expression in cells from patients with sideroblastic anemia, a disease associated with iron accumulation within the mitochondria as a result of dysfunctional heme synthesis. This indicated the protein expression to have potential to respond to cellular iron levels and a possible role for MtF in iron detoxification [Drysdale *et al*, 2002].

Divalent metal transporter 1

The divalent metal transporter 1 (DMT1) is an integral membrane protein, extensively modified by glycosylations having a molecular mass of 90-100 kDa. It is involved in both the dietary iron uptake in the duodenum and has a function as an iron exporter in the endosomes [Fleming *et al*, 1997, Gruenheid *et al*, 1999]. DMT1 is a proton coupled transporter of several metals including iron, zinc, manganese, cobalt, cadmium, nickel and lead [Gruenheid *et al*, 1995, Gunshin *et al*, 1997]. DMT1 localizes mainly in the recycling endosomes but to a lower extent it is also found on the plasma membrane where it colocalizes with the TfR. To be a functional metal transporter, DMT1 needs acidic pH and therefore it is only active as a transporter when located on the apical membrane of duodenal enterocytes or in acidic

endosomal compartments when endocytosed together with the Tf/TfR complex [Gruenheid *et al*, 1999].

Dietary iron absorption

Since Fe^{2+} readily oxidizes in contact with oxygen in the atmosphere, most of the inorganic iron in our diet is in the insoluble Fe^{3+} form, but the acidic pH in the intestine and the presence of the reductase duodenal cytochrome b (Dcytb) helps to dissolve and reduce the iron back to Fe^{2+} that can be taken up by the DMT1 located on the duodenal enterocytes [McKie *et al*, 2001]. Two thirds of the iron in our diet is heme iron, and heme is also taken up by the enterocytes but its transporter is not identified. Within the enterocyte iron is liberated from the heme structure and joins the fate of inorganic iron, where it can be stored in ferritin, utilized or exported through the basolateral side of the enterocyte by ferroportin-1, and reoxidized to Fe^{3+} by the extracellular and membrane anchored ferroxidase hephaestin or the soluble homolog ceruloplasmin. The Fe^{3+} is transported from enterocytes and delivered to circulating transferrins. The amount of iron taken up by the enterocytes is believed to be determined by regulators on different levels (i) dietary regulators constituted by the IRE and IRP system of the enterocytes, and hypothesized (ii) stores regulator and (iii) erythropoietic regulator. One suggested model is that the crypt cells get programmed during their differentiation to meet the demand of the body for iron and when they migrate to the villus as mature enterocytes they take up the iron required until they finally exfoliate. Recently, the peptide hepcidin expressed by the liver is suggested to be both stores and erythropoietic regulator. When iron stores of the body are low the expression of hepcidin is found to be increased and is suggested to stimulate iron absorption and simultaneously iron release from iron storing macrophages, and the opposite is believed to occur when iron stores of the body are high and hepcidin expression is decreased [Papanikolaou and Pantopoulos, 2005].

Cellular iron uptake and the labile iron pool

Two diferric transferrins bind the TfR on the cell surface and are together with the colocalizing DMT1 endocytosed by clathrin-coated pits. The early endosomes get acidified by H^+ -ATPase, a proton pump, facilitating the release of Fe^{3+} from the Tf/TfR complex.

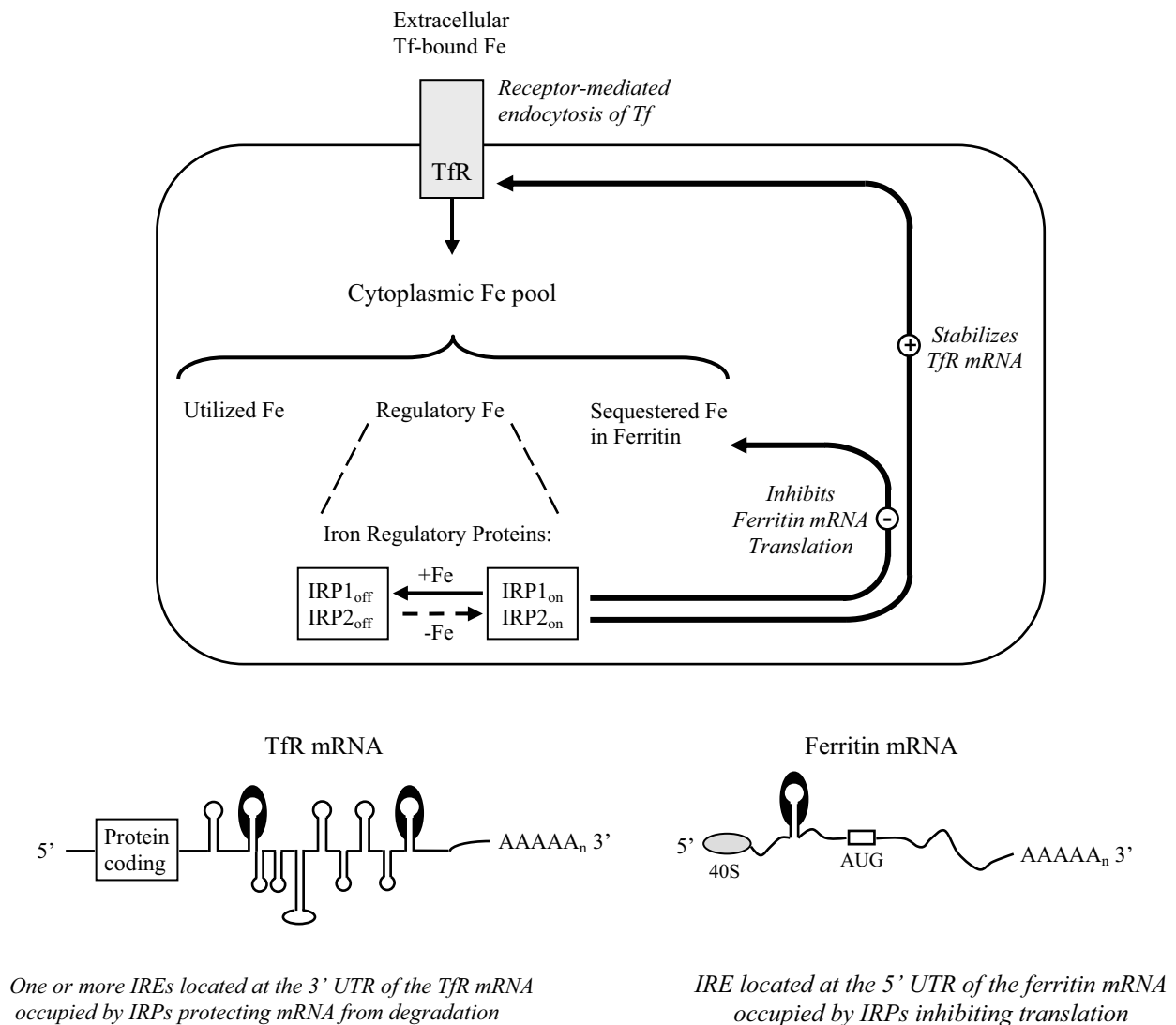


Figure 1. Cellular iron uptake and iron regulation by IRP1 and IRP2.

Before the iron is exported from the endosome it is reduced to Fe^{2+} by a so far unidentified reductase. DMT1 exports the divalent iron to the cytosol where the iron can be stored in ferritin, utilized by iron containing proteins or take part of the labile iron pool or LIP. The LIP constitutes a small fraction of about 5% of the total cellular iron. It is described as a low molecular iron pool of Fe^{2+} and Fe^{3+} , bound to low molecular weight ligands like phosphates, carboxylates but also to polypeptides and surface components of membranes. This iron is also defined as free iron accessible to cell permeant iron chelators. The LIP is supposed to be proportional to the total cellular iron and to be the iron that is sensed by the IRPs (Fig. 1) [Kakhlon and Cabantchik, 2002].

Prions and prion diseases

The annual incidence of human cases of prion diseases, also known as transmissible spongiform encephalopathies (TSEs), is low and over the years the public interest for these diseases has been sparse. Not until the breakout of the bovine spongiform encephalopathy (BSE) epidemic in the United Kingdom in the middle of the 1980's and later in 1996, when a new prion disease arose, the new variant Creutzfeldt-Jacob Disease (vCJD) affecting young people and obtained after ingestion of BSE infected meat, the mass medial interest increased. Scrapie in sheep, described as early as in the middle of 18th century, has never been considered a threat against human health since no cases of scrapie transmission to humans have been reported. In 1959, Gajdusek reported of a new epidemic neurodegenerative disease named kuru among the Fore people in Papua New Guinea [Gajdusek and Zigas, 1957]. Resemblances were observed between scrapie, kuru and familial Creutzfeldt-Jacob disease (fCJD). These studies further led to transmission studies of kuru and CJD to chimpanzees [Gajdusek *et al*, 1966, Gibbs *et al*, 1968]. With indications that these diseases all were transmissible, the theory of a slow virus arose. However, the scrapie agent was found to be highly resistant to standard methods used to inactivate viruses containing DNA or RNA. Instead, the infectious agent was sensitive and declined under denaturing conditions or proteolytic treatment [Diener *et al*, 1982]. In 1982, Stanley Prusiner introduced the term prion, a word jumble of its definition; a *proteinaceous infectious* particle resistant to inactivation of nucleic acid-modifying procedures [Prusiner, 1982]. In the middle of the 1980s a protein that co-purified with the infectious agent was partly sequenced leading on to the identification and cloning of the gene. The gene was found to encode a normal protein, referred to as the cellular prion protein or PrP^C, and it was suggested that PrP^C was post-translationally modified into a disease associated form, referred to as the scrapie prion or PrP^{Sc} [Basler *et al*, 1986, Kretzschmar *et al*, 1986, Oesch *et al*, 1985, Prusiner *et al*, 1984]. The choice of the Sc-denotation is made because scrapie is considered to be an archetype prion disease.

Human prion diseases and neuropathology

Human prion diseases are often categorized according to their different etiologies being sporadic, inherited or acquired. The classical histopathological lesions include spongiform

degeneration, astrocytosis and neuronal loss. Some subtypes are also associated with deposition of extracellular amyloid plaques containing high levels of PrP^{Sc}. The diseases are highly variable in their onset, duration, pathology and clinical signs but unified by the presence of PrP^{Sc} and by the property of being transmissible within and/or between species. There is also a high variability in the brain areas affected between the diseases, including the entire or only certain areas or layers of cortex, and sometimes also including basal ganglia and cerebellum. Clinical signs of prion diseases can be cognitive impairments, gait, limb ataxia, mental signs, visual signs, myoclonus, insomnia, hallucinations and dementia. Prion diseases are rare with sporadic CJD (sCJD) being the most common of them, with a yearly incidence of 1-2 per million worldwide. Sporadic and familial prion diseases are associated with methionine/valine polymorphism in codon 129, affecting the onset, severity of clinical signs and pathological lesions. The most common forms of sCJD have an onset at 60-65 years of age and clinical duration of 4-6 month [Gambetti *et al*, 2003].

The familial human prion diseases are fCJD, Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI). Today a large number of mutations are identified in the prion protein gene (*Prnp*), associated with the familial TSEs, including missense point mutations, octarepeat mutations resulting in addition or deletion of repeats leading to increased formation of the pathological form of the prion protein.

The acquired prion diseases include iatrogenic CJD (iCJD), vCJD and kuru. Iatrogenic transmission of prion diseases can occur through treatment with prion contaminated human growth hormones, human dura mater grafts, neurosurgical instruments, depth electrodes and corneal transplants. vCJD is a new prion disease acquired after ingestion of prion-infected meat from cattle having BSE. The first cases arose 1995 in the United Kingdom and until 2003 there were approximately 150 confirmed cases of vCJD world wide, all of them methionine homozygotes in codon 129. vCJD shows different peripheral pattern as compared to other CJDs, which might increase the risk for transmitting vCJD through blood transfusions, surgical instruments and organ transplants also before clinical onset of the disease [Will, 2003]. Kuru had an epidemic spreading among people of the Fore tribe in Papua New Guinea during the mid-1950s. The disease is believed to be transmitted through cannibalism [Collins *et al*, 2001].

The normal prion protein and the pathological prion

The function of the normal cellular prion protein PrP^C is still unknown but often studied in prion deficient mice models. The studied loss of function in these models may also provide keys to understand the neuropathology observed in prion diseases, since PrP^C when converted into PrP^{Sc} is likely to lose its normal function. Therefore it is also difficult to separate the discussion of PrP^C function from the pathological mechanisms of the prion diseases.

Structure and expression of the normal prion protein, PrP^C

PrP^C consists of approximately 250 amino acids and after post-translational modifications the final mature protein consists of approximately 210 amino acids with a molecular weight of 35 kDa. The unprocessed sequence of PrP^C includes an N-terminal signal sequence and a glycine-rich octamer repeat domain. Further, PrP^C consists of a highly conserved hydrophobic core sequence and a C-terminal hydrophobic segment that is a signal for a glycosylphosphatidylinositol (GPI) anchor addition [Harris, 1999, Millhauser, 2004, Riesner, 2003]. PrP^C is synthesized in rough endoplasmic reticulum (ER), transits through Golgi and on its way to maturation PrP^C undergoes several post-translational modifications. Attachment of the GPI-anchor associates the protein to the cell surface in micro domains known as lipid rafts that are short lived membrane structures rich in cholesterol. As part of the normal biosynthesis, small amounts of transmembrane spanning molecules of PrP are formed. They have two different orientations with the N-terminal (^{Ntm}PrP) or the C-terminal part (^{Ctm}PrP) of the polypeptide chain on the extracellular side of the membrane [Hegde *et al*, 1998, Holscher C, *et al*, 2001]. After attachment to the cell surface, PrP^C may also be post-translationally cleaved within the GPI-anchor, probably by a cell surface phospholipase and released into the extracellular space and by a protease within the hydrophobic core [Harris *et al*, 1999].

Conformational and biochemical differences between PrP^C and PrP^{Sc}

Structural analysis by Pan *et al* of purified PrP^C revealed a content of 42% α -helical structure and 3% β -sheet, while PrP^{Sc} consists of 43% β -sheet and approximately 30% α -helices [Pan *et al*, 1993]. The precise content of the secondary conformations like α -helical,

β -sheet, β -turns and random coils is still uncertain; however there is an agreement that the PrP^{Sc} form has a higher β -sheet content compared to PrP^C. The levels of PrP^C are similar in both healthy and scrapie-infected hamster brains, but can be separated from PrP^{Sc} due to different biochemical properties. Proteinase K (PK) digestion completely degrades PrP^C whereas digestion of PrP^{Sc} leads to an approximately 90-231 prototypically resistant core with a molecular size of 27-30 kDa (PrP²⁷⁻³⁰). PrP^C and PrP^{Sc} can also be separated based on different solubility in detergents like sarkosyl, where PrP^C is soluble and found in the supernatant whereas PrP^{Sc} precipitates to the pellet fraction [Meyer *et al*, 1986]. Furthermore, PrP^{Sc} is less sensitive to cleavage of the GPI anchor by phosphatidylinositol-specific phospholipase C (PIPLC) and is retained to the plasma membrane to a higher extent as compared to PrP^C [Lehmann and Harris, 1996].

Cellular location and mechanism of conversion of PrP^C to PrP^{Sc}

The site of conversion of PrP^C to PrP^{Sc} is still under debate, and it is possible that the site and the mechanism of conversion differ between prion diseases with different etiologies. In the familial diseases, point mutations or insertions in *Prnp* are suggested to thermodynamically favor a spontaneous conversion, or influence the contact of PrP^C with other components resulting in a misfolding, possibly during maturation in the ER [Ivanova *et al*, 2001]. In sporadic cases the *de novo* formation might be considered as a rare stochastic event, or possibly a consequence of other still unidentified factors inducing or favoring the conversion. In a cell culture study, PrP^C was shown to be subject for ubiquitination and degradation by the proteasome. When proteasomes are blocked or when misfolded proteins delivered to the cytosol exceed the capacity of protein quality control, PrP^C precursors might be converted to PrP^{Sc} and result in aggregates. If these aggresomes are long lived they could provide a seed for igniting sporadic or familial forms of prion diseases often appearing late in life [Cohen and Taraboulos, 2003, Yedidia *et al*, 2001]. In prion diseases associated with infection by exogenous prions the conformational change could take place on the cell membrane or after internalization to endosomal and lysosomal compartments [Borchelt *et al*, 1992, Prado *et al*, 2004, Taraboulos *et al*, 1992].

PrP^{Sc} formation is suggested to be self replicative according to one of the following mechanisms:

- Refolding model: Spontaneous refolding of PrP^{C} to PrP^{Sc} is prevented by a high energy barrier, but certain conditions could promote PrP^{C} unfolding, and contact with an incoming PrP^{Sc} molecule results in the adoption of the PrP^{Sc} conformation. PrP^{Sc} is considered as the template and catalyst of the reaction. The refolding might be facilitated by chaperones (Fig.2A.) [Cohen and Prusiner, 1998].

- Nucleation or seeding model: PrP^{C} and PrP^{Sc} are in equilibrium, strongly in favor of PrP^{C} . PrP^{Sc} can be stabilized if it aggregates. Formation of the aggregates is suggested to be slow, but when the aggregates are formed, monomer additions to the aggregates occur rapidly. The aggregates are assumed to occasionally be fragmented, further seeding the reaction, possibly explaining the exponential production of PrP^{Sc} observed once the reaction is initiated (Fig.2B.) [Caughey *et al*, 1997, Come *et al*, 1993, Krishnan and Lindquist, 2005].

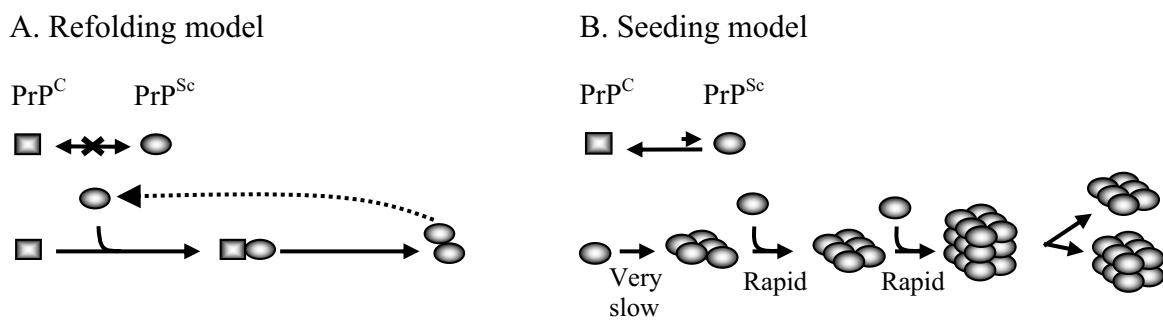


Figure 2. Models for the conformational conversion of PrP^{C} to PrP^{Sc}

TSE strains and the species barrier of TSE transmission

The conformational change of PrP^{C} in different species, individuals and populations does not result in just a single form of PrP^{Sc} , but also in so called strains of PrP^{Sc} giving them specific characteristics obtained from their conformations. The strains have characteristic features like specific incubation periods preceding the onset of the disease, neuroanatomic distribution, degree of PK resistance, resistance to chemicals and heat, causing different neuropathological lesions and ease of transmission to new species. Differences in glycosylation and amino acid polymorphism may add to the diversity [Bruce, 2003]. Some interspecies transmissions are prevented, e.g. scrapie from sheep to humans, whereas others like BSE transmission to humans may occur if the recipients are methionine homozygotes at codon 129. The species barrier has also been observed to be reduced in transgenic mice

producing human, ovine and bovine PrPs when infected with the respective PrP^{Sc} containing inoculums [Crozet *et al*, 2001, Scott *et al*, 1997, Telling *et al*, 1994].

Function of PrP^C and possible links to the prion pathology

PrP^C is mainly expressed in the central nervous system (CNS) in neurons, and glia cells where it is concentrated to synapses. It is also found to be ubiquitously expressed in peripheral tissues including spleen, lymph nodes and leukocytes. The concentration of PrP^C to synapses suggests a possible role in signal transduction or in electrical excitability and the possibility of implications of synapses in the TSE pathology [Fournier *et al*, 1995]. A study on chicken PrP^C has also demonstrated the protein to be constitutively endocytosed; perhaps a mechanism to inhibit a signaling pathway, or possibly the endocytosis offers an uptake mechanism of copper, as will be discussed below [Pauly and Harris, 1998, Shyng *et al*, 1993].

Mice homozygous for genetic disruption in the open reading frame, *Prnp*^{0/0} (Zürich I) and *Prnp*^{-/-} (Edinburgh), were reported to develop and reproduce normally and showed no clinical symptoms or any significant behavioral differences as compared to wild type mice [Büeler *et al*, 1992, Manson *et al*, 1994]. Later reports have demonstrated changes in the circadian rhythm, disruption of calcium activated K-currents and electrophysiological studies have verified weakened gamma aminobutyric acid (GABA) -ergic inhibition and impaired long term potentiation, however, others have presented conflicting data showing normal synaptic activity and excitability in the hippocampus [Colling *et al*, 1996, Collinge *et al*, 1994, Lledo *et al*, 1996, Tobler *et al*, 1996]. Since the list of these later observed phenotypes of the *Prnp* knock out mice is growing, it seems reasonable that a part of the pathology could be explained by loss of function of PrP^C in prion diseases, but does not explain the severe neuropathology observed in prion diseases. It is also conceivable that during the prion diseases, PrP^C still has a residual function, a distinction from the total deletions in the mice discussed above. In addition, there is always a risk that the true functional loss after ablation of a gene expression gets compensated for in the embryo and during the path of development. In an attempt to avoid this scenario Mallucci *et al* made a transgenic mouse in which the *Prnp* knockout was targeted to neurons in the adult mouse [Mallucci *et al*, 2002]. During the generation of this mouse, made on a *Prnp*^{0/0} genetic background, they found that

reintroduction of PrP expression rescued the neurophysiologic phenotype of the *Prnp*^{0/0} mouse, supporting previous findings by Colling *et al* [Colling *et al*, 1996]. After the adult-onset knock-out of *Prnp* in neurons, completed after the 12th week, the mouse continued to show normal phenotype and brain morphology. However, they displayed neurophysiological changes in hippocampal CA1 cells, again supporting the role of PrP^C as a modulator of synaptic transmission [Colling *et al*, 1996, Mallucci *et al*, 2002]. Prion protein deficient mice have also revealed PrP^C to be crucial for the infection and pathology of prion diseases. *Prnp*^{0/0} mice were shown to be resistant against scrapie disease after intracerebral injections with scrapie prion containing brain homogenate. Animals carrying a single allele *Prnp*^{0/+} had a later onset of the disease 253-322 days after infection compared to *Prnp*^{+/+} with an onset 134-146 days after infection [Büeler *et al*, 1993]. Mallucci *et al* showed that PrP^C expression in neurons is a prerequisite for the neurotoxicity during scrapie infection. Using the conditional *Prnp* knockout mouse they demonstrated that neuronal depletion of PrP^C in animals with established scrapie infection rescued the mice and reversed the neurodegeneration, despite the generation of PrP^C and subsequently PrP^{Sc} in non-neuronal cells [Mallucci *et al*, 2003]. PrP^C has also been shown to protect against Bax-mediated apoptosis, and the octa-repeat region to be necessary for this function [Bounhar *et al*, 2001]. In addition, since PrP^C seems to be essential for propagation of PrP^{Sc} and neurodegeneration, PrP^C may participate in the neurodegenerative cascade. Solforosi *et al* found cross linking of PrP^C with monoclonal antibodies to trigger apoptosis, suggesting the possibility that PrP^{Sc} similarly binds PrP^C and promotes cell death through such mechanism [Solforosi *et al*, 2004].

PrP^C binds metals -implications in oxidative stress in prion diseases

PrP^C and copper

Hornshaw *et al* were the first to demonstrate the property of PrP^C to bind copper (Cu). In an *in vitro* study they found copper to bind to peptides corresponding to three or four octarepeats of the mammalian PrP^C as analyzed by mass spectrometry [Hornshaw *et al*, 1995]. Later, an *in vivo* study supported the copper binding property of PrP^C. Membrane preparations of brains from wild-type and *Prnp* ablated (*Prnp*^{0/0}) mice were analyzed using

X-ray fluorescence, and the wild type preparations contained 15-fold higher copper levels as compared to *Prnp*^{0/0} mice [Brown *et al*, 1997c]. Another study using recombinant Syrian hamster prion protein PrP^C29-231 verified that PrP^C selectively binds copper as compared to other metal ions, the binding to be pH dependent, and at physiological pH two cupric ions (Cu²⁺) were complexed to four octarepeats by histidine residues [Stöckel *et al*, 1998]. Further, it was demonstrated that Cu²⁺ stimulated endocytosis of PrP^C, and the N-terminal sequence including the octarepeats region was essential for this function, as studied in mouse neuroblastoma cells (N2a) stably transfected with wild type and N-terminal deleted sequences of chicken prion protein, analyzed after surface labeling with iodine or biotin and immunoprecipitation [Pauly and Harris, 1998].

Brown *et al* found PrP^C deficient cerebellar cells from the *Prnp*^{0/0} mice, derived by Büeler *et al*, to have decreased cytosolic copper/zinc superoxide dismutase (Cu/Zn SOD) activity as analyzed by a gel-shift assay. Brown *et al* suggested the decrease in the enzyme activity to be a consequence of interrupted copper metabolism since PrP^C could function as a copper reservoir or shuttle [Brown *et al*, 1997b, Brown *et al*, 1997c, Büeler *et al*, 1992]. In addition, Brown *et al* found prion protein deficiency in cerebellar cells to correlate with increased susceptibility to oxidative stress induced by xanthine oxidase, and higher frequency of cell death, as analyzed by a cell viability test, an effect observed to be reversed by vitamin E [Brown *et al*, 1997b]. Also, clones of rat pheochromocytoma cells (PC12 cells) selected for their increased resistance to copper and oxidative stress had increased expression of PrP^C according to Western-blotting, indicating a possible function of PrP^C in copper metabolism and oxidative cellular balance [Brown *et al*, 1997a]. Brown and Besinger further analyzed mice expressing different levels of PrP^C and found the expression to correlate with increased levels of Cu/Zn SOD activity, without affecting the mRNA or protein expression of Cu/Zn SOD according to Northern and Western-blotting, and suggested that PrP^C aids the incorporation of copper into Cu/Zn SOD. In addition, they found mice overexpressing PrP^C to have increased glutathione peroxidase activity [Brown and Besinger, 1998]. In 1999, Brown *et al* broadened their concept and claimed recombinant mouse and chicken PrP^C to have an activity like that of SOD. The increased SOD activity was analyzed as the decreased formation of a formazan product after the addition of xanthine oxidase generating superoxide [Brown *et al*, 1999]. The mechanism behind how PrP^C

affects SOD activity has so far not been revealed and data are conflicting. Opposing data was presented in a study comparing mice with different PrP^C expression: wild type mice, Prnp^{0/0} mice and mice overexpressing PrP^C 10 times the normal level. When analyzing synaptic preparations of brains from these mice using inductively coupled plasma mass spectrometry it was found that the mice have similar levels of copper in their brains, similar Cu/Zn SOD protein levels and activities independent of the expressed level of PrP^C [Waggoner *et al*, 2000]. Further, Hutter and colleagues analyzed crosses of mice lacking the Cu/Zn SOD gene, PrP^C gene and mice overexpressing PrP^C, without detecting any contribution of PrP^C to the total SOD activity or support for PrP^C to possess SOD like activity as analyzed by inhibition of formazan production in synaptosome enriched brain fractions [Hutter *et al*, 2003].

Metals and oxidative stress in prion diseases

Several investigators have reported the presence of oxidative stress in a variety of prion diseases and scrapie-infected cells or animals, as indicated by changes in levels of antioxidative enzymes, increased levels of markers for lipid peroxidation and protein oxidation. Studies of neuronal cells from the Zürich I and Edinburgh PrP^C deficient mice have demonstrated increased sensitivity to hydrogen peroxide, decreased activity of glutathione reductase (GR) and increased levels of protein oxidation and lipid peroxidation [White *et al*, 1999, Wong *et al*, 2001a]. Choi *et al*, used scrapie-infected hamsters to analyze tissue homogenates of cerebral cortex, cerebellum and brainstem, and reported decreased manganese SOD (Mn SOD) activity, thus increasing the superoxide anion (O₂^{•-}) production in the mitochondria, perhaps explaining the detected lipid peroxidation and mitochondrial dysfunction, analyzed as decreased ATPase and cytochrome c activities. The activities of Cu/Zn SOD and catalase were unchanged whereas glutathione peroxidase (GPX) and GR activities were increased [Choi *et al*, 1998]. Augmented oxidative stress as well as increased susceptibility to oxidative stress has also been verified in scrapie-infected hypothalamic cells (GT1). Treatment of the cells with the glutathione depleting drug buthione sulfoxide or the nitric oxide donor SIN-1, decreased cell viability as analyzed by the MTT test in scrapie-infected GT1 (ScGT1) cells as compared to control cells. Further, ScGT1 cells had decreased GR and GPX activities opposing the data from the scrapie-infected hamster

described above. This discrepancy can perhaps be explained by the presence of activated microglia in tissue homogenates [Choi *et al*, 1998, Milhavet *et al*, 2000]. Decreased activities of glutathione-enzymes were followed by decreased levels of SOD activities as well as decreased protein levels of total SOD suggesting increased production of $O_2^{\bullet-}$, perhaps reacting with NO forming the reactive nitrogen species peroxynitrite ($ONOO^-$) [Milhavet *et al*, 2000]. A possible involvement of iron in oxidative stress was demonstrated in a study in scrapie-infected mice where increased total iron level, decreased ratio of Fe^{2+}/Fe^{3+} and the occurrence of lipid peroxidation was demonstrated in cerebral cortex, striatum and brainstem, as analyzed by atomic absorption, spectrophotometric measurements of iron, malondialdehyde and thiobarbituric acid, respectively [Kim *et al*, 2000].

Metal perturbations were also detected by Wong *et al* in scrapie-infected mice, but they also reported that strain-variations in the infected mice were observed. Mice infected with 139A strain had decreased levels of Fe^{2+} , whereas the ME7 strain induced slightly increased Fe^{2+} levels, as analyzed by mass spectrometry. Both strains induced decreased GPX activities and increased levels of NO and $O_2^{\bullet-}$, analyzed spectrophotometrically [Wong *et al*, 2001b]. A recent study on brains from prion disease affected individuals analyzed markers of oxidative damage, by immunocytochemistry, and the presence of redox active iron, by modified Perl staining. They reported a pattern where sCJD patients differed from GSS and vCJD patients. Sporadic CJD, perhaps as a result of the shorter duration of the disease, showed no redox active metals associated with PrP^{Sc} deposits as found in both GSS and vCJD patients. Neither were ROS, lipid peroxidation, elevated heme oxygenase 1 levels or glycation found in sCJD, whereas these changes were found to be associated with GSS and vCJD. In GSS, ferritin was also found associated with some amyloid plaque formations [Petersen *et al*, 2005]. These findings suggest there are resemblances with observations from Alzheimer's disease, where iron is associated with deposits like senile plaque and neurofibrillary tangles [Smith *et al*, 1997].

To analyze the mechanism of the PrP^{Sc} specific toxicity in prion diseases, a synthetic peptide fragment of the human prion protein; PrP106-126, shown to mimic properties of PrP^{Sc} like forming aggregates and fibrils, has often been used, even though this fragment is not found *in vivo*. PrP106-126 is reported to produce hydrogen peroxide in the presence of Cu^{2+} and further with addition of Fe^{2+} to produce hydroxyl radicals (OH^{\bullet})

through the iron catalyzed Fenton reaction (Fenton reaction will be discussed below) [Turnbull *et al*, 2003]. This is analogous with findings that amyloid- β (A β) 1-40, 1-42 and α -synuclein, localized to aggregates in Alzheimer's disease and Parkinson's disease, respectively, also produce OH \cdot in the presence of Fe $^{2+}$, probably as a secondary event after prior production of H $_2$ O $_2$, a reaction also believed to be metal catalyzed [Tabner *et al*, 2002]. Interestingly, Huang *et al* have shown that the A β peptide binds Fe $^{3+}$ and produces H $_2$ O $_2$, while simultaneously reducing the iron to Fe $^{2+}$ [Huang *et al*, 1999].

Iron and its role in neurotoxicity

Understanding iron-metabolism in the CNS will provide insights to how and to which extent iron deficiency, overload or misregulation of iron metabolism have a role in the development of the nervous system and neurodegeneration as well as in acute diseases as a result of trauma, hemorrhage and ischemia. The iron regulation in the brain is quite complex. The iron is heterogeneously distributed and the proteins of iron regulation are differently expressed in various brain cells reflecting cell specific demand for iron usage and storage. The concentration of iron normally increases with age, especially in certain brain areas coinciding with regions that are also affected by pathological changes in several neurodegenerative diseases.

Production of reactive oxygen species

Electrons occupy a space known as molecular orbitals. Each orbital can hold only two electrons of opposite spin-directions, this is known as the *spin restriction*. Free radicals are defined as a molecular species with unpaired electrons, thus qualifying molecular oxygen O $_2$ as a free-radical since it holds two spin-aligned electrons in the π -orbitals. Because of the spin restriction O $_2$ can only accept one electron at a time in a step-wise manner. When oxygen accepts only one electron, the O $_2^{\cdot-}$ is formed. The O $_2^{\cdot-}$ is not a highly reactive radical, but if its production is increased and not removed by cellular antioxidative mechanisms, it accumulates and can be converted into ROS that are more reactive and able to damage the cell. The brain has a very high metabolic activity and high consumption of O $_2$. The oxygen is to 95% consumed during the respiration occurring in the mitochondria of the cell where electrons flow from relatively reduced molecules to O $_2$ reducing it to

water. Since the brain only constitutes approximately 2% of the body weight but consumes 20% of the oxygen respired, it makes it a potentially high producer of ROS. Other factors putting the brain at risk for oxidative damaging mechanisms are its high content of polyunsaturated fatty acids (PUFA), high content of iron and a low antioxidative defence relative to other organs [Crichton *et al*, 2002, Halliwell, 1992, Reiter, 1995].

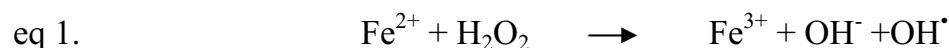
The main part of the ROS is produced by cells during normal respiration in the mitochondria. Briefly, the electron transport begins with electrons delivered to complex I and II from reduced nicotinamide adenine dinucleotide (NADH) or reduced flavin adenine dinucleotide (FADH₂), respectively, and the electrons are further transferred to the ubiquinol/ubiquinone (UQH₂/UQ) cycle. UQH₂ transfers electrons to complex III and reduces cytochrome c, which further delivers electrons to complex IV, also known as cytochrome c oxidase, finally passing four electrons, one by one, to molecular oxygen creating water. The electron flow is spontaneous and exergonic, and potential energy is built up and stored in a proton gradient over the inner membrane of the mitochondria, since the electron-flow is accompanied by proton transport into the intermembrane space by complex I, III and IV. This energy is transformed into biological work when ATP is synthesized by ATP synthase, and captured as chemical energy in the ATP molecule to be used in several metabolic pathways [Nicholls and Budd, 2000]. Generally, partially reduced intermediates of oxygen are tightly bound to cytochrome c, however occasionally single electrons leak directly to O₂ resulting in the formation O₂^{•-}.

O₂^{•-} is also produced by macrophages and in the brain by microglia upon activation by endogenous or exogenous pathological stimuli, like neuronal dysfunction/death, protein aggregation or invading bacteria [Vilhardt, 2005]. Cells are normally equipped with SOD, enzymes able to scavenge the O₂^{•-} and convert it to H₂O₂. There are two isoenzymes with SOD activity; mitochondrial Mn SOD and cytosolic Cu/Zn SOD. A different source of production of H₂O₂ specific for neurons is the oxidative deamination of dopamine by monoamine oxidase, possibly contributing to the oxidative stress observed in dopaminergic neurons in patients with Parkinson's disease [Halliwell, 1992, Reiter, 1995].

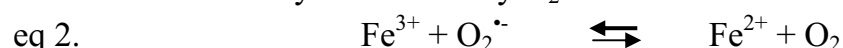
H₂O₂ is due to the stability of the dioxygen bond, not considered as a strong oxidant. However, electrons of the d-orbitals of transition metals like iron and copper may interfere with the double bond and catalyze the formation of the OH[•] [Imlay, 2003]. The LIP

constitutes a readily available pool of redox active iron as demonstrated by Kahklon *et al.* The repression of ferritin expression in human erythroleukemia cells by antisense technique, resulted in the increase of the calcein accessible and chelatable LIP and the increased free iron was accompanied by an increased ROS production [Kahklon *et al.*, 2001].

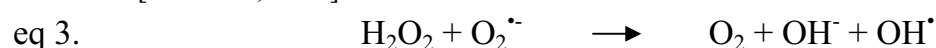
Fenton reaction:



The Fe^{3+} formed may be reduced by $\text{O}_2^{\bullet-}$:



When combining eq 1 with eq 2, the result is the overall iron catalyzed *Haber-Weiss reaction* [Halliwell, 1992]:



Reactivity of reactive oxygen species

$\text{O}_2^{\bullet-}$ is considered a weak oxidant of biomolecules like proteins and nucleic acids since its anionic charge decreases its reactivity towards electron rich biomolecules, however, both $\text{O}_2^{\bullet-}$ and H_2O_2 are able to react with transition metals and both have been shown to oxidize accessible [4Fe-4S] clusters resulting in $[3\text{Fe-4S}]^+$ clusters and the liberation of one potentially toxic Fe^{2+} ion [Imlay, 2003]. As shown in eq 2, $\text{O}_2^{\bullet-}$ can also act as a reductant and has also been shown to release iron stored within ferritin as Fe^{3+} [Biemond *et al.*, 1984].

The most reactive radical of all radicals is the hydroxyl radical, able to oxidize most of the biomolecules in its vicinity immediately after its formation. The DNA itself is an excellent platform for Fenton chemistry, since nucleic acids have been shown to have efficient iron chelating properties [Kruszewski and Iwanenko, 2003, Rai *et al.*, 2001]. The OH^\bullet formed reacts with the DNA and causes changes, being potentially mutagenic or lethal, like strand breaks in the phosphate back-bone, alterations of the deoxyribose and the purine and pyrimidine bases [Cadet *et al.*, 2003, Reiter, 1995].

The OH^\bullet also reacts with lipids like PUFA by removing one hydrogen atom hence creating a carbon centred radical. After rearrangements into a conjugated diene, it reacts with molecular O_2 and forms a peroxy radical (ROO^\bullet). The peroxy radical can abstract a hydrogen atom from another PUFA and a chain reaction is initiated, and unless it is

stopped by an antioxidant like vitamin E, lipid peroxidation may impair or destroy the integrity of the cell membrane and result in ion permeability of e.g. calcium ions [Farooqui and Horrocks, 1998, Halliwell, 1992].

Protein oxidation by OH^\bullet can result in cross-linking of proteins, and especially vulnerable are metal containing proteins, where H_2O_2 may produce OH^\bullet by Fenton reaction, and give rise to site-specific damage resulting in possible loss of protein functionality [Reiter, 1995].

Oxidative stress and regulation of the IRPs

IRP1 is not only regulated by iron, but can also be modulated by ROS and nitric oxygen species; the latter will be discussed below. IRP1 activity was found to be increased in Chinese hamster ovary cells and in murine fibroblasts after H_2O_2 exposure, as analysed by the band-shift assay, however, this effect was only observed in intact cells and not after addition of H_2O_2 to cell lysates [Martins *et al*, 1995, Pantopoulos and Hentze, 1995]. The latter finding has also been observed and confirmed by others [Bouton *et al*, 1996, Brazzolotto *et al*, 1999, Pantopoulos and Hentze, 1998]. In a step to resolve the mechanism to the activation of IRP1 and to address the question whether H_2O_2 directly could interact and disrupt the Fe-S cluster of IRP1 or if other components were required for the activation, Pantopoulos and Hentze used streptolysin-O permeabilized murine fibroblasts and found them to increase their IRP1 activity in response to H_2O_2 similarly as non-permeabilized control cells. Next, when treating membrane-free cytosol fractions with H_2O_2 , they could conclude that component(s) localized in the cell membrane were essential for the activation of IRP1. They further showed the activation of IRP1 to be temperature dependent, and to be abolished by the addition of calf intestinal alkaline phosphatase and non-hydrolyzable nucleotide triphosphate analogs. Consequently they proposed that extracellular administration of H_2O_2 activates IRP1 through receptor mediated signaling possibly involving kinases, not through direct interaction with the Fe-S cluster of IRP1 [Pantopoulos and Hentze, 1998].

Opposite to above discussed data, Cairo *et al* performed an *in vivo* study using rats treated with the glutathione depleting drug phorone to induce oxidative stress, that IRPs, and preferentially IRP2 (formerly known as IRF_B), had reduced activities. They also

detected increased mRNA levels of both H-Ft and L-Ft and increased ferritin protein synthesis. In an initial stage of the phorone treatment they also observed a drop in the ferritin levels, as a result of Ft degradation contributing to increased levels of free iron in the liver tissue [Cairo *et al*, 1995]. In a second study by Cairo *et al*, it was found that the combination of $O_2^{\bullet -}$ and H_2O_2 , produced by xantine oxidase in rat liver lysate, decreased the activity of IRP1. The addition of 2-mercapto ethanol (2-ME) abolished the decreased IRP1 activity, hence implicating the existence of a disulfide bridge between cysteine residues in the IRE binding cleft of the protein resulting in inhibition of the IRE binding [Cairo *et al*, 1996]. Further, Orino *et al* in a study of H_2O_2 -treated HeLa cells also found increased mRNA expression of H-Ft and increased protein levels of both H-Ft and L-Ft. In addition, they reported of a transient increment in the total IRP activity induced by H_2O_2 [Orino *et al*, 2001]. Interestingly, a recent report by Mehrlhase *et al*, suggests a mechanism for iron release from oxidative-ferritin degradation by the proteasome, possibly providing a mechanism for decreased activities of IRPs observed during oxidative stress [Mehrlhase *et al*, 2005].

Antioxidative defence in the central nervous system

Astrocytes have a guardian role, protecting neurons against oxidative stress in the brain. The astrocyte contains high levels of the antioxidative enzyme GPX and is the only cell type to express the metal chelating proteins ceruloplasmin and metallothionein in the nervous system [Hussain *et al*, 1996, Patel *et al*, 2002, Wilson, 1997]. In addition, the antioxidative defence of the brain relies on low molecular weight reductants like lipid soluble vitamin E, the tripeptide glutathione (GSH) and ascorbate. The enzymes Cu/Zn SOD and Mn SOD are included in the antioxidative defence, catalyzing the conversion of $O_2^{\bullet -}$ into H_2O_2 . In addition, there are catalase and GPX, two enzymes that degrade H_2O_2 to H_2O . The reduction of H_2O_2 to H_2O by GPX, mainly occurring in the mitochondria, involves electron donation by GSH and its oxidation to glutathione disulfide (GSSG). GSH is regenerated by GR using NADPH as electron donor. Catalase residing within the peroxisomes converts H_2O_2 into H_2O and O_2 [Dringen *et al*, 2005]. Both catalase and GPX activities increase during brain injury [Goss *et al*, 1997].

Iron and the Central Nervous System

Iron must pass the blood brain barrier (BBB) or the blood cerebrospinal fluid (CSF) barrier and, similar to the uptake by the intestinal mucosa, the passage involves transfer across the apical side of the endothelial cell, through the cytosolic compartment and across the basolateral side into the interstitium of the brain or the CSF. The blood vessels of the brain are formed by capillary endothelial cells joined together by tight junctions. End-feet processes of perivascular astrocytes with the endothelial cells along with interactions of other neuronal cells contribute to the properties of the BBB, ensuring specificity of the nutrients transported to the brain [Abbott, 2005].

The main-part of the iron enters the brain through the blood-brain barrier, and most of it by TfR-mediated endocytosis of the Tf-Fe complex. The iron is released within the endosomes of the capillary endothelial cells, and probably removed from the endosomes by the DMT1 and further exported into the interstitium of the brain by ferroportin-1 [Wu *et al*, 2004, Burdo *et al*, 2001]. Alternative mechanisms of iron transport through the BBB are suggested, like transferrin and non-transferrin mediated transcytosis across the capillary endothelial cells [Burdo *et al*, 2003]. A minor part of the total brain iron may enter the CSF from the blood through the choroid plexus epithelial cells by mechanisms including transport of Tf-Fe complex by both receptor-mediated endocytosis and Tf-Fe transcytosis [Moos and Morgan, 2000]. After the iron has entered the interstitium of the brain or the CSF of the ventricles it is complexed mainly by Tf, synthesized within the brain by oligodendrocytes and presumably the choroid plexus epithelial cells [Connor and Fine, 1987, Gerber and Connor, 1989]. Tf bound iron may next be transported to the different brain cells and internalized by TfR mediated endocytosis. The TfR is localised and expressed throughout the brain and found on brain capillary endothelial cells, in neurons, choroid plexus epithelial cells and on glial cells [Giometto *et al*, 1990, Jefferies *et al*, 1984].

Different roles of the brain cells in the brain iron homeostasis:

- Oligodendrocytes play an important role for the brain iron homeostasis, they contain the highest amount of iron of all brain cells and the iron is pivotal for myelin formation. They contain abundant levels of both L-Ft and H-Ft subunits, and since they also are the main producers of Tf in the brain, they have an important role for the total brain iron homeostasis. However, their iron uptake mechanism is not fully understood, since white

matter, in relative terms, shows low immunoreaction for TfR antibodies [Dickinson and Connor, 1998].

- Microglia, containing high levels of iron, expresses both chains of Ft, but predominantly the L-Ft, have a pivotal role in detoxification and storage of iron [Barron, 1995].
- Neurons take readily up iron for utilization, contain relative to other brain cells abundant levels TfR. Neurons contain relatively low levels of total Ft, but mainly the H-Ft isoform is expressed, this implicates low iron storage, high iron utilisation and possibly export of excess iron by ferroportin-1 [Connor *et al*, 1994, Wu *et al*, 2004].
- Astrocytes are relatively low in iron and express low levels of TfR and Ft, but interestingly they do express a GPI-anchored form of ceruloplasmin, which has been shown to be necessary for neuronal iron export [Patel *et al*, 2002].

Iron and neurodegenerative diseases

Alzheimer's and Parkinson's disease

Iron imbalance and oxidative stress are well documented pathological changes in both Alzheimer's (AD) and Parkinson's disease (PD). Analysis of iron levels and iron related proteins in various brain tissues of AD and PD diseased patients as compared to age-matched control persons revealed a pattern where the expression of ferritin did not increase in an age dependent manner as it did when comparing young controls to elderly control persons, and in addition this lack of ferritin was found to correlate with increased iron levels in globus pallidus in both PD and AD patients and in frontal cortex for AD patients [Connor *et al*, 1995, Loeffler *et al*, 1995]. Decreased iron storage capacity along with the normal age related iron accumulation may form the prerequisites for oxidative stress induced neurodegeneration in both AD and PD.

Oligodendrocytes and microglia contain the highest iron levels of the brain cells, and one of the reasons for age-related iron accumulation in the brain is the differentiation of oligodendrocytes to myelinate axons of cortex, a process occurring until the fifth decade of life. Myelin requires cholesterol and lipids, and their biosynthesis is iron dependent. High metabolic activity in these cells also requires higher mitochondrial respiration and, hence, increased production of superoxide anions and hydrogen peroxide, the combination

with iron makes the myelin susceptible for breakdown during inflammation, a considered risk-factor for the pathology of ageing as well as development of AD [Bartzokis, 2004].

Furthermore, iron might modulate the expression of amyloid precursor protein (APP), since APP transcript harbours an IRE at the 5'-UTR. A β can bind iron and copper, form hydrogen peroxide and hydroxyl radicals in the presence of iron [Tabner *et al*, 2002], and iron and other metals like zinc and copper can promote A β aggregation *in vitro* [Garzon-Rodriguez *et al*, 1999, Huang *et al*, 2004, Maynard *et al*, 2002, Rogers *et al*, 2002].

In PD large amounts of iron are complexed to neuromelanin in dopaminergic neurons of the substantia nigra, and iron is associated with Lewy bodies, one of the pathological hallmarks of PD consisting of neuronal inclusion bodies of α -synuclein and ubiquitin. Further, iron can promote the aggregation of Lewy bodies [Ostrerova-Golts *et al*, 2000]. The substantia nigra of PD patients have been shown to express lower levels of TfR, so it is suggested that substantia nigra instead is susceptible for serum iron, diffusing through a damaged BBB [Faucheux *et al*, 1997].

Friedreichs ataxia

Friedreichs ataxia is an autosomal recessive disease, caused by a genetic mutation in the first intron of the gene encoding the mitochondrial protein frataxin. The mutation is a hyperexpansion of GAA repeats, impairing the transcription of the gene and decreasing the normal levels of the frataxin protein. The onset of the disease may occur during childhood with clinical signs of ataxia, dysarthria, gaze instability and deep sensory loss. The disease causes neurodegeneration in the CNS and the peripheral nervous system, and cardiomyopathy. Loss of frataxin causes impaired Fe-S cluster synthesis and iron accumulation in the mitochondria. The respiration in the mitochondria relies on proper iron sulfur cluster synthesis since they constitute essential cofactors in complex I-III, and impairments of respiration may elevate the ROS production and iron catalyzed OH \cdot formation [Pandolfo, 2002].

Neurodegeneration with brain iron accumulation

Pantothenate kinase-associated neurodegeneration (PKAN), neuroferritinopathy and aceruloplasminemia belong to a group of autosomal recessive neurodegenerative diseases

classified as neurodegeneration with brain iron accumulation (NBIA). PKAN occurs as a progressive disease with onset in the early childhood or in a second form with later onset during the second decade. Clinical signs are dystonia, loss of ambulation, motor impairments, retinopathy and speech defects. Mutation in the gene encoding the protein pantothenate kinase 2 involved in the regulation of the synthesis of coenzyme A, results in accumulation of cysteines, which in turn due to its affinity for iron promote the iron accumulation observed in globus pallidus and substantia nigra [Hayflick, 2003, Zhou *et al*, 2001]. In aceruloplasminemia astrocytes lack the ferroxidase ceruloplasmin, believed to be necessary for iron export in neurons, thus the lack of the enzyme results in iron loaded neurons as observed in the basal ganglia of patients with the mutation [Patel *et al*, 2002]. Neuroferritinopathy is a recently described disease caused by mutations in the light chain of ferritin, altering its carboxyl terminus and the function of ferritin. Iron accumulation is observed in substantia nigra and globus pallidus along with abnormal ferritin accumulation in neurons and glial cells. Increased release of iron from mutated ferritin may cause oxidative damage and neurodegeneration [Curtis *et al*, 2001].

Lipopolysaccharide and nitric oxide regulation of iron metabolism

Production of reactive nitrogen species

Nitric oxide (NO^\bullet) is a gaseous radical able to diffuse through cell membranes and exert signaling or regulatory activities in adjacent cells. Besides being in its radical state nitric oxide may also exist as the nitrosonium ion (NO^+) and after a reaction with $\text{O}_2^{\bullet-}$ also as ONOO^- . When referring to nitric oxide in general without addressing its redox state, NO will be used as the common designation. NO is produced by a family of enzymes known as nitric oxide synthases (NOS) catalyzing the conversion of L-arginine into NO and L-citrulline [Preli *et al*, 2002]. The NO found in the circulation is produced by endothelial NOS (eNOS) and functions as a vasodilator, angiogenesis- and survival factor. In the brain neuronal NOS (nNOS) is the major isoform, but the epithelial form is also present in the capillary endothelial cells of the cerebral blood vessels. The effects of NO in the brain can be beneficial or destructive. When low doses of NO are released it mostly functions as a second messenger mediating e.g. neurotransmission, neuronal excitability, stimulates neuronal outgrowth and differentiation, and offer cytoprotection whereas high

concentrations created by the bursts of NO seen after injury and inflammation, can cause cell damage [Bishop and Anderson, 2005]. eNOS and nNOS are both constitutively expressed, but dormant until activated by the binding of calmodulin in a calcium dependent manner. There is also the inducible NOS (iNOS), normally expressed at very low levels. It is induced in response to inflammatory cytokines and lipopolysaccharide (LPS) by a mechanism involving NF- κ B [Kwon *et al*, 1995]. When expressed iNOS has continuous activity independent of the calcium levels [Li and Poulos, 2005]. iNOS can be detected in astrocytes, granular leukocytes, rodent cerebellar neurons, rodent microglia but it is still unclear whether iNOS is expressed in human microglia and oligodendrocytes [Brosnan *et al*, 1994, Endoh *et al*, 1994, Galea *et al*, 1994, Loihl *et al*, 1999, Minc-Golomb *et al*, 1996, Murphy, 2000].

Toxicity of nitric oxide

iNOS produces bursts of NO to eliminate invading pathogens, however, its expression is also implicated as a risk factor in several neurodegenerative diseases, where iNOS has been found to be upregulated [Heneka and Feinstein, 2001, Xie *et al*, 1993]. NO production is elevated in (i) in Alzheimer's and Huntington's disease, when glutamate neurotoxicity increases intracellular Ca^{2+} levels, activating Ca^{2+} dependent nNOS production of NO, and (ii) in e.g. in brain injury and Alzheimer's disease, where iNOS expression is increased, thus producing higher NO levels [Dawson and Dawson, 1996]. In addition, ONOO^- is able to react with thiol groups and decompose to toxic OH^\bullet , damage mitochondria, result in ATP depletion, DNA damage and possibly inducing cell death [Dawson and Dawson, 1996, Radi *et al*, 1991].

Nitric oxide and iron regulation

NO has a role in the regulation of iron homeostasis primary by modulating the activities of IRP1 and IRP2. The data in this field are diverse and sometimes conflicting and methodological variations cause further difficulties in the search for mechanistic explanations. The majority of the studies are made in murine macrophages since they are prototypic NO-producing cells that process a large part of the body iron when ingesting senescent red blood cells. The source of NO in these reports is endogenously produced NO in response to LPS and/or IFN- γ treatment of cells, or supplemented in the form of various

NO-donors to cells or cell lysates delivering the different NO-species like NO[•], NO⁺ and ONOO⁻. In the majority of current data it has been reported that NO can activate IRP1 and decrease IRP2 activity. IRP1 activation is suggested to involve a direct attack by NO compounds on the Fe-S cluster residing on the inactive form of the protein [Cairo *et al*, 2002, Drapier *et al*, 1993, Richardson *et al*, 1995, Weiss *et al*, 1993]. NO compounds have also by some authors, been reported to decrease the activity of IRP1 [Bouton *et al*, 1997, Richardson *et al*, 1995]. Bouton *et al* performed an *in vitro* study, treating cell lysates or recombinant IRP1 with ONOO⁻, and found IRP1 to lose the cluster without gaining the IRE binding capacity. When a reducing agent was added, the IRE binding activity increased. ONOO⁻ promoted, besides disrupting the cluster, the formation of a disulfide bridge between two cysteine residues crucial for binding of the Fe-S cluster, thus inhibiting IRP1 from binding to the IRE stem-loop. In the same study, IRP2 activity was found to be decreased as well, and that was also explained by the formation of a disulfide bridge between two cysteine residues in the IRE binding cleft, since IRP2 activity could be regained by reductive treatment [Bouton *et al*, 1997].

NO-mediated decrease of IRP2 is also reported in several studies in murine macrophages using different NO donors as well as using endogenously produced NO [Bouton *et al*, 1997, Bouton *et al*, 1998, Cairo *et al*, 2002, Kim and Ponka, 1999, Kim and Ponka, 2000, Kim *et al*, 2004, Recalcati *et al*, 1998]. Kim *et al* recently proposed a novel degradation mechanism of IRP2, involving nitrosylation by NO⁺ of cysteine 178 in the 73-amino acid stretch of IRP2, promoting ubiquitination and degradation of IRP2 by the proteasomes [Kim *et al*, 2004]. Further, Wang *et al* performed a study using human lung cancer cells and murine fibroblasts, treating them with the NO[•] donor SNAP or inducing physiologically synthesized NO. They found NO[•] to increase the IRP2 protein levels according to Western-blot, and to stabilize IRP2 against degradation, according to metabolic labelling, pulse-chase and immunoprecipitation. The authors suggest a mechanism involving NO-mediated decrease of the labile iron pool to support the stabilisation of IRP2 [Wang *et al*, 2005]. As mentioned, macrophages are often used when analyzing iron regulation by NO produced in response to inflammatory cytokines or LPS. Several authors have reported of decreased TfR expression and in parallel increased expression of ferritin, which in a pathological situation would reduce the uptake of potentially toxic iron and promote the storage, effects

attributed to an elevated role for IRP2 in macrophages as compared to IRP1 [Kim and Ponka, 1999, Kim and Ponka, 2000, Recalcati *et al*,1998].

The pathophysiological relevance of increased IRP1 activity might be more difficult to explain, however, IRP1 activity might also be under control of its gene expression. Oliveira and Drapier showed when treating macrophages with IFN- γ /LPS, increased IRP1 activity and a simultaneous decrease in the gene expression, as analysed by Western-blot, Northern-blot and band-shift assay. The repressed gene expression might provide a mechanism by which the cell can reduce the potential toxicity of the increased IRP1 activity [Oliveira and Drapier, 2000].

The LPS receptor signaling

LPS are major constituents of the cell wall of Gram-negative bacteria. It is toxic to various species, and in humans it can cause septic shock resulting in dangerously lowered blood-pressure. The host responds to LPS toxicity by producing inflammatory cytokines like TNF- α , IFN- β and pro-inflammatory species like iNOS [Pålsson-McDermott *et al*, 2004]. The mammalian innate immune system can recognize a large variety of invading pathogens by the Toll-like receptor family. Today there are ten identified receptors (TLR1-TLR10) each specialized to recognize special patterns conserved among pathogens, not found in mammals [Takeda and Akira, 2004]. The signal transduction from the TLRs is characterized by protein-protein interactions. LPS first binds to the circulating plasma LPS binding protein (LBP), which further forms a ternary complex with soluble or membrane attached CD14 protein. The function of CD14 is to load LPS to its receptor complex, consisting of the extracellular adaptor protein MD-2 and the TLR4, and to promote the TLR4 to dimerize. The downstream signaling of LPS can pursue in two directions involving or not involving the adaptor protein MyD88. MyD88-dependent signaling activates NF- κ B, the p38 and JNK pathways. NF- κ B activation leads to an early response and transcription of genes like iNOS, TNF and cyclooxygenase 2. MyD88-independent signaling activates the transcription factors IRF-3 and also NF- κ B, resulting in a late phase response and transcription of e.g. IFN- β [Pålsson-McDermott *et al*, 2004, Takeda and Akira, 2004].

MATERIALS AND METHODS

Materials

Anti-IRP1, anti-IRP2 and anti-ferritin antibodies were generously provided by Dr. Tracey A. Rouault. Anti-TfR antibodies were purchased from Zymed Laboratories Inc. Anti-Tubulin antibodies were purchased from Sigma. Secondary horseradish peroxidase conjugated goat anti-rabbit IgG and anti-mouse IgG were purchased from Amersham Pharmacia Biotech. TRIzol[®] Reagent and all cell culture reagents were from Invitrogen. The iron chelator salicylaldehyde (SIH) was generously provided by Prof. Prem Ponka. Calcein-AM and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) were purchased from Molecular Probes. All other reagents were from Sigma.

Cell cultures

The N2a cells are a clone of the tumour line C1300, which was established from a spontaneous brain neuroblastoma of an albino mouse [Schubert *et al*, 1969]. The GT1 cell line is a subclone of a cell line originally generated by SV40 T-antigen immortalized gonadotropin releasing hormone neurons [Mellon *et al*, 1990]. A chronically scrapie-infected N2a cell line were generated by Prusiner in the late 80s, shortly after the discovery of the PrP gene [Butler *et al*, 1988], with the main goal at the time to gain a system to perform structural analysis of PrP^C and PrP^{Sc}, and to study the mechanism of conversion but have since also been used to study cytopathological changes resulting from the prion infection. Apoptosis is typically observed during scrapie infection in mammals, but so far the only cell line known to undergo apoptosis is scrapie-infected GT1 cells. On the other hand, since PrP^{Sc} is shown not to be directly toxic to neurons the lack of apoptosis in a cultured cell line is not necessarily to be expected. In the brain during prion infection, apoptosis might be a secondary consequence resulting from activation of microglia and astrocytes, producing pro-inflammatory cytokines, ROS and nitric oxide compounds. In addition, it is possible that N2a cells with their rapid proliferation rate compared to GT1 cells, do not accumulate PrP^{Sc} to similar extent as the GT1 cells do. Further, cell treatments with ER-stress inducers have been demonstrated to induce apoptosis in ScN2a cells [Hetz *et al*, 2003]. We have mainly used N2a and ScN2a cells to study molecular changes in iron regulation

and as control cells, and to verify that the observations are not clonal artefacts but true consequences of the scrapie infection, we also used the GT1 and ScGT1 cells.

ScN2a cells were generated as previously described [Butler *et al*, 1988] and were together with non-infected N2a cells generously provided by Dr. Stanley B. Prusiner. N2a cells were also purchased from ATCC (Rockville, MD). Scrapie-infected ScGT1 cells were also generated according to the protocol of the ScN2a cells and generously provided by Dr. Katarina Bedecs. Briefly, the scrapie-infected cells were generated by inoculation of N2a and GT1 cells with brain homogenate from CD-1 mice infected with the Rocky Mountain Laboratory strain of mouse prions of the Chandler isolate (RML prions).

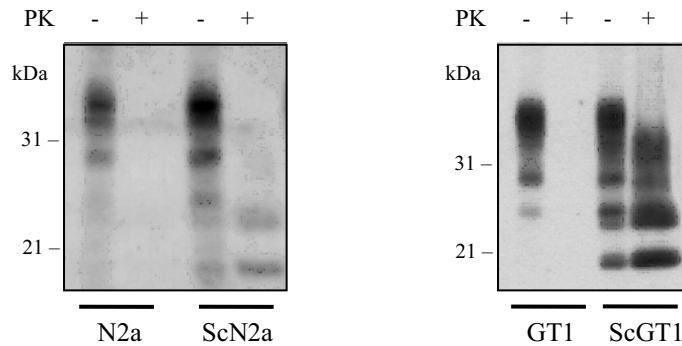


Figure 3. Western-blot detection of the PK-resistant core of PrP^{Sc}. PK digested (+) and non PK digested (-) lysates from N2a, ScN2a, GT1 and ScGT1 cells.

The ScN2a and ScGT1 cells were routinely checked for their scrapie infection as described by Östlund *et al*. [Östlund *et al*, 2001]. Briefly, cells were lysed and the supernatant was divided, and one sample was PK treated and the other sample was not. After termination of the digestion, the samples were analyzed by Western-blotting and immunodetection was performed using prion protein specific anti bodies. PrP^C is completely digested by PK, whereas PrP^{Sc} is only partly degraded, resulting in PK resistant PrP species (Fig. 3).

Cell treatments

For iron treatments cells were treated with ferric ammonium citrate (FAC) 100 µg/ml or 100 µM. For iron chelator treatments cells were treated with 100 µg/ml or 100 µM desferrioxamine (DFO), 100 µM Ferrozine and 100 µM 2,2'-bipyridyl (BIP). Cell treatments with hydrogen peroxide were performed using freshly prepared dilutions, 2.5-100 µM, from a 30% stock-solution. Other reagents used for cell treatment were the microbial agent LPS, 1 and 5 µg/ml, the free radical scavenger N-acetyl-cysteine (NAC) 10 mM and the iNOS inhibitor aminoguanidine 2mM.

Cellular iron measurements

Total cellular iron, including heme and non-heme iron, was measured using a modified version of the protocol described by Torrance and Bothwell [Torrance and Bothwell, 1968]. Briefly, samples of 5×10^6 cells were suspended in 1 ml HBS buffer (150 mM NaCl, 20 mM HEPES), mixed with 1 ml of an acid mixture (3 M HCl, 10% (v/v) trichloroacetic acid, 3% (v/v) thioglycolic acid) reducing Fe^{3+} to Fe^{2+} during an incubation at 37°C for 2 h. Samples were further cooled, centrifuged at $3,000 \times g$ for 30 min and mixed with 0.2 ml of the ferrous chelator bathophenanthroline sulfonate (0.045% (w/v) in 4.5 M Na-acetate, 0.2% (v/v) thioglycolic acid). The colored iron-bathophenanthroline sulfonate complex was measured by determining the absorption at 535 nm.

LIP measurements

The LIP was determined as described [Epsztejn *et al*, 1997] using a calcein fluorescence based method. Briefly, 4×10^6 cells were loaded with 100 nM of the calcein-acetoxymethyl (AM) in PBS at 37°C for 15 min. The calcein-AM probe remains non-fluorescent until it is internalized and the ester bond is cleaved by non-specific esterases. Calcein binds both Fe^{2+} and Fe^{3+} iron with high affinity and its fluorescence is quenched after binding of iron. After washing to remove non-internalized calcein, the cells were transferred to a stirred cuvette and the basal calcein fluorescence was recorded (excitation 488 nm, emission 517 nm). The fluorescence of the calcein-iron complex was de-quenched by the addition of 100 µM BIP or 100 µM SIH, that chelated calcein bound iron and increased the amount of free

fluorescent calcein, and the increase in fluorescence was monitored until a steady signal was obtained (<5 min).

Band-shift assay

IRP activities were determined by a gel-retardation assay performed as described by Haile *et al* using 10 µg of cell extract and a ³²P-labeled ferritin IRE probe. 2-ME was used at 1% (v/v) when noted to reduce the Fe-S cluster to achieve the apo-form of IRP1. RNA-protein complexes were resolved on 10% nondenaturing polyacrylamide gels and visualized by autoradiography [Haile *et al*, 1989]. Quantification of IRP activity was performed using a Molecular Dynamics PhosphorImager and Image Gauge Software (Fuji).

Western and Northern blotting

Protein levels were analyzed by Western-blotting and detected with antibodies against IRP1, IRP2, TfR, ferritin or tubulin. mRNA levels were analyzed by Northern-blotting and detected with ³²P-radiolabeled mouse TfR, ferritin or β-actin cDNA probe. Quantification was performed by densitometric analysis was performed using the Image Gauge software (Fuji).

Since tubulin levels were found to be twofold higher in ScN2a cells as compared to N2a cells, tubulin detection could not be used to confirm even loading between N2a and ScN2a samples, but could be used as internal standard within N2a and ScN2a samples, respectively. To assure even loading between N2a and ScN2a cells, their protein concentration was determined with the Coomassie assay using bovine serum albumin (BSA) as a protein standard. In addition, even loading was confirmed by Ponceau staining of the nitrocellulose membrane and quantified by densitometric analysis. We further counted the N2a and ScN2a cells to analyse the protein content/cell, and the data verified that the scrapie infection did not alter the total cellular protein content.

ROS measurements

Intracellular ROS levels were measured by incubating 500,000 cells with 200 or 800 nM CM-H₂DCFDA, (Molecular Probes) for 15 min. After washing the cells twice with PBS, 2',7'-dichlorofluorescein (DCF) fluorescence was measured at 488 nm (excitation) and 520

nm (emission). The CM-H₂DCFDA probe becomes fluorescent after oxidation by H₂O₂, OH[•], ROO[•] and ONOO⁻. In paper II, the ROS measurements were performed using 200 nM of the probe, however, after the paper was accepted we realized higher amounts of the probe was recommended. Therefore, in paper III, higher concentrations of the probe were tested and 800 nM was found to be sufficient to detect full ROS levels in ScN2a cells. This might explain the higher basal ROS levels in ScN2a cells detected in paper III as compared to the levels reported in paper II.

Cell viability assay

Cell viability was assessed by using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. This method measures cell growth or kills, detected as the formation of formazan from a tetrazolium salt. Briefly, around 10,000 cells per well were plated in 96-well microtiter plates with 100 µl of medium. After cell treatment, the medium was changed and 10 µl of MTT (5 mg/ml stock in PBS) was added to each well for 1 h at 37°C. 100 µl of solubilization buffer consisting of 50% DMSO/5% SDS or 20% SDS/50% DMF of pH 4.7 was added to solubilize the colored crystals and absorption readings were performed at 540 nm with reference at 690 nm. The latter buffer containing DMF was found to immediately dissolve the crystals, omitting pipetting step to dissolve the crystals [Hansen *et al*, 1989]. The increased efficiency in this step might explain the more profound difference in basal cell viability found in paper III as compared to the previously reported non-significant difference in paper II.

The MTT assay measures living cells and uses the ability of the enzyme dehydrogenase, residing inside intact mitochondria, to cleave the tetrazolium ring of MTT, creating blue solid crystals within the intact cells. Addition of a detergent-containing buffer will lyse the cells and solubilize the crystals. The absorption of the resulting colored solution is spectrophotometrically analyzed [Mosmann, 1983].

AIMS OF THE STUDY

Changed iron levels and iron induced oxidative stress are found in several neurodegenerative diseases but its contribution to the pathology of prion diseases is less characterized. This study aims to analyze possible changes in the iron regulation during prion infection in ScN2a cells as compared to N2a cells.

- In the first study we characterized the N2a and ScN2a cells with respect to their iron levels, activities and expression of key proteins involved in iron regulation (paper I).
- Since the ferritin expression is decreased in the ScN2a cells as compared to N2a cells (paper I) and ferritin is known to confer cytoprotection, we analyzed whether the scrapie infection rendered the cells more susceptible to administration of extracellular iron. We monitored the ferritin expression, changes in the LIP and effects on the cellular ROS level and cell viability (paper II).
- During inflammation neuronal cells commonly experience increased oxidative stress as a normal response to destroy and render invading pathogen harmless. Studies by others have demonstrated attenuated antioxidative defense in scrapie-infected animals and cell lines. We therefore challenged the cells with H₂O₂ to increase the oxidative stress and monitored the effect on the intracellular iron level by measuring LIP and in addition the effect of iron chelators on H₂O₂-induced toxicity and the ROS production (paper III).
- LPS induces expression of the NO producing enzyme iNOS in N2a but not in ScN2a cells. NO is well known to regulate the activities and turnover of the IRPs and the expression of TfR and ferritin post-translationally. We therefore aimed to analyze the effect of LPS treatment on the expression of TfR and ferritin in N2a and ScN2a cells. In addition, we co-treated the cells with a free radical scavenger and iNOS inhibitor to study the mechanisms of LPS-induced changes in TfR and ferritin expression (paper IV).

RESULTS AND DISCUSSION

The studies of paper I-III will be discussed where suitable, under the same sub-title. In these studies we have analyzed how various iron-related protein levels and their activities are changed between N2a and ScN2a cells. Sometimes cells were treated with iron or iron chelators, well known modulators of the IRP system, affecting TfR and ferritin expression on a post-transcriptional level. The discussion of paper IV will be held separately, because the objective and results of this work are distinguished from the first three papers. In the latter study we treated the N2a and ScN2a cells with LPS, and found this to modify the ferritin expression also on a transcriptional level.

Lower activities and protein levels of IRPs in ScN2a cells (paper I)

The activities of IRP1 and IRP2 as analyzed by band-shift assay using a radiolabelled ^{32}P -IRE probe were found to be 30% lower in ScN2a cells as compared to N2a cells (Fig. 2). The ratio of the IRP1 activity and its activity in the presence of 2-ME, which removes the Fe-S cluster and reveals the full IRE binding capacity of IRP1, did not differ significantly between N2a and ScN2a, reflecting the presence of the similar proportions of the apo- and holoforms of the protein. The functionality of IRP1 and IRP2 were analyzed after treating the cells for 16 h with the iron source FAC (100 $\mu\text{g/ml}$) or the iron chelator DFO (100 $\mu\text{g/ml}$). Chelation of iron led to significantly increased activities of both IRP1 and IRP2. The iron treatment significantly decreased the activity of IRP2 in both N2a and ScN2a cells, whereas the IRP1 activity was not significantly changed in either of the cells. The protein levels of IRP1 and IRP2 were further found to be decreased by approximately 40% and 50%, respectively, according to Western-blot analysis (Fig. 3). The protein levels of IRP2 were also analyzed in response to FAC and DFO since IRP2 protein levels are regulated through iron induced degradation. Western blot analysis showed that IRP2 levels increased and decreased normally, accordingly to the iron status in both N2a and ScN2a cells (Fig. 3B).

Considering that the total iron level as well as the level of LIP was two fold lower in ScN2a as compared to N2a cells, the lower activities of the IRPs and lower protein levels of IRP2 in particular, may seem contradictory. Low iron levels normally increase the total IRP activity and promote increased protein levels of IRP2. However, low activities of

IRPs are consistent with the finding of decreased TfR levels since the lack of IRPs increases degradation of TfR mRNA. Decreased TfRs result in decreased iron uptake and therefore less need for iron storage in ferritin, which was also found to be lower in ScN2a cells as compared to the N2a cells. However, the question still remains: Why are the expression and activities of the IRPs decreased in the first place and not increased when iron levels are so low?

According to band-shift analysis approximately 90% of IRP1 is in the inactive holoform likely harboring a Fe-S cluster in the ScN2a cells as well as in N2a cells. In the situation of low intracellular iron levels it could be expected that IRP1 would loose the cluster and achieve the IRE binding activity and thus promoting TfR expression. Recent data suggest IRP2 to be predominant over IRP1, especially in the brain. Mouse with genetic ablation of IRP1 develops and reproduces normally whereas mouse with genetic ablation of IRP2 develops a neurodegenerative disease with misregulation of iron metabolism in several tissues [LaVaute *et al*, 2001, Meyron-Holtz *et al*, 2004]. Furthermore, when analyzing IRP1 activity in the wild type mouse, Meyron-Holtz *et al* found the majority of the IRP1 to be in aconitase form, and only 4-18% were active IRE binding proteins. They also analyzed normal mice on low iron diet and found that iron starvation enough to activate IRP2 was insufficient to activate IRP1 [Meyron-Holtz *et al*, 2004]. This scenario could offer an explanation to the lack of activation of IRP1 in the iron deficient ScN2a cells. To explain the weak but non-significant decrease of IRP1 activity after FAC treatment, it is likely that the potential for Fe-S cluster assembly on IRP1 is reduced. In both N2a and ScN2a cells the majority of the IRP1 already harbors Fe-S clusters, and therefore more detectable decrease of IRP1 activity as a consequence of increased iron supply would not be expected.

In paper II we observed increased levels of ROS in ScN2a cells as compared to N2a cells, that could destroy the free radical-sensitive [4Fe-4S] cluster on IRP1, but the disruption of the cluster is not necessarily complete and may result in an [3Fe-4S]⁺ cluster and decreased activity of cytosolic aconitase, without increasing the IRE binding activity [Brazzolotto *et al*, 1999]. It has also been shown that when treating rat liver lysates or human recombinant IRP1 with xantine and xantine oxidase to produce O₂⁻ and H₂O₂, the activity of IRP1 is decreased, and that SOD and/or catalase when co-added to the liver samples

could abolish this decrease, showing that O_2^- and H_2O_2 in concert interfered with the IRE binding activity [Cairo *et al*, 1996]. This second scenario could also offer an explanation for the observed decreased activity of IRP1 in ScN2a cells, instead of the expected increased activity induced by the iron-deficiency.

Since IRP2 degrades by an iron dependent mechanism, the low iron status in ScN2a cells could be expected to provide protection against such a mechanism and increased IRP2 levels would be expected instead of the observed decreased protein levels [Iwai *et al*, 1998]. The explanation for the reduced IRP levels might also be lower expression of transcript or increased degradation through an iron independent mechanism. Reactive nitric species produced by macrophages as an inflammatory response may regulate activities and levels of IRPs. Cysteine residue 178 in the murine IRP2 polypeptide has been shown to be target for nitrosylation by the NO^+ , marking the protein for ubiquitination and degradation by the proteasome [Kim *et al*, 2004]. The NO status in N2a and ScN2a cells have not been studied in any of the projects included in this thesis and its involvement in the changes of iron regulation presented here is highly speculative but may be the subject for future analysis.

Lower TfR levels in ScN2a cells (paper I, III)

In paper I, the basal TfR protein level was fourfold reduced in ScN2a cells as compared to N2a cells, as analyzed by Western-blot (Fig. 4A). The mRNA levels were in agreement found to be significantly lower, according to Northern blot analysis (Fig. 5). When treating the cells with the iron source FAC (100 μ g/ml) for 16 h the expression of TfR decreased more than 50% in both cell lines. Treatment with the iron chelator DFO (100 μ g/ml) increased the expression of TfR by 75% and 100% in N2a and ScN2a cells, respectively, demonstrating the functionality of the IRPs, but despite the markedly increased expression of the TfR in ScN2a cells after DFO treatment it only constitutes 30% of the amount expressed in N2a cells (Fig. 4A). The biosynthesis of the TfR in response to intracellular iron levels is mainly controlled on the level of translation rather than at transcriptional level. Since the activities of IRPs were found to be lower in ScN2a cells, their capacity to stabilize the TfR transcript was reduced and the TfR expression could therefore be expected to be decreased as compared to N2a cells, as observed in paper I and confirmed in paper III.

In paper III, ScN2a but not N2a cells treated with 50 μ M H₂O₂ for 2 h were shown to increase their LIP. To address whether this iron originated from intra or extracellular compartments we studied whether the protein levels of the TfR were increased, but found the TfR levels not to be affected by H₂O₂ treatment (Fig. 2).

Lower ferritin expression in ScN2a cells (paper I, II)

In paper I, the H and L ferritin levels in ScN2a were threefold lower as compared to N2a cells according to Western-blot analysis. In cells treated for 16 h with DFO (100 μ g/ml) a decreased expression of ferritin protein levels was observed in both cell lines. In cells treated for 16 h with FAC (100 μ g/ml) the ferritin expression increased strongly in both cell lines, but the ferritin level was still twofold lower in ScN2a cells as compared to N2a cells, indicating the possibility that the ferritin mRNA levels could be reduced in ScN2a cells (Fig. 4B). Reduced basal levels of H ferritin mRNA in ScN2a cells was also confirmed by Northern blot analysis (Fig. 5).

Since the ferritin levels were found to be decreased in ScN2a cells and ferritin has a function to keep free intra cellular redox active iron at a low and safe level, we continued to analyze how these cells would respond to iron induced stress by challenging the cells to FAC for 1-16 h (paper II). As expected, iron treatment resulted in a rapid and significant increase in the expression of ferritin in the N2a cells (Fig. 3A), being twofold higher after 2 h and fourfold higher after 6 h than the basal level (Fig. 3B, white bars). In ScN2a cells, the basal level of ferritin was fourfold below the amount observed in N2a cells (Fig. 3B), and a twofold increase was not observed until 4 h of iron treatment, followed by a slow but continuous increase up to tenfold of its basal level after 16 hours treatment (Fig. 3A and 3B, grey bars). However, the expression of ferritin was significantly lower in ScN2a cells as compared to the N2a cells in all iron treatments, as highest being 30% lower as compared to the N2a cells after 16 h of FAC treatment. A similar expression pattern of ferritin was observed in scrapie-infected hypothalamic GT1 cells as compared to uninfected GT1 cells, demonstrating that scrapie-induced reduction of ferritin expression is not specific for N2a cells. Misregulation of iron metabolism and increased iron accumulation in specific brain regions is observed in several neurodegenerative diseases associated with neurodegeneration. Whether or not iron is the primary cause of the

neurodegeneration or if the misregulation of iron metabolism is a consequence of the neuropathology, redistribution or increased iron uptake to the CNS may damage cells and especially neurons that normally have the weakest antioxidative defence as compared to other brain cells. To avoid the generation of toxic free radicals the increase in ferritin expression must be quick to restore normal levels of the free iron pool. Ferritin expression has also been found to interfere with the JNK cascade and thereby has a role in inhibition of apoptosis. Ferritin was shown to exert its action by inhibiting TNF- α induced ROS formation in a NF- κ B dependent manner [Pham *et al*, 2004]. We suggest the decreased levels of ferritin protein and mRNA to be a result of the scrapie infection that reduces the capacity of the ScN2a cells to control iron induced stress.

Decreased iron levels in ScN2a cells (paper I)

Since iron is suggested to play a role in the pathophysiology of several neurodegenerative diseases and oxidative stress events have been shown to be present in prion diseases along with brain metal imbalances, we measured iron levels in N2a and ScN2a cells. The total cellular iron concentration was determined as acid-extracted iron and measured colorimetrically. Measurements of total cellular iron show that ScN2a cells have twofold lower iron levels than N2a cells (Fig. 1A).

Since IRPs are sensors of cytosolic free iron levels we next measured calcein-sensitive LIP in the cells. Analysis of calcein fluorescence shows that the LIP level is decreased by 60% in ScN2a cells as compared to the basal LIP in N2a cells (Fig. 1C). We also measured the LIP level in ScGT1 cells and observed that the LIP level was reduced twofold in ScGT1 cells as compared to the uninfected cells, showing that scrapie-induced reduction of LIP is not specific for N2a cells, but can be demonstrated in various neuronal cell-lines.

To our knowledge, this is the first study of intracellular iron levels in scrapie-infected cell lines. Others have reported of changed iron levels in scrapie-infected mice [Kim *et al*, 2000, Wong *et al*, 2001b]. They have analyzed iron in tissue samples of certain brain areas and their reports are to some extent conflicting. Kim *et al* reported of increased Fe³⁺ and total iron levels in the ME7 prion strain infected mice, whereas Wong *et al* measured Fe²⁺ and reported it to be significantly decreased in mice infected with the 139A scrapie

strain but to be unchanged in ME7 infected mice. In both studies brain homogenates were used for the iron measurements, so questions regarding in which cell type or to which subcellular place the iron is localized *in vivo* in scrapie-infected brains are still unanswered. Future immuno-histochemical studies in these mice will hopefully reveal these questions.

In paper I, we also studied how changes in iron concentration in culture media affected the LIP in neuroblastoma cells. We observed as shown in Fig. 1D, the addition of the iron source FAC (100 µg/ml) to the culture medium to result in a rapid increase of LIP, demonstrating that increased iron concentration in the extracellular culture medium resulted in increased LIP, and that the LIP measurements reflect the cellular iron status. Further interpretations of the latter experiment led us to the next study; paper II, since this experiment also revealed distinctions between N2a and ScN2a cells regarding time of normalization to basal level of LIP after iron treatment.

Increased susceptibility to induced stress in ScN2a cells is connected to changes in the LIP (paper II, III)

In paper II, we further analyzed the LIP and its change after treatment with FAC (100 µg/ml) during 1 to 16 h in ScN2a cells as compared to N2a cells, and in addition we analyzed how the supplied iron affected the levels of ROS, by the use of the fluorescent probe CM-H₂DCFDA, and the cell viability assay, as analyzed using the tetrazolium salt MTT. The ferritin expression induced by the addition of iron was also analyzed as already discussed above. One hour after the addition of iron, the LIP increased approximately twofold in the N2a cells and then gradually decreased until normal level was reached after four to five hours (Fig.1, white bars). In the ScN2a cells, LIP also increased twofold during the first hour after iron treatment, but then retained at a 1.7-fold higher level during the 16 h time-course of the experiment (Fig.1, grey bars). The basal level of ROS in untreated ScN2a cells was 1.8-fold higher as compared to the N2a cells. One hour iron treatment induced a threefold increase in ROS level in ScN2a cells that retained constant during the time-course of the experiment (Fig. 2A). Iron induced a 1.8-fold increase in ROS production after three hours of treatment in N2a cells, but the ROS level was normalized after six hours (Fig. 2A). After 24 h of iron treatment, we observed a 25% loss of the cell viability, according to the MTT assay, in ScN2a cells as compared to untreated ScN2a cells

(Fig. 2B). The cell viability of the N2a cells was not significantly affected by iron treatment (Fig. 2B).

Studies in scrapie-infected hypothalamic GT1 cells have shown that the scrapie infection renders the cells more susceptible to oxidative stress [Milhavet *et al*, 2000]. In paper III, we therefore increased the oxidative stress by treating the N2a and ScN2a cells with 50 μM H_2O_2 for 2h to analyze whether possible changes in the LIP could contribute to the neurotoxicity. H_2O_2 treatment resulted in a rapid and significant increase in LIP in ScN2a cells (Fig. 2A, grey bars), while the LIP levels were unaffected in N2a cells (Fig. 2A, white bars). ScGT1 cells experienced, like the ScN2a cells, a similar increase in the LIP as a result of the H_2O_2 treatment, while the LIP in the GT1 cells was unchanged. The H_2O_2 -induced increase in LIP in ScN2a cells was not affected by nonpermeant iron chelator ferrozine (Fig. 2A), nor was the basal TfR protein level affected by H_2O_2 treatment (Fig. 2B), showing that iron is mobilized from intracellular sources rather than taken up from the medium. Cell viability was analyzed, using the MTT assay, after treatment of cells with 2.5-100 μM H_2O_2 for 2 h. ScN2a cells were significantly more sensitive to the H_2O_2 toxicity than N2a cells as shown in a dose-response curve (Fig. 1A). Analysis of H_2O_2 toxicity revealed that ScN2a cells were susceptible to H_2O_2 induced oxidative stress already at the lower concentration range of H_2O_2 , 5 – 10 μM (Fig. 1B, grey bars), whereas no significant reduction in cell viability was measured in N2a cells at 10 μM concentration of H_2O_2 (Fig. 1B, white bars). Treatment of cells with highly permeant Fe^{2+} chelator BIP prevented H_2O_2 -induced reduction in cell viability in ScN2a cells (Fig. 1B, grey bars), whereas no significant effect on cell viability by BIP was observed in N2a cells (Fig. 1B, white bars). Fe^{3+} chelator DFO did not affect H_2O_2 -induced toxicity in N2a or ScN2a cells (Fig. 1B). The basal ROS level in ScN2a cells was found to be reduced when cells were treated with iron chelators, using DFO (100 μM) or BIP (100 μM) for 2h, to result in 30% reduction in basal ROS levels in ScN2a cells (Fig. 3), while the chelator treatment had no effect on the ROS levels in N2a cells (Fig.3), demonstrating that iron is partially involved in production of free radicals in ScN2a cells. A similar decrease in ROS production was also observed ScGT1 cells when treated with DFO (data not shown).

In paper II and in paper III, the basal ROS level in ScN2a cells was found to be markedly higher as compared to N2a cells, and in paper III we found the ROS production

to be partly iron catalyzed since it was reduced by the iron chelators DFO and BIP. Since CM-H₂DCFDA used to analyze the level of ROS, detects both H₂O₂ and OH[•], we suggest the lower ROS level detected after addition of the chelators to result from decreased levels of OH[•], since iron is well known to catalyze the dioxygen cleavage of H₂O₂ resulting in the formation OH[•]. Hence, the ROS level in ScN2a cells was found to be partially iron induced, nonetheless, the remaining ROS in ScN2a cells is still higher as compared to the low basal ROS level detected in N2a cells, and is likely to be the outcome of other ROS promoting mechanisms. Increased susceptibility to oxidative stress and increased oxidative stress is observed in scrapie-infected animals and cell lines, and could be attributed to attenuations of the antioxidative defense [Choi *et al*, 1998, Milhavet *et al*, 2000, Wong *et al*, 2001a, Wong *et al*, 2001b]. The mitochondria in brains of scrapie-infected hamster have decreased activities of mitochondrial MnSOD, thus promoting the accumulation of O₂^{•-}. Further, the mitochondria were found to be dysfunctional having decreased ATPase and cytochrome C activities [Choi *et al*, 1998]. Others have found the fragment of PrP106-126 to produce H₂O₂ and OH[•] in the presence of copper and iron, respectively [Turnbull *et al*, 2003].

In paper II, iron treatment resulted in sustained increased levels of ROS and LIP in ScN2a cells while the N2a cells were unaffected by the iron treatment apart from a swift change in the ROS level. We suggest the increased ROS formation to result from iron catalyzed formation of OH[•] from a surplus of H₂O₂ molecules and that the OH[•] may further catalyze the formation of lipid peroxides, molecules also detected by the CM-H₂DCFDA probe.

In paper III, the H₂O₂-mediated toxicity was found to be iron mediated, since the decreased cell viability in ScN2a cells was rescued by the addition of the iron chelator BIP. Here we suggest H₂O₂ to liberate iron from intracellular sources and increase the redox active free iron pool, further nourishing the oxidative stress, by forming OH[•] from H₂O₂.

Possible sources contributing to the LIP in response to oxidative stress could be cytosolic aconitase and ferritin, heme and Fe-S cluster containing proteins, and proteasomal or lysosomal compartments containing iron from degraded ferritin. Mehlhase *et al* recently demonstrated ferritin-oxidation when treating a mouse macrophage cell line with H₂O₂, as analyzed by Ft immunoprecipitation and immunodetection of protein carbonylations, with consequential degradation by the proteasome, as observed when

measuring degradation of metabolically labeled proteins [Mehlhase *et al*, 2005]. This could be a potential mechanism of oxidative stress to increase free cytosolic iron as a direct effect of ferritin degradation. Yu *et al* also suggest intracellular iron to be released from ferritin by H_2O_2 , but through a slightly different mechanism. Ferritin is during normal and non-oxidative conditions degraded by proteases in the lysosomes. They showed when treating a mouse macrophage cell line with H_2O_2 and simultaneously blocking protease activities in the lysosomes by increasing the pH, a resulting decrease in intra-lysosomal iron, reduced oxidative stress and decreased H_2O_2 -mediated OH^\bullet production, and concluded based on this data that redox active iron could be released by the lysosomes of oxidative stressed cells [Yu *et al*, 2003]. Furthermore, it is possible that the H_2O_2 treatment initiates an oxidative cascade ultimately damaging the mitochondrial respiration, thus inducing increased $O_2^{\bullet-}$ production. Part of the $O_2^{\bullet-}$ could be released to the cytosol and mobilize iron from ferritin acting as a reductant converting Fe^{3+} to Fe^{2+} [Biemond *et al*, 1984, Muller *et al*, 2004]. However, iron released by this mechanism is probably of lesser magnitude as compared to iron release from degraded ferritins by the above discussed mechanisms, and when considering the substantially lower expression of ferritin in ScN2a cells, we suggest iron is liberated mostly as a result of ferritin degradation. Iron can also be released from heme in cells under oxidative stress [Vile and Tyrell, 1993]. Liver cells from rats increased their heme oxygenase mRNA levels after ROS induction by phorone treatment, according to immunoprecipitation of radiolabelled proteins [Cairo *et al*, 1995]. Heme oxygenase mediates catabolism of heme and converts the tetrapyrrole heme ring to biliverdin, carbon monoxide and liberates Fe^{2+} . The iron may enter and increase the free pool of redox active iron unless the heme oxygenase expression is followed by an increased ferritin expression, scavenging the iron [Vile and Tyrell, 1993]. In the cytosol, there is also the Fe-S cluster containing protein IRP1. An *in vitro* study by Brazzolotto *et al* using recombinant human IRP1, H_2O_2 was found to extract one iron from the $[4Fe-4S]$ cluster creating a $[3Fe-4S]^+$ cluster as recorded by electron paramagnetic resonance, further resulting in decreased cytosolic aconitase activity without achievement of the IRE binding activity [Brazzolotto *et al*, 1999]. The released iron could contribute to increased levels of the LIP.

In paper III, the DFO treatment did not protect the ScN2a cells against H_2O_2 induced iron toxicity but was in the same study shown to be able to decrease the basal ROS

level in ScN2a cells. BIP is a Fe^{2+} chelator whereas DFO chelates Fe^{3+} . It is possible that the ratio of Fe^{2+} vs. Fe^{3+} during the basal ROS formation differs from the experimental situation where the LIP is increased as a consequence of the H_2O_2 treatment, explaining why DFO may decrease the basal ROS level, but not rescue against H_2O_2 induced iron toxicity. In addition, the cellular iron uptake of DFO is slower than the uptake of BIP, and it is therefore possible that increased concentrations of DFO or pretreatment of cells with DFO prior to challenge with H_2O_2 would have given a similar result as the treatment with BIP did.

To conclude the findings in paper II and III, we suggest that the increased susceptibility to oxidative stress observed in the ScN2a cells partly depends on inadequate potential to safely store and scavenge sudden increased levels of iron, regardless whether the iron enters the cell from extracellular compartments or if it is released from intracellular stores, thus further fuelling the oxidative stress.

LPS induces different expression of TfR and ferritin in N2a and ScN2a cells (paper IV)

At first this project aimed to analyze how NO affected the expression of iron-related proteins in N2a as compared to ScN2a cells, and the idea originated from the finding of Lindegren *et al* that LPS induced iNOS expression in N2a cells but not in ScN2a cells [Lindegren *et al*, 2003]. Since NO is well known to modify the activities and protein levels of IRPs and thus affect the expression of TfR and ferritin, we first intended to treat cells with LPS and analyze the effects on the TfR, ferritin, IRP1 and IRP2 in N2a as compared to ScN2a cells, effects we foresighted to be NO-mediated. Next aim would have been to mimic these changes in the cells by the use of various NO-donors, thus to provide further insights to the importance of a functional iNOS expression for iron metabolism and in addition to gain information of how the lack of iNOS affected the ScN2a cells. However, to our surprise the LPS treatment did not affect the ferritin or the TfR expression through iNOS and NO, instead the transcription of H-Ft mRNA and TfR protein level were found to be changed by a NO-independent mechanism. The data reported in paper IV, is preliminary and need to be further validated and evaluated.

Treatment of N2a cells with LPS resulted in time-dependent increase in H-Ft mRNA levels in N2a cells from a minor increase observed after 4 h up to a nearly twofold

increase after 16 h, according to Northern blot analysis (Fig. 1A, B, white bars). The LPS treatment had no effect on the ferritin mRNA levels in ScN2a cells (Fig.1A and 1B, grey bars).

L-Ft protein level, as analyzed by Western-blot, was also increased 1.5 fold in N2a cells, but this was not apparent until after 16 h of LPS treatment (Fig.2A and 2B, white bars). The LPS treatment had no effect on the ferritin protein levels in ScN2a cells (Fig.2A and B, grey bars).

After finding that ferritin expression was affected by LPS in N2a cells, we measured TfR protein levels by Western-blot in cells treated with LPS. As shown in Fig. 2A and 2C, TfR protein levels were significantly decreased in N2a cells, whereas LPS induced a slight increase of the TfR in ScN2a cells. However, this increase of the TfR in ScN2a cells has not been obvious in repeated experiments, so if it is a true effect or not remains to be resolved.

Next, we focused on the N2a cells and analyzed the possible involvement of iNOS and NO in the expression of the TfR. N2a cells were co-treated with LPS and the iNOS inhibitor, aminoguanidine. As shown by Western-blot in Fig. 3, aminoguanidine had no effect on the TfR expression by itself and neither did it abolish the effect of LPS in the N2a cells, indicating that NO produced by iNOS was not involved in LPS induced decrease of the TfR expression in N2a cells.

N2a cells were also treated with LPS together with the anti-oxidant NAC, to analyze the possibility that LPS-induced ROS could mediate the diminished expression of the TfR protein level. As shown in Fig 3, NAC totally abolished the effect of LPS on TfR expression in N2a cells, suggesting that mechanism(s) involving ROS are (partially) responsible for the LPS-mediated effects on iron regulation.

The effect of LPS/IFN- γ induced changes on iron homeostasis is extensively studied in macrophages where the effects of the treatment are mainly reported to be mediated by iNOS expression and NO production, affecting the activities of the IRPs. Recalcati *et al* observed a slight increase in H-Ft mRNA, according to Northern blot analysis, after LPS/IFN- γ treatment of macrophages, but the predominant cause to the increased protein expression was found to be due to decreased activity of IRP2, possibly a consequence of NO mediated increase of intracellular iron levels [Recalcati *et al*, 1998]. Unfortunately data

regarding changes in the protein level of H-Ft in LPS treated N2a cells have not yet been produced, for this reason; it is not yet possible to formulate conclusions regarding the significance of the twofold increase of the H-Ft mRNA. Further, the H-Ft gene have also been shown to be activated in non-neuronal cells by several cytokines e.g. TNF- α , IL-1 α and IFN- γ [Fahmy *et al*, 1993, Kwak *et al*, 1995, Torti *et al*, 1988, Wei *et al*, 1990]. Less is known about LPS induction of the ferritin gene expression and particularly in neuronal cells. However, since LPS signaling activates NF- κ B it is possible that the increase in ferritin mRNA levels as observed in paper IV, could be an effect of increased NF- κ B activation but this requires to be further investigated in future studies.

The decreased expression of TfR protein levels and the increase of L-Ft in N2a cells can be a reflection of decreased IRP-activity, but band-shift analysis of their activities will be performed to verify this assumption. NO has been shown to nitrosylate and deactivate both IRP1 and IRP2, but since the addition of the iNOS inhibitor aminoguanidine did not prevent the LPS effect, the mechanism of LPS signaling in N2a cells seems to be NO-independent [Kim and Ponka, 1999, Richardson *et al*, 1995]. Instead, the effect of the ROS scavenger NAC abolished the LPS induced decrease in TfR expression, pointing towards an oxidative mechanism. In an *in vivo* study by Cairo *et al*, the induction of oxidative stress initially caused degradation of ferritin, release of iron to the free iron pool and decreased activity of IRP2 finally resulting in increased rate of protein synthesis [Cairo *et al*, 1995]. Since LPS activation of NF- κ B is a ROS-mediated mechanism, the antioxidant NAC may also reverse the LPS effect and diminish the H-Ft mRNA expression [Asehnoune, 2004, Sanlioglu *et al*, 2001]. Determinations of the LIP and ROS-levels along with band-shift analysis in response to LPS treatment are required to study and elucidate the mechanisms.

In agreement with the findings of Lindegren *et al* that LPS did not induce expression of iNOS in ScN2a cells, we here report that ferritin H mRNA expression could not be increased by LPS [Lindegren *et al*, 2003]. A plausible explanation might be a dysfunctional LPS-signaling in ScN2a cells following from the scrapie infection. Further studies by Lindegren *et al* (unpublished data) revealed a complete absence of the expression of CD14 mRNA in ScN2a cells, as analyzed by reverse transcription and PCR analysis, to explain the abolished response to LPS treatment. However, the CD14 expression must be further analyzed since its expression also can be induced by LPS

treatment [Hopkins *et al*, 1995, Mahnke *et al*, 1997]. Even though LPS induced production of cytokines serves beneficial purposes, overproduction of cytokines may be harmful to cells and tissues. Endotoxin tolerance may be a protective mechanism. Dysfunctions in the LPS signaling pathway might be a defense mechanism to prevent overexpression of cell damaging cytokines in response to the scrapie infection. In support to this view, the expression of CD14 is decreased by cytomegalovirus infection of human macrophages and human resident intestinal macrophages [Hopkins *et al*, 1996, Smith *et al*, 2001]. Furthermore, studies in CD-14 knock-out mice have showed that LPS signaling may occur through a CD-14 independent mechanism [Cohen *et al*, 1995].

An additional possible mechanism to LPS-induced reduction of TfR expression can be suggested to be the decreased the activity of IRP1. Recent data from our lab demonstrated another LPS mediated effect on the cysteine desulfurase (IscS) involved in Fe-S cluster assembly in mitochondria and possibly in the cytosol. According to Northern blotting the expression of IscS was induced by LPS in N2a cells. We hypothesize that increased IscS synthesis participates in the anti-inflammatory response. The (possibly) resulting increase in Fe-S cluster synthesis and insertion into IRP1 causes decreased IRP1 activity and the resulting decrease in TfRs and cellular iron uptake, limiting the risk for iron induced toxicity.

CONCLUSIONS

- i) The scrapie infection results in changed iron metabolism in ScN2a cells.
- ii) IRP1 and IRP2 are functional, but their protein levels and activities are decreased in ScN2a cells as compared to N2a cells.
- iii) Decreased iron level in ScN2a cells can be a reflection of decreased TfR level, consistent with decreased IRP activities.
- iv) Low expression of ferritin in ScN2a cells may contribute to the attenuation of the antioxidative defense resulting in iron induced stress.
- v) ScN2a cells are susceptible to iron exposure, and respond with increased oxidative stress followed by decreased cell viability, as compared to N2a cells.
- vi) ScN2a cells respond to extracellular oxidative stress by increasing intracellular free iron levels, resulting in reduced cell viability, as compared to N2a cells.
- vii) The mechanism behind the effect of LPS on ferritin and TfR expression involves reactive oxygen species, but not nitric oxide in N2a cells.

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REFERENCES

A

- Abbott NJ.** Dynamics of CNS barriers: evolution, differentiation, and modulation. *Cell Mol Neurobiol.* 2005 25:5-23
- Aisen P, Leibman A and Zweier J.** Stoichiometric and site characteristics of the binding of iron to human transferrin. *J Biol Chem.* 1978 253:1930-1937
- Asehnoune K, Strassheim D, Mitra S, Kim JY and Abraham E.** Involvement of reactive oxygen species in Toll-like receptor 4-dependent activation of NF-kappa B. *J J Immunol.* 2004 172:2522-2529
- Aziz N and Munro HN.** Iron regulates ferritin mRNA translation through a segment of its 5' untranslated region. *Proc Natl Acad Sci U S A.* 1987 84:8478-8482

B

- Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW and Vercellotti GM.** Ferritin: a cytoprotective antioxidant strategem of endothelium. *J Biol Chem* 1992 267:18148-18153
- Barron KD.** The microglial cell. A historical review. *J Neurol Sci.* 1995 134:57-68.
- Bartzokis G.** Age-related myelin breakdown: a developmental model of cognitive decline and Alzheimer's disease. *Neurobiol Aging.* 2004 25:5-18
- Basler K, Oesch B, Scott M, Westaway D, Walchli M, Groth DF, McKinley MP, Prusiner SB and Weissmann C.** Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. *1986 Cell* 46: 417-428
- Biemond P, van Eijk HG, Swaak AJ and Koster JF.** Iron mobilization from ferritin by superoxide derived from stimulated polymorphonuclear leukocytes. Possible mechanism in inflammation diseases. *J Clin Invest.* 1984 73:1576-1579
- Bishop A and Anderson JE.** NO signaling in the CNS: from the physiological to the pathological. *Toxicology.* 2005 208:193-205
- Borchelt DR, Taraboulos A and Prusiner SB.** Evidence for synthesis of scrapie prion proteins in the endocytic pathway. *1992 J Biol Chem* 267: 16188-16199
- Bounhar Y, Zhang Y, Goodyer CG, LeBlanc A.** Prion protein protects human neurons against Bax-mediated apoptosis. *J Biol Chem.* 2001 276:39145-39149
- Bouton C, Hirling H and Drapier JC.** Redox modulation of iron regulatory proteins by peroxynitrite. *J Biol Chem.* 1997 272:19969-19975

- Bouton C, Oliveira L and Drapier JC.** Converse modulation of IRP1 and IRP2 by immunological stimuli in murine RAW. 264.7 macrophages. *J Biol Chem.* 1998 273:9403-9408.
- Bouton C, Raveau M and Drapier JC.** Modulation of iron regulatory protein functions. Further insights into the role of nitrogen- and oxygen-derived reactive species. *J Biol Chem.* 1996 271:2300-2306
- Brazzolotto X, Gaillard J, Pantopoulos K, Hentze MW and Moulis JM.** Human cytoplasmic aconitase (Iron regulatory protein 1) is converted into its [3Fe-4S] form by hydrogen peroxide in vitro but is not activated for iron-responsive element binding. *J Biol Chem.* 1999 274:21625-2163
- Brosnan CF, Battistini L, Raine CS, Dickson DW, Casadevall A and Lee SC.** Reactive nitrogen intermediates in human neuropathology: an overview. *Dev Neurosci.* 1994 16:152-161
- Brown DR and Besinger A.** Prion protein expression and superoxide dismutase activity. *Biochem J.* 1998 334:423-429
- Brown DR, Schmidt B and Kretzschmar HA.** Effects of oxidative stress on prion protein expression in PC12 cells. *Int J Dev Neurosci.* 1997a 15:961-972
- Brown DR, Schulz-Schaeffer WJ, Schmidt B and Kretzschmar HA.** Prion protein-deficient cells show altered response to oxidative stress due to decreased SOD-1 activity. *Exp Neurol.* 1997b 146:104-112
- Brown DR, Qin K, Herms JW, Madlung A, Manson J, Strome R, Fraser PE, Kruck T, von Bohlen A, Schulz-Schaeffer W, Giese A, Westaway D and Kretzschmar H.** The cellular prion protein binds copper in vivo. *Nature.* 1997c 390:684-687
- Brown DR, Wong BS, Hafiz F, Clive C, Haswell SJ and Jones IM.** Normal prion protein has an activity like that of superoxide dismutase. *Biochem J.* 1999 344:1-5
- Bruce ME.** TSE strain variation. *2003 Br Med Bull* 66: 99-108
- Bueler H, Aguzzi A, Sailer A, Greiner RA, Autenried P, Aguet M and Weissmann C.** Mice devoid of PrP are resistant to scrapie. *1993 Cell* 73: 1339-13347
- Bueler H, Fischer M, Lang Y, Bluethmann H, Lipp HP, DeArmond SJ, Prusiner SB, Aguet M and Weissmann C.** Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. *1992 Nature* 356: 577-582

Burdo JR, Antonetti DA, Wolpert EB and Connor JR. Mechanisms and regulation of transferrin and iron transport in a model blood-brain barrier system. *Neuroscience*. 2003 121:883-889

Burdo JR, Menzies SL, Simpson IA, Garrick LM, Garrick MD, Dolan KG, Haile DJ, Beard JL and Connor JR. Distribution of divalent metal transporter 1 and metal transport protein 1 in the normal and Belgrade rat. *J Neurosci Res*. 2001 66:1198-1207

Butler DA, Scott MR, Bockman JM, Borchelt DR, Taraboulos A, Hsiao KK, Kingsbury DT and Prusiner SB. Scrapie-infected murine neuroblastoma cells produce protease-resistant prion proteins. *J Virol*. 1988 62:1558-1564

C

Cadet J, Douki T, Gasparutto D and Ravanat JL. Oxidative damage to DNA: formation, measurement and biochemical features. *Mutat Res*. 2003 531:5-23

Cairo G, Castrusini E, Minotti G and Bernelli-Zazzera A. Superoxide and hydrogen peroxide-dependent inhibition of iron regulatory protein activity: a protective stratagem against oxidative injury. *FASEB J*. 1996 10:1326-1335

Cairo G, Rappocciolo E, Tacchini L and Schiaffonati L. Expression of the genes for the ferritin H and L subunits in rat liver and heart. Evidence for tissue-specific regulations at pre- and post-translational levels. *Biochem J*. 1991 275:813-816

Cairo G, Ronchi R, Recalcatti S, Campanella A and Minotti G Nitric oxide and peroxynitrite activate the iron regulatory protein-1 of J774A.1 macrophages by direct disassembly of the Fe-S cluster of cytoplasmic aconitase. *Biochemistry*. 2002 41:7435-7442

Cairo G, Tacchini L, Pogliaghi G, Anzon E, Tomasi A and Bernelli-Zazzera A. Induction of ferritin synthesis by oxidative stress. Transcriptional and post-transcriptional regulation by expansion of the "free" iron pool. *J Biol Chem*. 1995 270:700-703

Casey JL, Hentze MW, Koeller DM, Caughman SW, Rouault TA, Klausner RD and Harford JB. Iron-responsive elements: regulatory RNA sequences that control mRNA levels and translation. *Science*. 1988 240:924-928

Caughey B, Raymond GJ, Kocisko DA and Lansbury PT Jr. Scrapie infectivity correlates with converting activity protease resistance and aggregation of scrapie-associated prion protein in guanidine denaturation studies. *J Virol* 1997 71:4107-4110

Chasteen ND and Harrison PM. Mineralization in ferritin: an efficient means of iron storage. *J Struct Biol*. 1999 126:182-194

Cheng Y, Zak O, Aisen P, Harrison SC and Walz T. Structure of the human transferrin receptor-transferrin complex. *Cell*. 2004 116:565-576

Choi SI, Ju WK, Choi EK, Kim J, Lea HZ, Carp RI, Wisniewski HM and Kim YS. Mitochondrial dysfunction induced by oxidative stress in the brains of hamsters infected with the 263 K scrapie agent. *Acta Neuropathol (Berl)* 1998 96:279-286

Chou CC, Gatti RA, Fuller ML, Concannon P, Wong A, Chada S, Davis RC and Salser WA. Structure and expression of ferritin genes in a human promyelocytic cell line that differentiates in vitro. *Mol Cell Biol*. 1986 6:566-573

Cohen E and Taraboulos A. Scrapie-like prion protein accumulates in aggresomes of cyclosporin A-treated cells. *EMBO J* 2003 22: 404-417

Cohen FE and Prusiner SB. Pathologic conformations of prion proteins. *Annu Rev Biochem* 1998 67: 793-819

Cohen L, Haziot A, Shen DR, Lin XY, Sia C, Harper R, Silver J and Goyert SM. CD14-independent responses to LPS require a serum factor that is absent from neonates. *J Immunol* 1995 155:5337-5342

Colling SB, Collinge J and Jefferys JG. Hippocampal slices from prion protein null mice: disrupted Ca(2+)-activated K⁺ currents. *Neurosci Lett* 1996 209: 49-52

Collinge J, Whittington MA, Sidle KC, Smith CJ, Palmer MS, Clarke AR and Jefferys JG. Prion protein is necessary for normal synaptic function. *Nature*. 1994 370:295-297

Collins S, McLean CA and Masters CL. Gerstmann-Straussler-Scheinker syndrome fatal familial insomnia and kuru: a review of these less common human transmissible spongiform encephalopathies. *J Clin Neurosci* 2001 8: 387-397

Come JH, Fraser PE and Lansbury PT Jr. A kinetic model for amyloid formation in the prion diseases: importance of seeding. *Proc Natl Acad Sci U S A*. 1993 90:5959-5963

Connor JR, Boeshore KL, Benkovic SA and Menzies SL. Isoforms of ferritin have a specific cellular distribution in the brain. *J Neurosci Res*. 1994 37:461-465

Connor JR and Fine RE. Development of transferrin-positive oligodendrocytes in the rat central nervous system. *J Neurosci Res*. 1987 17:51-59

Connor JR, Snyder BS, Arosio P, Loeffler DA and LeWitt P. A quantitative analysis of isoform ferritins in select regions of aged, parkinsonian, and Alzheimer's diseased brains. *J Neurochem*. 1995 65:717-724

Crichton RR, Wilmet S, Legssyer R and Ward RJ. Molecular and cellular mechanisms of iron

- homeostasis and toxicity in mammalian cells. *J Inorg Biochem.* 2002 91:9-18
- Crozet C, Flamant F, Bencsik A, Aubert D, Samarut J and Baron T.** Efficient transmission of two different sheep scrapie isolates in transgenic mice expressing the ovine PrP gene. *J Virol* 2001 75: 5328-5334
- Curtis AR, Fey C, Morris CM, Bindoff LA, Ince PG, Chinnery PF, Coulthard A, Jackson MJ, Jackson AP, McHale DP, Hay D, Barker WA, Markham AF, Bates D, Curtis A and Burn J.** Mutation in the gene encoding ferritin light polypeptide causes dominant adult-onset basal ganglia disease. *Nat Genet.* 2001 28:350-354
- D**
- Dawson TM and Dawson VL.** Nitric oxide synthase: role as a transmitter/mediator in the brain and endocrine system. *Annu Rev Med.* 1996 47:219-227
- Dickinson TK and Connor JR.** Immunohistochemical analysis of transferrin receptor: regional and cellular distribution in the hypotransferrinemic (hpx) mouse brain. *Brain Res.* 1998 801:171-181
- Diener TO, McKinley MP and Prusiner SB.** Viroids and prions. *Proc Natl Acad Sci U S A.* 1982 79:5220-5204
- Drapier JC, Hirling H, Wietzerbin J, Kaldy P and Kuhn LC.** Biosynthesis of nitric oxide activates iron regulatory factor in macrophages. *EMBO J.* 1993 12:3643-3649
- Dringen R, Pawlowski PG and Hirrlinger J.** Peroxide detoxification by brain cells. *J Neurosci Res.* 2005 79:157-165
- Drysdale J, Arosio P, Invernizzi R, Cazzola M, Volz A, Corsi B, Biasiotto G and Levi S.** Mitochondrial ferritin: a new player in iron metabolism. *Blood Cells Mol Dis.* 2002 29:376-383
- E**
- Endoh M, Maiese K and Wagner J.** Expression of the inducible form of nitric oxide synthase by reactive astrocytes after transient global ischemia. *Brain Res.* 1994 651:92-100
- Epsztejn S, Kakhlon O, Glickstein H, Breuer W and Cabantchik I.** Fluorescence analysis of the labile iron pool of mammalian cells. *Anal Biochem.* 1997 248:31-40
- F**
- Fahmy M and Young SP.** Modulation of iron metabolism in monocyte cell line U937 by inflammatory cytokines: changes in transferrin uptake iron handling and ferritin mRNA. *Biochem J* 1993 296:175-181
- Farooqui AA and Horrocks LA.** Lipid peroxides in the free radical. *Cell Mol Neurobiol.* 1998 18:599-608
- Faucheux BA, Hauw JJ, Agid Y and Hirsch EC.** The density of [125I]-transferrin binding sites on perikarya of melanized neurons of the substantia nigra is decreased in Parkinson's disease. *Brain Res.* 1997 749:170-174
- Fleming MD, Trenor CC 3rd, Su MA, Foernzler D, Beier DR, Dietrich WF and Andrews NC.** Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nat Genet.* 1997 16:383-386
- Fournier JG, Escaig-Haye F, Billette de Villemeur T and Robain O.** Ultrastructural localization of cellular prion protein (PrPc) in synaptic boutons of normal hamster hippocampus. *C R Acad Sci III* 1995 318 : 339-344
- G**
- Gajdusek and Zigas** Degenerative disease of the central nervous system in New Guinea; the endemic occurrence of kuru in the native population. *N Engl J Med.* 1957 257:974-978
- Gajdusek DC, Gibbs CJ and Alpers M.** Experimental transmission of a Kuru-like syndrome to chimpanzees. *Nature.* 1966 209:794-796
- Galea E, Reis DJ and Feinstein DL.** Cloning and expression of inducible nitric oxide synthase from rat astrocytes. *J Neurosci Res.* 1994 37:406-414.
- Gambetti P, Kong Q, Zou W, Parchi P and Chen SG.** Sporadic and familial CJD: classification and characterization. *Br Med Bull* 2003 66: 213-239
- Garzon-Rodriguez W, Yatsimirsky AK and Glabe CG.** Binding of Zn(II), Cu(II), and Fe(II) ions to Alzheimer's A beta peptide studied by fluorescence. *Bioorg Med Chem Lett.* 1999 9:2243-224
- Gerber MR and Connor JR.** Do oligodendrocytes mediate iron regulation in the human brain? *Ann Neurol.* 1989 26:95-98
- Gibbs CJ Jr, Gajdusek DC, Asher DM, Alpers MP, Beck E and Daniel PM, Matthews WB.** Creutzfeldt-Jakob disease (spongiform encephalopathy): transmission to the chimpanzee. *Science.* 1968 161:388-389
- Giometto B, Bozza F, Argentiero V, Gallo P, Pagni S, Piccinno MG and Tavolato B** Transferrin receptors in rat central nervous system. An immunocytochemical study. *J Neurol Sci.* 1990 98:81-90
- Goss JR, Taffe KM, Kochanek PM and DeKosky ST.** The antioxidant enzymes glutathione peroxidase and catalase increase following traumatic brain injury in the rat. *Exp Neurol.* 1997 146:291-294
- Gruenheid S, Canonne-Hergaux F, Gauthier S, Hackam DJ, Grinstein S and Gros P.** The iron transport protein NRAMP2 is an integral membrane glycoprotein that colocalizes with transferrin in recycling endosomes. *J Exp Med.* 1999 189:831-884

Gruenheid S, Cellier M, Vidal S and Gros P Identification and characterization of a second mouse Nramp gene. *Genomics*. 1995 25:514-525

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL and Hediger MA. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature*. 1997 388:482-488

Guo B, Phillips JD, Yu Y and Leibold EA. Iron regulates the intracellular degradation of iron regulatory protein 2 by the proteasome. *J Biol Chem*. 1995 270:21645-21651.

H

Haile DJ, Hentze MW, Rouault TA, Harford JB and Klausner RD. Regulation of interaction of the iron-responsive element binding protein with iron-responsive RNA elements. *Mol Cell Biol*. 1989 9:5055-5061

Haile DJ, Rouault TA, Tang CK, Chin J, Harford JB and Klausner RD. Reciprocal control of RNA-binding and aconitase activity in the regulation of the iron-responsive element binding protein: role of the iron-sulfur cluster. *Proc Natl Acad Sci U S A*. 1992 89:7536-7540

Halliwell B. Reactive oxygen species and the central nervous system. *J Neurochem*. 1992 59:1609-1623

Hansen MB, Nielsen SE and Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods*. 1989 119:203-210

Harris DA. Cellular biology of prion diseases. *Clin Microbiol Rev* 1999 12: 429-444

Hayflick SJ. Pantothenate kinase-associated neurodegeneration (formerly Hallervorden-Spatz syndrome). *J Neurol Sci*. 2003 207:106-107

Hegde RS, Tremblay P, Groth D, DeArmond SJ, Prusiner SB and Lingappa VR. Transmissible and genetic prion diseases share a common pathway of neurodegeneration. *Nature* 1999 402:822-826

Heneka MT and Feinstein DL. Expression and function of inducible nitric oxide synthase in neurons. *J Neuroimmunol*. 2001 114:8-18

Hentze MW and Argos P. Homology between IRE-BP, a regulatory RNA-binding protein, aconitase, and isopropylmalate isomerase. *Nucleic Acids Res*. 1991 19:1739-1740

Hetz C, Russelakis-Carneiro M, Maundrell K, Castilla J and Soto C. Caspase-12 and endoplasmic reticulum stress mediate neurotoxicity of pathological prion protein. *EMBO J*. 2003 22:5435-5445

Holscher C, Bach UC and Dobberstein B. Prion protein contains a second endoplasmic reticulum targeting signal sequence located at its C terminus. *J Biol Chem* 2001 276: 13388-13394

Hopkins HA, Monick MM and Hunninghake GW. Lipopolysaccharide upregulates surface

expression of CD14 on human alveolar macrophages. *Am J Physiol*. 1995 269 :849-854

Hopkins HA, Monick MM and Hunninghake GW. Cytomegalovirus inhibits CD14 expression on human alveolar macrophages. *J Infect Dis* 1996 174:69-74

Hornshaw MP, McDermott JR, Candy JM and Lakey JH. Copper binding to the N-terminal tandem repeat region of mammalian and avian prion protein: structural studies using synthetic peptides. *Biochem Biophys Res Commun*. 1995 214:993-999

Huang X, Atwood CS, Hartshorn MA, Multhaup G, Goldstein LE, Scarpa RC, Cuajungco MP, Gray DN, Lim J, Moir RD, Tanzi RE and Bush AI. The A beta peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction. *Biochemistry*. 1999 38:7609-7616

Huang X, Atwood CS, Moir RD, Hartshorn MA, Tanzi RE and Bush AI. Trace metal contamination initiates the apparent auto-aggregation, amyloidosis, and oligomerization of Alzheimer's A beta peptides. *J Biol Inorg Chem*. 2004 9:954-960

Hussain S, Slikker W Jr and Ali SF. Role of metallothionein and other antioxidants in scavenging superoxide radicals and their possible role in neuroprotection. *Neurochem Int*. 1996 29:145-152

Hutter G, Heppner FL and Aguzzi A. No superoxide dismutase activity of cellular prion protein in vivo. *Biol Chem*. 2003 384:1279-1285

Imlay JA. Pathways of oxidative damage. *Annu Rev Microbiol*. 2003 57:395-418.

Ivanova L, Barmada S, Kummer T and Harris D. Mutant prion proteins are partially retained in the endoplasmic reticulum. *J Biol Chem* 2001 276:42409-42421

Iwai K, Drake SK, Wehr NB, Weissman AM, LaVaute T, Minato N, Klausner RD, Levine RL and Rouault TA. Iron-dependent oxidation ubiquitination and degradation of iron regulatory protein 2: implications for degradation of oxidized proteins. *Proc Natl Acad Sci U S A* 1998 95:4924-4928

Jain SK, Barrett KJ, Boyd D, Favreau MF, Crampton J and Drysdale JW. Ferritin H and L chains are derived from different multigene families. *J Biol Chem*. 1985 260:11762-11768

Jefferies WA, Brandon MR, Hunt SV, Williams AF, Gatter KC and Mason DY. Transferrin receptor on endothelium of brain capillaries. *Nature*. 1984 312:162-163

Kakhlon O and Cabantchik ZI. The labile iron pool: characterization, measurement, and

- participation in cellular processes(1). *Free Radic Biol Med.* 2002 33:1037-1046
- Kakhlon O, Gruenbaum Y and Cabantchik ZI.** Repression of the heavy ferritin chain increases the labile iron pool of human K562 cells. *Biochem J.* 2001 356:311-316
- Kaptain S, Downey WE, Tang C, Philpott C, Haile D, Orloff DG, Harford JB, Rouault TA and Klausner RD.** A regulated RNA binding protein also possesses aconitase activity. *Proc Natl Acad Sci U S A.* 1991 88:10109-10113
- Kawabata H, Yang R, Hiramata T, Vuong PT, Kawano S, Gombart AF and Koeffler HP.** Molecular cloning of transferrin receptor 2. A new member of the transferrin receptor-like family. *J Biol Chem.* 1999 274:20826-20832
- Kennedy MC, Mende-Mueller L, Blondin GA and Beinert H.** Purification and characterization of cytosolic aconitase from beef liver and its relationship to the iron-responsive element binding protein. *Proc Natl Acad Sci U S A.* 1992 89:11730-11734
- Kim NH, Park SJ, Jin JK, Kwon MS, Choi EK, Carp RI and Kim YS.** Increased ferric iron content and iron-induced oxidative stress in the brains of scrapie-infected mice. *Brain Res* 2000 884:98-103
- Kim S and Ponka P.** Control of transferrin receptor expression via nitric oxide-mediated modulation of iron-regulatory protein 2. *J Biol Chem.* 1999 274:33035-33042
- Kim S and Ponka P.** Effects of interferon-gamma and lipopolysaccharide on macrophage iron metabolism are mediated by nitric oxide-induced degradation of iron regulatory protein 2. *J Biol Chem.* 2000 275:6220-6206
- Kim S, Wing SS and Ponka P.** S-nitrosylation of IRP2 regulates its stability via the ubiquitin-proteasome pathway. *Mol Cell Biol* 2004 24:330-337
- Klausner RD and Rouault TA.** A double life: cytosolic aconitase as a regulatory RNA binding protein. *Mol Biol Cell.* 1993 4:1-5
- Kretzschmar HA, Stowring LE, Westaway D, Stubblebine WH, Prusiner SB and Dearmond SJ.** Molecular cloning of a human prion protein cDNA. *DNA* 1986 5: 315-324
- Krishnan R and Lindquist SL.** Structural insights into a yeast prion illuminate nucleation and strain diversity. *Nature* 2005 435: 765-772
- Kruszewski M and Iwanenko T.** Labile iron pool correlates with iron content in the nucleus and the formation of oxidative DNA damage in mouse lymphoma L5178Y cell lines. *Acta Biochim Pol.* 2003 50:211-215
- Kwak EL, Laroche DA, Beaumont C, Torti SV and Torti FM.** Role for NF-kappa B in the regulation of ferritin H by tumor necrosis factor-alpha. *J Biol Chem* 1995 270:15285-15293
- Kwon G, Corbett JA, Rodi CP, Sullivan P and McDaniel ML.** Interleukin-1 beta-induced nitric oxide synthase expression by rat pancreatic beta-cells: evidence for the involvement of nuclear factor kappa B in the signaling mechanism. *Endocrinology.* 1995 136:4790-4795
- ## L
- Langlois d'Estaintot B, Santambrogio P, Granier T, Gallois B, Chevalier JM, Precigoux G, Levi S and Arosio P.** Crystal structure and biochemical properties of the human mitochondrial ferritin and its mutant Ser144Ala. *J Mol Biol.* 2004 340:277-293
- LaVaute T, Smith S, Cooperman S, Iwai K, Land W, Meyron-Holtz E, Drake SK, Miller G, Abu-Asab M, Tsokos M, Switzer R 3rd, Grinberg A, Love P, Tresser N and Rouault TA.** Targeted deletion of the gene encoding iron regulatory protein-2 causes misregulation of iron metabolism and neurodegenerative disease in mice. *Nat Genet.* 2001 27:209-214
- Lehmann S and Harris DA.** Mutant and infectious prion proteins display common biochemical properties in cultured cells. *J Biol Chem* 1996 271: 1633-1637
- Leibold EA and Munro HN.** Cytoplasmic protein binds in vitro to a highly conserved sequence in the 5' untranslated region of ferritin heavy- and light-subunit mRNAs. *Proc Natl Acad Sci U S A.* 1988 85:2171-2175
- Levi S, Corsi B, Bosisio M, Invernizzi R, Volz A, Sanford D, Arosio P and Drysdale J.** A human mitochondrial ferritin encoded by an intronless gene. *J Biol Chem.* 2001 276:24437-24440
- Li H and Poulos TL.** Structure-function studies on nitric oxide synthases. *J Inorg Biochem.* 2005 99:293-305
- Lindgren H, Ostlund P, Gyllberg H and Bedecs K.** Loss of lipopolysaccharide-induced nitric oxide production and inducible nitric oxide synthase expression in scrapie-infected N2a cells. *J Neurosci Res.* 2003 71:291-299
- Lledo PM, Tremblay P, DeArmond SJ, Prusiner SB and Nicoll RA.** Mice deficient for prion protein exhibit normal neuronal excitability and synaptic transmission in the hippocampus. *Proc Natl Acad Sci U S A.* 1996 93:2403-2407
- Loeffler DA, Connor JR, Juneau PL, Snyder BS, Kanaley L, DeMaggio AJ, Nguyen H, Brickman CM and LeWitt PA.** Transferrin and iron in normal, Alzheimer's disease, and Parkinson's disease brain regions. *J Neurochem.* 1995 65:710-724
- Loihs AK, Asensio V, Campbell IL and Murphy S.** Expression of nitric oxide synthase (NOS)-2 following permanent focal ischemia and the role of nitric oxide in infarct generation in male, female and NOS-2 gene-deficient mice. *Brain Res.* 1999 830:155-164

M

- Mahnke K, Becher E, Ricciardi-Castagnoli P, Luger TA, Schwarz T and Grabbe S.** CD14 is expressed by subsets of murine dendritic cells and upregulated by lipopolysaccharide. *Adv Exp Med Biol* 1997 417:145-159
- Mallucci G, Dickinson A, Linehan J, Kohn PC, Brandner S and Collinge J.** Depleting neuronal PrP in prion infection prevents disease and reverses spongiosis. *Science* 2003 302: 871-874
- Mallucci GR, Ratte S, Asante EA, Linehan J, Gowland I and Jefferys JG Collinge J.** Post-natal knockout of prion protein alters hippocampal CA1 properties but does not result in neurodegeneration. *EMBO J* 2002 21: 202-210
- Manson JC, Clarke AR, Hooper ML, Aitchison L, McConnell I and Hope J** 129/Ola mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal. *Mol Neurobiol* 1994 8: 121-127
- Martins EA, Robalinho RL and Meneghini R.** Oxidative stress induces activation of a cytosolic protein responsible for control of iron uptake. *Arch Biochem Biophys.* 1995 316:128-134
- Maynard CJ, Cappai R, Volitakis I, Cherny RA, White AR, Beyreuther K, Masters CL, Bush AI and Li QX.** Overexpression of Alzheimer's disease amyloid-beta opposes the age-dependent elevations of brain copper and iron. *J Biol Chem.* 2002 277:44670-44676
- McKie AT, Barrow D, Latunde-Dada GO, Rolfs A, Sager G, Mudaly E, Mudaly M, Richardson C, Barlow D, Bomford A, Peters TJ, Raja KB, Shirali S, Hediger MA, Farzaneh F and Simpson RJ.** An iron-regulated ferric reductase associated with the absorption of dietary iron. *Science.* 2001 291:1755-1759
- Mehlhase J, Sandig G, Pantopoulos K and Grune T** Oxidation-induced ferritin turnover in microglial cells: role of proteasome. *Free Radic Biol Med.* 2005 38:276-285
- Mellon PL, Windle JJ, Goldsmith PC, Padula CA, Roberts JL and Weiner RI.** Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. *Neuron.* 1990 5:1-10
- Meyer RK, McKinley MP, Bowman KA, Braunfeld MB, Barry RA and Prusiner SB.** Separation and properties of cellular and scrapie prion proteins. *Proc Natl Acad Sci U S A* 1986 83: 2310-2314
- Meyron-Holtz EG, Ghosh MC, Iwai K, LaVaute T, Brazzolotto X, Berger UV, Land W, Ollivierre-Wilson H, Grinberg A, Love P and Rouault TA.** Genetic ablations of iron regulatory proteins 1 and 2 reveal why iron

regulatory protein 2 dominates iron homeostasis. *EMBO J.* 2004 23:386-395

- Milhavet O, McMahon HE, Rachidi W, Nishida N, Katamine S, Mange A, Arlotto M, Casanova D, Riondel J, Favier A and Lehmann S.** Prion infection impairs the cellular response to oxidative stress. *Proc Natl Acad Sci U S A* 2000 97:13937-13942
- Miller LL, Miller SC, Torti SV, Tsuji Y and Torti FM.** Iron-independent induction of ferritin H chain by tumor necrosis factor. *Proc Natl Acad Sci U S A.* 1991 88:4946-4950
- Millhauser GL.** Copper binding in the prion protein. *Acc Chem Res.*(2004 37:79-85.
- Minc-Golomb D, Yadid G, Tsarfaty I, Resau JH and Schwartz JP.** In vivo expression of inducible nitric oxide synthase in cerebellar neurons. *J Neurochem.* 1996 66:1504-1509
- Moos T and Morgan EH.** Transferrin and transferrin receptor function in brain barrier systems. *Cell Mol Neurobiol.* 2000 20:77-95
- Mosmann T.** Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983 65:55-63
- Muller FL, Liu Y and Van Remmen H.** Complex III releases superoxide to both sides of the inner mitochondrial membrane. *J Biol Chem.* 2004 279:49064-49073
- Murphy S.** Production of nitric oxide by glial cells: regulation and potential roles in the CNS. *Glia.* 2000 29:1-13

N

- Nicholls DG and Budd SL.** Mitochondria and neuronal survival. *Physiol Rev.* 2000 80:315-360

O

- Oesch B, Westaway D, Walchli M, McKinley MP, Kent SB, Aebersold R, Barry RA, Tempst P, Teplow DB and Hood LE.** A cellular gene encodes scrapie PrP 27-30 protein. *Cell* 1985 40: 735-746
- Oliveira L and Drapier JC.** Down-regulation of iron regulatory protein 1 gene expression by nitric oxide. *Proc Natl Acad Sci U S A.* 2000 97:6550-6555
- Orino K, Lehman L, Tsuji Y, Ayaki H, Torti SV and Torti FM.** Ferritin and the response to oxidative stress. *Biochem J.* 2001 357:241-247
- Ostlund P, Lindegren H, Pettersson C and Bedecs K.** Altered insulin receptor processing and function in scrapie-infected neuroblastoma cell lines. *Brain Res Mol* 200197:161-170
- Ostrerova-Golts N, Petrucelli L, Hardy J, Lee JM, Farer M and Woloizin B.** The A53T alpha-synuclein mutation increases iron-dependent aggregation and toxicity. *J Neurosci.* 2000 20:6048-6054

P

- Palsson-McDermott EM and O'Neill LA.** Signal transduction by the lipopolysaccharide receptor,

- Toll-like receptor-4. *Immunology*. 2004 113:153-162
- Pan KM, Baldwin M, Nguyen J, Gasset M, Serban A, Groth D, Mehlhorn I, Huang Z, Fletterick RJ and Cohen FE.** Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc Natl Acad Sci U S A* 1993 90:10962-10966
- Pandolfo M.** Iron metabolism and mitochondrial abnormalities in Friedreich ataxia. *Blood Cells Mol Dis*. 2002 29:536-547
- Pantopoulos K and Hentze MW.** Rapid responses to oxidative stress mediated by iron regulatory protein. *EMBO J*. 1995 14:2917-2924
- Pantopoulos K and Hentze MW.** Activation of iron regulatory protein-1 by oxidative stress in vitro. *Proc Natl Acad Sci U S A*. 1998 95:10559-10563
- Papanikolaou G and Pantopoulos K.** Iron metabolism and toxicity. *Toxicol Appl Pharmacol*. 2005 202:199-211
- Patel BN, Dunn RJ, Jeong SY, Zhu Q, Julien JP and David S.** Ceruloplasmin regulates iron levels in the CNS and prevents free radical injury. *J Neurosci*. 2002 22:6578-6586
- Pauly PC and Harris DA.** Copper stimulates endocytosis of the prion protein. *J Biol Chem*. 1998 273:33107-33110
- Petersen RB, Siedlak SL, Lee HG, Kim YS, Nunomura A, Tagliavini F, Ghetti B, Cras P, Moreira PI, Castellani RJ, Guentchev M, Budka H, Ironside JW, Gambetti P, Smith MA and Perry G.** Redox metals and oxidative abnormalities in human prion diseases. *Acta Neuropathol (Berl)*. 2005. In press
- Pham CG, Bubici C, Zazzeroni F, Papa S, Jones J, Alvarez K, Jayawardena S, De Smaele E, Cong R, Beaumont C, Torti FM, Torti SV and Franzoso G.** Ferritin heavy chain upregulation by NF-kappaB inhibits TNFalpha-induced apoptosis by suppressing reactive oxygen species. *Cell* 2004 119:529-542
- Prado Ma, Alves-Silva J, Magalhaes AC, Prado VF, Linden R, Martins VR and Bretani RR .** PrPc on the road: trafficking of the cellular prion protein. *J Neurochem*. 2004 88:769-781
- Preli RB, Klein KP and Herrington DM.** Vascular effects of dietary L-arginine supplementation. *Atherosclerosis*. 2002 162:1-15
- Prusiner SB.** Novel proteinaceous infectious particles cause scrapie. *Science* 1982 216: 136-144
- Prusiner SB, Groth DF, Bolton DC, Kent SB and Hood LE.** Purification and structural studies of a major scrapie prion protein. *Cell* 1984 38: 127-134
- R**
- Radi R, Beckman JS, Bush KM and Freeman BA.** Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J Biol Chem*. 1991 266:4244-4250
- Rai P, Cole TD, Wemmer DE and Linn S.** Localization of Fe(2+) at an RTGR sequence within a DNA duplex explains preferential cleavage by Fe(2+) and H2O2. *J Mol Biol*. 2001 312:1089-1101
- Recalcati S, Taramelli D, Conte D and Cairo G.** Nitric oxide-mediated induction of ferritin synthesis in J774 macrophages by inflammatory cytokines: role of selective iron regulatory protein-2 downregulation. *Blood* 1998 91:1059-1066
- Reiter RJ.** Oxidative processes and antioxidative defense mechanisms in the aging brain. *FASEB J*. 1995 9:526-533
- Richardson DR, Neumannova V, Nagy E, Ponka P.** The effect of redox-related species of nitrogen monoxide on transferrin and iron uptake and cellular proliferation of erythroleukemia (K562) cells. *Blood*. 1995 86:3211-3219.
- Riesner D.** Biochemistry and structure of PrP(C) and PrP(Sc). *Br Med Bull*. 2003 66:21-33
- Rittling SR and Woodworth RC.** The synthesis and turnover of ferritin in rat L-6 cells. Rates and response to iron, actinomycin D, and desferrioxamine. *J Biol Chem*. 1984 259:5561-5566
- Rogers JT, Randall JD, Cahill CM, Eder PS, Huang X, Gunshin H, Leiter L, McPhee J, Sarang SS, Utsuki T, Greig NH, Lahiri DK, Tanzi RE, Bush AI, Giordano T and Gullans SR.** An iron-responsive element type II in the 5'-untranslated region of the Alzheimer's amyloid precursor protein transcript. *J Biol Chem*. 2002 277:45518-45528
- Rouault TA, Hentze MW, Haile DJ, Harford JB and Klausner RD.** The iron-responsive element binding protein: a method for the affinity purification of a regulatory RNA-binding protein. *Proc Natl Acad Sci U S A*. 1989 86:5768-5772
- Rouault TA, Tang CK, Kaptain S, Burgess WH, Haile DJ, Samaniego F, McBride OW, Harford JB and Klausner RD.** Cloning of the cDNA encoding an RNA regulatory protein--the human iron-responsive element-binding protein. *Proc Natl Acad Sci U S A*. 1990 87:7958-7962
- Rouault TA and Tong WH.** Iron-sulphur cluster biogenesis and mitochondrial iron homeostasis. *Nat Rev Mol Cell Biol* 2005 6:345-351
- S**
- Samaniego F, Chin J, Iwai K, Rouault TA and Klausner RD.** Molecular characterization of a second iron-responsive element binding protein, iron regulatory protein 2. Structure, function, and post-translational regulation. *J Biol Chem*. 1994 269:30904-30910
- Sanlioglu S, Williams CM, Samavati L, Butler NS, Wang G, McCray PB Jr, Ritchie TC,**

- Hunninghake GW, Zandi E and Engelhardt JF.** Lipopolysaccharide induces Rac1-dependent reactive oxygen species formation and coordinates tumor necrosis factor- α secretion through IKK regulation of NF- κ B. *J Biol Chem.* 2001 276:30188-30198
- Schubert D, Humphreys S, Baroni C and Cohn M.** In vitro differentiation of a mouse neuroblastoma. *Proc Natl Acad Sci U S A.* 1969 64:316-32
- Scott MR, Safar J, Telling G, Nguyen O, Groth D, Torchia M, Koehler R, Tremblay P, Walther D, Cohen FE, DeArmond SJ and Prusiner SB.** Identification of a prion protein epitope modulating transmission of bovine spongiform encephalopathy prions to transgenic mice. *Proc Natl Acad Sci U S A.* 1997 94:14279-14284
- Shyng SL, Huber MT and Harris DA.** A prion protein cycles between the cell surface and an endocytic compartment in cultured neuroblastoma cells. *J Biol Chem.* 1993 268:15922-15928
- Smith MA, Harris PL, Sayre LM and Perry G.** Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. *Proc Natl Acad Sci U S A.* 1997 94:9866-986
- Smith PD, Smythies LE, Mosteller-Barnum M, Sibley DA, Russell MW, Merger M, Sellers MT, Orenstein JM, Shimada T, Graham MF and Kubagawa H.** Intestinal macrophages lack CD14 and CD89 and consequently are down-regulated for LPS- and IgA-mediated activities. *J Immunol.* 2001 167:2651-2656
- Solfarosi L, Criado JR, McGavern DB, Wirz S, Sanchez-Alavez M, Sugama S, DeGiorgio LA, Volpe BT, Wiseman E, Abalos G, Masliah E, Gilden D, Oldstone MB, Conti B and Williamson RA.** Cross-linking cellular prion protein triggers neuronal apoptosis in vivo. *Science.* 2004 303:1514-1516
- Stockel J, Safar J, Wallace AC, Cohen FE and Prusiner SB.** Prion protein selectively binds copper(II) ions. *Biochemistry.* 1998 37:7185-7193
- T**
- Tabner BJ, Turnbull S, El-Agnaf OM and Allsop D.** Formation of hydrogen peroxide and hydroxyl radicals from A(β) and α -synuclein as a possible mechanism of cell death in Alzheimer's disease and Parkinson's disease. *Free Radic Biol Med.* 2002 32:1076-1083
- Takeda K and Akira S.** TLR signaling pathways. *Semin Immunol.* 2004 6:3-9
- Taraboulos A, Raeber AJ, Borchelt DR, Serban D and Prusiner SB.** Synthesis and trafficking of prion proteins in cultured cells. *Mol Biol Cell.* 1992 3: 851-863
- Telling GC, Scott M, Hsiao KK, Foster D, Yang SL, Torchia M, Sidle KC, Collinge J, DeArmond SJ and Prusiner SB.** Transmission of Creutzfeldt-Jakob disease from humans to transgenic mice expressing chimeric human-mouse prion protein. *Proc Natl Acad Sci U S A.* 1994 91: 9936-9940
- Theil EC.** Ferritin: at the crossroads of iron and oxygen metabolism. *J Nutr.* 2003 133:1549-15453
- Theil EC and Eisenstein RS.** Combinatorial mRNA regulation: iron regulatory proteins and iso-iron-responsive elements (Iso-IREs). *J Biol Chem.* 2000 275:40659-40662
- Tobler I, Gaus SE, Deboer T, Achermann P, Fischer M, Rulicke T, Moser M, Oesch B, McBride PA and Manson JC.** Altered circadian activity rhythms and sleep in mice devoid of prion protein. *Nature.* 1996 380: 639-642
- Torrance JD and Bothwell TH.** A simple technique for measuring storage iron concentrations in formalinised liver samples. *S Afr J Med Sci.* 1968 33:9-11
- Torti SV, Kwak EL, Miller SC, Miller LL, Ringold GM, Myambo KB, Young AP and Torti FM.** The molecular cloning and characterization of murine ferritin heavy chain a tumor necrosis factor-inducible gene. *J Biol Chem.* 1988 263:12638-12644
- Trinder D and Baker E.** Transferrin receptor 2: a new molecule in iron metabolism. *Int J Biochem Cell Biol.* 2003 35:292-296
- Turnbull S, Tabner BJ, Brown DR and Allsop D.** Copper-dependent generation of hydrogen peroxide from toxic prion protein fragment PrP106-126. *Neuroscience Lett.* 2003 336: 159-162
- V**
- Vile GF and Tyrrell RM.** Oxidative stress resulting from ultraviolet A irradiation of human skin fibroblasts leads to a heme oxygenase-dependent increase in ferritin. *J Biol Chem.* 1993 268:14678-14681
- Vilhardt F.** Microglia: phagocyte and glia cell. *Int J Biochem Cell Biol.* 2005 37:17-21
- W**
- Waggoner DJ, Drisaldi B, Bartnikas TB, Casareno RL, Prohaska JR, Gitlin JD and Harris DA.** Brain copper content and cuproenzyme activity do not vary with prion protein expression level. *J Biol Chem.* 2000 275:7455-7458
- Wang J, Chen G and Pantopoulos K.** Nitric oxide inhibits the degradation of IRP2. *Mol Cell Biol.* 2005 25:1347-1353
- Wei Y, Miller SC, Tsuji Y, Torti SV and Torti FM.** Interleukin 1 induces ferritin heavy chain in human muscle cells. *Biochem Biophys Res Commun.* 1990 169:289-296
- Weiss G, Goossen B, Doppler W, Fuchs D, Pantopoulos K, Werner-Felmayer G,**

Wachter H and Hentze MW Translational regulation via iron-responsive elements by the nitric oxide/NO-synthase pathway. *EMBO J*. 1993 12:3651-3657

White AR, Collins SJ, Maher F, Jobling MF, Stewart LR, Thyer JM, Beyreuther K, Masters CL and Cappai R. Prion protein-deficient neurons reveal lower glutathione reductase activity and increased susceptibility to hydrogen peroxide toxicity. *Am J Pathol*. 1999 155:1723-1730

Will RG. Acquired prion disease: iatrogenic CJD, variant CJD, kuru. *Br Med Bull*. 2003 66:255-265

Wilson JX. Antioxidant defense of the brain: a role for astrocytes. *Can J Physiol Pharmacol*. 1997 75:1149-1163

Wong BS, Brown DR, Pan T, Whiteman M, Liu T, Bu X, Li R, Gambetti P, Olesik J, Rubenstein R and Sy MS . Oxidative impairment in scrapie-infected mice is associated with brain metals perturbations and altered antioxidant activities. *J Neurochem* 2001b 79:689-698

Wong BS, Liu T, Li R, Pan T, Petersen RB, Smith MA, Gambetti P, Perry G, Manson JC, Brown DR and Sy MS. Increased levels of oxidative stress markers detected in the brains of mice devoid of prion protein. *J Neurochem* 2001a 76:565-572

Wu LJ, Leenders AG, Cooperman S, Meyron-Holtz E, Smith S, Land W, Tsai RY, Berger UV, Sheng ZH and Rouault TA. Expression of the iron transporter ferroportin in synaptic vesicles and the blood-brain barrier. *Brain Res*. 2004 1001:108-117

X

Xie QW, Whisnant R and Nathan C. Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. *J Exp Med*. 1993 177:1779-1784

Y

Yedidia Y, Horonchik L, Tzaban S, Yanai A and Taraboulos A. Proteasomes and ubiquitin are involved in the turnover of the wild-type prion protein. *EMBO J* 2001 20: 5383-5391

Yu Z, Persson HL, Eaton JW and Brunk UT. Intralysosomal iron: a major determinant of oxidant-induced cell death. *Free Radic Biol Med*. 2003 34:1243-1252.

Z

Zhou B, Westaway SK, Levinson B, Johnson MA, Gitschier J and Hayflick SJ. A novel pantothenate kinase gene (PANK2) is defective in Hallervorden-Spatz syndrome. *Nat Genet*. 2001 28:345-349