CHAPTER 8

# CEREBRAL CREATINE DEFICIENCY SYNDROMES: CLINICAL ASPECTS, TREATMENT AND PATHOPHYSIOLOGY

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**Abstract:** Cerebral creatine deficiency syndromes (CCDSs) are a group of inborn errors of creatine metabolism comprising two autosomal recessive disorders that affect the biosynthesis of creatine – i.e. arginine:glycine amidinotransferase deficiency (AGAT; MIM 602360) and guanidinoacetate methyltransferase deficiency (GAMT; MIM 601240) – and an X-linked defect that affects the creatine transporter, SLC6A8 deficiency (SLC6A8; MIM 300036). The biochemical hallmarks of these disorders include cerebral creatine deficiency as detected *in vivo* by $^{1}$H magnetic resonance spectroscopy (MRS) of the brain, and specific disturbances in metabolites of creatine metabolism in body fluids. In urine and plasma, abnormal guanidinoacetic acid (GAA) levels are found in AGAT deficiency (reduced GAA) and in GAMT deficiency (increased GAA). In urine of males with SLC6A8 deficiency, an increased creatine/creatinine ratio is detected. The common clinical presentation in CCDS includes mental retardation, expressive speech and language delay, autistic like behaviour and epilepsy. Treatment of the creatine biosynthesis defects has yielded clinical improvement, while for creatine transporter deficiency, successful treatment strategies still need to be discovered. CCDSs may be responsible for a considerable fraction of children and adults affected with mental retardation of unknown etiology. Thus, screening for this group of disorders should be included in the differential diagnosis of this population. In this review, also the importance of CCDSs for the unravelling of the (patho)physiology of cerebral creatine metabolism is discussed



*G.S. Salomons and M. Wyss (eds.), Creatine and Creatine Kinase in Health and Disease*, 149–166.

## 1. INTRODUCTION

Over the last decade, a novel group of inborn errors affecting proteins involved in creatine biosynthesis and its transport (Figure 1) has been identified, the cerebral creatine deficiency syndromes (CCDSs). Creatine is synthesized in a two-step process: 1) transfer of the amidino group from arginine to glycine, yielding guanidinoacetic acid (GAA) and ornithine. This reaction is catalyzed by L-arginine:glycine amidinotransferase (AGAT); 2) transfer of a methyl group from *S*-adenosylmethionine to GAA, yielding creatine and *S*-adenosylhomocysteine. This reaction is catalyzed by *S*-adenosyl-L-methionine:*N*-guanidinoacetate methyltransferase (GAMT) (Walker, 1979; Wyss and Kaddurah-Daouk, 2000). Creatine synthesis primarily occurs in the kidney and pancreas which have high AGAT activity, and in liver which has high GAMT activity. From these organs and from nutritional sources (e.g. meat and fish), creatine is distributed via the bloodstream to the organs of usage – mainly muscle and brain – and is taken up into these tissues by a $Na^+$- and $Cl^-$-dependent creatine transporter (SLC6A8). The rate-limiting step in creatine biosynthesis is catalyzed by the AGAT enzyme. AGAT activity is repressed by high creatine concentrations at the pretranslational level. Additional allosteric inhibition is effected by high ornithine concentrations (Wyss and Kaddurah-Daouk, 2000). *In vitro*, GAMT activity is inhibited allosterically by high *S*-adenosylhomocysteine concentrations. However, no *in vivo* regulatory mechanism is known for GAMT activity. Understanding the regulation of the creatine transporter by investigating its expression and its activity is of significant importance, especially in the development or improvement of treatment strategies for CCDSs. Unfortunately, there is only limited information available so far on this topic, and particularly on creatine transporter regulation in brain. Early experiments suggested that creatine uptake is down-regulated in cultured rat myoblasts in the presence of high levels of extracellular creatine (Loike *et al.*, 1988). However, this has not been proven at the protein level due to the lack of specific antibodies against the creatine transporter (Speer *et al.*, 2004). In human skeletal muscle, creatine-monohydrate supplementation resulted in increased muscular creatine content without affecting the *SLC6A8* mRNA levels (Tarnopolsky *et al.*, 2003). Various factors may be involved in the activation and regulation of the creatine transporter, including signal transduction proteins, hormones and nutrition as discussed in chapter 6 of this book (Christie, 2007). Intracellularly, creatine is reversibly converted into the high-energy compound phosphocreatine by the action of creatine kinase (CK). Cytosolic CK transphosphorylates glycolytically generated ATP to phosphocreatine, or it uses phosphocreatine of either cytosolic or mitochondrial origin to regenerate ATP in the vicinity of energy-expending ATPases. Mitochondrial CK catalyses phosphate transfer from mitochondrial ATP to creatine in the mitochondrial intermembrane space, in a concerted manner with the ATP/ADP translocator of the inner mitochondrial membrane. The reversible transfer of high-energy phosphate groups to creatine as storage and carrier vehicle facilitates intracellular delivery of high-energy phosphates and provides additional energy resources during peak energy demands (Wyss and Kaddurah-Daouk, 2000).

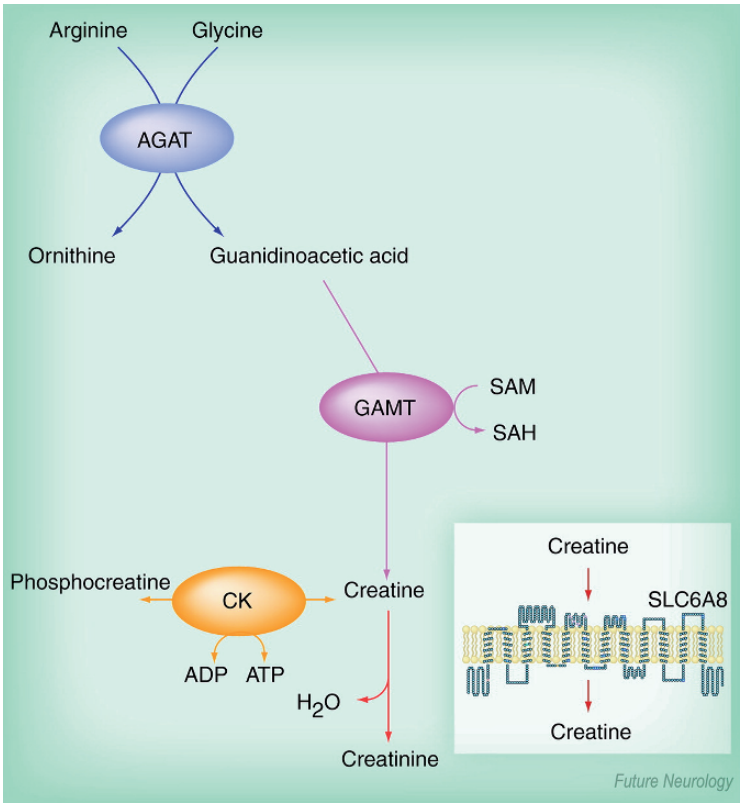


*Figure 1.* Schematic representation of creatine metabolism and transport, illustrating the proteins involved in the cerebral creatine deficiency syndromes. Creatine biosynthesis is a two-step process: L-arginine:glycine amidinotransferase (AGAT) catalyses the formation of guanidinoacetic acid from the amino acids arginine and glycine. If AGAT is impaired due to the presence of homozygous or compound heterozygous mutations, patients are affected with AGAT deficiency (MIM 602360). The second step involves the methylation of guanidinoacetic acid by *S*-adenosyl-L-methionine:*N*-guanidinoacetate methyltransferase (GAMT), which results in the formation of creatine and *S*-adenosylhomocysteine (SAH). *S*-Adenosylmethionine (SAM) functions as the donor of the methyl group. In case GAMT is impaired due to the presence of homozygous or compound heterozygous mutations, patients are affected with GAMT deficiency (MIM 601240). Creatine is distributed via the bloodstream and is subsequently taken up by cells via the creatine transporter (SLC6A8). The X-linked form of CCDSs – SLC6A8 deficiency (SLC6A8; MIM 300036) – is caused by hemizygous mutations in the *SLC6A8* gene in males, resulting in impaired creatine uptake. Due to skewed X-inactivation, the presence of a heterozygous *SLC6A8* mutation in females is associated with clinical symptoms of varying degrees. Creatine kinase (CK) catalyzes the (de)phosphorylation of creatine and phosphocreatine during ATP synthesis and usage. Reproduced from Almeida *et al.* (2006) with kind permission of Future Medicine Ltd.

Creatine and phosphocreatine are non-enzymatically converted into creatinine, with a constant daily turnover of approximately 1.5% of body creatine. Creatinine is mainly excreted in urine. Its daily excretion is directly proportional to total body creatine and, thus, in good approximation to muscle mass (i.e., 20–25 mg/kg/24 h in children and adults, and somewhat lower in infants younger than 2 years; Stöckler-Ipsiroglu and Salomons, 2006). Until recently, the group of creatine biosynthesis defects and the creatine transporter defect were referred to as creatine deficiency syndromes (CDSs). However, in body fluids, no creatine deficiency exists in creatine transporter deficient patients; thus, this term may be misleading. Therefore, it may be more appropriate to use the term cerebral creatine deficiency syndromes (CCDSs), which correlates better to the main clinical hallmarks that are related to CNS involvement. The discovery of CCDSs has brought new diagnostic options in patients with unexplained mental retardation, speech and language disorders, autism and epilepsy. Moreover, these defects are important for the unravelling of the physiologic functions and pharmacologic potential of creatine as well as of intermediates of creatine biosynthesis.

## 2. GAMT DEFICIENCY

GAMT deficiency was the first CCDS to be recognized in humans in 1994 (Stöckler *et al.*, 1994). The disorder affects the second step in creatine biosynthesis. The first patient, a German boy, seemed to develop normally until 4 months of age, when he was noted to have developmental arrest, hypotonia, hyperkinetic (hemiballistic) extra-pyramidal movements and head nodding. A diagnostic hint was given by *in vivo* $^{1}$H MRS of the brain, showing a spectrum lacking the creatine signal but positive for a signal which finally was identified as GAA. The combination of deficient creatine with a high GAA signal was suggestive of GAMT deficiency. Subsequent studies confirmed the absence of GAMT activity in liver and identified the pathogenic mutations in the *GAMT* gene (Stöckler *et al.*, 1996b). The *GAMT* gene has been mapped to chromosome 15q15.3, thus confirming that GAMT deficiency is an autosomal recessive disorder. Oral supplementation with creatine monohydrate resulted in a rise of cerebral creatine levels up to 50% of normal within the first 3 months of treatment. After 24 months of treatment, cerebral creatine levels had reached up to 90% of normal levels. The restoration of cerebral creatine levels was accompanied by significant clinical improvement: extra-pyramidal movement disorder and head nodding resolved, and the patient was able to walk at the age of 4 years. The EEG, which had shown theta delta background activity with multifocal spikes, normalised, as did the bilateral pathologic signal intensities in the globus pallidus (Stöckler *et al.*, 1996a). Disappointingly, the long-term outcome revealed that the severe mental retardation remained, and there was no improvement in speech development and progressive autistic and aggressive behaviour disorder. One reason for the incomplete clinical improvement seems to be the accumulation of GAA which is not normalised by creatine supplementation (Stöckler *et al.*, 1997).

Shortly after the description of the first patient with GAMT deficiency, a second patient was described (Schulze *et al.*, 1998). In this patient, the authors could show convincingly that dietary restriction of arginine, the immediate precursor of GAA, results in significant reduction in GAA accumulation (Schulze *et al.*, 1998). Since the description of the first patient with GAMT deficiency, approximately 29 patients have been diagnosed worldwide (Almeida *et al.*, 2007; Mercimek-Mahmutoglu *et al.*, 2006). An overview of 27 cases shows that mental retardation and epilepsy are the most consistent clinical features (Mercimek-Mahmutoglu *et al.*, 2006). The severity may range from mild to severe mental retardation and from occasional to drug-resistant seizures. Additional extra-pyramidal movement disorder and pathologic signal intensities in the basal ganglia on brain MRI are observed in the most severe cases. The clinical onset of the disease is between an age of four months to three years. Treatment with creatine monohydrate consistently resulted in improvement of epilepsy and movement disorder. However, this treatment did not have an impact on the intellectual deficit of the patients. A few patients were treated with a combination of creatine monohydrate supplementation and dietary arginine restriction (i.e., protein restriction), resulting in reduction in GAA accumulation, but in all patients taking this diet, complete normalisation of GAA levels could not be achieved. On such a case-by-case basis, final conclusions about the clinical effects of additional dietary arginine restriction cannot be made. However, as GAA is considered neurotoxic, it is now a general consensus to treat all patients with the combined approach and to aim at reduction in GAA accumulation as much as possible. Besides dietary arginine restriction, ornithine and sodium benzoate supplementation were proposed as strategies for reducing GAA accumulation. Experience with the only patient diagnosed at birth and treated at a pre-symptomatic stage of the disease suggests that early treatment might widely prevent neurological manifestations (Schulze *et al.*, 2006; see also chapter 9; Schulze and Battini, 2007).

So far, 15 different GAMT mutations have been described (Almeida *et al.*, 2007; Mercimek-Mahmutoglu *et al.*, 2006), including nonsense and missense mutations, splice errors, insertions, deletions and frameshifts. There is no evidence for a hotspot region, although certain mutations appear to occur more frequently than others (c.59G > A; p.Trp20Ser and a mutation that results in erroneous splicing: c.327G > A). A relatively high carrier rate for the c.59G > A mutation has been detected in certain areas of Portugal, and 10 out of the 29 patients affected with GAMT deficiency are of Portuguese origin (Almeida *et al.*, 2007). Comparison of the type and severity of clinical presentation of the patients who are homozygous for any one of these mutations does not show any genotype-phenotype correlation yet (Mercimek-Mahmutoglu *et al.*, 2006).

## 3. AGAT DEFICIENCY

AGAT deficiency affects the first enzyme in creatine biosynthesis. This disorder was first described in two Italian sisters with unspecific developmental and speech delay, and occasional (fever-induced) seizures in one of them. *In vivo*

$^{1}$H MRS of the brain revealed creatine deficiency, but opposite to GAMT deficiency, GAA levels were low in body fluids. This combination pointed to a defect in GAA biosynthesis, and AGAT deficiency was finally confirmed by deficient enzyme activity and identification of a homozygous nonsense mutation in the *AGAT* gene (Item *et al.*, 2001). The *AGAT* gene has been mapped to chromosome 19p13.3, thus confirming that AGAT deficiency is an autosomal recessive disorder. Supplementation with creatine monohydrate resulted in almost complete normalisation of cerebral creatine levels and a catch-up in psychomental development (Bianchi *et al.*, 2000). Although, after 6 years of treatment, the 13- and 11-year-old sisters still have a moderate intellectual deficit, there was improvement and stabilisation of their condition. In a younger sibling, AGAT deficiency was diagnosed prenatally and creatine supplementation was started at the age of 4 months. This patient revealed normal development at the age of 18 months, while his sisters at this age already showed signs of retardation (Battini *et al.*, 2006; see also chapter 9; Schulze and Battini, 2007). Observations on this limited number of patients (n = 3) suggest that, compared to GAMT deficiency, AGAT deficiency is associated with a milder phenotype, and/or with a better response to creatine supplementation. More importantly, early neurological sequelae may be prevented by early treatment. Currently, five patients are known worldwide with this condition, of whom four are from one Italian family. Only two mutations have been found so far: a nonsense mutation (in the Italian family; Battini *et al.*, 2002; Item *et al.*, 2001), and a splice error mutation (Almeida *et al.*, 2006a). Both mutations are associated with impaired AGAT activity in lymphoblasts.

## 4. CREATINE TRANSPORTER (SLC6A8) DEFICIENCY

### 4.1. Creatine Transporter (SLC6A8) Deficiency in Males

SLC6A8 deficiency – a defect of the creatine transporter – was originally described in a 6-year-old American boy with a history of central hypotonia, one episode of status epilepticus, multifocal epileptiform discharges in his EEG recordings, delayed speech and language development, and creatine deficiency in the brain. In contrast to GAMT and AGAT deficiency, GAA levels were normal and oral creatine supplementation was not effective in restoring cerebral creatine levels (Cecil *et al.*, 2001). Due to these findings, and the X-linked pattern of inheritance in the family, cerebral creatine transporter deficiency was suspected. This hypothesis was confirmed by demonstration of deficient creatine uptake in fibroblasts and by identification of a hemizygous mutation in the *SLC6A8* gene mapped to the X chromosome (Salomons *et al.*, 2001).

Since the first description of SLC6A8 deficiency in 2001, more than 150 patients from 60 families have been diagnosed. In boys as well as adult males (age range 2–66 years), the diagnosis of SLC6A8 deficiency has been ascertained. The main biochemical hallmark is an increased urinary creatine to creatinine ratio in males (Stöckler-Ipsiroglu and Salomons, 2006). The clinical phenotype of males

with SLC6A8 deficiency varies from mild to severe mental retardation associated with speech and language delay, expressive language disorder, epileptic seizures, behaviour disorders and gastrointestinal problems in adult patients (Kleefstra *et al.*, 2005). A neuropsychological profile was obtained for four affected Dutch boys from two unrelated families and revealed hyperactive impulsive attention deficit and a semantic-pragmatic language disorder with oral dyspraxia (Mancini *et al.*, 2005). Growth retardation, dysmorphic features, microcephaly, and brain atrophy have been described as accompanying structural characteristics in some, but not all patients (Kleefstra *et al.*, 2005). Brain atrophy may become more marked during the course of the disease, as may the behavioural symptoms (Degrauw *et al.*, 2002). Muscular hypotrophy and hypotonia and secondary mitochondriopathy have been reported as additional features in selected SLC6A8 deficient patients (Anselm *et al.*, 2006).

According to numerous reports, SLC6A8 deficiency is responsible for a substantial number of males with mental retardation of unknown cause. In a recently studied cohort of males presenting with risk symptoms, the urinary creatine to creatinine ratio was used to analyze SLC6A8 frequency. The frequency of SLC6A8 deficiency was confirmed by molecular and functional studies (i.e., creatine uptake in fibroblasts) to be 2.1% (2 out of 96 males; Mercimek-Mahmutoglu *et al.*, 2007). This frequency is consistent with results from other studies. SLC6A8 deficiency was detected in 5.4% (2 out of 37) and 2.1% (6 out of 288) of males with non-syndromic X-linked mental retardation (Lion-Francois *et al.*, 2006; Rosenberg *et al.*, 2004), in 2.2% (2 out of 92) of males with global developmental delay (IQ < 70) (Newmeyer *et al.*, 2005), as well as in 0.8% (4 out of 478) and 3.5% (4 out of 114) of males with mental retardation of unknown causes and negative for fragile X syndrome (Clark *et al.*, 2006; Lion-Francois *et al.*, 2006).

SLC6A8 deficiency appears not to be treatable by any of the approaches described above. Treatment of both males and females affected with SLC6A8 deficiency with creatine monohydrate has not proven to be successful (Anselm *et al.*, 2006; Poo-Arguelles *et al.*, 2006; Stöckler-Ipsiroglu and Salomons, 2006). Currently, supplementation with high doses of arginine and glycine, which are the primary substrates for creatine biosynthesis, combined with high doses of creatine monohydrate is being investigated. The rationale for this protocol is based on an increased cerebral uptake of both amino acids with the aim to enhance intracerebral creatine synthesis (Gracia M. Mancini, Marjo S. van der Knaap, and Gajja S. Salomons, unpublished data). In addition, alternative strategies may be developed that facilitate creatine transport into the brain either by modified transport via carrier molecules (e.g., peptides) or by supplementation with suitable creatine analogs.

Since the first description of SLC6A8 deficiency in 2001, 24 families have been described at the molecular level, and 20 different mutations have been identified. Four mutations have been identified in at least two unrelated families (c.321_323delCTT;p.Phe107del, c.1169C > T;p.Pro390Leu, c.1222_1224delTTC; p.Phe408del; c.1631C > T;p.Pro544Leu; Almeida *et al.*, 2006a; Stöckler-Ipsiroglu

and Salomons, 2006). The most frequently identified type of mutations in *SLC6A8* are missense mutations (i.e., single amino acid substitutions) and single amino acid deletions located throughout the gene. Especially in case of novel missense mutations, the diagnosis should be confirmed by investigating creatine uptake in cultured fibroblasts or by $^{1}$H-MRS of the brain. The pathogenicity of the mutation can be studied by overexpression of the mutant allele in SLC6A8-deficient cells, as recently described by Rosenberg *et al.* (2007). Approximately 10% of the mutations were shown to occur *de novo*, indicating that the mothers of children with such a *de novo* mutation will not have any clinical phenotype. Therefore, also in sporadic mental retardation, SLC6A8 deficiency should be considered in the differential diagnosis. Analyses of genotype-phenotype correlation have not been made so far. Characterization of a variety of missense mutations and one-amino acid deletions may provide insights into the structure and function of the protein.

### 4.2. Creatine Transporter (SLC6A8) Deficiency in Females

In addition to affected males who have a hemizygous mutation in *SLC6A8*, at least 80 female relatives as well as three index girls with a heterozygous mutation (carriers) have been identified so far; however, for the majority of them, no neuropsychological profiles have been obtained. Clinical symptoms of the index girls included learning disabilities and therapy-resistant epilepsy. Based on data on the first described females (Degrauw *et al.*, 2002, 2003), it is expected that approximately 50% of heterozygous females have learning and behavioural problems. Skewed X-inactivation may cause pronounced clinical manifestations in these cases that are similar to the male phenotype, whereas others remain seemingly asymptomatic. This phenomenon also applies to the biochemical hallmarks of the disease, which makes screening for a creatine transporter defect in females more difficult: 1) the urinary creatine to creatinine ratio is usually not informative; 2) the creatine signal in brain may be reduced only slightly; and 3) uptake studies in fibroblasts are also not informative and, thus, cannot be used as primary screening. Therefore, a combination of diagnostic tests may be needed, including molecular analysis of the *SLC6A8* gene. Treatment data is available for only one heterozygous female patient with learning disability and mildly decreased creatine concentration as revealed by brain $^{1}$H MRS. This data showed mild improvement on neuropsychological testing after 18 weeks of treatment with creatine monohydrate (250–750 mg/kg/day; Cecil *et al.*, 2001).

## 5. BIOCHEMICAL PATHOLOGY AND LABORATORY DIAGNOSIS

CCDSs are characterized by an almost complete lack of creatine in the brain, which is shown *in vivo* by brain $^{1}$H MRS. It should be noted that in females with a heterozygous mutation in the *SLC6A8* gene, a milder reduction in cerebral creatine is usually detected. However, cerebral creatine can vary in heterozygous females

from being absent to normal levels, depending on the skewing in X-inactivation. Interestingly, in CCDS patients, creatine deficiency is much less severe in muscle than in brain. Although creatine levels in skeletal muscle have not been investigated extensively in CCDS patients and insights are based on case studies only, creatine content of skeletal muscle was measured *in vivo* by proton MRS and *in vitro* in a muscle biopsy of a SLC6A8-deficient patient (Pyne-Geithman *et al.*, 2004). This case report revealed normal creatine levels, suggesting that endogenous creatine biosynthesis is able to maintain proper creatine levels, and/or that another creatine transporter is able to take up creatine. It is worthy of note that phosphorus MRS of skeletal muscle of a GAMT-deficient patient showed reduced phosphocreatine levels (Schulze *et al.*, 2003). Alternatively, the high levels of GAA in GAMT deficiency may interfere with sufficient uptake of creatine via the creatine transporter. This data is in agreement with GAMT knock-out mice that show reduced creatine levels in muscle and high levels of GAA (Renema *et al.*, 2003; Schmidt *et al.*, 2004). No data are available on creatine levels in heart muscle of affected patients. Clinically, however, there is no evidence of cardiac dysfunction in the patients who underwent cardiologic evaluation.

GAA is the main intermediary product in creatine biosynthesis. In GAMT deficiency, GAA accumulates 2- to 30-fold in urine and plasma, and about 200-fold in CSF (Mercimek-Mahmutoglu *et al.*, 2006). The high GAA concentrations in GAMT deficiency are due to at least two mechanisms: firstly, defective GAMT activity causes an accumulation of GAA *per se*. Secondly, creatine deficiency and consequent absence of AGAT repression cause an increased rate of GAA biosynthesis. Creatine's regulatory feedback mechanism on GAA synthesis is confirmed in GAMT-deficient patients who show a significant decrease (but not normalisation) in GAA concentrations as a response to oral creatine supplementation (Mercimek-Mahmutoglu *et al.*, 2006; Stöckler *et al.*, 1997). In AGAT deficiency, GAA is low as a result of defective synthesis (Battini *et al.*, 2002; Item *et al.*, 2001). In SLC6A8 deficiency, the main site of metabolic disturbance is the intracellular creatine pool, whereas plasma GAA and creatine levels are in the normal range. However, the ratio of creatine to creatinine concentrations in urine of affected males is increased (Stöckler-Ipsiroglu and Salomons, 2006). This could be the result of an elevated creatine excretion due to impaired tubular reabsorption and a reduced creatinine excretion due to reduced intracellular creatine pools (e.g. in muscle and brain). Since the urinary creatine to creatinine ratio is usually only mildly increased, it is important to realize that there is an inverse relationship between this ratio and age (Almeida *et al.*, 2004). Moreover, as mentioned above, the creatine to creatinine ratio is usually not increased in urine of females with a heterozygous mutation. The biochemical profile of the individual CCDS determines its laboratory diagnosis. In practical terms, this means that determination of urinary GAA concentrations allows to differentiate between AGAT (low concentration) and GAMT deficiency (high concentration). The determination of the urinary creatine/creatinine ratio has been found to be a useful diagnostic marker of SLC6A8 deficiency in males. Laboratory diagnosis is confirmed by demonstration of the enzyme or transporter

activity in either fibroblasts or lymphoblasts, and by DNA analysis of the relevant gene. Recently, we reviewed the biochemical changes in CCDSs (Stöckler-Ipsiroglu and Salomons, 2006).

## 6. PATHOPHYSIOLOGICAL CONSIDERATIONS

Present evidence suggests that each of the CCDSs has potential effects on several aspects of creatine physiology, including the intermediates of creatine biosynthesis and their biochemical reactions, the transfer of creatine from biosynthesis to usage compartments, intracellular creatine phosphorylation cycles, and all cellular functions normally supported by creatine in developing and mature brain.

### 6.1. Disrupted Creatine Transport

The concept of an organ-specific transfer of creatine from sites of biosynthesis in liver, pancreas and kidney to sites of usage in brain and muscle must be refined, at least with regard to the brain. Immunohistochemical and functional studies have established the presence of AGAT and GAMT in all types of brain cells *in vivo* (Braissant *et al.*, 2001) and the capacity of cultured astrocytes to synthesise creatine (Dringen *et al.*, 1998). This suggests a dual origin of cerebral creatine: from intracerebral biosynthesis and from transport across the blood-brain barrier. The creatine transporter, SLC6A8, is found in capillary endothelial cells, neurons, and oligodendrocytes (Braissant *et al.*, 2001). Based on these findings, creatine is thought to be transferred between different cell types within the brain, for instance from astrocytes to neurons (Tachikawa *et al.*, 2004), but also among the same cell type (Braissant *et al.*, 2005; see also chapters 4 and 5; Braissant *et al.*, 2007; Tachikawa *et al.*, 2007). Creatine transport across the blood-brain barrier and its transfer within the brain are the basis of our understanding of the pattern of cerebral creatine concentrations upon replacement therapy in the creatine biosynthesis deficiency syndromes, and of our understanding of the pathogenesis of SLC6A8 deficiency. Oral supplementation with creatine in patients with AGAT and GAMT deficiency results in a biphasic restoration of the cerebral creatine pool, with initially fast creatine accumulation followed by a slow, but continuous increase over several months to 80–90% of normal levels. Increasing the dosage or changing the time intervals between creatine administration do not result in any further increase in brain creatine levels (Stöckler *et al.*, 1997). The biphasic and incomplete restoration of the cerebral creatine pool suggests different intracerebral compartments of creatine metabolism, which may reflect creatine pools predominantly supplied by intracerebral creatine biosynthesis as opposed to those predominantly supplied by blood. In SLC6A8 deficiency, cerebral creatine levels are very low or even undetectable in *in vivo* $^{1}$H MRS measurements. This indicates that the fraction of intracerebral creatine biosynthesis under these conditions is rather low. SLC6A8 may be indispensable for the intracerebral redistribution of locally synthesised GAA and/or creatine. GAA is a known competitive inhibitor of the creatine transporter and is thought to be taken up via

the SLC6A8 transporter (Wyss and Kaddurah-Daouk, 2000). Therefore, due to the absence of a functional creatine transporter, GAA uptake might not be possible either. This may interfere with GAA methylation and, thus, creatine biosynthesis. In addition, low creatine levels may also result from disrupted intracerebral transport of creatine.

### 6.2. Intracellular Creatine Depletion in the Developing Brain

An important role for creatine during embryogenesis is suggested by the appearance of *AGAT* and *SLC6A8* transcripts in rat brain from as early as embryonal day 12.5 (Braissant *et al.*, 2005). Cell culture experiments on developing neurons suggest that creatine is needed for regular axonal growth (Braissant *et al.*, 2002; Wang *et al.*, 1998). Taken together, these findings indicate that creatine deficiency during brain development may cause impoverished axonal networks and reduced synaptic density. Microstructural anomalies of dendrites and neuronal networks are thought to be a morphological hallmark of mental retardation in Down syndrome and fragile X syndrome (Volpe, 2001). These considerations support the view that microstructural defects may be responsible, at least in part, for those clinical symptoms that are not reversible upon restoration of creatine content after a CCDS has manifested itself. Detailed morphological studies will be necessary to evaluate this hypothesis. Should these mechanisms indeed contribute to the clinical phenotype, pre-symptomatic treatment may be obligatory.

### 6.3. Intracellular Creatine Depletion in the Mature Brain

Creatine deficiency may cause a disruption of cellular energy homeostasis. The absence of creatine and phosphocreatine in the cytosol is expected to reduce the buffering capacity for peak ATP demands and the transport capacity for high-energy phosphate compounds. Since phosphocreatine is in part responsible for high-energy phosphate transport from mitochondria to ATPases elsewhere in the cell, its lack may resemble mitochondrial dysfunction. Its lack may also result in chronic energy failure. Such energy failure may explain pathologic signal intensities in the globus pallidus as observed in severe cases of GAMT deficiency (Mercimek-Mahmutoglu *et al.*, 2006). Comparable changes are found in patients with mitochondrial encephalopathies (e.g., cytochrome C oxidase deficiency), and in other cases of disrupted maintenance of cerebral energy state such as in hypoxic-ischemic events in newborns and in carbon monoxide intoxication. Recent observations suggest that oxidative phosphorylation may be impaired in patients affected by GAMT or SLC6A8 deficiency, as some patients with these conditions present with signs of myopathy and, at the biochemical level, mitochondrial dysfunction (e.g., lactic acidemia, or decreased ATP production in muscle biopsy samples; Anselm *et al.*, 2006; De Vries *et al.*, 2005; Stöckler *et al.*, 1996a).

Creatine has a protective effect on neuronal survival. This has been shown in neuronal cultures during exposure to high glutamate concentrations and in

animal models of various neurodegenerative diseases (Brewer and Wallimann, 2000; Klivenyi *et al.*, 1999; Matthews *et al.*, 1998, 1999). The mechanisms by which intracellular creatine reduces excitotoxic neuronal death have been studied, including effects on the mitochondrial permeability transition (Dolder *et al.*, 2003) and on apoptotic signalling (Juravleva *et al.*, 2005). However, no definitive conclusions can yet be drawn. Regardless of the cellular mediators, if the presence of creatine confers advantages for neuronal survival, its absence may facilitate cellular apoptotic pathways. This could result in increased apoptotic rates during the regressive phase of brain development, or in an enhanced apoptotic response to excitotoxic or oxidative stress. The latter may contribute to the brain atrophy observed in some SLC6A8 patients.

### 6.4. Interstitial and Intracellular Accumulation of Guanidino Compounds and Other Metabolites

Accumulation of guanidino compounds in tissues and bodily fluids is a characteristic of GAMT deficiency. GAA is most prominent among these, but changes in other intermediates of guanidino compound metabolism have also been described and may contribute to the neurotoxic load. GAA displays epileptogenic potential and is held to be the main cause of intractable seizures which occur in about one third of GAMT patients (Mercimek-Mahmutoglu *et al.*, 2006). A clear correlation between seizure activity and GAA levels in plasma was demonstrated in one patient in whom intractable seizures were independent of cerebral creatine status, but responded promptly to a reduction in plasma GAA by dietary arginine restriction (which is the rate-limiting substrate for GAA synthesis; Schulze *et al.*, 1998). On a molecular level, GAA interacts as partial agonist with $GABA_A$ receptors at pathophysiologically relevant concentrations (Neu *et al.*, 2002). Based on these findings, it has been speculated that GAA may also be responsible in part for the development of the extra-pyramidal movement disorder, which manifests in about 50% of GAMT-deficient patients, primarily as athetosis, chorea, and/or ataxia (Mercimek-Mahmutoglu *et al.*, 2006).

Changes in guanidino compounds secondary to GAA accumulation may also play a role in the development of neurological manifestations in GAMT deficiency. Analyses of samples from a GAMT-deficient patient (Stöckler *et al.*, 1997) and of brain homogenates from a GAMT knock-out mouse model (Torremans *et al.*, 2005) revealed additional accumulation of β-guanidinosuccinate, β-guanidinopropionate, and γ-guanidinobutyrate in the brain. Guanidinosuccinate may cause seizures and activate NMDA receptors (De Deyn *et al.*, 2001), β-guanidinopropionate is a competitive inhibitor of the creatine transporter, SLC6A8 (Peral *et al.*, 2002), whereas convulsive properties have been ascribed to γ-guanidinobutyrate (Jinnai *et al.*, 1966). The relative contribution of these substances to the neurological symptoms in GAMT deficiency is unclear, and synergistic effects cannot be excluded at the present stage.

Creatine synthesis requires a high percentage of total body 'labile' methyl groups. Therefore, one might expect that in GAMT deficiency, *S*-adenosylmethionine accumulates, which acts as a methyl donor for the GAMT reaction. Analysis of this compound in CSF from GAMT-deficient patients and in brain homogenates of the GAMT knock-out mouse model will answer the question whether changes in the methylation/remethylation pathway contribute to the pathophysiology of GAMT deficiency.

### 6.5. A Role for Creatine in Neurotransmission?

In stress-induced anxiety studies in chickens, creatine was reported to mediate an anxiolytic (sedative-hypnotic) effect as partial agonist at the central $GABA_A$ receptors (Koga *et al.*, 2005). These findings suggest that, *in vivo*, creatine modulates GABAergic neurotransmission throughout the brain. However, it should be noted that conflicting data regarding this effect of creatine has been published in *in vitro* studies on cultured cortex neurons of neonatal mice (Neu *et al.*, 2002). Nevertheless, the ubiquitous presence of creatine as well as of its biosynthesis enzymes in brain, including central neurons (Braissant *et al.*, 2005), and the possible effects at the $GABA_A$ receptors suggest a putative role of creatine as a neuromodulator in the brain. In agreement with this putative role, it was shown that [$^3$H]creatine is taken up into rat brain (neocortex) slices in a $Na^+$-dependent manner, likely mediated by the SLC6A8 transporter. Additionally, both [$^3$H]creatine and endogenous creatine were shown to be released upon electrical field stimulation from superfused neocortex slices (Almeida *et al.*, 2006b). This release was $Na^+$- and $Ca^{2+}$-dependent, indicating the involvement of an exocytotic release mechanism. Moreover, this electrically evoked creatine release appeared to depend not only on the activation of voltage-gated $Na^+$-channels (i.e., electrically evoked creatine release blocked by tetrodotoxin), but also on that of $K^+$-channels (i.e., electrically evoked creatine release strongly increased by 4-aminopyridine), demonstrating that the release is action potential dependent. Similar findings have previously been reported for radiolabelled classical neurotransmitters (e.g., Limberger *et al.*, 1986; Schoffelmeer *et al.*, 1981). These novel findings in brain may very well contribute to an explanation of the clinical phenotype in CCDSs, and await further studies.

## 7. PROSPECTS

The knowledge of the natural history of CCDSs is limited to the clinical presentations of a few patients only. Most of the known patients are children, but also a few young adults as well as a few elderly have been diagnosed. As these patients have been followed for a few years only, no definite conclusions can be drawn in terms of disease progression and life expectancy. Furthermore, only patients with the most severe clinical phenotypes may have been diagnosed so far, while milder phenotypes might still be under-recognised.

CCDSs are potentially treatable disorders. However, progress in understanding efficacy of treatment and development of new treatment strategies has been delayed due to the rareness of the single disorders. As with other rare inborn errors of metabolism, worldwide networks and orphan disease registries are needed to facilitate progress in understanding the natural history and in the development of strategies for treatment and prevention. Creatine deficiency syndromes should be investigated using these tools in the future.

Development of treatment strategies depends on the understanding of pathophysiology. Our understanding of pathophysiology and pathobiochemistry in CCDSs is still incomplete. The pathobiochemical actions and pathophysiological consequences of accumulation of GAA, GAA's role in brain function, and pharmacological inhibition of GAA's action in the brain need to be understood fundamentally in order to develop more effective treatment strategies for GAMT deficiency. The lack of neurological symptoms in the GAMT knock-out mouse model makes the exploration of this issue even more difficult (Torremans *et al.*, 2005). New concepts are also needed for understanding the pathogenesis of brain dysfunction in creatine transporter or AGAT deficiency. What is the pathogenetic impact of creatine deficiency? Knowledge about the regulation of creatine transporter activity and its interaction with other genes might provide promising targets for alternative treatment strategies such as pharmacological gene therapy.

The development of SLC6A8 knock-out mice will yield further insights into these questions. Better understanding of creatine's neuroprotective role may be another source of inspiration for the development of new treatment strategies. An AGAT knock-out mouse model would represent a biological system of creatine depletion and would be fundamental for understanding creatine's effects beyond its energy-buffering function. This would also allow investigations into a possible neuroprotective role of creatine which still has not been proven convincingly in humans. The final goal of diagnosis and treatment of CCDSs is the prevention of the clinical phenotype. Therefore, it is mandatory that CCDSs are part of the routine diagnostic work-up in patients with mental retardation and/or epilepsy, and determination of marker substances (urinary GAA and the urinary creatine to creatinine ratio) needs to be introduced in metabolic laboratory panels.

## ACKNOWLEDGEMENTS

G.S. Salomons is supported by the Dutch Society for Scientific Research (ZonMW/NWO), VIDI grant number 917.56.349.

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