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Post-transcriptional regulation of the creatine transporter gene: Functional relevance of alternative splicing

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ABSTRACT

Background: Aberrations in about 10–15% of X-chromosome genes account for intellectual disability (ID); with a prevalence of 1–3% (Gécz et al., 2009 [1]). The *SLC6A8* gene, mapped to Xq28, encodes the creatine transporter (CTR1). Mutations in *SLC6A8*, and the ensuing decrease in brain creatine, lead to co-occurrence of speech/language delay, autism-like behaviors and epilepsy with ID. A splice variant of *SLC6A8*–*SLC6A8C*, containing intron 4 and exons 5–13, was identified. Herein, we report the identification of a novel variant — *SLC6A8D*, and functional relevance of these isoforms.
Methods: Via (quantitative) RT-PCR, uptake assays, and confocal microscopy, we investigated their expression and function vis-à-vis creatine transport.
Results: SLC6A8D is homologous to *SLC6A8C* except for a deletion of exon 9 (without occurrence of a frame shift). Both contain an open reading frame encoding a truncated protein but otherwise identical to CTR1. Like *SLC6A8*, both variants are predominantly expressed in tissues with high energy requirement. Our experiments reveal that these truncated isoforms do not transport creatine. However, in *SLC6A8* (CTR1)-overexpressing cells, a subsequent infection (transduction) with viral constructs encoding either the *SLC6A8C* (CTR4) or *SLC6A8D* (CTR5) isoform resulted in a significant increase in creatine accumulation compared to CTR1 cells re-infected with viral constructs containing the empty vector. Moreover, transient transfection of CTR4 or CTR5 into HEK293 cells resulted in significantly higher creatine uptake.
Conclusions: CTR4 and CTR5 are possible regulators of the creatine transporter since their overexpression results in upregulated CTR1 protein and creatine uptake.
General significance: Provides added insight into the mechanism(s) of creatine transport regulation.

1. Introduction

Genetic deficiencies involving *AGAT* (OMIM ID: 602360), *GAMT* (OMIM ID: 601240) or *SLC6A8* (OMIM ID: 300036), make up the creatine deficiency syndromes — a group of inborn errors of metabolism with symptoms such as intellectual disability, autism-like behavior and epilepsy [2–4]. Intellectual disability, with a prevalence of 1-3% [1], is also the main symptomatic of defective creatine synthesis or transport. Creatine supplementation is the main therapeutic approach, with success (notably improved cognitive function) in treating AGAT and GAMT deficiencies [5]. The poor permeability of the blood–brain barrier to creatine (reviewed in [6]) renders creatine supplementation ineffective for treating *SLC6A8* deficiency. A recent comprehensive overview of creatine metabolism and transport in relation to CNS function is reviewed in Braissant et al. [45]. Congenital creatine deficiency aside, creatine has also been used to treat mitochondrial and muscular diseases, to alleviate brain and spinal cord injuries and to improve mental performance [7]. Creatine has also been employed as a neuroprotective agent in animal models of Parkinson's and Huntington's diseases [8]. In a more recent study [9], overexpression of the creatine transporter in mice resulted in protection against acute myocardial infarction. Consequently insight into the mechanisms of how the transporter is regulated is valuable.

Alternative splicing is one of the most important mechanisms regulating gene expression. It enables diversification of one gene into different protein products and is thought to provide a molecular mechanism for fine-tuning the gene functions of a single locus [10,11]. Contrary to the expected minor role of alternative splicing in functional regulation, large scale sequencing- and bioinformatics-based studies have reported that it occurs in up to 94% of human genes [12,13]. The presence of a novel *SLC6A8* splice variant (*SLC6A8C*), was revealed in primary fibroblasts of different individuals and in different human tissues [14]. The authors were however unable to detect *SLC6A8B* (GenBank: U17986) — the first identified splice variant

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of *SLC6A8*. In the present study, we report the identification in human and mouse, of a new variant (*SLC6A8D*) of presumably the *SLC6A8C* mRNA, with an in-frame deletion of exon 9. In order to investigate the functional relevance of alternative splicing of *SLC6A8* in terms of creatine uptake and/or regulation of the full-length creatine transporter, we generated recombinant mouse 3T3 Swiss cells overexpressing each splice variant, the full length transporter, as well as cells co-expressing the full length transporter with either one or the other splice isoform.

2. Materials and methods

2.1. Identification of SLC6A8D transcript

Total RNA was extracted (RNA isolation kit; Promega) from HEK-293 cells (since *SLC6A8* expression is high in kidney) and from primary human fibroblasts obtained from both controls and a patient with a genomic deletion encompassing the entire *SLC6A8* gene. cDNA synthesis (Fermentas) was performed (with and without reverse transcriptase, to check for gDNA contamination) using 1 μg of RNA. A forward primer complementary to intron 4 and a reverse primer complementary to the 3′UTR of the *SLC6A8* locus were designed to amplify both *SLC6A8C* and *SLC6A8D* from HEK293 cDNA (Table 1, №. 1). Subsequently nested primers were used to detect presence of *SLC6A8C* and *SLC6A8D* transcripts in the amplified PCR products. *SLC6A8D*-specific primers were designed complementary to intron 4 and spanning exon 8/10 such that only messages lacking exon 9 will be amplified (Table 1, №. 2). For detection of specific *SLC6A8C* and *SLC6A8* transcripts, previously designed primers [14] were used (Table 1, №. 3 and №. 4). RT-PCR of all transcripts was performed using KAPA HiFi™ Hotstart DNA polymerase with GC buffer (Kapa Biosystems). The general PCR conditions used were as specified by the manufacturer, except for the inclusion of 0.2 M Betaine in each reaction. Thermocycling was as follows: initial denaturation for 5 min at 95 °C; followed by 38 cycles of 20 s at 98 °C, followed by 15 s at 66 °C (*SLC6A8*, *SLC6A8C*, *SLC6A8D*) or 61 °C (*GAPDH*), and 2 min at 72 °C; with a final extension step of 10 min at 72 °C. Transcript identities were confirmed by sequencing (ABI 3130XL Genetic Analyser; Applied Biosystems).

2.2. Detection of SLC6A8D expression in monkey and mouse

Slc6a8d expression in mouse was investigated in NIH-3T3 2.2 fibroblasts and primary mouse embryonic fibroblasts (MEFs) of FVB mice. Two monkey cell lines (CP132 and Vero) were also included. RT-PCR for the monkey cell lines was performed with same primers and PCR conditions as for the human samples. For screening of the mouse cells, the primers were specifically designed to amplify mouse *slc6a8*-derived sequences (Table 1, №. 6, №. 7, №. 8); consequently the PCR annealing temperature (Ta) was adjusted to 61 °C for *slc6a8d*. As control, amplification of *Slc6a8c* and *Slc6a8* (Ta, 59 °C) was included. All PCR products were gel-purified and sequenced.

2.3. Quantitative real-time PCR (Q-PCR)

Investigation of differential splice variant expression was carried out on RNA isolated from 20 different tissues (FirstChoice™ Human Total RNA survey panel; Ambion). Gene-specific primers and probes used for amplification of each transcript are shown in Table 2.

To investigate if upregulation of creatine uptake in 3T3 Swiss cells co-expressing CTR1 with a splice isoform compared to cells co-expressing CTR1 with an empty vector, was as a result of enhanced *SLC6A8* transcription, standard Q-PCR was performed on an ABI7300 (Applied Biosystems) using a probe (5′-FAM 3′-TAMRA labeled probe: 5′-TGGGTGCTGGTCTACT TCTGTGTC-3′) and primers (5′-AAGTCTTGAGGCTGTCTGG-3′ and 5′-ACGATCTTTCCCGTGGAT-3′) specific for exon 4 of human *SLC6A8*.

All reactions were performed in the presence of 1 M Betaine and ROX reference dye, and corrected for input by normalizing to *GAPDH* (human tissues) or *Gapdh* (mouse 3T3 Swiss cells) gene expression assay (PrimeTime™ Std qPCR Assay; IDT Technologies). Quantification (threshold cycle number, C_T) of both target and reference genes was carried out in triplicate and in independent wells using the $2^{-\Delta Ct}$ method. Analysis was done using the Q-Gene™ software package [15]. The mean normalized expression was obtained by averaging the C_T values of target and reference genes, respectively and subsequent calculation of the standard error of the mean normalized expression [16].

2.4. Construction of pBABE-hygro-SLC6A8-EGFP, pBABE-puro-SLC6A8C-EGFP and pBABE-puro-SLC6A8D-EGFP expression vectors

The open reading frame (ORF) of all isoforms was cloned in-frame to the N-terminal of EGFP (enhanced green fluorescent protein). In order to obtain a *pBP-SLC6A8D-EGFP* construct; RNA was isolated from HEK293 cells followed by cDNA synthesis. The ORF of *SLC6A8D* (exons 7–13) was amplified by RT-PCR using primers with *HindIII* and *EcoRI* restriction site overhangs (Table 1, №. 9), and then cloned into pEGFPN1 by standard cloning techniques. Via PCR (Table 1, №. 11) and site directed mutagenesis, an *EcoRI-SLC6A8D-EGFP-SalI* construct was then shuttled from its pEGFPN1 vector into a *pBABE-puro* (pBP) destination vector. The ORFs

Table 1
PCR and cloning primers.

No.	Species	Forward/reverse sequence	mRNA sequence	*SLC6A8* location	Amplicon size (bp)
1	Human	5′-GAGGTACTGAAAGCCAAGCAATGC-3′ 5′-GCTGGTGATGTGAGCTGAGT-3′	*SLC6A8C/SLC6A8D*	Intron 4 3′ UTR	1969/1831
2	Human/monkey	5′-CTCCCACACCTGCACTGCCC-3′ 5′-GACGTACATCCCGCCCTGGC-3′	*SLC6A8D*	Intron 4 Exon 8/10	827
3	Human/monkey	5′-CTCCCACACCTGCACTGCCC-3′ 5′-GGAGAGATCGATGACAAAGCAG-3′	*SLC6A8C*	Intron 4 Exon 9	938
4	Human/monkey	5′-ATGGCGAAGAAGAGCGCCGAG-3′ 5′-GCTGGTGATGTGAGCTGAGT-3′	*SLC6A8*	Exon 1 3′ UTR	1930
5	Human	5′-ATGGCGAAGAAGAGCGCCGAG-3′ 5′-GCTGGTGATGTGAGCTGAGT-3′	*GAPDH*		819
6	Mouse	5′-CAAGAATGATCTGGAGTTTGGG-3′ 5′-GACGTACATTCCACCCTGGC-3′	*Slc6a8d*	Intron 4 Exon 8/10	944
7	Mouse	5′-CAAGAATGATCTGGAGTTTGGG-3′ 5′-GGAGAGATCGATGACAAAGCAG-3′	*Slc6a8c*	Intron 4 Exon 9	1056
8	Mouse	5′-GGTATCTATAGCGTGTCTGG-3′ 5′-TTACATGACACTCTCCACCACG-3′	*Slc6a8*	Exon 2 Exon 13	1884
9	Human	5′-CCCAAGCTTCCACCATGGCTGCAGAGCAGGGCGTGC-3′ 5′-CGGAATTCGCATGACACTCTCCACCACG-3′	*SLC6A8C/SLC6A8D*	Exon 7 Exon 13	809/671
10	Human	5′-CGGGATCCACCATGGCGAAGAAGAGC-3′ 5′-CGGGATCCCATGACACTCTCCACCACG-3′	*SLC6A8*	Exon 1 Exon 13	1905
11	Recombinant	5′-CGCGAATTCCACCATGGCTGCAGAGCAGGGCGTGC-3′ 5′-CGCGTCGACCTCTACAAATGTGGTATGGCTG-3′	*SLC6A8C-/SLC6A8D-(EGFP)*	Exon 7	1650/1515