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ORIGINAL PAPER

The anaplerotic flux and ammonia detoxification in hepatic encephalopathy

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Abstract Metabolic alterations in the brain underly many of the mechanisms leading to acute and chronic Hepatic Encephalopathy (HE). Controversy exists about the role of glutamine accumulation as a causal factor in HE. Glutamine formation contributes to detoxify ammonia, whereby anaplerotic mechanisms in the astrocytes have to be sufficient to replenish Krebs cycle intermediates. The application of *ex vivo* high-resolution nuclear magnetic resonance (NMR) spectroscopy permits direct measurements of metabolites and different metabolic pathways. *Ex vivo* ^{13}C-NMR studies in experimental animal models of acute and chronic HE have provided new insights. In an experimental rat model of ALF, ^{13}C isotopomer analysis of glucose metabolism showed that alterations of glucose flux through astrocytic pyruvate carboxylase might be linked to the pathogenesis of ALF as a limited anaplerotic flux in the brain, but not in the muscle, correlates with the development of brain edema. Moreover, ^{13}C-NMR data from a rat model of mild HE demonstrated relative differences in the pathway of glucose through pyruvate carboxylase in thalamus compared to frontal cortex, which might explain the vulnerability of this brain region compared to thalamus. These findings further support that glutamine accumulation might be not the primary cause of neurological symptoms in HE, and show that anaplerotic mechanisms could be essential for ammonia detoxification in HE.

Keywords Ammonia · Anaplerosis · Glutamine · Hepatic encephalopathy · NMR spectroscopy · Pyruvate carboxylase

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Introduction

Hepatic Encephalopathy (HE) defines neuropsychiatric abnormalities in patients with liver diseases (Adams and Foley 1953; Butterworth 2003), which range from altered personality to neuromuscular incoordination, stupor and coma. Whereas the chronic form progresses slowly, in acute liver failure (ALF) rapid death occur with the principal cause of mortality being brain herniation caused by a progressive increase of brain water content (Cordoba and Blei 1995). The pathophysiologic mechanisms in HE have not been fully elucidated. Hyperammonemia, due to failure of the liver to detoxify ammonia, is considered a key factor in both forms of liver failure, but its relation to neurological damage and brain edema is poorly understood.

Pathogenetic mechanisms leading to brain edema in HE due to ALF include neuronal dysfunction due to an imbalance between excitatory and inhibitory neurotransmitter systems and disturbed astrocytic-neuronal interactions (Butterworth 2000). Abnormalities in the excitatory amino acids glutamate (Glu) and aspartate (Asp) in brain tissue, cerebrospinal fluid (CSF) and extracellular fluid (ECF) of the brain may be implicated in the pathogenesis of both acute and chronic HE. The changes in brain Glu and Asp are generally associated with ammonia-induced disturbances in their synthesis and degradation (Hertz *et al.* 2000), and with changes in their efflux from and/or reuptake by astrocytes (Michalak *et al.* 1996), which are suggested to be the primary cellular target in HE. The conversion of Glu to glutamine (Gln) by the activity of glutamine synthetase (GS), a predominantly astrocytic enzyme (Norenberg and Martinez-Hernandez 1979), is the brain's primary mechanism of ammonia detoxification. One prevailing hypothesis has been that the osmotic disturbance induced by astrocytic accumulation of Gln due to ammonia detoxification lead to astrocyte swelling and consequently brain edema (Albrecht and Dolinska 2001; Blei *et al.* 1994). However, studies on the role of Gln accumulation as major factor leading to cell swelling have been questioned (Chatauret *et al.* 2003; Larsen *et al.* 2001; Cordoba *et al.* 1999; Albrecht 2003; Zwingmann *et al.* 2003; Zwingmann and Butterworth 2005). In fact, ammonia might exert direct effects on neuronal function, and Gln synthesis could be essential to avoid cerebral ammonia toxicity, rather than being the primary cause of neurological manifestations in HE.

Gln is synthesized in the astrocytes through amidation of Glu. Gln is transferred back to the neuron to be hydrolyzed by phosphate-activated glutaminase (PAG) to Glu, and to close the so-called "glutamine–glutamate-cycle" in the brain. This cycle is tightly coupled to brain energy metabolism and neurotransmission (Fig. 1). However, the precise nature of this cycle in relation to cerebral glucose metabolism remains not clearly resolved. Glucose, entering the brain from the bloodstream, has to cross the blood–brain barrier (BBB) through the endothelial cells. After crossing the BBB, glucose may enter both astrocytes and neurons through the intercellular space. The oxidation of glucose occurs in the mitochondrial TCA cycle after pyruvate-dehydrogenase (PDH)-mediated conversion of pyruvate to acetyl-CoA. On the other hand, the continuous drain of tricarboxylic acid (TCA) cycle intermediates due to biosynthetic processes as well as the entry of acetyl-CoA into the TCA cycle must be complemented by an anaplerotic mechanism (Patel 1974; Fig. 2). The principal anaplerotic enzyme in the brain is pyruvate carboxylase (PC; Patel 1974), which is, like GS, selectively localized in astrocytes. In particular, the continuous

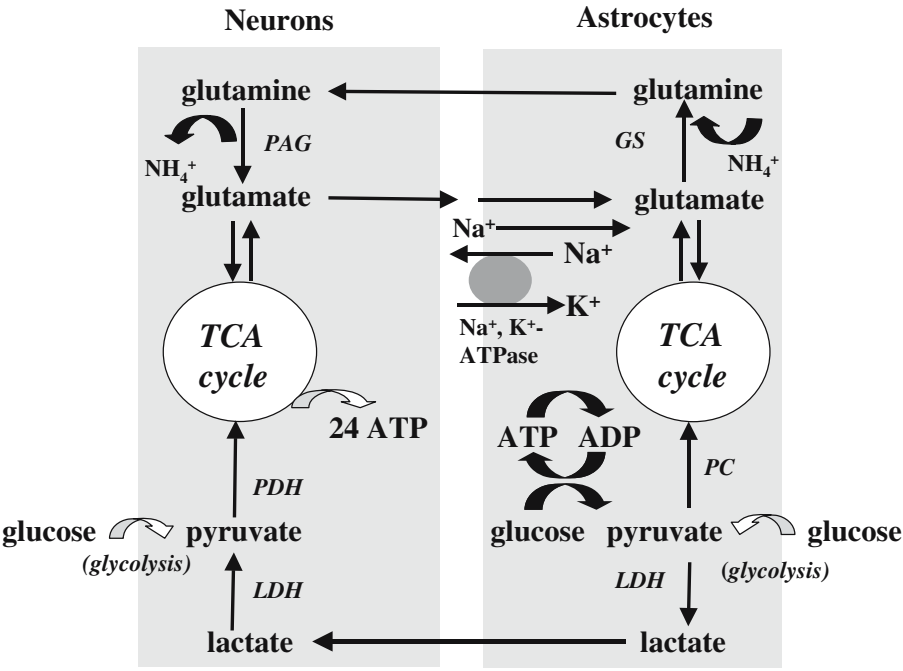

Fig. 1 Schematic presentation of the coupling of the glutamine–glutamate cycle between astrocytes and neurons with brain energy metabolism. Astrocytic glutamate transporters ensure removal of glutamate from the synaptic cleft. The entry of Na^+, cotransproted with glutamate, activates the Na^+, K^+–ATPase, hence decreasing ATP levels. The demand for ATP activates glycolysis in the cytosol. Glucose is then processed to pyruvate, which is reduced to lactate or enters the mitochondrial TCA cycle of both astrocytes and neurons to produce ATP and to synthesize glutamate. GS, as well as the anaplerotic enzyme, PC, are selectively localized in astrocytes. Lactate and glutamine, synthesized in the astrocytes, are transported to the neurons as energy substrates or precursors for neuronal glutamate. *ADP* Adenosine diphosphate; *ATP* adenosine triphosphate; *GS* glutamine synthetase; *LDH* lactate dehydrogenase; *PC* pyruvate carboxylase; *PAG* phosphate-activated glutaminase; *PDH* pyruvate dehydrogenase

drain of the TCA cycle intermediate α-ketoglutarate due to net Glu and subsequently Glu formation must be complemented by provision of oxaloacetate to condense with acetyl-CoA, which requires pyruvate carboxylation. Due to the specific glial localization of PC, astrocytes also play a "nutritional" role by controlling the delivery of metabolic substrate to neurons. Glial pyruvate, therefore, may contribute significantly to the de novo synthesis of Gln and ammonia detoxification, and thereby to the interplay between cerebral glucose metabolism and glutamatergic neurotransmission in HE.

Brain energy metabolism and anaplerosis

Oxidative glucose metabolism in the brain

Glucose has been previously thought to be metabolized mainly in the neuronal TCA cycle (Minchin and Beart 1975). Furthermore, the high rates of oxygen consumption

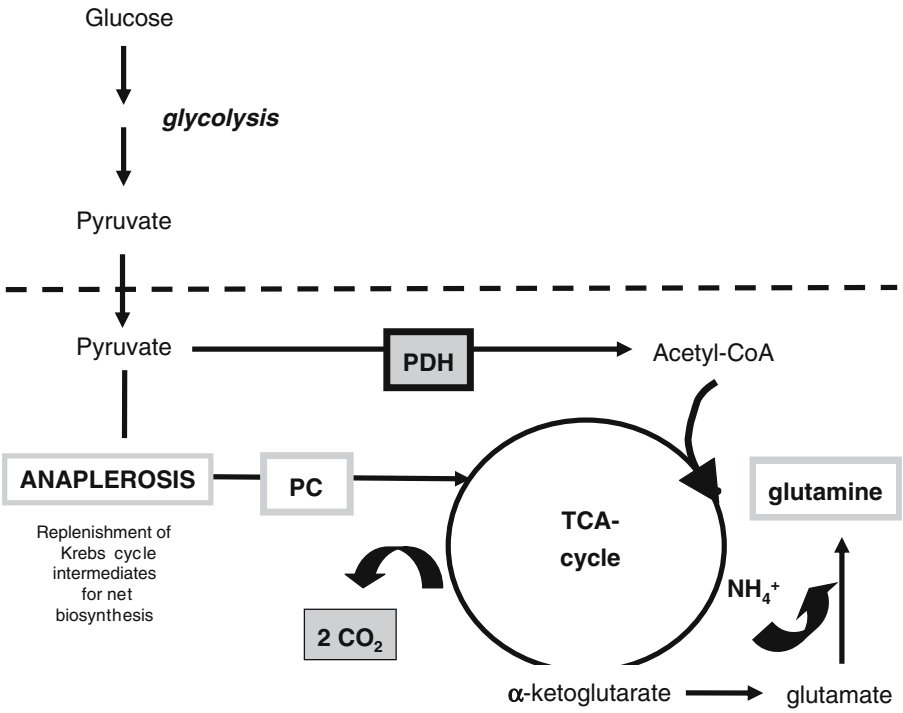

Fig. 2 Schematic presentation of the association of glutamine synthesis and ammonia detoxification with the TCA cycle and anaplerosis (pyruvate carboxylase; *PC*). The anaplerotic pathway in the astrocytes is needed to replenish TCA cycle intermediates as it provides oxaloacetate to condense with acetyl-CoA, provided by pyruvate dehydrogenase (*PDH*)

and glucose utilization by the brain have been devoted to neuronal signaling (Attwell and Laughlin 2001) and therefore to be dependent on the energy needs of the neurons. In addition, cellular estimations concluded that less than 5% of the brain energy usage can be attributed to the function of astrocytes (see Pellerin and Magistretti 2003 and references therein). However, this not necessarily implies that the majority of glucose is taken up by neurons, and evidences were provided that astrocytes could be the primary site of glucose uptake during neuronal activity (for review see Magistretti *et al*. 1993). Furthermore, glucose has to pass through the astrocytes, whose endfeet are located on blood vessels and neuronal processes. Furthermore, glucose may be metabolized in the astrocytes to other substrates, which are supplied to neurons. However, controversy exists about the relative contribution of released astrocytic metabolites and neuronal glucose uptake to neuronal function (Gjedde and Marrett 2001). The oxidation of glucose occurs in the mitochondrial TCA cycle of both neurons and astrocytes after pyruvate-dehydrogenase (PDH)-mediated conversion of pyruvate to acetyl-CoA. PDH, leading to acetyl-CoA entering the TCA cycle, is considered to be the key enzyme for oxidative and energy metabolism in the brain.

Definition of anaplerosis

There are two general definitions of anaplerosis : (1) A filling up; restoring a defective part; especially, promoting granulation of wounds or ulcers, and (2) The process of restoring the depleted cellular metabolic intermediate pools; most commonly used to refer to the restoration of the citric acid cycle intermediates pool. The most important key enzyme for anaplerosis is pyruvate carboxylase, an enzyme, which is highly concentrated in several metabolically active organs, such as the brain, the liver, the muscle and the heart. In the following, the role of anaplerosis for brain glucose metabolism and for the pathogenesis of HE, as well as the analytical NMR methods for its analysis, will be discussed.

Brain glucose metabolism via anaplerosis in the astrocytes

As mentioned above, PDH is the key enzyme for oxidative energy metabolism in the brain. Approximately 50–60% of the energy derived through PDH and subsequent oxidative phosphorylation is spent in maintaining ionic gradients across the membranes necessary for neuronal excitability. Approximately 30% of the energy is used for transmitter turnover, which includes several energy-requiring processes such as neurotransmitter synthesis, release and uptake. Nonoxidated glucose, not used for mitochondrial energy metabolism, is mainly used for biosynthetic processes. However, neurons are continuously drained from neurotransmitter synthesis and release, but can not sustain their TCA cycle intermediates by providing oxaloacetate as substrate for the citrate synthase reaction. In particular, neurons depend on the metabolic support from astrocytes (Shank *et al*. 1985; Kaufman and Driscoll 1993), as the loss of neuronal α-ketoglutarate stores has to be replenished by an anaplerotic mechanism, i.e., by carboxylation of pyruvate to oxaloacetate via PC. In addition, Glu (and GABA (γ-aminobutyric acid)) is not completely recycled by Gln formation in astrocytes, but is also catabolized in both the neuronal and the astrocytic TCA cycle, and used for synthesis of other metabolic compounds. Gln, formed in the astrocytes due to ammonia detoxification, also continually releases the brain with a rate of approximately 10 nmol/g tissue/min (Grill *et al*. 1992), which leads to a loss of TCA cycle intermediates in these cells.

It should be mentioned that pyruvate carboxylation in the brain depends mainly on the activity of PC, but is mediated also via malic enzyme, although to a much lower extent (Patel 1974). Whereas the exact inter- and intracellular localizations of ME and phosphoenolpyruvate carboxykinase (PEPCK) are not fully elucidated, and both were found in either neurons and astrocytes, PC activity has been demonstrated to be glial both in vivo and in vitro (Shank *et al*. 1985; Yu *et al*. 1983; Kaufman and Driscoll 1993), which was confirmed immunocytochemically by Cesar and Hamprecht (1995).

Previous studies measuring the rate of $^{14}CO_2$ fixation relative to glucose oxidation to CO_2 in rat brain (Naruse *et al*. 1966a, b) reveals an approximately 10% contribution of the anaplerotic pathway to the total glucose metabolism. However, in measurements based on CO_2 fixation or production alone (without considering the metabolic intermediates), it should be kept in mind that CO_2 can be fixed also nonanaplerotically through several reactions, such as by formation of

malonyl-CoA from acetyl-CoA during fatty acid synthesis, by purine- and pyrimidine synthesis, through carboxylation of glutamyl-residues of proteins etc. Later metabolic studies using ^{14}C-labeled glucose and pyruvate confirmed this 10% value (Shank and Aprison 1981). By use of ^{13}C-NMR spectroscopy in the intact brain and brain cells in primary cultures, metabolic fluxes through acetyl-CoA and pyruvate carboxylation can be investigated separately, because the labeling pattern of the citrate molecule, and thus of α-ketoglutarate and subsequently Glu, Gln and GABA, differs according to the relative operation of both pathways (Zwingmann and Leibfritz 2003). After systemic administration of ^{13}C-labeled glucose the flux of carbon through PC for net formation of Glu, Gln and GABA in brain *in situ* corresponds to approximately 10% and 20% of the total turnover in the TCA cycle in rats and humans, respectively (Aureli *et al.* 1997; Cruz and Cerdan 1999, Hertz *et al.* 1999, Gruetter *et al.* 2001). The astrocytic-selective metabolism of ^{13}C-labeled acetate in human in vivo (Bluml *et al.* 2002, Lebon *et al.* 2002) revealed that the astrocytic TCA cycle contributes to 15–20% of total brain oxidative metabolism, and that up to 30% of the Gln transferred to the neurons by the cycle may derive from astrocytic anaplerosis, supporting an active role of astrocytes for neurotransmitter activity. This reflects somewhat the four times higher rate of Gln formation compared to pyruvate carboxylation measured by in vivo ^{13}C-MRS in the «resting» human brain (Gruetter *et al.* 1998).

The rate of TCA cycle turnover amounts to 6 nmol/min per milligram of protein in human brain and 14 nmol min^{-1} mg^{-1} in rat brain under normal, resting conditions (Hertz and Dienel 2002 and references therein). Considering the relative contribution of pyruvate carboxylation to the TCA cycle flux, the rate of pyruvate carboxylation amounts to approximately 1–1.5 nmol/min per milligram of protein. Under the assumption of neuronal and glial TCA cycle of approximately 13 and 3 nmol min^{-1} mg^{-1}, reported in mouse brain in vivo (van den Berg and Garfinkel 1971), approximately one third to one half of astrocytic metabolism may account for pyruvate carboxylation. As astrocytes constitute 20–30% of total brain volume, a value of approximately 5 nmol/min per milligram of astrocytic protein is very similar to the rate of pyruvate carboxylation in cultured astrocytes (4 nmol/min per milligram of protein; Yu *et al.* 1983).

Glutamine synthesis and the glutamine-cycle: relation to anaplerotic activity

Glia pyruvate carboxylation and Gln synthesis have been shown to be prerequisites for the replenishment of neuronal Glu (Gruetter 2002). The association of the astrocytic TCA cycle with PC confirms the early notion of pyruvate carboxylation as an important prerequisite for Gln formation (Waelsch *et al.* 1964; Gruetter 2002). Consistent with a strong association of Gln synthesis with PC, pyruvate carboxylation supports glial formation and export of Gln both in vivo and in vitro (Shank *et al.* 1985; Martin *et al.* 1995, 1997; Gamberino *et al.* 1997; Lieth *et al.* 2001; Oz *et al.* 2004).

Pyruvate carboxylase in Hyperammonemia and Hepatic Encephalopathy

The enhanced metabolic activity associated with ammonia detoxification by Gln formation should be reflected also by an increased flux through astrocytic PC.

Conversely, a decreased enzymatic activity has been observed in nonsynaptic mitochondria isolated from brain tissue of three different models of hyperammonemia (Faff-Michalak and Albrecht 1991). However, the activity of an enzyme, measured in tissue homogenates, do not necessarily reflect the actual metabolic flux through the enzyme. NMR spectroscopy using the stable isotope ^{13}C is an unequivocal method, which enables to differentiate between fluxes through PDH and PC through detection of specifically labeled metabolites (Zwingmann and Leibfritz 2003; see below for theoretical explanation).

Using the NMR method, an increased metabolic flux through PC concomitant to stimulated Gln de novo synthesis has been observed in vivo and *ex vivo* in the brain of hyperammonemic animal models (Lapidot and Gopher 1994; Kanamatsu and Tsukada 1999), as well as in cultured astrocytes exposed to pathophysiological concentrations of ammonia (Gamberino *et al*. 1997; Sibson *et al*. 2001; Zwingmann *et al*. 1998). Figure 3 shows the percentage alterations of fluxes through PC contributing to Gln de novo synthesis after 3 or 24 h incubation of primary astrocytes in culture with 5 mM ammonium chloride for 24 h (unpublished results) Although the flux through PC was not further increased after 24 h incubation, which points to a limited capacity of the astrocytes to increase PC flux and/or flux through GS, these findings clearly indicate that the anaplerotic flux is coupled to nitrogen removal from the brain or from the astrocytes (ammonia detoxification) under hyperammonemic conditions.

Furthermore, in hyperammonemic rats existed after inhibition of GS by methionine sulfoximine (MSO) rather a correlation between the grade of encephalopathy with further increasing ammonia, whereas the binding of ^{15}N-labeled ammonia to cerebral Gln seem to be saturated (Kanamori *et al*. 1996). These observations are consistent with the findings that GS works at maximal capacity

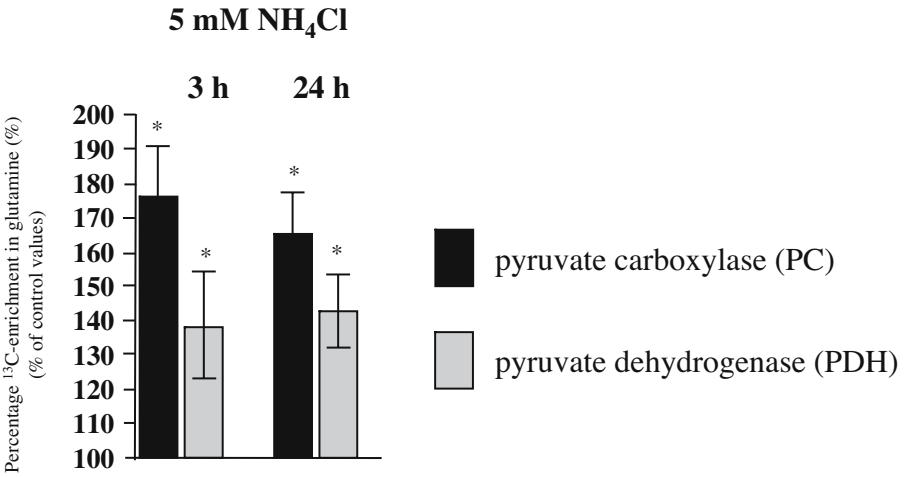

Fig. 3 Relative changes in the percentage ^{13}C enrichment in glutamine synthesized from [U–^{13}C]glucose through flux via PC or PDH after incubation of primary astrocytes in culture with 5 mM ammonium chloride (NH_4Cl) for 3 or 24 hours. The *values* represent percentage alterations compared to control values. (*$p<0.05$; nonpublished results)

(Cooper and Lai 1987), and its enzymatic activity is even decreased in hyperammonemia (Kanamori *et al*. 1996; Lavoie *et al*. 1987).

In view of the metabolic exchange between neurons and astrocytes via the Gln–Glu-cycle, the concomitant use of Gln as energy substrate in the neuronal and astrocytic TCA cycle, and the neuronal need for energy substrates, changes in the flux through astrocytic PC might show a better correlation with hyperammonemic states than brain Gln concentrations per se.

Ex vivo ^{13}C NMR spectroscopy to measure the anaplerotic flux in the brain

Metabolic compartmentation in the brain has been studied traditionally using different radioactive substrates which label either a large pool of Glu (in neurons) with a low turnover or the small glial Glu pool with a high turnover rate (Berl and Clarke 1969; Balanzs *et al*. 1970; Berl *et al*. 1970; Minchin and Beart 1975; Shank and Campbell 1983; Hassel *et al*. 1992). ^{13}C-NMR spectroscopic studies have the advantage of being noninvasive and nondestructive, and do not require metabolite isolation. In particular, the high chemical specificity of ^{13}C-NMR permits the identification of ^{13}C-label in specific positions of given compounds (isotopomers) and allows the calculation of various metabolic fluxes and substrate concentrations simultaneously (Zwingmann and Leibfritz 2003, and references therein).

A detailed description of the isotopomers arising via the main pathways of the most common used glucose isotopomers, i.e., of [1-$^{13}C_1$]–, [2-$^{13}C_1$]– and [U–$^{13}C_6$] glucose is given in Zwingmann and Leibfritz (2003). The metabolites in the brain/brain cells are multiple labeled with ^{13}C in specific carbon positions, depending on the enzymatic pathway. As an example, Fig. 4 shows the isotopomers of Glu and Glu, formed from [1-$^{13}C_1$]glucose via the first passage through PC or via the first turn through PDH. Briefly, [1-$^{13}C_1$]glucose is transformed via the glycolytic pathway to [3-$^{13}C_1$]pyruvate (and subsequently to [3-$^{13}C_1$]alanine via alanine aminotransferase (ALAT; EC 2.6.1.2) and to [3-$^{13}C_1$]lactate via lactate dehydrogenase (LDH; EC 1.1.1.27)). Than [3-$^{13}C_1$]pyruvate enters the TCA cycle via the anaplerotic pathway (PC; EC 6.4.1.1) or the oxidative pathway (PDH; EC 1.2.4.1). In the anaplerotic and oxidative pathway, [2-$^{13}C_2$]– and [4-$^{13}C_1$]Glu will be formed, respectively, during the first TCA cycle turn. Different isotopomers are formed during subsequent TCA cycle turns (not shown). The same isotopomer pattern as in Glu is seen in Gln. For a more detailed isotopomer analysis and the isotopomer pattern in GABA see (Zwingmann and Leibfritz 2003). These ^{13}C-labeled isotopomers are observed in ^{13}C-NMR spectra (Fig. 5).

Anaplerosis in experimental acute Hepatic Encephalopathy

Ex vivo ^{13}C NMR studies in an ischemic rat model of ALF have provided new information on the role of brain Gln synthesis and pyruvate carboxylation vs an ammonia-induced energy failure (Zwingmann *et al*. 2003; Chatauret *et al*. 2003). In these studies, the de novo synthesis of lactate and Gln was measured in the brain by *ex vivo* NMR spectroscopy. These study challenged the convention that astrocytic synthesis and accumulation of Gln is the major cause of brain edema and encephalopathy. It rather emphasized a concerted contribution of energy failure

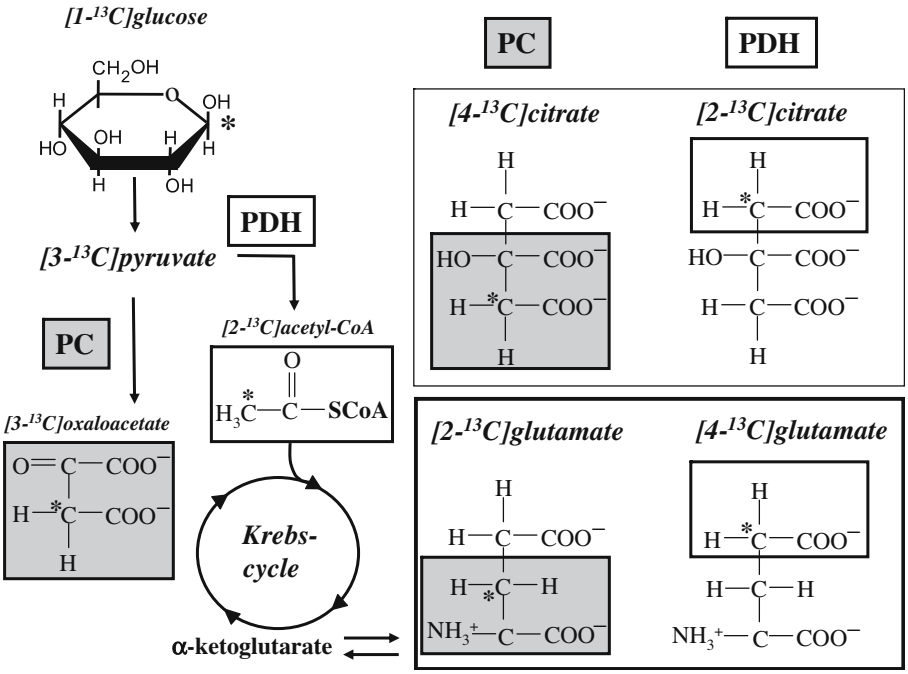

Fig. 4 Metabolic pathways of [1–^{13}C]glucose. Label distribution (*stars*) during [1–^{13}C]glucose metabolism via PDH (pyruvate dehydrogenase) and PC (pyruvate carboxylase), measured by the isotopomer pattern of glutamate. For description of the pathways see the text

resulting from impaired brain glucose metabolism and lactate accumulation due to metabolic impairment. In particular, the de novo synthesis of ^{13}C-labeled Gln from glucose was increased at both precoma and coma stages of encephalopathy compared with sham-operated controls. The enhanced metabolic activity associated with ammonia detoxification by Gln formation should be reflected also by an increased flux through astrocytic PC, which was found to occur already at precoma stages (Fig. 5). These findings clearly further indicate that in vivo anaplerotic flux is coupled to nitrogen removal from the brain. However, the biosynthesis of Gln from glucose via entry of pyruvate into the TCA cycle through were not further increased at coma stages, when brain edema has developed, vs precoma stages of encephalopathy (Zwingmann *et al*. 2003). It is interesting, however, that both Gln synthesis and flux through PC considerably further increased in the muscle (Fig. 5; unpublished results). These findings suggest that the capacity of the brain to synthesize Gln is limited in ALF, and that the skeletal muscle becomes the major organ responsible for ammonia removal. This assumption is in accordance with studies in the same experimental rat model of ALF demonstrating that the GS messenger RNA and its activity in brain were unchanged despite increased circulating ammonia concentrations (Desjardins *et al*; unpublished results). A limited capacity of GS was also concluded by Deutz *et al*. (1988), who demonstrated by in vivo ^{1}H-NMR spectroscopy during acute HE a faster increase of brain ammonia compared to Gln, and by Bosman *et al*. (1992), who observed, after an initial increase of extracellular Gln during mild HE, a subsequent decrease

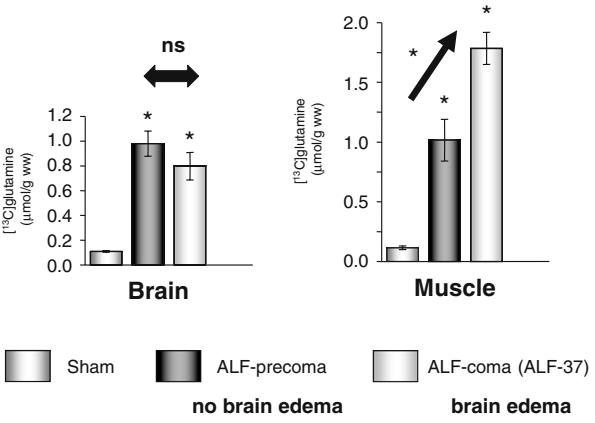

Fig. 5 Fractional ^{13}C-enrichments ($[^{13}C]/[^{12}C]$) in the glutamine isotopomer synthesized from $[1-^{13}C]$glucose via pyruvate carboxylase. The values were calculated from ^{13}C-NMR spectra of brain extracts from sham-operated controls, and from rats with ALF at precoma- and coma stages. Values are given as % of sham-operated controls and represent means±SD for $n=4$ (*$p<0.05$; *ns* not significant; adapted from Zwingmann *et al*. 2003)

during severe HE. Direct demonstration of a lack of correlation between the grade of HE and GS activity was initially reported by Kanamori *et al*. (1996) using in vivo ^{1}H-NMR in a hyperammonemic rat model. In a further study using the ischemic (PCS-HAL) rat model of ALF, it was shown that hypothermia sufficient to abolish brain edema in these animals did not prevent either the increase in brain Gln concentration or its de novo synthesis from glucose in astrocytes, i.e., via the astrocytic enzyme GS (Chatauret *et al*. 2003). Furthermore, similar to the noncorrelation of astrocytic flux through GS with the development of encephalopathy and brain edema, the increased flux through the astrocyte-specific enzyme PC flux was not prevented by hypothermia (Chatauret *et al*. 2003).

Anaplerosis in experimental chronic Hepatic Encephalopathy

It is interesting, that brain edema has been observed rarely in low-grade HA associated with chronic liver failure (CLF), although, like in ALF, brain Gln concentrations increased several-fold. The similar concentrations of brain Gln in ALF and CLF with the lack of brain edema in CLF is therefore one major shortcoming of the Gln hypothesis in ALF.

To date, in vivo ^{1}H-MRS studies were not performed in experimental animal models with CLF, possibly because only a few experimental animal models of CLF exist. Cognitive impairments characteristic of early HE in CLF have mostly been attributed to basal ganglia. However recent reports suggest that also cortical brain dysfunction as well as other brain structures such as thalamus may be implicated. In this regard, it is interesting that positron emission tomography (PET), a technique used to examine basic metabolic brain processes, shows significantly decreased glucose utilization as well as CBF in the cerebral cortex and concomitant increased utilization in the thalamus in patients with liver disease and minimal HE (Lockwood *et al*. 1991). These findings suggested that hypometabolism in the frontal cortex brains of patients with CLF could explain the neuropsychiatric abnormalities characteristic of HE. Furthermore, and in support of this assumption, the cerebral ammonia uptake rate and the cerebral ammonia extraction fraction (K1/CBF) decreased relative in frontal cortex compared to thalamus in cirrhotic patients (Ahl *et al*. 2004).

In order to further evaluate these relationships on a metabolic basis, high resolution ^{1}H- and ^{13}C-NMR spectroscopy was used to measure metabolite concentrations as well as glucose metabolism in frontal cortex compared to thalamus of rats 4 weeks and 3 months following end-to-side PCS, a model of mild HE (unpublished results). An advantage of *ex vivo* NMR spectroscopy in experimental chronic HE is to obtain high-resolution NMR spectra. For example, in most in vivo ^{1}H-NMR studies no clear distinction between cerebral Glu and Gln concentration could be made, and determinations of Gln as well as other metabolites such as choline and *myo*-inositol are only semiquantitative and made relatively to the creatine signal. For weeks following PCS, when all animals showed decreased locomotor activity and altered day–night rhythms compared to sham-operated controls, ^{1}H-NMR analysis revealed 3–5 fold increased Gln concentrations in both frontal cortex and thalamus (not shown). The organic osmolytes *myo*-inositol and taurine were concomitantly decreased. Interestingly, in thalamus, *myo*-inositol further decreased 3 months after PCS, when rats have recovered from neurological symptoms, whereas changes in *myo*-inositol were not significant in frontal cortex at this time point of PCS. These *ex vivo* results confirm the role of brain Gln as an accumulating osmolyte in CLF.

^{13}C-NMR spectroscopy on extracts of frontal cortex and thalamus were additionally performed to obtain more insight into metabolic activities in these brain regions. The flux of glucose through PC is reflected in the C-2 isotopomer of Gln (Fig. 6). ^{13}C-isotopomer analysis showed that the increased thalamic Gln content 4 weeks after PCS was principally due to stimulation of the PC pathway, which was not observed in frontal cortex. These data are consistent with decreased GS activities and disproportionately high ammonia levels in frontal cortex (Butterworth *et al*. 1988). This study suggests that the metabolic activity, i.e., the flux through the anaplerotic enzyme PC contributing to Gln formation, in frontal cortex is decreased relative to thalamus. These data might provide a plausible explanation for previous observations of selective decreases of cerebral cortical glucose utilization and blood flow and underscore the selective vulnerability of cerebral cortical structures in experimental and human chronic liver failure.

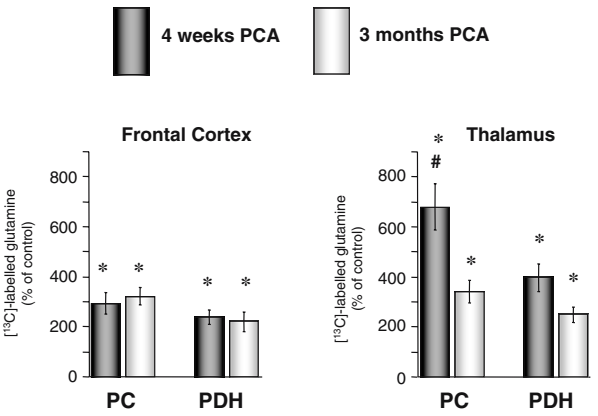

Fig. 6 Fractional ^{13}C-enrichments in the glutamine isotopomer synthesized from [U–^{13}C]glucose via pyruvate carboxylase (*PC*) and pyruvate dehydrogenase (*PDH*). The values were calculated from ^{13}C-NMR spectra of brain extracts from sham-operated controls, and from rats 4 weeks and 3 months after PCA. (*$p<0.05$; nonpublished results)

Concluding remarks

Recently, *ex vivo* ^{13}C NMR studies in experimental animal models of acute and chronic HE have provided new insights in the pathogenesis of HE and ammonia detoxification. For example, in an experimental rat model of ALF, ^{13}C isotopomer analysis of glucose metabolism has shown that alterations of glucose flux through the anaplerotic astrocytic enzyme PC might be linked to the pathogenesis of advanced hepatic coma in ALF due to limited capacity for Gln synthesis and ammonia detoxification. In particular, a limited metabolic/anaplerotic flux through PC in the brain, but not in the muscle, correlates with the development of brain edema in ALF. Moreover, *ex vivo* ^{13}C-NMR data obtained on a rat model of mild HE demonstrated relative differences in glucose metabolic activity and the specific pathway of glucose through PC in thalamus compared to frontal cortex. This means, a limited metabolic/anaplerotic flux through PC in the frontal cortex parallels the vulnerability of this brain region compared to thalamus. These studies also show that ex vivo NMR spectroscopy using stable isotopes (^{13}C) is a unique and favorable method to study cell-specific metabolic pathways under physiological and pathological conditions. Future studies of metabolic rates using NMR spectroscopy and the stable isotope ^{13}C may be potentially useful in the study of the dynamics of ammonia detoxification processes coupled to mitochondrial glucose metabolism to elucidate the relative role of Gln accumulation and of Gln-independent components contributing to brain edema.

These findings further support that Gln synthesis in astrocytes might be not the primary cause of brain edema or neurological symptoms in both acute and chronic HE. In fact, PC-mediated provision of carbons is essential for ammonia detoxification via Gln synthesis in the brain. Considering the already known limited brain capacity for Gln synthesis, increasing the anaplerotic flux and/or administration of nonnitrogenous anaplerotic compounds might be of therapeutic value to decrease brain ammonia concentrations in HE. Further studies using *ex vivo* NMR spectroscopy may provide novel insights into these relations between ammonia detoxification and yet unknown mechanisms contributing to the pathophysiology of acute and chronic HE.

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