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A MODEL OF CREATINE DEFICIENCY SYNDROMES IN 3D BRAIN CELL CULTURES BY KNOCKDOWN OF GAMT AND SLC6A8 GENES

BEARD Elidie

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Service de Biomédecine

A MODEL OF CREATINE DEFICIENCY SYNDROMES IN 3D BRAIN CELL CULTURES BY KNOCKDOWN OF GAMT AND SLC6A8 GENES

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par

Elidie BEARD

Biologiste diplômée de l'Université de Besançon, France

Jury

Prof. Vincent Mooser, Président
Dr. Olivier Braissant, PD MER, Directeur
Prof. Peter Clarke, Co-Directeur
Dr. Vassili Valayannopoulos, Expert
Prof. Eric Raddatz, Expert

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	Monsieur Dr Vassili **Valayannopoulos**

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Madame Elidie Beard

Master en Neurosciences de l'Université de Franche-Comté, France

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DEVELOPING OF A MODEL OF CREATINE DEFICIENCY SYNDROMES IN RAT BRAIN CELLS 3D CULTURES BY KNOCKDOWN OF GAMT AND SLC6A8 GENES

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Prof. Vincent Mooser

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ABSTRACT

Creatine plays essential roles in energy metabolism by the interconversion, by creatine kinase, to its phosphorylated analogue, phosphocreatine, allowing the regeneration of ATP. Creatine is synthesized in mammals by a two step mechanism involving arginine:glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT). Creatine is taken up by cells by a specific transporter, SLC6A8.

Creatine deficiency syndromes, due to defects in GAMT, AGAT and SLC6A8, are among the most frequent inborn errors of metabolism, and are characterized by an absence or a severe decrease of creatine in central nervous system, which is the main tissue affected. While it is known that AGAT, GAMT and SLC6A8 are expressed in CNS, many questions remain on the specific effects of AGAT, GAMT and SLC6A8 deficiencies on brain cells. Our aim was to develop new experimental models of creatine deficiencies by knockdown of GAMT and SLC6A8 genes by RNAi in 3D organotypic rat brain cell cultures in aggregates.

Specific shRNAs for the GAMT and SLC6A8 genes were transduced in brain cell aggregates by adeno-associated viruses (AAV). The AAV-transduced shRNAs were able to efficiently knockdown the expression of our genes of interest, as shown by a strong decrease of protein by western blotting, a decrease of mRNA by qPCR or characteristic variations of creatine and guanidinoacetate by tandem mass spectrometry.

After having validated our experimental models, we have also shown that GAMT and SLC6A8 knockdown affected the development of astrocytes and neurons or oligodendrocytes and astrocytes, respectively. We also observed an increase of cell death and variations in activation pattern of caspase 3 and p38 MAPK pathways, involved in apoptosis, in our experimental model.

RESUME

La créatine joue un rôle essentiel dans le métabolisme cellulaire par sa conversion, par la creatine kinase, en phosphocreatine permettant la régénération de l'ATP. La synthèse de créatine, chez les mammifères, s'effectue par une réaction en deux étapes impliquant l'arginine:glycine amidinotransférase (AGAT) et la guanidinoacétate méthyltransférase (GAMT). L'entrée de créatine dans les cellules s'effectue par son transporteur, SLC6A8.

Les déficiences en créatine, dues au déficit en GAMT, AGAT ou SLC6A8, sont fréquentes et caractérisées par une absence ou une forte baisse de créatine dans le système nerveux central. Alors qu'il est connu que AGAT, GAMT et SLC6A8 sont exprimés par le cerveau, les conséquences des déficiences en créatine sur les cellules nerveuses sont peu comprises. Le but de ce travail était de développer de nouveaux modèles expérimentaux des déficiences en Cr dans des cultures 3D de cellules nerveuses de rat en agrégats au moyen de l'interférence à l'ARN appliquée aux gènes GAMT et SLC6A8.

Des séquences interférentes (shRNAs) pour les gènes GAMT et SLC6A8 ont été transduites par des vecteurs viraux AAV (virus adéno-associés), dans les cellules nerveuses en agrégats. Nous avons ainsi démontré une baisse de l'expression de GAMT au niveau protéique (mesuré par western blot), et ARN messager (mesuré par qPCR) ainsi qu'une variation caractérisitique de créatine et guanidinoacétate (mesuré par spectrométrie de masse).

Après avoir validé nos modèles, nous avons montré que les knockdown de GAMT ou SLC6A8 affectent le développement des astrocytes et des neurones ou des oligodendrocytes et des astrocytes, respectivement, ainsi qu'une augmentation de la mort cellulaire et des modifications dans le pattern d'activation des voies de signalisation impliquant caspase 3 et p38 MAPK, ayant un rôle dans le processus d'apoptose.

PUBLICATIONS

- **E.Béard**, L. Hanna-el-Daher, H.Henry and O.Braissant (**manuscript in preparation**). A new model of GAMT deficiency in developing brain cells by AAV2-transduced RNAi.

- **E.Béard** and O.Braissant (**2010**). **Journal of Neurochemistry**, **115**:297-313. Synthesis and transport of creatine in the central nervous system: Importance for cerebral functions.

- O.Braissant, **E.Béard**, C.Torrent and H.Henry (**2010**). **Neurobiology of Disease**, **37**:423-433. Dissociation of AGAT, GAMT and SLC6A8 in CNS: Relevance to creatine deficiency syndromes.

- O.Braissant, H.Henry, **E.Béard** and J.Uldry (**2011**). **Amino Acids, 40**:1315-1324. Creatine deficiency syndromes and the importance of creatine synthesis in the brain.

- O.Braissant, J.Uldry, **E.Béard** (**2011**). Creatine, central nervous system and creatine deficiency syndromes. In: *Recent Research in Modern Medicine*, O.Braissant, H.Wakamatsu, I.Kuo-Kang, K.Allegaert, Y.Lenbury, A.Wachholtz, eds. (WSEAS press, Cambridge, UK), pp. 189-196.

LIST OF ABBREVIATIONS

5-HT: serotonin
AAV: adeno-associated virus
AD: Alzheimer's disease
AGAT: L-arginine:glycine amidinotransferase
BBB: blood-brain barrier
B-CK: brain creatine kinase
bp: base pairs
C6: rat astroglioma cells
cDNA: complementary DNA
cGFP: coral GFP
CK:creatine kinase
Cr: creatine
CSF: cerebrospinal fluid
DIV: day in vitro
dsRNA: double-stranded RNA
GAA: guanidinoacetate
GAMT: guanidinoacetate methyltransferase
GAMT $^{-/-}$: GAMT KO mouse
GFAP: glial fibrillary astrocyte protein
HRP: horseradish peroxydase
IEM: inborn errors of metabolism
ITRs: inverted terminal repeats
KO: knock-out
MAPK: mitogen-activated protein kinase
MBP: myelin basic protein
MCEC: microcapillary endothelial cells
M-CK: muscle creatine kinase
miRNAs: micro RNAs
MOI: multiplicity of infection
mPTP: mitochondrial permeability transition pores
MRS: magnetic resonance spectroscopy
O/N: overnight
ORF: open reading frame
PAZ: piwi/argonaute/zwille
PBS: phosphate buffered saline
PCr: phosphocreatine
PCR: polymerase chain reaction
pNFM: phosphorylated medium-weight neurofilament
PVDF: Immobilon polyvinylidene difluoride
qPCR: real-time quantitative PCR
rAAV: recombinant AAV
RISC: RNA induced silencing complex
RNAi: RNA interference
ROC: Rat oligodendroglia-C6 astroglioma hybridoma cells
ROS: reactive oxygen species
RT: room temperature
SDS: sodium dodecyl sulfate
shRNAs: short hairpin RNAs
siRNAs: small interfering RNAs
SLC6A8: Cr-specific transporter
sMtCK: sarcomeric mitochondrial creatine kinase
TdT: terminal deoxynucleotidyl transferase
TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labelin
uMtCK: ubiquitous mitochondrial creatine kina

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CHAPTER I: General introduction

I. Creatine deficiency syndromes

1. Creatine metabolism and transport

Creatine (Cr) (α-N-methylguanidino acetic acid) is a nitrogenous organic amino acid playing essential roles to maintain energy levels in most tissues, and is highly active in particular in those with high and/or fluctuating energy demand such as skeletal muscle, heart and brain (Wallimann et al., 1992). The Cr/phosphocreatine (PCr)/creatine kinase (CK) system not only serves as intracellular buffer for ATP, but also as high-energy phosphate shuttle from mitochondrial sites of production to cytoplasmic sites of consumption (Figure 1).

Part of intracellular Cr is converted by CK into the high-energy compound PCr. Four CK isoforms have been described, based on tissue expression and subcellular distribution: two cytosolic forms, M-CK (in muscle) and B-CK (in brain) and two mitochondrial forms, sarcomeric muscle form (sMtCK) and brain form called ubiquitous MtCK (uMtCK) (Schlattner et al., 2006; Wallimann et al., 1992). Each CK isoform has a specific function, mitochondrial CKs using ATP to convert Cr to its high energy phosphorylated analogue PCr for export to cytoplasm, and cytosolic CKs using PCr to convert ADP to ATP by the transfer of N-phosphoryl group at sites of energy demand, and to convert excess ATP to PCr for energy storage (Wallimann et al., 1992; Wallimann et al., 1998).

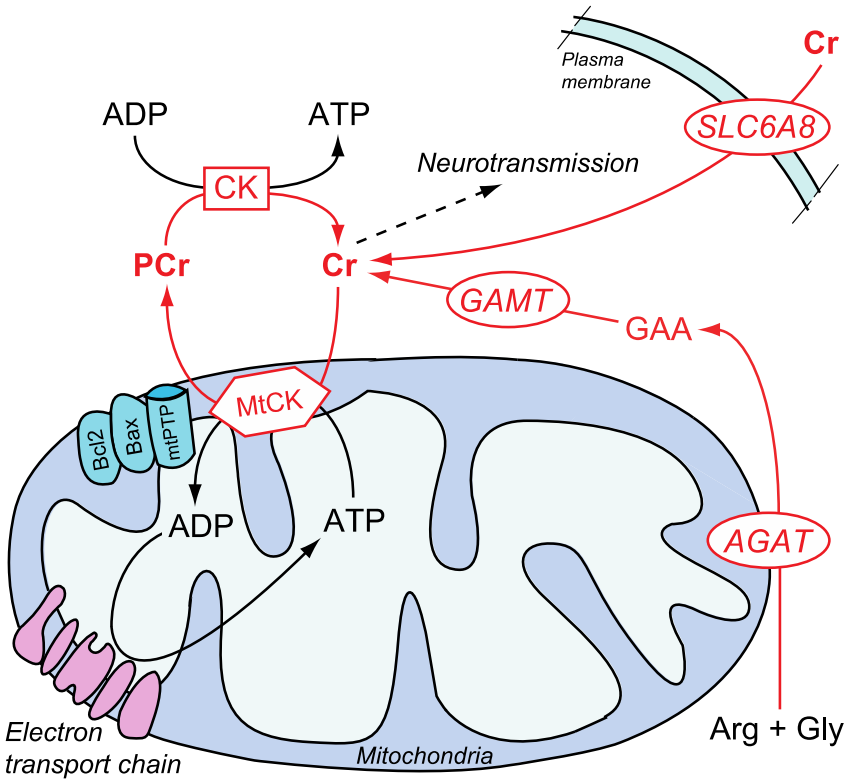


Figure 1 : Synthesis and function of creatine (Cr). Cr synthesis requires the presence of two enzymes, L-arginine: glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT); cells take up Cr by a specific transporter, SLC6A8. Mitochondrial or cytosolic creatine kinase (CK) convert Cr to its high-energy counterpart phosphocreatine (PCr). PCr dephosphorylation yields energy, as ADP is converted to ATP. Besides its function in cellular energy, Cr may also be involved in neurotransmission (figure taken from Béard and Braissant, 2010).

Total Cr (Cr + PCr) in 70kg young adults amounts for approximately 120g. Both Cr and PCr are non-enzymatically and irreversibly degraded to creatinine at a rate of about 1.7% of total body pool per day (Wyss and Kaddurah-Daouk, 2000). Creatinine is excreted via kidneys, the amount of creatinine eliminated being proportional to muscle. The amount of Cr needed to renew the degraded one, depends on creatinine excretion but accounts for about 2g/day (Casey and Greenhaff, 2000).

In human, half of Cr stores originate from food, mainly fresh meat, fish and dairy products, while the other half is biosynthesized endogenously through a pathway involving two enzymes: L-arginine:glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT). From the precursors arginine (limiting factor) and glycine, AGAT catalyzes the formation of guanidinoacetate (GAA) and ornithine. This step occurs mostly in kidney where Cr level exerts a negative feedback loop on AGAT gene regulation at the transcriptional level (Brosnan et al., 2009; McGuire et al., 1984). The second reaction, catalyzed by GAMT and occurring mostly in liver, uses S-adenosylmethionine to methylate GAA, producing Cr and S-adenosylhomocysteine (Brosnan et al., 2009). AGAT and GAMT expression are positively regulated by growth hormone, thyroid hormone and sex hormones (Carlson and Van Pilsum, 1973; Guthmiller et al., 1994; Lee et al., 1994; McGuire et al., 1984). While AGAT and GAMT highest expression is found in kidney and liver, respectively, they are also expressed at lower levels in various other tissues, including CNS (Braissant et al., 2001; Lee et al., 1998; Wyss and Kaddurah-Daouk, 2000).

Creatine is transported by blood to Cr-requiring tissues, and taken up in cells with high energy demand by a Cr-specific transporter, SLC6A8. SLC6A8 is a member of the solute carrier family 6, a large family of membrane transporters that mediate the transport of various neurotransmitters and/or amino acids across plasma membrane with the co-transport of two Na^+ and one Cl^- (Chen et al., 2004). This transport is electrogenic and driven by the sodium gradient established by Na^+/K^+-ATPase (Dai et al., 1999). SLC6A8 expression is important in tissues with high energy demand, such as skeletal muscle, heart, brain, retina, or with important (re)absorptive functions, such as kidney and intestine (Braissant et al., 2001; Guimbal and Kilimann, 1993; Mak et al., 2009; Peral et al., 2002). Cr uptake is regulated by different factors, like insulin which activates Na^+/K^+-ATPase and presumably increases the

driving force for Cr uptake (Snow and Murphy, 2001), or the Na^+ gradient and intracellular Cr concentration (Brosnan and Brosnan, 2007).

2. Creatine metabolism, transport and functions in the brain

2.1. Functions of creatine in CNS

The Cr/PCr/CK system plays essential roles in the brain to maintain the high energy levels necessary for CNS (maintenance of membrane potential and ions gradient, Ca^{++} homeostasis, neurotransmission, intracellular signaling systems as well as axonal and dendritic transport) (Wyss and Kaddurah-Daouk, 2000). The brain represents only 2% of the body mass but may spend up to 20% of total energy consumption. The Cr/PCr/CK system also plays essential roles in CNS during development. Different studies showed that CK isoforms are found highly concentrated in cerebellum (especially in glomeruli structures of cerebellar granular layer), choroid plexus and in hippocampal granular and pyramidal cells (Hemmer et al., 1994). It must be noted that hippocampus is important for learning and memory function and can be severely affected in Alzheimer's disease (AD). B-CK is much higher than uMtCK in cerebellar Bergmann glial cells and hypothalamus, where it plays essential functions in regenerating ATP for glutamate clearance during excitatory synaptic transmission (Oliet et al., 2001). Knock-out for one CK isoform (B-CK or uMt-CK) showed behavioral abnormalities and defects in formation and maintenance of hippocampal mossy fiber connections. Double knockout mice displayed decreased body weight and severely impaired spatial learning, lower nest building activity and reduction of hippocampal size (Jost et al., 2002; Streijger et al., 2005). All these studies demonstrate the key function of CK in brain energy metabolism (Hemmer and Wallimann, 1993).

Apart of its functions in energy, Cr may play other roles, as recently been suggested in particular in CNS. Cr was suggested as essential CNS osmolyte. Astrocytes placed in hyperosmotic shock significantly increase their Cr uptake, suggesting that Cr can work as compensatory osmolyte (Alfieri et al., 2006). Conversely, astrocytes exposed to hypo-osmotic swelling conditions stimulate the release of their osmotically active Cr (Bothwell et al., 2001). In contrast, ammonium-exposed microcapillary endothelial cells (MCEC) *in vitro* stimulate their Cr uptake (Belanger et al., 2007), suggesting that the cells making blood-brain barrier (BBB) (MCEC and astrocytes lining them) behave differentially during swelling. Cr was also proposed as appetite and weight regulator, by acting on specific hypothalamic nuclei (Galbraith et al., 2006).

2.2. Creatine: a co-transmitter in CNS?

Creatine and GAA can affect GABAergic neurotransmission as partial agonists on post-synaptic $GABA_A$ receptors, depending on local GABA concentration (Cupello et al., 2008; De Deyn et al., 1991; Neu et al., 2002). These data stimulated research showing that in organotypic cultures of rat cortex, caudate putamen and hippocampus slices, Cr is released from neurons in a similar manner as classical neurotransmitters. This electrically-evoked exocytotic Cr release mechanism is action potential-dependent, being dependent from Ca^{2+}, inhibited by the Na^+-channel blocker tetrodotoxin and enhanced by the K^+-channel blocker 4-amino-pyridine (Almeida et al., 2006b). According to these *in vitro* studies, Cr may thus also be considered as a neuromodulator or co-transmitter in CNS, which may modulate the activity of post-synaptic receptors such as $GABA_A$ (Almeida et al., 2006a). Interestingly, rat brain

synaptosomes were identified recently as expressing SLC6A8, which allows their active accumulation of Cr (Peral et al., 2010). This suggests the presence of a Cr recapture mechanism in axon terminal membrane, which would fit with a neurotransmitter/co-transmitter function of Cr in CNS (Almeida et al., 2006a).

2.3. AGAT, GAMT and SLC6A8 in the adult brain

It has long been thought that most of the brain Cr was of peripheral origin, be it taken from the diet or synthesized endogenously through AGAT and GAMT activities in kidney and liver respectively (Brosnan and Brosnan, 2007; da Silva et al., 2009; Wyss and Kaddurah-Daouk, 2000). However, Cr is synthesized in the mammalian brain (Van Pilsum et al., 1972) as well as in primary brain cell cultures and nerve cell lines (Braissant et al., 2002; Daly, 1985; Dringen et al., 1998). AGAT and GAMT are expressed in CNS, for which we provided the first detailed analysis demonstrating their expression in all the main structures of the adult rat brain, in every main cell types (neurons, astrocytes and oligodendrocytes; Braissant et al., 2001) (Figure 2). Particularly high levels were found in telencephalon and cerebellum. AGAT was further shown in rat retina (Nakashima et al., 2005), while our data on GAMT were confirmed in mouse and human (Schmidt et al., 2004; Tachikawa et al., 2004).

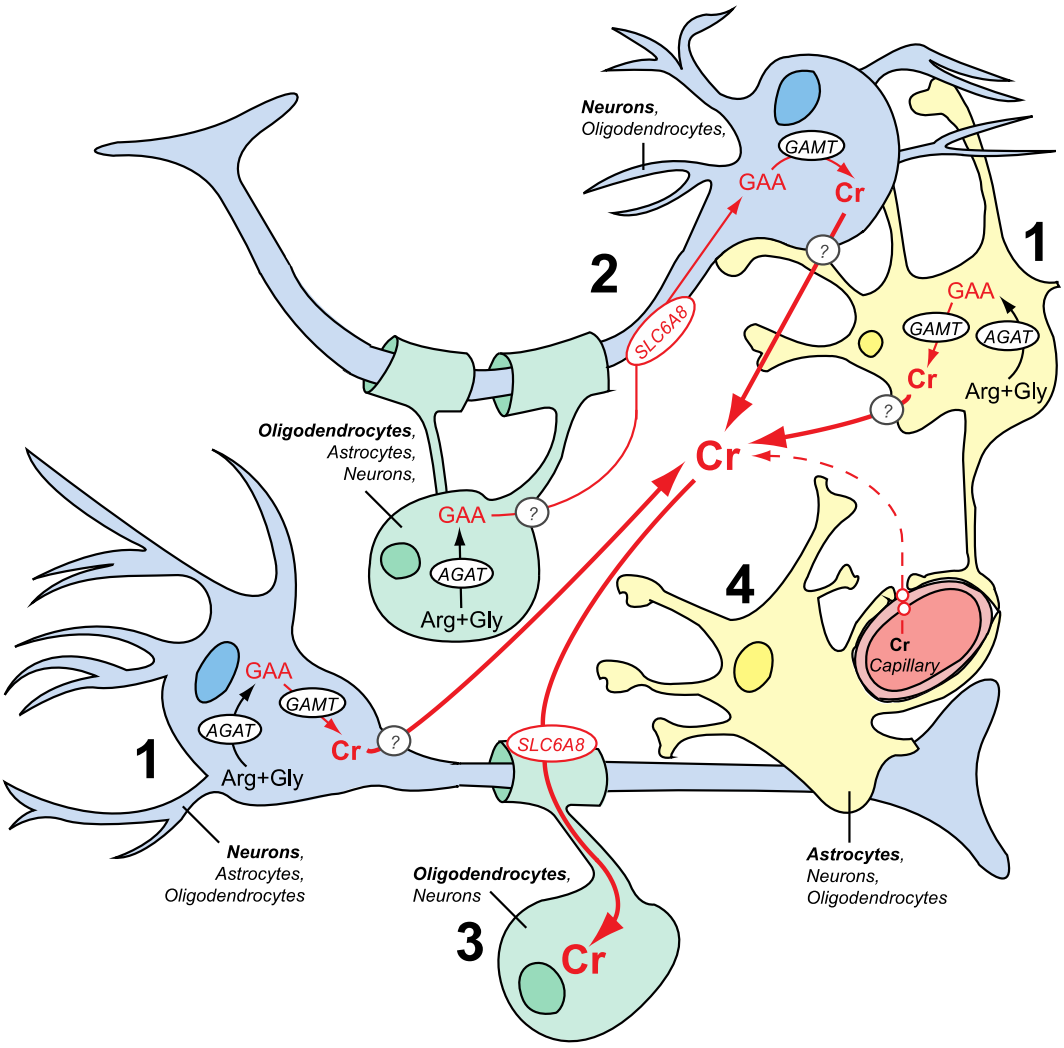


Figure 2 : Model of Cr synthesis and transport in CNS, illustrating the diversity of AGAT, GAMT and SLC6A8 expression by brain cells (figure taken from Béard and Braissant, 2010). **1)** Cr endogenous synthesis within cells co-expressing AGAT and GAMT. **2)** Cr endogenous synthesis through AGAT-expressing cells synthesizing GAA, and GAA uptake by SLC6A8 in GAMT-expressing cells. **3)** Cell expressing only SLC6A8 ("users" of Cr). **4)** Cells silent for AGAT, GAMT and SLC6A8. While microcapillaries express SLC6A8, astrocytic feet lining them do not. This implies that only low amounts of peripheral Cr can enter the brain through the limited endothelial surface that is free of astrocytic feet, and that CNS must also ensure its own endogenous synthesis of Cr. So far, the way Cr (and GAA) can leave cells is poorly known. Cr, creatine; AGAT, L-arginine: glycine amidinotransferase; GAMT, guanidinoacetate methyltransferase; GAA, guanidinoacetate; SLC6A8, Cr transporter.

Organotypic rat cortical cultures, primary brain cell cultures (neuronal, glial or mixed) and neuroblastoma cell lines have a Cr transport activity (Almeida et al., 2006b; Braissant and Henry, 2008; Daly, 1985; Möller and Hamprecht, 1989). *In vivo*, mouse and rat CNS can take up Cr from the blood against its concentration gradient (Ohtsuki et al., 2002; Perasso et al.,

2003). SLC6A8 is expressed throughout the main regions of the adult mammalian brain, particularly in those associated with learning, memory and general limbic functions (Guimbal and Kilimann, 1993; Happe and Murrin, 1995; Saltarelli et al., 1996; Schloss et al., 1994). We provided the first detailed analysis demonstrating that SLC6A8 is found in neurons and oligodendrocytes but, in contrast to AGAT and GAMT, cannot be detected in astrocytes (Braissant et al., 2001). We also showed that in contrast to its absence in astrocytes lining microcapillaries, SLC6A8 is present in MCEC (BBB; Figure 2). These data were confirmed later in rat and mouse (Acosta et al., 2005; Mak et al., 2009; Nakashima et al., 2004; Ohtsuki et al., 2002; Tachikawa et al., 2004; Tachikawa et al., 2008).

2.4. AGAT, GAMT and SLC6A8 in the developing brain

The Cr/PCr/CK system plays essential roles in energy homeostasis during vertebrate embryonic development (Wallimann et al., 1992). Many structures of the vertebrate embryo express CKs at early stages (Dickmeis et al., 2001; Lyons et al., 1991), and Cr concentrations between 5 and 8 mmol/kg wet weight have been measured in CNS of rat and human fetus (Kreis et al., 2002; Miller et al., 2000). Parts of CNS developmental needs for Cr are provided by active transport of Cr from mother to embryo, Cr accumulating in chorioallantoic placenta and yolk sac at concentrations higher than found in maternal and fetal blood, then diffusing down its concentration gradient into fetal circulation (Davis et al., 1978).

AGAT, GAMT and SLC6A8 are well expressed during vertebrate embryogenesis (Braissant et al., 2005; Ireland et al., 2009; Sandell et al., 2003; Schloss et al., 1994; Schmidt et al., 2004; Wang et al., 2007), and probably play essential roles in developing CNS as their

deficiencies lead to neurological symptoms in early infancy and severe neurodevelopmental delay (see below).

Working on the rat, we have provided the first detailed analysis of AGAT, GAMT and SLC6A8 expression in the developing embryonic CNS (Braissant et al., 2005). AGAT and GAMT are expressed in the whole developing CNS parenchyma. However, their low level (GAMT in particular) at early developmental stages suggests that embryonic CNS depends on external Cr supply, be it from embryonic periphery or from maternal origin. This is coherent with SLC6A8 expression in whole embryonic CNS already at early stages (E12.5 in the rat), with particularly high levels in ventricular zone and in choroid plexus, the predominant metabolic exchange zone of fetal brain before differentiation of BBB (Braissant et al., 2005; Braissant et al., 2007).

2.5. Functions of AGAT, GAMT and SLC6A8 in CNS: synthesis or uptake of creatine by the brain?

Total Cr levels and CK activity are well correlated in mammalian CNS (Wyss and Kaddurah-Daouk, 2000), their highest levels being reached in brain cells described with high and fluctuating energy demands, where AGAT, GAMT and SLC6A8 are expressed (Braissant et al., 2007; Hemmer et al., 1994; Wang and Li, 1998).

SLC6A8 absence in astrocytes, particularly in their feet sheathing MCEC, made us suggest that in the mature brain, BBB has a limited permeability for Cr, despite SLC6A8 expression by MCEC and their capacity to import Cr (Braissant et al., 2001; Braissant et al., 2007;

Braissant, 2012). *In vivo* data confirmed this hypothesis: the blood to brain transport of Cr is effective in rodents, but is relatively inefficient (Ohtsuki et al., 2002; Perasso et al., 2003), and long term treatment of AGAT- and GAMT-deficient patients with high doses of Cr allows a very slow and in most cases partial replenishement of their CNS Cr (Schulze and Battini, 2007; Stöckler et al., 2007). Consequently, the brain may depend more on its own Cr synthesis through AGAT and GAMT expression than on Cr supply from the blood (Braissant et al., 2007; Braissant and Henry, 2008). The effective but limited passage of Cr from blood to CNS through BBB may occur through the limited surface of CNS microcapillary endothelium that is free of astrocytic feet (Ohtsuki, 2004; Virgintino et al., 1997) (Figure 2).

One strong argument in favor of the "brain endogenous Cr synthesis" hypothesis comes from Cr measures in CSF of Cr-deficient patient (see Braissant and Henry 2008; and references therein). SLC6A8-deficient patients present normal Cr levels in CSF, but cannot import it from periphery (Cecil et al., 2001; de Grauw et al., 2002). In contrast, GAMT-deficient patients show strongly decreased Cr levels in CSF, but can import it from blood (Schulze et al., 1997). This also suggests that CNS Cr synthesis might still remain operational, although very partially, under SLC6A8 deficiency, while it is completely blocked in AGAT and GAMT deficiencies. Endogenous synthesis or a very efficient uptake from periphery are the two ways available for the brain to secure Cr homeostasis for its energy and functions. As uptake from periphery does not appear efficient, CNS might privilege Cr endogenous synthesis (Braissant, 2012).

The "brain endogenous Cr synthesis" hypothesis might seem contradictory with *in vivo* characteristics of SLC6A8 deficiency, which, despite AGAT and GAMT expression in CNS,

shows an absence (or a very low level) of brain Cr by magnetic resonance spectroscopy (MRS) (Salomons et al., 2001). This apparent contradiction is probably explained by AGAT, GAMT and SLC6A8 expression patterns in CNS. AGAT and GAMT are found in every CNS cell type (Braissant et al., 2001), but appear rarely co-expressed within the same cell (Braissant et al., 2010). This suggests that to allow Cr synthesis in the brain, GAA must be transported from AGAT to GAMT-expressing cells (Braissant and Henry, 2008) (Figure 2). This GAA transfer most propably occurs through SLC6A8, as recently shown by Cr and GAA competition studies, and the use of stable isotope-labelled GAA demonstrating its conversion to Cr by GAMT activity (Braissant et al., 2010). These observations may explain the absence of Cr in CNS of SLC6A8-deficient patient, despite normal expression of AGAT and GAMT in their brain (Braissant and Henry, 2008). Recent studies also demonstrate the importance of SLC6A8 (and taurine transporter) for the GAA transport across BBB and at blood-cerebrospinal fluid (CSF) barrier, as well as in brain parenchymal cells (Tachikawa et al., 2008; Tachikawa et al., 2009; Braissant, 2012).

While we have shown that AGAT and GAMT can be found in all brain cell types (Braissant et al., 2009), various studies demonstrated high levels of GAMT within glial cells (Braissant et al., 2008; Schmidt et al., 2004; Tachikawa et al., 2004), suggesting that the final CNS step for Cr synthesis may predominantly by glial. However, this probably depends on the CNS region considered, as in cortex, only 20% of astrocytes express GAMT, in comparison with 48% of neurons (Braissant et al., 2010).

3. Creatine deficiency syndromes

Inborn errors of Cr biosynthesis and transport, called Cr deficiency syndromes and due to deficiencies in AGAT, GAMT and SLC6A8 (Figures 3-5), are characterized by an absence or a severe decrease of Cr in CNS, as measured by MRS (Item et al., 2001; Salomons et al., 2001; Stöckler et al., 1994; Stromberger et al., 2003). AGAT and GAMT deficiencies are autosomal recessive diseases, while SLC6A8 deficiency is a X-linked disorder. Cr deficiency syndromes appear among the most frequent inborn errors of metabolism (IEM), the prevalence of SLC6A8 deficiency being estimated at 2% of all X-linked mental retardations (Rosenberg et al., 2004) and at 1% of males with mental retardation of unknown etiology (Clark et al., 2006). AGAT and GAMT deficiencies appear rarer. The prevalence of all combined Cr deficiencies was estimated at 2.7% of all mental retardation (Lion-François et al., 2006). CNS is the main organ affected in Cr deficiency syndromes, whose patients show severe neurodevelopmental delay and develop, in early infancy, mental retardation, disturbance of active and comprehensible speech, autism, and automutilating behavior (Battini et al., 2002; de Grauw et al., 2002; Schulze et al., 1997; Stöckler et al., 1996b). No significative effects have been observed in peripheral tissues with high-energy demand (e.g. muscle and heart) in creatine deficient patients, except a faint muscular hypotonia in very rare cases of GAMT deficiency. Patients with GAMT deficiency exhibit a more complex phenotype, including intractable epilepsy, extrapyramidal movement syndromes and abnormalities in basal ganglia (Mercimek-Mahmutoglu et al., 2006; Schulze, 2003; Stromberger et al., 2003). GAMT-deficient patients accumulates GAA because of the block in GAMT enzymatic activity, including in the brain where GAA accumulation is probably due to the combined CNS endogenous AGAT activity (Braissant and Henry, 2008), as well as to a facilitated crossing of BBB by GAA due to increased GAA versus decreased Cr in their blood

(Tachikawa et al., 2004) (Figure 4). GAA toxicity in CNS, and particularly its epileptogenic action (Schulze et al., 2001), may occur through disturbances of GABAergic neurotransmission (see above) (Neu et al., 2002). GAA may also inhibit the complex between Na^+/K^+-ATPase and CK (Zugno et al., 2006). Severe epilepsy may also appear in SLC6A8-deficient patients (Mancardi et al., 2007). This may be due to the observed CNS GAA accumulation in some SLC6A8-deficient patients (Sijens et al., 2005), that could be caused by impairment of GAA transport, through deficient SLC6A8, from AGAT- to GAMT-expressing cells (Braissant et al., 2010) (Figure 5).

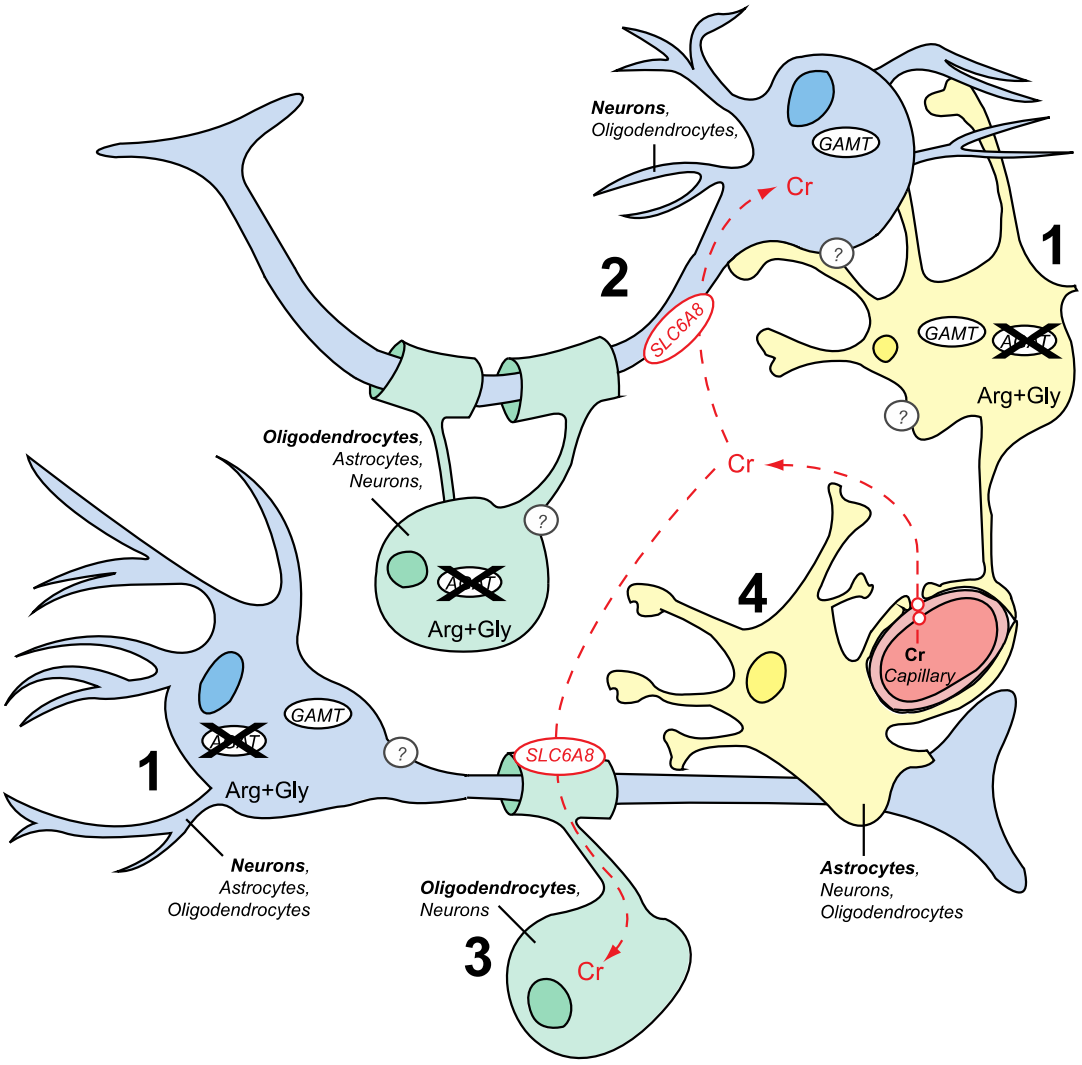


Figure 3 : Model of AGAT deficiency in CNS. See Fig. 2 for abbreviations (figure taken from Béard and Braissant, 2010).

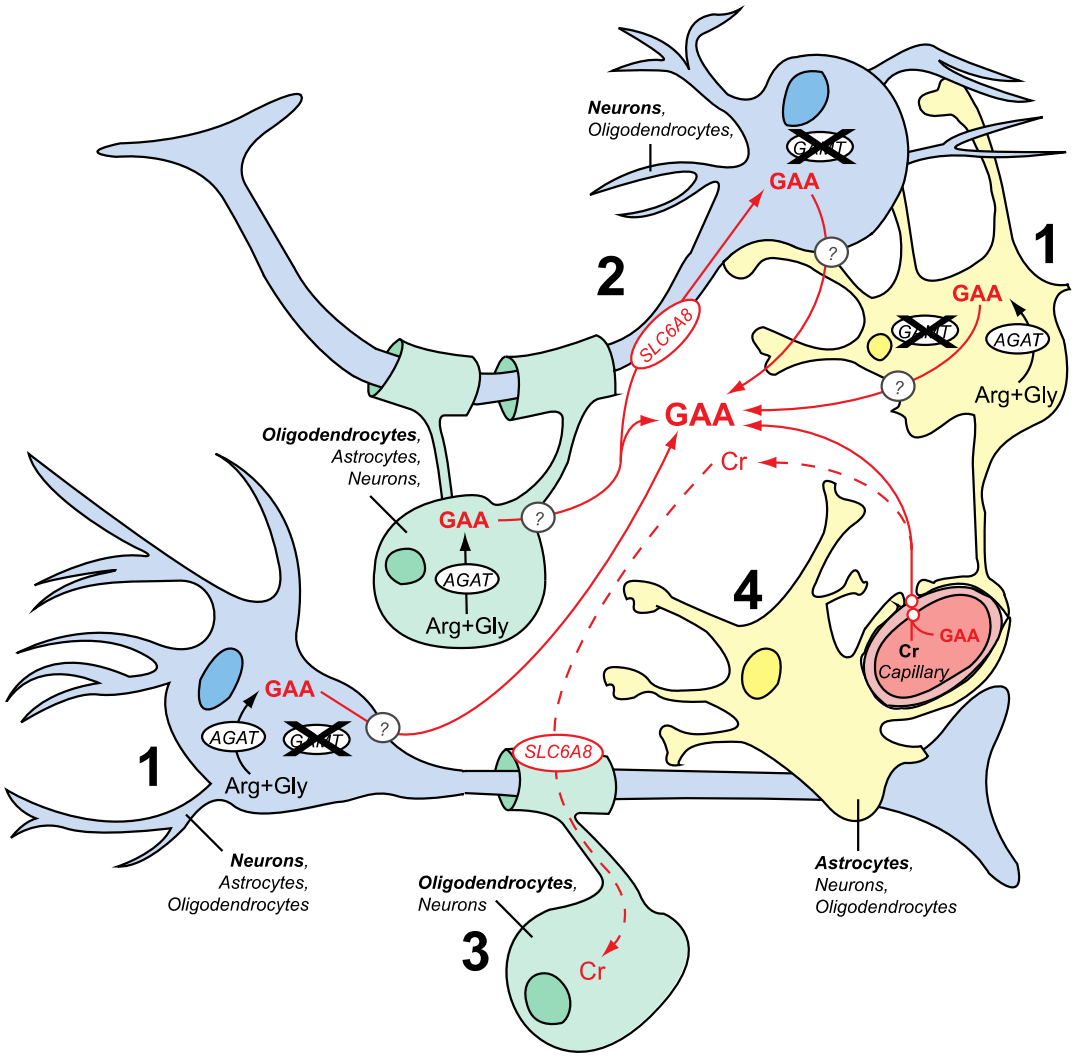


Figure 4: Model of GAMT deficiency in CNS. See Fig. 2 for abbreviations (figure taken from Béard and Braissant, 2010).

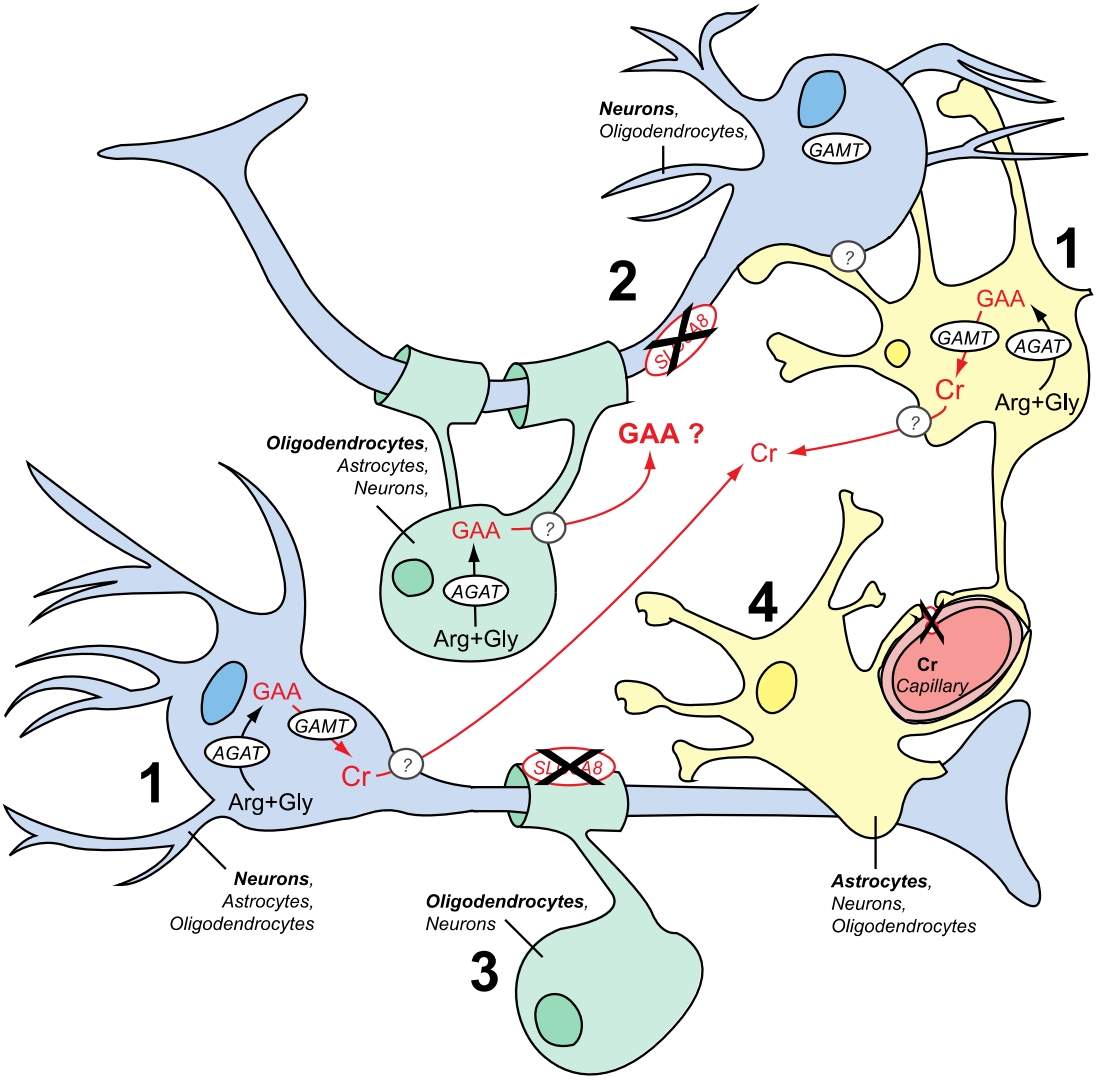


Figure 5: Model of SLC6A8 deficiency in CNS. See Fig. 2 for abbreviations (figure taken from Béard and Braissant, 2010).

The diverse phenotypic neurological spectrum observed in Cr deficiency syndromes show the importance of Cr for psychomotor development and cognitive functions and might be explained by the wide pattern of AGAT, GAMT and SLC6A8 genes in the mammalian brain (Figures 2-5), which has been documented in every main regions of rat (AGAT, GAMT and SLC6A8), mouse (GAMT and SLC6A8) and human (GAMT) CNS (see above). The potential

role of Cr as co-transmitter on the widely distributed GABA post-synaptic receptors (Almeida et al., 2006b) might also contribute to this phenotypic diversity.

AGAT and GAMT patients can be treated with Cr, which strongly improves their neurological status and CNS development (Battini et al., 2002; Item et al., 2001; Schulze et al., 1998; Stöckler et al., 1996a) (Figures 3 and 4). For GAMT-deficient patients, combined arginine restriction and ornithine substitution coupled to Cr treatment decrease GAA and improve clinical outcomes (Schulze et al., 1998; Schulze et al., 2001; Schulze, 2003). However, despite improvement of clinical outcome by Cr supplementation, most AGAT- and GAMT-deficient patients remain with CNS developmental problems. Oral supplementation of Cr is inefficient in replenishing CNS Cr in SLC6A8-deficient patients (Figure 5), who remain with mental retardation, severe speech impairment, and progressive brain atrophy (Bizzi et al., 2002; Cecil et al., 2001; de Grauw et al., 2002). Attempts to treat several SLC6A8-deficient patients with arginine and glycine as precursor of Cr gave encouraging results in the muscular symptoms of the disease during the first months of treatment (Chilosi A. et al., 2008; Valayannopoulos et al., 2011; van de Kamp et al., 2011; Wilcken B. et al., 2008), while it failed to improve the neurological status of four others patients (Fons et al., 2008). The use of a lipophilic Cr-derived compound, creatine ethyl ester, failed to replenish brain Cr concentration in SLC6A8-deficient patients, as well as to improve their neurological status (Fons et al., 2010).

4. Pre-symptomatic treatment of AGAT- and GAMT-deficient patients

Two recent studies have shown that the pre-symptomatic treatment of AGAT and GAMT deficiencies appears to prevent the phenotypic expression of these diseases (Schulze and Battini, 2007). An AGAT-deficient boy, brother of two already affected AGAT-deficient sisters, was diagnosed at birth with the same homozygous mutation as his sisters, and treated orally since the age of 4 months with Cr monohydrate (Battini et al., 2006). Similarly, a GAMT-deficient girl, sister of an already affected GAMT-deficient brother, was diagnosed at birth with the same heterozygous mutations as her brother, and treated orally since the age of 22 days with Cr monohydrate (Schulze et al., 2006). Both patients, over a follow-up of more than 2 years, did not develop the characteristic CNS phenotypic expression of AGAT and GAMT deficiencies (Schulze and Battini, 2007). These two cases suggest that Cr plays essential roles in the development of CNS higher cognitive functions, like speech acquisition, during the first months and years of life, and that treatment with Cr before irreversible damage occurs may prevent clinical symptoms of AGAT and GAMT deficiencies permanently. As described above, the pre-symptomatic treatment with Cr in postnatal stages and during the first years of life may also facilitate the entry of Cr into the brain, at stages where BBB is not as tightly regulated as in more mature stages (Engelhardt, 2003; Virgintino et al., 1997; Braissant, 2012), and where SLC6A8 expression on BBB and choroid plexus may still facilitate entry of peripheral Cr into the brain (Braissant et al., 2005; Ireland et al., 2009), in contrast to adulthood (Braissant et al., 2001).

5. The GAMT $^{-/-}$ mouse

Two *in vivo* models of Cr deficiencies have been described so far: the GAMT and SLC6A8 knock-out (KO) mice.

The GAMT KO mouse (GAMT $^{-/-}$) was generated by removing the first exon of the murine GAMT gene (Schmidt et al., 2004). As GAMT-deficient patients, GAMT$^{-/-}$ mice have markedly decreased Cr and increased GAA levels in CNS and in body fluids (urine, serum and CSF) (Renema et al., 2003), and slowly replenish their brain Cr when fed with Cr.

GAMT $^{-/-}$ mice show increased neonatal mortality, muscular hypotonia and decreased male fertility. The most obvious symptom observed is a reduction of body weight throughout life, more pronounced in females than males. While biochemical alterations of GAMT $^{-/-}$ mice are comparable to those found in GAMT-deficient patients, their neurological and behavioral analysis reveals only mild cognitive impairment and no severe neurological symptoms despite the important accumulation of GAA in the brain (Schmidt et al., 2004; Torremans et al., 2005). In particular, no severe symptoms like epileptic seizures or ataxia are observed. One explanation for this contrast to GAMT-deficient patients may be the use of GAA as CK substrate, leading to the formation of phosphorylated GAA which may play the same role as PCr in providing high-energy phosphates (Ellington, 2001; Renema et al., 2003). If this were true, the same compensation mechanism by GAA may also occur in GAMT-deficient patients, who can accumulate phosphorylated GAA at least in their muscle (Ensenauer et al., 2004; Schulze, 2003).

6. The SLC6A8 knock-out mouse

An *in vivo* model of SLC6A8 deficiency was very recently generated by Skelton et al. (2011). This transgenic mouse was generated with the Cre/loxP recombination system, and consists of the removal of exons 2-4 of SLC6A8 gene. As SLC6A8-deficient patients, SLC6A8 KO mice have no detectable levels of Cr in CNS and show cognitive impairments across a variety of learning and memory tests (spatial and memory deficits during Morris water maze as well as novel object recognition tests). In these mice, the only pathway that was found significantly affected was a disruption of the serotoninergic neurotransmitter system, with an increase of serotonin (5-hydroxytryptamine or 5-HT) in hippocampus and prefrontal cortex (Skelton et al., 2011). This may explain some of the learning and memory deficits observed in SLC6A8-deficient patients, as an increase of 5-HT may alter neuronal structure as well as behavior (Alvarez et al., 2002; Scott et al., 2008).

7. The AGAT $^{-/-}$ mice

AGAT $^{-/-}$ KO mice have been developed recently but nothing has been published so far on the neurological and behavioral effects due to their Cr deficiency (Craigen et al., 2011; Sinha et al., 2011).

II. RNA interference

1. Origins of RNA interference

RNA interference is an evolutionary-conserved process found in many eukaryotic organisms, by which double stranded small interfering RNAs (siRNAs) lead to the degradation of their complementary messenger RNAs. Subsequently, this pathway leads to a decrease of the proteins translated from their respective mRNAs (Almeida and Allshire, 2005). RNAi is a cellular surveillance phenomenon that can repress viral infection, interfere with transposable elements and avoid the occurrence of repetitive genes (transgenes). This mechanism can also regulate gene expression and normal cell development (Bitko and Barik, 2001).

Napoli and Jorgensen were the first to report an RNAi-type of phenomenon in 1990, named "cosuppression" (Napoli et al., 1990). In petunias, they overexpressed an enzyme, chalcone synthase, involved in the anthocyanin biosynthesis pathway and responsible for the petunia's deep violet coloration. They expected to generate violet petunias but the flowers unexpectedly flourished in white. After molecular analysis, they showed that levels of endogenous chalcone synthase were 50-fold lower than in wild-type petunias, leading them to hypothesize that introduced transgene was "cosuppressing" endogenous chalcone synthase (Napoli et al., 1990). In 1992, Romano and Macino reported a similar phenomenon in *Neurospora crassa*, noting that introduction of homologous RNA sequences caused repression of the endogenous gene (Romano and Macino, 1992).

In 1998, the phenomenon of RNA interference (RNAi) was studied in another organism, *Caenorhabditis elegans*, by Fire et al., who reported that injection of double-stranded RNA

(dsRNA) led to an efficient sequence-specific gene silencing in the whole organism (Fire et al., 1998).

The RNAi mechanism was first elucidated by using extracts of *Drosophila melanogaster* embryo which led to the identification of the key factors of RNAi response, the dsRNA processing enzyme Dicer and the RNA-induced silencing complex (RISC) (Hammond et al., 2000). Briefly, the first step consists in generating small specific dsRNAs by Dicer (Figure 6), while in the second step RISC executes RNAi by using dsRNAs as guidance molecules to target the homologous, endogenous mRNA for degradation (Elbashir et al., 2001b; Hannon, 2002; Zamore et al., 2000). In mammalian cells, dsRNA longer than 30 base pairs (bp) trigger the antiviral/interferon pathways, which results in global shut-down of protein synthesis (Gil and Esteban, 2000). RNAi-mediated gene silencing can be obtained in mammalian cell cultures by delivery of either chemically-synthesized short (< 30 nucleotides) double-stranded siRNAs (Elbashir et al., 2001a) or by endogenous expression of short hairpin RNAs (shRNAs) with fold-back stem-loop structure (Brummelkamp et al., 2002; Elbashir et al., 2002; Paddison et al., 2002).

2. Mechanisms of RNA interference

The precise mechanism of RNAi has not been fully elucidated. Hannon et al. have described this mechanism as a dsRNA-mediated process of a sequence-specific mRNA degradation of the target gene, which involves initiation and execution steps (Aagaard and Rossi, 2007; Angaji et al., 2010; Hannon, 2002; Sharp, 2001).

2.1. Initiation step

Depending on the organism, RNAi is triggered by various types of molecule, including experimentally long dsRNAs, aberrantly expressed transgenes, transposons, viral DNA, plasmid-based shRNAs or endogenous micro RNAs (miRNAs) (Hannon, 2002). In the initiation step, these molecules are processed in Dicer by its ribonuclease-III activity (Figure 6), to generate 21-22 nucleotides small interfering RNAs (siRNAs), usually with 2-3 nucleotides 3'-overhangs on each strand (Figure 7) (Aagaard and Rossi, 2007; Bernstein et al., 2001; Elbashir et al., 2001a).

Dicer is a member of the ribonuclease III superfamily of dsRNA-specific endoribonuclease and contains two RNase III domains and one Piwi/Argonaute/Zwille (PAZ) domain (characteristic domain of RISC) (Bernstein et al., 2003); the distance between these two regions of the molecule determines the length of the siRNAs it produces (Figure 6) (Macrae et al., 2006). Dicer is present in many eukaryotic organisms including plants (Golden et al., 2002), *C. elegans* (Knight and Bass, 2001), *Drosophila* (Bernstein et al., 2001), mouse (Bernstein et al., 2003) and human (Provost et al., 2002), suggesting that all these organisms use the same basic mechanism to initiate the RNAi pathway.

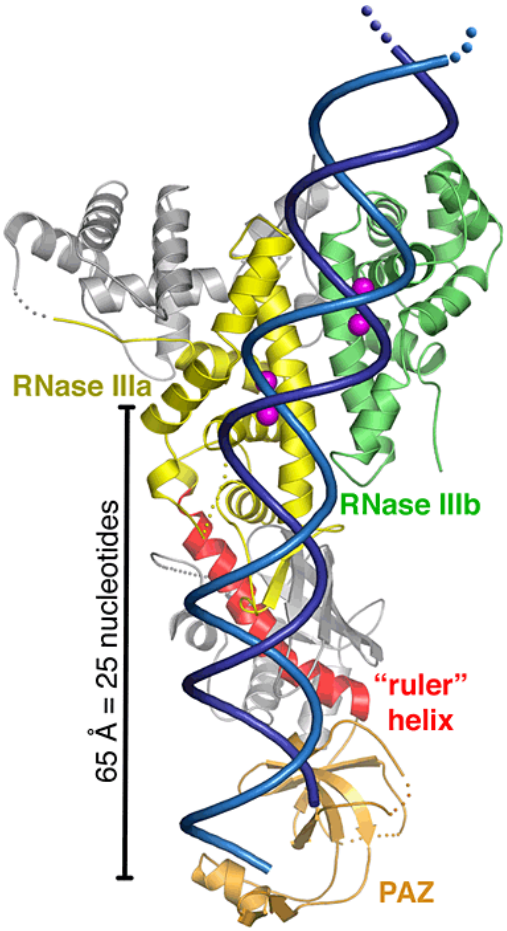


Figure 6: The image shows the structure of Dicer, an enzyme that cleaves RNA. New studies have revealed that Dicer carefully measures and snips strands of RNA into precise increments. When Dicer cuts large strands of RNA into smaller fragments, it initiates the process of RNA interference, which can turn genes off and thereby dictate key developmental events (figure taken from Macrae *et al*, 2006).

2.2. Execution step

In this next step, the siRNA generated by Dicer gets incorporated into the multiprotein RISC present in cytosol (Hannon, 2002). RISC contains a helicase protein and becomes activated after unwinding the double-stranded siRNA into single-stranded RNA. RISC uses the sequence of the antisense strand of the siRNA, retained in this complex, to identify the complementary mRNA, and to promote its endonucleolytic specific cleavage (Nykanen et al., 2001; Tang, 2005). The target mRNA is cleaved at a single site in the centre of the duplex

region between the guide siRNA and the target mRNA, 10 nucleotides from the 5' end of the siRNA (Elbashir et al., 2001b).

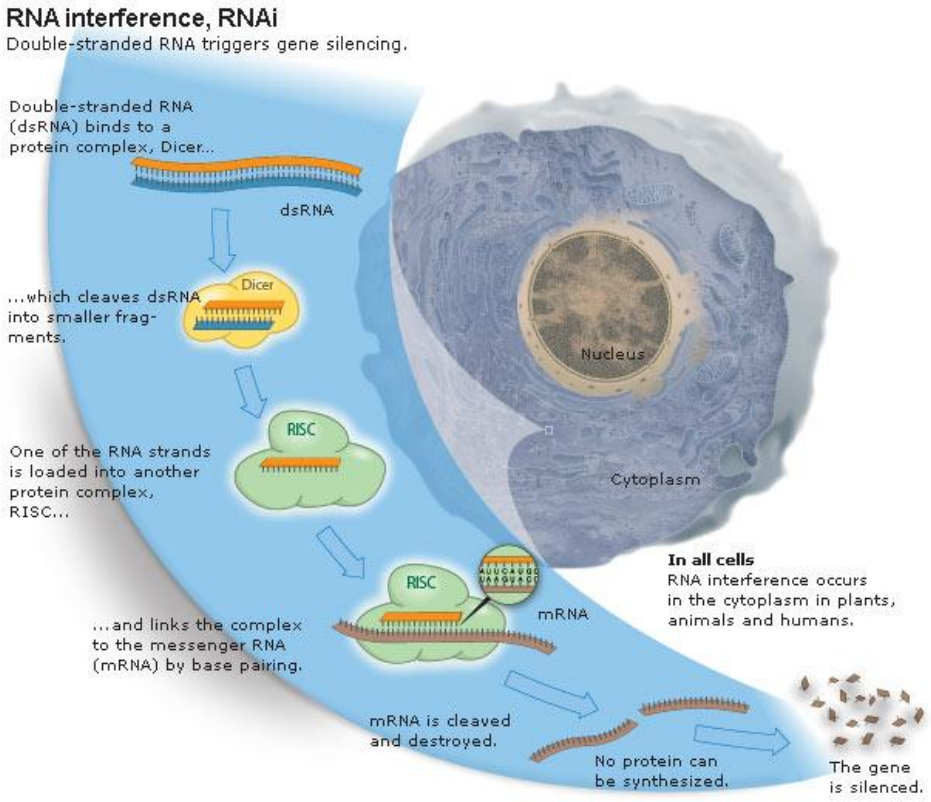


Figure 7: The RNA interference pathway. Long double-stranded RNA (dsRNA) or small hairpin RNA (shRNA) are processed by Dicer to form a small interfering RNA (siRNA), which associates with RNA-induced silencing protein complex (RISC) and mediates target sequence specificity for subsequent mRNA cleavage (see text for further details, figure taken from nobelprize.org).

2.3. RNAi in the brain

RNAi is now widely used to explore the functions of genes in the nervous system of both invertebrates, such as *C. elegans* and *Drosophila,* and vertebrates (mammals, fishes…).

Experimental studies of RNAi against specific genes is also being exploited to understand the pathogenesis of neurological disorders and to develop novel therapeutic strategies for incurable diseases of the nervous system (Davidson and Paulson, 2004; Wood et al., 2003). Despite the ability of siRNA to reduce gene expression in mammalian neuron and glial cell cultures, we must recognize that the brain poses two problems with RNAi technique: the first is the restrictive passive entry of materials through BBB, thus rendering the systemic approach almost ineffective to generate siRNA-mediated gene knock-down in the brain. Secondly, a high degree of cellular diversity as well as networking adds to the complexity in achieving the desired gene knock-down within a specific type of cells in the brain.

A critical goal for clinical neuroscience is the development of techniques with RNAi to understand the several activated pathways causing diseases and the development of effective therapies to prevent the neurological loss and suppressing the gene involved in neurodegeneration process that occurs in neurodegenerative diseases (Davidson and Paulson, 2004; Thakker et al., 2006; Wood et al., 2003).

2.4. shRNAs

In mammalian cells, a global response similar to an antiviral reaction was observed upon the introduction of long dsRNA into the cells (Elbashir et al., 2002). This mechanism is called the off-target effect. dsRNAs longer than 30 nucleotides trigger a dsRNA-mediated cell signaling pathway, including the induction of type I interferon synthesis and activation of interferon-induced enzymes, such as dsRNA-dependent protein kinase and 2', 5'-oligoadenylate synthetase. This global response results in nonspecific inhibition of gene expression, rather than specific knock-down of the targeted protein (Schiffelers et al., 2004). This obstacle has

been overcome by Elbashir and colleagues who found that gene-specific suppression in mammalian cells can be achieved by *in vitro* synthesized siRNA that are 19-22 nucleotides in length, long enough to induce gene-specific suppression, but short enough to avoid the host interferon response (Elbashir et al., 2001a). There are two different approaches to siRNA synthesis: exogenous chemical or enzymatic siRNA synthesis (Elbashir et al., 2001b). They observed a successful knock-down of exogenous gene expression (firefly luciferase expressed from plasmid), and endogenous gene expression (lamin A/C) in mammalian cell lines transfected with siRNA (Elbashir et al., 2001a). The second approach is an intracellular expression using plasmid or viral vectors producing short-hairpin RNAs (shRNAs) and achieve a transient or a permanent knock-down effect (McManus and Sharp, 2002; Tomar et al., 2003). shRNA is processed to siRNA through Dicer-mediated cleavage of the loop portion of the RNA hairpin (Paddison et al., 2002) and with the protein complex RISC allows the destruction of specific mRNA (for more details see section 2.). The design of shRNA is based on naturally occurring miRNAs and their sequence is composed of inverted repeat 19-29 nucleotides with the desired sequence separated by a short loop of 6-9 base pairs (Figure 8) (Siolas et al., 2005). The design of shRNAs is primordial to obtain an efficient knock-down and no deleterious or unwanted effects in the cells. There are several web-based tools available for the design of effective siRNAs, based on various factors, like thermodynamic property, length of the shRNA target site (21 nt), GC content of the target site (range of 30-60% as sequences with <60% GC content have a better chance to be functional shRNAs than those with higher GC content), sequence region (preferably 50-100 nt downstream of the "ATG" start codon), and control of off-target efficiency with BLAST search (Hajeri and Singh, 2009).

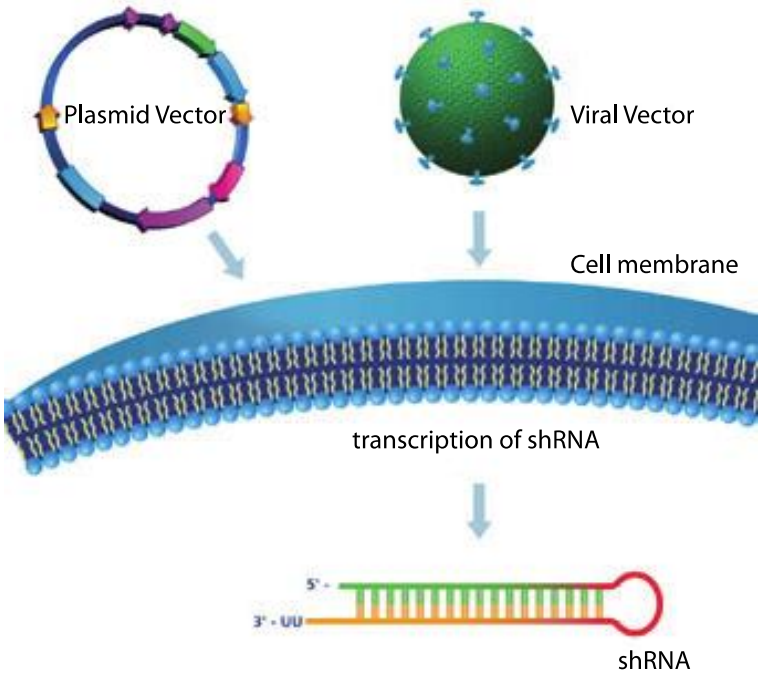


Figure 8: Different delivery system, plasmid or viral vectors, allow the transcription of shRNA in cells to achieve a knock-down effect (figure taken from www.genengnews.com)**.**

2.5. Viral vectors as tools for mediating RNAi

In vivo use of siRNA to target CNS is difficult, due to the complexity of this organ, with its high degree of cellular diversity and to the tightness of BBB. Oral or intravenous administration of siRNA is not feasible because short nucleic acids do not cross BBB and are not efficiently internalized into target cells. Different methods were used like transfection of siRNA with lipidic reagents (Hassani et al., 2005), infusion aided by osmotic minipumps (Thakker et al., 2004), direct injection of siRNA into the brain parenchyma (Isacson et al., 2003) including in combination with electroporation (Akaneya et al., 2005). However, the most promising method so far has been viral delivery of shRNAs (lentivirus, adenovirus,

adeno-associated virus or AAVs) due to the viral ability to transduce both dividing and non-dividing cells (Flotte et al., 1994). AAVs have many advantages, including the lack of pathogenic and immunogenic effects, a long-term expression due to both persistent episomal status and their ability to integrate into the genome of transduced cells, as well as their capacity to transduce both dividing and non-dividing brain cells (Mah et al., 2002; Shi et al., 2009; Stilwell and Samulski, 2003). AAV viral vectors are increasingly employed to develop experimental models of disease or in therapeutic purposes in clinical trials. It was shown that AAV-based delivery of shRNAs partially prevented the clinical, pathological and cytological aspects of the disease in a mouse model of spinocerebellar ataxia type 1 (Xia et al., 2004).

2.6. Adeno-associated virus (AAV)

AAV is a small, non-enveloped virus, member of the *Parvoviridae* family (genus *Dependovirus*), which is not associated with mammal or human diseases. In absence of a helper virus, like adenovirus, human cytomegalovirus or papillomavirus, AAV cannot replicate and establishes a latent infection within the cell, either by a site-specific integration into the host genome (in human, on chromosome 19, designated AAVS1) or by persisting as episomal forms (Buller et al., 1981). The virion shell is approximatively 25 nm in diameter and encapsidates a single-stranded DNA genome of 4.7 kb, converted into a double-stranded DNA after infection. Three functional regions are important in its genome: 2 large open reading frames (ORF), *rep* and *cap*, flanked by inverted terminal repeats (ITRs). The *rep* ORF encodes four replication proteins responsible for viral genome replication, transcriptional control, integration and encapsidation of AAV genome into capsid. The *cap* ORF encodes the viral structural proteins VP1, VP2, and VP3, allowing the formation of the icosahedral virion

shell. ITRs are the only cis-acting elements required for extrachromosomal replication and genomic integration of the transgene into the host cell genome (figure 9) (Wu et al., 2006). At least ten different AAV serotypes (AAV-1 to AAV-10) have been described, with different cell tropisms and antigenic properties (Cearley et al., 2008; Cearley and Wolfe, 2006; Zincarelli et al., 2008). A special feature of AAV is that its genome can be packaged into a capsid of its own serotype (isotype), or alternatively can be "cross-packaged" into capsid derived from another serotype (pseudotype).

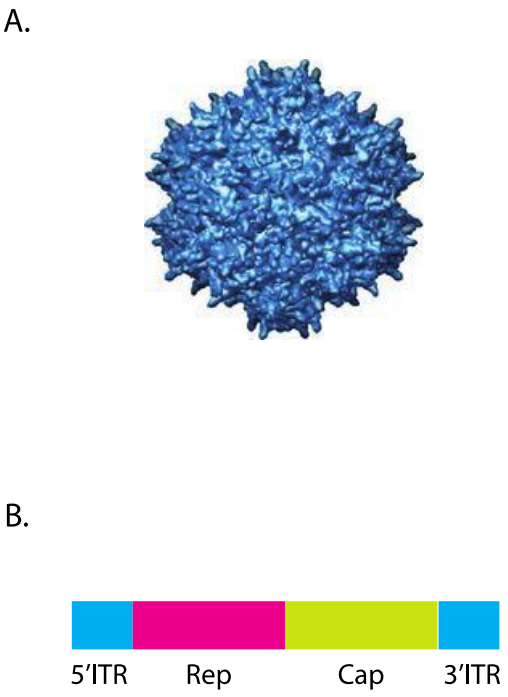


Figure 9: Representation of the adeno-associated-virus. A) Three-dimensional structure of the AAV capsid from VIPER database (http:// viperdb.scripps.edu). **B)** Schematic organization of the wild-type viral genome.

AAV viral infection is a multistep process that requires virions to pass through a series of barriers: receptor binding, cell entry, intracellular trafficking, endosomal release, nuclear entry or viral uncoating. The cellular entry of non-enveloped viruses is often initiated by an

interaction of the capsid proteins and the cell surface glycosaminoglycan receptors. Subsequent secondary interaction of the viral capsid with coreceptors (fibroblast growth factor receptor 1, hepatocyte growth factor receptor and laminin receptor) appears to dictate the virus endocytosis and its intracellular trafficking pathway. The next step is the trafficking of AAV into the nucleus through a mechanism independent of the nuclear pore complex, the uncoating event taking place in nucleus (Wu et al., 2006). Inside the nucleus, the presence or absence of a helper virus dictates whether AAV enters a lytic or latent life cycle. In the absence of helper functions, AAV enters a latent cycle and integrates at a specific locus known as AAVS1 on chromosome 19 (in human) or persist in episomal form. The AAVS1 sequence has been found only in humans and higher primates (McCarty et al., 2004). In contrast, in presence of a helper virus, induction of gene expression and replication take place immediately (Buning et al., 2008).

The efficient transduction may involve cell entry process associated with receptor binding, internalisation and post-entry events such as cellular processing pathways, intracellular transport, or nuclear entry.

For this study, we used recombinant AAV (rAAV) where all viral genes have been deleted, with only the two ITRs remaining. This vector has been used for delivery and expression of shRNA with enhanced safety and reduced immunogenicity.

III. Aims of the thesis

To date no *in vivo* model of Cr deficiency syndrome show neurological symptoms and pathophysiology comparable with those found in Cr deficient patients. Their analysis on the neurological and behavioral points of view demonstrates only mild cognitive impairments, especially for the GAMT KO mouse. At the start of this work, the existing mouse KO models appeared inefficient to reproduce the neurological symptoms of Cr deficiency syndromes, and no study had been performed on the cellular and molecular perturbations observed in these diseases. We therefore chose to develop another, strategically new, experimental model of Cr deficiencies in 3D reaggregated brain cell cultures, which are are composed of all the types of brain cells, are organized in a 3D network resembling that of the *in vivo* brain, and therefore are considered as organotypic brain cell cultures (Honegger and Monnet-Tschudi, 2001). This model should allow to better understand the mechanisms at the cellular and molecular levels underlying the CNS pathology of creatine deficiency syndromes.

The aim of this project was to develop new experimental models using RNAi in 3D reaggregated developing brain cell cultures to better understand the pathophysiology of Cr deficiency syndromes in the developing CNS. This project was divided in three parts:

- In a first part, we developed new experimental models of Cr deficiencies in the 3D reaggregated brain cell cultures, by gene knockdown of GAMT and SLC6A8 genes using RNAi and a viral vector to transduce the cultures.

- In a second part, we validated this model by measuring the variation of proteins, mRNAs and metabolites that are characteristics of the Cr-deficient patients.

- In a third part, we were interested in the consequences of the knockdown of GAMT or SLC6A8 genes on the integrity of the cultures and on the development of brain cells.

Chapter II: Materials and methods

I. Cloning

1. Digestion with restriction endonucleases

Plasmids containing the ORFs of GAMT and SLC6A8 or specific shRNAs were first digested with appropriate restriction endonucleases to remove the fragment of interest and to clone it in another plasmid. In parallel, the respective plasmids psiCHECK™-1 (for Dual-Luciferase® Reporter assay system; Promega, Madison, WI, USA) or pAAV-hrGFP (for AAVs production; Agilent Technologies, Palo Alto, CA, USA) were digested with the appropriate restriction endonucleases to allow their linearization. Typically, 10 µg of DNA was supplemented with 1 µl of defined enzyme (Promega, Madison, WI, USA) and 1 µl of enzyme buffer 10x in total volume of 10 µl. The digestion mixture was placed in a water bath at a specific temperature (depending on the enzyme) for at least one hour.

2. DNA gel electrophoresis

The digestion products or PCR products were analysed by agarose gel electrophoresis to check the purity and size of digestion. Agarose at 1% (Roche Diagnostics Gmbh, Indianapolis, IN, USA) was dissolved in 200 ml TBE buffer 0.5X (Invitrogen, Carlsbad, CA, USA) in a microwave oven, then added with 20 µl of Gel Red Nucleic acid stain (Biotium). The agarose gel was polymerized in the electrophoresis tray at room temperature (RT). Before loading the gel, 6x-loading dye (Promega, Madison, WI, USA) was added into the DNA samples and vortexed gently. The samples were loaded into wells and the gel was run in TBE

buffer 0.5x at 150-200 volts during approximately 2 hours. DNA bands were visualized by placing the gel on an UV-light Transilluminator ECX-26.M (Vilber Lourmat).

3. DNA gel extraction

Linearized plasmids and DNA fragments generated by restriction endonucleases digestion were excised from agarose gel under UV-light, using a scalpel blade and purified with Gel extraction kit (peqLAB, Germany), according to manufacturer's instructions.

4. Klenow enzyme

When the termini of digested DNA fragments and vectors were not compatible for direct ligation, the large Klenow fragment of DNA Polymerase I was used to fill in 5' or remove 3'overhangs to form blunt ends. DNA was incubated with 25 units of Klenow large fragment (Promega, Madison, WI, USA), 1x Klenow buffer and 200 μM of each dNTP (dATP, dGTP, dTTP, dCTP) in a total volume of 40 μl, for 15 min at RT. The enzyme was inactivated at 75°C for 10 min.

5. DNA dephosphorylation

Dephosphorylation is a general process of removing phosphate monoester on the 5' end of DNA provided by a specific enzyme, alkaline phosphatase. This process avoids the self-ligation of DNA fragments or plasmids. According to manufacturer's instructions, 5 μg of DNA vector was supplemented with 1 unit of alkaline phosphatase and 1x enzyme buffer (Roche Diagnostics Gmbh, Indiapolis, IN, USA). The mixture was placed at 37°C during 20 min and the enzyme was inactivated for 15 min at 75°C.

6. DNA ligation

DNA fragments were ligated into the linearized plasmid vector in the presence of 1x ligation buffer and 1 unit of T4 DNA ligase (Promega, Madison, WI, USA) in a total volume of 10 μl. Several molar ratios were used during this study: 1:1, 1:3 and 3:1 (vector: insert). The ligation reaction was incubated overnight (O/N) at 16°C.

7. Bacterial transformation

The ligated plasmids were transformed into Subcloning efficiency DH5α competent cells (Invitrogen, Carlsbad, CA, USA). In a 15 ml tube, 5 μl of the ligation reaction was incubated with 50 μl of DH5α on ice for 30 min. The tube was placed for 30 seconds in water bath at 42°C without shaking and then on ice for 2 minutes. The transformed bacteria were allowed

to propagate in 950 µl SOC medium for 1 hour in a 37°C shaking incubator at 250 rpm. The tube was centrifugated for 2 min in a centrifuge at 1200 rpm. The bacteria pellet was then plated on LB agar plates supplemented with 100 µg/ml of ampicillin (Applichem, Germany) and incubated O/N at 37°C.

8. Screening of colonies

On LB agar plates, few colonies were chosen to verify the cloning with restriction endonuclease digestion. For this, a colony picked with a sterile tip was added in a tube containing 5 ml of LB medium + Ampicillin (Applichem, Germany), and amplified by O/N incubation at 37°C in a shaking incubator (250rpm). The next day, the plasmid DNA was extracted by peqGOLD plasmid miniprep kit (peqLAB, Germany) (see below for explanation of method). To verify the insert of interest in the plasmid vector, several restriction endonuclease digestions were performed.

9. DNA amplification

Small- or large-scale preparations of DNA plasmids were performed using the mini- or maxiprep kits respectively (MP Biomedicals, France), according to manufacturer's instructions. Briefly, the procedure consists of alkaline lysis of the bacterial cell membrane and RNA degradation with RNase buffer. After centrifugation, the supernatant containing nucleic acids is loaded in an anion-exchange resin column, on which the DNA is bound. Several wash steps are realized and DNA is eluted with elution buffer containing a low salt

concentration. For maxi-preps only, plasmid DNA was finally desalted by isopropanol precipitation followed by ethanol precipitation, and finally concentrated by resuspension in TE buffer or MilliQ H_2O. The concentration of DNA elution was determined with the Nanodrop 2000c (Thermo Fisher Scientific Inc., Waltham, MA, USA).

II. Polymerase chain reaction (PCR)

Amplification of specific DNA fragments was performed by using the following PCR protocols. The PCR reaction mix was composed of 100 ng DNA, 40 ng primers (forward and reverse, Microsynth AG, Switzerland), 6 µl MilliQ H_2O and 12.5 µl TaqPCR Mastermix (Taq DNA Polymerase, PCR buffer, 200 µM of each dNTP and 1,5 mM MgCl2; Qiagen, Germany) in a total volume of 25 µl. Two different PCR programs were used with the GeneAmp PCR system 9700 (Applied Biosystem, Carlsbad, CA, USA):

(i) Classic program with a first denaturation step at 94°C for 5 min; 40 cycles of denaturation (30 seconds, 94°C), annealing (30 seconds, temperature determined for each PCR depending of primers) and elongation (2 min, 72°C); and a final elongation step at 72°C for 5 min; final hold at 4°C.

And (ii) program touchdown with first denaturation step at 95°C for 10 min; 35 cycles of denaturation (30 seconds, 95°C), annealing (30 seconds, 65°C to 55°C: temperature was decreased by 1°C for each of the first 10 cycles, and was kept constant at 55°C for the remaining cycles), and elongation (30 seconds, 72°C); and a final elongation step at 72°C for 10 min; final hold at 4°C.

PCR products were analysed by agarose gel electrophoresis, as described in section 1.2.

III. DNA sequencing

The sequencing reaction mix was composed of 0.8 µl purified PCR products, 0.8 µl primer (50 ng/µl, forward or reverse; Microsynth AG, Switzerland), 0.8 µl BigDye® Terminator V1.1, 0.4 µl 5X buffer (Applied Biosystem, Carlsbad, CA, USA) and 2.2 µl milliQ H_2O in a total volume of 5 µl. The plate was placed into a thermocycler GeneAmp PCR system 9700 (Applied Biosystem, Carlsbad, CA, USA), and the sequencing process was composed of the following steps: a denaturation step at 96°C for 1 min; 25 cycles of denaturation (10 seconds, 96°C), annealing (5 seconds, 50°C), and elongation (4 min, 60°C); followed by the final hold at 4°C for 7 min.

The sequencing reaction was then purified with Montage^{TM} SEQ96 Sequencing Reaction Cleanup Kit (for more details see manufacturer's instructions ; Millipore Corporation, Billerica, MA, USA) and the purified reaction was placed into the 3100-Avant Genetic Analyzer (Applied Biosystem, Carlsbad, CA, USA). After the run, the sequence was analyzed with the program Sequencing Analyses version 3.7 (Applied Biosystem, Carlsbad, CA, USA).

IV. Cell culture

1. ROC, C6 and AAV-293 cells in monolayer cultures

Three different cell lines were used: ROC cells (rat hybridoma between olidodendrocytes and astroglioma), C6 cells (derived from a rat astroglioma), and AAV-293 cells (derived from the

human embryonic kidney cell line HEK-293 and used to produce adeno-associated viruses). Cells were cultured under sterile conditions in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (PAA laboratories GmbH, Pasching, Austria), 1% Penicillin-Streptomycin (Invitrogen, Carlsbad, CA, USA) and 0.5% Hepes (Sigma-Aldrich, St-Louis, NO, USA; ROC and C6 only) in T75 culture flasks (Becton Dickinson Labware, England) at 37°C, 5% CO_2 in a humidified atmosphere. Cell splitting was performed at 90% confluence (ROC and C6) or 50% confluence (AAV-293) by removing culture medium, washing with 10 ml of pre-warmed PBS, followed by 2 ml of 1% trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) for 2 min. Trypsin activity was stopped by addition of 8 ml of fresh medium. 1 ml (1:10) of the cell suspension was then added into a new T75 culture flask previously filled with 10 ml of fresh DMEM.

2. 3D primary brain cell cultures in aggregates

3D primary brain cell cutures in aggregates were prepared from mechanically dissociated brains from 15-day old fetal rats (Sprague-Dawley, Harlan, Netherlands; animals handled according to the rules of the Swiss Academy for Medical Science, pregnant females and embryos killed by decapitation). These cultures were grown in a serum-free, chemically-defined medium and cultured in flask under constant gyratory agitation as previously described (Honegger and Monnet-Tschudi, 2001; Braissant et al., 2002). These cultures develop with neurons, astrocytes, oligodendrocytes and microglia 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 (Honegger and Monnet-Tschudi, 2001; Cagnon and Braissant, 2007). More specifically, these aggregates develop mature synapses, myelin

with fully formed nodes of Ranvier, spontaneous bioelectrical activity as well as biosynthesis, storage and release of neurotransmitters. We have shown that these cultures express AGAT, GAMT and SLC6A8 in the same cell types as the *in vivo* brain and synthesize their own Cr, suggesting that they behave as the *in vivo* CNS for Cr synthesis and transport (Braissant et al., 2008; Braissant et al., 2001). Besides their histotypic cellular organization and maturation, these cultures offer the possibility to use multiple cultures replicates of high cell density, allowing a high reproducibility of experiments.

2.1. Reagents for aggregate cultures

DMEM powder without sodium pyruvate and sodium bicarbonate was purchased from Invitrogen (Carlsbad, CA, USA). Gentamicin sulfate, insulin, linoleic acid, α-tocopherol, 3,3',5-triiodo-L-thyronine, apo-transferrin, choline chloride, hydrocortisone 21-hemisuccinate, alpha-lipoic acid and Basal Medium Eagle vitamin solution were purchased from Sigma (St Louis, MO, USA). Retinol, sodium bicarbonate, L-carnitine and vitamin B12 were purchased from Fluka Chemie AG (Buchs, Switzerland).

2.2. Culture of brain cell aggregates

Embryos were removed from the uterus of pregnant rat females, and their whole brain was dissected out. Fetal brains were pooled in 50 ml of dissection medium (1g/L glucose, 20g/L sucrose, 25 mg/L Gentamicin sulfate, 160g/L NaCl, 8g/L KCl, and 0.9g/L Na_2HPO_4) and were dissociated mechanically, washed and resuspended in serum-free, chemically-defined

medium consisting of DMEM with high glucose (25mM) (Invitrogen, Carlsbad, CA, USA) supplemented with insulin (0.8 μM), triiodo-L-thyronine (30 nM), vitamin B12 (1 μM), L-carnitine (10 μM), choline chloride (2.5 g/l), lipoic acid (1 μM), sodium chloride (3.7 g/l), hydrocortisone-21-phosphate (20 nM), transferrin (1 μg/ml), linoleic acid (10 μM), biotin (4 μM) and trace of vitamins (retinol, tocopherol) and ions (silicium, selenium, cadnium, cuivre, manganese, molybdene, nickel, etain, zinc). Gentamicin sulfate (25 μg/ml) was used as an antibiotic. Cultures were incubated at 3.6 x 10^7 cells per flask in 8 ml culture medium and maintained under constant gyratory agitation at 37°C, in an atmosphere of 10% CO2 and 90% humidified air in a Hera Cell 240 incubator (Thermo Scientific). The rotary speed was progressively increased depending on the day of culture, DIV 0 (DIV: day *in vitro*; DIV 0 being the day of dissection); DIV 0 end of dissection: 68 rpm; DIV 0 evening: 70 rpm; DIV 1 morning: 74 rpm; DIV 1 evening: 77 rpm; DIV 4 morning and after: 80 rpm.

Culture medium was removed every 3 days from DIV 5 onward by removing 5 ml of medium per flask and replenishing by 5 ml of fresh medium. Depending on experiments, aggregates were harvested at DIV 5, 6, 7, 8, 11, or 13. They were then transferred in a 15 ml tube, pelleted and the medium was removed. Aggregates were washed three times with ice-cold PBS and either embedded in Tissue-Tek O.C.T. cryo medium (Digitina, Switzerland) for histology analysis, or frozen in liquid nitrogen for protein (Western-Blot), RNA (real-time quantitative PCR) or metabolite analysis. Samples were kept at -80°.

V. Techniques with shRNAs

1. Dual-luciferase® reporter assay system

Dual-luciferase® reporter assay system is a genetic reporter system which allows a quantitative and rapid approach for the optimization of RNAi site selection. The term « dual reporter » refers to the simultaneous expression and measurement of two individual reporter enzymes (*Renilla* and *Firefly* luciferases) within a single system. Typically, a target gene of interest is cloned in psiCHECK plasmid downstream of the stop codon for the *Renilla* luciferase gene, such that a hybrid mRNA is expressed (but not a hybrid protein). Initiation of RNAi toward the target gene results in the cleavage and subsequent degradation of the fusion mRNA including the *Renilla* luciferase coding sequence. This results in a decrease in *Renilla* luciferase activity that can be easily monitored as an indicator of the RNAi effect. The presence of a second reporter gene, *Firefly* luciferase, allows the normalization of transfection efficiency.

During this work, the Dual-luciferase® reporter assay system was used to determine the efficiency of different GAMT and SLC6A8 shRNAs.

2. Co-transfection of several plasmids

To test the efficiency of each shRNAs, several co-transfections were performed with the jetPEI^{TM} (Polyplus transfection, Strasbourg, France) method. Briefly, jetPEI^{TM} compacts

DNA into positively charged particles capable of interacting with anionic proteoglycans at the cell surface and entering cells by endocytosis. For every condition, several plasmids were transfected into ROC cells:

- psiCHECK in which our cDNA of interest has been cloned downstream of the Renilla luciferase coding sequence (1 µg)
- Plasmids expressing our shRNA sequence (0,5 µg)
- Control plasmid expressing the Firefly luciferase coding sequence (1µg)

On the day of transfection, cells were plated in 12-well plates at a confluence of 50%. For each well, 50 µl of NaCl 150 mM in which all 3 plasmids were diluted as well as a mix of 5 µl of jetPEI^{TM} and 50 µl of NaCl 150 mM, were mixed together and incubated 30 min at RT. The DNA/jetPEI^{TM} solution was then distributed in each well and the 12-well plates were incubated at 37°C, 5% CO_2 in a humidified atmosphere during 48 hours. After 6 hours, the transfection culture medium was removed and replaced by fresh DMEM.

3. Measure of luciferases activites

After 48 hours incubation time, cells were washed with phosphate buffered saline (PBS) and incubated at RT for 15 min on a rocking platform with 250 µl of passive lysis buffer (Promega, Madison, WI, USA). 20 µl of cell extract were added in a tube containing 50 µl of Luciferase assay reagent II (Promega, Madison, WI, USA), and the tube was placed in the luminometer (TD-20/20, Turner Designs, Sunnyvale, CA, USA) to measure the activity of *Firelfy* luciferase. After the first measure, the tube was removed from the luminometer, and 50 µl of Stop and Glo reagent (Promega, Madison, WI, USA) was added. The tube was replaced in the luminometer and the *Renilla* luciferase activity was measured. Each condition

was performed in triplicate at least two times. The *Renilla* luciferase activity was divided by the *Firefly* luciferase activity in order to normalize for transfection efficiencies of cells, and multiplied by 100% to obtain efficiencies for each recording.

4. Transfection of C6 or ROC cells with shRNAs

To test the efficiency of each interferent or control shRNAs with an other method than the Dual-luciferase® reporter assay system, we analyzed the knock-down of the endogenous GAMT gene in C6 or ROC cells, by transfection with the jetPEI™ method of plasmids expressing the shRNAs. The plasmid, expressing the sequence of each shRNA, expressed a resistance gene, the neomycin gene. The treatment of cells with the neomycin allowed to select the cells with a stable transfection.

The day before transfection, C6 or ROC cells, expressing the GAMT gene, were plated in 6-well plates at 150 000 cells per dish in 4 ml of DMEM culture medium supplemented with 10% FBS and 1% Penicillin-Streptomycin (Invitrogen, Carlsbad, CA, USA). The day of transfection, 3 µg of plasmid were diluted in 100 µl of NaCl 150 mM as well as a mix of 6 µl of jetPEI™ and 100 µl of NaCl 150 mM, were mixed together and incubated 30 min at RT. The DNA/jetPEI™ solution was then distributed in each well and the 6-well plates were incubated at 37°C, 5% CO_2 in a humidified atmosphere. After 6 hours, the transfection culture medium was removed and replaced by fresh DMEM. After 24 hours, the cells were treated with 2000 µg/ml of neomycin for the C6 cells and 300 µg/ml for the ROC cells and, to select the cells expressing the plasmid. Then the cells were harvested after 6 days of transfection and the GAMT protein level was quantified by western blot.

VI. Techniques with adeno-associated-virus (AAV)

The 3D primary brain cell culture in aggregates is a system with a high cellular complexicity, in which a transfection of shRNAs with phosphate calcium or polycationic reagent showed a low efficiency due to the presence of already post-mitotic cells. The AAVs serotype 2 and 5 are known to efficiently transduce these types of brain cells. In this study, these AAV 2 and 5 serotypes were used to transduce the best shRNA for our gene of interest in aggregates.

The AAV Helper-Free System from Stratagene (Agilent Technologies, Santa Clara, CA, USA) allows the production of infectious AAV particles without the presence of helper adenovirus or herpes virus. This method is based on the co-transfection of several plasmids in AAV-293 host cells. The pHelper plasmid expresses the adenoviral E2A, E4, and VA genes; pAAV-RC expresses the AAV rep/cap-gene for AAV of serotype 2; pDP5rs expresses the AAV rep2/cap5-gene for AAV of serotype 5; pAAV-hrGFP contains the AAV inverted terminal repeats (ITRs) and the AAV-293 host cells stably express the adenovirus E1 gene, necessary to produce active viruses.

To produce AAV serotype 2, pAAV-RC, pHelper and pAAV-hrGFP or recombinant pAAV-hrGFP, in which our best shRNA and its promoter have been cloned, were required. For AAV serotype 2/5 it was pDP5rs, pHelper and pAAV-hrGFP or recombinant pAAV-hrGFP, in which our best shRNA and its promoter have been cloned.

1. AAVs production

The plasmids pHelper, pAAV-RC and pAAV-hrGFP were purchased from Stratagene (Agilent Technologies, Santa Clara, CA, USA); pRep2/5 was purchased from Aldevron (Fargo, ND, USA).

In this study, the following AAVs were produced by co-transfecting the following respective plasmids in AAV-293 cells:

1. AAV2-control → pHelper + pAAV-RC + pAAV-hrGFP
2. AAV2-shRNA GAMT → pHelper + pAAV-RC + pAAV-hrGFP in which shRNA for GAMT gene has been cloned (pAAV-shRNA GAMT)
3. AAV2-shRNA SLC6A8 → pHelper + pAAV-RC + pAAV-hrGFP in which shRNA for SLC6A8 gene has been cloned (pAAV-shRNA SLC6A8)
4. AAV2/5-control → pHelper + pRep2/5 + pAAV-hrGFP
5. AAV2/5-shRNA GAMT → pHelper + pRep2/5 + pAAV-shRNA GAMT
6. AAV2/5-shRNA SLC6A8 → pHelper + pRep2/5 + pAAV-shRNA SLC6A8

48 hours before transfection, AAV-293 cells were plated at $2x10^6$ cells per 100 mm diameter culture dish in 10 ml of DMEM culture medium supplemented with 10% FBS and 1% Penicillin-Streptomycin (Invitrogen, Carlsbad, CA, USA). Each AAV production necessitated the culture of 11 dishes.

On the day of transfection, cells were approximately 70-80% confluent to allow an efficient AAV production. Into a 15 ml conical tube containing 11 ml of 0.3M CaCl2 (Invitrogen, Carlsbad, CA, USA), 110 μg of each of the three plasmid DNA solutions were added and the solution was mixed gently by inversion. This DNA/CaCl2 mixture was added in a dropwise

fashion in a second tube containing 11 ml of 2x HBS (Invitrogen, Carlsbad, CA, USA). The transfection mixture was mixed by inversion and 2 ml/plate were immediately applied on cells. The dishes were swirled gently to distribute the DNA suspension evenly in the medium and were placed in a 37°C incubator for 6 hours. At the end of the incubation time, the medium was removed and replaced with 10 ml of fresh DMEM medium complemented with 10% FBS and 1% Penicillin-Streptomycin (Invitrogen, Carlsbad, CA, USA). The dishes were returned in the 37°C incubator for 72 hours.

Three days after transfection, the cells and the medium were collected into a tube and four rounds of freeze/thaw were performed by alterning the tubes between dry-ice and a 37°C water bath. Each round was of 10 min. The supernatant containing the viral particles was transferred into a tube after a 10,000 x g centrifugation for 10 min at RT. The viral stock was stored at -80°C before purification.

2. AAVs purification

Before purification, the viral stock was treated with 0.1 µl / ml Benzonase® (Roche) for 1 hour at 37°C in water bath. This treatment allows to remove much of the contaminating DNAs or RNAs (simple or double stranded, linear or circular).

The AAV Purification Virakit™ (Virapur, San Diego, CA, USA; for more details see the manufacturer's intructions) was used for purification of AAV serotype 2 and 5. Briefly, the viral suspension was purified by passing it through a 0.45 µm filter. After adding a dilution buffer, the virus solution was slowly passed over a treated filter, which absorbs the viral particles, allowing much of cellular debris to pass through the filter. The washing buffer was passed over the column to remove any bound debris, and the virus was eluted off the filter

with elution buffer. The viral stock was stored at -20°C until the utilisation in several cell cultures (ROC, C6, aggregates).

3. AAVs titration

AAV particles in purified viral stocks were quantified with an enzyme immunoassay, AAV2 or 5 Titration ELISA (Progen Biotechnik GMBH, Heidelberg, Germany). The assay is based on the sandwich ELISA technique. A monoclonal antibody specific for a conformational epitope on assembled AAV2 or AAV5 capsid was coated onto microtiter strips in 96 well plates and was used to capture AAV2 or AAV5 particles from the samples. Captured AAV particles were detected in two steps. First a biotin-conjugated monoclonal antibody to AAV2 or AAV5 was bound to the immune complex. In the second step streptavidin peroxidase conjugate was reacted with the biotin molecules. Addition of substrate solution resulted in a color reaction which was proportional to the amount of specifically bound viral particles. The absorbance was measured photometrically at 450 nm (see manufacturer's intruction). Briefly, 100 µl of the serial dilutions of kit control (empty AAV capsids for titration curve) and samples were added into the wells of the microtiter strips for 1 hour at 37°C in an incubator. After 3 washing steps with 200 µl of washing buffer, all wells were complemented with 100 µl of biotin conjugate for 1 hour at 37°C. After 3 washing steps with 200 µl of washing buffer, 100 µl of streptavidin was added into the wells for 1 hour at 37°C. After the last washing step, 100 µl of substrate were added into the wells for 10 min at RT and the reaction was stopped with 100 µl of stop solution. Intensity of color reaction was measured with a Nanodrop 2000c (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 450 nm.

VII. Techniques with RNA

1. RNA extraction

RNA extraction was performed on aggregated brain cells harvested at day 5, 6, 7, 8 or 11 and preserved at -80°C with AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Germany). According to manufacturer's protocol, the cells were first lysed and homogenized in Buffer RLT, which inactivated RNases to ensure isolation of intact RNA. The lysate was then passed through an AllPrep DNA spin column. After centrifuge, ethanol was added to the flow-through of AllPrep DNA spin column to provide appropriate binding conditions for RNA, and the sample was applied to an RNeasy spin column, where total RNA was binded to the membrane, which contaminants were washed away. RNA was then eluted in RNase-free water. The concentration of the total RNA was measured with the Nanodrop 2000c (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2. Reverse transcription reaction

The mRNAs extracted as above were transcribed by reverse transcription into complementary DNA (cDNA) strand by using poly-dT oligonucleotide primer hybridizing on the poly-A tail of the mature mRNA, and random 6-mers primers setting anywhere on the RNA. Reverse transcription reaction was performed with PrimeScript^{TM} RT kit (Takara Bio Inc., Japan) with the following conditions: the reaction mix was composed of 2 μg total RNA, 4 μl 5 x Prime Script buffer, 1 μl Prime Script RT enzyme, 1 μl oligo-dT primers (50 μM), 4 μl 6-mers

random (100 µM) in a total volume of 20 µl. The tubes were placed into a thermocycler GeneAmp PCR system 9700 (Applied Biosystem, Carlsbad, CA, USA) and the program was composed of two steps: reverse transcription for 15 min at 37°C and inactivation of enzyme for 5 secondes at 95°C. RT products were stored at -20°C before performing real time quantitative PCR.

3. Real-time quantitative PCR (qPCR)

The real-time PCR is used to amplify and simultaneously quantify a targeted DNA molecule. The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is detected as the reaction progresses in real time, a new approach compared to standard PCR, where the product of the reaction is detected at its end. In this study, we have used the methods with non-specific fluorescent dyes that intercalate with any double-stranded DNA.

Before starting the qPCR, cDNA samples were diluted to 50 ng with RNase free water, and one sample was diluted at different concentrations (100 ng, 50 ng, 25 ng, 12.5 ng) to form the standard curve. The qPCR reaction mix is composed 1 µl of diluted cDNA, 10 µM of each forward and reverse primers, 12.5 µl of ABsolute QPCR SYBR Green Mix 2x (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 10.5 µl RNase free water in a total volume of 25 µl. Samples were placed into a thermocycler Rotor-Gene 6000 (Qiagen, Germany) and run with the following steps: enzyme activation at 95°C for 15 min; 40 cycles of denaturation (15 secondes, 95°C), annealing (30 seconds, 50-60°C), and elongation (30 seconds, 72°C). After the run, the results were analysed with the Corbett Rotor-Gene 6000 Application Software, version 1.7 (Qiagen, Germany).

VIII. Western blot analysis of proteins

During this work, the expression of several specific proteins were analyzed by western blotting, making use of the following specific primary antibodies (table n°1) and specific secondary antibodies (table n°2).

Table 1: List of the proteins (antigens) analyzed in this work, and their specific antibodies used for western blotting analysis.

ANTIGEN	DILUTION	MOLECULAR WEIGHT	SPECIES	SUPPLIER
SLC6A8	1 :500	61 kDa	rabbit	Homemade Braissant *et al*., 2005
GAMT	1 :500	26 kDa	rabbit	Homemade Braissant *et al*., 2005
α - tubulin	1 :1000	55 kDa	rabbit	Santa Cruz
Caspase 3	1:1000	Full 35kDa Cleaved 17 kDa	rabbit	Cell Signaling
Phospho p38 MAPK	1:1000	43 kDa	rabbit	Cell Signaling

Table 2: List of secondary antibodies used in this study

Conjugation	Dilution	Species	Supplier
HRP-conjugated IgG	1 :3000	Goat anti-rabbit	BioRad
HRP-conjugated IgG	1 :3000	Goat anti-mouse	BioRad
HRP-conjugated IgG	1 :3000	Donkey anti-goat	Santa Cruz

1. Preparation of samples

Once harvested, cell samples were homogenized in lysis buffer containing 8M urea, 25mM Tris-HCl pH8, 1% SDS and Protease inhibitor Complete (Roche, Switzerland), and sonicated for 5 secondes. Homogenates were centrifuged at 12,000 x g for 10 min at 4°C in a microcentrifuge, and supernatants were recovered. Supernatant proteins were quantified by the bicinchoninic acid BCA™ Protein assay (Pierce, RockfordIL, USA). All samples were diluted at the same concentration in NuPage® sample buffer containing 0,14 M Tris Base, 0,5 mM EDTA, 10% glycerol, 2% lithium dodecyl sulphate, 500 mM dithiothreitol (Invitrogen, Carlsbad, CA, USA) and heated at 70°C for 10 minutes (denaturation of protein by heat).

2. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide NuPage 12% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) were used to separate the proteins of interest according to their molecular weight. The migration was

performed in 3-N-morpholino-propanesulfonic acid or MOPS running buffer containing 50 mM of MOPS, 50 mM Tris Base, 0.1% sodium dodecyl sulphate (SDS), 1 mM EDTA (Invitrogen, Carlsbad, CA, USA), at a constant voltage (200V) during 58 minutes.

3. Immunoblotting

After electrophoresis, polyacrylamide gel and Whatman filter papers were placed in Bjerrum buffer (48mM Trizma Base, 39 mM Glycine, 0,13 mM SDS, 15% methanol) for a few minutes under agitation. A « sandwich » was performed in the Transblot SD semi-dry transfer cell (Biorad, Hercules, CA, USA) with Whatman filter papers, an Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA), and the polyacrylamide gel. Protein samples were transferred from gel to the PVDF membrane under an electric current of 13V during 70 minutes.

After tansfer, PVDF membranes were washed 2 x 10 minutes with TBS-Tween (20 mM Trizma Base pH 7.5, 137 mM NaCl, 0,05% Tween 20), and then blocked with 5% non-fat dry milk in TBS-Tween for 2 hours at room temperature (RT) to avoid unspecific binding of the antibody. After blocking, PVDF membranes were incubated overnight (O/N) with primary antibodies diluted in 3% dry milk and TBS-Tween. The respective dilutions of primary antibodies used in this work are listed in table 1. On next day, PVDF membranes were washed 3 x 10 minutes with TBS-Tween and incubated with horseradish peroxydase (HRP)-coupled goat anti-rabbit IgG or goat anti-mouse IgG (1:3000; Vector laboratories, Burlingame, CA, USA; table 2). A final wash of membranes was performed during 2 hours with TBS-Tween and Amersham ECL™ Western Blotting Detection Reagents (Amersham Biosciences, Buckinghamshire, UK) were applied on the PVDF membranes to allow signal

revelation by chemiluminescence (Kodak M35 X-OMAT Processor). Blots could be stripped with Re-Blot Plus Mild antibody stripping solution (Chemicon, Temecula, CA, USA) and reprobed with other antibodies. The autoradiograms were scanned with an ImageScanner (Amersham Biosciences, Buckinghamshire, UK) and analyzed with the ImageJ image-processing program. Data were acquired in arbitrary densitometric units and transformed to percentages of controls.

IX. Immunohistochemistry

Immunohistochemistry was used to follow the expression of specific cell type markers in brain cell aggregates and analyze the morphology of brain cells.

1. Antibodies

The following antibodies were used for immunohistochemistry during this work (tables 3, 4).

Table 3: List of primary antibodies used in this work

Antigen	Dilution	Species	Supplier
GFAP	1 :100	mouse	Millipore
pNFM	1 :100	mouse	Sigma
MBP	1 :100	goat	Santa Cruz

Table 4: List of secondary antibodies used in this work

Conjugation	Dilution	Species	Supplier
HRP-conjugated IgG	1 :200	Goat anti-mouse	BioRad
HRP-conjugated IgG	1 :100	Rabbit anti-goat	Dako Cytomation

2. Method

16 µm-thick cryosections of aggregate pellets were performed on a CM1850 UV cryostat (Leica) and fixed by incubation in 4% paraformaldehyde for 1 hour at RT. After 3 washing steps with PBS 1x for 5 min at RT, sections were incubated in 1% H_2O_2 (TraceSelect 30%, Fluka Chemie AG, Buchs, Switzerland) for 15 min at RT to quench peroxidase activity. The non-specific antibody binding sites were blocked by incubation in 1% BSA (Sigma-Aldrich, St-Louis, NO, USA) for 15 min at RT. The primary antibody diluted in appropriate concentration in 1% BSA was applied on slide for 2 hours at RT, excepted for the anti-MBP antibody which was incubated O/N at 4°C. After 3 washing steps with 1x PBS for 5 min at RT, the HRP-coupled secondary antibody diluted in 1x PBS was added for 1 hour. Peroxidase staining was performed using 3-Amino-9-Ethylcarbazole or AEC Substrate Set (BD Biosciences, San Diego, CA, USA) and the reaction was stopped by immersing the slides in H_2O. For all experiments, the negative control was performed with no primary antibody incubation. Sections were mounted under FluorSave™ Reagent (Calbiochem, San Diego, CA, USA).

Slides were observed by the use of an Olympus BX50 microscope and photos were taken with an Olympus Color View 2 camera. Images were analyzed using image-processing software (Cell Imaging Software, Olympus).

X. *In situ* cell death detection

The cleavage of genomic DNA in cell generated by apoptosis can be detected and quantified with the method of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling or TUNEL. This staining was performed on cryosections (16 µm) of aggregates, according to supplier recommandations using *In Situ* Cell Death detection kit Fluorescein (Roche Diagnostics, Switzerland). Briefly, sections were fixed by incubation in 4% paraformaldehyde for 20 min at RT. After 3 washing steps with PBS 1x for 30 min at RT, sections were permeabilized 2 min with 0.1% Triton X-100, 0.1% sodium citrate at 2-8°C. After 3 washing steps with PBS 1x, sections were incubated with a mix of TdT from calf thymus and fluorescein-dUTP for 1 hour at 37°C in humid chamber. Slides were observed under microscopy with a specific filter of fluorescence (excitation at 450-500 nm; emission at 515-565 nm), resulting in green fluorescence within apopototic cells. For negative control, TdT was omitted resulting in no nuclear staining.

XI. Measure of extracellular GAA and Cr by tandem mass spectrometry

Brain cell aggregates were homogenized in H_2O at 4°C using a FastPrep Cell Disrupter (Qbiogene, France) and centrifuged at 10 000g during 5 min at 4°C. Creatine and guanidinoacetic acid determinations in the cell supernatants were performed by electrospray tandem mass spectrometry. 15 µl d_3-creatine and $^{13}C_2$-Guanidinoacetic acid each at 10 µM (CDN Isotopes Inc, Quebec Canada), 4 µl formic acid was added to 600 µl of cell supernatant. The analytes were purified by micro solid phase extraction (µ-SPE) using the Oasis MCX µElution Plate (Waters, MA USA). The procedure comprised of what is typical for SPE, conditioning of the µ-SPE wells by methanol (MeOH), application of the diluted samples, wash steps by 2 % (v/v) formic acid and MeOH and final elution with 5 % (v/v) NH_4OH in H_2O.

Chromatographic analysis was performed using a Rheos CPS-LC (Flux Instruments, Switzerland) and a PAL SYSTEM autosampler (CTC Analysis, Switzerland).

Separation of the analytes was achieved at 30 °C using an Atlantis HILIC silica 2.1 x 50 mm (Waters, MA USA). The mobile phases used for the chromatographic separation were composed of 20 mM ammonium formate, 0.4 % (v/v) formic acid in H_2O and acetonitrile. The column effluent was monitored using a Triple Quadrupole TSQ Quantum Discovery (Thermo Scientific, CA USA). The instrument was equiped with an electrospray interface and was controlled by the Xcalibur software (Thermo Scientific, CA USA). The sample were analyzed in the positive ionization mode operating in a cone voltage of 4 kV. The tandem mass spectrometer was programmed using the selected reaction monitoring mode (SRM) to allow the $[MH^+]$ ions of creatine at *m/z* 132.1 and that of guanidinoacetic acid at *m/z* 118.1 to

pass through the first quadrupole (Q1) and into the collision cell (Q2). The daughter ions for creatine and guanidinoacetic acid were of *m/z* 90.1 and 76 respectively. Calibration curves were computed using the ratio of the peak area of the analytes and internal standard using a weighted (1/x2) least squares linear-regression analysis.

Chapter III: Results

Part I: Study of GAMT deficiency syndrome

I. Knockdown of GAMT expression

The open reading frame of the GAMT gene is composed of 711 nucleotides and located on chromosome 19 (19p13.3) in human (Almeida et al., 2007) and on chromosome 7 (7q11) in *Rattus norvegicus*. The protein encoded by this gene is a methyltransferase that converts guanidinoacetate to creatine, using S-adenosylmethionine as the methyl donor. Mutations in this gene are implicated in the first described of the three Cr deficiency syndromes, GAMT deficiency (Stöckler et al., 1994). In these syndromes, CNS is the main organ affected, in particular during development (Beard and Braissant, 2010; Braissant et al., 2011; Braissant and Henry, 2008).

To study the consequences of GAMT deficiency in developing brain cells, our aim was to develop an experimental model *in vitro* of GAMT deficiency syndrome by using the technique of RNA interference.

1. Choice of the best interfering sequences

In order to generate a new experimental model of GAMT deficiency by RNA interference, we have designed several shRNA sequences with the Genscript's software incorporating several different rules such as thermodynamic property, length of the shRNA target site (21 nt), GC content of the target site (range of 30-60%, as sequences with < 60% GC content have a better

chance to be functional shRNAs than those with higher GC content), sequence region (preferably 50–100 nt downstream of the "ATG" start codon), and control of off-target efficiency with BLAST search.

Each shRNA was composed by 21 nt of the antisense (reverse complement) GAMT coding sequence, followed by a hairpin sequence and the corresponding sense 21 nt of the GAMT coding sequence. For shRNA 1, 2 and 3, the coding sequence represented nucleotides 309-329, 576-596, and 467-487, respectively, downstream from the start codon of the rat GAMT ORF (GenBank accession no. NM_012793) (figure 10).

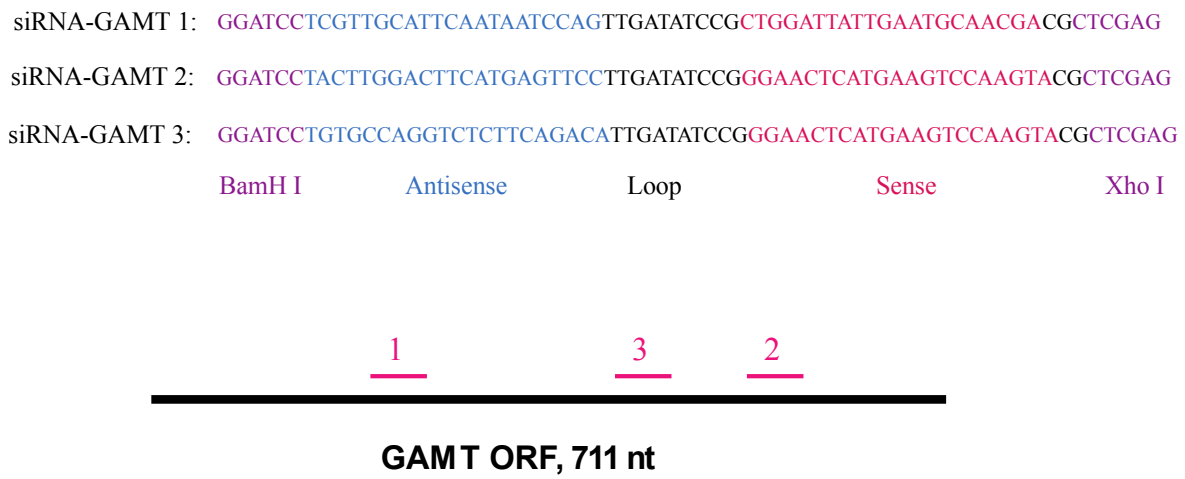


Figure 10: Sequences of each selectioned shRNA and their respective position on GAMT ORF.

The 3 shRNAs selectioned were inserted as DNA into the pRNAT-CMV3.2/Neo vector between the restriction sites BamHI and XhoI. In this vector, CMV promoter drives the expression of shRNA, and SV40 promoter drives the expression of the neomycin gene, a resistance gene, and cGFP (coral GFP) for tracking the transfection efficiency (see below figure 11).

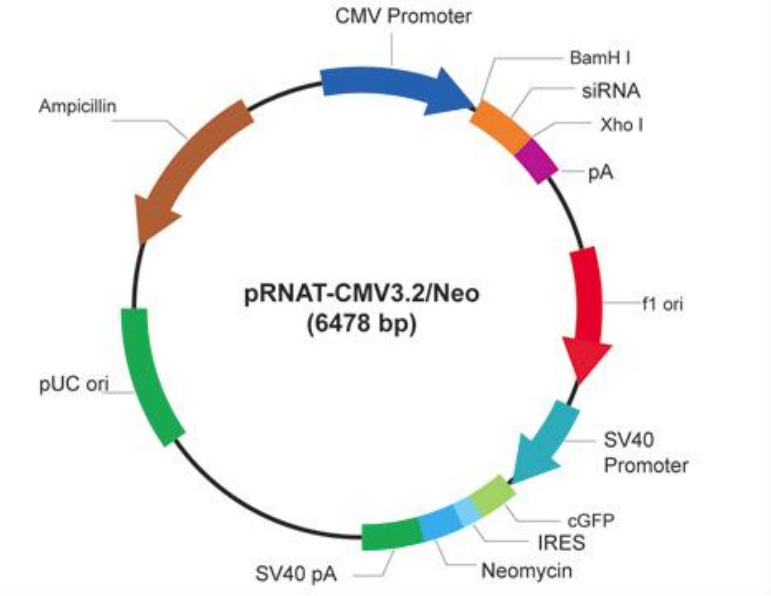


Figure 11: Circle map of pRNAT-CMV3.2/Neo (figure taken from www.genscript.com).

1.1. Dual-luciferase® reporter assay system

The goal of the first part of the study was to determine the efficiency of each shRNA on the GAMT expression in monolayer cell cultures. For this, we used the Dual-luciferase® reporter assay system which is a quantitative and rapid approach for the optimization of RNAi selection (for more explanation see chapter II). Briefly, the ORF of GAMT was cloned into the psiCHECK^{TM} plasmid downstream of the *Renilla* luciferase gene, allowing the transcription of a hybrid mRNA. If the GAMT shRNA is not efficient, the traduction of the hybrid mRNA containing the *Renilla* luciferase and the GAMT ORF can occur and the bioluminescence activity emitted by the *Renilla* luciferase protein can be monitored. On the other side, if the GAMT shRNA is efficient to knockdown GAMT, the cleavage and the degradation of the hybrid mRNA containing the *Renilla* luciferase and the GAMT ORF is

initiated, and therefore no activity of *Renilla* luciferase can be monitored. The decrease in *Renilla* luciferase activity is a good indicator for the RNAi efficiency (figure 12).

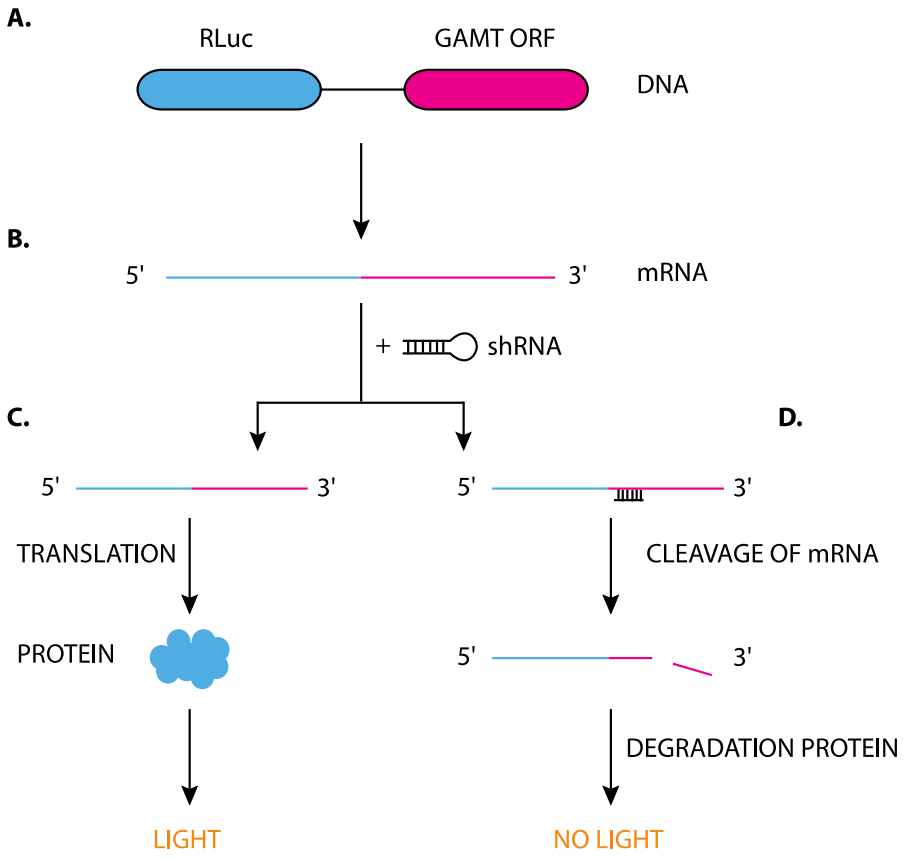


Figure 12: Schematic outline of the Dual-luciferase® reporter assay system. A) Vector expressing the Renilla luciferase gene fused to GAMT ORF. **B)** To determine the efficiency of different shRNAs, the plasmid expressing Renilla luciferase and GAMT ORF is co-transfected with the plasmid expressing shRNAs in cell culture. **C)** shRNA is not efficient to knockdown the target mRNA sequence, and the Renilla luciferase protein is translated and high luciferase activity is recorded. **D)** shRNA is very efficient to knockdown the target mRNA sequence, and high level of degradation of the fusion mRNA leads to low or no luciferase activity.

1.1.1. Clonage of GAMT ORF in the psiCHECK™ vector

The ORF of GAMT was excised from the pBS-KS$^-$ vector by digestion with Xho I and Sma I, purified by agarose gel electrophoresis and ligated into the psiCHECK™ vector, pre-digested

with Xho I and Sma I (figure 13). To determine the presence of our insert into the plasmid, we screened the colonies obtained after transformation of DH5α bacteria with 2 restriction enzymes, BamH I and Pst I. The expected result of this digestion was 3 bands with different molecular weight: 2284 bp, 1279 bp, and 648 bp. The cloned product was amplified by maxiprep and sequenced with two primers (forward: 5'- CAG CCA GGA GGA CGC TCC AG -3'; reverse: 5'- GCC ACC TGG ATC CTC ACA C -3') to ensure that no mutations had been introduced in the GAMT ORF.

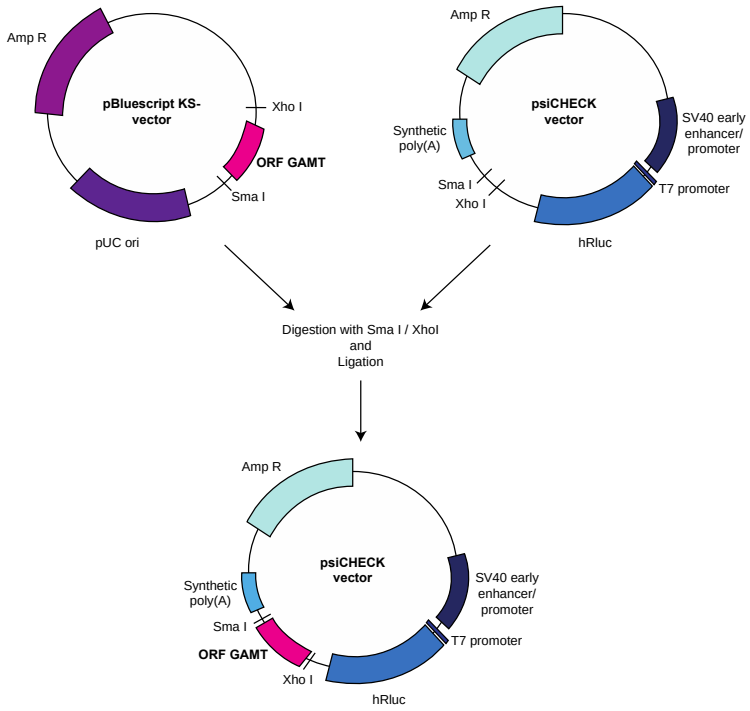


Figure 13: Schematic outline of the clonage of GAMT ORF in psiCHECK^{TM} vector. The pBluescript KS^{-} vector containing the GAMT ORF was digested by two restriction enzymes, SmaI and XhoI, to excise the fragment of interest. After the digestion of psiCHECK^{TM} vector by SmaI and Xho I, the GAMT ORF was ligated into the psiCHECK^{TM} vector. The expression of the hybrid mRNA (*Renilla* luciferase and GAMT ORF) was driven by the T7 promoter. hRLuc, *Renilla* luciferase ; Amp R, Ampicillin resistance gene ; pUC ori, pUC origin of replication sequence.

1.1.2. Determination of RNAi efficacy in ROC cells

The quantitative assessment of shRNA efficiency was analyzed as follows 24 hours after seeding in 12 well plates. Rat oligodendroglia-C6 astroglioma hybridoma cells (or ROC cells) were transfected with three differents plasmids, the reporter psiCHECK^{TM} containing the GAMT ORF, the Genscript plasmid expressing the GAMT shRNA, and p-Firefly as transfection control. A shRNA for synaptotagmin 4 gene, as well as the psiCHECK^{TM} vector containing the synaptotagmin 4 ORF, were used as positive control. The luciferase activity was recorded 48 hours after transfection. As shown in figure 14, shRNAs 2 and 3 significantly down-regulated the expression of the luciferase, with a decrease of approximatively 80% of luciferase activity for the GAMT-2 shRNA (figure 14).

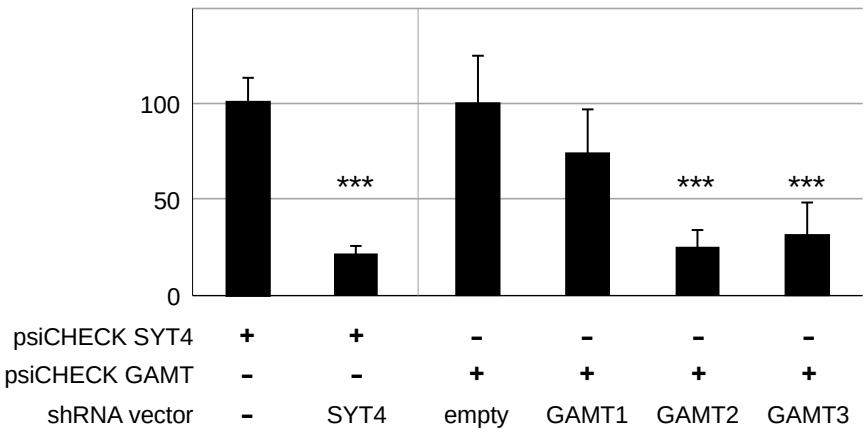


Figure 14: Silencing of luciferase activity by different shRNAs specific for GAMT ORFs, and Synaptotagmin 4 (SYT4) as positive control. ROC cells were transfected with a plasmid expressing firefly luciferase as a reporter for normalization of transfection, a psiCHECK^{TM} vector expressing GAMT open reading frame (or SYT4 open reading frame) downstream of the *Renilla* luciferase open reading frame, and plasmids expressing shRNAs specific for GAMT or SYT4. Luciferase activity is shown on the Y axis in arbitrary units (controls for SYT4 and GAMT: 100%). Mean ± SD (n=8; NS: non-significative; ***: $p<0.001$).

Our results based on the luciferase activity test suggested that the GAMT-2 shRNA had the best efficiency to knock down the expression of GAMT mRNA.

1.2. Off-target effect

In the first part of the study, we have demonstrated, by the technique of Dual-luciferase® reporter assay system, that the GAMT-2 shRNA had the best potential to knock-down the GAMT gene. Several studies have reported that the siRNA-mediated gene silencing could be less specific than originally thought (Jackson et al., 2006). The interpretation of data from RNA interference has become complicated due to unintended interactions between the siRNA and non specific cellular components, the so-called off-target effects (Echeverri and Perrimon, 2006). Briefly off-target effects include the induction of the antiviral interferon pathway machinery, the insertion of the sense siRNA into the RISC complex instead of the antisense strand or the nucleotide sequence similarity between the siRNA molecule and short motifs in mRNAs of other genes not intended to be knocked-down (Jackson et al., 2003; Tschuch et al., 2008). To avoid these off-target effects on our results, we have designed two supplementary negative controls in the form of mismatched and scrambled sequences. The mismatched sequence had the same sequence than GAMT-2 shRNA except 4 mutated bases, while the scrambled sequence had the same composition of bases than GAMT-2 shRNA but in a different randomized order (figure 15).

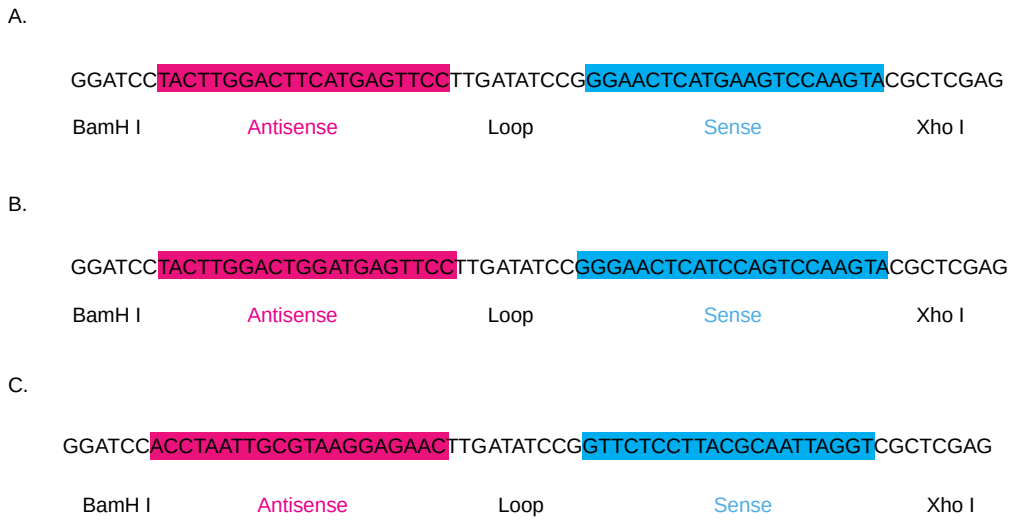


Figure 15: Schematic representation of GAMT-2 A), mismatched B) and **scrambled C)** GAMT shRNA sequences cloned into pRNAT-CMV3.2/Neo vector.

These sequences were inserted into pRNAT-CMV3.2/Neo vector between the restriction site BamHI and XhoI (figure 11). This vector expresses cGFP and a resistance gene under the control of SV40 promoter. This gene allows the selection of transfected cells with an antibiotic, the neomycin.

To demonstrate that these sequences have no effects on GAMT gene expression, we have transfected rat astroglioma C6 cells cells which do express the GAMT gene with the plasmid vectors expressing mismatched or scrambled GAMT-shRNA sequences. In parallel, in order to confirm the results obtained with the Dual-luciferase® reporter assay system, we quantified the expression of the GAMT protein in the presence of GAMT-2 shRNAs. Briefly, the cells were plated in 6 well plates at 150 000 cells/dish. The next day, the cells were transfected with plasmids expressing the mismatched GAMT-shRNA or the scrambled GAMT-shRNA, and the GAMT-2 shRNA. 24 hours after transfection, the cells were treated with 2000 µg/ml of neomycin to select the transfected cells. One week after the transfection, the cells were harvested and the protein were extracted to realize western blot and to quantify the GAMT

protein. Our preliminary results show that the GAMT protein level is not decreased under mismatched or scrambled shRNAs as compared to the empty vector, while GAMT-2 shRNA induced pronounced decrease of the GAMT protein (figure 16). However, work is still going on to better document the absence of off-target effects of GAMT RNAi.

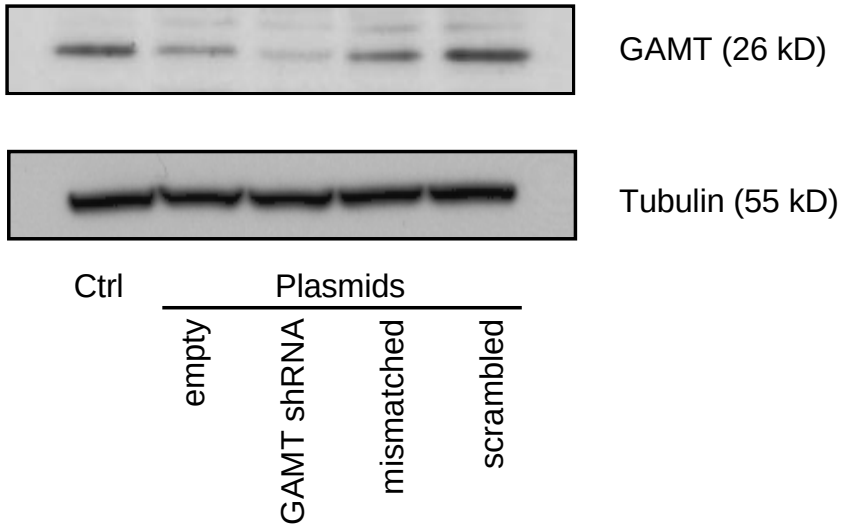


Figure 16: GAMT protein expression level after transfection with plasmids expressing GAMT-2, mismatched or scrambled shRNAs in ROC cells. The cells were harvested 120 hours after the transfection with the plasmids expressing shRNA GAMT-2, shRNA mismatched, shRNA scrambled or not (Empty). Western blot showing the GAMT protein level from control cells, or cells transfected with plamids Empty or expressing GAMT-2 shRNA or mismatched and scrambled shRNA for GAMT-2.

These preliminary results show that both mismatched and scrambled sequences for GAMT-2 shRNA have not effect on the GAMT protein level, suggesting that the GAMT-2 shRNA is efficient to down-regulate the GAMT gene in C6 cells.

II. The experimental model of GAMT deficiency syndrome in 3D reaggregated brain cell cultures

After having selected the best GAMT shRNA interfering sequence in monolayer cell cultures, our aim was to develop a new experimental model of GAMT deficiency in the organotypic *in vitro* system of 3D reaggregated brain cell cultures. Due to the 3D complexity of brain cell aggregates, and to the fact that most of the cells are already post-mitotic at the start of the culture, classical transfection protocols (e.g. JetPEI or $CaPO_4$) could not be used. Viral vectors appear as an efficient tool for gene transfer into brain cells, essentially due to their ability to transduce both dividing and non-dividing cells. We have decided to use AAV viruses as vectors to transduce the best GAMT shRNA into 3D reaggregated brain cell cultures, as AAVs are known to transduce all type of brain cells, whichever they are mitotic or not.

1. Construction of AAV vectors

The vector containing GAMT-2 shRNA sequence was digested by two restriction enzymes, BglII and XbaI, to remove the CMV promoter and the shRNA sequence. This fragment was purified by agarose gel electrophoresis and ligated in the pAAV-hrGFP vector, pre-digested with BstEII (figure 17). To determine the presence of our insert into the plasmid, we screened the colonies obtained after transformation of DH5α bacteria with 2 restriction enzymes, EcoRI and EcoRV. The result expected of this digestion was 2 bands with different molecular weight: 4160 bp and 1806 bp. The cloned product was amplified by maxiprep and sequenced

with two primers (forward: 5'- TCC GCG TTA CAT AAC TTA CGG -3'; reverse: 5'- GGG CGT ACT TGG CAT ATG AT -3') to ensure that no mutations had been introduced.

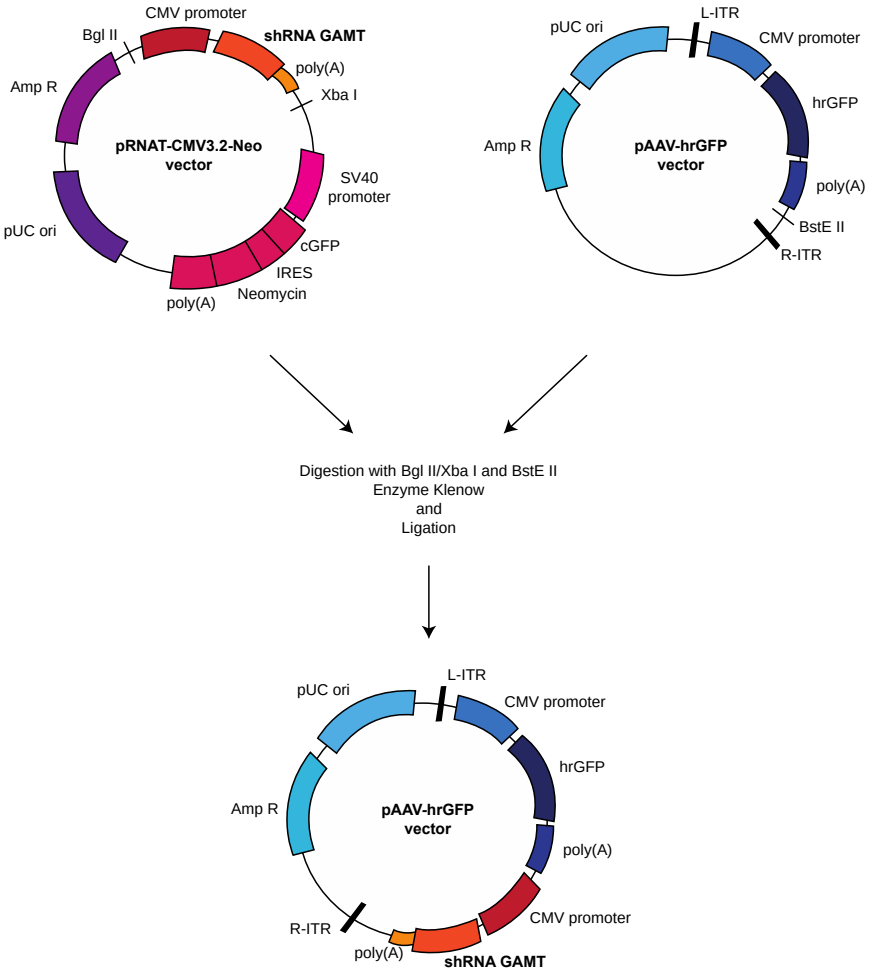


Figure 17: Schematic outline of the clonage of GAMT-2 shRNA and its CMV promoter in pAAV-hrGFP vector. The pRNAT-CMV3.2-Neo vector containing the CMV promoter and GAMT-2 shRNA was digested by two restriction enzymes, BglII and XbaI, to excise the fragment of interest. After the digestion of pAAV-hrGFP vector by BstEII and the reaction with the Klenow enzyme, the ligation of fragment of interest with pAAV-hrGFP was performed to obtain the insertion of CMV promoter and shRNA GAMT in pAAV-hrGFP vector. Amp R, Ampicillin resistance gene ; pUC ori, pUC origin of replication sequence; cGFP, corail green fluorescent protein; poly(A), poly-adenylation; hrGFP, humanized recombinant green fluorescent protein; L-ITR, left inverted terminal repeat; R-ITR, right inverted terminal repeat.

To produce the GAMT-2 shRNA-transducing AAV particles, we have transfected three plasmids in AAV-293 cell cultures, (i) pHelper, (ii) pAAV-RC to produce AAV serotype 2 or

pDP5rs to produce AAV pseudotype 2/5, and (iii) pAAV-hrGFP empty or expressing the GAMT-2 shRNA. Finally, we obtained two types of AAV viruses: 1) the AAV control virus without shRNA sequence, with a single cassette in its genome (figure 18-A), and 2) the interferent AAV virus expressing the GAMT-2 shRNA sequence, with a bi-cistronic expression cassette in its genome (figure 18-B).

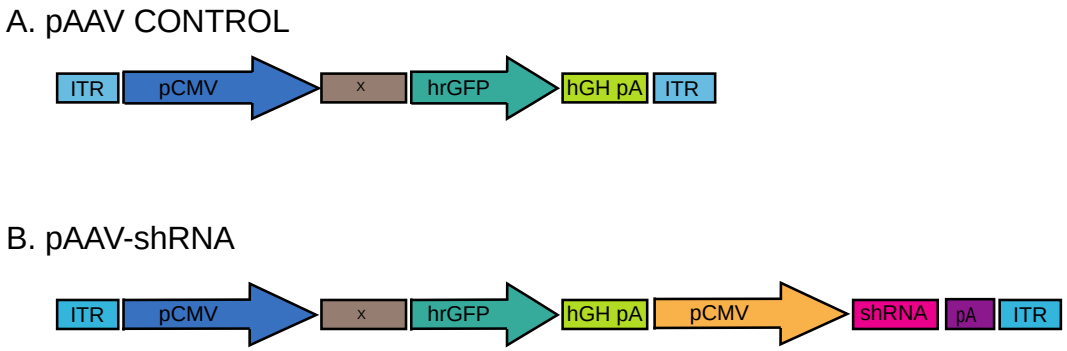
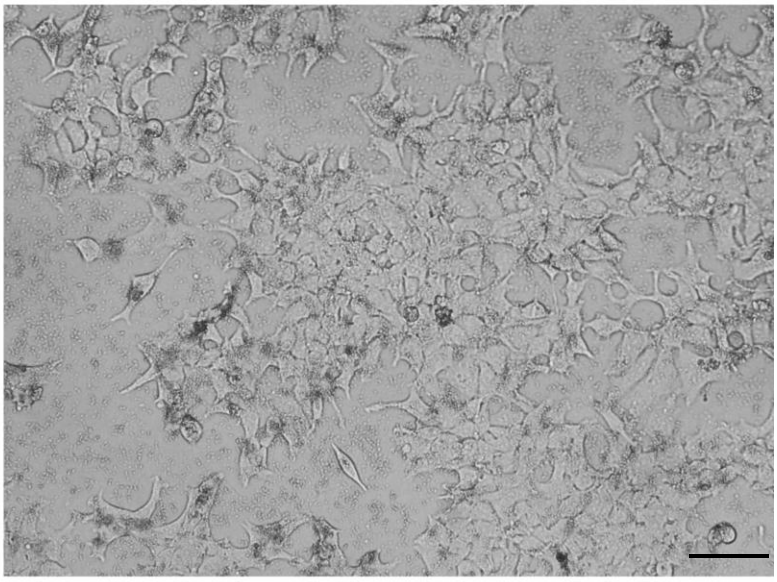


Figure 18: Schematic representation of the single (A) and bis-cistronic (B) expression vectors used in this study. ITR, inverted terminal repeat ; pCMV, Cytomegalovirus promoter; X beta-globin intron; hrGFP humanized recombinant green fluorescent protein ; hGH pA, human growth hormone polyA signal ; shRNA, short hairpin RNA ; pA, poly-adenylation.

The expression of a fluorescent reporter gene, hrGFP, was under the CMV promoter. The expression of the GAMT-2 shRNA was also under the control of the CMV promoter. The progress of AAV production could be monitored by observing phenotypic changes of the AAV-293 cell culture. The most obvious sign of viral production was a color change in the medium from red to orange/yellow, and the cells rounding up and detaching from the dish (figure 19).

A



B

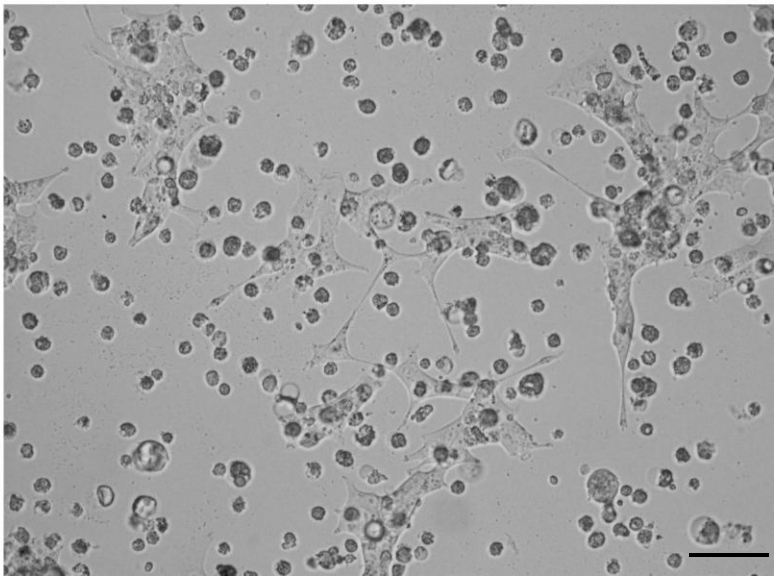


Figure 19: AAV particles production by the AAV-293 producer cells. A) AAV-293 cell morphology 6 hours after the co-transfection with the calcium-phosphate method of pHelper, pAAV-RC or pDP5rs, pAAV-hrGFP empty or expressing the GAMT-2 shRNA. **B)** AAV-293 cell morphology 3 days after the transfection. The cells round up and detach from the dish. The pictures were taken at 10x magnification without the removal of media and floating cells (Bar = 100 μm).

Four days after transfection, the cell supernatant containing the produced AAV particles was harvested. The viral particles were purified and then titrated by an ELISA method. The range of viral particles was between $1x10^9$ and $1x10^{11}$ depending on the viral production.

2. Transduction of monolayer cell cultures with AAV

AAVs isotype 2 and pseudotype 2/5 are the most employed AAV particles to transduce neural cells (Burger et al., 2005; Howard et al., 2008; Xu et al., 2001) and were thus chosen for this study. AAV isotype 2 has a genome and a capsid of serotype 2, while AAV pseudotype 2/5 has a genome of serotype 2 and a capsid of serotype 5.

To test the efficiency of AAV serotype 2 and 2/5 to down regulate the expression of the GAMT gene, first we investigated the transduction of AAV vectors in monolayer cell cultures. We have chosen the C6 monolayer cell cultures as these cells expressed GAMT. The efficiency of the transduction was evaluated by the observation of the hrGFP expression, the reporter gene expressed by AAV, with a fluorescent microscope, and a western blotting was realized to compare the expression of GAMT protein in C6 cell cultures treated with AAV control or AAV expressing the best selected GAMT-2 shRNA.

Briefly, the day before viral transduction, C6 cells were plated in dishes at 100 000 cell/dish. After 24 hours, the cells were transduced with AAV serotypes 2 or 2/5, either control or interferent to GAMT gene (AAV2-CTRL, AAV2-GAMT-2, AAV2/5-CTRL or AAV2/5-GAMT-2). The multiplicity of infection (MOI) is the ratio of viral particles to the cells to be infected, and in this study we have chosen a MOI of 1000. From the fifth day, we have observed the cells under a fluorescent microscope, figure 20 showing representative pictures of transduced cells. The morphology of infected cells, observed under Nomarsky microscopy, was unchanged as compared to the uninfected cells.

Depending on the cultures the proportion of AAV infected cells varied between 40 to 50% of the total number of cells.

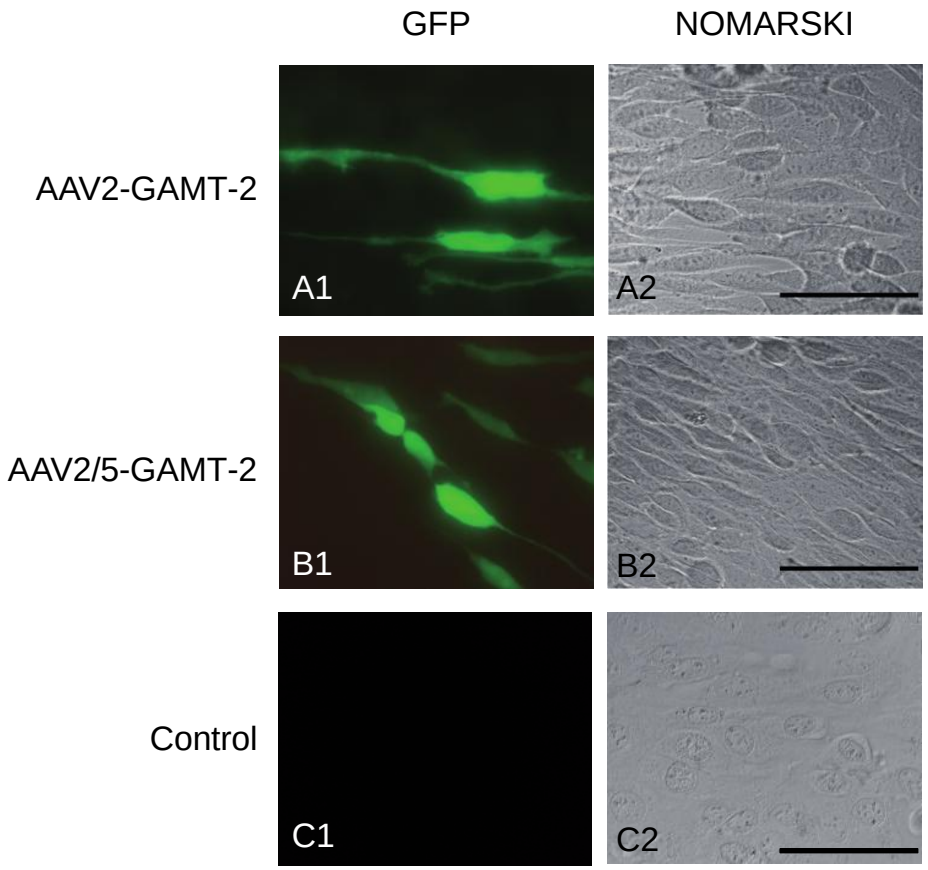


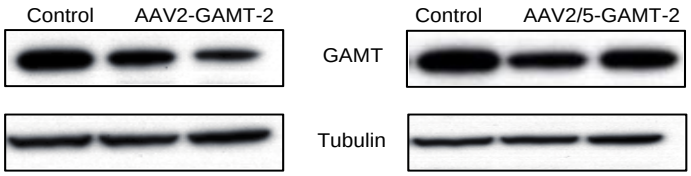
Figure 20: hrGFP expression in C6 monolayer cell cultures transduced with AAV2-GAMT-2 or AAV2/5-GAMT-2. Cells (100 000 cells/dish) were transduced with AAV2-GAMT-2 with a MOI of 1000 **(A)**, and AAV2/5-GAMT, with a MOI 1000 **(B)** or not transduced **(C)**.The fluorescent **(A1, B1 and C1)** or the Nomarsky pictures were recorded seven days after the transduction with AAV particles (Bar = 100 µm).

AAV2 and AAV2/5 vectors were equally able to transduce C6 cells.

To investigate the potential decrease of GAMT protein level due to the RNA interference, we performed western blotting to quantify the GAMT protein. For this, 15 days after the viral infection with AAV2 and AAV2/5 control or interferent vectors, the cells were harvested and the proteins were extracted. Compared to the cells infected with AAV control, the protein level of GAMT was decreased in cells infected with AAV2-GAMT-2 or AAV2/5-GAMT-2.

AAV2-GAMT-2 was more efficient than the AAV2/5-GAMT-2, with a decrease of GAMT protein level of about 50% for AAV2, instead of 25% for serotype 2/5 (figure 21).

A.



B.

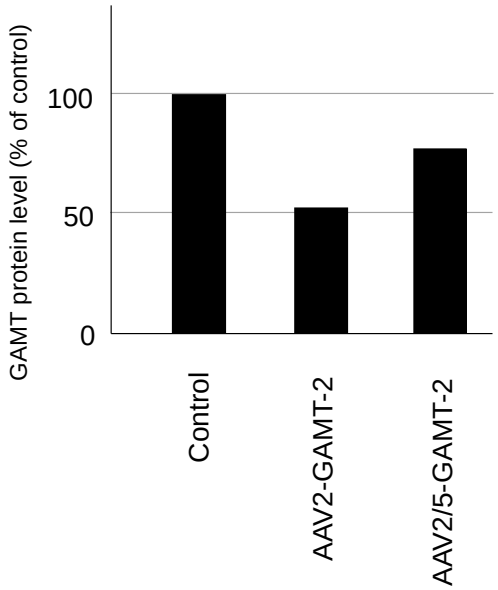


Figure 21: Quantification of GAMT protein expression after 15 days of AAV infection in C6 cell cultures. **A)** Representative western blot showing the GAMT and tubulin protein levels from control cells or cells infected by AAV2-GAMT-2 or AAV2/5-GAMT-2. **B)** The level of GAMT protein was normalized with tubulin to correct for minor differences in total protein loading from different samples. The values represent the mean from 2 replicate cultures.

AAV2 serotype appears the most effective serotype to transduce GAMT shRNA in C6 cells.

3. GAMT knock down in 3D reaggregated brain cell cultures

In the first part of this study, we realized prelimary tests in C6 monolayer cell cultures to investigate the efficiency of several shRNAs to induce the knock-down of GAMT gene, before to develop our new experimental model in brain cell aggregates. We selectioned the best sequence GAMT-2 shRNA to produce serotype 2 and 2/5 AAVs to transduce brain cell cultures. We have demonstrated in a simple model of C6 monolayer cell cultures expressing GAMT that AAV serotype 2 induced the maximal knock down as compared with the results obtained with serotype 2/5. After having demonstrated the knockdown effect of GAMT-2 shRNA on GAMT protein expression in C6 cells, we turned to the 3D reaggregated brain cell cultures. These cultures are composed of all types of brain cells, that are organized in a 3D network acquiring a tissue-specific pattern resembling that of the *in vivo* brain, and therefore are considered as organotypic brain cell cultures (Honegger and Monnet-Tschudi, 2001).

As 3D primary brain cell cultures in aggregates, which are made of all the different brain cell types, may behave differently as compared to C6 cells, AAV serotypes and the optimal MOI employed may also differ to obtain a maximal knock down. Several tests were performed to determine the best AAV serotypes and the adequate MOI to transduce reaggregated developing brain cell cultures. The cultures were infected at DIV 0 by AAV-GAMT-2 (expressing GAMT-2 shRNA) or control AAV-Empty viruses, with different MOI (10, 50, 100, 200 or 1000). The effect of viruses on GAMT expression level was evaluated at DIV 5, 8, 11 and 13 of culture by western blotting and qPCR.

3.1. Quantification of GAMT down-regulation by western-blot

The effect of AAV on GAMT expression in 3D primary brain cell cultures was assessed by measuring the GAMT protein by western blotting. While at DIV 5, level of GAMT protein was very low making difficult any measure of GAMT knock down, later stages showed a clear expression of GAMT protein, thus allowing the evaluation of the possible decrease of GAMT protein. At DIV 8, the infection of AAV2- GAMT-2 with a MOI of 100 provided a decrease of GAMT protein of about 40%, instead of about 25% for the infection with AAV2/5-GAMT-2 (figure 22). Moreover, AAV2/5 appeared to have a "toxic" effect per se, without GAMT-2 shRNA expression, as shown on figure 22 with a decrease of about 30 % of GAMT protein expression in aggregates infected with AAV2/5-Empty.

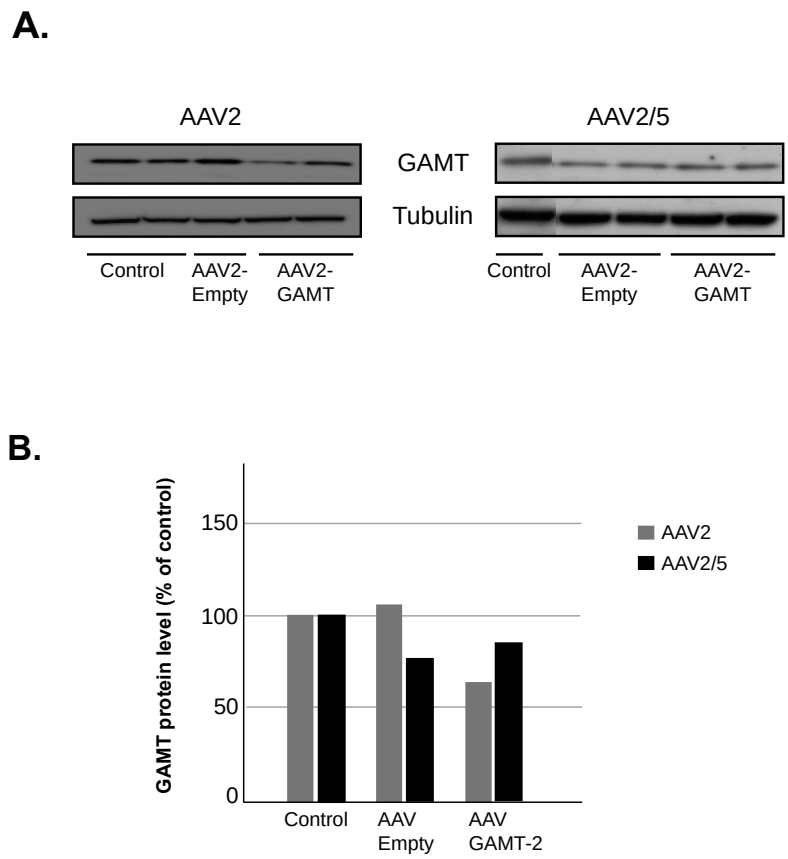


Figure 22: GAMT protein expression level after infection by serotype 2 or 2/5 AAVs (MOI: 100). Aggregates were harvested at DIV 8 of culture and the proteins were extracted to determine the GAMT protein level by western blotting. **A)** Representative western blot showing the GAMT level of cultures infected with AAV2 or AAV2/5. Samples under AAV2/5 condition come from the same blot. **B)** Quantification of western blots for GAMT protein. The level of GAMT protein was normalized with tubulin to correct for minor differences in total protein loading from different samples. The values represent the mean from 2 replicate cultures.

Therefore, as in C6 cultures, AAV2 appeared the best serotype to allow the strongest GAMT RNAi effect in aggregates at day 8 of culture.

In the first part of study with aggregates infected by AAV, we selected the serotype 2 of AAV vectors expressing GAMT-2 shRNA to down-regulate the endogenous levels of GAMT. As the expression pattern of transducing AAV-based vectors in aggregates brain cell cultures are not known, we investigated the stability of expression of shRNA from AAV vectors by the harvest of aggregates at several stages after the dissection (DIV 0). The harvests were performed at DIV 5, 8, 11 and 13, and the proteins were extracted to realize the quantification of GAMT protein level by western blotting. The maximal knock down of GAMT was obtained at DIV 8. An infection with a MOI 100 allowed a 55% decrease of the GAMT protein as compared to the aggregates infected with AAV2-Empty (figure 23). At DIV 5, the GAMT protein was very low and we could not see any difference between the infection with AAV2-Empty or AAV2 transducing the GAMT-2 shRNA (data not shown). At DIV 11, the GAMT protein returned progressively to normal levels in aggregates infected with AAV2-GAMT-2 (figure 23). At DIV 13, the levels of GAMT protein in aggregates infected with AAV2-Empty and AAV2-GAMT-2 were superior to the control condition. This observation might be due to an effect of AAVs on tubulin expression, as suggested by the decrease of tubulin in AAV-infected aggregates (figure 23). In the future, other genes not-affected by AAVs might be tested to normalize our experiments.

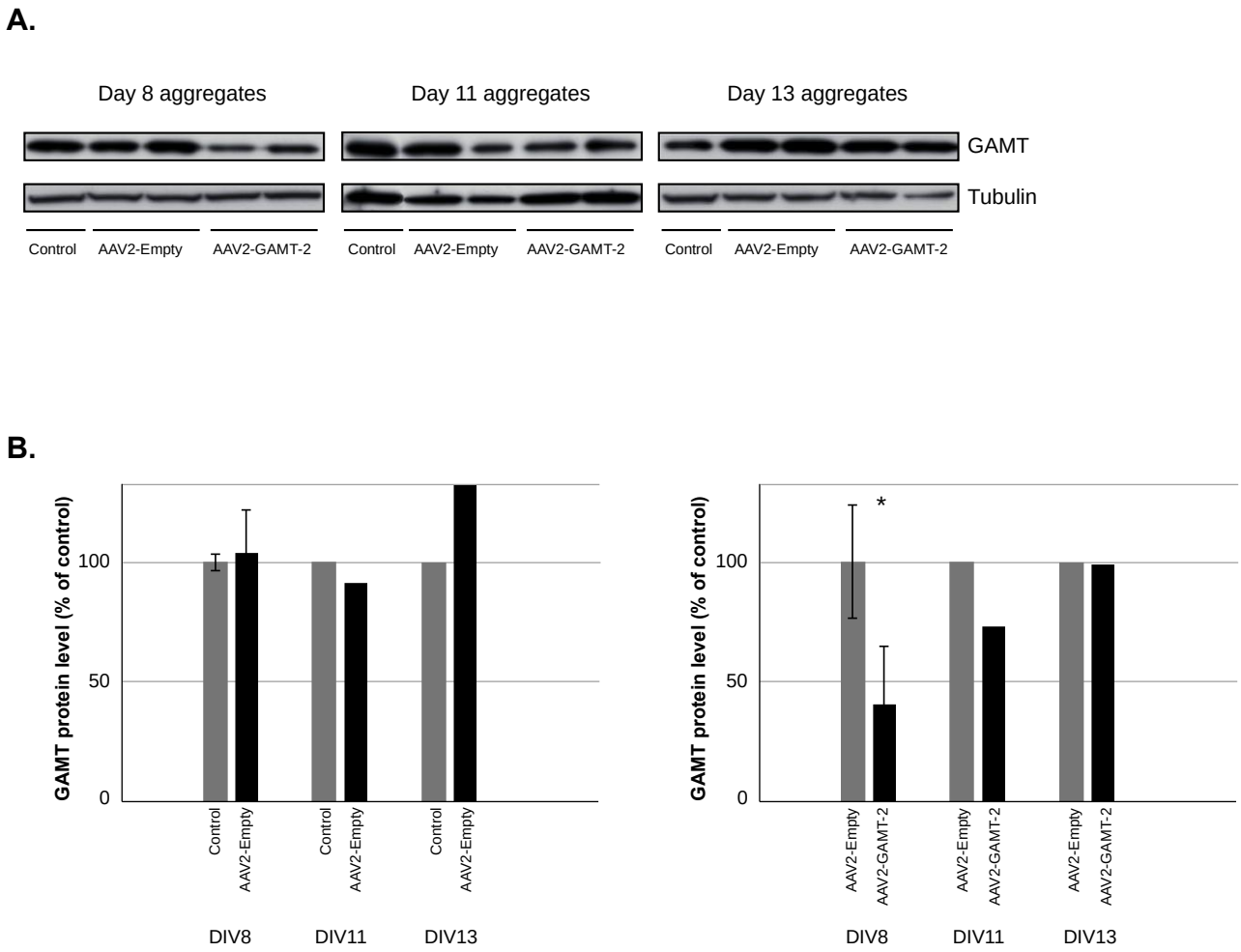


Figure 23: Knockdown effect on GAMT protein expression level at DIV 8, 11 and 13 after the infection by AAV2 with a MOI of 100. The aggregates were harvested at DIV 8, 11 or 13 of culture and the proteins were extracted to determine the GAMT protein level by western blotting. **A)** Representative western blot showing the GAMT level of samples infected by AAV2 at DIV 8, 11, and 13. **B)** Quantification of western blots for GAMT protein. The level of GAMT is normalized with tubulin to correct the minor differences in total protein loading from different samples. At DIV8 mean ± SD ($3<n<6$; NS: non-significative; *: $p<0.05$). At DIV11 and DIV13, the values represent the mean from 2 replicate cultures.

The maximal GAMT RNAi effect occurs at DIV 8 of culture, while at DIV 11 and 13, aggregates return to a higher level of GAMT.

After the selection of the best serotype and the observation of the day with the more important knockdown of GAMT protein, we analyzed which was the best MOI to allow the maximal knock down effect on the GAMT expression level. The aggregates were infected by AAV2 at

DIV 0 of culture and harvested at DIV 8. Immunoblot analysis revealed that the MOI of 10 and 50 were not efficient to decrease the GAMT protein level (data are not shown). A MOI of 200 allowed the most important decrease of GAMT protein level, at about 83% as compared to 40% with a MOI of 100 (figure 24). Moreover, we also tested a MOI of 1000, which led to the death of aggregates (data not shown).

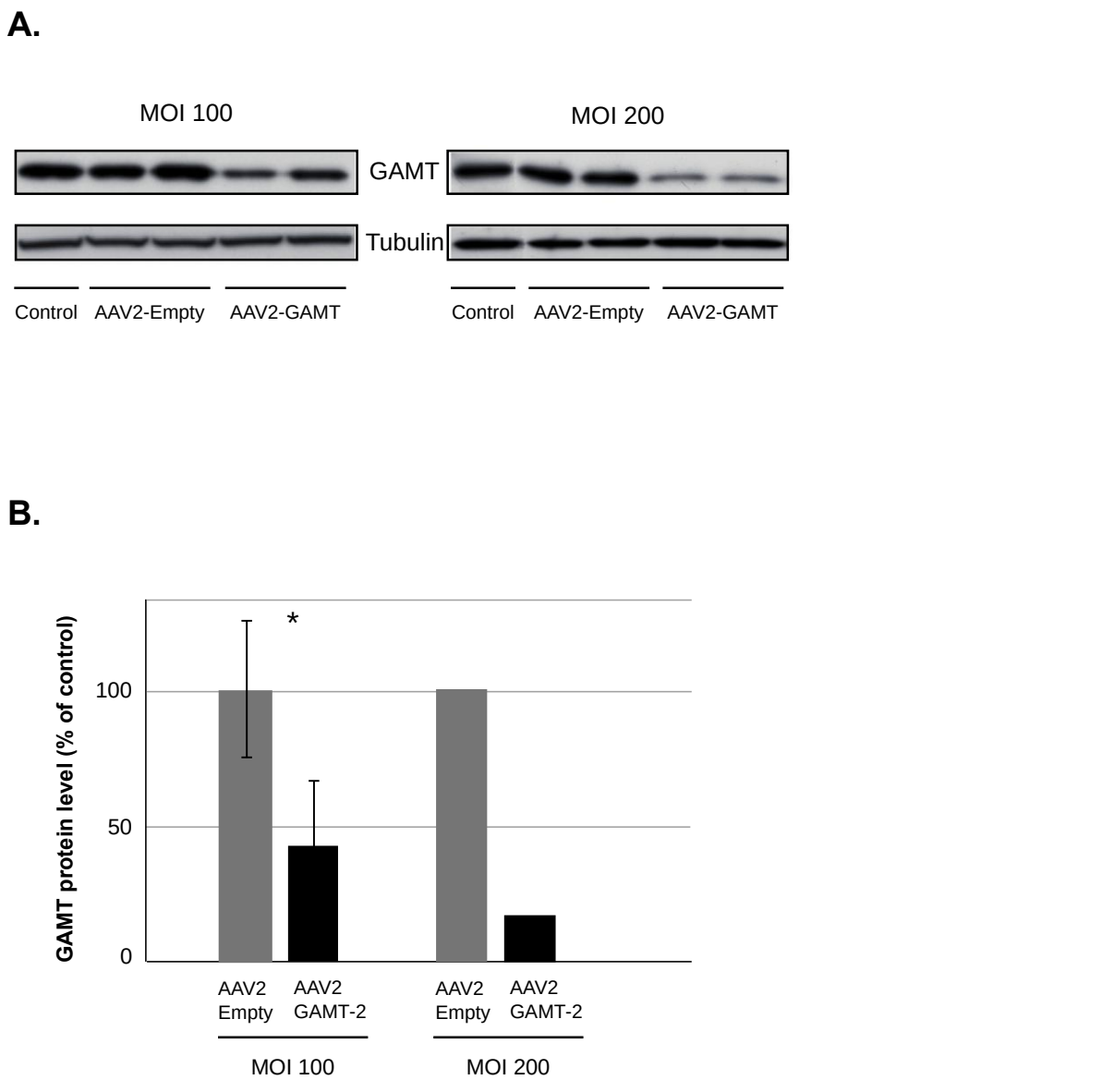
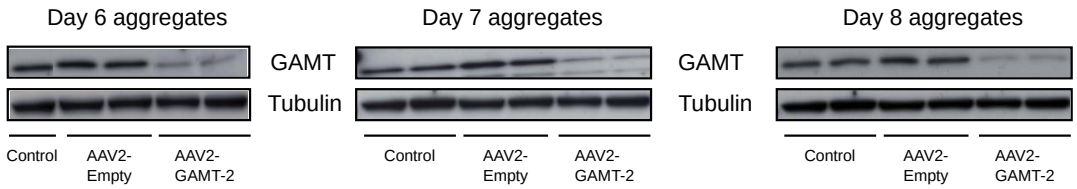


Figure 24: GAMT protein expression level after infection by serotype 2 AAV with a MOI of 100 or 200. The aggregates were harvested at DIV 8 of culture and the proteins were extracted to determine the GAMT protein level by western blotting. **A)** Representative western blot showing the GAMT level of samples infected by AAV2 with different MOIs, 100 or 200. **B)** Quantification of western blots for GAMT protein. The level of GAMT is normalized with tubulin to correct the minor differences in total protein loading from different samples. Infection with a MOI 100 mean ± SD ($3<n<6$; *: $p<0.05$). Infection with MOI 200, the values represent the mean from 2 replicate cultures.

Our results showed that the MOI of 200 was more efficient that the MOI of 100 to obtain the maximal decrease of GAMT protein level.

Finally we decided to observe precisely the pattern of GAMT down-regulation on consecutive days. The harvests were performed at DIV 5, 6, 7 and 8 after the infection by AAV2 with a MOI of 200, and the protein were extracted to realize quantification of GAMT protein level by western blotting. The results obtained at DIV 5 were not showed because the GAMT protein level was very low and we could not observed precisely a difference between the several conditions. From DIV 6 the decrease of GAMT protein level was progressive, reaching its maximum at DIV 8, with a decrease of 83% of GAMT protein level (figure 25).

A.



B.

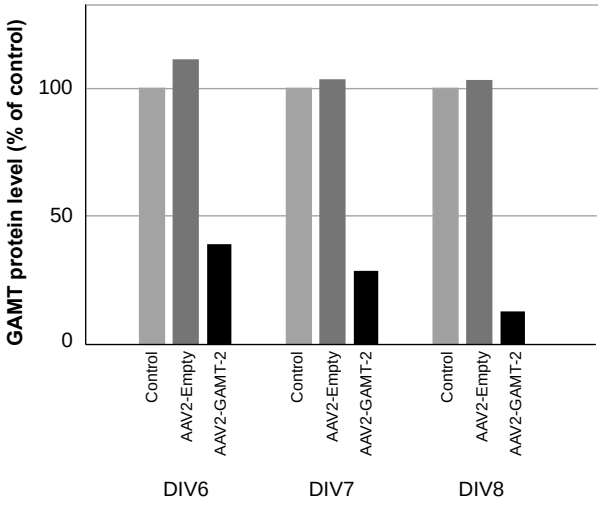


Figure 25 : GAMT protein expression level at DIV 6, 7 and 8 after the infection by AAV2 with a MOI of 200. The aggregates were harvested at DIV 6, 7 or 8 of culture and the proteins were extracted to determine the GAMT protein level by western blotting. **A :** Representative western blot showing the GAMT level of samples infected by AAV2 at DIV 6, 7, and 8. At DIV 6, the samples come from the same blot. **B :** Quantification of western blots for GAMT protein. The level of GAMT is normalized with tubulin to correct the minor differences in total protein loading from different samples. The values represent the mean from 2 replicate cultures.

Our results showed the progressive decrease of the GAMT protein level during three consecutive days and confirmed that the best decrease of GAMT protein was obtained at DIV 8 after the infection by AAV2.

3.2. Quantification of GAMT down-regulation by qPCR

In the first part of the study, we have shown the efficiency of RNAi on the GAMT protein level. We were also interested at analyzing the efficiency of RNAi on the GAMT mRNA expression. Real-time PCR, or quantitative PCR (qPCR), is a sensitive gene analysis technique, which combined to reverse transcription, allows the quantification of mRNA: qPCR allowed to evaluate the impact of shRNA on GAMT mRNA expression. The aggregates brain cell cultures were infected by control AAV2-Empty or AAV2-GAMT-2 at DIV 0 of culture with a MOI of 200, and harvested at DIV 8. The RNA was extracted and the cDNA was synthesized by reverse transcription to realize the qPCR (couple of primers: forward 5'-TAC AAA ACT GGG CCC TGA AG-3' and reverse 5'-AGC AAA CGG AAA GCA TGA GT-3').

The results showed a decrease of 30% of GAMT mRNA in aggregates infected by AAV2-GAMT-2 as compared to the aggregates infected by AAV2-Empty (figure 26).

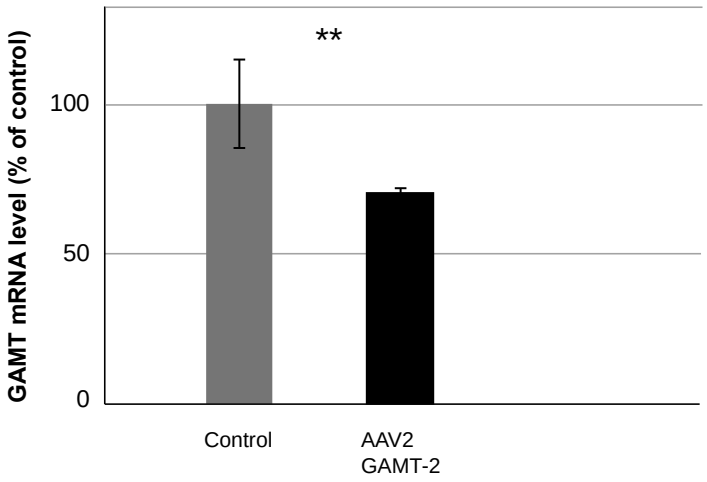


Figure 26: Quantification of GAMT mRNA expression after 8 days of AAV infection in aggregates brain cell cultures. The qPCR was realized on the cDNA obtained by reverse transcription of aggregates harvested at DIV 8 of cultures. The level of GAMT gene was normalized with GAPDH gene to correct the minor differences in sample loading. Mean ± SD (n=4; **: $p<0.01$).

AAV2 transducing GAMT-2 shRNA induced a decrease of 30% of GAMT mRNA in aggregates.

3.3. Measure of extracellular GAA and Cr by tandem mass spectrometry

After having demonstrated the knockdown effect of AAV2-GAMT-2 on GAMT protein expression and on GAMT mRNA in 3D reaggregated brain cell cultures, we were interested on the measure of two metabolites, GAA and Cr, to validate our new experimental model of GAMT deficiency in 3D reaggregated brain cell cultures. It is known that GAMT-deficient patients accumulate GAA in brain due to the block of GAMT enzymatic activity, with a concomittent decrease of their brain Cr. We wanted to verify in aggregates infected with AAV2-GAMT-2 if this could also be observed, with a decrease of Cr and an increase of GAA. The 3D reaggregated brain cell cultures were infected by control AAV2-Empty or AAV2-GAMT-2 at DIV 0 of culture with a MOI of 100, and harvested at DIV 5, 8, 11 and 13 to measure extracellular GAA and Cr by the tandem mass spectrometry.

As already demonstrated (Braissant et al., 2008), control conditions with unaffected aggregates showed the progressive increase of the Cr synthetic pathway along the time of culture, Cr levels released by brain cells in the medium being measured at 6 µM at DIV 5 but reaching 100 µM at DIV 13 in control condition (figure 27, A). In parallel, GAA released in the medium was measured at 5 µM at DIV 5, but reached 55 µM at DIV 13 in control condition (figure 27, B). The Cr/GAA ratio measured here (between 2 and 3.5 depending of the DIV) is in good correlation with the ratios measured in previous experiments in brain cell aggregates under control conditions (Braissant et al., 2008).

AAV viral toxicity (tested by TUNEL assay) could be observed, in particular at DIV 8, with very important increased levels of GAA (and to a less extend of Cr) in the culture medium in AAV2-Empty and AAV2-GAMT-2 cultures as compared to unaffected controls (figure 27, B and C). Viral toxicity was still visible at DIV 11, in particular on GAA level. This increase of released GAA and Cr in the medium may illustrate cell death induced by viral infection. No significant viral toxicity could be observed anymore at DIV 13 (figure 27, A and B).

A specific effect of GAMT RNAi was observed at DIV 8 (however non-significative at this stage) as well as at DIV 11 and 13 (with high significativity; $p<0.01$) between AAV2-GAMT-2 and AAV2-Empty cultures, with the characteristic increased GAA level due to the GAMT block induced by RNAi, and the concomittent decrease of Cr. Most interestingly, the increase of GAA and decrease of Cr at DIV 11 and DIV 13 were in stoechiometric proportions, which is expected also under GAMT activity inhibition (figure 27, C).

These results validated our experimental of GAMT-deficiency model in 3D reaggregated brain cell cultures, the same characteristic metabolite changes being observed as in GAMT-deficient patients.

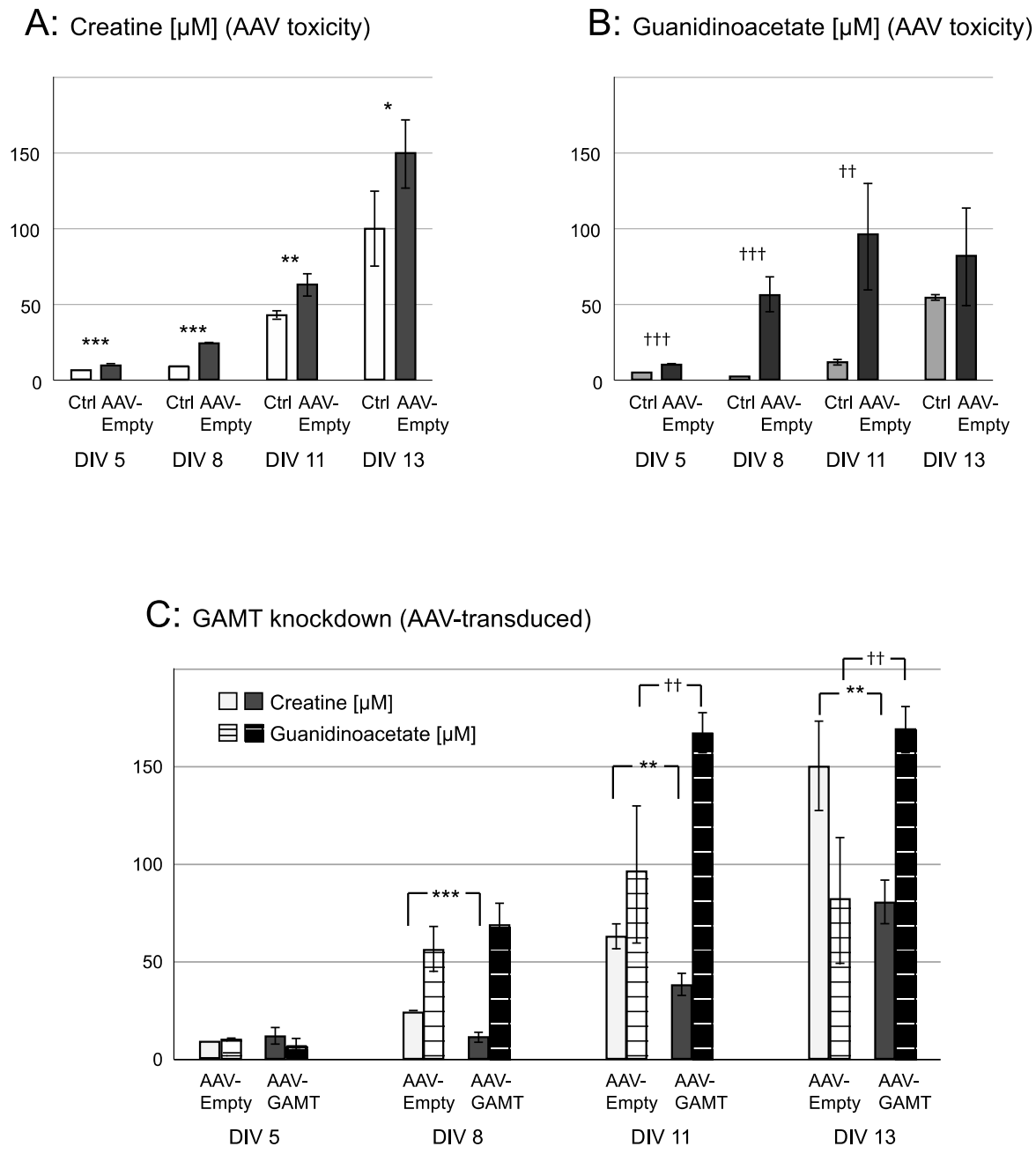


Figure 27: Measure of extracellular GAA and Cr by tandem mass spectrometry within 3D reaggregated brain cell cultures at DIV 5, 8, 11 and 13 after infection or not by AAV2 with a MOI of 100. Measures were realized on aggregates harvested at DIV 5, 8, 11 and 13 of culture. Measure of Cr (**A**) and GAA (**B**) in aggregates infected or not by AAV2-Empty. Panels **A** and **B** illustrate the effect of AAV2 per se on Cr and GAA release, including a potential toxicity to brain cells ; mean ± SD (n=4; NS: non-significative; for **Cr**: * $p<0.05$, ** $p<0.01$, *** $p<0.001$; for **GAA**: † $p<0.05$, †† $p<0.01$, ††† $p<0.001$). **C**: Measure of Cr and GAA in aggregates infected by AAV2-Empty or AAV2-GAMT-2. Panel **C** illustrates the effect of GAMT knockdown on Cr and GAA release by brain cells ; mean ± SD (n=4; NS: non-significative; for **Cr**: * $p<0.05$, **, $p<0.01$ *** $p<0.001$; for **GAA**: † $p<0.05$, †† $p<0.01$, ††† $p<0.001$).

Our results validated the new experimental model of GAMT deficiency with a decrease of extracellular Cr level and an increase of extracellular GAA level in aggregates infected with AAV2-GAMT-2 as compared to the aggregates infected with AAV2-Empty.

After validating the GAMT knockdown, as shown above, in our experimental model, we aimed at observing the effects of GAMT knockdown in 3D reaggregated brain cell cultures.

III. Consequences of GAMT knockdown on 3D reaggregated brain cell cultures

1. Effect of GAMT knockdown on developing brain cells

To analyse the effect of AAV2-GAMT-2 on 3D reaggregated brain cell cultures, we first examined the morphology of brain cell aggregates by immunohistochemistry against different cell-specific markers, namely phosphorylated medium-weight neurofilament (p-NFM, neurons), glial fibrillary astrocyte protein (GFAP, astrocytes) and myelin basic protein (MBP, oligodendrocytes). The cryosections (16 μm) were realized from aggregates harvested at DIV 13. It appeared that the AAV2-GAMT-2 viruses had no effect on MBP expression in oligodendrocytes, while a strong decrease of p-NFM expression in neurons and of GFAP expression in astrocytes was observed (figure 28).

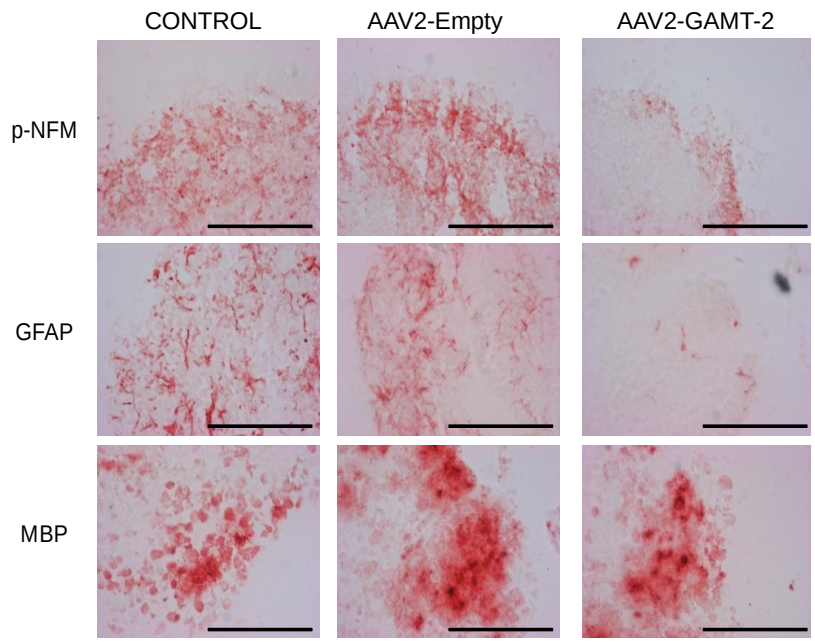


Figure 28: Perturbation in brain cell development in aggregates infected with AAVs with a MOI of 200. Aggregates were infected by AAVs at DIV 0 and harvested at DIV 13. Immunostaining of MBP, p-NFM and GFAP was realized on cryosections and revealed that the AAV2-GAMT-2 had no effect on oligodendrocytes but strongly affected the signal in neurons and astrocytes (Bar = 100 µm).

GAMT RNAi appears to affect neurons and astrocytes predominantly in aggregates infected by AAV2-GAMT-2, but not oligodendrocytes.

2. Activation of neuronal cell death pathway by GAMT knockdown

The effect of GAMT RNAi on cell death in 3D reaggregated brain cell cultures was assessed by *in situ* detection of apoptotic cell death using terminal desoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) in samples harvested at DIV 8 and 11 after the infection by control AAV2-Empty and AAV2-GAMT-2. At DIV 8 of culture, the number of TUNEL-positive cells was very low in control condition not infected by AAV2 (figure 29-A). The infection with AAV2-Empty increased the number of apoptotic cells, this result

showing the death caused by the toxicity of viruses (figure 29-B). Finally, TUNEL-positive cells were much more numerous in cultures infected with AAV2-GAMT-2 suggesting that the interference of the GAMT gene induced an increase of apoptosis (figure 29-C).

The harvest at DIV 11 showed the same results for the control condition and the aggregates infected with control AAV2-Empty (figure 30-A & B), with a very low number of TUNEL-positive cells as compared to the aggregates treated with AAV2-GAMT-2. On the other side, the number of TUNEL-positive cells in aggregates treated with AAV2-GAMT-2 was less important at DIV 11 than the number of apoptotic cells at DIV 8 (figure 30-C).

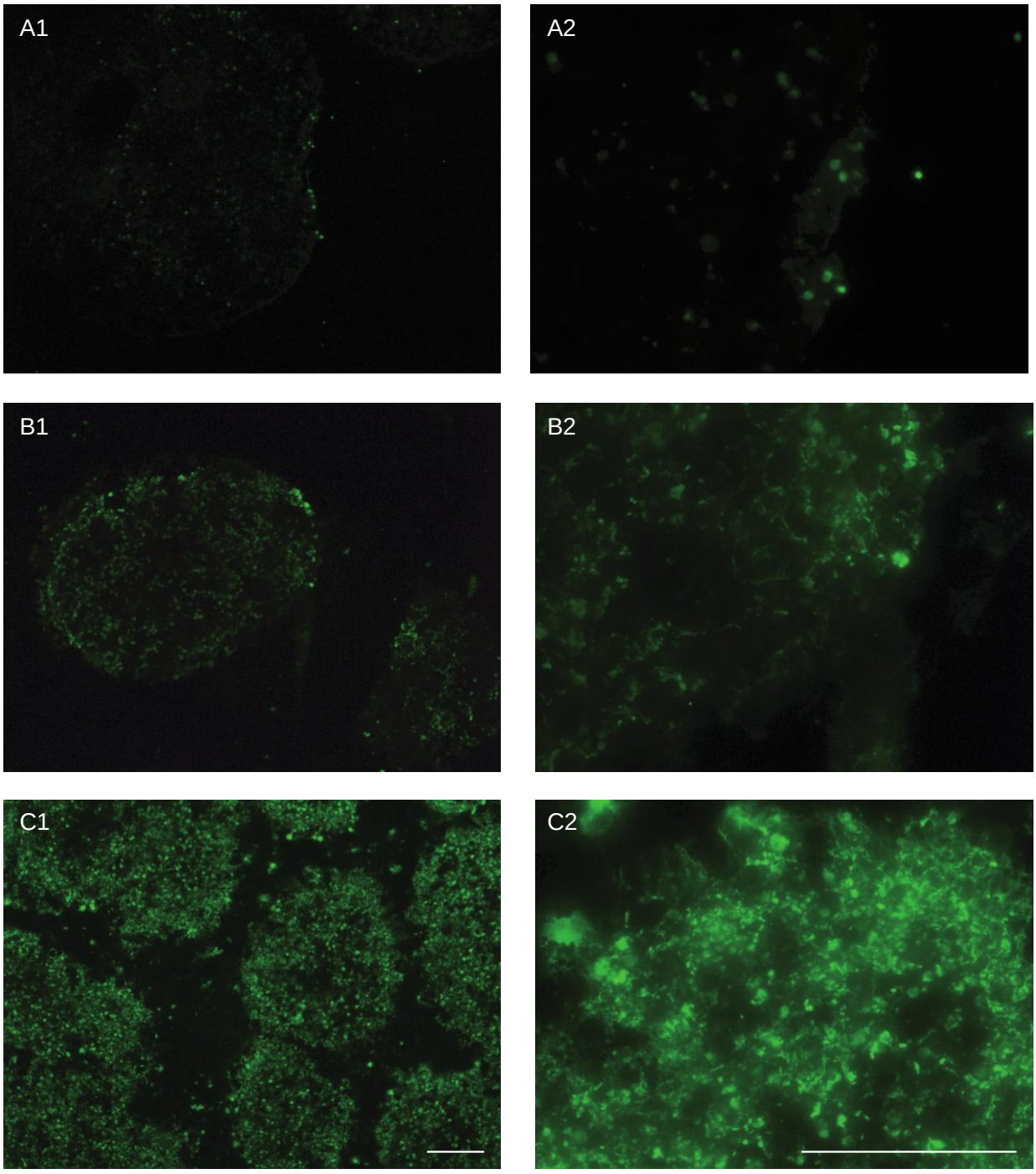


Figure 29: Neurotoxic effects (apoptosis, necrosis) of infection by AAVs on reaggregated developing brain cell cultures at day 8. Untreated cultures and cultures infected by AAVs at DIV 0 and harvested at DIV 8 were fixed and labeled with the TUNEL method. **A)** control condition. **B)** aggregates infected by control AAV2-Empty with a MOI of 200. **C)** aggregates infected by AAV2-GAMT-2 with a MOI of 200. **A2**, **B2** and **C2** are higher magnifications (40x) of the pictures presented in **A1**, **B1** and **C1** respectively (10x) (Bar = 100 µm).

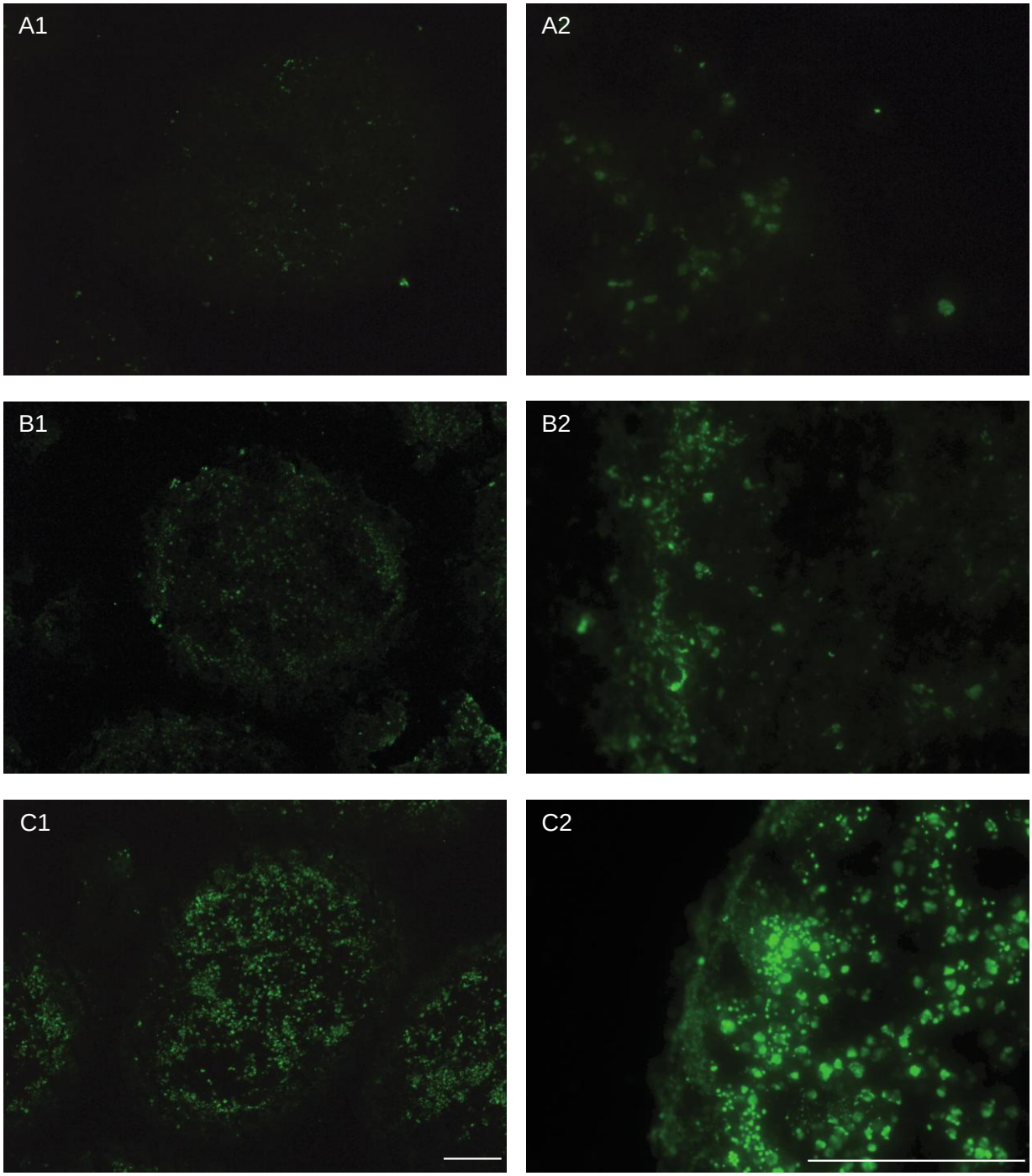


Figure 30: Neurotoxic effects (apoptosis, necrosis) of infection by AAVs on reaggregated developing brain cell cultures at DIV 11. Untreated cultures and cultures infected by AAVs at DIV 0 and harvested at DIV 11 were fixed and labeled with the TUNEL method. **A)** control condition. **B)** aggregates infected by control AAV2-Empty with a MOI of 200. **C)** aggregates infected by AAV2-GAMT-2 with a MOI of 200. **A2**, **B2** and **C2** are higher magnifications (40x) of the pictures presented in **A1**, **B1** and **C1** respectively (10x) (Bar = 100 µm).

A significant increase of apoptotic cells was observed by TUNEL labeling after 8 days of infection by AAV2-GAMT-2. After 11 days of infection, the amount of apoptotic cells was lower than at DIV 8.

To confirm the increase of apoptosis in aggregates infected with AAV2-GAMT-2, we analyzed by western blotting the full length and cleaved caspase 3 (e.g. activated caspase 3). Aggregates were harvested at day 8 of culture when the maximal effect of apoptosis was observed by TUNEL labeling. Immunoblots revealed that the cleaved caspase 3 increased in aggregates infected by AAV2-GAMT-2 and not in samples treated with AAV2-Empty (figure 31). These results showed that the apoptosis induced by the activation of caspase 3 pathway was due to the interference on the GAMT gene and not by the toxicity of AAV viruses.

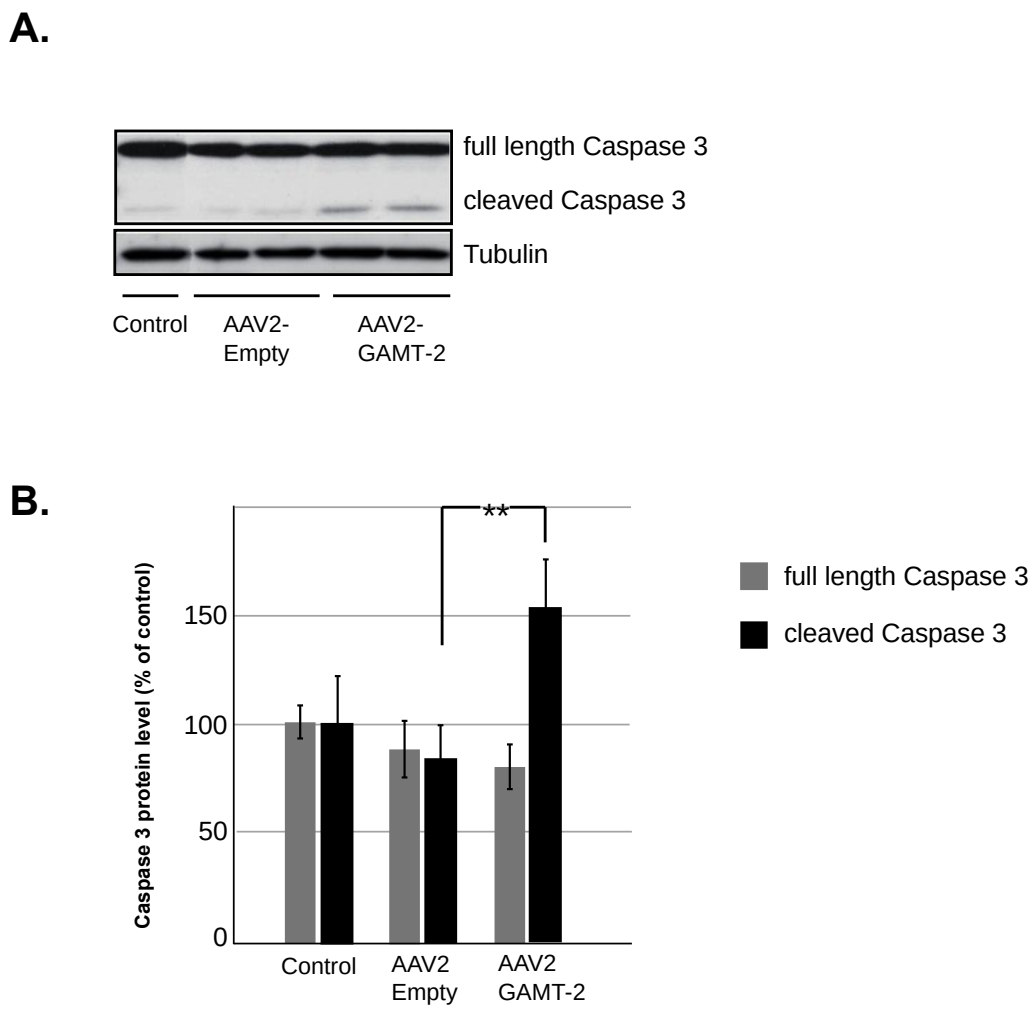


Figure 31: Full length and cleaved caspase 3 protein level at DIV 8 after the infection by AAV2 with a MOI of 200 in aggregates. The aggregates were harvested at DIV 8 of culture and the proteins were extracted to determine the full length and cleaved caspase 3 protein level by western blotting. **A)** Representative western blot showing the full length and cleaved caspase 3 level of samples infected by AAV2. **B)** Quantification of western blots for the two forms of caspase 3 protein. The level of caspase 3 was normalized with tubulin to correct the minor differences in total protein loading from different samples. Mean ± SD (n=4; NS: non-significative; **: $p<0.01$).

Increase in activated caspase 3 confirmed the increase of apoptosis in aggregates infected by AAV2-GAMT-2

Many of the external signals encountered by cells are transduced to the nucleus by a highly conserved eukaryotic signaling mechanism, the mitogen-activated protein kinase (MAPK) cascades. Activated MAPKs phosphorylate specific substrate such as transcription factors, cytoskeletal elements and other protein kinases, which in turn control physiological processes such as gene expression, mitosis, proliferation, motility, metabolism or programmed cell death (Aouadi et al., 2006). We were interested in one of the major conserved groups of MAPK, the p38 MAPK, in 3D reaggregated brain cell cultures infected by AAV2-Empty or AAV2-GAMT-2 or not. The p38 MAPK pathway is activated by cytokines, UV irradiation, osmotic and temperature stress and growth factors (Aouadi et al., 2006). The p38 MAPK is also involved in apoptosis and many function both upstream and downstream of caspases in the apoptosis response (Ono and Han, 2000).

On western blot, we showed that the infection with AAV2-Empty and AAV2-GAMT-2 increased the phosphorylation of p38 MAPK as compared with the control condition (figure 32). Consequently the AAV particles have an effect of toxicity on aggregates and caused the activation of cell death pathways. However, the increase of phosphorylated p38 MAPK was higher in aggregates treated with AAV2-Empty than in aggregates treated with AAV2-GAMT-2 (figure 32). This result might be explained by the potential decrease of astrocytes and neurons under AAV2-GAMT-2, as suggested by the strong decrease observed in GFAP and p-NFM proteins (figure 28). This observation might also be explained by the fact that the activation of p38 MAPK was principally located in reactive astrocytes (Cagnon and Braissant, 2009), the potential decrease of astrocytes under AAV2-GAMT-2 leading thus to a decrease

of phopho p38MAPK in aggregates infected by AAV2-GAMT-2 as compared to AAV-Empty.

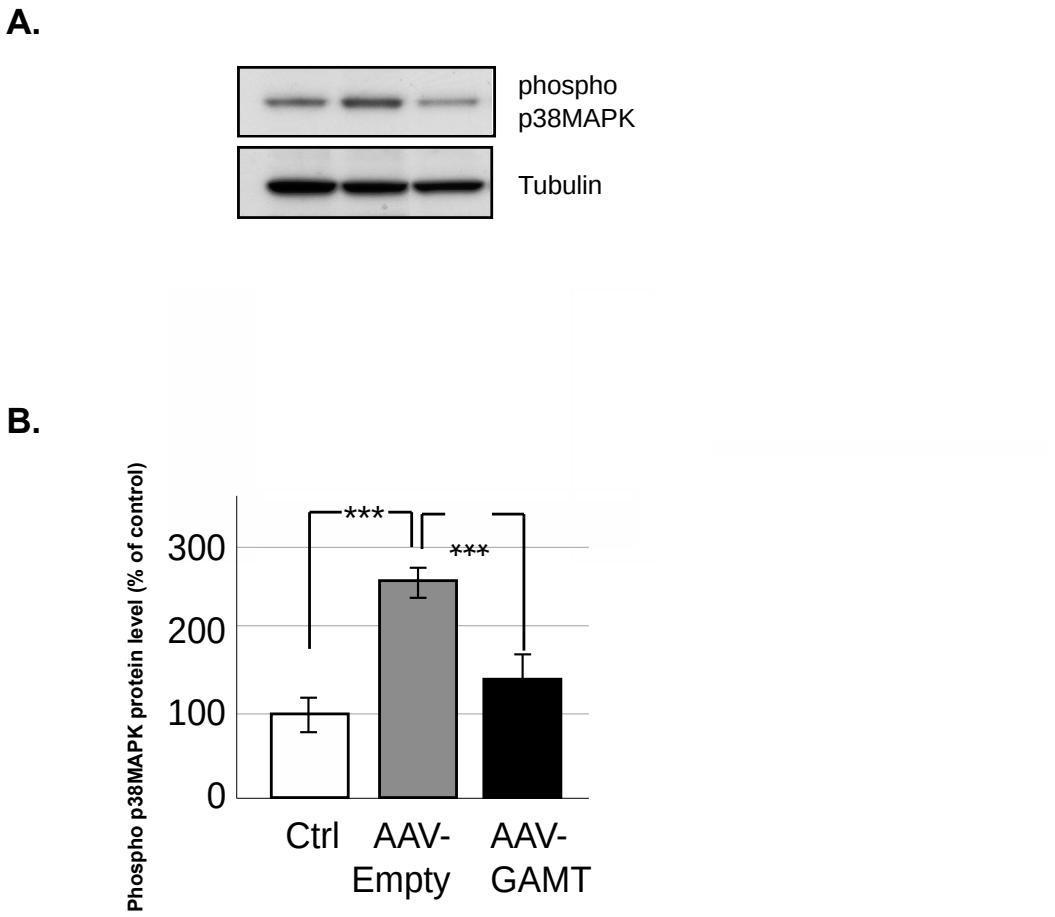


Figure 32: Activated p38MAPK protein level at DIV 5 after the infection by AAV2 with a MOI of 200 in aggregates. The aggregates were harvested at DIV 5 of culture and the proteins were extracted to determine the activated p38 MAPK protein level by western blotting. **A)** Representative western blot showing the phospho p38MAPK level of samples infected by AAV2 or not. **B)** Quantification of western blots for phospho p38MAPK protein. The level of phospho p38MAPK was normalized with tubulin to correct the minor differences in total protein loading from different samples. Mean ± SD (n=4; NS: non-significative; ***: $p<0.001$).

Our results showed an increase of the phosphorylation of p38 MAPK in aggregates infected by AAV2-Empty and AAV2-GAMT-2 and the difference between the aggregates infected by AAV2-Empty and AAV2-GAMT-2 might be explained by the potential decrease of astrocytes under AAV2-GAMT-2.

Part II: Study of SLC6A8 deficiency syndrome

In parallel of the first part of this work based on the development of a model of GAMT deficiency in developing brain cells, we were also interested in developing an experimental model for SLC6A8 deficiency. The same strategy as the one used for GAMT deficiency was applied: (i) test of several SLC6A8 specific shRNAs with the Dual-luciferase® assay system and selection of the best interfering sequence to knock down SLC6A8 gene expression, (ii) production of AAVs expressing SLC6A8 shRNA, and (iii) analysis of the consequences of SLC6A8 knockdown on brain cell development.

I. Knockdown of SLC6A8 expression

The design of SLC6A8 RNAi in 3D rat reaggregated brain cell cultures was performed as described in part I, chapter I.1. (GAMT RNAi). Rat SLC6A8 ORF is composed of 1908 nt (genBank accession no. NM_017348.2). Three specific shRNAs for SLC6A8 ORF were selected with the same algorithm than used for GAMT. The 21 nt SLC6A8 shRNAs spanned nucleotides 1056-1076, 1462-1482, and 1002-1022, respectively, downstream from the start codon of SLC6A8 ORF (figure 33).

siRNA-SLC6A8-1 GGATCCTAAAGGACTTGACCAGGTAATTTGATATCCGATTACCTGGTCAAGTCCTTTACGCTCGAG

siRNA-SLC6A8-2 GGATCCTTTGACCAGTCAGGCTTGAGATTGATATCCGTCTCAAGCCTGACTGGTCAAACGCTCGAG

BamHI Antisense Loop Sense XhoI

siRNA-SLC6A8-3 GGATCCCGCCTGACTGGTCAAAGCTATTCAAGAGATAGCTTTGACCAGTCAGGCTTTTTTCCAAAAGCTT

BamHI Antisense Loop Sense HindIII

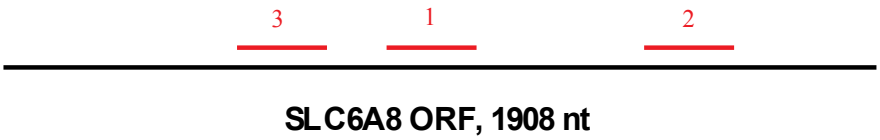


Figure 33: Sequences of each shRNA selectioned and their representative position on the rat SLC6A8 ORF.

SLC6A8-1 and -2 shRNAs were cloned into pRNAT-CMV3.2/Neo between the restriction site BamHI and XhoI as for the GAMT knockdown in figure 11, while SLC6A8-3 shRNA was cloned into pRNA-H1.3/Neo between the restriction site BamHI and HindIII (figure 11 et 34).

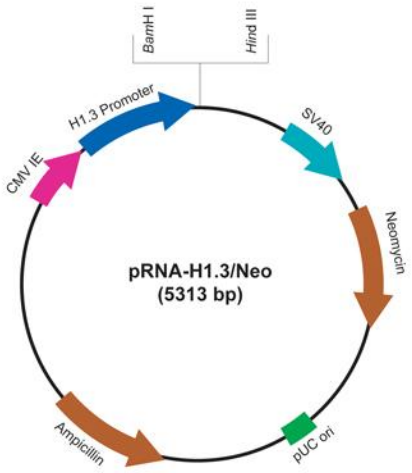


Figure 34: Circle map of pRNAT-H1.3/Neo (figure taken from www.genscript.com).

CMV (SLC6A8-1 and -2) or H1 (SLC6A8-3) promoters drived the expression of shRNA, and SV40 promoter drived the expression of the neomycin gene and cGFP.

As in the GAMT part of the study, the different SLC6A8 shRNAs were evaluated by the Dual-luciferase® assay system, which required the cloning of SLC6A8 ORF downstream of the *Renilla* luciferase gene in psiCHECK^{TM} vector (see figure 12; as for GAMT).

To achieve the quantitative assessment of SLC6A8 shRNAs efficiency, ROC cells were transfected 24 hours after seeding in 12 well plates with three differents plasmids, the reporter psiCHECK^{TM} containing the SLC6A8 ORF, the Genscript plasmid expressing the SLC6A8 shRNA, and p-Firefly as transfection control. A shRNA for synaptotagmin 4 gene, as well as the psiCHECK^{TM} vector containing the synaptotagmin 4 ORF, were used as positive control. The luciferase activity was recorded 48 hours after transfection. As shown in figure 35, SLC6A8-3 shRNA significantly down-regulated luciferase activity with a >84% decrease.

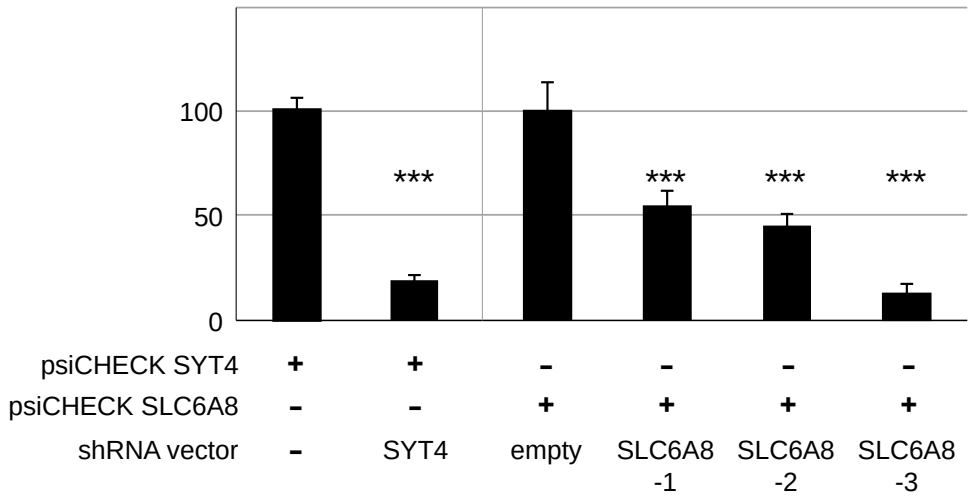


Figure 35: Silencing of luciferase activity by different shRNAs specific for SLC6A8 open reading frame, and Synaptotagmin 4 (SYT4) as positive control. ROC cells were transfected with a plasmid expressing firefly luciferase as a reporter for normalization of transfection, a psiCHECK^{TM} vector expressing SLC6A8 open reading frame (or SYT4 open reading frame) downstream of the *Renilla* luciferase open reading frame, and plasmids expressing shRNAs specific for SLC6A8 or SYT4. Luciferase activity is shown on the Y axis in arbitrary units (controls for SYT4 and SLC6A8: 100%). Mean ± SD (n=6; ***: $p<0.001$).

Our results suggests that SLC6A8-3 shRNA has the best efficiency to knockdown the expression of SLC6A8.

After having selected the best SLC6A8 shRNA interfering sequence in monolayer cell cultures, our aim was to develop a new experimental model of SLC6A8 deficiency in the organotypic *in vitro* system of 3D reaggregated brain cell cultures. Like the first part for GAMT deficiency, we used the AAV vector system to transduce the brain cell aggregates. We found ourselves faced with a problem to quantify the SLC6A8 down-regulation because our SLC6A8 antibody became inefficient, and a lot of time was lost to change protocols or use other antibodies to obtain a result on western blot. We found however interesting to show the preliminary results obtained on the consequences of SLC6A8 knockdown on 3D reaggregated brain cell cultures. The experiments for SLC6A8 knockdown were realized in parallel with these of the GAMT knockdown.

II. Consequences of SLC6A8 knockdown on 3D reaggregated brain cell cultures

1. Effect of SLC6A8 knockdown on developing brain cells

Like for the GAMT deficiency, we analyzed the effect of AAV2-SLC6A8-3 on 3D reaggregated brain cell cultures, by immunohistochemistry against different cell-specific markers, namely p-NFM (neurons), GFAP (astrocytes) and MBP (oligodendrocytes). The

cryosections (16 µm) were realized from aggregates harvested at DIV 13. It appeared that the AAV2-SLC6A8-3 viruses had no effect on pNFM expression in neurons, while a strong decrease of GFAP expression in astrocytes and of MBP expression in oligodendrocytes was observed (figure 36).

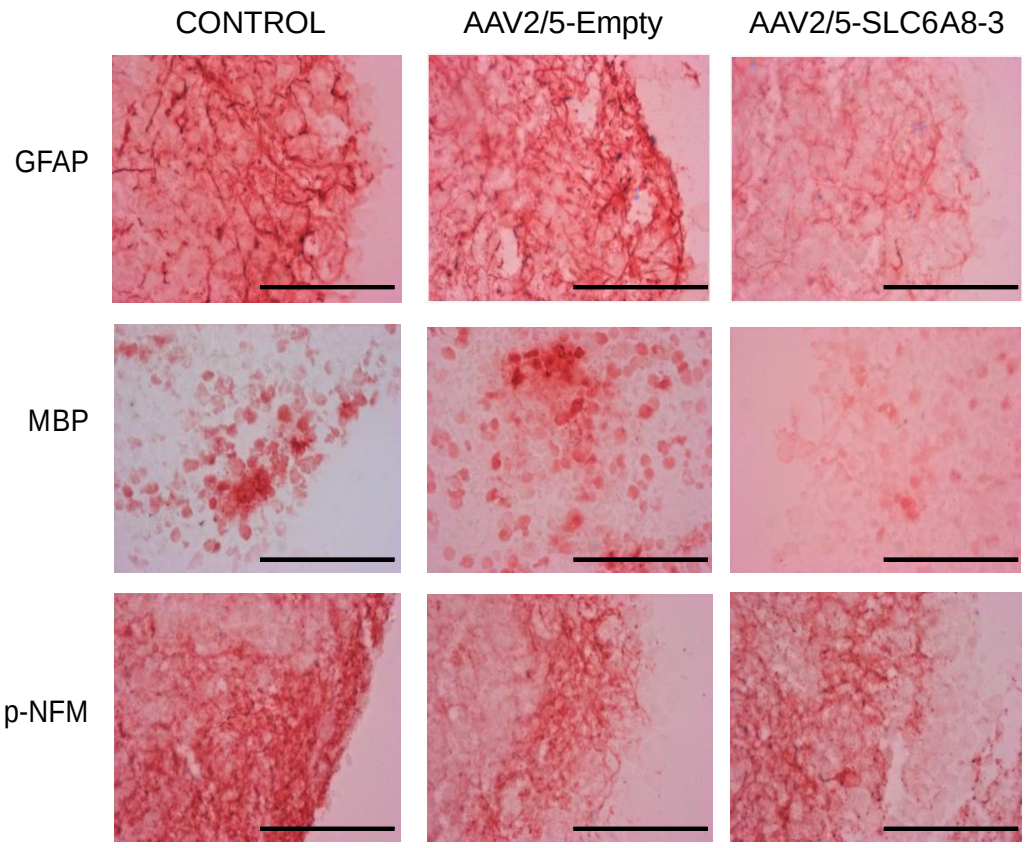


Figure 36: Perturbation in brain cell development in aggregates infected with AAVs with a MOI of 100. Aggregates were infected by AAVs at DIV 0 and harvested at DIV 13. Immunostaining of MBP, p-NFM and GFAP was realized on cryosections and revealed that the AAV2-SLC6A8-3 had no effect on neurons but strongly affected the signal in astrocytes and oligodendrocytes (Bar = 100 µm).

SLC6A8 RNAi appears to affect astrocytes and oligodendrocytes predominantly in aggregates infected by AAV2/5-SLC6A8-3, but not neurons.

2. Activation of neuronal cell death pathway by SLC6A8 knockdown

The same strategy to detect the activation of neuronal cell death as the one used for GAMT deficiency was applied. The TUNEL labeling was realized in samples harvested at DIV 8 and 11 after the infection by control AAV2-Empty and AAV2-SLC6A8-3. At DIV 8 of culture, the number of TUNEL-positive cells was very low in control conditions not infected by AAV2 (figure 37-A). The infection with AAV2-Empty increased the number of apoptotic cells, this result showing the death caused by the toxicity of viruses (figure 37-B). Finally, TUNEL-positive cells were much more numerous in cultures infected with AAV2-SLC6A8-3 suggesting that the interference of the SLC6A8 gene induced an increase of apoptosis (figure 37-C).

The harvest at DIV 11 showed the same results for the control condition and the aggregates infected with control AAV2-Empty (figure 38-A & B), with a low number of TUNEL-positive cells as compared to the aggregates treated with AAV2-SLC6A8-3. On the other side, the number of TUNEL-positive cells in aggregates treated with AAV2-SLC6A8-3 was less important at DIV 11 than the number of apoptotic cells at DIV 8 (figure 38-C).

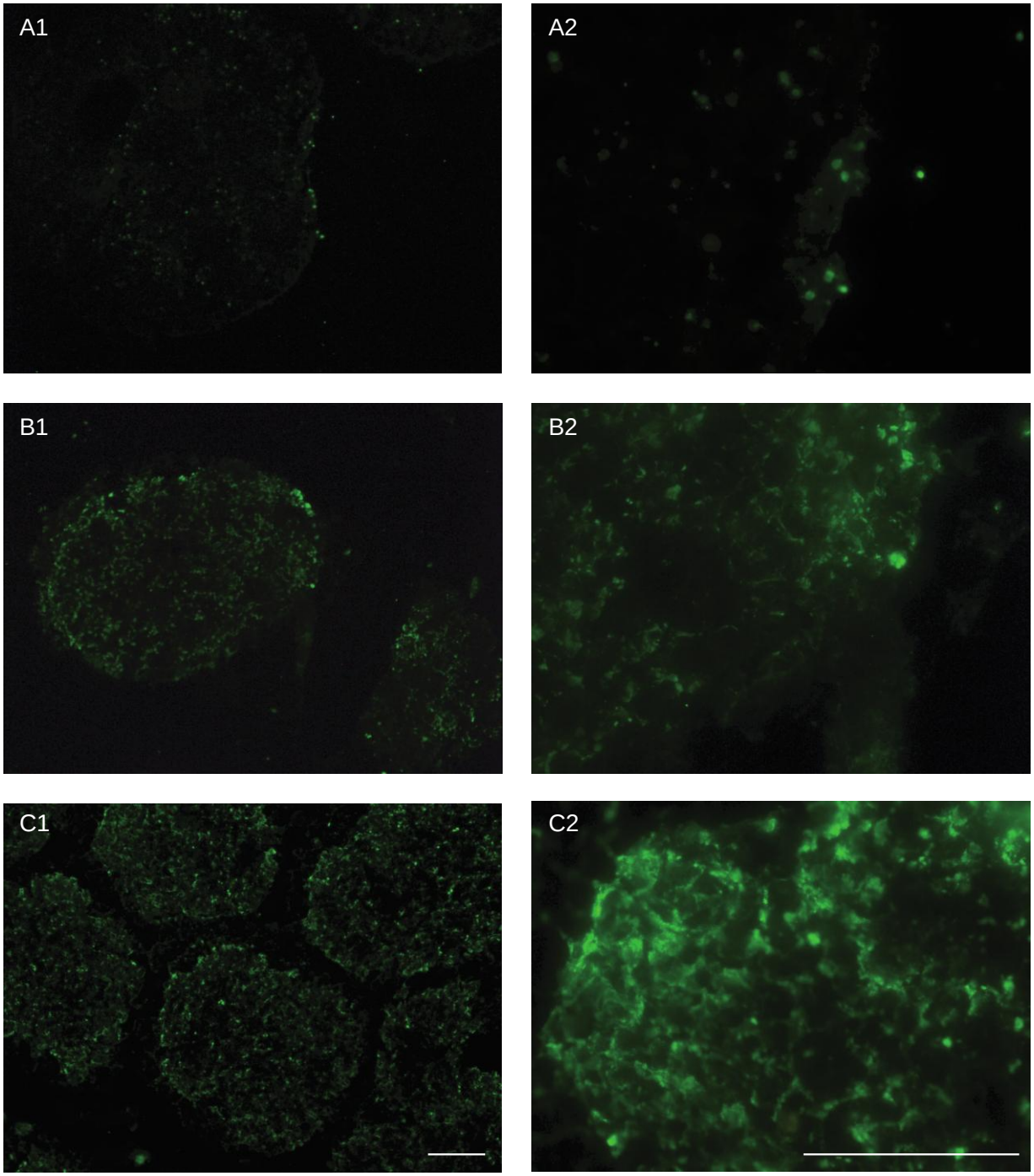


Figure 37: Neurotoxic effects (apoptosis, necrosis) of infection by AAVs on reaggregated developing brain cell cultures at DIV 8. Untreated cultures and cultures infected by AAVs at DIV 0 and harvested at DIV 8 were fixed and immunostained with the TUNEL labeling. **A)** control condition. **B)** aggregates infected by control AAV2-Empty with a MOI of 200. **C)** aggregates infected by AAV2-SLC6A8-3 with a MOI of 200. **A2**, **B2** and **C2** are higher magnifications (40x) of the pictures presented in **A1**, **B1** and **C1** respectively (10x) (Bar = 100 μm).

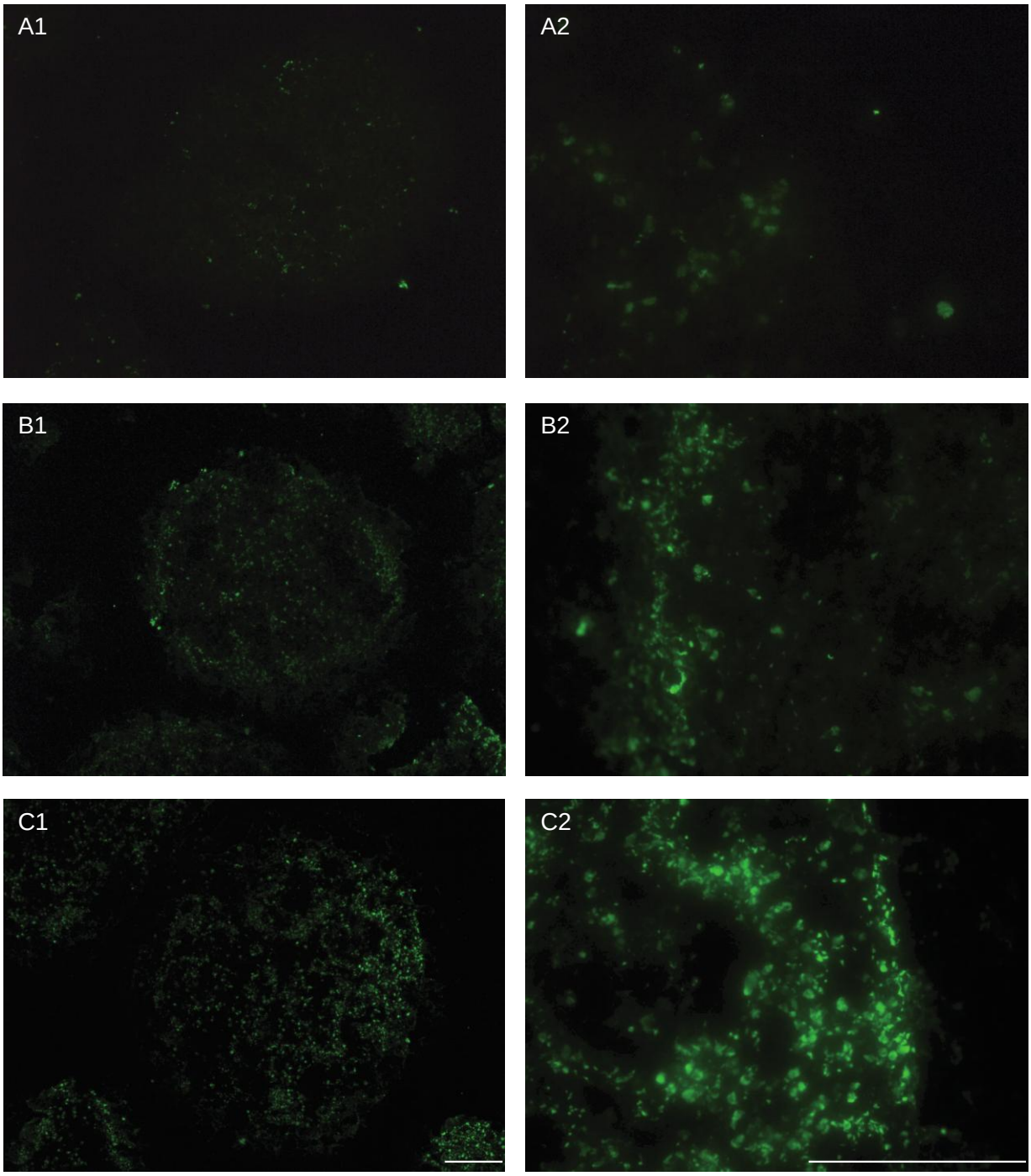


Figure 38: Neurotoxic effects (apoptosis, necrosis) of infection by AAVs on reaggregated developing brain cell cultures at DIV 11. Untreated cultures and cultures infected by AAVs at DIV 0 and harvested at DIV 11 were fixed and immunostained with the TUNEL labeling. **A)** control condition. **B)** aggregates infected by control AAV2-Empty with a MOI of 200. **C)** aggregates infected by AAV2-SLC6A8-3 with a MOI of 200. **A2**, **B2** and **C2** are higher magnifications (40x) of the pictures presented in **A1**, **B1** and **C1** respectively (10x) (Bar = 100 µm).

A significant increase of apoptotic cells was observed by TUNEL labeling after 8 days of infection by AAV2-SLC6A8-3. After 11 days of infection, the amount of apoptotic cells was lower than at day 8.

CHAPTER IV: Discussion

Cr deficiency syndromes due to AGAT, GAMT or SLC6A8 deficiencies are characterized by an absence or a strong decrease of Cr in CNS (Item et al., 2001; Salomons et al., 2001; Stöckler et al., 1994; Stromberger et al., 2003), which is the main organ affected. Indeed, no significative effects have been observed in peripheral tissues with high-energy demand (e.g. muscle and heart) in creatine deficient patients, except a faint muscular hypotonia in very rare cases of GAMT deficiency. So far, the mechanisms leading to the main neurological symptoms of these diseases are poorly understood. To understand the physiopathology of Cr deficiency syndromes, several *in vivo* models have been described so far, but their neurological symptoms and behavioral comportment are not comparable to those observed in patients. In the different mouse models, namely GAMT $^{-/-}$, SLC6A8 $^{-/-}$ and very recently AGAT $^{-/-}$ KO mice, nothing is known so far on the neurological and behavioral effects due to their Cr deficiency (Schmidt et al., 2004; Skelton et al., 2011; Torremans et al., 2005; Craigen et al., 2011; Sinha et al., 2011). No experience has been realized at the morphological level to see the effect of Cr deficiency syndromes on brain cell development.

We aimed at developing new experimental models of Cr deficiency syndromes in 3D primary brain cell cultures in aggregates in order to understand the disturbance in brain cell development, which might be responsible of some of the symptoms in patients. We observed the different cell types of CNS to see which were the most affected by the Cr deficiency, and what were the disturbances in the activation or inactivation of several pathways implicated in cell development or cell death. For this purpose, viral vector-mediated RNA interference was used to down-regulate GAMT and SLC6A8 genes in rat 3D reaggregated primary brain cell cultures.

As 3D reaggregated brain cell cultures are composed of all brain cell types, most of them being post-mitotic already at the culture start, we have decided to use viral vectors to

transduce these cultures with the best GAMT and SLC6A8 shRNAs. We were confronted with some difficulties to obtain effective viral production, as most of the time the viruses produced were too few for the infection of the primary brain cell cultures in aggregates. We also suspected that sometimes the transduction was ineffective to produce a decrease of our gene of interest, probably because of the insufficient proportion of AAVs containing a genome. These two points might explain the low number of positive results obtained in this study. Several modifications are currently being tested in our viral production protocol to obtain better yields of viral particles (modifications in the proportion of plasmids allowing the viral production, as well as in the incubation time of the transfection solution in dishes), as the quality of the viral production is the major point to improve in the future to obtain a systematic efficient knockdown of our gene of interest.

In the culture infected with AAV where we have observed a decrease of GAMT protein, we were confronted with another problem in the observation of GFP by microscopy. We were not able to observe GFP on aggregate sections while could observe the same GFP in monolayer cell cultures, thus proving that AAVs were able to transduce nerve cells with GFP expression. We have tested several methods, like fixation, or not, of our samples with paraformaldehyde at different %, fixation at the moment of harvest or after the cryosection. This point is the second point to improve in our laboratory to observe which types of cells were infected with AAV.

The main part of this work describes our proof of principle that GAMT could be knocked down by RNAi through AAV transduction in 3D reaggregated brain cell cultures. We have shown that the serotype 2 of AAV appears more efficient to knockdown the expression of GAMT in brain cell cultures as compared to pseudotype 2/5, and we have demonstrated that

this knockdown of GAMT seems due to the specific effect of GAMT-shRNA and not to an off-target effect. We have validated our experimental model by measuring the GAMT protein and mRNA levels, as well as the concentration Cr and GAA in the extracellular medium. We finally have analyzed the effect of the GAMT knockdown on the development of brain cells, by analyzing various intracellular signaling pathways implicated in cell death, as well as brain cell reactivity and differenciation.

Preliminary results were also obtained for the SLC6A8 knockdown, despite the fact that we had difficulties to fully demonstrate this knockdown. Interesting results were obtained on the morphology of brain cells or on the activation of cell death in aggregates infected by AAV-SLC6A8 shRNA, which were performed at the same time as those of the aggregates infected by AAV-GAMT shRNA. We thought it was interesting to show here these preliminary results as the infection with AAV-SLC6A8 modified brain cell development differently as the GAMT knockdown. Ongoing work continues to clearly demonstrate the knockdown of SLC6A8 in brain cell aggregates.

Part I: RNAi as an efficient tool

I. shRNA silencing efficacy

In order to realize an efficient down regulation of our gene of interest by shRNAs in neuronal cells cultures, we have analyzed the potential of several interfering sequences to decrease the

expression of GAMT and SLC6A8 genes. Despite considerable efforts to improve the selection of effective RNAi target sequences, including the development of various algorithms and the use of favourable thermodynamic properties, several shRNA sequences against a particular target mRNA need to be screened to obtain efficient knockdown. The dual luciferase reporter assay system was used to quantify the efficiency of shRNAs to down-regulate our genes of interest in monolayer cell cultures. This system has been reported to be a robust siRNA screening system and to have many benefits. First, the assay can be performed rapidly and allow the screening of various shRNA molecules. Second, the presence of the control reporter allows normalization for experimental variations such as cell number, pipetting volume errors, and cell lysis efficiency (McNabb et al., 2005; Xu et al., 2003). Nevertheless, this system has some disadvantages, like the fact that the tested mRNA, being composed of the luciferase ORF combined to our gene of interest ORF, is a hybrid with a different configuration as compared with the endogenous mRNA. The secondary and tertiary mRNA structures are different between the extrinsic and the endogenous mRNAs, and this may influence the ability of the shRNA to interfere with our gene of interest. Among several shRNA sequences, the dual luciferase assay system allowed us to select the best sequence to downregulate the GAMT or SLC6A8 gene. To confirm these results, we transfected the plasmid expressing the selected shRNAs in monolayer cell cultures, normally expressing GAMT, and we observed the GAMT protein level by western blot. We obtained the same results as those with the dual luciferase assay system with a downregulation of GAMT gene expression of 83% at the protein level.

II. AAV as a viral vector

We aimed at investigating the consequences of GAMT and SLC6A8 deficiency in 3D reaggregated brain cell cultures because the brain is the main organ affected by these pathologies. These cultures are composed of all types of brain cells, are organized in a 3D network resembling that of the *in vivo* brain, and therefore are considered as organotypic brain cell cultures (Honegger and Monnet-Tschudi, 2001). Given the complexity of this system growing as 3D spheres containing many different brain cells including already post-mitotic cells, a simple transfection of siRNA would not allow to obtain an efficient knockdown of our gene of interest. Only the mitotic cells on the surface of the spheres would be transfected with the siRNA. To obtain better results in the down-regulation of our gene, we decided to use an AAV viral vector as a tool for shRNA transfer in all cell types of the 3D reaggregated brain cell cultures, with the aim also of getting a longer effect of RNAi. In summary, AAV has many advantages including: (1) The ability to transduce dividing and non-dividing cells, (2) broad tropism, (3) lack of pathogenic and immunogenic effects and (4) long-term expression due to persistent episomal status (Mah et al., 2002; Shi et al., 2009; Stilwell and Samulski, 2003). Finally, the AAV vector has the advantage of long-term RNAi effect upon single administration (Grimm et al., 2005; Grimm, 2009; Shevtsova et al., 2005; Xu et al., 2001). The major disadvantage of AAV is the complex production of virus, and the difficulty to obtain high titers of viral particles (Mah et al., 2002; Stilwell and Samulski, 2003). AAVs isotype 2 and pseudotype 2/5 are the most employed AAV particles to transduce neural cells due to their high efficiency to transduce these cells (Blits et al., 2010; Burger et al., 2005; Howard et al., 2008; Mason et al., 2010; Xu et al., 2001), and were therefore chosen for our study.

Our next aim was thus to develop methods to produce, purify and titrate the AAV particles. Briefly, for the production, three essential components are used to produce recombinant AAV vectors. The first is a transgene expression cassette flanked by two AAV2 ITRs and constructed in a plasmid. The second is the AAV helper function of Rep and Cap proteins. The third is the adenoviral helper function provided by the products of the adenovirus E2A, E4 and VA genes. The production involves co-transfection of AAV-293 cells with these three plasmids (Shi et al., 2009). The potential of AAV viral vectors is however limited by difficulties to produce high vector yields. We were regularly confronted with low yields that did not allow the following infection of aggregate cultures. Work is going on in the laboratory to obtain better yields in AAV production.

The AAV titration was performed with an ELISA detection method, which recognizes a protein expressed by the capsid of the AAV particle. The limitation of this method is its recognition of two types of AAV particles, AAVs containing a genome and AAVs empty particles. Work is now going on to develop a precise qPCR test to allow the quantification of the AAV particles containing a genome, which are the effective ones to transduce shRNAs.
Once the production, purification and titration of viruses were realized, we have obtained AAV particles ready to infect the cell cultures. Before testing the interfering potential of AAV vectors in the 3D reaggregated brain cell cultures, we have tested the AAV viruses on simple monolayer cell cultures expressing the GAMT gene to observe the potential of AAV to knockdown the GAMT gene expression. We observed that AAV2 and AAV2/5 particles were equally able to transduce C6 cells. However, AAV2 particles were more effective to knockdown the GAMT gene as compared with AAV2/5. Thus our production of viruses was able to transduce the cells and to decrease the expression of our gene of interest. After having demonstrated that our production of AAV virus was able to down regulate the GAMT gene,

we used this vector on 3D reaggregated brain cell cultures to realize an experimental model of GAMT deficiency.

Part II: GAMT knockdown in 3D reaggregated brain cell cultures.

I. Choice of optimal conditions to obtain a maximal knockdown of GAMT

In this part of the study, we have developed the optimal conditions of AAV infections to obtain the maximal knockdown of GAMT gene in 3D reaggregated brain cell cultures.

We have first compared the efficiency of AAV isotype 2 and pseudotype 2/5 to downregulate the GAMT gene in aggregates, with an infection at DIV 0 of culture and a MOI of 100. AAV2-GAMT-2 appeared as the best serotype to allow the strongest GAMT RNAi effect in aggregates with a decrease of about 40% as compared to pseudotype 2/5. The next experiments were thus performed with AAV2 vector.

A decrease of 40% being considered as low, we have decided to improve the MOI used to obtain better knockdown of GAMT protein. While a MOI of 200 was more efficient than 10, 50, or 100, and allowed a decrease of the GAMT protein of 83%, a higher MOI (e.g. 1000) caused the death of aggregates. It has been reported that cell death due to AAV toxicity can have different explanations. First, the cell loss triggered by injecting recombinant AAV

vectors expressing shRNA can be interpreted as a result of an off-target knockdown of other mRNAs or saturation of the miRNA pathway (Ehlert et al., 2010). Briefly the saturation of the miRNA machinery by overexpressing shRNAs inhibits endogenous miRNA processing (Boudreau et al., 2009; Grimm et al., 2006; Lund et al., 2004; Yi et al., 2005) with concomitant adverse effects on the transduced cells (McBride et al., 2008; Ulusoy et al., 2009). In the nucleus, exogenous shRNAs can saturate the function of Exportin-5, a factor required for nuclear export of pre-miRNAs and shRNAs (Lund et al., 2004). Exportin-5 expression is relatively low in brain tissue (Yi et al., 2005) as compared to other tissues, rendering the brain particularly sensitive to Exportin-5 function saturation.

Secondly, AAV toxicity can be a result of a nonspecific effect due to overdosing, which can be seen with overexpression of any transgene and not related to the shRNA constructs (Ehlert et al., 2010).

In our study the second explanation should be considered because the toxicity due to high titer of AAV infection was similar in aggregates treated with AAV2-Empty and with AAV2-GAMT-2, suggesting that the phenomenon was unlikely to be a result of the shRNA expression. With a lower MOI, we could see this AAV toxicity in 3D reaggregated brain cell cultures by *in situ* cell death detection (TUNEL). The number of apoptotic cells in aggregates infected by AAV2-Empty and AAV2-GAMT-2 was higher than in the aggregates non-infected by AAV particles. This observation of the toxicity effect of AAV might be correlated with the measure of metabolites in extracellular medium of 3D reaggregated brain cell cultures. Indeed, aggregates infected with both AAV2-Empty and AAV2-GAMT-2 showed, in comparison with uninfected aggregates, significative increased release of GAA and Cr in the culture medium at DIV 5, 8 and 11, with a maximal effect at DIV 8. This increased released of metabolites in the medium may illustrate the cell death observed by TUNEL experiments. At DIV 13, no significant change in extracellular GAA and Cr could be

observed anymore between AAV-infected and uninfected aggregates, as also illustrated by the decrease of TUNEL-labelled cells observed from DIV 11 on in AAV-infected aggregates. This decrease of AAV toxicity at later stages of the culture (DIV 11, DIV 13) might be due either to a decrease of AAV efficiency after more than 10 days of infection (transiency of AAV effect), a recovery of aggregates from AAV infection, or simply to the natural increase of the Cr synthesis pathway along time of culture (Braissant et al., 2008), which might allow neuroprotective effects (Braissant et al., 2002).

We next determined the time-course of the RNAi efficiency in brain cell aggregates. We have performed harvests at DIV 5, 6, 7, 8, 11, and 13, and shown that the maximal knockdown of our gene of interest was observed at DIV 8 after the infection with AAV2-GAMT-2. From DIV 11 on aggregates returned to a normal level of GAMT.

In conclusion, we have developed a recombinant AAV vector expressing a GAMT-specific shRNA (shRNA-GAMT-2), able to efficiently initiate RNAi against the GAMT gene, and we have determined the better conditions of AAV infection in 3D reaggregated brain cell cultures to obtain the maximal down-regulation of GAMT gene. In summary, we used the AAV serotype 2 with a MOI of 200, to have the maximal effect of GAMT knockdown at DIV 8 after the viral infection.

II. Validation of our model of GAMT deficiency in brain cell aggregates

GAMT-deficient patients show an absence of Cr and an accumulation of GAA, the intermediate of Cr synthesis, in the brain due to the absence of the GAMT enzyme activity. In order to validate our new experimental model of GAMT deficiency with the RNAi method,

we had to show that it presents the same characteristics as those observed in the brain tissue of patients.

In the optimal conditions of AAV infection, we have demonstrated that the GAMT-2 shRNA transduced by AAV2 was efficient to decrease the GAMT protein level from 83% at DIV 8 of culture in brain cell aggregates cultures. With these experiments we confirmed that AAV is an efficient vector to transduce shRNAs in primary 3D reaggregated brain cell cultures composed of different types of cells, eather dividing or already post-mitotic. On the other side, we also showed that this AAV-transduced knockdown was a transient mechanism as the GAMT protein returned to a normal level at DIV 11 and 13 post-infection.

We were also interested to analyse the knockdown affect on the GAMT mRNA with qPCR method. The results obtained at DIV 8 after AAV infection were not as strong as compared with the results obtained by western blot. The decrease of the GAMT mRNA was approximately of 30% in 3D reaggregated brain cell cultures at DIV 8. Measures of GAMT mRNA level should be realized by qPCR at earlier days of harvest to see whether a time lag of the knockdown effect is observable in brain cell aggregates (figure 38). Indeed RNAi effects first affect mRNA, and then in turn the protein level.

Our results by western blot and qPCR suggest the presence of this time lag in changes in GAMT mRNA and protein levels by RNAi, the level of GAMT mRNA at DIV 8 being already returning to normal levels while the effect on GAMT protein is still clearly visible, and while both GAMT protein have normal levels from DIV 11 onward.

The final step to validate our new experimental model of GAMT deficiency was the measure of Cr and GAA in the culture medium by tandem mass spectrometry at DIV 5, 8, 11 and 13 after the infection with AAV2-GAMT-2 and AAV2-Empty. The results of this measure confirmed that our system was a good experimental model, as the level of Cr was decreased

in aggregates infected with AAV2-GAMT-2 as compare to aggregates infected with AAV2-Empty, and as the level of GAA was increased in aggregates infected with AAV2-GAMT-2. Interestingly moreover and as expected, we could observe, at DIV 11 and 13, an almost perfect stoichiometric balance between the decrease in Cr and the increase in GAA, in AAV2-GAMT-2 as compared to AAV2-Empty. The 3D reaggregated brain cell cultures behaved thus as the brain tissues in GAMT-deficient patients. To complete and confirm these results obtained by tandem mass spectrometry, ongoing work will allow the measure of intracellular Cr and GAA in the aggregates infected by AAVs.

After having validated our experimental model of GAMT deficiency, we focused on the effects of GAMT knockdown both on brain cell development and death.

Part III: Effect of GAMT and SLC6A8 knockdown in 3D reaggregated brain cell cultures

In our new experimental model of Cr deficiency, we observed by immunohistology a perturbation in brain cell development with an important potential loss of neurons and astrocytes in aggregates infected with AAV-GAMT as compared with aggregates infected with AAV-Empty. On the other side, we have observed an important potential loss of oligodendrocytes and astrocytes in aggregates infected with AAV-SLC6A8 as compared with aggregates infected with AAV-Empty. We could conclude that the presence of Cr seems to be essential for the brain development and the cell maturation. The effects observed in our brain cell aggregate model may appear much more deleterious than those observed in Cr-deficient patients, who would not survive such a potential loss of neurons and astrocytes for GAMT

deficiency or oligodendrocytes and astrocytes for SLC6A8 deficiency. However, we may speculate that similar effects may affect the brain regions that are the most affected in Cr deficiencies, namely cortex and basal ganglia.

Our laboratory has realized the first study showing the localization of AGAT, GAMT and SLC6A8 genes in the rat brain and in different CNS cells, at the mRNA and protein levels. Both in the *in vivo* brain and in 3D reaggregated brain cell cultures, GAMT mRNA and protein were found expressed in neurons, astrocytes and oligodendrocytes, but particularly high levels were localized in oligodendrocytes (Braissant et al., 2001; Braissant et al., 2008). On the other side, when brain cells are under a stress or toxic environment (e.g. hyperammonemia), they become reactive and a modification in the expression pattern of GAMT can be observed, GAMT expression increasing in particular in oligodendrocytes while no change is observed in neurons and astrocytes (Braissant et al., 2008).

Several hypotheses might explain the perturbation in brain cell development observed in our experimental model of GAMT deficiency with a potential loss of neurons and astrocytes but nothing on oligodendrocytes. First, the infection with AAVs might increase the reactivity of the cultures and increase GAMT expression in oligodendrocytes, thus rendering less effective on these cells. Second, AAVs might not infect oligodendrocytes, and thus no effect of RNAi could be observed on this type of cells. We could exclude this explanation because we observed an effect on oligodendrocytes in our experimental model of SLC6A8 deficiency. Third, the differentiation of oligodendroblast in oligodendrocytes occurs later in the maturation of aggregates as compared with those of neurons and astrocytes. We have observed that the AAV-transduced RNAi is transient, with a peak at DIV 8, a stage when oligodendrocytes are not mature at all. The peak of RNAi at DIV 8 might thus affect neurons and astrocytes in priority, while leaving oligodendrocytes non-affected. As for the second

hypothesis, this should probably be excluded as oligodendrocytes are affected in the SLC6A8 deficiency model.

In conclusion, reactive oligodendrocytes might be less affected by GAMT RNAi as suggested by the results observed by immunohistology. On the other side, GAMT RNAi might affect reactive neurons and astrocytes in priority, leading to accumulated GAA and thus cell toxicity.

SLC6A8 mRNA and protein are found in neurons and oligodendrocytes, but are normally absent from astrocytes. In contrast SLC6A8 expression is induced in reactive aggregates (Braissant et al., 2008). These results might explain the potential loss of astrocytes in our SLC6A8-deficient model. Brain cells in aggregates might react following the viral infection by activating several mechanisms of defense, in particular an induction of SLC6A8 in astrocytes, leading to a susceptibility of astrocytes to SLC6A8 RNAi and the potential loss of astrocytes.

After having shown the perturbation in brain cell development, we were interested in several cells signaling pathways implicated in cell death. First, we have observed by in situ cell death detection (TUNEL) that the infection with AAV-GAMT and AAV-SLC6A8 increased the number of apoptotic cells in 3D reaggregated brain cell cultures. This harmful effect due to the knockdown of GAMT could be explained by the increase of GAA generating a perturbation in neurotransmission of GABA, and by the decrease of Cr generating a decrease in high energy phosphates production and perturbation in neurotransmission. The loss of Cr leads to a failure in most energy-dependent processes necessary for cell survival, such as ion pumping, neuronal depolarization or pre-synaptic re-uptake of excitatory amino acids (Nicholls and Attwell, 1990). Previous research showed that GAA inhibits Na^+-K^+-ATPase activity in brain cells, which is deleterious for the cell survival (Zugno et al., 2004), and that an accumulation of GAA can reduce the brain antioxidant capacity, indicating the possible

involvement of oxidative injury in brain GAMT-deficient patient and offering an explanation for the pathogenic mechanisms of brain damage (Zugno et al., 2008). The loss of Cr and accumulation of GAA due to the knockdown of GAMT may cause neuronal death in aggregates by necrosis or apoptosis (Beard and Braissant, 2010; Dirnagl et al., 1999; Zhu et al., 2004). While the important cell loss observed at DIV8 after AAV infection may lead to the observed decrease of GAMT protein at this stage independently of RNAi, the normalizing levels of GAMT protein at DIV11 and 13 speak in favor of a transient, but efficient, specific GAMT RNAi at the younger stages.

A study of our laboratory has brought the first evidence that GAA is taken up by brain cells through the same transporter as Cr: SLC6A8 (Braissant and Henry, 2010). A decrease of SLC6A8 expression may lead as under GAMT deficiency to an accumulation of GAA, which is very toxic for the cells, and a decrease of synthesis and transport of Cr, resulting in an increase of the number of apoptotic cells in cultures.

We were also interested in the intracellular signaling pathway that may be altered by Cr deficiencies. We have observed the increase of the cleaved caspase 3 in aggregates infected with AAV-GAMT, by the quantification of the activated form of caspase 3 by western blot. The caspase family, composed of proteases, is the core component of the apoptotic machinery involving a proteolytic system. Two major caspase-3 activating pathways have been identified: an extrinsic pathway involving cell-surface receptors, and an intrinsic pathway resulting from alterations at the level of mitochondria (Rupinder et al., 2007). The first is triggered by signals extrinsic to the cell through ligation of death receptors such as Fas or tumor necrosis factor-alpha receptor. These ligand-activated receptors recruit a number of adaptor molecules to the intracellular domain of the receptor, which culminate in activation of the initiator caspase 8. This caspase cleaves executioner caspases such as caspase-3, which

cleaves a wide range of intracellular substrates to trigger the apoptotic response. The second major pathway activating the caspase cascade is an intrinsic cellular response to a range of stimuli including oxidative stress, growth factor withdrawal, UV radiations and chemotherapeutic agents (figure 40).

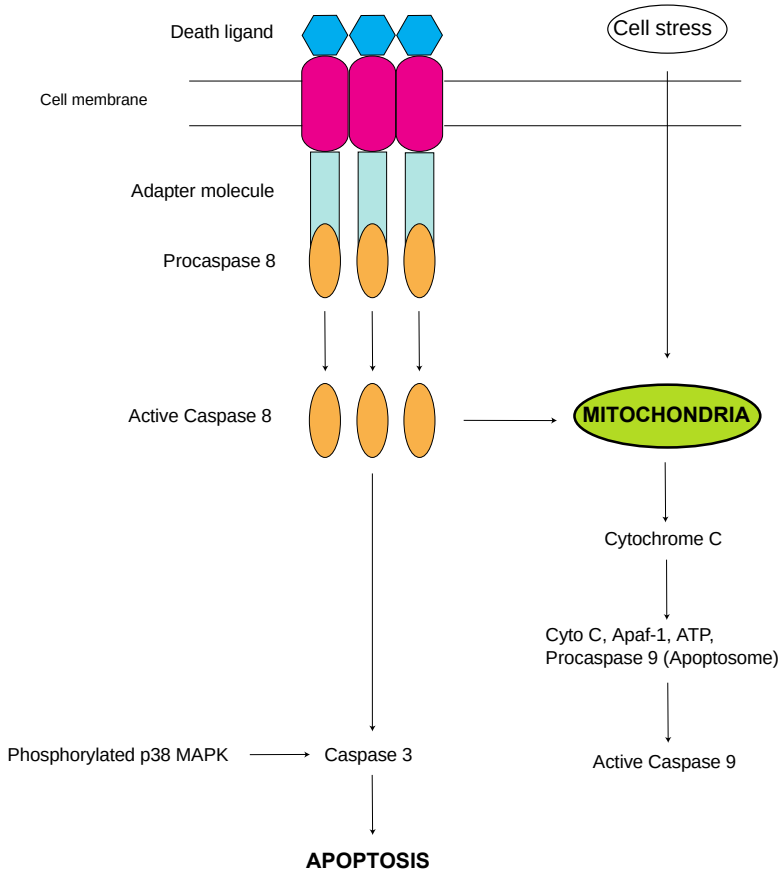


Figure 39: Steps in signaling of extrinsic and intrinsic pathways of apoptosis (inspired by Rupinder et al., 2007)

The loss of Cr has consequences at the mitochondrial level because Cr plays essential roles in stabilizing mitochondrial function and in decreasing neuronal death. The loss of Cr, as observed in our experimental model, causes the dysregulation of the mitochondria with alterations in Ca^{2+} homeostasis, production of reactive oxygen species (ROS) and cell death (apoptosis) (Beard and Braissant, 2010; Green and Reed, 1998; McBride et al., 2006; Steeghs et al., 1997). A recent study has shown an overproduction of ROS due to an accumulation of

GAA in GAMT-deficient patient fibroblast cell lines, which may also occur in our experimental model and explain the very important number of apoptotic cells (Alcaide et al., 2011). Mitochondria can release several pro-apoptotic proteins into cytosol, which in turn can induce cell death (Primeau et al., 2002). This release is under control of ROS, allowing formation of mitochondrial permeability transition pores (mPTP), a continuum between inner and outer mitochondrial membranes (Adhihetty and Beal, 2008). mPTP are associated with different death mechanisms leading to apoptosis and necrosis (Bernardi et al., 1998). mPTP formation and opening is facilated by several factors, like accumulation of Ca2+, reduction in membrane potential, increase in inorganic phosphate, decrease in ATP and ADP, and elevation in oxidative stress (Di Lisa and Bernardi, 2005). In particular, mPTP are localized on the mitochondrial membrane beside MtCK, with which they interact. MtCK suppresses pore opening and potentially decreases apoptotic susceptibility, which is itself stabilized by the presence of Cr (Adhihetty and Beal, 2008; Beard and Braissant, 2010; O'Gorman et al., 1997). In our GAMT-deficient model with a strong decrease of Cr, the MtCK might not be stabilized and the mPTP might be open. Consequently the pro-apoptotic proteins might be released in cytosol causing the cell death (figure 41). This suggests important roles of Cr to protect neurons from cell death, and might explain some of the consequences of Cr deficiency in CNS.

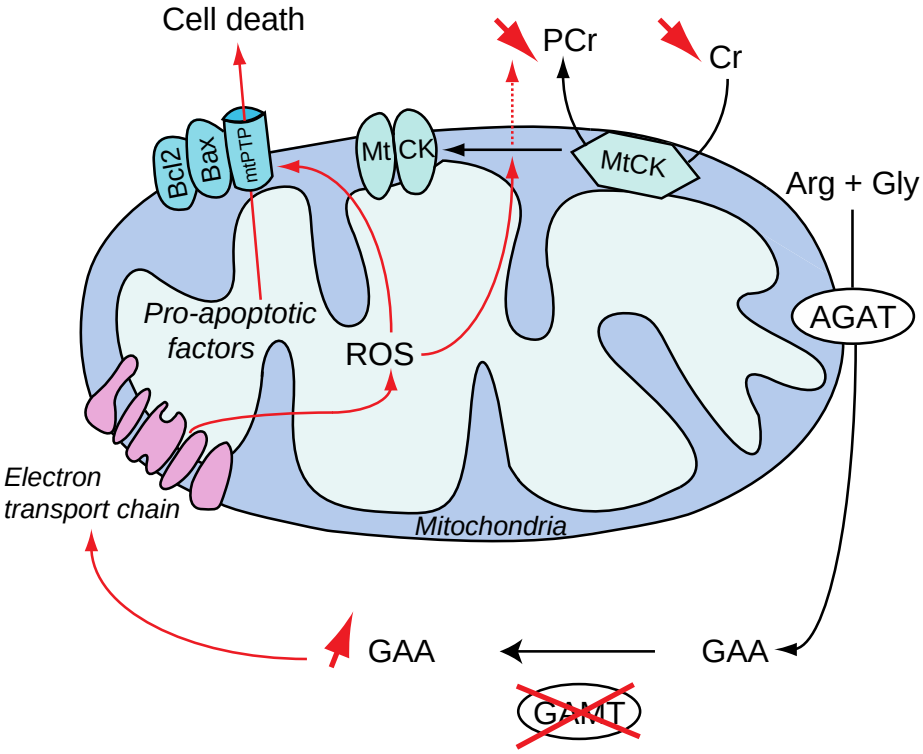


Figure 40: Involvement of mitochondria and the Cr/PCr system in brain cell death. In GAMT-deficient cells, the absence of GAMT-activity causes an increase of GAA in culture medium and intracellularly, and a decrease of creatine (Cr) levels. The accumulation of GAA induces the impairment of mitochondria and the production of reactive oxygen species (ROS) by the electron transport chain. ROS inactivate mitochondrial creatine kinase (MtCK) by changing its octameric conformation to a dimeric inactivated form, leading to a secondary PCr depletion. Dimeric MtCK and ROS modify the structure of mitochondrial permeability transition pores (mPTP) and allow its opening, leading to the release of pro-apoptotic factors in cytosol and to cell death (Béard and Braissant, 2011).

In a second part, we have observed the activation of a member of the mitogen-ativated protein kinase (MAPK), p38, by the quantification of the phosphorylated p38 MAPK form. The measure at DIV 8 after the infection with AAV vectors in 3D aggregates brain cell cultures showed an increase of the phosphorylated p38 MAPK in cultures infected by AAV-Empty and AAV-GAMT. On the other side the increase of phosphorylated p38 MAPK was higher in aggregates infected by AAV-Empty as compared with cultures infected by AAV-GAMT. This result may be reflecting the potential cellular loss in aggregates infected by AAV-GAMT, as described above.

MAPKs are serine-threonine kinases that, when activated by phosphorylation, phosphorylate specific substrates such as transcription factors, cytoskeletal elements and other protein kinases, which in turn control physiological processes such as gene expression, mitosis, proliferation, motility, metabolism or programmed cell death (Aouadi et al., 2006). The p38 MAPK is composed of 4 isoforms and is associated with stress responses such as cytokines stimulation, DNA damage and oxidative stress (Johnson and Lapadat, 2002). The p38 MAPK pathway is also involved in apoptotic response and is associated with the upstream and downstream activation of effector caspases, including caspase 3 (figure 40) (Ono and Han, 2000).

This interconnection between the caspase 3 and p38 MAPK signaling pathways might contribute to the strong increase of cleaved caspase 3 and the important number of apoptotic cells observed in aggregates infected with AAV-GAMT.

Chapter V: Conclusion and perspectives

At the start of the project, in 2007, the only available *in vivo* model for Cr deficiencies was the $GAMT^{-/-}$ KO mouse (Schmidt et al., 2004), which revealed only mild cognitive impairment and no severe neurological symptoms. We thus chose another strategy with the aim of developing new *in vitro* models of Cr deficiencies making use of organotypic 3D reaggregated developing brain cell cultures in which AGAT, GAMT and SLC6A8 genes would be knocked down by using RNAi. These models would allow a better understanding of the cellular, molecular and biochemical pathways altered in the developing brain cells under Cr deficiency. It must be emphasized that these brain cell aggregates are devoid of vascularization and do not develop BBB, thus limiting the analysis of Cr deficiencies to CNS intercellular interactions.

The first step of the project was thus the development of the RNAi technique to knockdown GAMT or SLC6A8 genes in the brain cell aggregates, making use of AAVs expressing GFP to transduce specific shRNAs. The effective decrease in GAMT or SLC6A8 mRNAs and proteins were analyzed during the whole lifetime of the cultures, and we could determine the culture stages showing the maximal decrease of GAMT or SLC6A8 expression. During this work, a lot of difficulties were encountered, in particular to obtain important yields of efficient AAVs that would be sufficient to transduce the cultures, and to be able to observe GFP expression in the AAV-transduced cells. These technical difficulties explain the low number of valid knocked down experiments obtained, and consequently the low number of samples which, in many of the data presented, did not allow a statistical analysis of the data. Our work however demonstrates the proof of principle that GAMT and SLC6A8 genes can be knocked down by RNAi through AAV transduction in 3D reaggregated brain cell cultures.

The second step of the project allowed the validation of our GAMT deficiency model, first by showing the semi-quantitative decrease of GAMT mRNA and protein in AAV-transduced brain cell aggregates, and second by measuring the awaited increased GAA and decreased Cr by tandem mass spectrometry, as observed in the brain tissue of GAMT-deficient patients.

The third part of our work allowed to further analyze the developmental effects of GAMT or SLC6A8 knockdowns in brain cells, demonstrating in particular differential effects of GAMT and SLC6A8 deficiencies on the different types of brain cells, an increase of apoptosis in brain cells under Cr deficiencies (increase of activated caspase 3), and an alteration of intracellular signaling pathways (p38 MAPK).

The main objective in the near future is the improvement of the developed tools to allow a better knock-down of AGAT, GAMT or SLC6A8 genes in brain cell aggregates:

The production of AAV particles has to be improved to allow efficient infections and transduction of brain cell aggregates. In particular, another way of titrating AAV viral particles at the level of their genome, making use of RT-PCR, complementary to the ELISA technique used in this work to titrate the viral particles based on their capsides, will allow an estimation of the yield of AAVs that will be efficient to transduce RNAi. AAV2 and AAV2/5 particles have been used in this work, and other AAV serotypes will be tested to analyze which AAV serotypes are the most efficient to transduce brain cells, and whether they transduce the different brain cell types differentially. This work is actually going on in the laboratory.

The development of a method to observe GFP in AAV-transduced brain cell aggregates is actually going on also in the laboratory, that will allow a better follow-up of the AAV-transduced brain cells undergoing RNAi. Preliminary results show now that GFP is clearly detectable in AAV-transduced brain cell aggregates.

Finally, to improve the validation of the GAMT and SLC6A8 deficiency models developed in this work, intracellular and extracellular measures of Cr and GAA will be realized by tandem mass spectrometry, in complement of the extracellular measures performed for GAMT deficiency in this study.

In GAMT (and AGAT) RNAi, treatment of AAV-transduced aggregates with Cr will be performed to try to correct Cr deficiency, as it is done for AGAT- and GAMT-deficient patients.

The use of AAV-transduced brain cell aggregates that knock down the expression of AGAT, GAMT or SLC6A8 opens new perspectives in the understanding of primary creatine deficiencies, and in their therapeutic approach:

AGAT, GAMT and SLC6A8 RNAi in 3D brain cell aggregates will be used to further understand the physiopathology of Cr deficiencies, to know which CNS cell types are the most affected, and which downstream signaling pathways are altered. As Cr plays essential roles in energy homeostasis, focus will be put on the alterations of energetic metabolism in brain cells induced by Cr deficiency, and how it affects brain cell development and differentiation. As Cr and GAA affect GABAergic neurotransmission and as Cr has been

suggested recently even as a true neurotransmitter, the ways Cr deficiencies affect the different neurotransmission systems will also be investigated.

Our experimental model will also be used in therapeutic perspectives. Apart of the Cr treatments of AGAT and GAMT-deficient aggregates (as described above) performed to try the correction of the Cr deficiency (as in patients), Cr-deficient 3D brain cell aggregates will be used to screen Cr analogs that are developed to bypass the transport of Cr through SLC6A8, and analyze their therapeutical potential on Cr-deficient brain cells. In that case, only the effects on brain cells will be tested, as aggregates are devoid of BBB.

Finally, our experimental models making use of AAV may be further develop to test a future gene therapy for Cr deficient patients. AAVs are currently developed in many studies as tools of gene transfer for the treatment of genetic neurological disorders. AAVs appear to allow gene delivery into CNS and brain post-mitotic cells for long periods with minimal to no toxicity. AAV vectors as developed in our study may be further engineered to transduce Cr-deficient brain cells in order to correct them with the normal AGAT, GAMT or SLC6A8 genes.

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Synthesis and transport of creatine in the central nervous system : Importance of cerebral functions.

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REVIEW

Synthesis and transport of creatine in the CNS: importance for cerebral functions

Elidie Béard and Olivier Braissant

Inborn Errors of Metabolism, Clinical Chemistry Laboratory, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland

Abstract

Apart of its well known function of 'energetic buffer' through the creatine/phosphocreatine/creatine kinase system allowing the regeneration of ATP, creatine has been recently suggested as a potential neuromodulator of even true neurotransmitter. Moreover, the recent discovery of primary creatine deficiency syndromes, due to deficiencies in L-arginine : glycine amidinotransferase or guanidinoacetate methyltransferase (the two enzymes allowing creatine synthesis) or in the creatine transporter, has shed new light on creatine synthesis, metabolism and transport, in particular in CNS which appears as the main tissue affected by these creatine deficiencies. Recent data suggest that creatine can cross blood-brain barrier but only with a poor efficiency, and that the brain must ensure parts of its needs in creatine by its own endogenous synthesis. Finally, the recent years have demonstrated the interest to use creatine as a neuroprotective agent in a growing number of neurodegenerative diseases, including Parkinson's and Huntington's diseases. This article aims at reviewing the latest data on creatine metabolism and transport in the brain, in relation to creatine deficiencies and to the potential use of creatine as neuroprotective molecule. Emphasis is also given to the importance of creatine for cerebral function.

Keywords: brain, creatine, creatine deficiency, guanidinoacetate, mitochondria, neuroprotection.

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Creatine (Cr) (α-*N*-methylguanidino acetic acid) is a nitrogenous organic amino acid playing essential roles in energy metabolism by interconversion to its high energy phosphorylated analogue phosphocreatine (PCr). This reaction is catalyzed by the ubiquitous enzyme creatine kinase (CK). CK isoforms are highly expressed in tissues with high and fluctuating energy demands, such as muscle and brain (Wallimann *et al.* 1992; Wyss and Kaddurah-Daouk 2000). PCr dephosphorylation yields energy, as ADP is converted to ATP by the transfer of *N*-phosphoryl group from PCr to ADP. The Cr/PCr system also allows the shuttle of high-energy phosphates from mitochondria to cytoplasmic sites of utilization (Wallimann *et al.* 2007) (Fig. 1).

Pools of Cr in vertebrates are maintained through uptake from diet and endogenous synthesis. This biosynthetic pathway involves two enzymes: L-arginine : glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT). Cr is distributed by blood to tissues, where cells take it up by a specific transporter, SLC6A8, also called CRT1, CT1, CreaT or CRT (Wyss and Kaddurah-Daouk 2000).

It has long been thought that cerebral Cr was principally of peripheral origin (Wyss and Kaddurah-Daouk 2000). However, AGAT and GAMT are expressed in CNS (Braissant *et al.* 2001, 2005) suggesting that the brain is able of its own Cr synthesis. While SLC6A8 is expressed by microcapillary endothelial cells (MCEC) at blood-brain barrier (BBB),

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Address correspondence and reprint requests to Olivier Braissant, Inborn Errors of Metabolism, Clinical Chemistry Laboratory, Centre Hospitalier Universitaire Vaudois and University of Lausanne, CH-1011 Lausanne, Switzerland. E-mail: olivier.braissant@chuv.ch

Abbreviations used: AD, Alzheimer's disease; AGAT, L-arginine : glycine amidinotransferase; ALS, amyotrophic lateral sclerosis; BBB, blood-brain barrier; B-CK, brain creatine kinase; CK, creatine kinase; Cr, creatine; GA, gyrate atrophy of the choroid and retina; GAA, guanidinoacetate; GAMT, guanidinoacetate methyltransferase; HD, Huntington's disease; MCEC, microcapillary endothelial cells; mPTP, mitochondrial permeability transition pores; NH_4^+, ammonium; PCr, phosphocreatine; PD, Parkinson's disease; ROS, reactive oxygen species; SLC6A8, Cr transporter; uMtCK, ubiquitous mitochondrial creatine kinase.

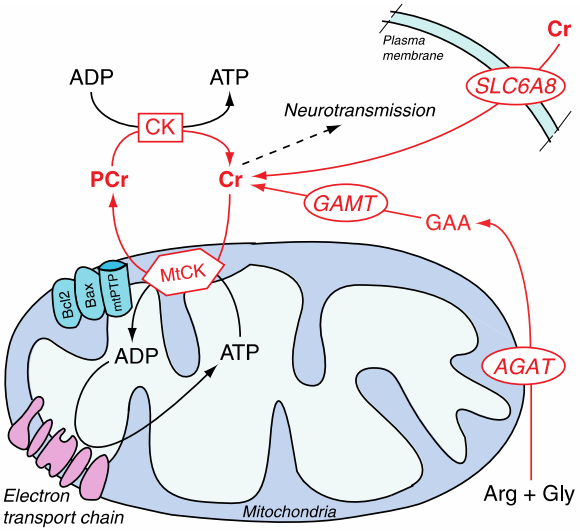

Fig. 1 Synthesis and function of creatine (Cr). Cr synthesis requires the presence of two enzymes, L-arginine : glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT); cells take up Cr by a specific transporter, SLC6A8. Mitochondrial or cytosolic creatine kinases (CK) convert Cr to its high-energy counterpart phosphocreatine (PCr). PCr dephosphorylation yields energy, as ADP is converted to ATP. Besides its function in cellular energy, Cr may also be involved in neurotransmission.

allowing CNS to import Cr from periphery, it is absent from astrocytes, and particularly from their feet lining MCEC (Braissant *et al.* 2001; Ohtsuki *et al.* 2002; Tachikawa *et al.* 2004). This suggested that BBB has a limited permeability for peripheral Cr, and that CNS must supply an important part of its Cr needs by endogenous synthesis (Braissant *et al.* 2001; Braissant and Henry 2008). Considering that CNS ensures parts, if not all, of its Cr needs, and thus does not depend only on Cr issued from periphery, is coherent with the essential roles played by the Cr/PCr system in CNS energy homeostasis, and by Cr as potential neuromodulator or neurotransmitter (see below) (Wallimann *et al.* 1992; Wyss and Kaddurah-Daouk 2000; Brosnan and Brosnan 2007; Andres *et al.* 2008).

Creatine deficiency syndromes, caused by mutations in AGAT, GAMT and SLC6A8 genes, have been identified in human (Stöckler *et al.* 1994; Item *et al.* 2001; Salomons *et al.* 2001). CNS is the main organ affected in patients suffering from Cr deficiency syndromes. Their common phenotype is an almost complete lack of Cr in the brain and the development of several neurological symptoms like mental retardation, delays in speech acquisition or epilepsy (Stöckler *et al.* 2007). AGAT- and GAMT-deficient patients can be treated by oral Cr supplementation (Stöckler *et al.* 1996a; Schulze *et al.* 1998; Battini *et al.* 2002; Schulze and Battini 2007), while Cr supplementation of SLC6A8-deficient patients is inefficient (Bizzi *et al.* 2002; Póo-Argüelles *et al.* 2006; Arias *et al.* 2007). Several other brain pathological states can also lead to secondary Cr deficiencies in brain cells, like stroke, hyperammonemic states or gyrate atrophy of the choroid and retina (GA) (Valle *et al.* 1981; Braissant *et al.* 2008; Lei *et al.* 2009).

Recent studies have shown that Cr administration has a therapeutic potential for neurodegenerative disorders with bioenergetic deficits like Huntington's (HD) or Parkinson's (PD) diseases (Gualano *et al.* 2010). Cr supplementation may also play important protective roles in a number of other pathological conditions, including brain ischemia and stroke, brain and spinal cord trauma, epilepsy or hyperammonemia (Balestrino *et al.* 1999; Tarnopolsky and Beal 2001; Braissant *et al.* 2002; Klein and Ferrante 2007).

This review is focused on the latest data on Cr synthesis and transport in CNS and their functions for brain cells. Cr deficiency syndromes will be discussed, as well as other brain pathologies leading to secondary Cr deficiencies in brain cells. Finally, the therapeutic potential of Cr for various brain pathologies will be considered.

Creatine metabolism and transport

The Cr/PCr/CK system is essential to maintain energy levels in most tissues, and is highly active in particular in those with high and/or fluctuating energy demand such as skeletal muscle, heart and brain (Wallimann *et al.* 1992). The Cr/PCr/CK system not only serves as intracellular buffer for ATP, but also as high-energy phosphate shuttle from mitochondrial sites of production to cytoplasmic sites of consumption (Fig. 1).

Part of intracellular Cr is converted by CK into the high-energy compound PCr. Four CK isoforms have been described, based on tissue expression and subcellular distribution: two cytosolic forms, M-CK (muscle) and B-CK (brain) and two mitochondrial forms, sarcomeric muscle form (sMtCK) and brain form called ubiquitous MtCK (uMtCK) (Wallimann *et al.* 1992; Schlattner *et al.* 2006). Each CK isoform has a specific function, mitochondrial CKs using ATP to convert Cr to PCr for export to cytoplasm, and cytosolic CKs using PCr to convert ADP to ATP at sites of energy demand, and to convert excess ATP to PCr for energy storage (Wallimann *et al.* 1992, 1998).

Total Cr (Cr + PCr) in 70 kg young adults amounts for approximately 120 g. Both Cr and PCr are non-enzymatically and irreversibly degraded to creatinine at a rate of about 1.7% of total body pool per day (Wyss and Kaddurah-Daouk 2000). Creatinine is excreted via kidneys, the amount of creatinine eliminated being proportional to muscle. The amount of Cr provided by diet or by endogenous synthesis depends on creatinine excretion but accounts for about 2 g/day (Casey and Greenhaff 2000).

In human, half of Cr stores originate from food, mainly fresh meat, fish and dairy products, while the other half is

biosynthesized endogenously through the AGAT/GAMT pathway. From the precursors arginine (limiting factor) and glycine, AGAT catalyzes the formation of guanidinoacetate (GAA) and ornithine. This step occurs mostly in kidney where Cr level exerts a negative feedback loop on AGAT gene regulation at transcriptional level (McGuire *et al.* 1984; Brosnan *et al.* 2009). The second reaction, catalyzed by GAMT and occurring mostly in liver, uses *S*-adenosylmethionine to methylate GAA, producing Cr and *S*-adenosylhomocysteine (Brosnan *et al.* 2009). AGAT and GAMT expression are positively regulated by growth hormone, thyroid hormone and sex hormones (Carlson and Van Pilsum 1973; McGuire *et al.* 1984; Guthmiller *et al.* 1994; Lee *et al.* 1994). While AGAT and GAMT highest expression is found in kidney and liver, respectively, they are also expressed at lower levels in various other tissues, including CNS (Lee *et al.* 1998; Wyss and Kaddurah-Daouk 2000; Braissant *et al.* 2001).

Creatine is transported by blood to Cr-requiring tissues and taken up in cells with high energy demand by a Cr-specific transporter, SLC6A8. SLC6A8 is a member of the solute carrier family 6, a large family of membrane transporters that mediate the transport of various neurotransmitters and/or amino acids across plasma membrane with the co-transport of two Na^+ and one Cl^- (Chen *et al.* 2004). This transport is electrogenic and driven by the sodium gradient established by Na^+/K^+-ATPase (Dai *et al.* 1999). SLC6A8 expression is important in tissues with high energy demand, such as skeletal muscle, heart, brain, retina, or with important (re)absorptive functions, such as kidney and intestine (Guimbal and Kilimann 1993; Braissant *et al.* 2001; Peral *et al.* 2002; Mak *et al.* 2009). Cr uptake is regulated by different factors, like insulin which activates Na^+/K^+-ATPase and presumably increases the driving force for Cr uptake (Snow and Murphy 2001), or the Na^+ gradient and intracellular Cr concentrations (Brosnan and Brosnan 2007).

Creatine metabolism, transport and functions in the brain

Functions of creatine in CNS

The Cr/PCr/CK system plays essential roles to maintain the high energy levels necessary for CNS (maintenance of membrane potential and ions gradients, Ca^{2+} homeostasis, neurotransmission, intracellular signaling systems as well as axonal and dendritic transport) (Wyss and Kaddurah-Daouk 2000). The brain represents only 2% of body mass but may spend up to 20% of total energy consumption. The Cr/PCr/CK system also plays essential roles in CNS development. Different studies showed that CK isoforms are found highly concentrated in cerebellum (especially glomeruli structures of granular layer), choroid plexus and hippocampal granular and pyramidal cells (Hemmer *et al.* 1994). It must be noted that hippocampus is important for learning and memory function and can be severely affected in Alzheimer's disease (AD). B-CK is much higher than uMtCK in cerebellar Bergmann glial cells and hypothalamus, where it plays essential functions in regenerating ATP for glutamate clearance during excitatory synaptic transmission (Oliet *et al.* 2001). Knock-out for one CK isoform (B-CK or uMtCK) showed behavioral abnormalities and defects in formation and maintenance of hippocampal mossy fiber connections. Double knock-out mice displayed decreased body weight and severely impaired spatial learning, lower nest building activity and reduction of hippocampal size (Jost *et al.* 2002; Streijger *et al.* 2005). All these studies demonstrate the key function of CK in brain energy metabolism (Hemmer and Wallimann 1993).

Apart of its functions in energy, Cr may play other roles, as recently suggested in particular in CNS. Cr was suggested as essential CNS osmolyte. Astrocytes placed in hyperosmotic shock significantly increase their Cr uptake, suggesting that Cr can work as compensatory osmolyte (Alfieri *et al.* 2006). Conversely, astrocytes exposed to hypo-osmotic swelling conditions stimulate the release of their osmotically active Cr (Bothwell *et al.* 2001). In contrast, ammonium-exposed MCEC *in vitro* stimulate their Cr uptake (Bélanger *et al.* 2007), suggesting that cells making BBB (MCEC and astrocytes lining them) behave differentially during swelling. Cr was also proposed as appetite and weight regulator, by acting on specific hypothalamic nuclei (Galbraith *et al.* 2006).

Creatine: a co-transmitter in CNS?

Creatine and GAA can affect GABAergic neurotransmission as partial agonists or antagonists on post-synaptic $GABA_A$ receptors, depending on local GABA concentration (De Deyn *et al.* 1991; Neu *et al.* 2002; Cupello *et al.* 2008). These data stimulated research showing that in organotypic cultures of rat cortex, caudate putamen and hippocampus slices, Cr is released from neurons in a similar manner as classical neurotransmitters. This electrically-evoked exocytotic Cr release mechanism is action potential-dependent, being dependent from Ca^{2+}, inhibited by the Na^+-channel blocker tetrodotoxin and enhanced by the K^+-channel blocker 4-amino-pyridine (Almeida *et al.* 2006b). According to these *in vitro* studies, Cr may thus also be considered as a neuromodulator or co-transmitter in CNS, which may modulate the activity of post-synaptic receptors such as $GABA_A$ (Almeida *et al.* 2006a). Interestingly, rat brain synaptosomes were identified recently as expressing SLC6A8, which allows their active accumulation of Cr (Peral *et al.* 2010). This suggests the presence of a Cr recapture mechanism in axon terminal membrane, which would fit with a neurotransmitter/co-transmitter function of Cr in CNS (Almeida *et al.* 2006a).

AGAT, GAMT and SLC6A8 in adult brain

It has long been thought that most of brain Cr was of peripheral origin, be it taken from the diet or synthesized endogenously through AGAT and GAMT activities in kidney and liver, respectively (Wyss and Kaddurah-Daouk 2000; Brosnan and Brosnan 2007; da Silva *et al.* 2009). However, Cr is synthesized in the mammalian brain (Van Pilsum *et al.* 1972) as well as in primary brain cell cultures and nerve cell lines (Daly 1985; Dringen *et al.* 1998; Braissant *et al.* 2002). AGAT and GAMT are expressed in CNS, for which we provided the first detailed analysis demonstrating their expression in all the main structures of the adult rat brain, in every main cell types (neurons, astrocytes and oligodendrocytes; Braissant *et al.* 2001) (Fig. 2). Particularly high levels were found in telencephalon and cerebellum. AGAT was further shown in rat retina (Nakashima *et al.* 2005), while our data on GAMT were confirmed in mouse and human (Schmidt *et al.* 2004; Tachikawa *et al.* 2004).

Organotypic rat cortical cultures, primary brain cell cultures (neuronal, glial or mixed) and neuroblastoma cell lines have a Cr transporter activity (Daly 1985; Möller and Hamprecht 1989; Almeida *et al.* 2006b; Braissant *et al.* 2008). *In vivo*, mouse and rat CNS can take up Cr from the blood against its concentration gradient (Ohtsuki *et al.* 2002; Perasso *et al.* 2003). SLC6A8 is expressed throughout the main regions of adult mammalian brain, particularly in those associated with learning, memory and general limbic functions (Guimbal and Kilimann 1993; Schloss *et al.* 1994; Happe and Murrin 1995; Saltarelli *et al.* 1996). We provided the first detailed analysis demonstrating that SLC6A8 is found in neurons and oligodendrocytes but, in contrast to AGAT and GAMT, cannot be detected in astrocytes (Braissant *et al.* 2001). We also showed that in contrast to its absence in astrocytes lining microcapillaries, SLC6A8 is present in MCEC (BBB; Fig. 2). These data were confirmed later in rat and mouse (Ohtsuki *et al.* 2002; Nakashima *et al.* 2004; Tachikawa *et al.* 2004, 2008; Acosta *et al.* 2005; Mak *et al.* 2009).

AGAT, GAMT and SLC6A8 in developing brain

The Cr/PCr/CK system plays essential roles in energy homeostasis during vertebrate embryonic development (Wallimann *et al.* 1992). Many structures of vertebrate embryo express CKs at early stages (Lyons *et al.* 1991; Dickmeis *et al.* 2001), and Cr concentrations between 5 and 8 mmol/kg wet weight were measured in CNS of rat and human fetus (Miller *et al.* 2000; Kreis *et al.* 2002). Parts of CNS developmental needs for Cr are provided by active transport of Cr from mother to embryo, Cr accumulating in chorioallantoic placenta and yolk sac at concentrations higher than found in maternal and fetal blood, then diffusing down its concentration gradient into fetal circulation (Davis *et al.* 1978).

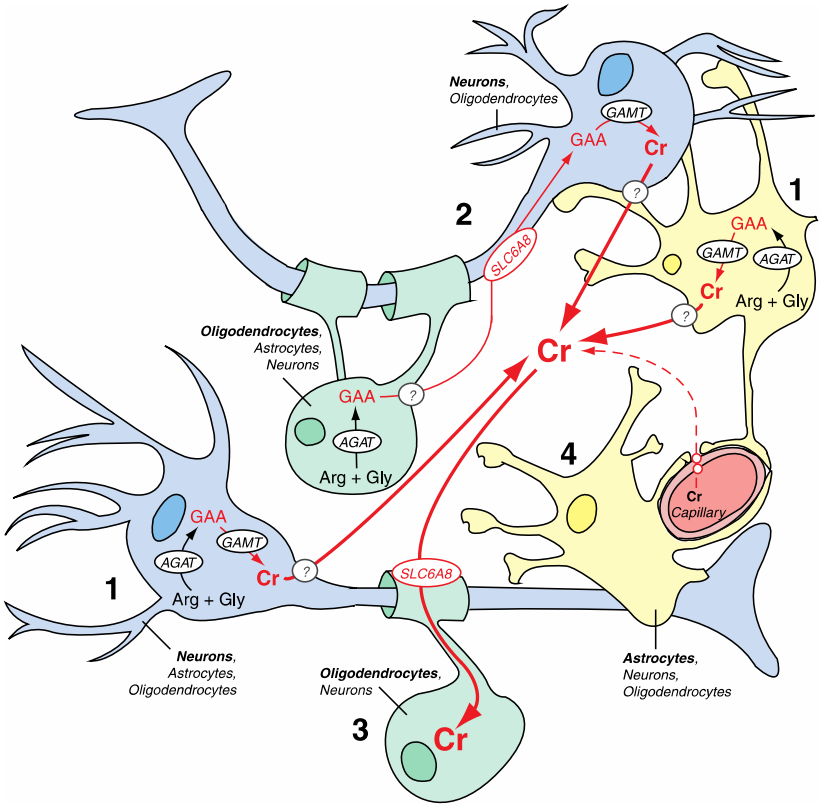

Fig. 2 Model of Cr synthesis and transport in CNS, illustrating the diversity of AGAT, GAMT and SLC6A8 expression by brain cells (Braissant *et al.* 2010). 1) Cr endogenous synthesis within cells co-expressing AGAT and GAMT. 2) Cr endogenous synthesis through AGAT-expressing cells synthesizing GAA, and GAA uptake by SLC6A8 in GAMT-expressing cells. 3) Cell expressing only SLC6A8 ('users' of Cr). 4) Cells silent for AGAT, GAMT and SLC6A8. While microcapillaries express SLC6A8, astrocytic feet lining them do not. This implies that only low amounts of peripheral Cr can enter the brain through the limited endothelial surface that is free of astrocytic feet, and that CNS must also ensure its own endogenous synthesis of Cr. So far, the way Cr (and GAA) can leave cells is poorly known. Cr, creatine; AGAT, L-arginine : glycine amidinotransferase; GAMT, guanidinoacetate methyltransferase; GAA, guanidinoacetate; SLC6A8, Cr transporter.

AGAT, GAMT and SLC6A8 are well expressed during vertebrate embryogenesis (Schloss *et al.* 1994; Sandell *et al.* 2003; Schmidt *et al.* 2004; Braissant *et al.* 2005; Wang *et al.* 2007; Ireland *et al.* 2009), and probably play essential roles in developing CNS as their deficiencies lead to neurological symptoms in early infancy and severe neurodevelopmental delay (see below).

Working on rat, we have provided the first detailed analysis of AGAT, GAMT and SLC6A8 expression in developing embryonic CNS (Braissant *et al.* 2005). AGAT and GAMT are expressed in the whole developing CNS parenchyma. However, their low level (GAMT in particular) at early developmental stages suggests that embryonic CNS depends on external Cr supply, be it from embryonic periphery or from maternal origin. This is coherent with SLC6A8 expression in whole embryonic CNS already at early stages (E12.5 in rat), with particularly high levels in periventricular zone and choroid plexus, the predominant metabolic exchange zones of fetal brain before differentiation of BBB (Braissant *et al.* 2005, 2007).

Functions of AGAT, GAMT and SLC6A8 in CNS: synthesis or uptake of creatine by the brain?

Total Cr levels and CK activity are well correlated in mammalian CNS (Wyss and Kaddurah-Daouk 2000), their highest levels being reached in brain cells described with high and fluctuating energy demands, where AGAT, GAMT and SLC6A8 are expressed (Hemmer *et al.* 1994; Wang and Li 1998; Braissant *et al.* 2007).

SLC6A8 absence in astrocytes, particularly in their feet sheathing MCEC, made us suggest that in mature brain, BBB has a limited permeability for Cr, despite SLC6A8 expression by MCEC and their capacity to import Cr (Braissant *et al.* 2001, 2007). *In vivo* data confirmed this hypothesis: the blood to brain transport of Cr is effective in rodents, but is relatively inefficient (Ohtsuki *et al.* 2002; Perasso *et al.* 2003), and long term treatment of AGAT- and GAMT-deficient patients with high doses of Cr allows only a slow and in most cases partial replenishment of their CNS Cr (Schulze and Battini 2007; Stöckler *et al.* 2007). Consequently, the brain may depend more on its own Cr synthesis through AGAT and GAMT expression than on Cr supply from blood (Braissant *et al.* 2007; Braissant and Henry 2008). The effective but limited passage of Cr from blood to CNS through BBB may occur through the limited surface of CNS microcapillary endothelium that is free of astrocytic feet (Virgintino *et al.* 1997; Ohtsuki 2004) (Fig. 2).

One strong argument in favor of the 'brain endogenous Cr synthesis' hypothesis comes from Cr measures in CSF of Cr-deficient patients (see Braissant and Henry 2008; and references therein). SLC6A8-deficient patients present normal Cr levels in CSF, but cannot import it from periphery (Cecil *et al.* 2001; de Grauw *et al.* 2002). In contrast, GAMT-deficient patients show strongly decreased Cr levels in CSF, but can import it from blood (Schulze *et al.* 1997). This also suggests that CNS Cr synthesis might still remain operational, although very partially, under SLC6A8 deficiency, while it is completely blocked in AGAT and GAMT deficiencies. Endogenous synthesis, or a very efficient uptake from periphery, are the two ways available for the brain to secure Cr homeostasis for its energy and functions. As uptake from periphery does not appear efficient, CNS might privilege Cr endogenous synthesis.

The 'brain endogenous Cr synthesis' hypothesis might seem contradictory with *in vivo* characteristics of SLC6A8 deficiency, which, despite AGAT and GAMT expression in CNS, shows absence (or very low level) of brain Cr by magnetic resonance spectroscopy (Salomons *et al.* 2001). This apparent contradiction is probably explained by AGAT, GAMT and SLC6A8 expression patterns in CNS. AGAT and GAMT are found in every CNS cell type (Braissant *et al.* 2001), but appear rarely co-expressed within the same cell (Braissant *et al.* 2010). This suggests that to allow Cr synthesis in the brain, GAA must be transported from AGAT- to GAMT-expressing cells (Braissant and Henry 2008) (Fig. 2). This GAA transfer most probably occurs through SLC6A8, as recently shown by Cr and GAA competition studies, and the use of stable isotope-labeled GAA demonstrating its conversion to Cr by GAMT activity (Braissant *et al.* 2010). These observations may explain Cr absence in CNS of SLC6A8-deficient patient, despite normal expression of AGAT and GAMT in their brain (Braissant and Henry 2008). Recent studies also demonstrate the potential role of SLC6A8 (and taurine transporter) for GAA transport across BBB and at blood-cerebrospinal fluid barrier, as well as in brain parenchymal cells (Tachikawa *et al.* 2008, 2009).

While we have shown that AGAT and GAMT can be found in all brain cell types (Braissant *et al.* 2010), various studies demonstrated high levels of GAMT within glial cells (Schmidt *et al.* 2004; Tachikawa *et al.* 2004; Braissant *et al.* 2008), suggesting that the final CNS step for Cr synthesis may predominantly by glial. However, this probably depends on the CNS region considered, as in cortex, only 20% of astrocytes express GAMT, in comparison with 48% of neurons (Braissant *et al.* 2010).

Creatine deficiency syndromes

Inborn errors of Cr biosynthesis and transport, called Cr deficiency syndromes and due to deficiencies in AGAT, GAMT and SLC6A8 (Figs 3–5), are characterized by an absence or a severe decrease of Cr in CNS, as measured by magnetic resonance spectroscopy (Stöckler *et al.* 1994; Item *et al.* 2001; Salomons *et al.* 2001; Stromberger *et al.* 2003). AGAT and GAMT deficiencies are autosomal recessive diseases, while SLC6A8 deficiency is a X-linked disorder. Cr deficiency syndromes appear among the most frequent inborn errors of metabolism, the prevalence of SLC6A8

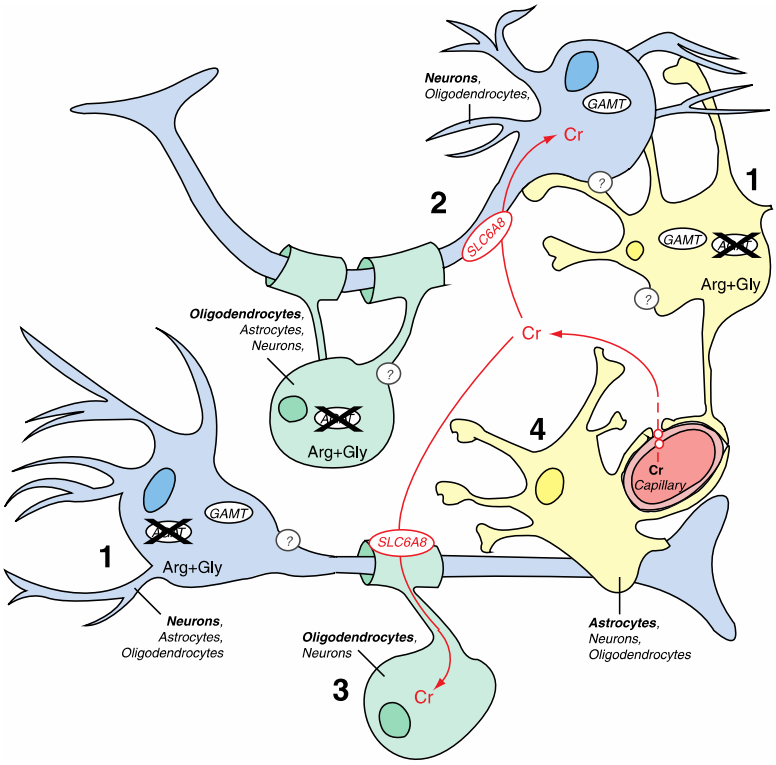

Fig. 3 Model of AGAT deficiency in CNS. See Fig. 2 for abbreviations.

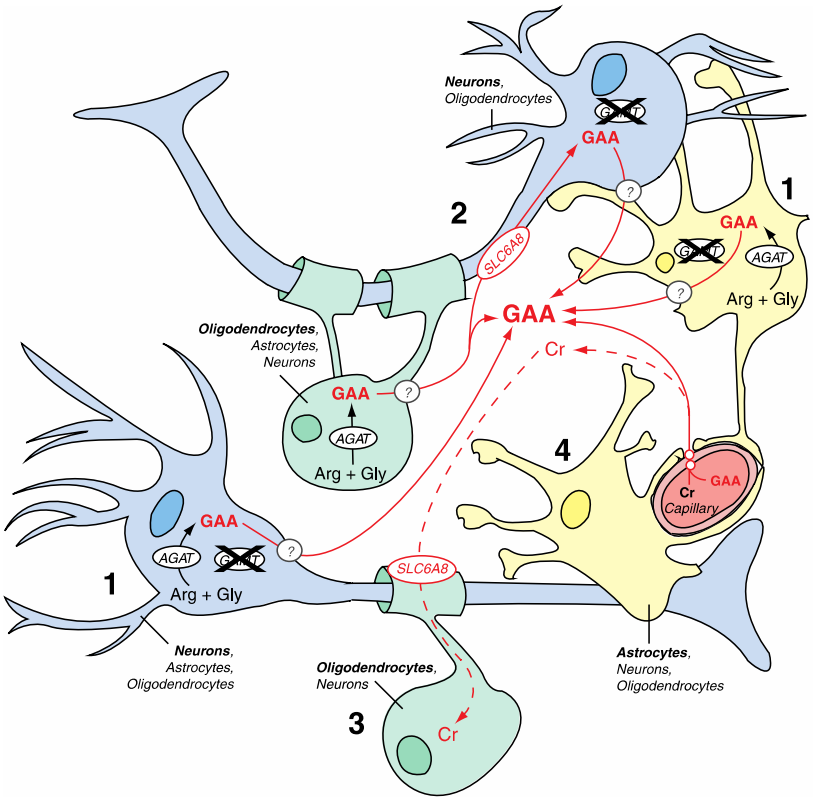

Fig. 4 Model of GAMT deficiency in CNS. See Fig. 2 for abbreviations.

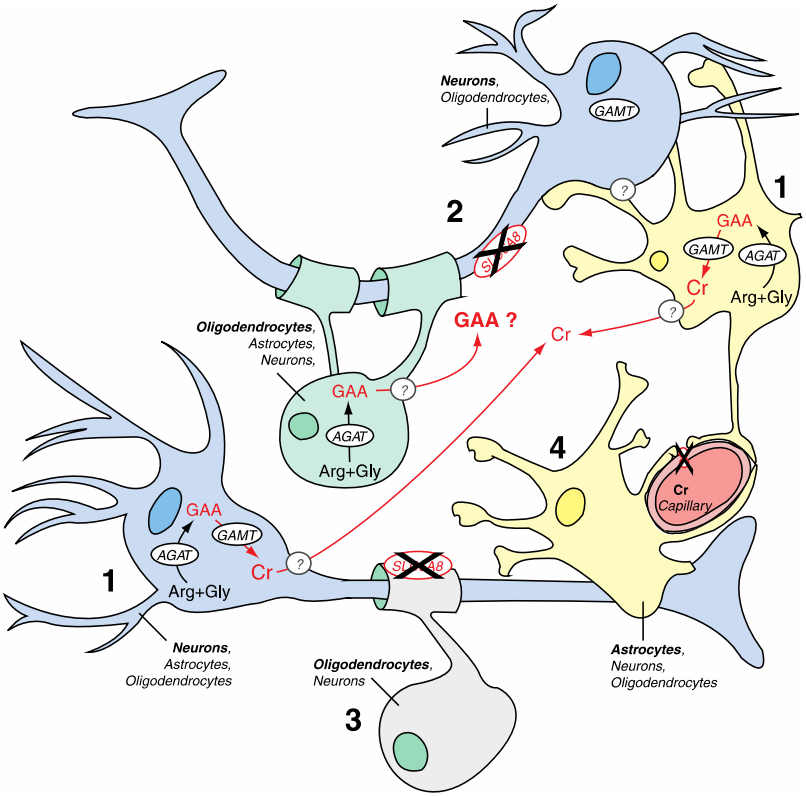

Fig. 5 Model of SLC6A8 deficiency in CNS.
See Fig. 2 for abbreviations.

deficiency being estimated at 2% of all X-linked mental retardations (Rosenberg *et al.* 2004) and at 1% of males with mental retardation of unknown etiology (Clark *et al.* 2006). AGAT and GAMT deficiencies appear rarer. The prevalence of all combined Cr deficiencies was estimated at 2.7% of all mental retardation (Lion-François *et al.* 2006). CNS is the main organ affected in Cr deficiency syndromes, whose patients show severe neurodevelopmental delay and develop, in early infancy, mental retardation, disturbance of active and comprehensible speech, autism, automutilating behavior and hypotonia (Stöckler *et al.* 1996b; Schulze *et al.* 1997; Battini *et al.* 2002; de Grauw *et al.* 2002). Patients with GAMT deficiency exhibit a more complex phenotype, including intractable epilepsy, extrapyramidal movement syndromes and abnormalities in basal ganglia (Schulze 2003; Stromberger *et al.* 2003; Mercimek-Mahmutoglu *et al.* 2006). GAMT-deficient patients accumulates GAA because of the block in GAMT enzymatic activity, including in the brain where GAA accumulation is probably due to the combined CNS endogenous AGAT activity (Braissant and Henry 2008), as well as to a facilitated crossing of BBB by GAA due to increased GAA versus decreased Cr in their blood (Tachikawa *et al.* 2009) (Fig. 4). GAA toxicity in CNS, and particularly its epileptogenic action (Schulze *et al.* 2001), may occur through disturbances of GABAergic neurotransmission (see above; Neu *et al.* 2002). GAA may also inhibit the complex between Na^+/K^+-ATPase and CK (Zugno *et al.* 2006). Severe epilepsy may also appear in SLC6A8-deficient patients (Mancardi *et al.* 2007). This may be due to the observed CNS GAA accumulation in some SLC6A8-deficient patients (Sijens *et al.* 2005), that could be caused by impairment of GAA transport, through deficient SLC6A8, from AGAT- to GAMT-expressing cells (Braissant *et al.* 2010) (Fig. 5).

The diverse phenotypic neurological spectrum observed in Cr deficiency syndromes show the importance of Cr for psychomotor development and cognitive functions and might be explained by the wide pattern of AGAT, GAMT and SLC6A8 genes in mammalian brain (Figs 2–5), which has been documented in every main regions of rat (AGAT, GAMT and SLC6A8), mouse (GAMT and SLC6A8) and human (GAMT) CNS (see above). The potential role of Cr as co-transmitter on the widely distributed GABA post-synaptic receptors (Almeida *et al.* 2006b) might also contribute to this phenotypic diversity.

AGAT- and GAMT-deficient patients can be treated with Cr, which strongly improves their neurological status and CNS development (Stöckler *et al.* 1996a; Schulze *et al.* 1998; Item *et al.* 2001; Battini *et al.* 2002) (Figs 3 and 4). For GAMT-deficient patients, combined arginine restriction and ornithine substitution coupled to Cr treatment decrease GAA and improve clinical outcome (Schulze *et al.* 1998,

2001; Schulze 2003). However, despite improvement of clinical outcome by Cr supplementation, most AGAT- and GAMT-deficient patients remain with CNS developmental problems. Oral supplementation of Cr is inefficient in replenishing CNS Cr in SLC6A8-deficient patients (Fig. 5), who remain with mental retardation, severe speech impairment, and progressive brain atrophy (Cecil *et al.* 2001; Bizzi *et al.* 2002; de Grauw *et al.* 2002). Attempts to treat SLC6A8-deficient patients with arginine and glycine as precursors of Cr gave encouraging results in two SLC6A8-deficient patients (Chilosi *et al.* 2008; Wilcken *et al.* 2008), while it failed to improve the neurological status of four others (Fons *et al.* 2008). The use of a lipophilic Cr-derived compound, creatine ethyl ester, failed to replenish brain Cr concentration in SLC6A8-deficient patients, as well as to improve their neurological status (Fons *et al.* 2010).

Pre-symptomatic treatment of AGAT- and GAMT-deficient patients

Two recent studies have shown that the pre-symptomatic treatment of AGAT and GAMT deficiencies appears to prevent the phenotypic expression of these diseases (Schulze and Battini 2007). An AGAT-deficient boy, brother of two already affected AGAT-deficient sisters, was diagnosed at birth with the same homozygous mutation as his sisters, and treated orally since the age of 4 months with Cr monohydrate (Battini *et al.* 2006). Similarly, a GAMT-deficient girl, sister of an already affected GAMT-deficient brother, was diagnosed at birth with the same heterozygous mutations as her brother, and treated orally since the age of 22 days with Cr monohydrate (Schulze *et al.* 2006). Both patients, over a follow-up of more than 2 years, did not develop the characteristic CNS phenotypic expression of AGAT and GAMT deficiencies (Schulze and Battini 2007). These two cases suggest that Cr plays essential roles in the development of CNS higher cognitive functions, like speech acquisition, during the first months and years of life, and that treatment with Cr before irreversible damage occurs may prevent clinical symptoms of AGAT and GAMT deficiencies permanently. As described above, the pre-symptomatic treatment with Cr in postnatal stages and during the first years of life may also facilitate the entry of Cr into the brain, at stages where BBB is not as tightly regulated as in more mature stages (Virgintino *et al.* 1997; Engelhardt 2003), and where SLC6A8 expression on BBB and choroid plexus may still facilitate entry of peripheral Cr into the brain (Braissant *et al.* 2005; Ireland *et al.* 2009), in contrast to adulthood (Braissant *et al.* 2001).

The GAMT−/− mouse

So far, only one *in vivo* model of Cr deficiencies has been described: the GAMT knock-out mouse (GAMT−/−) in which the first exon of the murine GAMT gene has been disrupted (Schmidt *et al.* 2004). As GAMT-deficient patients, GAMT−/− mice have markedly decreased Cr and increased GAA levels in CNS and in body fluids (urine, serum, CSF) (Renema *et al.* 2003), and slowly replenish their brain Cr when fed with Cr. GAMT−/− mice show increased neonatal mortality, muscular hypotonia and decreased male fertility. The most obvious symptom observed is a reduction of body weight throughout life, more pronounced in females than males. While biochemical alterations of GAMT−/− mice are comparable to those found in GAMT-deficient patients, their neurological and behavioral analysis reveals only mild cognitive impairment and no severe neurological symptoms despite the important accumulation of GAA in CNS (Schmidt *et al.* 2004; Torremans *et al.* 2005). In particular, no severe symptoms like epileptic seizures or ataxia are observed. One explanation for this contrast to GAMT patients may be the use of GAA as CK substrate, leading to the formation of phosphorylated GAA which may play the same role as PCr in providing high-energy phosphates (Ellington 2001; Renema *et al.* 2003). If this were true, the same compensation mechanism by GAA may also occur in GAMT-deficient patients, who can accumulate phosphorylated GAA at least in their muscles (Schulze *et al.* 2003; Ensenauer *et al.* 2004). No *in vivo* models of AGAT and SLC6A8 deficiencies have been published so far.

Secondary creatine deficiencies in CNS

Apart of the primary Cr deficiency syndromes, several other CNS pathologies cause a secondary Cr deficiency in brain cells.

Hyperammonemia

Excess of ammonium (NH_4^+) is toxic for CNS. In adults, liver failure can result in hyperammonemia and lead to a potentially severe neuropsychiatric disorder named hepatic encephalopathy, which progressively leads to altered mental status and coma (Beal and Martin 1998). In pediatric patients, hyperammonemia can be caused by various inherited or acquired disorders, the most frequent being urea cycle diseases, which can cause irreversible damages to the developing brain with presentation symptoms such as cognitive impairment, seizures and cerebral palsy (Leonard and Morris 2002; Gropman and Batshaw 2004). In CNS, NH_4^+ exposure alters several amino acid pathways and neurotransmitter systems, cerebral energy, nitric oxide synthesis, axonal and dendritic growth and signal transduction pathways (Cagnon and Braissant 2007, 2008, 2009) eventually leading to energy deficit, oxidative stress and cell death (Braissant 2010a). In particular, NH_4^+ exposure generates a secondary Cr deficiency in brain cells, both *in vivo* and *in vitro* (Ratnakumari *et al.* 1996; Choi and Yoo 2001;

Braissant *et al.* 2002). NH_4^+ appears to inhibit AGAT enzymatic activity and to differentially alter AGAT, GAMT and SLC6A8 gene expression in a cell type-specific manner, which may alter the energy requirements of brain cells (Braissant *et al.* 2008; Braissant 2010b).

Stroke

Stroke is the rapidly developing loss of brain functions due to disturbances in CNS blood flow, and resulting in insufficient oxygen and glucose delivery to support brain cell homeostasis (Donnan *et al.* 2008). Distinction is made between ischemic stroke, due to thrombotic or embolic events interrupting blood supply to the brain, and hemorrhagic stroke, due to the rupture of a blood vessel or an abnormal vascular structure. Ischemic stroke is the most frequent, representing about 87% of all cases of stroke (Donnan *et al.* 2008). The Cr/PCr system is known to allow the regeneration of ATP even in absence of oxygen and glucose, but for a very limited amount of time. In the brain in particular, PCr levels are limited, and rapidly become depleted after anoxia or ischemia, the PCr decrease preceding the fall in ATP (Lipton and Whittingham 1982; Obrenovitch *et al.* 1988). Moreover, studies on different *in vivo* models for brain ischemia have demonstrated a rapid diminution in CNS total Cr (Gideon *et al.* 1992; Peres *et al.* 1992; Lei *et al.* 2009). In ischemic patients, total Cr levels are also significantly lower than in normal volunteers (Mathews *et al.* 1995). This lower Cr level causes a decrease in high energy phosphates production, and leads to a failure in most energy-dependent processes necessary for cell survival, such as ion pumping, neuronal depolarization or pre-synaptic re-uptake of excitatory amino acids (Nicholls and Attwell 1990). This in turn favors the accumulation of excitotoxic glutamate in CNS extracellular space, eventually leading to neuronal death by necrosis or apoptosis (Dirnagl *et al.* 1999; Zhu *et al.* 2004).

Gyrate atrophy of the choroid and retina

Gyrate atrophy of the choroid and retina, an inborn error of metabolism causing chorioretinal dystrophy starting in childhood and that can lead to blindness in the fourth to seventh decade of life, is caused by mutations in ornithine δ-aminotransferase (Valle *et al.* 1981). GA generates a secondary Cr deficiency in skeletal muscle as well as in brain cells, as ornithine δ-aminotransferase deficiency leads to an important accumulation of ornithine which inhibits AGAT reaction, therefore depleting GAA for Cr synthesis (Sipila 1980), as shown in brain and skeletal muscle of GA animal models and patients (Wang *et al.* 1996; Valayannopoulos *et al.* 2009). While GA patients may develop with normal intelligence, electroencephalography and magnetic resonance imaging analysis have, however, demonstrated unspecific abnormalities and premature degenerative changes in CNS (Näntö-Salonen *et al.* 1999; Valtonen *et al.* 1999). GA neurological symptoms may thus possibly be related to a secondary Cr deficiency in CNS (Näntö-Salonen *et al.* 1999; Valayannopoulos *et al.* 2009).

Therapeutic potential of creatine for brain diseases

Troubles in CNS energy metabolism due to mitochondrial dysfunction, either from oxidative stress, mitochondrial DNA deletions, pathological mutations or altered mitochondria morphology, play critical roles in the progression of neurological diseases as a primary or secondary mechanism in neuronal death cascade (Beal 2000; Chaturvedi and Beal 2008). The cellular energy state plays key roles in regulating and initiating necrosis and apoptosis in brain cells, as mitochondria are known as essential in controlling specific apoptotic pathways (Green and Reed 1998) (Fig. 6).

The dominant role of mitochondria is to supply and regulate energy, in the form of ATP, for the cell. In addition, mitochondria are involved in a range of other processes, such as cellular growth and differentiation, and cell cycle control (McBride *et al.* 2006). Their dysregulation can lead to alterations in Ca^{2+} homeostasis, production of reactive oxygen species (ROS) and cell death (apoptosis) (Steeghs *et al.* 1997; Green and Reed 1998; McBride *et al.* 2006). Mitochondria can release several pro-apoptotic proteins into cytosol which in turn can induce cell death (Primeau *et al.* 2002). This release is under control of ROS, allowing formation of mitochondrial permeability transition pores (mPTP), a continuum between inner and outer mitochondrial membranes (Adhihetty and Beal 2008) (Fig. 6). mPTP are associated with different death mechanisms leading to apoptosis and necrosis (Bernardi *et al.* 1998). mPTP formation and opening is facilitated by several factors, like accumulation of Ca^{2+}, reduction in membrane potential, increase in inorganic phosphate, decrease in ATP and ADP, and elevation in oxidative stress (Di Lisa and Bernardi 2005). In particular, mPTP are localized on the mitochondrial membrane beside MtCK, with which they interact. MtCK suppresses pore opening and potentially decreases apoptotic susceptibility, which is itself stabilized by the presence of Cr (O'Gorman *et al.* 1997; Adhihetty and Beal 2008). Thus, Cr can play essential roles in stabilizing mitochondrial function and in decreasing neuronal cell death (Fig. 6).

The mechanisms of neuroprotection by Cr differ depending on the brain pathology, but several studies have shown that Cr supplementation can improve the bioenergetic deficit associated with these disorders (Gualano *et al.* 2010).

Huntington's disease

Huntington's disease is caused by a cytidine-adenosine-guanosine (CAG) triplet expansion in exon 1 of huntingtin gene, resulting in an elongated polyglutamine expansion in huntingtin protein. The precise roles of huntingtin are unknown so far, but hypotheses have been made for functions in intracellular transport, autophagy, transcription,

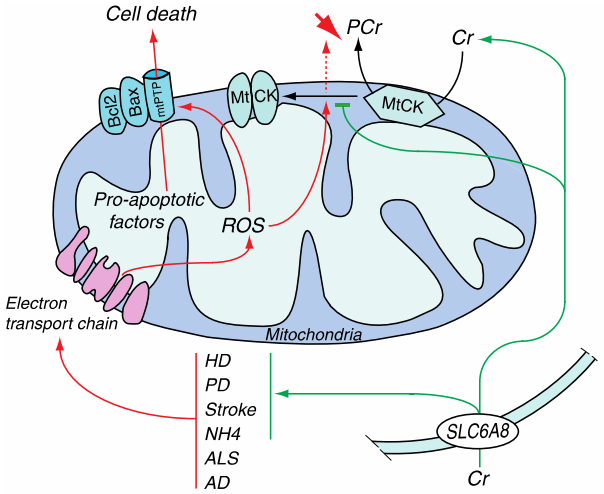

Fig. 6 Involvement of mitochondria and the Cr/PCr system in brain cell death. In Huntington's (HD), Parkinson's (PD) and Alzheimer's (AD) diseases, as well as in amyotrophic lateral sclerosis (ALS), stroke and hyperammonemia (NH_4), the impairment of mitochondria produces reactive oxygen species (ROS) by the electron transport chain. ROS inactivate mitochondrial creatine kinase (MtCK) by changing its octameric conformation to a dimeric inactivated form, leading to phosphocreatine (PCr) depletion. Dimeric MtCK and ROS modify the structure and open mitochondrial permeability transition pores (mPTP), leading to the release of pro-apoptotic factors in cytosol and to cell death. Creatine (Cr) supplementation allows the regeneration of the cell Cr pool. Moreover, in HD, PD, stroke and hyperammonemia, Cr supplementation may stabilize octameric MtCK and prevent the opening of mPTP, thus avoiding cell death.

signal transduction and mitochondrial function (Beal and Ferrante 2004; Gauthier *et al.* 2004; Ross 2004). Huntingtin is a cytosolic protein expressed ubiquitously in vertebrates, including in CNS (Bender *et al.* 2005). HD symptoms are progressive motor dysfunction, emotional disturbance, dementia and weight loss (Klein and Ferrante 2007).

Mutated huntingtin has a toxic effect in neural tissue, with transcriptional dysregulation, pro-apoptotic signaling, oxidative injury, inflammatory reactions and mitochondrial dysfunctions (Ryu and Ferrante 2005). HD−/− mice showed an important interaction between energy metabolism dysfunction, mitochondrial abnormalities and excitotoxicity in HD pathogenesis (Brouillet and Beal 1993; Beal 1995, 2000), and that Cr plays important roles in stabilizing intracellular Ca^{2+}, buffering intracellular energy reserves, inhibiting mPTP and decreasing extracellular glutamate (Ferrante *et al.* 2000; Andreassen *et al.* 2001; Dedeoglu *et al.* 2003; Ryu and Ferrante 2005) (Fig. 6). Cr supplementation of HD−/− mice increased their life span, decreased their brain atrophy and delayed the formation of mutant huntingtin aggregates (Ferrante *et al.* 2000). Recently, a phase II clinical trial on safety and tolerability of Cr in HD patients showed that Cr supplementation made an indicator of oxidative-induced damage to DNA (8-hydroxy-2′-deoxyguanosine) undetectable in the serum (Hersch *et al.* 2006). A phase III clinical trial has now been approved and is currently ongoing in various centers.

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is caused by a loss of motor neurons in CNS, particularly in brainstem and motor cortex, which leads to skeletal muscle atrophy, paralysis and death. ALS is caused by a variety of genetic mutations, the most common being located in superoxide dismutase 1 gene (Hervias *et al.* 2006). Different studies with G93A transgenic mice, an animal model for ALS, have shown decreased ATP levels and impairment in respiratory chain activity, inducing a significant decrease in mitochondrial Ca^{2+} loading capacity, oxygen consumption, and ATP synthesis in CNS mitochondria (Mattiazzi *et al.* 2002; Damiano *et al.* 2006) (Fig. 6). Cr supplementation in G93A mice improved their motor performance, extended their survival, protected against neuronal loss in substantia nigra and motor cortex, and finally decreased oxidative damage in mitochondria (Klivenyi *et al.* 1999).

Despite these promising results, human clinical trials testing the efficacy of Cr in ALS patients showed no evidence for Cr therapeutic potential on survival and/or disease progression in patients (Groeneveld *et al.* 2003; Shefner *et al.* 2004). While mitochondrial dysfunction is essential in the motor neuron death cascade in G93A mice, it is not known whether it plays similar roles in inducing motor neuron degeneration in ALS patients (Wong *et al.* 1995; Kong and Xu 1998; Swerdlow *et al.* 1998; Borthwick *et al.* 1999). Other reasons for the discrepancy in Cr responsiveness between G93A mice and ALS patients may be the time of starting Cr treatment (40 days before onset of disease in mice, as compared to an average of 500 days after onset of symptoms in patients), as well as the Cr dose given to ALS patients that may have been inefficient as compared to the dose given to G93A mice (Groeneveld *et al.* 2003; Shefner *et al.* 2004; Rosenfeld *et al.* 2008).

Parkinson's disease

Mitochondrial dysfunction and oxidative damage play important roles in the pathogenesis of PD, which manifests by a loss and/or dysfunction of dopaminergic neurons in substantia nigra (Beal 1995, 2003) and intraneural protein inclusions called Lewy bodies (Lin and Beal 2006). Principal symptoms are progressive bradykinesia, rigidity, tremor and gait abnormalities (Adhihetty and Beal 2008). Mitochondrial dysfunction in PD decreases ATP synthesis and increases ROS production. ROS inactivate MtCK and decrease cytosolic CK activities, thus shutting down energy metabolism (Bindoff *et al.* 1989; Parker *et al.* 1989) (Fig. 6). Mutations in several genes also appear to affect mitochondrial metabolism in PD (Thomas and Beal 2007). Cr

supplementation in PD animal models resulted in significant protection against both CNS dopamine depletion and neuronal loss of neurons in substantia nigra (Matthews *et al.* 1999). Finally, several double-blinded, phase II clinical trials of Cr in early PD patients indicated that Cr supplementation is not futile and should be considered for phase III clinical trials (Bender *et al.* 2006).

Alzheimer's disease

Alzheimer's disease, the most common form of dementia, is characterized by a loss of neurons in cerebral cortex and specific subcortical regions (Wenk 2003). This neuronal loss is associated with deposits of extracellular plaques (amyloid-β peptide and cellular material) outside and around neurons, and deposits of intracellular neurofibrillary tangles (aggregation of the microtubule-associated protein tau in a hyperphosphorylated form) (Bouras *et al.* 1994; Tiraboschi *et al.* 2004).

At the molecular level, AD lesions show inactive CKs in association with depositions enriched in Cr (Bürklen *et al.* 2006). This loss of bioenergetic function appears due to an excessive production of ROS by mitochondria and the absence of translocation of MtCK from cytosol to mitochondria due to lack of a protein chaperone-like activity (Li *et al.* 2006) (Fig. 6). Cr supplementation does not improve cellular bioenergetics at late stages of AD, and the question remains open whether improvement can occur earlier. Other Cr functions have been considered, like protection against oxidative-induced CK inactivation by a delay in ROS action (Aksenov *et al.* 2000). Cr supplementation appears neuroprotective against glutamate and β-amyloid toxicity in rat hippocampal neurons (Brewer and Wallimann 2000).

Ischemic stroke

As described above, stroke generates a significant decrease in CNS total Cr pools. Several studies have investigated the potential neuroprotective effects of Cr supplementation to protect CNS against stroke deleterious mechanisms. Cr supplementation of organotypic cultures of hippocampal slices placed in anoxic conditions appears to replenish their PCr content, protect synaptic transmission and enhance survival of hippocampal neurons (Whittingham and Lipton 1981; Balestrino *et al.* 1999, 2002) (Fig. 6). Total Cr is also increased *in vivo* in the ischemic CNS of rat supplemented with Cr (Wick *et al.* 1999). Moreover, Cr supplementation exerts neuroprotective effects against cerebral ischemia in mice, by inhibiting mitochondrial cytochrome *c* release and downstream caspase 3 activation (Zhu *et al.* 2004). To counteract the poor penetration of Cr from periphery to CNS, the direct administration of Cr into cerebral ventricles, aimed at bypassing BBB, protected CNS from damage of global ischemia in rat (Lensman *et al.* 2006). Similarly, Cr-derived compounds that can cross biological membranes in a Cr transporter-independent manner also showed neuroprotective effects against brain tissue anoxia (Lunardi *et al.* 2006; Perasso *et al.* 2008).

Hyperammonemia

As described above, NH_4^+ exposure generates a secondary Cr deficiency in brain cells, both *in vitro* and *in vivo*. As Cr is essential, during CNS development, to buffer the energetic levels necessary, in growth cones, for axonal and dendritic elongation, and as NH_4^+ exposure impairs axonal growth (Braissant *et al.* 2002), we investigated whether a Cr co-treatment under NH_4^+ exposure could be neuroprotective. We could show that Cr supplementation can protect axonal growth under NH_4^+ exposure (Braissant *et al.* 2002). This protection by Cr depends on the presence of glial cells. As NH_4^+ exposure inhibits axonal growth and decreases Cr, while Cr co-treatment under NH_4^+ protects axonal growth, methods to efficiently sustain Cr concentration in the developing hyperammonemic CNS should be assessed. As described above, Cr can cross from blood to brain through BBB under physiological conditions, but with a low permeability, partly because astrocytes lining BBB do not express SLC6A8. MCEC, at BBB, express SLC6A8 (Braissant *et al.* 2001; Ohtsuki *et al.* 2002). NH_4^+ exposure increases both SLC6A8 and Cr uptake in MCEC (Bélanger *et al.* 2007). As we demonstrated that SLC6A8 is induced in NH_4^+-exposed astrocytes (Braissant *et al.* 2008), BBB of the hyperammonemic CNS might thus be more permeable to Cr than under physiological conditions, and supplying oral Cr to hyperammonemic neonates or infants might likely contribute to protect their brain (Braissant 2010a,b).

Conclusion

The main function of Cr, in energy metabolism, is to allow ATP regeneration through CK enzymatic activity. In recent years, new roles of Cr have been suggested in CNS, like a function of central neuromodulator or even true neurotransmitter and roles in appetite and weight regulation by acting on specific hypothalamic nuclei.

Several studies investigated the brain biosynthetic pathway and transport of Cr, and suggested that because of a poor permeability of BBB for Cr, CNS must secure parts of its needs in Cr by endogenous synthesis. We have recently shown that in many brain structures, AGAT and GAMT are dissociated between different cells, suggesting that to allow brain synthesis of Cr, GAA must be transported from AGAT- to GAMT-expressing cells, most probably through SLC6A8 (Braissant and Henry 2008; Braissant *et al.* 2010).

Given the essential functions of Cr played in CNS, several studies have investigated its neuroprotective potential in numerous brain pathologies, both on neurodegenerative animal models and in patients, with ongoing clinical trials

in phase II and III. Cr supplementation appears to exert neuroprotective effects in HD and PD, but not in AD nor in ALS. Cr may also be used as neuroprotective agent under stroke, ischemia or hyperammonemic states, for which further studies are needed.

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Dissociation of AGAT, GAMT and SLC6A8 in CNS: Relevance to creatine deficiency syndromes

Olivier Braissant *, Elidie Béard, Céline Torrent, Hugues Henry

Inborn Errors of metabolism, Clinical Chemistry Laboratory, Centre Hospitalier Universitaire Vaudois and University of Lausanne, CI 02/33, Avenue Pierre-Decker 2, CH-1011 Lausanne, Switzerland

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ABSTRACT

AGAT and GAMT, the two enzymes of the creatine synthesis pathway, are well expressed within CNS, suggesting autonomous brain creatine synthesis. This contradicts SLC6A8 deficiency, which causes creatine deficiency despite CNS expression of AGAT and GAMT. We hypothesized that AGAT and GAMT were not co-expressed by brain cells, and that guanidinoacetate must be transported between cells to allow creatine synthesis. We finely analyzed the cell-to-cell co-expression of AGAT, GAMT and SLC6A8 in various regions of rat CNS, and showed that in most structures, cells co-expressing AGAT + GAMT (equipped for autonomous creatine synthesis) were in low proportions (<20%). Using reaggregating brain cell cultures, we also showed that brain cells take up guanidinoacetate and convert it to creatine. Guanidinoacetate uptake was competed by creatine. This suggests that in most brain regions, guanidinoacetate is transported from AGAT- to GAMT-expressing cells through SLC6A8 to allow creatine synthesis, thereby explaining creatine deficiency in SLC6A8-deficient CNS.

Introduction

In mammals, creatine (Cr) is taken from diet or synthesized by a two-step mechanism involving L-arginine:glycine amidinotransferase (AGAT) yielding guanidinoacetate (GAA) as intermediate, and guanidinoacetate methyltransferase (GAMT) converting GAA to Cr. Cells take up Cr through a specific transporter, SLC6A8 (Wyss and Kaddurah-Daouk, 2000).

Central nervous system (CNS) is the main organ affected in Cr deficiency syndromes caused by AGAT, GAMT or SLC6A8 deficiency (Stöckler et al., 1994; Schulze et al., 1997; Salomons et al., 2001; Item et al., 2001). These patients present neurological symptoms in infancy, including mental retardation and delays in speech acquisition. GAMT, and often SLC6A8 deficiencies, also cause epilepsy. Cr deficiencies are characterized by strongly decreased Cr levels in CNS, as measured by magnetic resonance spectroscopy (MRS). AGAT- and GAMT-deficient patients can be treated with oral Cr supplementation, while Cr supplementation of SLC6A8-deficient patients does not restore brain Cr (Stöckler et al., 2007; Schulze and Battini, 2007).

Brain cells synthesize Cr, as shown in organotypic cultures (Braissant et al., 2002; Almeida et al., 2006). *In vivo*, AGAT and GAMT are found in all brain cell types (neurons, astrocytes, oligodendrocytes); SLC6A8 is expressed in neurons and oligodendrocytes but not in astrocytes, and is present in microcapillary endothelial cells (MCEC) forming blood–brain barrier (BBB) (Braissant et al., 2001a; Ohtsuki et al., 2002; Schmidt et al., 2004; Tachikawa et al., 2004). Apart of BBB, astrocyte feet around MCEC are more and more recognized as true regulators of water and metabolite exchanges between periphery and CNS (Nedergaard et al., 2003). SLC6A8 absence from astrocytes suggested thus that BBB has a limited permeability for Cr (Braissant et al., 2001a). This is supported *in vivo*: the blood to brain Cr transport appears relatively inefficient in rodents (Ohtsuki et al., 2002; Perasso et al., 2003) and the replenishment of CNS Cr pools in AGAT- and GAMT-deficient patients supplemented with high doses of Cr is slow and only partial (Schulze and Battini, 2007). Moreover, Cr levels are normal in CSF of SLC6A8-deficient patients unable of Cr import from periphery, while they are strongly decreased in CSF of GAMT-deficient patients who can import Cr from periphery (Braissant and Henry, 2008).

These observations suggest that CNS depends more on autonomous Cr synthesis than on supply from periphery. This seems contradictory with SLC6A8-deficient patients who, despite AGAT and GAMT expression in CNS, show strongly decreased levels of brain Cr. We hypothesized that the brain cell-to-cell AGAT, GAMT and SLC6A8 expression pattern might explain this contradiction: AGAT and GAMT, which can be found in every CNS cell types, might not be co-expressed within the same cells. That would also imply that GAA must be transported between brain cells for Cr synthesis to occur. This work aimed at dissecting the cell-to-cell co-expression of AGAT, GAMT and SLC6A8 within various regions of rat CNS. Moreover, brain cell GAA uptake and its conversion to Cr were analyzed by tandem mass

* Corresponding author. Fax: +41 21 314 35 46.
E-mail address: Olivier.Braissant@chuv.ch (O. Braissant).
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spectrometry in reaggregated brain cell primary cultures, which synthesize Cr and express AGAT, GAMT and SLC6A8 as CNS *in vivo* (Braissant et al., 2008).

Materials and methods

Preparation of adult rat brains

Animal procedures were in compliance with the directives of the Swiss Academy of Medical Science. Adult rats (Sprague–Dawley, 300 g, Harlan, Netherlands) were sacrificed by decapitation. Their brain was extracted within 2 min, rinsed in diethylpyrocarbonate-treated PBS, and immediately embedded in tissue freezing medium (Tissue-Tek, Sakura Finetek, Netherlands), frozen in liquid nitrogen-cooled isopentane and stored at −80 °C.

In situ hybridization and immunohistochemistry

AGAT, GAMT and SLC6A8 expression was analyzed within various representative regions of the rat brain (Fig. 1 and Supplementary Table 1) by *in situ* hybridization (ISH) coupled to immunohistochemistry, allowing the detection of both mRNA and protein for AGAT, GAMT and SLC6A8 genes. To achieve this, specific RNA probes and antibodies were applied to rat brain cryosections (12 μm thick). ISH was performed by hybridizing digoxigenin-labeled antisense and sense riboprobes specific for rat AGAT, GAMT and SLC6A8 as described in details (Braissant et al., 2001a). Sections were stained with alkaline-phosphatase using nitroblue-tetrazolium and 5-bromo-4-chloro-3-indolyl-phopshate (blue signal). After staining, sections were dehydrated. The hybridization specificity was ascertained by the negative staining of ISH sense control probes-hybridized sections for AGAT, GAMT and SLC6A8 (data not shown). Sense probes-hybridized sections were mounted (Eukitt, O.Kindler, Germany). Antisense probes-hybridized sections were processed for immunohistochemistry as described in details (Braissant, 2004), using specific anti-AGAT, -GAMT and -SLC6A8 rabbit polyclonal antibodies already characterized (Braissant et al., 2005), or alternatively anti-glial fibrillary acidic protein (GFAP) or anti-NeuN mouse monoclonal antibodies (Chemicon International). Sections were stained using horse radish peroxidase, the Histostain-Plus kit (Zymed Laboratories) and aminoethyl-carbazole/H_2O_2 (red signal).

The double-stained sections (ISH blue, immunohistochemistry red) were mounted in glycerol. AGAT, GAMT and SLC6A8 patterns were considered specific and validated by the excellent correlation, in all CNS regions, between signals observed by ISH (mRNA) and immunohistochemistry (protein), the negative signals observed with ISH sense probes, and the absence of labeling in immunohistochemical controls without primary antibodies or in presence of pre-immune serum (data not shown, and Braissant et al., 2005). The ISH protocol sensitivity, which can detect as low as 15 transcripts per cell, has been described elsewhere (Braissant et al., 2001b; Braissant, 2004).

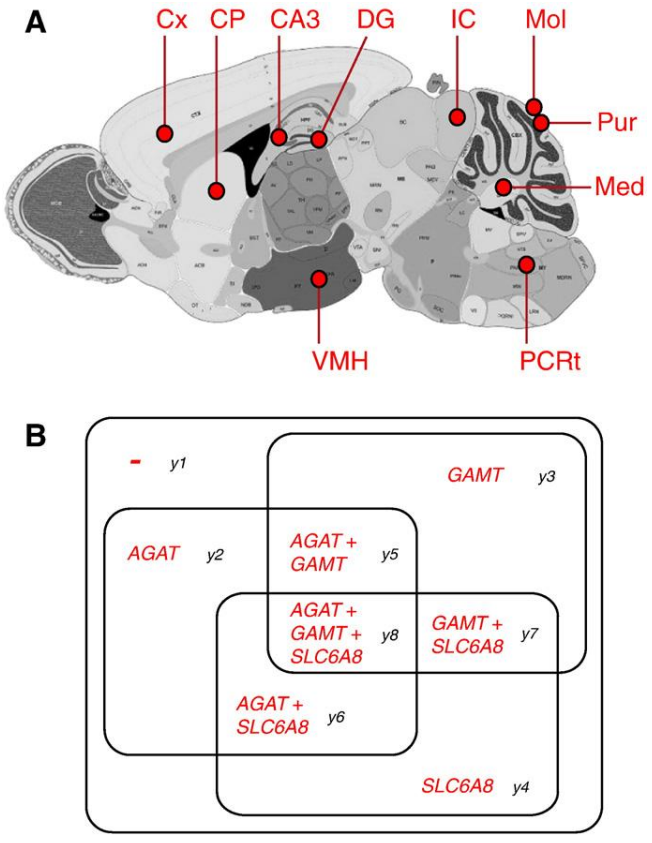

Fig. 1. Rat brain structures analyzed for AGAT, GAMT and SLC6A8 co-expression. (A) 10 different structures of the rat brain were analyzed: Cortex (layer V, Cx); caudate putamen (CP); hippocampus (CA3 and dentate gyrus neurons, DG); hypothalamus (ventromedial nucleus, VMH); inferior colliculus (IC); pons (parvocellular reticular nucleus, PCRt); cerebellum (molecular layer, Mol; Purkinje neurons, Pur; and deep medial nucleus, Med) (sagittal view of rat brain adapted from http://www.brain-map.org; lateral level for rat brain: 1.20 mm). (B) The proportions of cells (co-) expressing AGAT alone (y2), GAMT alone (y3), SLC6A8 alone (y4), AGAT + GAMT (y5), AGAT + SLC6A8 (y6), GAMT + SLC6A8 (y7), AGAT + GAMT + SLC6A8 (y8), or none (y1), were determined in each brain structures (see also Supplementary Tables 2–4).

Histological analysis and cell counting

Sections were observed on an Olympus BX50 microscope equipped with a ColorView-II camera (Olympus Opticals, Japan). Brain structures were identified according to Paxinos and Watson (1986) (Supplementary Table 1). In each brain structure, cells were counted within a square surface of 200 μm × 160 μm (12 μm thick cryosections), except for CA3 pyramids and dentate gyrus granular neurons (hippocampus), and for Purkinje neurons (cerebellum), where cells were counted along 200 μm (hippocampus) or 500 μm (Purkinje neurons) of these linear structures. Cell counting was achieved with the help of the Cell^F software (Olympus). The average number of cells counted per brain and per co-labeling experiment is indicated in Supplementary Table 1. While AGAT, GAMT and SLC6A8 proteins can be localized far from soma (e.g.: cell processes), they are also present, when expressed, in soma (AGAT and GAMT) or at its surface (SLC6A8) (Tachikawa et al., 2004; Braissant et al., 2008; Tachikawa et al., 2008). Therefore, to be coherent with ISH counting (mRNA, essentially localized around nucleus), brain cells were counted positive for AGAT, GAMT or SLC6A8 proteins only if their soma showed the immunohistochemistry signal.

The 3 different "2 by 2" combinations (AGAT + GAMT; AGAT + SLC6A8; GAMT + SLC6A8) were unraveled in each brain analyzed ($n = 3$; 2 females, 1 male). For each combination, mRNA no. 1 was revealed by ISH coupled to immunohistochemistry for protein no. 2, followed on adjacent section by ISH detection of mRNA no. 2 coupled to immunohistochemistry for protein no. 1. Particular care was taken to consider identical cells on adjacent sections. All combinations were repeated twice, allowing, for each brain, a total of 4 labeling "2 by 2" of each combinations of the 3 genes (12 adjacent sections covering 144 μm depth of brain parenchyma). The series of (co-)labeling, distributed among the three rat brains used, were designed to allow the observation of the largest neuronal soma, by the continuous observation of each of the three genes through a depth of 96 μm (i.e.: 8 adjacent sections). With each "2 by 2" combination and for each brain structure, the cell proportions with no expression of genes 1 or 2, expression of gene 1 only, expression of gene 2 only, or co-expression of genes 1 and 2, were obtained (= values x_i, also defined in Supplementary Tables 2 and 3). Each combination of "2 by 2" co-

labeling ($n=4$) gave extremely coherent results (see standard deviations in Supplementary Table 3). Thus, for each gene in each couple of genes, in each of the 10 different brain regions analyzed from 3 different rat brains, mRNA and protein data gave identical proportions: When a specific mRNA was detected on one slice, its respective protein was always detected also in the same cell on the adjacent slice, and no presentation of mRNA without its respective protein occurred. Conversely, when a specific protein was detected on one slice, its respective mRNA was always detected also in the same cell on the adjacent slice, and no presentation of protein without its respective mRNA occurred. Thus, these very coherent data between mRNA (perinuclear region) and protein (considered only in the cell soma) validated the gene expression observation in the brain cell soma as truly representative of expression in the whole brain cells.

This allowed then the determination of AGAT, GAMT and SLC6A8 (co-)expression patterns taken "3 by 3" (AGAT + GAMT + SLC6A8 co-expression; see below).

The determination of the "3 by 3" AGAT + GAMT + SLC6A8 co-expression proportions (= values y_j, also defined in Fig. 1B and Supplementary Table 2) was obtained using two different approaches:

The first consisted in determining the smallest intervals containing each of the y_1 to y_8 "3 by 3" expression proportions. To achieve this, we determined x_i as the lowest x_1–x_{12} experimental measure in each brain structure. We chose one of the two y_j unknowns giving x_i as sum, to express all the other y_1 to y_8 unknowns, allowing finally the determination of the smallest intervals fitting for y_1 to y_8 (see their complete determination, illustrated for dentate gyrus, in Supplementary Table 3). These intervals are presented in the left columns of Figs. 3A–C ("experimental").

The second was based on the hypothesis, true or not, that AGAT, GAMT and SLC6A8 might be expressed independent of each other in brain cells, both at transcriptional and translational levels. y_1 to y_8 were calculated, in each brain structure, by developing, based on Fig. 1 and Supplementary Table 2, the following equations, exemplified here for y_2 (AGAT only) :

$$y_2 = x_2 - y_6 = x_2 - [y_6 / (y_2 + y_6)*(y_2 + y_6)]. \quad (1)$$

Then, if (and only if) AGAT, GAMT and SLC6A8 genes are expressed independent of each other in the brain, both at transcriptional and translational levels :

$$y_6 / (y_2 + y_6) = (y_6 + y_8) / (y_2 + y_6 + y_5 + y_8). \quad (2)$$

After replacement of "$y_6 / (y_2 + y_6)$" in (1) :

$$y_2 = x_2 - [(y_6 + y_8) / (y_2 + y_6 + y_5 + y_8)*(y_2 + y_6)] \quad (3)$$
$$= x_2 - [x_8 / (x_8 + x_6)*x_2].$$

For each y_j, 3 different (but equivalent) equations can be developed, the 2 equations remaining for y_2 being:

$$y_2 = x_6 - y_5 = x_6 - [x_{10} / (x_{10} + x_9)*x_6], \quad (4)$$

$$y_2 = x_9 - y_1 = x_9 - [x_1 / (x_1 + x_2)*x_9], \quad (5)$$

By analogy, the same calculation procedure was applied to y_1 and y_3 to y_8 (see their complete calculation, illustrated for dentate gyrus, in Supplementary Table 4). y_j values for each brain structure using this second method is presented in the right columns of Figs. 3A–C ("calculated").

Reaggregated brain cell cultures

Reaggregated brain cell primary cultures were prepared from mechanically dissociated telencephalon of 16-day rat embryos, grown in serum-free, Cr-free, chemically-defined medium (Braissant et al., 2008). These cultures 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 (Honegger and Monnet-Tschudi, 2001; Cagnon and Braissant, 2007). These cultures express AGAT, GAMT and SLC6A8 in the same cells as *in vivo* brain and synthesize their own Cr, suggesting that they behave as *in vivo* CNS for Cr synthesis and transport (Braissant et al., 2008). Brain cell aggregates were grown for 13 days *in vitro* (DIV), then exposed for 12 h or 24 h to various concentrations of GAA (GAA or $^{13}C_2$-GAA; 200, 600 or 1000 μM) and/or Cr (Cr or ^{13}C-Cr; 200 or 1000 μM). Aggregates were harvested at DIV13.5 (12 h exposure) or DIV14 (24 h exposure), washed three times with ice-cold PBS, frozen in liquid nitrogen, and stored at −80 °C.

Creatine and guanidinoacetate determination, and GAMT activity

Brain cell aggregates were homogenized in H_2O at 4 °C using a FastPrep Cell Disrupter (Qbiogene, France) and centrifuged (10,000*g*, 5 min, 4 °C). The supernatant was used to measure Cr and GAA by electrospray tandem mass spectrometry, as described in details (Braissant et al., 2008). D_3-Cr (CDN Isotopes, Canada) and $^{13}C_2$-GAA (Dr Herman J ten Brink, Amsterdam, Netherlands) were used as internal standards. Cr and GAA were purified by micro solid phase extraction (Oasis MCX μElution Plate, Waters, MA USA). Separation of Cr and GAA was achieved at 30 °C using an Atlantis HILIC silica 2.1 × 50 mm 3 μm (Waters, MA USA). The column effluent was monitored using a Triple Quadrupole TSQ Quantum Discovery (Thermo Scientific, CA USA). The instrument was equipped with an electrospray interface and was controlled by the Xcalibur software (Thermo Scientific, CA USA). Samples were analyzed in positive ionization mode operating in a cone voltage of 4 kV. The tandem mass spectrometer was programmed using the selected reaction monitoring mode (SRM) to allow the [MH^+] ions of Cr and GAA respectively at m/z 132 and 118 and that of the internal standards D_3-Cr and $^{13}C_2$-GAA at m/z 135 and 120 to pass through the first quadrupole (Q1) and into the collision cell (Q2). The daughter ions for Cr and GAA are of m/z 90 and 76 respectively, and of m/z 93 and 78 for D_3-Cr and $^{13}C_2$-GAA respectively. Calibration curves were computed using the peak area ratio of analytes and internal standards.

For GAA uptake, brain cell aggregates were incubated for 12 h or 24 h with 200 or 600 μM GAA. Intracellular GAA was measured as described above using SRM with the transition of m/z 118 to m/z 76. For Cr uptake, brain cell aggregates were incubated for 12 h in presence of 200 μM ^{13}C-Cr (Cambridge Isotope Laboratories, MA USA). Intracellular ^{13}C-Cr was measured as above using SRM with transition of m/z 133 to m/z 91. The relative abundance of m/z 133 calculated at a ratio of 3.8 % of endogenous Cr was deduced from the measured transported ^{13}C-Cr. Uptakes of GAA and ^{13}C-Cr (12 h incubation, 200 μM) were competed by 5-fold excess (1 mM) of Cr and GAA, respectively.

GAMT activity of brain cell aggregates *in vivo* was accessed by the synthesis of endogenous $^{13}C_2$-Cr when incubated with $^{13}C_2$-GAA (12 h incubation, 200 μM $^{13}C_2$-GAA). Newly synthesized $^{13}C_2$-Cr was measured using SRM with transition of m/z 134 to m/z 92.

Statistical analysis

The statistical significance of intracellular GAA and Cr variations (Fig. 6) was evaluated by Student's t test. $p<0.05$ was considered significant. Data were expressed as mean ± standard deviation.

Results

Using ISH coupled to immunohistochemistry, the 3 different "2 by 2" combinations (AGAT + GAMT; AGAT + SLC6A8; GAMT + SLC6A8) were unraveled on adjacent sections of the rat brain. For each

combination, mRNA no. 1 was revealed by ISH coupled to immunohistochemistry for protein no. 2, followed on adjacent section by ISH detection of mRNA no. 2 coupled to immunohistochemistry for protein no. 1. Particular care was taken to consider identical cells on adjacent sections. When a specific mRNA was detected on one slice, its respective protein was always detected also in the same cell on the adjacent slice, and no presentation of mRNA without its respective protein occurred. Conversely, when a specific protein was detected on one slice, its respective mRNA was always detected also in the same cell on the adjacent slice, and no presentation of protein without its respective mRNA occurred.

AGAT, GAMT and SLC6A8 are expressed independent of each other in rat CNS

Two different approaches were used to determine the "3 by 3" AGAT + GAMT + SLC6A8 co-expression proportions ($=y_j$; as also defined in Fig. 1B and Supplementary Figure 2) of brain cells. The 1st was the simple estimation of the minimal interval containing each y_j proportion based on the direct measure (=cell counting) of the "2 by 2" AGAT, GAMT and SLC6A8 co-expression proportions ($=x_i$; as also defined in Supplementary Figure 2). The 2nd was based on the hypothesis, true or not, that AGAT, GAMT and SLC6A8 might be expressed independent of each other in brain cells, both at the level of transcription and translation. This hypothesis allowed to calculate the "3 by 3" expression proportions (y_j) from the "2 by 2" expression proportions (x_i). Two strikingly identical expression patterns were found using these two independent approaches (Figs. 3A–C), in each brain structure analyzed. As the 1st approach is a true measure of the "3 by 3" expression proportions, and as the 2nd approach is valid only if AGAT, GAMT and SLC6A8 are expressed independent of each other, we conclude that in rat CNS, AGAT, GAMT and SLC6A8 genes are expressed independent of each other, both at the level of transcription and translation.

Four different AGAT, GAMT and SLC6A8 co-expression patterns were found depending of brain structures (Figs. 2 and 3). Figs. 2–5, as well as Supplementary Tables 1, 3 and 4, show the data from one representative adult rat brain, the same patterns having been obtained in two other adult rat brains.

Cortex, inferior colliculus and cerebellum (molecular layer and deep medial cerebellar nucleus)

In cortex (layer V), 31% of brain cells were silent for AGAT, GAMT or SLC6A8 (Figs. 2B, E, F and 3A). Cells expressing AGAT, GAMT or SLC6A8 alone were 15%, 14% and 14% respectively. Cells co-expressing AGAT and GAMT (but not SLC6A8) were 8%, cells expressing AGAT and SLC6A8 (but not GAMT) were 7%, and cells co-expressing GAMT and SLC6A8 (but not AGAT) were 8%. Finally, cells co-expressing AGAT, GAMT and SLC6A8 were 4%. Thus, in cortex, cells silent for the three genes were 31%, cells equipped for autonomous Cr synthesis (AGAT + GAMT) were 12%, and cells equipped for Cr synthesis from imported GAA (SLC6A8 + GAMT) were 12%. Cells with only SLC6A8 ("users" of Cr, but not participating to its synthesis) were 14%.

Similar expression patterns were found in inferior colliculus as well as in cerebellar molecular layer and deep medial nucleus (Fig. 3A). In inferior colliculus, cells silent for the three genes were 33%, cells equipped for autonomous Cr synthesis were 14%, and cells equipped for Cr synthesis from imported GAA were 16%. Cells with only SLC6A8 were 12%. In molecular layer of cerebellum, cells silent for the three genes were 32%, cells equipped for autonomous Cr synthesis were 18%, and cells equipped for Cr synthesis from imported GAA were 17%. Cells with only SLC6A8 were 9%. In deep medial nucleus of cerebellum, cells silent for the three genes were 21%, cells equipped for autonomous Cr synthesis were 22%, and cells equipped for Cr synthesis from imported GAA were 26%. Cells with only SLC6A8 were 11%.

Hippocampus (CA3 and dentate gyrus granular neurons) and hypothalamus

Hippocampus (neurons of CA3 layer and dentate gyrus) and ventromedial nucleus of hypothalamus presented similar patterns (Figs. 2C, D and 3B). In hippocampal CA3 layer, 40% of neurons co-expressed AGAT, GAMT and SLC6A8, 20% being silent for the three genes. 47% of cells were equipped for autonomous Cr synthesis, cells equipped for Cr synthesis from imported GAA were 49%, and cells with SLC6A8 only were 6%. In granular neurons of dentate gyrus, 32% of cells co-expressed AGAT, GAMT and SLC6A8, 35% being silent for the three genes. 38% of cells were equipped for autonomous Cr synthesis, cells equipped for Cr synthesis from imported GAA were 37%, and cells with SLC6A8 only were 5%. In hypothalamic ventromedial nucleus, 29% of cells co-expressed AGAT, GAMT and SLC6A8, 30% being silent for the three genes. 37% of cells were equipped for autonomous Cr synthesis, cells equipped for Cr synthesis from imported GAA were 37%, and cells with SLC6A8 only were 4%.

Purkinje neurons

37% of Purkinje neurons co-expressed AGAT, GAMT and SLC6A8, 9% only being silent for the three genes. 61% of Purkinje neurons were equipped for autonomous Cr synthesis, cells equipped for Cr synthesis from imported GAA were 44%, and cells with SLC6A8 only were 5% (Fig. 3B).

Caudate putamen and pons (parvocellular reticular nucleus)

Caudate putamen and parvocellular reticular nucleus of pons showed very peculiar patterns (Figs. 2A and 3C). In caudate putamen, 50% of cells were silent for AGAT, GAMT or SLC6A8, and 43% expressed GAMT only. Cells equipped for autonomous Cr synthesis were 1%, cells equipped for Cr synthesis from imported GAA were 1%, and cells with SLC6A8 only were 2%. In parvocellular reticular nucleus of pons, 44% of cells were silent for AGAT, GAMT or SLC6A8, and 37% expressed AGAT only. Cells equipped for autonomous Cr synthesis were 7%, cells equipped for Cr synthesis from imported GAA were 5%, and cells with SLC6A8 only were 4%.

Proportions of neurons and astrocytes expressing AGAT, GAMT and SLC6A8 in cortex

We analyzed within layer V of cortex whether AGAT and GAMT were homogeneously expressed between neurons and glia. This was achieved by ISH for AGAT, GAMT or SLC6A8 mRNAs, coupled to immunohistochemistry for the neuronal marker NeuN or the astrocytic marker GFAP (Fig. 4). The abovementioned cell counting method was applied to determine the proportion of specific cell types expressing AGAT, GAMT or SLC6A8 (Fig. 5A). We showed that only 22% of NeuN-positive neurons were positive for AGAT, while 48% were positive for GAMT. In contrast, 55% of GFAP-positive astrocytes were positive for AGAT, while only 21% were positive for GAMT. 27% of NeuN-positive neurons expressed SLC6A8, while, as expected, no astrocyte was found expressing SLC6A8. As AGAT, GAMT and SLC6A8 are expressed independent of each other (see above and Fig. 3), applying the calculation method described in Methods and Supplementary Tables 2 to 4 allowed to reconstitute the differential "3 by 3" AGAT + GAMT + SLC6A8 co-expression in cortical neurons and astrocytes (Fig. 5B). We show in particular that important proportions of both neurons (25%) and astrocytes (35%) were silent for the three genes. Based on the 1:0.4 ratio of neurons versus astrocytes in the rat cortex (Nedergaard et al., 2003), we could reconstitute the cumulative proportions of cortical neurons and astrocytes co-expressing AGAT, GAMT and SLC6A8, which fitted remarkably well with the direct measure of expression ranges presented in Fig. 3A (Fig. 5C). The slight

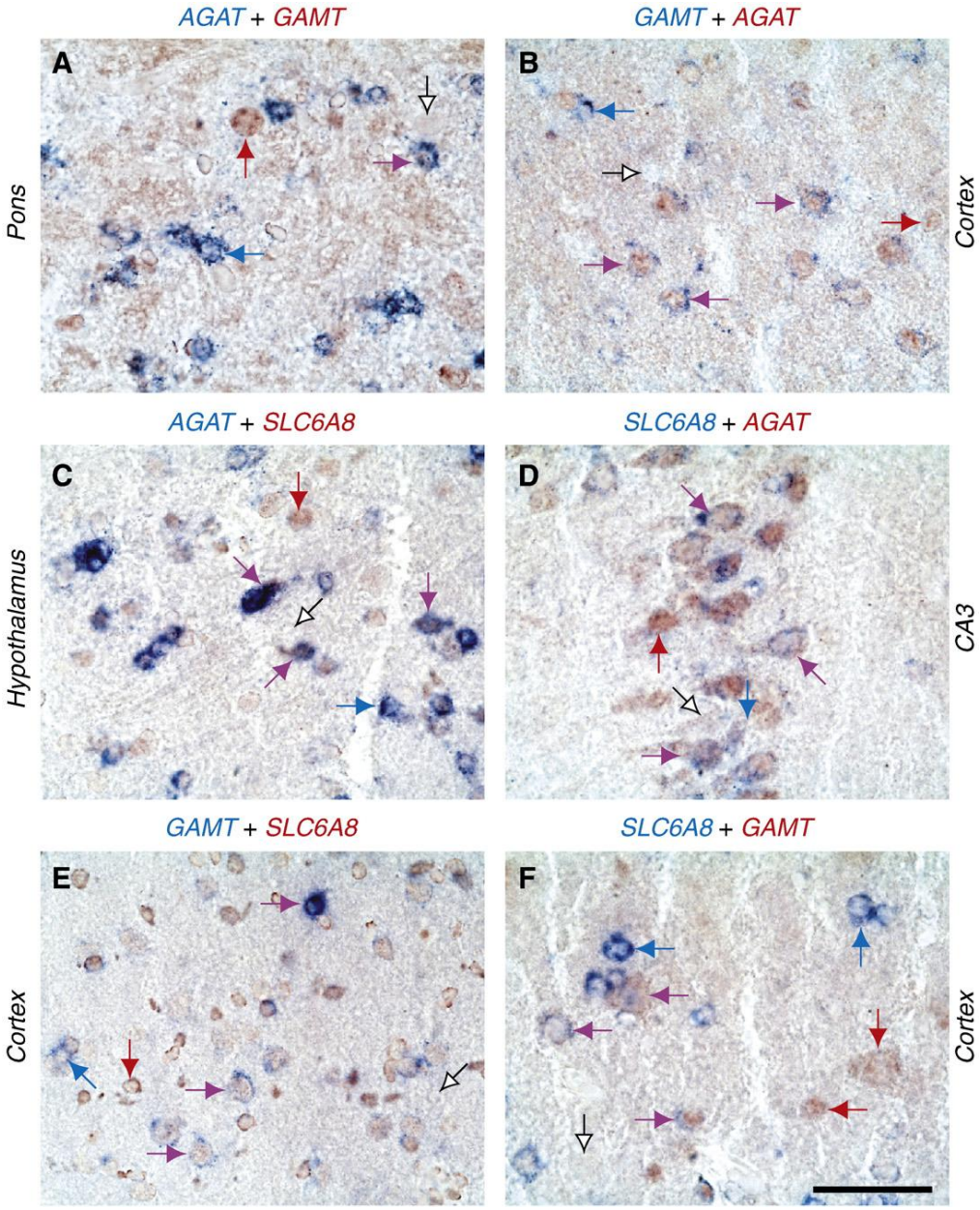

Fig. 2. Co-expression of AGAT, GAMT and SLC6A8 in the rat brain. *In situ* hybridization (mRNA, blue signal) coupled to immunohistochemistry (protein, red signal), showing "2 by 2" co-expression of (A) AGAT mRNA and GAMT protein in pons, (B) GAMT mRNA and AGAT protein in layer V of cortex, (C) AGAT mRNA and SLC6A8 protein in hypothalamus, (D) SLC6A8 mRNA and AGAT protein in CA3 neurons, (E) GAMT mRNA and SLC6A8 protein in layer V of cortex, and (F) SLC6A8 mRNA and GAMT protein in layer V of cortex. Examples of cells without any labeling are indicated by empty arrows; examples of cells with gene 1 mRNA expression only are indicated by blue arrows; examples of cells with gene 2 protein expression only are indicated by red arrows; examples of cells with co-labeling for gene 1 mRNA and gene 2 protein are indicated by purple arrows. Bar: 50 μm.

variations between both patterns are likely due to other cell types present in lower proportions within cortex (oligodendrocytes, microglia), which were not evaluated here.

Brains cells take up guanidinoacetate and convert it to creatine

As most rat brain structures showed a high proportion of cellular dissociation between AGAT- and GAMT-expressing cells, we hypothesized that brain cells were able to import GAA, and convert it to Cr. Using rat brain cell primary cultures in aggregates, we first demonstrated by tandem mass spectrometry that brain cells can import GAA (Fig. 6A). Exposure up to 200 μM GAA for 12 h did not disturb the endogenous Cr synthesis pathway, which occurs in this system as in CNS *in vivo* (Braissant et al., 2008); a higher GAA concentration (600 μM) was toxic to brain cells, as shown by the 30% decrease in intracellular Cr (Fig. 6A), as well as by axonal growth impairment and induction of apoptosis (data not shown). 24 h GAA exposure had the same effects (data not shown). We further showed that imported GAA could be converted to Cr, by exposing brain cell aggregates to ^{13}C stable isotope-labelled $^{13}C_2$-GAA, and measuring intracellular $^{13}C_2$-Cr. After 12 h exposure to 200 μM $^{13}C_2$-GAA, 29% of imported $^{13}C_2$-GAA was converted to $^{13}C_2$-Cr (Fig. 6B). Finally, we showed that 1000 μM Cr completely competed GAA uptake by brain cells (200 μM GAA exposure), with the same efficiency as 1000 μM

GAA competed Cr uptake (200 μM ^{13}C-Cr exposure, to avoid influence of the endogenous Cr synthesis pathway) (Fig. 6C).

Discussion

Brain AGAT, GAMT and SLC6A8 are expressed independent of each other

This work demonstrates that AGAT, GAMT and SLC6A8 are expressed independent of each other in CNS, at the level of transcription and translation. Cr exerts a negative pre-translational feedback on AGAT regulation in kidney (McGuire et al., 1984). Brain appears to behave differently, where Cr rather alters AGAT enzymatic activity (Braissant et al., 2008). Human muscle behave similarly, where Cr does not influence SLC6A8 expression (Tarnopolsky et al., 2003). Thus, brain AGAT, GAMT and SLC6A8 expression does not seem influenced by their substrate or reaction products, probably allowing CNS to be permanently equipped with Cr synthesis and transport machineries, something crucial knowing that Cr is essential in CNS development and functions (Braissant and Henry, 2008; Andres et al., 2008).

Dissociation of AGAT and GAMT, and GAA uptake by brain cells: Relevance to Cr deficiency syndromes

Brain Cr was thought to come principally from periphery through BBB (Wyss and Kaddurah-Daouk, 2000). However, mammalian CNS synthesizes Cr and express AGAT and GAMT in every cell types (Pisano et al., 1963; Braissant et al., 2001a; Schmidt et al., 2004; Tachikawa et al., 2004). SLC6A8 is expressed in CNS, but only by neurons and oligodendrocytes. The SLC6A8 absence from astrocytes (particularly astrocyte feet lining MCEC; Braissant et al., 2001a; Ohtsuki et al., 2002; Tachikawa et al., 2004) made us suggest that BBB has a limited permeability for Cr, and that CNS depends more on autonomous Cr synthesis than on supply from periphery (Braissant et al., 2007; Braissant and Henry, 2008).

Brain autonomous Cr synthesis seems contradictory with *in vivo* characteristics of SLC6A8 deficiency, which, despite normal CNS expression of AGAT and GAMT, shows no, or very low levels of, brain Cr by MRS, as in AGAT and GAMT deficiencies. We hypothesized that this apparent contradiction might be explained by brain patterns of

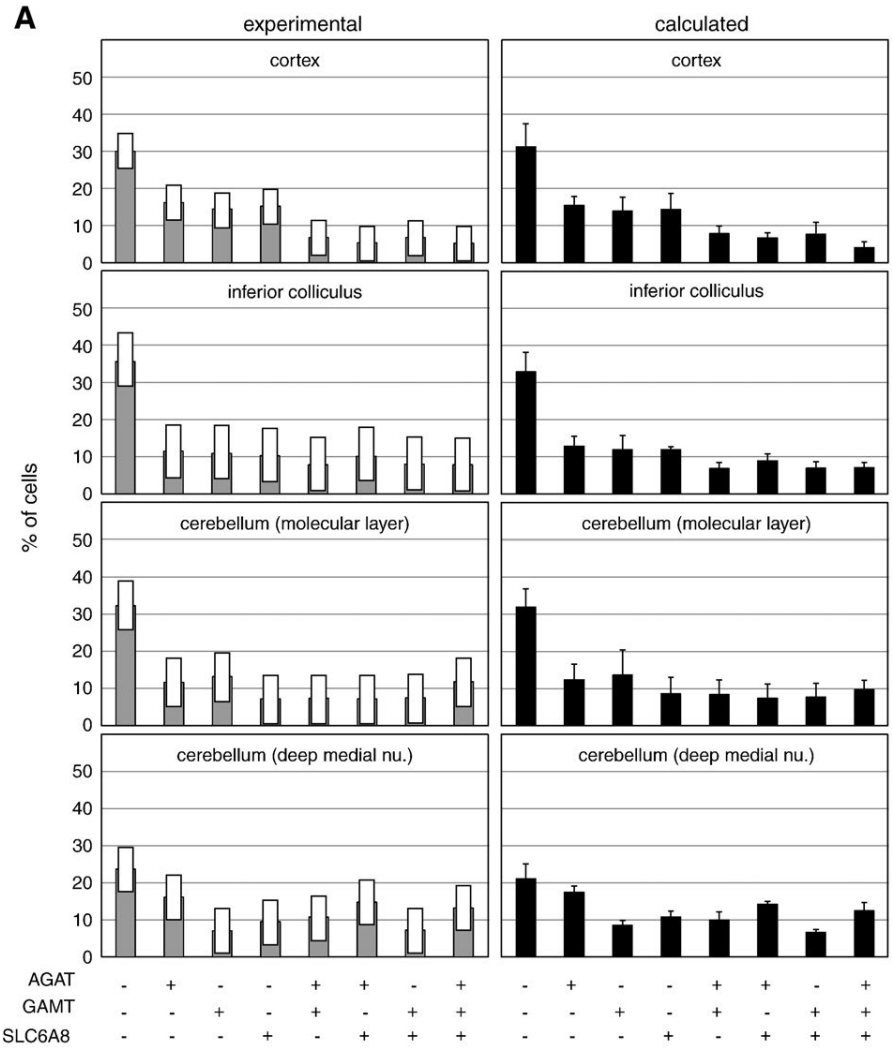

Fig. 3. Co-expression of AGAT, GAMT and SLC6A8 in the different structures of the rat brain. (A) cortex, inferior colliculus and cerebellum (molecular layer and deep medial nucleus). (B) hippocampus (CA3 and dentate gyrus), hypothalamus (ventromedial nucleus) and cerebellum (Purkinje neurons). (C): caudate putamen and pons (parvoreticular nucleus). The percentage of cells (co-)expressing AGAT alone, GAMT alone, SLC6A8 alone, AGAT + GAMT, AGAT + SLC6A8, GAMT + SLC6A8, AGAT + GAMT + SLC6A8, or none, is indicated, for both methods used (experimental and calculated) to estimate these proportions (see also Fig. 2 for an illustration of the co-labeling used). For the experimental measure of co-expression proportions (left column), the ranges between minimal and maximal proportions are represented (open white rectangle). For the calculated proportions (right column), means ± standard deviations ($n = 3$) are represented.

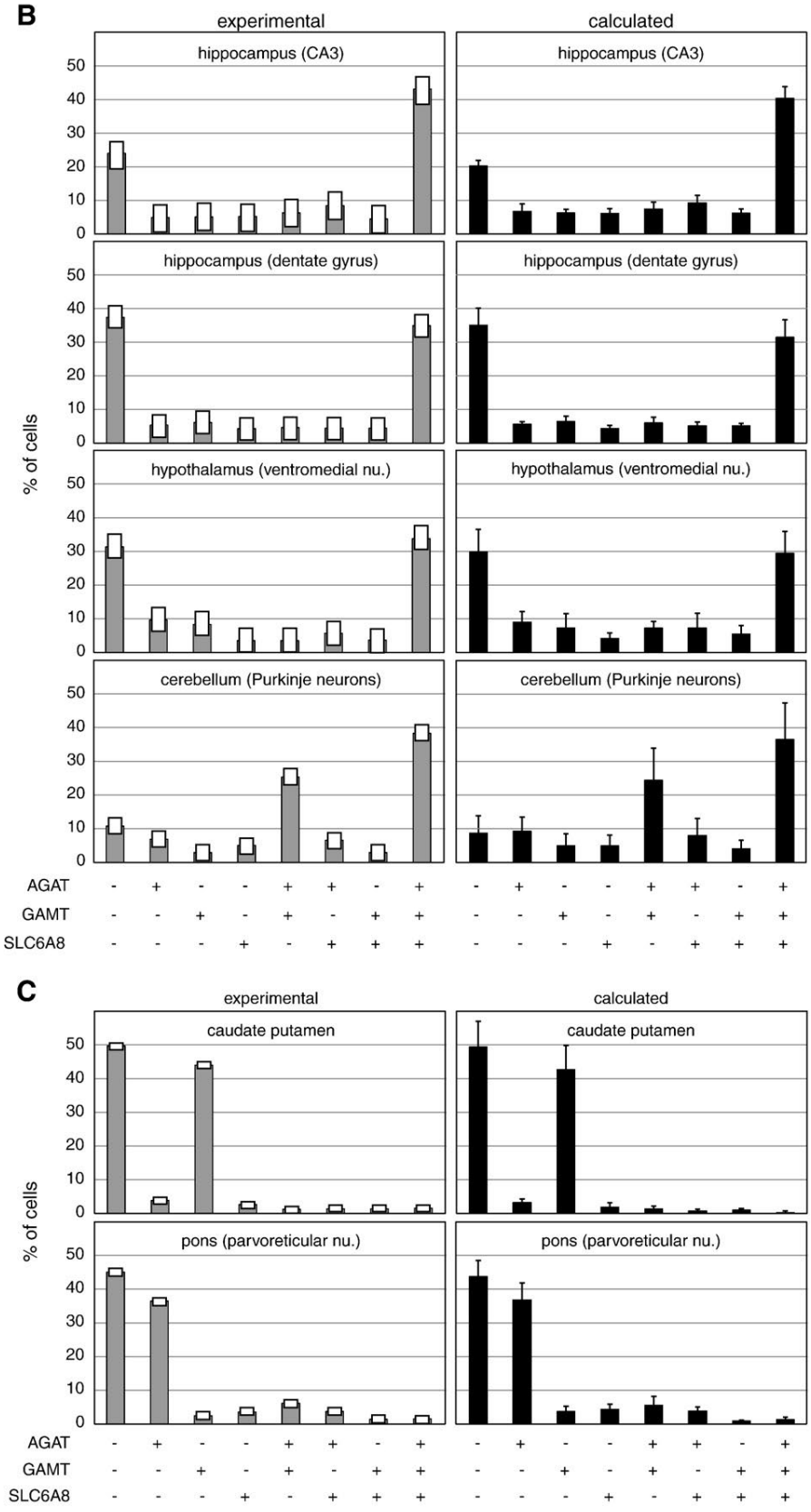

Fig. 3 (*continued*).

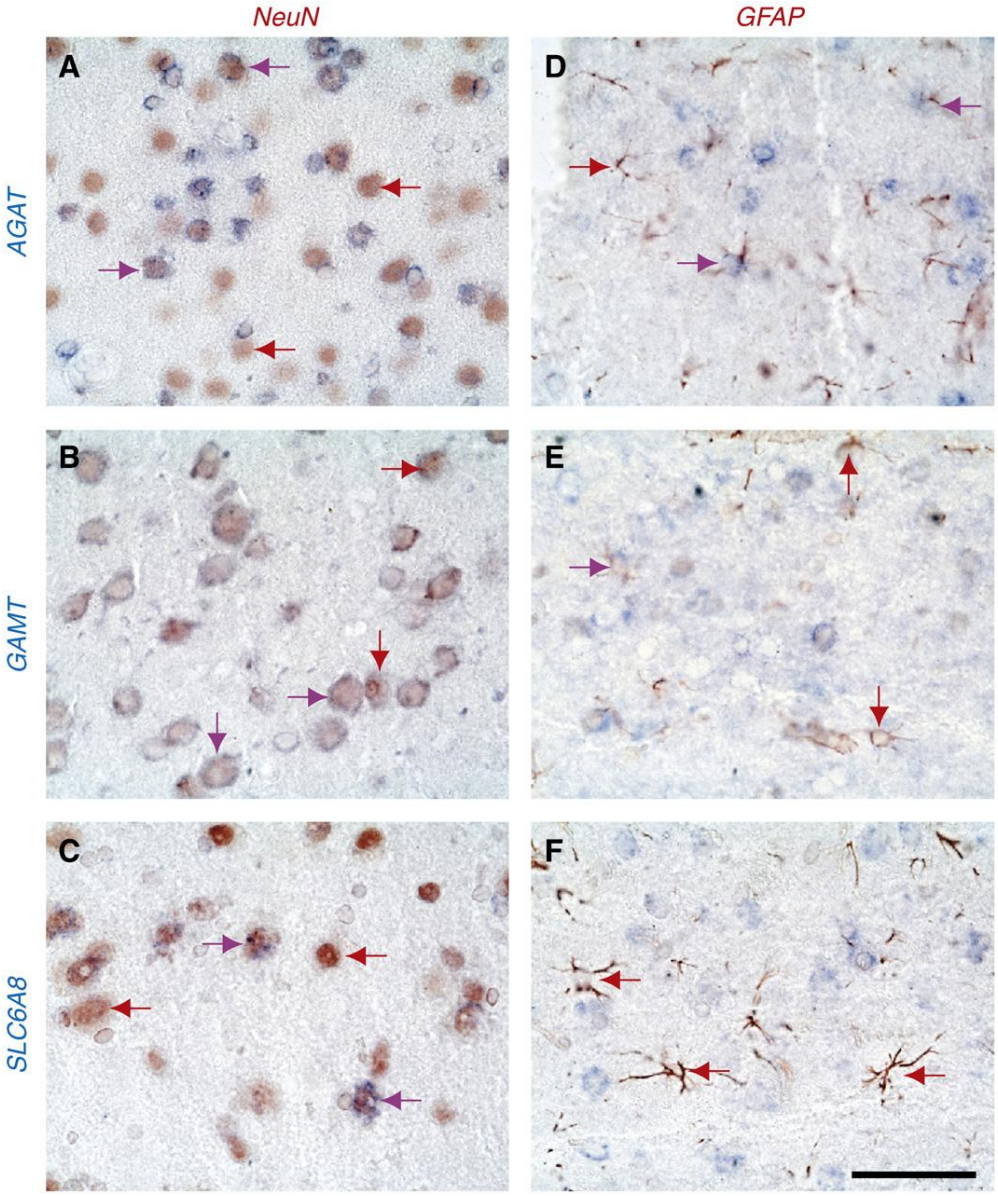

Fig. 4. Expression of AGAT, GAMT and SLC6A8 in cortical neurons and astrocytes. *In situ* hybridization for AGAT (A, D), GAMT (B, E) and SLC6A8 (C, F) mRNAs (blue signal), coupled to immunohistochemistry for neuronal (NeuN; A–C) and astrocytic (GFAP; D–F) markers respectively (red signal). Examples of neurons and astrocytes without co-labelling for AGAT, GAMT or SLC6A8 are indicated by red arrows, while examples of neurons and astrocytes with co-labelling for AGAT, GAMT or SLC6A8 are indicated by purple arrows. Bar: 50 μm.

AGAT, GAMT and SLC6A8, which might be expressed in a dissociated way (Braissant and Henry, 2008).

This work demonstrates that in many brain structures, including these usually observed by MRS, cells fully equipped for Cr synthesis (co-expressing AGAT + GAMT) are <20% (cortex: 12%, striatum: 1%), and that a higher proportion of cells expresses AGAT without GAMT, or GAMT without AGAT. This suggested that for Cr synthesis to occur, GAA must be transported from AGAT- to GAMT-expressing cells. If GAA is transported by SLC6A8 (see below), it would explain the absence of Cr measured by MRS in CNS of SLC6A8-deficient patients. This recently proposed model (Braissant and Henry, 2008) is also supported *in vivo* by the attempt to treat SLC6A8-deficient patients with arginine to stimulate replenishment of their cerebral Cr pool by brain endogenous synthesis, which failed to increase CNS Cr (Fons et al., 2008). Thus, according to the above-mentioned hypothesis, in human brain also, GAA must be transported from AGAT- to GAMT-expressing cells, through SLC6A8, for Cr synthesis to occur.

Whilst in most regions of AGAT- and GAMT-deficient brain, the MRS measure of Cr should show an absence or strong decrease of Cr (partly due to the lack of brain autonomous Cr synthesis), the situation may be more contrasted in SLC6A8 deficiency. Indeed, the MRS Cr measure is usually performed in cortical areas and basal ganglia (striatum), where SLC6A8-deficient patients present an absence or strong decrease of Cr (DeGrauw et al., 2002) (excepted for females heterozygous for SLC6A8 mutation, who usually present a milder cerebral Cr deficiency, as SLC6A8 deficiency is an X-linked disorder). If AGAT, GAMT and SLC6A8 patterns, as described here in rat CNS, are also true in human, a normal or only attenuated Cr peak should be detected in brain regions of SLC6A8-deficient patients presenting high proportions of cells co-expressing AGAT + GAMT, like hippocampus, hypothalamus or Purkinje cells.

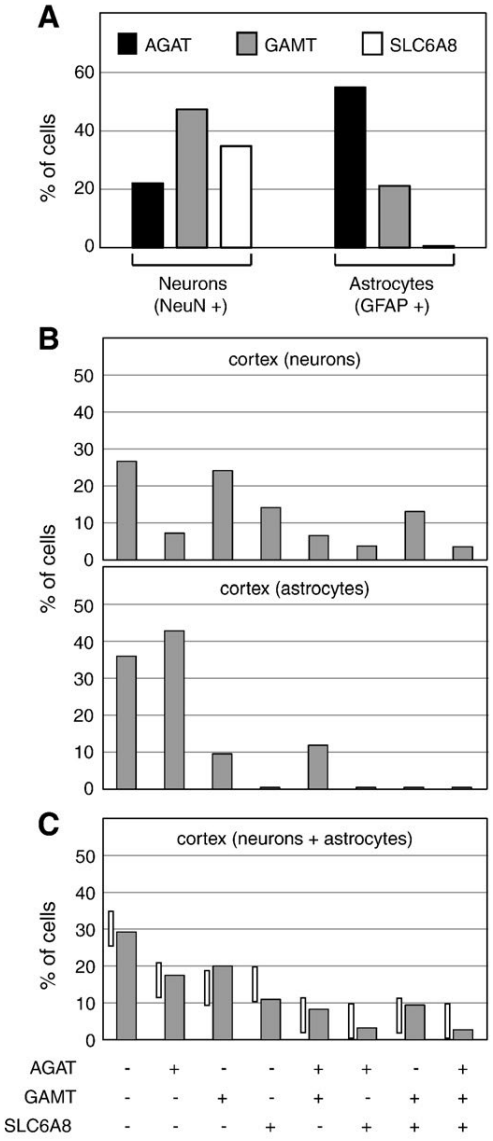

Fig. 5. Proportions of neurons and astrocytes expressing AGAT, GAMT and SLC6A8, respectively, within layer V of cortex. Cell counting as described in Materials and methods, from co-labeling experiments as shown in Fig. 4 (ISH for AGAT, GAMT or SLC6A8, coupled to immunohistochemistry for NeuN or GFAP to identify neurons and astrocytes, respectively). Data from a representative adult rat cortex; 6 adjacent sections observed for each co-labeling experiment; total number of NeuN-positive neurons: 441; total number of GFAP-positive astrocytes: 337. The cell percentages are based on the cumulative counting of the 6 adjacent sections, for each co-labeling experiment. (A) Proportions of cortical neurons, respectively astrocytes, expressing AGAT, GAMT or SLC6A8; (B) reconstitution of the "3 by 3" AGAT, GAMT and SLC6A8 expression pattern within cortical neurons and astrocytes; (C) reconstitution of the cumulative, neuronal and astrocytic, "3 by 3" AGAT, GAMT and SLC6A8 expression pattern, based on the ratio of 1 neuron per 0.4 astrocyte (Nedergaard et al., 2003). Measured ranges between minimal and maximal proportions (taken from Fig. 3A) are shown in white rectangles, for comparison.

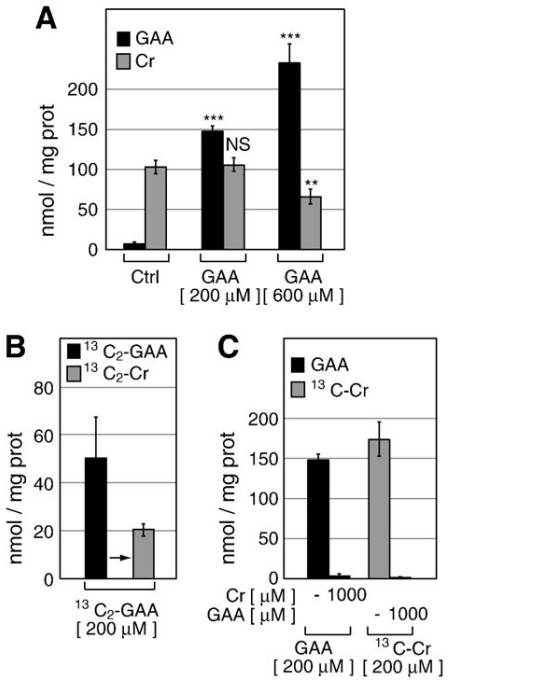

Fig. 6. Uptake of guanidinoacetate by brain cells, and conversion to creatine. Measure of intracellular GAA and Cr within brain cell 3D cultures (DIV13) by tandem mass spectrometry, after 12 h of exposure to GAA (GAA or $^{13}C_2$-GAA) and/or Cr (Cr or ^{13}C-Cr). (A) GAA Uptake by brain cells, and influence on endogenous Cr synthesis pathway. Cultures exposed for 12h to 200 and 600 µM GAA, respectively, and measure of intracellular GAA and Cr. (B) Conversion of GAA taken up by brain cells to Cr. Cultures exposed for 12 h to 200 µM $^{13}C_2$-GAA, and measure of intracellular $^{13}C_2$-GAA and $^{13}C_2$-Cr. (C) Competition of GAA uptake by Cr, respectively of Cr uptake by GAA, in brain cells. Cultures exposed 12 h to 200 µM GAA (± competition with 1000 µM Cr), respectively, 200 µM ^{13}C-Cr (± competition with 1000 µM GAA), and measure of intracellular GAA, respectively ^{13}C-Cr. A–C show representative experiments, with mean ± standard deviations for each point of measure. Significant increase in GAA, respectively decrease in Cr (A) are indicated, as compared to control conditions (200 µM GAA), respectively, to control conditions and 200 µM GAA (600 µM GAA). Student's t test; $n=3$; NS: not significant; $^{**}p<0.01$; $^{***}p<0.001$).

The striking patterns found for AGAT in pons and GAMT in caudate putamen are difficult to interpret. However, the GAMT-only expression of caudate putamen may fit with GAMT deficiency, which impairs striatum development (Von Figura et al., 2001).

GAA uptake by brain cells, and conversion to Cr

GAA competes for Cr uptake by SLC6A8 (Ohtsuki et al., 2002). Our work brings the first evidence that cells of brain parenchyma (neurons, astrocytes, oligodendrocytes) can take up GAA. GAA uptake was completely competed by 5-fold excess Cr, as efficiently as Cr uptake was competed by 5-fold excess GAA. This strongly suggests that GAA is taken up by brain cells through the same transporter as Cr: SLC6A8. This is in good agreement with SLC6A8 overexpression studies in HEK293 cells and *Xenopus* oocytes (Tachikawa et al., 2008). Finally, using $^{13}C_2$-GAA, our work is the first to demonstrate that GAA taken-up by brain cells is readily converted to Cr, in line with our hypothesis that for Cr synthesis to occur in CNS, GAA must be transported from AGAT- to GAMT-expressing cells. The low proportion of GAA conversion to Cr (29%) was due to the diluting effect of endogenous Cr synthesis pathway. One important question remaining to elucidate is how GAA can leave AGAT-expressing cells to be imported by GAMT-expressing cells through SLC6A8.

A recent work demonstrated GAA uptake by TR-CSFB cells (blood–CSF barrier model) and isolated choroid plexus *in vitro*, probably by SLC6A8, but concluded that due to GAA:Cr ratio in CSF (1:1000; 0.015–0.560 µM versus 17–90 µM in human; Braissant and Henry, 2008), SLC6A8 was unlikely to transport GAA in choroid plexus *in vivo* (Tachikawa et al., 2008). Cr and GAA concentrations are much higher in brain parenchyma (5–6 mM and 0.9–1.6 mM in human, respectively), but nothing is known on their concentrations in extracellular fluid. In rat brain parenchyma, Cr was measured at 8.5 mM (Renema et al., 2003), and we measured GAA at levels 5- to 10-fold lower than Cr in rat brain cell organotypic cultures (Braissant et al., 2008; and this study). We may thus extrapolate levels of GAA in rat brain parenchyma at

0.85–1.7 mM, comparable to human. 200 μM GAA, as used in this study, may thus be relevant in CNS extracellular fluid, at least locally, in vicinity of GAA transfer from AGAT- to GAMT-expressing cells.

AGAT and GAMT dissociation, as well as Cr and GAA uptake in CNS: A role for neuromodulation?

Dissociation of AGAT and GAMT activities between different brain cells may appear expensive for CNS in terms of both the energy and machinery needed to transfer GAA, and GAA toxicity to neurons if released in the extracellular space at too high concentrations. Cr is exocytotically released from central neurons (Almeida et al., 2006) and acts as partial agonist on central $GABA_A$ post-synaptic receptors (Koga et al., 2005), suggesting a role of co-transmitter modulating postsynaptic GABA receptors. On the other hand, GAA can activate $GABA_A$ receptors in cortical and cerebellar neurons (Neu et al., 2002; Cupello et al., 2008). In cortex in particular, GAA can activate $GABA_A$ receptors at concentrations compatible with its levels in CNS extracellular fluid (see above; and Neu et al., 2002; Braissant and Henry, 2008). Thus, both Cr and GAA can act as neuromodulators. Keeping AGAT and GAMT expression separated in different brain cells might thus facilitate the fine tuning of the respective synthesis of GAA and Cr for their specific roles of neuromodulation, while the efficient uptake of Cr and GAA by brain cells might be essential for keeping their extracellular concentrations in low ranges, to allow neuromodulation (Cr and GAA) while avoiding toxic effects of GAA.

Fine tuning versus high flux of Cr synthesis and transport in the brain

Two strikingly coherent main expression patterns are found in the different brain structures. In 1st one, present in most brain regions, high proportions of cells do not express either AGAT, GAMT or SLC6A8 (20–50%), the proportion of cells expressing SLC6A8 only (Cr "users") remains below 15%, while AGAT and GAMT are not co-expressed. These structures, distributed throughout the whole CNS, may need the dissociation between AGAT and GAMT expression, as well as the low proportion of cells expressing SLC6A8, for the fine tuning of GAA and Cr synthesis described above, and their respective roles in neuromodulation.

In 2nd one, specific structures (hippocampus, Purkinje neurons) present high proportions of cells co-expressing AGAT + GAMT (40–60%), and high proportions of cells expressing SLC6A8 (45–65%). It suggests that these neuronal layers permanently need high Cr levels, in line with their high creatine kinase activity (Wallimann and Hemmer, 1994; Kaldis et al., 1996).

Neuronal versus glial synthesis of creatine

While GAMT is expressed by rat neurons and glia (Braissant et al., 2001a), data showing GAMT predominantly (but not exclusively) in mouse glia (Schmidt et al., 2004; Tachikawa et al., 2004) suggested that Cr synthesis final step may principally be glial. Brain mixed-cell or astrocyte primary cultures synthesize Cr (Dringen et al., 1998; Braissant et al., 2008), but we also showed that neuron-enriched primary cultures synthesize Cr (Braissant et al., 2002). This study demonstrates that depending on brain structure, Cr synthesis may occur both in neurons and glia. In cortex, 48% of NeuN-positive neurons and 21% of GFAP-positive astrocytes expressed GAMT. CA3, dentate gyrus and Purkinje neurons were 50%–70% to express GAMT. Both neurons and astrocytes presented every possibility of AGAT, GAMT and SLC6A8 co-expression patterns (apart of SLC6A8 absence from astrocytes), and both neurons and astrocytes could be silent for the three genes. It is emphasized that oligodendrocytes, not evaluated in this study, highly express GAMT (Schmidt et al., 2004; Tachikawa et al., 2004; Braissant et al., 2008), and may be high Cr producers. It remains also to elucidate how Cr can leave "Cr producers" to be taken up by "Cr users" through SLC6A8.

Conclusion

We show that in numerous rat brain structures, including cortex and caudate putamen, AGAT and GAMT are expressed in a dissociated way, suggesting GAA transport from AGAT- to GAMT-expressing cells to allow Cr synthesis. Accordingly, this work is the first to demonstrate that brain cells can take up GAA probably through SLC6A8, and convert it to Cr. Taken together, our results explain the absence of Cr in the brain of SLC6A8-deficient patients, despite normal AGAT and GAMT expression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbd.2009.10.022.

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Annex 3: O.Braissant, H.Henry, E.Béard and J.Uldry , 2011

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REVIEW ARTICLE

Creatine deficiency syndromes and the importance of creatine synthesis in the brain

Olivier Braissant · Hugues Henry · Elidie Béard · Joséphine Uldry

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Abstract Creatine deficiency syndromes, due to deficiencies in AGAT, GAMT (creatine synthesis pathway) or SLC6A8 (creatine transporter), lead to complete absence or very strong decrease of creatine in CNS as measured by magnetic resonance spectroscopy. Brain is the main organ affected in creatine-deficient patients, who show severe neurodevelopmental delay and present neurological symptoms in early infancy. AGAT- and GAMT-deficient patients can be treated by oral creatine supplementation which improves their neurological status, while this treatment is inefficient on SLC6A8-deficient patients. While it has long been thought that most, if not all, of brain creatine was of peripheral origin, the past years have brought evidence that creatine can cross blood–brain barrier, however, only with poor efficiency, and that CNS must ensure parts of its creatine needs by its own endogenous synthesis. Moreover, we showed very recently that in many brain structures, including cortex and basal ganglia, AGAT and GAMT, while found in every brain cell types, are not co-expressed but are rather expressed in a dissociated way. This suggests that to allow creatine synthesis in these structures, guanidinoacetate must be transported from AGAT- to GAMT-expressing cells, most probably through SLC6A8. This new understanding of creatine metabolism and transport in CNS will not only allow a better comprehension of brain consequences of creatine deficiency syndromes, but will also contribute to better decipher creatine roles in CNS, not only in energy as ATP regeneration and buffering, but also in its recently suggested functions as neurotransmitter or osmolyte.

Keywords Creatine deficiency syndromes · Creatine · Guanidinoacetate · Brain · AGAT · GAMT · SLC6A8

O. Braissant (✉) · H. Henry · E. Béard · J. Uldry
Inborn Errors of Metabolism, Clinical Chemistry Laboratory, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Avenue Pierre-Decker 2, CI 02/33, 1011 Lausanne, Switzerland
e-mail: Olivier.Braissant@chuv.ch

Introduction

The creatine (Cr)/phosphocreatine (PCr)/creatine kinase (CK) system plays essential roles in maintaining the high energy levels necessary for brain development and functions, through regeneration and buffering of ATP levels (Wallimann et al. 1992, 2007; Wyss and Kaddurah-Daouk 2000; Brosnan and Brosnan 2007; Andres et al. 2008). Recent works suggest that creatine in CNS may also act as true neurotransmitter and one of the main CNS osmolytes (Bothwell et al. 2002; Almeida et al. 2006). In mammals, half of Cr is obtained from diet, the other half being synthesized endogenously by a two-step mechanism involving L-arginine:glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT). Cr is distributed by blood to tissues and taken up by cells through a specific Cr transporter, SLC6A8, also abbreviated CT1, CRTR, CTR or CreaT (Wyss and Kaddurah-Daouk 2000).

Cr deficiency syndromes are caused by mutations in AGAT, GAMT and SLC6A8 genes (Stöckler et al. 1994; Salomons et al. 2001; Item et al. 2001). Their common phenotype is an almost complete lack of Cr in CNS, as measured by magnetic resonance spectroscopy (MRS), CNS appearing as the main organ affected in these primary Cr deficiencies. Patients develop severe neurodevelopmental delay and present neurological symptoms in early infancy, such as mental retardation, delays in speech acquisition or epilepsy (Stöckler et al. 2007). Oral Cr

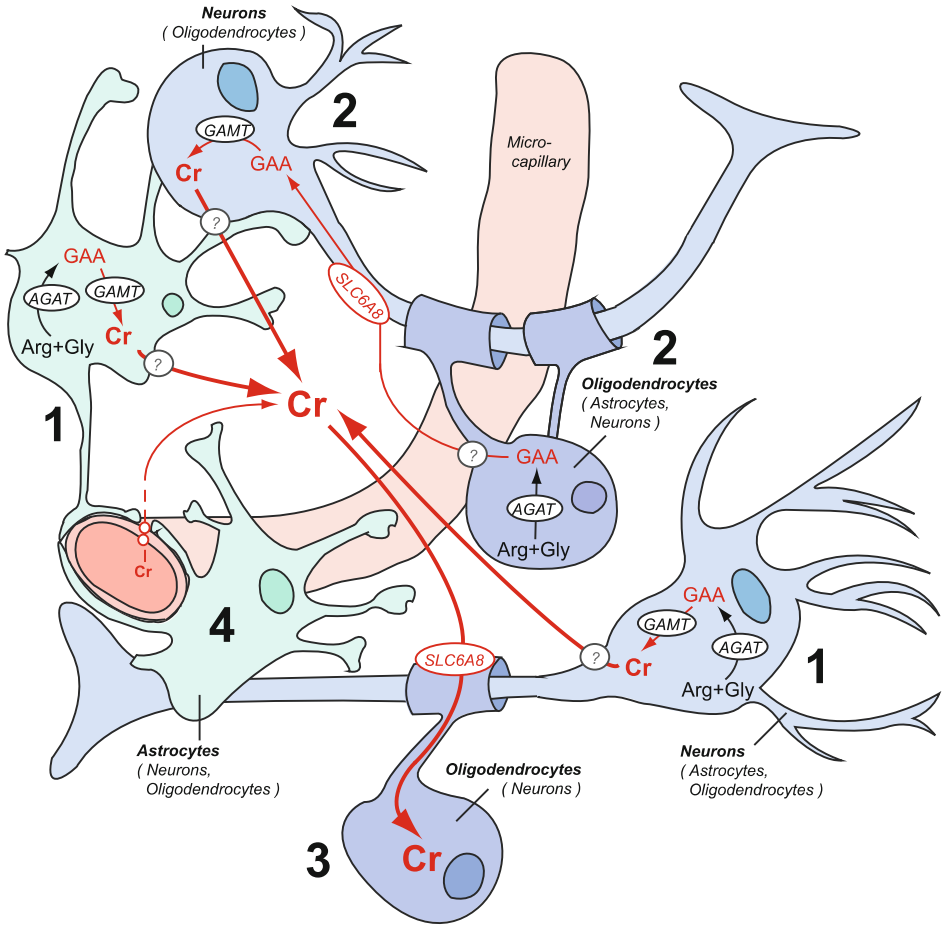

Fig. 1 Creatine synthesis and transport in central nervous system: model illustrating endogenous synthesis versus uptake from periphery, including the various combinations of AGAT, GAMT and SLC6A8 expression within brain cells (Braissant et al. 2010). (*1*) Cr endogenous synthesis within cells co-expressing AGAT and GAMT. (*2*) Cr endogenous synthesis through AGAT-expressing cells synthesizing GAA, and GAA uptake by SLC6A8 in GAMT-expressing cells; this conformation appears the prevalent one in most brain regions. (*3*) Cells expressing only SLC6A8 ("users" of Cr). (*4*) Cells silent for AGAT, GAMT and SLC6A8. While microcapillaries express SLC6A8, astrocytic feet lining them do not. This implies that only low amounts of peripheral Cr can enter the brain through the limited endothelial surface that is free of astrocytic feet and that CNS must also ensure its own endogenous synthesis of Cr. So far, the way Cr (and GAA) can leave cells is poorly known. Cr: creatine; AGAT: L-arginine:glycine amidinotransferase; GAMT: guanidinoacetate methyltransferase; GAA: guanidinoacetate; SLC6A8: Cr transporter

supplementation strongly improves the neurological status of AGAT- and GAMT-deficient patients (Stöckler et al. 1996; Schulze et al. 1998; Battini et al. 2002; Schulze and Battini 2007), while this treatment is inefficient on SLC6A8-deficient patients (Bizzi et al. 2002; Póo-Argüelles et al. 2006; Arias et al. 2007). Secondary Cr deficiencies are also observed in other pathological states of the brain, like stroke, hyperammonemic states or gyrate atrophy of the choroid and retina (GA) (Valle et al. 1981; Braissant et al. 2008; Lei et al. 2009).

It has long been thought that most, if not all, cerebral Cr was of peripheral origin (Wyss and Kaddurah-Daouk 2000). However, AGAT and GAMT are expressed in CNS and brain cells synthesize their own Cr (Braissant et al. 2001b; Braissant et al. 2002). In contrast, while SLC6A8 is expressed by microcapillary endothelial cells (MCEC) at blood–brain barrier (BBB), allowing CNS to import Cr from periphery, it is absent from astrocytes, at least in physiological conditions, and particularly from their feet lining MCEC (Fig. 1; Braissant et al. 2001b; Ohtsuki et al. 2002; Tachikawa et al. 2004). This made us suggest that BBB has a limited permeability for peripheral Cr and that CNS must supply an important part of its Cr needs by endogenous synthesis rather than on an exclusive supply from the blood (Braissant et al. 2001b; Braissant and Henry 2008). Similar to periphery with predominant expression of AGAT in kidney and GAMT in liver (Edison et al. 2007; da Silva et al. 2009), recent data also suggest that the Cr synthesis pathway may be dissociated in CNS, guanidinoacetate (GAA) being transported through SLC6A8 from

AGAT- to GAMT-expressing cells to allow synthesis of Cr in the brain (Braissant et al. 2010).

This review is focused on Cr deficiency syndromes and their effects on the brain in view of the latest data on Cr synthesis and transport in CNS, in order to delineate a comprehensive frame on Cr metabolism and transport in CNS, both in normal and Cr-deficient conditions.

Creatine deficiency syndromes

CNS is the main organ affected in patients suffering from the Cr deficiency syndromes, inborn errors of Cr biosynthesis and transport caused by AGAT, GAMT or SLC6A8 deficiency which are characterized by an absence or a severe decrease of Cr in CNS as measured by MRS (Stöckler et al. 1994; Salomons et al. 2001; Item et al. 2001). AGAT and GAMT deficiencies are autosomal recessive diseases, while SLC6A8 deficiency is an X-linked disorder. Cr deficiency syndromes appear among the most frequent inborn errors of metabolism (IEM), the prevalence of SLC6A8 deficiency being estimated at 2% of all X-linked mental retardations (Rosenberg et al. 2004) and at 1% of males with mental retardation of unknown etiology (Clark et al. 2006). AGAT and GAMT deficiencies are rarer, but the prevalence of all combined Cr deficiencies was estimated between 0.3% and 2.7% of all mental retardation (Lion-François et al. 2006; Arias et al. 2007).

Cr-deficient patients present neurological symptoms in infancy (Schulze et al. 1997; Battini et al. 2002; DeGrauw et al. 2002). In particular, mental retardation and delays in speech acquisition can be observed (AGAT, GAMT and SLC6A8 deficiencies), as well as intractable epilepsy (GAMT and SLC6A8 deficiencies), autism, automutilating behavior, extrapyramidal syndrome and hypotonia (GAMT deficiency) (Stöckler et al. 2007). The diverse phenotypic spectrum of neurological symptoms observed in AGAT-, GAMT- and SLC6A8-deficient patients shows the importance of Cr for psychomotor development and cognitive functions and is probably explained by the wide pattern of AGAT, GAMT and SLC6A8 expression in the mammalian brain (see below). The recently proposed roles of Cr as co-transmitter on GABA postsynaptic receptors (Almeida et al. 2006) and of regulator of appetite and weight on specific hypothalamic nuclei (Galbraith et al. 2006) might also contribute to this phenotypic diversity. The more complex phenotype of GAMT deficiency, including intractable epilepsy, extrapyramidal movement syndromes and abnormalities in basal ganglia is probably due to the toxicity, and particularly the epileptogenic action, of brain GAA accumulation characteristic of GAMT deficiency (Schulze et al. 2001), which may occur through activation of $GABA_A$ receptors by GAA (Neu et al. 2002). GAA may also inhibit the complex between Na^+/K^+-ATPase and CK (Zugno et al. 2006). Severe epilepsy may also appear in SLC6A8-deficient patients (Mancardi et al. 2007). This may be due to the observed CNS GAA accumulation in some SLC6A8-deficient patients (Sijens et al. 2005), which could be caused by impairment of GAA transport through deficient SLC6A8, from AGAT- to GAMT-expressing brain cells (see below) (Braissant et al. 2010).

Treatments and outcome of Cr deficiency syndromes

AGAT- and GAMT-deficient patients can be treated by oral supplementation of Cr. While this strongly improves their neurological status and CNS development, very high doses of Cr must be used, and replenishment of cerebral Cr takes months and only results, in most cases, in partial restoration of cerebral Cr pools (Stöckler et al. 1996; Ganesan et al. 1997; Item et al. 2001; Battini et al. 2002). For GAMT-deficient patients, combined arginine restriction and ornithine substitution coupled with Cr treatment decreases GAA and also improves clinical outcome (Schulze et al. 1998, 2001). Despite improvement of clinical outcome by Cr supplementation, many AGAT- and GAMT-deficient patients remain with CNS developmental problems. However, the pre-symptomatic treatment of AGAT- and GAMT-deficient patients appears to prevent, so far, most of the Cr-deficiency effects on their brain (Battini et al. 2006; Schulze et al. 2006; Schulze and Battini 2007). Oral supplementation of Cr is inefficient in replenishing brain Cr in SLC6A8-deficient patients, who remain with mental retardation, severe speech impairment and progressive brain atrophy (Cecil et al. 2001; Bizzi et al. 2002; DeGrauw et al. 2002; Póo-Argüelles et al. 2006). Attempts to treat SLC6A8-deficient patients with arginine as precursor of Cr also failed to improve their neurological status (Fons et al. 2008). Similarly, the use of a lipophilic Cr-derived compound, creatine ethyl ester, failed to replenish brain Cr concentration in SLC6A8-deficient patients, as well as to improve their neurological status (Fons et al. 2010).

Secondary creatine deficiencies in CNS

Several other CNS pathologies that cause a secondary Cr deficiency in brain cells have been identified. Excess of ammonium (NH_4^+) is toxic for CNS. In pediatric patients, hyperammonemia can be caused by various acquired or inherited disorders, the most frequent being urea cycle diseases, which can cause irreversible damages to the developing brain (Leonard and Morris 2002) by altering

several amino acid pathways and neurotransmitter systems, nitric oxide synthesis, axonal and dendritic growth and signal transduction pathways (Cagnon and Braissant 2007, 2008, 2009) eventually leading to energy deficit, oxidative stress and cell death (Braissant 2010b). In particular, NH_4^+ exposure generates a secondary Cr deficiency in brain cells (Ratnakumari et al. 1996; Choi and Yoo 2001; Braissant et al. 2002). NH_4^+ appears to inhibit AGAT enzymatic activity and differentially alters AGAT, GAMT and SLC6A8 gene expression in a cell type-specific manner, which may alter the energy requirements of brain cells (Braissant et al. 2008; Braissant 2010a). Ischemic stroke in CNS leads to a rapid diminution in brain total Cr (Cr + PCr) (Obrenovitch et al. 1988; Gideon et al. 1992; Mathews et al. 1995; Lei et al. 2009). This lower Cr level in stroke causes a decrease in high-energy phosphate production and leads to a failure in most energy-dependent processes necessary for cell survival. Gyrate atrophy of the choroid and retina (GA) is an inborn error of metabolism leading to blindness in the first 10 years of life and is caused by mutations in ornithine δ-aminotransferase (OAT) (Valle et al. 1981). OAT deficiency generates a secondary Cr deficiency, as it generates an important ornithine accumulation facilitating the reversed AGAT reaction, therefore depleting GAA for Cr synthesis (Sipilä 1980). This is particularly true in CNS, where GA neurological symptoms may be related to this secondary Cr deficiency (Näntö-Salonen et al. 1999; Valayannopoulos et al. 2009).

AGAT, GAMT and SLC6A8 in CNS

Cr is synthesized in the mammalian brain (Pisano et al. 1963; Van Pilsum et al. 1972), in nerve cell lines as well as in primary and organotypic brain cell cultures (Daly 1985; Dringen et al. 1998; Braissant et al. 2002). AGAT and GAMT are expressed in CNS, where they are found in all the main structures of the brain, in every main cell types (neurons, astrocytes and oligodendrocytes, Fig. 1; Braissant et al. 2001b; Tachikawa et al. 2004; Schmidt et al. 2004; Nakashima et al. 2005). Moreover, we have shown very recently that in most regions of the rat brain, AGAT and GAMT are rarely co-expressed within the same cell (see below; Braissant et al. 2010). Organotypic rat cortical cultures, primary brain cell cultures (neuronal, glial or mixed) and neuroblastoma cell lines have a Cr transporter activity (Möller and Hamprecht 1989; Almeida et al. 2006; Braissant et al. 2008). In vivo, mouse and rat CNS can take up Cr from the blood against its concentration gradient (Ohtsuki et al. 2002; Perasso et al. 2003). SLC6A8 is expressed throughout the main regions of adult mammalian brain (Schloss et al. 1994; Happe and Murrin 1995; Braissant et al. 2001b; Tachikawa et al. 2008; Mak et al. 2009). In the first detailed analyses of Cr transporter expression in CNS, it was demonstrated that SLC6A8 is found in neurons and oligodendrocytes but, in contrast to AGAT and GAMT, cannot be detected in astrocytes (Fig. 1; Braissant et al. 2001b), except for very rare ones in cerebellum (Mak et al. 2009). This holds true also for the retina, where SLC6A8 is expressed in retinal neurons, but not in astrocytes (Nakashima et al. 2004; Acosta et al. 2005). In contrast to its absence in astrocytes lining microcapillaries, SLC6A8 is present in MCEC making BBB (Braissant et al. 2001b; Ohtsuki et al. 2002; Tachikawa et al. 2004).

It must be emphasized also that AGAT, GAMT and SLC6A8 are expressed very differently by brain cells, depending on whether the analysis is made in vivo or in vitro, as well as in vitro depending on the type of cultures. 20 years ago, an important paper by Möller and Hamprecht showed a detailed description of the in vitro Cr uptake capacity of numerous types of primary brain cells, as well as immortalized cell lines, all cultured in the presence of serum. They concluded that astrocytes have the highest activity of Cr transporter (Möller and Hamprecht 1989). We showed that in vivo AGAT and GAMT can be found in all brain cell types, while, in contrast to what was demonstrated in primary cultures of astrocytes, SLC6A8 is not expressed by astrocytes (Braissant et al. 2001b). We further demonstrated that organotypic cultures such as brain 3D mixed-cell aggregates cultured in the absence of serum synthesize their own Cr and express AGAT, GAMT and SLC6A8 as the in vivo CNS, including the absence of SLC6A8 from astrocytes (Braissant et al. 2008). In contrast, when we analyzed brain 3D neuron-enriched aggregates also cultured in the absence of serum, from which astrocytes and oligodendrocytes have been eliminated, we showed that in the absence of glial cells, AGAT and GAMT are totally silent from neurons and from the very few remaining astrocytes, while both genes are well expressed in both cell types when glial cells are present (Braissant et al. 2008). It is known that brain cells cultured as 2D monolayers or in the presence of serum develop a pathological reactive state that completely alters their behavior in terms of gene expression and protein activities. This is particularly true for astrocytes, which develop constant reactive gliosis as illustrated by their increase in GFAP and vimentin expression as compared with in vivo conditions (Langan and Slater 1992; F.Tschudi-Monnet and P.Honegger, personal communication; and unpublished results). We have shown that SLC6A8, which is silent in astrocytes in vivo as well as in 3D brain cells aggregates cultured in the absence of serum, is activated in these same astrocytes when they are placed in a reactive state (in that case exposure to ammonium; Cagnon and

Braissant 2007; Braissant et al. 2008; Braissant 2010b). It appears, thus, that most models of cultured brain cells, in particular when cultured in the presence of serum, do not behave as the in vivo CNS for Cr metabolism and transport. Thus, to analyze Cr in brain cells in conditions as near as possible of the in vivo CNS, complex 3D, organotypic and mixed-cell culture systems in the absence of serum should be used (Braissant 2010a).

Creatine in CNS: endogenous synthesis versus uptake from periphery?

The in vivo expression of AGAT and GAMT within mammalian CNS, as well as the in vitro endogenous synthesis of Cr by various types of cultured brain cells, including primary and organotypic cultures, suggest that brain synthesizes Cr (Braissant et al. 2007). However, it was thought for a long time that most, if not all, of the Cr needed by CNS comes from periphery through BBB (Wyss and Kaddurah-Daouk 2000).

The discovery that SLC6A8 cannot be detected in astrocytes, particularly in their feet sheathing MCEC made us suggest, however, that in mature CNS, BBB has a limited permeability for Cr, despite SLC6A8 expression by MCEC and their capacity to import Cr (Braissant et al. 2001b; Ohtsuki et al. 2002; Tachikawa et al. 2004; Nakashima et al. 2004; Acosta et al. 2005). In vivo data confirmed this hypothesis: the blood to brain transport of Cr through BBB is effective in rats and mice but is relatively inefficient (Ohtsuki et al. 2002; Perasso et al. 2003), and long-term treatment of AGAT- and GAMT-deficient patients with high doses of Cr allows only a slow and in most cases partial replenishment of their brain Cr pools (Stöckler et al. 2007; Schulze and Battini 2007). Similarly, $GAMT^{-/-}$ KO mice treated with high doses of Cr replenish their brain Cr, but only slowly (Kan et al. 2007). The effective but limited entry of Cr from blood to CNS through MCEC but without going through astrocytes may occur through the limited surface of CNS microcapillary endothelium that is free of astrocytic feet (Fig. 1; Virgintino et al. 1997; Ohtsuki 2004).

One strong argument in favor of the "brain endogenous Cr synthesis" hypothesis comes from Cr measures in the CSF of Cr-deficient patients (see Braissant and Henry 2008, and references therein). SLC6A8-deficient patients present normal Cr levels in CSF, but are strictly unable to import Cr from periphery (Cecil et al. 2001; DeGrauw et al. 2002; Bizzi et al. 2002; Póo-Argüelles et al. 2006). In contrast, GAMT-deficient patients show strongly decreased levels of Cr in CSF but are able to import Cr from the blood (Stöckler et al. 1994; Schulze et al. 1997). This also suggests that Cr synthesis in the brain might still remain operational, although very partially, under SLC6A8 deficiency, while it is completely blocked in AGAT and GAMT deficiencies. Endogenous synthesis or a very efficient uptake from the periphery are the two ways available for the brain to secure Cr homeostasis for its energy and functions. As uptake from the periphery does not appear efficient, CNS might privilege Cr endogenous synthesis. The brain capacity for Cr synthesis would thus depend on the efficient supply of arginine, the limiting substrate for Cr synthesis, from blood to CNS, and then also on local trafficking of arginine between brain cells. We and others have shown that cationic amino acid transporters (CATs) might fulfill these roles in the brain, as CAT1 is expressed in MCEC as well as ubiquitously in neuronal and glial cells, as CAT2(B) is present in neurons and oligodendrocytes, and as CAT3 is restricted to neurons (Braissant et al. 1999; Hosokawa et al. 1999; Braissant et al. 2001a).

The hypothesis of endogenous Cr synthesis in the brain might seem to contradict the in vivo characteristics of SLC6A8 deficiency, which, despite AGAT and GAMT expression in CNS, presents an absence (or a very low level) of brain Cr by MRS (Salomons et al. 2001). This apparent contradiction is probably explained by our very recent data on AGAT, GAMT and SLC6A8 expression patterns in the brain. AGAT and GAMT are found in every CNS cell type (Braissant et al. 2001b), but appear rarely co-expressed within the same cell (Braissant et al. 2010). This suggests that to allow Cr synthesis in the brain, GAA must be transported from AGAT- to GAMT-expressing cells (Fig. 1). This GAA transfer most probably occurs through SLC6A8, as shown in the same study by Cr and GAA competition studies and the use of stable isotope-labeled GAA demonstrating its uptake by brain cells followed by its conversion to Cr by GAMT activity (Braissant et al. 2010). These observations may explain the absence of Cr in CNS of SLC6A8-deficient patient, despite normal expression of AGAT and GAMT in their brain (Braissant and Henry 2008), as well as the lack of effect of treatment of SLC6A8-deficient patients with arginine as a precursor of Cr (Fons et al. 2008). Recent studies also demonstrated the potential role of SLC6A8 (and taurine transporter) for GAA transport across BBB and in brain parenchymal cells (Tachikawa et al. 2008, 2009).

While we have shown that AGAT and GAMT can be found in all brain cell types (Braissant et al. 2001b), other studies demonstrated particularly high levels of GAMT in glial cells (Schmidt et al. 2004; Tachikawa et al. 2004; Braissant et al. 2008), suggesting that the final CNS step for Cr synthesis may predominantly be glial. However, this probably depends on the brain region considered, as in cortex only 20% of astrocytes express GAMT in comparison with 48% of neurons (Braissant et al. 2010).

Adult versus developmental CNS

As described above, the adult (or mature) brain might privilege Cr endogenous synthesis versus uptake from periphery, due to low permeability of BBB for Cr and the expression of AGAT and GAMT in CNS parenchyma. Fetal and perinatal (or immature) CNS probably behaves differently for its Cr needs.

The Cr/PCr/CK system plays essential roles in energy homeostasis during vertebrate embryonic development (Wallimann et al. 1992), the fetal needs in Cr being partly supported by active transport of Cr from mother to fetus (Davis et al. 1978; Ireland et al. 2008). On the other hand, AGAT, GAMT and SLC6A8 are also well expressed during vertebrate embryogenesis, including in the brain (Schloss et al. 1994; Sandell et al. 2003; Schmidt et al. 2004; Braissant et al. 2005; Wang et al. 2007; Ireland et al. 2009). We have shown that AGAT and GAMT are expressed in the whole developing CNS parenchyma (Braissant et al. 2005). However, their low level (GAMT in particular) at early developmental stages suggests that in contrast to adult brain, embryonic CNS depends predominantly on external Cr supply, be it from embryonic periphery or from maternal origin. This is coherent with SLC6A8 expression in the whole embryonic CNS already at early stages (E12.5 in rat), with particularly high levels in the periventricular zone and choroid plexus, the predominant metabolic exchange zones of fetal CNS before microcapillary angiogenesis and differentiation of BBB (Braissant et al. 2005, 2007).

Creatine and guanidinoacetate within normal versus creatine-deficient CNS

In normal conditions, Cr within human CSF is maintained in the 17–90 μM range, while GAA is maintained at a 1000× lower level (0.015–0.114 μM). By MRS, total Cr is measured between 5.5 and 6.4 mM in the cortical gray matter, while GAA was estimated to 1.6 mM (see Braissant and Henry 2008, and references therein).

With the exception of SLC6A8-deficient heterozygous females where brain Cr deficiency is partial (Cecil et al. 2003), all three Cr deficiencies present the virtual absence of the Cr peak measured by MRS in cortex and basal ganglia (Stöckler et al. 2007). However, despite this lack of Cr detection by MRS, Cr remains present within the brain of Cr-deficient patients. In SLC6A8 deficiency, Cr CSF levels do not differ from age-matched controls (Cecil et al. 2001; Salomons et al. 2001; DeGrauw et al. 2002). In AGAT deficiency, total Cr levels in cortical gray matter are decreased to 12% of age-matched controls (Battini et al. 2002). In GAMT deficiency, CSF Cr levels are strongly decreased (<2 μM) (Schulze et al. 1997, 2003; Ensenauer et al. 2004), while in cortical gray matter total Cr was measured in the 0.2-1.5mM range (Stöckler et al. 1994; Mancini et al. 2005).

GAA accumulation in body fluids is characteristic of GAMT deficiency, where its toxicity is responsible for the more complex and specific phenotype of GAMT-deficiency (see above). GAA CSF levels in GAMT-deficient patients are 60–1000× higher than in age-matched controls, while GAA was estimated to 3.6 mM within cortical gray matter. No precise data are available on GAA levels within AGAT- and SLC6A8-deficient CNS, but it was shown that GAA can also accumulate in the brain of SLC6A8-deficient patients (Sijens et al. 2005). As described above, GAA may appear as a key intermediate player for endogenous Cr synthesis in CNS, as it must most probably be transported from AGAT- to GAMT-expressing cells for Cr synthesis to occur (Braissant et al. 2010), just as it does in periphery between AGAT in kidney and GAMT in liver (Edison et al. 2007; da Silva et al. 2009). It was recently demonstrated that both BBB endothelial cells (Tachikawa et al. 2009) and CNS parenchymal cells (Tachikawa et al. 2008; Braissant et al. 2010) are able to take up GAA by SLC6A8. The Km value of SLC6A8 for GAA appears ten times lower than that for Cr (Tachikawa et al. 2008). Thus, entry of GAA into CNS in normal conditions must be inhibited by blood Cr levels (1–3.5 μM for GAA versus 6–50 μM for Cr). This entry might, however, be facilitated under GAMT deficiency, blood GAA levels becoming higher than Cr levels (12–39 μM for GAA versus 1–5 μM for Cr) (Almeida et al. 2004), therefore contributing to GAA accumulation into the GAMT-deficient brain.

Models for creatine synthesis and trafficking in CNS

Taken together, (i) the absence of Cr within the brain of Cr-deficient patients, (ii) the CNS expression patterns of AGAT, GAMT and SLC6A8, (iii) the low permeability of BBB for Cr and (iv) the measures of Cr and GAA within CNS both in normal and Cr-deficient conditions, lead us to propose the following model to understand Cr synthesis and trafficking within the brain (Fig. 1) (Braissant and Henry 2008):

In normal conditions, SLC6A8 is expressed by MCEC, but not by the surrounding astrocytic feet, implying that limited amounts of Cr can enter the brain through BBB. In most brain regions (including cortex and basal ganglia, where most MRS measures are performed), brain cells express AGAT and GAMT in a cell-dissociated way, and GAA must be transported from AGAT- to GAMT-expressing cells by SLC6A8 for Cr synthesis to occur. In AGAT and GAMT deficiency, no Cr can be synthesized

within CNS, but SLC6A8 expression in MCEC allows the limited entry of Cr within the brain and thus their treatment by oral Cr and the partial replenishment of the brain Cr pools. Moreover, the brain of GAMT-deficient patients accumulates GAA. Cr transporter-deficient patients lack functional SLC6A8 on MCEC and thus cannot be treated by oral Cr. Moreover, their endogenous CNS Cr synthesis pathway is also deficient, as in most brain regions, GAA cannot cross from AGAT- to GAMT-expressing cells due to their lack in functional SLC6A8.

Conclusion

Brain is the main organ affected in Cr deficiency syndromes due to deficiencies in AGAT, GAMT or SLC6A8, which lead to a complete absence or a very strong decrease of Cr in CNS. AGAT- and GAMT-deficient patients can be treated by oral creatine supplementation which improves their neurological status, while this treatment is inefficient on SLC6A8-deficient patients. The recent years have brought new knowledge on Cr metabolism and transport in the brain, allowing a better understanding on the pathophysiology of Cr deficiency syndromes in brain cells. In particular, there is evidence that BBB presents a low permeability for Cr and that CNS must ensure parts of its needs in Cr by endogenous synthesis. Moreover, in many regions of the brain, Cr endogenous synthesis appears to be dissociated, GAA needing to be transported by SLC6A8 from AGAT- to GAMT-expressing cells for Cr synthesis to occur. This probably explains why, despite AGAT and GAMT expression in their brain, SLC6A8-deficient patients remain with a Cr-depleted CNS.

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Creatine, central nervous system and creatine deficiency syndromes.

Creatine, central nervous system and creatine deficiency syndromes

OLIVIER BRAISSANT, JOSÉPHINE ULDRY, ELIDIE BÉARD
Inborn Errors of Metabolism, Clinical Chemistry Laboratory
Centre Hospitalier Universitaire Vaudois and University of Lausanne
Avenue Pierre-Decker 2, 1011 – Lausanne
SWITZERLAND
Olivier.Braissant@chuv.ch http://www.unil.ch/fbm/page21581.html

Abstract: It was long thought that most of brain creatine was of peripheral origin. However, recent works have demonstrated that creatine crosses blood-brain barrier only with poor efficiency, and that CNS must ensure parts of its creatine needs by its own creatine synthesis pathway, thank to the brain expression of AGAT and GAMT (creatine synthesis) and SLC6A8 (creatine transporter). This new understanding of creatine metabolism and transport in CNS allows a better comprehension of creatine deficiency syndromes, which are due to deficiencies in AGAT, GAMT and SLC6A8 and mainly affect the brain of patients who show severe neurodevelopmental delay and present neurological symptoms in early infancy.

Keywords: Creatine, brain, AGAT, GAMT, SLC6A8, guanidinoacetate, creatine deficiency syndromes.

1 Introduction

The creatine (Cr) / phosphocreatine (PCr) / creatine kinase (CK) system plays essential roles to maintain the high energy levels necessary for brain development and functions, through regeneration and buffering of ATP levels [21,72]. The Cr/PCr/CK system also allows the shuttle of high-energy phosphates from mitochondria to their cytoplasmic sites of utilization [70]. Cr was also suggested recently as true neurotransmitter and one of the main central nervous system (CNS) osmolytes [3,9]. In mammals, pools of Cr are maintained through uptake from diet and endogenous synthesis. Cr biosynthesis involves L-arginine:glycine amidinotransferase (AGAT) yielding guanidinoacetate (GAA) from arginine and glycine, and guanidinoacetate methyl-transferase (GAMT), yielding Cr from GAA. Cr is distributed by blood to tissues, where cells take it up by a specific transporter, SLC6A8, also called CRT1, CT1, CreaT or CRT [72].

It has long been thought that most, if not all, cerebral Cr was of peripheral origin [72]. However, AGAT and GAMT are expressed in CNS and brain cells synthesize their own Cr [11,20]. In contrast, while SLC6A8 is expressed by microcapillary endothelial cells (MCEC) at blood-brain barrier (BBB), allowing CNS to import Cr from periphery, it is absent from astrocytes and particularly from their feet lining MCEC [11,47,65]. This suggested that BBB has a limited permeability for peripheral Cr, and that CNS must supply an important part of its Cr needs by endogenous synthesis rather than on exclusive supply from the blood [6,11,18]. Recent data also suggest that in the brain, the Cr synthesis pathway may be dissociated as it is in periphery, the intermediate GAA being transported through SLC6A8 from AGAT- to GAMT-expressing cells for Cr synthesis to occur in CNS [15].

Cr deficiency syndromes are caused by mutations in AGAT, GAMT and SLC6A8 genes [38,53,62]. Their common phenotype is an almost complete lack of Cr in CNS, which appears as the main organ affected in these primary Cr deficiencies. Patients develop severe neurodevelopmental delay and present neurological symptoms in early infancy, like mental retardation, delays in speech acquisition or epilepsy [63]. Oral Cr supplementation strongly improves the neurological status of AGAT- and GAMT-deficient patients [5,55,58,61], while this treatment is inefficient on SLC6A8-deficient patients [4,7,51]. Secondary Cr deficiencies are also observed in other CNS pathological states, like stroke, hyperammonemic states or gyrate atrophy of the choroid and retina (GA) [6].

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This review is focused on the latest data on Cr synthesis and transport in CNS, in order to delineate a comprehensive frame on Cr metabolism and transport in the brain, both in normal and in the Cr-deficient conditions characteristic of Cr deficiency syndromes.

2 Creatine in the brain

2.1 Functions of creatine in CNS

The Cr/PCr/CK system plays essential roles to maintain the high energy phosphates levels necessary for CNS (maintenance of membrane potential and ions gradients, Ca^{++} homeostasis, neurotransmission, intracellular signaling systems as well as axonal and dendritic transport, axonal and dendritic growth) [6]. Apart of its main functions in energy, Cr was recently suggested as neurotransmitter or neuromodulator. Indeed, neurons can release Cr in an action potential-dependent manner [3], and a mechanism of Cr recapture from the synaptic cleft may exist through SLC6A8 [48]. Cr was also suggested as one of the essential CNS osmolytes [2,8], and as one potential appetite and weight regulator through action on specific hypothalamic nuclei [32].

2.2 AGAT, GAMT and SLC6A8 in CNS

It has long been thought that most of brain Cr was of peripheral origin, be it taken from the diet or synthesized endogenously through AGAT and GAMT activities in kidney and liver respectively [21,72]. However, Cr is synthesized in the mammalian brain [50,69], in nerve cell lines as well as in primary and organotypic brain cell cultures [20,27,30]. AGAT and GAMT are expressed in all the main structures of the brain, in every main cell types (neurons, astrocytes and oligodendrocytes; [11,54,65]). Moreover, we have shown that in most region of the rat CNS, AGAT and GAMT rarely appear co-expressed within the same cell [15]. Organotypic rat cortical cultures, primary brain cell cultures (neuronal, glial or mixed) and neuroblastoma cell lines have a Cr transporter activity [3,16,44]. *In vivo*, mouse and rat CNS can take up Cr from the blood against its concentration gradient [47,49]. SLC6A8 is expressed throughout the main regions of the adult mammalian brain [11,42,65]. It was demonstrated that SLC6A8 is found in neurons and oligodendrocytes but, in contrast to AGAT and GAMT, cannot be detected in astrocytes [11], except for very rare ones in cerebellum [42]. In contrast to its absence in astrocytes lining microcapillaries, SLC6A8 is present in MCEC making BBB [11,47,65].

2.3 Brain creatine: endogenous synthesis or uptake from periphery?

The discovery that SLC6A8 cannot be detected in astrocytes, particularly in their feet sheathing MCEC, made us suggest that in mature CNS, BBB has a limited permeability for Cr, despite SLC6A8 expression by MCEC and their capacity to import Cr [1,11,45,47,65]. *In vivo* data confirmed this hypothesis: the blood to brain transport of Cr through BBB is effective in rats and mice but is relatively inefficient [47,49], and long term treatment of AGAT- and GAMT-deficient patients with high doses of Cr allows only a slow and in most cases partial replenishment of their brain Cr pools (see below) [55,63]. One strong argument in favor of the "brain endogenous Cr synthesis" hypothesis comes from Cr measures in the cerebrospinal fluid (CSF) of Cr-deficient patients (see below) [18]. SLC6A8 deficient patients present normal Cr levels in CSF, but are unable to import Cr from periphery [7,23,29,51]. In contrast, GAMT-deficient patients show strongly decreased levels of Cr in CSF but are able to import Cr from the blood [57,62]. This also suggests that Cr synthesis in the brain might still remain operational, although very partially, under SLC6A8 deficiency, while it is completely blocked in AGAT and GAMT deficiencies. Endogenous synthesis, or a very efficient uptake from periphery, are the two ways available for the brain to secure Cr homeostasis for its energy and functions. As uptake from periphery does not appear efficient, CNS might privilege Cr endogenous synthesis. The brain capacity for Cr synthesis would thus depend on the efficient supply of arginine, the limiting substrate for Cr synthesis, from blood to CNS, and then also on local trafficking of arginine between brain cells. We and others have shown that cationic amino acid transporters (CATs) CAT1, CAT2(B) and CAT3 might fulfill these roles in the brain [10,17,35].

The hypothesis of endogenous Cr synthesis in the brain might seem contradictory with the *in vivo* characteristics of SLC6A8 deficiency (see below), which, despite AGAT and GAMT expression in CNS, presents an absence (or a very low level) of brain Cr by magnetic resonance spectroscopy (MRS) [53]. This

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apparent contradiction is probably explained by our recent data on AGAT, GAMT and SLC6A8 expression patterns in the brain. AGAT and GAMT are found in every CNS cell type [11], but appear rarely co-expressed within the same cell [15]. This suggests that to allow Cr synthesis in the brain, GAA must be transported from AGAT- to GAMT-expressing cells. This GAA transfer most probably occurs through SLC6A8, as shown in the same study by Cr and GAA competition studies and the use of stable isotope-labeled GAA, demonstrating its uptake by brain cells followed by its conversion to Cr by GAMT activity [15]. These observations may explain the Cr absence in CNS of SLC6A8-deficient patient, despite normal expression of AGAT and GAMT in their brain [6,18]. Recent studies also demonstrate the potential role of SLC6A8 for GAA transport across BBB and in brain parenchymal cells [64,66].

2.4 Cr in developmental versus adult CNS

As described above, the adult (or mature) brain might privilege Cr endogenous synthesis *versus* uptake from periphery, due to low permeability of BBB for Cr and thank to the expression of AGAT and GAMT in CNS parenchyma. Fetal and perinatal (or immature) CNS probably behaves differently for its Cr needs. Fetal needs in Cr are partly supported by active transport of Cr from mother and embryo [28,36]. AGAT, GAMT and SLC6A8 are also well expressed during vertebrate embryogenesis, including in the brain [19,37,54]. We have shown that AGAT and GAMT are expressed in the whole developing CNS parenchyma [19]. However, their low level (GAMT in particular) at early developmental stages suggests that in contrast to adult brain, embryonic CNS depends predominantly on external Cr supply, be it from embryonic periphery or from maternal origin. This is coherent with SLC6A8 expression in the whole embryonic CNS already at early stages (E12.5 in rat), with particularly high levels in periventricular zone and choroid plexus, the predominant metabolic exchange zones of fetal CNS before microcapillary angiogenesis and differentiation of BBB [14,19].

3 Creatine deficiency syndromes

CNS is the main organ affected in patients suffering from Cr deficiency syndromes, inborn errors of Cr biosynthesis and transport caused by AGAT, GAMT or SLC6A8 deficiency which are characterized by an absence or a severe decrease of Cr in CNS as measured by MRS [38,53,62]. As the prevalence of SLC6A8 deficiency was estimated at 2% of all X-linked mental retardations [52] and at 1% of males with mental retardation of unknown etiology [26], while all combined Cr deficiencies were estimated between 0.3% and 2.7% of all mental retardation [4,41], Cr deficiency syndromes appear as some of the most frequent inborn errors of metabolism (IEM).

Cr-deficient patients present neurological symptoms in infancy, such as mental retardation and delays in speech acquisition; GAMT deficiency exhibits a more complex phenotype, including intractable epilepsy, extrapyramidal movement syndromes and abnormalities in basal ganglia [63]. The diverse phenotypic spectrum of neurological symptoms observed in Cr deficiency syndromes demonstrate the importance of Cr for psychomotor development and cognitive functions. The more complex phenotype of GAMT deficiency is probably due to the toxicity of brain GAA accumulation [56], which may occur through activation of $GABA_A$ receptors by GAA [46] or inhibition of the complex between Na^+/K^+-ATPase and CK [73]. Severe epilepsy is also observed sometimes in SLC6A8-deficient patients [43]. This may be due to the observed CNS GAA accumulation in some SLC6A8-deficient patients [59], that could be caused by impairment of GAA transport through deficient SLC6A8, from AGAT- to GAMT-expressing brain cells (see below) [15].

AGAT- and GAMT-deficient patients can be treated by oral supplementation of Cr. While this strongly improves their neurological status and CNS development, very high doses of Cr must be used, and replenishment of cerebral Cr takes months and only results, in most cases, in partial restoration of cerebral Cr pools [5,33,38,61]. The pre-symptomatic treatment of AGAT- and GAMT-deficient patients appears to improve even more their clinical outcome [55]. For GAMT-deficient patients, combined arginine restriction and ornithine substitution coupled to Cr treatment decrease GAA and also improve clinical outcome [56,58]. However, despite improvement of clinical outcome by Cr supplementation, most AGAT- and GAMT-deficient patients remain with CNS developmental problems. Oral supplementation of Cr is inefficient in replenishing brain Cr in SLC6A8-deficient patients, who remain with

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mental retardation, severe speech impairment and progressive brain atrophy [7,23,29,51]. Attempts to treat SLC6A8-deficient patients with arginine and glycine as precursors of Cr gave encouraging results in two SLC6A8-deficient patients [25,71], while it failed to improve the neurological status of four others [31].

4 Secondary creatine deficiencies in CNS

Several other CNS pathologies can lead to a secondary Cr deficiency in brain cells. Excess of ammonium (NH_4^+) in CNS, as seen in pediatric patients under various acquired or inherited disorders like urea cycle diseases, can cause irreversible damages to the developing brain [13,22,40]. NH_4^+ exposure generates a secondary Cr deficiency in brain cells [16,20], eventually leading to energy deficit, oxidative stress and cell death [12,13]. Ischemic stroke in CNS leads to a rapid diminution in brain total Cr (Cr + PCr), causing a decrease in high energy phosphates production which leads to a failure in most energy-dependent processes necessary for cell survival [39]. Gyrate atrophy of the choroid and retina, an IEM caused by mutations in ornithine δ-aminotransferase (OAT) [68], generates a secondary Cr deficiency [60] which in CNS may contribute to GA neurological symptoms [67].

5 Model for creatine synthesis and trafficking in CNS

Altogether, (i) the absence of Cr within the brain of Cr-deficient patients, (ii) the CNS expression patterns of AGAT, GAMT and SLC6A8, (iii) the low permeability of BBB for Cr, and (iv) the brain levels of Cr and GAA both in normal and Cr-deficient conditions, lead us to propose the following concept for Cr synthesis and trafficking within CNS [6]. In normal conditions, SLC6A8 is expressed by MCEC, but not by the surrounding astrocytic feet, implying that limited amounts of Cr enter the brain through BBB. In most brain regions, brain cells express AGAT and GAMT in a cell-dissociated way, and GAA must be transported from AGAT- to GAMT-expressing cells by SLC6A8 for Cr synthesis to occur. In AGAT and GAMT deficiency, no Cr can be synthesized within CNS, but SLC6A8 expression in MCEC allows the limited entry of Cr within the brain, and thus their treatment by oral Cr and the partial replenishment of the brain Cr pools. The GAMT-deficient brain accumulates GAA. Cr transporter-deficient patients lack functional SLC6A8 on MCEC, and thus cannot be treated by oral Cr. Their endogenous CNS Cr synthesis pathway is also deficient, as in most brain regions, GAA cannot cross from AGAT- to GAMT-expressing cells due to their lack in functional SLC6A8.

6 Creatine as therapeutic potential for brain diseases

Troubles in CNS energy metabolism due to mitochondrial dysfunction, either from oxidative stress, mitochondrial DNA deletions, pathological mutations or altered mitochondria morphology, play critical roles in the progression of neurological diseases as a primary or secondary mechanism in neuronal death cascade [24]. Cr is known to play essential roles in stabilizing mitochondrial function and in decreasing neuronal cell death, and Cr supplementation was shown to improve the bioenergetic deficit associated with several brain pathologies, including Huntington's, Parkinson's and Alzheimer's diseases, amyotrophic lateral sclerosis, stroke and hyperammonemia [6,34].

7 Conclusions

Cr plays its main role in energy metabolism, allowing ATP regeneration through CK enzymatic activity. In recent years, new roles of Cr have been suggested in CNS, like a function of neuromodulator or even true neurotransmitter. The recent years have brought new knowledge on Cr metabolism and transport in the brain, allowing a better understanding on the pathophysiology of Cr deficiency syndromes in brain cells [6]. In particular, there is evidence that BBB presents a low permeability for Cr, and that CNS must ensure parts of its needs in Cr by endogenous synthesis. Moreover, in many regions of the brain, Cr endogenous synthesis appears to be dissociated, GAA needing to be transported by SLC6A8 from AGAT- to GAMT-expressing cells for Cr synthesis to occur [15,18].

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