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Transferrin response in normal and iron-deficient mice heterozygotic for hypotransferrinemia; effects on iron and manganese accumulation

Elise A. Malecki*,**, Attila G. Devenyi*, John L. Beard*** & James R. Connor **,***

*Departments of *Pediatrics and **Neuroscience and Anatomy, College of Medicine, The Pennsylvania State University, and ***Department of Nutrition, College of Health and Human Development, The Pennsylvania State University, Pennsylvania, USA*

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Hypotransferrinemia is a genetic defect in mice resulting in <1% of normal plasma transferrin (Tf) concentrations; heterozygotes for this mutation (+/hpx) have low circulating Tf concentrations. These mice provide a unique opportunity to examine the developmental pattern and response of Tf to iron-deficient diets, and furthermore, to address the controversial role of Tf in Mn transport. Twenty-three weanling +/hpx mice and forty-five wild-type BALB/cJ mice were either killed at weaning or fed diets containing either 13 or 72 mg kg^{-1} Fe, and killed after four or eight weeks. Plasma Tf concentrations were lower in +/hpx mice, plasma Tf nearly doubled and liver Tf was only 50% of normal in response to iron deficiency. Brain iron concentration did not correlate significantly with either plasma Tf or TIBC. However, iron accumulation into brain continued with iron deficiency whereas most other organs had less iron. These results imply that either there is a selected targeting of iron to the brain by plasma Tf or there is an alternative iron delivery system to the brain. Furthermore, we observed no differences in tissue distribution of ^{54}Mn despite the differences in circulating Tf concentrations and body iron stores; this suggests that there are non-Tf dependent mechanisms for Mn transport.

Keywords: brain, ferritin, liver, plasma, TIBC

Abbreviations: Fe (iron); Mn (manganese); Tf (transferrin); TIBC (total iron binding capacity); +/+ (wild-type BALB/cJ mice); +/hpx (mice heterozygotic for the hypotransferrinemia mutation).

Introduction

Transferrin (Tf) is the major iron transport protein in plasma. Tf concentrations in plasma are maintained by liver synthesis and secretion (Zakin 1992). Plasma concentrations of Tf (Idzerda *et al*. 1986) and synthesis in liver (Morgan 1969) may be upregulated during dietary iron deficiency, but the mechanism of this regulation is not well understood. Other tissues also express Tf mRNA and synthesize Tf (Morgan 1969). In the brain, Tf is synthesized in choroid plexus and oligodendrocytes (Aldred *et al*. 1987). The synthesis of Tf in brain may be involved in transporting iron from endothelial cells to other cells (Jeffries *et al*. 1984, Fishman *et al*. 1987), and may also function as a paracrine growth factor or as an antioxidant. The expression of Tf mRNA in brain is unique in that it increases between weaning and adulthood (Levin *et al*. 1984, Bartlett *et al*. 1991) whereas Tf mRNA expression decreases postnatally in other organs. Factors that influence the synthesis of Tf in organs other than liver have received minimal attention.

A hypotransferrinemic (hpx) mouse has been identified, which should provide insight into the function and perhaps regulation of transferrin. The mutation involves a Tf mRNA splicing defect

Address for correspondence: J.R. Connor, Department of Neuroscience & Anatomy, College of Medicine, The Pennsylvania State University, Hershey, PA 17033, USA. Tel: (+1) 717 531 6408; Fax: (+1) 717 531 5184; e-mail: jrc3@email.psu.edu

(Huggenvik *et al*. 1989) whereby mature Tf mRNA and therefore Tf protein are not produced, leading to <1% of normal plasma Tf in homozygotes and intermediate values in heterozygotes (Bernstein 1987, Raja *et al*. 1995). The mice that are heterozygotic for the hpx mutation could be expected to provide insight into Tf regulation. Previous work with mice homozygotic for the mutation has shown that they are severely anemic, yet accumulate iron in the liver (Bernstein 1987).

In addition to iron, Tf may bind manganese (Mn; Davidsson *et al*. 1989, Critchfield & Keen 1992), to the extent that Mn^{2+} is oxidized *in vivo* to the Mn^{3+} ion. Mn can thus potentially be delivered to tissues via Tf receptors; Tf-mediated uptake of Mn has been observed in neuroblastoma cells in culture (Suárez & Erikson 1993). Regulation of Mn transport is critical, because although Mn is an essential nutrient (Keen & Zidenberg-Cherr 1996), Mn in excess is neurotoxic. Mn toxicity presents as a Parkinsonian-like movement disorder due to the death of neurons of the basal ganglia (Aschner 1997), and has been observed in patients receiving total parenteral nutrition supplemented with Mn (Fell *et al*. 1996, Alves *et al*. 1997). Because Tf may be involved in Mn transport, hypotransferrinemic mice should provide insight into the role of Tf in Mn delivery to tissues. Hypotransferrinemic mice have been used to study the role of Tf in the tissue uptake of aluminium and gallium (Radunovic *et al*. 1997).

The purpose of this study was three-fold: (1) to determine the developmental changes in tissue levels of Tf in wild-type mice and +/hpx mice in organs capable of synthesizing Tf; (2) to determine whether dietary iron deficiency would induce an increase in plasma Tf concentrations in +/hpx mice; and (3) to determine if variations in plasma Tf concentration and Tf saturation modify Mn tissue accumulation from an intraperitoneal dose. An experiment was conducted with wild-type and +/hpx mice from weaning to 12 weeks of age with manipulation of dietary iron.

Materials and methods

Animals and diets

Colonies of wild-type BALB/cJ and hypotransferrinemic BALB/cJ -hpx/hpx mice were maintained at the Hershey Medical Center Animal Research Facility. Heterozygotes were mated, and from their offspring, +/hpx were identified from their wild-type (+/+) littermates by plasma Tf < 2 g l^{-1} at weaning. Homozygotes were readily distinguished by their anemic appearance. Mice were housed up to five per cage in plastic shoe box cages, and allowed free access to the diets and distilled water. Animal facilities were accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and protocols were approved by an Institutional Animal Care and Use Committee.

Forty-five wild type and 23 +/hpx mice were used in this study. Seven wild-type and four +/hpx mice were killed at weaning (post-natal day 24–28). The remainder were fed purified diets based on the AIN 93-G formulation (Reeves *et al*. 1993): cornstarch, 397.5 g kg^{-1}; low trace element casein, 200 g kg^{-1}; dextrinized cornstarch, 132 g kg^{-1}; sucrose, 100 g kg^{-1}; soybean oil 70 g kg^{-1}; cellulose, 50 g kg^{-1}; mineral mix (AIN-93G without iron), 35 g kg^{-1}; vitamin mix (AIN-93), 10 g kg^{-1}; L-cystine, 3 g kg^{-1}; choline bitartrate, 2.5 g kg^{-1}; *t*-butylhydroquinone, 0.014 g kg^{-1}. All diet ingredients were purchased from ICN (Cleveland, OH), except for soybean oil (Foodhold USA, Inc., Windsor Locks, CT) and minerals (Fisher, Pittsburgh, PA; Sigma, St Louis, MO; and Aldrich, Milwaukee, WI). These diets contained either an adequate concentration of iron as ferric citrate (Fe+) or no added iron (Fe–), and were found to contain 72 ± 12 and 13 ± 1 μg Fe g^{-1} by analysis, respectively. This gave four strain by diet treatments: +/+ Fe+ (wild-type iron-adequate), +/+ Fe– (wild-type iron-deficient, +/hpx Fe+ (heterozygote iron-adequate), and +/hpx Fe– (heterozygote iron-deficient).

Mice were killed by exsanguination by cardiac puncture under CO_2 anesthesia. Blood was collected into heparinized syringes, and plasma was obtained by centrifugation for 10 min at 15 000 × *g* and 4°C. Carcasses were perfused with 5 ml PBS. Brain, heart, spleen, liver and gastrocnemius muscle were collected, rinsed with cold PBS, weighed, and divided for determination of radioactivity, analysis of iron concentrations, or Tf measurements.

Transferrin immunoblotting and ferritin Western blotting

Mouse plasma and mouse transferrin standards (Cappel, West Chester, PA) were diluted 1:5000 in Tris-buffered saline and loaded onto a nitrocellulose membrane (0.45 μm pore size; Schleicher & Schuell, Keene, NH) in triplicate. Immunoblotting was performed as described previously (Dickinson *et al*. 1996); the primary antibody was goat anti-mouse transferrin (Cappel) diluted 1:1000, and the secondary antibody was rabbit anti-goat IgG coupled to horseradish peroxidase (Sigma, St. Louis, MO) diluted 1:12 000. Enhanced chemoluminescence Western blotting detection reagents (Amersham, Buckinghamshire, UK) were used as the substrate, and the blots were then exposed to Kodak X-OMAT film and bands quantitated by densitometry (Molecular Dynamics laser densitometer, Sunnyvale, CA and Quantity One software, PDI, Huntington, NY).

Frozen (–70°C) tissue samples were homogenized in 4.5 volumes of PBS and centrifuged for 30 min at 17 500 × *g*. Protein centrations of supernatants were measured by the BioRad dye binding assay (Hercules, CA). Supernatants (5 μg protein per well for liver and heart; 30 μg protein

Table 1. Body weights of mice either wild-type (+_/+) or heterozygotic for the hypotransferrinemia mutation (+/hpx) fed diets either deficient in (Fe–) or adequate in (Fe+) iron[1]

Strain	Dietary iron	Sex	Body weight (g)		
			0 weeks	4 weeks	8 weeks
+/+	Fe+	M	15.3 ± 0.3 (3)^{gh}	22.4 ± 1.2(7)^{ef}	32.4 ± 1.6(3)^{a}
+/+	Fe+	F	13.6 ± 0.4(4)^{h}	20.2 ± 0.97(7)^{fg}	24.0 ± 1.4(3)^{cde}
+/+	Fe–	M		25.9 ± 0.8(12)^{bc}	29.1 ± 0.6(3)^{ab}
+/+	Fe–	F		22.1 ± 1.5(3)^{def}	23.5 ± 1.1(3)^{cde}
+/hpx	Fe+	M	17.2(1)^{fgh}	22.5 ± 2.2(2)^{cdef}	29.2 ± 1.0(2)^{ab}
+/hpx	Fe+	F	15.6 ± 1.3(3)^{gh}	20.2(1)^{defg}	21.9 ± 1.1(3)^{def}
+/hpx	Fe–	M		21.9 ± 0.5(3)^{def}	25.1 ± 0.6(5)^{cd}
+/hpx	Fe–	F		19.1 ± 0.6(2)^{fg}	20.4(1)^{defg}

[1]Values are means ± SEM with number of mice in parentheses. Values not sharing a common superscript letter are significantly different ($P < 0.05$). Statistical effects determined by ANOVA: time, $P < 0.0001$; strain, $P < 0.05$; sex, $P < 0.0001$; time × sex, $P < 0.01$; time × dietary iron, $P < 0.05$; dietary iron main effect, time × strain, strain × dietary iron, strain × sex, and three-and four-way interactions not significant.

per well for spleen, brain and muscle) were also loaded onto nitrocellulose membranes in triplicate and analyzed for transferrin concentrations as above.

Supernatants of liver were pooled, and samples containing 200 μg of total protein were loaded onto a 10% SDS-polyacrylamide gel with a 4% stacking gel. Electrophoresis was accomplished with a discontinuous Tris/tricine system for optimal resolution of the ferritin subunits (Chen *et al*. 1997). Proteins were transferred to a PVDF membrane (Amersham); the membrane was then blocked for 1 h in 5% nonfat dried milk in Tris-buffered saline. The primary antibody was rabbit anti-human liver ferritin (1:2000; Boehringer-Mannheim, Indianapolis, IN); the secondary antibody was goat anti-rabbit IgG coupled to horseradish peroxidase (1:96 000; BioRad). Enhanced chemoluminescence Plus Western blotting detection reagents (Amersham) were used as the substrate.

^{54}Manganese injections

Twenty-four hours before killing, mice were injected intraperitoneally (i.p.) with 37 kBq $^{54}MnCl_2$ (NEN, Boston, MA) in 0.1 ml 0.9% NaCl. Tissue samples were counted in a GammaTrac 1191 counter (TM Analytic, Elk Grove, IN) for measurement of ^{54}Mn uptake. A series of standards were counted simultaneously for construction of a standard curve and to correct for decay.

Iron concentrations

Iron in plasma and and ashed diet and tissues (two hours digestion with 70% HNO_3) was measured electrochemically with a Ferrochem II analyzer (ESA, Inc., Bedford, MA). Total iron binding capacity (TIBC) was measured as plasma iron concentration after incubation with a low-affinity iron-binding resin (ESA, Inc.).

Calculations and statistical analysis

TIBC saturation equals plasma Fe divided by TIBC. Transferrin saturation equals plasma Fe divided by the number of metal-binding sites on Tf, the concentration of which was determined by immunoblot.

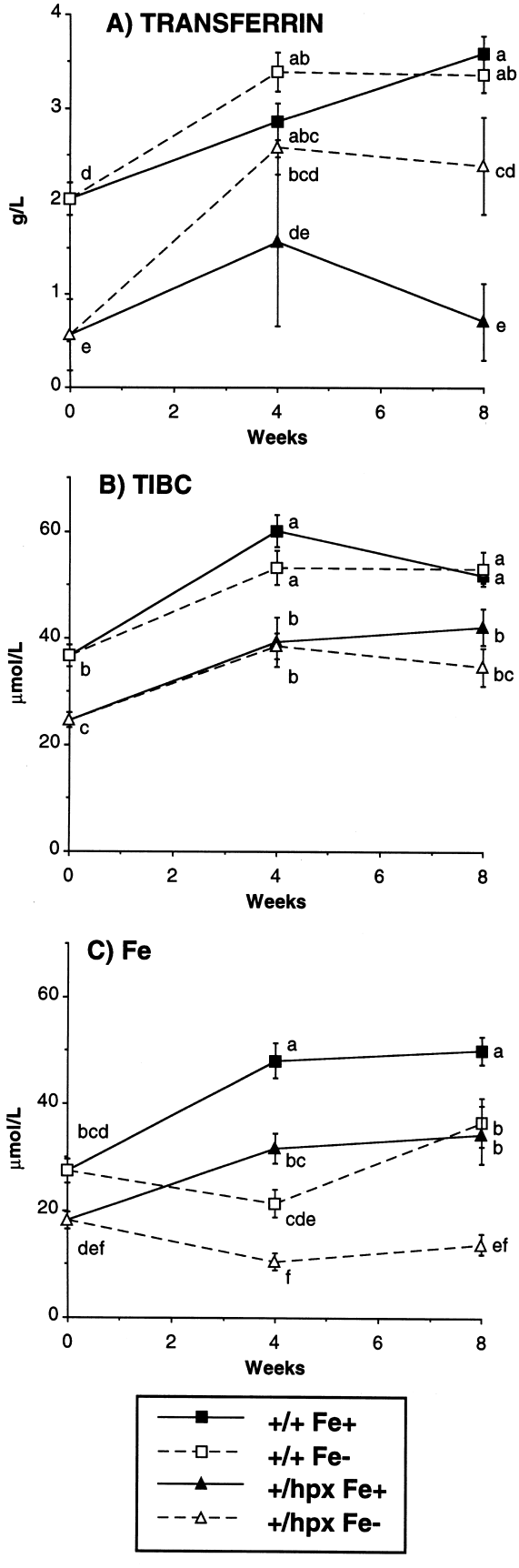
Skeletal muscle was assumed to make up 38.8% of the mouse body weight (Durbin *et al*. 1992). ^{54}Mn tracer uptake in muscle was extrapolated from a sample of gastrocnemius muscle. Although severe iron deficiency will affect body composition (Tobin *et al*. 1993), we did not measure body composition in this study; the slight changes in body composition anticipated with the relatively mild iron deficiency in this study would not affect our interpretation of the ^{54}Mn uptake data.

Four-way ANOVA (time × strain × dietary iron × sex) was accomplished using the general linear models procedure of SAS verson 6.08 (SAS Institute, Cary, NC). The factorial was collapsed into a three-way ANOVA (time × strain × dietary iron) if no sex main effects or interactions were found. Post-hoc analyses of differences between means (Duncan's multiple range test) were also obtained with SAS. Additionally, Pearson's correlation coefficients were obtained between plasma Tf and TIBC, and tissue iron concentrations and Mn accumulation.

Results

Body weights of mice were lower in heterozygotes than in normal mice (Table 1). As expected, iron-deficient mice showed less weight gain than iron-adequate mice, and females weighed less than males.

Overall, manipulation of dietary iron in wild-type and heterozygotic hypotransferrinemic mice resulted in alterations in plasma Tf saturations (Figure 1) and in tissue Tf concentrations (Figure 2). Dietary iron deficiency was effective in reducing hematocrits (Table 2) and tissue iron concentrations (Tables 3 and 4). The iron-deficient mice were borderline anemic, in a range of iron nutriture comparable with that of a healthy human population. There were no differences in tissue accumulation of Mn despite



the differences in Tf concentrations and tissue iron concentrations (Figure 3).

Plasma

Depressed plasma Tf was evident in +/hpx mice at weaning, when concentrations were 28% of those of wild-type mice (Figure 1A). Plasma Tf concentrations then rose in the wild-type mice fed the iron-adequate diet, but remained at the same low level in the +/hpx mice fed the iron-adequate diet. After eight weeks, the +/hpx mice fed an iron-adequate diet had only 20% of the plasma Tf of wild-type mice. After four weeks, there were 64% higher plasma Tf concentrations in +/hpx mice fed the iron-deficient diet versus the iron-adequate diet, but only a 19% higher concentration in the wild-type mice fed the iron-deficient diet versus the iron-adequate diet. No further change was seen between four and eight weeks. The eight week plasma Tf concentrations in iron-deficient +/hpx mice were 67% of those of iron-adequate wild-type mice, but 236% higher than those of iron-adequate +/hpx mice.

Plasma TIBC (Figure 1B) was less in the +/hpx mice compared with the wild-type mice at every time point. Both wild-type and +/hpx mice had increases in plasma TIBC with age, and this increase was not affected by dietary iron. Iron concentrations in plasma (Figure 1C) were significantly higher in the wild-type mice than in the +/hpx mice at weaning. Plasma iron levels increased over time in the wild-type mice but not in the +/hpx mice. Iron deficiency resulted in decreased plasma iron concentrations in both +/hpx and wild-type mice.

Brain

At weaning, Tf concentrations in the brain were identical for both the wild-type and +/hpx mice

Figure 1. (A) Transferrin; (B) total iron binding capacity; and (C) iron concentrations of plasma from wild-type (+/+) mice or mice heterozygotic for the hypotransferrinemia mutation (+/hpx) fed diets either deficient in (Fe–) or adequate in (Fe+) iron. Statistical effects determined by ANOVA for (A) transferrin: time, $P < 0.05$; strain, $P < 0.0001$; dietary iron, $P < 0.005$; strain × dietary iron, $P < 0.05$; time × strain, time × dietary iron, and three-way interaction effects not significant; (B) TIBC: time, $P < 0.0001$; strain, $P < 0.0001$; dietary iron, and two and three-way interaction effects not significant; and (C) Fe: time, $P < 0.0001$; strain, $P < 0.0001$; dietary iron, $P < 0.0001$; two- and three-way interaction effects not significant. $n = 3$–14 mice per treatment per time point.

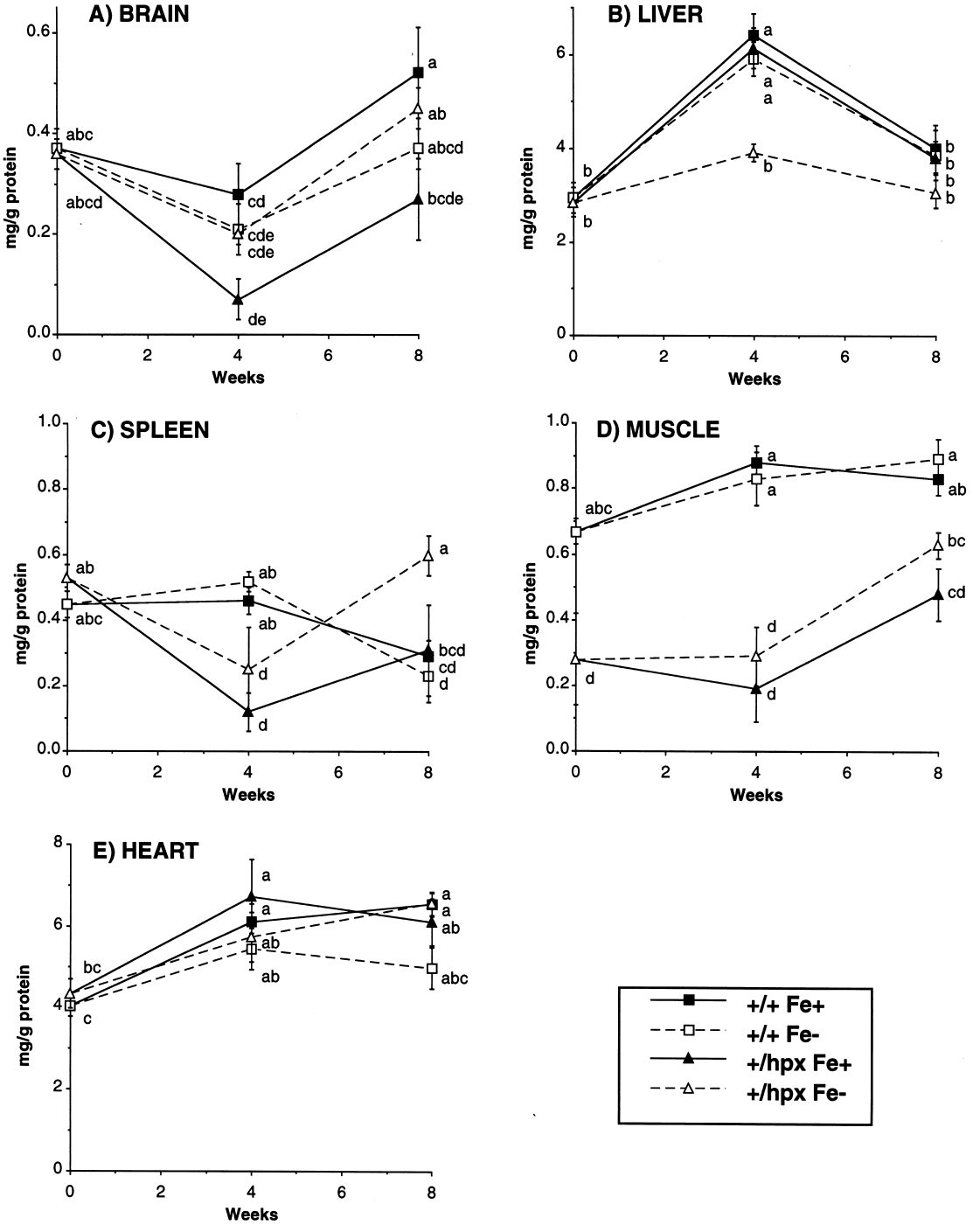


Figure 2. Transferrin concentrations in: (A) brain; (B) liver; (C) spleen; (D) muscle; and (E) heart of wild-type (+/+) mice or mice heterozygotic for the hypotransferrinemia mutation (+/hpx) fed diets either deficient in (Fe–) or adequate in (Fe+) iron. Statistical effects determined by ANOVA for (A) brain: time, $P < 0.0001$; strain × dietary iron, $P < 0.01$; strain, dietary iron, time × strain, time × dietary iron, and three-way interaction effects not significant; (B) liver: time, $P < 0.0001$; strain, $P < 0.05$; dietary iron, $P < 0.05$; two- and three-way interaction effects not significant; (C) spleen: time, $P < 0.05$; dietary iron, $P < 0.05$; time × strain, $P < 0.0001$; strain × dietary iron, $P < 0.05$; strain, time × dietary iron, and three-way interaction effects not significant; (D) muscle: time, $P < 0.05$; strain, $P < 0.0001$; time × strain, $P < 0.05$; dietary iron, time × dietary iron, strain × dietary iron, and three-way interaction effects not significant; (E) heart: time, $P < 0.005$; strain, dietary iron, and two- and three-way interaction effects not significant. n = 3–14 mice per treatment per time point.

Table 2. Hematocrits of mice either wild-type (+/+) or heterozygotic for the hypotransferrinemia mutation (+/hpx) fed diets either deficient in (Fe–) or adequate in (Fe+) iron[1]

Strain	Dietary iron	Hematocrits (%)		
		0 weeks	4 weeks	8 weeks
+/+	Fe+	$38.8 \pm 0.9(7)^{c}$	$44.8 \pm 1.3(10)^{a}$	$42.5 \pm 2.6(6)^{ab}$
+/+	Fe–		$40.7 \pm 0.7(12)^{bc}$	$43.3 \pm 1.6(6)^{ab}$
+/hpx	Fe+	$39.6 \pm 4.4(2)^{abc}$	-	$45.8 \pm 1.1(5)^{a}$
+/hpx	Fe–		-	$40.1 \pm 1.0(6)^{bc}$

[1]Values are means ± SEM with number of mice in parentheses. Missing data are indicated by a hyphen. Values not sharing a common superscript letter are significantly different ($P < 0.05$). Statistical effects determined by ANOVA: time, $P < 0.005$; dietary iron, $P < 0.005$; strain × dietary iron, $P < 0.05$; strain main effect, time × strain, time × dietary iron, and three-way interaction not significant.

Table 3. Brain and heart iron concentrations of mice either wild-type (+/+) or heterozygotic for the hypotransferrinemia mutation (+/hpx) fed diets either deficient in (Fe–) or adequate in (Fe+) iron[1]

Strain	Dietary iron	Brain iron concentration (nmol g^{-1} wet weight)			Heart iron concentration (μmol g^{1} wet weight)		
		0 weeks	4 weeks	8 weeks	0 weeks	4 weeks	8 weeks
+/+	Fe+	$323 \pm 6(7)^{de}$	$320 \pm 10(14)^{de}$	$405 \pm 13(6)^{a}$	$1.40 \pm 0.10(7)^{ab}$	$1.37 \pm 0.03(14)^{b}$	$1.55 \pm 0.10(6)^{a}$
+/+	Fe–		$326 \pm 5(12)^{de}$	$377 \pm 5(6)^{ab}$		$1.25 \pm 0.03(12)^{cd}$	$1.36 \pm 0.05(6)^{bc}$
+/hpx	Fe+	$307 \pm 24(4)^{e}$	$315 \pm 3(3)^{de}$	$348 \pm 6(5)^{cd}$	$1.20 \pm 0.09(4)^{cd}$	$1.36 \pm 0.02(3)^{abc}$	$1.36 \pm 0.06(5)^{abc}$
+/hpx	Fe–		$359 \pm 21(5)^{bc}$	$303 \pm 6(6)^{e}$		$1.14 \pm 0.05(5)^{d}$	$1.27 \pm 0.03(6)^{bcd}$

[1]Values are means ± SEM with number of mice in parentheses. Values for each organ not sharing a common superscript letter are significantly different ($P < 0.05$). Statistical effects determined by ANOVA (brain): time, $P < 0.0005$; strain, $P < 0.01$; time × strain, $P < 0.0001$; time × dietary iron, $P < 0.001$; dietary iron, strain × dietary iron, and three-way interaction effects not significant. Statistical effects determined by ANOVA (heart): time, $P < 0.05$; strain, $P < 0.005$; dietary iron, $P < 0.001$; two- and three-way interaction effects not significant.

(Figure 2A). Brain Tf concentrations after four and eight weeks were highest in wild-type iron-adequate mice, and lowest in +/hpx iron-adequate mice. A strain × dietary iron interaction was evident: in iron deficiency, brain Tf tended to be depressed in +/+ mice but elevated in +/hpx mice.

At weaning, brain iron levels did not differ significantly between wild-type and +/hpx mice (Table 3). Brain iron concentrations increased with time in the wild-type mice, whether fed the iron-adequate or iron-deficient diet. The +/hpx mice on the iron-adequate diet also accumulated brain iron but at a slower pace than the iron-adequate wild-type mice. Despite the increase in brain Tf in +/hpx mice on iron-deficient diets, brain iron concentrations in these mice were significantly decreased compared with iron-adequate +/hpx mice after eight weeks. Overall, brain iron concentrations did not correlate significantly with either plasma Tf or TIBC.

No strain or dietary iron differences were seen with regard to total brain ^{54}Mn uptake (Figure 3A). Brain ^{54}Mn uptake was not related to plasma Tf concentrations at any time, but correlated positively with plasma TIBC ($r = 0.26$, $P < 0.05$).

Liver

Liver Tf concentrations (Figure 2B) were identical between +/hpx and wild-type mice at weaning, despite the lower plasma Tf concentrations in +/hpx mice at that time. After four weeks, Tf concentrations increased over weanling values in both iron-adequate and iron-deficient wild-type mice, and +/hpx mice fed the iron-adequate diet. After four weeks on the iron-deficient diet liver Tf concentrations in +/hpx mice were significantly lower that in either wild-type mice or +/hpx mice fed the iron-adequate diet. By eight weeks, liver Tf concentrations were the same in all four groups.

Liver iron concentrations (Table 4) in male wild-type mice fed the iron-adequate diet remained similar from weaning throughout the study period; in female wild-type mice fed the iron-adequate diet, liver accumulated iron over the study period. Liver iron concentrations were clearly depressed by dietary iron deficiency in both wild-type and +/hpx mice. Iron-adequate +/hpx mice had higher liver iron concentrations than did wild-type mice at weaning and after four weeks. Liver iron concentrations cor-

Table 4. Liver and spleen iron concentrations of mice either wild-type (+/+) or heterozygotic for the hypotransferrinemia mutation (+/hpx) fed diets either deficient in (Fe–) or adequate in (Fe+) iron[1]

Strain	Dietary iron	Sex	Liver iron concentration (μmol g^{-1} wet weight)			Spleen iron concentration (μmol g^{-1} wet weight)		
			0 weeks	4 weeks	8 weeks	0 weeks	4 weeks	8 weeks
+/+	Fe+	M	$1.37 \pm 0.13(3)^{bc}$	$1.02 \pm 0.07(7)^{cd}$	$1.10 \pm 0.12(3)^{cde}$	$2.49 \pm 0.19(3)^{defg}$	$2.72 \pm 0.12(7)^{def}$	$3.68 \pm 0.49(3)^{cd}$
+/+	Fe+	F	$1.24 \pm 0.07(4)^{bcd}$	$1.50 \pm 0.12(7)^{b}$	$1.92 \pm 0.17(3)^{a}$	$3.51 \pm 0.75(3)^{cde}$	$4.31 \pm 0.44(7)^{c}$	$6.79 \pm 0.48(2)^{ab}$
+/+	Fe–	M		$0.57 \pm 0.02(9)^{f}$	$0.71 \pm 0.05(3)^{ef}$		$2.21 \pm 0.19(8)^{fg}$	$2.15 \pm 0.21(3)^{fg}$
+/+	Fe–	F		$0.64 \pm 0.03(3)^{f}$	$0.87 \pm 0.09(3)^{def}$		$2.54 \pm 0.18(3)^{defg}$	$2.60 \pm 0.42(3)^{defg}$
+/hpx	Fe+	M	$2.16(1)^{a}$	$1.40 \pm 0.24(2)^{bc}$	$1.42 \pm 0.12(2)^{bc}$	$2.53(1)^{defg}$	$3.00 \pm 0.14(2)^{def}$	$5.93 \pm 0.54(2)^{b}$
+/hpx	Fe+	F	$2.06 \pm 0.06(3)^{a}$	$2.50(1)^{a}$	$2.09 \pm 0.50(3)^{a}$	$2.38 \pm 0.29(3)^{efg}$	$4.10(1)^{cd}$	$7.48 \pm 0.13(3)^{fg}$
+/hpx	Fe–	M		$0.57 \pm 0.02(3)^{f}$	$0.80 \pm 0.05(5)^{ef}$		$1.60 \pm 0.22(3)^{g}$	$2.31 \pm 0.41(5)^{fg}$
+/hpx	Fe–	F		$0.62 \pm 0.01(2)^{f}$	$0.78(1)^{def}$		$2.19 \pm 0.17(2)^{fg}$	$2.62(1)^{defg}$

[1]Values are means ± SEM with number of mice in parentheses. Values for each organ not sharing a common superscript letter are significantly different ($P < 0.05$). Statistical effects determined by ANOVA (liver): strain, $P < 0.0001$; dietary iron, $P < 0.0001$; sex, $P < 0.01$; strain × dietary iron, $P < 0.01$; time × sex, $P < 0.001$; dietary iron × sex, $P < 0.0001$; time, time × strain, time × dietary iron, strain × sex, time × strain × sex, time × strain × dietary iron, time × dietary iron × sex, strain × dietary iron × sex, and four-way interaction effects not significant. Statistical effects determined by ANOVA (spleen): time, $P < 0.0001$; dietary iron, $P < 0.0001$; sex, $P < 0.0005$; time × strain, $P < 0.05$; time × dietary iron, $P < 0.0001$; strain × dietary iron, $P < 0.05$; time × sex, $P < 0.05$; dietary iron × sex, $P < 0.005$; strain, strain × sex, time × strain × dietary iron, time × strain × sex, strain × dietary iron × sex, time × dietary iron × sex, and four-way interaction effects not significant.

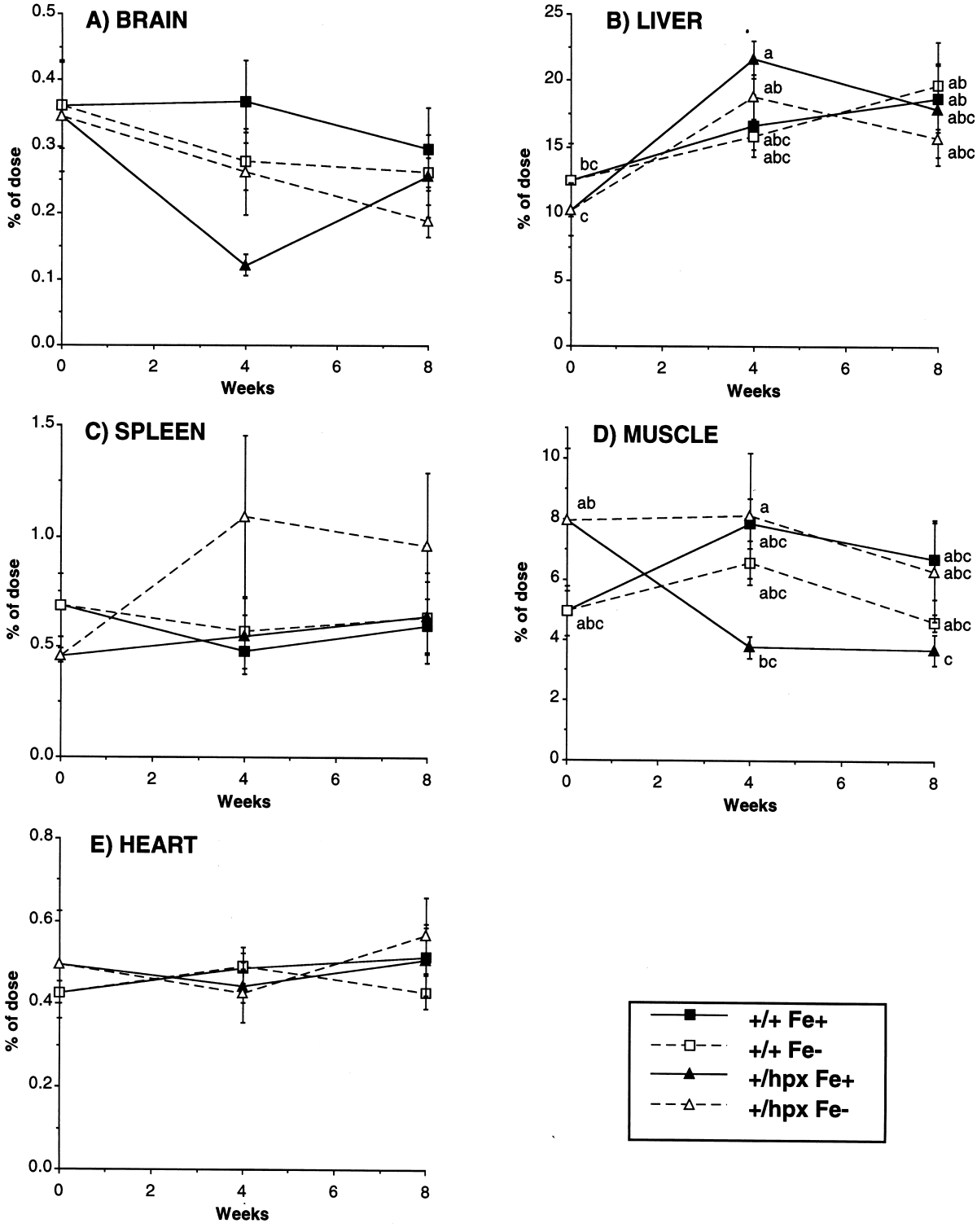


Figure 3. ^{54}Mn accumulation 24 h after an i.p. injection in: (A) brain; (B) liver; (C) spleen; (D) muscle; and (E) heart of wild-type (+/+) mice or mice heterozygotic for the hypotransferrinemia mutation (+/hpx) fed diets either deficient in (Fe–) or adequate in (Fe+) iron. Statistical effects determined by ANOVA for (B) liver: time, $P < 0.05$; strain, dietary iron, and two- and three-way interaction effects not signficiant; (D) muscle: time × strain, $P < 0.05$; main effects, time × dietary iron, strain × dietary iron, and three-way interaction effects not significant. $n = 3$–14 mice per treatment per time point.

related negatively with both plasma Tf ($r = -0.57$, $P < 0.0001$) and TIBC ($r = -0.28$, $P < 0.05$). No significant strain or dietary iron effects were seen in liver ^{54}Mn uptake at any time point (Figure 3B).

There appeared to be more liver ferritin in +/hpx than in wild-type mice at weaning (Figure 4). Liver ferritin then declined from weaning over the eight weeks of the study in both wild-type and +/hpx mice. Liver ferritin was greatly diminished in both wild-type and +/hpx mice fed the iron-deficient diet.

Spleen

Spleen Tf concentrations (Figure 2C) fell in wild-type mice over the study period, and were not affected by dietary iron. Spleen Tf concentrations in +/hpx mice fell even more than those of wild-type mice after four weeks. By contrast to the wild-type mice, spleen Tf concentrations dramatically increased in iron-deficient +/hpx mice between four and eight weeks.

Spleen iron concentrations (Table 4) showed similar dietary iron and sex effects to those of liver. Both wild-type and +/hpx mice fed the iron-adequate diet accumulated iron in spleen over the study period, especially the female +/hpx mice. Spleen iron concentrations were clearly depressed by dietary iron deficiency in both wild-type and +/hpx mice. We observed no significant strain or dietary iron effects on spleen ^{54}Mn uptake (Figure 3C).

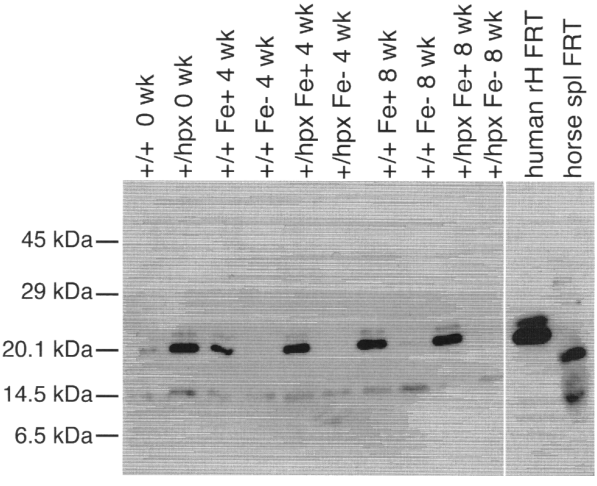


Figure 4. Western blot of liver ferritin in mice either wild-type (+/+) mice or mice heterozygotic for the hypotransferrinemia mutation (+/hpx) fed diets either deficient in (Fe–) or adequate in (Fe+) iron, killed either at weaning (0 weeks) or after 4 or 8 weeks of dietary treatment. Pooled liver post-nuclear supernatant (200 μg protein per lane) and ferritin standards (16 μg per lane) were subjected to 10% SDS-PAGE; the primary antibody was rabbit anti-human liver ferritin.

Muscle

Muscle Tf (Figure 2D) concentrations in +/hpx mice were one-third that of wild-type mice at weaning, and remained lower throughout the study. In wild-type mice, muscle Tf concentrations remained similar throughout the study period, whereas in the +/hpx mice we observed an increase over time. Muscle iron concentrations were not affected by time, strain or iron deficiency, and averaged 0.30 ± 0.01 μmol g^{-1} wet weight.

We observed a strain × dietary iron interaction on muscle ^{54}Mn uptake after four weeks of feeding (Figure 3D). At that time point, iron-adequate +/hpx mice had less ^{54}Mn uptake in muscle than did iron-adequate wild-type mice.

Heart

Heart Tf concentrations (Figure 2E) rose over the study period in both wild-type and +/hpx mice. There were no strain or dietary iron effects on heart Tf concentrations.

Heart iron concentrations (Table 3) increased over time in both wild-type and +/hpx iron-adequate mice. Iron deficiency resulted in lower heart iron concentrations in both wild-type and +/hpx mice. We observed no significant time, strain or dietary iron effects on heart ^{54}Mn uptake (Figure 3E).

Discussion

In general, we observed significant differences in Tf (in plasma and muscle) and Fe concentrations (in liver and heart) in wild-type versus +/hpx mice; these differences were present as early as weaning. In addition, we found differences in the dynamics of the response between wild-type and +/hpx mice to iron-deficient diets; these differences were organ specific. Most notably, our study revealed that the Tf level in plasma of +/hpx mice can increase from its initial level (less than half of wild-type) if challenged with an iron-deficient diet. The increase in plasma Tf in +/hpx mice in response to an iron-deficient diet places the Tf concentration in the range of normal. This observation raises a major question about what regulates Tf plasma levels and why the +/hpx mice would have less than half of normal levels. Different strains of mice are able to achieve iron homeostasis with a wide range of TIBC values and saturations (Leboeuf *et al.* 1995). One possible explanation for our observations is that plasma Tf is removed at a greater rate in the +/hpx mice because it is more highly saturated with iron. Indeed,

when apo-Tf is injected into +/hpx mice its $t_{1/2}$ is higher than normal (Raja *et al.* 1995).

Tf developmental response

Plasma Tf concentrations during postnatal development are not static; in wild-type mice they increased from weaning over the eight weeks examined. In the +/hpx mice, Tf plasma concentrations were initially at 28% of normal and decreased to 20% of normal at the end of the eight weeks of analysis. This observation is in general agreement with reports that adult +/hpx mice have lower than normal circulating Tf levels (Bernstein 1987).

At weaning, muscle Tf concentrations are lower than normal in +/hpx mice, but brain, liver, spleen and heart Tf concentrations are the same in wild-type and +/hpx mice. Each of these organs synthesize Tf (Morgan 1969, Levin *et al.* 1984) and Tf concentrations measured in this study reflect both Tf which has been endocytosed and endogenously synthesized Tf. The key observation is that normal Tf, and perhaps more importantly, normal iron concentrations are present in the tissues despite the lower than normal levels of Tf in the plasma. The results suggest that differic Tf is removed more rapidly from plasma in the +/hpx mice, presumably because diferric Tf has a higher affinity for the Tf receptor. There is no up-regulation of the transferrin receptor on brain endothelial cells of +/hpx or hpx/hpx mice (Dickinson & Connor 1998).

The tissue iron distribution at weaning likely reflects contribution from maternal sources including lactoferrin in the milk obtained by the suckling pups. Thus, the analysis at four to eight weeks after weaning is a more direct assessment of the endogenous transport mechanisms of iron and Mn in the +/hpx mouse. Four weeks after weaning, Tf was decreased in brain, muscle and spleen of +/hpx mice, but iron levels were similar to normal. After eight weeks of the dietary treatments, Tf concentrations were still depressed in brain and spleen of +/hpx mice, but only brain showed decreased iron concentrations. Although the brain has the capacity to synthesize Tf as other organs, the other organs (except muscle) do not have a deficit in Tf concentrations. Furthermore, only the brain has a deficit in iron concentration. Mn accumulation in brain was not affected. The explanation for this observation may lie in the fact that plasma Tf has limited access to the brain because of the blood–brain barrier. This limited access of Tf (and consequently iron) could explain the slower rate of iron accumulation than normal in the +/hpx mouse between four and eight weeks postweaning. Tf in brain could be stimulated by iron, thus accounting for the lower than normal Tf levels in brain because there is less iron. The ability of iron to stimulate Tf synthesis may be true of all organs, but is only a factor in brain Tf concentrations because of the lack of direct delivery of plasma iron via plasma Tf to neurons. Furthermore, iron and Tf mRNA are predominantly found in ologodendrocytes in brain (Bartlett *et al.* 1991, Dickinson & Connor 1995) but Tf receptors are found predominantly on endothelial cells and neurons (Fishman *et al.* 1987).

Tf iron deficiency response

The increase in plasma Tf in the +/hpx mice in response to dietary iron deficiency that we observed indicates that there is a mechanism for plasma levels to increase in +/hpx mice. The ability of the +/hpx mice to increase plasma Tf levels in iron deficiency may come at the expense of iron levels in the liver, which were significantly lower in the +/hpx iron-deficient mice four weeks after feeding the iron-deficient diet. These data are consistent with the notion that iron saturated Tf is cleared more quickly from the plasma.

In wild-type mice, plasma Tf concentrations were unaffected by dietary iron intake even though iron-deficient diets resulted in a significant loss of plasma iron. Tissue Tf concentrations were not affected by dietary iron deficiency, except for the decrease in liver Tf in the +/hpx mice at four weeks. Liver, heart and spleen, but not brain iron concentrations were decreased in the wild-type mice following iron-deficient diets.

After four weeks on the iron-deficient diet, brain iron concentrations in +/hpx mice were elevated compared with +/hpx mice on normal iron diets. Because plasma Tf also increased in the +/hpx mice in response to iron-deficient diets, it is possible that the increased plasma Tf targeted iron to the brain preferentially, similar in concept to the 'glucose-sparing' effect. The elevated plasma Tf concentrations were not sufficient to maintain brain iron accumulation, because by eight weeks of iron-deficient diets, brain (and all other organs but muscle) had lower than normal iron concentrations. However, brain iron content continued to increase during iron deficiency, suggesting either a continued selective targeting or implying a non-Tf dependent contribution to brain iron. We have previously reported that iron injected systemically in the hpx/hpx mice, which have no circulating Tf, can be found in brain after 24 h (Dickinson *et al.* 1996), and may even enter the brain at higher rates (Ueda *et al.* 1993). In cultured glial cells from hpx/hpx mice (as well as wild-type controls) there is also

uptake and release of iron in the absence of Tf (Takeda *et al*. 1998). These studies imply that a non-Tf mediated transport system for iron may exist. Identification of the existence of non-Tf mediated transport systems is critical to understanding the mechanism behind iron accumulation in numerous neurological disorders such as Alzheimer's, Parkinson's, Frederich' ataxia and Huntington's diseases (Connor 1997). Candidates for non-Tf dependent iron uptake systems include p97 or melanotransferrin (Kennard *et al* . 1995) and Nramp2 (natural resistance-associated macrophage protein) or DCT-1 (divalent cation transporter; Fleming *et al*. 1997, Gunshin *et al*. 1997).

Mn accumulation

Twenty-four hour accumulation of Mn from the i.p. dose correlated with plasma TIBC but not with circulating Tf concentrations, thus implying that acute Mn uptake into the brain is not Tf dependent. A previous study from this laboratory showed similar Mn accumulation 24 h after a subcutaneous dose in chow-fed wild-type mice, +/hpx mice and homozygotes for hypotransferrinemia (Dickinson *et al*. 1996). Furthermore, Mn uptake and release was not affected by the presence or absence of Tf in a study using glial cell cultures of hpx/hpx mice (Takeda *et al*. 1998). A study showing increased Mn in hypothalamus in iron deficiency (Shukla *et al*. 1989) was confounded by the well-known upregulation of both Fe and Mn intestinal uptake in Fe deficiency (Thomson & Valberg 1972, Davis *et al*. 1992). Chua & Morgan have shown (1996) that Mn uptake in brain from intravenous (i.v.) dose was not affected by dietary iron deficiency in 15 day or 63 day old rats, but was actually potentiated by iron overload in 15 day old rats. We show in this study that when intestinal uptake is circumvented by an i.p. injection, total brain Mn accumulation after 24 h is not affected by iron deficiency. If Tf-mediated uptake is not the major mechanism for Mn entry into the brain, other mechanisms must be operating. The identity and role of the non-Tf binding species in plasma for Mn delivery to tissues remains to be further studied. Mn transport could be facilitated by complexation with a small molecule such as ascorbic acid; guinea pigs supplemented with vitamin C had increased brain Mn and Fe concentrations (Seaborn *et al*. 1994).

In conclusion, this study establishes the mice heterozygotic for hypotransferrinemia as a model for studying the Tf dependence of metal transport. Our data suggest that one or more non-Tf mediated transport systems must exist for Mn and may exist for iron. These mice also provide a sensitive system for the study of Tf regulation in iron deficiency and reveal the potential for organ specific regulation.

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