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Review article

Current and potential new treatment strategies for creatine deficiency syndromes

Gabriella Fernandes-Pires, Olivier Braissant *

Service of Clinical Chemistry, University of Lausanne and Lausanne University Hospital, Lausanne, Switzerland

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ABSTRACT

Creatine deficiency syndromes (CDS) are inherited metabolic disorders caused by mutations in *GATM*, *GAMT* and *SLC6A8* and mainly affect central nervous system (CNS). AGAT- and GAMT-deficient patients lack the functional brain endogenous creatine (Cr) synthesis pathway but express the Cr transporter SLC6A8 at blood-brain barrier (BBB), and can thus be treated by oral supplementation of high doses of Cr. For Cr transporter deficiency (SLC6A8 deficiency or CTD), current treatment strategies benefit one-third of patients. However, as their phenotype is not completely reversed, and for the other two-thirds of CTD patients, the development of novel more effective therapies is needed. This article aims to review the current knowledge on Cr metabolism and CDS clinical aspects, highlighting their current treatment possibilities and the most recent research perspectives on CDS potential therapeutics designed, in particular, to bring new options for the treatment of CTD.

Contents

* Corresponding author at: Service of Clinical Chemistry, Lausanne University Hospital and University of Lausanne, Rue du Bugnon 46, 1011 Lausanne, Switzerland.
E-mail address: Olivier.Braissant@chuv.ch (O. Braissant).

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1. Introduction

1.1. Creatine

Creatine (Cr), or α-*N*-methyl-guanidino-acetic acid, is a nitrogenous organic acid playing an essential role in ATP regeneration and buffering, as well as transport of high-energy phosphates within the cell through the Cr / Phosphocreatine (PCr) / Creatine kinase (CK) system [1–3] (Fig. 1A). Based on tissue expression and subcellular distribution, four CK isoforms have been described: two cytosolic forms (M-CK enriched in muscle and B-CK enriched in brain) and two mitochondrial forms (sarcomeric muscle form / sMtCK) and brain form called ubiquitous MtCK / uMtCK) [4,5]. Each isoform has specific roles, as mitochondrial CKs consume the mitochondrially-formed ATP to produce PCr to export it to the cytoplasm, while cytoplasmic CKs control energy demand through PCr consumption for ATP production and convert ATP into PCr for energy storage [4,6]. High Cr levels are found in tissues with high ATP demand like heart, muscle and brain.

In a young adult of 70 kg body weight, the total Cr content amounts to 120 g of which about 2% has to be replaced daily due to the non-enzymatic conversion of Cr to creatinine (Crn) [7,8]. In humans, about half of the daily needs in Cr is obtained through diet, the other half being synthesized endogenously by a two-step enzymatic pathway involving arginine:glycine amidinotransferase (AGAT / EC 2.1 4.1) and guanidinoacetate methyltransferase (GAMT / EC 2.1.1.2). AGAT converts arginine (Arg, limiting factor) and glycine (Gly) into the intermediate guanidinoacetate (GAA) and ornithine (Orn). The second enzyme, GAMT, uses S-adenosylmethionine as a methyl group donor to convert GAA into Cr. Cells take up Cr by a specific transporter, SLC6A8, also known as CT1, CRT, CRTR or CreaT. SLC6A8 is a member of the solute carrier family 6, a large family of membrane transporters mediating the transport of various neurotransmitters and amino acids across plasma membrane with the co-transport of two Na^+ and one Cl^- [7,9,10] (Fig. 1A).

While in periphery the main Cr synthesis pathway occurs through the expression of AGAT in the kidney, which releases GAA as substrate for GAMT expressed in the liver, AGAT and GAMT are also expressed in most other tissues, including the brain [7,8,11–13]. It was long considered that brain Cr was synthetized in peripheral tissues followed by transport to the brain. While the microcapillary endothelial cells (MCEC) at the blood-brain barrier (BBB) express SLC6A8, allowing peripheral Cr entry into CNS, the astrocytes, particularly their feet ensheathing BBB, do not express the Cr transporter [12,14,15]. This leads to a low permeability of BBB for Cr implying that the brain must ensure a part of endogenous synthesis through AGAT and GAMT expression to meet its Cr needs [12,16]. AGAT and GAMT are found expressed in neurons, oligodendrocytes and astrocytes [12,17] (Fig. 1B). However, in most brain structures, AGAT and GAMT are rarely co-expressed in the same cell (particularly in cortex and basal ganglia), implying that GAA must be transported from AGAT- to GAMT-expressing cells to complete the Cr synthesis pathway (Fig. 1B); this uptake of GAA in brain GAMT-expressing cells appears to occur through the same transporter as for Cr, SLC6A8 [16].

Apart of its functions in cellular energy, other roles have been attributed to Cr.

Several studies have proposed that Cr may act as neuromodulator or even true neurotransmitter in CNS, in particular through modulation of GABAergic neurons [18,19] (Fig. 1A). Indeed, Cr and GAA were reported to act as partial agonist of post-synaptic neuronal γ-aminobutyric acid receptors type A ($GABA_A$-R) (but not $GABA_B$-R) in *in vivo* experiments in the chicken CNS as well as in GAMT-deficient mice, and in *in vitro* experiments on mice primary neuronal cultures and organotypic brain slices [19–22]. Furthermore, from experiments in organotypic cultures of rat brain slices, Cr was proposed to act as true neurotransmitter, being electrically-released from vesicles in an action potential-dependent manner including dependency from Ca^{2+}, inhibition by the Na^+ channel blocker tetrodotoxin (TTX) and enhancement by the K^+ channel blocker 4-amino-pyridine. It was also demonstrated that synaptosomes extracted from suckling and adult rat brains were able to take up Cr, suggesting a re-uptake mechanism to recycle Cr in the presynaptic terminal [23,24].

Cr is also considered as one of the main cellular osmolytes in brain cells [25,26]. *In vitro* studies on rat cortical brain slices and in primary astrocytic cultures demonstrated, during hypo-osmotic shock, the decrease of intracellular Cr concentration, and the opposite effect during hyper-osmotic conditions [25,27] (Fig. 1A). A similar observation has been made in a rat *in vivo* model of hepatic encephalopathy (HE), in which the HE-induced increase in brain glutamine, increasing CNS osmotic pressure, is paralleled for homeostatic compensation by a decrease of several other brain osmolytes including Cr [28]. These findings suggest that Cr works as a compensatory osmolyte to maintain the correct CNS osmolar environment.

Cr was also suggested as appetite and weight regulator in hypothalamic nuclei. Intracerebroventricular administration, in adult rats, of cobaltic protoporphyrin IX (CoPP, an anorectic compound), resulted in decreased Cr concentrations paralleled by increased expression of SLC6A8 in several hypothalamic areas involved in the regulation of food intake, as compared to vehicle-treated fed or fasted controls animal [29].

Recent studies also proposed new roles for Cr in peripheral tissues. Cr appears essential for cardiac function, as recently shown in Cr transporter-deficient patients and mice [64]. In thermogenesis, Cr metabolism may provide an alternative mechanism of heat production following a futile cycle, as a main UCP1-independent thermogenic pathways in brown and beige adipose tissue (BAT) [30–32]. Cr further emerges as a key player in immune cell function by regulating the macrophage-mediated immune response [33]. Moreover, Cr was identified as an important "molecular battery" conserving bioenergy to enhance antitumor T cell immunity [34]. Finally, Cr appears active in mucosal environment as the SLC6A8 transporter acts as regulator of intracellular Cr playing role in barrier formation and wound healing in organoids from *Slc6a8* KO mice [35].

These multiple roles of Cr in the organism, as well as its anabolic potential, have led to the investigation of therapeutic interventions by Cr supplementation for pathological conditions within many different fields, including (but not restricted to) oncology, immune system, as well as involving musculoskeletal, cardiopulmonary or nervous systems [36].

1.2. Creatine deficiency syndromes

Creatine deficiency syndromes (CDS) are inherited metabolic disorders caused by mutations in *GATM*, *GAMT* and *SLC6A8* (AGAT, GAMT and Cr transporter deficiencies, respectively; see summary of CDS features in Table 1). AGAT and GAMT deficiencies affect males and females comparably as they are autosomal recessive. SLC6A8 deficiency is X-linked. Male SLC6A8-deficient patients are therefore affected, while female heterozygous SLC6A8-deficient patients can present a wide phenotypic variability, from mild symptoms to severe disease, due to the random inactivation of the X chromosomes [37–40]. While about 120 GAMT-deficient patients and less than 20 AGAT deficiencies have been documented, SLC6A8 deficiency appears to represent the second largest cause of X-linked intellectual disability behind Fragile X syndrome with a prevalence of 1–2% of males with intellectual disability [41–45]. These three diseases are characterized by the absence, or very strong decrease, of Cr in the brain when measured by ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS) [43,46,47]. CNS is the primary tissue affected and patients show neurological symptoms already in infancy, presenting intellectual and developmental delays (ID/DD) and problems of speech acquisition. Additional seizures, extrapyramidal movement and behavioral disorders including autistic and auto-mutilating behaviors as well as hyperaggressivity are present especially for GAMT- and

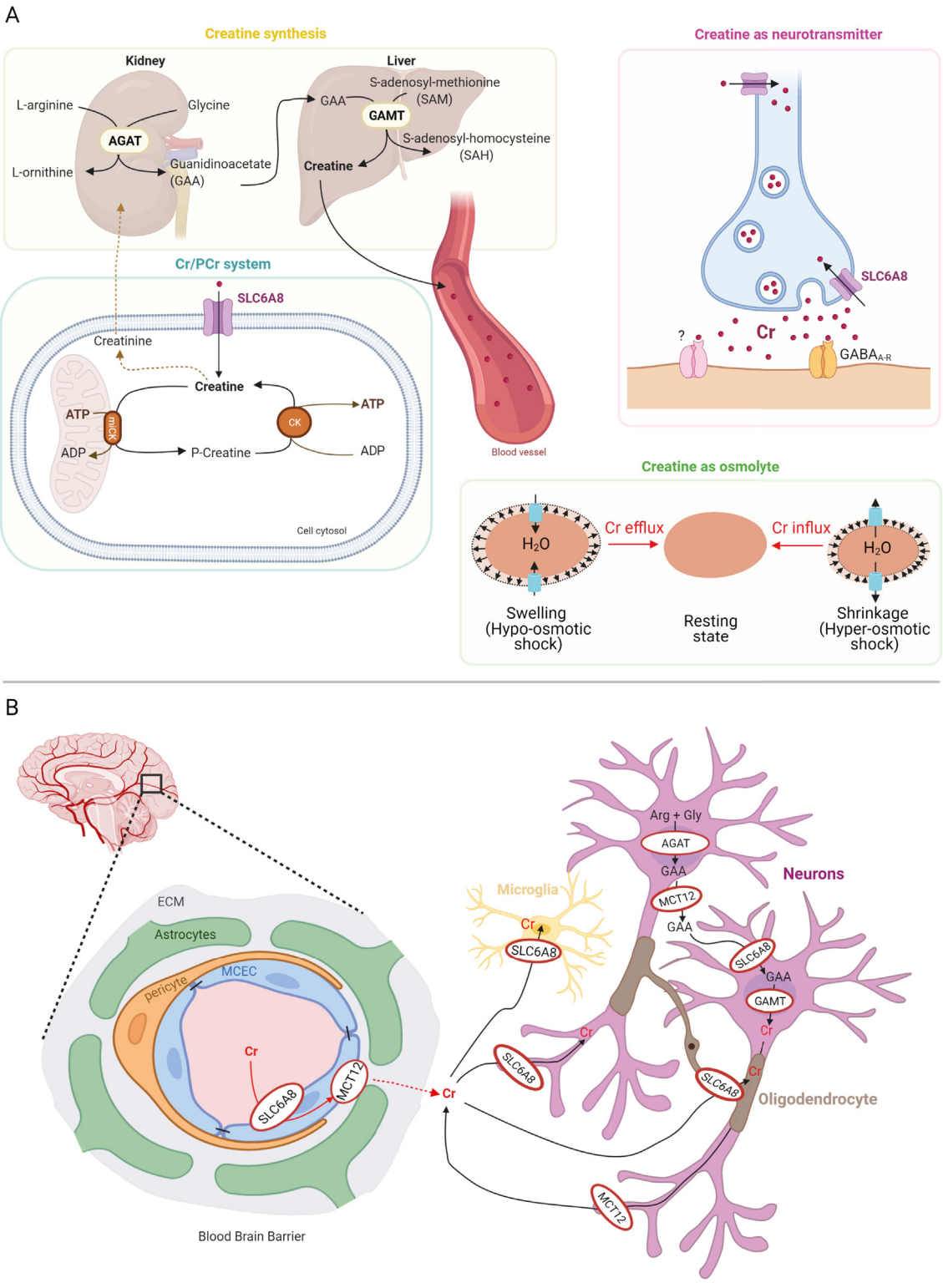

Fig. 1. A: Cr synthesis and transport and its roles in Cr/PCr/CK system, neurotransmission and osmoregulation. Within the kidneys, AGAT allows the formation of GAA, which is transferred and processed in the liver by GAMT resulting to the formation of Cr. Cr is released into circulation to reach the different tissue such as skeletal muscle, brain, kidney and heart. Cells take up Cr by a specific transporter (SLC6A8). Cr can be converted into phosphocreatine (PCr) by mitochondrial (mtCK) or cytosolic (CK) creatine kinase. To maintain the adenosine triphosphate (ATP) / adenosine diphosphate (ADP) ratio through ATP resynthesis or buffering, the cellular Cr/PCr pool is utilized. Cr is metabolized into Crn *via* a non-enzymatic spontaneous reaction. Crn is transported to the kidney from where it is excreted in the urine. Cr is also suggested to be a neurotrasmotter as it can be synthesized in neurons, released in an action-potential manner and act as partial agonist of post-synaptic GABAA-R and possibly other receptors. Cr is one of the main cellular osmolytes in CNS. B: Cr transport and synthesis in CNS. Because of the very low permeability of BBB for Cr (absence of SLC6A8 in the astrocytic feet ensheathing BBB), the brain must ensure its own endogenous synthesis through AGAT and GAMT expression to meet its Cr needs. AGAT and GAMT are found expressed in neurons (in violet), oligodendrocytes (in brown) and astrocytes (in green) but are rarely co-expressed in the same cell, implying that GAA must be transported (through the same SLC6A8 transporter) from AGAT- to GAMT-expressing cells to complete the Cr synthesis pathway. Created with BioRender.com.

Table 1
Clinical and biochemical features of AGAT, GAMT and SLC6A8 deficiencies.

	GAMT deficiency	AGAT deficiency	SLC6A8 deficiency
Date of 1st publication	1994[a]	2001[b]	2001[c]
Chromosome localization	19p 13.3	15q 15.3	Xq28
Inheritance pattern	Autosomal recessive	Autosomal recessive	X-linked
Number of cases	~120	~20	1−2 % of male ID
Symptoms			
ID/DD	+++	+++	+++
Speech delay	+++	+++	+++
Autism spectrum	+++	−	+++
Epilepsy	++	−	+
Movement disorder	+	−	±
Myopathy	−	+	+
Biochemical features	CNS Cr deficiency ↑ GAA (P, U, CSF)	CNS Cr deficiency ↓ GAA (P, U, CSF)	CNS Cr deficiency ↑ Cr/Crn (U)
Treatment available	High Cr doses (400 mg/kg/day) Orn supplementation Arg restriction Pre-symptomatic treatment possible	High Cr doses (200–400 mg/kg/day) Pre-symptomatic treatment possible	No satisfactory treatment so far. However, Cr (100–400 mg/kg/day) beneficial for some patients (females in particular), combined or not with Arg and Gly

Arg, arginine; *CNS*, Central nervous system; *Cr*, creatine; *Crn*, creatinine; *CSF*, cerebrospinal fluid; *GAA*, guanidinoacetate; *Gly*, glycine; *ID/DD*, intellectual delay and developmental delay; *Orn*, ornithine; *P*, plasma; *U*, urine. Modified from Salomons et al. 2003 [127] and Stöckler et al. 2014 [57].
[a] Stöckler et al. 1994 [37].
[b] Salomons et al. 2001 [39].
[c] Item et al. 2001 [38].

sometime for SLC6A8-deficient patients [48–51]. Non-neurological features of CDS are described below for each disease.

1.3. GAMT deficiency

The first CDS discovered was GAMT deficiency (OMIM #612736) [37,43,48,52,53]. GAMT deficiency results in a lack of brain Cr and an accumulation of GAA observable in plasma, urine and CSF. Patients with GAMT deficiency exhibit the most complex phenotype of CDS, including intractable seizures, extrapyramidal disorders and behavioral disorders such as autistic-like or self-aggressive behaviors, and hyperactivity [46,54,55]. These severe symptoms are due to the accumulation of GAA, in particular in cerebrospinal fluid (CSF) and brain parenchyma (Table 1). GAMT-deficient patients can present with muscular hypotonia and reduced muscle mass.

1.4. SLC6A8 deficiency

SLC6A8 deficiency (CTD; OMIM #300352) was the second discovered CDS [39]. As for other CDS and despite AGAT and GAMT expression in their CNS, brain Cr deficiency is observed in SLC6A8-deficient patients by ^{1}H-MRS. This is due to the brain dissociated expression of AGAT and GAMT (as in periphery), and the need of SLC6A8 to complete brain Cr synthesis [16]. Interestingly normal levels of Cr are usually measured in CSF of these patients, which appears to be due to the small proportion of brain cells co-expressing AGAT and GAMT [56,57,59]. Cr and GAA levels are generally normal in plasma of SLC6A8-deficient patients, while the Cr/Crn ratio in urine is typically elevated [60] (Table 1). ID/DD and speech development retardation are also observed in SLC6A8 deficiency. Moreover, as in GAMT deficiency, some SLC6A8-deficient patients may also present seizures and behavioral disorders probably caused by the increased GAA brain levels in absence of a functional SLC6A8 to complete the Cr synthetic pathway [10,61] (Table 1). Other clinical manifestations including failure to thrive, skinny physique/low muscle mass, hypotonia, gastrointestinal features (feeding difficulties, vomiting, diarrhea) and dysmorphic facial features (broad forehead, mid-face hypoplasia, ptosis, short nose) can be observed in these patients [62]. It was also suggested that SLC6A8 deficiency may protect from the development of metabolic syndrome caused by diet-induced obesity [63]. Recently, prolonged QTc and abnormal echocardiographic parameters were observed in some male subjects with SLC6A8 deficiency as well as in a *Slc6a8* KO mice [64].

1.5. AGAT deficiency

AGAT deficiency (OMIM #612718) was the third and last discovered CDS [38]. Very low GAA levels in plasma, urine and CSF are characteristic markers of the disease [49,57,65]. Myopathy is observed in AGAT deficiency. In contrast to GAMT deficiency, no behavioral or extrapyramidal disorders are found in AGAT-deficient patients [57,65] (Table 1). From studies in AGAT-deficient mice, it has been suggested that AGAT deficiency may also protect from metabolic syndrome [63].

2. Treatments of CDS

2.1. Creatine supplementation to treat AGAT and GAMT deficiencies

AGAT and GAMT deficiencies are treatable by oral Cr administration. Since AGAT- and GAMT-deficient patients lack the functional brain endogenous Cr synthesis pathway, but still express SLC6A8 at BBB, this treatment restore their brain Cr and clinically improves their neurological status. However, due to low permeability of BBB for Cr, a long period of treatment needs to be carried out with high doses of Cr (200–400 mg Cr/kg/day; 50–100 times the normal needs in Cr), allowing to partially restore their brain Cr pool [38,49,62,66,67]. These high doses of Cr supplementation are well tolerated in general. However, as urinary Cr crystals can be observed in patients supplemented with higher doses of Cr (800 mg/kg/day), patients should be monitored regularly for the presence of Cr crystals and urinary tract infection [43,68].

Upon Cr supplementation, the majority of GAMT-deficient patients show important clinical improvements in several domains of ID (behavior, language and self-supportive skill) as well as in epilepsy and movement disorders. Importantly, these improvements were reported in patients with severe, moderate and mild ID [43]. Similar outcomes were reported for AGAT-deficient patients, resulting in almost complete restoration of brain Cr levels and significant improvement of myopathy [68]. For GAMT-deficient patients, Cr supplementation has the interesting property to lower GAA levels, through retroregulation on AGAT expression and/or activity, depending on the tissue. This has been demonstrated *in vivo* in chicken liver as well as human and rat kidney [58,128,129], and in organotypic cultures of brain tissue including a GAMT-deficient model [130,131].

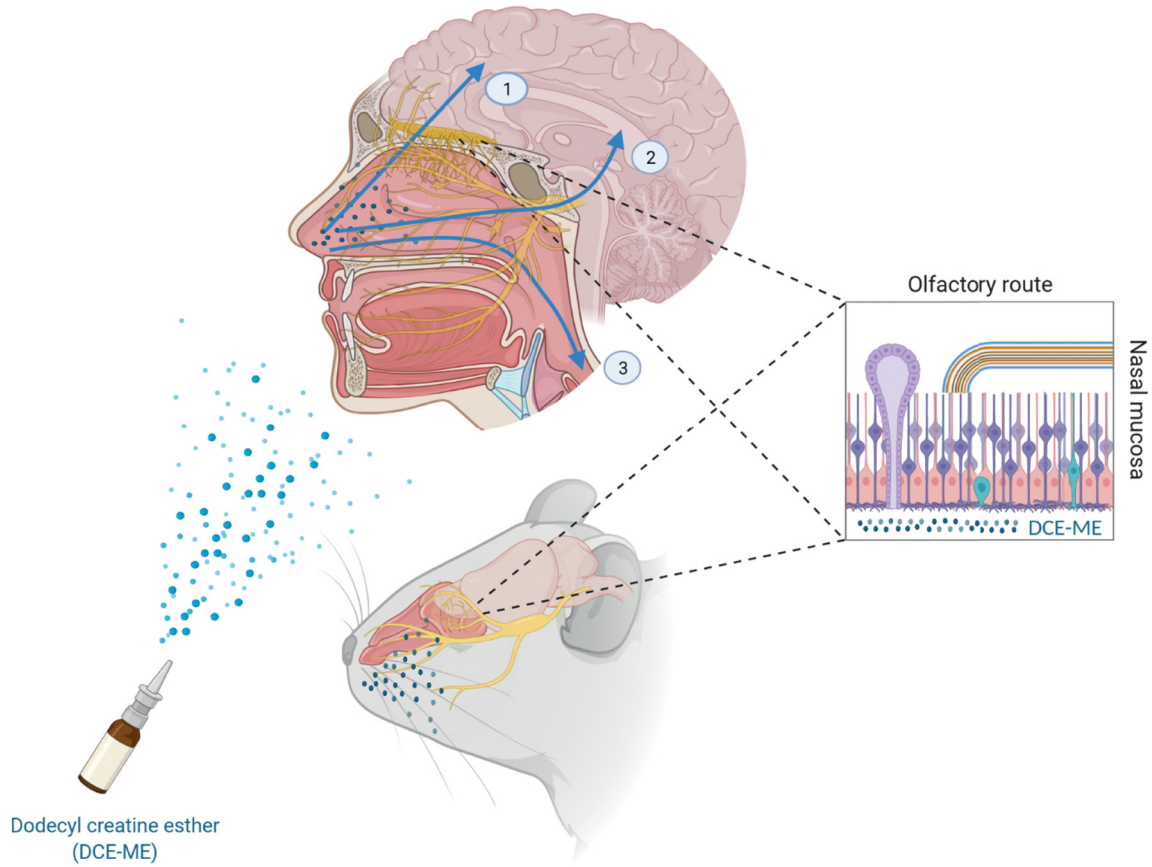

Fig. 2. : Nose-to-brain drug delivery in both the human and rodent brain.
The replenishment of Cr followed by improvement of cognitive function might be achieved through the transport of DCE-loaded vesicles by either (1) the direct pathway through olfactory bulbs, (2) the trigeminal nerve or (3) through the indirect pathway involving the transport of DCE-loaded vesicles *via* the systemic pathway. *DCE*: dodecyl-creatine ester. Created with BioRender.com.

2.2. Additional GAA-lowering strategy in GAMT deficiency

The neurological outcome of GAMT-deficient patients can further be improved thanks to the development of GAA-lowering strategies through Orn supplementation and/or Arg or dietary protein restriction in order to inhibit or reverse AGAT reaction. This allows, in particular, the almost complete elimination of severe seizures [69].

2.3. Pre-symptomatic treatment for AGAT and GAMT deficiencies, and newborn screening

As for many IEM affecting CNS development, therapy, if available, needs to be started early in life to prevent irreversible brain damage. The prenatal diagnosis of AGAT- and GAMT-deficient patients (performed due to older affected siblings) allows the pre-symptomatic treatment with oral Cr and appears to prevent the installation of symptoms while protecting brain development [43,70,71]. Since the pre-symptomatic treatments appear to improve the outcome of AGAT- and GAMT-deficient patients, even leading to complete normal development, GAMT deficiency has been proposed to enter newborn screening (NBS) programs in several countries (currently performed in parts of Australia, Canada and some states in USA such as Utah, New York and Michigan; foreseen in others such as The Netherlands) [72]. AGAT deficiency has not been proposed for NBS because of its rarity and of its difficulty of detection on dry blood spots (extremely low or undetectable levels of GAA), while GAMT deficiency is more frequent and detected through increased GAA [72]. The rise and progresses in genomic NBS, currently in development in several countries, may well prove very useful for CDS, particularly for CTD.

As for SLC6A8 deficiency (see below), new treatment strategies are also developed for GAMT and AGAT deficiencies, in particular making use of gene therapy through adeno-associated virus (AAV) vectors.

2.4. The challenge of treating SLC6A8 deficiency

Despite 20 years of research, no efficient treatment for SLC6A8-deficient patients has been found so far. Indeed, oral supplementation of Cr is completely inefficient in replenishing their brain Cr [62,73–75]. The main cause for this is probably that SLC6A8, expressed on BBB, appears as the only mean for Cr to enter into CNS from periphery [59]. Treating SLC6A8 deficiency is in fact a difficult challenge, due to the necessity, for the treating molecules (be it Cr or others) to cross up to three (without astrocytes) or five (through astrocytes) membranes, from microcapillary endothelial cells at BBB to surrounding astrocytes and to brain cells within CNS parenchyma (*e.g.* neurons and oligodendrocytes): 3 uptake and 2 release mechanisms (Fig. 1).

2.5. Creatine, creatine precursors, creatine derivatives and betaine

In contrast to the other two CDS, dietary supplementation in SLC6A8 patients showed limited success to rescue Cr levels in the brain. Cr supplementation (100–400 mg/kg/day), alone or combined with Arg, Gly and/or S-adenosylmethionine precursors, failed to restore brain Cr content and to ameliorate clinical parameters in the majority of patients with SLC6A8 deficiency [50,73,74,76–81]. However, a systematic review on 28 CTD patients showed that 10 of them reported improvement of their condition after combined Cr, Arg and Gly therapy [82]. Among these patients, increase in cerebral Cr ($n = 5$) as well as improved

cognitive and developmental abilities ($n = 7$), psychiatric disturbance ($n = 3$), epilepsy ($n = 4$) and gross motor functions (n = 7) were observed. Moreover, three male patients reported muscle mass change with weight increase. All the patients showing increased cerebral Cr demonstrated improved development and/or adaptive functions, supporting the relationship between increased cerebral Cr and improved clinical outcome. Furthermore, a recent international retrospective cohort study demonstrated that among patients under combined treatment with Cr, Arg and Gly, two female patients improved their clinical severity score while only subjective improvements were reported for the behavior of male patients. It was concluded that this combined treatment might have stopped the disease progression in males, while the phenotypic improvement in females could be due to their *SLC6A8* heterozygous status keeping some residual activity of Cr transporter at BBB [83]. For those patients who appear to respond to combined Cr, Arg, Gly and/or S-adenosylmethionine supplementation,

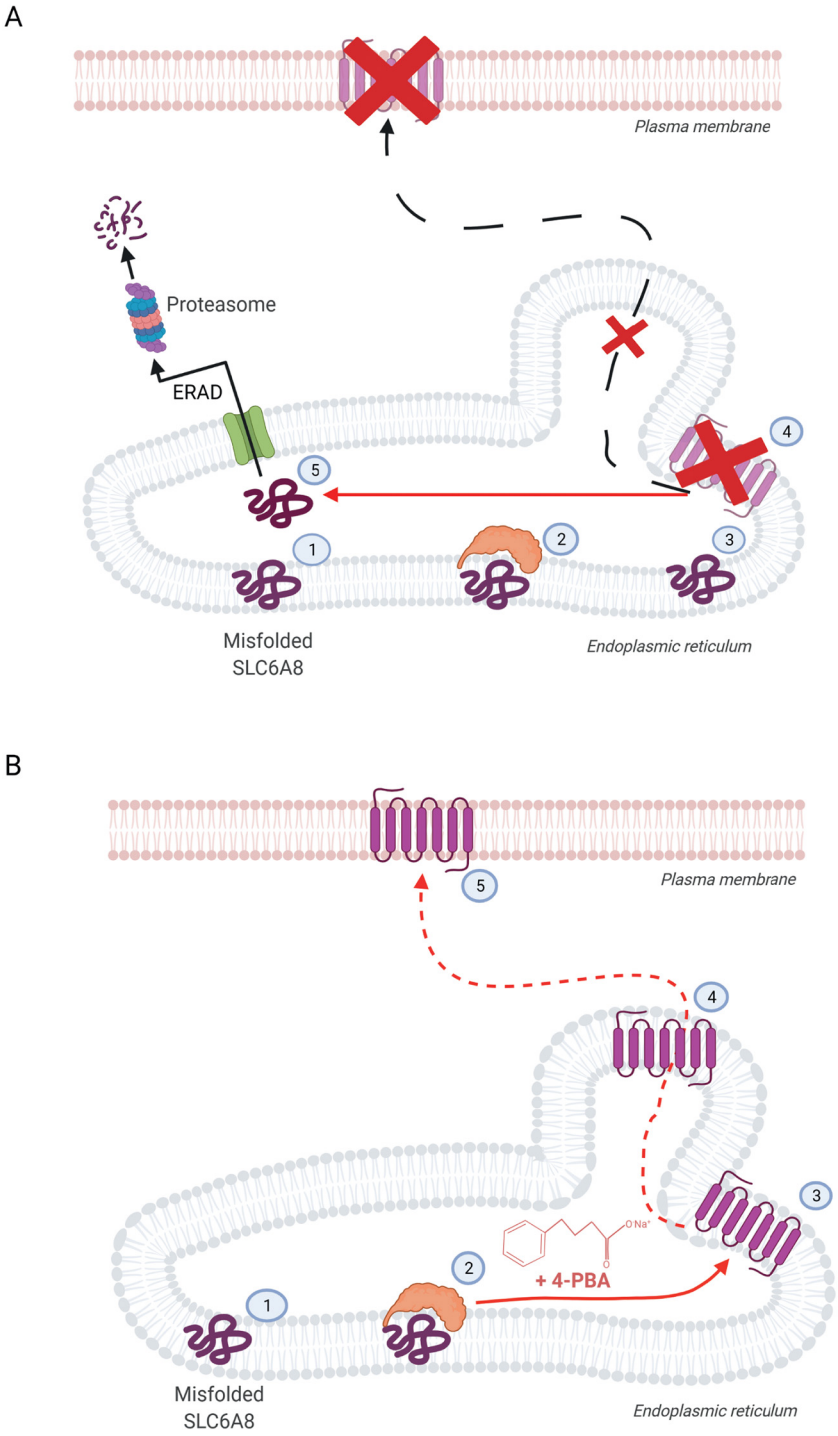

Fig. 3. Treatment by pharmacochaperones.
A: Misfolded proteins remain bound to calnexin (in orange) and then are degraded by the ER-associated degradation systems (ERAD) towards proteasome.
B: Pharmacological chaperones, as 4-PBA, are small-molecules ligands that selectively bind and stabilize the misfolding of the mutated protein (SLC6A8 transporter), allow its translocation to the appropriate cellular localization (i.e plasma membrane) and may make it regain its activity. Created with BioRender.com.

Table 2
Main features of the most commonly used viral vectors for CNS gene therapy.

	Adeno-associated virus (AAV) [a]	Adenovirus (Ad)	Lentivirus	Herpes simplex virus (HSV)
Genome	4.8 Kb (ssDNA)	36 Kb (dsDNA)	9 Kb (ssRNA)	152 kb (dsDNA)
Packaging capacity	4.7 Kb	7.5 Kb	9 Kb	>30 Kb
Infection	Most dividing and non-dividing cells	Most dividing and non-dividing cells	Most dividing and non-dividing cells	Most dividing and non-dividing cells
Transduction efficiency	Moderate	High	Moderate	High
Host genome integration	No	No	Yes	No
Expression	Potentially long lasting	Transient	Long lasting	Potentially long lasting
Immunogenicity	Very low	High	Low	Low

[a] The most frequently used serotypes for gene transfer to the CNS have been AAV1, AAV2, AAV5, AAV9 and AAVrh.10; Piguet et al. 2017 [118].

new treatment modalities should be developed for their long-term administration, as it was shown that combined Arg and Gly supplementation can induce adverse effects in CTD patients, like hyperhomocysteinemia [84]. Besides the positive effects of Cr supplementation, safety on a long-term administration should be carefully monitored with the assessment of liver and kidney functions, plasma amino acids analysis, as well as Cr, GAA, folate and homocysteine status.

Importantly, future research should also focus on the dose-effect relationship in children and in adult patients to assess Cr potential adverse effects. This last point is essential and should be carefully evaluated, as CDS therapeutic strategies aiming at reestablishing sufficient amounts of Cr (in CNS in particular) may lead to local (central and peripheral) excessive levels of Cr being toxic or playing adverse effects.

To find a treatment for SLC6A8-deficient patients, a lot of effort has been performed to develop more lipophilic derivatives of Cr that would cross BBB more easily, possibly by simple trans-membrane diffusion, independently of SLC6A8 transporter. Even though some derivatives (N-amidino-piperidine) were harmful, others (Cr-Mg-complex acetate, PCr-Mg- complex acetate, Cr ethyl ester, Cr amino acids methyl esters, Cr ascorbate) showed interesting neuroprotective effects in hippocampal organotypic cultures [85–87]. PCr-Mg-complex acetate and Cr benzyl ester were demonstrated to cross membranes without the help of SLC6A8 [88]. More interestingly, PCr-Mg-complex acetate and creatinyl amino acids showed neuroprotective effects *in vivo* in rodents, suggesting a crossing of BBB [89,90]. Cyclocreatine, another Cr derivative, appeared promising to treat CNS-specific *Slc6a8*$^{-/y}$ KO mice, since it improved their cognitive abilities [91] and ameliorated the cognitive, autistic and epileptic phenotype in *Slc6a8*$^{-/y}$ KO mice [92]. Among Cr derivatives, only Cr ethyl ester has been assayed so far in treatment trial on SLC6A8-deficient patients, and it failed to replenish brain Cr concentration as well as to improve patients' neurological status [93].

Recently betaine supplementation was reported to have positive effects on two adult SLC6A8-deficient patients, who improved their verbal and motor functions, including feeding and balance. However, further studies are needed on a larger number of patients to evaluate the potential beneficial effects of betaine [94].

Altogether, these data emphasize the need for novel more effective treatment strategies for SLC6A8-deficient patients (Table 3). For this reason, actual research lines develop several therapeutic approaches, in particular through use of the available *in vivo* models for SLC6A8 deficiency: nose-to-brain drug delivery, use of chemical chaperones and gene therapy.

2.6. Potential new therapeutic avenues for SLC6A8 deficiency

2.6.1. Nose-to brain drug delivery

Dodecyl creatine esther (DCE), as Cr derivative encapsulated in lipid nanovesicles, was demonstrated as a promising drug to treat SLC6A8 deficiency in an *vitro* cell-based BBB model [95,96]. Moreover, intracerebroventricular administration of DCE alone increased Cr content in different brain regions and improved object recognition in *Slc6a8*$^{-/y}$

Table 3
Tested and promising treatments for SLC6A8 deficiency.

SLC6A8 deficiency		
		Outcome
Tested treatments		
Cr, Cr derivatives and betaine	Cr	Treatment of male and female CTD patients not successful (no brain Cr restoration); only 1 heterozygous female showed mild improvement on neuropsychological testing after 18 months of treatment[a]
	Cr derivatives	No improvement of neurological status[a]
	Betaine	Improvement of verbal and motor function as well as feeding and balance in two adults patients[a]
Enhancing Cr synthesis in CNS	Arg and Gly	Successful in one heterozygous female patient with intractable epilepsy[a]
	Combined Cr, Arg and Gly	In most cases no normalization of intracerebral Cr or clinical outcome. However 36% CTD patients showed clinical response to treatment (18% increased brain Cr / 36% clinical improvement / on a total of 28 patients)[a]
	Combined Cr, Arg, Gly and S-adenosylmethionine	No brain Cr restoration. However improvement in speech/language skills and muscle mass[a]
Promising treatment		
Overcoming BBB	Nose to brain drug delivery	Intranasal administration of DCE-ME improves object recognition learning in Slc6a8$^{-/y}$ mice[b]
	Pharmacochaperones	Treatment with 4-PBA restores the activity of several CTD-inducing variants[c]
Correcting SLC6A8 gene defect	Gene therapies with AAV vectors	Several approaches in progress

CTD, Creatine transporter deficiency; DCE-ME, micro-emulsion of dodecylcreatine esther; 4-PBA, 4-phenylbutyric acid.
[a] DeGrauw et al. 2002 [50]; Van de Kamp et al. 2014 [53]; Jaggumantri et al. 2015 [73]; Van de Kamp et al. 2012 [75]; Cecil et al. 2001 [76]; Bizzi et al. 2002 [77]; Póo-Argüelles et al. 2006 [78]; Fons et al. 2008 [79]; Valayannopoulos et al. 2012 [74]; Chilosi et al. 2008 [80]; Wilcken et al. 2008 [81]; Dunbar et al. 2014 [82]; Schjelderup et al. 2021 [94].
[b] Ullio-Gamboa et al. 2019 [97].
[c] El-Kasaby et al. 2019 [100].

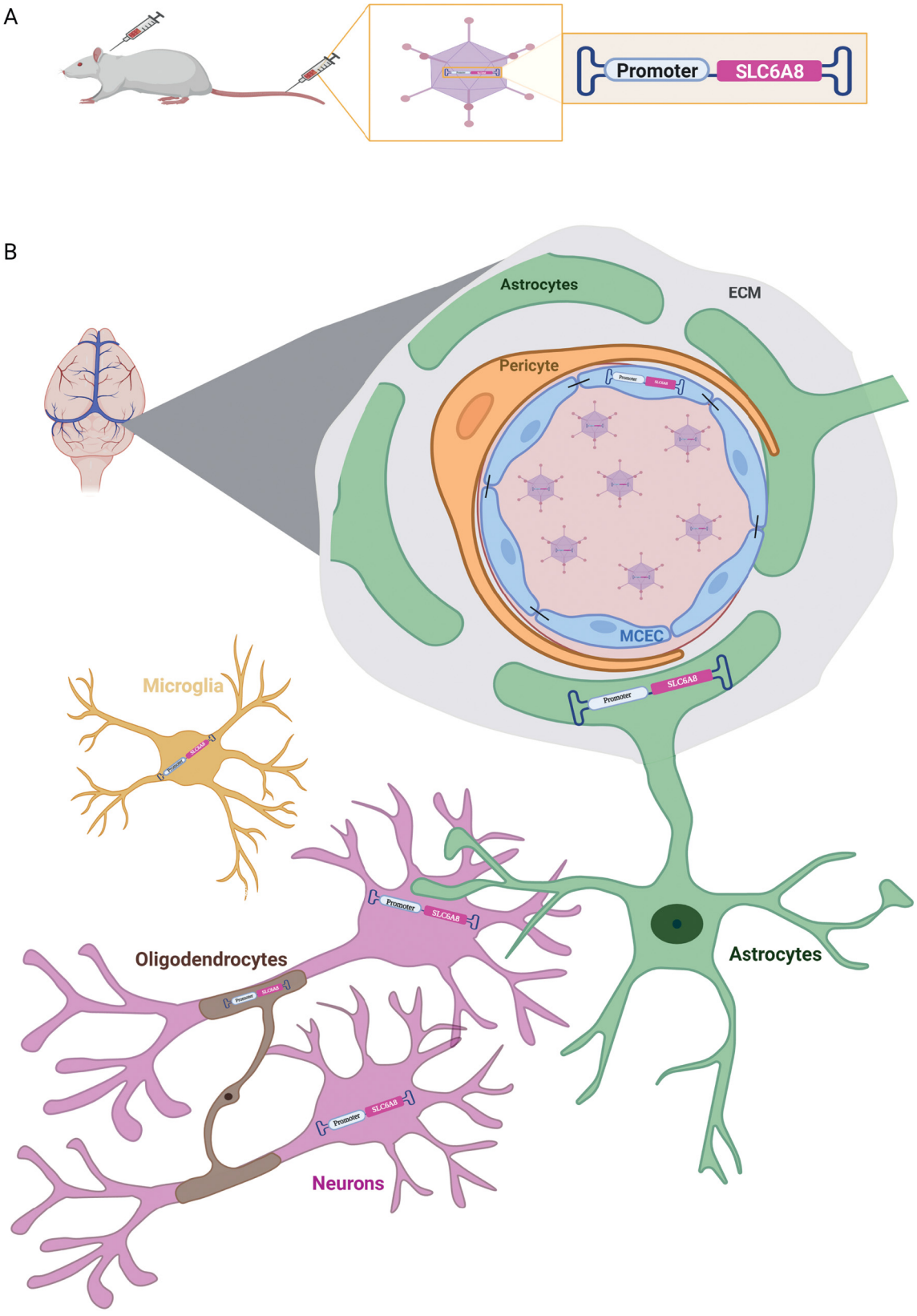

Fig. 4. AAV-based CNS treatment for SLC6A8 transporter by systemic or intrathecal injection.
A: A schematic representation of the AAV vector containing the creatine transporter (SLC6A8) injected by systemic or intrathecal delivery route.
B: A schematic representation of blood-brain barrier (BBB) and AAV delivery from the systemic or intrathecal injection in a rat to target the different brain cells: astrocytes (green), neurons (violet), oligodendrocytes (brown), microglia (yellow) and micro-capillary endothelial cells (MCEC, blue). Created with BioRender.com.

KO mice. Furthermore, Ullio-Gamboa *et al* [86] developed a new therapeutic approach based on a microemulsion of DCE (DCE-ME) in nanovesicles to improved DCE delivery and membrane transport using intranasal administration to take advantage of the potential nose-to-brain drug delivery (Fig. 2 and Table 3). Intranasal treatment with DCE-ME for 10 days improved novel object performance deficits in *Slc6a8*$^{-/y}$ mice and increased synaptic hippocampus markers such as PSD95, BDNF and CREB. These results suggest DCE-ME as a potential promising drug for the treatment of SLC6A8 deficiency [97].

2.6.2. Pharmacochaperones

Missense mutations producing misfolded proteins mutants account for a wide variety of genetic diseases. These misfolded mutant proteins are often not addressed to their correct localization, and often remain trapped in the endoplasmic reticulum (ER) [98]. Pharmacological and chemical chaperones are small molecules that prevent/correct the misfolding of the mutated protein, allow its translocation to the appropriate cellular localization and may make it regain its activity [99] (Fig. 3). The use of pharmacochaperones holds great promises as a novel therapeutic venue for the patients with missense mutation of SLC6A8 variants (Table 3). So far, the chemical chaperone 4-phenylbutyric acid (4-PBA) was the most effective molecule tested *in vitro* on several folding deficient SLC6A8 variants. Over a third of 16 known misfolded SLC6A8 variants were responsive to the functional rescue by 4-PBA treatment, the Cr transporter being re-addressed on the plasma membrane and regaining is Cr uptake activity [100,101].

2.6.3. Gene therapy

Gene therapy also appears as one of the promising avenues to treat CDS, and CTD in particular. Strategies making use of viral vectors, gene editing, iPSCs/cellular therapies or RNA therapy are being developed to treat inherited metabolic diseases. Some of these techniques (*e.g.* gene editing, iPSCs/cellular therapies and RNA therapy) are already tested for diseases such as ornithine transcarbamylase or methylmalonic acidemia, in which selected tissues (*e.g.* liver and/or kidney) are targeted. For CDS, and in particular SLC6A8 deficiency, the aim of targeting CNS (with its difficult access) as well as peripheral tissues renders the use of viral vectors transducing the corrected gene expression more suitable. The viral vectors most commonly used for targeting CNS are herpes simplex virus (HSV), adenovirus (Ad), adeno-associated viruses (AAV) as well as retroviral and lentiviral (LV) vectors [102–105]. These vectors differ in payload capacity, cell tropism and ability to integrate into the host genome, which may affect the duration of transgene expression (Table 2).

2.6.4. Adeno-associated viruses

Adeno-associated viruses (AAV) have emerged as some of the safest and most commonly used vectors for the delivery of therapeutic genes due to their high safety *versus* low elicited immune response and low inflammatory properties. They have reached the clinical trials with promising results in a number of pathologies such as Parkinson's disease or spinal muscular atrophy (SMA) [106,107].

AAV are small, non-enveloped viruses belonging to the *Parvoviridae* family. In absence of another helper virus, AAV cannot replicate and establish a latent infection within the cell [108]. They are easily manipulated to package a transgene of interest into a recombinant AAV for the purpose of gene delivery. However, due to their small genome, the size of the transgene that can be packaged is limited. More precisely, the use of a single-stranded AAV (ssAAV) vector allow the delivery of a transgene of 4.4 kb maximal length, while using a double-stranded self-complementary AAV (scAAV) vector only authorizes half this length (2.2 kb) [109]. A wide variety of AAV serotypes exist (AAV1–10), each with their own specific cellular tropism and efficiency both depending on genome composition and delivery route [110].

For SLC6A8 deficiency, the main goal should be to re-establish the expression of a functional SLC6A8 transporter in the different brain cells normally expressing it (BBB, neurons and oligodendrocytes), allowing the brain of CTD patients to recover sufficient amounts of Cr in order to improve their neurological outcome (Fig. 4 and Table 3).

One of the challenges for gene delivery is to identify vectors targeting the brain that are able to cross the BBB, so that a gene therapy can be administered peripherally. Thanks to the works of Duque *et al* and Foust *et al* [111,112], it is known that AAV9 can cross BBB in mice and cats when injected intravenously in both neonatal and adult animals. Moreover, after intravenous injection of AAV9 vectors, both neurons and astrocytes were transduced. This demonstrated that it is possible to deliver gene therapy to larger portion of the brain and spinal cord without having to inject directly into the CNS. Translation of AAV9 gene therapy from small animals to humans leads to additional challenges due to the occurrence of anti-AAV9 neutralizing antibodies in the human population and to the potential off-target distribution of the virus in peripheral tissues. However, for IEM diseases affecting the brain, an off-target distribution of virus to peripheral tissues may not be detrimental since in most cases these diseases also affect peripheral tissues. In the recent years, another important point emerging concerns the safety use of AAV. High vector copy dose-related hepatotoxicity has been reported in gene therapy trials for X-linked myotubular myopathy, SMA and Duchenne muscular dystrophy [113,114]. Additional preclinical studies are thus needed to determine the safest AAV doses to use, in particular for intravenous injections.

To avoid peripheral off-target effects, one strategy is the use of direct intra-CSF viral delivery *via* intrathecal injection into the lumbar cistern or cisterna magna [109,115–118]. In a comparative study of AAV9 delivery routes in non-human primates, a greater CNS transduction was demonstrated by using low dose intra-CSF injection than through higher dose intravascular injections [119]. However a viral distribution to peripheral organs was also detected, although at much lower levels than seen with intravascular injection [117,119]. Overall, multiple groups have demonstrated that intra-CSF delivery of AAV9 vectors results in widespread expression of transgenes in large animals and support the use of intra-CSF AAV9 vector delivery for gene therapy in humans [120].

However, the systemic injection of therapeutic AAV also appears as a promising treatment approach for brain diseases. It is less invasive than the intrathecal delivery routes, and allows the transduction of the global CNS, which can be an advantage to treat diseases involving multiple brain lesions. Preclinical and clinical trials have already been conducted with the use of systemic injections of therapeutic AAV to treat diseases affecting nervous tissue such as late-onset Pompe disease, GM1 gangliosidosis, spinal muscular atrophy (SMA) and phenylketonuria [121–124].

To achieve sufficient levels of transgene expression across target tissues, especially within CNS, the AAV vector doses to use vary depending on the delivery route. For intravenous injection, an average dose of 10^{13} vg/kg was demonstrated efficient in different animal models (rodents and non-human primates), while a 10 times lower dose is generally chosen for intra-thecal delivery route [103,111,112,115,116,119,125,126].

3. Conclusion

In conclusion, several new promising routes are being developed to ameliorate existing therapies for CDS, and to find new treatments for the so far challenging CTD (Table 3). These new strategies may involve the use of the nose-to-brain delivery, pharmacochaperones as well as gene therapy through AAV vectors.

Declaration of Competing Interest

None.

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