Published in final edited form as:

Neuroscience. 2014 March 28; 263: 54–59. doi:10.1016/j.neuroscience.2013.12.056.

Diurnal Variations in Brain Iron Concentrations in BXD RI Mice

Erica L. Unger², Byron C. Jones¹, Laura E Bianco², Richard P. Allen³, and Christopher J. Earley³

¹Department of Biobehavioral Health, The Pennsylvania State University, University Park, PA, 16802

²Department of Nutritional Sciences, The Pennsylvania State University, University Park, PA, 16802

³Johns Hopkins University School of Medicine, Baltimore, MD, USA

Abstract

Under normal and dietary iron deficiency conditions, the BXD recombinant inbred (RI) strains of mice show large variations in regional brain iron concentration, particularly in the ventral midbrain (VMB). In a study utilizing just one of the BXD strains, diurnal changes in subregional brain iron concentration were found, which were dependent on the brain region and sex of the mice. The focus of this study was to determine if diurnal changes in VMB can be found across other BXD RI strains and whether a diurnal effect would be common to all strains or variable across strains similar to the large strain variability in iron concentrations determined during the first part of the light phase. Eight RI (BXD type) strains of mice of both sexes were selected for this study. Mice were sacrificed at postnatal day 120: half in the light phase (LP) and half in the dark phase (DP) of the light-dark cycle. Iron concentrations were determined in VMB, which was the primary region of interest, and five other brain regions. Exploratory analysis was also done on liver and spleen iron concentrations to assess for diurnal changes. Three strains showed clear diurnal variation in iron in the VMB and the others strains showed diurnal variations in other regions. These changes were not equally apparent in both sexes. Exploratory analysis also found strain $\times$ sex dependent diurnal differences in spleen and liver iron. In conclusion, significant brain-regional-specific diurnal changes in total iron concentrations were found in a selection of BXD RI mice. Sex and strain are functional determinates of which regions will be affected and in what direction the affect will be. The study provides an animal model for future work into determining the biological and genetic basis of circadian influences on VMB iron homeostasis.

Keywords

diurnal; liver; spleen; brain; ventral midbrain; iron

© 2014 IBRO. Published by Elsevier Ltd. All rights reserved.

Address correspondences to: Christopher J Earley, Johns Hopkins Bayview Medical Center, 5501 Hopkins Bayview Circle, Room 1B-82, Baltimore, Maryland 21224, USA. TEL: 410-550-0572; FAX: 410-550-3364. cearley@jhmi.edu.

The authors have no conflicts of interest to declare.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

In rodents and humans, several studies have shown circadian or diurnal variations of iron and its associated proteins in peripheral tissue or serum (Lynch et al., 1973, Unger et al., 2009), but whether similar changes occur in the brain is less clear. Several studies have found that changes in brain iron, if present, occur relatively slowly, suggesting that diurnal variations in regional tissue iron concentrations would seem unlikely (Dallman and Spirito, 1977, Banks et al., 1988, Bradbury, 1997). In fact, very little has been done to study the diurnal or circadian nature of iron homeostasis in peripheral tissues let alone in the brain. The first reported study of CNS diurnal iron variation was in human using cerebrospinal fluid analyses of iron-related proteins. The study found that patients with Restless Legs Syndrome, compared to age- and sex-matched control subjects, showed a 2-fold greater diurnal change in cerebrospinal fluid ferritin and 4–5 fold greater diurnal change in cerebrospinal fluid transferrin (Earley et al., 2005). These findings suggest a nocturnal dip in brain iron stores specifically in Restless Legs Syndrome (Earley et al., 2005), which interestingly parallels the well-known, day-night drop in serum iron seen in humans (Lynch et al., 1973). This disease-related change in brain iron is an important finding in itself as it raises several important questions. Does the brain have natural circadian changes in iron concentration? If so, are there regional differences in diurnal changes as there are in iron concentrations found at a single time point noted previously in mice (Jones et al., 2003, Jellen et al., 2012)? At the time of this study, there was only one published study that had explored region-specific diurnal changes in brain iron. That study had used only one strain from the broader BXD recombinant inbred (RI) strains of mice (RI strain 40) and had shown regionally-specific diurnal changes in iron tissue concentration (Unger et al., 2009).

The BXD RI strains of mice have been used extensively to look for natural variations in biological and behavioral phenotypes with the emphasis on linking those findings to genetic traits (Williams et al., 2001). Understanding the neurobiology and genetics of iron homeostasis in the VMB has been a primary interest of our research group as we work towards a better understanding of the pathology of iron dysregulation found in the substantia nigra of patients with Restless Legs Syndrome (Jones et al., 2008, Earley, 2009). Therefore the primary brain region of interest for much of our work has been the ventral midbrain (VMB) containing the substantia nigra. Using a panel of BXD RI mice, we have demonstrated that there is wide, across-strain, variations in the iron concentration of VMB under both normal dietary iron conditions and under dietary iron-insufficiency conditions (Jones et al., 2003, Jellen et al., 2012). We have also shown that the VMB iron concentrations did not correlate with peripheral iron stores, indicating the organ- or region-specific nature of iron homeostasis (Jones et al., 2003, Jellen et al., 2012).

The focus of this study was to determine if diurnal changes in regional brain iron concentrations, particularly in the VMB, can be found across several strains from the BXD RI lineage, and whether the diurnal effect would be common to all strains or variable across strains as was found with the absolute iron concentrations when tested once during the light (inactive) period.

Materials and Methods

Animals

All experimental protocols were conducted in accordance with the National Institutes of Health Animal Care guidelines and were approved by the Penn State Institutional Animal Care and Use Committee. All animal care, tissue collection, brain dissection and iron determination have been described in more detail in a previous publication (Unger et al., 2009).

Mice were recombinant inbred (RI) strains derived from the C57BL/6J and DBA/2J (BXD type) parental strains (The Jackson Laboratory, Bar Harbor, ME). Mice were reared and housed under a constant light-dark cycle (Light-On schedule: 06:00–18:00 hrs), ambient temperature (23 ± 2 °C) and relative humidity (40%). Eight strains of mice were selected for this study from the twenty-two strains of RI BXD mice that were being used in the assessment of genetic variables associated with VMB iron concentration (Jellen et al., 2012). As our primary interest was on VMB iron, the eight strains were selected to provide a spectrum of high-to-low VMB iron determined from samples obtained at 3–5 hours after onset of the light cycle. Breeding effectiveness and similar VMB iron concentration between the sexes of any given strain were also taken into account in the strain selection. The eight RI BXD strains (S) selected were: S-1, S-14, S-21, S-31, S-34, S-39, S-40 and S-42. Upon weaning at postnatal day 21, same-sex littermates were co-housed (maximum 3 males or four females) and maintained on an *ad libitum* diet containing 240 µg/g iron (Harlan Laboratory, Teklad Customized Rodent diet).

Blood and Tissue Collection and Processing

At postnatal day 120, all mice were sacrificed via CO₂ inhalation and the brains were immediately harvested for tissue iron analysis. Half of the animals of equal sex and strains were sacrificed between 0900–1100 hrs (3–5 hours after start of the light phase (light-phase (LP) group)) and half between 2000 – 2200 hrs (2–4 hours after the start of the dark phase (dark-phase (DP) group)). Whole blood was collected by cardiac puncture, centrifuged and frozen. Livers and spleens were removed and frozen. The brains were dissected into VMB, dorsal striatum (caudate-putamen (DST)), ventral striatum (nucleus accumbens (NAC)), prefrontal cortex (PFC), pons (PON) and cerebellum (CBM) as described previously (Jones et al., 2003, Unger et al., 2007) and frozen. All tissue samples were weighed and stored at –80°C until assayed. Hemoglobin values were determined photometrically using cyanmethemoglobin standard solutions. Liver and spleen non-heme iron was measured using the techniques described previously (Cook et al., 1980). Brain tissue iron concentrations were determined by graphite furnace atomic absorption spectrophotometry (Perkin Elmer Analyst 600, Perkin Elmer, Norwalk, CT). Spectrophotometric values for iron concentration were normalized to tissue weight (µg/g) (Unger et al., 2009).

Statistical Analysis

The differences in total tissue iron concentration in the VMB (our primary region of interest) between LP and DP groups were analyzed for each of the eight RI strains using a two-tailed, t-test with significance set at $p < 0.05$. The other five brain regions were considered secondary regions of interest to ascertain how localized or wide spread any VMB diurnal effect, if found, was likely to be across the eight strains. Differences in iron concentrations between LP and DP groups in each of these five brain regions were tested independently for each sex-strain grouping using a two-tailed, t-test with significant set at $p < 0.05$. Spleen and liver iron concentrations were analyzed to explore diurnal changes in body iron stores. Regional brain iron concentrations determined 3–5 hours after onset of the light cycle under normal and dietary iron deficient conditions showed sex differences (Jellen et al., 2012). It is reasonable to assume that diurnal changes, if present, may also show significant sex differences. Therefore, all analyses were done separately on males and females. We establish *a priori* minimal sample size requirements ($N=5$) for each strain $\times$ sex $\times$ time grouping that would be required for statistical analysis to be performed. The only strain $\times$ sex $\times$ time group with inadequate sample size for analyses was S-39 males with $n = 4$. Therefore data on S-39 males are not presented in the Results Section.

Results

Iron concentration data presented in the text are presented as LP group versus DP group mean $\pm$ SD ($\mu\text{g/g}$) with t-test-derived probability value (p).

Diurnal differences in VMB

The primary brain region of interest, VMB, showed both strain- and sex-dependent differences (Figure 1). Overall, the mean VMB iron concentrations during the DP were less than that found in the LP group for the majority of female strains (14, 21, 31, 34, 40, 42), but not for the majority of males strains (21,31,40). Diurnal changes in VMB iron concentrations were significant, however, only for four strain-by-sex groups. VMB iron concentrations were found to be at least 25% lower during the DP compared to LP in the S-40 females (20.5 ± 6.5 vs 15.0 ± 2.5 , $p < 0.02$), but not S-40 males, and in the S-31 males (22.6 ± 5.2 vs 16.4 ± 3.3 , $p < 0.04$), but not S-31 females (Figure 1). On the other hand, S-42 showed sex-dependent divergence effects (Figure 1). The VMB iron concentrations during DP relative to the LP for S-42 were 23% lower (23.3 ± 3.4 vs 17.9 ± 2.4 , $p < 0.01$) for females while being 21% higher (15.8 ± 2.2 vs 20.0 ± 2.2 , $p < 0.02$) for males.

Diurnal differences in other brain regions

Of the four, strain-by-sex groups that showed diurnal changes in the VMB, only three showed changes in other brain regions (Table 1). S-40 females showed significantly lower iron concentrations during the DP in the pons (23.9 ± 6.7 vs 17.8 ± 3.2 , $p < 0.01$) and in the nucleus accumbens (18.7 ± 5.5 vs 13.8 ± 4.6 , $p < 0.02$). S-31 males also had lower iron in the pons during the DP (25.9 ± 4.7 vs 20.3 ± 2.7 , $p < 0.02$) but showed no change in other regions. During the DP compared to LP, S-42 males, but not S-42 females, had higher iron concentrations in the dorsal striatum (13.9 ± 1.6 vs 19.4 ± 4.5 , $p < 0.03$) but lower iron in the nucleus accumbens (23.0 ± 5.5 vs 17.6 ± 2.6 , $p < 0.04$).

Several of the other strains, though not showing diurnal changes in VMB iron, did show diurnal changes in iron concentrations in other brain regions (Table 1). In this regards, the findings in S-14 females is most notable. Despite showing no changes in VMB, this group had significantly lower iron concentrations during the DP relative to the LP in the nucleus accumbens ($p = 0.001$), dorsal striatum ($p = 0.016$) and cerebellum ($p = 0.004$). In fact, the largest diurnal difference (42% lower in the DP) found in this study was in the cerebellum of S-14 females (42.8 ± 7.3 vs 24.7 ± 6.9 , $p < 0.004$). In Strain 1, the females had higher iron in the cerebellum (18.1 ± 1.7 vs 21.0 ± 2.4 , $p < 0.04$) and males had lower iron in the prefrontal cortex (17.0 ± 4.0 vs 12.2 ± 2.8 , $p = 0.01$) during the DP relative to the LP. Of note, Strain 21 and 34, irrespective of sex, showed no diurnal changes in any brain regions.

Diurnal differences in spleen and liver

Table 2 shows the means and standard deviations for the spleen and liver iron concentrations ($\mu\text{g/g}$ of tissue) for male and female mice across the eight mice strains. S-14 mice were the only strain that showed no diurnal change in iron for either organ. The only consistency in the data was that liver iron was significantly higher during the DP. This was seen in female S-1, 31, and 40 and in male S-34 and 40. Also the higher liver iron concentrations in the DP for the S-40 male (73 ± 14 vs 88 ± 24 , $p < 0.05$) and female (101 ± 22 vs 122 ± 24 , $p = 0.012$), were similar to diurnal changes reported previously (Unger et al., 2009). The diurnal changes in splenic iron were more variable and, unlike liver iron, in both directions. Both S-42 sexes showed similar decrease in splenic iron during the DP, while S-31 females showed higher and males showed lower DP iron. Female mice from the S-39 and S-21 strains showed an increase in splenic iron while females from the S-34 strain showed a decrease in the DP.

There were no significant diurnal differences in hemoglobin concentrations for female or for male BXD RI strains (data not shown).

Discussion

Statistically significant ($p < 0.05$) diurnal differences were found in VMB iron concentrations for three of eight BXD RI strains. The S-42 mice were the only strain in which both sexes showed diurnal differences in VMB iron; however, day-night differences were in the opposite direction for female and males of this strain. Statistically significant diurnal differences were also found in one or more of the brain regions for six strains with strains 21 and 34 showing no changes in any brain region. The regional diurnal changes in iron concentration differed between the sexes as much as they differed across the strains. S-40 females and S-42 males both had several other brain regions other than the VMB which showed significant diurnal changes. In the previous (Unger et al., 2009) and current studies, the S-40 females showed significantly lower iron concentrations in the nucleus accumbens during the DP but not in the VMB or pons as was found in the current study. In the previous study, the primary cellular iron transport protein, transferrin receptor, was evaluated just in the VMB and was found to be lower during the DP. This would indicate that there exists, at some level, diurnal changes in VMB iron homeostasis for S-40 female strain (Unger et al., 2009). Also in support of the current findings, a recent study using an indwelling dialysis probe in the VMB of S-40 females showed, over a 72-hour collection period, a clear circadian oscillation in extracellular iron, with peak and trough matching the LP-DP diurnal finding in the current study (Unger et al., 2013).

Diurnal changes in iron homeostasis appear to be sensitive to dietary iron status. What is not clear is the range of iron concentrations that can still have an effect on diurnal changes in iron homeostasis. The diurnal changes in VMB transferrin receptor expression and in nucleus accumbens iron concentrations seen in S-40 female mice under dietary iron-sufficient conditions (50 $\mu\text{g/g}$ iron) were negated or reversed under a dietary iron-insufficient condition (4 $\mu\text{g/g}$ iron) (Unger et al., 2009). Clearly reducing the dietary iron from 50 to 4 $\mu\text{g/g}$ has an effect but what about increasing the dietary iron from 50 to 240 $\mu\text{g/g}$, which was the only notable difference between the current study (240 $\mu\text{g/g}$ iron diet) and the previous study (50 $\mu\text{g/g}$ iron diet) using just the S-40 female mice (Unger et al., 2009). We know that iron-overload diets (5000 $\mu\text{g/g}$) do alter brain iron concentrations in a strain $\times$ region manner (Unger et al., 2007). A recent study in S-40 female mice has also shown that a large intravenous iron infusion selectively increased iron only in the VMB and nucleus accumbens (Unger et al., 2013). It is, therefore, possible that the significant diurnal changes found in VMB and pons iron in this study but not in the prior study were a result of the greater amount of dietary iron in this study. If true, it would suggest the diurnal changes in VMB iron homeostasis in S-40 females is highly sensitive to a very broad range of systemic iron concentrations.

The diurnal iron changes in the liver and spleen were, as anticipated, organ $\times$ sex dependent. The only consistency in the data was that significant changes in diurnal liver iron, when found, were higher during the DP. The greater variability in the splenic iron, more than that seen for liver, suggests more complex genetics is at play in regulating iron homeostasis in the spleen (Jones et al., 2003, Jones et al., 2007). A crude visual assessment of the liver and spleen iron concentrations do not show any obvious link between body iron stores and brain iron, which is not unexpected given the lack of correlation between liver and VMB iron concentrations across the BXD RI strains (Jones et al., 2003). The current data also do not indicate any obvious relation between diurnal changes in liver or spleen iron and diurnal changes in VMB iron.

Conclusion

Significant diurnal changes in brain iron concentrations can be found across a selection of BXD RI mice. Sex and strain are functional determinates of which regions will be affected and in what direction the affect will be. As total tissue iron concentrations were determined in this study, the significant diurnal changes seen in the VMB or other brain regions indicates total tissue iron concentrations are changing over a 12-hour period by as much as 25% in some strains. Thus there must be some level of relatively rapid iron transport increasing and decreasing iron in some regions of the brain over a 24 hour period, possibly via blood-brain-barrier mechanism. As brain iron homeostasis in general is poorly understood, it is difficult at this time even to speculate on what mechanisms may be involved (Connor et al., 2001). Across a larger cohort of BXD RI mice, we have found a two-fold variation in VMB iron with normal iron diet and a threefold variation with an iron-insufficient diet (Jones et al., 2003, Jellen et al., 2012). We have now demonstrated in a smaller cohort of BXD RI mice, diurnal change in total VMB iron concentration occurs in several strains. Strain 40 females mice have also been shown to have circadian change in VMB extracellular iron concentrations (Unger et al., 2013). Our interest in VMB iron is driven by our interest in understanding why patients with Restless Legs Syndrome have low iron concentrations in the substantia nigra. The BXD RI mice strains provide an experimental model in which to examine mechanisms for VMB iron homeostasis, which seem likely to provide insight into the biological basis of Restless Legs Syndrome.

Acknowledgments

This research was supported by a grant from The National Institutes of Health, PO1-AG21190.

References

- Banks WA, Kastin AJ, Fasold MB, Barrera CM, Augereau G. Studies of the slow bidirectional transport of iron and transferrin across the blood-brain barrier. *Brain Res Bull.* 1988; 21:881–885. [PubMed: 3224280]
- Bradbury MWB. Transport of iron in the blood-brain-cerebrospinal fluid system. *J Neurochem.* 1997; 69:443–454. [PubMed: 9231702]
- Connor JR, Menzies SL, Burdo JR, Boyer PJ. Iron and iron management proteins in neurobiology. *Pediatr Neurol.* 2001; 25:118–129. [PubMed: 11551742]
- Cook GA, King MT, Veech RL. Changes in liver inorganic pyrophosphate content during ethanol metabolism. *Adv Exp Med Biol.* 1980; 132:433–440. [PubMed: 6252769]
- Dallman PR, Spirito RA. Brain iron in the rat: extremely slow turnover in normal rats may explain long-lasting effects of early iron deficiency. *J Nutr.* 1977; 107:1075–1081. [PubMed: 864518]
- Earley, CJ. Iron dysregulation in restless legs syndrome. In: Hening, WA., et al., editors. *Restless Legs Syndrome*. Philadelphia: Saunders Elsevier; 2009. p. 61-68.
- Earley CJ, Connor JR, Beard JL, Clardy SL, Allen RP. Ferritin levels in the cerebrospinal fluid and restless legs syndrome: effects of different clinical phenotypes. *Sleep.* 2005; 28:1069–1075. [PubMed: 16268375]
- Jellen LC, Unger EL, Lu L, Williams R, Rousseau S, Wang X, Earley CJ, Allen RP, Miles M, Jones BC. Systems genetic analysis of the effects of iron deficiency in mouse brain. *Neurogenetics.* 2012; 13:147–157. [PubMed: 22457016]
- Jones BC, Beard JL, Gibson JN, Unger EL, Allen RP, McCarthy KA, Earley CJ. Systems genetic analysis of peripheral iron parameters in the mouse. *Am J Physiol Regul Integr Comp Physiol.* 2007; 293:R116–124. [PubMed: 17475678]
- Jones BC, Reed CL, Hitzemann R, Wiesinger JA, McCarthy KA, Buwen JP, Beard JL. Quantitative genetic analysis of ventral midbrain and liver iron in BXD recombinant inbred mice. *Nutr Neurosci.* 2003; 6:369–377. [PubMed: 14744041]

- Jones LC, Earley CJ, Allen RP, Jones BC. Of mice and men, periodic limb movements and iron: how the human genome informs the mouse genome. *Genes Brain Behav.* 2008; 7:513–514. [PubMed: 18363860]
- Lynch SR, Simon M, Bothwell TH, Charlton RW. Circadian variation in plasma iron concentration and reticuloendothelial iron release in the rat. *Clin Sci Mol Med.* 1973; 45:331–336. [PubMed: 4785135]
- Unger EL, Beard JL, Jones BC. Iron regulation in C57BL/6 and DBA/2 mice subjected to iron overload. *Nutr Neurosci.* 2007; 10:89–95. [PubMed: 17539487]
- Unger EL, Earley CJ, Beard JL. Diurnal cycle influences peripheral and brain iron levels in mice. *J Appl Physiol.* 2009; 106:187–193. [PubMed: 18988764]
- Unger EL, Earley CJ, Thomsen LL, Jones BC, Allen RP. Effects of IV iron isomaltoside-1000 treatment on regional brain iron status in an iron-deficient animal. *Neuroscience.* 2013; 246:179–185. [PubMed: 23660192]
- Williams RW, Gu J, Qi S, Lu L. The genetic structure of recombinant inbred mice: high-resolution consensus maps for complex trait analysis. *Genome Biol.* 2001; 2:RESEARCH0046. [PubMed: 11737945]

Highlights

1. Brain-regional-specific diurnal changes in tissue iron were found in across BXD mice
2. Total tissue iron concentration in the ventral midbrain changed as much as 25%.
3. Diurnal changes in liver and spleen iron were not related to brain iron changes.
4. Diurnal changes in tissues iron were dependent on sex and strain of mice.

Figure 1. Male and female BXD RI mice strains (1,14, 21,31, 34,39, 40 and 42) and their mean ($\pm$ sem) iron concentrations ($\mu\text{g/g}$ of tissue) in ventral midbrain (VMB) during the light and dark phase of the light dark-cycle

The range of sample sizes for female strain $\times$ time groups was 5–15 with a median sample size of 9. The range of sample sizes for male strain $\times$ time groups (excluding strain 39) was 6–13 with a median sample size of 9.

Male and Female RI Mice Strains and Their Mean ($\pm$ SD) Iron Concentrations (μ g/g of tissue) in Several Brain Regions during the Light (LP) and Dark (DP) Phases of the Light–Dark Cycle

TABLE 1

Mice Strain	Brain Region	Female Mice			Male Mice		
		LP	DP	P value	LP	DP	P value
		Mean $\pm$ SD	Mean $\pm$ SD		Mean $\pm$ SD	Mean $\pm$ SD	
1	NAC	13.43 $\pm$ 3.72	14.35 $\pm$ 1.50	0.594	19.34 $\pm$ 5.04	14.00 $\pm$ 2.62	0.059
	DST	17.35 $\pm$ 4.28	14.47 $\pm$ 2.31	0.186	18.61 $\pm$ 4.09	15.96 $\pm$ 2.74	0.153
	PFC	17.18 $\pm$ 6.74	13.67 $\pm$ 3.13	0.301	17.01 $\pm$ 3.98	12.15 $\pm$ 2.77	<u>0.010</u>
	PON	19.02 $\pm$ 6.15	20.27 $\pm$ 1.98	0.652	21.15 $\pm$ 6.99	22.15 $\pm$ 6.09	0.765
	CBM	18.08 $\pm$ 1.74	21.03 $\pm$ 2.44	<u>0.039</u>	24.20 $\pm$ 6.45	21.46 $\pm$ 3.30	0.285
	(N=)	(6)	(6)		(9)	(8)	
14	NAC	26.24 $\pm$ 2.91	18.28 $\pm$ 1.75	<u>0.001</u>	15.87 $\pm$ 2.32	18.46 $\pm$ 3.86	0.167
	DST	17.96 $\pm$ 2.38	13.58 $\pm$ 2.15	<u>0.016</u>	18.77 $\pm$ 5.36	17.05 $\pm$ 6.33	0.594
	PFC	23.92 $\pm$ 6.47	18.15 $\pm$ 5.68	0.158	19.70 $\pm$ 3.58	20.99 $\pm$ 8.98	0.721
	PON	24.92 $\pm$ 2.20	22.37 $\pm$ 9.12	0.533	20.15 $\pm$ 3.97	24.11 $\pm$ 6.07	0.167
	CBM	42.77 $\pm$ 7.26	24.73 $\pm$ 6.88	<u>0.035</u>	21.04 $\pm$ 1.93	22.38 $\pm$ 4.67	0.490
	(N=)	(5)	(6)		(6)	(8)	
21	NAC	16.55 $\pm$ 2.35	15.47 $\pm$ 3.41	0.522	17.33 $\pm$ 4.25	17.70 $\pm$ 5.21	0.882
	DST	18.84 $\pm$ 4.26	15.82 $\pm$ 2.78	0.107	20.31 $\pm$ 7.53	16.73 $\pm$ 3.58	0.251
	PFC	15.30 $\pm$ 2.53	13.80 $\pm$ 2.46	0.278	17.96 $\pm$ 6.03	24.20 $\pm$ 10.8	0.176
	PON	19.15 $\pm$ 3.27	18.71 $\pm$ 4.52	0.828	23.32 $\pm$ 5.02	19.49 $\pm$ 3.59	0.089
	CBM	22.04 $\pm$ 5.83	18.11 $\pm$ 6.19	0.193	25.17 $\pm$ 5.99	22.15 $\pm$ 4.40	0.252
	(N=)	(9)	(7)		(9)	(8)	
31	NAC	19.69 $\pm$ 3.04	20.48 $\pm$ 4.18	0.664	17.29 $\pm$ 3.33	19.13 $\pm$ 4.15	0.304
	DST	18.71 $\pm$ 3.25	18.07 $\pm$ 4.63	0.729	15.63 $\pm$ 3.00	15.45 $\pm$ 4.35	0.918
	PFC	17.63 $\pm$ 5.31	16.68 $\pm$ 5.41	0.696	17.98 $\pm$ 5.29	17.30 $\pm$ 7.55	0.817
	PON	23.09 $\pm$ 3.13	20.59 $\pm$ 3.36	0.102	25.86 $\pm$ 4.71	20.28 $\pm$ 2.74	<u>0.012</u>
	CBM	31.44 $\pm$ 13.3	21.42 $\pm$ 4.29	<u>0.045</u>	23.44 $\pm$ 4.41	24.10 $\pm$ 5.58	0.777
	(N=)	(10)	(10)		(8)	(13)	

Mice Strain	Brain Region	Female Mice			Male Mice		
		LP	DP	P value	LP	DP	P value
		Mean $\pm$ SD	Mean $\pm$ SD		Mean $\pm$ SD	Mean $\pm$ SD	
34	NAC	18.87 $\pm$ 5.35	16.13 $\pm$ 4.23	0.217	16.37 $\pm$ 3.43	17.77 $\pm$ 3.21	0.372
	DST	18.96 $\pm$ 3.66	19.91 $\pm$ 5.10	0.638	18.49 $\pm$ 3.44	17.36 $\pm$ 5.32	0.606
	PFC	18.51 $\pm$ 6.14	17.37 $\pm$ 7.64	0.717	19.61 $\pm$ 5.10	20.61 $\pm$ 6.59	0.718
	PON	24.79 $\pm$ 8.78	23.80 $\pm$ 5.85	0.774	24.47 $\pm$ 6.21	27.43 $\pm$ 9.97	0.471
	CBM	29.04 $\pm$ 10.0	24.73 $\pm$ 12.7	0.404	24.13 $\pm$ 11.3	24.81 $\pm$ 5.93	0.871
	(N=)	(11)	(9)		(12)	(9)	
39	NAC	24.37 $\pm$ 4.31	19.28 $\pm$ 4.07	<u>0.017</u>	-----	-----	-----
	DST	22.77 $\pm$ 7.29	21.84 $\pm$ 8.01	0.796	-----	-----	-----
	PFC	19.74 $\pm$ 6.13	22.30 $\pm$ 5.78	0.359	-----	-----	-----
	PON	21.46 $\pm$ 4.01	21.54 $\pm$ 3.64	0.965	-----	-----	-----
	CBM	25.93 $\pm$ 4.09	26.59 $\pm$ 6.32	0.798	-----	-----	-----
	(N=)	(12)	(8)				
40	NAC	18.65 $\pm$ 5.53	13.82 $\pm$ 4.60	<u>0.038</u>	16.37 $\pm$ 4.70	15.99 $\pm$ 1.57	0.814
	DST	17.82 $\pm$ 2.93	17.26 $\pm$ 4.01	0.737	15.40 $\pm$ 3.81	16.15 $\pm$ 5.14	0.737
	PFC	17.90 $\pm$ 4.56	14.98 $\pm$ 5.92	0.225	17.15 $\pm$ 6.94	16.06 $\pm$ 4.77	0.681
	PON	23.94 $\pm$ 6.70	17.81 $\pm$ 3.22	<u>0.007</u>	22.09 $\pm$ 5.47	19.25 $\pm$ 5.35	0.266
	CBM	29.05 $\pm$ 7.69	23.34 $\pm$ 5.91	0.055	22.90 $\pm$ 7.09	24.78 $\pm$ 6.96	0.552
	(N=)	(15)	(9)		(12)	(9)	
42	NAC	22.19 $\pm$ 5.26	22.11 $\pm$ 3.89	0.977	22.99 $\pm$ 5.54	17.60 $\pm$ 2.61	<u>0.035</u>
	DST	15.88 $\pm$ 2.54	19.13 $\pm$ 3.89	0.100	13.90 $\pm$ 1.55	19.38 $\pm$ 4.47	<u>0.029</u>
	PFC	14.84 $\pm$ 3.49	16.24 $\pm$ 3.63	0.476	20.03 $\pm$ 5.78	19.13 $\pm$ 10.2	0.854
	PON	22.25 $\pm$ 5.41	23.22 $\pm$ 3.73	0.727	21.73 $\pm$ 5.51	23.03 $\pm$ 3.88	0.628
	CBM	22.62 $\pm$ 3.04	24.88 $\pm$ 4.03	0.299	25.91 $\pm$ 7.62	21.57 $\pm$ 3.70	0.214
	(N=)	(7)	(7)		(8)	(6)	

Abbreviation: nucleus accumbens (NAC), caudate-putamen/dorsal striatum (DST), prefrontal cortex (PFC), pons (PON), cerebellum (CBM). Using Student's t-test, p-values <0.05 for differences between LP and DP are marked in ***bold/italic/underlined***. The cells containing no data [-----] indicates that the sample size was < 5 and not considered adequate for analysis. Sample size (N=) for each strain $\times$ gender $\times$ time grouping is given in parenthesis.

Male and Female RI Mice Strains and Their Mean ($\pm$ SD) Spleen and Liver Iron Concentrations ($\mu\text{g/g}$ of tissue) during the Light (LP) and Dark (DP) Phases of the Light-Dark Cycle

TABLE 2

Mice Strain	Body Organ	Female Mice			Male Mice		
		LP	DP	P Value	LP	DP	P Value
		Mean $\pm$ SD	Mean $\pm$ SD		Mean $\pm$ SD	Mean $\pm$ SD	
1	Spleen	346 $\pm$ 73	311 $\pm$ 56	0.344	292 $\pm$ 42	279 $\pm$ 50	0.516
	Liver	93 $\pm$ 13	115 $\pm$ 122	<u>0.010</u>	66 $\pm$ 18	61 $\pm$ 9	0.389
	(N=)	(7)	(7)		(14)	(11)	
14	Spleen	491 $\pm$ 164	441 $\pm$ 115	0.548	303 $\pm$ 111	256 $\pm$ 81	0.272
	Liver	110 $\pm$ 24	103 $\pm$ 9	0.477	84 $\pm$ 25	82 $\pm$ 8	0.838
	(N=)	(7)	(7)		(11)	(14)	
21	Spleen	519 $\pm$ 114	671 $\pm$ 201	<u>0.046</u>	347 $\pm$ 116	350 $\pm$ 73	0.923
	Liver	86 $\pm$ 15	100 $\pm$ 22	0.112	59 $\pm$ 8	58 $\pm$ 6	0.878
	(N=)	(11)	(11)		(14)	(11)	
31	Spleen	978 $\pm$ 185	1197 $\pm$ 279	<u>0.016</u>	595 $\pm$ 200	410 $\pm$ 104	<u>0.007</u>
	Liver	120 $\pm$ 24	158 $\pm$ 37	<u>0.001</u>	93 $\pm$ 22	91 $\pm$ 18	0.823
	(N=)	(18)	(17)		(14)	(13)	
34	Spleen	723 $\pm$ 130	633 $\pm$ 106	<u>0.039</u>	638 $\pm$ 274	570 $\pm$ 167	0.361
	Liver	168 $\pm$ 31	165 $\pm$ 27	0.800	110 $\pm$ 22	124 $\pm$ 21	<u>0.043</u>
	(N=)	(19)	(16)		(20)	(22)	
39	Spleen	721 $\pm$ 68	976 $\pm$ 131	<u>0.010</u>	-----	-----	-----
	Liver	89 $\pm$ 5.2	106 $\pm$ 23	0.155	-----	-----	-----
	(N=)	(5)	(5)				
40	Spleen	819 $\pm$ 129	908 $\pm$ 273	0.267	315 $\pm$ 111	392 $\pm$ 162	0.126
	Liver	101 $\pm$ 22	122 $\pm$ 24	<u>0.012</u>	73 $\pm$ 14	88 $\pm$ 24	<u>0.046</u>
	(N=)	(23)	(17)		(21)	(16)	
42	Spleen	307 $\pm$ 61	235 $\pm$ 47	<u>0.010</u>	266 $\pm$ 88	183 $\pm$ 58	<u>0.014</u>
	Liver	96 $\pm$ 22	85 $\pm$ 15	0.212	46 $\pm$ 10	53 $\pm$ 5	0.091

Mice Strain	Body Organ	Female Mice			Male Mice		
		LP	DP	P Value	LP	DP	P Value
		Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	
		(N=)			(13)	(9)	

Abbreviation: Using Student's t-test, p-values <0.05 for differences between LP and DP are marked in ***bold/italic/underlined***. The cells containing no data [-----] indicates that the sample size was < 5 and not considered adequate for analysis. Sample size (N=) for each strain × gender × time grouping is given in parenthesis.