MR Spectroscopy of Muscle and Brain in Guanidinoacetate Methyltransferase (GAMT)-Deficient Mice: Validation of an Animal Model to Study Creatine Deficiency

W. Klaas Jan Renema,†* Andreas Schmidt,‡ Jack J.A. van Asten,† Frank Oerlemans,§ Kurt Ullrich,¶ Bé Wieringa,∥ Dirk Isbrandt,‡ and Arend Heerschap†

As a model for guanidinoacetate methyltransferase deficiency (GAMT deficiency) in humans, a gene knockout mouse model was generated. Here we report on several metabolic abnormalities in these mice, observed by in vivo and in vitro MR spectroscopy. In 1H MR spectra of brain and hindleg muscle a clearly reduced signal of creatine (Cr) was observed in GAMT–/– animals. Analysis of the 1H MR spectra of GAMT–/– brain indicated little or no increase of a signal for guanidinoacetate (Gua). In proton MR spectra of muscle, a broad signal of low intensity was observed for Gua. However, substantial Gua accumulation in intact muscle tissue was unequivocally confirmed in high-resolution magic angle spinning spectra, in which the Gua signal was resolved as one clear sharp singlet. In 31P MR analysis of brain and hindleg muscle a strongly reduced phosphocreatine (PCr) content was shown. In addition, a signal of phosphorylated Gua at 0.5 ppm upfield of PCr was observed, which was assigned to PCr. The second signal decreased when ischemia was applied to the muscle and recovered after ischemia was released. Overall, the in vivo 31P and 1H MR spectroscopy of GAMT–/– mice is similar to that of human GAMT deficiency. This opens up new avenues for the fundamental study of tissue-type dependence of creatine synthesis and transport and for diagnostic and therapeutic aspects of creatine deficiencies in humans. Magn Reson Med 50: 936–943, 2003. © 2003 Wiley-Liss, Inc.

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Guanidinoacetate methyltransferase (GAMT) is a key enzyme in the biosynthesis of creatine (Cr), a process that mainly occurs in pancreas and liver (1). The first step on this synthetic route involves transfer of the amidino group of arginine to glycine, to yield ornithine and guanidinoacetate (Gua). Subsequently, GAMT facilitates the methylation of Gua to Cr.

Cr is a central compound in the energy metabolism of tissues such as muscle and brain. In these tissues it can be phosphorylated to phosphocreatine (PCr) using one of the five isoenzymes of creatine kinase (CK). Finally, Cr and PCr are catabolized to creatinine (Crn), which is excreted through the urine. To maintain a constant body pool of Cr, its breakdown and build-up via dietary intake and endogenous synthesis must be balanced and its transport across membranes of cells in various tissues must be tightly regulated (2).

GAMT deficiency was described for the first time as a new inborn error of metabolism (3) in an infant with extrapyramidal movement disorder, who had low Crn in serum and urine (3–6). To date, several cases of GAMT deficiency have been reported (7–11). Patients show symptoms in a wide range of severity (12) including developmental retardation (4,7–9,11), muscle hypotonia (3,5,8,11), extrapyramidal movement abnormalities (3–5,7), and epileptic seizures (7,8,11).

Localized MR spectroscopy (MRS) of the brain of young patients has played a decisive role in the elucidation of the pathophysiology of GAMT deficiency. A strongly reduced Cr signal was revealed by 1H MRS (3,4,7–11,13). In 31P MR spectra PCr was decreased and a new resonance appeared, which was assigned to phosphorylated Gua (PGua) (7,10,13). This assignment was confirmed by an additional resonance in the 1H MR spectrum at 3.8 ppm, which is likely to arise from Gua protons (3,9,10,13).

Oral intake of arginine resulted in an increase of the Gua signal intensity in the proton MR spectra (3,13), while intake of Cr led to a partial restoration of the Cr signal (3,4,7,9–13), decrease of the Gua signal (3,7,10,13), and improvement of clinical symptoms (3–13).

To our knowledge, in only two patients with GAMT-deficiency was MRS of muscle performed. The first case study reported on a considerable signal for Cr in 1H MR spectra of muscle but not in brain, and no peak at 3.8 ppm (10). In the 31P MR spectrum only one peak was observed around 0 ppm, which was assigned to PCr. The second case study showed both PCr and PGua in 31P MR spectra of muscle, while no 1H Cr resonance was observed in brain (14).
Detailed study of the pathophysiology of GAMT deficiency and possible treatment strategies is difficult in humans, as the disease is very rare. Therefore, GAMT-deficient knockout mice (GAMT−/−) were generated (15). This article presents the first results of 31P and 1H MRS of hindleg muscle and brain in these mice. The purpose of this work was to validate the GAMT−/− mice as a useful model for Cr deficiency in human patients and to initiate a study into tissue-type and dietary-composition dependent accumulation of Gua derivatives.

**MATERIALS AND METHODS**

**Animals**

GAMT−/− mice were generated by homologous recombination in embryonic stem cells (15). As a reference, heterozygous (+/-) and homozygous (+/+ wildtype-like (wt) littermates of the GAMT−/− animals were used. In previous studies (not published), Isbrandt et al. showed that no differences could be observed between these groups and that animals of both genotypes can serve as control. For the determination of the T2 of water and methyl Cr protons in hindleg muscle, six wt-like mice with a randomly inbred mixed background of C57Bl/6 and 129/Ola were used.

During the MR experiments, all animals were anesthetized using 1% isoflurane in a gas mixture of 50% O2 and 50% N2O delivered through a facemask. Breathing frequency was monitored and a warm waterbed was used to keep the animals at normal body temperature. All experiments were approved by the local animal ethics committee.

**MR Equipment**

The in vivo MR experiments were performed on a 7.0 T magnet (Magnex Scientific, Abingdon, UK) interfaced to an S.M.I.S. spectrometer (Surrey Medical Systems, Surrey, UK) operating at 300.22 MHz for 1H and at 121.53 MHz for 31P. The horizontal magnet was equipped with a 150 mT/m shielded gradient set and had a free bore size of 120 mm.

High-resolution magic angle spinning (HRMAS) spectroscopy experiments were performed on muscular tissues using a Bruker DRX 500 MHz spectrometer.

**MR Spectroscopy Studies**

To get a good overview of the phenotype of the GAMT−/− mice, 1H and 31P MR spectroscopy studies of both hindleg muscle and of brain were performed. In the 1H MR experiments Cr refers to the sum of Cr and PCr.

For the 1H MR studies on the mouse brain, a 12 mm surface coil anatomically shaped for mouse brain was used. Additional perspex earbars provided stereotactic fixation. Localized spectroscopy was performed using a STEAM sequence (TE = 10 ms, TM = 15 ms, TR = 5000 ms, 64 averages) with VAPOR water suppression (16) and outer volume suppression. A 3 × 3 × 3 mm3 (27 μl) voxel was positioned in the center of the brain guided by gradient echo images. In this study, measurements were performed in seven GAMT−/− and five wt animals.

The 31P MR brain measurements were done using two surface coils, tuned to the 31P frequency and working in quadrature mode. A large 1H surface coil was utilized for shimming, imaging, and localization. To prevent contamination with signal from the masseter muscles, an ISIS sequence (17) was used (TR = 7000 ms, 8 step phase cycle, 512 averages), consisting of a 6.5 kHz adiabatic hyperbolic secant pulse for inversion, together with a 50 μs rectangular 90° RF pulse for excitation. A voxel with size 6.5 × 6.5 × 4.5 mm3 (190 μl) was positioned in the brain guided by gradient echo images. The mouse head was fixated using the quadrature surface coil and earbars.

1H MR measurements on the mouse hindleg were done using an Alderman-Grant type of coil (18), oriented at the magic angle (55°) with respect to the main magnetic field to reduce dipolar interactions (19). The same STEAM sequence was used as in the 1H brain measurements (voxel size 1.8 × 1.8 × 3.4 mm3, TR = 5 sec, 128 averages). For absolute quantification, the T2 for water and Cr protons was determined by acquiring a suppressed and water-suppressed 1H spectra at six different echo times in a separate experiment.

31P MR pulse acquire experiments (TR = 7000 ms, 64 averages) on the hindleg were done using a three-turn solenoid coil, together with a proton Alderman-Grant type of coil for shimming. 31P MR experiments on hindleg muscle and brain and 1H MR experiments on hindleg muscle were performed in seven GAMT−/− and seven wt animals.

Ischemic occlusion of skeletal muscle was applied to two GAMT−/− animals by clamping with a diaphragm plate, which allowed reversible and reproducible obstruction of blood flow through the hindlimb (20). 31P MR spectra were obtained using a pulse-acquire sequence (TR = 1400 ms, 76 averages). After four reference spectra an ischemic period of 25 min was applied (14 spectra), followed by a recovery period of 16 min (9 spectra).

Three GAMT−/− and three wt animals were sacrificed and their hindleg muscles were removed and snapshot frozen in liquid nitrogen. The intact tissue was measured in a 12 μl rotor using HRMAS spectroscopy at 4°C with a spinning rate of 5 kHz. Residual rotor space was filled with a PBS-buffer/D2O mixture (30/70). For 1H MR a CPMG pulse sequence was employed with a 90° pulse of 7.2 μs, while the time between the 180° pulses was one rotor period. Water was suppressed by presaturation and a T2 filter of 50 ms was applied.

**Chemical Analysis of Total Cr Pool**

To serve as a reference to the 1H MR spectroscopy brain metabolite values, the size of the total Cr pool (i.e., Cr plus PCr) was determined by chemical analysis. Five C57Bl/6 mice and five mice with a randomly inbred mixed background of C57Bl/6 and 129/Ola were sacrificed, after which their brains were snapshot frozen in liquid nitrogen. These frozen brains were crushed and extracted with 0.6 M HClO4. The extracts were neutralized with 3 M KOH and the PCr and Cr were assayed via coupled enzymatic reactions (21). PCr and Cr concentrations were added to yield the total Cr pool size.
Data Processing

The $^1$H MR data of brain were analyzed using the LCModel 5.2-2 software package (22). The unsuppressed water signal was used for eddy current correction, phasing, and normalization. The chemically determined Cr value was used to scale the MR determined metabolite values of the wt mice such that Cr values agreed. This scaling factor was also applied to the MR spectra of the GAMT mice, yielding absolute quantitative levels for all metabolites in the $^1$H MR brain spectra.

The $^{31}$P MR data of brain and muscle and the $^1$H MR data of hindleg muscle were analyzed using the MRUI software package (23). In the data processing of $^{31}$P MR brain spectra, the first-order phase was constrained. In GAMT−/− animals the damping of the PGua signal was set equal to the damping of the PCr signal. All signals were fitted assuming a Lorentzian line shape model function and normalized to the $^1$H $\beta$-NTP signal since the $^1$H $\beta$-NTP signal was affected by a chemical shift artifact caused by the 6.5 kHz excitation pulse.

For the $^1$H MR spectra of muscle, the first-order phase was constrained to zero because the first acquired data point was obtained at the top of the echo. Signals were eddy current-corrected using the unsuppressed water signal. The ratio of the linewidths of taurine and Cr was determined in wt animals. Cr linewidths were constrained to this ratio in both GAMT−/− and wt animals. All signals were fitted assuming a Gaussian line shape model function.

Absolute quantification of the Cr concentration in muscle was performed using the unsuppressed water signal. To correct for $T_2$ relaxation, the obtained water and methyl Cr spectra at six different echo times were fitted using MRUI (23) and the results at different echo times were fitted using a monoeXponential curve. The proton signals in hindleg muscle are considered to be fully relaxed at a TR of 5 sec, and hence no $T_1$ corrections needed to be applied. Tissue water content was assumed to be 77% (24).

In the fitting procedure of the $^{31}$P MR spectra data of hindleg muscle, the first-order phase was constrained. No other prior knowledge was used. Signals were fitted assuming a Lorentzian line shape model function and normalized to the $^1$H $\beta$-NTP signal.

For brain and muscle, the pH was calculated from the shift in resonance position (S) of the inorganic phosphate (Pi) signal compared to the resonance position of PCr (25) using the equation $pH = 6.75 + \log((3.27-S)/(S-5.63))$.

A two-tailed Mann-Whitney test was used to test if GAMT−/− from wt metabolite levels were significantly different. Differences were considered significant at $P < 0.05$.

RESULTS

Muscle

The $^{31}$P MR spectra of GAMT−/− animals showed striking differences with respect to wt spectra: PCr was significantly reduced and a new resonance appeared 0.5 ppm upfield of PCr (Fig. 1), which was assigned to PGua (13). Signal intensities of the residual PCr and newly formed PGua showed large fluctuations between animals. However, in all individual mice the ratio PGua/PCr was larger than 1, ranging from 1.4–8.8 (mean 3.4 ± 3). We observed that with higher PGua levels PCr levels tended to be equally lower; however, significance was not reached for this observation. Other metabolite levels (normalized to

### Table 1

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<td>wt</td>
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<td>0.40 ± 0.08</td>
<td>0.76 ± 0.33</td>
<td>1.78 ± 0.42</td>
<td>3.4 ± 3</td>
<td>7.18 ± 0.06</td>
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<tr>
<td>wt</td>
<td>1.09 ± 0.52</td>
<td>0.59 ± 0.16</td>
<td>1.36 ± 0.46</td>
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<td>7.03 ± 0.08</td>
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<td>GAMT−/−</td>
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<td>0.71 ± 0.26</td>
<td>0.31 ± 0.10</td>
<td>0.28 ± 0.07</td>
<td>0.90 ± 0.2</td>
<td>7.11 ± 0.04</td>
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All values are presented as mean ± SD. PME: phosphomonoesters.

*Muscle metabolite signal values are normalized to the resonance of $\beta$-NTP, brain metabolite signals to $\gamma$-NTP (see Materials and Methods section for details).
the β-NTP signal) and tissue pH were not significantly different between GAMT−/− animals and their littermates (Table 1).

In order to find out whether PGua was metabolically active, ischemia of hindleg muscle was applied and monitored by 31P MRS (Fig. 2). The spectrum before ischemia showed hardly any PCr signal, but a large PGua signal was apparent, especially in animals that were completely deprived of Cr. During ischemia the PGua signal decreased while Pi signals increased. After ischemia PGua increased again to its initial value. The time course of PGua depletion and recovery was of similar order as that of PCr in wt animals during and after ischemia (20).

The mono exponential fits of the water and Cr signals at different echo times yielded $T_2$ values for water and Cr proton spins of 21.9 ± 0.1 ms and 83 ± 4 ms, respectively. Those values were used to calculate absolute Cr concentrations.

Cr was significantly reduced in hindleg muscle of GAMT−/− animals (8.9 ± 3.8 mM) compared to wt animals (28.4 ± 2.6 mM) (Fig. 3). Despite the presence of PGua in the 31P spectra, we observed hardly any signal for Gua in the 1H MR spectra. Even though the spectra were obtained at the magic angle (55°) to reduce dipolar interactions, the Gua signal appeared at best as a broad line (Fig. 3c) in the spectra where Cr was largely reduced. It is of note that, although we did not explicitly study the relation between Cr content of the food and (P)Cr and PGua signals in muscle, the muscle 1H MR spectrum showing largely reduced Cr (Fig. 3c) was taken in the same period, i.e., in mice eating the same batch of chow, as the 31P MR spectrum, which shows hardly any PCr (Fig. 2a).

In an attempt to clarify the apparent discrepancy of the broad Gua signal, HRMAS experiments on muscular tissue of GAMT−/− and wt animals were performed (Fig. 4). This approach again yielded a clearly reduced Cr signal, but now at 3.78 ppm a clear signal for Gua appeared as a distinct singlet (Fig. 3c).

Brain

Next we used 1H and 31P MRS to establish whether the brains of GAMT−/− animals show the same metabolic features as brains of GAMT-deficient patients.

The chemical-determined values of the total Cr pool were 8.7 ± 1.0 and 8.6 ± 1.4 µmol/g wet weight for C56Bl/6 and mice with a randomly inbred mixed background of C57Bl/6 and 129/Ola, respectively. Using a brain density of 1.05 g/cm³ (26), this could be converted to
tissue concentrations of 8.3 ± 1.0 and 8.2 ± 1.3 mM, respectively.

Compared to wt, the Cr signal was significantly reduced in brain of GAMT–/– mice, although some broad signals remained present at 3.0 and 3.9 ppm (Fig. 5). No other metabolites analyzed from the 1H MR brain spectra were significantly different between wt and GAMT–/– animals (Table 2). In addition, no significant increase could be observed at 3.8 ppm, where Gua resonates. Other metabolites resonate around this position and might obscure the Gua signal. However, in some wt and GAMT–/– animals there was a residual signal present at ~3.8 ppm after fitting the spectra. This residue was of low SNR and could not be quantified properly.

To see whether the residual Cr in GAMT–/– animals could result from intake via the mother milk of heterozygous mother animals, 1H MR spectra of brain of GAMT–/– animals born to GAMT–/– mothers were also obtained. There was no distinction between 1H MR spectra from GAMT–/– animals nourished by heterozygous or homozygous mutant females (data not shown), indicating that the genotype of the mother is not an important parameter for the metabolic profile of the offspring.

In 31P MR spectra of the brain of GAMT–/– animals, a strongly reduced PCr signal was evident compared to the 31P MR spectra of brain of wt animals (Fig. 6). In addition, a signal for PGua was observed 0.5 ppm upfield of PCr in GAMT–/– animals, at a much lower intensity than in muscle. The mean ratio of PGua/PCr in brain was smaller than 1 (0.9 ± 0.2). Other metabolite levels, normalized to γ-NTP, did not differ between GAMT–/– and wt animals in brain (Table 1).

## DISCUSSION

In this study we showed that combined use of in vivo and in vitro MR spectroscopy is a valuable tool in elucidating the metabolic status of tissues of animal models for Cr deficiency. In combination with the introduction of the new GAMT knockout mouse model, this opens up many possibilities for studying the integral-physiological fate of precursors and products at various steps in the Cr-biosynthetic pathway, in much more detail than is possible in human patients.

### MR Spectra of Brain and Muscle of GAMT-Deficient Mice

1H MR spectra of both brain and muscle of GAMT–/– mice showed reduced levels compared to wt animals, but no complete absence of Cr. The Cr concentration in the food of the mice likely accounts for the continued presence of Cr.

There is evidence that the permeability of the blood-brain barrier for Cr is limited and that the brain partially relies on its own Cr synthesis (1,27,28). Therefore, it is...
interesting to note that the reduction of Cr in GAMT−/− mice relative to the signal in wt animals appeared to be larger in brain than in muscle, which could be explained by a slower uptake of orally ingested Cr in the brain. As absolute concentration data from proton MR spectroscopy of mouse brain metabolites have, to our knowledge, not been published, we compared our inferred values to metabolite concentrations measured in rat brain (16). Cr concentrations found in wt animals are almost similar to those found in rat. Strikingly, NAA and glutamate values are significantly lower in mouse brain than in rat brain. NAA is considered a neuronal marker (29) and the observed concentration is in agreement with other biochemical studies done in mice (29). The difference in rat and mouse NAA levels corresponds well with the factor of about 2-fold difference in cholinergic neurons per section of equal thickness in mouse and rat brain (30). Glutamate, important for synaptic signaling activity in brain, is stored mainly in neurons (31, and references therein), which explains the lower concentration found in mouse brain compared to rat brain. Although the concentration of choline compounds is in good agreement with values found in the human brain (32), it is higher than the concentration observed in rat brain. Why the concentration of choline compounds occurs at higher levels in mouse brain than in rat brain is currently not known.

Visibility of Guanidinoacetate

If brain tissue ATP concentration is assumed to be ~3 mM (35) also in GAMT mice, PGua levels in brain can be estimated from the observed signal ratios (Table 1) to be around 1 mM. Assuming that about 75% of the total pool is phosphorylated, as is the case for Cr, the total Gua pool in the brain would be on the order of 1.3 mM. At this low concentration the signal of Gua may be hard to detect, especially as it occurs at 3.8 ppm in a spectral area with several other overlapping resonances.

In muscle, by using analogous reasoning, Gua levels can be estimated to be ~19 mM, based on an assumed ATP concentration of 8 mM (36) and a phosphorylated fraction of 75%. Despite this high Gua tissue concentration, hardly any signal could be observed at the resonance position of Gua in our in vivo 1H muscle spectra. In cases where a signal for Gua was observed, this signal appeared at best as a broad line. Because the muscle 1H spectra of the GAMT−/− animals were obtained at the magic angle, dipolar interactions cannot account for the large linewidth (19). However, HRMAS spectra of intact muscle of GAMT−/− animals showed a clear singlet for Gua at 3.78 ppm. As HRMAS spectra were obtained at a temperature of 4°C, the difference could possibly be due to conformational or environmental changes in vivo.

Remarkably, the Gua signal appeared to be more distinct in the 1H MR spectra of muscle if a low Cr signal was present, and sometimes very low PCr and high PGua concentrations were observed in the 31P spectrum of muscle. Whether the PGua concentration decreases on increasing PCr concentration should be investigated further using controlled Cr feeding of GAMT−/− animals.

A remarkable observation from MR spectra of GAMT−/− compared to wt was that the PGua content in muscle was much higher than in brain, although in brain we observed a more constant level of PGua and PCr. GAMT transcript and protein was found to be present in several normal human and mouse tissues, including in muscle at high levels (15). In muscle also, the enzyme AGAT, involved in synthesis of Gua, occurs at high levels (33). However, since the GAMT enzyme is lacking in GAMT−/− mice this does not necessarily mean that Gua will accumulate. Altered transport or change in feedback control of metabolic steps may also affect the rate of accumulation of precursors, and these factors may vary between tissues. Currently, we lack proper explanations for the differences in PGua and PCr levels between tissues. It has been suggested that brain is relatively self-sufficient in terms of Cr biosynthesis (34), whereas muscle is not. However, no data are available about the relative capacities of the different biosynthetic steps leading to the formation of Gua (and the conversion to Cr) or for the relative import capacities for Cr in different tissues. The permeability of the blood–brain barrier is suggested to be limited for Cr (1) and, throughout the brain, Cr transporters are not equally distributed. Cr uptake capacity almost surely differs between neural and glial cell types (28). In muscle fibers Cr transporters may be more equally distributed.

**FIG. 6.** Localized 31P MRS of brain of wt (a) and GAMT−/− mice (b) in vivo. Peak assignments: phosphomonoesters (PME); inorganic phosphate (Pi); phosphocreatine (PCr); phosphorylated guanidinoacetate (PGua). Vertical scaling is arbitrary.
Phosphorylated Guanidinoacetate as a Substitute for Phosphocreatine

The large variation in the amount of PGua and residual PCr observed in GAMT–/– muscle is most likely caused by fluctuations in Cr intake via the food, or fluctuation in cellular import capacity, which in muscle is dependent on the feeding status and presumably other physiological parameters (2).

Based on the observation that CK can play a role in the phosphorylation of Cr analogs (37–39) it is likely that CK activity is the source of PGua in GAMT–/– animals. The utilization of PGua during ischemia was very similar to that of PCr in wt mice (20), despite the fact that the conversion rate may be lower, as shown in brain (13). We do not know, however, if the cytosolic BB-CK in brain and MM-CK in muscle have equal in vivo capacity to convert Gua to PGua. A role of mitochondrial CKs (which also occur at quite different concentrations in muscle and CNS) in this process can be excluded. Study of the utilization of Cr analogs by mitochondrial CKs in (permeabilized) tissue has demonstrated that these isoforms have no role in Gua phosphorylation (37,38).

Comparison With Patient Case Studies

The reduction of (P)Cr in the 31P and 1H MR spectra, and the appearance of PGua in the 31P MR spectra of the brain in our mouse model, revealed a striking similarity to the metabolic findings in human patients (3,4,7–11,13). For muscle, however, only two case studies have been reported so far to our knowledge (10,14). In one case the 31P MR spectrum showed PCr and PGua, as observed in our study in GAMT–/– mice (14). In the other case, however, a large signal at about 0 ppm in the 31P MR spectrum was assigned to PCr (10). No signal for Gua was found in the human 1H MR spectrum (10). As shown in the present study, Gua in muscle gives rise to a broad signal which is hard to detect in vivo even at very low Cr concentrations. Brain MRS in GAMT-deficient patients showed different levels of PCr after oral administration of Cr (3,4,7,9–13). Variable Cr levels were also found in GAMT–/– mice and levels of Cr after oral administration of Cr (3,4,7,9–13). Brain MRS in GAMT-deficient patients showed different levels of Cr (3,4,7,9–13). Variable Cr levels were also found in GAMT–/– mice and levels of Cr after oral administration of Cr (3,4,7,9–13).

Although different inborn errors of Cr metabolism give rise to a reduction of the Cr signal in MR spectra (40–43), the simultaneous observation of reduced Cr signals and new signals for PGua is highly indicative of GAMT deficiency and can be diagnostically applied. In summary, our results indicate that Cr synthesis is blocked in GAMT-deficient mice, providing evidence for the existence of only one biosynthetic pathway for Cr production in mammals. We can now use this model for an in-depth study of the effects of oral intake of Cr, the pathophysiological thresholds in the Gua levels in different tissues, and the monitoring of treatment of this disease using MRS.

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