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Iron Deficiency Alters Expression of Genes Implicated in Alzheimer Disease Pathogenesis

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Abstract

Neonatal brain iron deficiency occurs after insufficient maternal dietary iron intake, maternal hypertension, and maternal diabetes mellitus and results in short and long-term neurologic and behavioral deficits. Early iron deficiency affects the genomic profile of the developing hippocampus that persists despite iron repletion. The purpose of the present study was threefold: 1) quantitative PCR confirmation of our previous microarray results, demonstrating upregulation of a network of genes leading to beta amyloid production and implicated in Alzheimer disease etiology in iron deficient anemic rat pups at the time of hippocampal differentiation; 2) investigation of the potential contributions of iron deficiency anemia and iron treatment to this differential gene expression in the hippocampus; and 3) investigation of these genes over a developmental time course in a mouse model where iron deficiency is limited to hippocampus, is not accompanied by anemia and is not repletable. Quantitative PCR confirmed altered regulation in 6 of 7 Alzheimer-related genes (*Apbb1*, *C1qa*, *Clu*, *App*, *Cst3*, *Fn1*, *Htatip*) in iron deficient rats relative to iron sufficient controls at P15. Comparison of untreated to treated iron deficient animals at this age suggested the strong role of iron deficiency, not treatment, in the upregulation of this gene network. The non-anemic hippocampal iron deficient mouse demonstrated upregulation of all 7 genes in this pathway from P5 to P25. Our results suggest a role for neonatal iron deficiency in dysregulation of genes that may set the stage for long-term neurodegenerative disease and that this may occur through a histone modification mechanism.

Keywords

Iron deficiency; Alzheimer Disease; Genes; Amyloid precursor protein; Hippocampus

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1. Introduction

Environmental influences that induce alterations in gene expression during critical periods of brain development are being recognized as having potentially negative effects on acute brain function or contributing to neurodegeneration during the senescence of an organism. For example, exposure to neurotoxins such as lead or ethanol, variation in maternal care, and dietary manipulations of critical nutrients such as choline during gestation have been shown to alter patterns of gene expression and gene imprinting in a programmatic fashion in different brain regions. Recently, we identified similar potential long-term effects on the hippocampus due to gestational iron deficiency (Carlson et al., 2007).

Iron deficiency is the most common form of malnutrition worldwide, affecting an estimated 30% of the world's population, and is prevalent in developing and industrialized countries alike (World Health Organization, 2008). Maternal iron deficiency affects 30–50% of pregnancies and is the most common cause of newborn iron deficiency worldwide (World Health Organization, 2008). More than 80% of pregnant women in certain developing countries are affected (Sesma and Georgieff, 2003). The impact of iron deficiency on the developing fetal brain is of particular concern since iron plays a critical role in tissue oxygenation and in virtually all energy-dependent developmental processes.

Neonatal brain iron deficiency is caused by insufficient maternal dietary intake during pregnancy, maternal hypertension causing intrauterine growth restriction, and uncontrolled maternal diabetes mellitus (Lozoff and Georgieff, 2006; Rao and Georgieff, 2007). The third trimester is characterized by accelerated brain growth, high iron needs and increased vulnerability to deficits in nourishment (Sesma and Georgieff, 2003). Maternal iron deficiency anemia and maternal hypertension result in decreased fetal iron accretion during gestation with a consequent reduction in brain iron concentration (Georgieff et al., 1996). During pregnancies complicated by diabetes mellitus, fetal brain iron deficiency results from a combination of a 30% increase in fetal iron demand for erythropoiesis coupled with reduced maternal-fetal iron transport (Georgieff et al., 1992; Petry et al., 1992; Petry et al., 1994). The rapid expansion of the red cell mass induces a mobilization of iron from fetal tissue pools, including those in the brain, to support hemoglobin synthesis. Brain iron loss averages 32 to 40% (Georgieff et al., 1996; Petry et al., 1992).

The neurodevelopmental effects of neonatal iron deficiency in humans and animals are significant and may be irreversible in spite of therapy. Rodent models of iron deficiency anemia during gestation and in the early postnatal period have demonstrated deficits in genes regulating synaptic plasticity (Jorgenson et al., 2005) and apical dendrite growth in the hippocampus (Jorgenson et al., 2003), as well as impairment in striatally and hippocampally mediated behaviors (Felt et al., 2006; Ward et al., 2007). The abnormal behaviors persist into adulthood in spite of complete iron rehabilitation (Schmidt et al., 2007). Iron deficient human newborns have similar adverse hippocampal behavioral effects (Siddappa et al., 2004) that also persist in spite of recovery from the iron deficient state (DeBoer et al., 2005). These long-term findings in animals and humans imply irreparable structural changes, altered regulation of genes involved in synaptic plasticity or both.

Changes in expression of particular gene products that result from environmental influences prime the brain for either protective (e.g., choline) or deleterious (e.g., lead) effects during senescence (Wong-Goodrich et al., 2008; Wu et al., 2008; Zeisel, 2006). The question of whether gestational iron deficiency may be involved in a similar deleterious mechanism recently arose in our laboratory as a result of microarray findings in a rat model of neonatal iron deficiency anemia (Carlson et al., 2007). Among the genes that were differentially regulated in postnatal day (P) 15 iron deficient rats was a network of nine genes that are

implicated in Alzheimer Disease (AD) etiology. In the current study we tested whether seven of these genes were dysregulated due to iron deficiency per se (in iron deficient, non-treated [IDNT] rats), or whether they were induced by treatment with iron that the iron deficient (ID) animals received from P7 through P15 compared to iron-sufficient (IS) controls. Additionally, we examined their developmental expression trajectories in a mouse genetic model of hippocampal-neuron specific non-anemic iron deficiency that we have recently developed in which iron cannot be replenished to neurons.

2. Results

IDNT rats were severely anemic, with hematocrits measured at 12.5 ± 1.5 % at P15. ID rats had hematocrits of 25.2±1.3% and IS rats had hematocrits of 40.4±2.1% at P15. The *Slc11a2*$^{hipp/hipp}$ mice had hematocrits of 48.44 ± 1.17%, no different than the *Slc11a2*$^{WT/WT}$ hematocrits of 48.14 ± 1.05%.

Seven transcripts (*Apbb1*, amyloid beta (A4) precursor protein-binding, family B, member 1 (Fe65); *App*, amyloid beta (A4) precursor protein; *Clu*, clusterin; *C1qa*, complement component 1, q subcomponent, alpha polypeptide; *Cst3*, cystatin c; *Fn1*, fibronectin 1; *Htatip*, HIV-1 tat interactive protein, homolog (human) [Tip60]) in the AD pathway identified by microarray (Carlson et al., 2007) were assessed by qPCR in the 3 rat dietary groups and the 2 mouse groups at P15 (Table 1, Figure 1). Six (Apbb1, C1qa, Cst3, Fn1, App, and Htatip) were differentially expressed across rat dietary groups. Post-hoc analysis demonstrated that gene expression in the IDNT group was upregulated compared to IS controls in 5 genes (Apbb1 [$p < 0.05$], C1qa [$p < 0.05$], Cst3 [$p < 0.05$], Fn1 [$p < 0.001$], and Htatip [$p < 0.001$]) and downregulated in 1 gene (App [$p < 0.05$]) (Figure 1). Iron treatment (ID group) resulted in expression patterns that were no different than IS controls in 4 of the 7 genes examined (Apbb1, C1qa, Clu, and Cst3).

The genetic mouse model removes the confounding variable of anemia resulting in an untreated model of specifically hippocampal iron deficiency from E18 onward. Expression patterns in the *Slc11a2*$^{hipp/hipp}$ mutant at P15 confirmed the findings in the IDNT rat in 5 of the 7 genes (Apbb1 [$p < 0.05$], C1qa [$p < 0.05$], Cst3 [$p < 0.05$], Fn1 [$p < 0.05$], and Htatip [$p < 0.01$]), suggesting that the changes in these transcripts was driven by iron deficiency, per se (Figure 1). Two genes had dissimilar expression between the models. App, which was downregulated in the iron deficient anemic rat, was upregulated in the iron deficient non-anemic mouse ($p < 0.05$), and Clu, which showed no difference among conditions in the rat, was upregulated in the iron deficient non-anemic mouse ($p < 0.05$).

Genetically-induced iron deficiency resulted in alterations in developmental trajectories of expression in this subset of genes implicated in AD pathogenesis (Table 2, Figure 2). In the *Slc11a2*$^{hipp/hipp}$ mice, persistence of iron deficiency resulted in consistent elevation of all 7 tested genes in the early differentiative time period compared to control, with a return to normal levels in early adulthood.

3. Discussion

Iron deficiency during gestation and early postnatal life has been shown to alter gene expression in total brain and hippocampus acutely and after iron repletion. In this study, we confirmed that early iron deficiency alters developmental gene expression of a subset of genes whose dysregulation is implicated in AD etiology, including *Fn1*, *Cst3*, *Apbb1*, *Clu*, *App*, and *Htatip* (Bing et al., 1982; Hall et al., 1995; Kim et al., 2004; Lidstrom et al., 1998; Palmert et al., 1988; Zambrano et al., 1998).

Many observations suggest a potential role for altered iron homeostasis in the etiology of AD pathogenesis, through the generation of oxidative stress. Oxidative damage and antioxidant response are well characterized in Alzheimer Disease, and may be pivotal in its pathogenesis (Pappolla et al., 1992; Smith et al., 1991; Smith et al., 1996). Transcription of some gene products (e.g. ferritin) involved in iron metabolism is redox-responsive through antioxidant response elements in the promoter regions of their genes (Hintze and Theil, 2005; Theil, 2007). Iron accumulation has been shown in both senile plaques and neurofibrillary tangles, and may be a source of reactive hydroxyl radicals generated by the Fenton reaction (Smith et al., 1997). This accumulation of iron was also shown to participate in oxidation *in situ*, and completely removed with iron chelation by deferoxamine (Smith et al., 1997).

The possibility that gestational iron deficiency alters expression of genes involved in AD was first noted in our previous study using a broad microarray approach (Carlson et al., 2007). The current study used the same rat dietary model to confirm these findings at P15 using qPCR. This dietary model of gestational iron deficiency has been used in multiple studies (Carlson et al., 2007; de Ungria et al., 2000; Jorgenson et al., 2003; Jorgenson et al., 2005; Schmidt et al., 2007) because it induces a degree of total brain iron deficiency found in the human (Georgieff et al., 1996; Petry et al., 1992). The iron deficient diet is continued in the mother until the pups are seven days old, the developmental equivalent of term human birth with respect to the hippocampus, after which iron supplemented chow is provided. While this mimics the human condition, it introduces two potential confounders into the determination of whether iron deficiency was responsible for the P15 findings; anemia with its attendant tissue hypoxia, and iron treatment with its potential generation of reactive oxygen species. Either or both may have been responsible for the changes noted in the original study (Carlson et al., 2007) and in the ID group in the current study.

We first eliminated the treatment confound by extending the iron deficient diet until P15. The comparison of the IDNT, ID and IS groups revealed striking differences between the IDNT and the IS groups, with lesser or no effect differences between the ID and IS groups. The findings suggest that iron treatment did not exacerbate dysregulation of these genes and that the findings were primarily due to iron deficiency with anemia. Furthermore, it suggests that these genes are rapidly responsive to iron treatment since most were no different than IS after only one week of treatment. The exception to this pattern, however, was App in that downregulation of this gene was greater in the ID with treatment group compared to the IDNT group. The potential long-term ramifications of this finding need to be studied.

The genetic mouse model provided us with an opportunity to determine whether transcript alterations implicated in AD pathogenesis were due to untreated ID per se or to the more general condition of iron deficiency anemia. The similar directional responses of Apbb1, C1qa, Cst3 and Fn1 suggest that these genes are iron responsive. Conversely, the down-regulation of App in the rat may well have been due to anemia, glucocorticoid activation, or toxicity from other divalent cations such as manganese, all of which are present in the iron deficiency anemia rat but not in the *Slc11a2*$^{hipp/hipp}$ mouse (Carlson et al, 2008). Indeed, the upregulation of App transcript by iron deficiency alone in the mouse model is concerning since its protein is the final step prior to activation of betaamyloid (Schmechel et al., 1988). Overall, the genetic mouse demonstrated that much of the upregulation of the pathway early in life is due to iron deficiency alone.

The developmental trajectories of these genes in the *Slc11a2*$^{hipp/hipp}$ mouse indicate that the greatest activation of these genes by iron deficiency occurs during the period of hippocampal dendritogenesis and synaptogenesis between P5 and P25. This time period is characterized by high rates of cellular metabolism and iron utilization in rodents. Iron deficiency during this time period results in altered energy status, morphologic development, and synaptic plasticity

(Carlson et al., 2007; de Ungria et al., 2000; Jorgenson et al., 2003; Jorgenson et al., 2005; Rao et al., 2003). Recently, alterations in the expression of growth factors such as BDNF required for both differentiation and plasticity of hippocampal neurons were demonstrated in rats with early iron deficiency (Tran et al., 2008).

Iron deficiency altered gene expression during this highly metabolic time period by either increasing or maintaining higher transcript levels at a time when they normally decline in the IS animals (e.g., Apbb1, C1qa, Fn, Htatip, App) or increasing transcript levels beyond iron sufficient levels when expression in the IS animals are also increasing (e.g., Clu, Cst 3). Hippocampal metabolic rate slows after P25 and it was interesting to note that ongoing iron deficiency in the mouse did not continue to activate these genes abnormally compared to controls during early adulthood. While this may be interpreted as reassuring with respect to long term morbidities associated with the early activation of these genes, there still may be cause for concern. Basha and colleagues demonstrated a similar upregulation of App during development in the cortex of rats exposed to lead during the early postnatal period (Basha et al., 2005). Interestingly, App expression returned to normal during early adulthood, but was subsequently reactivated during senescence without further exposure to the toxin. Further studies of our model will be important to determine if a similar process occurs and if it is accompanied by neuropathological and behavioral findings consistent with AD pathogenesis.

The mechanism by which early activation of this pathway may alter gene expression for life resides in the role of App/Fe65/Tip60 in histone modification. Many studies have suggested that adverse events during development may increase disease vulnerability during adulthood and/or senescence (Barker et al., 1989; Barker, 2002; Basha et al., 2005; Ozanne, 2001; Wu et al., 2008). Some dietary factors or environmental influences (maternal stress, lead, bisphenol A) at particular times in development have been associated with adverse adult brain function and vulnerability to neurodegenerative diseases (Basha et al., 2005; Miyagawa et al., 2007; Weaver et al., 2004). Other dietary factors, such as folic acid, choline, and caloric restriction appear to benefit brain function, and may prevent neurodegeneration (Kruman et al., 2002; Maswood et al., 2004; Zeisel, 2006). While the mechanism(s) that meditate these processes are still not entirely clear, one possibility is that early energy and/or oxidative stress may lead to genomic programming changes through by enzymatic alteration of chromatin. Many enzymes are capable of modifying histones (the proteins that package and fold DNA into chromatin) by acetylation or methylation, for example, or modifying DNA itself by methylation during brain development (Mattson, 2003).

Previously, App has been shown to interact with Fe65 (Apbb1) and Tip60 (Htatip) to form a complex that translocates to the nucleus, and potently induces transcription by its acetyltransferase activity (Cao and Sudhof, 2001). This complex has been shown to alter cell cycle progression *in vitro* by down-regulating the gene for thymidylate synthase, an enzyme required for DNA synthesis (Bruni et al., 2002). This mechanism is potentially active during hippocampal development, as Apbb1, Htatip, and App are all expressed during this time period, and act together to play a role in actin cytoskeleton remodeling, cell motility, and growth cone formation (Guenette et al., 2006; Kesavapany et al., 2002; McLoughlin and Miller, 2008; Neve et al., 1996). Here, we show that all three of these genes have altered expression during development in the context of iron deficiency. This raises the possibility that iron deficiency-induced alterations in energy metabolism and neuronal oxidative state may lead to alterations in App/Fe65/Tip60 histone modification potentially increasing long-term susceptibility/ vulnerability to AD pathogenesis.

The data we present here suggests that iron deficiency, and not anemia or iron repletion, alters expression of genes implicated in AD pathogenesis during a potentially vulnerable time in hippocampal development. Future studies will examine whether programmatic changes in the

genome consistent with altered App/Fe65/Tip60 expression and function occur due to neuronal iron deficiency, whether these changes cause functional impairment, last into senescence, and if they may be relieved by pharmacologic intervention.

4. Experimental Procedures

Animals

The animals in this study were handled in accordance with all guidelines set by the University of Minnesota and the NRC Guide for the Care and Use of Laboratory Animals. Both of the animal models used in this study model the degree of human neonatal brain ID seen with maternal anemia, diabetes mellitus, and intrauterine growth restriction (Jorgenson et al., 2005; Petry et al., 1992). Only male animals (larger anogenital distance on P1 day of birth) were used for real time polymerase chain reaction (RT-PCR) experiments. Pups were weaned on P21. Pups were anesthetized with pentobarbital and their hippocampi dissected out in ice-cold phosphate-buffered saline pH 7.4, flash-frozen in dry ice, and stored at −80°C.

Rats

Tissue from pups from 22 litters of Sprague-Dawley rats (Harlan, IN) were used in this study. As before, iron deficient rat pups were generated by feeding an iron-deficient diet (3– 6 ppm Fe) to the single-housed dams from E2 to P7, followed by an iron supplemented diet (198 ppm Fe) thereafter in the IS and ID groups (Carlson et al., 2007; Jorgenson et al., 2005), but not the IDNT group. IDNT animals were reared on the iron deficient diet until P15, the age at which we previously performed the microarray screen (Carlson et al., 2007) and then killed for tissue collection.

Mice

Pups from 8 litters of targeted *Slc11a2* flox/flox mice in a 129 J1 background strain (Gunshin et al., 2005) were crossed with *CaMKIIa-cre* (L7ag#13 line (Dragatsis and Zeitlin, 2000) transgenic mice in a C57BL/6J background to generate double mutant, hippocampal neuron specific knockout of *Slc11a2* (*Slc11a2*$^{hipp/hipp}$ mice) in a mixed 129 J1/C57BL/6J background. These mice are non-anemic, and have a 50% reduction of iron concentration specifically in the hippocampus starting at E18.5 (Carlson, et al., 2008).

Hematocrit Measurement

Blood samples for hematocrit were taken from IS, IDNT, and ID rats at age P15, and from *Slc11a2*$^{WT/WT}$ and *Slc11a2*$^{hipp/hipp}$ mice at P25 with heparinized capillary tubes from right cardiac atrium before the animals were perfused for brain tissue preparation. The tubes were then spun at 3,000×g for 20 min and measured with a standard hematocrit reader.

Tissue dissection and RNA collection

Male rats at P15 and mice at P5, P10, P15, P20, P25 and P45 were euthanized by an i.p. injection of Beuthanasia (10mg/Kg). For each time point, N = 4–10 rats (per age and condition), N = 3–5 *Slc11a2*$^{hipp/hipp}$ mice, and N = 3–5 *Slc11a2*$^{WT/WT}$ mice were utilized. The specific postnatal ages were selected on the basis of the ontogeny of hippocampal formation as well as on the hippocampal iron status implications in the two models. Specifically, P5 represents the end of the proliferative stage prior to hippocampal differentiation in both mice and rats. P10 and P25 in mice represent early and late differentiation phases in the hippocampus. P45 in mice represents a time of synaptic plasticity during a post-differentiation stage. Brains were removed from the cranium and bisected along the midline. Hippocampus was dissected and flash-frozen in liquid Nitrogen. Total RNA was isolated from dissected hippocampus using RNA-isolation

kit (Stratagene, La Jolla, CA) and concentrations were measured by Absorbance at 260nm ($A_{260/280}$) using a NanoDrop ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE).

Quantitative Real Time (qRT) PCR

Hippocampal mRNA was isolated using the MO BIO Laboratories (Carlsbad, CA) UltraClean Tissue RNA Isolation Kit. Template cDNA was synthesized from 1 μg of total RNA using the Stratagene (La Jolla, CA) AffinityScript QPCR cDNA Synthesis Kit and quantified by UV spectroscopy. qRT-PCR was performed using Applied Biosystems (Foster City, CA) TaqMan 2X Universal PCR Master Mix and TaqMan Gene Expression Assay primers, with each sample represented twice. Primers were chosen for the Alzheimer-related genes indicated in Table 1. Amplifications were performed using a Stratagene Mx3000P RT-PCR system for 45 cycles using parameters of 95°C for 15 s and 60°C for 60 s. qRT-PCR data was normalized using 18S rRNA as control. Iron sufficient P15 data was used as a “calibrator" for each gene analyzed, to allow comparison of effects of age and iron status on expression data.

Statistical methods

Data for transcript levels were collected from 4–10 animals per postnatal age for each rat dietary group, and 3–4 animals per mouse group. Because the majority of the current data were at P15, all expression fold-changes are shown relative to P15. P15 rat data were analyzed by one-way ANOVA across the 3 groups. All developmental data were analyzed by 2-way analysis of variance (group by age) in both rats and mice. IDNT rats were not included in the developmental analyses. Post-hoc analyses between iron groups at each time point were assessed by Bonferroni-corrected t-tests or Student’s t-test. Significance was set at an alpha of 0.05.

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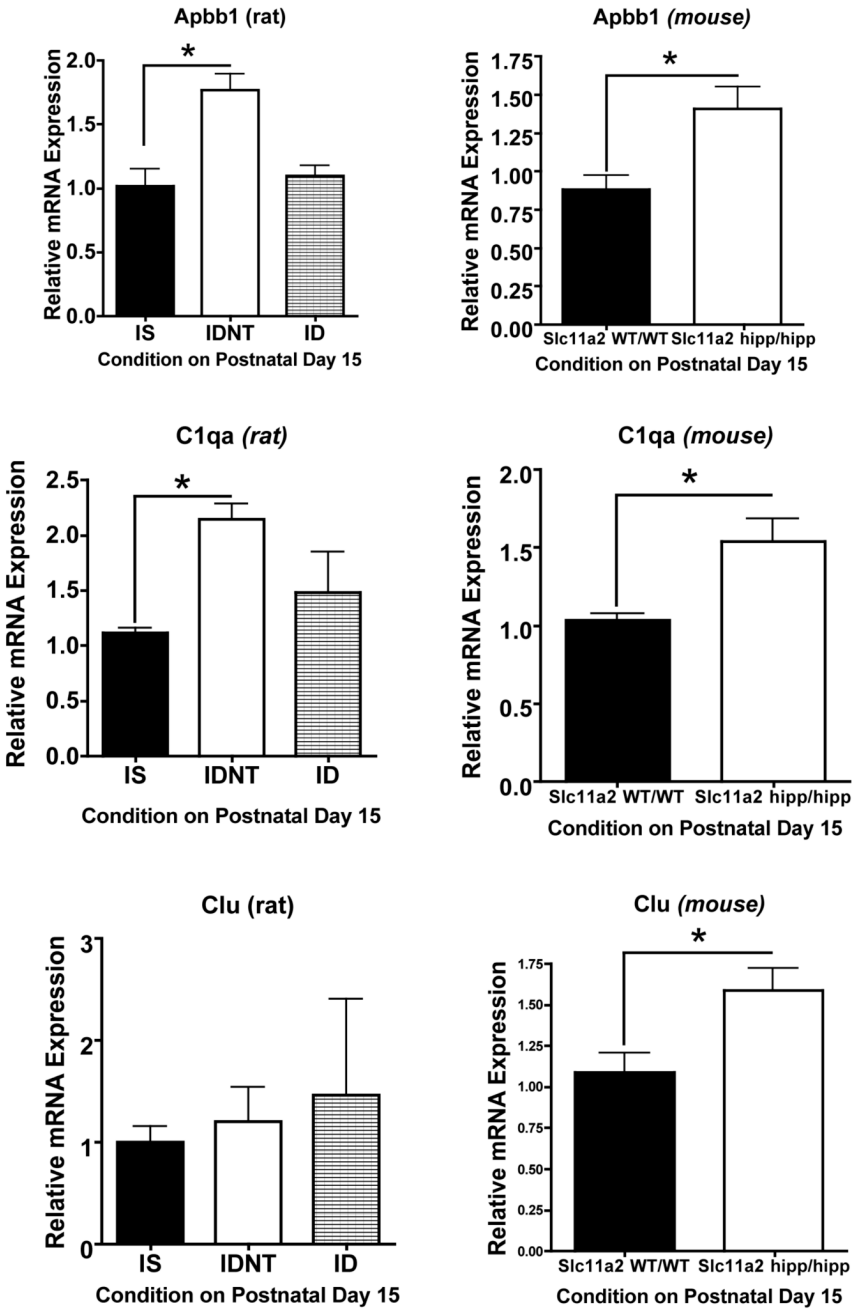

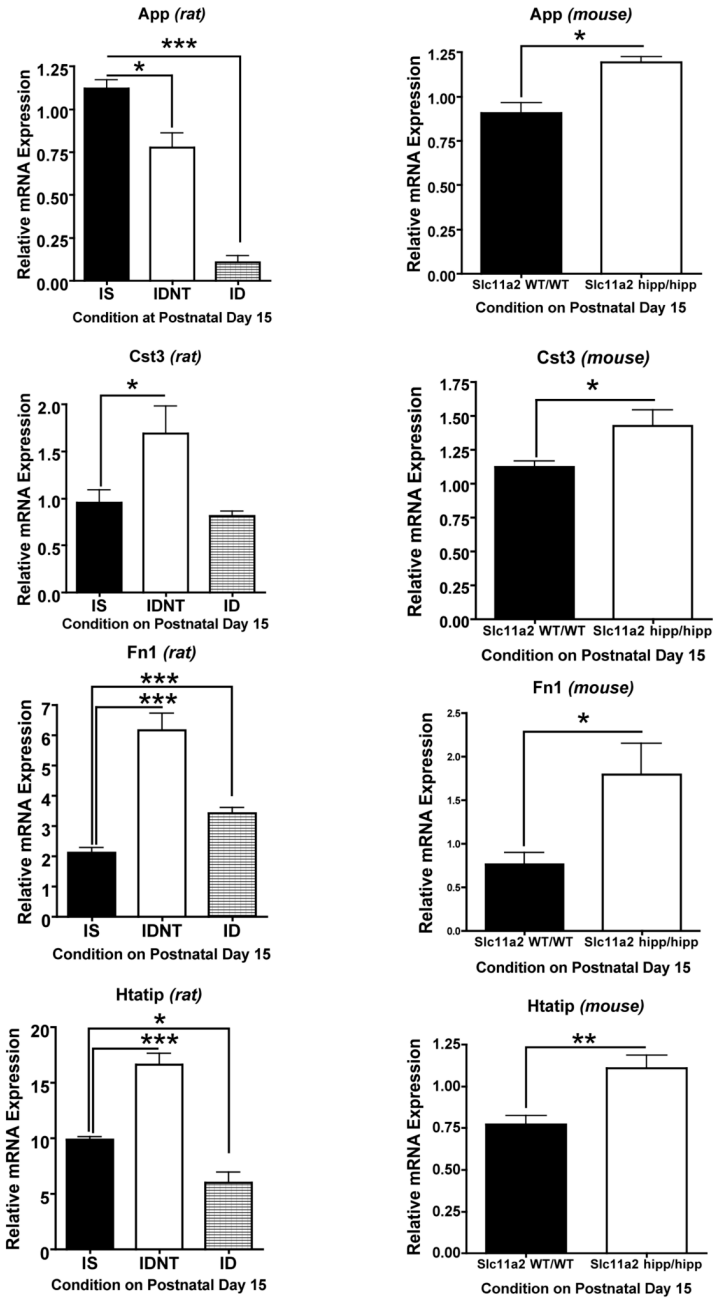

Figure 1.

Expression of genes of interest in P15 IS, IDNT, and ID rats, and P15 *Slc11a2*$^{WT/WT}$ and *Slc11a2*$^{hipp/hipp}$ mice, quantified by qPCR. Graphs show patterns of expression for each gene in both *Slc11a2*$^{WT/WT}$ and *Slc11a2*$^{hipp/hipp}$ mice. The *Y*-axis is in units of relative mRNA expression normalized to P15 IS controls. The *X*-axis is the postnatal day on which gene expression was measured. For each time point/condition, N = 3–5. Significance values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

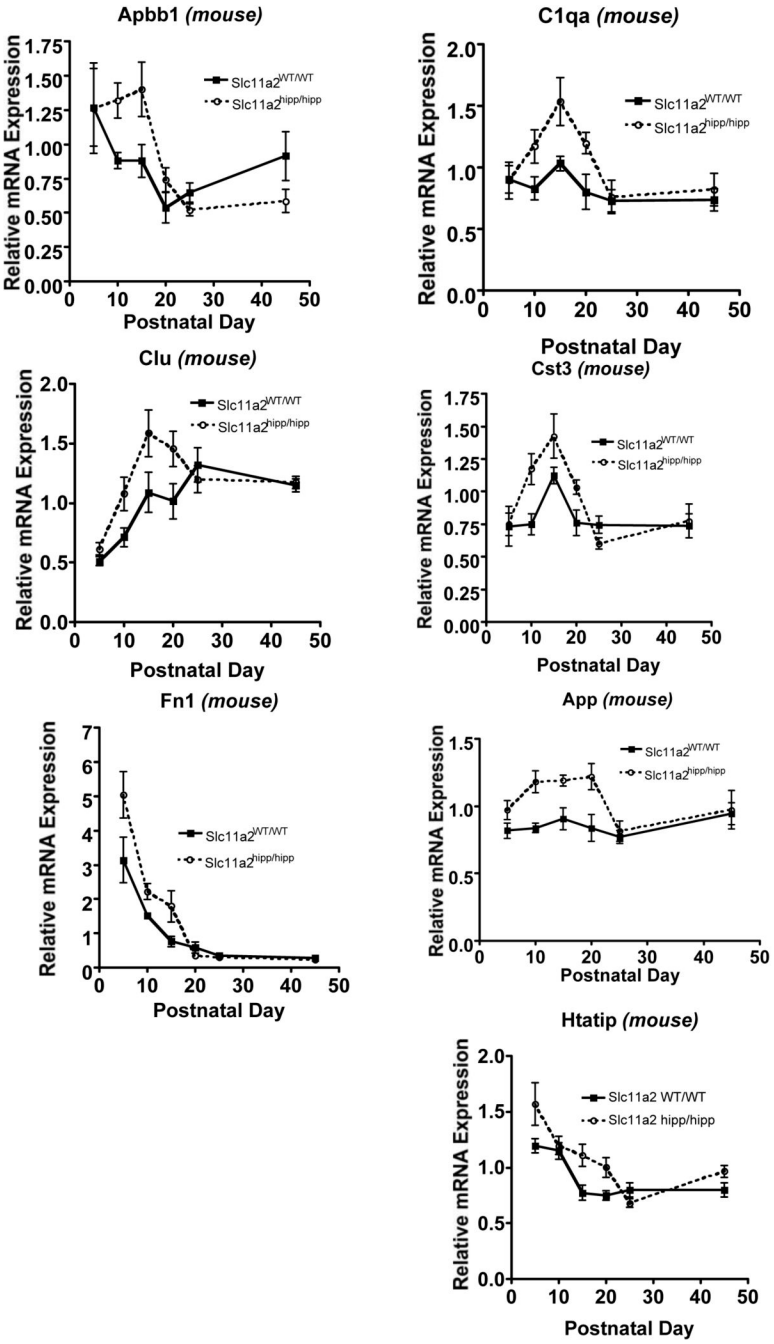

Figure 2.

Expression of genes of interest in both *Slc11a2*$^{WT/WT}$ and *Slc11a2*$^{hipp/hipp}$ mice, quantified by qPCR from P5 to P45. Graphs show patterns of expression for each gene in both *Slc11a2*$^{WT/WT}$ and *Slc11a2*$^{hipp/hipp}$ mice. The *Y*-axis is in units of relative mRNA expression normalized to P15 IS controls. The *X*-axis is the postnatal day on which gene expression was measured. For each time point/condition, N = 3–5.

Table 1

Rat and mouse genes examined by quantitative PCR.

Gene symbol and name	Rat Unigene ID	Mouse Unigene ID
Apbb1, amyloid beta (A4) precursor protein-binding, family B, member 1 (Fe65)	Rn.19953	Mm.38469
App, amyloid beta (A4) precursor protein	Rn.2104	Mm.277585, Mm.472971
Clu, clusterin	Rn.1780	Mm.200608
C1qa, complement component 1, q subcomponent, alpha polypeptide	Rn.105647	Mm.439957
Cst3, cystatin c	Rn.106351	Mm.4263
Fn1, fibronectin 1	Rn.1604	Mm.193099
Htatip, HIV-1 tat interactive protein, homolog (human) (Tip60)	Rn.6629	Mm.228930

Table 2

ANOVA Statistics of Mouse Developmental Gene Expression Data.

Gene symbol	F-Statistic (Iron Status)	F-statistic (Developmental Age)	F-Statistic (Interaction)
Apbb1 (mouse)	1.39	5.68***	1.85
Clu (mouse)	10.64**	12.68***	2.31
C1qa (mouse)	10.18**	5.14**	1.59
Cst3 (mouse)	6.13*	8.49***	2.05
Fn1 (mouse)	9.40**	44.52***	3.56*
App (mouse)	19.71***	2.92*	1.78
Htatip (mouse)	11.1**	13.02***	2.05

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$