Review

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Pathomechanisms in hepatic encephalopathy

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Abstract: Hepatic encephalopathy (HE) is a frequent neuropsychiatric complication in patients with acute or chronic liver failure. Symptoms of HE in particular include disturbances of sensory and motor functions and cognition. HE is triggered by heterogeneous factors such as ammonia being a main toxin, benzodiazepines, proinflammatory cytokines and hyponatremia. HE in patients with liver cirrhosis is triggered by a low-grade cerebral edema and cerebral oxidative/nitrosative stress which bring about a number of functionally relevant alterations including post-translational protein modifications, oxidation of RNA, gene expression changes and senescence. These alterations are suggested to impair astrocyte/neuronal functions and communication. On the system level, a global slowing of oscillatory brain activity and networks can be observed paralleling behavioral perceptual and motor impairments. Moreover, these changes are related to increased cerebral ammonia, alterations in neurometabolite and neurotransmitter concentrations and cortical excitability in HE patients.

Keywords: astrocytes; MR spectroscopy; neurophysiology; oscillatory activity; osmotic and oxidative stress; transcranial magnetic stimulation.

Introduction

Hepatic encephalopathy (HE) is a neuropsychiatric complication which develops in the majority of patients with liver cirrhosis and which significantly worsens the prognosis and increases the mortality of the patients (Jepsen et al. 2010). The symptoms of HE in patients with liver cirrhosis are manifold and comprise impaired motor, sensory and cognitive functions of varying severity (for a review see Häussinger and Blei (2007)). Although these symptoms are in general reversible, cognitive disturbances may not fully reverse upon resolution of HE (Bajaj et al. 2010; Riggio et al. 2011).

Current evidence suggests that HE in patients with liver cirrhosis develops as a consequence of a low-grade cerebral edema (Häussinger et al. 1994; Häussinger et al. 2000) and cerebral oxidative/nitrosative stress (Görg et al. 2010b). A mutual amplification between osmotic and oxidative/nitrosative stress in astrocytes triggers a number of functionally relevant alterations including protein and RNA modifications, gene expression changes and senescence (for a review see Häussinger and Görg (2019)). Most importantly, these alterations, first discovered in cell culture and animal models of HE have also been observed in post-mortem brain samples from patients with liver cirrhosis and HE (Görg et al. 2010b, 2013, 2019).

These alterations may underlie cerebral dysfunction as reflected by behavioural impairments and neurophysiological changes on the systems level. Thus, a global slowing of oscillatory activity can be observed which comprises spontaneous brain activity, stimulus related activity, and oscillatory networks underlying motor symptoms such as (mini-) asterixis (Butz et al. 2013). Behavioral perceptual and motor impairments parallel these findings and are related to increased cerebral ammonia, alterations in neurometabolite and neurotransmitter concentrations, and changes in cortical excitability in HE patients (Butz et al. 2010; Groiss et al. 2019; Kircheis et al. 2002; Lazar et al. 2018; Oeltzschner et al. 2015; Zöllner et al. 2019).

Low-grade cerebral edema in the pathogenesis of HE

The first evidence for the development of a low-grade cerebral edema in HE in patients with liver cirrhosis came from studies by Häussinger and colleagues using in vivo proton magnetic resonance spectroscopy (1H-MRS) (Häussinger et al. 1994).

Their findings suggested that in human brain glutamine and myo-inositol serve as organic osmolytes and...
demonstrated elevated glutamine and decreased myo-inositol levels in the brain of patients with liver cirrhosis and HE (Häussinger et al. 1994). Since in the brain, glutamine synthetase is largely confined to astrocytes (Norenberg 1987), cerebral myo-inositol depletion in the HE patients was interpreted to reflect a volume regulatory response compensating for glutamine synthesis-induced osmotic imbalances in the astrocytes (Häussinger et al. 1994). Clear evidence for glutamine synthesis-dependent induction of osmotic stress in astrocytes in brain was also provided by animal studies from Brusilow and colleagues (for a review see Brusilow et al. (2010)). Here, the ammonia-induced astrocyte swelling and increase in brain water content were completely prevented by the glutamine synthetase inhibitor methionine sulfoxime (Willard-Mack et al. 1996).

These observations strengthened the central role of astrocytes in the pathogenesis of HE and gave rise to a paradigm, according to which HE in patients with liver cirrhosis is triggered by a low-grade cerebral edema (for reviews see Häussinger et al. (2000), Häussinger and Sies (2013), and Cudalbu and Taylor-Robinson (2019)). This edema develops as a consequence of a hyperammonemia-induced exhaustion of the volume regulatory capacity of the astrocyte (Häussinger et al. 2000). Here, the astrocyte may no longer compensate for any osmotic disturbance introduced by HE-relevant factors, which are all known to trigger astrocyte swelling and which then will aggravate the low-grade cerebral edema (Häussinger et al. 2000). Such HE-relevant factors include not only ammonia, but also hyponatremia, benzodiazepines and inflammatory cytokines and explain why HE episodes in cirrhotic patients are precipitated by high dietary protein intake, infections, sedatives, trauma, electrolyte disturbances and gastrointestinal bleeding.

Quantitative water imaging confirmed the 1H-MRS findings on the presence of a low-grade cerebral edema in patients with liver cirrhosis with HE and allowed discrimination of region-specific changes in brain water content (Shah et al. 2008). Here, an elevated brain water content was observed in the globus pallidus, the caudate nucleus and the thalamus of patients with liver cirrhosis and HE (Shah et al. 2008; Winterdahl et al. 2019). However, consequences for the specific functions of the respective brain regions remain to be established.

**Interaction between osmotic and oxidative/nitrosative stress in HE**

With the beginning of this century many studies on cultured rat brain cells and animal models of HE indicated an important role of oxidative/nitrosative stress for the pathogenesis of HE (Brück et al. 2011; Görg et al. 2003, 2008; Jayakumar et al. 2002; Kosenko et al. 1999, 2017; Kručzek et al. 2011; Murthy et al. 2001; Qvartskhava et al. 2015; Reinehr et al. 2007; Schliess et al. 2002, 2004; Suarez et al. 2006).

The significance of these findings for the pathogenesis of HE in patients with liver cirrhosis was established by studies on post-mortem human brain tissue which demonstrated an upregulation of surrogate markers of oxidative/nitrosative stress in patients with liver cirrhosis with but not in those without HE (Görg et al. 2010b, 2013). These included heat shock protein 27, protein tyrosine nitration, RNA oxidation and oxidative/nitrosative stress-related genes (Görg et al. 2010b). Importantly, nitration of the astrocytic protein glutamine synthetase (GS) confirmed the presence of oxidative/nitrosative stress in astrocytes in brain in patients with liver cirrhosis with HE (Görg et al. 2010b).

Mechanistic studies on cultured rat astrocytes revealed the common property of the HE-inducing factors to trigger both, astrocyte swelling as well as formation of reactive nitrogen and oxygen species (RNOS, Figure 1). Since osmotic and oxidative/nitrosative stress are interrelated in astrocytes (Lachmann et al. 2013; Moriyama et al. 2010; Schliess et al. 2004), HE-relevant factors were proposed to trigger a self-perpetuating cycle (Häussinger and Schliess 2008; Schliess et al. 2006). This reciprocal enhancement leads to a number of alterations in the astrocytes which are outlined in detail in the following sections. These alterations compromise astrocytic and neuronal functions and thereby impair the astrocytic/neuronal communication which disturbs oscillatory networks in brain as reflected by the symptoms of HE (Figure 1; for a review see Häussinger and Görg (2019)).

The interaction between osmotic and oxidative/nitrosative stress in astrocytes in HE is also strongly reflected by osmolyte transporter expression changes (Oenarto et al. 2014) which counteract osmotic stress through the rapid uptake or release of osmolytes.

In astrocytes in vitro and in rat and human brain, this is in part accomplished by the sodium-dependent myo-inositol transporter (SMIT) (Fu et al. 2012; Oenarto et al. 2014). In vitro studies on rat astrocytes showed that the swelling induced by HE-triggering factors is paralleled by a downregulation of SMIT and/or the taurine transporter (TAUT) mRNAs thereby counteracting the uptake of the organic osmolytes myo-inositol and taurine (Oenarto et al. 2014). In line with a role of RNOS formation for osmotic stress, astrocyte swelling (Jayakumar et al. 2009; Lachmann et al. 2013) as well as downregulation of SMIT and TAUT mRNA (Oenarto et al. 2014) were ameliorated by
NADPH oxidase inhibitors in ammonia-exposed astrocytes 
in vitro.

Downregulation of SMIT mRNA which may be indicative for astrocyte swelling was also observed in rat cerebral cortex in acute or chronic hyperammonemia after injection of ammonium acetate or partial portal vein ligation, respectively (Oenarto et al. 2014). However, the expression of cerebral osmolyte transporters in patients with liver cirrhosis and HE remains to be investigated.

The interaction between osmotic and oxidative/nitrosative stress in astrocytes is further supported by studies showing that ammonia enhances the water uptake in astrocytes through oxidative/nitrosative stress-dependent upregulation of aquaporin (AQ) 4 in the plasma membrane (Rama Rao et al. 2003). Whether brain edema formation in animal models of HE also relates to AQ4, is currently a matter of debate. While the knockout of AQ4 in mice prevented brain edema formation in an acute liver failure model (Rama Rao et al. 2014), AQ4 membrane polarization was fully preserved in brains in rat models of acute or chronic HE (Wright et al. 2010).

Unfortunately, no data on cerebral AQ4 expression in patients with liver cirrhosis and HE are available.

While astrocytes were clearly established as an important source of RNOS, further in vitro data suggest that also neurons, microglia, fibroblasts and endothelial cells may contribute to RNOS production in HE (Jayakumar et al. 2012; Kruczek et al. 2011; Rao et al. 2013; Zemtsova et al. 2011). However, the in vivo relevance of these findings remains to be established.

Apart from the brain, also reactive oxygen species (ROS) derived from outside the brain may further contribute to cerebral dysfunction in HE. This was suggested by studies showing increased levels of hydrogen peroxide in the arterial plasma of bile duct-ligated rats (BDL) (Bosoi et al. 2012, 2014). However, cellular sources of ROS in peripheral blood were not identified in these studies. Moreover, scavenging ROS or elevating ROS levels in peripheral blood of BDL or portacaval-shunted rats either prevented or triggered brain edema formation, respectively (Bosoi et al. 2012, 2014). These findings suggest that ROS derived from outside the brain and liver dysfunction and/or hyperammonemia may trigger cerebral osmotic stress in a synergistic way. Importantly, levels of the oxidative stress surrogate marker nitrotyrosine were also strongly elevated in peripheral blood of patients with liver cirrhosis with HE (Felipo et al. 2013; Montoliu et al. 2011).

Whether oxidative/nitrosative stress also relates to nuclear swelling in ammonia-exposed astrocytes in vitro (Lachmann et al. 2013), in animal models of HE (Dhanda et al. 2018; Willard-Mack et al. 1996) and in HE patients (Norenberg 1987) is currently unclear. However, nuclear swelling may affect nuclear transport processes and gene transcription (Oberleithner et al. 2000).

Mechanisms of oxidative/nitrosative stress in astrocytes in HE

The formation of oxidative/nitrosative stress in astrocytes in vitro as induced by the HE-relevant factors ammonia, benzodiazepines, proinflammatory cytokines and hypotonicity is tightly coupled to an N-methyl-D-aspartate receptor (NMDAR)-dependent elevation of the intracellular calcium concentration $[Ca^{2+}]_i$ (Görg et al. 2003, 2006; Schliess et al. 2002, 2004). In ammonia-exposed astrocytes $[Ca^{2+}]_i$ is further amplified by a prostanoid synthesis-dependent vesicular glutamate release (Görg et al. 2010a). The elevated $[Ca^{2+}]_i$, in turn activates nitric oxide synthases (NOS) and NADPH oxidase (NOX)-dependent RNOS formation (Görg et al. 2006; Jayakumar et al. 2009; Schliess et al. 2002).
The two NOX isozymes 2 and 4 were identified to contribute to the ammonia-induced ROS formation in astrocytes in vitro. NOX2 becomes activated within minutes in cultured astrocytes exposed to ammonia or hypoosmotic cell culture media through protein kinase Cδ-dependent phosphorylation of p47<sup>phox</sup> (Reinehr et al. 2007). Twenty four hours after ammonia exposure NOX4 protein was upregulated in cultured astrocytes (Görg et al. 2019). Therefore, NOX2 and NOX4-dependent ROS formation may contribute to the rapid (Lachmann et al. 2013) and late swelling in ammonia-exposed astrocytes (Jayakumar et al. 2009), respectively.

Likewise, two nitric oxide synthase (NOS) isozymes were identified to contribute to nitric oxide (NO) formation in ammonia-exposed astrocytes. The neuronal isozyme (nNOS) is constitutively expressed in astrocytes in vitro and was suggested to contribute to the rapid NO formation in astrocytes exposed to ammonia and hypoosmotic cell culture media (Kruczek et al. 2009; Schliess et al. 2002). However, long term exposure of astrocytes to ammonia also upregulates nuclear factor κB-dependent inducible NOS isozyme (iNOS) as a powerful source for NO (Schliess et al. 2002; Sinke et al. 2008). Importantly, both nitric oxide isozymes were shown to contribute to ammonia-induced astrocyte swelling (Lachmann et al. 2013; Sinke et al. 2008) in which again may point to roles of nNOS and iNOS-derived NO for the early and late swelling of the astrocytes.

Also mitochondria were shown to contribute to ROS formation in ammonia-exposed astrocytes in vitro (Görg et al. 2015; Jayakumar et al. 2004). Here, mitochondrial ROS formation was shown to depend on glutamine synthase (Görg et al. 2015) and was suggested to be a consequence of a glutaminase-dependent hydrolysis of glutamine, whereby the exact mechanisms remained unclear (for a review see (Rama Rao and Norenberg 2014)). Interestingly, the ammonia-induced mitochondrial ROS formation is paralleled by a fragmentation and swelling of the mitochondria (Drews et al. 2020; Görg et al. 2015) which both are inhibited by a siRNA-mediated knockdown of GS and kidney-type glutaminase (KGA) in the astrocytes (own unpublished results). These findings point to an interrelation between mitochondrial swelling and ROS formation in ammonia-exposed astrocytes in vitro. For further information on the role of mitochondria for the ammonia toxicity in HE, the reader is referred to Zimmermann and Reichert (2021) in this issue.

**Consequences of oxidative/nitrosative stress in HE**

The oxidative/nitrosative stress induced by HE-relevant factors triggers a number of alterations in astrocytes in vitro which are summarized in Figures 1 and 2 and in detail described in the following sections. These include posttranslational protein modifications such as nitration of protein tyrosine residues (Görg et al. 2006, 2003; Jayakumar et al. 2008; Schliess et al. 2002, 2004), the phosphorylation of signaling proteins (Moriyama et al. 2010; Schliess et al. 2002), protein carbonylation and ubiquitination (Klose et al. 2014; Widmer et al. 2007). Furthermore, RNA becomes oxidized (Görg et al. 2008; Qvartskhava et al. 2015), gene transcription is altered (Kruczek et al. 2009, 2011; Warskulat et al. 2001) and astrocytes become senescent (Bobemín et al. 2020; Bodega et al. 2015; Görg et al. 2015, 2018, 2019; Oenarto et al. 2016).

Most importantly, the majority of these observations was also confirmed in post-mortem brain samples from patients with liver cirrhosis with HE but not in those without HE (for a review see Häussinger and Görg 2019).

**Posttranslational protein and posttranscriptional RNA modifications**

Protein phosphorylation and tyrosine nitration are well known to modulate the protein function (Sabadashka et al. 2020). Proteins which become phosphorylated upon exposure to ammonia in cultured astrocytes in a RNOS-dependent way include the mitogen-activated protein kinases (MAPK) p38<sup>MAPK</sup>, extracellular signal-regulated kinases (ERK) 1,2 (Moriyama et al. 2010; Schliess et al. 2002) and c-Jun N-terminal kinase (JNK) 1 and 2 (Moriyama et al. 2010). The activation of these MAP-kinases triggers the oxidative/nitrosative stress response in astrocytes exposed to hypoosmolarity, ammonia, diazepam, and proinflammatory cytokines triggers the nitration of tyrosine residues in a variety of proteins in vitro (Jayakumar et al. 2008; Görg et al. 2003, 2006; Schliess et al. 2002, 2004). Increased protein tyrosine nitration was also found in different animal models of HE (Brück et al. 2011; Ding et al. 2014; Qvartskhava et al. 2015; Schliess et al. 2002; Suarez et al. 2006) and in diazepam- (Görg et al. 2003) or lipopolysaccharide (LPS)-treated rats (Görg et al. 2006).

Most importantly, enhanced protein tyrosine nitration was also found in post-mortem brains from the cerebral cortex of patients with liver cirrhosis with HE but not in those without HE (Görg et al. 2010b).
Proteins identified to become tyrosine-nitrated in ammonia-exposed astrocytes were GS, ERK1, the peripheral-type benzodiazepine receptor (PBR) (Schliess et al. 2002) and the NKCC1 (Jayakumar et al. 2008).

Importantly, an enhanced tyrosine nitration of GS paralleled by a decrease in the specific activity of GS was not only found in ammonia-exposed astrocytes \textit{in vitro} (Schliess et al. 2002), but also \textit{post-mortem} brain tissue from patients with liver cirrhosis with HE (Görg et al. 2010b). An enhanced tyrosine nitration of GS may also underlie the reduced GS activity in different brain regions of portacaval anastomized rats (Butterworth et al. 1988; Girard et al. 1993).

\textit{In vitro} studies on peroxynitrite-treated purified GS suggested nitration of Tyr336 at the active center of GS to interfere with the binding of adenosine triphosphate and thereby impair GS activity (Frieg et al. 2020). For further information on the molecular mechanism of tyrosine nitration-dependent inactivation of GS the reader is referred to Frieg et al. (2021) in this issue.

Interestingly, nitration as well as inactivation of \textit{in vitro} peroxynitrite-treated purified GS was found to be reversed upon incubation with spleen protein lysates from LPS-treated rats (Görg et al. 2007). A similar “denitrase” activity was also present in brain extracts. This suggests that GS nitration represents a mechanism regulating GS activity under conditions of oxidative stress in brain and does not simply reflect RNS-mediated protein damage (Görg et al. 2007). In this respect, it should be noted that in acidic environments (pH about 5) the enzymatic activity of GS is restored despite nitration (Frieg et al. 2020). However, GS enzyme activity may not be optimal at this pH.

Increased GS tyrosine nitration was also found in brains (Görg et al. 2006) and livers (Görg et al. 2005a, 2005b) from LPS-intoxicated rats suggesting that septic conditions may further impair ammonia detoxification in liver and brain of patients with liver cirrhosis and thereby aggravate HE. Whether GS tyrosine nitration also occurs in skeletal muscle under such conditions has not yet been investigated. In the context of the impaired ammonia disposal by the dysfunctional liver, skeletal muscle was suggested to become an important site of glutamine synthesis-dependent ammonia detoxification (Lockwood et al. 1979). Studies in portacaval shunted rats further suggested, that liver dysfunction may even increase GS activity in skeletal muscle (Girard and Butterworth 1992).

Nitration and carbonylation of the NKCC1 was also observed in ammonia-exposed rat astrocytes \textit{in vitro} and similar to NKCC1 phosphorylation both modifications enhanced NKCC1 transport activity (Jayakumar et al. 2008). Importantly, the activation of the NKCC1 by tyrosine nitration was suggested to contribute to ammonia-induced astrocyte swelling (Jayakumar et al. 2008).

Oxidative/nitrosative stress in astrocytes exposed to HE-relevant factors or in the brain from animal models of HE also triggers the oxidation of guanine in ribosomal and messenger RNA to form 8-oxo-guanosine (Brück et al. 2011; Görg et al. 2008; Qvartskhava et al. 2015). While oxidation of ribosomal RNA was suggested to impair protein translation, messenger RNA oxidation may lead to the synthesis of truncated or misfolded proteins or the degradation of RNA (Nunomura et al. 2017). In view of the latter, degradation of oxidized glutamate/aspartate co-transporter (GLAST) mRNA...
A prominent RNA oxidation was also found in RNA granules in the soma and at synapses in brain of animal models of HE (Görg et al. 2008). Since RNA oxidation may disturb the protein synthesis-dependent remodelling of synapses, neuronal RNA oxidation was suggested to impair cerebral neurotransmission in HE (Görg et al. 2008).

Most importantly, increased levels of oxidized RNA were also found in post-mortem human brain tissue from patients with liver cirrhosis with HE but not in those without HE (Görg et al. 2010b). However, further research is required to clarify consequences of oxidized RNA for cerebral dysfunction in the pathogenesis of HE.

**Altered gene expression in HE**

Osmotic and oxidative/nitrosative stress also alter the levels of a large variety of mRNA species in rat astrocytes in vitro (for a review see Häussinger and Görg (2019)).

Here, the nitric oxide-mediated liberation of zinc ions from zinc thiolate clusters in proteins was identified as a mechanism by which astrocyte swelling triggers the nuclear accumulation of metal responsive transcription factor (MTF) 1/2 and specificity protein (SP) 1 and consequently activates the transcription of metallothioneins (MT) 1/2 and the peripheral type benzodiazepine receptor (PBR) mRNA (Kruczek et al. 2009). Importantly, mRNA levels of several metallothioneins were also upregulated in post-mortem brain samples from patients with liver cirrhosis and HE (Görg et al. 2013).

While upregulation of MT1/2 was considered to counteract toxic effects of the hypoosmolarity- and ammonia-induced elevation of intracellular free zinc ions, upregulation of the PBR may enhance the synthesis of neurosteroids (Kruczek et al. 2009). Interestingly, upregulation of the PBR (Lavoie et al. 1990) and increased neurosteroid levels (Abhoucha et al. 2005) were also observed in post-mortem brain tissue from patients with liver cirrhosis with HE and were suggested to underlie the enhanced γ-aminobutyric acid (GABA)-ergic neurotransmission in HE (Abhoucha et al. 2005).

Neurosteroids are substrates of the multidrug resistance protein 4 (MRP4) which is upregulated by ammonia in cultured astrocytes through NROS-mediated activation of the peroxisome-proliferator activated receptor (PPAR) α (Jördens et al. 2015). Importantly, MRP4 mRNA and protein were also significantly elevated in post-mortem brain tissue from patients with liver cirrhosis with but not in those without HE (Jördens et al. 2015). This raises the possibility that a MRP4-mediated neurosteroid release from astrocytes may contribute to the enhanced GABA-ergic neurotransmission in HE (Jördens et al. 2015).

Neurosteroids were also shown to be ligands of the bile acid receptor TGR5 which is expressed in astrocytes and neurons in the brain (Keitel et al. 2010). Interestingly, activation of the TGR5 by neurosteroids triggers ROS formation in astrocytes in vitro (Keitel et al. 2010). Therefore, downregulation of the TGR5 in ammonia-exposed astrocytes and in post-mortem brain tissue from patients with liver cirrhosis and HE may serve to counteract the neurosteroid and TGR5-mediated ROS formation (Keitel et al. 2010).

In addition to the genes mentioned above, more than 600 further genes were recently identified in a transcriptome analysis on post-mortem brain tissue to be selectively altered in patients with liver cirrhosis and HE compared to controls without liver cirrhosis (Görg et al. 2013). Here, bioinformatic analyses revealed an enrichment of genes implicated in biological processes for which a role in the pathogenesis of HE was established by several in vitro and animal studies before. These include genes related to oxidative stress (e.g. peroxiredoxin 4), proliferation (e.g. lamin A/C) and microglia activation (e.g. cluster of differentiation 14) (Görg et al. 2013). Surprisingly, this study also revealed a number of gene expression changes which may counteract pro-inflammatory pathways in brain of patients with liver cirrhosis and HE such as PPARα (Görg et al. 2013). However, it remains to be determined which cell types are affected by the identified gene expression changes and whether these also manifest at the protein level.

Recent studies also identified a number of microRNAs (miRNAs) which were downregulated by ammonia in an oxidative stress-dependent way in astrocytes in vitro (Oenarto et al. 2016). Interestingly, some of these mRNA species such as miR-326-3p were shown to target heme oxygenase 1 (HO1) and NOX4 which both contribute to oxidative stress and senescence in cultured astrocytes (Görg et al. 2019; Oenarto et al. 2016). Moreover, downregulation of the KGA mRNA-targeting miRNAs miR-23a-3p and miR-23b-3p may explain the upregulation of KGA protein in ammonia-exposed astrocytes. Furthermore, downregulation of miR-326-3p, miR-221-3p and miR-221-5p may underlie the upregulation of the alanine-serine-cysteine transporter 2 (ASCT2) by ammonia in astrocytes in vitro (own unpublished results). These findings suggest that downregulation of specific miRNAs may enhance the mitochondrial hydrolysis of glutamine (KGA) and glutamine transport (ASCT2) in astrocytes and thereby contribute to mitochondrial oxidative stress in HE.
Astrocyte senescence in HE

Recent evidence suggested an important role of astrocyte senescence for cerebral dysfunction in neurodegenerative diseases (Bussian et al. 2018). The underlying mechanisms are not fully understood yet, but may include an impaired growth factor signalling, disturbed synaptic glutamate homeostasis and destabilization of synaptic contacts (Bussian et al. 2018; Kawano et al. 2012).

Contrary to long-held beliefs, symptoms of HE in patients with liver cirrhosis may not fully reverse after resolution of an acute episode of overt HE (Bajaj et al. 2010; Rigio et al. 2011). This was recently suggested to be a consequence of cerebral senescence (Görg et al. 2015) as evidenced by the upregulation of growth arrest and DNA damage-inducible 45a (GADD45a), p21 and p53 in post-mortem human brain samples from patients with liver cirrhosis with HE but not in those without HE (Görg et al. 2015).

In vitro studies on rat astrocytes revealed that ammonia triggers senescence through a glutamine synthesis-dependent O-GlcNAcylation of yet unknown proteins (Görg et al. 2019). These studies offered an additional explanation for the long-known association between glutamine formation and ammonia toxicity in HE.

Similar to phosphorylation, O-GlcNAcylation is a dynamic posttranslational modification which affects the individual functions of the respective modified proteins (Yang and Qian 2017). Unfortunately, except for glyceraldehyde-3-phosphate dehydrogenase, no other case of the many proteins that become O-GlcNAcylated in ammonia-exposed rat astrocytes has been identified yet (Görg et al. 2019; Karababa et al. 2014).

Further in vitro studies revealed that the ammonia-induced protein O-GlcNAcylation inhibited the transcription of the heme oxygenase 1 (HO1) and NOX4 mRNA-repressing microRNA miR-326-3p and upregulated HO1 and NOX4 protein (Görg et al. 2019). While the underlying mechanisms are currently unknown, downregulation of miR-326-3p may be triggered by an O-GlcNAcylation-dependent inactivation of RNA polymerase II at the miR-326-3p transcription site (Görg et al. 2019). Upregulation of HO1 was paralleled by an elevation of intracellular levels of free ferrous iron and siRNA-mediated knockdown of either HO1 or NOX4 abolished the ammonia-induced oxidative stress (Görg et al. 2019). Thus, HO1 and NOX4 may contribute to ammonia-induced oxidative stress through liberation of iron from heme and through H$_2$O$_2$ both of which may lead to hydroxyl radical formation in the so-called Fenton reaction (Görg et al. 2019).

The ammonia-induced oxidative stress subsequently activated the p53-dependent transcription of the cell cycle inhibitory genes p21 and GADD45a and triggers senescence in the astrocytes (Görg et al. 2015, 2019). These findings highlight an important role of ammonia-induced oxidative stress for the induction of astrocyte senescence in vitro (Figure 3).

Both, upregulation of HO1 and oxidative stress were also consistently observed in brains from different animal models of HE (Chastre et al. 2010; Schliess et al. 2002; Wang et al. 2013; Warskulat et al. 2002) and inhibition of HO1 by zinc protoporphyrin prevented cerebral oxidative stress in bile duct-ligated rats (Wang et al. 2013). While these findings also support a role of HO1 for cerebral oxidative stress, it remains to be established whether upregulation of HO1 also triggers astrocyte senescence in these HE animal models.

Most importantly, the pathogenetic relevance of these findings was clearly confirmed by studies showing an enhanced protein O-GlcNAcylation and upregulation of HO1 and genes related to iron metabolism in post-mortem brain samples from patients with liver cirrhosis with but not in those without HE (Görg et al. 2019).

Central and peripheral inflammation in HE

In brain, microglia are a powerful source of RNOS and inflammatory factors and play a central role for cerebral inflammation. Depending on the stimulus, the so-called “resting” microglia may either become activated or adopt a reactive phenotype and produce large amounts of proinflammatory cytokines. While activated microglia may confer neuroprotection (Graeber and Streit 2010), reactive microglia-derived proinflammatory cytokines may trigger cerebral dysfunction and are considered a hallmark of neuroinflammation (Estes and McAllister 2014).

Evidence for microglia activation was provided in cerebrocortical post-mortem brain samples from patients with liver cirrhosis and HE (Dennis et al. 2014; Görg et al. 2013; Zemtsova et al. 2011). This was associated with an upregulation of surrogate markers for the anti-inflammatory so-called type 2 microglia phenotype (Görg et al. 2013). Interestingly, in four out of nine analyzed patients with liver cirrhosis and HE, activated microglia showed an upregulation of the proliferating cell nuclear antigen (PCNA) and Ki-67 which are both characteristic for proliferating cells (Dennis et al. 2014). As this was paralleled by an increased neuronal density, it was proposed that microglia proliferation in these cases may have played a neuroprotective role which however failed to prevent the progression of the disease (Dennis et al. 2014).
Importantly, these studies found no evidence for neuroinflammation in patients with liver cirrhosis and HE when defined by enhanced levels of proinflammatory cytokines (Dennis et al. 2014; Görg et al. 2013; Zemtsova et al. 2011). This was evidenced by unchanged mRNA levels of proinflammatory cytokines (Görg et al. 2013) and of IL-1β and TNF-α mRNA and protein (Zemtsova et al. 2011) and of IL-4, IL-10 and IFN-γ protein (Dennis et al. 2014).

This indicates that microglia were activated but not reactive in the cerebral cortex of cirrhotic patients with HE (Dennis et al. 2014; Görg et al. 2013; Zemtsova et al. 2011) and therefore may serve to protect from cerebral dysfunction in patients with liver cirrhosis and HE. Importantly, these findings do not rule out that microglia become reactive in other brain regions of patients with liver cirrhosis and HE which were not yet investigated.

Evidence for microglia activation was also obtained from studies on ammonia-exposed cultured microglia (Zemtsova et al. 2011) and different HE animal models (Balzano et al. 2020; Hernandez-Rabaza et al. 2016; Jiang et al. 2009; Rodrigo et al. 2010).

As opposed to animal models of HE and acute liver failure (for a review see Butterworth (2016)), neuroinflammation was not consistently observed in different animal models. While IL-1β and TNF-α protein levels were higher in brain of bile duct-ligated rats (Balzano et al. 2020; Rodrigo et al. 2010), IL-1β and TNFα mRNA levels were unchanged in partially portal vein-ligated rats (Brück et al. 2011) and IL-1β and TNFα mRNA and protein levels were unchanged in liver-specific GS knockout mice (Qvartskhava et al. 2015), respectively. The reasons for these inconsistencies are currently unclear and may involve model-, species- or brain region-specific differences.

Further in vitro studies investigated effects of ammonia on the LPS-induced microglia reactivity and found that ammonia decreased the LPS-induced activation and synthesis of proinflammatory but not of anti-inflammatory cytokines in microglia in presence, but not in absence of astrocytes (Karababa et al. 2017). This astrocyte-dependent anti-inflammatory effect was explained by an ammonia-induced synthesis and MRP4-mediated release of neurosteroids from astrocytes (Jördens et al. 2015) which subsequently activate the neurosteroid receptor TGR5 (Keitel et al. 2010) on microglia (Karababa et al. 2017). Interestingly, TGR5 mRNA levels were downregulated in brains from patients with liver cirrhosis and HE (Keitel et al. 2010). However, TGR5 protein levels and cell type specific expression changes remain to be determined in these patients (Keitel et al. 2010). Thus, the exact role of
TGR5 in ameliorating microglia reactivity requires further investigation and other factors may antagonize the synthesis of proinflammatory cytokines by microglia in cerebral cortex from patients with liver cirrhosis and HE.

More recent investigations also point to an important role of peripheral inflammation and elevated levels of circulating cytokines for cerebral dysfunction in animal models of acute or chronic liver failure and HE (Balzano et al. 2020; Chastre et al. 2012). The underlying mechanisms are not yet fully understood but may include a cytokine-induced weakening of the blood brain barrier (for reviews see Butterworth (2016) and Azhari and Swain (2018)).

Behavioral impairments and slowed oscillatory activity in HE

The dysfunctions at the molecular and cellular level outlined above finally lead to changes at the system level (Häussinger and Sies 2013). These changes can be assessed and observed both as behavioural impairments (Brenner et al. 2015; Butz et al. 2010; Heiser et al. 2018; Kircheis et al. 2002; Lazar et al. 2018) and as changes in neurophysiological activity (Butz et al. 2013; Timmermann et al. 2005).

A very intensively studied behavioral impairment in patients suffering from HE is the slowing of the so-called critical flicker frequency (CFF) (Kircheis et al. 2002; Romero-Gomez et al. 2007; Sharma et al. 2007), an impairment of temporal visual perception. Here, a gradual slowing of the CFF with disease severity could be demonstrated. These findings stimulated the usage of the CFF as an increasingly applied yet still debated diagnostic tool (Bedioux et al. 2014; Goldbecker et al. 2013; Kircheis et al. 2014; Lauridsen et al. 2011; Torlot et al. 2013).

A similar impairment in temporal perception could also be shown in the tactile modality (Lazar et al. 2018). HE patients need a significantly longer time delay (on average 120 ms) between two tactile stimuli to perceive the two stimuli as temporally distinct events than healthy controls (on average 70 ms). This impairment in temporal discrimination ability in the tactile modality correlates negatively with the CFF (Lazar et al. 2018), i.e. patients with a lower CFF need longer time delays between two tactile stimuli to perceive the two stimuli as temporally distinct events. Thus, the temporal discrimination ability of HE patients is slowed in the tactile and visual modality in parallel.

In addition, also motor performance assessed by clinical tremor and ataxia scales as well as fastest alternating finger movements revealed motor impairments in parallel to the slowed CFF (Butz et al. 2010). Moreover, olfactory perception was found to be reduced in cirrhotic patients, and among cirrhotic patients, the prevalence of olfactory deficits increased with the severity of HE as assessed by the CFF (Heiser et al. 2018). Finally, tests of thermal processing revealed that patients with severe HE perceive cold at lower temperatures and need a higher temperature difference to distinguish between warm and cold than controls. Again, these impairments correlated with the CFF (Brenner et al. 2015). These close correlations between behavioral impairments and the CFF underpin the usefulness and relevance of the CFF both, in the clinics and in basic research, addressing HE pathophysiology.

Studying neurophysiological activity in HE patients using magnetoencephalography (MEG) has revealed stage-dependent slowing of spontaneous and stimulus-induced oscillatory activity across different frequency bands and across different cortical systems (Butz et al. 2013; Timmermann et al. 2005). Consistent with the early descriptions of a general slowing of the spontaneous EEG in HE patients (Foley et al. 1950; Parsons-Smith et al. 1957), recent studies report a slowing of spontaneous MEG activity and could additionally demonstrate a correlation of this slowing with the CFF (Baumgarten et al. 2018; Götz et al. 2013). This is also in line with more recent EEG studies demonstrating a slowing of EEG activity (e.g. (Olesen et al. 2016)). Both EEG and MEG recordings are suggested to support the study and diagnosis of HE (Guerit et al. 2009; Hari et al. 2018; Schiff et al. 2016).

Not only spontaneous but also stimulus related oscillatory activity is slowed in HE patients and this was demonstrated in the visual system (Kahlbrock et al. 2012) as well as in the somatosensory system (May et al. 2014). Again, the slowing in different cortical subsystems correlated with the slowing of the CFF.

Another intensively studied aspect of neurophysiological alterations in HE is oscillatory coupling within the motor system. Thus, it could be shown that motor symptoms in HE, i.e. mini-asterixis and asterixis (flapping tremor), are associated with altered oscillatory coupling between the involved muscles and the brain (Butz et al. 2014; Timmermann et al. 2002) and also between cortical and subcortical brain structures (Timmermann et al. 2003). Again, a correlation between slowing of this cerebro-muscular coupling and the slowed CFF was reported (Timmermann et al. 2008). Also these findings substantiate the notion that oscillatory activity is slowed across different frequency bands and across different cortical systems in parallel with the behavioral impairments. Hence, global slowing of oscillatory activity can be regarded as a pathophysiological hallmark of HE.
Altered cerebral excitability in HE

A longstanding concept advocates a generally increased GABA-ergic tone in HE patients (Schafer and Jones 1982). However, more recent experimental animal studies suggest a more complex picture of regionally specific changes in GABA levels (Cauli et al. 2009a, 2009b). Challenging the classical hypothesis of a generally increased cortical GABA-ergic tone in HE (Schafer and Jones 1982), Groiss and colleagues used a paired-pulse transcranial magnetic stimulation (TMS) paradigm to investigate short-interval intracortical inhibition (SICI) as a well-established marker of GABA-ergic neurotransmission. Contrary to the traditional GABA hypothesis, a significantly reduced GABA-ergic tone in the primary motor cortex of HE patients was observed (Groiss et al. 2019). Furthermore, there was a significant negative correlation between GABA-ergic inhibition and HE disease severity as quantified by CFF. In contrast to this, Nardonne and co-workers reported increased GABA-ergic inhibition measured with SICI, which would be in line with the GABA hypothesis (Nardone et al. 2016). However, in this study only minimal HE patients were studied, while Groiss et al. studied manifest HE patients. These findings suggest that the motor cortical GABA-ergic tone switches from increased to decreased as clinical HE symptoms evolve.

Another earlier study by Nolano et al. investigated cirrhosis with and without HE and reported an increase of central motor conduction time and motor evoked potential (MEP) thresholds at rest in patients with HE. A shortening of the central silent period, however, was observed in all cirrhotic patients. The authors interpreted their findings as evidence that the damage to the cortico-spinal pathways is related to the development and progression of HE, and that cirrhotic patients present a dysfunction of the inhibitory motor mechanisms even before HE is clinically manifest (Nolano et al. 1997). These results and notion tally with later findings by Cordoba et al. (Cordoba et al. 2003). Moreover, Golaszewski et al. also provided evidence for impaired cortical plasticity already in minimal HE (Golaszewski et al. 2016).

In another study, Hassan et al. employed the TMS paired-pulse cerebellar inhibition (CBI) paradigm in HE patients to investigate the functional integrity of the GABA-ergic cerebello-thalamo-cortical pathway (Hassan et al. 2019). In this paradigm, a conditioning TMS pulse over the cerebellum decreases the size of MEPs evoked by the test TMS pulse over the contralateral primary motor cortex at short interstimulus intervals. CBI is assumed to be mediated by activation of cerebellar Purkinje cells and consecutive inhibition of the dentato-thalamo-cortical pathway (Groiss and Ugawa 2013). On average, less cerebellar inhibition was observed in HE patients when compared to healthy individuals. Again, the degree of CBI within HE patients correlated with disease severity captured with CFF. These results suggest a dysfunctional cerebello-thalamocortical pathway in HE and demonstrate an increasing GABA-ergic tone in Purkinje cells with increasing HE severity. Taken together, these results from TMS works confirm recent animal studies suggesting that alterations of GABA-ergic neurotransmission in the motor system of HE patients vary between different brain regions.

Brain water mapping and CEST in HE

Magnetic resonance (MR) imaging has been used to scrutinise, if the postulated chronic cerebral edema can be observed and quantified in HE patients in vivo. Shah and colleagues reported a significant global increase in cerebral water content in cortical white matter (Shah et al. 2008). Recently, this finding could be replicated with novel techniques and at higher magnetic field strength (Winterdahl et al. 2019), however, other studies observed no difference when comparing healthy individuals with minimal HE (mHE) and HE1 patients (Oeltzschner et al. 2016). More research with highly sensitive MR methods is needed to clarify this issue.

Another MR study investigated the relationships between GABA, glutamate, glutamine and myo-inositol with disease severity and blood ammonia levels in HE patients. Here, decreased levels of GABA in the visual cortex were demonstrated which correlated with blood ammonia levels, CFF, and the brain osmolytes myo-inositol and glutamine. The authors interpreted their findings as evidence for a regional specificity of alterations in GABA-ergic tone in HE (Oeltzschner et al. 2015).

Hyperammonemia plays a pivotal role in the pathogenesis of HE. However, correlation studies on blood ammonia with HE severity have yielded inconsistent results. Probably, this is the case because blood ammonia levels do not reliably reflect brain region specific pathological processes. To address this problem, Zöllner and colleagues have developed a method of cerebral ammonia imaging using the magnetic resonance technique Chemical Exchange Saturation Transfer (CEST), which allows for non-invasive MR imaging of target molecules in the brain (Zöllner et al. 2018; Zöllner et al. 2019). The CEST contrast is based on the indirect observation of the exchange of protons in the target molecule with protons of the surrounding water. In a study on HE patients using CEST with ammonia as target
molecule, patients with manifest HE presented significant CEST contrast changes both in the cerebellum and in the visual cortex. These contrast changes measured in vivo can thus be attributed to increased brain ammonia concentrations and agree well with previous studies reporting an involvement of the visual cortex in HE (Oeltzschner et al. 2015). In addition, HE patients demonstrate an impaired processing of temporal tactile stimuli (Lazar et al. 2018) and thermal perception (Brenner et al. 2015). Cerebellum: A TMS study demonstrated less cerebellar inhibition in HE (Hassan et al. 2019) and a MR spectroscopy study suggested an increase of ammonia levels in the cerebellum in HE patients (Zöllner et al. 2019).

Outlook

Investigations of the past decades have uncovered a variety of disturbances at the molecular, cell biology and behavioral level, which are characteristic for HE and most likely relevant for the pathogenesis of HE. Such hallmarks are the low grade cerebral edema with oxidative/nitrosative stress and disturbances of oscillatory activity in the brain. Driven by molecular pathologies disturbed oscillatory activity and oscillatory networks in different brain regions result in a variety of clinical HE symptoms (Figure 4). These discoveries have identified potential targets for the development of novel specific and effective therapeutic strategies. It is hoped that with advancing pathophysiological understanding of HE, novel therapeutic options may emerge. Until now all forms of HE treatment focus on the elimination of so-called precipitating factors, however therapeutic approaches which directly target the pathophysiological processes in the brain are missing. Potential sites of intervention could be counteracting oxidative/nitrosative stress in the brain and its sequelae or could target pathological oscillations by neuromodulatory methods.

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