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Creatine in the central nervous system: From magnetic resonance spectroscopy to creatine deficiencies

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ABSTRACT

Creatine (Cr) is an important organic compound acting as intracellular high-energy phosphate shuttle and in energy storage. While located in most cells where it plays its main roles in energy metabolism and cytoprotection, Cr is highly concentrated in muscle and brain tissues, in which Cr also appears to act in osmoregulation and neurotransmission. This review discusses the basis of Cr metabolism, synthesis and transport within brain cells. The importance of Cr in brain function and the consequences of its impaired metabolism in primary and secondary Cr deficiencies are also discussed. Cr and phosphocreatine (PCr) in living systems can be well characterized using *in vivo* magnetic resonance spectroscopy (MRS). This review describes how ^{1}H MRS allows the measurement of Cr and PCr, and how ^{31}P MRS makes it possible to estimate the creatine kinase (CK) rate constant and so detect dynamic changes in the Cr/PCr/CK system. Absolute quantification by MRS using creatine as internal reference is also debated. The use of *in vivo* MRS to study brain Cr in a non-invasive way is presented, as well as its use in clinical and pre-clinical studies, including diagnosis and treatment follow-up in patients.

1. Creatine

Creatine (Cr), or α-N-methylguanidino acetic acid, is a nitrogenous organic acid playing an important role in the regeneration of ATP through the Cr/phosphocreatine/creatine kinase (Cr/PCr/CK) system (Fig. 1A [1,2]). Four different CKs have been described based on their tissue expression and subcellular localization: two mitochondrial, sMtCK (sarcomeric muscle) and uMtCK (ubiquitous/brain), consuming mitochondrial ATP to produce PCr for export to the cytoplasm, or in brown/beige adipose tissue for the control of thermogenesis; and two cytosolic, B-CK (brain) and M-CK (muscle), for local energy control through ATP regeneration by PCr consumption [3,4]. Tissues with high ATP demand (heart, muscle and brain) show elevated Cr levels.

Dietary intake is responsible for about half of the daily Cr needs, while the remainder is synthesized endogenously through a two-step enzymatic pathway: Arginine:glycine amidinotransferase (AGAT or GATM; EC 2.1.4.1) converts arginine (limiting substrate) and glycine into ornithine and the intermediate guanidinoacetate (GAA), while guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2) uses *S*-adenosylmethionine as co-factor to convert GAA into Cr (also releasing *S*-adenosylhomocysteine). Total Cr content amounts to ~120 g in a young adult of 70 kg body weight, of which about 2% is replaced daily due to the non-enzymatic conversion of Cr to creatinine [5,6]. Cr is transported into cells with high-energy demand (including cells within CNS) by the Na^{+}-Cl^{-}-dependent Cr transporter, SLC6A8 (also known as CT1, CRT, CRTR or CreaT) [5].

Abbreviations: AD, Alzheimer's disease; AGAT, arginine:glycine amidinotransferase; ALS, amyotrophic lateral sclerosis; ATP, adenosine triphosphate; BBB, blood-brain barrier; CCDS, cerebral creatine deficiency syndromes; CK, creatine kinase; CNS, central nervous system; Cr, creatine; CSF, cerebrospinal fluid; FID, free induction decay; GA, gyrate atrophy; GAA, guanidinoacetate; GAD, glutamic acid decarboxylase; GAMT, guanidinoacetate methyltransferase; HD, Huntington's disease; ID/DD, intellectual and developmental delays; IEM, inborn error of metabolism; mIns, myo-inositol; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; NMR, nuclear magnetic resonance; OAT, ornithine δ-aminotransferase; PCr, phosphocreatine; PD, Parkinson's disease; ppm, parts per million; ROS, reactive oxygen species; T1, longitudinal relaxation time constant; T2, transverse relaxation time constant; tCr, total creatine; TE, echo time; TR, repetition time; VOI, volume of interest.

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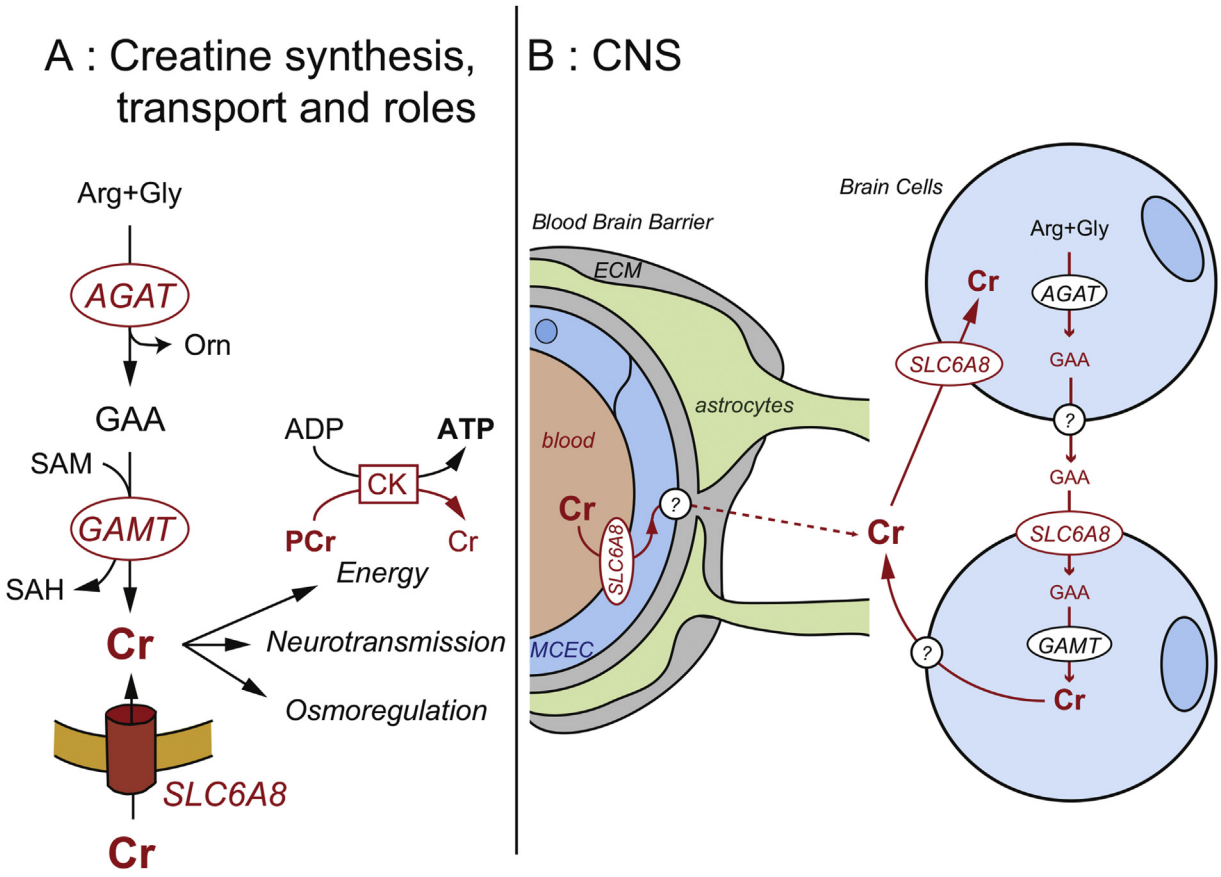

Fig. 1. Creatine synthesis, transport and functions in CNS. A: Cr is synthesized through a two-step successive enzymatic pathway involving AGAT and GAMT, and is taken up by cells through a specific transporter, SLC6A8. The main function of Cr allows ATP regeneration and high-energy phosphate shuttling through the Cr/PCr/CK system; Cr is also suggested to be a neurotransmitter, and is an important CNS osmolyte. **B**: While microcapillary endothelial cells (MCEC) express SLC6A8, astrocytic feet lining them do not, implying that only low amounts of peripheral Cr enter the brain through blood-brain barrier (BBB) and that CNS must accomplish part of its own endogenous synthesis of Cr. In most brain regions, the Cr synthesis pathway is dissociated, with AGAT-expressing cells synthesizing GAA followed by GAA uptake through SLC6A8 in GAMT-expressing cells. The cell exit of Cr and GAA occurs through so far unknown transporters or channels. **Abbreviations: AGAT** arginine:glycine amidinotransferase; **Arg** arginine; **Cr** creatine; **ECM** extracellular matrix; **GAA** guanidinoacetate; **GAMT** guanidinoacetate methyltransferase; **Gly** glycine; **MCEC** microcapillary endothelial cells; **Orn** ornithine; **PCr** phosphocreatine; **SAH** S-adenosyl homocysteine; **SAM** S-adenosyl methionine; **SLC6A8** Cr transporter.

2. Creatine synthesis and transport in CNS

While in the periphery the main Cr synthesis pathway occurs through the dissociated expression of AGAT in the kidney, which releases GAA as substrate for GAMT expressed in liver, AGAT and GAMT are also expressed in most other tissues, including the brain [5–9]. Brain Cr was long-considered to be synthetized in peripheral tissues followed by transport to the brain. Although microcapillary endothelial cells at the blood-brain-barrier (BBB) express SLC6A8, which allows peripherally synthesized Cr entry into the CNS, the astrocytes, and particularly their end-feet ensheathing the BBB, do not express the Cr transporter [7,10,11]. This leads to a very low permeability of BBB for Cr. Thus, the brain is dependent on its own endogenous synthesis of Cr through AGAT and GAMT expression (Fig. 1B [7,8,12]).

In the CNS, neurons and oligodendrocytes can contain AGAT, GAMT and SLC6A8 but astrocytes express only AGAT or GAMT as described above [7,11,13]. In most brain structures, AGAT and GAMT are rarely co-expressed in the same cell (particularly in cortex and basal ganglia), and thus, GAA must be transported from AGAT- to GAMT-expressing cells to complete the Cr synthesis pathway (Fig. 1B [8,14]). Moreover, this transport of GAA into brain GAMT-expressing cells appears to occur through the same transporter as that used for Cr, namely SLC6A8 [8]. While it was proposed that the final Cr synthetic step mostly occurs in glial cells [15,16], this does not seem to be the case in many brain structures, especially in cortex where neurons rather that astrocytes have the highest GAMT expression, or in hippocampal CA1-4 and dentate gyrus neurons, as well as in cerebellar Purkinje cells which express GAMT in very high proportions [8].

Two coherent patterns of expression are thus found in the different brain structures. First, as observed in most brain regions, AGAT and GAMT are not co-expressed. This separation of AGAT and GAMT expression might facilitate the fine tuning of GAA and Cr synthesis, in regard to the essential function of Cr in cellular energy metabolism. In addition, the efficient uptake of Cr and GAA by brain cells might be essential for maintaining low extracellular concentrations to allow neuromodulation/neurotransmission by Cr while avoiding the toxic effects of GAA (see below) [8,17,18]. It is noteworthy that the dissociated expression of AGAT and GAMT in CNS at the *intercellular* level is similar to the tissular separation of these enzymes between kidney and liver in the periphery [5,9,19]. Secondly, the cellular co-expression of AGAT and GAMT, sometimes also with SLC6A8, may indicate cells with permanent high Cr needs. Some specific brain structures, such as hippocampus, cerebellar Purkinje neurons and hypothalamic nuclei contain up to 60% of cells co-expressing AGAT + GAMT, and up to 65% of cells expressing SLC6A8. The presumed elevated demands of Cr in these neuronal layers are in line with their high creatine kinase activity [8,20].

During embryogenesis, the fetal needs for Cr are partly supported by active transport of Cr from the mother to the fetus [21].

However, AGAT, GAMT and SLC6A8 are also expressed during vertebrate embryogenesis in tissues that include the CNS [15,22,23]. We have shown that AGAT and GAMT are expressed in the whole developing brain [22]. However, their low level (GAMT in particular) at early developmental stages suggests that in contrast to adulthood, the embryonic CNS predominantly depends on an external Cr supply. This is probably sustained by the early expression of SLC6A8 in the whole fetal brain, with particularly high levels of expression in the periventricular zone and choroid plexus, before microcapillary angiogenesis and differentiation of the BBB [12].

3. Creatine: a multifunctional molecule in the brain

The essential role of Cr in energy metabolism has been known for a long time. Through the Cr/PCr/CK system, Cr serves in ATP regeneration as well as the shuttling and buffering of high energy phosphates (Fig. 1A) [1,2]. In the CNS, the function of Cr in energy metabolism is particularly important for maintaining membrane potentials, ion gradients and Ca^{2+} homeostasis [5,24]. Cr has also been described as a potent antioxidant molecule, in particular through scavenging of reactive oxygen species (ROS) [25]. Cr was shown to be neuroprotective in *in vivo* and *in vitro* rodent models of ischemia/anoxia [26], and was also suggested to protect the CNS under neurodegenerative conditions such as Parkinson's (PD) or Huntington's (HD) disease, in particular through cell energy support [27]. However, the first therapeutic trials for these two diseases were disappointing, possibly due to the poor permeability of the BBB for Cr (see above and Fig. 1B). Finally, the Cr/PCr/CK system is essential during brain development to provide enough ATP in neuronal growth cones to ensure normal growth of axons and dendrites [28].

In addition to its well-known function in energy metabolism (see above), Cr has recently been suggested to play roles in neuromodulation or even true neurotransmission (Fig. 1A). Cr and GAA can act as neuromodulators of GABAergic neurons as partial agonists of the post-synaptic $GABA_A$ receptor ($GABA_AR$) [29,30], and accumulated GAA under partial GAMT deficiency alters the expression of the pre-synaptic GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) as well as that of the post-synaptic $GABA_AR$ [18]. Moreover, a potential role for Cr as a true neurotransmitter was suggested by data showing that Cr can be released from neurons in an action-potential dependent manner, while rat brain synaptosomes are able to take up Cr [31,32]. This suggests that Cr may act as a vesicle-harboured neurotransmitter, which may also be recaptured/recycled through SLC6A8 expression in axonal terminals [17]. This suggestion is reinforced also by the fact that the Cr transporter SLC6A8 is a member of the Na^+- and Cl^--dependent neurotransmitter transporter BETA family (together with SLC6A6, SLC6A11, SLC6A12 and SLC6A13). More data are needed however to demonstrate this potential role of Cr as true neurotransmitter, in particular by discovering a so far unknown specific postsynaptic Cr receptor. Cr is also a major osmolyte of the CNS (Fig. 1A), as shown by ^1H and ^{31}P magnetic resonance spectroscopy (MRS) in rat cortical brain slices and primary astrocytic cultures [33,34]. A decrease of brain Cr was shown in an *in vivo* model of hepatic encephalopathy induced by bile duct ligation in the rat, in parallel with an increase of glutamine [35,36]. Finally, Cr was also suggested to be a potential regulator of appetite and weight by acting on hypothalamic nuclei [37].

4. Measurement of brain creatine by *in vivo* magnetic resonance spectroscopy

MRS offers a non-invasive opportunity to assess Cr content in various tissues/organs *in vivo*, including brain. In particular, MRS allows the study of the various metabolites which are involved in the multifunctional role of Cr in the CNS: energy metabolism (e.g. Cr, PCr, lactate, ATP, NADP), neuromodulation/neurotransmission (e.g. Gln, Glu, GABA, Asp), osmolality (Gln, mIns, Tau, tCho, tCr), and neuroprotection (NAA, GSH). Moreover, MRS measurements can be performed in a longitudinal way to assess metabolic changes during disease or treatment. Metabolite concentrations are measured in a volume of interest (VOI) as a mean value containing intra- and extracellular space and are usually compared between pathologic and control groups or before and after disease/treatment if the study design allows it.

4.1. ^1H MRS

In ^1H MR spectra, hydrogen nuclei (called protons) of Cr resonate at three different frequencies: CH_3 protons at 3.027 ppm, CH_2 protons at 3.913 ppm and NH_2 protons at 6.649 ppm (ppm = parts per million) [38]. The NH_2 of Cr is often not detected *in vivo* because of its short T_2 and fast exchange of its protons with water [39]. In PCr the chemical shifts are slightly different from those observed with Cr with CH_3 protons resonating at 3.029 ppm (chemical $shift_{Cr-PCr}$ = 0.002 ppm), CH_2 protons at 3.930 ppm (chemical $shift_{Cr-PCr}$ = 0.017 ppm) and NH_2 protons (when observed) at 6.581 and 7.296 ppm [38]. This difference between Cr and PCr resonant frequencies is too small to be reliably distinguished at low magnetic field (up to 3 T), thus often leading to the estimation of the sum of these metabolites as total Cr (tCr = Cr + PCr). At higher magnetic field, especially in animal studies, Cr and PCr concentrations can be assessed separately [36,40], as illustrated in Fig. 2. This is particularly useful when imbalance of the Cr/PCr ratio needs to be observed. In the case of GAMT deficiency, the Cr precursor GAA can be detected by ^1H MRS as a singlet peak from CH_2 protons resonating at 3.78 ppm which may be misidentified as the Cr peak at 3.913 ppm [41,42]. The chemical shift position of this GAA peak in the ^1H MR spectrum is shown in Fig. 2.

All resonances of Cr and PCr are singlets without any J-coupling, thus facilitating their detection and quantification (Fig. 3). The absence of J-coupling, together with relatively high brain tissue concentration, makes Cr an easily detectable metabolite with few technical demands on localization pulse sequence (e.g. PRESS, STEAM, LASER, SPECIAL). Although Cr and PCr (or tCr) can reasonably be quantified using long echo times (TEs), Ke et al. [43] showed a difference in T_2 relaxation of Cr and PCr CH_3 groups (3.03 ppm) at 1.5 T indicating that the contributions of Cr and PCr in the tCr peak change when acquired with different TEs [44]. It means that a short TE sequence is important to minimize T_2 weighting of Cr and PCr in the tCr peak. On the other hand, comparison between short and long TE acquisition allows the assessment of Cr/PCr ratio even in magnetic fields where Cr and PCr peaks cannot be separately distinguished [43,44].

4.2. Brain Cr concentration in ^1H MRS

The concentration of Cr measured by *in vivo* ^1H MRS in the CNS under physiologically normal conditions varies between 4.5 and 5.6 mmol/l in human and 3.5–5.5 mmol/l in rat; PCr reaches 3–5.5 mmol/l in human and 3–5 mmol/l in rat [36,40,45,46]. By using off-resonance magnetization transfer experiments in rats, it was shown that almost all brain metabolites have immobile pools of variable size, suggesting specific interactions with macromolecules [47]. Thus, the measurement of tCr by *in vivo* ^1H MRS appears to result in an underestimate by approximately 13–18%, as part of Cr present in brain tissue is MR invisible due to its immobilization by binding to large molecules [48,49].

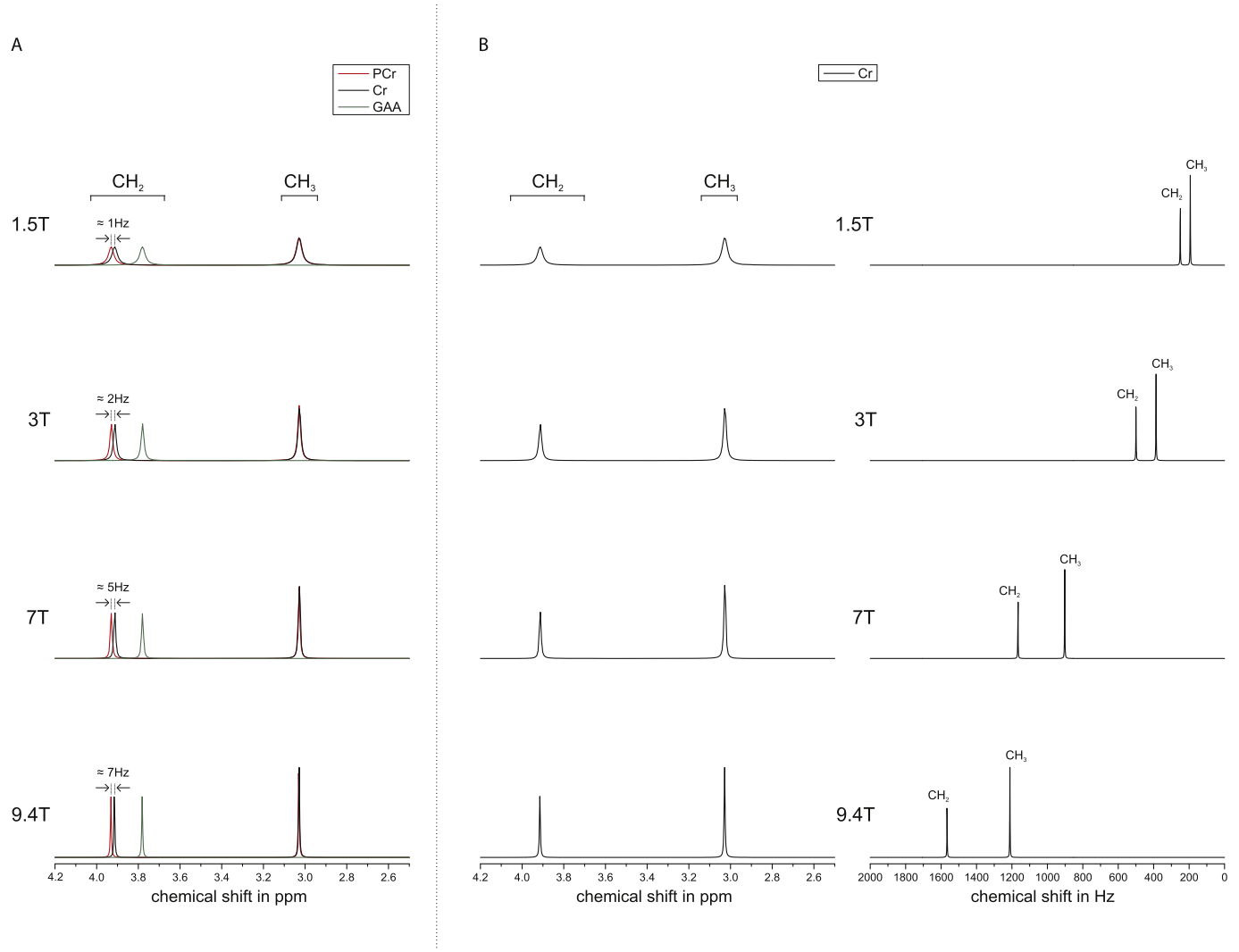

Fig. 2. PCr, Cr and GAA in ^{1}H MR spectra. A: Simulated spectra of phosphocreatine (PCr), creatine (Cr) and guanidinoacetate (GAA) at different static magnetic fields (1.5 T, 3 T, 7 T and 9.4 T). With increasing magnetic field, the CH_2 peaks of Cr and PCr can be better separated. As indicated in the figure, the chemical shift between CH_2 peaks of Cr and PCr is equal to 0.017 ppm and corresponds to 1.086 Hz, 2.171 Hz, 5.067 Hz and 6.804 Hz at 1.5 T, 3 T, 7 T and 9.4 T respectively. In contrast, CH_3 peaks of Cr and PCr are indistinguishable even at 9.4 T (the chemical shift between Cr and PCr is 0.002 ppm and corresponds only to 0.8 Hz at 9.4 T). The GAA peak (at 3.78 ppm) is undetectable under physiological conditions due to its low concentration and is typically observed only in the brain spectra from GAMT-deficient patients, sometimes wrongly reported as Cr CH_2 peak due to its close position. **B**: Simulated spectrum of Cr at different static magnetic fields (1.5 T, 3 T, 7 T and 9.4 T). The left column shows the chemical shift in ppm units, the CH_3 peak at 3.027 ppm and the CH_2 peak at 3.913 ppm for every magnetic field strength. The right column shows the chemical shift in Hz units demonstrating a shift of resonant frequencies at different magnetic fields. 0 ppm or 0 Hz is equivalent to no chemical shift and so corresponds to resonant Larmor frequency at each magnetic field strength (63.864 MHz at 1.5 T, 127.728 MHz at 3 T, 298.032 MHz at 7 T and 400.214 MHz at 9.4 T). **Note**: All simulated peaks have fixed linewidth to 2 Hz which results in apparently larger peaks at lower magnetic fields in ppm scale (1 ppm at 1.5 T corresponds to 63.864 Hz whereas 1 ppm at 9.4 T corresponds to 400.214 Hz). All metabolites were simulated with NMRSCOPE B plug-in in jMRUI software (http://www.mrui.uab.es/mrui/). **Abbreviations: Cr** creatine; **GAA** guanidinoacetate; **PCr** phosphocreatine.

4.3. Use of Cr as a concentration reference in ^{1}H MRS

The facile detection of Cr, in combination with relatively constant levels in the brain under physiological conditions, is attractive for the use of tCr as an internal concentration reference (by providing the tCr concentration as internal reference against which a metabolite concentration may be compared or as a ratio of metabolite concentration to tCr concentration).

However, this approach has to be taken with caution. While no significant change of tCr was reported in intrasubject daily variability [50] or due to aging [51] in adult human brain, tCr varies considerably during brain development (with an increase of tCr from 5.7 μmol/g at 7 days postnatal to 8.5 μmol/g at 28 days postnatal in rats [52]). Moreover, regional changes between gray and white matters were also observed in humans (tCr varying from 4.4 to 6.3 mmol/l in white matter to 5.7–10.9 mmol/l in gray matter [53,54]).

While in many diseases brain tCr stays constant, pathologies that disturb cell energetics may disturb tCr content. Examples of such pathologies are not surprisingly primary creatine deficiencies, but also stroke, tumors, as well as neurodegenerative and psychiatric diseases (see below).

An alternative would be to use water concentration as internal reference [60,61]. This would not only eliminate possible bias in concentrations of other brain metabolites due to changes in tCr brain concentration but also allow precise detection of Cr and PCr (or tCr) changes. In most CNS pathologies, changes of water content in brain tissue are negligible. Changes due to various brain edemas are in the range of 1–2% [55–57]. However in some disorders, water content may vary to a greater extent (up to 10%), e.g. multiple

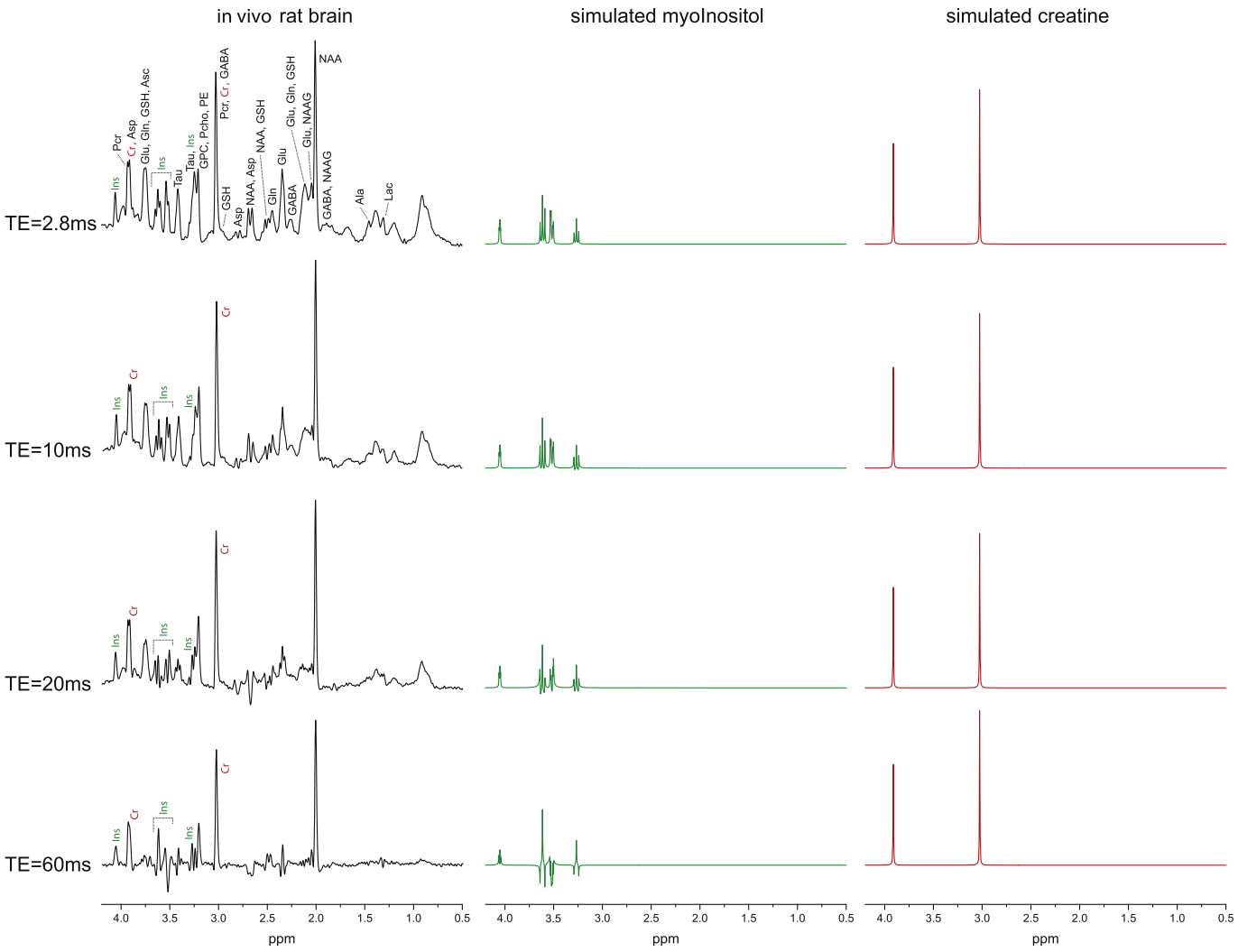

Fig. 3. ***In vivo*** **and simulated ^{1}H MR spectra at different echo times (TE). Left column**: Evolution of *in vivo* ^{1}H MR brain spectra at different echo times. Cr peaks (marked in red) are very well quantifiable even at TE = 60 ms compared to those of myo-Inositol (marked in green). Spectra were acquired in a rat brain *in vivo* at 9.4 T using the SPECIAL spectroscopic sequence [178] in a voxel of 27 mm^3 with 240 averages. **Middle column**: Simulated spectra of myo-Inositol as an example of a metabolite showing a complex J-coupled spectral pattern with increasing TE, and thus, making the multiplets more difficult to quantify. **Right column**: Simulated singlets of Cr show no change due to J-evolution with increasing TE and are very clear in the spectra even at longer TE. **Note**: Decrease of signal due to T_2 relaxation is not taken into account in the simulated spectra. All metabolites were simulated with NMRSCOPE B plug-in in jMRUI software (http://www.mrui.uab.es/mrui/). **Abbreviations**: **Ala** alanine; **Asc** ascorbate; **Asp** aspartate; **Cr** creatine; **GABA** γ-aminobutyric acid; **Glc** glucose; **Gln** glutamine; **Glu** glutamate; **GSH** glutathione; **GPC** glycerophosphocholine; **PCho** phosphocholine; **PCr** phosphocreatine; **PE** phosphoethanolamine; **Lac** lactate; **Ins** myo-Inositol; **NAA** N-acetylaspartate; **NAAG** N-acetylaspartylglutamate; **Tau** taurine.

sclerosis [58] or tumors [59]. This needs to be considered when water signal representing water concentration is chosen as an internal concentration reference. Internal water referencing requires the separate acquisition of the water signal under the conditions identical to those used for acquisition of metabolite signals. In addition, water concentration in the VOI needs to be known, which depends on the tissue composition within that volume. The tissue composition (cerebrospinal fluid (CSF), gray and white matter) can be determined by performing tissue segmentation of a high resolution structural image. Once the tissue composition in the VOI is known, the water concentration can be estimated using published values for the water content of various tissues types. A second non-invasive method is the *in vivo* estimation of brain water versus structural, NMR-invisible material (often called dry weight) and presence of CSF by measuring the multi-compartment T_2 decay curve of the water signal [60,61]. The obtained information on the water content in the VOI can be further used for quantitative estimation of brain metabolites. For important advice on data acquisition, processing and appropriate calculations of brain metabolite concentrations in different units, consult ref [61]. However, it must be emphasized that regardless of the referencing method used, corrections for relaxation of both metabolite and reference signals need to be performed. The effects of relaxation are considered minimal for acquisitions performed at long TR and short TE (fully relaxed spectra) and therefore, acquisitions obtained under these conditions are considered to be good choices for absolute quantification. However, in clinical practice these types of acquisitions are very difficult to perform (i.e. long TR implies long scanning time while on clinical scanners the typical short TEs are around 20–30ms).

4.4. ^{31}P MRS

Another way to measure PCr is to detect its phosphorus nucleus by *in vivo* ^{31}P MRS. ^{31}P MRS is however less sensitive (~7% relative to ^{1}H MRS) requiring longer acquisition times and/or bigger measured

volumes but offers insight into cellular high energy metabolites (ATP, PCr), phospho-monoesters and diesters as well as some other phosphorus containing compounds (Fig. 4) which are difficult to study *ex vivo* due to high metabolic rates.

The PCr peak is a singlet, dominating ^{31}P brain spectra under normal physiological conditions and often used as a chemical shift reference assigned to 0 ppm. In the physiological range, its resonant frequency does not change with change of pH or [Mg^{2+}] [62] unlike many other ^{31}P metabolites. The resonance frequency of inorganic phosphate (Pi) can be used to determine the intracellular pH (considering that the Pi signal is due to Pi mainly in the intracellular space) by utilizing the Henderson-Hasselbalch equation as adapted for MRS:

$$pH = 6.75 + \log[(\delta_{Pi} - 3.29)/(5.68 - \delta_{Pi})]$$

δ_{Pi} being a chemical shift between Pi and PCr in ppm [63]. Similarly, chemical shift between β-ATP and PCr signals (δβ-ATP) in ppm can be used to assess [Mg2+] [64]:

$$pMg^{2+} = 4.24 - \log\left[\left(\delta_{\beta-ATP} + 18.58\right)^{0.42} \Big/ \left(-15.74 - \delta_{\beta-ATP}\right)^{0.84}\right]$$

In the quantification of ^{31}P MR spectra attention needs to be paid to the correction due to T_1 relaxation, as phosphorus metabolites have longer T_1 relaxation times and sufficiently long TR (MR sequence repetition time) is rarely used in order to maximize the signal-to-noise ratio. On the other hand, there is no need for the correction of T_2 relaxation since ^{31}P MR spectra are often acquired as the FID (free induction decay) and not echos.

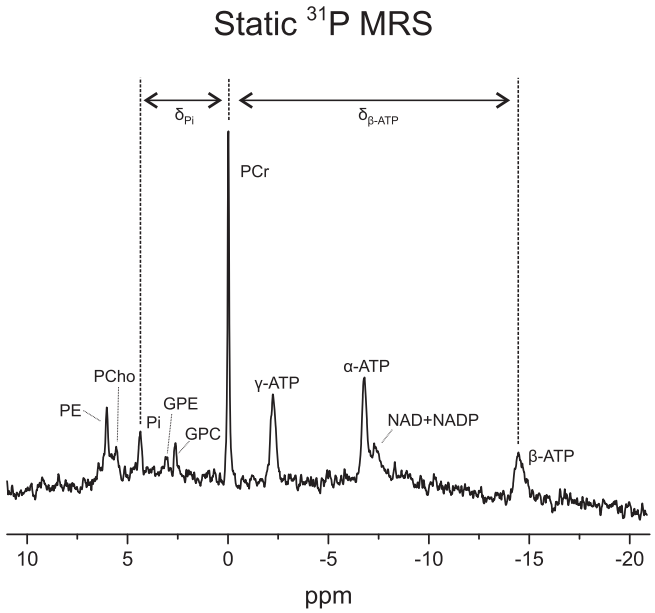

Fig. 4. *In vivo* ^{31}P MR spectrum. *In vivo* ^{31}P MR spectrum acquired in a rat brain at 9.4 T (a non-selective adiabatic half passage pulse was used for excitation, localization was performed with OVS in the x-, y- and z-direction, together with a one dimensional ISIS in the y-direction [179], voxel size 195 mm^3, TR = 5 s, 640 averages). **Abbreviations**: **ATP** adenosine triphosphate; **GPC** glycerophosphocholine; **GPE** glycerophosphoethanolamine; **Pi** inorganic phosphate; **NAD** nicotinamide adenine dinucleotide; **NADP** nicotinamide adenine dinucleotide phosphate; **PCho** phosphocholine; **PCr** phosphocreatine; **PE** phosphoethanolamine. **δ_{Pi}**: difference between resonant frequencies of PCr and Pi, used for determination of intracellular pH. **$\delta_{\beta\text{-ATP}}$**: difference between resonance frequencies of PCr and β-ATP, used for determination of [Mg^{2+}].

Concentration referencing in *in vivo* ^{31}P MRS is more difficult than that for ^{1}H MRS due to the lack of a suitable, stable internal concentration reference (such as tissue water content for ^{1}H MRS). Very often ATP or PCr concentration is fixed to a certain constant value in the studied subjects. However this approach may lead to inaccuracies if cell energetics are perturbed during disease. Another possibility is the use of an external concentration reference [65,66] with the correction/calibration for coil loading or metabolite quantitation based on the reciprocity principle [67]. A heteronuclear concentration reference is also possible, by using the PCr concentration from the same volume if measured by ^{1}H MRS at high field [36], or by using directly tissue water as an internal reference after correction to different detection sensitivities of the proton and phosphorus spectrometer channels [68,69].

The brain PCr concentrations reported from *in vivo* ^{31}P MRS vary between 2.5 and 5.1 mmol/l in adult human [66,67,70,71], 1.4–2.8 mmol/l in neonatal and infant human [68,72] and 3.4–5.9 mmol/l in adult rat [36,73]. Reported values are in agreement with those noted above as measured by *in vivo* ^{1}H MRS.

4.5. Saturation transfer in ^{31}P MRS

In many pathologies, the static concentrations of metabolites do not change while the dynamic properties of metabolism vary, often in order to maintain the metabolite concentrations stable for as long as possible. This is in particular true for ATP, as mirrored in the rate of CK reaction:

$$PCr^{2-} + MgADP^{-} + H^{+} \underset{k_{ATP-PCr}}{\overset{k_{PCr-ATP}}{\rightleftarrows}} Cr + MgATP^{2-}$$

(k: creatine kinase rate constant)

The high-energy phosphate exchange between ATP and PCr can be studied using a saturation transfer experiment (magnetization transfer technique, Fig. 5) [45,74,75]. Selective saturation of the γ-phosphorus in ATP molecule by a narrow-band pulse scheme such as BISTRO [76] will result in reduction of PCr signal due to chemical exchange of the phosphorus nucleus with saturated magnetization between ATP and PCr (Fig. 5). However this selective saturation pulse applied to γ-ATP is not perfect and can also partially saturate PCr. This decrease of PCr signal due to bleedover of the saturation pulse and not chemical exchange of saturated phosphorus nuclei from γ-ATP is unwanted. So-called mirrored spectra need to be acquired to perform the appropriate correction. For this acquisition, a saturation radiofrequency (RF) pulse is applied, not at the γ-ATP resonance but at the mirrored position in respect to PCr. Using this approach the PCr signal should decrease as a function of the length of the saturation time (t_{sat}) applied to the γ-ATP according to the equation:

$$M(t_{sat})/M(0) = (1 - k_{ATP-PCr}T_{1sat}) + k_{ATP-PCr}T_{1sat}\, exp(-t_{sat}/T_{1sat})$$

where M(0) is the PCr signal intensity in the mirrored spectrum and T_{1sat} is the apparent T_1 relaxation time of PCr during the γ-ATP saturation. Since T_{1sat} is often unknown, the equation needs to be sampled with enough t_{sat} times in order to fit both T_{1sat} and $k_{ATP\text{-}PCr}$, which can provide an indication of metabolic rates of high-energy phosphate buffer system/metabolism. Brain CK activity seems to be altered in some psychiatric disorders and in some neurodegenerative diseases [77,78]. Under physiological conditions, brain CK activity increases during swine brain development [79], while in contrast Smith et al. [80] showed reduced CK flux in aged rats.

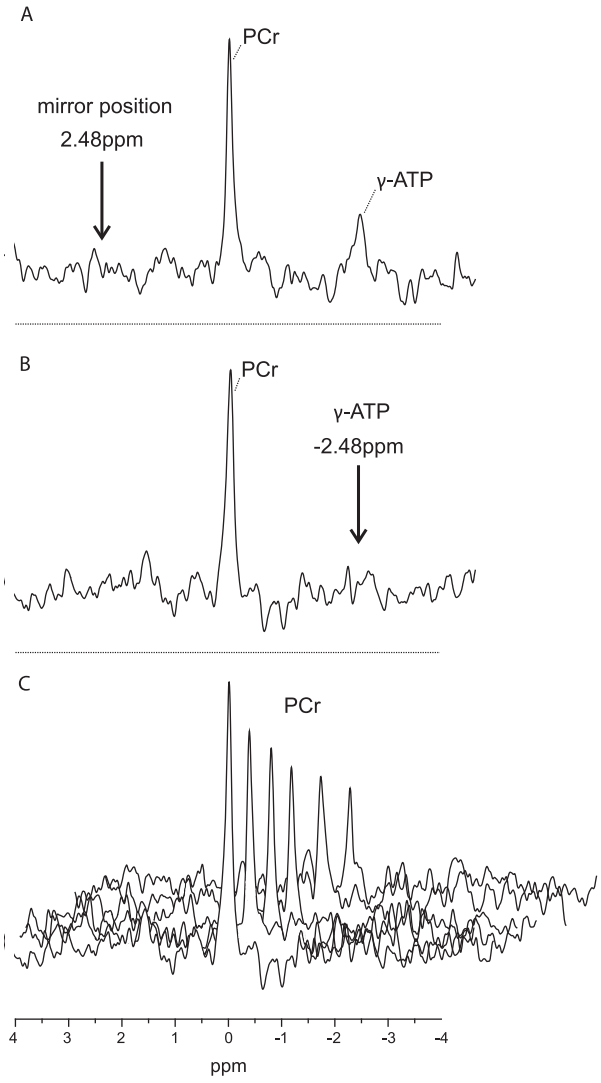

Fig. 5. ^{31}P MR saturation transfer spectra. *In vivo* ^{31}P MR saturation transfer spectra acquired in a rat brain at 9.4 T (a non-selective adiabatic half passage pulse was used for excitation, localization was performed with OVS in the x-, y- and z-direction, together with a one dimensional ISIS in the y-direction [179], the γ-ATP signal was saturated by a BISTRO pulse train [76] interleaved with OVS, voxel size = 195 mm^3, TR = 8 s, 80 averages). **A: Mirrored acquisition**; saturation BISTRO pulse train applied on the mirrored position of γ-ATP in respect to PCr (at 2.48 ppm). **B: γ-ATP saturation**; saturation BISTRO pulse train applied on γ-ATP (at -2.48 ppm). The γ-ATP resonance is suppressed as expected. **C: γ-ATP saturation with different saturation times** (number of saturation blocks from 1 to 16); Decrease of PCr due to transfer of saturated phosphate from the γ-ATP is observed. **Abbreviations**: **ATP** adenosine triphosphate; **PCr** phosphocreatine; **ppm** parts per million.

5. Cerebral creatine deficiency syndromes

As described above, the CNS levels of Cr (and PCr) measured by MRS can be impaired in several neuropathological conditions. Twenty-two years ago the first patient with an inborn error of metabolism (IEM) affecting Cr synthesis or transport, or cerebral Cr deficiency syndromes (CCDS), was identified. This GAMT-deficient patient, described by Stöckler et al. [42,81,82], was rigorously examined with ^{1}H and ^{31}P MRS, which helped to pave the way for the diagnosis of CCDS and possible treatment. CCDS are caused by mutations in *GAMT* [42], *GATM* (AGAT [83]) and *SLC6A8* [84] genes. AGAT and GAMT deficiencies are autosomal recessive, while SLC6A8 deficiency is X-linked. These three diseases are characterized by the absence, or a strong decrease, of Cr in the brain when measured by ^{1}H MRS. The CNS is the primary tissue affected and patients show neurological symptoms beginning in early infancy, in particular presenting with intellectual and developmental delays (ID/DD), speech acquisition delay and, specifically for GAMT but sometimes also for SLC6A8 deficiencies, additional intractable seizures as well as extrapyramidal movement and behavioural disorders [17,85–89].

The first CCDS discovered, GAMT deficiency, has been described so far in about 110 patients [42,80–82,86]. GAMT deficiency shows the CCDS-characteristic brain Cr deficiency with absent Cr resonances in ^{1}H MRS (Fig. 6). GAMT deficiency appears to be the most severe of the CCDS due, apart from Cr deficiency, to GAA accumulation in body fluids (plasma, serum, CSF) and tissues, including CNS in which the endogenous brain Cr synthetic pathway is inoperative. GAA levels in CSF of GAMT-deficient patient may thus exceed >100x the upper reference value [85,90,91], and a unique GAA peak at 3.78 ppm (very close to the CH_2 resonance of Cr, and normally undetectable in the healthy brain), may be observed in the brain of GAMT-deficient patients (Figs. 2 and 6 [41,42]). Some GAMT-deficient patients also show hyperintensity in globus pallidus on T_2 weighted MRI, probably reflecting neuronal injury due to GAA accumulation [42,92,93] as well as to a differential CNS distribution of GAA, with higher GAA signals in gray than in white matter using a ^{1}H chemical shift imaging method [94]. The more severe neurological phenotypes observed in GAMT deficiency are due to the neurotoxic effects of GAA, which impairs GABA-ergic, glutamatergic and cholinergic neurotransmission systems and can have a direct depressing effect on brain energy metabolism by inhibiting plasma membrane Na^+/K^+-ATPase [29,95,96], and may also affect neuronal differentiation during CNS development [18].

AGAT deficiency was the third discovered CCDS and the rarest, with so far only 16 patients described worldwide [83,97]. AGAT deficiency is characterized, as in the case of GAMT deficiency, by low or undetectable brain Cr levels measured by ^{1}H MRS, but also by very low levels of GAA in all body fluids, including CSF [85,87,97–100].

Finally, the second discovered CCDS, SLC6A8 deficiency [84], appears to be the most frequent, and may account for 1–2% of male intellectual disabilities [85,101–104]. As it is X-linked, SLC6A8-deficient males are more severely affected than heterozygous females [105–108]. Cr and GAA levels are generally normal in plasma of SLC6A8-deficient patients, while the Cr/creatinine ration in urine is typically elevated [98]. As noted for the two other CCDS, brain Cr virtual absence in ^{1}H MRS is characteristic of SLC6A8 deficiency (Fig. 6), while interestingly normal levels of Cr are usually measured in CSF of these patients [14,85]. As in GAMT deficiency, some SLC6A8-deficient patients may also present increased levels of GAA in their brain due to the absence of a functional SLC6A8 to complete the endogenous brain Cr synthetic pathway, as described above, and this GAA accumulation can be observed by ^{1}H MRS [12,109].

6. Treatment and follow-up of creatine deficiency syndromes by MRS

Brain ^{1}H and ^{31}P MRS in the first-described GAMT-deficient patient [42] helped to follow up the treatments. Oral supplementation with arginine did not change the brain tCr resonance, while GAA resonance increased (stimulation of AGAT activity); arginine + Cr supplementation increased CNS tCr but maintained an elevated GAA; finally, Cr supplementation alone also increased brain tCr. The ^{31}P MRS was in agreement with the ^{1}H MRS, with PCr resonance following the behaviour of ^{1}H tCr resonance and phospho-GAA resonance following the behaviour of ^{1}H GAA

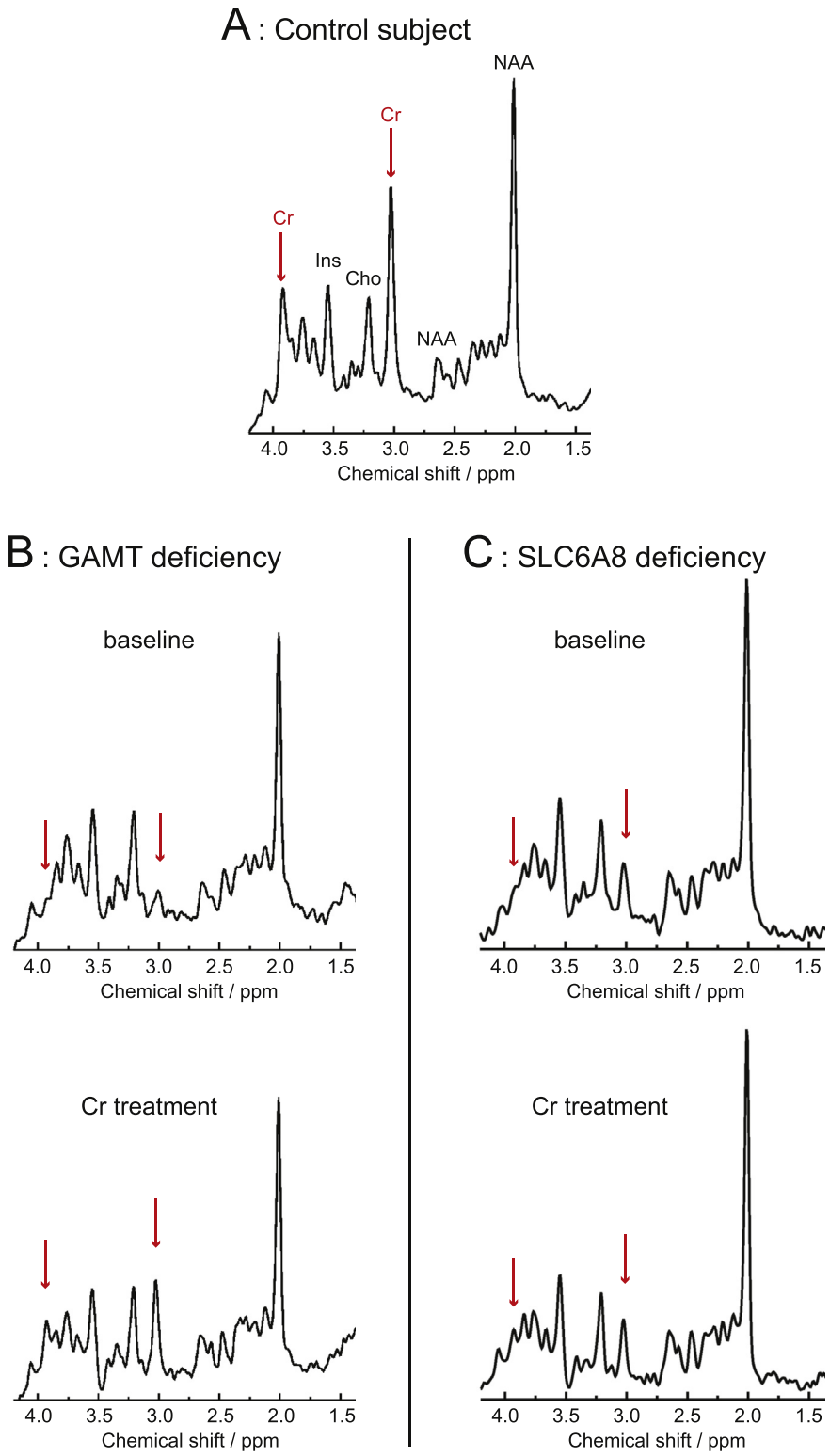

Fig. 6. ^{1}H MRS spectra in the cortex of patients suffering from creatine deficiency syndromes. Acquisition of ^{1}H MRS spectra was obtained in the parietal gray matter, with the following sequence parameters: 1.5 T MR scanner (Siemens Vision, Erlangen, Germany); single voxel MRS (short-echo time stimulated echo acquisition mode "STEAM" sequence, with TR/TE/TM = 6000/20/10 ms, 64 accumulations). **A: Control subject** (normal spectrum), 4.1 years old, showing the main choline (Cho), creatine (Cr, red arrows), myoinositol (Ins) and N-acetylaspartate (NAA) peaks. **B: GAMT-deficient patient**, 2.6 years old (baseline) and 3.1 years old at follow up after Cr treatment. Note the cortical Cr deficiency at baseline, followed by partial Cr replenishment after 6 months of Cr treatment. **C: SLC6A8-deficient patient**, 5.9 years old (baseline) and 8.6 years old at follow up after Cr treatment. Note the cortical Cr deficiency at baseline, with no Cr replenishment after 2.7 years of Cr treatment.

resonance. Both GAMT- and AGAT-deficient patients often respond positively to Cr monohydrate supplementation, which strongly improves their neurological status and CNS development [81,97]. Cr supplementation is generally performed long-term with high doses of Cr (100–800 mg/kg/day), to slowly replenish their brain Cr pools. This supplementation is monitored by ^{1}H MRS measurements [81,97], demonstrating both active SLC6A8 transporter on microcapillary endothelial cells and the low permeability of BBB for Cr (see above and Fig. 1B [12,110]). For GAMT deficiency and its more severe phenotype due to CNS accumulation of GAA (see above), coupling Cr supplementation to a strategy of GAA lowering is generally proposed [12,81,111]. This is achieved by restricting arginine in the diet together with ornithine supplementation, leading to the reversal of AGAT reaction and thus helping to reduce GAA. Despite clinical improvements, most GAMT- and AGAT-deficient patients remain with irreversible brain damage, the level of phenotype reversion under Cr therapy being highly dependent on the age of the patient at the beginning of treatment, with limited improvement in cognitive functions for late treated patients [81,97]. In particular, the brain of GAMT- and AGAT-deficient patients appears to develop normally if patients can be treated pre-symptomatically with oral Cr (for GAMT deficiency, coupled to the GAA-lowering strategy described above) [112–114]. For both GAMT and AGAT deficiencies, ^{1}H MRS appears to be an essential non-invasive tool for measuring the efficacy of treatment follow-up of patients. In particular, as oral supplementation with Cr has to be applied on the life-long range, as CNS Cr replenishment is very slow due to the low permeability of BBB for Cr, and as it has been shown that patients respond differentially to the treatment, ^{1}H MRS allows for the adjustment of the most effective daily dose of Cr, which depending on the patient may vary from 100 to 800 mg/kg/day [85,86,97].

Up to now, attempts to successfully treat SLC6A8-deficient patients have failed. Cr supplementation is inefficient in replenishing CNS Cr in male patients, and has no effect on their neurological phenotype [88,115–117] showing that SLC6A8 is the only Cr transporter at the BBB [12]. Heterozygous females may respond more positively due to the mosaical X-chromosome differential inactivation [115]. Trials with the Cr precursors arginine and glycine gave only modest results in two SLC6A8-deficient patients (slight improvement in intellectual ability and amelioration of muscular symptoms) but failed in many others [105,106,118–120], due to AGAT and GAMT dissociated expression in the CNS (Fig. 1B) [12,110]. As a functional Cr transporter is absent on the BBB of SLC6A8-deficient patients, considerable research has been performed over these last years to identify Cr derivatives which would cross plasma membrane independently of SLC6A8 while maintaining Cr functions within the brain [121–128]. So far, however, none of these derivatives have been found to be efficacious in the treatment of SLC6A8 deficiency.

7. Secondary creatine deficiencies

7.1. Gyrate atrophy of the choroid and retina

Gyrate atrophy of the choroid and retina (GA) is an IEM caused by mutations in the ornithine δ-aminotransferase (OAT) gene generating a secondary Cr deficiency by accumulation of ornithine, which inhibits the AGAT reaction, thereby depleting GAA for Cr synthesis [129–131]. Patients may show normal cognition, but electroencephalography and magnetic resonance imaging (MRI) analysis can reveal unspecific abnormalities and premature degenerative brain changes, possibly related to the OAT deficiency-induced secondary brain Cr deficiency [131,132].

7.2. Hyperammonemia

Hyperammonemia can be caused by several IEM (urea cycle disorders being the most common) as well as by acute or chronic liver disease, leading to high ammonium ion accumulation in the CNS. Hyperammonemia is toxic to the brain through alteration of neurotransmission and nitric oxide synthesis, oxidative stress and cerebral energy metabolism, as well as osmotic stress due to accumulation of glutamine in astrocytes [35,133–135,180]. High ammonium ion exposure alters AGAT, GAMT and SLC6A8 gene expression and leads to a secondary Cr deficiency in developing brain cells [28,136]. We also recently showed by ^{1}H MRS that brain tCr is decreased in an *in vivo* rodent model of chronic liver disease [137]. During development, while high ammonium exposure impairs axonal growth, Cr supplementation under ammonium exposure protects axonal elongation [28]. As described above, the BBB has a limited permeability for Cr in physiological conditions. However, high ammonium exposure induces SLC6A8 in astrocytes surrounding the BBB [136] while microcapillary endothelial cells at the BBB increase SLC6A8 expression as well as uptake of Cr [138]. It is therefore suggested that Cr supplementation could be beneficial and neuroprotective for hyperammonemic patients [139,140].

7.3. Stroke

The loss of brain functions in stroke is due to compromised blood flow leading to insufficient oxygen and glucose delivery to brain cells [141]. The Cr/PCr system can regenerate ATP even in the absence of oxygen and glucose, but for a very limited time. In the CNS PCr is present in limited amounts and is rapidly depleted after anoxia or ischemia, preceding the ATP fall [142,143]. A fast diminution in CNS total Cr by MRS has also been demonstrated in different *in vivo* models for brain ischemia [144–146] and in ischemic patients [147], eventually leading to neuronal death by necrosis or apoptosis [148,149].

7.4. Psychiatric disorders

It is thought that impairment of brain energy metabolism may contribute to the pathogenesis of several psychiatric disorders [150–152], in particular by disturbance of mitochondrial structure and function, which can result in a perturbation of Cr/PCr metabolism [77]. In schizophrenia, altered Cr metabolism in the brain was observed, sometimes with contradictory results, Cr/PCr levels being either decreased or elevated, including an asymmetrical distribution of PCr between left and right temporal lobes [153–157]. However no beneficial effect of Cr supplementation could be observed in schizophrenic patients [158]. Decreased levels of Cr can be observed in the CSF of individuals with mood disorders or depression, with however Cr MRS measurements showing differentially affected CNS structures between patients, as well as sex-dependent differences [159–162]. A correlation between PCr levels in frontal lobe (detected by ^{31}P MRS) and the degree of depression was observed [163]. In animal studies, Cr supplementation seemed to have positive antidepressant-like effects [77,164]. In depressed patients, relatively low doses of Cr (3–5 g/day) ameliorated symptoms of depression even in patients resistant to standard anti-depressants [165,166], and increased brain PCr, as measured by ^{31}P MRS [167].

8. Creatine as neuroprotective molecule

In addition to some of the neuropathologies described above, Cr supplementation has been demonstrated to be neuroprotective in several neurodegenerative conditions (see Ref. [24] for a review).

In a mouse model of HD, Cr supplementation was shown to stabilize intracellular Ca^{2+}, buffer intracellular energy reserves, inhibit opening of the mitochondrial permeability transition pore and decrease extracellular glutamate [168]. Cr supplementation also increased life span, decreased brain atrophy and delayed the formation of mutant huntingtin aggregates [169]. Positive effects of Cr supplementation were also shown in HD patients under clinical trial [170]. Similarly, Cr supplementation in animal models of PD protected against both CNS dopamine depletion and neuronal loss in the substantia nigra [171] while effects on PD patients have so far been less convincing, only leading to a decrease in the necessary dose of dopaminergic therapy [172].

In contrast, while Cr supplementation showed neuroprotective effects in an *in vivo* model of amyotrophic lateral sclerosis (ALS; e.g. the G93A mouse) and attenuated its clinical symptoms [173], the same was not true in ALS patients, possibly due to insufficiency of Cr dosage as well as to treatments having started too late in the progression of the disease [174,175].

In Alzheimer's disease (AD), inactive CKs and depositions enriched in Cr have been observed [176]. However, while Cr supplementation appeared neuroprotective against β-amyloid toxicity in rat hippocampal neurons [177], this did not improve cellular bioenergetics at late stages of AD [24], and the question remains open whether Cr supplementation may improve AD outcome if treatment start at much earlier stages.

9. Conclusion

In the CNS, Cr not only plays roles in energy metabolism within the Cr/PCr/CK system, but is also one of the main brain osmolytes and may also act as a possible neuromodulator/neurotransmitter. Cr and PCr are easily detected in the *in vivo* brain by ^{1}H and ^{31}P MRS, and have long been used as concentration references to help estimate other CNS metabolites measured by MRS. However, Cr (and PCr) can change in the CNS under several neuropathological conditions, including primary Cr deficiencies (or CCDS), some neurodegenerative and neuropsychiatric diseases and conditions such as stroke or ischemia. Therefore, the early detection of change of CNS Cr concentration by MRS can help in diagnosis, offer prognostic features and guide potential treatment. Moreover, the non-invasive character of MRS offers an opportunity to follow the course of the disease with time, verify the efficiency of a treatment and personalize it. Reliable measurement of tCr is possible even with a 1.5 T scanner, which is available in most hospital centers. Detection of brain Cr variations in pre-clinical and clinical studies may also help to understand new mechanisms of disease, in particular through the use of *in vivo* animal models (e.g. rodents) and MRS at higher magnetic fields ranging from 7 T to 17 T.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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