Protein polyubiquitination and oxidative damage in hepatic encephalopathy

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"Damit das Mögliche entsteht,

muss immer wieder das

Unmögliche versucht werden."

Hermann Hesse

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1. Introduction

1.1 Hepatic Encephalopathy

Hepatic encephalopathy (HE) is a neuropsychiatric disorder resulting from either acute or chronic liver failure. Acute liver failure (ALF) most frequently results from acute hepatitis B infection or intoxication with liver-toxic drugs such as paracetamol and may subsequently lead to HE (Lee, 2003; Butterworth, 2014). However, in most HE cases, chronic liver failure represents the origin of the disease. Chronic liver failure often arises from persistent alcohol abuse or chronic hepatitis virus infection leading to hepatocyte loss and fibrosis which destroys the physiological function and liver architecture (Romero-Gómez et al., 2014; Vilstrup et al., 2014). When the liver fails to properly process and metabolize toxic compounds or when blood is shunted past the liver, detrimental metabolites can accumulate, reach the central nervous system (CNS) and thereby affect cerebral function. Patients display a large variability of symptoms, clinically manifesting in impairment of the sleep-wake cycle, cognitive and intellectual performance, motor functions as well as abnormal emotional and behavioral patterns that can progressively worsen (Häussinger and Schliess, 2008). Manifestation of HE is categorized into different grades, depending on the severity of symptoms. Minimal HE appears with rather subtle alterations that may only be detectable by psychometric tests. Overt HE is traditionally graded into the West Haven criteria in HE grades I-IV (Conn et al., 1977; Häussinger and Sies, 2013). Although the pathogenic factors that cause the progress from minimal HE to overt HE are not completely understood, elevated levels of circulating ammonia resulting from liver failure have been strongly associated with induction of manifest HE. In patients, the reduction of liver function correlates with their plasma ammonia but is however not always clearly correlated with the severity of the disease (Ong et al., 2003; Lockwood, 2004). Nevertheless decreasing the systemic ammonia concentration is a therapeutic intervention that improves HE severity with partial reversion of the symptoms (Blei, 2000). Hence, ammonia is thought to be the most important factor and the key toxin leading to HE (Norenberg, 1996; Butterworth, 2002).

Table 1

West Haven criteria in HE

Grade	Symptoms
Low grade	
Minimal HE	
	no overt symptoms, detectable by psychometric tests
Manifest HE	
	Grade I: trivial lack of awareness/attention, irritability, postural tremor
	Grade II: lethargy, apathy, fatigue, disorientation, altered sleep-wake cycle, personality/behavioral changes, flapping tremor
High grade	
	Grade III: somnolence, stupor
	Grade IV: coma

1.1.1 Ammonia metabolism

The gross of systemic ammonia is generated from the breakdown of nitrogenous components from the diet by intestinal bacteria (Huizenga et al., 1996; Gerber and Schomerus, 2000). From the gut, ammonia is delivered to the liver via the portal vein circulation and, under physiological conditions, converted to urea in the hepatic urea cycle (Morris, 2002). Thereby blood ammonia is maintained at low concentrations (<50µM in adults) (Donn and Thoene, 1985). During ALF or cirrhosis, a dysregulation of the liver-gut axis and a deranged hepatic metabolism lead to rise of ammonia levels. In the blood, ammonia is mostly present in its ionized form (NH_4^+). Changes of the pH affect the concentration of un-ionized ammonia (NH_3) which is thought to be capable to diffuse across the blood brain barrier (BBB). Also, some studies suggest an active transport of ionized NH_4^+ over the BBB (Wetterling, 2002; Ott and Larsen, 2004). Notably, the brain is unable to convert ammonia (in the following termed as ammonia or NH_4) to urea due to its lack of carbamoylphosphate synthetase-1 and ornithine transcarbamoylase. For this reason, in the CNS the levels of ammonia are maintained and regulated by astrocytes expressing the enzyme glutamine synthetase (GS) which converts glutamate and NH_4 to glutamine (Felipo and Butterworth, 2002). However, an osmotic effect occurs when intracerebellar ammonia

levels rise to an extend that exceeds the capacity of astrocytes to detoxify the substances. As a consequence, astrocytes swell and by this represent a main contributor to development of brain edema causing a drastic elevation of the intracranial pressure (Blei and Larsen, 1999; Butterworth, 2002; Vaquero et al., 2003a). Thus, it is not surprising that ammonia toxicity is assumed a central component for the development of HE. Ammonia possibly sensitizes the brain for so-called precipitating factors that are thought initiate overt HE phase and even represents a precipitating factor itself (Ong et al., 2003). In addition to ammonia, inflammatory cytokines and benzodiazepines are other characteristic precipitating factors which provoke astrocyte swelling. Consequently, oxidative and nitrosative stress in the CNS emerges (Görg et al., 2003; Häussinger and Schliess, 2005). There is a substantial body of literature addressing oxidative and nitrosative stress and its association with the pathogenesis of HE (Rao et al., 1991; Rao et al., 1992; Kosenko et al., 1997; Schliess et al., 2002; Häussinger and Schliess, 2005; Reinehr et al., 2007; Brück et al., 2011; Görg et al., 2013b).

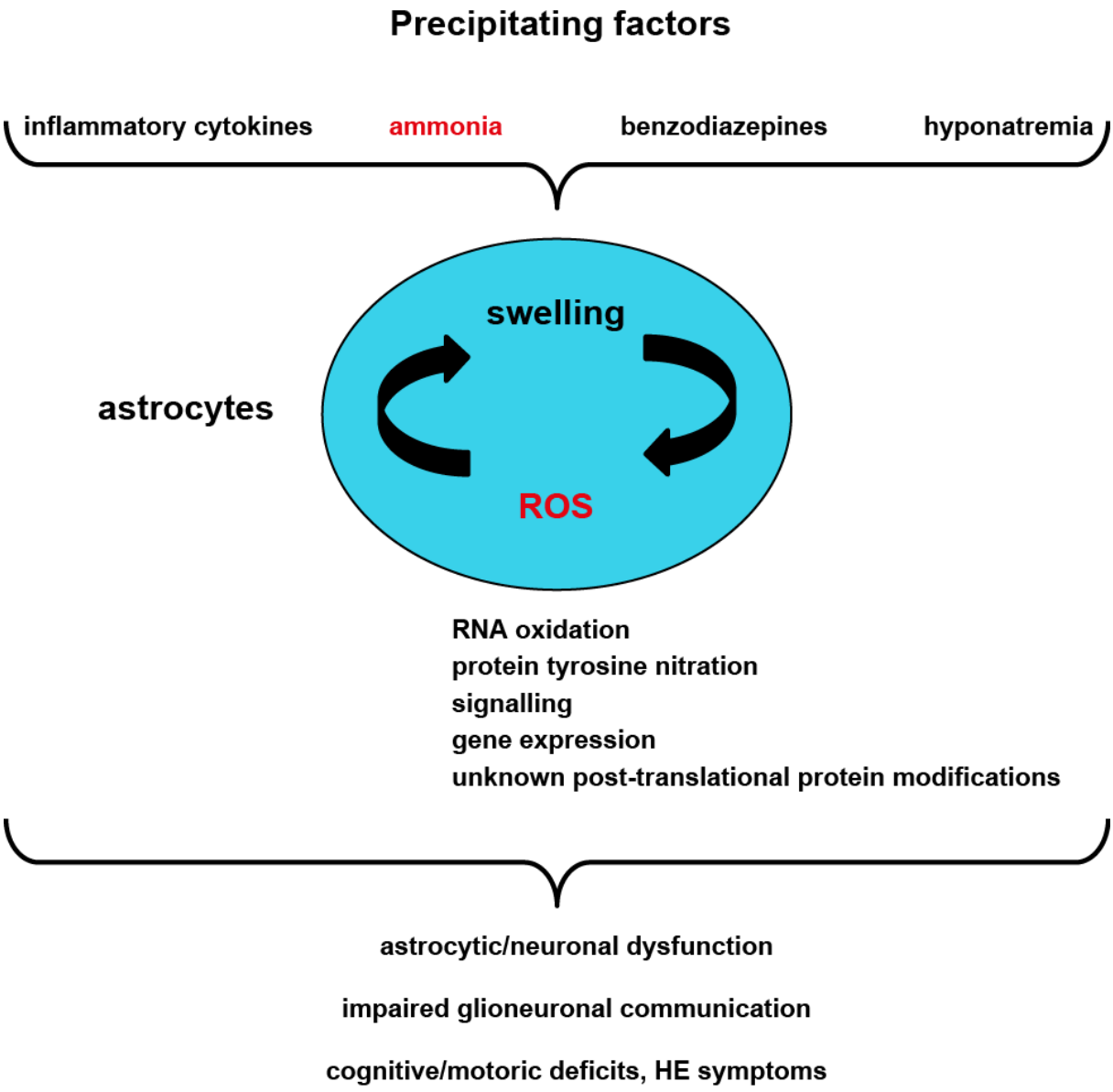

Figure 1.1. Pathophysiological model of hepatic encephalopathy HE. Heterogeneous precipitating factors are thought to induce swelling of astrocytes and putatively impact on microglia. Cell swelling promotes production of reactive oxygen species (ROS) which further reinforce cell swelling. Thereby a fatal self-amplificatory loop of swelling and ROS generation in the brain is induced resulting in modifications of proteins and RNA, gene expression and signalling alterations. Finally these effects may lead to dysfunction of neural cells and contribute to symptoms and cognitive deficits evident in HE patients (modified after Häussinger and Sies, 2013).

1.1.2 Oxidative and nitrosative stress in HE

There is considerable evidence that ammonia is implicated in causing oxidative and nitrosative stress in the CNS of HE patients and in HE animal models. This includes generation of free radicals, protein tyrosine nitration (PTN) and oxidation of both, proteins and RNA (Norenberg, 2003; Schliess et al., 2009; Görg et al., 2010; Montoliu et al., 2011; Görg et al., 2013b). Reaching the brain, precipitating factors such as ammonia, benzodiazepines and inflammatory cytokines lead to swelling of astrocytes and to subsequent formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Figure 1.1), (Murthy et al., 2001; Schliess et al., 2002; Reinehr et al., 2007; Lachmann et al., 2013). *N*-methyl-*D*-aspartate (NDMA) receptors and Ca^{2+}-dependent mechanisms are involved in this process. The astrocytic NMDA receptor is thought to be activated by removal of Mg^{2+} that is beforehand bound to the receptor. Depolarization removes this Mg^{2+}-blockade and causes a subsequent release of glutamine-containing vesicles from the astrocytes. Thereby an autocrine amplification loop of NMDA receptor activation is created (Häussinger and Schliess, 2008; Schliess et al., 2009; Görg et al., 2013b). Hence, activation of NMDA receptors enhances the intracellular Ca^{2+}-concentration which activates NADPH-oxidase (NOX) and nitric oxide synthase (NOS). These enzymes lead to formation of superoxide-anion radicals (O_2^-) and nitric oxide (NO) respectively. NOX-derived O_2^- was shown to enhance RNA oxidation in astrocytes and neurons and by this possibly affecting protein expression and turnover (Görg et al., 2008; Schliess et al., 2009). Astrocyte swelling and induction of such oxidative stress reinforce each other and produce a self-amplifying signalling loop (Häussinger, 2006). Ammonia detoxification by the GS-catalyzed reaction is almost exclusively performed in astrocytes but the generation of ROS was recently shown to take place in microglial cells as well (Martinez-Hernandez et al., 1977; Rama Rao, Kakulavarapu V et al., 2010; Zemtsova et al., 2011; Rao, Kakulavarapu V Rama et al., 2013). In addition to ammonia, release of ROS originating from microglia cells may further contribute to astrocyte swelling and, thus, progression of HE (Zemtsova et al., 2011; Rao, Kakulavarapu V Rama et al., 2013). Systemic inflammation and presence of bacteria-derived endotoxins such as lipopolysaccharide (LPS) lead to activation of immune cells and may act in concert with ammonia to generate ROS/RNS in the CNS (Shawcross et al., 2010; Bosoi et al., 2012). Persistent ROS/RNS stress in the brain has functional consequences. As mentioned earlier, RNA oxidation represents one modification induced by an increased ammonia load. Further, in cultured astrocytes, PTN was identified as another implication of ammonia exposure with. Thus, GS activity was shown to be abolished by tyrosine nitration while astrocytic Na-K-Cl co-transporter show an enhanced activity (Görg et al., 2007; Jayakumar et al., 2008; Görg et al., 2010). Overall increase of PTN as well as high levels of RNA oxidation were also found in *post mortem* cortex samples from cirrhotic HE

patients (Häussinger and Görg, 2010; Görg et al., 2010). Another recently identified post translational modification induced by ammonia is *O*-GlcNAcylation of proteins (Karababa et al., 2013). *O*-GlcNAcylation participates in several cellular mechanisms such as signalling pathways, transcription and also protein degradation. Nevertheless its importance for the pathogenesis of HE remains to be investigated. Accordingly, other post translational modifications such as ubiquitination or oxidation of proteins may be relevant for HE. Oxidation of proteins involves alteration of amino acid chains, for instance introduction of carbonyl groups which can be measured as a reference for ongoing protein oxidation (Levine et al., 1994; Nyström, 2005). Since oxidative protein modifications occur continuously during physiological conditions, the cell has evolved mechanisms to reduce oxidized proteins in order to restore their function. Such systems are crucial for protein homeostasis within the cell; examples are the thiol repair systems that require glutathione or thioredoxin (Holmgren, 1989). However, in case repair fails, protein degradation involving ubiquitination and proteasomal lysis, remains the only way to remove damaged proteins. In pathological conditions, exorbitant intensities of oxidative stress produce high levels of such damaged proteins. In this situation the ubiquitin proteasome system (UPS) and the unfolded protein response (UPR) of the endoplasmic reticulum (ER) may fail to clear damaged proteins. As a consequence, oxidized and misfolded proteins are prone to form non-functional aggregates that remain in the cell. In diverse neurodegenerative diseases aggregate formation is frequently observed (Ardley et al., 2005; Ciechanover, 2005). At present there is no data available on the accumulation of oxidized, misfolded or ubiquitinated proteins in the HE-affected brain.

1.1.3 Experimental models to study the effects of hyperammonemia

Pathogenic animal models of HE are of particular interest since the molecular mechanisms that underlie HE pathology and ammonia toxicity are still incompletely understood. It is challenging to model HE and its origin considering that the disease is based on varying causes. HE involves different degrees of liver damage, portacaval shunting or association with precipitating factors such as intoxication, systemic inflammation with subsequent cytokine release or presence of LPS among many other aspects. For that reason, it is fairly impossible to reproduce the entirety of aspects in a single experimental model. Thus, the experimental paradigm chosen for a study should fit to the main research objectives. Some models are based on administration of hepatotoxic compounds to induce ALF. Toxic substances such as galactosamine, thioacetamine, acetaminophen or azoxymethane are administered to mice and rats and less frequent to rabbits and dogs. The hepatitis resulting from these hepatotoxins leads to diverse pathologies that do not always reflect common

human HE symptoms. Hence, for induction of hyperammonemia *in vivo* other acute and chronic models for mice and rats have been established (Boulton et al., 1992; Butterworth et al., 2009; Bhatnagar and Majumdar, 2003). Among the chronic models, use of portacaval anastomosis is a common one. Another option offers the bile duct ligation (BDL), a model for biliary cirrhosis in rats. Contrary to portal-systemic shunting, animals develop liver cirrhosis, jaundice and immune system dysfunction (Chan et al., 2004; Jover et al., 2006; Wright et al., 2007). BDL rats exhibit elevated ammonia levels and detectable oxidative stress in the brain; nonetheless, only subtle symptoms of HE are evident (Chan et al., 2004; Chroni et al., 2006). Feeding BDL rats with an ammonium acetate (NH_4^+Ac) containing diet results in further elevation of ammonia levels and produces an induction of HE with a neuropathology similar to humans, including motor deficits, brain edema and inflammation (Jover et al., 2006). The model of portacaval anastomosis in rats is widely applied as a method to study the reduced detoxifying capacity of the liver on the brain function without actual presence of a parenchymal liver disease as present in cirrhosis. Different from BDL rats, shunted rats instantly develop a manifest encephalopathy with brain edema, astrocyte swelling and elevated ammonia levels in the brain (Blei et al., 1994; Chung et al., 2001). Moreover, the animals display a disturbed circadian rhythm, show reduced cognitive performance and exhibit oxidative and nitrosative stress as well as increased glutamine levels (Méndez et al., 2011; Desjardins et al., 2012; Llansola et al., 2012; Bosoi et al., 2014). Similarly to BDL, shunted rats are sensitive to an additional increase of ammonia levels. Thus, systemic administration of ammonia results in progression of HE symptoms and may finally lead to coma and death of the animals. Recently, the conditional GS knockout mouse was established as a model for hyperammonemia. This mouse has a specific deletion of the hepatic GS which leads to elevated blood ammonia levels and induction of oxidative stress in the brain (Qvartskhava et al., 2015).

A pure acute model for hyperammonemia is the systemic application of pathophysiological relevant concentrations of ammonia. This model aims to study the *per se* effect of acutely elevated ammonia levels on the brain in disregard of the hepatic function. Intraperitoneal (i.p.) injection of NH_4^+Ac rapidly exposes the brain to ammonia, thereby serving as an appropriate model to study the instant effects triggered by an acute rise of ammonia levels. Acute hyperammonemia was shown to have numerous metabolic and neurophysiologic consequences including alterations in synaptic function, impaired long-term potentiation (LTP) or NMDA receptor-mediated excitotoxicity (Szerb and Butterworth, 1992; Robinson et al., 1995; Hermenegildo et al., 1998; Aguilar et al., 2000; Muñoz et al., 2000; Izumi et al., 2005; Chepkova et al., 2006). Studies demonstrated an impairment of mitochondrial function due to acute hyperammonemia (Lores-Arnaiz et al., 2005; Perazzo et al., 1998). In this model, oxidative stress is evident due to induction of NO und superoxide production and a

decrease in activities of antioxidant enzymes (Rao et al., 1995; Kosenko et al., 1998; Kosenko et al., 1997). However, the effect of ammonia-related oxidative stress on protein integrity is largely unknown. Furthermore, only few studies focus on the effect of acute hyperammonemia on specific brain regions (Muñoz et al., 2000; Arias et al., 2015) and rather examine global brain consequences.

In vitro, ammonia toxicity has been extensively studied on primary cultures with a focus on astrocytes (Schliess et al., 2002; Jayakumar et al., 2006). Astrocyte cultures exhibit metabolic and pathological alterations similar to those found in HE (Murthy et al., 2001; Warskulat et al., 2002; Häussinger and Schliess, 2005; Rao, Kakulavarapu V Rama et al., 2013). Recently, neuronal, microglial and mixed microglia-astrocyte cultures have been involved in the analysis of ammonia toxicity (Zemtsova et al., 2011; Rao, Kakulavarapu V Rama et al., 2013; Görg et al., 2015), since the interplay between different neural cell types has to be considered. Another culture model to elucidate HE underlying mechanisms is the use of organotypic slice cultures such as hippocampal brain slice cultures. In this model the tissue architecture is preserved, which permits the analysis of cell-cell interactions. Brain slice cultures have been used to study the effect of ammonia on astrocyte swelling, LTP, neuronal injury and cell signalling (Zielinska et al., 2003; Chepkova et al., 2006; Monfort et al., 2007; Reinehr et al., 2007; Görg et al., 2008; Back et al., 2011; Haack et al., 2014; Izumi et al., 2005).

1.2 Ubiquitin and the ubiquitin proteasome system

Impairment of the neural UPS function has been linked to various pathological conditions. An important task of the UPS is to eliminate oxidatively-damaged proteins, thus the UPS represents a crucial and vital mechanism to ensure a physiological CNS function. For that reason, the UPS may display a link between ammonia-induced oxidative stress and neural dysfunction. In various neurodegenerative disorders and neuroinflammatory disturbances the protein homeostasis is affected (Ciechanover and Brundin, 2003; Dantuma and Bott, 2014; Jansen, Anne H P et al., 2014). The UPS represents the main cellular machinery involved in protein homeostasis and consists of two main components: the protein ubiquitin-ligation system and the proteolytic system. In order to be recognized and subsequently degraded by the proteasome, damaged proteins first need to be marked. This occurs by attachment of ubiquitin moieties to target substrates.

1.2.1 Ubiquitin and ubiquitination

Ubiquitin is a small protein with a molecular mass of 8.5 kDa and 76 amino acids length. Transcription occurs from four different genes (*RPS27a*, *UBCEP2, UBB* and *UBS*) either fused to ribosomal proteins (RPS27a and UBCEP2) or as a polyubiquitin precursor protein (UBB and UBC). Seven lysines (K) play a key role in the amino acid sequence of ubiquitin located at positions 6, 11, 27, 29, 33, 48 and 63. A C-terminal glycine provides the possibility to conjugate a single ubiquitin to an internal lysine of another ubiquitin molecule (Chau et al., 1989). This posttranslational modification is termed polyubiquitination. Polyubiquitination is a crucial step for directing proteins towards proteasomal degradation and thus it preserves the overall intracellular protein homeostasis (Kleiger and Mayor, 2014). Additionally, polyubiquitination and monoubiquitination are involved in numerous other cellular processes such as ER-associated protein degradation, immune response, lysosomal degradation, autophagy and various signalling pathways (Liu, 2004; Chen and Sun, 2009; Ebrahimi-Fakhari et al., 2011; Lemus and Goder, 2014). The process of ubiquitination is regulated by the activity of three different enzymes. The E1 enzyme activates a single ubiquitin molecule by forming a bond between the enzyme's active site cysteine and an internal carboxyl group of ubiquitin in an ATP-dependent manner. Transfer to the E2 ubiquitin conjugating enzyme includes a transthioesterification reaction. Subsequent transfer to E3 ubiquitin ligase enzyme finally leads to formation of an isopeptide bond between ubiquitin and a target protein. (Pickart, 2001). There are different variants of E1, E2 and E3 enzymes, ranging from two to 600 different isoforms (Semple, Colin A M, 2003). Principally, all lysines of the ubiquitin chain can be connected to another ubiquitin molecule. Nevertheless, K48-linkage is the most abundant one, especially in terms of labelling a target protein for proteasomal degradation. A chain of at least four ubiquitin moieties is necessary to ensure recognition and degradation by the proteasome (Thrower et al., 2000). K63-linkage is the only polyubiquitin chain that does not mark a target substrate for proteasomal degradation. Interestingly, K63-linkage was found to be increased in neural tissue during neurodegenerative processes (Lim and Lim, Grace G Y, 2011; Tan, Jeanne M M et al., 2008) and seems to play a role in cell death, inflammation and ER-associated protein degradation (Martinez-Forero et al., 2009; Humphrey et al., 2013; Lemus and Goder, 2014). Deubiquitination as a reverse process to ubiquitination is mediated by linkage-specific deubiquitinating enzymes (DUBs) (Larsen et al., 1998). DUBs are essential for preventing the proteasomal degradation and thus important for signalling cascades and neuronal functioning (Eletr and Wilkinson, 2014; Ristic et al., 2014; Todi and Paulson, 2011; Wolberger, 2014). Polyubiquitinated proteins with at least four ubiquitin moieties are recognized by specialized shuttling proteins and subsequently transferred to the proteasome for degradation (Thrower et al., 2000). However,

there is also evidence for an ubiquitin-independent degradation which may play a role in conditions of oxidative stress (Reinheckel et al., 1998; Pickering et al., 2010).

1.2.2 Proteasome and immunoproteasome

Polyubiquitinated proteins are degraded by the 26S proteasome. The 26S complex is composed of a barrel-shaped 20S core and the 19S regulator. Binding of a substrate to the 19S regulatory particles of the proteasome initiates the proteolytic process. Structurally, the 20S core is composed of two identical outer rings with seven α1-α7 subunits, flanking two identical inner rings of seven β-subunits of which β1, β2 and β5 represent the catalytically active parts (Figure 1.2). The β1 subunit holds caspase-like activity with a cleavage specificity after acidic residues. β2 and β5 show trypsin- and chymotrypsin-like activities with cleavage preferences after basic and hydrophobic residues respectively (Kloetzel et al., 1999; Rock et al., 2002; Pickering and Davies, Kelvin J A, 2012). On either end of the 20S core complex, one 19S regulatory “lid” is attached, which contains a hexameric ring that includes six proteins with ATPase activity (Pickart and Cohen, 2004; Finley, 2009). The 19S regulator identifies, binds and unfolds the substrates and subsequently transfers them to the 20S core complex in an ATP-dependent fashion (Kloetzel, 2004a; Ebstein et al., 2012). In addition to the 19S complex, other regulatory proteasome activators (PA) exist, including PA28αβ as well as PI31 and PA200 (Lilienbaum, 2013). Among these activators, PA28αβ has an outstanding role. During the immune response the release of pro-inflammatory cytokines such as interferon-γ (IFN-γ) induces PA28αβ expression and its binding to the 20S core. Interaction leads to formation of hybrid proteasomes (19S-20S-PA28) which increase the proteolytic activity of all β-subunits and thus enhance the proteasomal capacity to produce peptides for the major histocompatibility complex (MHC) class I antigen presentation (Tanahashi et al., 2000; Kloetzel, 2004a; Kloetzel, 2004b; Sijts, E J A M and Kloetzel, 2011). Besides incorporation of PA28αβ subunits to the proteasome, pro-inflammatory cytokine IFN-γ was shown to induce expression of immunoproteasome (iP) subunits LMP2 (β1i, Psmb9), Mecl1 (β2i; Psmb10) and LMP7 (β5i; Psmb8). Thus, replacement of the constitutive subunits β1, β2 and β5 leads to an alternative proteasome composition and further enhances the proteasome’s activity (Kloetzel, 2001). The iP exhibits an altered cleavage pattern resulting in a more efficient and diverse generation of peptide fragments for MHC class I presentation (Kloetzel and Ossendorp, 2004; Kruger and Kloetzel, 2012). The formation of iPs is transient, occurs exclusively *de novo* and is strongly accelerated during an immune response (Heink et al., 2005; Seifert and Krüger, 2008). In addition to pure iPs, generation of mixed proteasomes can occur, which are comprised of both, constitutive and iP subunits (Dahlmann et al., 2000; Drews et al., 2007). In lymphoid tissues, iPs are constitutively expressed. T-cells, B-cells, macrophages and immature dendritic cells show high expression

levels of iP even under basal conditions, emphasizing their importance for the immune response (Macagno et al., 1999; Noda et al., 2000; Sijts, E J A M and Kloetzel, 2011; Ebstein et al., 2012). Upon oxidative stress, the UPS represents a pivotal cellular machinery to react on an increased amount of misfolded and oxidized proteins.

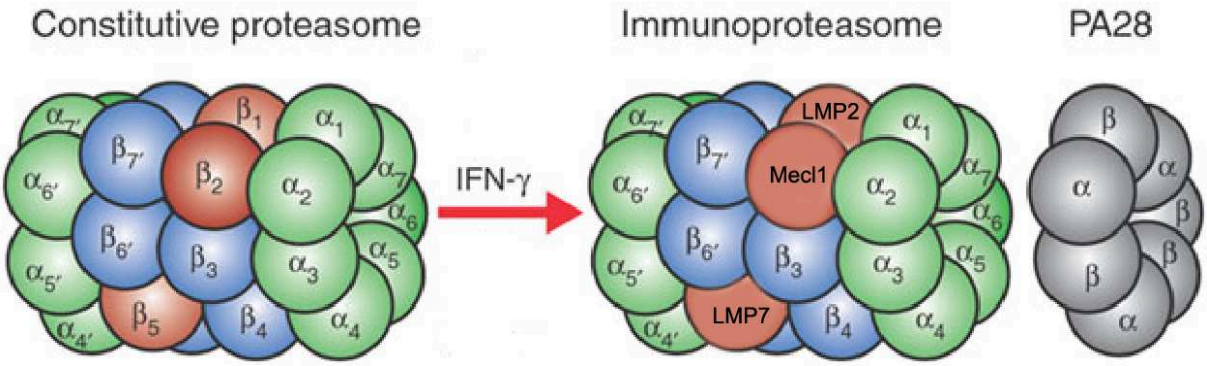

Figure 1.2. Formation of immunoproteasomes. The 20S proteasome is composed of 28 different subunits arranged as a barrel-shaped complex. The two outer rings consist of α1-α7 subunits, the two inner rings of β1-β7. The active sites are located in subunits β1, β2 and β5. Upon inflammatory challenge (IFN-γ) immunoproteasome subunits LMP2 (β1i, Psmb9), Mecl-1 (β2i; Psmb10) and LMP7 (β5i; Psmb8) are synthesized *de novo* and incorporated into the immunoproteasome. Moreover, IFN-γ induces synthesis and binding of PA28αβ to 20S both proteasomes and immunoproteasomes thereby enhancing the proteasomal activity (modified after Kloetzel, 2004).

1.2.3 Endoplasmic reticulum stress

In addition to the UPS, protein homeostasis is regulated in the ER compartment. The ER is crucial for the cellular protein quality control by guidance of protein folding, post-translational modifications and transport of nascent proteins to target destinations (Ellgaard and Helenius, 2003; English and Voeltz, 2013). Furthermore, ER residing proteins prevent protein misfolding and aggregation. Upon various cellular stress conditions, the ER activation and the unfolded protein response (UPR) are initiated via modulation of translational and transcriptional events (Walter and Ron, 2011, 2011, 2011). Hence, loss of ER integrity results in ER stress and is another source for accumulation of misfolded proteins. The normal ER response involves translational attenuation to reduce the amount of damaged proteins, induction of ER chaperones and subsequent degradation of disarranged proteins. ER chaperones are molecules that facilitate proper folding of proteins and ensure degradation of misfolded proteins. Thus, ER chaperones such as Calreticulin, GRP94 and GRP78 are vital for the reaction on stress conditions that perturb ER function and protein homeostasis (Lee, 2005; Eletto et al., 2010).

However, when stress conditions persist over a longer period of time, ER stress leads to induction of pro-apoptotic pathways and by this promotes cell death including neuronal cells (Breckenridge et al., 2003; Rao et al., 2004). Therefore, it is suggested that ER stress occurs during neuroinflammation and plays a role in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease or multiple sclerosis (Katayama et al., 2004; Holtz and O'Malley, 2003; Mháille et al., 2008). In addition to chronic CNS diseases, a role for the ER stress response was proposed for acute CNS distortions such as cerebral ischemia and brain trauma (Larner et al., 2004; Hayashi et al., 2005; Zhang and Kaufman, 2008). In cerebral stress conditions, the UPS is known to be misregulated as well, leading to further accumulation of protein aggregates and promotion of pro-inflammatory toxic conditions in the CNS tissue (Ciechanover and Brundin, 2003; Soto, 2003). Interestingly, ER stress in combination with abnormal protein degradation due to an impaired UPS, was shown to promote the pathophysiology of neurodegenerative diseases (Ryu et al., 2002; Holtz and O'Malley, 2003; Sakagami et al., 2013). Thus, dysfunction of the UPS and ER stress may critically modulate outcome and pathophysiology of neurological diseases in oxidative stress conditions. However, to date, nothing is known about a possible role of ER stress in HE.

2. 1 Aspects of neurodegeneration in HE

2.1.2 Hippocampal functionality and ammonia toxicity

To date, ammonia in HE is accepted to hold a key role as a neurotoxin. However the molecular reasons for the cognitive dysfunctions during HE and/or hyperammonemic episodes still remain to be defined. The hippocampus displays the crucial brain structure for certain types of memory and learning (Broadbent et al., 2004; Nakazawa et al., 2004; Neves et al., 2008). Damage in this region causes a reduction in performance of cognitive tasks, recognition and severely impairs spatial memory (Moser et al., 1995; Duva et al., 1997; Eichenbaum and Cohen, 2004). The toxic effect of ammonia on the hippocampus is poorly investigated. In rodent hippocampal slice cultures, an acute ammonia load was demonstrated to decrease the LTP and lead to reduced cognitive performance which is also evident in human HE patients (D'Hooge et al., 2000; Muñoz et al., 2000; Izumi et al., 2005; Chepkova et al., 2006). The impaired LTP may be one reason for variations in cognitive performance found in animals as well as in patients during episodes of hyperammonemia, since the LTP is considered to be the basis for some forms of memory and learning (Rodrigo et al., 2010). In addition to LTP, NMDA receptor activity in the hippocampus is needed for spatial learning (Morris et al., 1986). Interestingly, in conditions of hyperammonemia in primary neuronal cultures and in animals *in vivo*, NMDA receptor activity was restricted (Madison et al., 1991; Hermenegildo et al., 1998; Muñoz et al., 2000). Cognitive impairment

is also found in congenital defects of the urea cycle causing neonatal hyperammonemia. Undetected and untreated this disease leads to death of the newborn within a few days (Cagnon and Braissant, 2007). Surviving children exhibit mental retardation correlating with the levels and extent of the neonatal hyperammonemia (Msall et al., 1984; Leonard et al., 2008). The mental retardation clearly demonstrates the irreversible damage on the developing brain evoked by high ammonia levels. In case studies, congenital urea cycle deficiencies were connected to a volume reduction of the brain, defects of myelination and improper development of the oligodendro-axonal unit. Importantly, neuronal abnormalities and an unphysiological low number of neurons were evident, possibly due to an impaired neuronal migration (Msall et al., 1988; Harding et al., 1984; Yamanouchi et al., 2002; Takanashi et al., 2003). In adults, HE during episodes of hyperammonemia may also involve neurotoxic effects (Norenberg, 2003) and act on neurogenesis. Neuroprotective strategies may be a future prospective for the therapy of HE patients.

In this context, the hippocampus is of particular interest. There are several lines of evidence showing that this brain region is particularly sensitive for toxic agents such as ammonia. Notably, hyperammonemia due to liver damage was shown to promote mitochondrial dysfunction in hippocampal endothelial cells, astrocytes, neurons and related synapses. Examined animals revealed morphological changes of astrocytes and mitochondria (Perazzo et al., 1998; Felipo, 2002; Lores-Arnaiz et al., 2005). GS activity increased in hippocampi of rats with portal hypertension. At the same time glutamate uptake into the cell was decreased leading to toxic accumulation of extracellular glutamate (Schmidt et al., 1990; Acosta et al., 2009). Furthermore, elevated ammonia levels were linked to provoke oxidative stress in the hippocampus (Roselló et al., 2008).

2.1.2 Adult hippocampal neurogenesis

New neurons are continuously added throughout adulthood in discrete regions of the mammalian brain (Alvarez-Buylla and Lim, 2004; Lie et al., 2004, Seki et al., 2011a, 2011b). This process is dynamic and outermost complex. It is influenced by various intrinsic and extrinsic factors (Zhao et al., 2008; Ming and Song, 2005). Intrinsic factors are hormones, growth factors, neurotransmitters or aging (Schmidt and Duman, 2007; Couillard-Despres et al., 2011; Kempermann, 2011; Okamoto et al., 2011; Schoenfeld and Gould, 2012). Contrariwise, extrinsic factors such as environment, behaviour, stress, drugs and pathological conditions impact on adult neurogenesis (Duman et al., 2001; Farmer et al., 2004; Lie et al., 2004; Cho and Kim, 2010; Kempermann, 2011).

The subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus are the brain regions where adult neurogenesis originating from neural precursor cells (NPCs) has been observed (Zhao et al., 2008; Faigle and Song, 2013). Neurons arising from the SVZ become granule neurons and periglomerular neurons of the olfactory bulb after travelling through the rostral migratory stream. Neurons originating from the SGZ differentiate to granule cells of the granular cell layer and thereby form the neural network of the dentate gyrus. Due to the resemblance to embryonic radial glia, these stem cells of the adult SGZ are also termed radial-glial-like cells. Initially, they are located with their cell bodies within the SGZ projecting with their radial processes through the granular cell layer. From these progenitors transiently dividing cells arise that have the potential to proliferate and differentiate into immature neurons. Subsequent migration right into the granular cell layer and axonal growth connects the cells with the CA3 pyramidal cell layer (Hastings and Gould, 1999). Their dendrites grow into the opposite direction and associate with the molecular layer. Finally, the new granule neurons undergo synaptic integration into the pre-existing hippocampal circuitry and contribute to the neuronal signalling network (van Praag et al., 2002). The number of neuronal progenitors in the adult brain that undergo cell division and differentiation is low. To identify this distinct cell population, administration of nucleotide analogues such as bromodeoxyuridine (BrdU) is generally used. BrdU is incorporated into the DNA during DNA replication in the S phase of the cell cycle. Subsequent detection by immunofluorescence offers the opportunity to trace adult neurogenesis.

Pathological conditions influence the process of adult neurogenesis. Brain injuries mainly increase the proliferation rate causing migration of new neurons into the damaged brain region for instance after ischemic brain injury or seizures (Nakatomi et al., 2002; Jessberger and Parent, 2011; Zheng et al., 2013; Althaus and Parent, 2014). Neurogenesis was also demonstrated to be altered in neurodegenerative diseases (Winner et al., 2011a). However, here the findings are to a certain extent conflicting between human studies and rodent models (Sierra et al., 2011). Histological examinations of *post mortem* brain tissue and studies using animal models of Huntington's disease (HD), Alzheimer's disease (AD) and Parkinson's disease (PD) show an increase in neurogenesis for HD and AD (Jin et al., 2004a; Jin et al., 2004b; Tattersfield et al., 2004). Contrarily, PD was predominantly connected to an impairment of hippocampal neurogenesis (Höglinger et al., 2004; Winner et al., 2011b). Regardless of conflicting findings, evidence suggests that there is an impact on adult neurogenesis in degenerative neurological diseases, demonstrating the brains compensatory response to self-repair. Systemic and CNS inflammation is also associated with neurodegenerative diseases. Pro-inflammatory mediators such as cytokines and increased production of ROS and NO act in concert and lead to augmented intracranial blood

flow, extravasation of circulating immune cells and influence residing microglia cells (Newton and Dixit, 2012). During systemic inflammation, bacterial LPS can reach the CNS by ventricular infusion and promote an inflammatory milieu. In this context, LPS was shown to reduce the formation of new neurons in the adult hippocampus, presumably mediated by effects of pro-inflammatory cytokines on neuronal precursor cells, induction of apoptosis or effects on microglial cells (Fujioka and Akema, 2010; Sierra et al., 2010; Valero et al., 2014). Interestingly, the number of activated microglia is negatively correlated with the numbers of newborn neurons (Ekdahl et al., 2003). At the cellular level, ER stress, for instance caused by increased levels of misfolded proteins, could initiate inflammatory pathways and thereby putatively activate microglia.

2.1.3 Microglia activation and neuroinflammation in HE

Apart from extensive studies on the role of astrocytes in HE, recently microglia cells have come into the focus of research. Activated microglial cells are potent producers of inflammatory cytokines leading to neuroinflammation and by this may also influence the pathogenesis of HE (Nakamura, 2002). However, to date there is conflicting data on the connection between ammonia-induced microglia activation, release of pro-inflammatory cytokines and subsequent induction of neuroinflammation. Diverse results seem also to be dependent on the experimental paradigm used to provoke a hyperammonemic state (Jiang et al., 2009a; Rodrigo et al., 2010; Brück et al., 2011; Zemtsova et al., 2011). In animal models of ALF, microglia activation and release of pro-inflammatory cytokines was evident. Microglial activation marker CD11b was upregulated in an early response to ALF and further increased with manifestation of encephalopathy and brain edema (Jiang et al., 2009b). At the same time, brain concentrations of pro-inflammatory cytokines were elevated promoting microglial activation and a pro-neuroinflammatory milieu. Experimental data resulting from *in vitro* cell cultures and from acute hyperammonemia *in vivo*, as well indicate an activation of microglia (Jiang et al., 2009a; Rodrigo et al., 2010; Zemtsova et al., 2011). Moreover, in microglia cells, presence of ammonia was shown to inhibit phagocytosis, stimulate cell migration and produce ROS (Zemtsova et al., 2011). Microarray and gene array data from *post mortem* HE brain samples support these experimental findings. In HE patient samples, markers associated with microglia were induced (Warskulat et al., 2002; Görg et al., 2013a). Microglial activation was not only detectable in acute but in chronic HE as well. Microglial activation was not detectable in human samples from cirrhotic patients without HE (Jiang et al., 2009a; Rodrigo et al., 2010; Agusti et al., 2011; Zemtsova et al., 2011). In chronic animal models using portacaval shunts and BDL to provoke HE, microglia activation was also evident (D'Mello et al., 2009; Rodrigo et al., 2010). Despite an emerging body of data, in

chronic HE patients, the involvement of neuroinflammation is still under discussion. For instance, analysis of *post mortem* cortex HE samples could not verify a change in pro-inflammatory cytokine levels, nevertheless this may be the case for other brain regions (Görg et al., 2013a). The microglial activation found in these human cortex samples was related to the anti-inflammatory phenotype (M2), rather than to the pro-inflammatory phenotype (M1). It was suggested that in chronic HE microglia cells are activated but do not produce pro-inflammatory cytokines to an extend that is sufficient to promote neuroinflammation (Warskulat et al., 2002). Again, it has to be considered that a microglial reaction may be brain region specific. Still, neuroinflammation in HE is a topic of research and it was proposed that hyperammonemia and neuroinflammation act in concert to evoke HE symptoms and promote its progression possibly involving microglia (Shawcross et al., 2004).

2. Material & Methods

2.1 Animals

WT Mice with C57BL/6 background at 8—12 weeks of age with an average weight of 20-25g were bred in the animal care facility center of the Heinrich-Heine University Düsseldorf, Germany and housed in a 12h light/dark cycle with food and water available *a libitum*. All experimental procedures were conducted following the guidelines and protocols approved by the German animal protection law and approved by the responsible state office Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV) under protocol number 84-02.04.2011.A204. Handling of mice occurred according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) and the national animal welfare body Gesellschaft für Versuchstierkunde - Society for Laboratory Animal Science (GV-SOLAS).

2.2 LPS and NH_4Ac treatment of mice

LPS stock of 10mg/ml (Sigma, strain 026:B6) was dissolved in sterile H_2O with a final concentration of 0.5mg/ml. LPS was injected i.p. at a non-lethal dose of 5mg/kg body weight (Maio et al., 2005). NH_4Ac (Sigma) was diluted in sterile H_2O as vehicle and injected i.p. at a non-lethal dose of 10mM/kg body weight. Control mice, referred to vehicle group, were injected i.p. with sterile H_2O only. After injection animals had free access to food and water for duration of the experiment. 6h or 24h post-injection mice were deeply narcotized with isoflurane (Actavis) and perfused transcardially through the left ventricle with cold PBS (Life Technologies). For analyzes tissue was removed, dissected and immediately frozen in liquid oxygen.

2.3 BrdU treatment

BrdU was dissolved in H_2O for injections and injected i.p. at a non-lethal concentration of 50mg/kg body weight subsequently to NH_4Ac treatment.

2.4 Western blot analysis and oxyblot

Cells and tissue were lysed at 4°C with 1x RIPA buffer (Thermo Scientific) and 10mM protease inhibitor cocktail (Roche Diagnostics). The homogenized lysates were centrifuged at 18000g for 40min at 4°C. Protein concentration of supernatants was estimated via BCA (Interchim). For western blot analysis, cell and tissue lysates were added to an identical amount of LDS sample loading buffer 4x (Invitrogen) and sample reducing agent 10x (Invitrogen). After boiling samples at 95°C for 5min, SDS gel electrophoresis was performed at 170V using AnykD and 4-20% gradient Mini-PROTEAN TGX Precast gels (BioRad) (30-100µg protein per lane) in Tris/Glycine/SDS buffer (BioRad). Following gel electrophoresis, proteins were blotted with Trans-Blot Turbo Transfer System (BioRad) on

PVDF membranes (BioRad). Successful protein transfer was proofed by Ponceau red (Sigma) staining. After washing with PBS-T 0.05% to remove Ponceau Red staining, membranes were blocked with 5% skim milk in PBS-T 0.05%. Antibodies used for Western blot and oxyblot are listed in Table 2. Antibodies were diluted in 5% milk/PBS-T 0.05% and incubated overnight shaking at 4°C. Following three times 10min washing, membranes were incubated with IRDye secondary antibodies (LI-Cor) (Table 2) for 1h shaking at RT. Subsequent three times 10min washing with PBS-T 0.05%, detection of protein bands was performed at 800nm and 680nm with Odyssey Infrared Imaging Systems (LI-Cor). Band intensities were analyzed using densitometric analysis with ImageJ (Rasband, W.S., USA) and normalized to β-actin bands as a housekeeping protein. For detection of protein carbonyls, lysates were subjected to Oxyblot protein oxidation detection kit (Millipore) according to the manufacturer's instructions. In brief, samples were adjusted to 5-15µg protein in 5µl. After denaturation with 12% SDS, samples were derivatized by addition of DNPH solution and incubated for 15mins at RT. Addition of neutralization solution stopped the reaction. For visualization of protein carbonyls, samples were separated and blotted according the western blot procedure. The membranes were blocked for 1h in 1%BSA/PBS-T and incubated 1h with α-DNP antibody diluted 1:150 in 1%BSA/PBS-T shaking at RT. Detection and washing were carried out as described for western blot procedure. Optical densities of protein bands were calculated by densitometric analysis using ImageJ software (Rasband, W.S., USA). Values were normalized to housekeeping gene β-actin and expressed as fold to control or vehicle samples.

Table 2

Antibodies for western blot and oxyblot

Primary antibody	Host	Company	Dilution
anti-DNP	rabbit	Millipore	1:150
anti-HO1	rabbit	Abcam	1:1000
anti-LMP7	rabbit	kind gift of AG Prof.Kloetzel, Charité Berlin, Germany	1:10000
anti-NO_2-Tyrosine	rabbit	Millipore	1:1000
anti-Trx1	rabbit	(Godoy et al., 2011)	1:200
anti-Ubiquitin	rabbit	Dako	1:200

Secondary antibody	Host	Company	Dilution
anti-rabbit IgG 800	donkey	LI-Cor	1:20000
anti-mouse IgG 680	donkey	LI-Cor	1:20000

2.5 RNA isolation and quantitative RT-PCR (qPCR)

RNA isolation from cells and tissue samples was performed using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Quality and amount of RNA was measured with Nanodrop 2000 (Thermo Scientific). 1-2µg of RNA was used to synthesize cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems) in a T Gradient Thermocycler (Biometra). Primers used for qPCR analysis are listed in Table 3. The applied programme was the following: 10min at 25°C, 45min at 48°C and 5min at 95°C. For qRT-PCR, depending in the primers Power SYBRGreen (Applied Biosystems) or TaqMan probe (5'Fam, 3'TAMRA) were used and measured in the 7500 Real Time PCR System (Applied Biosystems). The programme was the following: 2min at 50°C, 10min at 95°C and 40 cycles with 15s at 95°C and 60s at 60°C. For SYBRGreen primers a dissociation curve was acquired from 95°C to 60°C. Primers were designed with Primer Express software (Applied Biosystems) and ordered from Eurofins MWG Operon, Germany. Primer stocks were diluted in DNase-RNase-free H_2O (Life Technologies) and used at a concentration of 10mM. For primer sequences see table below. All measurements were performed in duplicates and the relative amount of mRNA was normalized to the housekeeping gene GAPDH by means of the ΔCT method.

Table 3

Primers for qPCR analysis

Primer	Sequence 5'→3'
Calreticulin	F: TGCTGTACTGGGCCTAGATCTCT
	R: GCTTCTCTGCAGCCTTGGTAA
CD68	F: TGACCTGCTCTCTCTAAGGCTACAG
	R: AGGACCAGGCCAATGATGAG
Chop	F: TCTTGAGCCTAACACGTCGATTAT
	R: GGGACTCAGCTGCCATGACT
EIF2α	F: GGTTTCTCTTTCCGGGACAAG
	R: CTGGGAAGCACCGTGCTT
ERDj4	F: AAAATAAAAGCCCTGATGCTGAA
	R: CATTGCCTCTTTGTCCTTTGC
FCγR	F: GCCTCAGCGACCCTGTAGATC
	R: CCCTTAGCGTGATGGTTTCC
GAPDH	F: CCAGCCTCGTCCCGTAGAC
	R: CCATTCTCGGCCTTGACTGT
	probe: (Fam) CGGATTTGGCCGTATTGGGCG (TAMRA)
GRP78	F: TGCAGCAGGACATCAAGTTCTT
	R: GCTGGGCATCATTGAAGTAAGC
GRP94	F: GACCCAAGAGGAAACACACTAGGT
	R: AGGGCTCCTCAACAGTCTCTGT

Continued from Table 3

Primers for qPCR analysis

Primer	Sequence 5'→3'
HO1	F: CCAAGCCGAGAATGCTGAGT
	R: CTCTTCCAGGGCCGTGTAGAT
IL6	F: AAGTCGGAGGCTTAATTACACATGT
	R: AAGTGCATCATCGTTGTTCATACA
iNOS	F: GGAAGTGGGCCGAAGGAT
	R: ACTGGAGGGACCAGCCAAAT
LAMP1	F: ACATGAAGAATGTGACCGTTGTG
	R: TCCATCCTGTGTGCAGTGTGT
LAMP2	F: AGGTGCAACCTTTTAATGTGACAA
	R: GCCTGAAAGACCAGCACCAA
LMP2	F: AGGAGTGCCGGCGTTTC
	R: TCCCAGGATGACTCGATGGT
	probe: (Fam)CATCACTCTGGCCATGAACCGAGA(TAMRA)
LMP7	F: CTCCGGAGGTCGCACTTC
	R: GGGCCATCTCAATTTGAACATT
	probe: (Fam)TGCAGCCCACCGCATTCCTGA(TAMRA)

Continued from Table 3

Primers for qPCR analysis

Primer	Sequence 5'→3'
MECL1	F: AGACCGGTTCCAGCCAAAC
	R: CTCAGGATCCCTGCTGTGATG
	probe: (Fam)TGACGCTGGAGGCTGCGCA(TAMRA)
MSR1	F: ACAGAGGGCTTACTGGACAAACTG
	R: ACCTCCAGGGAAGCCAATTT
SIRPα	F: TGCTCTCCGCGTCCTGTT
	R: CAGTTCAGAACGGTCGAATCC
SOD1	F: TGGGTTCCACGTCCATCAGT
	R: TGCCCAGGTCTCCAACATG
SOD2	F: TCTGTGGGAGTCCAAGGTTCA
	R: ATCCCCAGCAGCGGAATAA
SOD3	F: TGGGTGCTGGCCTGAACT
	R: ACAGGCCGCCAGTAGCAA
TNFα	F: GGGCCACCACGCTCTTC
	R: GGCTTGTCACTCGAATTTTGAGA
Trx1	F: GGATGTTGCTGCAGACTGTGA
	R: TCAGAGCATGATTAGGCATATTCAG

2.6 Cell culture

Murine cell line BV2 (microglia) were cultured in DMEM (Invitrogen) supplemented with 1%P/S (Life Technologies) with 10%FCS (Life Technologies) and maintained at 37°C with 5%CO_2 in a humidified incubator (Thermo Scientific). For experiments, cells were exposed to 1µg/ml LPS (Sigma), 5mM NH_4Cl (Sigma) or 10µM MG132 (Millipore) diluted in cell culture medium without FCS for the indicated time points.

2.7 Proteasome activity assay

Proteasomal activity in cytosolic and brain tissue lysates was measured using the 20S proteasome activity assay kit (Millipore) according the manufacturer's instructions. In brief, BV2 cytosolic extract (50µg), mouse hippocampus lysate (100µg), or human cortex lysate (50µg) were mixed in a 100µl reaction with 10x assay buffer and the fluorogenic proteasome substrate peptide Suc-Leu-Leu-Val-Tyr-AMC. The mixture was incubated for 1h at 37°C and fluorescence was subsequently measured at 380/460nm in a microplate fluorescence reader. Proteasome activity values were calculated according to the AMC fluorogenic standard curve and subtraction of blank values. Assays were performed in triplicates and statistical significance was determined using a paired student's *t*-test.

2.8 Phagocytosis assay

To assess the phagocytosis, BV2 microglia cells were seeded in 24-well plates one day prior to the experiment in DMEM with 10%FCS. Cells were treated in DMEM without FCS for the indicated time points. DiI-labelled myelin (lab stock) and/or 5mM NH_4Cl with or without NAC (Millipore) was diluted in DMEM (Invitrogen) and added to the cells at 37°C. After 2 h, medium was discarded and cells fixed with PFA for 20min shaking at RT. For direct detection of the phagocytic activity, the fluorescent signal of DiI-labelled myelin was measured at 485/535nm in a microplate fluorescence reader (Tecan Group Ltd). For further staining of the cells, wells were washed with washing buffer for 5min and permeabilized with PBS/1% Triton for 1min. After that, blocking buffer was added to each well for 1-3h shaking at RT. Primary antibodies were diluted in washing buffer and added to the cells. The 24-well plate was incubated in a wet chamber shaking at 4°C overnight. The next day, the staining procedure was continued as described in 2.9.

2.9 Immunocytochemistry

Buffers and antibodies used for immunocytochemistry are listed in Tables 4 and 5, respectively. Cell were cultured and treated in absence of FCS with NH_4Cl (5mM). After the indicated time points, cells were fixed with PFA for 20min shaking at RT, washed once with washing buffer and permeabilized for 1min with 1% Triton (Merck) in PBS (Life Technologies). Blocking was carried out by incubation with blocking buffer for 1-3 h. Primary antibodies (Table 5) were diluted in washing buffer and added to the cells overnight shaking at 4°C. The next day, cells were washed three times 10min with washing buffer. The secondary antibody was diluted in washing buffer and incubated with the cells for 1h shaking at RT. Hoechst dye 33258 (Sigma) at a dilution of 1:5000 was included in the last of three washing steps to counterstain nuclei. Analysis of stained sections was carried out with Olympus BX51 microscope and Photoshop 5.0 software (Adobe).

Table 4

Buffer for immunocytochemistry

Buffer	Composition
Washing buffer	0.1% Triton (Merck) in PBS (Life Technologies)
Blocking buffer	10% BSA (Carl Roth) 5% NGS (Invitrogen) 0.5% Triton (Merck)

Table 5

Antibodies for immunocytochemistry

Primary antibody	**Host**	**Company**	**Dilution**
anti-HO1	rabbit	Abcam	1:1000
anti-NO_2-Tyrosine	rabbit	Millipore	1:1000
anti-Trx1	rabbit	(Godoy et al., 2011)	1:200
anti-Ubiquitin	rabbit	Dako	1:200
Secondary antibody	**Host**	**Company**	**Dilution**
anti-rabbit cy2	goat	Millipore	1:500
anti-rabbit cy3	goat	Millipore	1:500

2.10 Immunohistochemistry

Antibodies used for immunohistochemistry are listed in Table 6. After transcardial perfusion of mice with cold PBS, brain halves were fixed overnight in 4% PFA, dehydraded in a 30% sucrose solution and subsequently embedded in Tissue Tek (Sakura Fintek). Brains were stored at -80°C and afterwards cut in 10 µm thick slices with a cryostat (Leica Microsystems). Sections were permeabilized with 0.5% Triton and blocked with 5% NGS (Invitrogen) and 1% BSA (Carl Roth). Primary antibodies were incubated overnight shaking at 4°C. After washing 3x10 min with 0.1% Triton/PBS, the secondary antibody was incubated for 1 h shaking at RT. Hoechst dye 33258 (Sigma) at a dilution of 1:5000 was included in the last of three washing steps to counterstain nuclei. Analysis of stained sections was carried out with Olympus BX51 microscope and Photoshop 5.0 software (Adobe).

Table 6

Antibodies for immunohistochemistry

Primary antibody	Host	Company	Dilution
anti-BrdU	rat	AbDSerotec	1:200
anti-GFAP	guinea pig	SySy	1:1000
anti-Ubiquitin	rabbit	Dako	1:200
anti-BrdU	rat	AbDSerotec	1:200

Secondary antibody	Host	Company	Dilution
anti-guinea pig cy5	donkey	Millipore	1:500
anti-rabbit cy3	goat	Millipore	1:500
anti-rat cy3	goat	Millipore	1:500

2.11 P*ost mortem* human brain tissue and microarray analysis

Post mortem human brain tissue was a kindly offered by Prof.Dr. Häussinger and Dr. Boris Görg (Clinic for Gastroenterology, Hepatology and Infectious Diseases, Heinrich-Heine-University Düsseldorf, Germany). The tissue was obtained from autopsies from patients who died while in hepatic coma (HE grade IV according to West Haven criteria (Conn et al., 1977). The control samples were free from hepatic or neurological diseases. The patient characteristics and histories have been previously described (Görg et al., 2013a) and brain samples have also been analysed in (Görg et al., 2010). Samples were obtained from the Australian Brain Donor Programs NSW Tissue Resource Centre, supported by the University of Sydney, National Health and Medical Research Council of Australia, Schizophrenia Research Institute and the National Institute of Alcohol Abuse and Alcoholism and the NSW Department of Health. All brain samples included in this study were taken from

the intersection parietal to occipital cortex area. Microarray analysis was carried out by order of Prof. Dr. Häussinger and Dr. Boris Görg (Clinic for Gastroenterology, Hepatology and Infectious Diseases, Heinrich-Heine-University Düsseldorf, Germany). A detailed description of the microarray analysis is published in the supplementary information in (Görg et al., 2013a).

2.12 Mass spectrometry

Hippocampi from vehicle or 24h NH_4Ac treated mice were lysed at 4°C with 1x RIPA buffer (Thermo Scientific) and 10mM protease inhibitor cocktail (Roche Diagnostics). The homogenized lysates were centrifuged at 18000g for 40min at 4°C and supernatants were collected. Samples for vehicle (n=3) and 24h NH_4Ac (n=3) were pooled and protein concentration of lysates were estimated via BCA (Interchim). Lysates (5µg protein per sample) were subjected to mass spectrometry carried out at the Molecular Proteomics Laboratory (MPL), Heinrich-Heine-University Düsseldorf. Briefly, samples were tryptically digested and resulting peptides were separated on a C18 column by nano HPLC using a two hour gradient. MS and MS/MS spectra of eluting peptides were acquired by an online coupled Orbitrap Elite mass spectrometer (Thermo Scientific).

2.13 Kits

Table 7

Kits used for analysis

Kit	Company
BC Assay Protein Quantification Kit	Interchim
Oxyblot Protein Oxidation Detection Kit	Millipore
20S Proteasome Activity Assay	Millipore

2.14 Software

Table 8

Software used for data analysis

Software	Company
Adobe Acrobat Reader 11.0	Adobe System Inc.
Adobe Photoshop CS5	Adobe System Inc.
Adobe Illustrator CS5	Adobe System Inc.
GraphPad Prism 5	GraphPad Software
ImageJ	Rasband, W.S.
Magellan Data Analysis Software	Tecan Group Ltd.
Odyssey Imaging System Software	LI-Cor
Office Professional Plus 2010	Microsoft Corporation
Primer Express software	Applied Biosystems

2.15 Statistical analysis

Data from independent experiments are expressed as mean +SEM. Statistical analysis was performed with GraphPad Prism 5 (GraphPad Software). A p-value of less than 0.05 was determined to be statistically significant by Student's *t*-test.

3. Results

The UPS is the crucial machinery for protein homeostasis in the cell (Sijts, E J A M and Kloetzel, 2011). Removal of misfolded and damaged proteins occurs by tagging target molecules with ubiquitin moieties following their subsequent degradation by the proteasome thereby protecting the cell from accumulation of protein debris. However, if the proteasomal capacity exceeds the amount of polyubiquitinated proteins, cell viability is threatened by an aggregation of polyubiquitinated proteins which have been shown to impair the function of the UPS (Bence et al., 2001). Accumulation of such damaged, ubiquitinated proteins is a feature of different neurodegenerative diseases (Keller et al., 2000; Ardley et al., 2005). In models of HE, post translational modifications such as protein tyrosine nitration and *O*-GlcNAcylation occur (Schliess et al., 2002; Häussinger et al., 2005; Karababa et al., 2013). Whether these post-translational modifications associate with alterations of protein-folding and, therefore, with protein ubiquitination is unknown. For this reason, we investigated the role of protein aggregates that are modified by post-translational processes, such as ubiquitination, to shed light on their possible role for the pathogenesis of HE.

3.1 HE patient brains exhibit polyubiquitinated protein aggregates and induction of immunoproteasome subunits

To investigate protein ubiquitination in the context of HE, we subjected *post mortem* biopsy material from patients with and without HE (Görg et al., 2013a) to western blot analysis. As depicted in Figure 1A, we observed that high molecular polyubiquitinated proteins accumulated in cortical samples from HE patients as compared to control patients. HE is associated with oxidative stress and upregulation of antioxidant enzyme HO1 in CNS tissue as demonstrated in human HE and HE animal models (Warskulat et al., 2002; Görg et al., 2010; Görg et al., 2013a). The antioxidant enzyme Trx1 is involved in the reduction of thiol modifications of oxidized proteins in oxidative stress conditions (Holmgren, 1989; Ohashi et al., 2006; Berndt et al., 2007). For this reason, we studied its expression in the human HE brain tissue. However, Trx1 expression appeared to be heterogeneous among control and HE samples (Figure 1A). Accordingly, densitometric quantification confirmed a significant increase for polyubiquitinated proteins (Figure 1B; upper graph) but not for Trx1 expression in these cortical samples (Figure 1B; lower graph). Since the proteasome is responsible for the degradation of damaged and ubiquitin-labeled proteins, we assayed the proteasomal chymotrypsin-like activity in the same patient samples. Interestingly, in HE patients a decreased activity of the chymotrypsin-like proteasomal activity was evident (Figure 1C). This effect is in line with previous findings, demonstrating a connection between impaired proteasomal lysis and accumulation of misfolded and polyubiquitinated proteins (Bence et al., 2001).

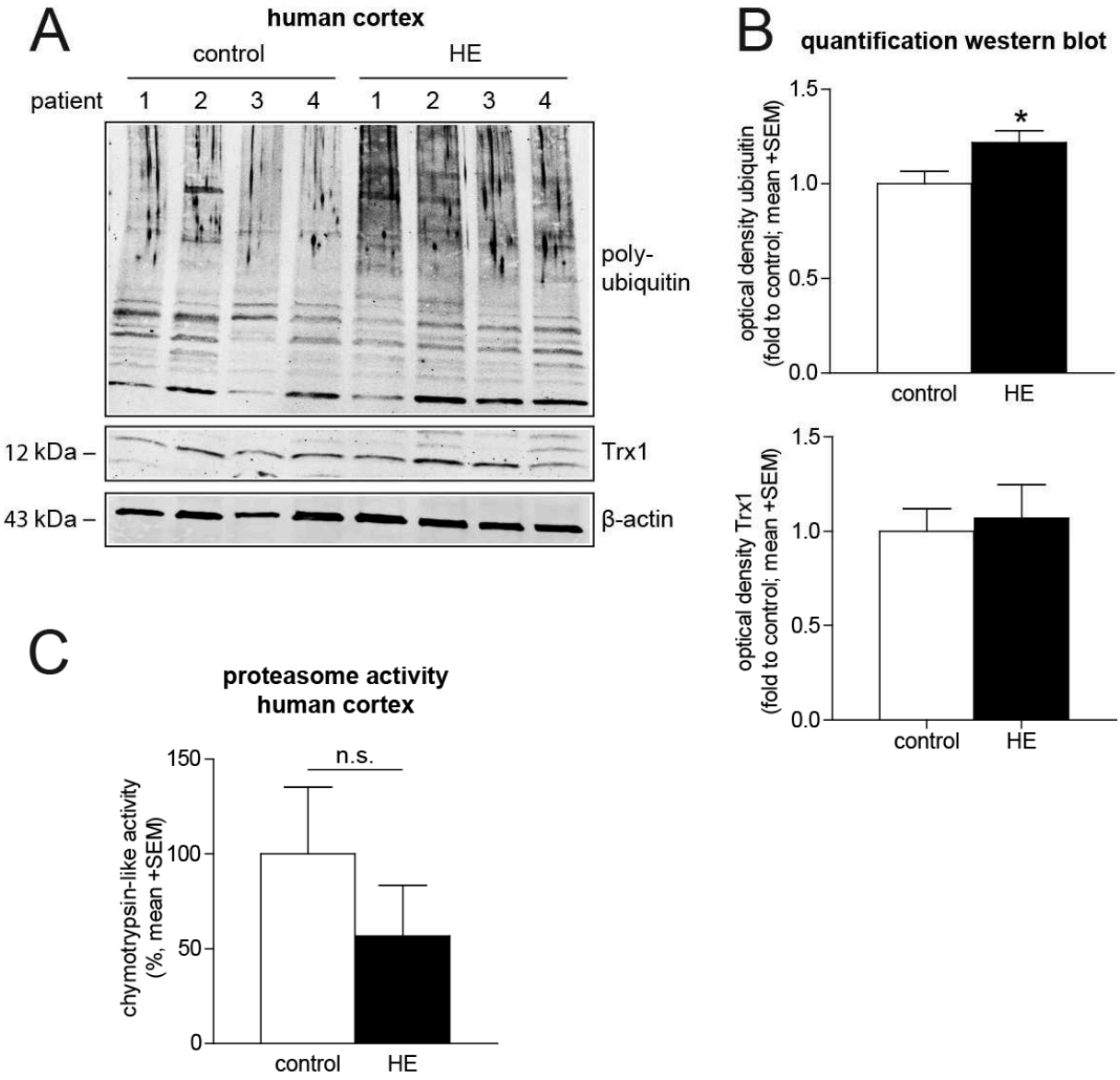

Figure 1. Increased protein polyubiquitination in the CNS of HE patients. (A) Representative western blot analysis of total protein lysates from cortical samples of control (n=8) and HE (n=8) patients. Protein samples were assessed for accumulation of polyubiquitinated proteins and Trx1. Normalization of protein loading was performed by analysis of β-actin protein. (B) Densitometric quantification of western blot analysis (fold to control patients; mean +SEM) and (C) measurement of the chymotrypsin-like 20S proteasome activity in human cortex lysates (% to control patients, mean +SEM). Data shown as mean +SEM. Student's *t*-test, *p <0.05.

The decrease in proteasomal activity (Figure 1C) may be connected to a lower overall expression of proteasome-related genes. To test this hypothesis, patient cortex samples from two independent patient cohorts (Figure 2A upper and lower heatmap) were subjected to a microarray gene analysis. Because HE is thought to closely associate with systemic inflammation (Seyan et al., 2010; Montoliu et al., 2011; Shawcross et al., 2011; Tranah et al., 2013), for the microarray analysis (Figure 2A), we considered not only genes of the

constitutive proteasome but also immunoproteasome genes, known to be regulated by inflammatory stimuli (Seifert et al., 2010; Ebstein et al., 2013).

Previously, our group revealed that immunoproteasomal subunit LMP7 essentially contributes to removal of misfolded proteins resulting from oxidative and inflammatory stress conditions *in vivo* and *in vitro.* Here, we found that the expression of the human constitutive proteasome subunits *PSMB5, PSMB6* and *PSMB7* remained unchanged between control and HE patients. Interestingly, the immunoproteasome subunits *LMP7/PSMB8* and *LMP2/PSMB9* were significantly increased (1.7 and 1.57 fold in cohort 1; 2.54 and 2.23 fold in cohort 2 respectively) in patients with liver cirrhosis with HE but not in patients with cirrhosis without HE (Figure 2A). Contrariwise, another immunoproteasome subunit *Mecl1/PSMB10* remained unchanged in both patient cohorts. The significant upregulation of *LMP7/PSMB8* and *LMP2/PSMB9* (Figure 2A) implicate a yet unknown role of these immunoproteasome subunits in the context of a disturbed protein homeostasis (Figure 1A-C) in human HE.

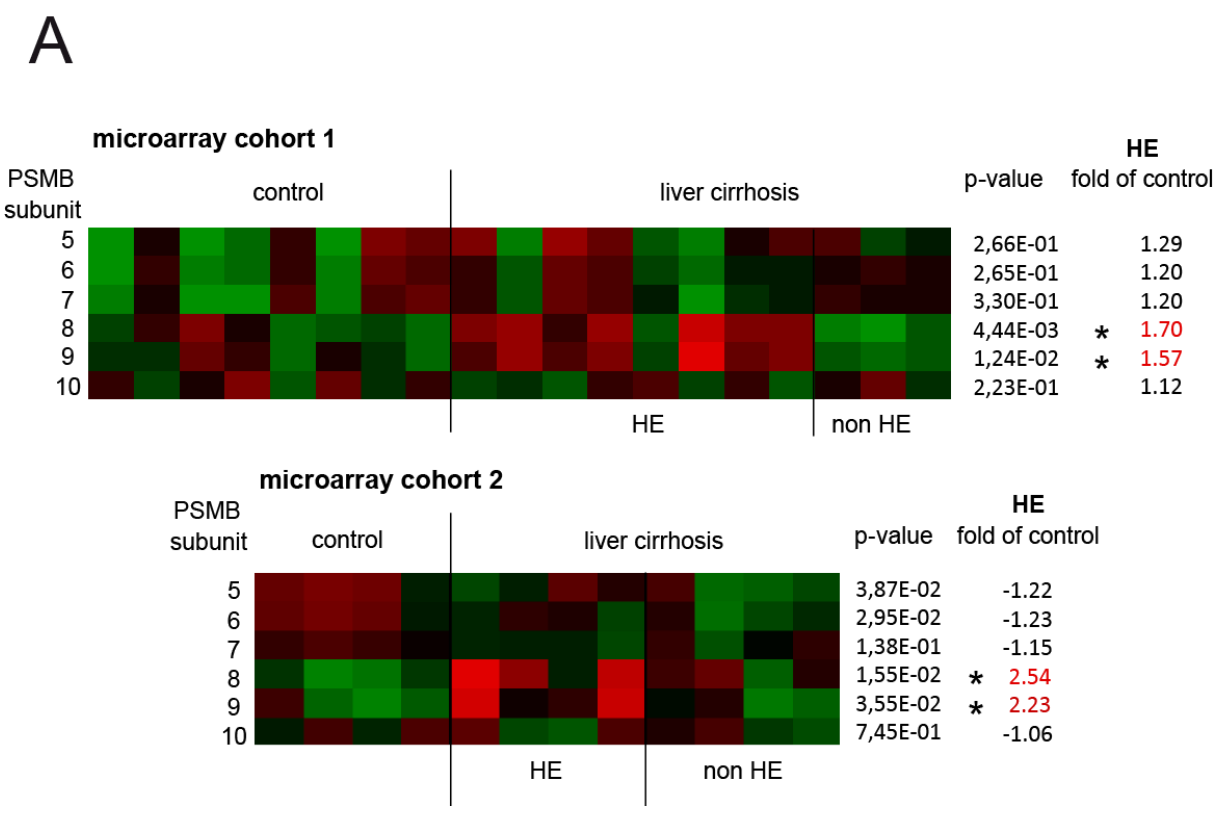

Figure 2. Proteasome expression in the CNS of HE patients. (A) Microarray data from two different patient cohorts (upper and lower heatmap). Each box shows the individual expression of constitutive and immunoproteasome subunits *PSMB 5-10* in the cortex of cirrhotic patients. The relative expression levels are color coded; red refers to higher and green to lower expression levels compared with the median of the expression levels of all patients analyzed (microarray cohort 1: control n=8, cirrhosis with HE n=8, cirrhosis without HE n=3 and microarray cohort 2: control n=4, cirrhosis with HE n=4, cirrhosis without HE n=4). Student's *t*-test, *p <0.05.

3.2 Ammonia induces accumulation of polyubiquitinated proteins and protein oxidation in the hippocampus

In HE, ammonia is considered the key toxin and its levels in patients play a critical role for the course of the disease (Häussinger and Schliess, 2008). Hyperammonemia has been connected to induce protein tyrosine nitration and oxidation of both, proteins and RNA, in brains of HE patients and primary brain cell culture, leading to persisting oxidative stress (Schliess et al., 2002; Norenberg, 2003; Häussinger and Görg, 2010; Görg et al., 2013a; Görg et al., 2013b). To assess the impact of acutely elevated ammonia concentrations in the brain, we next applied an animal model for acute HE. We induced a hyperammonemic state in adult mice by i.p. administration of NH_4Ac (10mM/kg body weight) and sacrificed the animals after 6h or 24h. Previously, hyperammonemia was connected to the cognitive impairment of HE patients and in animal models (Bajaj et al., 2010; Rodrigo et al., 2010). This led us to investigate the impact of an acute ammonia load on the hippocampus, the brain area fundamental for learning and memory (Neves et al., 2008). To date, few data is available on the effect of ammonia toxicity on this brain region known to be susceptible to various pathological external stimuli.

Firstly, we detected a significant elevation of high molecular polyubiquitinated proteins in the hippocampus of ammonia treated mice after 24h (Figure 3A and 3B). A 6h ammonia treatment seemed to be insufficient to produce a noticeable accumulation of polyubiquitinated conjugates (Figure 3B). Previously, aggregates of misfolded proteins were shown to induce oxidative stress (Szeto et al., 2014). Since oxidative stress is evident in human HE and HE animal models, we speculated that hyperammonemia leads to a pro-oxidative milieu in the hippocampus which may thereby contribute to alterations in protein folding. Therefore, we subsequently analyzed hippocampal samples from ammonia challenged mice for the presence of protein carbonyls which is recognized as a hallmark for protein oxidation (Levine et al., 1994; Nyström, 2005). As depicted in Figure 3C and 3D, we found that hyperammonemic animals exhibit a higher level of oxidatively modified proteins which, like protein ubiquitination, became significantly evident after 24h but not after 6h. Importantly, similar to accumulation of polyubiquitinated proteins with a high molecular weight (Figure 3A), oxidative modifications, evident by protein carbonylation, occur as well in high molecular weight proteins (Figure 3C). These results link the accumulation of polyubiquitinated proteins after acute hyperammonemia to oxidative stress in the hippocampus and may, thus, argue for persistent oxidation-related changes over 24h after a single ammonium injection.

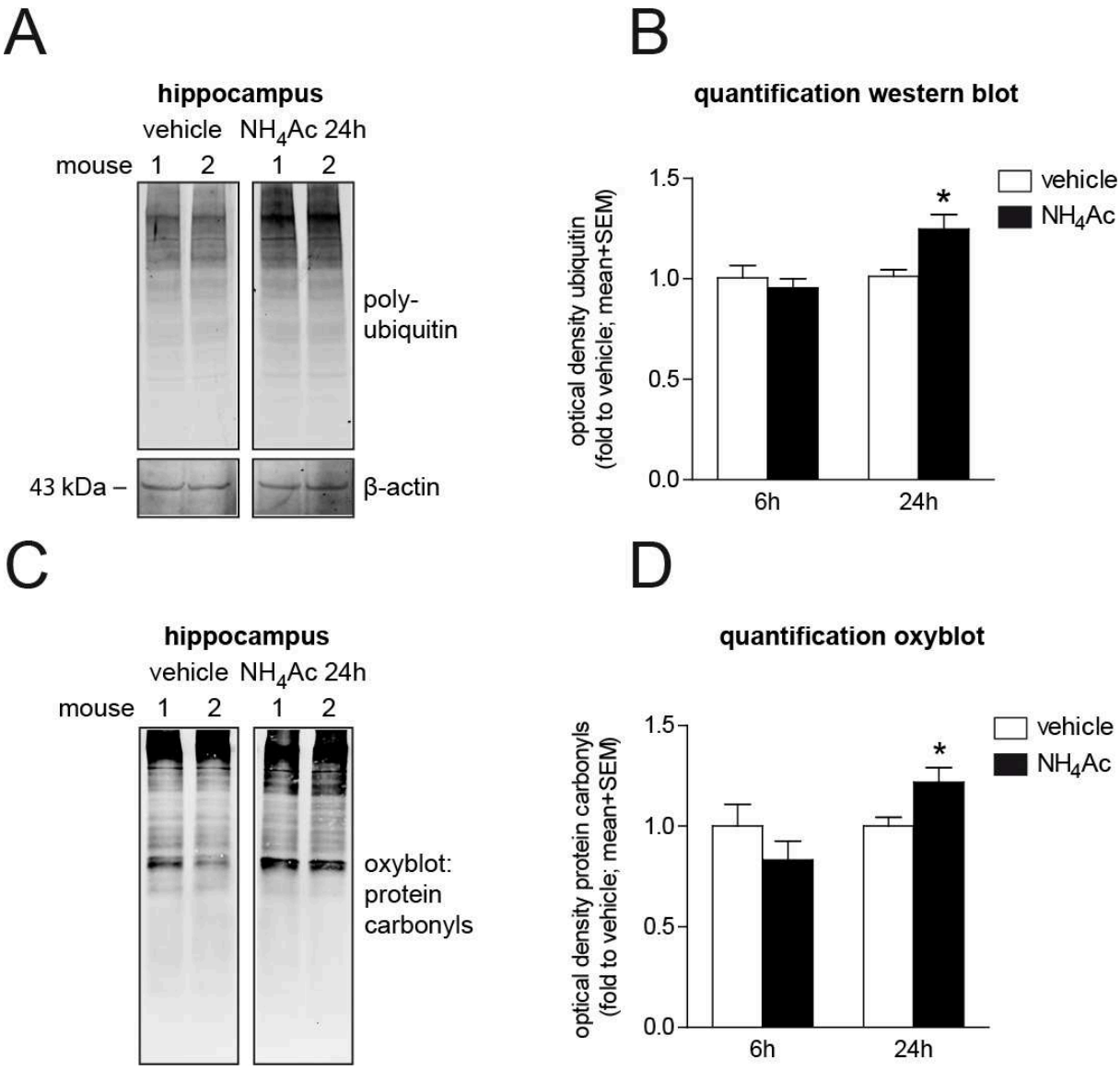

Figure 3. Increased protein polyubiquitination and oxidation in the hippocampus of mice upon acute hyperammonemia. (A) Animals were treated with NH_4Ac (10mM/kg body weight) for 6h (vehicle n=4; NH_4Ac n=6) or 24h (vehicle n=7; NH_4Ac n=11) and hippocampus lysates were analyzed by western blot for accumulation of polyubiquitinated proteins. Representative western blot is shown. (B) Western blot results were quantified by densitometry. Data represent fold increase of polyubiquitin immunoreactivity to vehicle animals. (C) Oxyblot analysis revealed protein carbonylation in the hippocampal lysates as a hallmark of oxidative stress. Representative oxyblot is shown. (D) Band intensities were quantified by densitometry and expressed as fold to vehicle treated animals (vehicle 6h n=4, NH_4Ac 6h n=5 and vehicle 24h n=6, NH_4Ac 24h n=6). Data shown as mean +SEM. Student's *t*-test, *p <0.05.

The increase in the amount of ubiquitinated proteins was also detectable by means of immunohistochemistry. Figure 4A illustrates a representative image of the hippocampal region from 24h vehicle and ammonia treated mice. Notably, ubiquitin (red) does not colocalize with astrocytic marker GFAP (green) but was mostly abundant in neuronal cells in the dentate gyrus of the hippocampus (Figure 4A).

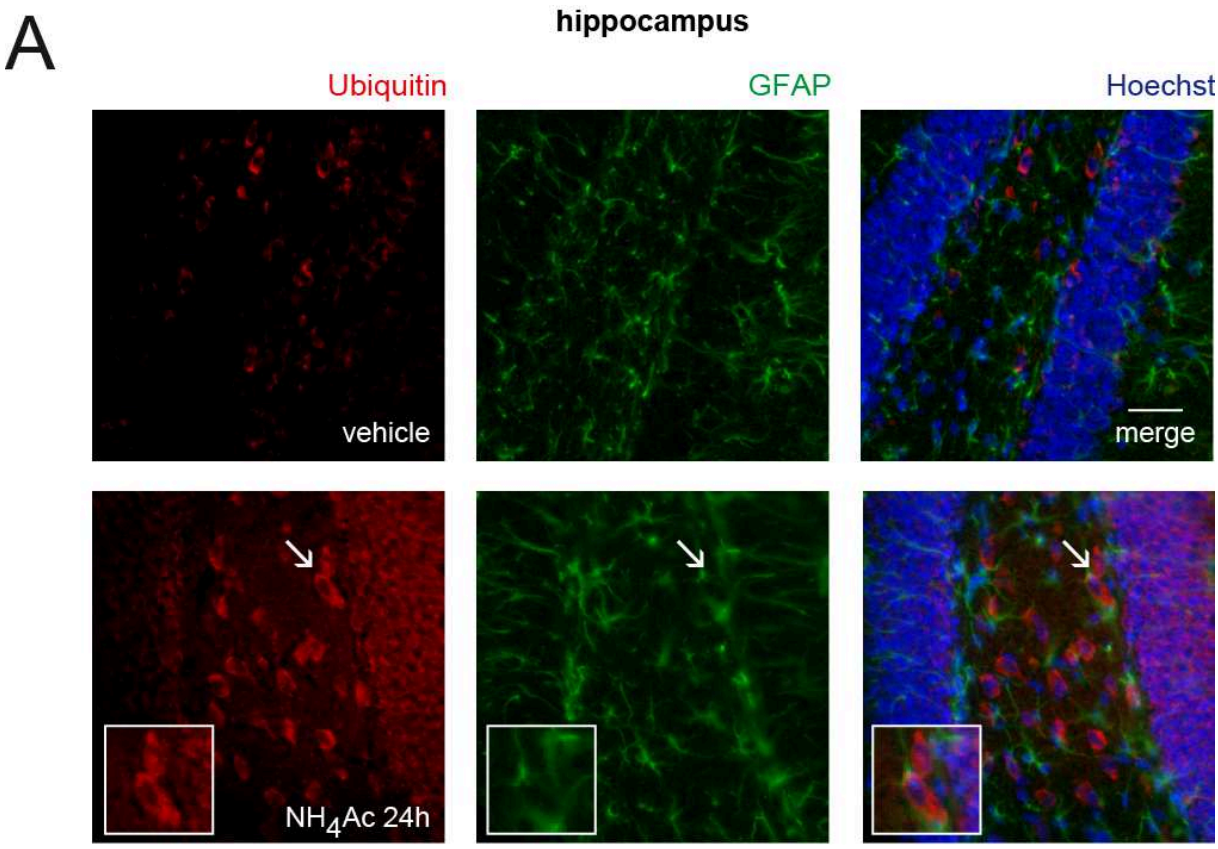

Figure 4. Protein ubiquitination in the hippocampus of mice upon acute hyperammonemia. Mice were treated with NH_4Ac (10mM/kg body weight) for 24h. (A) Representative image of hippocampal cryosections stained for ubiquitin (red) and GFAP (green) after vehicle (upper row) or 24h ammonium (NH_4Ac) treatment (lower row) of mice. Nuclear staining: Hoechst 33258 (blue). Scale bar: 100 µm.

3.3 Acute hyperammonemia increases the proteasomal activity independently from induction of the immunoproteasome

Our data show that treatment with ammonia leads to accumulation of proteins marked for proteasomal degradation. Thus, we next aimed to further investigate whether ammonia influences the proteasomal activity. To this end, we measured the chymotrypsin-like activity in hippocampal samples. In contrast to the observation in human HE brain tissue (Figure 1C), acutely hyperammonemic mice displayed a significant elevation of proteasomal enzymatic activity after 24h (Figure 5C) which is the same timepoint when a significant increase of ubiquitinated proteins was observed (Figure 3A). This may indicate that acute HE does not inhibit, but rather modulate the proteolytic response towards an elimination of oxidized proteins. Also, the effect of HE does not primarily involve the inhibition of the UPS.

Beforehand, hyperammonemia was linked to neuroinflammation in certain types of HE models (Chung et al., 2001; Shawcross et al., 2004; Cauli et al., 2007) and we here show that expression of *LMP7* is altered in the human HE brain (Figure 2A). Thus, we next analyzed the expression profile of the immunoproteasomal subunits in hyperammonemic mice by western blotting and qPCR technique.

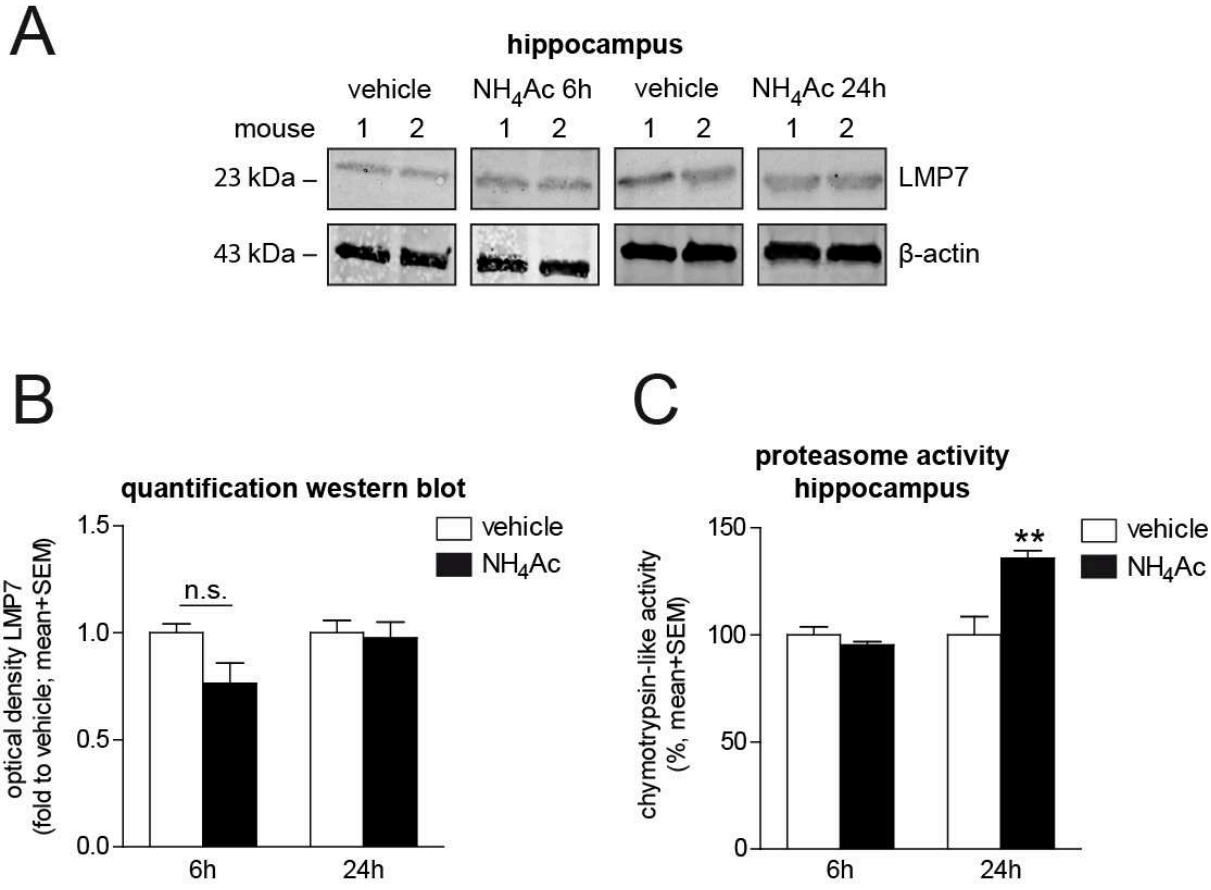

Figure 5. Increased proteasome activity is not associated with induction of LMP7 upon acute HE. (A) Animals were treated with NH_4Ac (10mM/kg body weight) for 6h or 24h and hippocampus lysates were analyzed by western blot for LMP7 expression. Representative western blot is shown. (B) Band intensities were quantified by densitometry and expressed as fold to vehicle group (6h and 24h: vehicle n=3, NH_4Ac n=6). (C) Chymotrypsin-like 20S proteasome activity was measured in hippocampal lysates after 6h and 24h treatment (vehicle 6h n=6, NH_4Ac 6h n=6 and vehicle 24h n=3, NH_4Ac 24h n=5). Data shown as mean, +SEM. Student's *t*-test, **p<0.01.

Our results revealed that the hippocampal protein and RNA expression of crucial immunoproteasome subunit LMP7 (Figure 5A, 5B and 6B) remained unchanged after an acute ammonia load. Also, hippocampal gene expression of immunoproteasome subunits *LMP2* (Figure 6A) and Mecl1 (Figure 6C) were not altered upon ammonia treatment.

Nonetheless, in control experiments, induction of immunoproteasome subunits *LMP7, LMP2* and *MECL1* was observed upon LPS challenge of mice (Figure 6A-C), demonstrating the principle responsiveness of the immunoproteasome in the hippocampus. Additionally, we assessed the gene expression of the constitutive proteasomal subunits *Psmb5, Psmb6* and *Psmb7*. These subunits were neither modified by acute hyperammonemia nor by LPS treatment (Figure 6D). Taken together, the findings indicate that an acute ammonia load is associated with increased proteasomal activity in the hippocampus that at the same time correlates with elevated levels of polyubiquitinated proteins. Furthermore, we found that an increase in proteasomal activity was not linked to an induced expression of proteasome subunits (Figure 5).

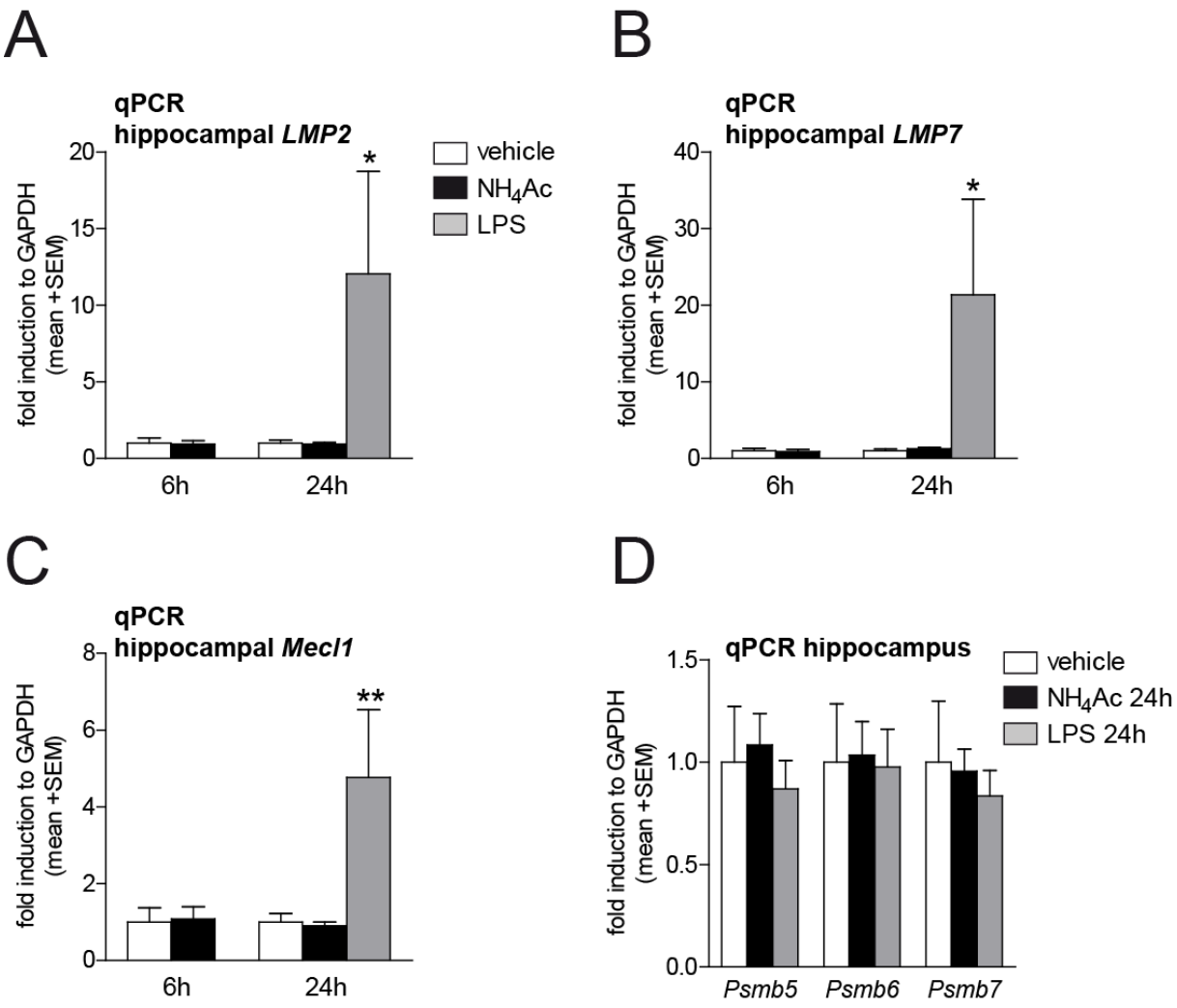

Figure 6. Acute hyperammonemia does not change immunoproteasome expression. qPCR analysis of immunoproteasome subunits (A) *LMP2*, (B) *LMP7* and (C) *Mecl1* expression in the hippocampus after 6h and 24h (vehicle 6h n=3-4; NH_4Ac 6h n=5 and vehicle 24h n=10; NH_4Ac 24h n=6-8; LPS 24h n=4). (D) Expression of constitutive proteasome subunits *Psmb5*, *Psmb6* and *Psmb7* in the hippocampus after 24h of ammonium treatment (vehicle n=3; NH_4Ac n=5; LPS n=4). Data shown as mean, +SEM. Student's *t*-test, *p<0.05; **p<0.01.

3.4. Acute hyperammonemia is not associated with the induction of antioxidant enzymes in the hippocampus

The previous experiments showed that hippocampi from acutely hyperammonemic mice exhibit an accumulation of polyubiquitinated proteins (Figure 3A and 3B) and display carbonylation, a hallmark of oxidative stress (Figure 3C and 3D). We next examined whether ammonia-induced oxidation is associated with upregulation of antioxidant enzymes HO1 and Trx1 as a possible compensatory mechanism. To this end, hippocampal tissue from mice challenged with ammonia for 6h or 24h was subjected to western blot and qPCR analysis (Figure 7 and Figure 8).

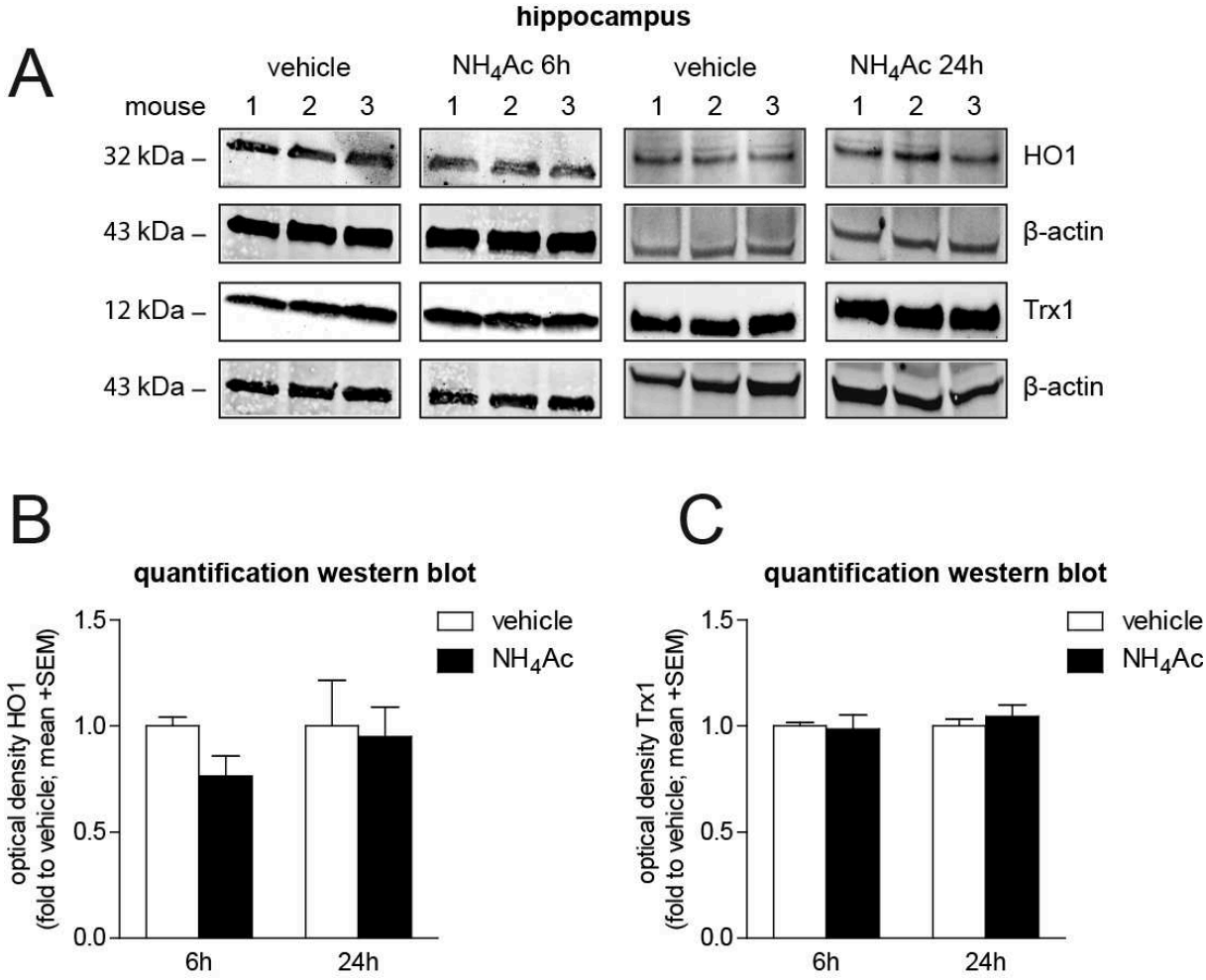

Figure 7. HO1 and Trx1 protein expression in the hippocampus of mice after acute hyperammonemia. Mice were injected i.p. with NH_4Ac (10mM/kg body weight) for 6h and 24h. (A) Representative western blot analysis of antioxidant enzymes HO1 and Trx1 expression in the hippocampus. (B+C) Band intensities were quantified by densitometry and expressed as fold to vehicle group (B: vehicle n=3; NH_4Ac n=6 and C: vehicle n=4; NH_4Ac n=5). Data shown as mean, +SEM.

Figure 7A depicts representative western blots revealing that HO1 and Trx1 protein expression is not changed upon ammonia challenge after 6h or 24h (Figure 7A; quantification in Figure 7B and 7C).

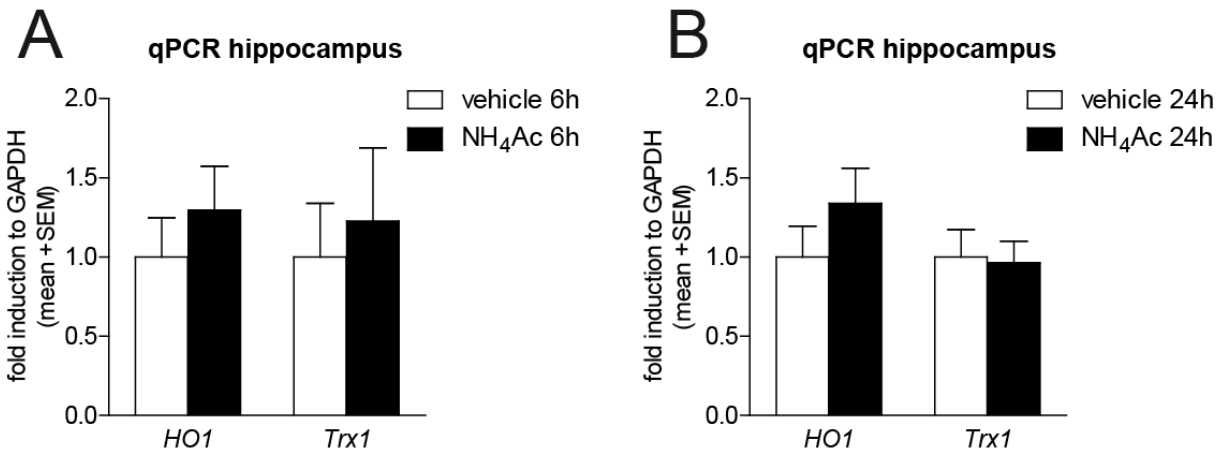

Figure 8. Antioxidant enzyme gene response in the hippocampus of mice after acute hyperammonemia. Mice were injected i.p. with NH_4Ac (10mM/kg body weight) for 6h and 24h. qPCR analysis of (A) *HO1* and (B) *Trx1* gene expression after 6h and 24h of hyperammonemia (6h vehicle n=3-4; NH_4Ac n=5 and 24h vehicle n=10; NH_4Ac n=8). Data shown as mean, +SEM. Student's *t*-test, *p <0.05.

We also assessed gene expression of *HO1* and *Trx1* for 6h and 24h treatment periods by qPCR. Additionally, we analyzed the expression of superoxide dismutase isoforms *SOD1, SOD2* and *SOD3* which are involved in the response to oxidative stress (Zelko et al., 2002; Méthy et al., 2004). For *HO1* we detected a nonsignificant gene induction already after 6h, which persisted until the 24h timepoint. *Trx1* was slightly upregulated after 6h (Figure 8A and 8B). Among the SOD genes analyzed, *SOD1* expression was significantly declined after 24h (Figure 9B). *SOD2* and *SOD3* gene expression were not altered (Figure 9A and 9A). To consider inflammation as a potential consequence following an acute ammonia load, qPCR measurements of hippocampal tissue were carried out for inflammatory cytokine gene expression. We could not detect *TNFα* after 6h and *IL6* was not significantly changed (Figure 9C). However, after 24h of treatment, a significant upregulation of *TNFα* was evident, accompanied by a still unchanged gene expression of *IL6* gene (Figure 9D). These results may point to selective changes in the induction of cytokines in response to an acute ammonia load.

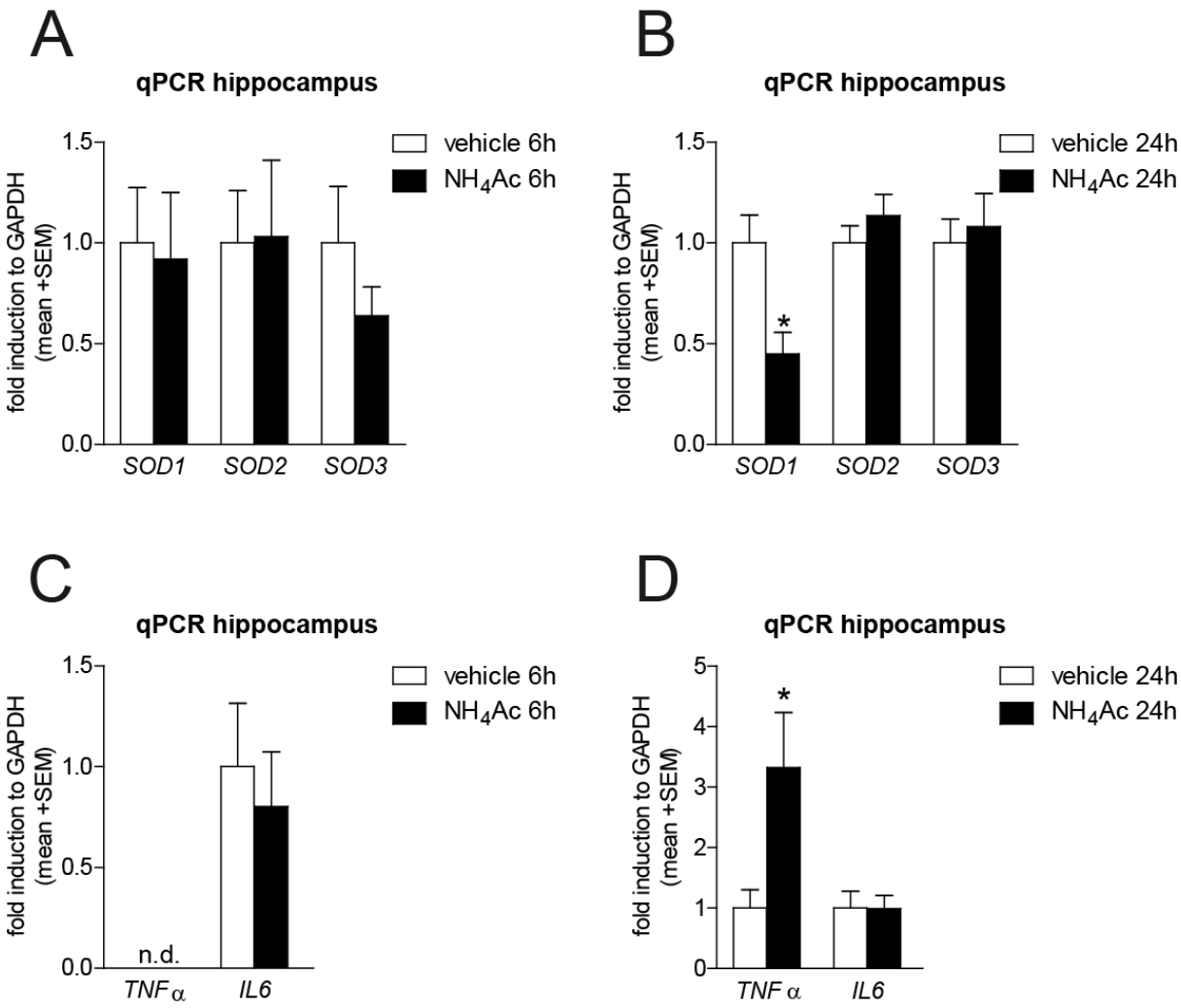

Figure 9. Superoxide dismutase and inflammatory cytokine gene response in the hippocampus of mice after acute hyperammonemia. Mice were injected i.p. with NH_4Ac (10mM/kg body weight) for 6h and 24h. (A+B) qPCR analysis of *SOD1*, *SOD2* and *SOD3* gene expression after 6h and 24h of hyperammonemia (6h vehicle n=4; NH_4Ac n=5 and 24h vehicle n=6; NH_4Ac n=5-8). (C+D) qPCR analysis of *TNFα* and *IL6* gene expression after 6h and 24h of hyperammonemia (6h vehicle n=4; NH_4Ac n=5 and 24h vehicle n=10; NH_4Ac n=8). Data shown as mean, +SEM. Student's *t*-test, *p <0.05.

3.5 Acute hyperammonemia is associated with induction of ER stress response genes

While we were able to show that acute hyperammonemia is associated with an accumulation of oxidized proteins, to date, the mechanisms and origin of protein oxidation remains unknown. Presumably, our observation is not related to a global oxidative stress, since expression of antioxidant enzymes remained unchanged (Figure 8). To further explore the molecular mechanism, we assessed whether the accumulation of oxidized and polyubiquitinated proteins in the hippocampi of acute hyperammonemic mice (Figure 3 A-D) may lie in the impact of ammonia on the ER and protein folding of *de novo* synthesized proteins. ER stress is implicated in various pathological conditions in the CNS (Yu et al., 1999; Hayashi et al., 2005; Sokka et al., 2007; Roussel et al., 2013) and can be depicted by the induction of specific ER stress-related genes. Therefore, we were interested in the effect of an acute systemic ammonia load on the hippocampal ER stress response gene expression.

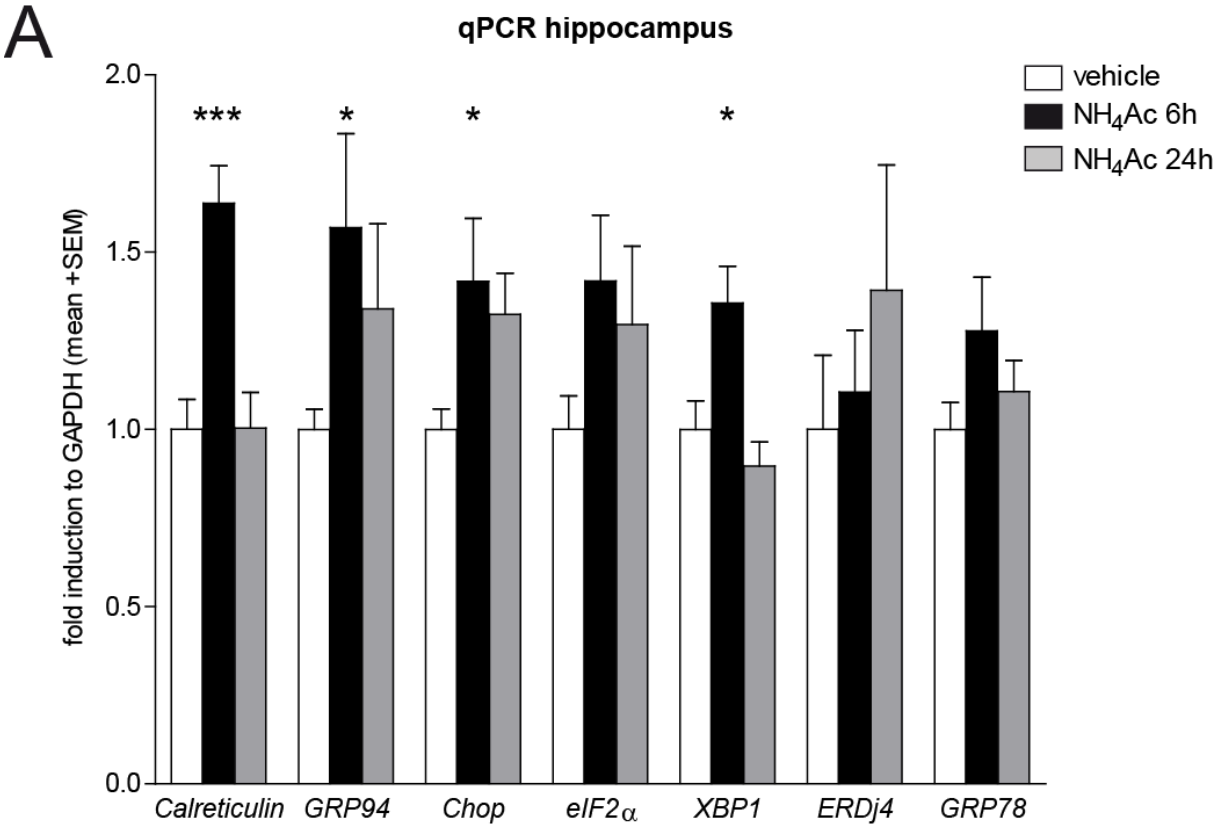

Figure 10. Induction of ER stress proteins in the hippocampus after acute hyperammonemia. Mice were treated with NH_4Ac (10mM/kg body weight) for 6h or 24h. (A) qPCR analysis of ER stress gene expression in the hippocampus (vehicle n=7; NH_4Ac 6h n=6; NH_4Ac 24h n=4). Data shown as mean, +SEM. Student's *t*-test, *p <0.05; ***p <0.001.

By means of qPCR we detected that hippocampal ER stress genes *Calreticulin, GRP94, Chop* and *XBP1* were significantly upregulated after 6h of hyperammonemia (Figure 10A). Notably, prior to the accumulation of oxidized proteins (Figure 3C). These genes are part of the ER quality control and protein folding machinery. The effect was transient and declined after 24h of hyperammonemia. This finding suggests a quick, compensatory response of the ER to stress resulting from increased intracerebellar ammonia concentrations. Thus, ammonia triggers a hippocampal ER stress response after 6h and prior to the accumulation of ubiquitinated proteins (Figure 3A). Increased expression of Calreticulin on protein level (red) was also observed by means of immunohistochemistry in hippocampal slices derived from mice which were treated for 24h with ammonia (Figure 11A, left panel). Calreticulin expression did not colocalize to microglia cells (CD68, green). Also, expression of CD68 as a microglial activation marker was not changed upon hyperammonemia in the hippocampus (Figure 11A, center panel).

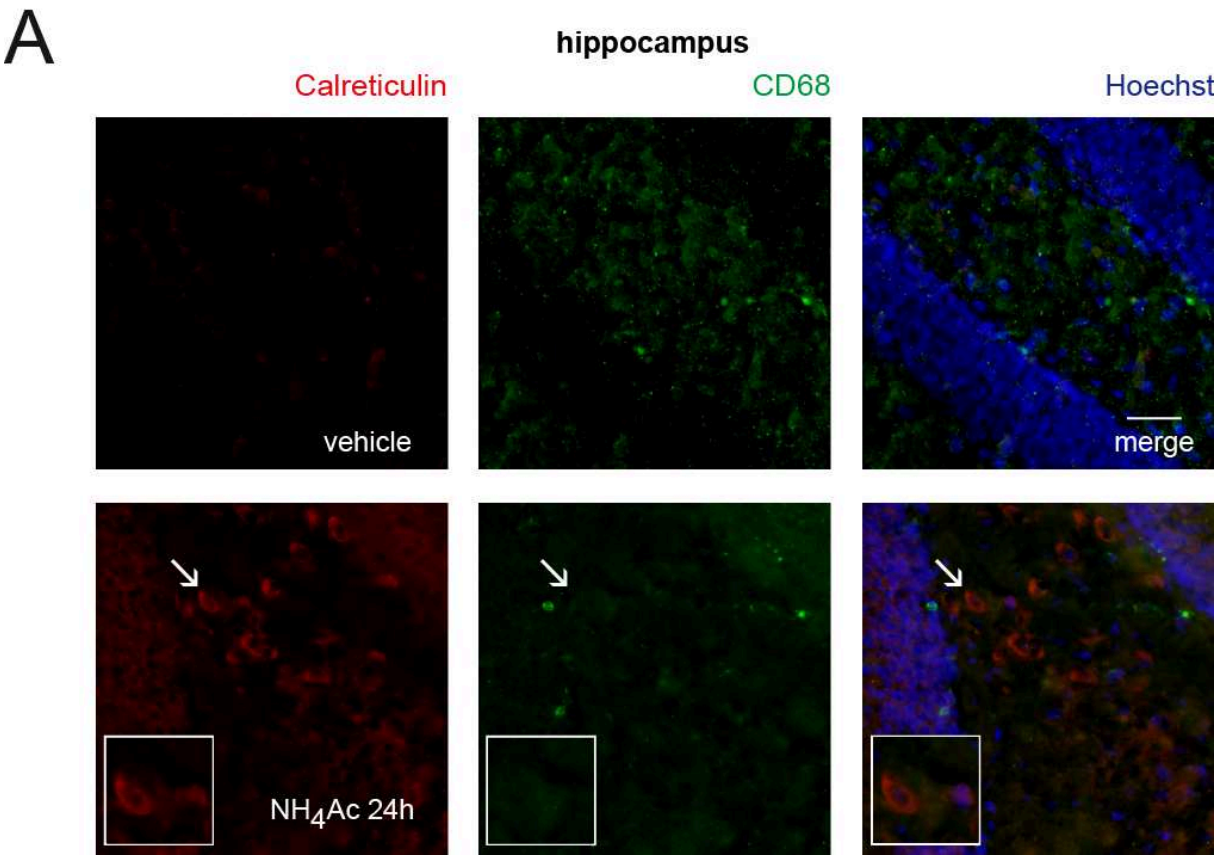

Figure 11. Calreticulin in the hippocampus after acute hyperammonemia. Mice were treated with NH_4Ac (10mM/kg body weight) for 24h. (A) Representative image of hippocampal cryosections stained for Calreticulin (red) and microglial marker CD68 (green) after vehicle (upper row) or 24h ammonium (NH_4Ac) treatment (lower row) of mice. Nuclear staining Hoechst 33258 (blue). Scale bar: 100 µm.

3.6 Acute hyperammonemia impairs hippocampal neurogenesis

Diverse studies demonstrated that hippocampal neurogenesis is affected by various pathological conditions in the CNS (Vallières et al., 2002; Ekdahl et al., 2003; Zheng et al., 2013; Valero et al., 2014). However, to date, there is no data available on how HE and hyperammonemia particularly influence neurogenesis and thereby putatively contribute to HE pathology and symptoms.

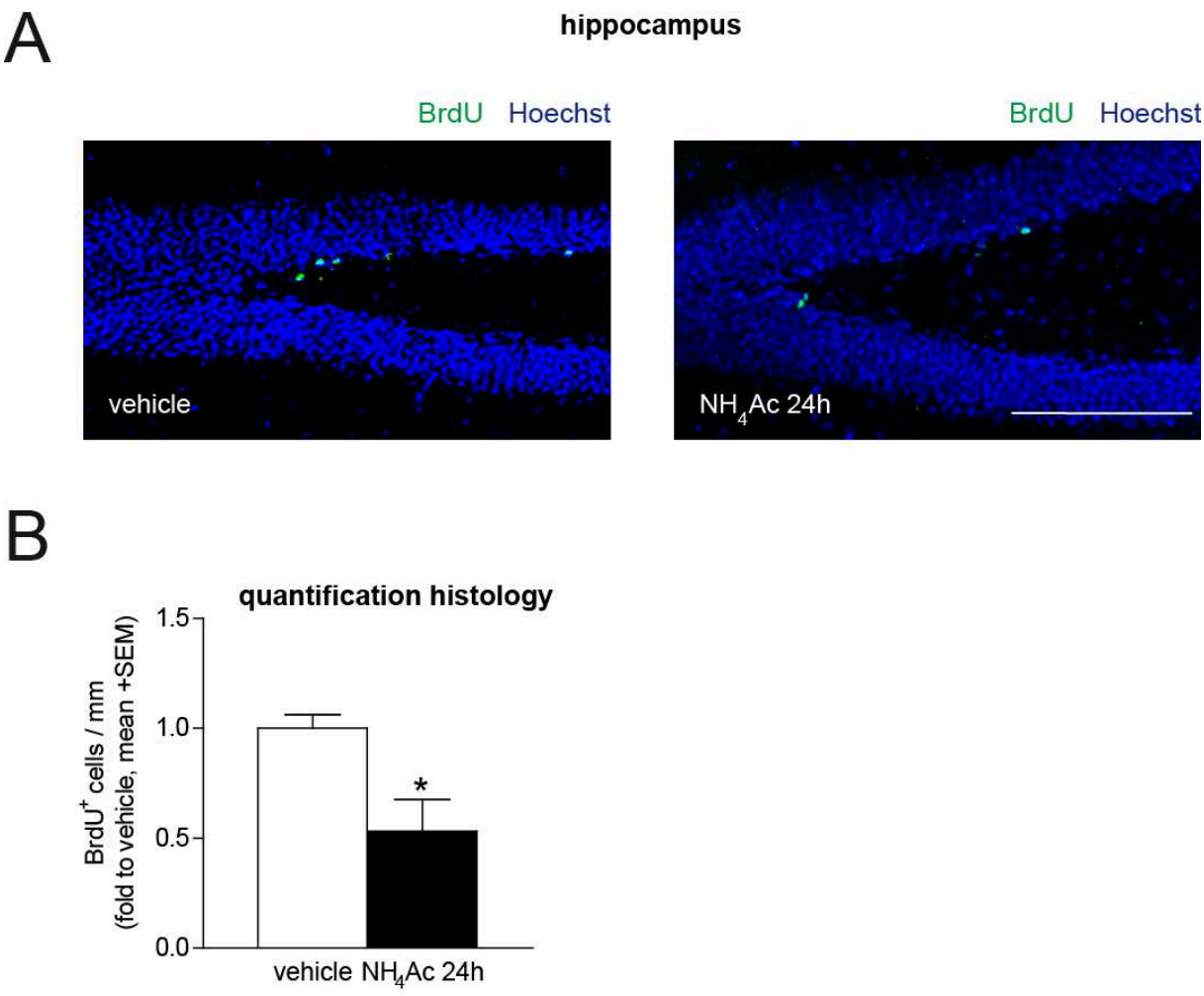

Figure 12. Inhibition of neurogenesis in the hippocampus after acute hyperammonemia. Mice were treated with NH_4Ac (10mM/kg body weight) for 24h (A) Representative image of hippocampal cryosections stained for BrdU (green) and nuclear staining Hoechst 33258 (blue). Scale bar: 200 µm. (B) BrdU positive cells per mm were counted in the dentate gyrus of the hippocampus of vehicle (n=3; 31 hippocampal slices) and NH_4Ac treated mice (n=4; 54 hippocampal slices). Data shown as mean, +SEM. Student's *t*-test, *p <0.05.

To address this issue, we treated mice with ammonia for 24h and coinjected the animals with the thymidine analog BrdU to mark proliferating cells. Figure 12A displays a representative image from hippocampal cryosections derived from vehicle and ammonia treated mice. Quantification of the immunohistological stainings revealed a significant decrease of BrdU-positive cells in the SGZ of the dentate gyrus, indicating an inhibition of proliferation in the hippocampus of hyperammonemic mice (Figure 12B). These data further argue for an increased sensitivity of the hippocampus to ammonia toxicity and may thus provide a possible mechanism for the cognitive dysfunction present in HE patients.

3.7 Acute hyperammonemia leads to changes in hippocampal protein content

Accumulation of polyubiquitinated proteins (Figure 3A and 3B) and increased proteasomal activity (Figure 5C) may alter the protein composition in hippocampal cells. To address this hypothesis, we subjected hippocampal samples of 24h ammonia-treated mice to mass spectrometry analysis. After comparison of vehicle versus ammonia-treatment group, we found a distinct profile of proteins present in hyperammonemic mice (Figure 13A). We detected proteins implicated in synaptic transmission to be diminished after ammonia treatment. Among them, glutamate receptor GRIA1, Cacnb3, a subunit of voltage-dependent calcium channels, and GABA receptor component Gabrg2 (Caddick et al., 1999; Saras et al., 2008; Granger et al., 2013) were undetectable in hyperammonemic mice. Moreover, ammonia led to decreased levels of hippocampal Psmc4, a regulatory subunit of the 26S proteasome, involved in the degradation of ubiquitinated proteins. Furthermore, ammonia treatment was associated with upregulation of certain proteins primarily implicated in mitochondrial activity, metabolism and in the UPS. Slc25a13/Citrin and Slc25a18 are mitochondrial membrane proteins required for mitochondrial aspartate and glutamate transport (Fiermonte et al., 2002; Palmieri, 2013). Slc25a13/Citrin participates in the urea cycle and is thus crucial for ammonia detoxification (Ruder et al., 2014). Furthermore, an upregulation of mitochondrial proteins TOM22 and Ndufa7 was evident. These proteins are involved in central mitochondrial mechanisms such as folding of imported proteins and in the mitochondrial respiratory chain, suggesting an involvement of ammonia in the cellular energy metabolism. With Cand1 and Ube2v2 two proteins related to the UPS (Franko et al., 2001; Zheng et al., 2002; Goldenberg et al., 2004) were specifically upregulated in the hippocampus after ammonia exposition. Mecp2 protein binds to methylated DNA, thereby mediating a transcriptional repression of various genes (Koch and Strätling, 2004; Klose and Bird, 2006). In summary, this mass spectrometry data from hippocampi of mice subjected to acute ammonia intoxication point to an altered hippocampal protein composition.

The proteins identified may play a yet unknown role for the cellular response to hyperammonemia and, thus, possibly for the pathogenesis of HE.

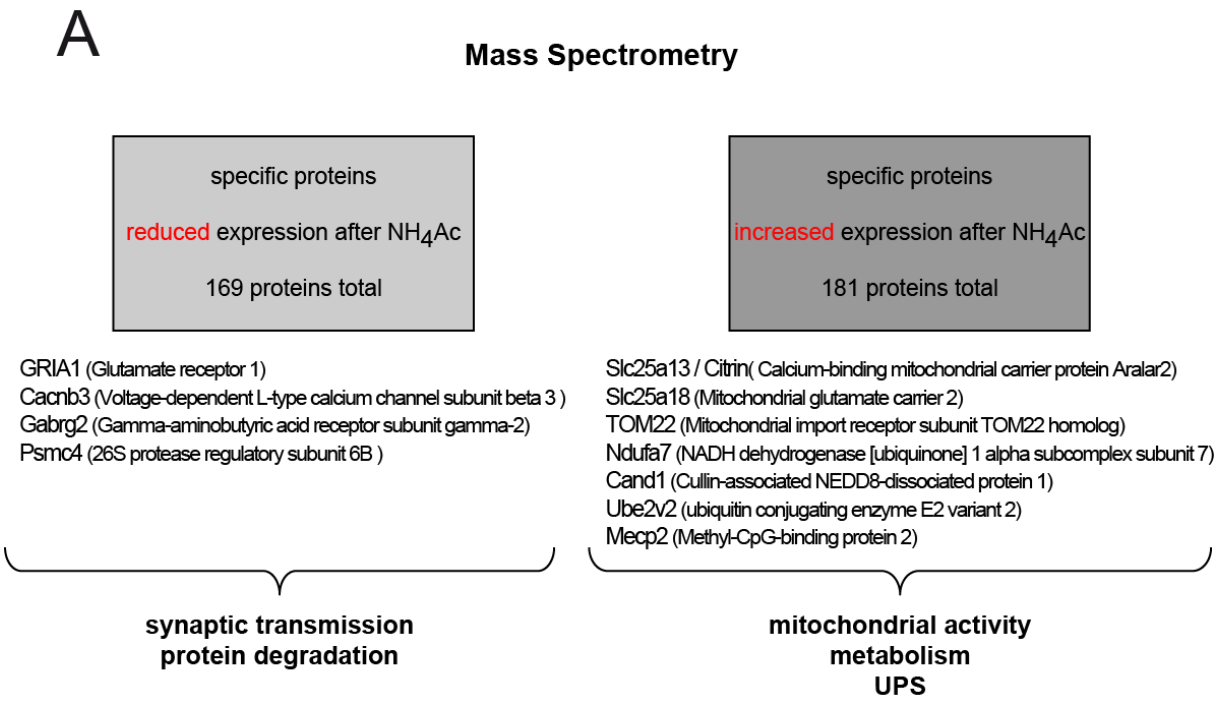

Figure 13. Acute hyperammonemia alters protein content in the hippocampus. Mice were injected i.p. with NH_4Ac (10mM/kg body weight). (A) Mass spectrometry analysis of hippocampal tissue from vehicle (n=3) and NH_4Ac (n=3) treated mice revealed either decrease or induction of specific proteins as compared to vehicle.

3.8 Ammonia induces accumulation of polyubiquitinated proteins and protein oxidation in microglia

Microglia are the resident immune cells in the brain and involved in the CNS immune response (Rock et al., 2004; Garden and Möller, 2006). To date, their significance for neurodegenerative diseases and association with neuroinflammation is an emerging topic of investigation (Hauwel et al., 2005; Hanisch and Kettenmann, 2007; Mandrekar-Colucci and Landreth, 2010). However, microglia cells are not well characterized in the context of ammonia toxicity and HE. Therefore, we focused on microglia cells to investigate how ammonia affects this specific cell type and thus possibly affects the pathogenesis of HE.

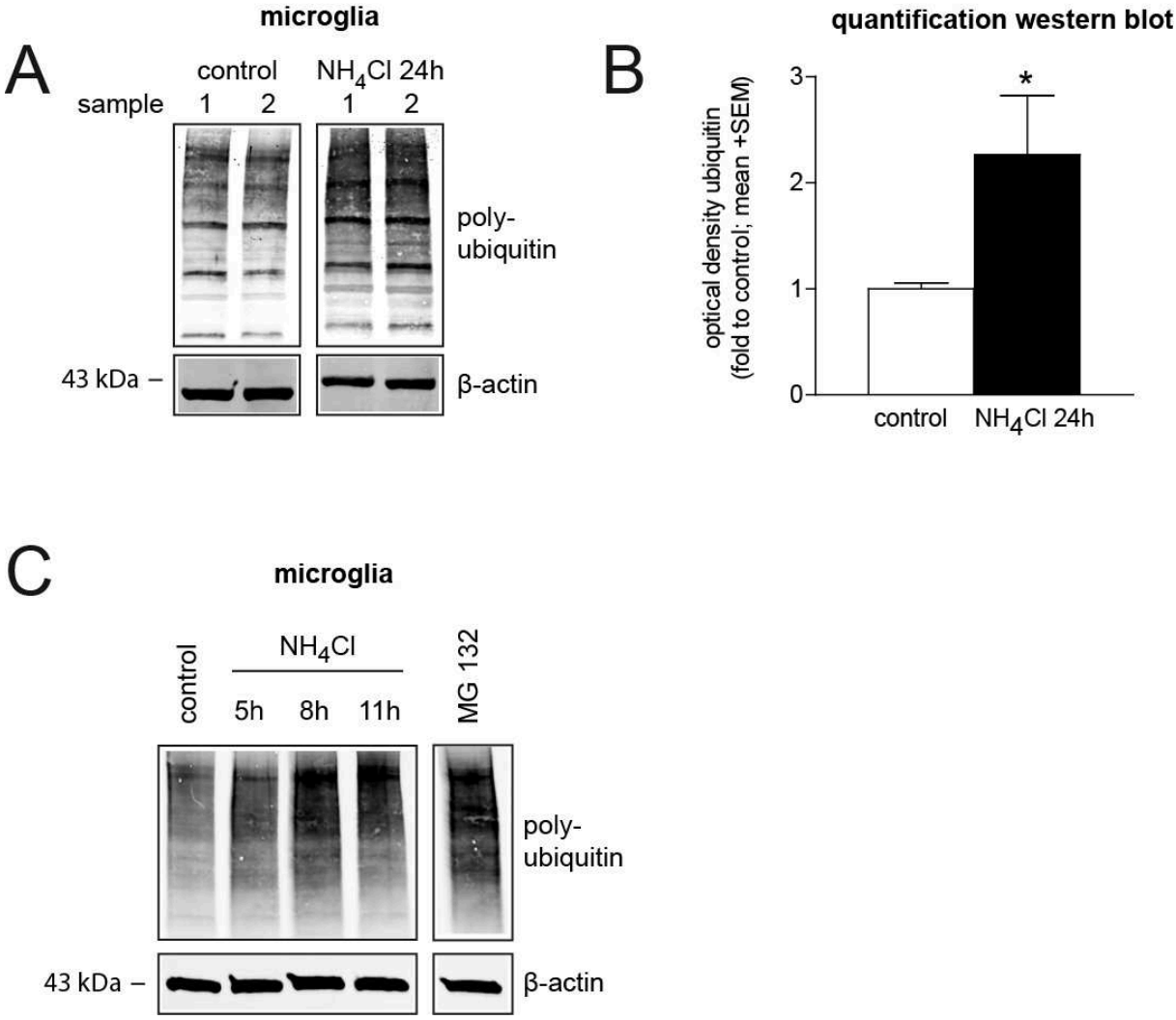

Figure 14. Accumulation of polyubiquitinated proteins in microglia after ammonia exposure. BV2 microglia were cultured with or without NH_4Cl (5mM) for the indicated timepoints. Proteasome inhibitor MG 132 (10µM) served as a positive control for accumulation of ubiquitin. (A) Representative western blot image shows accumulation of polyubiquitinated proteins in microglia cells. (B) Densitometric quantification of western blot analysis; normalization to β-actin served as a loading control (control: n=7; NH_4Cl: n=4). (C) Formation of polyubiquitinated proteins over time detected by western blotting. Data shown as mean, +SEM. Student's t-test, *$p<0.05$.

We cultured BV2 microglia in presence of NH_4Cl (5mM) and analyzed the accumulation of polyubiquitinated proteins. Figure 14A shows a representative western blot analysis, revealing a significant elevation of polyubiquitinated proteins in microglia after 24h of ammonia treatment (Figure 14A and 14B). Accumulation of these polyubiquitinated proteins gradually increased over time (Figure 14C). The proteasome inhibitor MG132 was applied as a positive control for proteasome-dependent accumulation of polyubiquitinated proteins (Ebstein et al., 2009; Seifert et al., 2010). By immunocytochemistry, we detected an increased intracellular ubiquitin content in microglial cells (Figure 15A). For quantification, we measured ubiquitin levels in a microplate fluorescence reader and thereby detected a highly significant accumulation after 24h. Notably, this effect was reversible when BV2 cells were cultured in presence of the antioxidant *N*-acetylcysteine (NAC), indicating the role of oxidative stress for protein damage and accumulation of polyubiquitinated proteins (Figure 15B).

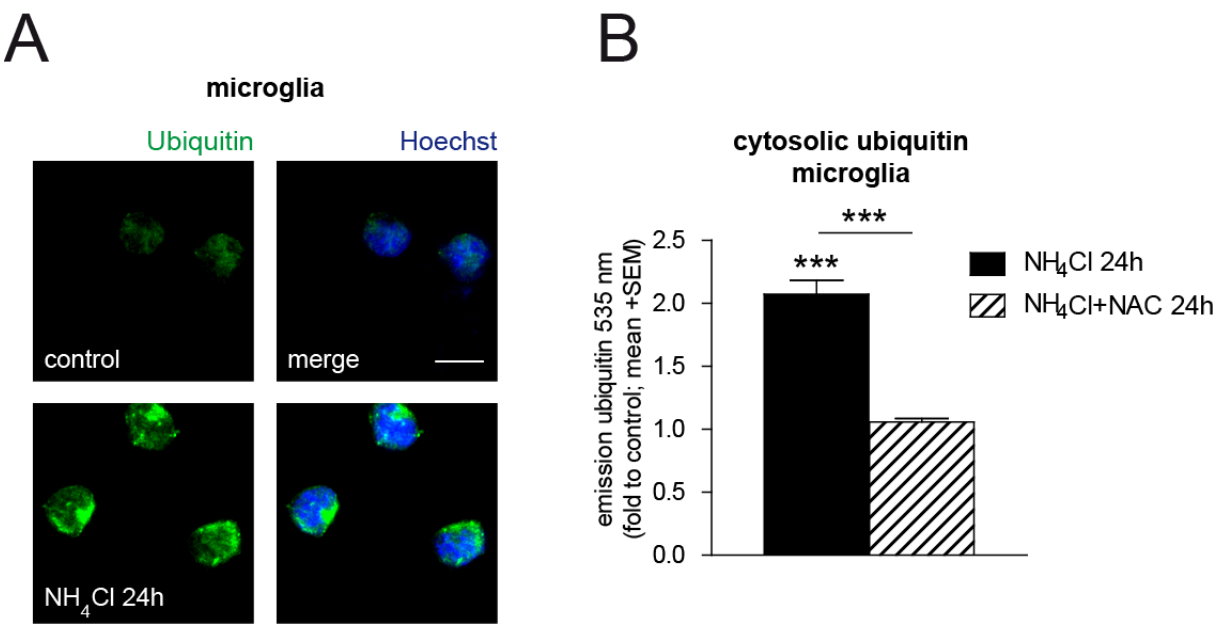

Figure 15. Ubiquitination in microglia after ammonia exposure. BV2 microglia were cultured with or without NH_4Cl (5mM) for 24h and in presence of antioxidant *N*-acetylcysteine (NAC; 1mM). (A) Representative image of cytosolic ubiquitin aggregates detected by fluorescence staining. Scale bar: 15 µm. (B) Cytosolic ubiquitinated proteins were stained and emission was measured in a microplate fluorescence reader at 535 nm (control: n=8; NH_4Cl: n=8; NH_4Cl+NAC n=8 from three individual measurements). Data shown as mean, +SEM. Student's t-test, ***p<0.001.

To analyze whether an elevated content of polyubiquitinated proteins is connected to protein oxidation, we subjected microglia samples cultured for 24h with ammonia to oxyblot

technique. Indeed, we detected a significant formation of protein carbonyls as a hallmark of oxidatively-damaged proteins (Figure 16A and 16B). To explore whether nitrosative stress is involved, we measured protein nitrotyrosinylation. This posttranslational modification has been described in oxidative stress conditions in HE and HE animal models (Norenberg, 2003; Häussinger et al., 2005; Görg et al., 2013b). Similar to the elevated polyubiquitination evident after 24h (Figure 14A and 14B), we detected presence of nitrotyrosinylated proteins by immunocytochemistry (Figure 16C). Also, a time-dependent increase of protein nitrotyrosine formation was evident in microglia cells in response to ammonia treatment (Figure 16D).

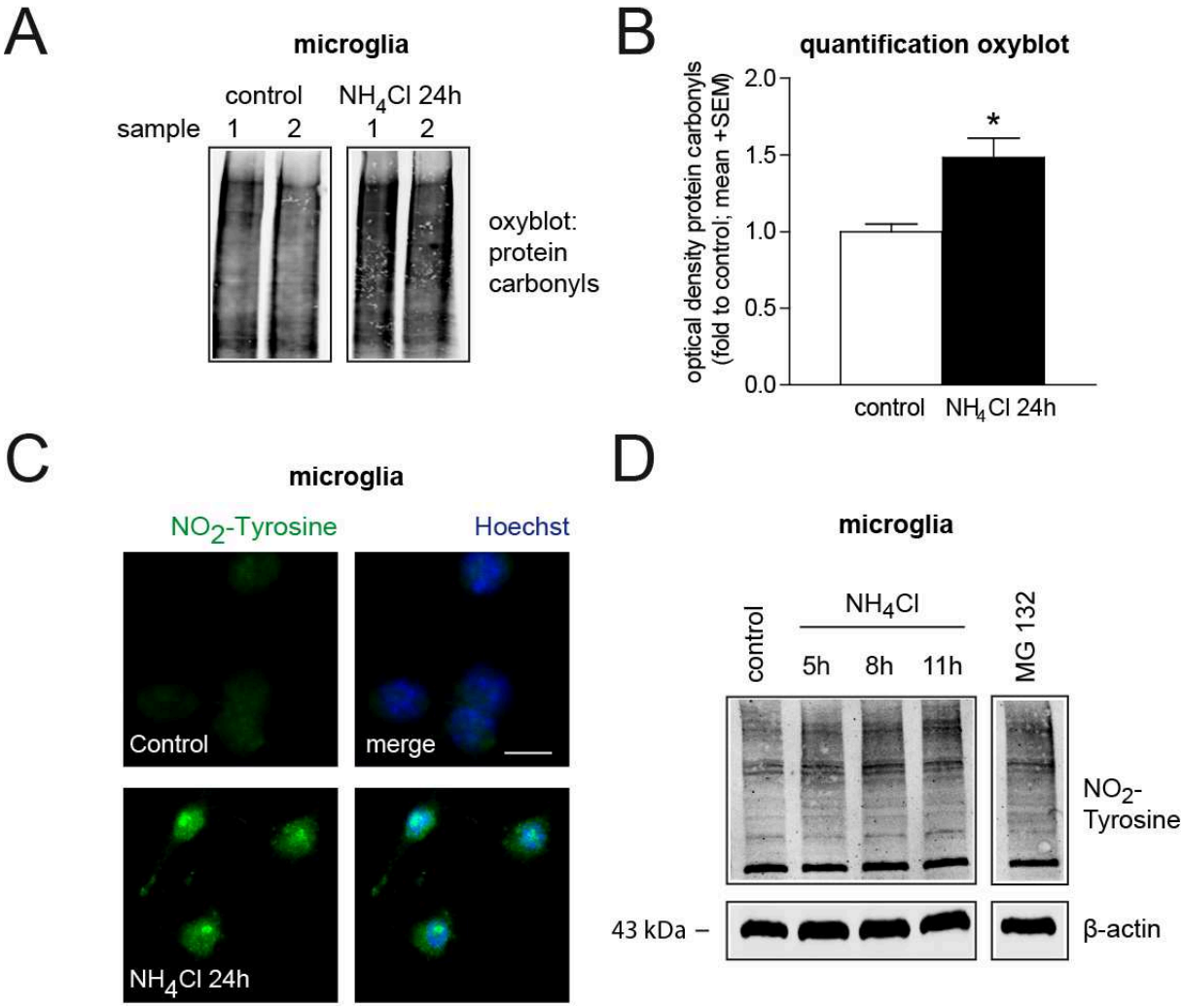

Figure 16. Ammonia produces oxidative stress and protein nitrotyrosinylation in microglia. BV2 microglia were cultured with or without NH_4Cl (5mM) for 24h. (A) Representative oxyblot analysis to detect protein carbonyls as a hallmark for oxidative stress in microglia. (B) Densitometric quantification of oxyblot analysis. Normalization to β-actin served as loading control (control: n=4; NH_4Cl: n=4). (C) Presence of protein nitrotyrosinylation (NO_2-Tyrosine) as a marker for oxidative stress was detected by immunofluorescence microscopy (representative image; scale bar: 15µm). (D) Formation of protein nitrotyrosinylation (NO_2-Tyrosine) over time detected by western blotting Data shown as mean, +SEM. Student's t-test, *$p<0.05$.

3.9 Ammonia increases microglial proteasome activity and antioxidant enzyme expression

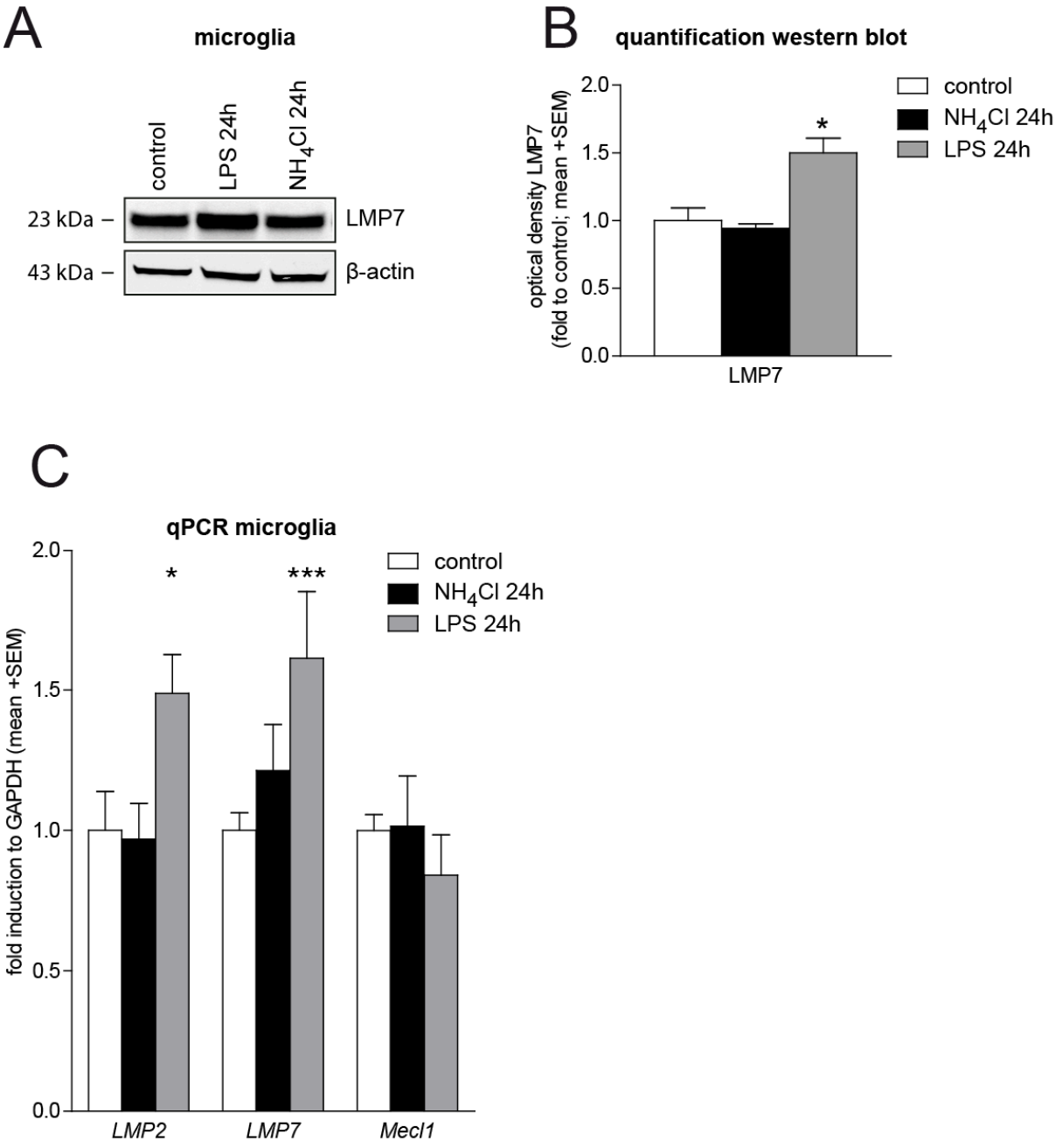

Figure 17. Immunoproteasome expression remains unchanged in ammonia treated microglia. BV2 microglia were cultured with or without NH_4Cl (5mM) or LPS (1mg/ml) for 24h. (A) Representative western blot shows LMP7 protein expression upon LPS and NH_4Cl exposure. (B) Densitometric quantification of western blot analysis (control: n=3; NH_4Cl: n=3; LPS: n=3). (C) qPCR analysis of microglial immunoproteasome subunits. LPS served as a positive control for immunoproteasome induction (control: n=6; NH_4Cl: n=6; LPS: n=3). Data shown as mean, +SEM. Student's t-test, *$p<0.05$, ***$p<0.001$.

It is well accepted that microglia are involved in the CNS immune response during systemic inflammation (Rock et al., 2004; Garden and Möller, 2006; Hanisch and Kettenmann, 2007; D'Mello et al., 2009; Mandrekar-Colucci and Landreth, 2010). HE and elevated ammonia levels are closely linked to systemic inflammation *in vivo*, involving inflammatory cytokine production among other effects (Jiang et al., 2009a; Rama Rao, Kakulavarapu V et al., 2010; Rodrigo et al., 2010; Görg et al., 2013a). Presence of such inflammatory cytokines is capable to induce the immunoproteasome (Ebstein et al., 2012). Therefore, we next investigated how ammonia challenge may impact on immunoproteasome expression in microglial cells. However, we found that ammonia treatment alone was not sufficient to induce immunoproteasomal subunit LMP7 on protein level (Figure 17A and 17B). Also, gene expression of *LMP7* and other immunoproteasome subunits *LMP2* and *Mecl1* remained unchanged after 24h of ammonia challenge (Figure 17D). LPS as a known inducer of immunoproteasome expression (Kruger and Kloetzel, 2012) was applied as a positive control. Accordingly, LPS potently induced LMP7 on protein and RNA level and *LMP2* on RNA level (Figure 17C). Interestingly and relevant for hippocampal samples from hyperammonemic mice (Figure 5C), we assessed an augmented chymotrypsin-like activity of the 20S proteasome in microglia (Figure 18A).

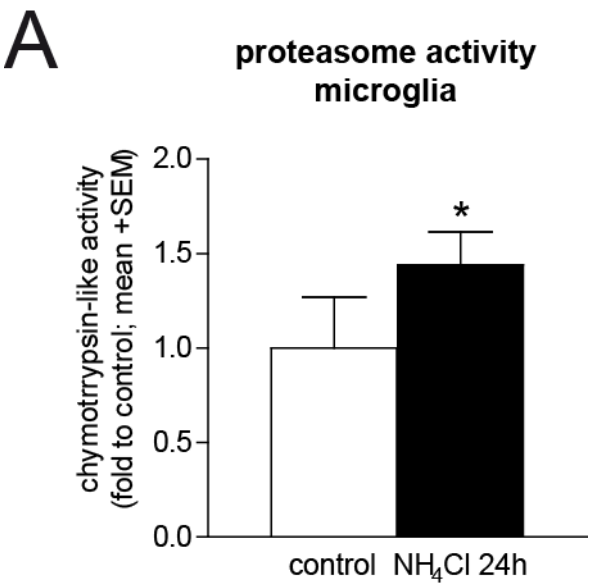

Figure 18. Proteasomal activity in microglia after ammonia exposure. BV2 microglia were cultured with or without NH_4Cl (5mM) for 24h. (A) Chymotrypsin-like 20S proteasome activity was measured in BV2 microglia (control: n=3; NH_4Cl: n=3). Data shown as mean, +SEM. Student's t-test, *p<0.05.

We afterwards investigated how ammonia affects expression of antioxidant enzymes and inflammatory cytokines in microglia. Figure 19 illustrates how the microglial expression of antioxidant enzymes HO1 and Trx1 is influenced by high ammonia levels. Culturing microglia in presence of 5mM ammonia led to elevated protein levels of both, HO1 and Trx1 (Figure 19A and Figure 19B). This augmented HO1 and Trx1 content was also confirmed by immunocytochemistry (Figure 11C).

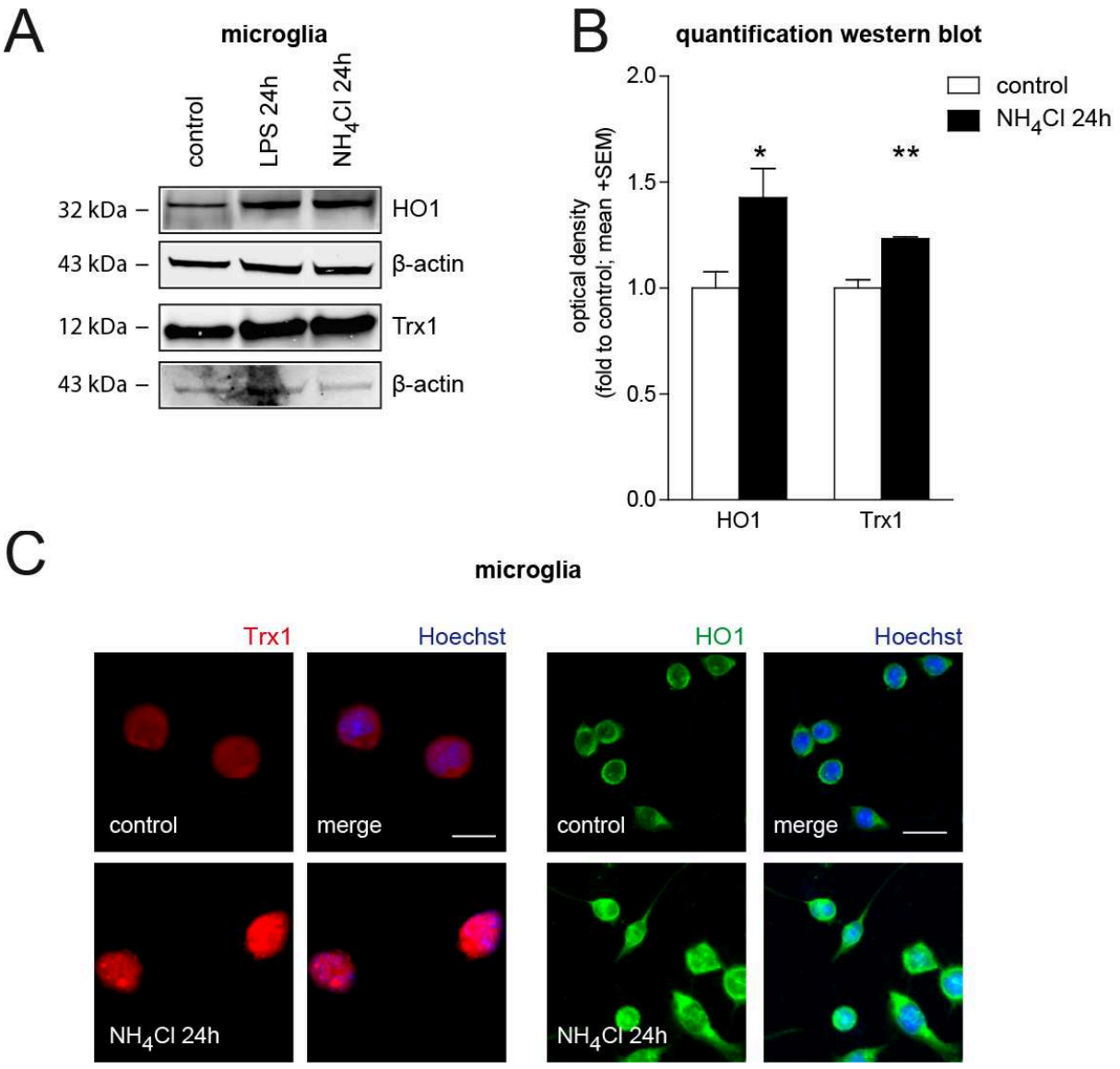

Figure 19. Antioxidant enzyme response in ammonia treated microglia. BV2 microglia were cultured with or without NH_4Cl (5mM) or LPS (1mg/ml) for 24h. (A) Representative western blot shows protein expression of antioxidant enzymes HO1 and Trx1 upon LPS and NH_4Cl exposure. (B) Densitometric quantification of western blot analysis (control: n=4-7; NH_4Cl: n=4-5). (C) Induction of HO1 and Trx1 expression was detected by immunofluorescence staining (representative image; scale bar: 15µm). Data shown as mean, +SEM. Student's t-test, *p<0.05, **p<0.01.

Examination of gene expression by qPCR technique revealed a tendency towards an elevated *Trx1* gene expression which was enhanced after 6h of ammonia challenge (Figure 20A). However, isoforms of superoxide dismutase *SOD1*, *SOD2* or *SOD3* genes were not induced after 6h or 24h of ammonia challenge in microglia (Figure 21A and 21B). Similarly, inflammatory cytokines TNFα and IL6 were not changed in a short term response after 6h (Figure 21C).

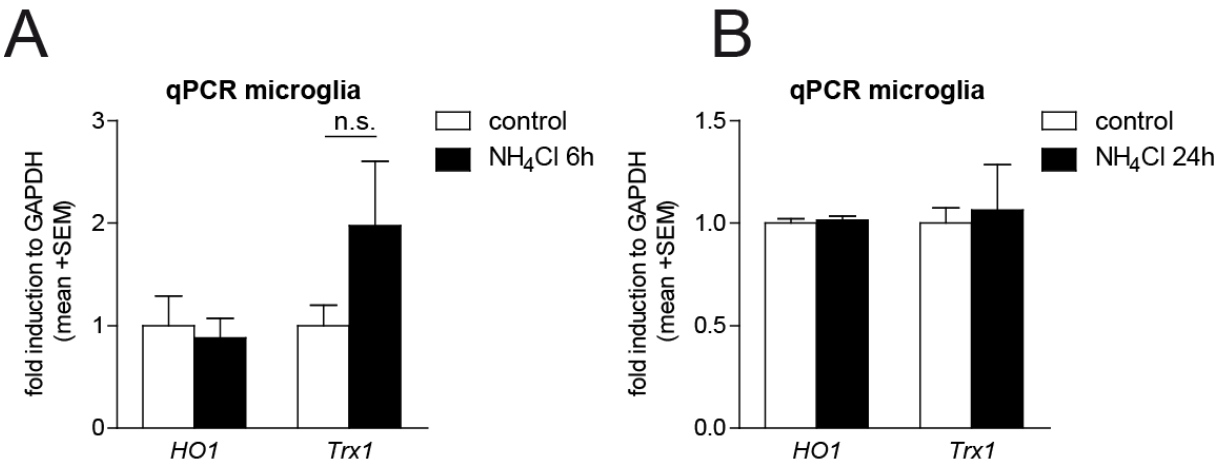

Figure 20. Antioxidant enzyme gene response in ammonia treated microglia. BV2 microglia were cultured with or without NH_4Cl (5mM) for 6h and 24h. qPCR analysis of (A) *HO1* and (B) *Trx1* gene expression after 6h and 24h ammonia treatment (HO1 control n=3; NH_4Cl n=3 and Trx1 control n=6; NH_4Cl n=6). Data shown as mean, +SEM.

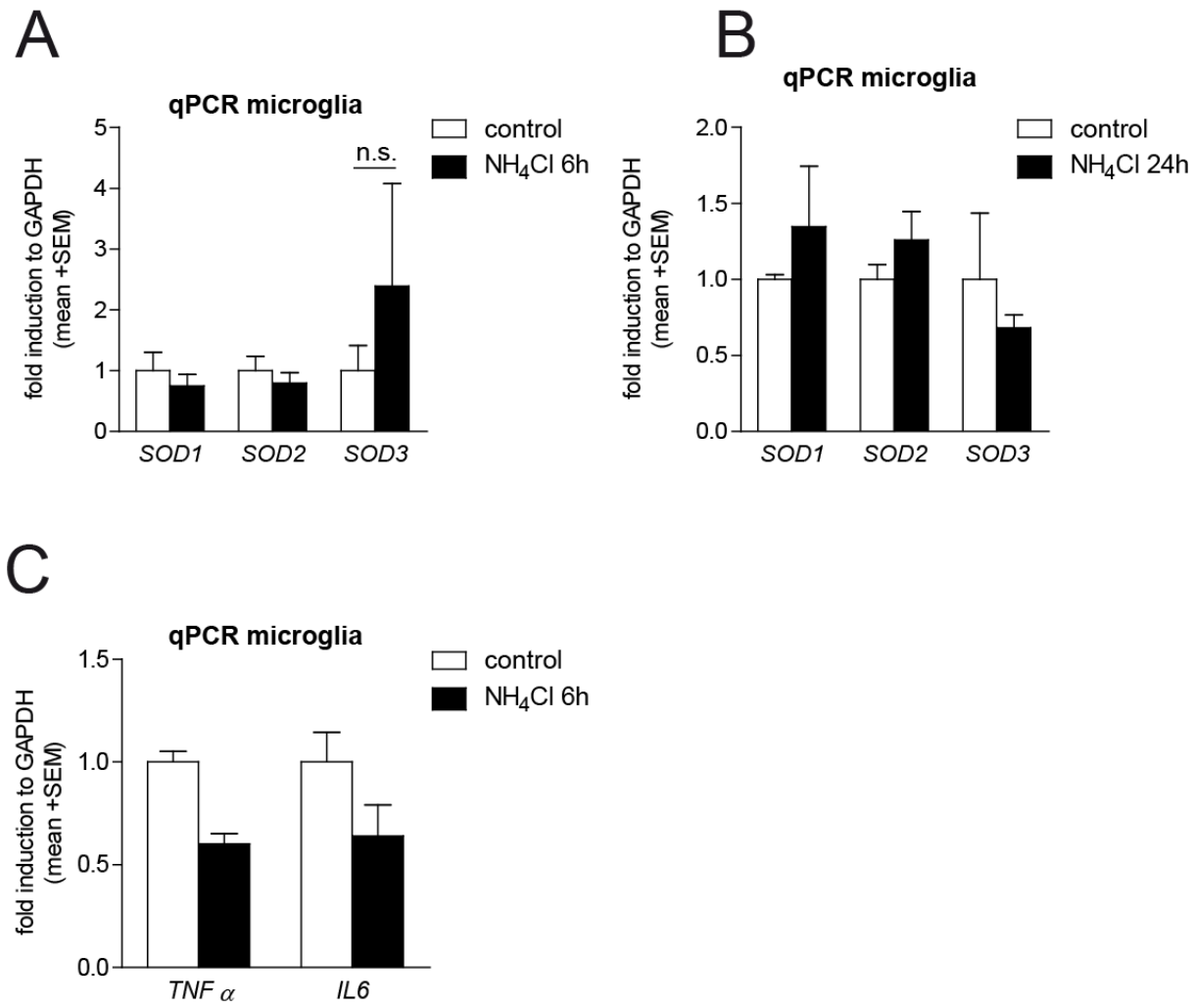

Figure 21. Superoxide dismutases (SODs) and inflammatory cytokine gene response in ammonia treated microglia. BV2 microglia were cultured with or without NH_4Cl (5mM) for 6h and 24h. (A+B) qPCR analysis of *SOD1*, *SOD2* and *SOD3* gene expression after 6h and 24h of ammonia treatment (control n=3; NH_4Cl n=3). (C) qPCR analysis of *TNFα* and *IL6* gene expression after 6h of ammonia treatment (control n=3; NH_4Cl n=3). Data shown as mean, +SEM.

The key feature of microglial cells is their ability to phagocyte cell debris (Hanisch and Kettenmann, 2007; Sierra et al., 2014). Thus, we next asked whether ammonia may impact on the microglial phagocytic activity. For this reason, we cultured microglia in presence of fluorescently-labelled myelin as a physiological stimulus to induce phagocytosis (Figure 22A and 22B). Already after 6h of incubation, we observed a significantly decreased ability of microglia to phagocyte myelin debris which was measured by detection of fluorescent DiI-coupled myelin in a microplate fluorescence reader. This phagocytic deficit remained evident after 24h of ammonia treatment. Interestingly, the inhibitory effect was reversible when cells where cultured in presence of the antioxidant NAC which restored the phagocytic ability to control levels (Figure 22A).

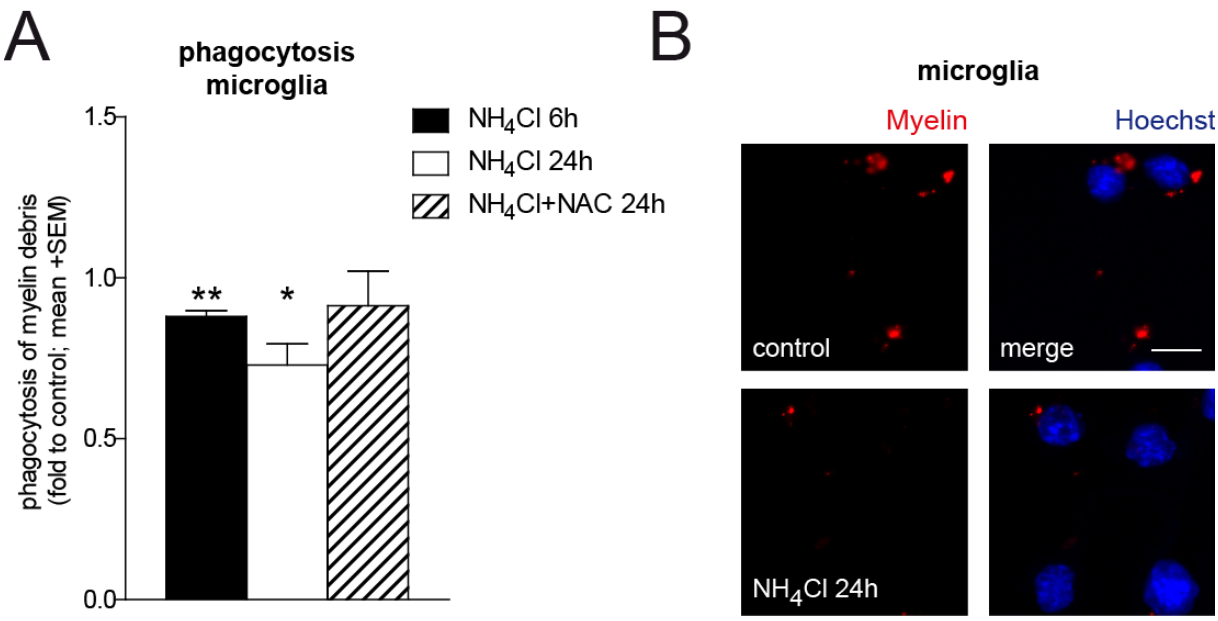

Figure 22. Ammonia impairs microglial phagocytosis of myelin debris. BV2 microglia were cultured with or without NH_4Cl (5mM) and antioxidant *N*-acetylcysteine (NAC; 1mM) for 6h and 24h. (A) Microglial phagocytosis of myelin debris upon presence of NH_4Cl and antioxidant NAC was measured by detection of fluorescent DiI-coupled myelin in a microplate fluorescence reader at 590 nm emission. (B) Representative immunofluorescence image of myelin debris phagocytosis after 24h NH_4Cl (scale bar: 15µm). Data shown as mean, +SEM. Student's t-test, *p<0.05; **p <0.01.

Additionally, we examined the expression of genes involved in myelin debris recognition and phagocytosis. We observed that all genes tested exhibited a tendency towards gene induction after 24h of ammonia challenge, becoming significantly apparent in *MSR1* gene expression (Figure 23A).

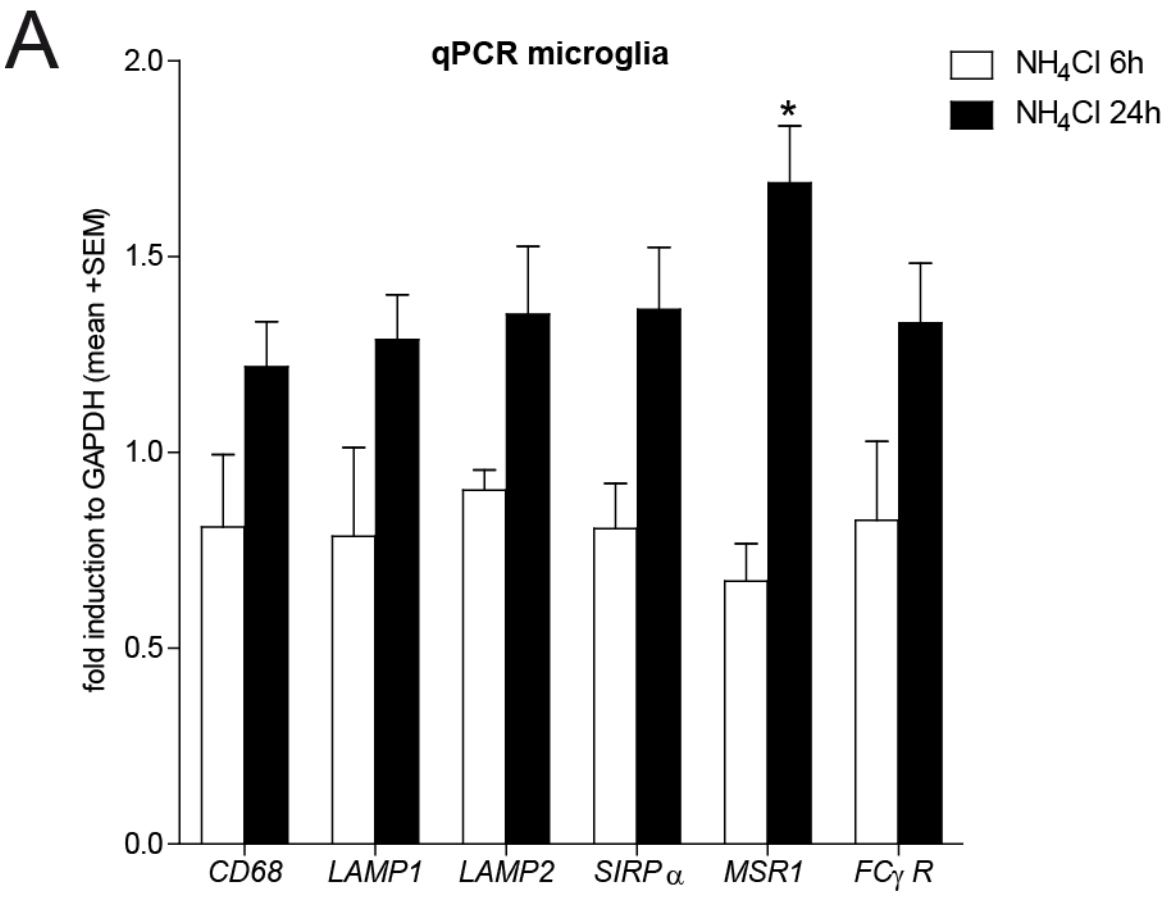

Figure 23. Ammonia affects microglial expression of phagocytosis-related genes. BV2 microglia were cultured with or without NH_4Cl (5mM) for 24h. (A) qPCR analysis of phagocytosis-related genes (control: n=3; NH_4Cl: n=3-6). Data shown as mean, +SEM. Student's t-test, *$p<0.05$

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4. Discussion

The present study aimed to clarify the knowledge about ammonia-mediated toxicity on the CNS and on microglial cells. In particular, we studied the effects of hyperammonemic conditions on protein homeostasis and the cellular oxidative stress response in the hippocampus. We investigated human brain tissue from HE patients and applied a mouse model for acute HE. Additionally to this *in vivo* approach, we carried out *in vitro* experiments with microglia cell culture. We characterized the microglial response upon an acute ammonia challenge. This cell type represents an important contributor to tissue homeostasis and plays a role in neuroinflammation, a component which is putatively associated with HE.

4.1 Involvement of the UPS in the pathogenesis of acute and chronic HE

In acute and chronic liver pathology, HE constitutes one of the most serious complications. In humans and animal models of liver disease, hyperammonemia represents a hallmark of HE. Numerous studies demonstrate that hyperammonemia potently increases free radical formation and may lead to oxidation-dependent structural modifications such as protein nitrotyrosinylation (NO_2-tyrosination) and RNA oxidation in the CNS and in cultured neural cells (Häussinger et al., 2005; Häussinger and Schliess, 2005; Schliess et al., 2009; Görg et al., 2010; Häussinger and Görg, 2010; Bosoi et al., 2012; Görg et al., 2013b). However, to date, the effect of ammonia-induced oxidation on the cellular protein homeostasis in the context of HE was not investigated.

The UPS ensures protein homeostasis and thus participates as a key element in the oxidative stress response resulting protein modifications. Most notably, the proteasomes represent the fundamental cellular machineries for the degradation of misfolded proteins resulting from oxidative damage (Pickering et al., 2010; Sijts, E J A M and Kloetzel, 2011).

In this study, we demonstrate that brain tissue from chronic HE patients exhibit an accumulation of polyubiquitinated proteins and show an upregulation of immunoproteasomal *LMP7* and *LMP2* genes. The involvement of accumulated polyubiquitinated proteins and participation of immunoproteasomal subunits in the pathogenesis of HE is a novelty. However, accumulation of polyubiquitinated proteins in neural tissue is frequent in various CNS diseases. Particularly, ubiquitin-containing protein aggregates are well documented in neurodegenerative diseases such as amyotrophic lateral sclerosis or Parkinson's, Alzheimer's and Huntington's disease, (Bendotti and Carrì, 2004; Gispert-Sanchez and Auburger, 2006; Dohm et al., 2008; Irvine et al., 2008; Pierre et al., 2009). Notably, there is strong evidence that aggregated proteins can inhibit the proteasome activity in neurodegenerative diseases (Keller et al., 2000; Stefanis et al., 2001; Junn et al., 2002;

Urushitani et al., 2002; Tseng et al., 2008; Zhang et al., 2008; Emmanouilidou et al., 2010; Ebrahimi-Fakhari et al., 2011). Accordingly, in addition to accumulation of polyubiquitinated proteins, we detected a reduced proteasomal activity in chronic HE patient brains. Considering the induction of *LMP7* and *LMP2* genes in brains of chronic HE patients and that LMP7 and LMP2 expression is regulated by inflammatory mediators (Seifert et al., 2010; Ebstein et al., 2012; Kruger and Kloetzel, 2012; Basler et al., 2013), we suggest an involvement of neuroinflammation for the pathogenesis of chronic HE. To date, especially the contribution of systemic inflammation to HE is topic of investigation while the role of neuroinflammation in HE is less understood (Vaquero et al., 2003b; Shawcross et al., 2004; Wright et al., 2007; Butterworth, 2011a; Tranah et al., 2013; McMillin et al., 2014). Regarding the function of immunoproteasome (iP) subunits, it is tempting to speculate about a yet unknown function of LMP7 and LMP2 in the context of neuroinflammation and a disturbed protein homeostasis in HE. In general, immunoproteasomes (iP) are involved in antigen processing and required for efficient MHC class I peptide supply. Beyond the iP's role for MHC class I antigen processing, our group has previously demonstrated that iPs, furthermore, represent major effectors for protein homeostasis (Seifert et al., 2010). Particularly, immunoproteasomal subunit LMP7 essentially contributes to removal of misfolded proteins resulting from oxidative stress conditions *in vivo* and *in vitro* (Seifert et al., 2010; Ebstein et al., 2012; Ebstein et al., 2013). Probably, induction of iPs in HE is dependent from inflammatory mediators but does not occur as a response to accumulation of polyubiquitinated proteins. Thus, in an acute model of HE, induction of iP would lack due to absence of inflammation. In line with this assumption, we found that in hippocampi of acute hyperammonemic mice and in ammonia-treated microglia cultures, accumulation of polyubiquitinated proteins is evident but iP subunit expression remains unchanged. Furthermore, in contrast to chronic disease stages, the proteasomal activity was enhanced in the acute HE model. This increased proteasomal activity upon an acute ammonia challenge can be regarded as a cellular response to the accumulation of polyubiquitinated proteins to ensure their proper degradation (Seifert et al., 2010; Basler et al., 2013). Contrarily, the decrease in proteasome activity evident in chronic HE brains, may point to an exhaustion of compensatory mechanisms which in turn results in reinforced accumulation of protein aggregates forming a vicious cycle. Thus, our results suggest the role of immunoproteasomes in chronic HE stages rather than during acute hyperammonemia. Putatively, the reduced proteasomal activity displays a consequence of the patient's chronic HE status resulting from recurrent episodes of hyperammonemia and accumulation of misfolded and ubiquitinated proteins.

In summary, for the first time the results of this work suggest the involvement of the UPS in the pathogenesis of HE. Our analyses indicate the accumulation of polyubiquitinated proteins

in the brain of chronic HE patients, in an acute animal model of HE and in microglia after exposure to ammonia. Thus, the accumulation of polyubiquitinated proteins may represent a common mark in HE due to ammonia toxicity. However, the response of the UPS differs, depending on disease progression since iP expression and proteasome activity vary between chronic and acute HE.

4.2 Oxidative stress in the HE brain and in microglia

The involvement of oxidative stress in the pathology of HE has been extensively discussed and studied (Norenberg, 2003; Norenberg et al., 2004; Görg et al., 2010; Lachmann et al., 2013; Bosoi et al., 2014). Protein nitrotyrosinylation (NO_2-tyrosination) and RNA oxidation seem to be prominent hallmarks of oxidative processes in the HE brain (Schliess et al., 2002; Görg et al., 2008; Schliess et al., 2009; Montoliu et al., 2011). However, to which extent such oxidative processes may alter cellular function remains to be elucidated. Here, we hypothesize that development of HE pathology involves the pro-oxidative effect of ammonia and is associated with alterations of protein folding, post-translational modifications and protein turnover.

In this study, we unveiled that ammonia leads to accumulation of protein carbonyls in the mouse hippocampus and in microglia cells as a hallmark of oxidative stress. Supporting these observations, different studies demonstrated the presence of oxidative stress as a consequence of elevated ammonia in other brain regions, neuronal cultures and most notably also in HE patients (Görg et al., 2010; Häussinger and Görg, 2010; Brück et al., 2011; Carbonero-Aguilar, 2012). In addition to protein carbonylation, in microglia oxidative stress, furthermore, became evident by NO_2-tyrosination of proteins. Previously, NO_2-tyrosinated proteins were linked to oxidative stress present in HE patients, in animal models and in *in vitro* cell culture studies (Schliess et al., 2002; Görg et al., 2010; Montoliu et al., 2011). Schliess and colleagues demonstrated that NO_2-tyrosinated proteins are evident in astrocytes after exposure to ammonia. Here, we reveal that this is also true for microglia cells and therefore conclude that NO_2-tyrosination due to ammonia is not unique to astrocytes.

Oxidative stress activates the antioxidant response involving antioxidant enzymes (Finkel and Holbrook, 2000; Görg et al., 2013a). We examined the impact of hyperammonemia on antioxidant enzymes HO1 and Trx1 in the brain. Induction of HO1 was demonstrated in HE patients, in HE animal models and *in vitro* using neural cell cultures. (Warskulat et al., 2002; Widmer et al., 2007; Brück et al., 2011; Zemtsova et al., 2011; Görg et al., 2013a). So far, the role for Trx1 in the context of HE was not investigated. We hypothesized about an

involvement of Trx1 since its ability to scavenge excess ROS protects against inflammation-induced oxidative damage (Ohashi et al., 2006; Berndt et al., 2007; Drechsel and Patel, 2010). Also, recent work has shown that Trx1 is implicated in microglia activation after LPS or IL-1β exposure (Wang et al., 2007; Sharma et al., 2007). In our study, we found that expression of Trx1 is heterogeneous among HE patients. Mice with acute ammonia intoxication lack induction of hippocampal HO1 or Trx1. However, the induction of both, Trx1 and HO1, was evident in microglial cultures upon ammonia treatment. In line with this observation, induction of HO1 was previously shown in microglia and astrocytes (Warskulat et al., 2002; Zemtsova et al., 2011; Görg et al., 2013b), suggesting a role for the activation of antioxidant defense mechanisms in microglia cells upon hyperammonemia. Thus, our results imply that, in addition to HO1, microglial Trx1 represents an antioxidant enzyme involved in the defense against oxidative stress resulting from ammonia. Superoxide dismutase isoforms (SODs) contribute to the antioxidant response. SOD1 participates in conversion and removal of excess superoxide radicals, thereby representing a central antioxidant which counteracts the deleterious effects of ROS. Previously, Song and colleagues reported a reduced SOD1 expression in hyperammonemic rats (Song et al., 2002). In line with this study, we found decreased hippocampal *SOD1*, but not *SOD2* or *SOD3*, gene expression in our acute HE model. Before, acute ammonia toxicity was shown to inhibit the catalytic activity of antioxidant enzymes such as SODs, catalase or peroxidase despite of increased ROS production, suggesting that ammonia may directly disarrange a physiological ROS detoxification (Kosenko et al., 1997; Kosenko et al., 1999). In microglia cells, *SOD1* and *SOD2* gene expression was not significantly regulated upon ammonia exposure suggesting a differential regulation in this specific cell type.

In addition to antioxidant enzymes, direct application of free radical scavenging agents may serve as a strategy to prevent the harmful effects of oxidative stress. Beforehand, studies analyzed the protective effect of antioxidants like NAC or resveratrol in the context of ammonia toxicity and neuroinflammation in HE models (Wang et al., 2007; Butterworth, 2011b; Bobermin et al., 2012). Accordingly, we provide evidence that NAC protects microglia against intracellular accumulation of polyubiquitinated proteins and furthermore, is able to prevent ammonia-induced inhibition of phagocytosis.

4.3 Acute hyperammonemia in the context of neuroinflammation and microglia activation

In addition to ammonia, inflammation represents a major contributor to the development of HE. Excess levels of intracranial ammonia may induce neuroinflammation, thereby contributing to cognitive dysfunction and negatively affecting the clinical outcome (Shawcross et al., 2004; Seyan et al., 2010). Previously, increased TNFα production was detected in the cortex of HE rodents (Bémeur et al., 2010; Brück et al., 2011). Therefore, we examined the expression of TNFα in the hippocampus. Upon hyperammonemia, hippocampal *TNFα* gene expression was increased suggesting that this gene induction may rely on oxidative stress. TNFα activation is regulated via redox-dependent NFκB signaling, a transcription factor induced by ROS and in response to oxidative and ER stress (Oyadomari and Mori, 2004; Negi et al., 2011; Chhunchha et al., 2013). Also, TNFα potentiates NO production in astrocytes (Hamby et al., 2008) and thereby putatively contributes to a pro-inflammatory milieu in the brain. Interestingly, the effect of hyperammonemia seems to be specific to TNFα production since no changes of pro-inflammatory cytokine IL6 were detectable. In patients, IL6 expression in the peripheral blood was shown to correlate with the severity of HE (Montoliu et al., 2009).

To complement our *in vivo* findings, this work also explored the impact of ammonia on cultured microglial cells. Oxidative stress or pro-inflammatory stimuli can activate microglia and consequently lead to production of ROS/RNS and release of inflammatory cytokines (Garden and Möller, 2006; Hanisch and Kettenmann, 2007; Norenberg et al., 2007; Wright et al., 2007). Thereby microglia may contribute to HE pathology. Indeed, several studies indicate an impact of microglia for HE models *in vivo* and *in vitro* and, notably, also in the human HE brain (Svoboda et al., 2007; Jiang et al., 2009a; Rodrigo et al., 2010; Zemtsova et al., 2011; Rao, Kakulavarapu V Rama et al., 2013; Görg et al., 2013a; McMillin et al., 2014). Our findings suggest that ammonia exposure *per se* is insufficient to induce microglial IL6 or TNFα expression. Recent data addressing the inflammatory response of microglia in the context of HE still remain conflicting, suggesting a tight dependency on disease state, applied HE model and the extent of hyperammonemia (Jiang et al., 2009a; Rodrigo et al., 2010; Brück et al., 2011; Zemtsova et al., 2011). Presumably, microglia activation may not be triggered by ammonia itself but rather occur as a secondary event in the context of systemic inflammation, neuroinflammation and by involvement of other toxic components such as bacterial LPS.

4.4 Role of hippocampal ER stress in acute hyperammonemia

Generation of ROS has been connected to induction of ER stress and activation of the UPR as a reaction to protein misfolding (Walter and Ron, 2011). Moreover, both, oxidative stress and the ER stress response participate in different neurodegenerative and CNS diseases such as Parkinson's disease, Alzheimer's diseases and multiple sclerosis (Holtz and O'Malley, 2003; Katayama et al., 2004; Mháille et al., 2008). To date, there is no knowledge whether ER stress is associated with HE pathogenesis. We here demonstrate upregulation of hippocampal ER stress genes *Calreticulin*, *Grp94*, *Chop* and *XPB1* upon acute hyperammonemia, suggesting a role of the ER stress response for HE. ER chaperones Calreticulin, GRP94 and transcription factor XBP1 are induced due to ER stress and thereby support the ER protein folding capacity (Yoshida et al., 1998; Lee, 2005; Eletto et al., 2010; Zhang et al., 2014). Calreticulin plays a central role in the intracellular calcium (Ca^{2+}) homeostasis (Rutkevich and Williams, 2011; Frasconi et al., 2012). It is believed that ER stress causes release of Ca^{2+} from the ER which may promote ROS production (Chinopoulos and Adam-Vizi, 2006). Related to HE, increased intracellular Ca^{2+} was associated with ammonia-induced cell swelling of astrocytes (Jayakumar et al., 2009). Also, exposure to ammonia leads to a calcium-dependent glutamate release which may cause alterations in glutamatergic synaptic transmission (Rose et al., 2005). Also, the induction of ER stress gene Chop was evident upon hyperammonemia. Involvement of Chop was previously linked to neurodegenerative diseases such as Parkinson's disease, the lipid storage disease GM1-gangliosidosis, to Huntington's disease and spinocerebellar ataxia (Nishitoh et al., 2002; Ryu et al., 2002; Holtz and O'Malley, 2003; Tessitore et al., 2004; Lindholm et al., 2006). Moreover, Calreticulin and Chop induction was present in ER stress induced apoptosis (Yamaguchi and Wang, 2004; Chao et al., 2010). Considering the functions of these ER stress genes, the here evident gene inductions underline our hypothesis that ammonia generates a pro-oxidative milieu in the hippocampus and thus may affect protein folding in the ER compartment and activation of the UPS-dependent degradation machinery.

4.5 Functional relevance of ammonia-induced stress

4.5.1 Hyperammonemia alters hippocampal protein composition

Mass spectrometry analysis of hippocampal tissue from ammonia challenged mice revealed a specific regulation of protein expression after hyperammonemia. In comparison to vehicle treated animals, we found that hyperammonemia specifically alters the protein content in the hippocampus. Certain proteins that were reduced in animals after acute ammonia intoxication are implicated in synaptic transmission. For instance, Cacnb3 is highly expressed in hippocampal cells (Pichler et al., 1997), important for calcium trafficking and thus implicated in the intracellular signal transduction (Joëls and Karst, 2012). Interestingly, Cacnb3 knockout mice exhibit an impaired learning ability (Murakami et al., 2007). GRIA1 and Gabrg2 proteins are required for proper neuronal signal transduction (Saras et al., 2008; Granger et al., 2013). Therefore, reduced levels may impede neuronal signal transduction in ammonia-treated mice. Our results suggest that ammonium may impair synaptic transmission and may thereby affect cognitive function known to be disturbed in HE patients (Häussinger and Schliess, 2008; Lachmann et al., 2013). Moreover, we found regulation of UPS-related proteins. The hippocampal content of Psmc4 was reduced by ammonium. Psmc4 is an essential ATPase subunit of the 26S proteasome and crucial for the degradation of ubiquitinated proteins (Gottesman et al., 1997). Importantly, Psmc4 expression was also reduced in Parkinson's disease (Grünblatt et al., 2004), a neurodegenerative disease in which accumulation of polyubiquitin protein aggregates is evident (Pierre et al., 2009). The decreased expression of hippocampal Psmc4 after ammonia treatment may be connected to the accumulation of polyubiquitinated proteins in the hippocampus upon hyperammonemia. Furthermore, hyperammonemia induced ubiquitin conjugating enzyme Ube2v2. This enzyme catalyzes the synthesis of K63-linked polyubiquitin chains (Eddins et al., 2006). K63 linkage of ubiquitin is the only type of polyubiquitin chain which does not lead to protein degradation by the proteasome but rather participates in signaling pathways. Interestingly, K63 linkage is abundant in neurodegenerative processes (Tan, Jeanne M M et al., 2008; Lim and Lim, Grace G Y, 2011) and was connected to apoptosis, inflammation and the DNA damage response (Franko et al., 2001; Martinez-Forero et al., 2009; Humphrey et al., 2013; Lemus and Goder, 2014). Furthermore, Ube2v2 participates in the degradation of proteins in the ER compartment and thus links the UPS response upon hyperammonemia to ER stress (Lemus and Goder, 2014). Additionally, we detected an involvement of mitochondrial proteins in response to ammonia. Slc25a13/Citrin and Slc25a18 were increased in the hippocampus after hyperammonemia. The Slc25a13/Citrin encoding gene is mutated in citrullinemia, a neonatal disorder. Deficiency of Slc25a13/Citrin function leads to a disturbed urea cycle in the infants, subsequently causing accumulation of systemic ammonia with symptoms that resemble those found in HE. Similar to HE, neonatal citrullinemia provokes

neurodegenerative changes, cognitive deficits and can result in coma and death (Leonard et al., 2008; Ruder et al., 2014). Upregulation of Slc25a13/Citrin may represent a compensatory mechanism to counteract elevated ammonia levels. Another mitochondrial protein induced by hyperammonemia is the mitochondrial receptor subunit TOM22. This receptor complex located in the outer mitochondrial membrane is important for translocation of mitochondrial proteins synthesized in the cytosol (Yano et al., 2000). There is evidence that TOM22 initiates apoptotic pathways via interaction with pro-apoptotic proteins (Bellot et al., 2007; Cassidy-Stone et al., 2008). In line with our findings, ammonia was shown to induce oxidative and nitrosative stress in mitochondria leading to mitochondrial dysfunction and apoptosis (Bai et al., 2001; Rama Rao, K V et al., 2005; Widmer et al., 2007).

In summary, protein oxidation and changes in protein turnover upon an acute ammonia load seem to be associated with specific changes in hippocampal protein composition, thereby affecting primary neurobiological processes. While the role of these proteins has to be examined in HE, our data indicate a general dysregulation of synaptic plasticity mechanisms, of the UPS and of mitochondria. Therefore, data from this mass spectrometry analysis supports our findings that hyperammonemia leads to alteration of protein homeostasis.

4.5.2 Hyperammonemia impairs hippocampal neurogenesis

Numerous studies demonstrate the severe impairment of cognitive function in HE patients and in HE animal models (Monfort et al., 2007; Häussinger and Schliess, 2008; Méndez et al., 2011; Rodrigo et al., 2010; Häussinger and Sies, 2013; Lachmann et al., 2013; Umapathy et al., 2014; Arias et al., 2015). Also, the effect of ammonia on neurons was topic of investigation and indicated that hyperammonemia impairs long-term potentiation in the hippocampus by inhibition of neuronal pathways (Hermenegildo et al., 1998; Aguilar et al., 2000; Muñoz et al., 2000; Monfort et al., 2001; Izumi et al., 2005). However, to date, the underlying mechanisms for the cognitive dysfunction in HE are not understood. The hippocampus plays a crucial role for cognitive processes and is particularly sensitive to CNS stress (Kempermann et al., 2004; Neves et al., 2008). Continuous adult neurogenesis in the hippocampus is known to contribute to cognitive function (Gould et al., 1999; Schoenfeld and Gould, 2012). Noteworthy, neurogenesis is also impaired in other CNS diseases such as Parkinson's disease or multiple sclerosis (Höglinger et al., 2004; Huehnchen et al., 2011; Winner et al., 2011a). So far, the impact of ammonia toxicity on adult hippocampal neurogenesis remains disregarded. For the first time we here reveal that hyperammonemia inhibits proliferation of neural progenitors in the SGZ of the dentate gyrus. In future, it has to be examined whether hyperammonemia acts as a toxic factor on neural progenitors. Before, ammonia toxicity was linked to cell death in developing brain cells underlining the detrimental effect of ammonia on neuronal cells (Höglinger et al., 2004; Cagnon and Braissant, 2008;

Winner et al., 2011a; Winner et al., 2011b). Our findings provide a novel aspect of ammonia toxicity that may contribute to the cognitive dysfunction present in acute and chronic HE.

4.5.3 Ammonia impairs microglial phagocytosis of myelin debris

Phagocytosis represents a fundamental homeostatic function of microglia in the brain (Hanisch and Kettenmann, 2007). We here demonstrate that ammonia inhibits the ability of microglia to phagocyte myelin debris. This observation is in line with previous publications showing an impaired microglial latex beads phagocytosis due to high ammonia concentrations (Zemtsova et al., 2011). At the same time we found a tendency to induction of microglial activation marker *CD68,* lysosomal markers *LAMP1* and *LAMP2* and phagocytosis receptor *FCγR*. These molecules are related to initiation and performance of phagocytosis (Fu et al., 2012; Voss et al., 2012). Interestingly, *SIRPα*, a gene associated with inhibition of microglial phagocytosis (Gitik et al., 2011), was induced as well. Putatively, the results display an imbalance between ammonia-provoked inhibition of phagocytosis and a compensatory upregulation of pro-phagocytic genes. Speculatively, the reason for the phagocytic inhibition is connected to the pro-oxidative effect of ammonia since we found that treatment with the antioxidant substance *N*-acetylcysteine was capable to restore the phagocytic impairment of microglial cells.

4.6 Summary

Taken together, with based on the analysis of human brain tissue and our mouse model for acute hyperammonemia, we provide novel insights in the impact of ammonia on the CNS. We found that HE patients and hyperammonemic mice exhibit an accumulation of polyubiquitinated and oxidatively modified proteins in the brain. The proteasomes as crucial components of the UPS are differentially regulated in chronic HE patients and acute experimental hyperammonemia. Microarray analysis revealed an induction of iP subunits LMP7 and LMP2 in chronic HE, which was absent in acute hyperammonemia. Also, the proteasomal activity was differentially regulated in chronic and acute HE. For the first time, we demonstrate that ammonia leads to a transient induction of genes involved in the UPR. This suggests implication of ER stress and misfolding of *de novo* synthesized proteins upon hyperammonemia. Mass spectrometry analysis of hippocampal tissue from ammonia treated mice identified ammonia-responsive proteins with a yet unknown role in the context of HE. Our results suggest that polyubiquitination as a post-translational modification acts in concert with the ER stress response and that these mechanisms participate in HE pathophysiology. Furthermore, ammonia-induced toxicity in the hippocampus was associated with a decreased proliferation of neural progenitors in the dentate gyrus, suggesting the involvement of a disturbed neurogenesis in HE-associated cognitive dysfunction.

Applying microglia cell cultures as an *in vitro* model of hyperammonemia, we demonstrate that microglia display a potential target for ammonia toxicity in HE. Elevated ammonia levels provoke an accumulation of both, polyubiquitinated and NO_2-tyrosinated proteins and give rise to carbonylation of proteins indicating protein oxidative damage mechanisms in microglial cells. Enhanced proteasomal activity was not associated with iP induction, a mechanism we beforehand identified in our HE mouse model. Lack of inflammatory cytokine expression suggests that inflammation is not primarily involved. However, expression of antioxidant enzymes was promoted in microglia, putatively displaying a compensatory mechanism to cope with the present accumulation of oxidized proteins. Furthermore, ammonia inhibited microglial myelin phagocytosis underlining the detrimental effects of ammonia toxicity on microglia. Importantly, scavenging ROS by *N*-acetylcysteine was sufficient to prevent both, accumulation of polyubiquitinated proteins and inhibition of phagocytosis. Thus, microglia participate in the response to ammonia and may in future serve as targets for novel therapeutic approaches in HE.

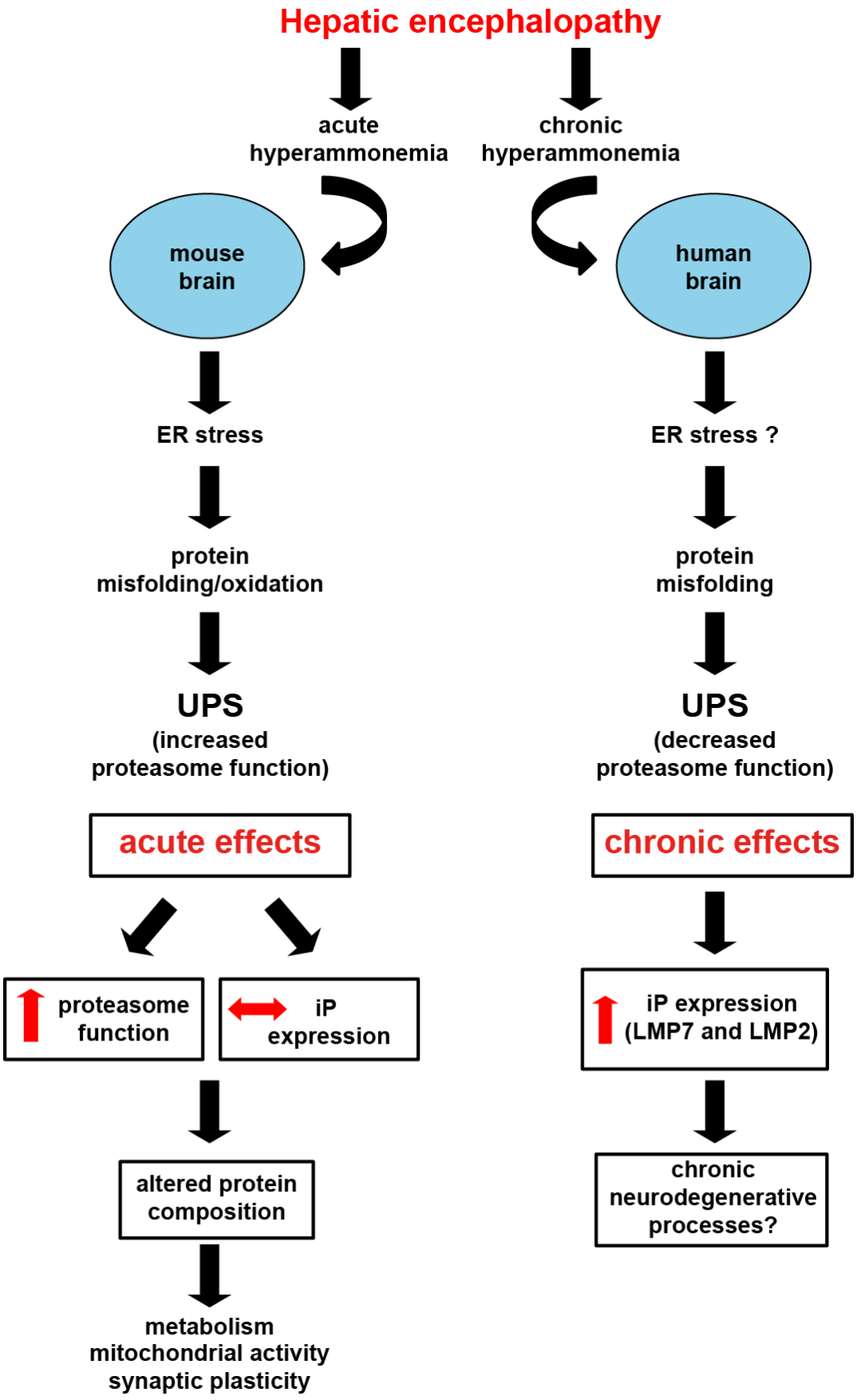

Figure 14. Schematic representation of differential regulation upon acute and chronic hyperammonemia and hypothetic consequences on protein homeostasis and proteasome.

ER (endoplasmic reticulum), UPS (ubiquitin proteasome system), iP (immunoproteasome)

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5. Appendix

5.1 Abstract

Hepatic encephalopathy (HE) is a frequent and serious neuropsychiatric disorder resulting from chronic or acute liver failure. Among a broad spectrum of symptoms, HE patients exhibit alterations of behaviour and personality, impaired cognition and motor dysfunction. Ultimately HE may result in coma and death. Although the exact pathophysiology remains to be resolved, ammonia is thought to be the central neurotoxin for development and pathogenesis of HE. A disturbed ammonia metabolism gives rise to systemic hyperammonemia. Reaching the brain, elevated ammonia levels provoke swelling of astrocytes along with generation of reactive oxygen species (ROS). Subsequently, oxidative stress leads to altered gene expression, RNA oxidation and post translational protein modifications among other potential detrimental effects. To date, oxidative stress is an emerging mechanistic concept for the pathogenesis of HE and may act in concert with neuroinflammation. Thereby it may contribute to glioneuronal complications and a deranged central nervous system (CNS) network. Although the implication of oxidative stress in HE is well accepted, the precise mechanisms involved are not understood. Recently an involvement of microglia cells for HE pathogenesis has been proposed since hyperammonemia affects microglia activation, phagocytosis and the inflammatory response.

In the present study we addressed the effect of hyperammonemia on protein oxidation and involvement of post translational protein modifications such as protein ubiquitination and nitrotyrosinylation. We also investigated the cellular antioxidant response in terms of antioxidant enzyme regulation and the endoplasmic reticulum (ER) stress response by application of three different approaches. We studied human *post mortem* brain samples, analyzed the hippocampi of acutely hyperammonemic mice and investigated the microglial response to ammonia. For the first time, we provide evidence for an accumulation of polyubiquitinated proteins in the brains of chronic HE patients. Notably, in the human *post mortem* brain samples gene expression of immunoproteasome subunits *LMP2* and *LMP7* was induced. As our group published earlier, LMP7 is critical for the turnover of damaged proteins under oxidative stress and inflammatory conditions. Hence, induction of LMP7 and accumulation of polyubiquitinated proteins may argue for a disturbed protein homeostasis and involvement of immunoproteasome function in the pathophysiology of HE. In the mouse hippocampus and in microglia cells we found that acute ammonia intoxication likewise favors accumulation of polyubiquitinated proteins, induces protein oxidation and increases proteasomal activity. Furthermore, we observed an involvement of the ER stress response as demonstrated by induction of the unfolded protein response (UPR) genes *Calreticulin*, *Grp94* and *Chop*. Additionally, we demonstrate an impairment of adult hippocampal neurogenesis in our HE animal model. *In vitro*, treatment with ammonia compromised microglial myelin phagocytosis and provoked protein polyubiquitination and nitrotyrosinylation in microglia cells. Impaired phagocytosis and accumulation of polyubiquitin conjugates were reversible by treatment with the antioxidant *N*-acetylcysteine. Moreover, ammonia promoted microglial expression of antioxidant enzymes HO1 and Trx1, however without involvement of immunoproteasome genes or inflammatory cytokines.

Taken together, this study provides further evidence for the detrimental effect of ammonia toxicity *in vivo* and *in vitro*. Our results suggest differential regulatory mechanisms in chronic and acute hyperammonemia. In both models ammonia promotes a pro-oxidative milieu and generates post translational protein modifications. We found evidence for protein oxidation and an altered proteasomal activity, thus impacting on the physiological cellular protein homeostasis. The oxidative damage in the hippocampus and in microglia cell which results from a disturbed protein turnover may in part contribute to HE pathogenesis.Hence, the results of this study may help to elucidate the underlying molecular mechanisms of neuronal cell dysfunction caused by hyperammonemia and may provide novel aspects to future therapeutic approaches for HE treatment.

5.2. Zusammenfassung

Die hepatische Enzephalopathie (HE) stellt eine häufige und gravierende neuropsychiatrische Störung dar, welche als Folge von chronischem oder akutem Leberversagen entsteht. Klinisch zeigen HE-Patienten ein weites Spektrum von Symptomen; vor allem kognitive Einschränkungen und Bewegungsstörungen bis hin zu Koma und zum Tode. Final kann HE zu Koma und zum Tode führen. Obgleich die genaue Pathophysiologie bisher nicht aufgeklärt ist, wird Ammonium als zentrales Neurotoxin für die Pathogenese der Erkrankung angesehen. So führt ein gestörter Ammonium-Metabolismus zu einer Erhöhung der systemischen und zentralnervösen Ammoniumspiegel im Patienten und damit klinisch zur Ausbildung einer Hyperammonemie. Im Gehirn bewirkt Ammonium eine Schwellung von Astrozyten und führt zur Bildung reaktiver Sauerstoffspezies. So entsteht oxidativer Stress, der im Gehirngewebe eine veränderte Genexpression, RNA-Oxidation sowie post-translationale Proteinmodifikationen zur Folge hat. Gegenwärtig wird oxidativer Stress als ein mechanistisches Konzept für die Pathogenese von HE diskutiert. Oxidativer Stress führt möglicherweise zu neuroinflammatorischen Prozessen und verursacht so glioneuronale Komplikationen im zentralen Nervensystem. Obwohl die Beteiligung von oxidativem Stress als gesichert gilt, sind die Mechanismen bisher nicht im Detail verstanden. Unlängst wurde über die Beteiligung von Mikroglia für die Pathogenese von HE spekuliert, da gezeigt werden konnte, dass Hyperammonemie die Aktivierung, Phagozytose und die inflammatorische Antwort von Mikroglia-Zellen beeinflusst.

Die vorliegende Arbeit beschäftigt sich mit den Effekten von Hyperammonemie auf Protein-Oxidation und post-translationale Proteinmodifikationen wie Protein-Ubiquitinierung und –Tyrosinnitrierung. Weiterhin beleuchteten wir die zelluläre antioxidative Reaktion in Bezug auf die Regulation von antioxidativen Enzymen sowie die Stress-Antwort des endoplasmatischen Retikulums (ER). Experimentell wendeten wir drei verschiedene Paradigmen an. Zum einen analysierten wir humane *post mortem* Gehirnbiopsien, zum anderen untersuchten wir *in vivo* die Hippocampi von akut hyperammonemischen Mäusen und erforschten mittels *in vitro* Versuchen die Antwort von Mikroglia auf Ammonium-Exposition. So erbringen wir zum erstmals den Nachweis für eine Akkumulation von polyubiquitinierten Proteinen in Gehirnen chronischer HE Patienten. Bemerkenswerterweise fanden wir in den humanen *post mortem* Gehirnbiopsien eine Geninduktion der Immunoproteasom-Untereinheiten *LMP2* und *LMP7*. Wie unsere Gruppe zuvor publizierte, stellt LMP7 eine entscheidende Untereinheit für den Umsatz beschädigter Proteinen dar, welche unter oxidativem Stress in inflammatorischem Milieu entstehen. Die Induktion von LMP7 und die Akkumulation polyubiquitinierter Proteine im Gehirn von HE Patienten sprechen daher für eine gestörte Proteinhomöostase in Folge der Pathogenese von HE. Ferner belegen wir, dass eine akute Ammonium Intoxikation im Hippocampus von Mäusen und in kultivierten Mikroglia-Zellen eine Akkumulation von polyubiquitinierten Proteinen, Protein-Oxidation sowie eine erhöhte proteasomale Aktivität begünstigt. Erstmals beschreiben wir in einem HE Modell eine Beteiligung der ER Stress-Antwort. Wir detektierten die hippocampale Induktion von *Calreticulin*, *Grp94* und *Chop*; Gene , die in der Antwort auf ungefaltete Proteine (unfolded protein response, UPR) des ER beteiligt sind. Weiterhin zeigen wir in unserem HE Tiermodell zum ersten Mal, dass eine Beeinträchtigung der adulten hippocampalen Neurogenese durch akute Hyperammonemie hervorgerufen wird. *In vitro* führte die Ammonium Behandlung von Mikroglia-Zellen zur Inhibierung der Phagozytose von Myelindebris. Überdies verursachte Ammonium eine Protein-Polyubiquitinierung und –Tyrosinnitrierung in Mikroglia. Die erwartetermaßen eingeschränkte Phagozytose sowie die Akkumulation polyubiquitinierter Proteine waren reversibel durch zeitgleiche Behandlung mit dem Antioxidans *N*-Acetylcystein. Des Weiteren förderte Ammonium die mikrogliäre Expression der antioxidativ wirksamen Enzyme HO1 und Trx1, jedoch ohne Regulation immunoproteasomaler Gene oder inflammatorischer Zytokine.

Zusammenfassend betrachtet unterstreicht diese Studie nachdrücklich den schädlichen Effekt der Ammonium-Toxizität *in vivo* und *in vitro*. Unsere Ergebnisse legen den Schluss nahe, dass für chronische und akute Hyperammonemie differentielle regulatorische

Mechanismen greifen. In beiden Modellen fördert Ammonium ein pro-oxidatives Milieu und generiert post-translationale Proteinmodifikationen. Wir erbringen den Nachweis für eine Protein-Oxidation und eine veränderte proteasomale Aktivität, welche gemeinsam Einfluss auf die physiologische zelluläre Proteinhomöostase nehmen. Der gestörte Umsatz von Proteinen resultiert in einer oxidativen Schädigung des Hippocampus und der Mikroglia-Zellen und könnte so zur Pathogenese der HE beitragen.

Die Erkenntnisse dieser Arbeit tragen zu einem besseren Verständnis der zugrunde liegenden molekularen Mechanismen der neuronalen Dysfunktion durch Hyperammonemie bei und implizieren neuartige Aspekte für zukünftige Therapieansätze in der Behandlung der HE.

5.3 List of abbreviations

AD	Alzheimer's disease
ALF	acute liver failure
BBB	blood brain barrier
BCA	bicinchoninic acid
BDL	bile duct ligation
bromodeoxyuridine	BrdU
CNS	central nervous system
DNP	2,4-dinitrophenol
DNPH	2,4-dinitrophenylhydrazine
DMEM	dulbecco´s modified eagle medium
DRIPs	defective ribosomal products
DUB	deubiquitinating enzyme
ER	endoplasmic reticulum
FCS	fetal calf serum
Fw	foward
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
gp	guinea pig
GS	glutamine synthetase
HC	hippocampus
HD	Huntington's disease
HE	hepatic encephalopathy
IFN-γ	interferon-γ
iP	immunoproteasome
i.p.	intraperitoneal
K	lysine
LPS	lipopolysaccharide
LTP	long-term potentiation
MHC	major histocompatibility complex
min	minute
MS	multiple sclerosis
ms	mouse
neural precursor cells	NPCs
NAC	*N*-acetylcysteine
NH_4Ac	ammonium acetate
NH_4Cl	ammonium chloride
NMDA	N-methyl-D-aspartate
NOS	nitric oxide synthase
NO	nitric oxide
NOX	NADPH-oxidase
PA	proteasome activator
PBS	phosphate buffered saline
PD	Parkinson's disease
PTN	protein tyrosine nitration
rb	rabbit
RNS	reactive nitrogen species
ROS	reactive oxygen species
rt	rat
RT	room temperature
s	second
SDS	sodium dodecyl sulphate
SEM	standard error mistake
SGZ	subgranular zone
SVZ	subventricular zone

TNFα	tumor necrosis factor α
UPR	unfolded protein response
UPS	ubiquitin proteasome system
WB	western blot
RT	room temperature

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Declaration

Ich versichere an Eides Statt, dass die Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der "Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf" erstellt worden ist.

Die Dissertation wurde noch nicht bereits einer anderen Fakultät vorgelegt.

Ich habe keine vorherigen erfolglosen Promotionsversuche gemacht.

Düsseldorf, den 24. Juni 2015

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Jasmin Mona Klose