Cyclocreatine treatment improves cognition in mice with creatine transporter deficiency

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The second-largest cause of X-linked mental retardation is a deficiency in creatine transporter (CRT; encoded by SLC6A8), which leads to speech and language disorders with severe cognitive impairment. This syndrome, caused by the absence of creatine in the brain, is currently untreatable because CRT is required for creatine entry into brain cells. Here, we developed a brain-specific Slc6a8 knockout mouse (Slc6a8–/y) as an animal model of human CRT deficiency in order to explore potential therapies for this syndrome. The phenotype of the Slc6a8–/y mouse was comparable to that of human patients. We successfully treated the Slc6a8–/y mice with the creatine analog cyclocreatine. Brain cyclocreatine and cyclocreatine phosphate were detected after 9 weeks of cyclocreatine treatment in Slc6a8–/y mice, in contrast to the same mice treated with creatine or placebo. Cyclocreatine-treated Slc6a8–/y mice also exhibited a profound improvement in cognitive abilities, as seen with novel object recognition as well as spatial learning and memory tests. Thus, cyclocreatine appears promising as a potential therapy for CRT deficiency.

Introduction
Creatine transporter (CRT; encoded by SLC6A8; geneID 6535) transports creatine with high specificity into cells via secondary active transport, using the Na+ gradient to drive the transport against creatine’s concentration gradient. Creatine and its phosphorylated form, phosphocreatine, are used as an energy reservoir by buffering ATP concentration via the creatine kinase (CK) reaction (1). This high-energy phosphate-buffering system is essential to maintain ATP levels. CK and CRT are coexpressed in many tissues with a dynamic and/or fluctuating energy demand. In the CNS, the creatine/phosphocreatine/CK system plays an important role in neurotransmitter release, membrane potential maintenance, Ca2+ homeostasis, and/or ion gradient restoration (1–3). SLC6A8, a member of the solute carrier 6 family, is expressed in many tissues, such as CNS, heart, and skeletal muscle (4), and is highly specific for creatine (5–7). Interestingly, the CNS is the main organ affected by creatine deficiency syndromes, including CRT deficiency. There appears to be normal cardiac function and normal creatine levels in the muscles of patients with CRT deficiency (8).

Of the 3 creatine deficiency syndromes, L-arginine:glycine amidinotransferase (AGAT) deficiency and guanidinoacetate N-methyltransferase (GAMT) deficiency are caused by defects in the enzymes that synthesize creatine, whereas CRT deficiency results from a defect in CRT caused by SLC6A8 deficiency. Thus, creatine cannot enter the brain’s cells. In patients, creatine deficiency syndromes have several common clinical manifestations, including cognitive dysfunction with mental retardation, poor language skills, and autism spectrum disorders (9–15). Proton magnetic resonance spectroscopy (MRS) of affected patients shows an absence or dramatic diminution of the creatine peak, with relatively normal levels of N-acetyl aspartate (9, 16, 17).

Whereas AGAT and GAMT deficiencies have been identified in about 100 patients worldwide, CRT deficiency is described as the second-most common cause of X-linked mental retardation, with an estimated 42,000 individuals affected in the US and approximately 1 million worldwide (17–20). Because SLC6A8 is located on human chromosome Xq28, mutations in this gene result in a more severe syndrome in males than in female carriers. Patients with AGAT deficiency or GAMT deficiency have been successfully treated with creatine supplementation, which reverses symptoms, as well as other supplements, which manage buildup of intermediates (13–15, 21, 22); however, patients with CRT deficiency are not successfully treated with creatine supplementation (10, 16, 23–25). Creatine is found in blood and cerebrospinal fluid (CSF), but is not able to enter brain cells—the cell membranes are an effective barrier to creatine transport. Some previous studies suggest that the rodent brain has the enzymes to synthesize creatine (4, 5, 26, 27). However, it appears that the synthesis system does not work in mice or humans in vivo, because no detectable brain creatine was observed in the Slc6a8 whole-body knockout mouse (28) or in human patients (9, 16, 17), as assessed by biochemical assays or MRS.

Normal brain function might primarily depend on its ability to transport creatine into neurons, as suggested by the presence of creatine in the CSF of patients with CRT deficiency. Despite its presence in the CSF, however, patients’ brain creatine levels are markedly reduced or not detectable when measured by 1H-MRS. Yet a lack of MRS signal does not prove that creatine is absent; it could be present at levels below the detection limit. SLC6A8 is highly expressed in neurons and oligodendrocytes, but not in astrocytes, and is present in microcapillary endothelial cells (MCECs), which form part of the blood-brain-barrier (BBB) (5). An additional part of the BBB is a barrier between the periphery and the CNS, formed by astrocytic end-feet around MCECs, which regulate water and metabolite exchange. Passive creatine diffusion into brain cells—against its concentration gradient—
Is not expected to generate significant brain creatine levels, and this is supported by data in patients.

Currently, there is no approved treatment for patients with CRT deficiency; treatment strategies are palliative for managing seizure and related sequelae. In order to identify a strategy for treatment that focuses on the cognitive deficiencies, we needed a valid animal model with the phenotype of the human disease. Here, we generated a brain-specific Slc6a8 knockout mouse (referred to herein as Slc6a8–/–) with specific knockout of target areas of cortex and hippocampus — the predominant areas of the brain for cognition and memory — and treated those mice with the creatine analog cyclocreatine (1-carboxymethyl-2-iminimidazolidine).

Cyclocreatine is a nearly planar creatine analog (Figure 1) with a maximum kinetic velocity approaching that of creatine itself (29). Because its planar characteristics aid in passive transport across membranes, cyclocreatine might therefore be useful in the treatment of CRT deficiency. Moreover, cyclocreatine is phosphorylated and dephosphorylated by mitochondrial and cytosolic CKs. It has already been demonstrated that cyclocreatine phosphate can indeed function as a phosphagen in mouse brain in vivo (31). Cyclocreatine was given to humans when it was investigated as a chemotherapeutic adjunct under an investigational new drug phase I safety study; thus, toxicity and cGMP data are known (32, 33). Here, we assessed the ability of cyclocreatine to treat Slc6a8–/– mice. We hypothesized that cyclocreatine would cross the BBB and pass through brain cell membranes, improving cognitive function in treated mice.

Our results showed that Slc6a8 was efficiently deleted in Slc6a8–/– mouse brains, and creatine content in these brains was somewhat consistent with that seen in human patients. Slc6a8–/– mice had impaired cognitive function, but normal balance and musculoskeletal control systems, also similar to human patients. Cognitive abilities were improved after 9 weeks of cyclocreatine treatment compared with Slc6a8–/– control littermates, with cyclocreatine and cyclocreatine phosphate seen in the mouse brain. Our results confirmed that in mice, cognitive deficiencies were caused by a lack of CRT in the brain, and that this cognitive deficit could be reversed by 9 weeks of treatment with cyclocreatine.

Results
Creatine concentrations in brains and other organs. The knockout strategy is outlined in Figure 2A. We found substantially decreased levels of creatine in the brains of Slc6a8–/– mice compared with littermate Slc6a8+/+ controls (2.8 ± 0.11 versus 11.2 ± 0.74 mmol/kg wet wt; P ≤ 0.001; Table 1), a diminution close to that measured in patients by 1H-MRS. The relative reduction of brain creatine was similar to the reduced Slc6a8 mRNA expression in Slc6a8–/– mice (Figure 2B and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI59373DS1). Furthermore, there were no differences in any of the tissues studied, excepting a statistically significant increase in the amount of creatine in the urine (Slc6a8–/–, 10.2 ± 2.57 mM; Slc6a8+/+, 4.0 ± 0.66 mM; P ≤ 0.05; Table 1).

Body morphometry. Although no significant difference in body weight or percent lean body mass was observed in Slc6a8–/– compared with Slc6a8+/+ mice before or after treatment, percent body fat was significantly lower at baseline (Slc6a8–/–, 15.8% ± 1.3%; Slc6a8+/+, 19.8% ± 1.4%; P ≤ 0.05), a trend that was still seen after treatment (Supplemental Figure 2, A–C). This may be due to increased activity in Slc6a8–/– compared with Slc6a8+/+ mice (Supplemental Figure 2D). Treatment with cyclocreatine, creatine, or placebo did not significantly change morphometric parameters in Slc6a8–/– or Slc6a8+/+ mice.

Cyclocreatine. We observed successful cyclocreatine entry into the brains of all mice treated with cyclocreatine for 9 weeks, with higher levels in Slc6a8–/– than in Slc6a8+/+ brains (3.1 ± 0.34 vs. 1.7 ± 0.2 mmol/kg wet wt; P ≤ 0.001; Figure 3). Brain cyclocreatine was also observed by chemical analysis as well as by nuclear MRS (Figure 4 and Supplemental Figure 3). We also found cyclocreatine in mouse hair (Slc6a8–/–, 34.8 ± 3.7 mmol/kg dry wt; Slc6a8–/–; 39.9 ± 6.7 mmol/kg dry wt; Supplemental Figure 4) and claws (data not shown). There were no deaths or overt health problems observed in any of the groups studied.

31P-MRS. We observed a decrease in 31P phosphocreatine in Slc6a8–/– versus control Slc6a8+/+ brains; however, there was a profound lack of phosphocreatine peak, with no detectable inorganic phosphate, in the former group (Supplemental Figure 3 and Figure 4, A and B). Treatment with cyclocreatine did result in metabolite changes (Figure 4B), and we observed a substantial peak corresponding to phosphocreatine (and/or phosphocreatine) in Slc6a8–/– mice (Supplemental Figure 3). The peak positions of phosphocreatine and cyclocreatine phosphate are separated by less than 0.2 ppm (34), which means that they cannot be resolved by in vivo nuclear MRS. Because our chemical analysis showed no significant change in creatine, we interpret this peak to represent cyclocreatine phosphate. The presumed cyclocreatine phosphate levels appeared comparable in Slc6a8–/– and Slc6a8+/+ mice, which suggests that cyclocreatine is phosphorylated in the brains of these mice.

Spatial learning and memory. Spatial learning and memory are executive functions for mice, with consequences for environment familiarity and food finding; therefore, we used the established Morris water maze and radial arm maze tests to assess these cognitive functions. In the Morris water maze, Slc6a8–/– mice took significantly longer to find the hidden platform in the last 3 training trials than did Slc6a8+/+ controls before treatment; 9 weeks of cyclocreatine treatment improved the performance of Slc6a8–/– mice so that they were not significantly impaired compared with controls, whereas creatine or placebo did not significantly change Slc6a8–/– mouse performance (Figure 5A). The percentage of time spent in platform area during the probe trial also improved in Slc6a8–/– mice after 9 weeks of cyclocreatine treatment (Figure 5B). The velocity of swimming in the platform area during the probe trial was not significantly different between groups before and after each treatment (Figure 5C), suggestive of normal motor function in the Slc6a8–/– group. We concluded that the impaired spatial learning and memory in Slc6a8–/– mice was normalized by 9 weeks of cyclocreatine treatment.
In the radial arm maze task, Slc6a8–/y mice consistently performed worse than their controls in all parameters measured at the baseline assessment (i.e., working memory error and reference memory error in both original baited arms and reversed baited arms; Supplemental Figure 5). In Slc6a8fl/y mice, working and reference errors decreased significantly in reversed baited arms compared with original baited arms (P < 0.001 for both). However, no significant difference was seen in Slc6a8–/y mice between original and reversed baited arms with respect to either error, suggestive of impairment.

Novel object recognition. Slc6a8–/y mice showed significant impairment in the novel object recognition test that was normalized after 9 weeks of cyclocreatine treatment (Figure 6). Whereas the discrimination index was significantly lower in Slc6a8–/y mice before treatment (–0.07 ± 0.06 versus 0.41 ± 0.06; P ≤ 0.001), cyclocreatine-treated Slc6a8–/y mice showed a discrimination index of 0.53 ± 0.16, not significantly different from that of littermate controls receiving the same treatment. The other treatments showed no significant change from pretreatment levels in the Slc6a8–/y mice (creatine, –0.03 ± 0.06; placebo, 0.05 ± 0.13).

Activity. Home cage activity increased in Slc6a8–/y mice, which were significantly more active than Slc6a8fl/y controls, especially in the dark phase (P < 0.01; Supplemental Figure 2D). The trend was still seen after cyclocreatine treatment (P = 0.06).

Motor function. All motor function parameters — duration to fall from a rotarod with maximal speed, latency to fall in the hanging wire test, and time taken to cross a 50-cm-long beam — were essentially identical between Slc6a8–/y and Slc6a8fl/y mice and were not significantly changed after cyclocreatine treatment (Supplemental Figure 6). Moreover, no missteps were observed with the beam walk test.
Table 1
Creatine content

<table>
<thead>
<tr>
<th></th>
<th>Slc6a8&lt;sup&gt;y&lt;/sup&gt; (n = 10)</th>
<th>Slc6a8&lt;sup&gt;y&lt;/sup&gt; (n = 9)</th>
</tr>
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<tbody>
<tr>
<td><strong>Brain</strong></td>
<td></td>
<td></td>
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<tr>
<td>Subcortical (mmol/kg wet wt)</td>
<td>12.3 ± 1.01</td>
<td>2.8 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cortex (mmol/kg wet wt)</td>
<td>11.2 ± 0.74</td>
<td>2.8 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Skeletal muscle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastrocnemius (mmol/kg wet wt)</td>
<td>37.6 ± 3.13</td>
<td>38.8 ± 2.92</td>
</tr>
<tr>
<td>Solus (mmol/kg wet wt)</td>
<td>30.2 ± 2.04</td>
<td>29.9 ± 2.24</td>
</tr>
<tr>
<td>Heart (mmol/kg wet wt)</td>
<td>13.5 ± 0.70</td>
<td>13.5 ± 1.97</td>
</tr>
<tr>
<td>Bladder (mmol/kg wet wt)</td>
<td>6.1 ± 0.33</td>
<td>5.5 ± 0.29</td>
</tr>
<tr>
<td>Kidney (mmol/kg wet wt)</td>
<td>1.1 ± 0.04</td>
<td>1.1 ± 0.10</td>
</tr>
<tr>
<td>Liver (mmol/kg wet wt)</td>
<td>0.24 ± 0.02</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>Lung (mmol/kg wet wt)</td>
<td>0.55 ± 0.04</td>
<td>0.49 ± 0.04</td>
</tr>
<tr>
<td>Serum (mM)</td>
<td>0.14 ± 0.01</td>
<td>0.12 ± 0.004</td>
</tr>
<tr>
<td>Urine (mM)</td>
<td>4.0 ± 0.66</td>
<td>10.2 ± 2.57&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. <sup>a</sup>P ≤ 0.001, <sup>b</sup>P < 0.05 vs. Slc6a8<sup>y</sup>, 2-tailed Student’s t test.

Discussion

In the present study, we validated our brain-specific CRT-knockout Slc6a8<sup>y</sup> mouse model of CRT deficiency as being comparable to the human disease. We also reported the effective treatment of cognitive deficits in Slc6a8<sup>y</sup> mice with 9 weeks of therapy with the repurposed drug cyclocreatine. We propose a 2-part model in which creatine and phosphocreatine prove central to the energy metabolism and function of brain cells (Figure 7, A and B): the creatine transport mechanism is interrupted, leading to impaired energy metabolism along with impaired function.

At baseline, the phenotype in Slc6a8<sup>y</sup> mice was pronounced for cognitive and executive function deficits, but normal for balance and musculoskeletal control systems. Creatine and phosphocreatine in the brain was significantly decreased, whereas creatine in skeletal muscle, heart, and serum was normal (Table 1). Unlike whole-body Slc6a8-knockout mice, in which body weight is normal and urine creatine is high (9, 11, 8, 35, 36), we observed normal creatine in Slc6a8<sup>y</sup> muscle, also modeling what is seen in patients. After 9 weeks of cyclocreatine treatment, cyclocreatine entered the brain and was phosphorylated, and the cognitive deficits of Slc6a8<sup>y</sup> mice were normalized compared with Slc6a8<sup>y</sup> mice. Creatine or placebo treatment did not show any cognitive or musculoskeletal benefits for Slc6a8<sup>y</sup> mice. These data suggest that cyclocreatine is a potential therapeutic for patients with CRT deficiency.

Justification of the Slc6a8<sup>y</sup> mouse model. A mouse model that resembles the human condition is an important tool with which to validate cyclocreatine as a therapy for patients with CRT deficiency. We had previously reported whole-body Slc6a8-knockout mice using different Cre recombinase (CMV promoter; ref. 28); these had a very severe phenotype distinct from that of patients with CRT deficiency, including motor deficits (8). We felt that cyclocreatine treatment of the whole-body knockout might produce results in which it would be difficult to distinguish cognitive and motor benefits. Thus, we here generated brain-specific Slc6a8<sup>y</sup> mice in order to focus on the cognitive and executive function deficiencies key to the patient pathology we sought to address. Slc6a8<sup>y</sup> mice had a phenotype with markedly reduced levels of creatine in the brain, similar to what is seen in human patients (37), as well as impaired cognitive function, normal motor function, and normal creatine levels in skeletal muscles, heart, and serum. With the current model and the results reported therein, we believe that cyclocreatine treatment improves brain function independent of muscle effects and therefore should be considered as a viable treatment of human CRT deficiency. However, the data do not tell us whether cyclocreatine enters the brain cells by diffusion or through an alternate transport mechanism.

Biochemical phenotype and human CRT deficiency. CRT deficiency was originally characterized as lacking creatine in the brain (9, 16); similarly, we reported here a substantial decrease in creatine in mice lacking CRT, as assessed by biochemical analysis (Table 1). After cyclocreatine treatment, Slc6a8<sup>y</sup> mouse brains showed substantial increases in cyclocreatine and cyclocreatine phosphate. This suggests that cyclocreatine phosphate is effectively replacing phosphocreatine in the brain and reversing the metabolic deficits, which results in cognitive normalization.

Mitochondrial CK is thought to modulate mitochondrial respiration by shifting the mitochondria’s sensitivity to ADP (38). Brain mitochondria are essential for energy metabolism and normal cellular function. In CNS, the creatine/phosphocreatine/CK system is involved in neurotransmitter release, membrane potential maintenance, Ca<sup>2+</sup> homeostasis, and/or ion gradient restoration.

Figure 3
Increased cyclocreatine content in brain after cyclocreatine treatment. (A) Cyclocreatine (cCr) and (B) creatine (Cr) content in brains of Slc6a8<sup>y</sup> mice (n = 7 [cyclocreatine]; 5 [creatine and placebo]) and Slc6a8<sup>y</sup> littermate controls (n = 5 per group) after 9 weeks of treatment, measured by biochemical assays. P, placebo. Data are mean ± SEM. ***P ≤ 0.001 vs. Slc6a8<sup>y</sup>, ***P ≤ 0.001 as indicated by brackets.
(1–3). One of the major energy-demanding activities of the CNS is action potentials. After depolarization of an action potential, which uses stored energy, the constitutive repolarization requires resynthesis of energy in the form of ATP and phosphocreatine. The ATP is used to reestablish the ionic gradients across the membrane. Action potentials often travel together along an axon as a train; the faster and longer the action potential train, the more energy the neuron uses (39). In Slc6a8−/− animals, it is possible that the ATP buffering is inadequate to meet the energy demands for certain action potential trains. Lack of phosphocreatine means the ATP concentration cannot be buffered, which could result in truncated or altered action potential trains with concomitant loss of message. Previous studies have suggested a relationship between impaired/improved cognitive function or hippocampal neurogenesis and changed brain energy metabolism that occurs upon consumption of a high-fat, low-carbohydrate diet (40, 41). Thus, creatine is not entering or being made in the brain, or at least in the brain regions in which it is required. Importantly, our present study also showed no significant changes in brain creatine levels or in functional parameters in creatine-treated Slc6a8−/− mice, which demonstrated that the creatine/phosphocreatine/CK system was inadequate to reverse the cognitive deficits.

Strikingly, cyclocreatine treatment normalized several of the cognitive parameters in adult Slc6a8−/− mice, suggestive of not just a palliative improvement, but the return of substantial neurocognitive function. This is quite intriguing, given that the animals were adults when they started cyclocreatine treatment. It is also noteworthy that home cage activity did not significantly change, an observation that requires further investigation.

**Cyclocreatine treatment.** CRT deficiency is caused by a mutation of CRT, but it is also a disease of the 2 CKs. In the absence of its substrate, mitochondrial CK can crystallize (47) and lead to mitochondrial paracrystalline inclusions and concomitant pathology. Therefore, any treatment of CRT deficiency needs to address CRT, UMTCK, and CKBB in the cytosol. Cyclocreatine is known to interact with both of the CKs, and our present observations suggest that it effectively bypasses the transporter, making it an ideal drug to treat the disease.

Cyclocreatine was first synthesized by Kenyon and colleagues in 1971 (48). It was then evaluated for its biochemical properties in vitro and in vivo by Walker and others (29, 31). These investigators revealed that cyclocreatine is the most kinetically similar analog of creatine in vitro, with Vmax, approaching that of creatine itself (relative Vmax: 90 and 100 nmol/s; Km: 25 and 5 mM, for cyclocreatine and creatine, respectively; ref. 29).

In mice fed cyclocreatine orally, the drug has been shown to be taken up by brains, be phosphorylated to cyclocreatine phosphate, and buffer ATP in vivo (31). These authors also demonstrated that maximal value for phosphorylation in the mouse brain is markedly higher for cyclocreatine than for the natural phosphagen, phosphocreatine. When mice were fed a cyclocreatine-containing diet, 98% of cyclocreatine observed in the brain was in the form of cyclocreatine phosphate, whereas 70% of creatine was phosphocreatine, a reflection of a distinct Vmax for the compounds.

Chemically, our present data demonstrated 2 things: (a) accumulation of cyclocreatine in the brain was slower in Slc6a8−/− than in Slc6a8fl/fl mice, likely due to lack of CRT; and (b) cyclocreatine
entered the Slc6a8–/y brain and was phosphorylated at levels similar to those in Slc6a8fl/y mice. One possible reason why cyclocreatine was taken up by the brains of Slc6a8–/y mice is that cyclocreatine is a nearly planar 5-membered ring. Although our studies cannot definitively prove how or where cyclocreatine entered the brain in our Slc6a8–/y model, they demonstrated that it was in fact entering and had a therapeutic effect.

Walker and colleagues also showed that in mice fed a diet containing large amounts of cyclocreatine, intraperitoneally inoculated Ehrlich ascites tumor cells grew more slowly than did those in mice fed a control diet (49). Studies followed concerning the effects of cyclocreatine on proliferation of various cancer cells, in vitro and in vivo, but did not lead to an approved drug. Reinforcing the possibility that cyclocreatine may be used to treat patients with CRT deficiency is the fact that it has already been given to humans; thus, the manufacture and toxicology have been established (32, 33). Using a repurposed drug may prove forcing the possibility that cyclocreatine may be used to treat patients with CRT deficiency is the fact that it has already been given to humans; thus, the manufacture and toxicology have been established (32, 33). Using a repurposed drug may prove forcing the possibility that cyclocreatine may be used to treat patients with CRT deficiency.

**Methods**

**Materials.** B. Tsao-Nivaggioli (Avicena Group, Palo Alto, California, USA) provided the cGMP-grade cyclocreatine used for this study. All other chemicals were reagent grade.

**Generation of Slc6a8–/y mice.** A Cre-lox system (51) was used to generate a conditional Slc6a8 knockout in the brain. We first generated Slc6a8 floxed mice. Homologous recombination in C57BL/6N cells was carried out by transfecting a targeting vector containing a loxp site within intron 1 and a positive selection cassette containing the neomycin phosphotransferase gene (neo) flanked by Frt sites and the second loxp site within intron 4 (Figure 2A). Neomycin-resistant ES cell clones were screened for homologous recombination by PCR, followed by Southern blot analysis of ES cell genomic DNA using probes located outside the 5′ and 3′ homology arms. Correctly targeted ES cell clones were injected into MF-1 blastocysts. Male chimeras were crossed with C57BL/6 females to produce N1F0 offspring, which was confirmed by Southern blot and PCR analysis. The conditional allele was generated by breeding the heterozygous mice to a germline Flp deleter strain (Jackson Laboratory) to delete the neo cassette, leaving a single loxp and Frt sites in intron 4. The resulting mice were further bred with C57BL/6 females to be free of Fipase. We then crossed Slc6a8fl/y female mice with male mice (C57BL/6) expressing a Cre recombinase driven by the CamKIIα promoter in the brain (52) to generate Slc6a8–/y mice and Slc6a8fl/y littermate controls. We chose this Cre construct because it drives expression throughout the mouse brain, but shows particularly dramatic
expression in the hippocampus and cortex, areas controlling cognitive function, which is an important aspect of the human phenotype. It is also known that Slc6a8 highly expressed in that area in mice (53). We did not observe problems with the floxed mice, so we used Slc6a8fl/y animals as control littermates. This minimizes the influence of genetic background on the observed phenotype. A total of 29 Slc6a8−/− mice and 28 male Slc6a8fl/y controls were used in this study. At 6 months of age, baseline assessment was performed for all mice. To assess the efficacy of cyclocreatine treatment, 17 of the 29 Slc6a8−/− mice and 15 of the 28 Slc6a8fl/y controls were further used for 9 weeks of treatment.

**Experimental design.** Mice were maintained on an ad libitum standard pellet diet (Teklad irradiated standard diet; Harlan animal research laboratory) and on ad libitum water intake with 12-hour dark/light cycles in 70°F ± 2°F through the study. At the conclusion of the study, mice were analyzed for Slc6a8 mRNA levels. Other tests — such as baseline values for working and reference memory, Morris water maze, body composition, home cage locomotor activity, rotarod, hanging wire grip, and beam walk tests — were done before and after treatment. At 12 months of age, Slc6a8−/− and Slc6a8fl/y mice were randomly assigned to groups and started on 1 of 3 treatments for 9 weeks: (a) cyclocreatine (n = 7 [Slc6a8−/−]; 5 [Slc6a8fl/y]), (b) creatine (n = 5 per group), and (c) maltodextrin as placebo (n = 5 per group). Each treatment group was supplemented in the drinking water, and the concentration was adjusted to deliver 0.28 mg/g body weight/d. This is a standard creatine dose for human subjects (20 g/70 kg body weight/d), intended to induce maximum creatine or phosphocreatine concentrations inside the body. Body weight and water and food intake volumes were monitored every other day throughout the treatment period. After 9 weeks of treatment, each parameter was evaluated and compared with baseline values or between groups.

**Cyclocreatine and creatine assay.** Animals were deeply anesthetized with 1%–2% isoflurane delivered with oxygen. Whole blood was harvested with a 1-ml syringe and 27-gauge, 0.625-inch needle from the heart and centrifuged at 3,000 g for 20 minutes; serum was subsequently placed in Eppendorf tubes. Brain, liver, heart, kidney, lung, bladder, and soleus and gastrocnemius muscles were rapidly removed and frozen in liquid nitrogen. Urine and hair samples were also collected. The samples were each dropped into 250 μL boiling water and boiled for 20 minutes to remove protein and lyse cells. The total creatine content of the protein-free extract was assayed using the fluorometric method of Conn (54). Total phosphocreatine content of brain and hair was assayed using the method of Griffith (55). All chemicals were obtained from Sigma-Aldrich unless otherwise stated.

**In vivo MRS.** All data were collected on a Bruker BioSpec 7T system (Bruker BioSpec 70/30) equipped with 400 mT/m actively shielded gradients. In total, 6 mice were used at baseline assessment, and 18 mice after 9 weeks of treatment. Mice were anesthetized by 1%–2% isoflurane delivered with oxygen. Whole blood was harvested with a 1-ml syringe and 27-gauge, 0.625-inch needle from the heart and centrifuged at 3,000 g for 20 minutes; serum was subsequently placed in Eppendorf tubes. Brain, liver, heart, kidney, lung, bladder, and soleus and gastrocnemius muscles were rapidly removed and frozen in liquid nitrogen. Urine and hair samples were also collected. The samples were each dropped into 250 μL boiling water and boiled for 20 minutes to remove protein and lyse cells. The total creatine content of the protein-free extract was assayed using the fluorometric method of Conn (54). Total phosphocreatine content of brain and hair was assayed using the method of Griffith (55). All chemicals were obtained from Sigma-Aldrich unless otherwise stated.

**In vivo MRS.** All data were collected on a Bruker BioSpec 7T system (Bruker BioSpec 70/30) equipped with 400 mT/m actively shielded gradients. In total, 6 mice were used at baseline assessment, and 18 mice after 9 weeks of treatment. Mice were anesthetized by 1%–2% isoflurane delivered with oxygen. Whole blood was harvested with a 1-ml syringe and 27-gauge, 0.625-inch needle from the heart and centrifuged at 3,000 g for 20 minutes; serum was subsequently placed in Eppendorf tubes. Brain, liver, heart, kidney, lung, bladder, and soleus and gastrocnemius muscles were rapidly removed and frozen in liquid nitrogen. Urine and hair samples were also collected. The samples were each dropped into 250 μL boiling water and boiled for 20 minutes to remove protein and lyse cells. The total creatine content of the protein-free extract was assayed using the fluorometric method of Conn (54). Total phosphocreatine content of brain and hair was assayed using the method of Griffith (55). All chemicals were obtained from Sigma-Aldrich unless otherwise stated.
were identified by chemical shift. Ratios of metabolites to total phosphorus were calculated by comparing the total peak amplitude of all phosphorylated metabolites with the individual peak heights. Metabolite peak heights were averaged and reported.

Morris water maze. Before and after 9 weeks of treatment, a total of 32 mice were subjected to Morris water maze testing, a hippocampal-dependent task of spatial learning and memory. The maze consisted of a circular fiberglass pool (122 cm diameter, 75 cm high; Rowland Fiberglass Inc.) filled with water (18 ± 1°C, 43 cm deep). A clear glass platform (10.5 cm × 10.5 cm square) was submerged 1 cm below the water surface. The pool was situated in a room containing extramaze cues (42-cm × 76-cm posters printed with contrasting patterns and shapes) that provide specific visual reference points for locating the submerged platform. A video camera mounted to the ceiling, directly above the center of the pool, was used for recording the probe trial. The recording was digitized by a computer and analyzed using CleverSystem Topscan software (Cleversys) for path analysis (distance traveled and percentage of time in the target platform area). Each mouse received 3 trials in the water maze on each of 5 days. The submerged platform remained in one quadrant of the pool throughout all trials, and latency to find the platform was recorded. If the mouse failed to reach the platform within 60 seconds, the trial was terminated, and the mouse was guided onto the platform for 5 seconds. On the sixth day, each mouse received a final 60-second probe trial in which the platform was removed from the pool.

Radial maze. An 8-arm radial maze was used to test spatial working and reference memory at baseline with a total of 32 mice (n = 17 [Slc6a8−/−]; 15 [Slc6a8fl/fl]). The maze consisted of an octagonal central platform (51.5 cm in diameter) with 8 radial arms (61 cm long, 12 cm wide, 10 cm high) extending outward (Lafayette Instrument Co.). The maze was elevated 70.5 cm above the floor in a room containing many extramaze visual cues. Mice must use these visual stimuli to navigate the maze, which lacked intramaze, nonspatial cues for navigation. First, a food deprivation schedule was carried out to reduce animals’ weight to 85% of baseline. During the entire training and test periods, water was available ad libitum. Next, an acclimation trial was conducted. On days 1 and 2, food pellets were placed near the end of all arms. From day 3, food pellets were located only in arms 3, 5, 7, and 8; the

Figure 7
Creatine and cyclocreatine in brain function. (A) The creatine/CK system is essential for shuttling energy from sites of energy production to sites of energy use. Creatine and phosphocreatine can modulate energy metabolism at the mitochondria and glycolytic pathways. When creatine is absent, energy supply can be insufficient or slow during energy demands. Having cyclocreatine and phosphorylated cyclocreatine keeps ATP levels more constant and decreases pathophysiological consequences. (B) In contrast to the transport and use of creatine by the normal brain, with CRT deficiency, creatine cannot enter the brain, resulting in poor speech and cognition. In the cyclocreatine-treated CRT deficiency brain, cyclocreatine enters brain cells and works with the cell’s metabolism to improve speech and cognition.
remaining 4 arms were empty. Mice received 1 or 2 training trials per day for 22 trials, and then were tested using reversed baited arms (food pellets in arms 1, 2, 4, and 6 only) for 28 trials, for a total of 50 trials. At the beginning of each trial, mice were placed in the central platform, then allowed to explore the maze. Trials were completed when the 4 food pellets were eaten or 15 minutes had elapsed. Arm entries for each mouse were recorded by an investigator. The total number of entries into unbaited arms was tabulated as an index of reference memory error, and the number of reentries into previously baited arms was used as an index of working memory error.

Novel object recognition. Before and after 9 weeks of treatment, the novel object recognition task was conducted in a Plexiglas open-field apparatus (60 cm × 50 cm × 21 cm high) to test short-term recognition. Mice were individually habituated to the open-field apparatus with no objects in the cage for 5 minutes at a time on 3 consecutive days. On the fourth day, 2 identical objects (50-ml falcon tubes, 11 cm high, 3.5 cm diameter, covered with yellow tape) were placed symmetrically 12 cm away from the wall. The mouse was placed near the wall at equal distance from both objects and observed for 5 minutes. A second 5-minute trial was done 3 hours later, in which one of the familiar objects was replaced by a novel one (tube 4 cm in diameter, covered with red tape). We defined exploration zones around the objects (10 cm diameter), and time spent inside the zones was used as an index of object exploration. The discrimination index was calculated by dividing the difference between exploration times of the new object and the familiar object by the total time spent in object zones.

Body composition analysis. Total lean tissue, fat tissue, and water of the 32 total SLC6A8<sup>+/−</sup> and SLC6A8<sup>−/−</sup> mice was determined by MRI (56) before and after 9 weeks of treatment. After system calibration, an unanesthetized mouse was weighed, placed in the restraint tube, and inserted into the Echo MRI whole-body composition analyzer (EchoMedical Systems) for 45 seconds. Fat mass and lean body mass were determined.

Home cage locomotor activity. We measured the locomotor activity expressed by each animal in its home cage before and after 9 weeks of treatment. This allowed us to capture general locomotor activity, unrelated to the animals’ activity in a novel, anxiogenic environment. The cage rack frame (Lafayette Instrument Co.) equipped with infrared photobeams was placed around each animal’s standard shoebox home cage. Infrared photobeam interruption sensors (7X and 15Y) mounted in the frame detected movement, which was recorded and analyzed using HMM100 Motor Monitor software (Lafayette Instrument Co.). Vertical and horizontal activity within the home cage was recorded for 48 hours, and events were collapsed into 60-minute bins. In order to discern short- and long-term activity–related circadian rhythms, results were expressed as the average number of beam interruptions per group per hour.

Rotarod. The rotarod test of motor coordination and motor learning was performed before and after 9 weeks of treatment. On day 1, mice were placed on a stationary rod (3.2 cm diameter) of the apparatus (Type ENV-576M; Med associates) for 30 seconds. Daily training trials were then administered for the next 7 consecutive days: mice were placed on the rod at increasing speeds, from 16 to 32 rpm, for up to 2 minutes. On day 9, mice were placed on the stationary rod at maximum speed (32 rpm). The duration in seconds until the animal fell from the rod was recorded as a measure of motor coordination; cutoff time was 120 seconds.

Hanging wire grip. Before and after 9 weeks of treatment, the hanging wire grip test was performed by placing a mouse on a wire net (2.5-cm × 2.5-cm grid, 30 cm2 wire), then turning the net upside-down at approximately 50 cm above the cage floor to prevent the animal from easily climbing down. The elapsed time until the animal fell was recorded. Tests consisted of 3 trials with 30-second intervals; cutoff time was 540 seconds.

Beam walk. The beam walk is a test of complex motor coordination. Before and after 9 weeks of treatment, animals were trained to ambulate across the beam (1 m long, 9 mm diameters) to a 14-cm<sup>2</sup> platform for 7 consecutive days. On day 8, mice were placed on the beam 50 cm from the platform, and latency to reach the platform was measured. Missed steps off of the beam were also recorded.

Statistics. Data are expressed as mean ± SEM. Statistical significance of mean differences for each parameter was determined by 2-tailed Student’s t test; by ANOVA followed by post-hoc Tukey test (treatment group as between-subject factor); or by repeated-measures ANOVA followed by post-hoc Bonferroni test for multiple comparisons (treatment group and treatment timing as between- and within-subject factors). A P value less than 0.05 was considered significant.

Study approval. The experimental procedures performed herein were in compliance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

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