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Deletion of the creatine transporter gene in neonatal, but not adult, mice lead to cognitive deficits.

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Abstract

Creatine (Cr) is a guanidino compound that provides readily-available phosphate pools for the regeneration of spent ATP. The lack of brain Cr causes moderate to severe intellectual disability, language impairment, and epilepsy. The most prevalent cause of Cr deficiency are mutations in the X-linked *SLC6A8* (Creatine transporter; CrT) gene, known as CrT deficiency (CTD). One of the most critical areas that need to be addressed is if Cr is necessary for brain development. To address this concern, the *Slc6a8* gene was knocked out in either neonatal (postnatal day (P)5) or adult (P60) mice using a tamoxifen-inducible Cre recombinase driven by the UBC promoter. Regardless of treatment age, mice were tested in the Morris water maze, novel, object recognition, and conditioned fear 60 days after Slc6a8 deletion. In addition, overnight locomotor activity was analyzed. Mice that had the gene deleted on P5 showed deficits in the Morris water maze and novel object recognition, while there were no deficits in P60 knockout mice. Interestingly, the P5 knockout mice showed hyperactivity during the dark phase; however, when examining control mice, the effect was due to the administration of tamoxifen from P5-10. Taken together, the results of this study show that Cr is necessary during periods of brain development involved in spatial and object learning. This study also highlights the continued importance of using proper control groups for behavioral testing.

INTRODUCTION

Creatine (Cr) is a guandino compound that serves as a phosphate reserve to replenish spent ATP (Wyss and Kaddurah-Daouk 2000). In the mitochondria, Cr kinase (CK) transfers a phosphate from ATP to Cr, creating phospho-Cr (pCr). At sites of energy utilization, CK transfers the phosphate from pCr to ADP, thereby regenerating ATP. The importance of Cr to

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Conflict of Interest statements:

Kenea C. Udobi, Nicholas Delcimmuto, Amanda N. Kokenge, Zuhair I. Abdulla, Marla K. Perna, and Matthew R. Skelton declare that they have no conflicts of interest.

Informed consent

No human subjects were used in this study.

Animal Rights

All institutional and national guidelines for the care and use of laboratory animals were followed.

cellular function is highlighted by a group of disorders resulting in the lack of brain Cr (Schulze 2003). Caused by a lack of Cr synthesis or transport, cerebral Cr deficiency leads to intellectual disability, a lack of language development, and epilepsy. The most common cause of Cr deficiency is due the loss of the *SLC6A8* gene (Cr transporter; Crt)(Cecil et al 2001; deGrauw et al 2003). The exact incidence of CTD has been difficult to ascertain due to limitations of public databases, but the limited number of studies have suggested that CTD accounts for 0.2-5.4% of males with intellectual disability (Newmeyer et al 2005; Clark et al 2006; Lion-Francois et al 2006; Cheillan et al 2012; DesRoches et al 2015). Unlike defects in Cr synthesis, CTD cannot be treated with oral Cr supplementation (van de Kamp et al 2013) or Cr synthesis precursors (Valayannopoulos et al 2012; Dunbar et al 2014; Bruun et al 2018). The inability to restore Cr in CTD makes the development of molecules that could deliver Cr past the blood-brain barrier (Trotier-Faurion et al 2013) or Cr mimetics (Kurosawa et al 2012) valuable as possible treatment strategies. However, the lack of understanding on the role of brain Cr may lead to an inadequate design for proof of concept studies.

One of the primary concerns in designing treatment protocols should be targeting the critical periods in which Cr is necessary to facilitate cognitive processes. It has been shown that early-life Cr supplementation provides a greater cognitive improvement than later life treatment in individuals with mutations of the Cr-synthesis genes *Arginine:glycine amidinotransferase* (*AGAT*) or *guanidinoacetate-methyltransferase (GAMT)* (Battini et al 2006; Stockler-Ipsiroglu et al 2014; Stockler-Ipsiroglu et al 2015). Mitochondrial dysfunction or CK inhibition reduces the growth and dendritic arborization of cerebellar Purkinje cells *in vitro* (Fukumitsu et al 2015). The neurons with dysfunctional mitochondria were rescued by Cr supplementation, highlighting the role Cr plays in cellular metabolism. Together, the human and *in vitro* data strongly suggest that Cr is necessary for brain development.

We developed transgenic mice with exons 2-4 of the *Slc6a8* gene flanked by loxP sites ($Slc6a8^{FLOX}$) that allow for conditional deletion of the gene (Skelton et al 2011). Ubiquitous ($Slc6a8^{-/y}$) and brain-specific knockout mice generated from this line have spatial learning, object recognition, and fear memory deficits (Skelton et al 2011; Udobi et al 2018). The purpose of this study was to determine if the neonatal or adult loss of the *Slc6a8* gene was sufficient to cause learning and memory deficits. Using a tamoxifen-inducible $CreER^{T2}$, we examined the cognitive effects caused by deletion of the *Slc6a8* gene on postnatal day (P)5 or P60.

METHODS

Full methods are available in the supplement.

Generation of conditional knockout mice

All institutional and national guidelines for the care and use of laboratory animals were followed. Female $Slc6a8^{FLOX/+}$ mice, generated and genotyped as described (Skelton et al 2011), were mated to mice expressing an inducible Cre recombinase driven by the *UBC* promoter (B6.Cg-$Ndor^{Tg(UBC-Cre/ERT2)Ejb}/1J$ *(UBC-CreERT2)*; Jackson laboratory, Bar

Harbor, ME). On either P5 or P60, mice were treated (1/d i.p.) with tamoxifen (75 mg/kg) or corn oil vehicle for 5 d, to generate mice lacking Slc6a8 in either neonatal (P5KO) or adult (P60KO) mice. Behavioral testing was performed 60 d following treatment (P65 for the P5KO group and P125 for the P60KO group). The groups (along with N's) used are outlined in supplemental table 1.

Slc6a8 expression and Cr determination

Tissue collection: For the P60KO mice, tissues were harvested at 0, 5, 10, 15, 30, 60, and 90 days following tamoxifen exposure to gain a better understanding of Cr content over time. For the P5KO group, tissue was harvested at 0, 60, and 90 days to determine the Cr content at the beginning and end of testing. Mice were lightly anesthetized with isoflurane inhalation and sacrificed by decapitation. The brain was removed from the skull, divided into hemispheres, and flash frozen.

Cr determination: Cr content was measured using reversed phase HPLC with UV detection (Tranberg et al 2005).

Quantitative RT-PCR: Real-time quantitative PCR (QPCR) was used to measure *Slc6a8* expression levels in the brain as described previously (Hautman et al 2014).

Morris water maze

The Morris water maze (MWM) is a test of spatial learning and reference memory (Vorhees and Williams 2006). Mice were tested as described (Skelton et al 2011; Udobi et al 2018). Mice were tested in three phases, a visible platform phase, a hidden platform acquisition phase and a reversal phase. Hidden platform testing was conducted over 4 days with a probe trial on day 5 of testing. 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) as previously described (Hautman et al 2014; Udobi et al 2018). On the test day, mice were presented with two identical objects and allow to explore until 30 s of observation time between objects was accrued. The maximum time for the trial was 10 min. 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. Percent time spent observing the novel object was the independent variable for this test.

Contextual and cued fear conditioning

Conditioned and contextual fear was assessed as described with modification (Peters et al 2010; Udobi et al 2018).

Overnight locomotor activity

Spontaneous locomotor activity (Brooks and Dunnett 2009) was tested in automated activity chambers. Mice were placed into the open-field arena at 1800 and left undisturbed for 14 h, with lights off at 2000 and on at 0600 the next day. The dependent measure was total number of photobeams interruptions.

Statistics

The hypothesis of this study was that the conditional knockout mice would differ from corn-oil treated *Slc6a8*$^{+/y}$*::Ubc-CreERT2*$^{-}$ mice (WT-VEH). Therefore, preplanned comparisons were designed regardless of the outcome of the omnibus ANOVA. As there were no interactions with the repeated measure, the Dunnett's tests were performed using the means of all trials. A $p<0.05$ is used to reject the null hypothesis that the groups are the same. The F values from the omnibus ANOVAs for each test are presented in Supplemental Data Table 2. Data are presented as LSMEANS±SEM and will be made freely available upon request.

RESULTS

Body weight, *Slc6a8* expression, Cr measurement

Expression of *Slc6a8* mRNA was measured following 5 d of tamoxifen administration. No *Slc6a8* expression was seen in the P5 or P60 groups (data not shown). Brain Cr levels were reduced in both P5KO (Figure 1A) and P60KO (Figure 1B) mice. No differences were observed in Cr levels between P5KO and P60KO mice at the time of testing ($t_{10}=1.293$, $p=0.225$). Main effects of gene were seen in P5KO ($F(1,175)=38.21$, $p<0.0001$; Figure 1C) and P60KO ($F(1,111)=167.23$, $p<0.0001$; Figure 1D) . KO mice from both groups weighed less than the control mice at the time of behavioral testing. No effects of tamoxifen were observed. We have shown that these body weight reductions do not affect cognitive function (Udobi et al 2018).

Morris Water Maze

On the first day of visible platform testing, P5KO and WT-VEH mice had a similar latency to the platform ($t_{130}=2.23$, $p=0.1386$, Figure 2A). On days 2 and 3, P5KO mice had longer latencies across days compared with the WT-VEH group ($t_{130}=3.03$, $p=0.0181$). For the P60-treated group, no effects were seen between P60KO and WT-VEH mice on any day ($t_{60.9}=2.04$ $p=0.2369$ for days 2-3; Figure 2B).

During the acquisition phase of hidden platform testing, P5KO mice had longer latencies to find the platform than the WT-VEH mice ($t_{174}=4.27$, $p=0.0002$; Figure 2C*).* Similar effects were seen in the distance taken to the platform and for path efficiency, which measures how close to a direct path the mouse takes to find the platform. No differences were observed between WT-VEH mice and the other control groups in any measure (Figure 2G). There were no differences in swim speed between groups. During the reversal phase, the P5KO mice had longer latencies than WT-VEH group ($t_{140}=-2.78$, $p=0.033$; Figure 2E) while no differences were observed between control groups.

No differences were observed during the acquisition or reversal phase in P60KO mice (Figures 2D, 2F, and 2H). All groups had a similar swim speed in the P60 groups. ($t_{97.5}$= −2.17, p=0.1727 P60KO vs WT-VEH).

A probe trial was conducted the day following the completion of each phase of MWM training as a test of spatial memory. (Supplemental Figure 1). There were no differences observed in the P5 or P60 groups in the acquisition phase. During the reversal phase, the P5KO had a greater average distance from the platform compared with WT-VEH (t_{137}=2.74, p=0.0408). No differences were observed in P60KO mice (t_{77}=0.78, p=0.4353).

Novel object recognition

The P5KO mice spent less time with the novel object compared with WT-VEH (t_{113}=−2.78, p=0.0384; Figure 3). No differences were observed in the P60 group or any of control groups from the P5 or P60 treatment.

Fear memory

No differences were observed in contextual or conditioned fear in either group (Supplemental Figure 2).

Overnight locomotor activy.

Locomotor activity was measured continuously for 2 h prior to the dark phase, the entire 10 h dark phase, and 2 h after the lights came back on (Figure 4). During the first 2 h, no differences were observed in either KO group. While the P5KO mice were hyperactive during the dark phase (t_{459}=4.26, p=0.0002) there were significant effects of all tamoxifen controls vs WT-VEH. During the overnight testing, the tamoxifen-treated (t_{211}=4.89. p<0.0001) and P5KO (t_{211}=−2.96, p=0.0034) mice were hyperactive compared with vehicle-treated mice but did not differ from each other. In the 2 h light period following the dark cycle, tamoxifen-treated (t_{144}=4.01. p<0.0001) and P5KO (t_{144}=−2.26, p=0.0253) mice showed hyperactivity. No differences were observed between tamoxifen-treated and P5KO mice (t_{211}=−0.31, p=0.7587). No differences in activity between the WT-VEH and the P60KO mice were observed during any phase.

DISCUSSION

The purpose of this study was to determine if there is a developmental component to the cognitive deficits seen in CTD patients and *Slc6a8* knockout mice. By eliminating the *Slc6a8* gene in adulthood and during the neonatal period, we were able to determine that the later-developing brain is still sensitive to the loss of Cr while the adult brain is not. Combined with data from human Cr-synthesis deficiencies supplemented with Cr and in vitro work showing Cr is involved in neuronal morphology, these findings suggest that Cr plays an important role in brain development. In addition, we show that tamoxifen exposure during the neonatal period may lead to changes in locomotor activity. This finding further affirms the need for adequate controls in behavioral studies.

As seen in Figure 1, tamoxifen induced a successful recombination in *Slc6a8*^{FLOX} with no brain *Slc6a8* expression after 5 d of treatment. By the time of behavioral testing both groups of mice had Cr levels that were similar to Cr levels seen in the ubiquitous *Slc6a8* knockout mice (Skelton et al 2011). In the P60 mice, a gradual reduction in brain Cr levels was observed suggesting that Slc6a8 is necessary for the maintenance of brain Cr levels, through peripheral transport and possible transport of Cr intermediates required for brain Cr synthesis, and not for the normal loss of Cr through degradation to creatinine and export to the CSF. This is important when designing treatments for CTD as it suggests that there is not a "trapping" mechanism that could lead to an excessive amount of Cr accumulating with Cr-derived treatments. While we observed a gradual loss of Cr in the brain, the data collected are not sufficient to determine the rate in which the brain loses Cr. The purpose of these measurements was to ensure that Cr levels were significantly reduced by the time of testing. In fact, the brain Cr levels in this study (3-5% of WT levels) were lower than the Cr levels from the brain-specific Slc6a8 used in Udobi et al (20% of WT), suggesting that Cr levels were adequately reduced prior to testing. The loss of *Slc6a8* in adult mice caused an almost immediate weight reduction. The musculoskeletal effects of Cr are well documented since most Cr-related studies are focused on its use as an athletic supplement, so while the immediacy of the weight loss was unexpected, it was expected that the Slc6a8-KO would lose weight or have reduced growth.

Translating rodent to human brain development is a multifactorial process without direct correlations in many cases. Many processes that happen simultaneously in the rodent do not occur simultaneously in humans. In the case of this study, we set out to understand why the lack of Cr leads to deficits in MWM, NOR, and conditioned fear performance. As MWM and NOR are largely mediated by the hippocampus, we concluded that the ideal treatment age for this study should be centered on the development of this region. In rodents, the peak development of the hippocampus occurs during the neonatal period. During this time, the hippocampus appears to be the most sensitive to hypoxia (Ikeda et al 2001) and drugs of abuse like methamphetamine (Williams et al 2003; Skelton et al 2007). The results of this study similarly suggest that the lack of Cr during peak levels of hippocampal development leads to cognitive deficits. Interestingly, conditioned fear behavior was not affected in either group of mice tested. Conditioned fear is thought to be based primarily in the amygdala (Butler et al 2017; Ressler and Maren 2018), which reaches its peak development around gestational day 12 in the mouse (Clancy et al 2007). The lack of a conditioned fear phenotype and the presence of deficits in hippocampally-based behaviors in the P5KO mice suggest that Cr may play an important role in the development of this brain region as well. Future studies could be designed to eliminate the *Slc6a8* gene in the developing amygdala and determine if the conditioned fear effect could be isolated.

The P5KO and the P60KO mice weighed less than their respective controls, which has been considered as a potential confound for interpreting behavioral data. The visible platform is frequently used to test for sensorimotor deficits (Vorhees and Williams 2006) and the P5KO mice perform this task slower than their WT-VEH counterparts. However, mice with a brain-specific *Slc6a8* deletion show visible platform deficits and do not have weight differences (Udobi et al 2018). In addition, the P5KO mice do show improvement over trials, suggesting that they are able to perform and learn the task. Finally, the P5KO mice do not show swim

speed deficits during the hidden platform trials suggesting that the loss of Cr does not directly impact physical performance. Spatial learning and memory as well as novel object recognition deficits were observed in mice lacking *Slc6a8* in *Camk2a*-expressing neurons (Kurosawa et al 2012). While the primary focus of the Kurosawa study was on the therapeutic potential of cyclocreatine, the use of the Camk2a-Cre leads to a perinatal deletion of the *Slc6a8* gene (Casanova et al 2001), though only in excitatory neurons. Interestingly, the lack of a probe trial effect during the acquisition phase of the MWM was evident in both models. A probe trial effect was seen during reversal, showing that this extra testing phase is important to fully assess spatial learning and memory. As with the brain-specific *Slc6a8* knockout mice, the *Camk2a-Cre::Slc6a8*^{FLOX} mice did not have changes in body weight or swim speed, supporting the hypothesis that the deficits in the MWM are not due to motor changes in the mice. Cyclocreatine treatment during adulthood was able to rescue the cognitive deficits in the *Camk2a::Slc6a8*^{FLOX} mice. This would suggest that even though early Cr loss is required for deficits to manifest, the brain is still amenable to later treatment. It should be noted that the *Camk2a::Slc6a8*^{FLOX} mice may not be ideal for treatment due to an intact Slc6a8 at the blood brain barrier. Further studies using ubiquitous knockouts and restoration of the *Slc6a8* gene could provide a greater insight into this critical issue.

Attention deficit hyperactivity disorder is a commonly reported finding in CTD though it is not present in all patients (van de Kamp et al 2013). Based on these findings, we have consistently evaluated locomotor behavior in our mice as an initial screen for hyperactivity in order to determine how well the phenotype of this model mimics the phenotype of the human disorder. Interestingly, to date there has not been a consistent locomotor phenotype in *Slc6a8* mice. The *Slc6a8*$^{-/y}$ mice showed an initial hypoactivity followed by similar activity levels to WT (Skelton et al 2011). Female heterozygous (Hautman et al 2014) and brain-specific knockouts were hyperactive (Udobi et al 2018). While P5KO mice were hyperactive during the dark phase compared with the WT-VEH mice, this was likely due to tamoxifen administration since all mice treated with tamoxifen from P5-10 were hyperactive. To our knowledge this is the first study that has shown that neonatal tamoxifen exposure leads to nocturnal hyperactivity. In a recent study, Mikelman et al show that tamoxifen interacts with the dopamine transporter (DAT) and can block amphetamine-stimulated dopamine release as well as blocking dopamine reuptake in rat synaptosomes (Mikelman et al 2018). While acute tamoxifen exposure did not change locomotor behavior, it was effective at blocking the locomotor-stimulating effects of amphetamine (Mikelman et al 2017; Mikelman et al 2018). It is possible that the neonatal DAT stimulation by tamoxifen caused the mild hyperactivity when the mice were tested as adults, though it is also possible that modulation of the estrogen receptor played a role. Together, these data add to the growing literature that behavioral studies employing tamoxifen, especially those looking at dopamine-modulated behaviors, should always include vehicle-treated controls. It should be noted that tamoxifen exposure did not lead to deficits in the cognitive tasks-showing that the loss of the *Slc6a8* was responsible for these deficits.

In conclusion, the findings of this paper show that Cr plays an important role in brain development in terms of cognition. Mice lacking *Slc6a8* from early life show spatial learning and memory deficits that were not present when the *Slc6a8* was knocked out in

adult mice. There were also deficits in object recognition memory in the P5KO mice while this memory was intact in P60KO mice. These data provide a valuable information when it comes to designing treatment strategies for CTD. Beyond the desire to provide maximal temporal benefit to the patient, full recovery of symptoms may rely on early-life treatment of this disorder.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Take-home message:

The learning and memory deficits seen in Slc6a8-deficient mice are likely due to the developmental loss of Cr.

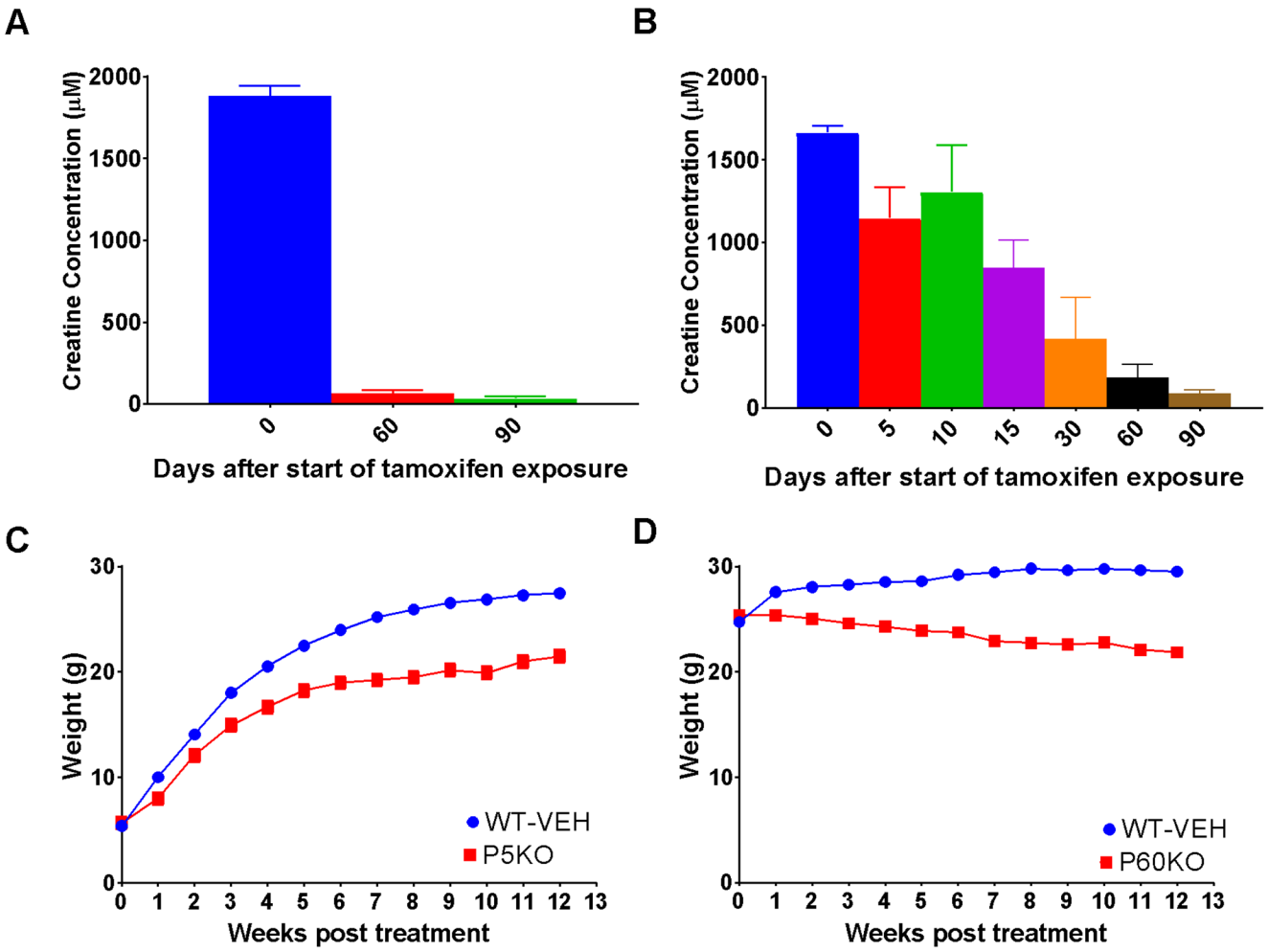

Figure 1. Reductions in body weight and brain Cr levels following P5 or P60 *Slc6a8* deletion.

A) Brain Cr levels in P5KO mice before and 60- or 90-days following tamoxifen exposure. By the time of behavioral testing (60 days) Cr levels were reduced to less than 10% of initial levels. B) Time course of Cr levels in P60KO mice. C) The P5KO mice gained weight as they aged however the growth did not match WT mice (main effect of group). D) In P60KO mice there was a body mass reduction following tamoxifen administration. Data are LSMEAN±SEM. N=13-18/group

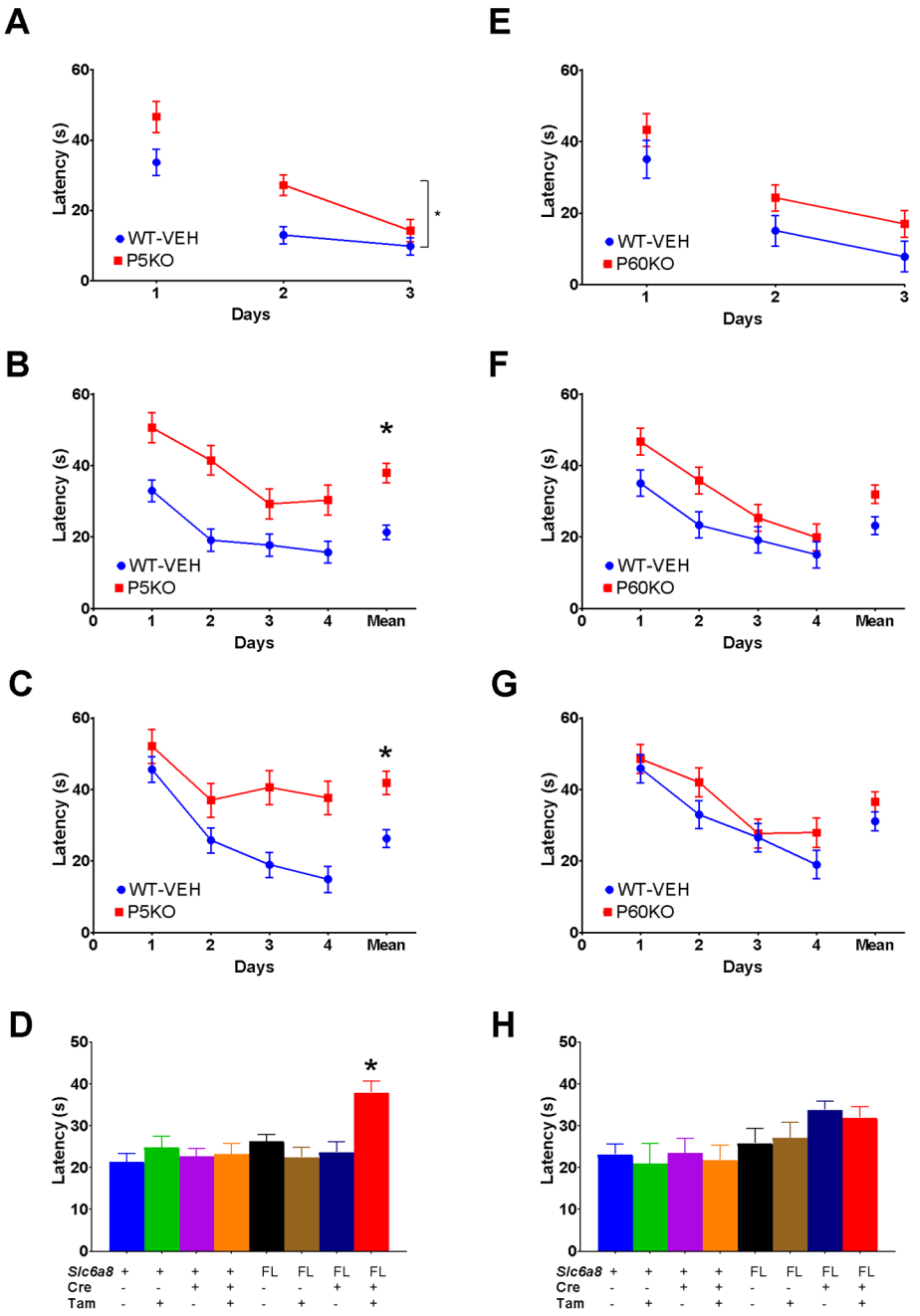

Figure 2. Spatial learning deficits in P5KO but not P60KO mice.

During visible platform training (A), P5KO mice show an increase latency on days 2 and 3 of training compared with WT-VEH mice. All mice improved across days. In the hidden platform training, the P5KO mice show higher mean latencies to the platform across all days of testing (Mean) during the (B) acquisition and (C) reversal phases of the MWM compared with the WT-VEH mice. (D) Shows the overall mean across days for all groups tested with only the P5KO mice differing from the WT-VEH mice. No differences were observed in the P60 during (E) visible platform, (F) acquisition, or (G) reversal testing. There were no

differences between any P60 group tested and WT-VEH during the acquisition phase (H). For panels D and H, *Slc6a8* refers to the Slc6a8 genotype*:* (*+*) is WT, (FL) is floxed; *Cre* refers to UBC-Cre: (−) means the mouse was not carrying a copy of UBC-Cre, (+) indicates expression; *Tam* refers to tamoxifen administration, (+) received tamoxifen, (−) received vehicle. The far-right bar in each panel shows the WT-VEH while the far-left bar of each panel shows the age-respective KO Data are LSMEAN±SEM, *p<0.05. N=13-18/group

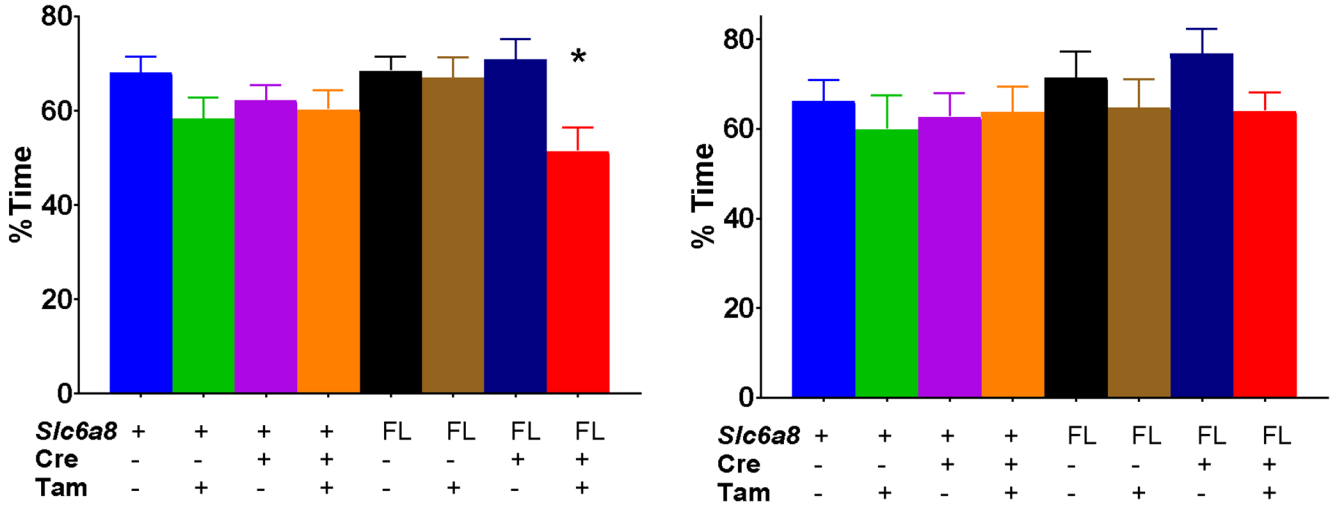

Figure 3. Novel object recognition deficits in P5KO mice.

Slc6a8 refers to the Slc6a8 genotype: (+) is WT, (FL) is floxed; *Cre* refers to UBC-Cre: (−) means the mouse was not carrying a copy of UBC-Cre, (+) indicates expression; *Tam* refers to tamoxifen administration, (+) received tamoxifen, (−) received vehicle. The far-right bar in each panel shows the WT-VEH while the far-left bar of each panel shows the age-respective KO. *Left:* In the P5-treated group, only the P5KO mice show a reduction in time spent with the novel object compared with the WT-VEH. *Right:* No differences from WT-VEH were observed in any of the groups treated at P60. Data are LSMEAN±SEM. N=9-18/group. *p<0.05

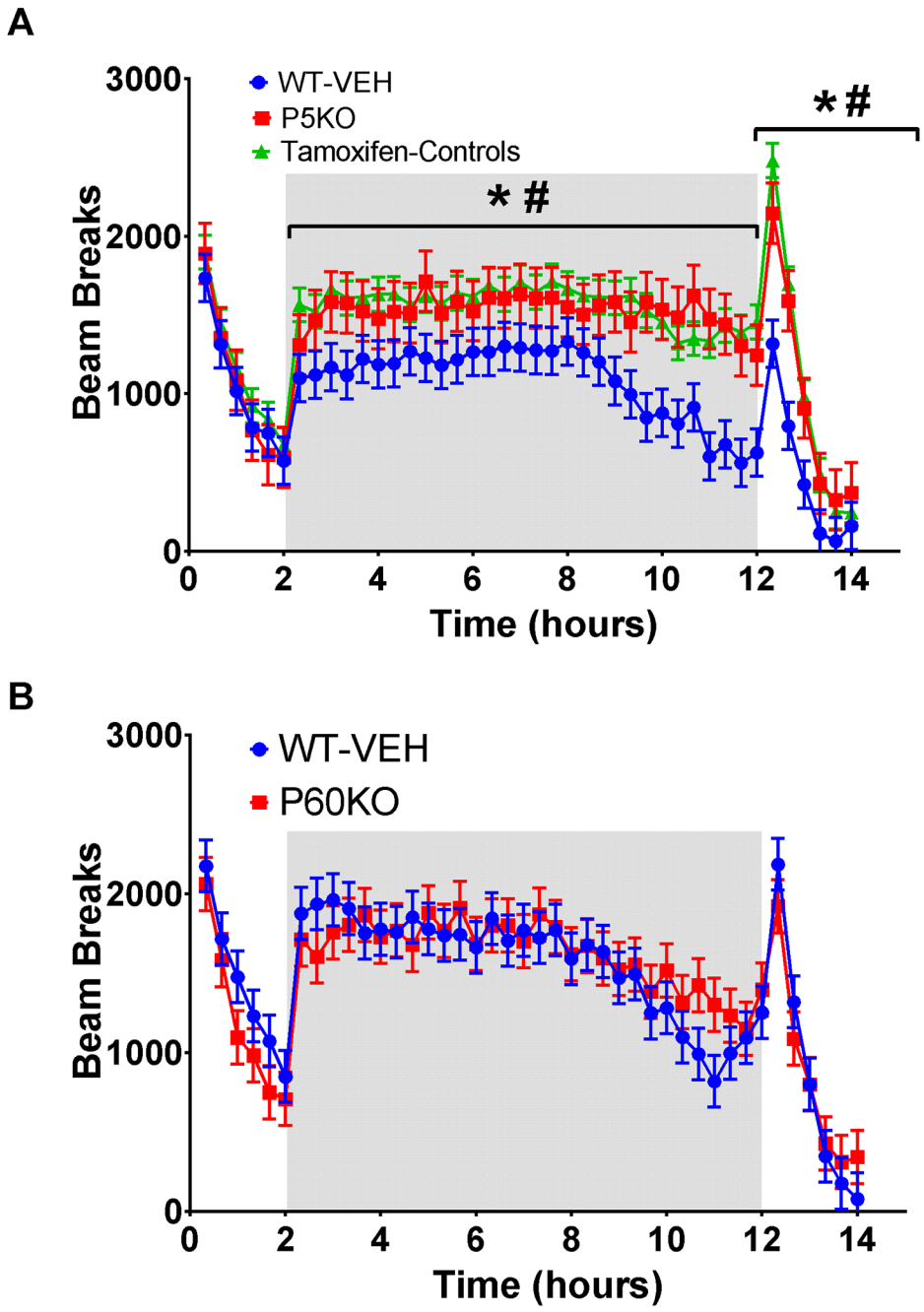

Figure 4. Neonatal tamoxifen administration leads to hyperactivity during adulthood.

Locomotor activity was assessed in mice for 14 hours with the shaded region representing testing during the lights-off phase. Data are presented as the total beam breaks during a 20-min period. (A) The P5KO mice show a hyperactivity compared with WT-VEH mice; however, the remaining tamoxifen treated mice show a similar hyperactivity. (B) No differences in activity were observed between WT-VEH and P60K0. Data are LSMEAN

±SEM. N=13-18/group. *p<0.05 P5KO vs WT-VEH; # p<0.05 Tamoxifen controls vs WT-VEH