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Hippocampus specific iron deficiency alters competition and cooperation between developing memory systems

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Abstract Iron deficiency (ID) is the most common gestational micronutrient deficiency in the world, targets the fetal hippocampus and striatum and results in long-term behavioral abnormalities. These structures primarily mediate spatial and procedural memory, respectively, in the rodent but have interconnections that result in competition or cooperation during cognitive tasks. We determined whether ID-induced impairment of one alters the function of the other by

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genetically inducing a 40% reduction of hippocampus iron content in late fetal life in mice and measuring dorsal striatal gene expression and metabolism and the behavioral balance between the two memory systems in adulthood. *Slc11a2*$^{hipp/hipp}$ mice had similar striatum iron content, but 18% lower glucose and 44% lower lactate levels, a 30% higher phosphocreatine:creatine ratio, and reduced iron transporter gene expression compared to wild type (WT) littermates, implying reduced striatal metabolic function. *Slc11a2*$^{hipp/hipp}$ mice had longer mean escape times on a cued task paradigm implying impaired procedural memory. Nevertheless, when hippocampal and striatal memory systems were placed in competition using a Morris Water Maze task that alternates spatial navigation and visual cued responses during training, and forces a choice between hippocampal and striatal strategies during probe trials, *Slc11a2*$^{hipp/hipp}$ mice used the hippocampus-dependent response less often (25%) and the visual cued response more often (75%) compared to WT littermates that used both strategies approximately equally. Hippocampal ID not only reduces spatial recognition memory performance but also affects systems that support procedural memory, suggesting an altered balance between memory systems.

Keywords Iron deficiency · Memory systems · Hippocampus · Striatum · DMT1, Slc11a2, Nuclear magnetic resonance spectroscopy · Morris water maze · Spatial memory · Procedural memory

Introduction

Iron deficiency is the most common nutrient deficiency in the world, and affects the developing fetal brain in humans in the context of gestations complicated by maternal iron deficiency, diabetes mellitus, or hypertension (Georgieff et al. 1990; Lozoff and Georgieff 2006). Behaviorally, iron deficient infants of diabetic mothers have abnormal recognition memory at birth (Siddappa et al. 2004), a finding that remains present at 3.5 years of age and is a function of the degree of ID at birth (Riggins et al. 2009). Fetal/neonatal ID has adverse long-term effects (despite iron repletion after birth) on both the hippocampus and the striatum, which are among the primary brain regions that contribute to spatial navigation and procedural memory, respectively (Felt and Lozoff 1996; Beard et al. 2002; Felt et al. 2006; Schmidt et al. 2007; Ward et al. 2007; Carlson et al. 2009). ID reduces energy metabolism, glutamatergic and GABAergic neurotransmission, neuronal dendrite extension, and pre- and postsynaptic function in the rodent hippocampus (Jorgenson et al. 2003; Rao et al. 2003; Jorgenson et al. 2005; Carlson et al. 2009). Early ID also profoundly and permanently alters monoamine and energy metabolism in the rodent striatum in spite of iron treatment (Youdim et al. 1980; Beard et al. 2003; Beard et al. 2006; Felt et al. 2006; Ward et al. 2007).

Optimal cognitive development and subsequent performance rely on the interactions among multiple memory systems including recognition memory based largely in the hippocampus, and procedural memory linked to the dorsal striatum (White and McDonald 2002). In the Multiple Parallel Memory Systems model (White and McDonald 2002), these systems are juxtaposed as competitive, while in the Interactive Memory Systems Theory these systems act cooperatively to mediate behavior (McDonald et al. 2004). Important connections among these systems allow detection of novelty and facilitate cognitive flexibility (McDonald et al. 2002; Lisman and Grace 2005; McDonald et al. 2008). Dampening of one input system can result in dominance of another, consequently eliciting a maladapted behavioral phenotype (McDonald et al. 2004; Lee et al. 2008).

A working framework of how these individually affected areas work together to produce abnormal early life and adult behavioral phenotypes seen in ID has not been explored despite an extensive literature on the adverse effects of early ID on the primary circuitry of each memory system (Lozoff and Georgieff 2006). One approach to create such a framework is to selectively cause ID in one memory system (and not the others) to understand the role of the first system in regulating the others. This has not been possible with the maternal dietary ID model because it induces ID throughout the brain and thus affects structures in both systems (deUngria et al. 2000). Recently we reported a model that generates early ID specifically in hippocampal neurons at embryonic day 18.5 by deleting the iron transporter *Slc11a2* in a conditional manner in neurons of the hippocampus (Carlson et al. 2009). The model has several advantages. It allows a reductionistic approach to examine the effects of ID in a tissue specific manner. It avoids several potential confounds of dietary models, such as anemia (which can cause fatigue and tissue hypoxia), altered maternal behavior, and increased uptake of other divalent metals. The targeted tissue knock-out obviates the problem with whole-animal *Slc11a2* knockouts, which are lethal in the first week of life (Gunshin et al. 2005).

Slc11a2 encodes a channel protein involved in the transfer of ferrous iron from a clathrin-coated endosome to the cytosol, after it has been taken up into the cell on the transferrin-transferrin receptor-1 complex (Gunshin et al. 2005). *Slc11a2*, in addition to other iron-sensitive mRNA transcripts, are upregulated in hippocampus after spatial navigation learning, suggesting that neuronal iron uptake is critical for such learning (Carlson et al. 2009; Haeger et al. 2009). Cre-recombinase mediated deletion of this gene specifically in neurons of the developing hippocampus reduces iron content and impairs spatial navigation learning and memory in two

versions of place learning in the Morris Water Maze. The effect is accompanied by altered hippocampal gene expression, energy metabolism and neuronal structure (Carlson et al. 2009). Here, we use this unique model to probe the effects of hippocampal-neuron specific ID on other brain regions and functions, in order to understand the interactions between developing spatial and procedural memory systems.

Methods

Animals All experiments were performed in accordance with the NRC Guide for the Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committee of the University of Minnesota. *Slc11a2*$^{hipp/hipp}$ mice and *Slc11a2*$^{WT/WT}$ littermates were bred as described previously; briefly: *Slc11a2* flox/flox mice (Gunshin et al. 2005) were crossed with *CaMKIIa-cre* (L7ag#13 line, (Dragatsis and Zeitlin 2000)) transgenic mice to generate hippocampal-neuron specific knockout of *Slc11a2* (Carlson et al. 2009). All animals were bred and housed in a specific pathogen free facility, and only male littermates were analyzed. Individual mouse tail DNA was genotyped by PCR, with previously published cycling conditions and primers (Carlson et al. 2009).

Tissue dissection, iron concentration analysis, and RNA collection Male mice (not used in other experiments) at age 3 months were killed by an intraperitoneal injection of Beuthanasia (10 mg/kg) for both iron content experiments and mRNA isolation as previously described (Carlson et al. 2009). The striatum, nucleus accumbens (NA), PFC, ventral midbrain (VMB, substantia nigra and ventral tegmentum), and hippocampus were dissected and flash-frozen in liquid nitrogen. Iron concentration was analyzed with atomic absorption spectroscopy as described previously (Beard et al. 2006). Total RNA was isolated and concentrations were measured as previously described (Carlson et al. 2009).

Quantitative real-time PCR Messenger RNA levels from the five regions were measured for 14 transcripts from P90 mice by real-time, quantitative PCR (qPCR) (Taqman) (Supplemental Table 1). Thirteen mRNA transcripts were used to assess iron metabolism, the neurometabalome, and dendritic structure. Reverse transcription was carried out using SuperScript III (Invitrogen) and random hexamers as described previously (Carlson et al. 2009).

In vivo ^{1}H NMR spectroscopy All spectroscopic experiments were performed as previously described (Carlson et al. 2009) on a horizontal bore 9.4 T/31 cm magnet (Varian/Magnex, Oxford, UK) equipped with a 15-cm I.D. gradient coil insert (450 mT/m, 200 μs) which included a strong second- order shim system (Resonance Research, Inc., Billerica, MA) interfaced to a Varian INOVA console (Varian, Inc., Palo Alto, CA, USA) (Tkac et al. 2004).

In vivo ^{1}H NMR spectra were collected using a previously described protocol (Rao et al. 2003; Tkac et al. 2004; Rao et al. 2007; Ward et al. 2007; Carlson et al. 2009). Briefly, all first- and second-order shims were automatically adjusted using FASTMAP (Gruetter 1993; Gruetter and Tkac 2000). Ultra-short echo-time STEAM (echo time TE=2 ms, repetition time TR=5 s, number of transients NT=240) combined with outer volume suppression and VAPOR water suppression (Tkac et al. 1999; Tkac et al. 2004) was used to acquire spetral data from 5.2 μL (1.6×1.8×1.8 mm^3) volume of interest (VOI) centered in the left dorsal striatum. Multi-slice coronal and sagittal RARE imaging technique (echo train length ETL = 8, echo spacing ESP = 15 ms, TE = 60 ms, matrix = 256×128, FOV = 20 mm×20 mm, slice thickness = 1 mm) was used for the selection of the VOI.

Metabolite concentrations were quantified from ^{1}H NMR spectra using LCModel software package (Provencher 1993). The LCModel analysis calculates the best fit to the experimental spectrum as a linear combination of model solution spectra of brain metabolites. The spectrum of fast relaxing macromolecules was also included in the LCModel basis set as previously described (Pfeuffer et al. 1999; Rao et al. 2003; Tkac et al. 2004; Rao et al. 2007; Ward et al. 2007; Carlson et al. 2009). Unsuppressed water signal was used as an internal reference assuming brain water content of 80%. Concentrations of the following 14 metabolites were consistently quantified from striatal ^{1}H NMR spectra: alanine (Ala), creatine (Cr), phosphocreatine (PCr), γ-aminobutyric acid (GABA), glucose (Glc), glutamate (Glu), glutamine (Gln), glutathione (GSH), the sum of glycerophosphorylcholine and phosphocholine (GPC + PC), *myo*-inositol (*myo*-Ins), lactate (Lac), *N*-acetylaspartate + *N*-acetylaspartylglutamate (NAA + NAAG), phosphoethanolamine (PE), and taurine (Tau). Estimated errors of metabolite concentrations (Cramer-Rao lower bounds) were 0.3–0.5 μmol/g.

Behavioral experiments

Apparatus The Morris Water Maze (MWM) consisted of a white circular water tank 120 cm in diameter and 45 cm high, filled to 30 cm. A transparent platform (10 cm in diameter) was placed so that its surface was either 1.5 cm below the water line (hidden escape platform) or 0.5 cm above the water line and locally cued with a visible flag. The pool was located in a large test room where there were many cues external to the maze (e.g., pictures, lamps): these were visible from the pool and could be used by the mice for spatial orientation. These extra-maze cues were kept

constant throughout the testing period. Water temperature was kept at 21–23°C. Animal behaviors were videotaped and analyzed by the Topscan system (Clever Systems, Reston, VA). For descriptive data collection, the pool was subdivided into four equal quadrants formed by imaginary lines.

Procedural memory experiment This experiment was performed in animals not used in the Competition Experiment. A single habituation trial was performed 1 day before the first water maze test, by placing each mouse on the hidden platform for 30 s. If the mouse fell or jumped from the platform, it was placed back on the platform. Four cued trials, in which the escape platform protruded above the water surface at different locations within the target quadrant, were performed a day after the habituation trial.

Competition experiment This experiment was performed in animals not used in the Procedural Memory Experiment. As with the first MWM experiment, a single habituation trial was performed 1 day before the first water maze test (Carlson et al. 2009). In this experiment, each mouse received four training trials per day for 8 consecutive days. On the first day of the experiment, mice were trained to swim to the visible platform located in the center of the target quadrant equivalent to the procedural memory experiment described above. Each daily training session consisted of four trials on which each mouse was released once from each of four start points. The trial began by placing a mouse in the pool facing the wall, at a randomly selected start position and ended when the mouse climbed onto the visible platform, or after 30 s had elapsed. If the mouse had not escaped after 30 s, it was gently placed on the platform. Each mouse was left on the platform for 30 s, and returned to its home cage. For each mouse, there was a 20–25 min delay between trials within a daily session. During the delay, the remaining mice were run on the same trial.

On the next day after the day of training on the visible platform, each mouse received a four-trial session in which the visible platform was replaced with the submerged platform in the same location. Identical training procedures were used during these hidden platform trials. Subsequently, this 2-day sequence of one visible platform session followed by one hidden platform session was repeated three more times, for a total of 32 training trials (16 visible, 16 hidden) over a total of 8 days. Escape latency (time to reach the available platform) were measured on these acquisition trials.

On day 6 and day 8, competition probes were given. The visible platform was moved to the center of the quadrant directly opposite the target quadrant used in the acquisition trials. Two trials were given from start positions equidistant to the center of the both the target quadrant used during training and the new location of the visible platform. Video recordings were used to determine whether the mice swam within 5 cm of the perimeter of the former platform location in the target quadrant before escaping to the visible platform, now located in the opposite quadrant. A point was scored for each animal each time it crossed within 5 cm of the perimeter of the former platform location in the target quadrant.

Statistical analysis For statistical comparisons between data from *Slc11a2*$^{hipp/hipp}$ and *Slc11a2*$^{WT/WT}$ mice in iron content, gene expression, and ^{1}H MRS studies, significance was determined using unpaired, two-tailed Student's *t* tests. For behavioral data, we performed two-way ANOVA with presence or absence of Cre recombinase expression (Cre +/− status), trial number, and their interaction as variables to examine the response of each group for each trial in the visual cued task and for analysis of competition probe trials. We analyzed the aggregate responses of competition trials between *Slc11a2*$^{hipp/hipp}$ and *Slc11a2*$^{WT/WT}$ mice with unpaired, two-tailed Student's *t*-test.

Results

Iron concentration in striatum and nucleus accumbens The iron concentration was not significantly reduced in either striatum (*Slc11a2*$^{hipp/hipp}$: 18.0±0.8 μg iron/g tissue ± SEM, n=8; *Slc11a2*$^{WT/WT}$: 20.9±1.0 μg iron/g tissue ± SEM, n = 12, P>0.05) or NA (*Slc11a2*$^{hipp/hipp}$: 13.5±0.6 μg iron/g tissue ± SEM, n=8, *Slc11a2*$^{WT/WT}$: 15.0±0.4 μg iron/g tissue ± SEM, n=12, P>0.05) in *Slc11a2*$^{hipp/hipp}$ mice at 3 months old.

Gene expression in five selected brain regions Three month old male mice (n=5/group) were used for all gene expression results. *Slc11a2* showed decreased expression in all brain regions measured (Fig. 1a). Since Cre is not expressed in all regions of the brain of *Slc11a2*$^{hipp/hipp}$ mice, the question arose of whether the decrease in gene expression in regions of the brain besides hippocampus was due to deletion of the gene by Cre recombinase or down-regulation, potentially due to decreased demand. Thus, we directly compared expression of deleted region/floxed Slc11a2 (detected by primer set "ex6-7") with nonfloxed/nondeleted Slc11a2 (detected by primer set "ex15-6") in each brain region of each animal. In areas where *Slc11a2* was conditionally knocked out (hippocampus, PFC, and NA), there was significant decrease in the floxed region

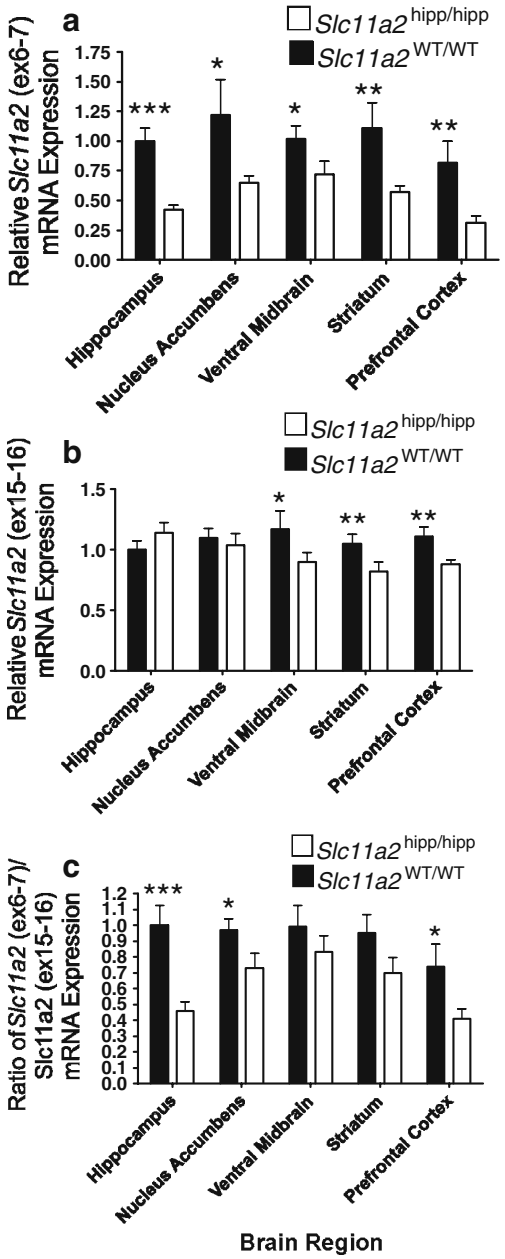

Fig. 1 Gene expression of *Slc11a2* exon 6–7 (floxed region) (**a**) *Slc11a2* exon 15–16 (unfloxed region) (**b**) and ratio of floxed to unfloxed expression (**c**) in selected brain regions. Values are means ± SEM, $n=5$ for each group. *Significantly different from $Slc11a2^{hipp/hipp}$ mice at $P<0.05$, ** $P<0.01$, *** $P<0.001$

of expression, relative to the unfloxed region (Fig. 1c). In areas (e.g., striatum, VMB) where iron demand was lower due to reduced metabolism (see ^{1}H NMR spectroscopy results below), there were no significant relative decreases in the floxed to unfloxed region of expression (Fig. 1b–c).

Twelve additional mRNA transcripts relevant to iron metabolism, neurometabolome, and dendritic and synaptic structure and function (Carlson et al. 2009) were different in $Slc11a2^{hipp/hipp}$ mice compared with $Slc11a2^{WT/WT}$ mice in at least one brain region (Table 1).

^{1}H NMR spectroscopy in striatum In vivo ^{1}H NMR spectra measured from striata of $Slc11a2^{hipp/hipp}$ ($n=6$) and $Slc11a2^{WT/WT}$ mice ($n=7$) are shown in Fig. 2a–b and were markedly different than spectra measured from the hippocampus in the same animals (Carlson et al. 2009). Neurochemical profiles quantified from 1H NMR spectra of $Slc11a2^{hipp/hipp}$ and $Slc11a2^{WT/WT}$ mice are compared in Fig. 3. Among the 14 neurometabolites that could be reliably quantified in the striatum, significant differences were observed in concentrations of metabolites involved in energy production (glucose, lactate, and phosphocreatine) and membrane metabolism (GPC + PC). Concentration of PCr was increased by 17% and concentrations of glucose and lactate were decreased in $Slc11a2^{hipp/hipp}$ mice by 18%, and 45% respectively ($P<0.05$) (Fig. 3). The PCr/Cr ratio in $Slc11a2^{hipp/hipp}$ mice was increased by 30% ($P<0.05$). Choline compounds (GPC + PC), a marker of membrane metabolism, was decreased in $Slc11a2^{hipp/hipp}$ mice by 19% ($P<0.05$) (Fig. 3). Markers of myelination (PE, *myo*-inositol), GABAergic neurotransmission, and neuronal integrity (NAA + NAAG) were unchanged.

Behavioral assessment We evaluated procedural memory function using the visual cue task of the MWM. Initially, a version of the visual cued task in which the visible platform is moved between training trials to random places within the target quadrant was used. $Slc11a2^{hipp/hipp}$ mice ($n=17$) had greater mean swim distances (ANOVA F-value=5.65 for Cre +/− status, $P<0.05$, F-value=3.1 for trial number $P<0.05$, and interaction, $P>0.05$) over all, compared to $Slc11a2^{WT/WT}$ mice ($n=13$), suggesting altered acquisition of procedural memory (Fig. 4). $Slc11a2^{hipp/hipp}$ and $Slc11a2^{WT/WT}$ mice had swim velocities that did not differ in this task (data not shown). We then determined relative contributions of spatial navigation memory and procedural memory deficits in $Slc11a2^{hipp/hipp}$ mice and WT littermates not used in the first experiment. We used a modified version of the MWM in which the visible platform and hidden platform training trials were alternated on a daily basis, and a competition test to probe for place and/or cued responses (McDonald and White 1994; Devan and White 1999). This version puts spatial navigation and procedural memory into direct competition (McDonald and White 1994). During training, there were no significant differences in escape latency (aggregated by training day) (data not shown) or distance traveled (aggregated by training day) between $Slc11a2^{hipp/hipp}$ ($n=8$) and $Slc11a2^{WT/WT}$ ($n=6$) mice on either the visible (cued) platform or the hidden platform (spatial navigation place learning) task suggesting

Table 1 Relative gene expression in each selected brain region. Numbers are fold expression changes relative to control, with control = 1

mRNA transcript level	Hippocampus (ID/IS)	Nucleus accumbens (ID/IS)	Ventral midbrain (ID/IS)	Striatum (ID/IS)	Prefrontal Cortex (ID/IS)
Tfrc-1 (transferrin receptor-1)	1.25*	1.35**	0.92 n.s.	1.12 n.s.	1.41*
Tf (transferrin)	1.33*	1.24*	0.99 n.s.	0.66***	1.32*
Ferritin, heavy	1.17**	1.21 n.s.	0.89 n.s.	0.92 n.s.	0.99 n.s.
Tyrosine hydroxylase	0.81 n.s.	0.90 n.s.	1.07 n.s.	0.71*	1.51 n.s.
Myelin basic protein	0.89 n.s.	0.96 n.s.	0.65*	0.65***	0.81 n.s.
Camk2a	0.91 n.s.	1.40*	0.82 n.s.	1.24*	0.73*
cFos	0.53*	0.96 n.s.	0.43**	0.19**	0.57*
Slc2a1 Blood-brain barrier glucose transporter	1.26*	1.40**	1.10 n.s.	1.03 n.s.	0.79*
Slc2a3, neuronal glucose transporter	0.99 n.s.	1.27*	0.98 n.s.	0.79*	1.00 n.s.
Slc40a1 (Ferroportin 1)	0.96 n.s.	0.72*	0.74*	0.75*	0.64*
Dlg4 (PSD-95)	1.29*	1.08 n.s.	0.96 n.s.	0.82**	0.85*
Creatine Kinase, brain	0.96 n.s.	0.98 n.s.	0.88 n.s.	0.71*	0.91 n.s.

n.s. = not significant. All animals are male, and 3 months old, n=5 for each group

*P<0.05, ** P<0.01, *** P<0.001

that there was no difference between *Slc11a2*$^{hipp/hipp}$ and *Slc11a2*$^{WT/WT}$ mice in terms of vision, motivation, or fatigue in the learning of this task (Fig. 5a). However, faced with the choice of escaping to the visible platform or searching the location for the previously hidden platform on the memory system competition probes, *Slc11a2*$^{hipp/hipp}$ mice showed fewer place responses during each trial (ANOVA F-value=4.11 for Cre +/− status, P<0.05; trial number and interaction P>0.05) (Fig. 5b) and in aggregate across trials (*Slc11a2*$^{WT/WT}$ had 0.58±0.18 place responses/ competition probe trial vs 0.25±0/10 place responses/ competition probe trial for *Slc11a2*$^{hipp/hipp}$ mice, P<0.05). Therefore, they spent significantly less time (ANOVA F-value=4.35 for Cre +/− status, P<0.05; trial number and interaction, P>0.05) and traveled a shorter distance (ANOVA F-value=4.17 Cre +/− status, P<0.05; trial number and interaction P>0.05) in the maze than *Slc11a2*$^{WT/WT}$ mice before escaping to the visible platform

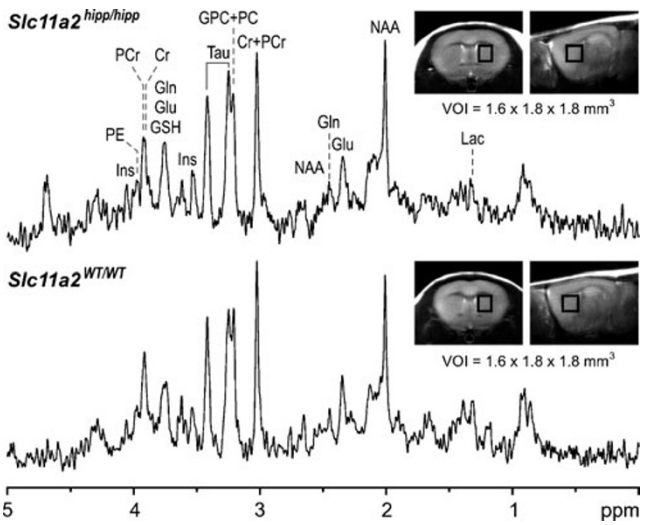

Fig. 2 In vivo ^{1}H NMR spectra measured from the left striatum of 3-month old *Slc11a2*$^{hipp/hipp}$ mice (n=6) and *Slc11a2*$^{WT/WT}$ mice (n=7). Inset: MR Images with the selected volume of interest centered in the left dorsal striatum. Abbreviations: *Cr*, creatine; *Gln*, glutamine; *Glu*, glutamate; *GPC*, glycerophosphocholine; *GSH*, glutathione; *Lac*, lactate; *Ins*, *myo*-inositol; *NAA*, *N*-acetylaspartate; *PC*, phosphorylcholine; *PCr*, phosphocreatine; *Tau*, taurine

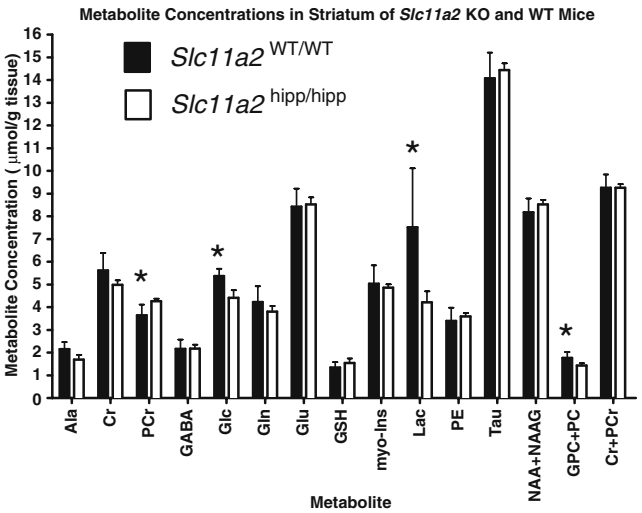

Fig. 3 Striatal neurochemical profiles of *Slc11a2*$^{hipp/hipp}$ mice (n=6) and *Slc11a2*$^{WT/WT}$ mice (n=7) at 3 months of age. Values are means ± SD. *Different from *Slc11a2*$^{hipp/hipp}$ mice, P<0.05. alanine (Ala), creatine (Cr), phosphocreatine (PCr), γ-aminobutyric acid (GABA), glucose (Glc), glutamate (Glu), glutamine (Gln), glutathione (GSH), the sum of glycerophosphorylcholine and phosphocholine (GPC + PC), *myo*-inositol (*myo*-Ins), lactate (Lac), *N*-acetylaspartate + *N*-acetylaspartylglutamate (NAA + NAAG), phosphoethanolamine (PE), and taurine (Tau)

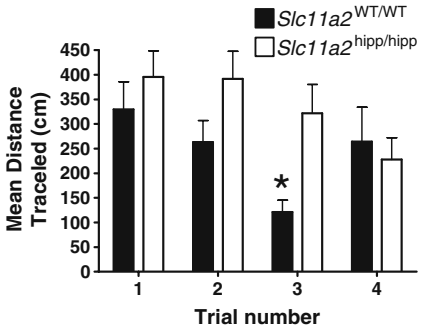

Fig. 4 Procedural memory performance in 3-month old *Slc11a2*$^{hipp/hipp}$ mice (n=17) and *Slc11a2*$^{WT/WT}$ mice (n=13) in the MWM visual (cued) task. Values are distance traveled (cm) ± SEM. Performance was significantly different between *Slc11a2*$^{hipp/hipp}$ mice and *Slc11a2*$^{WT/WT}$ mice by two-way ANOVA (F-value=5.65 for Cre +/− status, P<0.05, F-value=3.1 for trial number P<0.05, interaction P>0.05). *Significantly different from *Slc11a2*$^{hipp/hipp}$ mice on individual trial at P<0.05 by Bonferroni's post hoc test

(Fig. 5c), all of which are consistent with a strategy of escaping to the visible platform. They *Slc11a2*$^{hipp/hipp}$ and *Slc11a2*$^{WT/WT}$ mice had similar swim velocities during the probe trials (data not shown).

Discussion

In this study, *Slc11a2*$^{hipp/hipp}$ mice, which have a 40% reduction in hippocampal iron content and abnormal spatial navigation memory (Carlson et al. 2009), disproportionately resorted to procedural memory solutions to escape the MWM, when the spatial navigation memory and procedural memory systems were placed in competition. This occurred despite the finding that *Slc11a2*$^{hipp/hipp}$ mice appear to have reduced striatal metabolism and gene expression, and altered performance in a visual cued task that relies heavily on a normally functioning dorsal striatum when compared to wild type control mice. Since iron content in the striatum was not different between *Slc11a2*$^{hipp/hipp}$ mice and their wild type littermates, we interpret the striatal changes seen in this animal to be due to impaired hippocampal input resulting from hippocampal-neuron specific ID. These abnormalities appear to index a disturbance of the balance between two cooperating systems and demonstrate the dependence of optimal striatal performance on an intact hippocampus.

Approaches to dissociate multiple parallel memory systems through stereotaxically-lesioning either the dorsal lateral striatum or hippocampus were validated in the 1990s using similar water maze competition tasks to the ones used in the current experiment (McDonald and White 1994; Devan and White 1999). A similar cue/place competition water maze task was recently validated with a transgenic

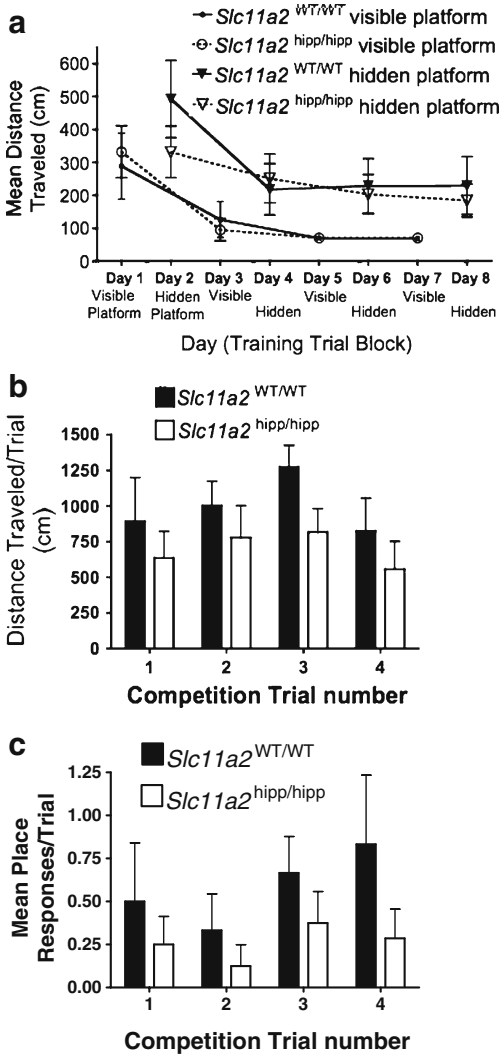

Fig. 5 Performance in memory system competition task experiment. Performance for 3-month old *Slc11a2*$^{hipp/hipp}$ mice (n=8) and *Slc11a2*$^{WT/WT}$ mice (n=6) in each training trial block (**a**) are expressed as distance traveled (cm) ± SEM. (**b**) Performance was significantly different between *Slc11a2*$^{hipp/hipp}$ mice and *Slc11a2*$^{WT/WT}$ mice over all probe trials by two-way ANOVA as measured by distance traveled (cm) ± SEM (Source of variation = Cre +/− status, F-value = 4.17, P<0.05, for trial number and interaction, P>0.05). (**c**) Performance was significantly different between *Slc11a2*$^{hipp/hipp}$ mice and *Slc11a2*$^{WT/WT}$ mice over all probe trials by two-way ANOVA as measured by place responses/trial (Source of variation = Cre +/− status, F-value = 4.11, P<0.05, for trial number and interaction, P>0.05)

mouse model and showed bidirectional competition between hippocampus and striatum during learning (Lee et al. 2008). Rats with sham surgeries show a striking balance between these two memory systems when these systems are forced to compete (McDonald and White 1994; Devan and White 1999). This balance can be altered experimentally by stereotaxically ablating one region or the other. Rats with a dorsal striatal lesion but a functioning hippocampus choose spatial navigation strategies, whereas rats with a hippocam-

pal lesion but intact dorsal striatum choose cued strategies to solve water mazes (Packard and McGaugh 1992; McDonald and White 1994; Devan and White 1999).

Nutritional deficiencies early in life affect the developing brain because of its high demand for substrates that support the metabolic requirements of growth and differentiation (Fugelstad et al. 2008). Early deficiencies can cause brain dysfunction during the period of deficiency but importantly can also alter the developmental trajectories of rapidly developing regions such that the functions subserved by those areas remain abnormal throughout the lifespan. Unlike stereotaxic lesions, nutritional deficiencies dampen but do not obliterate neural circuitry and thus result in impairment, but not complete loss of performance. Our model of 40% ID that is specific to the hippocampus is particularly relevant to the degree of ID seen in human neonates (Petry et al. 1992). ID, the most common single nutrient deficiency in the world, has been extensively studied in humans as well as rodent and monkey models. The effects of late fetal and early postnatal ID on the brain are protean and occur when several processes of archicortical and neocortical development are underway. Early brain-wide ID leads to impairments of learning and memory, mood and affect, and speed of processing through its effects on the hippocampus, on monoamine-rich areas like the striatum and on myelin. All three processes are undergoing rapid development in the late fetal and early neonatal period (Thompson and Nelson 2001; Georgieff and Innis 2005; Ward et al. 2007), but fetal ID tends to compromise the hippocampus disproportionately (deUngria et al. 2000; Golub et al. 2007) resulting in long term spatial learning and memory abnormalities (Schmidt et al. 2007). However, prior models that induced pan-brain ID were unable to provide any understanding of the relative contributions of the three processes to altered behavioral function during ID. Thus, it was unclear whether the extensive striatal dysfunction noted in early ID (Beard et al. 2003; Ward et al. 2007) is solely due to alterations in dopaminergic neurotransmission because tyrosine hydroxylase is iron dependent or whether hippocampal inputs also compromise this region's performance. By combining the temporal- and tissue-specific genetic manipulation with a set of molecular, biochemical and behavioral experiments designed to dissociate these memory systems, we were able in this study to differentiate the primary effects of neuronal ID as described in our previous report (Carlson et al. 2009) from secondary or downstream effects on extra-hippocampal brain regions subserving other memory systems. The current study suggests that ID-induced pathology in striatum is not solely due to direct effects (on dopamine) within that region (Beard et al. 2003), but is in part due to a direct, downstream impact from ID in another area of the brain involved in a complimentary/competitive type of cognitive function (the hippocampus). Therapeutically, strategies to shore up dopaminergic neurotransmission in the striatum are not likely to reverse all the behavioral manifestations of the disease.

Iron has an important role in neuronal metabolism through its incorporation into cytochromes (Dallman 1986). A 40% reduction in brain iron content leads to a 40% reduction in cytochrome c oxidase activity in the hippocampus, altered dendritic arborization, reduced long-term potentiation and poorer performance on spatial memory tasks (deUngria et al. 2000). Genes that are responsible for cellular iron uptake, including *Slc11a2* (encoding DMT1), *Tfrc*, *Aco1* (IRP-1), and *Ireb2* (IRP-2), are upregulated in hippocampus during spatial navigation learning tasks in a dose dependent manner, consistent with a response to the increased neuronal metabolic demands induced by learning (Carlson et al. 2009). Thus, it was interesting to note that despite its normal iron content, the dorsal striatum of $Slc11a2^{hipp/hipp}$ mice nevertheless showed reduced expression of genes related to iron uptake (e.g., *Slc11a2*, *Slc40a1*, and *Tf*), energy metabolism (*ckb* and *Slc2a3*, the neuronal glucose transporter), and dendrite formation/synaptic activity compared to wild type mice. We interpret these findings to be consistent with hypoactive neural circuits in the striatum. Reductions in the transcript for tyrosine hydroxylase, the rate-limiting (and iron-requiring) enzyme in dopamine synthesis, are also evident in the striatum of $Slc11a2^{hipp/hipp}$ mice and suggest potential alterations in that neurotransmitter system that are not directly due to ID in the that brain region. *Mbp*, a marker of myelination, is downregulated in the striatum and VMB of $Slc11a2^{hipp/hipp}$ mice, but not in hippocampus. Myelination is a neuronal activity-dependent process (Demerens et al. 1996; Zalc and Fields 2000), and in Slc11a2 mice, decreased *Mbp* expression in striatum may reflect decreased basal maintenance of myelin due to suppressed basal electrical activity due to the dampening of hippocampal activity. Expression of *cfos*, an immediate early gene whose expression is induced in an activity-dependent manner (Herdegen and Leah 1998) was downregulated in all regions examined except NA, with its greatest reduction in striatum. Basal levels of *Camk2a* are elevated, and basal levels of *Dlg4* (encoding PSD-95) are decreased in $Slc11a2^{hipp/hipp}$ mouse striatum. Camk2a and PSD-95 act in a differential manner to regulate the stability of NR2A and NR2B containing NMDA receptors in hippocampus (Park et al. 2008), and the altered basal differential expression of *Dlg4* and *Camk2a* maybe an adaptive response to maintain long term potentiation-type plasticity in this structure, at the cost of long term depression-type stability.

The NMR spectroscopy findings support the genomic findings and indicate dorsal striatal alterations in critical

metabolites such as lactate, glucose, phosphocreatine, and GPC + PC in *Slc11a2*$^{hipp/hipp}$ mice. Decreased lactate concentration and increased PCr/Cr ratio in *Slc11a2*$^{hipp/hipp}$ mouse striatum likely reflect decreased energy demands (Jost et al. 2002; Bartlett et al. 2004), and are consistent with a hypometabolic state (Mangia et al. 2007a; Mangia et al. 2007b). Lower glucose levels in the striatum are consistent with observed regional decreases in gene expression for neuronal and endothelial glucose transporters. Reductions in GPC + PC observed in *Slc11a2*$^{hipp/hipp}$ mice may represent decreased synthesis of membrane phospholipids (Ledeen 1984; Vance et al. 1994), which are required for synaptic plasticity and dendrite growth (Ehlers 2007).

A potential confounding factor in the model is that the PFC also shows significant cre deletion of *Slc11a2*. PFC-striatal connections are important in learning (McDonald et al. 2008), and cre-recombinase mediated reduction of *Slc11a2* in PFC could influence striatal metabolism, gene expression and behavior. However, the robustness of our behavioral dissociation of the hippocampal and striatal memory systems remains compelling. Furthermore, electrophysiologic coherence between striatum and hippocampus is observed in rats that are successful in a procedural memory task, evidence of the coordinated, dynamic interactions in hippocampus and striatum during experience-dependent acquisition and performance of learned behaviors despite relatively indirect connectivity of these structures (DeCoteau et al. 2007). Since the primary lesion in Slc11a2$^{hipp/hipp}$ mice is in the hippocampus, we interpret our results to indicate that the dorsal striatal system does not need to expend as much metabolic activity to overcome the hippocampal system. A future experiment could use a mouse model in which the hippocampal iron deficiency was repletable to determine whether the formerly iron deficient hippocampus induced metabolic changes in the striatum returns to normal with subsequent normalization of procedural memory behaviors.

These studies were designed to better understand the role of hippocampal iron in early cognitive development. However, the neurobehavioral consequences of early ID in humans appear to extend beyond disordered cognition. Fetal or early postnatal ID also results in abnormal motor development, slower auditory and visual system neurotransmission, altered sleep architecture, poorer maternal ratings of neonatal social and emotional behaviors, and an increased incidence of behavioral problems during childhood (Lozoff and Georgieff 2006; Burden et al. 2007; Lozoff et al. 2007; Peirano et al. 2007; Lozoff et al. 2008; Corapci et al. 2009; Shafir et al. 2009). Early ID is also linked to complex behavioral abnormalities later in life such as attention deficit disorder and schizophrenia (Insel et al. 2008; Van Lieshout and Voruganti 2008; Dopheide and Pliszka 2009; Sorensen et al. 2010). These latent effects are of particular interest because their fundamental pathologies may lie outside primary target areas of early ID and their symptoms may instead represent a failure of primary areas to interconnect and regulate each other in a cooperative manner from early life onward. Hippocampal dampening may predispose an organism to a schizophrenic endophenotype, with alterations in dopamine-related behavior being a secondary consequence of prenatal or neonatal alterations in hippocampus (McDonald et al. 2004). Hyper-dopaminergic states in one animal model of schizophrenia are dependent on hippocampal dysfunction induced prenatally (Lodge and Grace 2007; Lisman et al. 2008). Another major heuristic model of the etiology of schizophrenia induces a transient disruption of neural activity in the ventral hippocampus during the neonatal period, and results in several endophenotypic features of schizophrenia later in the life of the organism (Lipska 2004; Tseng et al. 2009). In a recent large cohort study, maternal gestational ID was associated with an increased risk of schizophrenia in offspring in a dose dependent manner (Insel et al. 2008). Diabetes mellitus (DM) during gestation also increases the risk of schizophrenia in the offspring seven-fold (Cannon et al. 2002; Van Lieshout and Voruganti 2008). A nutritional hallmark of DM during pregnancy is altered fetal iron metabolism that results in a 65% rate of ID and a 40% reduction in brain iron content in the offspring (Georgieff et al. 1990; Petry et al. 1992). Maternal ID and DM during gestation increase the risk of neonatal hypoxic-ischemic injury (Rao et al. 1999; Rao et al. 2007), a condition that also increases the risk of schizophrenia in adulthood (Cannon et al. 2002).Taken together, the altered behaviors observed in children with ID are remarkably similar to the motor, cognitive, and behavioral deficits that are observed in children who go on to develop schizophrenia (Erlenmeyer-Kimling 2000; Isohanni et al. 2004). We speculate that ID induced alterations of hippocampal, prefrontal cortex (PFC) and striatal development and function in early life may induce significant imbalances in developing memory systems that may contribute to the cognitive and behavioral abnormalities similar to those seen in pre-schizophrenic children.

The hope of identifying early disruptions of memory function is that intervention is most effective when instituted during maximal brain plasticity early in life. Nutritional deficiencies and glucose intolerance during pregnancy are eminently treatable. The critical periods for developing brain regions that are particularly susceptible to ID appear to span the late fetal and early neonatal periods, suggesting that both maternal and neonatal nutritional status and metabolic health are paramount to cognitive health. Specifically, screening pregnant women for ID and DM during the last trimester and

both recognizing and treating iron deficient newborns may reduce the risk to developing memory systems and of developing psychopathology later in life.

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