Cognitive deficits and increases in creatine precursors in a brain-specific knockout of the creatine transporter gene *Slc6a8*

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Abstract

Creatine transporter (CrT; SLC6A8) deficiency (CTD) is an X-linked disorder characterized by severe cognitive deficits, impairments in language, and an absence of brain creatine (Cr). In a previous study, we generated floxed *Slc6a8* (*Slc6a8*^{flox}) mice to create ubiquitous *Slc6a8* knockout (*Slc6a8*$^{-/y}$) mice. *Slc6a8*$^{-/y}$ mice lacked whole body Cr and exhibited cognitive deficits. While *Slc6a8*$^{-/y}$ mice have a similar biochemical phenotype to CTD patients, they also showed a reduction in size and reductions in swim speed that may have contributed to the observed deficits. To address this, we created brain-specific *Slc6a8* knockout (bKO) mice by crossing *Slc6a8*^{Flox} mice with *Nestin-cre* mice. bKO mice had reduced cerebral Cr levels while maintaining normal Cr levels in peripheral tissue. Interestingly, brain concentrations of the Cr synthesis precursor guanidinoacetic acid were increased in bKO mice. bKO mice had longer latencies and path lengths in the Morris water maze, without reductions in swim speed. In accordance with data from *Slc6a8*$^{-/y}$ mice, bKO mice showed deficits in novel object recognition as well as contextual and cued fear conditioning. bKO mice were also hyperactive, in contrast with data from the *Slc6a8*$^{-/y}$ mice. The results demonstrate that the loss of cerebral Cr is responsible for the learning and memory deficits seen in ubiquitous *Slc6a8*$^{-/y}$ mice.

Introduction

The absence of the high-energy phosphate buffer creatine (Cr) in the brain leads to intellectual disability (ID), aphasia, and epilepsy (Van De Kamp *et al.*, 2013). There are currently three known causes of cerebral Cr deficiency, of which Cr transporter (CrT; *SLC6A8*) deficiency (CTD) is the most prevalent. CTD has been estimated to be one of the leading causes of X-linked ID (XLID) in males (Almeida *et al.*, 2006, Clark *et al.*, 2006, Rosenberg *et al.*, 2004). Despite the relatively high prevalence of CTD in males with XLID, little is known about the mechanisms underlying the cognitive deficits. To better understand the underlying patholophysiology of CTD, we generated mice with exons 2-4 of the murine *Slc6a8* gene flanked with LoxP sites (*Slc6a8*^{Flox}*).* Ubiquitous *Slc6a8* knockout (*Slc6a8*$^{-/y}$) mice were derived from *Slc6a8*^{Flox} mice (Hautman *et al.*, 2013, Skelton *et al.*, 2011). Consistent with human CTD, the *Slc6a8*$^{-/y}$ mice show an absence of brain Cr and cognitive deficits. *Slc6a8*$^{-/y}$ mice also had reduced body mass and a near absence of Cr in most tissues, including the muscle (Skelton *et al.*, 2011). The reduction of muscle Cr in *Slc6a8*$^{-/y}$ mice may be inconsistent with the limited data from human CTD patients showing that they have normal muscle Cr levels, (Degrauw *et al.*, 2003, Pyne-Geithman *et al.*, 2004). The reduced size and loss of muscle Cr could influence the behavioral deficits observed in the *Slc6a8*$^{-/y}$ mice. For example, *Slc6a8*$^{-/y}$ mice swam slower than *Slc6a8*$^{+/y}$ mice in the Morris water maze, an effect that may have affected navigating to the hidden platform (Skelton *et al.*, 2011).

Using the floxed *Slc6a8* (*Slc6a8*$^{flox/y}$;FLOX) mouse line from the Skelton et al. (2011) study, Kurosawa et al. created a partial *Slc6a8* brain knockout mouse using a *Camk2a-cre* expressing mouse (Kurosawa *et al.*, 2012). Similar to the *Slc6a8*$^{-/y}$ mice, the *Slc6a8*$^{flox/}$-*Camk2a* mice showed deficits in the Morris water maze. While the *Slc6a8*$^{flox/}$-*Camk2a* mice showed deficits in the Morris water maze, these deficits were only observed in the last few days of testing wereas *Slc6a8*$^{-/y}$ mice showed a robust deficit throughout testing. *Camk2a-cre* expression is limited to excitatory neurons in the forebrain, potentially sparing Cr function (Tsien *et al.*, 1996). This incomplete brain knockout may explain differences between models. A more complete brain knockout, such as the *Nestin-cre* used in this study, may resolve this difference. The *Nestin* promoter drives Cre recombinase expression in neuronal precursor cells, creating a more complete brain-specific knockout (Tronche *et al.*, 1999).

Another mouse model of CTD has been created where exons 5-7 of the murine *Slc6a8* gene were flanked with LoxP sites (Baroncelli *et al.*, 2014). Similar to the *Slc6a8(2-4)*$^{-/y}$ mice of Skelton et al. (2011), ubiquitous knockout (*Slc6a8(5-7)*$^{-/y}$) mice from this line also showed reduced body weight and deficits in novel object recognition compared with *Slc6a8*$^{+/y}$ mice. While *Slc6a8(5-7)*$^{-/y}$ mice showed Morris water maze deficits the effect only occurred on the last 3 days of testing (Baroncelli *et al.*, 2014). In a subsequent study *Slc6a8(5-7)*$^{flox/y}$ mice were crossed to *Nestin-cre* mice to create brain specific knockout mice. These mice show deficits in novel object recognition and Y-maze performance but Morris water maze was not tested in these mice (Baroncelli *et al.*, 2016). While these data show that the object recognition deficits occur in both models, it does not address possible performance deficits in the *Slc6a8*$^{-/y}$ mice.

The purpose of this study was to test brain-specific *Slc6a8* knockout (bKO) mice using behaviors that were disrupted in *Slc6a8*$^{-/y}$ mice. The results of this study show that bKO mice have Morris water maze deficits similar to those seen in the ubiquitous *Slc6a8*$^{-/y}$ mice without reductions in swim spedd, suggesting that the deficits observed in the *Slc6a8*$^{-/y}$ mice are central cognitive impairments analogous to those seen in CTD patients.

Methods

Generation of bKO mice

The bKO mice were generated and maintained on a C57BL/6J background in a pathogen-free facility in microisolators. Female *Slc6a8*$^{FLOX/+}$ mice, generated as described (Skelton *et al.*, 2011), were mated to mice expressing Cre recombinase driven by the *Nestin* promoter (B6.Cg-Tg (Nes-cre)$^{1Kln/J}$; Jackson laboratory, Bar Harbor, ME) to create the mice used in this study. Genotyping was performed using a previously described touchdown PCR protocol (Skelton *et al.*, 2011). Testing was done during the light phase on *Slc6a8*$^{+/y}$*::Nes-Cre*$^{-}$ (WT), bKO, *Slc6a8*$^{+/y}$*::Nes-Cre*$^{+}$ (Nestin), and FLOX mice. No more than one mouse per genotype was taken from a litter. The vivarium is fully accredited by AAALAC International and protocols were approved by the Institutional Animal Care and Use Committee. Lights were on a 14:10 light:dark cycle, room temperature was maintained at 19±1º C, and food (NIH-007) and filtered, sterilized water were available *ad libitum*. All institutional and national guidelines for the care and use of laboratory animals were followed.

Behavioral Testing

Adult male mice of each genotype (n=14 WT; 15 bKO; 23 FLOX; 12 Nestin) were used. Mice were weighed on the first and last day of testing. Testing order consisted of: locomotor activity (1 day duration), MWM cued (6 days) and hidden platform phases (7 days of acquisition, 7 days of reversal), novel object recognition (5 days), and conditioned fear (3 days). Mice were tested in one task at a time with 1 day between tasks.

Locomotor activity

Spontaneous locomotor activity (Brooks & Dunnett, 2009) was tested in automated activity chambers, Photobeam Activity System (PAS) – Open-Field (San Diego Instruments, San Diego, CA) for 1 h. Chambers were 40 cm (W) × 40 cm (D) × 38 cm (H) with 16 LED-photodetector beams in X and Y planes. Photocells were spaced 2.5 cm apart. Mice were tested for 1 h. The dependent measure was total number of photobeams interruptions.

Morris water maze

The MWM is a test of spatial learning and reference memory (Vorhees & Williams, 2006); mice were tested as described (Skelton *et al.*, 2011). The tank was 122 cm in diameter, white, and filled with room temperature (21 ± 1˚C) water. Prior to hidden platform testing, visible platform training (cued) was conducted for 6 days. During this phase, a 10 cm diameter platform with an orange ball mounted above the water on a brass rod was placed in one quadrant. On day 1, mice were given 6 trials with identical start and platform positions as training for the task. Mice were then given 2 trials per day for 5 days with the start and platform positions randomized and curtains closed around the pool to obscure distal cues to assess proximal cue learning.

Hidden platform trials were conducted in 2 phases; each phase consisted of 4 trials per day for 6 days followed by a single probe trial without the platform on day 7. Platform diameters were 10 cm during acquisition and 7 cm during reversal (platform located in the opposite quadrant). The trial limit was 90 s and the intertrial interval was ~10 s. Performance was measured using ANY-maze® software (Stoelting Company, Wood Dale, IL).

Novel object recognition

Novel object recognition (NOR) is a test of incidental learning and memory (Clark *et al.*, 2000).

Mice were tested in the ANY-box apparatus (Stoelting Company, Wood Dale, IL). First, mice were habituated to the arena (40×40 cm) for 2 days (10 min/day); next they were then habituated to two identical objects (10 min/day) for 2 days. On the fifth day, mice were presented with two new identical objects until 30 s of observation time between objects was accrued. One hour later, memory was tested by presenting the mouse with an identical copy of one of the familiar objects along with a novel object. Time exploring an object was defined as entry into a 2 cm zone around the object. Performance was measured using ANY-maze® software. A discrimination index was calculated: time observing the novel object was subtracted from the time observing the familiar object, divided by total object observation time.

Conditioned fear

Conditioned fear was assessed as described with modification (Peters *et al.*, 2010). On day 1, mice were placed in a chamber for 10 min before exposure to three tone-footshock pairings (82 dB, 2 kHz, 30 s on/off cycle). Each pairing consisted of the 30 s tone accompanied during the last second by a scrambled footshock (0.3 mA for 1 s) delivered through the floor. On the second day, mice were returned to the chamber with no tone or shock as a test of contextual fear. On the third day, mice were placed in the chamber with a novel floor. Following 3 min of acclimation, the tone was presented for 3 min. On day 2 and 3 freezing behavior was scored. Freezeframe software and Coulbourn test chambers were used (Coulbourn Instruments, Allentown, PA). Percent time freezing was analyzed.

Creatine and guandinoacetic acid (GAA) determination

Following behavioral testing a subset of mice were sacrificed by decapitation. Brains were divided into hemispheres and flash frozen. Cr and GAA content were assayed in brain, heart, and kidney using a UPLC-MS/MS method(Trotier-Faurion *et al.*, 2013). First, 20 µL from each brain homogenate (diluted 1/10) was spiked with 5 µL of internal standard solution (creatine-D3-0.5 µg/mL) and 175µl of water were added to reach 200 µl final volume. For liquid–liquid extraction, 400 µL ethanol and 400 µL hexane were added and the mixture was centrifuged at 4 ºC for 15 min (10000g). Then, the upper phase was discarded and the hexane step was repeated one more time. Finally, the lower phase was transferred into a clean tube and evaporated to dryness under a gentle stream of nitrogen at 40 ºC. Then the samples were added with 200 µL of hydrogen chloride -1-butanol (≥99% HPLC Sigma-Aldrich, France) and placed in

a Thermomixer (xxx) at 65 ºC for 15 min followed by an evaporation step. The residue was reconstituted with 100 µL of water injected into the LC–MS/MS system. The analytical method was performed with a binary pumps LC-30AD (Shimadzu Nexera X2) and compounds (creatine, GAA, creatine-D3) were separated by injecting extracted samples (10µL) on a C18 symmetry column-Waters (50 x 2.1 mm), with a 3.5 µm particles size maintained at 30 ºC. The mobile phase consisted of phase A (0.1% formic acid in water) and phase B (0.1% formic acid in acetonitrile) on isocratic mode. Each analysis was carried out for 8 min and the flow rate of 0.4ml/min was used for the sample analysis. Detection was performed with a triple quadrupole mass spectrometer TSQ Quantum Ultra (Thermo) equipped with electrospray ionization source (ESI) in the positive ion mode. The equipment was set up in a multiple reaction monitoring (MRM) mode and the optimized setting parameters were: ion spray Voltage 3000V, source temperature 350 ºC, sheath gas pressure 23, followed transitions for quantification: Creatine derivative: 188.18 → 90.20; 132.07 and 146.15 (Collision energy: 20); Creatine-D3 derivative: 190.93 → 93.21; 134.87 and 149.23 (Collision energy: 20); GAA derivative: 174.09 → 72.75; 100.75 and 117.75 (Collision energy: 20). Results were recorded by Xcalibur and TSQ Tune Master (Thermo). The amount of creatine and GAA were standardized to the amount of protein in each homogenate tissue by the Bradford technique following the manufacturer's instruction (Sigma-Aldrich, France).

Statistics

Data were analyzed using one-way ANOVA except when there were repeated measurements on the same mouse (interval or day) in which case they were analyzed by repeated-measures ANOVA. Significant effects were analyzed using the method of Benjamini, Krieger, and Yekuteli (Benjamini *et al.*, 2006). Data were analyzed using GraphPad Prism.

Results

Body Weight

Mice were weighed on the first and the last day of testing. No significant differences were observed in body weight between WT (26.4±0.6 g), FLOX (25.6±0.6 g), Nestin (25.3±1.0 g) and bKO (25.3±0.6 g) mice.

Cr and GAA levels

Brain: There were main effects of gene for Cr ($F(3,13)=39.94$, $p<0.001$) and GAA

($F(3,13)=4.3$, $p<0.05$) levels (Figure 1A & B). Significant reductions in Cr were observed in bKO mice compared with WT, FLOX, and Nestin mice. There was a slight reduction in Cr levels in Nestin mice compared with WT mice ($p<0.05$) but not in FLOX mice. No differences were observed between FLOX and WT mice. GAA levels were increased in bKO mice compared with the control groups ($p<0.05$). No differences were observed between control groups for GAA.

Peripheral tissues: There was no effect of gene on Cr or GAA levels in the heart (Figure 1C & D). There was no effect of gene on Cr or GAA levels in the kidney (Figure 1E & F). There was no effect of gene on Cr or GAA levels in the muscle or liver (not shown for clarity).

Locomotor Activity

There was a significant main effect of gene ($F(3,54)=21.9$; $p<0.001$; Figure 2) for total activity. A gene x time interaction ($F(33, 594)=2.0$, $p<0.01$) was observed. bKO mice showed increased activity compared with all other groups ($p<0.05$) at all times. FLOX mice showed hyperactivity compared with WT and Nestin mice at 15, 35, and 60 min. No differences were observed between WT and Nestin groups.

Morris water maze

On day-1 cued training, there was trend towards a main effect of gene ($F(3,73)=2.6$, $p<0.07$). An increase in latency was seen on days 2-6 (main effect of gene: $F(3,73)=6.03$, $p<0.01$). No differences were observed between the control groups.

For hidden platform testing, all variables showed a similar pattern, therefore, path length is presented. During acquisition, bKO mice had an increased path length compared with control groups ($F(3,72)=13.4$, $p<0.001$; all post-hoc tests $p<0.01$ vs control groups; Figure 3A). No differences were observed between control groups. There were no significant effects of gene on swim speed. During the acquisition probe trial, bKO mice had a greater average distance from the former platform site compared with the WT mice (main effect of gene ($F(3,72)=5.0$, $p<0.001$; bKO vs WT $p<0.05$, Figure 3C). Learning curves for the WT vs bKO mice are shown in Figure 3E.

During reversal, there was a main effect of gene for path length ($F(3,69)=10.2$, $p<0.001$; Figure 3B). bKO mice travelled a longer path to the platform compared with the control groups ($p<0.05$). No differences were observed between control groups. There were no differences

in swim speed during reversal. During the reversal probe trial, there was a main effect of gene ($F(3,69)=4.3$, $p<0.01$; Figure 3D). FLOX mice had shorter average distance to the target site compared with WT and bKO mice ($p<0.05$). No other differences were observed. Learning curves for WT vs bKO mice are shown in Figure 3F.

Novel Object Recognition

For time spent observing the novel object, there was a main effect of gene ($F(3,56)=7.7$, $p<0.001$; Figure 4). Post-hoc tests showed that bKO mice spent less time with the novel object compared with control groups ($p<0.05$); the control groups did not differ from each other.

Conditioned Fear

On day 1, mice were conditioned to the tone-footshock pairing. On day 2, there was a main effect of gene ($F(3, 60)=7.7$, $p<0.01$, Figure 4) that was the result of bKO mice spending less time freezing than WT and FLOX mice in the training context (contextual fear). Nestin mice did not show a significant difference from other groups. On day 3, there was a main effect of gene ($F(3,64) = 10.3$, $p<0.001$,Figure 5);bKO mice spent less time freezing to the tone (cued fear) than WT and FLOX mice.

Discussion

The study was conducted to determine if cognitive deficits observed in ubiquitous *Slc6a8*$^{-/y}$ mice were influenced by sensorimotor deficits caused by the lack of peripheral Cr. The results show that *Nestin-cre*::*Slc6a8*$^{FLOX/y}$ mice have robust learning and memory deficits, similar to *Slc6a8*$^{-/y}$ mice but in the absence of body weight reductions or slow swimming. Using the same *Slc6a8*$^{FLOX/y}$ line, Kurosawa et al (2012) generated *Camk2a-cre*:: *Slc6a8*$^{FLOX/y}$ mice with forebrain-specific knockout of the *Slc6a8* gene (Tsien *et al.*, 1996). These mice showed a reduction in object recognition memory but modest deficits in the MWM that were only seen on the last three days of testing when these trials were analyzed separately from the overall ANOVA. The *Camk2a-cre*::*Slc6a8*$^{FLOX/y}$ mice were not tested in MWM reversal to assess cognitive flexibility. In contrast, our bKO mice showed learning deficits on both phases of the MWM. As can be seen in Figure 3E, the learning curves of the WT and bKO mice show that the deficits began on day 1 in the acquisition phase. This suggests that a whole-brain knockout

of the *Slc6a8* gene best recapitulates the learning deficits seen in the ubiquitous *Slc6a8*$^{-/y}$ mouse and in CTD. Along with the elimination of the *Slc6a8* gene in brain, the milder phenotype observed in the Kurosawa et al. paper may be due to the presence of some Cr during brain development in the *Camk2a-cre*:: *Slc6a8*$^{FLOX/y}$ mice. *Camk2a-cre* expression is not observed until approximately postnatal day 3 (Burgin *et al.*, 1990, Guo *et al.*, 2000) and even then only affects forebrain Cr. This postnatal elimination of *Slc6a8* could allow for the presence of Cr during critical periods of brain development. Further, since the elimination of Cr is ~3% per day (Wyss & Kaddurah-Daouk, 2000), Cr will be present at high levels for several weeks even after *Slc6a8* deletion in the *Camk2a-cre*:: *Slc6a8*$^{FLOX/y}$ mice. The importance of Cr in brain development has been highlighted recently in a study showing that the loss of Cr kinase leads to aberrant neuronal development (Fukumitsu *et al.*, 2015). In addition, maternal Cr supplementation in rats enhanced hippocampal neuron development and improved long-term potentiation (Sartini *et al.*, 2016). Taken together, the use of *Nestin-cre* mice to generate brain-specific *Slc6a8* disruption shows a phenotype more comparable to the ubiquitous *Slc6a8*$^{-/y}$ mice.

Slc6a8$^{-/y}$ mice are smaller than *Slc6a8*$^{+/y}$ mice with similar reductions in Cr as found in AGAT and GAMT-deficient mice (Choe *et al.*, 2013, Schmidt *et al.*, 2004). In addition, *Slc6a8*$^{-/y}$ mice have increased body fat percentages compared with *Slc6a8*$^{+/y}$ mice (Perna *et al.*, 2016). Both CNS and peripheral systems contribute to body mass. The finding of normal body mass in the bKO mice suggests that body fat changes are peripherally-mediated and not due to a loss of hypothalamic Cr. This is further supported by a recent study where adipose-tissue specific deletion of the Cr-synthesis precursor *Arginine:glycine amidinotransferase (Agat* or *Gatm)* increased body fat in mice (Kazak *et al.*, 2017).

Similar to the ubiquitous *Slc6a8*$^{-/y}$ mice, bKO mice show learning and memory deficits in the MWM, NOR, and conditioned fear. In contrast to *Slc6a8*$^{-/y}$ mice, the MWM deficits appeared without differences in swim speed. Interestingly, the bKO mice had deficits in the cued platform phase, which is a control procedure for sensorimotor deficits. However, given a lack of swim speed changes during hidden platform testing, it is unlikely that the cued effect is carrying over to the spatial learning phases of the test. The use of additional tests such as NOR and fear conditioning that assess other types of learning supports the view that lack of brain Cr has broad effects on higher cognitive functions.

Baroncelli et al. (2016) showed that a brain-specific knockout of exons 5-7 of the *Slc6a8* gene resulted in novel object recognition and spontaneous alternation deficits. Ubiquitous deletion of the same region results in Morris water maze deficits, but only during the last 3 days of testing (Baroncelli *et al.*, 2014), revealing that this mouse has a milder phenotype than both our ubiquitous and our bKO mice. Interestingly, this deficit did not fully replicate in a subsequent study when similarly-aged mice showed a deficit only on day 5 of testing (Baroncelli *et al.*, 2016). The brain-specific knockout of exons 5-7 mice were not evaluated in the Morris water maze. The difference in spatial learning between the two *Slc6a8* knockouts could be explained by differences in the test apparatus or procedures; for example, platform to arena size ratio in these studies differ. All of the studies in both models used 120 cm water tanks, but used different sized platforms. In this and our previous (Hautman *et al.*, 2013, Skelton *et al.*, 2011) studies, we used a 10 cm diameter for the acquisition phase, giving a 144:1 tank to platform ratio. In the Baroncelli studies, a square 11 x 11 cm platform was used, giving a 93.4:1 tank to platform ratio. Smaller search ratios simplify the task (Vorhees & Williams, 2006), perhaps making the lower ratio apparatus less sensitive.

Attention deficit/hyperactivity disorder has been reported in a majority of CTD patients (Dunbar *et al.*, 2014). Ubiquitous *Slc6a8*$^{-/y}$ mice showed initial hypoactivity followed by no difference in an open-field test compared with WT mice. In contrast, bKO mice were hyperactive. In these studies, a photobeam system was used to determine movement. It is possible that the smaller stature of the *Slc6a8*$^{-/y}$ mice lead to fewer beam interruptions but the exact cause is unknown. It is possible that housing played some role initially, the *Slc6a8*$^{-/y}$ mice were housed conventionally in wire top cages. Since then, both lines were moved to a pathogen-free barrier facility with individually ventilated cages (IVC). It is becoming increasingly apparent that IVCs alter the behavior of rodents (Pasquarelli *et al.*, 2017, Shan *et al.*, 2014) and results of this study suggest this. In our prior study, *Slc6a8*$^{+/y}$ and *Slc6a8*$^{-/y}$ mice had higher baseline activity than in the present study by a factor of two (Skelton *et al.*, 2011). In both studies locomotor activity was assessed prior to cognitive testing and all mice were assessed during the same 4 h window of each day. In addition to the lower baseline in this study, the mice had an increased percent time freezing in the conditioned fear test compared with the Skelton et al. (2011) study. While the magnitude of the cognitive deficits remained the same, the overall difference in affective behaviors warrant further study. Interestingly, the FLOX mice also showed hyperactivity, though not to the same degree as the bKO mice. FLOX

mice did not show a decrease in Cr levels compared with WT mice, making the interpretation of this effect unclear. The hyperactivity was the only difference between WT and FLOX mice, suggesting that the insertion of the loxP sites did not affect cognitive function.

Brain Cr levels were reduced in the bKO mice while Cr levels in other tissues were spared. This suggests that there was no carry-over effect using the *Nestin-cre* in other tissues. Interestingly, there was an increase in the Cr synthesis precursor GAA in the brains of the bKO mice. This supports the hypothesis that the brain can synthesize Cr and uses the *Slc6a8* to shuttle Cr intermediates between cells (Braissant *et al.*, 2011). A better understanding of this system is required to determine how the brain synthesizes Cr and what impact this has for CTD patients. CTD patients supplemented with Cr-synthesis precursors arginine and glycine did not show an increase in cerebral Cr levels (Jaggumantri *et al.*, 2015, Valayannopoulos *et al.*, 2012). If this system can be better understood, it is possible that future treatment could involve enhancing endogenous Cr synthesis in the brain.

The results show that the cognitive deficits in ubiquitous *Slc6a8*$^{-/y}$ mice are attributable to changes in cognitive function and are not caused by being smaller or having motor deficits. Based on this, we suggest that the *Slc6a8*$^{-/y}$ mouse is the best model of human CTD. While smaller stature is not always observed in human CTD, all CTD patients have ubiquitous deletion of the gene. Brain-specific models are generated using neuronal precursors that likely allow for the expression of the *Slc6a8* gene at the blood brain barrier (BBB). At the BBB, *Slc6a8* is expressed in microcapillary endothelial cells and not on astrocytic feet that encompass the majority of the BBB (Braissant & Henry, 2008). These cells derive from a mesodermal lineage and any neuronal model of Cre expression would not target them, allowing for *Slc6a8* expression at the BBB. In addition, recent case reports show that hypotonia is a prominent phenotype in many CTD patients (Dunbar *et al.*, 2014), suggesting that the reduced size of the *Slc6a8*$^{-/y}$ mouse muscle effects found in many cases of CTD. Taken together, the bKO data support the ubiquitous *Slc6a8*$^{-/y}$ mouse as best model of CTD; whereas the bKO models should be limited to studies to provide evidence that the ubiquitous model is not confounded. Therefore, the ubiquitous model should be utilized to develop potential therapeutics for this disorder.

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Figure 1. Creatine and GAA levels in the brain, heart and kidney. A) BKO mice show reduced brain Cr levels compared with all control groups. Nestin mice had lower Cr levels than WT mice. B) GAA levels are increased in the brain of bKO mice. In the heart and kidney, Cr (C and E respectively) and GAA (D and F) levels are unchanged. N=5/group Mean ±SEM *p<0.05 vs WT, FLOX, and NESTIN, #p<0.05 vs WT

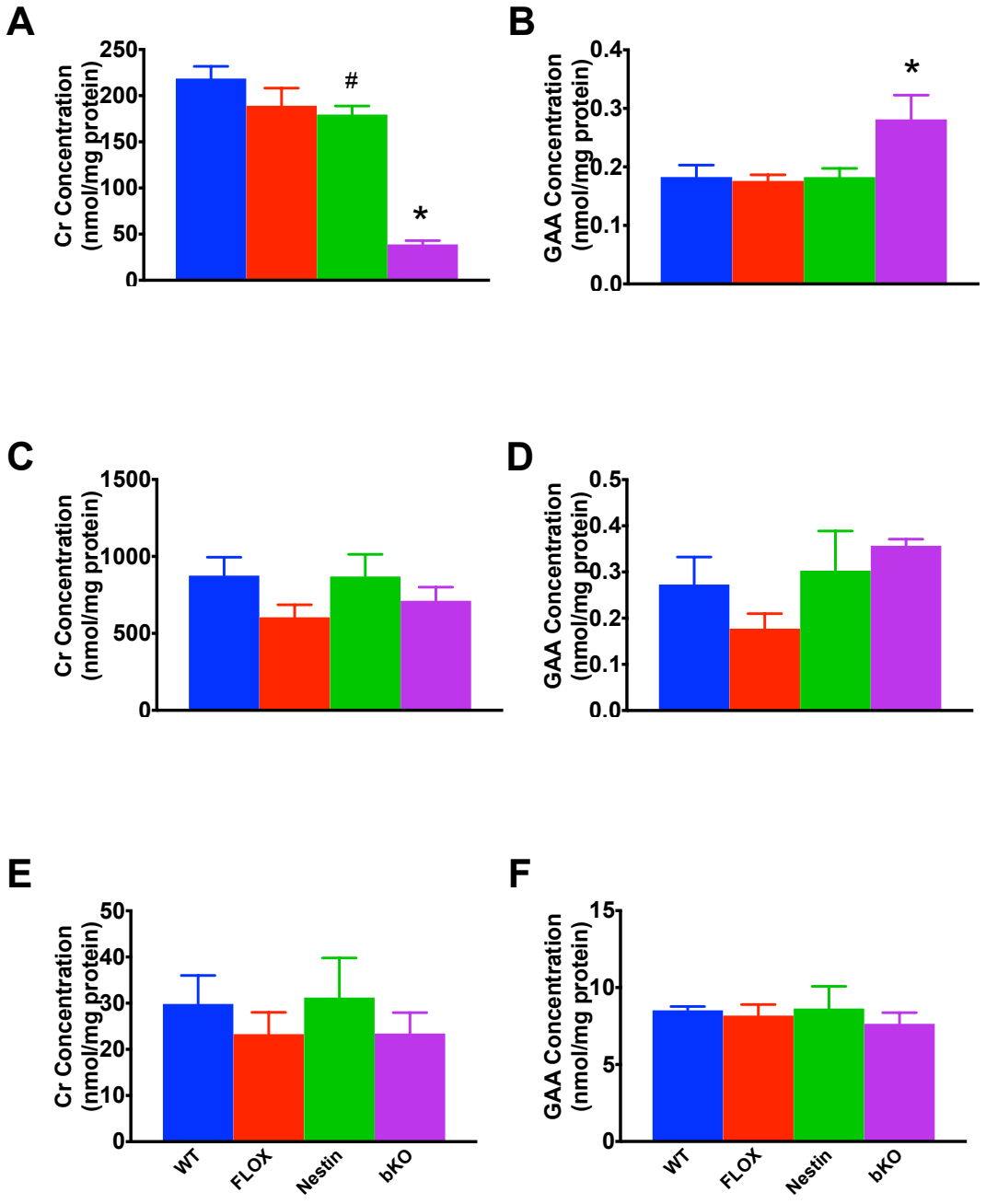
Figure 2. Hyperactivity in *Slc6a8* bKO mice. bKO mice show increased activity compared with FLOX and WT mice. FLOX mice showed increased activity compared with WT mice at 15 and 35 minutes. Data are presented as Mean ±SEM *p<0.05 vs FLOX, Nestin & WT, #p<0.05 vs WT & Nestin

Figure 3. Spatial learning deficits in the MWM. bKO mice have increased path length to the platform during the acquisition (A) and reversal (B) phases of the MWM compared with WT and FLOX mice. On probe trials, bKO mice showed a higher average distance from the platform compared with controls during the acquisition phase (C) but not during the reversal phase (D). Learning curves by day for the bKO and WT mice during the acquisition (E) and reversal (F) hidden platform trials. Data are presented as Mean ±SEM **p<0.01 vs FLOX & WT; *p<0.05 vs FLOX & WT

Figure 4. Object recognition memory deficits. bKO mice had a reduced discrimination index for the novel object compared with the control groups. Data are presented as Mean ±SEM *p<0.05 vs WT, FLOX & Nestin

Figure 5. Contextual and conditioned fear deficits in *Slc6a8* bKO mice. bKO mice showed reduced freezing compared with control groups during both the contextual and cued phases of testing. Data are presented as Mean ±SEM **p<0.01 vs FLOX & WT.

Figure 1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C
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WT
FLOX
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Figure 2

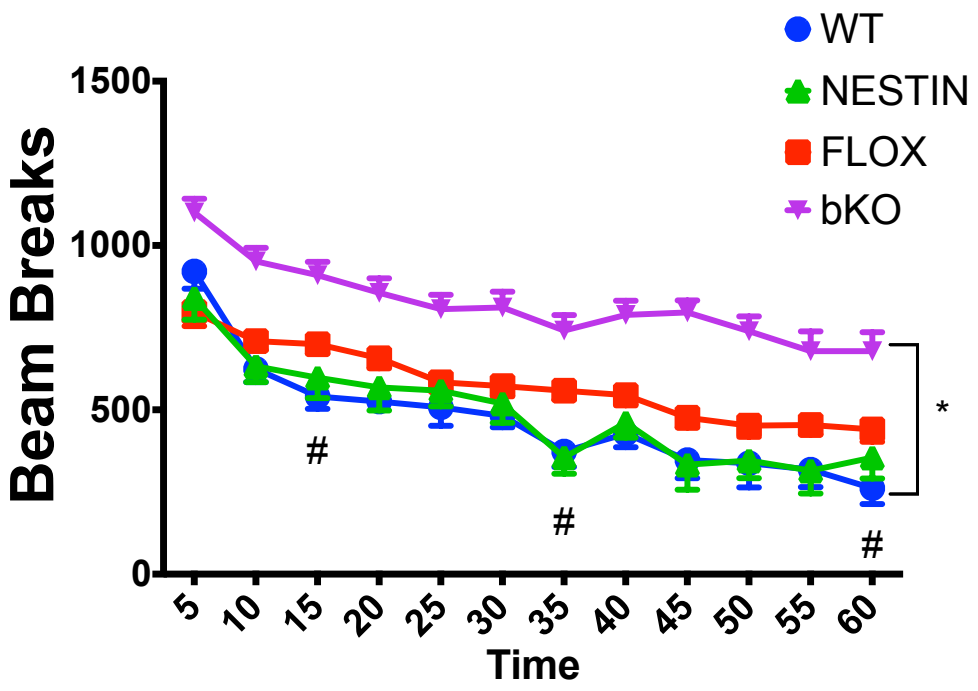
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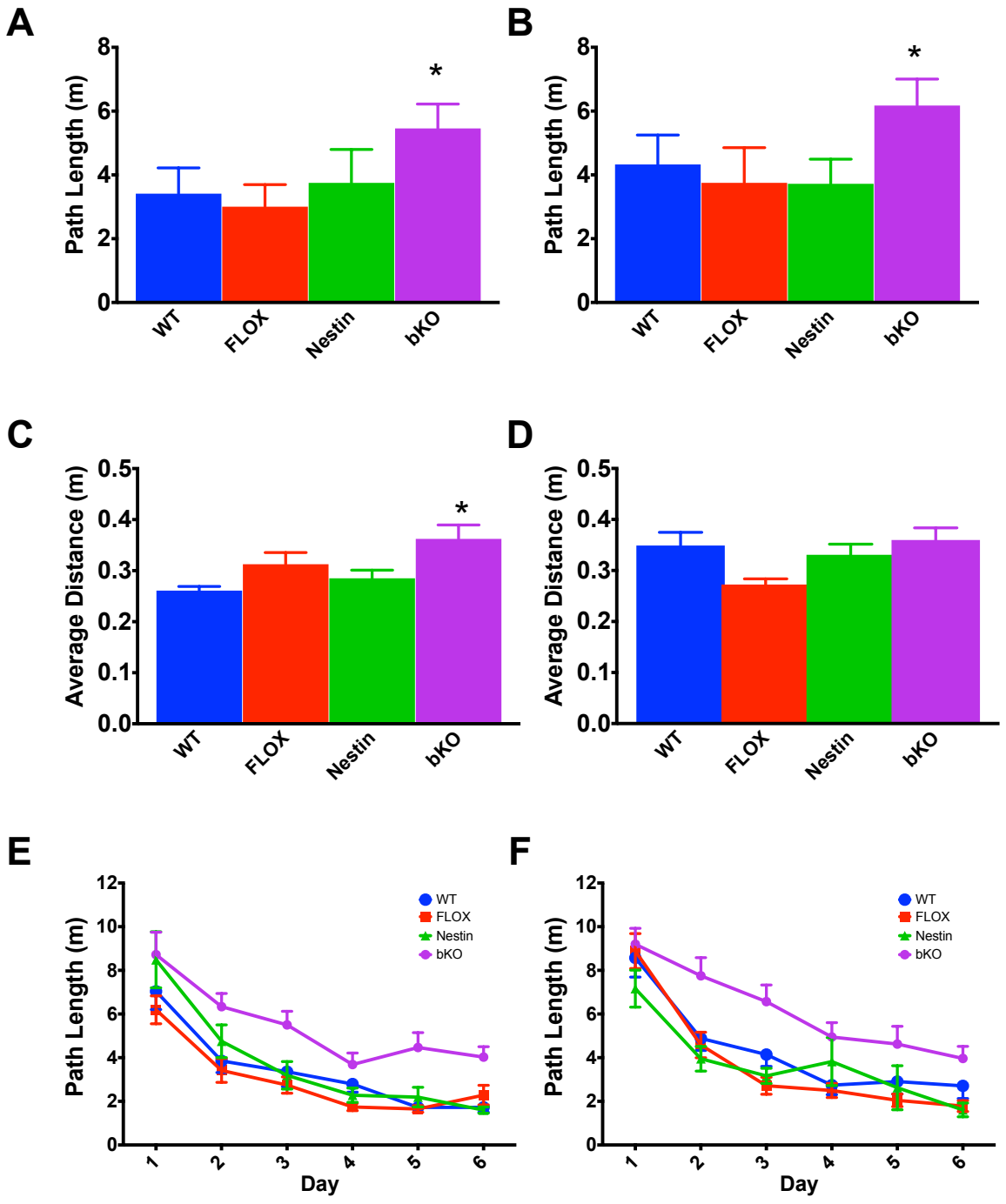
Figure 3.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WT
FLOX
Nestin
bKO
C
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0.5
*
WT
FLOX
Nestin
bKO
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bKO
E
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Figure 4.

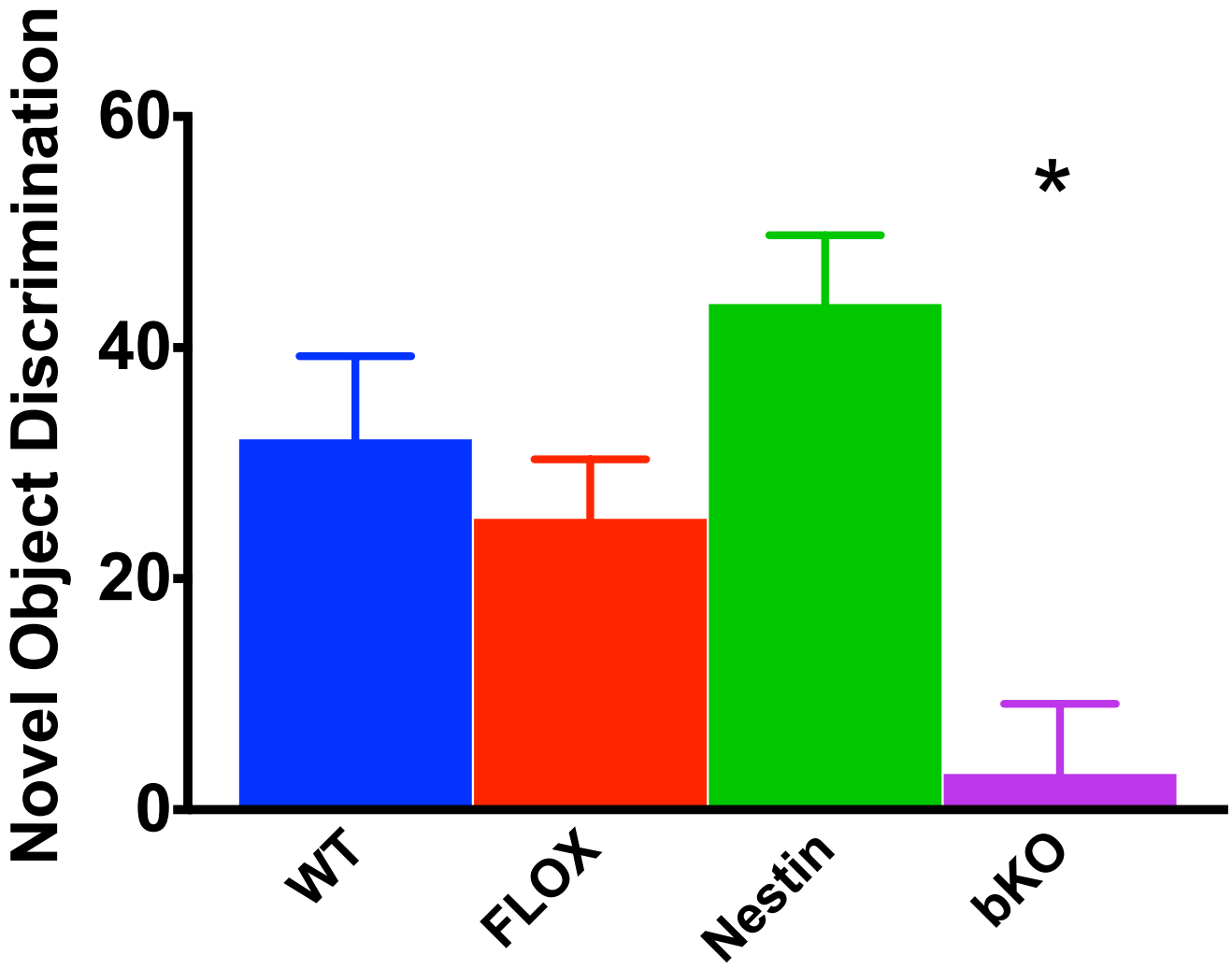
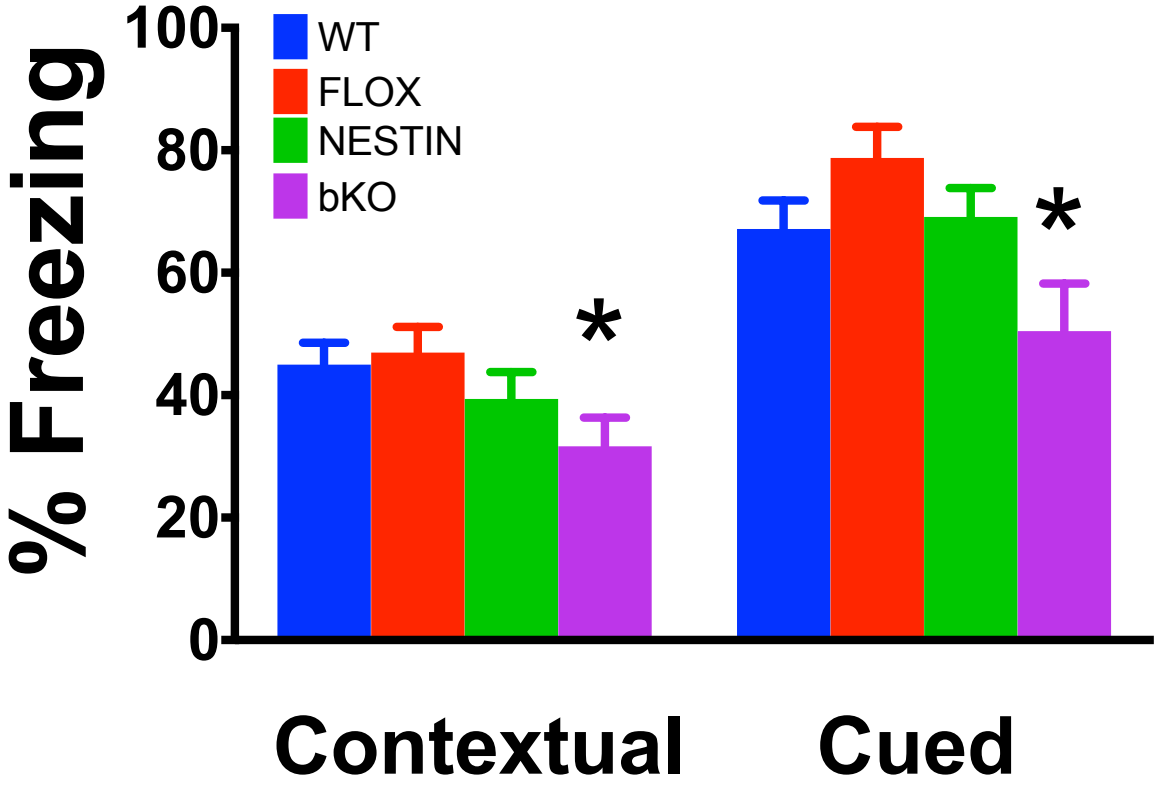
Figure 5.