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ORIGINAL PAPER

In vivo studies of brain metabolism in animal models of Hepatic Encephalopathy using ^{1}H Magnetic Resonance Spectroscopy

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Abstract Hepatic encephalopathy (HE) is a common and severe neuropsychiatric complication present in acute and chronic liver disease. The unique advantages of high field ^{1}H MRS provide a method for assessing pathogenic mechanism, diagnosis and monitoring of HE, as well as for treatment assessment or recovery after liver transplantation, in a reproducible and reliable non-invasive way. The purpose of the present review is to present some new features of in vivo proton Magnetic Resonance Spectroscopy (^{1}H MRS) at high magnetic fields combined with some basic requirements for reliable metabolic profiling. Finally, in vivo applications of ^{1}H MRS in different HE animal models are presented.

Keywords In vivo short echo time ^{1}H MRS · Quantification of neurochemical profile · Hepatic encephalopathy · Chronic liver disease · Acute liver failure · Hyperammonemia

Introduction

Hepatic encephalopathy (HE) is a common and severe neuropsychiatric complication present in acute and chronic liver disease (i.e. 30 to 50 % of patients with cirrhosis exhibit HE (Munoz 2008)), which is characterized by an array of cognitive and fine motor deficits progressively leading to altered mental status, coma and death (Norenberg et al. 2009; Caudle et al. 2010; Butterworth 2003). Minimal HE (mHE) occurs in approximately 70 % of the patients with chronic liver disease (CLD) and is defined by the presence of cognitive impairment on psychometric testing in absence of any overt clinical signs (McPhail and Taylor-Robinson 2010). The diagnosis of HE is traditionally done using neuropsychological tests together with the assessment of liver function, but it lacks consensus and has never been standardized especially in mHE (Rovira et al. 2008; McPhail and Taylor-Robinson 2010). Consequently, there is an urgent need for new unified tools for reliable diagnostic in HE.

Although, the molecular basis for the neurological disorder in HE remains elusive, increasing evidence points to ammonia as a key factor (Braissant et al. 2012; Cagnon and Braissant 2007; Felipo and Butterworth 2002; Norenberg et al. 2009; Butterworth 2003; Cooper and Plum 1987). Ammonia is mainly produced in the gut by intestinal urease-positive bacteria and amino acid metabolism, and under normal circumstances undergoes a high degree of extraction by the urea cycle in the liver. In addition to the gut sources, it was reported the ammonia can be liberated by kidney or muscle during and after vigorous exercise (Cooper and Plum 1987). In the diseased liver ammonia passes into the blood circulation and thereby enters the brain through the blood–brain barrier, where its main toxic effects occur. Cerebral ammonia removal relies on formation of glutamine by glutamine synthetase (GS) in astrocytes. It is hypothesized that the unique astrocytic expression of GS (Norenberg 1979) underlies the relative sensitivity of astrocytes to ammonia, leading to astrocytes swelling and finally to brain edema.

The precise mechanisms and their relative contributions leading to astrocytes swelling and consequently to brain edema are very complex and not fully elucidated, but recent studies showed that the main pathogenic mechanisms

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involved in HE are the following: amino acids disturbances; alterations in neurotransmission; cerebral energy disturbance; alteration of nitric oxide synthesis and oxidative stress which leads to induction of the mitochondrial permeability transition; impairment of axonal and dendritic growth during brain development; signaling transduction pathways; alterations in channels and transporters activity (Braissant et al. 2012; Cagnon and Braissant 2007; Butterworth 2003; Felipo and Butterworth 2002; Norenberg et al. 2009; Leke et al. 2011; Cooper and Plum 1987; Brusilow and Traystman 1986; Brusilow et al. 2010). In this context, several hypotheses have been proposed, suggesting that a byproduct of ammonia metabolism rather than ammonia per se could be the main cause of ammonia toxicity to the brain. Approximately 25 years ago the osmotic gliopathy theory (glutamine hypothesis) was elaborated, postulating that glutamine can act as an osmolyte and that its increase leads to a shift of water into astrocytes (Brusilow and Traystman 1986). Recently, another hypothesis was proposed, the "Trojan horse", where glutamine acts as a carrier of ammonia across the mitochondrial membrane. Once in the mitochondria, glutamine is hydrolyzed by phosphate-activated glutaminase to glutamate and ammonia, which further gives rise to excessive production of free radicals inducing opening of the mitochondrial permeability transition (Albrecht and Norenberg 2006). More details about the hypotheses, related evidences and controversies can be found in (Brusilow et al. 2010; Desjardins et al. 2012; Rama Rao et al. 2012).

Although there is extensive research examining the biochemistry of ammonia-induced glutamine accumulation and energy failure in cultured astrocytes and neurons, in vivo data are often missing. The importance of in vivo data cannot be understated when it comes to astrocyte and neuronal metabolism, as both cell types are exquisitely sensitive to certain drugs, and certainly to ischemia. Understanding the pathogenic mechanisms involved in HE, finding the reliable markers which would assess in a reproducible way the different degrees of HE or unraveling new therapeutic targets to protect the brain form ammonia toxicity, requires experimental models focusing on the brain in its cellular complexity, with neurons and glial cells interacting together (in vivo rodent models, ex-vivo CNS organotypic cultures, in vitro primary 3D brain cell cultures in aggregates).

In the last decades, Magnetic Resonance Imaging (MRI) and Magnetic Resonance Spectroscopy (MRS) became powerful and reliable diagnostic tools with unique advantages: they are applicable in vivo, non-invasively and consequently longitudinally, thus allowing to monitor disease progression and/or effect of treatment, and made a bridge between the clinical diagnostics and basic research. Therefore, MRI combined with MRS might provide a method for assessing pathogenic mechanisms, diagnosis and monitoring of HE, as well as for treatment assessment or recovery after liver transplantation in a reproducible and reliable non-invasive way.

The present review will focus on presenting some new features of in vivo proton Magnetic Resonance Spectroscopy (^{1}H MRS) at high magnetic fields combined with some basic requirements for reliable metabolic profiling. Finally, in vivo applications of ^{1}H MRS in different HE animal models will be presented.

In vivo Proton Magnetic Resonance Spectroscopy at high fields

Proton Magnetic Resonance Spectroscopy (^{1}H MRS) has proven to be a powerful tool to non-invasively investigate in vivo brain metabolism of animals and humans in different neurological disorders (Williams 1999). Some studies reported the in vivo investigation and monitoring of HE using ^{1}H MRS in humans and animal models (Cauli et al. 2011; Cauli et al. 2007; Cudalbu et al. 2012b; Cudalbu et al. 2010; Fitzpatrick et al. 1989; Nyberg et al. 1998; Shen et al. 1998; Braissant et al. 2012; Haussinger et al. 2000; Rovira et al. 2008; Spahr et al. 2002; Williams 1999; Bates et al. 1989; McPhail and Taylor-Robinson 2010; Butterworth 2003; Kreis et al. 1991; Kreis et al. 1992; Ross et al. 1994). The main finding of these studies was the increase in brain glutamine concentration.

In humans, previously published studies were performed at magnetic fields of 1.5 T and only few at 3 T, using echo times (TE) ≥20 ms and leading to the quantification of only few metabolites (i.e. sum of glutamine and glutamate, total creatine, total choline and myo-inositol) (Rovira et al. 2008; Haussinger et al. 2000; Spahr et al. 2002; Cordoba et al. 2002; Cordoba et al. 2001; Williams 1999; Butterworth 2003; Kreis et al. 1991; Kreis et al. 1992; Ross et al. 1994). As shown by these studies, several pathogenic mechanisms involved in HE can be explored in vivo using ^{1}H MRS, e.g. amino acid disturbances (e.g. modifications in the concentrations of glutamine, glutamate), osmoregulation (e.g. decrease of myo-inositol, total choline concentrations), cerebral energy disturbance (e.g. increase in lactate concentration). However, at magnetic fields of 1.5 T the separation of glutamine and glutamate is not possible, while at 3 T is difficult, therefore only the sum of these two metabolites (named as Glx) was reported. Additionally, at longer echo times (TE above 20 ms), the number of spectral lines, which can be used for quantification, is reduced. This is due to phase distortion of multiplets produced by coupled spin systems and to fast transversal relaxation (T_2 relaxation) leading to signal reduction, which prevents observation of small peaks in the spectrum. Moreover, correction factors for the T_2 relaxation times when performing absolute quantification are required.

In this context, the detection of additional brain metabolites is required to accurately assess the pathogenic mechanisms and for monitoring/diagnostic in HE. The high magnetic field strengths (≥7 T) combined with the possibility of acquiring spectra at very short echo time (<10 ms) have increased the number of in vivo detectable brain metabolites to about 20 metabolites in animal models and humans. This so-called neurochemical profile at high magnetic fields comprises: glucose (Glc), lactate (Lac), creatine (Cr), phosphocreatine (PCr), alanine (Ala) (markers of energy metabolism); phosphocholine (PCho), glycerophosphocholine (GPC), phosphoethanolamine (PE), N-acetylaspartate (NAA), N-acetylaspartylglutamate (NAAG) (markers of myelination/cell proliferation); glutamate (Glu), glutamine (Gln), aspartate (Asp), γ-aminobutyrate (GABA), glycine (Gly) (markers of neurotransmitter metabolism); taurine (Tau), myo-inositol (Ins) (markers of osmoregulation) and ascorbate (Asc), glutathione (GSH) (antioxidants) (Fig. 1b and c).

Overall, increased intrinsic signal-to-noise ratio (increased sensitivity), chemical shift dispersion (increased spectral resolution) and decreased strong coupling effects are the main benefits of high magnetic fields, which are expected to be useful for low concentration metabolites, strongly overlapped metabolites and for metabolites having complex multiplet patterns, leading to an improved quantification precision and accuracy (Mekle et al. 2009; Mlynarik et al. 2008a; Tkac et al. 2009). Moreover, acquisitions performed at a short echo time enable to obtain high quality spectra with resolved lines of many metabolites due to minimal phase distortions of multiplets of coupled spin systems such as Glu, Gln, Ins, Glc, Asp, Ala, GABA, Asc, PE, Gly and Tau (Mekle et al. 2009; Mlynarik et al. 2008a; Tkac et al. 2009), thus increasing the amount of biochemical information. Signal loss due to T_2 relaxation is reduced and consequently a more precise quantification is expected. Acquisitions at short TE and long repetition times (TR) (fully relaxed spectra) are considered to be a good choice for absolute quantification, since the effects of relaxation are minimized. Macromolecule contributions at short echo times need also to be considered. Several techniques have been used to overcome this issue and were presented more in details elsewhere (Cudalbu et al. 2012c).

These advantages of high magnetic field spectroscopy are also accompanied by some factors that can decrease the spectral quality and consequently the quantification precision. A main factor is related to the increased peak linewidths (in Hz) since magnetic field inhomogeneities increase with the magnetic field due to the fact that magnetic susceptibility differences between tissues, blood, bone and air (in Hz) are proportional to the static magnetic field (B_0). Resonance frequency of nuclear spins is proportional to a magnitude of the B_0. When B_0 is spatially inhomogeneous, resonance frequency of ^{1}H nuclei is not the same over the measured volume of a tissue, resulting in broadening spectral lines and in decreasing spectral resolution and signal-to-noise ratio. An adjustment of the B_0 inhomogeneity for ^{1}H MRS is technically rather challenging in rodent brain due to the small size of the brain, and powerful first- and second-order B_0 corrections (shims) are necessary (Tkac and Gruetter 2005). A second factor is the chemical shift displacement error which is related to frequency selective localization. The use of stronger magnetic fields gradients can limit this artefact; however, attention has to be paid to eddy current effects on the B_0 stability. T_1 relaxation times have been shown to increase with

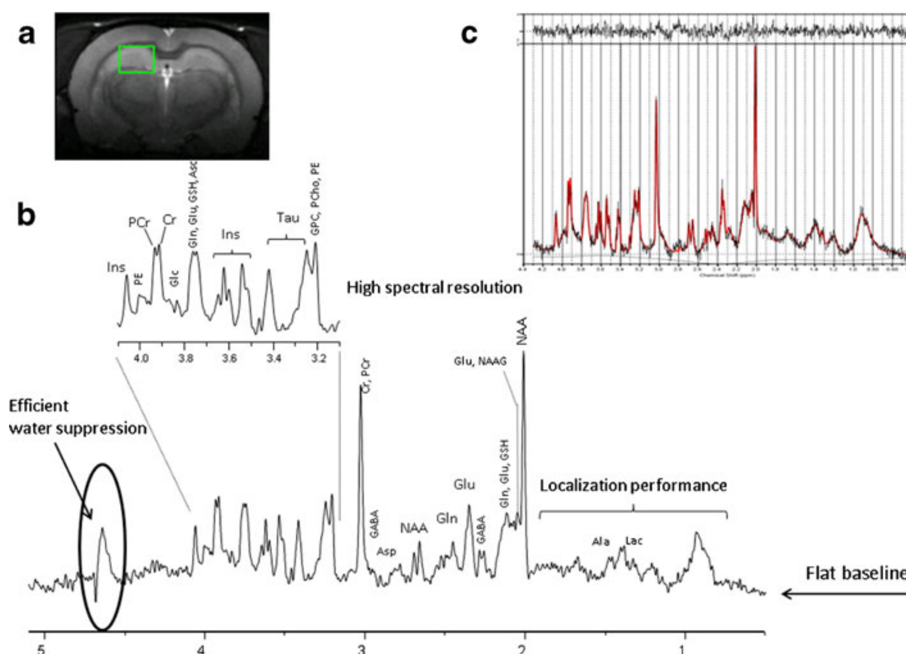

Fig. 1 **a** In vivo T_2 weighted image acquired in the rat brain at 9.4 T showing the localization of the volume of interest in the hippocampus; **b** In vivo ^{1}H MRS spectrum acquired using the SPECIAL sequence in the hippocampus of the rat brain at 9.4 T (TE=2.8 ms, TR=4 s, 160 averages, 10 min of acquisition). Note the high spectral resolution obtained with efficient B_0 shimming, the efficient water suppression, the localization performances and the flat baseline, **c** LCModel quantification result, from bottom to top: the raw in vivo spectrum (in *black*), the LCModel fit (in *red*) and the quantification residual

magnetic field (de Graaf et al. 2006) leading to increased signal saturation for shorter repetition times (TR). This can be overcome by increasing TR, which in turn would increase the total acquisition time or decrease signal-to-noise in the same total acquisition time. However, no further increase in T_1 relaxation times has been observed beyond 9.4 T (Cudalbu et al. 2009).

The main goal of localized in vivo ^{1}H MRS is the reliable quantification of the concentration of as many metabolites as possible in a well-defined region of the brain. Metabolite concentration ratios are often used as markers of changes in brain metabolite concentrations. However, for an accurate interpretation of these changes in different neurological diseases, absolute metabolite concentrations can be more valuable. Accurate and precise quantification of brain metabolites, independently of the magnetic field, is challenging and depends on: 1) spectral quality (signal–to-noise ratio, spectral resolution, water suppression, localization performances, baseline, chemical shift displacement error and eddy current correction), 2) reliable data processing (estimate of the signal amplitude or peak area); 3) quantification strategies (conversion into concentration units). Overall, the main features of a good quality spectrum acquired in the hippocampus of the rat brain are shown in Fig. 1b. The signal-to–noise ratio depends on: the efficiency of B_0 shimming, the performances of radiofrequency coils and localization sequences, the B_0 field strength, the precise adjustment of rephasing gradients in localization sequences and subject motion. Poor water suppression can lead to the presence of residual water signal in the acquired spectrum and consequently affects the spectral baseline (a flat baseline is critical for reliable quantification). Localization performance of the acquisition sequence represents the ability to provide a maximum signal from a selected volume with minimum contamination from outside. The subcutaneous lipids represent the main source of contamination in the region 0.5–2 ppm and their presence, typically as phase distorted signals, could lead to an overestimation of the metabolites present in this spectral region (e.g. lactate, alanine, GABA). The use of outer volume suppression bands (OVS) can limit this contamination (Mlynarik et al. 2008a; Tkac and Gruetter 2005). Any misadjustment of the parameters in the pulse sequence can affect the spectral quality leading to unreliable metabolite concentrations.

The choice of the data processing method is also important, since many algorithms depend on user input which leads to additional inaccuracies. Metabolite concentrations are usually determined by fitting the measured in vivo ^{1}H MRS spectrum to a linear combination of spectra of individual metabolites (the metabolite basis set), making feasible the quantification especially for low concentration metabolites (Cudalbu et al. 2012c; Mlynarik et al. 2008a; Tkac et al. 2009) (Fig. 1c). The metabolite basis set can be obtained by measuring aqueous solutions of pure metabolites (under the identical conditions as the in vivo spectra) or by quantum mechanics simulations, based on the density-matrix formalism, using published values of spin-spin coupling constants and chemical shifts, and the relevant acquisition parameters (Cudalbu et al. 2008; Cudalbu et al. 2012c). Well-known time and frequency–domain algorithms, based on metabolite basis sets, are currently used for accurate quantification (i.e. QUEST from jMRUI software working in the time domain, LCModel working in the frequency domain, AQSES working in time domain and TDFD working in both time and frequency domains) (Fig. 1c).

While ^{1}H MRS (also called single voxel spectroscopy) allows signal detection from a well-defined single volume, usually positioned in a specific brain region, proton spectroscopic imaging (^{1}H MRSI), also called chemical shift imaging (CSI), allows the simultaneous detection of multiple spectra from different brain regions and thus the spatial distribution of metabolites in various regions of the brain can be efficiently studied. Spectroscopic imaging can be performed in rodents with μL spatial resolution, which is comparable in resolution to animal positron emission tomography (PET) (Mlynarik et al. 2008b).

^{1}H MRS in models of hyperammonemia

Since ammonia is believed to be the main culprit in HE, several animal models of hyperammonemia (HA) were used to study the effect of ammonia per se without liver failure (e.g. single i.p. injection of ammonia or continuous infusion for several hours). Very few studies have used in vivo ^{1}H MRS and ^{1}H MRSI to investigate brain metabolism in animal models of HA without liver failure (Cudalbu et al. 2012b; Cauli et al. 2007; Fitzpatrick et al. 1989; Cudalbu et al. 2010). There are two ways in which the brain can metabolize ammonia: the reductive amination of α-ketoglutarate to glutamate via the glutamate dehydrogenase reaction (GDH) and the synthesis of glutamine from glutamate and ammonia by glutamine synthetase reaction (GS), the last being the major metabolic pathway for ammonia detoxification (Brusilow et al. 2010; Cooper 2011, 2012).

Our group recently studied the brain metabolism in animal models of HA without liver failure using in vivo ^{1}H MRS and ^{1}H MRSI (Cudalbu et al. 2012b; Cudalbu et al. 2010). Using ^{1}H MRS at high magnetic field (9.4 T) we showed that the Gln concentration increased immediately after the starting of ammonium infusion and continued to increase linearly over time (2.3±0.4 μmol/g before the infusion which increased to 17.7±4.0 μmol/g at the end of the infusion) without significant changes in the concentration of other brain metabolites (Fig. 2a). The linear and continuous increase of total Gln during the period of ammonium chloride infusion observed in our in vivo ^{1}H MRS

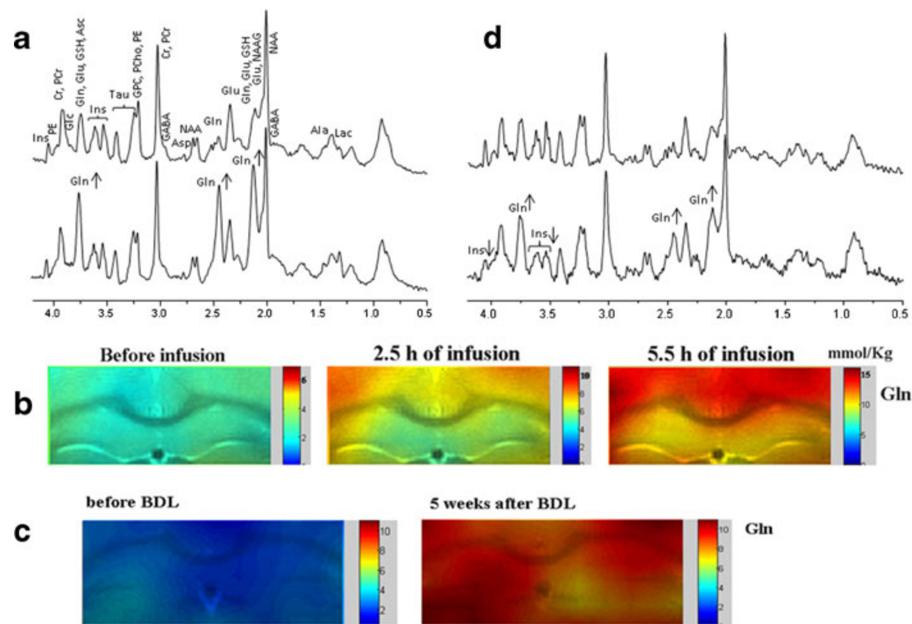

Fig. 2 **a** In vivo brain ^{1}H MRS spectra acquired in a rat model of HA at 9.4 T. From top to bottom spectrum acquired before ammonium infusion and spectrum acquired after 9 h of infusion; **b** In vivo metabolic maps of glutamine acquired in a rat brain during ammonium infusion, superimposed on the anatomical T_2 weighted images and acquired at different time points before and during infusion; **c** In vivo metabolic maps of glutamine acquired in a rat brain before and 5 weeks after BDL, and superimposed on the anatomical T_2 weighted images; **d** In vivo brain ^{1}H MRS spectra acquired in a rat model of CLD at 9.4 T. From top to bottom spectrum acquired before BDL and spectrum acquired 8 weeks after BDL

data implies increased anaplerosis (Berl et al. 1962; Shen et al. 1998; Zwingmann 2007), which appears to be coupled to the ammonia detoxification pathway. From the linear fit of the time courses of total Gln we obtained a net Gln accumulation of 0.033±0.001 μmol/g/min, suggesting no delay in Gln accumulation and consequently in anaplerosis. We further continued our studies on HA by mapping brain metabolism in different brain regions in the same rat model using ^{1}H MRSI (Cudalbu et al. 2010). Figure 2b illustrates the metabolic maps of Gln, superimposed on the anatomical T_{2w} images and acquired at different time points during ammonium chloride infusion. As for the data obtained using ^{1}H MRS, the increase in the Gln pool at different time points during infusion was apparent from the maps with no substantial differences for the other brain metabolites. Even though before infusion, the Gln map did not show any substantial differences among brain regions, during infusion the Gln increase showed a specific brain region pattern, i.e. higher in cortex than in hippocampus (at 5.5 h of NH_4^+infusion: 16.2±2.7 μmol/g in the cortex and 11.5±1.2 μmol/g in the hippocampus, p=0.03) (Fig. 2b). Consequently, these results showed a higher net glutamine synthesis flux in cortex than in hippocampus. In a similar way, Cauli et al (2007) performed single voxel ^{1}H MRS in cerebellum and substrantia nigra after i.p. injection of ammonium acetate. Surprisingly, they did not report the Gln concentrations, however they showed a decrease of NAA in cerebellum and an increase of Ins in both investigated brain regions, which were prevented by blocking the NMDA receptors. The decrease of NAA was reported to reflect neuronal damage, whereas Ins increase was suggestive of the presence of vasogenic edema. Moreover, the authors in (Fitzpatrick et al. 1989) showed a decrease in Glu concentrations and an increase in Lac concentrations additionally to Gln increase during ammonium acetate infusion using single voxel ^{1}H MRS. The authors suggested that the increase of Lac was more likely a result of a mismatch between the rate of glycolysis and the activity of tricarboxylic acid cycle (TCA), rather than an attempt by the brain to buffer incoming ammonia.

^{1}H MRS in models of acute liver failure

In vivo ^{1}H MRS has also been used to study brain metabolism in few experimental models of acute liver failure (ALF) (e.g. galactosamine or carbon tetrachloride administration, portocaval anastomosis followed by hepatic artery ligation, etc.) (Nyberg et al. 1998; Chavarria et al. 2010; Cauli et al. 2011; Bates et al. 1989; Bosman et al. 1990). As for HA models, these studies reported an increase of brain Gln concentration in ALF. In addition, these studies reported alterations in brain lactate concentration at later stages of the disease and the presence of brain edema. The authors reported that these changes in brain metabolism were probably caused by the exposure of the brain to ammonia (Chavarria et al. 2010). The mechanisms leading to an increase of Lac are not clear,

however this increase might be an indicator of brain energy impairment secondary to ammonia-induced brain edema (Chavarria et al. 2010). In the same context, it was shown ex-vivo (brain extracts) that lactate and alanine synthesis was increased in ALF using high resolution $^{1}H/^{13}C$ nuclear magnetic resonance spectroscopy (Chatauret et al. 2003). This study suggested that a disturbance in brain glucose metabolism rather than Gln accumulation might be the major cause of cerebral complications in ALF.

^{1}H MRS in models of chronic liver disease

While few studies using biochemical methods have been performed ex-vivo (brain extracts) or on cultured cells (astrocytes or neurons), no in vivo longitudinal ^{1}H MRS studies were performed, to our knowledge, in experimental animal models of chronic liver disease (CLD) to assess the kinetics of glutamine, brain osmolyte changes and/or brain edema in combination with brain energy metabolism using ^{1}H MRS. These previous ex-vivo studies reported increased cerebral glutamine and ammonia levels with sometimes reductions in brain osmolytes reflecting disturbances in cell-volume homeostasis. It has been suggested that the differences in brain osmolytes could partially explain the differences in frequency of brain edema between acute and chronic liver failure (Cordoba et al. 1998; 1996; Cordoba 1996). In a recent study, our group characterized for the first time the in vivo and longitudinal progression of HE in a rat model of CLD using ^{1}H MRS, ^{1}H MRSI (Fig. 2c and d) and Diffusion Tensor Imaging (DTI) (Cudalbu et al. 2012a). We imaged the in vivo spatial distribution of 12 metabolites (i.e. Gln, Glu, Cr, PCr, tCho, Ins, Tau, Lac, NAA + NAAG, PE, Glc, GABA) in various brain structures (cortex, hippocampus and thalamus) before and weekly (for 8 weeks) after CLD induced by bile duct ligation (BDL). Our preliminary data showed that Gln was increased at all time points after BDL reaching ~300 % at 8 weeks. Among the other brain osmolytes only Ins and tCho decreased significantly over time (~10 % in the first week, reached ~30 % and even~80 % for tCho 8 weeks after BDL), whereas Tau showed a smaller (~10–20 %) but significant decrease. The sum of the main brain osmolytes (Gln, Ins, tCho, Tau, Cr) was constant over time, presumably to compensate for the Gln increase. It is interesting to note the cellular localization of these brain osmolytes: Ins was reported to be highly concentrated in glial cells (Brand et al. 1993) and Tau to be present in both neurons and astrocytes (Urenjak et al. 1993). The osmotic imbalance created by the continuous increase of Gln is likely to be compensated by a concomitant decrease of other idiogenic osmolytes resulting in minimal brain edema (as suggested by the DTI measurements), prior to the appearance of severe neurological signs in CLD.

Finally, it has to be emphasized that the differences in the neurochemical profile between HA and CLD are clearly visible when comparing Fig. 2a and d. Even though Gln was increased in both cases, the decrease in the brain osmolytes was present only in CLD. Additionally, in CLD glutamine increased similarly in all brain regions, contrary to HA where we measured a higher increase of Gln in the cortex (Fig. 2b and c).

Conclusion and future perspectives

As can be seen from the present review, ^{1}H MRS has proven to be a very useful tool for assessing several pathological mechanisms involved in HE and also for performing an overall diagnostic in HE using the increase of brain glutamine as a bio-marker and the concomitant decrease of other brain osmolytes (e. Ins, tCho) in CLD. The non-invasive applicability of this technique combined with the unique advantages of high magnetic fields allows, as shown here, a longitudinal assessment of brain glutamine concentrations during disease progression (in humans and animal models), as well as of energy metabolism (Glc, Ala, Lac, Cr, PCr), osmoregulation, neurotransmitter metabolism (Asp, GABA, Gly, Glu) and oxidative stress (Asc, GSH). The extraordinary developments in ^{1}H MRS combined with other in vivo MR imaging/spectroscopy techniques (DTI, ^{13}C MRS, ^{31}P MRS, etc.) promises new perspectives for a better understanding of pathogenic mechanisms involved in HE but also for a more reliable diagnostic in HE by establishing precise correlations between different profiles of metabolites and the degrees of HE.

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