Review

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Rapid metabolic and bioenergetic adaptations of astrocytes under hyperammonemia – a novel perspective on hepatic encephalopathy

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Abstract: Hepatic encephalopathy (HE) is a well-studied, neurological syndrome caused by liver dysfunctions. Ammonia, the major toxin during HE pathogenesis, impairs many cellular processes within astrocytes. Yet, the molecular mechanisms causing HE are not fully understood. Here we will recapitulate possible underlying mechanisms with a clear focus on studies revealing a link between altered energy metabolism and HE in cellular models and *in vivo*. The role of the mitochondrial glutamate dehydrogenase and its role in metabolic rewiring of the TCA cycle will be discussed. We propose an updated model of ammonia-induced toxicity that may also be exploited for therapeutic strategies in the future.

Keywords: hepatic encephalopathy; mitochondrial dysfunction; TCA cycle; glutamine metabolism; autophagy; hyperammonemia.

Introduction

Liver cirrhosis, resulting from various types of chronic liver diseases (excluding hepatocellular carcinoma) is responsible for roughly 1.2 million deaths worldwide in 2015. This represents $\approx 2.1\%$ of global deaths with increasing tendency (Asrani et al. 2019). The majority (50–80%) of cirrhotic patients develop symptoms of at least mild forms of hepatic encephalopathy (HE) over time. HE denotes the progression of neurological symptoms caused by alterations in the

central nervous system as a consequence of various types of liver dysfunction e.g. cirrhosis, portosystemic shunt, or blood flow bypassing the liver and thereby mimicking liver failure. The severity of symptoms of HE ranges from mild i.e. confusion or lack of concentration abilities to very severe such as memory or intellectual impairment, coma, and ultimately death. In principle, HE is distinguished into two forms, minimal or covert HE where no or only very mild, often hard to detect symptoms are observable, yet the prerequisites of some type of liver dysfunction to develop HE are met (Zamora Nava and Delgadillo 2011). Opposed to this, in overt HE symptoms are clearly apparent. Here, certain grades according to the West Haven criteria are distinguished, ranging from changes in behaviour and consciousness (grade 1) to disorientation and asterixis (grade 2) to confusion, incoherent speech and aberrant sleep patterns (grade 3) to finally a comatose condition (grade 4) (Vilstrup et al. 2014). The only curative treatment options in most cases is liver transplantation, yet due the complications of HE many patients die before they can receive a transplant. Furthermore, some patients do not fully recover cognitive functions even after liver transplantation, indicating that HE is possibly not fully reversible (Atluri et al. 2010). Hence, to be able to ameliorate symptoms and disease progression, in particular as long as liver transplantations are limiting, it is of utmost interest to understand the pathomechanisms of HE in detail.

Although many mechanisms and precipitation factors linked to HE (e.g. hyponatremia, inflammatory cytokines, benzodiazepine, and ammonia) were proposed and pursued experimentally during more than 50 years of research (Häussinger and Sies 2013), the underlying molecular processes involved during the development of this common liver disease complication are still largely unclear. This is in particular true for early events occurring during the pathogenesis of HE in astrocytes and neurons. This minireview focuses on recent findings highlighting the role of early metabolic and bioenergetic alterations during HE.

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Role of ammonia as a major toxin in HE

Ammonia is one of several compounds which is often but not always markedly increased in concentration in the blood/plasma of HE patients and in multiple models of severe liver damage. It was one of the earliest identified biomarkers for HE and some studies observed a good correlation with disease severity (Kramer et al. 2000; Ong et al. 2003). Naturally, it was suspected early on to be one of the major toxins causing HE-related symptoms. Nonetheless, ammonia blood/plasma levels alone are not sufficient to predict disease progression or fate of the patients as there is considerable overlap in ammonia concentrations of patient with different stages of HE (Nicolao et al. 2003). Whether there is a meaningful correlation between ammonia blood levels and HE disease severity is quite controversially disputed (Jayakumar and Norenberg 2018). The concentrations of ammonia used in a number of *in vitro* HE models is between 1 and 5 mM (Drews et al. 2020; Görg et al. 2015; Lu et al. 2019; Warskulat et al. 2002), and a similar ammonia concentration (5.4 mM) was present in the brain of an *in vivo* rat model (Swain et al. 1992).

Ammonia can pass the blood-brain barrier in its gaseous, non-charged form (NH_3) relatively well, but, to a smaller extent, can also be transported in its cationic form (NH_4^+) as a potassium mimic (Kelly and Rose 2010). It can in principle enter both neurons and astrocytes, yet astrocytes are thought to initially take up the majority of ammonia as they are an integral part of the blood-brain barrier and metabolically well equipped to detoxify ammonia efficiently.

One fundamental chemical and physical feature of ammonia is the fact that it will increase the cellular pH by penetrating membranes with ease and accepting a proton to form the ammonia cation which can impair the function of all cellular compartments with a low pH, i.e. lysosomes or endosomes. Though ammonia was used in many HE-unrelated studies as an inhibitor of autophagy, the controlled degradation and recycling of cellular material, not much attention was given to this aspect in the context of HE. Our recent study showed that high ammonia concentrations (~3–5 mM) are able to inhibit autophagy in a cell culture and animal model as evidenced by microtubule-associated proteins 1A/1B light chain 3B (LC3) and sequestosome 1 (p62) accumulation (Lu et al. 2019). Low concentrations of ammonia (~1 mM), however, led to a stimulation of autophagic flux. In addition, this study showed that LC3 levels were indeed elevated in brain samples of HE patients but not in controls. The

ammonia-induced inhibition of autophagy is pH- and reactive oxygen species (ROS)-dependent and could be partially overcome by taurine administration, a compound well known to improve mitochondrial function (Schaffer et al. 2016). Based on these recent findings and since autophagy is integral to cellular and metabolic homeostasis, we propose that altered autophagic flux is linked to HE and possibly linked to ROS formation, senescence, inflammation, and mitochondrial dysfunctions (Lu et al. 2019). This is in line with another study that has convincingly demonstrated that autophagy of dysfunctional mitochondria, termed mitophagy, is pivotal to liver function and relevant to the pathogenesis of non-alcoholic fatty liver disease (Wang et al. 2015).

Thus, ammonia influences a number of cellular molecular functions in different cells, and it can directly participate in more than 15 biochemical reactions, many of which are oxidative deaminations which are considered unidirectional (Adeva et al. 2012). Of high interest are the anabolic reactions where ammonia takes part such as the synthesis of glutamine (Gln) from glutamate (Glu) via the enzyme glutamine synthetase (GS) and the reductive amination of α -ketoglutarate (α KG) to glutamate via the enzyme glutamate dehydrogenase (GDH) (Figure 1). Ammonia fixation in the liver via the urea cycle is well-known and it has been reported early that α KG levels are reduced, as well as glutamate and NADPH levels, upon addition of ammonia to the perfused liver (Sies et al. 1974). Further, since the synthesis and secretion of glutamine from astrocytes as a part of the glutamate-glutamine or gamma aminobutyric acid (GABA)-glutamine cycle supplying glutamatergic and GABA-ergic neurons is one of the major tasks of astrocytes, it is expected that ammonia has a robust impact on the GDH reaction. Glutamine levels are indeed elevated in cerebrospinal fluid (CSF) and blood in patients (Record et al. 1976; Tofteng et al. 2002) as well as in a number of animal models for HE indicating a strong shift towards glutamine via GS helping to detoxify ammonia in the brain. Indeed, ammonia was found to be primarily fixed into glutamine in rat brains as demonstrated by injection of small amounts of radioactive ^{13}N -labeled ammonia either into the blood or into the CSF (Cooper et al. 1979). While a small amount of ^{13}N was also found in the amino group of glutamate, the majority of ammonia is incorporated into glutamine via the GS reaction. This observation was confirmed for rats that underwent a portocaval shunt procedure inducing chronic hyperammonemia (Cooper et al. 1985). The fact that glutamate still showed a small amount of labelling was attributed to transamination reactions instead of a reversed GDH reaction (reviewed in Cooper 2011). Overall, these findings led to the proposal that *in vivo* ammonia fixation in the brain primarily

occurs via GS questioning the relative importance of ammonia fixation by GDH. More recent data however challenge this view and will be discussed in a later section. A detailed summary on the role of GS in the liver and extra-hepatic tissues is discussed by Frieg et al. (2021) and Zhou et al. (2020).

Glutamine – the trojan horse – is an important toxic mediator in HE

The ‘Trojan horse’ theory aims to explain the toxic effect of glutamine by proposing it to act as a ‘transient transporter’ for ammonia into mitochondria of astrocytes (reviewed in Albrecht and Norenberg 2006). In brief, it was suggested that

glutamine accumulated in the cytosol enters mitochondria where it is transformed into glutamate by glutaminase I (GLS I, also commonly called phosphate-activated glutaminase). Subsequently, glutamate is converted by oxidative deamination of α KG by the GDH fuelling the tricarboxylic acid (TCA) cycle for energy production, releasing free ammonia in both steps. It was postulated, that the intra-mitochondrial ammonia alters intramitochondrial pH, inhibit various mitochondrial enzymes and biochemical reactions including the respiratory chain, and thereby promotes ROS production. These mitochondrial alterations impact astrocyte function which over time might also impair neuronal functions as is the case for other neurodegenerative diseases (Oksanen et al. 2019), possibly contributing at least partially to the observed macroscopic effects e.g. cerebral edema and patient symptoms.

Figure 1: Ammonia and rewiring the TCA cycle.

Schematic representation of the TCA cycle and anaplerotic reactions relevant for HE. Ammonia directly participates in only a few reactions, but through its effect on the reactions involving α KG, Glu and Gln it indirectly influences transamination reactions catalysed by ALT, AST, TTA and BCAT. Amino acids found elevated as a result of HE in patients marked with a red arrow, as a result of ammonia treatments in cell culture models marked with a purple arrow, and as a result of acute or chronic hyperammonemia in animals models marked with a blue arrow. Triple arrows denote undepicted reactions. Three letter code for amino acids is used. aa, amino acid; α ka, α -keto acid; α KG, α -ketoglutarate; α KGM, α -ketoglutaramate; AcCoA, acetyl-Coenzyme A; ACO, aconitase; ALT, alanine transaminase; AS, asparagine synthetase; ASN, asparaginase; AST, aspartate transaminase; BCAT, branched chain amino acid transaminase; Cit, citrate; CS, citrate synthase; FH, fumarate hydratase; Fum, fumarate; GDH, glutamate dehydrogenase; GLS1/2, glutaminase 1/2; GS, glutamine synthetase; iCit, isocitrate; IDH, isocitrate dehydrogenase; KGDH, ketoglutarate dehydrogenase; Mal, malate; MDH, malate dehydrogenase; ME, malic enzyme; OA, oxaloacetate; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; SCS, succinyl-CoA synthetase; SDH, succinate dehydrogenase; Succ, succinate; SuCoA, succinyl-CoA; TTA, tyrosine transaminase.

This model is supported by several experimental observations e.g. the fact that elevated glutamine levels themselves and subsequent mitochondrial ammonia contribute to astrocyte swelling. Not only do glutamine levels correlated with the severity of edema (Swain et al. 1992), but also administration of methionine sulfoximine (MSO), a GS inhibitor, inhibits the occurrence of swelling in astrocytes (Willard-Mack et al. 1996) and reduces cerebral edema in portocaval shunted rats (Master et al. 1999). Glutamine-derived ammonia in mitochondria can then also lead to a number of downstream effects, including the production of ROS (Murthy et al. 2001) and the induction of mitochondrial permeability transition (MPT) (Bai et al. 2001), which could both be inhibited by MSO. Interestingly, MPT could even be induced in isolated mitochondria by high levels of glutamine but not ammonia (Ziemska et al. 2000). Additionally, inhibition of the glutamine transport into mitochondria was able to ameliorate cerebral edema in a hyperammonemic animal model (Rama Rao et al. 2010b). Though glutamine is an important contributing factor for brain edema formation, also other factors play significant roles e.g. lactate formation, oxidative/nitrosative stress, or ATP depletion. For a more thorough summary of the role of these factors with respect to astrocyte swelling and brain edema refer to a recent review (Sepehrinezhad et al. 2020) and reviews in this special issue. In summary, it is well established that astrocyte dysfunction is mechanistically linked to alterations of energy metabolism, impaired mitochondrial function and mitochondrial ROS formation, yet the molecular events leading to this are still under debate.

ROS, senescence and inflammation are additional mechanistic aspects of HE

The relevance of ROS and reactive nitrogen oxide species (RNOS) in the pathogenesis of HE was shown e.g. by demonstrating ammonia-induced protein tyrosine nitration in rat astrocytes (Schliess et al. 2002). Moreover, oxidative and nitrosative stress induce gene expression changes, including certain miRNA genes, that lead to downstream senescence in rat astrocytes (Görg et al. 2015; Oenarto et al. 2016). Senescence is thought to be another important factor contributing to the pathogenesis of HE. A recent study could for example show a simultaneous increase of senescence marker CDK-interacting protein 1 (p21), and activation of inflammatory response and p38 MAPK/NFκB pathways in astrocytes from adult rats,

linking senescence with inflammation (Bobermin et al. 2020). Astrocyte inflammation was proposed by several studies as an important HE promoting factor as e.g. tumor necrosis factor alpha (TNFα) contributes to HE (Odeh et al. 2005) and proinflammatory cytokines such as TNFα, interleukin 1 (IL-1) and interleukin 6 (IL-6) promote astrocyte swelling more when pretreated with ammonia than without (Rama Rao et al. 2010a). Interestingly, inflammatory cytokines were also able to induce protein tyrosine nitration in rat astrocytes (Görg et al. 2006) demonstrating the intricate connection of these different aspects of HE. Many of these precipitation factors are further linked to mitochondrial dysfunction. Hyponatremia is observed in patients suffering from various mitochondriopathies (Brecht et al. 2015; Kubota et al. 2005). Mitochondrial dysfunction is strongly promoting the production of inflammatory cytokines in various cells and is thought to play a critical role in neuroinflammation and autoimmune diseases (van der Burgh and Boes 2015). Also, benzodiazepines are known to bind and modulate the peripheral benzodiazepine receptor TSPO (named also PBR), which is a mitochondrial cholesterol transporter (Gut et al. 2015). The role of mitochondria in combination with these and other precipitation factors for HE needs to be resolved in future studies.

Ammonia modulates cellular energy metabolism

The indications for an altered energy state and a change in energy metabolism in the brain during HE are manifold. Reduced ATP levels were found in cell culture models acutely treated with ammonia, in acute or chronic HE animal models and in patient samples though in chronic models the effect was less severe and variable (Bernal et al. 2002; Haghighat and McCandless 1997). Another good indicator for a shift in brain energy metabolism during HE is the notion that lactate levels increased in HE patient samples after acute liver failure (Tofteng et al. 2002), in portocaval shunt animal models (Chavarria et al. 2015), and in cell culture models suggesting a shift from aerobic to anaerobic energy metabolism. Likewise, increased levels of lactate and alanine, indicating the accumulation of pyruvate, possibly due to increased glycolysis or reduced flux via the TCA cycle, have been reported in rats with acute liver failure (Zwingmann et al. 2003). Still, alterations in glucose metabolism have been inconsistent (Hawkins and Jessy 1991). These discrepancies could in part be explained by the use of different model systems or different

parameters and time-points analysed, yet it could also relate to the fact that alteration of brain metabolic activity in these HE models is controlled in a complex manner and involves variable compensatory mechanisms. Overall, it is clear that the energy metabolism in the brain is affected in HE patients which is demonstrated also by the observation that cerebral metabolic oxygen consumption and cerebral blood flow is strongly reduced (Iversen et al. 2009). What is less clear is the molecular mechanism of the observed metabolic shifts and also whether, and if so, when and how this contributes to the pathogenesis of HE.

Rapid alterations of mitochondrial energy metabolism in astrocytes and the role of glutamate dehydrogenase in hepatic encephalopathy

We observed that treating primary rat astrocytes and astrocytoma cells *in vitro* with ammonium chloride, a well-established *in vitro* HE model, led to an unexpected immediate response on mitochondrial morphology as well as mitochondrial oxygen consumption rates due to a very rapid impairment of mitochondrial oxidative phosphorylation (Drews et al. 2020). Within minutes we observed a concentration-dependent but pH-independent impact of ammonia on the spare respiratory capacity of mitochondria in primary rat astrocytes and a human astrocyte cell culture model. Surprisingly, the basal respiration is only marginally affected and both effects are most severe for short treatments, slowly recovering upon prolonged ammonia exposure, indicating the activation of compensatory mechanisms. Isotope metabolomics indicated an accumulation of certain amino acids e.g. Ile, Ser, and Trp over time and an increase of ammonia-derived heavy nitrogen labelling in glutamate, aspartate, and Pro, pointing towards the involvement of GDH, in particular human GDH2 (hGDH2), and downstream transaminases. Targeted metabolic flux analysis further revealed that ammonia is fixed very efficiently in glutamate within few minutes after administration (Drews et al. 2020). siRNA-mediated KD of hGDH2 as well as overexpression of a negative regulator of GDH, namely SIRT4, rescues rapid ammonia-induced impairment of mitochondrial respiration. Furthermore, the addition of glutamine and glutamate ameliorates ammonia-induced respiration impairment (Drews et al. 2020). These results demonstrate very rapid metabolic alterations and show that hGDH2 can in fact work in the

direction of reductive amination of α KG under hyperammonemic conditions. Another aspect that should not be neglected is that our study demonstrates that mitochondrial dysfunction can be prevented by inhibiting the activity of GDH2 despite the presence of high ammonia concentrations. Thus, at least with respect to mitochondrial respiration the primary problem under hyperammonemia is the depletion of α KG from the TCA cycle and not ammonia *per se*. This impact on cellular energy metabolism is possibly relevant in particular under elevated energy and GABA demand during high neuronal activity.

In the past the importance of GDH during hyperammonemia and in the underlying mechanism of acute or chronic HE was always controversially discussed. This enzyme, under normal conditions, is classically thought to catalyse the oxidative deamination of glutamate into α KG and ammonia acting as an anaplerotic reaction for the TCA cycle. However, under high ammonia concentrations it was speculated that it actually is reversed, namely fixing ammonia by fuelling glutamate (via GDH) and subsequently glutamine synthesis (via GS) while overall depleting α KG from the TCA cycle and thereby decreasing the flux in the TCA cycle (Bessman and Bessman 1955). Yet, due to radioactive ^{13}N -labelling studies of the late 1970s and 1980s the role of GDH was proposed to be minor (Cooper et al. 1979, 1985). The main argument was that after injection of small amounts of radioactive ^{13}N -labelled ammonia either into the blood or into the CSF only low concentrations of ^{13}N -labelled glutamate but high concentration of ^{13}N -labelled glutamine at early time points (~5 min) were observed (Cooper et al. 1979). However, one could alternatively in retrospect explain this as follows. Once GS has converted a fraction of glutamate to glutamine, glutamate levels become low which in turn would promote reductive amination of α KG generating ^{13}N -labelled glutamate via GDH, in particular when ammonia concentrations are high. This is in line with the general view that the net direction of the GDH reaction is strongly determined by the concentration of the different metabolites. Next, ^{13}N -labelled glutamate would further react with ^{13}N -labelled ammonia to generate double- ^{13}N -labelled glutamine which would further increase the apparent amount of labelled glutamine and reduce the amount of ^{13}N -labelled glutamate. This highlights the importance of metabolic flux analyses as opposed to steady-state metabolite analyses as discussed next.

An important contribution to our understanding of the metabolic effects of ammonia came from the Waagepetersen group, who for example investigated the TCA cycle flux under hyperammonemia in cultured mouse astrocytes. Feeding the cells with ^{13}C -glucose leads to an

increase of heavy carbon labelling in glutamate and glutamine and to some extent also aspartate, which suggests an increase/activation of TCA cycle flux towards α KG which is subsequently converted to glutamate (Johansen et al. 2007). Moreover, the labelling pattern pointed towards an increased pyruvate carboxylase (PC) activity (Figure 2) compensating for the reduced replenishment of oxaloacetate (OA). The low levels of OA are expected and can be explained either by a shift of the aspartate transaminase (AST) reaction (Figures 1 and 2) producing aspartate while replenishing α KG from glutamate, or by flux inhibition of a part of the oxidative TCA cycle (from α KG to OA) due to conversion of α KG to glutamate by GDH. Utilizing rat brains and astrocyte neuron cocultures, Dadsetan and collaborators could show that heavy nitrogen from ammonia is incorporated less into glutamine and more into glutamate, alanine, and aspartate when the GS inhibitor MSO is applied in model systems (Dadsetan et al. 2013). This indicated that the GDH reaction can in fact run in the opposite direction to a certain degree when GS is inhibited and that the concerted reactions of GDH and transaminases do impact the fate of ammonia within astrocytes. Apparently, there is an intricate connection between the general amino acid metabolism and elevated ammonia levels and this relationship was indeed investigated by a number of studies. Two studies focussing on the role of isoleucine (Ile) could show that carbon derived from this branched chain amino acid

(BCAA) is channelled into glutamate and aspartate in neuronal cell cultures and into glutamate, glutamine, GABA and aspartate in brain sections of bile-duct ligated (BDL) rats, an HE animal model, though not to a major degree (Bak et al. 2009). Interestingly, Ile-derived labelling of glutamate and aspartate was inhibited by ammonia in neuronal cell cultures while in astrocyte cultures ammonia apparently boosts Ile-derived carbon channelling into glutamate, alanine, aspartate, and glutamine (Bak et al. 2009) consistent with GDH stimulated formation of glutamate from α KG driving the BCAT reaction (Figure 1). A study by the same group using a GABAergic neuronal-astrocyte coculture could show that ammonia is mainly incorporated into glutamine when exogenous glutamate is added. The authors furthermore showed that also glucose-derived heavy carbon is channelled more strongly into glutamate, glutamine, aspartate, and alanine when ammonia is present compared to the situation without ammonia. Specific labelling of glucose revealed a shift in carbon channelling away from the pyruvate dehydrogenase (PDH) towards the PC reaction (Leke et al. 2011). These observations are in strong agreement with earlier observations of this group (Johansen et al. 2007), showing an activation of a part of the TCA cycle, by an enhancement of OA synthesis via PC (see above). An increased citrate synthesis enhances carbon flux towards α KG and glutamate via the GDH which incorporates free ammonium ions. The amino

Figure 2: TCA cycle and anaplerotic fluxes are modulated by ammonia and by GDH in astrocytes.

Under normal conditions anaplerotic reactions (e.g. Ala to Pyr, Asp to OA) dominate, PDH and PC fluxes are approximately the same. There is a net synthesis of Gln mainly via AST and GS. In hyperammonemia the net synthesis of Gln increases drastically, but also Glu synthesis increases mainly due to the action of GDH. The TCA cycle flux is partially depleted for α KG, leading to a reduced capacity of oxidative phosphorylation (OXPHOS). A shift via the PC reaction compensates partially depleted levels of OA. The shift of α KG/Glu ratio influences transaminase reactions (AST and ALT). A flux inhibition from Pyr towards the TCA cycle via PDH might explain increased Lac levels in many HE models. Knockdown of GDH in a human astrocyte cell culture model was able to rescue OXPHOS capacity indicating a reversion to normal TCA flux levels. For abbreviations see the legend of Figure 1.

group can in turn be transferred to aspartate and alanine by the respective transaminases: AST, alanine transaminase (ALT), and other transaminases. It seems that elevated amino acid levels are a direct consequence of altered steady-state concentrations catalysed by transamination reactions. Since α KG and glutamate are involved in all transamination reactions, it is conceivable that the main reason for the transamination effect must be an imbalance of the concentrations of α KG and glutamate, which further strongly suggest a substantial role of GDH.

Further studies focussing on the role of GDH found that RNA silencing of GDH decreases the amount of incorporation of ^{15}N from ammonia in glutamate, alanine, and aspartate, while increasing the ^{15}N incorporation of alanine and aspartate when glutamate was the source for heavy nitrogen (Skytt et al. 2012). This again nicely shows that the GDH reaction can in principle work in both directions and leads to an equilibrium distribution of heavy nitrogen among amino acids by subsequent transamination reactions and supports our findings that GDH is important for ammonia detoxification (Drews et al. 2020).

A study characterizing GDH KD mouse astrocytes, yet without ammonia intoxication, was able to further shed light on the general importance of GDH for astrocytes. Both glutamate and aspartate levels are increased in GDH KD astrocytes, but CO_2 production from isotope-labelled glutamate is unaltered. Glutamate-derived ^{13}C -labelling of glutamate, glutamine, aspartate, malate (Mal) and citrate (Cit) is slightly increased in GDH KD cells when glucose was the major carbon source, but not in its absence suggesting an enhanced channelling of glutamate into the TCA cycle via AST when GDH is silenced. Furthermore, shifts in glutamate-derived citrate and glucose-derived aspartate labelling pattern indicates a reduced carbon flux from glutamate towards OA and citrate under glucose-deprivation as well as an increased carbon flux from glucose towards OA and aspartate via PC in GDH KD cells (Nissen et al. 2015). Taken together these observations suggest that indeed without ammonia stress GDH primarily fuels the TCA cycle and a GDH KD leads to the upregulation of compensatory mechanisms, i.e. PC-driven anaplerosis of the TCA cycle.

Adding an extra level of complexity to the picture, another reaction might play a role in the equilibrium of glutamine, glutamate, α KG, and ammonia in the brain: glutaminase 2 (GLS 2). GLS 2 a two-enzyme complex converting glutamine to α -ketoglutarate (α KGM) in the first step, while transferring the amino group onto a keto acid, and deaminating α KGM to α KG in the second step, releasing free ammonia. This presents an alternative route from glutamine to α KG bypassing GLS 1 and GDH or

transaminases. Though there is no direct interaction of this pathway with GDH, elevated glutamine levels might also induce the transamination reaction via GLS 2 onto keto acids producing the corresponding amino acid. This will as well influence the equilibrium of all transamination reactions involving glutamate and α KG and therefore also indirectly the GDH reaction. Indeed, α KGM turns out to be a good biomarker for HE, as it correlates fairly well with the severity of symptoms in patients suffering from HE (reviewed in Cooper and Kuhara 2014).

The sum of direct and indirect influences of ammonia on the metabolism in astrocytes is no doubt highly complex and involves many reactions, yet the GDH reactions seems to be a central point and is in our opinion of high importance for the understanding of the energetic and metabolic effects and might be underestimated in many studies since evolution bestowed humans with a distinctive form of GDH.

Specific role of human GDH2 as a putative vulnerable target for ammonia-induced toxicity

While the majority of higher eukaryotes encode only one GDH, found in both the cytosol and mitochondria, in their genome, hominoids (i.e. humans, gorilla, common chimpanzee, pygmy chimpanzee, orang-utans, and gibbons) encode two separate forms of GDH, GDH1 and GDH2, representing a quite recent evolutionary change about 25 Mya. GDH2 is exclusively found in mitochondria and is expressed only in a few tissues, including testis and astrocytes. Most of the *in vivo* studies on HE were done in mice or rats, which do not express GDH2. Our findings that human GDH2 is required for ammonia-induced mitochondrial dysfunction in astrocytes (Drews et al. 2020) raise the interesting possibility that the vulnerability of brain functions under hyperammonemia is enhanced in hominoids compared to e.g. rodents. Future studies will have to address if indeed some parts of the picture are missing due to the lack of GDH2 in rodent HE models.

Interestingly, a recent study characterized astrocytes derived from a transgenic mouse model expressing hGDH2 (*hGLUD2* mouse). Upon glutamate addition to the medium astrocytes isolated from these mice showed enhanced glutamate uptake under normoglycemia and enhanced CO_2 production under aglycemia (Nissen et al. 2017). Glutamate and glutamine levels were increased in glutamate-supplemented hGDH2 astrocytes in the presence of glucose while other amino acids are unaffected.

Without glucose intracellular glutamate, glutamine, and BCAA levels decreased, while aspartate levels increased and glutamate-derived heavy carbon labelling was less prominent in citrate when glucose is limited in hGDH2 cells, indicating a lower flux via GDH2 into the TCA cycle. Lastly, glucose oxidation was negatively affected by hGDH2 expression (Nissen et al. 2017). Thus, hGDH2 expression shows a tendency of lower glutamate-derived anaplerosis of the TCA cycle and lower glucose oxidation but an increased anaplerosis from other sources such as BCAAs. The characterization of the *hGLUD2* mouse revealed remarkable parallels to human brain development with respect to metabolomic and transcriptomic parameters (Li et al. 2016). In particular, hGDH2 did not affect glutamate levels in the mouse cortex but altered carbon flux via the TCA cycle in a manner resembling differences between humans and macaques emphasizing the role of hGDH2 on energy metabolism and brain function.

Energy metabolism and TCA cycle under hyperammonemia – what is actually happening?

While the classical view of GDH is not disputed at all, namely the oxidative deamination of glutamate replenishing the TCA cycle, there are clearly conditions where GDH might actually work in reverse mode. One such condition is apparently given when ammonia levels are sufficiently high. High levels of ammonia in astrocytes can be primarily fixated by the GS reaction but as glutamate levels are low or become limiting rapidly, glutamate needs to be regenerated by other reactions. It appears that glutamate is almost instantly filled up by two main pathways: GDH and transaminases (Figures 1 and 2). Transamination reactions rely on the availability of amino acids and hence the AST reaction is by far the most important one, converting aspartate into OA, followed by the ALT reaction, connecting alanine and pyruvate. A recent review on the role of transaminases in the glutamate/glutamine cycle emphasized the importance of the AST for both directions of the α KG-glutamate axis (Hertz and Rothman 2017). We propose that amino acid availability might actually be limiting to a certain degree in chronic hyperammonemia since one of the cellular recycling pathways for amino acids, autophagy, is impaired in astrocytes under hyperammonemia (Lu et al. 2019). Isotope labelling clearly shows that glutamate, aspartate, and alanine are among those amino acids with the highest incorporation of ammonia-derived nitrogen

besides glutamine (Drews et al. 2020; Leke et al. 2011). This can be explained by substantial contribution of the GDH reaction towards glutamate and subsequent transamination reactions. The only possible alternative would be the strong involvement of GLS2, yet α KG is not a good acceptor for the amino group in the first step of the GLS2 reaction, but rather the keto acids of methionine, leucine and the aromatic amino acids. Since glutamate is labelled to a larger extent than aspartate and alanine in ^{15}N tracing experiments an important role for GDH is strongly favoured. We propose that high ammonia levels lead to shifts in several reactions resulting in a TCA cycle-driven net synthesis of glutamine, glutamate, aspartate, and alanine from their respective keto precursors and inhibition of anaplerosis in case of essential amino acids such as BCAAs (Figure 1). This effect can presumably be compensated when cells have a low energy demand but impacts their metabolic flexibility, in particular when higher energy demands prevail. Since many of these reactions potentially extract intermediates from the TCA cycle or pathways feeding into the TCA cycle, the maximal mitochondrial respiration is limited or in other words the cells have no capacity to increase their respiration in times of greater energy demand corresponding to enhanced neuronal activity *in vivo*. Astrocytes are possibly trying to compensate this by upregulating anaplerotic pathways (Figure 2), like shifting from PDH towards the PC reaction (Zwingmann et al. 2003). Both reactions, however, rely on a pyruvate availability, which itself is partially extracted by conversion to alanine. The cells could of course compensate a lack of pyruvate by increasing glycolytic rate. Yet, we rather observed an inhibition of glycolysis in astrocytes and macroscopic observations like the overall decreased brain energy metabolism question such compensation. Elevated lactate levels (together with elevated alanine) on the other hand indicate an enhanced glycolysis but apparently there might be a problem with supplementing the TCA cycle with glucose-derived carbon.

Overall, there are many open questions and dissecting the complex relationship of all reactions involved in cellular energy metabolism, the influence of ammonia on these processes in astrocytes, and their importance for the development of HE is an endeavour that just has begun. We recently showed that cellular energy metabolism of astrocytes is heavily influenced by ammonia and that the GDH reaction plays a pivotal role here (Drews et al. 2020). Transferring these cell culture-based observations into *in vivo* animal studies and also observations of human patient samples is the next important step to estimate the impact of this for the development of HE.

Possible implications for the development of new therapies against HE symptoms

Though therapies directed at the energy and amino acid metabolism of the brain have been discussed for a very long time, they are not used commonly in treating HE patients. One major exception is the so called LOLA therapy – the administration of L-ornithine and L-aspartate (Butterworth and McPhail 2019). This therapy however is not directly related to the energy metabolism of the brain or astrocytes, but it rather aims at directly lowering the ammonia load in the blood by inducing urea synthesis in residual hepatocytes and glutamine synthesis in skeletal muscle cells. Many of the toxic effects in HE are thought to be based on the strong induction of glutamine levels and hence MSO as a GS inhibitor is also considered as a treatment option. We suggest that monitoring and maintaining a healthy energy state in brain cells is essential to halt the progression of HE symptoms. Furthermore, enhancing anaplerosis of the TCA cycle (e.g. by BCAAs) or preventing depletion of TCA cycle intermediates (e.g. by inhibiting GDH) might help to stabilize intracellular energy levels and to capture spikes of high ammonia load. It will be very interesting to see future studies addressing the therapeutic potential of modulating the energy metabolism in patients suffering from HE.

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