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Mild guanidinoacetate increase under partial guanidinoacetate methyltransferase deficiency strongly affects brain cell development

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ABSTRACT

Among cerebral creatine deficiency syndromes, guanidinoacetate methyltransferase (GAMT) deficiency can present the most severe symptoms, and is characterized by neurocognitive dysfunction due to creatine deficiency and accumulation of guanidinoacetate in the brain. So far, every patient was found with negligible GAMT activity. However, GAMT deficiency is thought under-diagnosed, in particular due to unforeseen mutations allowing sufficient residual activity avoiding creatine deficiency, but enough guanidinoacetate accumulation to be toxic. With poorly known GAA-specific neuropathological mechanisms, we developed an RNAi-induced partial GAMT deficiency in organotypic rat brain cell cultures. As expected, the 85% decrease of GAMT protein was insufficient to cause creatine deficiency, but generated guanidinoacetate accumulation causing axonal hypersprouting and decrease in natural apoptosis, followed by induction of non-apoptotic cell death. Specific guanidinoacetate-induced effects were completely prevented by creatine co-treatment. We show that guanidinoacetate accumulation without creatine deficiency is sufficient to affect CNS development, and suggest that additional partial GAMT deficiencies, which may not show the classical brain creatine deficiency, may be discovered through guanidinoacetate measurement.

Introduction

Deficiency of guanidinoacetate methyltransferase (GAMT), creatine (Cr) synthesis second enzyme (gene *GAMT*), was the first identified cerebral creatine deficiency syndrome (Stöckler et al., 1994; Schulze et al., 1997; Braissant, 2014; Stöckler-Ipsiroglu et al., 2014). The two other creatine deficiencies are arginine:glycine amidinotransferase (AGAT; Cr synthesis first enzyme; gene *GATM*) (Item et al., 2001) and Cr transporter (gene *SLC6A8*) (Salomons et al., 2001) deficiencies. GAMT and AGAT deficiencies are autosomal recessive inherited metabolic diseases, while SLC6A8 deficiency is X-linked. Their hallmark is the virtual Cr absence when measured by ^{1}H-MRS in cortex and basal ganglia. Central nervous system (CNS) is the main tissue affected, with patients developing neurological symptoms in infancy, in particular intellectual disability/developmental delay and speech acquisition defects (Schulze, 2003). GAMT deficiency can present a wide variety of symptoms from mild forms to very severe neurological phenotypes (Mercimek-Mahmutoglu et al., 2006). Guanidinoacetate (GAA) accumulation upstream of the GAMT enzymatic block is thought to cause these severe phenotypes ranging from intractable epilepsies to autistic and automutilating behaviors as well as extrapyramidal syndrome (Schulze et al., 2001; Schulze, 2003).

Unlike microcapillary endothelial cells at blood–brain barrier (BBB), surrounding astrocytes do not express SLC6A8, resulting in low Cr uptake efficiency and forcing the brain to complete its Cr needs by expressing AGAT and GAMT (Braissant et al., 2001; Béard and Braissant, 2010; Tachikawa and Hosoya, 2011; Braissant, 2012; Lowe et al., in press). Under GAMT deficiency, cerebral Cr comes from periphery and the low BBB-crossing efficiency makes CNS Cr-deficient. Moreover, GAA accumulates in CNS (uptake from peripheral excess, endogenous AGAT activity with no functional GAMT; Braissant, 2012). Despite low BBB Cr permeability, SLC6A8 expression in microcapillary endothelial cells allows high Cr dosage treatment of GAMT-deficient patients who nevertheless often remain with severe intellectual disability/developmental

Abbreviations: AAV, adeno-associated viruses; AGAT, arginine:glycine amidinotransferase; BBB, blood–brain barrier; CNS, central nervous system; Cr, creatine; DAPI, 4′,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; GAA, guanidinoacetate; $GABA_AR$, $GABA_A$ receptor; GAD, glutamate decarboxylase; GalC, galactocerebroside; GAMT, guanidinoacetate methyltransferase; GAP43, growth-associated protein 43; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; ^{1}H-MRS, proton-coupled magnetic resonance spectroscopy; LC–MS/MS, liquid chromatography coupled to mass spectrometry in tandem; MAP2, microtubule-associated protein 2; MBP, myelin basic protein; MOI, multiplicity of infection; NeuN, neuronal nucleus protein; NFM, medium weight neurofilament; ORF, open reading frame; p-NFM, phosphorylated medium weight neurofilament; RNAi, RNA interference; ROC, rat hybridoma between olidodendrocytes and C6 astroglioma; scAAV, self-complementary AAV; shRNA, small hairpin RNA; SLC6A8, creatine transporter; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling.

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delay (Stöckler-Ipsiroglu et al., 2014). Few prenatally-diagnosed patients were treated pre-symptomatically and developed normally (Schulze and Battini, 2007; El-Gharbawy et al., 2013; Viau et al, 2013).

GAMT deficiency is treatable, potentially under-diagnosed due to limited awareness, and was thus suggested as a newborn screening candidate through GAA measure (Mercimek-Mahmutoglu et al., 2012; El-Gharbawy et al., 2013; Pasquali et al., 2014). All GAMT-deficient patients identified so far were diagnosed by ^{1}H-MRS-measured brain Cr deficiency and showed negligible residual GAMT activity (Mercimek-Mahmutoglu et al., 2006, 2014; Stöckler-Ipsiroglu et al., 2014; http://www.LOVD.nl/GAMT). Apart from identifying new patients with CNS Cr deficiency and negligible GAMT activity, newborn screening through GAA measurement may in addition identify GAMT-deficient patients with sufficient GAMT residual activity to escape diagnosis as Cr non-deficient, but sufficiently accumulating GAA to be toxic. Newborn screening may also identify other patients with levels of GAA altered independently of GAMT deficiency, as found recently for example for arginase deficiency (Amayreh et al., 2014).

While mostly known for its ATP regeneration and buffering role (Wallimann et al., 1992; Brosnan and Brosnan, 2007), Cr was recently suggested to act also as neurotransmitter (Almeida et al., 2006; van de Kamp et al., 2013). Although of poorly known pathophysiology, Cr deficiency probably affects brain energy and neurotransmission. In CNS, GAA is toxic through $GABA_A$ receptor ($GABA_AR$) activation which may explain its epileptogenic action (Neu et al., 2002), and disturbs energy through Na^+-K^+-ATPase/creatine kinase complex inhibition (Zugno et al., 2006). A *Gamt*$^{-/-}$ mouse was developed showing the biochemical GAMT deficiency characteristics (Cr deficiency and GAA accumulation), which however does not present the patients severe neurological symptoms (Schmidt et al., 2004).

To better understand GAA toxicity under GAMT deficiency on developing CNS, rat organotypic 3D brain cell cultures were transduced by adeno-associated viruses (AAV) driving *Gamt* gene knock-down by RNA interference (RNAi).

Materials and methods

Selection of GAMT shRNAs

Three 21 nt siRNA sequences specific for rat *Gamt* ORF (GenBank no. J03588) were selected according to the Wang and Mu (2004) algorithm and used to generate 66 bp small hairpin RNA (shRNA)-encoding DNA inserts, later cloned into pRNAT-CMV3.2/Neo under cytomegalovirus (CMV) promoter (Genscript) (Fig. 1A). GAMT-1, 2 and -3 shRNAs' potential RNAi efficiency was evaluated by Dual Luciferase Assay by co-transfection of pRNAT-CMV3.2/Neo/GAMT-1/2/3/empty with psiCHECK-GAMT (Promega; vector expressing GAMT ORF downstream of *Renilla* luciferase ORF) and a control firefly luciferase-expressing vector, into ROC cells (rat hybridoma between olidodendrocytes and C6 astroglioma) plated at 80% confluence using jetPEI reagent (Polyplus). Cells were washed and lysed after 48 h with lysis buffer, and cell extract was measured by Dual Luciferase Assay (Promega) using a TD-20/20 luminometer (Turner Designs). *Renilla* luciferase activity was normalized with firefly luciferase activity. GAMT-2 shRNA presented the highest RNAi potential, with 81% decrease of *Renilla* luciferase activity (Fig. 1B). pRNAT-CMV3.2/Neo/GAMT-2, mismatched and scrambled controls were used to demonstrate the specific GAMT knock-down. After 24 h, stably transfected ROC cells were selected with 300 μg/ml neomycin. Cells were harvested 6 days after transfection and their GAMT protein level was quantified by western blotting (Fig. 1C).

Adeno-associated virus production

Several AAV genotypes/serotypes were tested to uncover the most efficient vector for developing our GAMT deficiency model in immature brain cells (Tenenbaum et al., 2004): AAV2,2/1,2/5,2/8 (single-stranded) and scAAV2,2/5,2/8,2/9 (self-complementary) with multiplicity of infection (MOI) of 100, 300 and 1000 viral genomes per cell (data not shown). AAV2 and scAAV2 proved to be the most efficient in our cultures, where they were able to target neurons, astrocytes and oligodendrocytes (Fig. 2A). GAMT RNAi-expressing AAV2 viruses were prepared by cloning GAMT-2 shRNA and its mismatched and scrambled controls into pAAV-hrGFP (Agilent Technologies) under CMV promoter (pAAV-hrGFP/GAMT-2/mismatched/scrambled). pAAV-hrGFP co-expresses hrGFP, also under CMV promoter, for the follow-up of transduced cells. AAV2/GAMT-2/mismatched/scrambled viruses were prepared with AAV-293 cells and the Agilent AAV Helper Free system, according to supplier instructions (Agilent Technologies). Viral particles were purified with the AAV Purification Virakit according to manufacturer's instructions (Virapur) and stored at −20 °C until use. Titration of total AAV2 particles (empty and full viruses) was performed with the AAV2 Titration ELISA (Progen Biotechnik GMBH) according to manufacturer's instructions, using a Nanodrop 2000c at 450 nm (Thermo Fisher Scientific). Titration of efficient AAV2 particles was performed by qPCR. Purified AAV2/GAMT-2/mismatched/scrambled were obtained with titers of 10^{10}. Self complementary AAV2 (scAAV2) viruses transducing GAMT RNAi were customized under the construction ITR-(U6 promoter)-(GAMT-2/mismatched/scrambled)-(CMV promoter)-EGFP-PolyA-ITR (SignaGen). EGFP allowed the follow-up of transduced cells. Purified scAAV2/GAMT-2/mismatched/scrambled were obtained with titers of 10^{12}–10^{13} (SignaGen). AAV2/GAMT-2 particles were used to transduce ROC cells (MOI: 300) to validate the AAV2-transduced *Gamt* knock-down by GAMT-2 shRNA (Fig. 1C), while scAAV2/GAMT-2/mismatched/scrambled viruses were used to generate a GAMT deficiency model by transduction of GAMT-2 shRNA in 3D organotypic developing brain cell cultures in aggregates (Fig. 1D).

3D organotypic cultures of developing brain cells

Pregnant rats (Sprague–Dawley 300 g, Charles River) were handled according to the Swiss Academy for Medical Science rules. Their embryos were dissected out at E15.5 days to prepare 3D primary cultures of brain cells in aggregates from their mechanically-dissociated whole brains, as previously described (Braissant et al., 2002). Aggregates develop with neurons, astrocytes and oligodendrocytes organized in a 3D network acquiring a tissue-specific pattern resembling that of the *in vivo* brain, and are therefore considered as organotypic brain cell cultures (Fig. 2A). They express AGAT, GAMT and SLC6A8 in the same manner as the *in vivo* brain and synthesize their own Cr, suggesting that they behave as *in vivo* CNS for Cr synthesis and transport (Braissant et al., 2001, 2008, 2010). They are grown under continuous gyratory agitation (80 rpm) in a serum-free, Cr-free and chemically-defined medium, in which precursor amino acids for GAA and Cr synthesis (Arg, Gly and Met) are provided by the DMEM amino acid mix used. To generate RNAi-driven *Gamt* knock-down, cultures were infected at day *in vitro* 0 (DIV0) by either AAV2 or scAAV2 vectors co-transducing GFP (AAV2) or EGFP (scAAV2) expression and a specific GAMT/mismatched/scrambled shRNA with MOI of 100, 300 or 1000. Additional cultures were treated, from DIV 5 on, with a final concentration of 10 or 30 μM GAA at every medium change until harvest to verify GAA specific effects. Co-treated cultures were supplemented with 1 mM Cr with every media change until harvest. Aggregates were harvested at DIV8, 18 and 28. Aggregate pellets were collected by sedimentation, three rapid rinsing with cold PBS, and either frozen in liquid nitrogen for metabolite (liquid chromatography coupled to tandem–mass spectrometry, LC/MS–MS) and protein (western blotting) analysis, or embedded in cryo-medium (Tissue-Tek O.C.T., Digitana) and frozen in liquid nitrogen-cooled isopentane for immunohistological analysis. Samples were kept at −80 °C until use. Culture medium was also harvested at DIV8, 18 and 28 and kept at −80 °C for metabolite analysis by LC/MS–MS.