The Impact of Iron Deficiency During Development on Mammalian Target of Rapamycin Signaling, Neuronal Structure, and Learning and Memory Behavior

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Michael K. Georgieff, Advisor

November 2010

Acknowledgements

This dissertation is the product, not only of several years of my own hard work, but of the contributions of so many others who had the patience and willingness to teach, troubleshoot and encourage me to keep going. In particular I would like to acknowledge:

The strong foundation in basic neuroscience and welcoming intellectual environment provided by the staff, students and professors of the Graduate Program in Neuroscience

The outstanding family that I found in my classmates and the members of the Georgieff and Rao labs, your technical and intellectual support, and warm friendships have been invaluable

Technical and intellection collaborations with Mu Sun, Mark Peterson, Anna Petryk, Ragu Rao, Kathleen Storey, Erik Carlson and Phu Tran

Academic, technical, and career guidance from my advisory committee: Jonathan Gewirtz, Thomas Neufeld and Yasushi Nakagawa

The tremendous advisor I found in Michael Georgieff, thank you for everything

The unfailing support of my family and friends

The completion of my graduate education has only been possible through the support, guidance, laughter, tears, and friendship of these and countless other labmates, classmates, professors, friends and family, and I am ever grateful for your kindness.

Abstract

Iron deficiency (ID) is the most common micronutrient deficiency, affecting an estimated 2 billion people world wide including 20-30% of pregnant women and their offspring. Many human studies have demonstrated negative effects of early life ID on learning and memory which persist beyond the period of ID despite of prompt iron treatment, observations which are supported by rodent models of early iron deficiency anemia (IDA). In spite of a large, observational literature the mechanisms through which early ID causes acute and persistent brain dysfunction are largely unknown. Mammalian target of rapamycin (mTOR) signaling is an attractive candidate for mediating the effects of early ID because it integrates cellular metabolic status to regulate fundamental aspects of cellular growth and differentiation. The overall goal of the current studies is to understand the role of iron in regulating mTOR signaling during a critical period of development in the hippocampus by using unique genetic mouse models of hippocampal ID to: 1) Determine when iron is required for hippocampal development 2) Determine the role of iron in mTOR signaling 3) Manipulate iron and mTOR to determine effects on hippocampal structure and behavior. The findings from these experiments demonstrate that mTOR signaling is upregulated by neuronal ID during the same period that rapid hippocampal development requires large amounts of iron. Additionally, rescue of behavioral outcomes in adult animals following restoration of mTOR signaling (through either timely iron repletion or pharmacological suppression) provides functional evidence for a connection between mTOR and the persistent effects of early ID.

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Chapter 1

Introduction

Overview

Deficiencies of nutrients that affect brain development and function have been estimated to shift the world's IQ potential negatively by 10 points (Morris et al., 2008). Iron deficiency (ID) is the most common of these nutrient deficiencies, affecting an estimated 2 billion people world wide according to the World Health Organization, including 20-30% of pregnant women and their offspring. Many human studies have demonstrated the negative effects of ID on learning and memory, and affective and social behavior (Lozoff and Georgieff, 2006). In humans, early life ID (late gestation through 2-3 years of age), results in learning and memory deficits which persist beyond the period of ID in spite of prompt iron treatment (Burden et al., 2007; Riggins et al., 2009); findings supported by rodent models of early iron deficiency anemia (IDA) (Schmidt et al., 2007). In spite of a large, observational literature on early IDA effects on learning and memory in humans and rodent models, several significant gaps in knowledge exist, specifically how early ID causes acute brain dysfunction and why permanent deficits remain despite iron treatment.

Iron Biology

Iron is an essential micronutrient that has multiple biological functions, many of which are fundamental to cell survival. Most iron-containing proteins are evolutionarily highly conserved and are involved in a wide range of cellular processes including DNA replication, cell cycle regulation, oxidative phosphorylation, oxygen transport, lipid synthesis, and neurotransmitter synthesis (Beard and Connor, 2003; De Domenico et al.,

2008). Not surprisingly, therefore iron is required for cell growth and survival (Le and Richardson, 2002). In conditions when iron supply is insufficient to meet iron demand, iron prioritization determines which tissues will receive available iron. The red blood cell (RBC) mass has the highest priority, which is expected considering that it is where the majority of the body's iron resides (Andrews, 1999). The heart, brain, muscle and liver have lower priority than RBCs (Georgieff et al., 1992). Thus, it is not surprising that IDA results in brain ID, and subsequent acute and long-term neurocognitive deficits.

Disruptions in Human Iron Homeostasis

Despite regulatory mechanisms, iron homeostasis is often disrupted by inadequate iron supply (i.e. insufficient dietary iron intake, blood loss, parasites), and shifts in iron demand (i.e. rapid growth, hypoxia) (Andrews, 1999). ID most commonly results from insufficient dietary intake and can be exacerbated by additional environmental factors. There are three populations with especially high risk for developing ID including women of childbearing age (i.e., increased iron loss due to menstruation) infants and toddlers (i.e., rapid growth, insufficient dietary intake, iron loss due to intestinal parasites), and late gestation fetuses/neonates (i.e., rapid intrauterine growth, severe maternal ID, gestational complications). In addition to severe maternal ID, several common gestational complications disrupt the balance of fetal iron supply and demand, resulting in total body and/or tissue level ID. These conditions include uncontrolled diabetes during gestation, maternal high blood pressure, maternal smoking, infection, placental

insufficiency, and prematurity (Siddappa et al., 2007). Early ID is a term used to refer to both infant/toddler and fetal-neonatal populations.

In adults, ID increases fatigue, affects physical work performance, and impairs cognitive function. These deficits completely resolve following iron therapy with no residual physical or cognitive effects (Basta et al., 1979; Patterson et al., 2001). Infants and children with early ID also demonstrate acute learning and memory deficits. For example, 9 and 12 month old infants with IDA show altered event related potential processing of strangers vs. mothers face, indicating impaired development of recognition memory (Burden et al., 2007). Similarly, iron deficient newborn infants of diabetic mothers (IDMs) show impaired auditory recognition memory processing of mother's voice (Siddappa et al., 2004).

Compared to ID in adulthood, early ID populations continue to demonstrate wide-ranging learning and memory deficits following iron repletion. For example, at 2-3 years of age, formerly iron deficient IDMs, exhibit impaired recall memory during elicited imitation tasks which correlates with iron status at birth (Riggins et al., 2009). At 5 years of age, children with low iron stores at birth showed decreased language development, fine motor skills, and tractability relative to children with normal iron stores at birth (Tamura et al., 2002). Additionally, at 11-14 years old, compared to normal children, children who were IDA as toddlers had lower psychomotor development scores, increased incidence of repeating a grade in school, impaired performance on visual-spatial memory tasks and increased difficulties with anxiety, social situations, and attention (Lozoff et al., 2000; Lozoff et al., 2006; Shafir et al., 2006). These deficits

remain in early adulthood, despite normal iron status (Lozoff et al., 2006). Therefore, prevention of ID is paramount for preventing acute dysfunction of the brain as well as long-term sequelae.

Animal Models of Early IDA

Animal models have been utilized to access and identify developmental processes that underlie the persistent cognitive deficits observed in humans. Early IDA is induced in rodent models by restricting maternal dietary iron during gestation and lactation. Observations from early dietary IDA models mirror many behavioral, and learning and memory deficits from human studies. Acutely, IDA in developing rats impairs motor development and hippocampus-dependent trace conditioning (Beard et al., 2006; Ward et al., 2007; Gewirtz et al., 2008). Formerly iron deficient anemic animals demonstrate persistent neurocognitive deficits including impaired spatial memory and win-shift performance (Felt and Lozoff, 1996; Felt et al., 2006; Schmidt et al., 2007).

The most commonly used method to experimentally assess spatial memory in rodents is the Morris water maze (MWM). The water maze was developed in the 1980's by Richard Morris (Morris, 1984), and has been extensively validated as a measure of hippocampus-dependent learning and memory. There are many modifications of the task, but the general approach remains the same. An animal is placed into a large tank filled with opaque water and given the opportunity to navigate to a platform submerged below the surface of the water where the animal can escape from swimming. Since the platform is not visible, the animal must use visual cues surrounding the tank to locate the

submerged platform. Normal animals utilize the cues to develop a spatial strategy to locate the platform; but mechanical, pharmacological, and genetic legions to the hippocampus impair spatial learning and MWM performance (Maei et al., 2009). Formerly iron deficient anemic rats have impaired MWM performance and do not develop spatial search strategies as well as iron sufficient control rats, indicated by longer swimming distances and increased escape latencies during training, and less distance traveled in the platform quadrant during probe trials (Felt and Lozoff, 1996; Felt et al., 2006).

Furthermore, animal models have been valuable for examining structural, metabolic, and molecular consequences of early IDA that are not accessible in human research. In the developing brain, acute IDA impairs neurometabolism, alters gene expression, myelination, monoamine function, and dendrite structure, and reduces synaptic efficacy (Beard and Connor, 2003; Jorgenson et al., 2003; Rao et al., 2003; Jorgenson et al., 2005; Clardy et al., 2006; Carlson et al., 2007). Following iron repletion, many of these structural and functional deficits remain (Jorgenson et al., 2003; Jorgenson et al., 2005; Clardy et al., 2006; Carlson et al., 2007). Together with human studies, these findings from animal models suggest that adequate iron is necessary during brain development to establish the structural and functional basis for long-term cognitive function.

Iron Homeostasis and Neuronal Development

Three primary brain processes are postulated to be directly affected by ID in humans and animals models: myelination, monoamine metabolism and energy production. While all three processes are important for neurodevelopment, the effects of early ID on neuronal energy metabolism are particularly prominent. ID impairs the function of iron-containing hemoproteins (e.g., cytochromes) resulting in reduced ATP production and reduced neuronal energy capacity (Maguire et al., 1982; Dallman, 1986; de Ungria et al., 2000).

The hippocampus is selectively vulnerable to the effects of early ID on energy metabolism because of the high metabolic demands of rapid growth exhibited in the late fetal-early neonatal period (Rice and Barone, 2000). There are many aspects of hippocampal development including neurogenesis, migration, neurite formation, synapse generation, and synaptic refinement. Although early ID has the potential to affect many of these processes, dendritogenesis and synaptogenesis are highly susceptible to reduced energy availability because these processes require large amounts of energy (de Ungria et al., 2000). Furthermore, the hippocampus undergoes rapid dendritogenesis and synaptogenesis during late gestation/early neonatal development, coincident with the periods of highest risk for early IDA (Pokorny and Yamamoto, 1981b, a). The presence of iron is needed to support this rapid growth, as evidenced by the upregulation of iron transport and the mobilization of iron stores in the hippocampus that occurs concomitant with the growth spurt (Taylor and Morgan, 1990; Siddappa et al., 2002). Furthermore, the need for iron is reinforced by the vulnerability of the hippocampus to early ID as

evidenced by learning and memory deficits following iron repletion in humans and in animal models (Schmidt et al., 2007; Riggins et al., 2009).

These findings suggest that the relationship between iron and energy metabolism is important for neuronal growth and differentiation in the hippocampus. However, it is not known how ID-induced perturbations in energy metabolism affect the growth and differentiation of developing neurons.

In spite of a large amount of observational literature, the cellular pathways that mediate the relationship between iron's effect on energy production and the structural and functional deficits observed in ID are unknown. In part, this is due to a lack of information about signaling pathways that integrate metabolic information during cell growth. Recently, a particularly relevant candidate pathway has emerged, the mammalian target of rapamycin (mTOR) signaling pathway, which is ubiquitous in metabolically active cells. mTOR is a kinase which integrates cellular metabolic status to regulate fundamental aspects of cellular growth and differentiation including protein synthesis and actin organization (Wullschleger et al., 2006).

Thus, the overall goal of these studies is to understand the role of iron in regulating mTOR signaling during a critical period of development in the hippocampus by addressing the following specific aims:

1. Determine when iron is required for hippocampal development
2. Determine the role of iron in mTOR signaling
3. Manipulate iron and mTOR to determine effect on hippocampal structure and behavior

Chapter 2

Prevention of Long-term Behavioral Deficits in an Inducible Mouse Model of Hippocampal Fetal-Neonatal Iron Deficiency

Introduction

The role of iron in brain development is not well understood. Iron deficient infants and children demonstrate a wide range of neurological deficits while iron deficient, including increased motor activity, impaired auditory and facial recognition, and increased fearfulness and hesitancy (Lozoff et al., 1986; Angulo-Kinzler et al., 2002; Siddappa et al., 2004; Burden et al., 2007). Following iron repletion, these populations continue to demonstrate wide-ranging deficits including reduced processing speed, decreased language development, and impaired fine motor skills at 4-5 years old (Tamura et al., 2002; Algarin et al., 2003). By 11-14 years old, formerly iron deficient anemic children have persistently lower psychomotor development scores, increased incidence of repeating a grade in school, impaired visual-spatial memory and increased difficulties with anxiety, social situations, and attention (Lozoff et al., 2000; Shafir et al., 2006). Despite iron repletion, these deficits persist into young adulthood (Lozoff et al., 2006), suggesting a sensitive window or a critical period for iron availability during neurodevelopment.

Animal models of early life dietary iron deficiency (ID) have further demonstrated the importance of iron for neuronal development and function in adulthood. The most common models in rats restrict maternal dietary iron from early gestation through lactation, causing iron deficiency anemia (IDA) in pups and reducing brain iron by up to 40%, approximating human autopsy observations from infants of diabetic mothers (IDMs) and intrauterine growth restricted (IUGR) infants (Petry et al., 1992; Georgieff et al., 1996). This rat model has demonstrated acute behavioral alterations

including delays in developmental milestones and trace conditioning (Beard et al., 2006; Ward et al., 2007; Gewirtz et al., 2008). Like human conditions, behavioral deficits persist following iron repletion, including impaired spatial memory and win-shift task performance (Felt and Lozoff, 1996; Schmidt et al., 2007). Together with human findings, these observations suggest a period during brain development that depends on iron availability in order to ensure appropriate neurodevelopment.

Neurodevelopment is shaped by a variety of factors, including growth factors, synaptic activity and environment. Structures are most sensitive to these factors during rapid development (Rice and Barone, 2000). Humans are most vulnerable to early ID from late gestation through 2-3 years old, during the most rapid period of brain development. Since early ID disrupts long-term cognitive development in humans and animals, we can therefore postulate that iron is a critical substrate for brain development. Of all the brain regions impacted by ID, the hippocampus is particularly vulnerable. Early ID occurs most frequently during rapid hippocampal development. In humans, the hippocampus develops rapidly, both structurally and functionally during the first two years of life. Hippocampus-dependent memory appears and matures between 3-18 months of age (Nelson, 1995). In rodents, the hippocampus also has a period of rapid development between postnatal day (P)10-25. This rapid development involves extensive dendrite arborization, spine formation, and synaptogenesis (Pokorny and Yamamoto, 1981a, b), and requires adequate metabolic support. Iron is a necessary substrate for energy production and cellular metabolism due to its role in mitochondrial enzymes such as cytochromes (Dallman, 1986). ID reduces cytochrome c oxidase

activity in the hippocampus, ultimately reducing hippocampal metabolic activity (de Ungria et al., 2000; Rao et al., 2003). Furthermore, many of the cognitive deficits observed in formerly iron deficient humans and rodents depend in part on the hippocampus, including recognition memory and limbic function (Nelson, 1995). Together, the timing and energy demands of hippocampal development with long-term deficits support the vulnerability of the structure to early life ID and suggest a critical developmental requirement for iron.

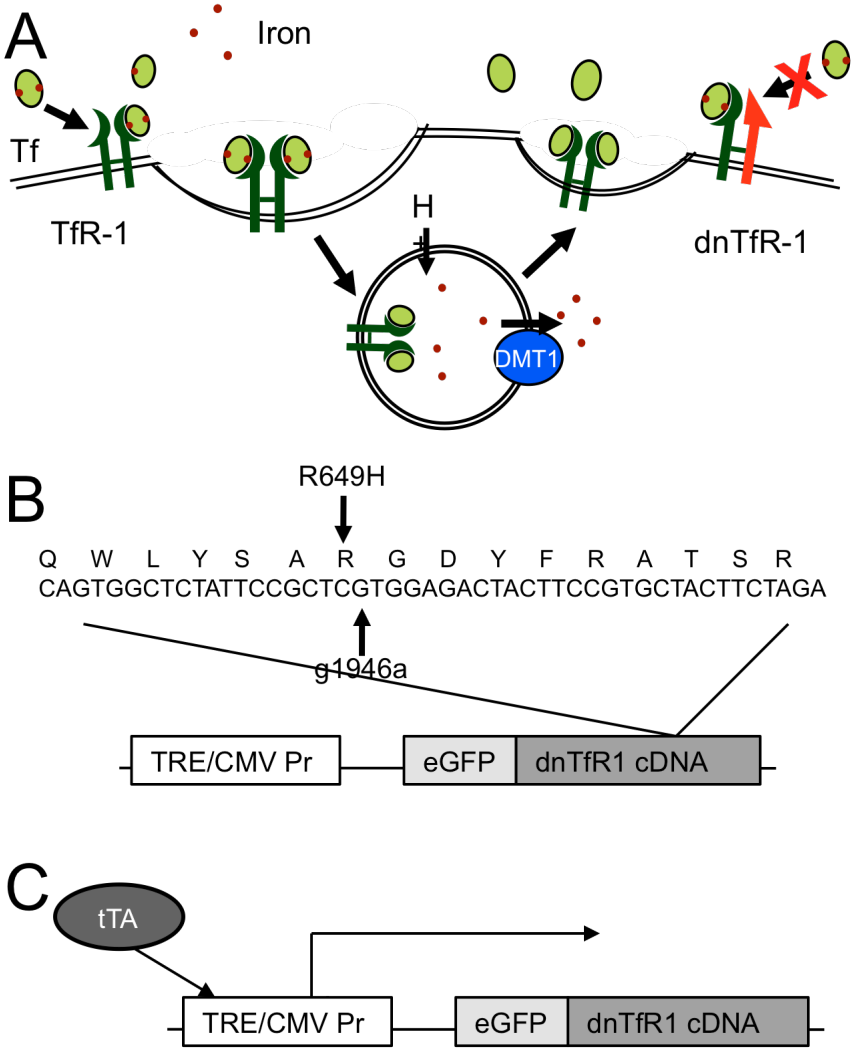
Animal models have shown that early IDA impairs hippocampal electrophysiology, CA1 apical dendrite structure and, gene expression during ID. In formerly iron deficient animals, many of these deficits persist including reduced long-term potentiation, dendrite structure abnormalities, and altered gene expression (Jorgenson et al., 2003; Jorgenson et al., 2005; Carlson et al., 2007). Despite what is known about the function of iron-containing proteins, the precise contribution of iron to these deficits has been difficult to determine because, in addition to creating neuronal ID, conventionally used dietary models of ID also produce maternal stress, total body ID and anemia in pups which may negatively alter long-term cognition independently of brain ID (Jorgenson et al., 2003; Carlson et al., 2009). Due to these additional consequences of dietary iron restriction, the specific normative role of iron in neurodevelopment remains elusive.

Recently, genetic manipulation of iron uptake genes has been utilized to generate non-dietary models of ID. The classic, most prevalent mechanism of cellular iron uptake is via transferrin receptor-1 (TfR-1) (Fig. 2.1*A*). Transferrin (Tf) is the primary

extracellular iron binding protein, and is present in plasma, cerebral spinal fluid and the extracellular fluid space (Moos and Morgan, 2000). Diferric transferrin binds TfR-1, which is expressed on the neuronal membrane in a soma- and dendrite-compartment specific manner, and this Tf-TfR-1 complex is taken up by a clathrin-coated endosome (Roberts et al., 1992; West et al., 1997). Upon endosomal acidification, iron is released from Tf and leaves the endosome through the divalent metal transporter 1 (DMT1), where it is stored or utilized by the cell. Point mutations in Tf cause hypotransferrinemia due to lack of Tf. In the brains of hypotransferrinemic mice the morphology and cellular distribution of iron is disrupted in the hippocampus, however these animals are also anemic (Bernstein, 1987; Dickinson and Connor, 1994). Total body genetic knock out (KO) of TfR-1 or DMT1 in mice result in severe anemia and death by P7 (Levy et al., 1999; Gunshin et al., 2005).

In the brain, ID has been restricted to hippocampal neurons using Cre-loxP conditional KO (CKO) of *Slc11a2* (the gene encoding DMT1) (Carlson et al., 2009). This model is not complicated by anemia or hypoxia and demonstrates the specific importance of iron for normal hippocampal development and function. Compared to rat IDA models, adult DMT1 CKO mice show remarkably similar alterations in recognition memory behavior, dendrite morphology and gene expression, suggesting that the main pathology of dietary IDA is mediated through the lack of iron delivery to the neurons. Nevertheless, gene expression differences do exist between the DMT1 CKO and dietary IDA models (Carlson et al., 2009), suggesting that anemia contributes to at least part of the neuronal pathology. A limitation of the DMT1 CKO model, however, is that it

Figure 2.1. Generation of dnTfR-1 transgene. **A,** Schematic of TfR-1 mediated cellular iron uptake. **B,** dnTfR-1 transgene contains a g1946a point mutation resulting in R649H substitution in the RGD Tf binding domain. **C,** tTA drives dnTfR-1 expression from the TRE CMV promoter.


A
Iron
Tf
TfR-1
H
+
DMT1
dnTfR-1
B
R649H
Q W L Y S A R G D Y F R A T S R
CAGTGGCTCTATTCCGCTCGTGGAGACTACTTCCGTGCTACTTCTAGA
g1946a
TRE/CMV Pr
eGFP
dnTfR1 cDNA
C
tTA
TRE/CMV Pr
eGFP
dnTfR1 cDNA

permanently disrupts DMT1 function in hippocampal pyramidal neurons, making it impossible to differentiate the developmental effects of ID from ongoing ID or to assess the efficacy of iron repletion.

Using a novel, reversible, genetic mouse model of hippocampal neuronal ID generated in our laboratory, the findings presented here provide evidence that there is a critical requirement for iron during hippocampal development. This model restricts ID to CA1 pyramidal neurons through reversible over-expression of a non-functional, dominant negative TfR-1 (dnTfR-1) using a tissue specific conditional, tetracycline responsive transgene system which can be regulated with dietary doxycyline (Gossen and Bujard, 1992; Mayford et al., 1996). This unique model isolates ID both spatially and temporally *in vivo* during hippocampal development. In this study, the lack of TfR1-mediated iron uptake between P21 and P42 leads to abnormal learning and memory in the adult.

Methods

Generation of transgenic mice.

dnTfR-1 Construct. Total brain RNA was isolated from C57/B6 mice and used to generate TfR-1 cDNA. A total brain cDNA library was generated using oligo-dT primers from an Invitrogen reverse-transcriptase-PCR (RT-PCR) kit. TfR-1 cDNA was then isolated via PCR with the forward primer 5'-CGGGATCCGATGATGGATCAAGCCAGATCA-3' (containing a BamHI restriction site) and the reverse primer 5'-CCATCGATGGTTAAAACTCATTGTCAATATT-3' (containing a ClaI site). This generated a ~2.3 kb cDNA of TfR-1, corresponding to its

predicted size. Site-directed mutagenesis (Stratagene) was used to generate a point mutation (g1946a) in the TfR-1 cDNA eliciting the amino acid substitution R649H in the conserved Arg-Gly-Asp transferrin binding motif in the TfR-1 protein (Fig. 2.1*B*). This fragment was then cut with BamHI and ClaI restriction enzymes, and subsequently ligated into the MCS of the CLONTECH eGFP vector, a PCR fragment for enhanced green fluorescent protein (EGFP) was isolated using the forward primer 5'-TCCCCGCGGGGACGCCACCATGGTGAGCAAGGGA-3' (containing a SacII restriction site and a Kozak consensus translation initiation site) and the reverse primer 5'-CGCGGATCCGCGCCTTGTACAGCTCGTCCATGCC-3' (containing a BamHI restriction site and omitting the stop codon). This ~700bp fragment was successfully ligated into the MCS of the pTRE2-TfRcDNA construct, downstream from the TRE, but upstream and in frame relative to the TfR-1 cDNA and linearized with a unique PvuI site for pronuclear injection. All cloning steps have been confirmed by automated DNA sequencing performed by the Advanced Genetic Analysis Center at the University of Minnesota.

Injection and confirmation of genomic integration of Tg(TRE-eGFP-dnTfR1).

The University of Minnesota Mouse Genetics Laboratory generated founder mice positive for Tg(TRE-eGFP-dnTfR1) by pronuclear injection. C57BL/6J female mice (21-25 days of age) were superovulated to synchronize ovulation and then mated to C57BL/6J fertile males. Fertilized embryos were collected at 0.5 days post conception. Isolated single cell embryos displaying two pronuclei were microinjected with the Tg. The 22-30 injected embryos were implanted into the left oviduct of a 0.5 dpc

pseudopregnant CD-1 female (6-7 weeks of age). Pups were born 19 days post implant. DNA was prepared from a tail snip for definitive genotyping by Southern blot and PCR. Genomic DNA isolated from tail snips was used to indentify the genotype of resultant offspring. Three pups positive for the transgene were unique founders; each was propagated and maintained as heterozygotes by backcrossing to WT C57BL/6J mice. Here findings from a single strain, *Mkg1,* are presented. Mkg1 was further mated with B6;CBA-Tg(Camk2a-tTA)1Mmay/J mice (purchased from Jackson Laboratories) which express tetracycline-OFF transactivator (tTA) under the regulatory control of a CaMKIIα promoter (Mayford et al., 1996). Animals positive for dnTfR-1 and Camk2a-tTA express dnTfR-1 at physiologic levels sufficient to affect iron uptake, in the absence of doxycycline (Fig. 2.1*C*). These doubly transgenic mice will be referred to as dominant negative (DN), while offspring positive for one or no transgenes will be referred to as wild type (WT) because they do not express dnTfR-1.

Animals

All experiments were performed in accordance with the NRC's Guide for Care and Use of Laboratory Mice, and with approval of the Institutional Animal Care and Use Committee of the University of Minnesota. Mice were housed in RAR facilities in a 12 light 12 dark cycle. All experimental animals were maintained on ad lib standard laboratory chow until weaning (P21). Following weaning, animals continued on standard diet (WT^{nodox}, DN^{nodox}) or were switched to ad lib doxycycline diet (0.625mg doxycycline/kg, TD.01306, Harlan-Teklad, Madison, WI) at P21 (WT^{P21dox} and DN^{P21dox})

or P42 (WT^{P42dox} and DN^{P42dox}). The doxycycline diet was identical to the standard diet in all other nutritional aspects.

To produce animals for morphological analysis, B6;CBA-Tg(Camk2a-tTA)1Mmay/J animals were crossed with B6.Cg-Tg(Thy1-YFP)16Jrs/J animals, which expresses yellow fluorescent protein (YFP) in random subsets of neurons (Feng et al., 2000). Animals positive for both Tg(Camk2a-tTA)1Mmay and Tg(Thy1-YFP)16Jrs were then mated with C57 BL/6-*Tg(TRE-eGFP-dnTfR1)Mkg1* animals.

Tissue Collection

Animals were euthanized by i.p. injection of Beuthanasia (10mg/kg). Whole brains used for Perl's staining and morphology analysis were then collected following transcardial perfusion with PBS, and by perfusion with 4% PFA. Hippocampal tissue used for mRNA and protein analysis was dissected following rapid decapitation and brain removal. Dissected hemispheres were then flash frozen in liquid nitrogen and stored at -80°C until use.

Experimental Procedures

<u>*CHO Culture*</u>. To test the ability of dnTfR-1 binding to transferrin in vitro, CHO cells (CHO AA8 Tet-Off Control Cell Line, Clontech), which stably express the Tet-OFF tetracycline transactivator, were transfected with *Pvu*I-linearized pCMV:eGFP-TfR1 or pCMV:eGFP-dnTfR1 plasmid using Lipofectamine per manufacturer's recommendation (Invitrogen). Following overnight incubation, transfected CHO cells were incubated for 1 hour with 25μg/mL texas-red labeled tranferrin (Invitrogen). Unbound transferrin was removed with PBS washes. Bound transferrin was visualized with light microscopy

equipped with a CCD camera. Images were captured and processed using Adobe Photoshop.

Hematocrit. Trunk blood samples for hematocrit measures were taken following rapid decapitation using heparinized capillary tubes to ensure that the animals were not anemic and that doxycycline administration did not alter the hematologic status. Samples were centrifuged at 10,000 g for 10 min and hematocrit was determined using a standard hematocrit reader.

Modified Perl's iron staining. 50μm vibratome brain sections from P70 WT^{nodox}, WT^{P21dox}, DN^{nodox}, and DN^{P21dox} mice were stained for storage iron using modified Perl's iron stain, as described previously (Carlson et al., 2009). Sections were imaged using a Nikon microscope (Eclipse 600) and staining density was measured using Photoshop. 20 pixel square boxes were used to record the average staining intensity from a non-stained region and from the CA1 pyramidal cell layer and the cortex. Background staining intensity was then subtracted from CA1 and cortical staining intensity to determine specific CA1 and cortex iron staining densities as previously described (Gewirtz et al., 2008).

Morris Water Maze. A modified version of the Morris water maze (MWM) was used to evaluate spatial memory, as previously described (Choi et al., 2006; Carlson et al., 2009). Briefly, 2-3 month old male $WT^{nodox, P21dox, P42dox}$ and $DN^{nodox, P21dox, P42dox}$ mice were tested across 6 consecutive days. A circular pool 120 cm in diameter was filled with water occluded with white non-toxic paint and divided into virtual quadrants (NE, NW, SE, SW). A 10 cm diameter escape platform was submerged 1 cm below the

surface in the NW quadrant. Salient visual cues were located on the walls surrounding the tank. The animals were habituated to the water on day 1. During the four test days that followed animals were given 5 training trials with an inter-trial interval of 30 minutes. For each training trial the escape platform was located in a unique position within the NW quadrant and animals were placed into the pool facing the wall from an entry location chosen randomly from N, NE, E, SE, S, SW positions along the pool. Animals were allowed to swim until they escaped onto the platform, if a mouse did not escape within 90 seconds, it was guided to the platform. Following the 5 training trials, the animals were given a probe trial during which the platform was removed from the pool and the animals were allowed to swim for 30 seconds before being removed from the pool. Performance was measured by the amount of time spent searching for the submerged platform in the target quadrant during probe trials. On the 6th day, the animals were given visual cued task (VCT) training where a visible flag was attached to the submerged platform, and animals were given three 45 second training trials. VCT performance was measured by escape latency as previously described (Carlson et al., 2009). The trials were video-captured and analyzed using Topscan software (Clever Systems, Reston, VA).

Morphological Analysis. 50μm brain sections were cut using a vibratome, mounted on slides with Dapi and imaged using a Nikon microscope (Eclipse E600).

Data Analysis

Analysis of Variance was used to compare the staining intensity and hematocrit values. Two-way ANOVA was used to assess genotype x time interactions for each

doxycycline conditions in MWM performance. α was set at 0.05 and Bonferroni post-hoc analysis was used.

Results

dnTfR-1 expression inhibits iron uptake.

To test dnTfR-1 binding to transferrin *in vitro*, eGFP-TfR-1 and eGFP-dnTfR-1 plasmids were transfected into CHO cells stably expressing tetracycline transactivator (Tet-Off) and incubated with Texas red labeled transferrin (Texas red-Tf) for one hour. Texas red-Tf co-localized with eGFP-TfR-1 (Fig. 2.2*A*) but not eGFP-dnTfR-1 (Fig. 2.2*B*).

To determine if dnTfR-1 expression is sufficient to reduce *in vivo* iron uptake in the hippocampus, storage iron was visualized with modified Perl's staining. dnTfR-1 expression significantly reduces storage iron in adult DN^{nodox} CA1 pyramidal neurons compared to WT^{nodox} (Fig. 2.3*A-B,E*). Iron staining in the cortex is not altered in DN^{nodox} animals (Fig. 2.3*A-B,E*). The mice are not anemic since the hematocrit is not altered in DN^{nodox} animals compared to WT^{nodox} controls (DN^{nodox} 43.5±0.7% vs. WT^{nodox} 41.2±3.6%, *p*=ns). Therefore, in this model dnTfR-1 expression specifically reduced iron in CA1.

Dietary doxycyline inhibits dnTfR-1 expression and restores iron status.

Dietary doxycyline beginning at P21 inhibits dnTfR-1 expression and restores normal iron status in CA1 pyramidal neurons in adult DN^{P21dox} animals (Fig 2.3 *C-D,E*).

Cortical iron status and hematocrit are not affected by doxycyline (Fig 2.3*C-D,E*; WT^{P21dox} 43.3±1.5% vs DN^{P21dox} 43.3±1.9%, *p*=ns).

ID in CA1 impairs adult learning and memory performance.

In the modified Morris water maze (Choi et al., 2006; Carlson et al., 2009) adult, hippocampally iron deficient DN^{nodox} mice spend less time in the target quadrant during probe trials across training days (Fig. 2.4*A; n=10-14, F=13.96, p<0.001*). Additionally, DN^{nodox} animals spend more time swimming in the perimeter of the maze during training trials than WT^{nodox} animals (Fig. 2.4*B, n=10-14, F=10.81, p<0.01*). VCT performance is not different between the groups (Table 2.1), indicating that motor and sensory deficits do not account for differences in spatial task performance.

In order to determine if structural abnormalities contribute to impaired spatial memory in DN^{nodox} animals, CA1 apical dendrites were visualized using transgenic thy-1 YFP. Compared to iron sufficient WT^{nodox} animals, apical dendrites in adult DN^{nodox} animals are truncated and disorganized (Fig. 2.5*A-B*). Consistent with previous findings, these data demonstrate that ongoing neuronal ID impairs spatial memory learning and CA1 apical dendrite structure.

Restoration of iron status at P21 but not P42 rescues learning and memory performance.

DN mice given doxycyline beginning at P21 (DN^{P21dox}) demonstrate similar acquisition of the spatial learning task as WT^{P21dox} littermates on the MWM spatial memory task when tested at P70 (Fig. 2.4*C, n=11-14, F=0.313, p=ns*). They spend the same amount of time swimming in the perimeter of the maze as the WT^{P21dox} mice (Fig.

Figure 2.2. dnTfR-1 expression inhibits Tf binding. In tetOFF CHO cells, Texas red-Tf co-localizes with TfR-1 in CHO cells transfected with **A,** wild type eGFP-TfR-1 but not with **B,** eGFP-dnTfR-1.

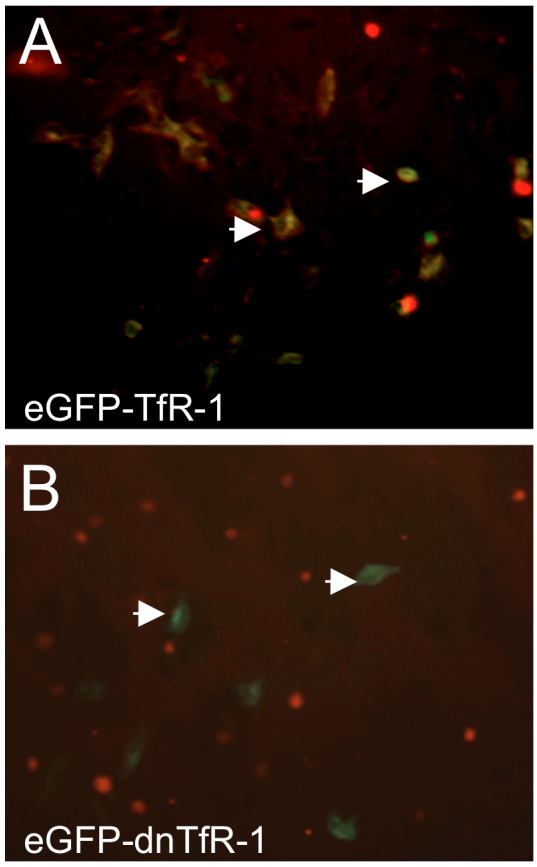

A
eGFP-TfR-1
B
eGFP-dnTfR-1

Figure 2.3. dnTfR-1 expression selectively and reversibly inhibits iron uptake in whole brain. Storage iron staining in adult hippocampus and cortex (inset, magnification of CA1 pyramidal cell layer indicated by arrowhead) **A,** WT^{nodox} **B,** DN^{nodox} **C,** WT^{P21dox} and **D,** DN^{P21dox} mice. **F,** Quantification of staining intensity. **p<0.01 Data are mean±SEM (n=4-6), scale bar=200μm

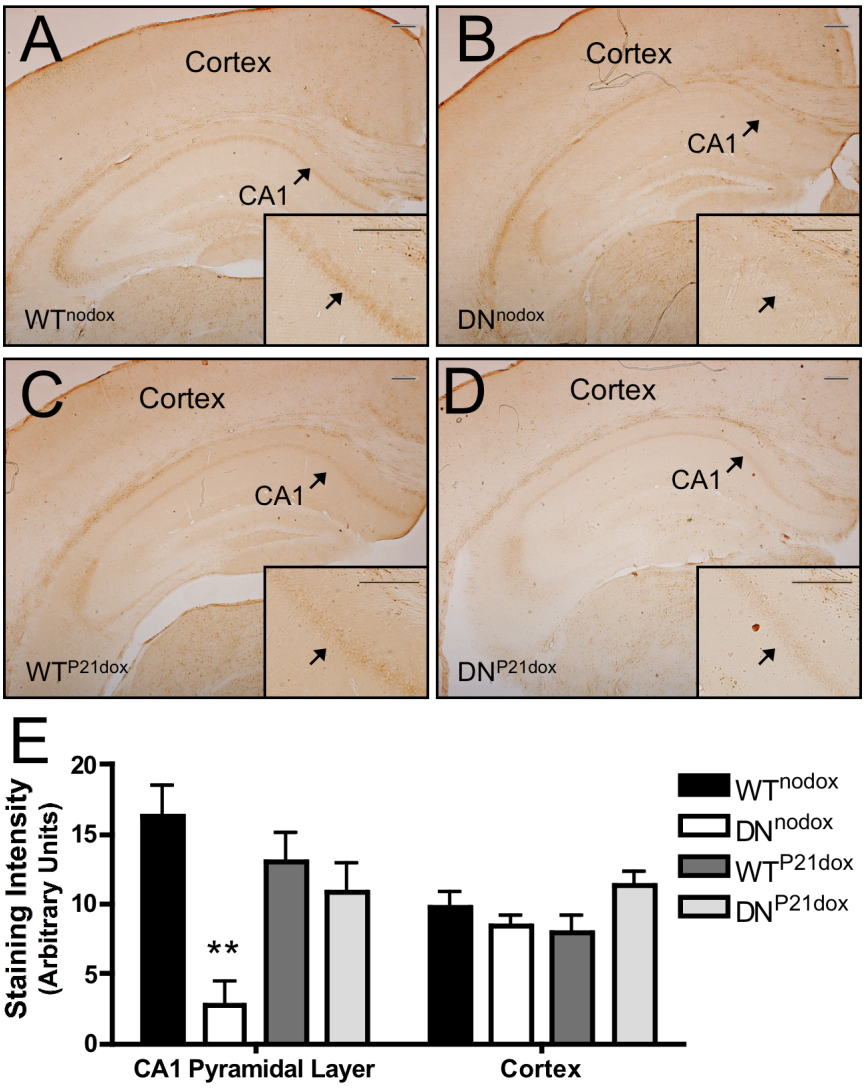

A
Cortex
CA1
WTnodox
B
Cortex
CA1
DNnodox
C
Cortex
CA1
WTP21dox
D
Cortex
CA1
DNP21dox
E
Staining Intensity (Arbitrary Units)
20
15
10
5
0
**
CA1 Pyramidal Layer
Cortex
WTnodox
DNnodox
WTP21dox
DNP21dox

Figure 2.4. Hippocampal ID impairs spatial learning. Percentage of time spent target quadrant during MWM probe trails for **A,** WT^{nodox} and DN^{nodox} **C,** WT^{P21dox} and DN^{P21dox} **E,** WT^{P42dox} and DN^{P42dox} animals. Percentage of time spent swimming in the perimeter of MWM during training trials for **B,** WT^{nodox} and DN^{nodox} **D,** WT^{P21dox} and DN^{P21dox} **F,** WT^{P42dox} and DN^{P42dox} animals. Data are mean±SEM (n=10-14)

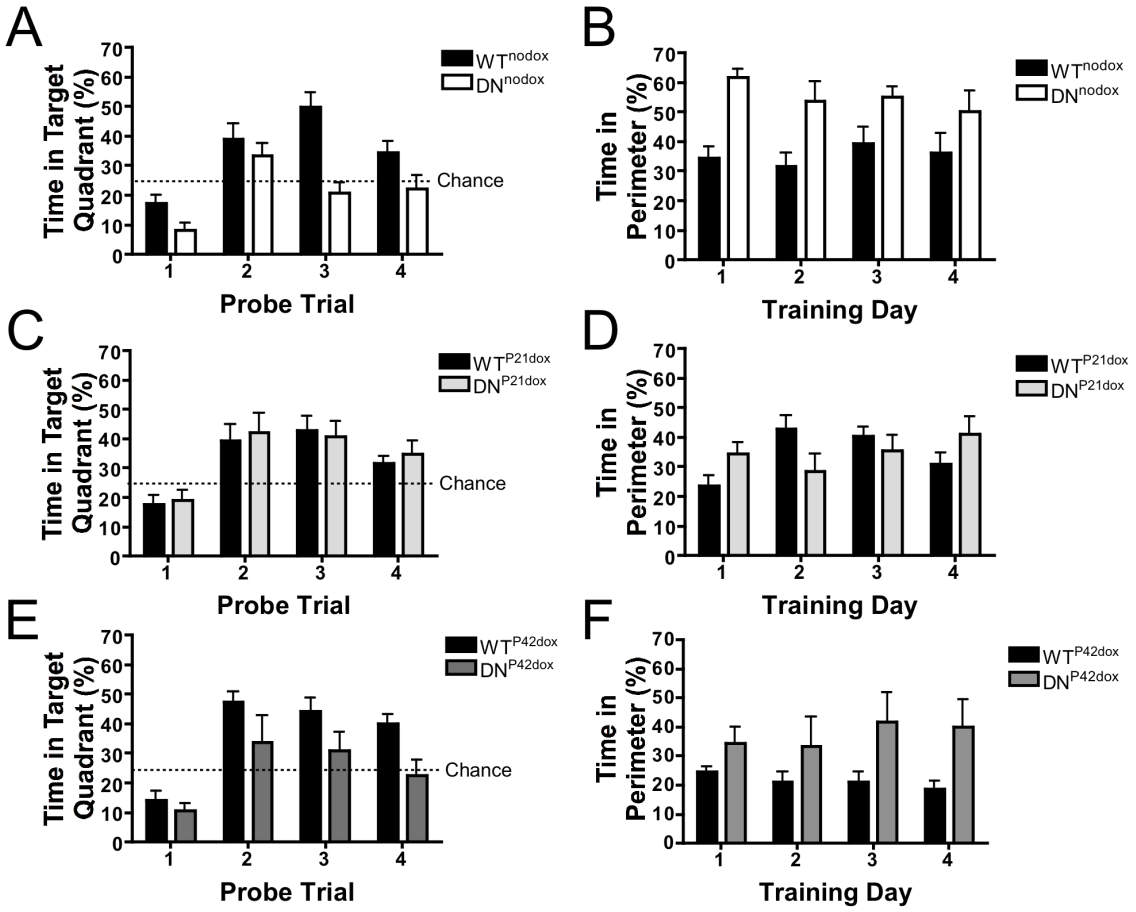

A
Time in Target Quadrant (%)
WT nodox
DN nodox
Chance
Probe Trial
B
Time in Perimeter (%)
WT nodox
DN nodox
Training Day
C
Time in Target Quadrant (%)
WT P21dox
DN P21dox
Chance
Probe Trial
D
Time in Perimeter (%)
WT P21dox
DN P21dox
Training Day
E
Time in Target Quadrant (%)
WT P42dox
DN P42dox
Chance
Probe Trial
F
Time in Perimeter (%)
WT P42dox
DN P42dox
Training Day

Figure 2.5. Hippocampal ID impairs CA1 apical dendrite morphology. CA1 apical dendrite morphology in A) WT^{nodox} B) DN^{nodox} C) WT^{P21dox} D) DN^{P21dox} E) WT^{P42dox} and F) DN^{P42dox}animals. Scale bar=200μm

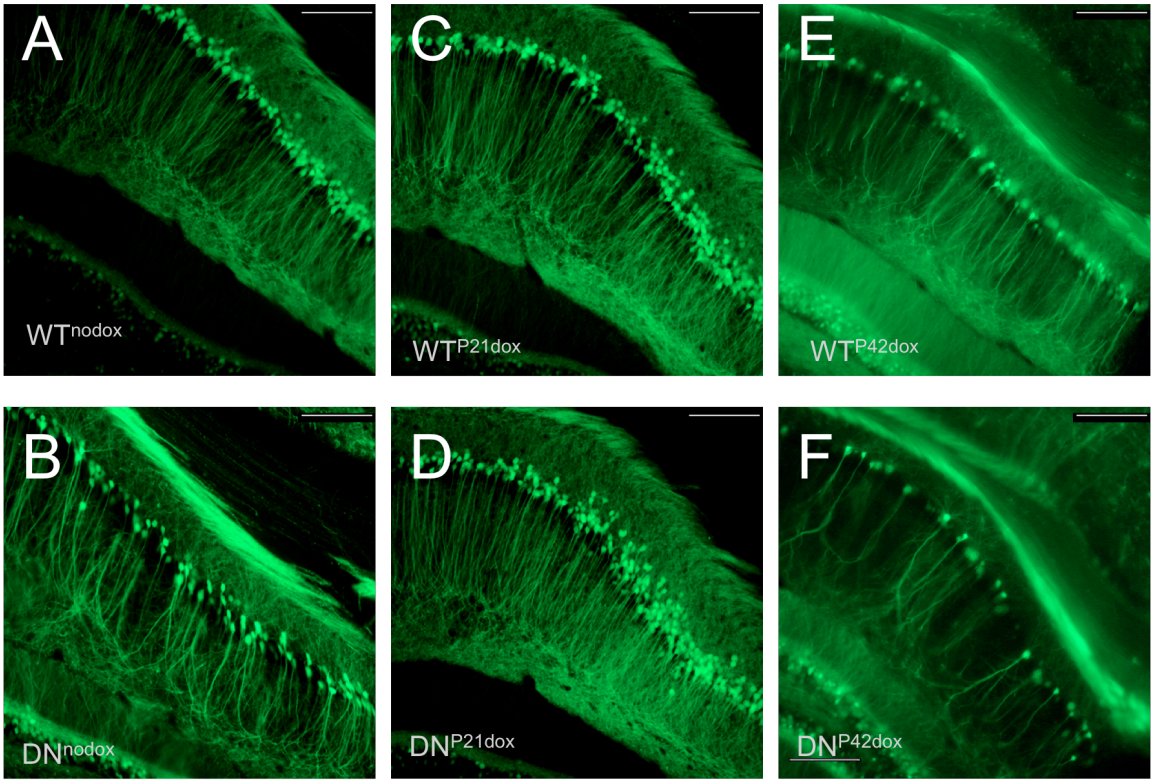

A
WTnodox
C
WTP21dox
E
WTP42dox
B
DNnodox
D
DNP21dox
F
DNP42dox

Table 2.1. Genotype and Doxycyeline Treatment do not Affect Visual Cued Trial Escape Latencies (values are mean seconds ± SD)

Treatment Group	Trial 1		Trial 2		Trial 3	
	WT	DN	WT	DN	WT	DN
No Dox (n=10-14)	10.5±8.5	15.3±8.8	9.9±6.8	9.6±4.9	6.3±2.5	8.8±3.6
P21 Dox (n=14-11)	8.1±4.4	12.2±7.3	5.9±1.9	7.8±3.0	8.7±9.6	11.2±10.6
P42 Dox (n=10-14)	7.5±3.0	11.9±/8.0	9.7±7.9	15.7±10.7	9.5±9.0	13.0±9.9

2.4*D*). CA1 apical dendrite structure in DN^{P21dox} animals is comparable to WT^{P21dox} (Fig. 2.5*C-D*). In contrast, adult DN mice given doxycyline beginning at P42 (DN^{P42dox}) spend less time in the target quadrant than WT^{P42dox} littermates (Fig. 2.4*E, n=10-14, F=11.79, p<0.001*) and continue to show increased time spent in the perimeter of the maze, similar to untreated DN^{nodox} animals (Fig. 2.4*F, n=10-14, F=4.49, p<0.05*). Moreover, DN^{P42dox} CA1 apical dendrite structure is disrupted, similar to untreated DN animals despite iron repletion (Fig. 2.5*E-F*). VCT performance was not altered between doxycyline treated DN and WT groups, regardless of age of iron repletion (Table 2.1). Restoration of iron beginning at P21, but not at P42, restores behavioral performance and dendrite structure in adult DN animals compared to iron deficient DN^{nodox} animals, indicating a developmental requirement for iron in establishing neural circuitry that underlies learning and memory behavior.

Discussion

Human and animal studies of early life ID have demonstrated that there is a critical requirement for iron during hippocampal development. However, due to the confounds of dietary animal models, determining precisely when iron is necessary to ensure appropriate hippocampal development has not been possible. Using this novel approach to modeling reversible cellular ID, we have more narrowly defined a critical requirement for iron between P21-42. Iron repletion beginning at P21 rescues the long-term effects of ID, whereas iron repletion beginning at P42 does not.

The rodent hippocampus undergoes rapid development between P10-P25 including dendritogenesis and synaptogenesis, which require large amounts of energy (Pokorny and Yamamoto, 1981a, b). Brain iron uptake and utilization also peaks between P10-25 (Taylor and Morgan, 1990; Blanpied et al., 2003; Cheah et al., 2006). The rapid development of dendrites and synapses and corresponding increase in iron uptake between P10-25 make this a very vulnerable period for ID. While it is logical that treatment at P21, near the end of this vulnerable window would be more likely to restore hippocampal health than treatment outside the window, it was unexpected that such late iron repletion could prevent long-term deficits.

Dietary rat models demonstrate that iron treatment as early as P7 is not sufficient to rescue long-term hippocampal behavioral and structural deficits. The ability of iron repletion late in the window of rapid hippocampal development to rescue behavioral and structural impairment in DN^{P21dox} animals suggests that early life ID may extend or delay the period of dendrite arborization and synaptogensis. Extension of this period of development would enable cells to complete dendritogenesis if iron is restored.

By extending the period of ID from 21 to 42 days, well beyond the end of rapid hippocampal dendritogenesis, iron repletion is no longer sufficient to restore behavior and structure in DN^{P42dox} mice, indicating that there is a critical neuronal requirement for sufficient iron during development between P21 and 42 for establishing functional hippocampal circuitry.

Chapter 3

Iron Deficiency Dysregulates mTOR Signaling During Hippocampal Development

Introduction

There are many consequences of early iron deficiency (ID), but one of the most puzzling is that cognitive deficits remain in spite of relatively prompt and complete iron repletion (Lozoff et al., 2006), suggesting that early ID interferes with long-term cognitive function. Human findings are supported by studies from rat models of early life dietary ID and genetic hippocampal mouse models of neuronal ID which demonstrate long-term cognitive deficits (Felt and Lozoff, 1996; Schmidt et al., 2007), as well as alterations in neuron structure (Jorgenson et al., 2003; Carlson et al., 2009). Neuronal structure, particularly dendrite arborization and complexity, defines the capacity of a neuron to integrate synaptic inputs (Spruston, 2008). It is likely, therefore, that the structural abnormalities observed in ID are in part responsible for and mediate the long-term cognitive deficits.

The successful construction of the hippocampus during development and its maintenance during adulthood (which allows for life-long plasticity and experience-dependent learning) is a complex process. At the most basic level, neuronal development requires appropriate guidance cues and growth factors, and sufficient substrates, oxygen, and energy (Erecinska et al., 2004; Conde and Caceres, 2009). Disrupting any of these factors can significantly alter neurodevelopment. Normative neuronal development, therefore, depends on the availability and integration of all these factors.

Iron contributes to many of these basic neurodevelopmental processes. Rapidly growing cells are characterized by increased iron uptake through expression of high levels of transferrin receptor-1 (TfR-1, an iron uptake protein) which can be stimulated

by exogenous growth factors and *c-myc* activation (Neckers and Trepel, 1986; O'Donnell et al., 2006). Furthermore, exogenous transferrin (Tf, the extracellular iron chaperon protein) promotes growth of lymphocytes; and heme stimulates neurite outgrowth in cultured neurons (Ishii and Maniatis, 1978; Neckers and Trepel, 1986). Iron uptake by rapidly growing cells is crucial for cellular responsiveness to oxygen availability and oxidative stress as well as energy production. Iron is a co-factor for globin oxygen transport proteins, and is essential for the activity of prolyl-hydroxylase (Prl-H) which regulates hypoxia inducible factor 1α (HIF1α) stability (Siddiq et al., 2008). HIF1α is a transcription factor which is stimulated by hypoxic conditions. Many mitochondrial enzymes integral for oxidative phosphorylation and ATP production require iron in the form of heme and iron-sulfur clusters, including cytochromes, NADPH, and flavoproteins (Maguire et al., 1982).

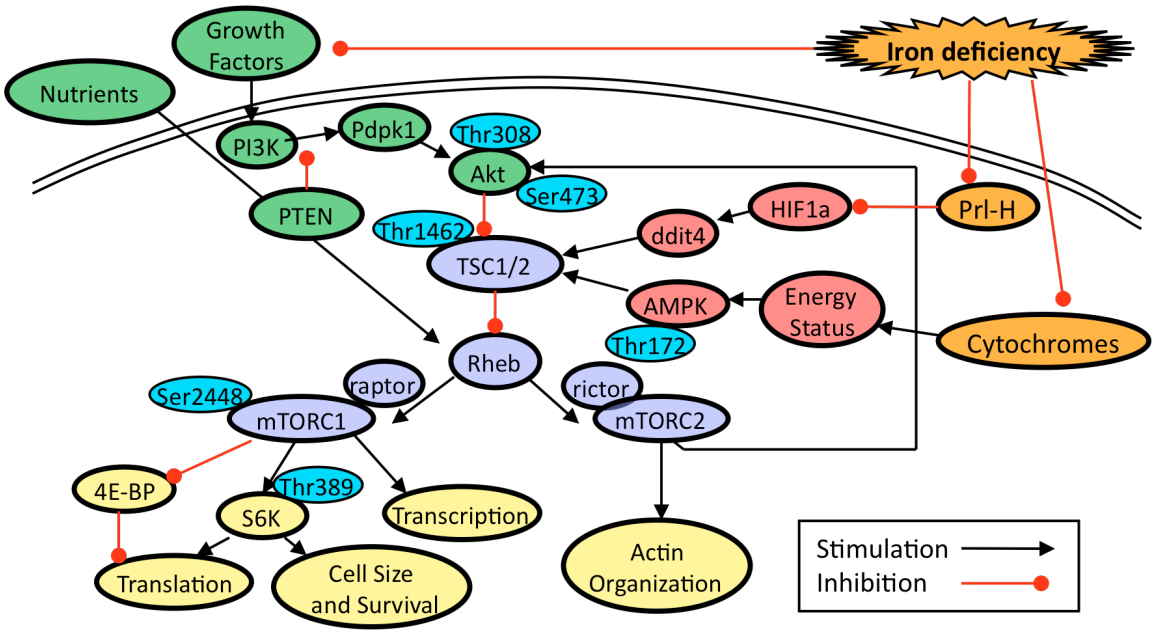
Given iron's role in cell growth, oxygen utilization and energy production, it is not surprising that dietary and genetic ID interferes with these neurodevelopmental processes, particularly in the highly metabolic, rapidly developing hippocampus. For example, acute IDA reduces expression of growth factors such as brain derived neurotrophic factor (BDNF) (Tran et al., 2008). The transcriptional activity of HIF1α is disrupted by both dietary and genetic ID (Carlson et al., 2007; Carlson et al., 2009). Dietary and genetic ID reduce cytochrome oxidase c activity, and alter PCr/Cr levels in hippocampus, indicating reduced energy availability (de Ungria et al., 2000; Rao et al., 2003; Carlson et al., 2009). Together, these effects are thought to mediate the long-term alterations in hippocampal behavior, structure, gene expression, and electrophysiology

observed in adult, iron replete animals (Felt and Lozoff, 1996; Jorgenson et al., 2003; Jorgenson et al., 2005; Felt et al., 2006; Carlson et al., 2007; Schmidt et al., 2007; Brunette et al., 2010).

These consequences of early life ID on hippocampal learning and memory have been largely ascribed to abnormalities in iron-containing hemoproteins (e.g., cytochromes) resulting in reduced neuronal energy capacity (Dallman, 1986; de Ungria et al., 2000; Rao et al., 2003). As described above however, iron availability also influences the activity of other proteins involved in neuronal cellular metabolism including HIF1α and BDNF (McDonough et al., 2005; Carlson et al., 2007; Tran et al., 2008; Carlson et al., 2009; Tran et al., 2009). It is unclear how these multiple iron dependent effects are integrated by neurons and translated into important, fundamental structural outputs such as dendrite arborization, which is closely linked to functional capacity in learning and memory behavior (Spruston, 2008).

During normal development, the activity of the mammalian target of rapamycin (mTOR) signaling pathway regulates many aspects of the growth of all cells by integrating growth factor stimulation and nutrient availability with energy and oxygen availability. mTOR is a highly conserved Ser/Thr kinase that forms two distinct functional complexes (mTORC1 and mTORC2) (Fig. 3.1). mTORC1 is sensitive to the drug rapamycin and its targets regulate protein translation, cell survival, gene transcription, and autophagy (Wullschleger et al., 2006). mTORC2 is involved in regulating actin organization as well as Akt and PKC activity (Jacinto et al., 2004; Facchinetti et al., 2008; Ikenoue et al., 2008). mTOR activity is determined by a balance

Figure 3.1. Schematic of mTOR signaling. Central components are highlighted in purple and downstream effectors in yellow. Stimulatory upstream growth factor signaling in green and inhibitory upstream metabolic signaling in red. Possible iron-dependent regulatory factors are noted in orange.


Growth Factors
Iron deficiency
Nutrients
PI3K
Pdpk1
Thr308
Akt
Ser473
PTEN
HIF1a
Prl-H
Thr1462
ddit4
TSC1/2
AMPK
Energy Status
Cytochromes
Thr172
Rheb
raptor
rictor
Ser2448
mTORC1
mTORC2
4E-BP
Thr389
S6K
Transcription
Translation
Cell Size and Survival
Actin Organization
Stimulation
Inhibition

of phosphorylation states and is stimulated by growth factors such as insulin and BDNF, and by branch chain amino acids. mTOR activity is inhibited by reduced energy status, and increased oxidative stress (Wullschleger et al., 2006). In neurons, mTOR's regulation of protein synthesis and actin organization is required for neuronal differentiation and dendrite arborization, which in turn determine cellular structure and function (Jaworski et al., 2005; Kumar et al., 2005). mTOR activity is also important for the maturation of oligodendrocytes and the formation of myelin which support neuronal structure and plasticity (Tyler et al., 2009). Genetic and pharmacologic manipulation of mTOR in animal models further demonstrates the importance of mTOR signaling for neuronal morphology, electrophysiology, and spatial learning (Knox et al., 2007; Ehninger et al., 2008; Zhou et al., 2009).

ID affects several important regulators of mTOR activity, including BDNF, oxidative signaling (through HIF1α), and energy availability. Previously, a hippocampal microarray study identified alterations in expression of genes in the mTOR pathway at P15 in a dietary ID anemia (IDA) rat model (Carlson et al., 2007). However, because mTOR signaling is ultimately regulated by a balance of phosphorylation states, the relationship between iron availability, permanent long-term hippocampal deficits, and mTOR signaling is unknown.

Here, in order to isolate the effect of ID, a permanent, genetic, hippocampus specific conditional knock out (CKO) of divalent metal transporter-1 (DMT1) was used to investigate mTOR signaling as a potential effector of disrupted hippocampal neuronal development in ID. Results showed that mTOR activity is developmentally regulated in

wild type (WT) animals, corresponding to both peak iron uptake and dendritogenesis, and that mTOR signaling is dysregulated by ID at the peak of this developmental period at P25.

Methods

Animals

All experiments were performed in accordance with the NRC's Guide for Care and Use of Laboratory Mice, and with approval of the Institutional Animal Care and Use Committee of the University of Minnesota. Mice were housed in RAR facilities in a 12 light 12 dark cycle and given *ad lib* access to water and standard laboratory diet.

The DMT1 CKO model was generated by crossing $Slc11a2^{flox/flox}$ mice (Gunshin et al., 2005) with CamKIIα-Cre mice as previously described (Carlson et al., 2009). Resulting offspring positive for Cre will be referred to as DMT1 CKO, and Cre negative littermates will be referred to as DMT1 WT. Animals were killed by i.p. injection of Beuthanasia (10mg/kg). Hippocampal tissue used for mRNA, polysome isolation, and protein analysis was dissected from DMT1 CKO and WT littermates at postnatal days 5, 10, 15, 20, 25, and 45, flash frozen in liquid nitrogen and stored at -80°C until use. Whole brains were collected following transcardial perfusion with PBS, followed by perfusion with 4% PFA in PBS. The brains were removed, and submerged O/N in 4% PFA.

Experimental Procedures

Western Blot. Protein samples were prepared from individual dissected hippocampal hemispheres by sonication in cytoskeletal lysis buffer. 30 μg of total protein was separated using NuPAGE 4-12%, or 12% Bis-Tris gels (Invitrogen). Protein was transferred onto nitrocellulose membrane (Pierce), blocked in Rockland Near-Fluorescence blocking solution, and incubated overnight with primary antibody diluted in blocking buffer. Following primary incubation, the blots were washed using PBS + 0.1% Tween-20, and incubated for 45 minutes in secondary antibody diluted in blocking buffer plus 0.01% Tween-20 and 0.001% SDS. Then blots were then washed again and imaged with Odyssey infrared scanning (LiCor Bioscience, Lincoln, NE). Unless otherwise noted, all primary antibodies were obtained from Cell Signal Technology (Danvers, CO) and used at a 1:1000.

qPCR. Quantative PCR (qPCR) was performed as previously described (Tran et al., 2009). Briefly, mRNA was isolated from individual hippocampal hemispheres using an RNA isolation kit (Applied Biosystems), and cDNA was synthesized using a kit from Applied Biosystems (Carlsbad, CA). qPCR was performed using MX3000P thermocycler (Stratagene) and TaqMan master mix and probes at according to manufacturer's recommendations (Applied Biosystems).

Polyribosome Analysis. Polyribosomes were isolated and fractionated as previously described (Larsson et al., 2006). Briefly, 6, P25 hippcampal hemispheres from each genotype were pooled together and homogenized. The homogenate was placed on a sucrose gradient, ultricentrifuged, and fractionated into 10 fractions. RNA

was isolated from each fraction, cDNA was synthesized, and qPCR was performed for targets of interest using Taqman probes as described above.

Data Analysis

Analysis of Variance, α=0.05, with Bonferroni post-hoc analysis, was used to compare soma size, developmental protein and mRNA expression. Student t-tests were used to compare individual proteins at P25

Results

ID disrupts developmental regulation of mTOR activation.

In iron sufficient WT animals, mTOR activity measured by S6K(Thr389) phosphorylation, a direct mTORC1 target, demonstrated developmental activation, with peak activity occurring at P10 (Fig. 3.2, solid bars). In iron deficient DMT1 CKO mice, S6K(Thr389) activity showed a higher and more sustained increase in activity throughout development with significantly higher levels of activation at P5 and P25 compared to DMT1 WT (Fig. 3.2, white bars; n=3-4, $F=16.82$ $p<0.001$). S6K(Thr389) levels normalize by P45.

Overall mTOR activity was increased by ID at P25.

Due to the rapid dendritic development occurring in the hippocampus, the effect of ID on mTOR signaling was examined in more detail at P25. Consistent with increased downstream S6K(Thr389) activation, central components were also activated by ID at P25, including mTOR(Ser2448) phosphorylation (Fig 3.3*A*), reflecting increased

Figure 3.2. Developmental profile of mTOR signaling. S6K(Thr389) phosphorylation relative to total S6K protein and actin in DMT1 WT (Black bars) and DMT1 CKO (white bars) mice over postnatal development. * $p<0.05$, ** $p<0.01$

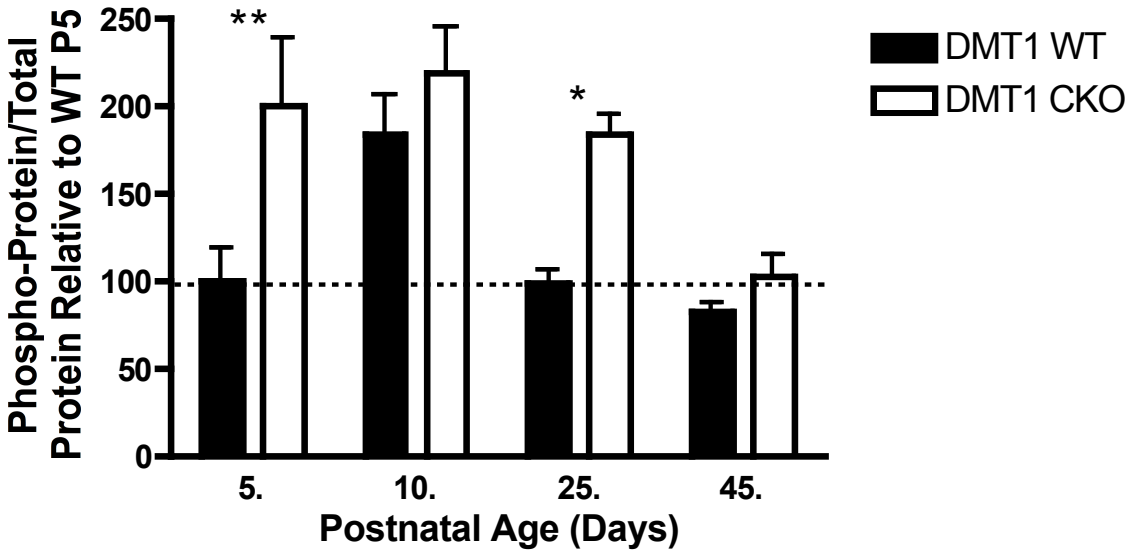

Phospho-Protein/Total Protein Relative to WT P5
250
200
150
100
50
0
**
*
5.
10.
25.
45.
Postnatal Age (Days)
DMT1 WT
DMT1 CKO

Figure 3.3. ID dysregulates mTOR signaling at P25. **A,** Protein phosphorylation of central components relative to total protein and actin of mTOR signaling **B,** Phosphorylation and total protein quantification of insulin signaling protein at P25 **C,** Relative mRNA expression of *ddit* at P25. **D,** Relative phosphorylation of AMPK protein at P25. *p<0.05, **p<0,01, ***p<0.001

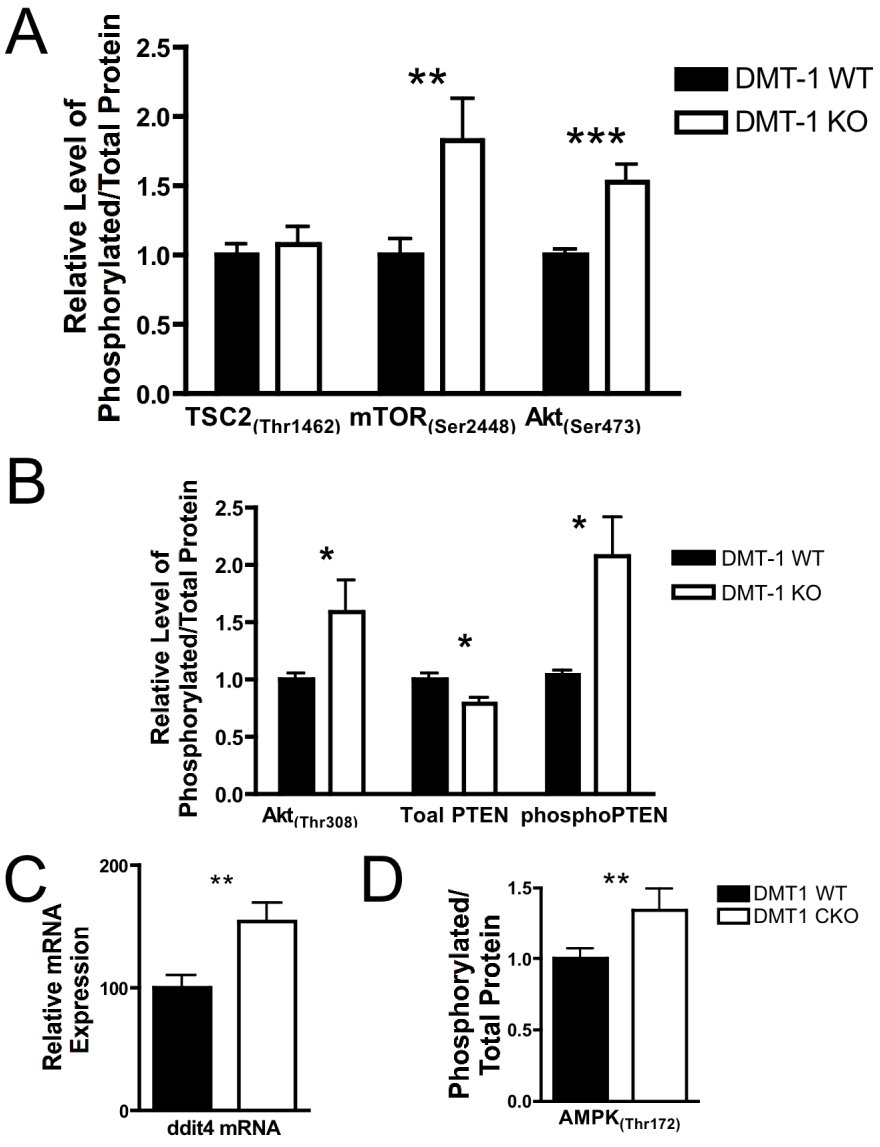
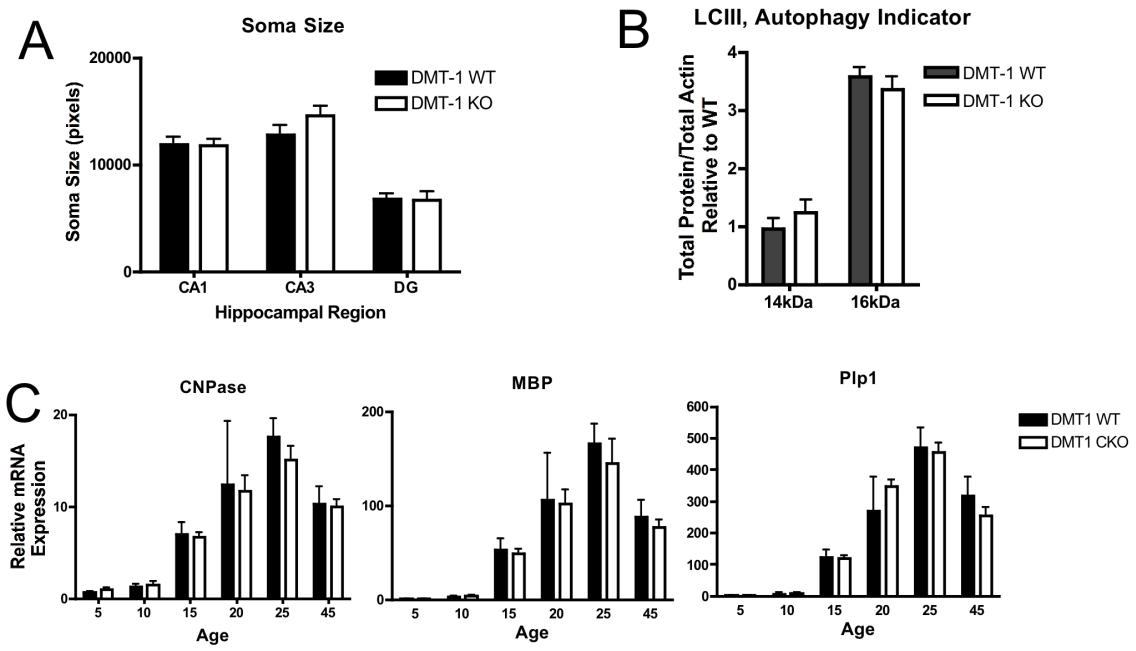

A
Relative Level of Phosphorylated/Total Protein
2.5
2.0
1.5
1.0
0.5
0.0
**
***
DMT-1 WT
DMT-1 KO
TSC2(Thr1462)
mTOR(Ser2448)
Akt(Ser473)
B
Relative Level of Phosphorylated/Total Protein
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
DMT-1 WT
DMT-1 KO
Akt(Thr308)
Toal PTEN
phosphoPTEN
C
Relative mRNA Expression
200
100
0
**
ddit4 mRNA
D
Phosphorylated/ Total Protein
1.5
1.0
0.5
0.0
**
DMT1 WT
DMT1 CKO
AMPK(Thr172)

Figure 3.4. Downstream mTOR effectors are not altered by ID at P25. **A,** Neuronal soma size across hippocampal region **B,** LCIII protein levels in hippocampus at P25 **C,** Developmental expression of mRNA transcripts important for myelination.


A
Soma Size
Soma Size (pixels)
20000
10000
0
CA1
CA3
DG
Hippocampal Region
DMT-1 WT
DMT-1 KO
B
LCIII, Autophagy Indicator
Total Protein/Total Actin Relative to WT
4
3
2
1
0
14kDa
16kDa
DMT-1 WT
DMT-1 KO
C
CNPase
MBP
Plp1
Relative mRNA Expression
Age
5
10
15
20
25
45
DMT1 WT
DMT1 CKO

mTORC1 activity. mTORC2 activity was also increased, measured by Akt(473) phosphorylation (Fig. 3.3*A*).

Positive and negative mTOR regulatory signaling was stimulated by ID at P25.

Insulin stimulated phosphorylation of Akt(Thr308) increased in DMT1 CKO animals compared to WT (Fig. 3.3*B*). In addition, ID reduced PTEN activity and stability, indicated by decreased total PTEN protein and increased PTEN phosphorylation (Fig. 3.3*B*, (Gericke et al., 2006)), which leads to increased Akt stimulation of mTOR. Iron dependent mTOR inputs were also activated in iron deficient DMT1 CKO animals compared to iron sufficient DMT1 WT, including increased *ddit4* expression (Fig. 3.3*C)*, a HIF1α transcriptional target that inhibits mTOR, and AMPK(Thr172) activation (Fig. 3.3*D)*, reflecting low ATP availability. Together, these findings indicated that despite the activation of iron-dependent negative mTOR regulators, the overall phosphorylation patters suggest that ID stimulates mTOR activity.

Downstream mTOR effectors were not altered in DMT1 KO hippocampus.

In order to determine if the alteration in phopshorylation patterns reflected functional outputs, several downstream mTOR effectors were assessed at P25. Protein translation rates were analyzed for mRNA transcripts known to be affected by mTOR activity (eif4, eEF2), transcripts involved in iron homeostasis (TfR-1, FPN, IRP1 and IRP2), and transcripts important for synaptic plasticity (CamKIIα, PSD95, Grin2a, Grin2b). While there were subtle genotype differences in translation rate profiles for some individual transcripts, there was no overall trend suggesting global shifts in protein translation rates in DMT1 CKO hippocampus (data not shown). Neuronal soma size,

which is maintained by mTOR activity (Kwon et al., 2003), was not altered by ID in any hippocampal region at P25 (Fig. 3.4*A*). LCIII protein levels, an indicator of autophagic activity, were not altered in DMT1 CKO hippocampus (Fig. 3.4*B*). In addition, mRNA expression of myelination markers (*MBP, Plp1, CNPase*) were not altered across development in CKO hippocampus (Fig. 3.4*C*). Despite consistent increases in phosphorylation indicative of mTOR activity, the direct downstream effectors assayed here were not obviously altered by ID.

Discussion

In iron sufficient WT animals, developmental activation of mTOR activity between P5-25 completely overlaps the period of peak brain iron uptake, hippocampal growth factor expression, and energy production (Dallman and Schwartz, 1964; Dallman and Spirito, 1977; Taylor and Morgan, 1990; Moos and Morgan, 2000; Siddappa et al., 2002; Erecinska et al., 2004; Tran et al., 2008). This pattern of mTOR activation is consistent with developmental increases in stimulatory growth factors, including insulin/Akt, and Ras/MAPK signaling (Ma et al., 2005; Wullschleger et al., 2006). mTOR activity serves to increase energy production by modulating mitochondrial activity through retrograde signaling (Liu and Butow, 2006; Schieke et al., 2006), and there is limited evidence that mTOR activity increases cellular iron uptake in HeLa cells by increasing surface expression of TfR-1 (Galvez et al., 2007). In this way, increased mTOR activity may serve to enlarge metabolic capacity necessary to respond to stimulation by growth factor signaling. Considering the importance of mTOR signaling

for supporting protein synthesis and actin organization needed for dendritogenesis and synaptogenesis, these observations suggest that mTOR is a central integrator of metabolic signaling during hippocampal development.

Iron is a critical substrate for cellular metabolism. Previous studies in cancer cells and bacteria have found that these rapidly dividing and differentiating cells require proportionately large amounts of iron to support their metabolism. For example, targeted blocking of TfR mediated Tf uptake is considered a viable strategy to "starve and kill" cancer cells (Crepin et al., 2010). Similarly, the role of iron in bacterial multiplication and differentiation was considered to be the reason iron supplementation in malaria endemic areas resulted in increased malarial incidence and virility (Prentice, 2008). The stimulation of mTOR by nutrient availability, growth factors, and oxygen (which are all acknowledged substrates for cellular metabolism) is well established (Wullschleger et al., 2006). The importance of iron as a metabolic substrate suggests that in normal conditions iron could be an additional metabolic regulator of mTOR. The results presented in this chapter examine the specific influence of iron on mTOR in developing neurons.

In the context of ID, there are at least three direct points in the mTOR pathway that ID is likely to affect: 1) BDNF stimulation of Akt activity (Patapoutian and Reichardt, 2001), 2) AMPK activity determined by ATP availability (Hardie, 2007), and 3) HIF1α transcription of *ddit4* (Peyssonnaux et al., 2007). Iron, therefore, likely exerts a complex effect on mTOR signaling, which has multiple feedback and feedforward loops (Wullschleger et al., 2006). Iron chelation studies in COS and myeloid leukemia cells

demonstrate that reduction of intracellular iron inhibits overall mTOR activity (Ndong et al., 2009; Ohyashiki et al., 2009). Additionally, expression of mTOR pathway genes is downregulated by IDA in rats at P15 (Carlson et al., 2007). These observations of mTOR downregulation by ID are consistent with the established impact of iron on these direct mTOR inputs. Furthermore, downregulation of mTOR signaling inhibits dendritogenesis and electrophysiology similarly to observations from early IDA models (Jorgenson et al., 2003; Jaworski et al., 2005; Jorgenson et al., 2005; Kumar et al., 2005; Ehninger et al., 2008). However, because early IDA also induces anemia (i.e., tissue hypoxia which can also influence mTOR signaling), it is not possible to distinguish the specific effect of iron in an early IDA model. Instead, we utilized the DMT1 CKO model of hippocampal neuronal ID to isolate at the effects of iron on mTOR signaling in the hippocampus.

Consistent with the known effects of ID on HIF1α transcriptional activity and ATP production, ddit4 mRNA and AMPK activation are increased by cellular ID at P25. Surprisingly, these iron-dependent effects were not sufficient to inhibit overall mTOR activity, which was increased throughout development by ID. Normative developmental activation of mTOR signaling is extended by cellular ID, with pronounced increases in activity at P25. Activity normalizes in iron deficient hippocampus by P45. Upstream, mTOR stimulation by insulin-Akt activity is also extended by ID at P25, and may mediate the observed increase in mTOR activity.

Despite upregulation of overall mTOR activity, however, downstream consequences of mTOR signaling including overall protein translation, soma size, autophagy, and markers of myelination, are not altered by ID. Therefore, increased

mTOR activity may be stimulated by and effectively compensate for more direct effects of iron on protein synthesis or other downstream processes. Alternatively, there are several genetic human conditions and animal models characterized by neurocognitive impairments that are caused by aberrant mTOR upregulation. Pharmacological inhibition of mTOR signaling in these models can resolve many of the negative effects of mTOR upreglation (Ehninger et al., 2008; Zhou et al., 2009), making it possible that mTOR upregulation is not a beneficial compensation of ID but instead may contribute to the structural and behavioral deficits observed in this model.

The upregulation of mTOR by ID, contrary to previous findings and lack of observed immediate downstream consequences presents the question of how tissue-level ID influences mTOR signaling. One major difference between the DMT-1 CKO model and other models used to examine mTOR signaling, is that the CKO model is less severe and decreases neuronal metabolic activity without creating total body anemia, hypoxia, or genetically ablating components of mTOR signaling. It is possible that, in the absence of additional negative factors common to previous studies, decreased metabolic activity may stimulate mTOR in order to generate energy production. In iron sufficient conditions, increased mTOR would facilitate energy production to meet the demands of hippocampal growth by increasing metabolic output and mitochondriogenesis as well as facilitating iron uptake (Liu and Butow, 2006; Cunningham et al., 2007; Galvez et al., 2007). In conditions of ID however, the cell may not be able to successfully increase metabolic activity in order to support the consequences of mTOR upregulation. This lack of balance between supply and demand created by increased mTOR activity concurrent with

ID could then impair neuronal differentiation and dendrite arborization. This impairment of structural development during early ID, particularly during the period of rapid hippocampal development, likely contributes to the persistent cognitive deficits which remain following iron repletion.

Ultimately, these findings highlight the interrelationship of cell growth, metabolic need, iron uptake, and the maintenance of optimal mTOR signaling as an important aspect of understanding the role of iron in the developing brain.

Chapter 4

mTOR Activity Responds to Cellular Iron Status and Contributes to Long-Term Hippocampal Behavioral Outcomes

Introduction

The hippocampus undergoes a period of rapid structural and functional development in humans (birth-2 years) and rodents (postnatal day (P)10-25). During this period of rapid growth, neurons establish complex dendritic arbors and form synaptic connections (Pokorny and Yamamoto, 1981b, a). Protein synthesis and actin organization are required to generate and maintain the cytoskeletal structure required for dendrite arbors and synapse formation. Supporting the demands of dendritogenesis and synaptogenesis requires sufficient substrates for protein synthesis (ie amino acids and ribosomes) and sustained energy production (oxygen, mitochondria, glucose, iron) (Dallman, 1986; Pollard et al., 2000). In addition, neurotrophic growth factors and guidance cues are necessary to stimulate dendrite growth and synapse formation. In the rodent hippocampus, the activity of all of these factors is increased during rapid postnatal growth (Dallman and Schwartz, 1964; Taylor and Morgan, 1990; Siddappa et al., 2002; Erecinska et al., 2004; Tran et al., 2008). Reduction of cellular metabolism during hippocampal development, through hypoxia or iron deficiency (ID), results in structural abnormalities including reduced dendrite complexity and altered spine morphology (Pokorny and Trojan, 1986; Brunette et al., 2010).

Together, these observations demonstrate that precise regulation of cellular metabolism is an essential requirement for hippocampal development. Mammalian target of rapamycin (mTOR) is an important signaling pathway that integrates metabolic demand (growth factor stimulation) and metabolic supply (oxygen, branch chain amino acid availability, energy status) in order to support cell growth and morphology

(Wullschleger et al., 2006). mTOR activity stimulates protein synthesis and actin organization and also increases metabolic activity by increasing mitochondrial gene expression, oxygen consumption and increasing iron uptake (Cunningham et al., 2007; Galvez et al., 2007). Through this coordinated regulation, mTOR activity not only promotes cell growth, but also modulates the metabolic activity required to support it.

Recently developed genetic models of hippocampal neuronal ID by our group have laid a foundation for assessing mTOR activity in ID. First, the divalent metal transporter1 conditional knock out (DMT1 CKO) model, which permanently disrupts *Slc11a2* (DMT1 gene, an iron transporter) expression in hippocampal neurons, demonstrates that neuronal ID upregulates mTOR signaling (Chapter 3), extending the period of developmental mTOR activation compared to iron sufficient animals. This sustained activation of mTOR extends throughout the peak of rapid hippocampal development. Second, the dominant negative (DN) model induces reversible ID in CA1 hippocampal pyramidal neurons through overexpression of a dominant negative non-functional transferrin receptor-1 (dnTfR-1, Chapter 2). Manipulation of the timing of iron repletion in this model identified a critical requirement for iron between P21-P42 to ensure appropriate hippocampal development. Iron repletion at P21, near the end of peak hippocampal development and during extended mTOR activity in the DMT1 CKO hippocampus, prevents long-term behavioral and structural deficits. These findings suggest that mTOR activity could be related to the ability of P21 repletion to prevent long-term deficits.

Sustained upregulation of mTOR activity during hippocampal development could be beneficial or detrimental to long-term outcomes in the context of ID. mTOR activity stimulates protein synthesis, increases metabolic activity and corresponds to increased transferrin (Tf) uptake (Liu and Butow, 2006; Wullschleger et al., 2006; Galvez et al., 2007). Therefore increased activity may compensate for direct effects of ID on protein synthesis/stability or actin organization, or even moderate ID by stimulating iron uptake and increasing metabolic activity. However, there are many genetic disorders and animal models characterized by increased mTOR activity which result in cognitive, structural, and electrophysiological impairments. Many of these impairments can be improved or even rescued through pharmacological normalization of mTOR signaling (Ehninger et al., 2008; Meikle et al., 2008; Zhou et al., 2009). Therefore, it is possible that ID-induced increased mTOR signaling during development could contribute to rather than ameliorate the structural and behavioral deficits which persist following iron repletion (Felt and Lozoff, 1996; Jorgenson et al., 2003).

Using two unique genetic models of cellular ID the relationship between iron and mTOR signaling can be explored. The data presented here first shows that ongoing ID in the DN model dysregulates mTOR signaling in CA1, confirming findings from the DMT1 CKO model (Chapter 3). Additionally, mTOR activity was reduced by iron repletion at P21 in DN animals. The functional importance of mTOR responsiveness to iron availability during hippocampal development to long-term behavioral outcomes was then examined by pharmacologically decreasing mTOR activity using rapamycin treatment between P10-42 in the iron deficient DMT1 CKO.

Methods

Animals

All experiments were performed in accordance with the NRC's Guide for Care and Use of Laboratory Mice, and with approval of the Institutional Animal Care and Use Committee of the University of Minnesota. Mice were housed in RAR facilities in a 12 light 12 dark cycle.

DN Model. Animals were generated and euthanized as described in Chapter 2. Male and female hippocampal region CA1 was isolated and collected from WT^{nodox} and DN^{nodox} animals at P21, P30, and P42, and from WT^{P21dox} and DN^{P21dox} animals at P30 and P42. Due to the restriction of ID to CA1 region neurons in the DN model (Chapter 2), CA1 tissue was microdissected from freshly removed brains by cutting thin sections with a razor blade and isolating CA1 using a dissection microscope and two fine gauge needles. Tissue was immediately sonicated in cytoskeletal buffer, aliquoted and stored at -80°C until use.

DMT Model. Animals were generated as described in Chapter 3. 6 mg/kg rapamycin or vehicle was delivered via i.p. injection every other day beginning at P10-12 and continuing through P40-42. Rapamycin was dissolved in 100% EtOH and diluted in 5% PEG and 5% Tween-80 as previously described (Meikle et al., 2008). Tissue was collected as described in Chapter 3 from male and female vehicle and rapamycin treated animals at P25, and from male and female adult (P60-P90) animals following behavioral testing.

Experimental Procedures

Behavioral Assays. An enabled Morris water maze (EMWM) was utilized as previously described (Carlson et al., 2009). Males and females were tested separately. Briefly, the animals were first habituated to handling, and then observed in an open field as previously described (Sun et al., 2007). Mice were then habituated to the water maze, and the following day were given 4, 1 minute, visual cued task (VCT) trials. In the VCT the animals could escape from swimming onto a 10 cm diameter platform with a visually salient flag attached, the animals were allowed to swim for one minute or until they escaped to the platform. On each of the next 5 days the animals were given 6, 90 second training trials, followed by a 30 second probe trial. In the EMWM trials, the platform location was fixed in the center of a predetermined target quadrant, however, to make the task less difficult the size of the platform changed across trials. During the first two days of training (training trials 1-12), trials 1-4 used a 20 cm diameter platform, trials 5-8 used a 15 cm platform, and trials 9-12 used a 10 cm diameter platform. For the remaining 3 days of training, the first two daily trials used the 20 cm platform, followed by two 15 cm platform trials, and finally two 10 cm platform trials. During probe trials, the platform was removed from the water and the animal was allowed to swim for 30 seconds before being removed by the experimenter. Video was captured and analyzed using Topscan (Clever Systems, Reston, VA).

Western Blot. Western blot analysis was performed as described in Chapter 3 using 20ug of total protein.

Hematocrit. Blood collected at euthanization was used to measure hematocrit as described in Chapter 2.

Data Analysis

Analysis of Variance, α=0.05, with Bonferroni post-hoc analysis, was used to compare demographic and behavioral data. Student t-tests were used to compare individual proteins at P21 and P42.

Results

mTOR signaling is disrupted in the DN model of hippocampal ID

Neither genotype nor doxycycline treatment affected body weight, total body iron status, or brain/body weight ratios (Table 4.1). Phosphorylation of several key mTOR proteins including S6K(Thr389), Akt(Ser473) and S6(Ser235/236) was increased at P21 in DN^{nodox} animals compared to WT^{nodox} (Fig. 4.1*A*, see Fig 3.1 for pathway reference) In addition, total mTOR and S6 protein was also increased in DN^{nodox} CA1 (Fig. 4.1*B*). The increase in active phosphorylation suggested increased mTOR activity resulting from cellular ID, consistent with results from DMT1 CKO (Chapter 3). To examine if increased active phosphorylation states are responsive to iron repletion, doxycycline treatment at P21 was used to restore normal iron status and phosphorylation was then examined at P42 in DN^{P21dox} CA1. Iron repletion normalized phosphorylation of S6K(Thr389), Akt(Thr308) and S6(Ser235/236) by P42 in DN^{P21dox} CA1 compared to DN^{nodox} CA1 (Fig. 4.2).

Table 4.1 Genotype and Doxycyline have no Effect on Body Weight, Brain/Body Weight Ratio, or Percentage of Red Blood Cells (values are mean±SD)

Postnatal Age, Genotype, and Treatment		Body Weight (g)	Brain/Body Weight (%)	Hematocrit (%)
P21	WT (n=15)	9.7 ± 0.9	4.2±0.3	38.6±3.6
	DN (n=4)	9.1 ± 1.2	4.3±0.2	38.1±1.7
P30	WT (n=8)	17.9 ± 1.6	2.4±0.2	39.8±4.8
	DN (n=6)	16.1 ±1.3	2.6±0.1	41.9±1.5
$P30^{P21dox}$	WT (n=9)	16.2±1.8	2.6±-.3	40.5±1.7
	DN (n=3)	14.5±4.1	2.5±0.4	39.5±2.8
P42	WT (n=4)	20.2±3.1	2.2±0.5	48.6±1.7
	DN (n=4)	20.4 ±3.2	2.1±0.2	45.8±1.9
$P42^{P21dox}$	WT (n=15)	22.3±3.4	2.1±0.3	44.0±4.9
	DN (n=4)	19.3±1.3	2.3±0.2	42.6±3.4

Figure 4.1. mTOR signaling is dysregulated at P21 in DN^{nodox} CA1. Quantification of **A,** phospho protein and **B,** total protein relative to actin from WT^{nodox} and DN^{nodox} CA1. *$p<0.05$

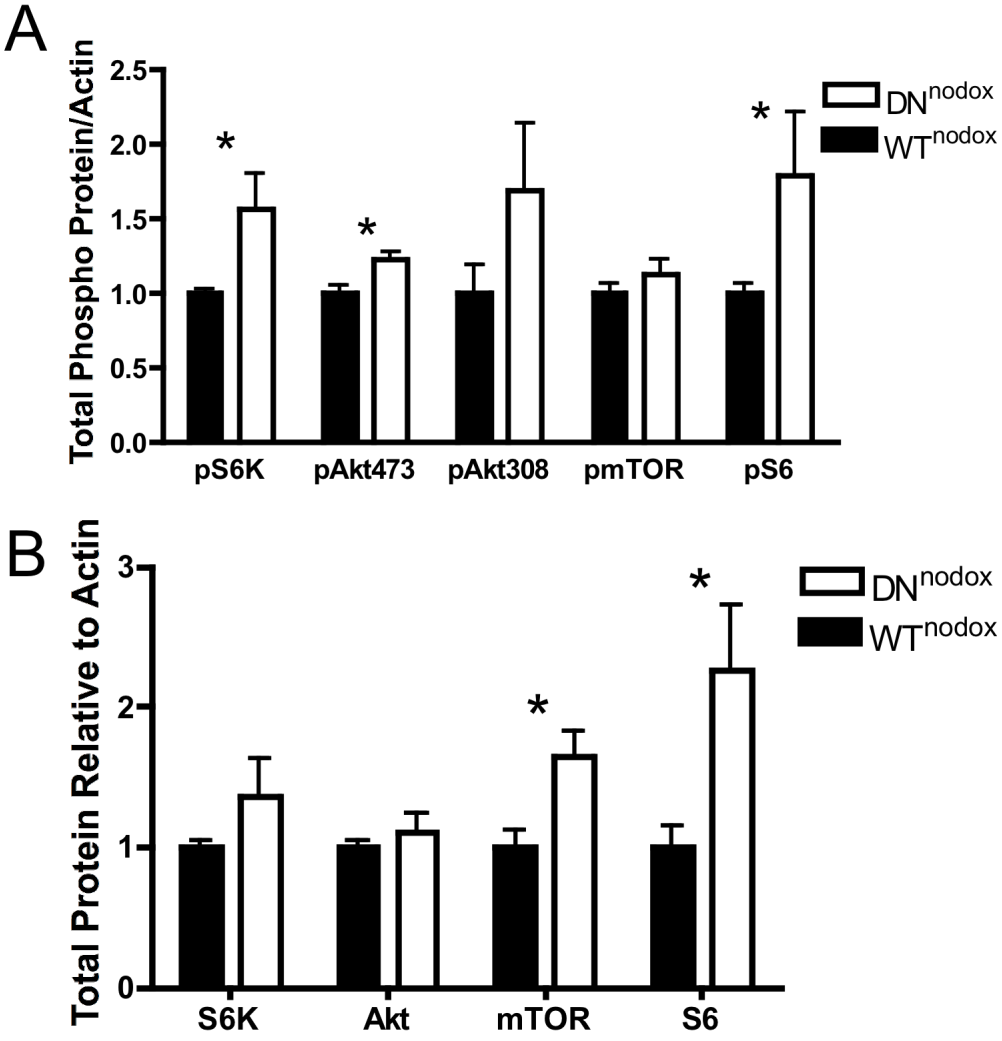
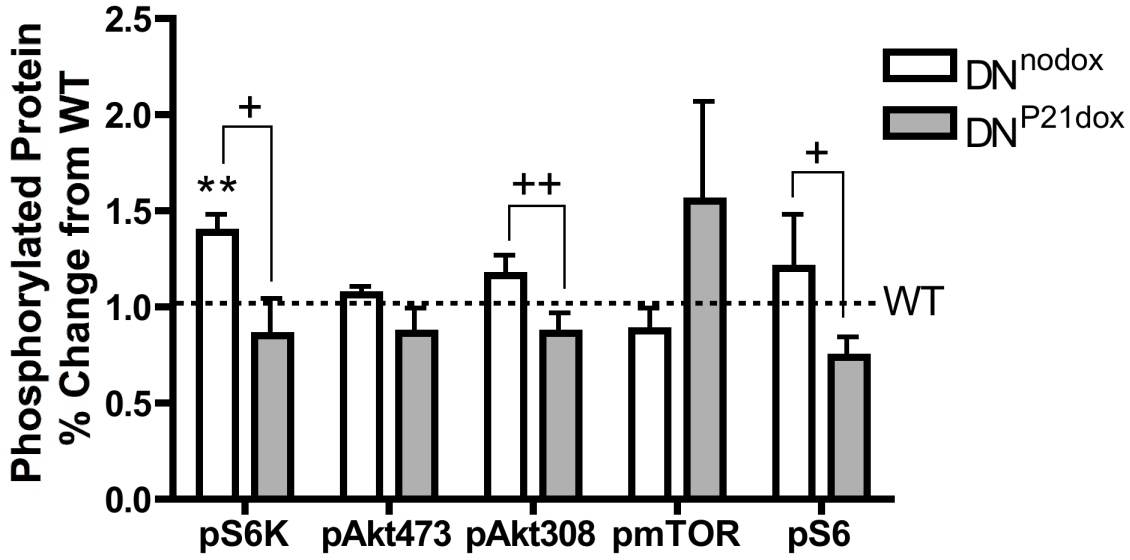

A
Total Phospho Protein/Actin
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
DN^nodox
WT^nodox
pS6K
pAkt473
pAkt308
pmTOR
pS6
B
Total Protein Relative to Actin
3
2
1
0
*
*
DN^nodox
WT^nodox
S6K
Akt
mTOR
S6

Figure 4.2. mTOR phosphorylation responds to iron repletion. White bars represent percent change of phosphorylated protein in iron deficient P42 DN^{nodox} compared to WT^{nodox} animals (dashed line), **$p<0.01$. Dark bars represent percent change of phosphorylated protien in iron repleted CA1 from P42 DN^{P21dox} animals compared to WT^{P21dox} animals (dashed line). +$p<0.1$, ++$p<0.05$ indicates differences in phosphorylation between iron deficient DN^{nodox} and iron replete DN^{P21dox} groups.


Phosphorylated Protein
% Change from WT
2.5
2.0
1.5
1.0
0.5
0.0
**
+
++
+
WT
DN nodox
DN P21dox
pS6K
pAkt473
pAkt308
pmTOR
pS6

Pharmacological inhibition of mTOR activity by rapamycyin between P10-42 improved behavioral outcomes in females.

Rapamycin treatment altered total body growth over development (Fig. 4.3), but brain growth was spared relative to body weight indicated by increased brain/body weight ratios in rapamycin treated animals (Table 4.2). There was no effect of genotype or treatment on total body iron status measured by hematocrit (Table 4.2). In order to verify that rapamycin treatment inhibited mTOR activity in the hippocampus, S6K(Thre389) phosphorylation was assessed. Consistent with previous findings from the DMT1 CKO, mTOR activity is increased in vehicle treated DMT1 CKO animals compared to vehicle treated DMT1 WT animals (Fig. 4.4). Rapamycin reduced S6K(Thr389) phosphorylation in both DMT1 WT and CKO mice (Fig. 4.4, n=7, $F=14.77$, $p<0.001$).

Vehicle treated DMT1 WT and CKO females both spent increasing amounts of time searching in the target quadrant across consecutive probe trials. However, DMT1 CKO mice had a slower improvement and did not reach WT performance levels, consistent with previous findings (Fig. 4.5*A*, solid bars, n=4-6, $F=3.227$, $p=0.08$). Rapamycin treatment impaired the ability of DMT1 WT females to learn the task compared to vehicle treated WT, (Fig. 4.5*A*, dark hatched bars, n=4, $F=10.31$, $p=0.06$). Rapamycin treatment in DMT1 CKO animals however, increased the percentage of time spent searching in the target quadrant compared to vehicle treated DMT1 CKO animals (Fig. 4.5*A*, light hatched bars, n=6-7, $F=2.868$, $p=0.1$). Male, vehicle treated DMT1 CKO and WT animals did not show any differences in performance on the maze (Fig.

4.5*B*, solid bars), making the interpretation any rapamycin findings difficult (Fig. 4.5*B,* hatched bars). Gender, genotype, and rapamycin treatment did not impair escape latencies on VCT (data not shown). Furthermore, there were no gender, genotype or treatment effects on open field measurements including time spent in center of field, total distance travelled (data not shown). In summary, rapamycin administration between P10-P42 effectively reduces mTOR signaling in the hippocampus and results in modest spatial memory behavior improvement in iron deficient adult female DMT1 CKO mice.

Discussion

mTOR is an important mediator of neuronal growth and function. mTOR signaling is altered by ID, and may play a role in the long-term consequences of early life ID. However, the relationship between iron and mTOR is complex. *In vitro* studies have shown that iron chelation suppresses mTOR activity (Ndong et al., 2009; Ohyashiki et al., 2009), consistent with mTOR inhibition by reduced energy availability and increased oxidative stress. *In vivo* evidence supports an inhibitory relationship between ID and mTOR signaling. Iron deficiency anemia (IDA) in rats suppresses hippocampal expression of genes in the mTOR pathway and reduces mTOR protein phosphorylation in total brain lysates (Carlson et al., 2007; Ndong et al., 2009). However, it has also been demonstrated *in vitro* that mTOR activity can stimulate iron uptake (Galvez et al., 2007), consistent with mTOR increasing metabolic activity to support cell growth. In a genetic mouse model, *in vivo* cellular ID increases hippocampal mTOR activity (Chapter 3),

Figure 4.3. Rapamycin inhibits growth (weight gain, g) over postnatal development.

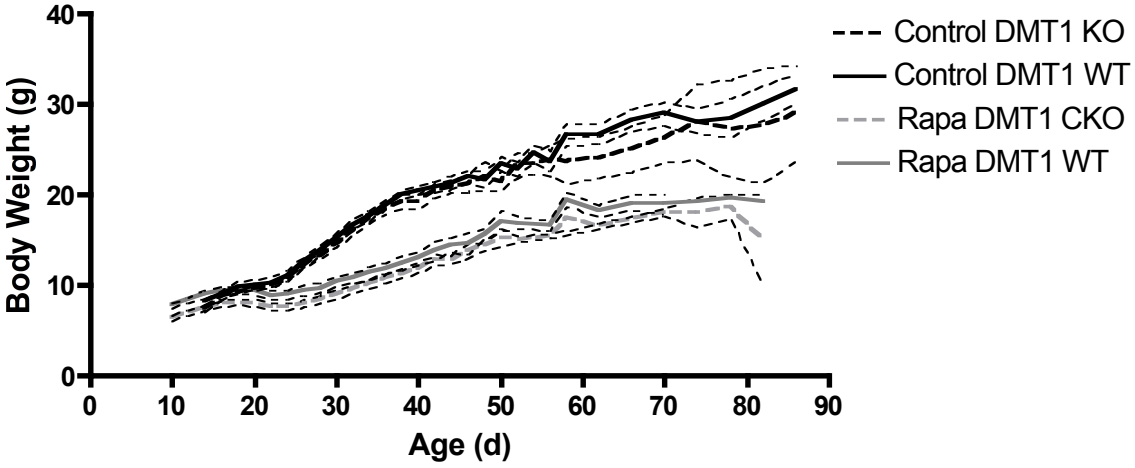

Body Weight (g)
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
Age (d)
Control DMT1 KO
Control DMT1 WT
Rapa DMT1 CKO
Rapa DMT1 WT

Table 4.2 Rapamycin Treatment and Genotype do not Affect Brain/Body Weight Ratio or Percentage of Red Blood Cells (values are mean±SD)

Postnatal Age and Genotype		Brain/Body Weight Ratio (%)		Hematocrit (%)	
		Vehicle	Rapamycin	Vehicle	Rapamycin
P25	DMT1 WT	3.0±0.4	3.1±0.3	40.5±1.8	44.8±2.3
	DMT1 CKO	3.0±0.4	3.5±0.3	41.3±1.5	44.7±1.7
Adult	DMT1 WT	1.6±0.2	1.8±0.2	47.5±1.0	51.2±2.4
	DMT1 KO	1.8±0.3	1.9±0.2	48.7±1.6	49.7±2.6

Figure 4.4. Rapamycin inhibits S6K(Thr389) phosphorylation. Quantification of phosphorylation in vehicle and rapamycin treated DMT1 WT and CKO animals at P25. + $p<0.1$, ** $p<0.01$

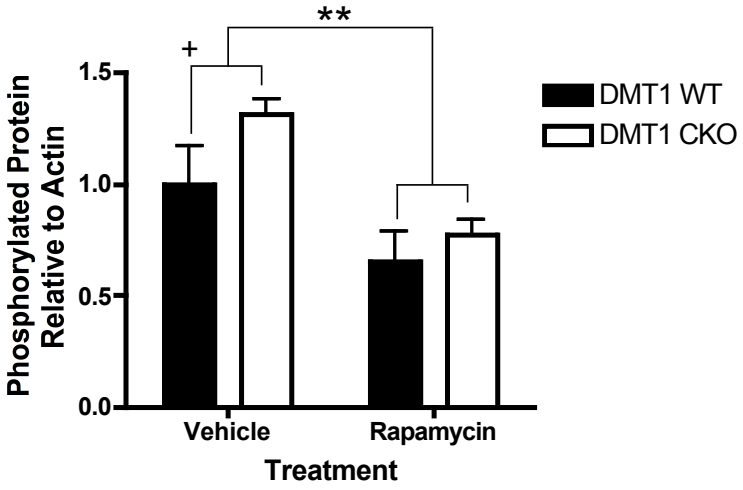
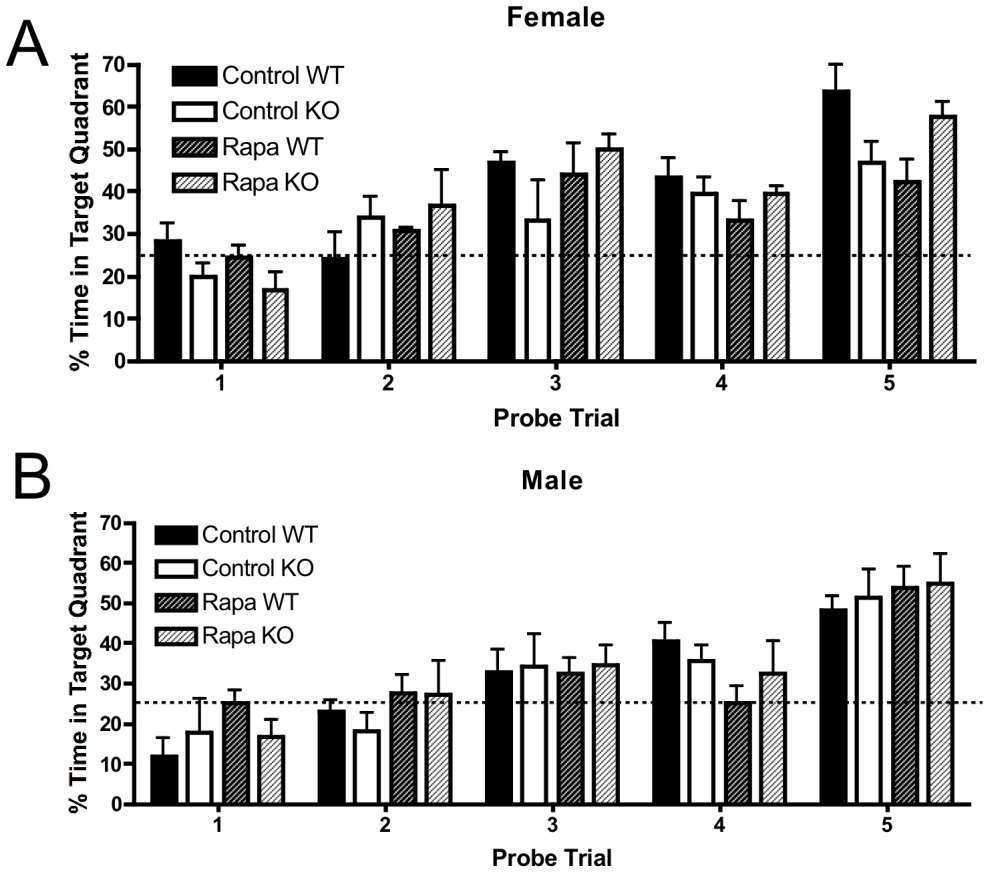

**
+
1.5
1.0
0.5
0.0
Phosphorylated Protein
Relative to Actin
DMT1 WT
DMT1 CKO
Vehicle
Rapamycin
Treatment

Figure 4.5. Rapamycin treatment during development affects spatial memory behavior in female DMT1 CKO mice. Percentage time spent searching in target quadrant during probe trials in EMWM for Vehicle treated (solid bars) and rapamycin treated (hatched bars) **A,** female mice and **B,** male mice.


A
Female
Control WT
Control KO
Rapa WT
Rapa KO
% Time in Target Quadrant
0
10
20
30
40
50
60
70
1
2
3
4
5
Probe Trial
B
Male
Control WT
Control KO
Rapa WT
Rapa KO
% Time in Target Quadrant
0
10
20
30
40
50
60
70
1
2
3
4
5
Probe Trial

which may reflect a compensatory mechanism to increase iron uptake. In the experiments presented here, iron and mTOR signaling were independently manipulated during development to examine both the impact of cellular iron status on mTOR activity and the functional significance of mTOR dysregulation during hippocampal development.

In the repletable DN model of ID, mTOR activity was increased in CA1 by neuronal ID, confirming findings from the permanent DMT1 CKO model (Chapter 3). mTOR activity normalizes following iron repletion beginning at P21, demonstrating that mTOR activity is responsive to cellular iron status. Galvez and colleagues (2007) demonstrated that mTOR activity stimulates iron uptake, and these findings suggest a bi-directional relationship, through which increased iron demand resulting from ID may stimulate mTOR activity. Restoration of mTOR activity following iron repletion could therefore reflect reduced iron demand.

The functional impact of the interaction between cellular iron homeostasis and mTOR on hippocampal development is not clear. It is possible that mTOR activation by ID could be beneficial—providing compensation for reduced energy and protein synthesis caused by ID, or harmful—taxing the cell beyond what it can metabolically support during this important window in development, causing more stress and contributing to lasting structural and behavioral deficits.

Reduction of mTOR activity from P10-42 using rapamycin improved spatial memory behavior in female DMT1 KO animals, despite ongoing ID. P10-P42 encompasses the most rapid period of hippocampal growth, iron uptake, and the

developmental increase in mTOR activity. Although the beneficial effects of normalizing mTOR signaling with rapamycin were only observed in female mice, these preliminary findings indicate that decreasing mTOR activity and preventing prolonged mTOR activation during development may be protective for long-term outcomes. mTOR dysregulation resulting from early ID is therefore detrimental, not compensatory, for the establishment of hippocampal circuitry, suggesting that optimal mTOR activity is fundamental for successful hippocampal development.

In conditions of ID, oxidative stress and hypoxia inducible factor 1α (HIF1α) activity are increased and energy production is decreased (Bianchi et al., 1999; Lee and Andersen, 2006), which have been observed in dietary and genetic models of early ID (Rao et al., 2003; Carlson et al., 2007; Carlson et al., 2009). These well-documented effects of reduced iron availability are known to suppress mTOR activity, contrary to our observations of increased mTOR activity during cellular ID. It is possible to speculate then, that in a tissue specific, cellular ID context, reduced energy availability and/or increased iron demand stimulates mTOR activity. This ID-induced mTOR activation could be detrimental because the iron deficient cell may not be able to support the consequences of increased mTOR activity, including increased protein synthesis and metabolic activity. Exuberant mTOR activity and its attendant metabolic demands on differentiating cells during this important window of rapid hippocampal growth may contribute to impaired structural and functional development.

It is possible, therefore, to postulate that restoration of mTOR activity may enable iron repletion at P21, well beyond the normal period for mTOR activation and peak

dendritogenesis, to successfully rescue behavioral and structural deficits in the DN model. The recovery of deficits following P21 repletion, suggests that early ID may extend the window of development, so that upon iron repletion, even late in development, the neurons are able to complete dendrite arborization and synaptogenesis. Normalization of mTOR signaling may facilitate this developmental extension by restoring sustainable metabolic supply and demand. mTOR activation is developmentally quiescent in both iron sufficient and iron deficient animals by P42, when iron repletion is no longer sufficient to rescue long-term outcomes (Chapter 3). This observation suggests that mTOR, in part, may regulate a definitive window during which iron repletion can restore the developmental processes involved in hippocampal dendritogenesis and synaptogenesis.

What is currently known about mTOR was discovered using very broad and often severe tools such as genetic ablation of mTOR pathway proteins, iron chelation, total body IDA, or hypoxia. This is the first time that it has been possible to examine the effect of subtle neurometabolic manipulation on mTOR activity. These *in vivo* findings suggest additional complexity for the regulation of mTOR activity and provide avenues for further investigation of the relationship between mTOR and cellular metabolism.

Chapter 5

Concluding remarks and future directions

Early life iron deficiency (ID) is a significant problem in humans that results in acute and long-term learning and memory deficits, indicating abnormal hippocampal function. The cellular mechanisms mediating both the acute and persistent cognitive deficits resulting from ID have been largely unknown. However, one repeated observation from multiple studies in animal models is that early ID induces significant structural abnormalities in pyramidal cell neurons of the hippocampus and a reduced overall hippocampal volume (Jorgenson et al., 2003; Ranade et al., 2008; Carlson et al., 2009; Brunette et al., 2010). It is not unreasonable to postulate that these gross and microscopic structural abnormalities have a significant role in mediating the concomitant behavioral and electrophysiological deficits seen in these models of early ID (Jorgenson et al., 2005). The overall goal my research was to understand how lack of iron causes these structural deficits. My unique contribution to iron deficiency and neurodevelopmental research is the identification of mammalian target of rapamycin (mTOR) as a candidate signaling pathway through which iron regulates neuronal structural development.

The experiments reported here demonstrated that mTOR signaling is dysregulated by neuronal ID during the same time period that rapid hippocampal development requires large amounts of iron. Previous genetic and pharmacologic studies established that normal mTOR activity is required for dendrite branching and complexity *in vitro* (Jaworski et al., 2005; Kumar et al., 2005), and that conditions that result in increased hippocampal mTOR activity are characterized by *in vivo* structural and functional (i.e., learning and memory) abnormalities (Kwon et al., 2003; Ehninger et al.,

2008; Zhou et al., 2009). Similarly, ID-induced upregulation of mTOR resulted in hippocampal structural, functional, and cognitive abnormalities comparable to those observed in the genetic models with mTOR upregulation (Chapters 2-4). The experiments demonstrating rescue of behavioral outcomes in adult animals following restoration of mTOR signaling (through either timely iron repletion or pharmacological suppression) provide functional evidence for a connection between mTOR and the long-term structural and cognitive effects of ID. Therefore, I conclude that mTOR is a likely cellular mechanism through which the metabolic effects of ID are translated into impaired neuronal development.

These findings are among the first to establish the responsiveness of neuronal mTOR signaling to cellular iron status during development. The mechanisms (i.e., the exact entry points into the mTOR pathway) which mediate this responsiveness are unknown but there are several likely ways ID might affect mTOR regulation, including reduced growth factor signaling, HIF1α activation, and activation of AMPK by low ATP availability (Lee et al., 2006; Hardie, 2007; Tran et al., 2008) (see Fig. 3.1). It is possible that ID might also influence mTOR through additional known or unknown regulatory signals, however, the most likely avenue connecting cellular iron status and mTOR is via the effects of ID on mitochondrial enzymes essential for energy production (Dallman, 1986). Reduced energy production resulting from ID would impair the ability of mTOR to successfully integrate cellular metabolism to provide optimal outputs.

Until now, the influence of cellular metabolism on the regulation of signaling pathways like mTOR has only been explored using drastic metabolic insults such as

hypoxia-ischemia, iron deficiency anemia (IDA), or glucose deprivation (Ndong et al., 2009; Carloni et al., 2010; Jezek et al., 2010). The general conclusion of previous findings is that conditions which suppress energy production and metabolic activity also suppress mTOR signaling. However, the models of restricted cellular ID utilized in the current studies are relatively mild manipulations of cellular energy production. Increased mTOR signaling in response to cellular ID demonstrates that the relationship between cellular metabolism and mTOR is not as straightforward as previous studies have indicated. Therefore it is possible, particularly during periods of rapid growth, that mTOR activity responds differentially to varying cellular metabolic activity and demand.

The regulatory relationship between neuronal metabolic activity and mTOR activity could be addressed in future studies using *in vitro* approaches in primary neuronal culture. Pharmacological agents make it is possible to independently manipulate signaling pathways that affect mTOR signaling (such as AMPK, HIF1α and PI3K) while simultaneously altering metabolic activity by varying oxygen, glucose, iron, or even neuronal activity. Such an approach to dissecting the regulation of mTOR signaling could provide molecular explanations for the discrepancies between the stimulatory effect of cellular ID and the inhibitory effect of total body IDA on mTOR signaling. A likely explanation for the differential response of mTOR to IDA (Carlson et al., 2007) and ID without anemia (Chapters 3-4) is the presence of tissue hypoxia in addition to ID in the former. Hypoxia may influence more regulatory entry points than ID alone, thus changing the balance of signaling with the pathway.

During hippocampal development, peak energy utilization and production, and increased iron uptake and storage utilization converge at the time when pyramidal cell dendrites are undergoing extensive arborization. Here, for the first time, it is clear that developmental mTOR activation coincides with these critical and interrelated cellular processes. Together, these observations indicate that mTOR signaling has an integral role in coordinating metabolic supply and demand required to support neuronal differentiation, as such, its optimal regulation is crucial for supporting successful development. In the instance of ID, the coordination of these developmental processes is disrupted, altering gene expression, neuronal structure, electrophysiological plasticity, and ultimately behavior. Future work should address how the regulatory relationship between mTOR and iron affects fundamental developmental processes.

In addition to the acute effects of ID on mTOR signaling, recent studies have demonstrated acute and lasting consequences of early ID on the expression of genes involved in establishing and maintaining neuronal structure such as BDNF (Tran et al., 2009). A potential mechanism through which these alterations in BDNF might act in concert or individually with dysregulation of mTOR signaling by ID is illustrated through the successful rescue of behavioral and structural impairments following iron repletion at P21 in the DN model. P21 is remarkably late in the traditional window of hippocampal apical dendrite differentiation (Pokorny and Yamamoto, 1981b, a), and thus it was surprising to observe such a robust recovery in animals treated at that time. These data suggest the possibility that early life neuronal ID adaptively extends or delays the primary period of dendrite arborization and synaptogenesis, enabling cells to complete

dendritogenesis if iron becomes available. Our group and others have shown that ID is capable of delaying aspects of neurodevelopment such as trace conditioning (Gewirtz et al., 2008) and eye opening (Beard et al., 2006). Such a delay in hippocampal development may provide a functional adaptation for the developing system wherein it would allow the system to retain plasticity at unexpectedly late times, thereby resulting in no or minimal long-term impairment. Thus, if the substrate is provided within an appropriate and, perhaps slightly extended period, allows for recovery even beyond traditional periods of development.

Recently, significant progress has been made in understanding mechanisms that regulate critical periods, particularly in the visual cortex. The balance of inhibitory and excitatory activity is a hallmark of critical periods, and the onset and duration of critical periods have been attributed to the maturation of parvalbumin positive GABA interneurons and the formation of extracellular perineuronal nets (Hensch, 2005). A recent study provides evidence that at P12 early IDA reduces parvalbumin mRNA expression in whole brain, suggestive of delayed GABA-ergic development (Bastian et al., 2010). In the visual cortex, BDNF has been shown to promote interneuron development, and chronic increases in BDNF expression result in precocious critical periods, whereas chronic reduction of BDNF reduces inhibitory synaptic transmission and impairs long term potentiation (Hanover et al., 1999; Huang et al., 1999; Abidin et al., 2008). Early IDA reduces developmental BDNF expression in both total brain and hippocampus (Tran et al., 2008; Bastian et al., 2010). In light of evidence from the visual

cortex, reductions in BDNF may mediate delayed hippocampal development resulting from early ID.

mTOR has recently been implicated in the differentiation and maturation of oligodendrocytes (Tyler et al., 2009). While it is unknown if mTOR has a role in interneuron maturation, it is not unlikely considering that BDNF can stimulate mTOR signaling. It is possible that the effects of ID on mTOR signaling and BDNF gene expression work in concert to delay hippocampal development. Alternatively, prolonged mTOR activation caused by cellular ID could reflect delayed interneuron maturation and subsequent excitatory/inhibitory imbalance caused by reduced BDNF. In order to determine the mechanisms underlying delayed development in ID, future studies could utilize the DN model alongside pharmacological treatments to independently manipulate BDNF, iron status, and mTOR activity and examine critical period markers such as perineuronal nets and parvalbumin expression.

In conclusion, the findings presented here demonstrate that mTOR is a mechanistic link between the acute iron-dependent effects of early ID and the hippocampal abnormalities that mediate long-term behavioral and cognitive deficits following iron repletion.

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