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Development and Iron-Dependent Expression of Hephaestin in Different Brain Regions of Rats

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Abstract It has been suggested that Hephaestin (Heph), a newly discovered ceruloplasmin homologue, is necessary for iron egress from the enterocytes into circulation via interacting with ferroportin1 (FP1). Based on the putative function of Heph, and the similarity between the process of iron transport in the enterocytes and that in the blood-brain barrier (BBB) cells, it has also been proposed that Heph plays a similar role in exporting iron from the BBB cells and other brain cells as it works in the enterocytes via interacting with FP1. The existence of FP1 in the brain has been demonstrated. In this study, we investigated Heph expression and effects of development and iron in the cortex, hippocampus, striatum, and substantia nigra. The data demonstrated that all the four regions we examined have the ability to express Heph mRNA and protein. The findings also showed that both the development and iron status have a significant effect on Heph expression and the effects of iron status are regionally specific. It was also suggested that Heph expression is probably regulated at the transcriptional level by the development and iron in these brain regions. These findings, together with other published data, support a putative role of Heph in the iron metabolism in the brain. J. Cell. Biochem. 102: 1225–1233, 2007.
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Key words: brain iron metabolism; hephaestin expression; iron transport; brain iron; development; brain regions

Hephaestin (Heph), a newly discovered ceruloplasmin homologue, is mutated in the sex-linked anemia (*sla*) mouse. Northern blot analysis in adult mouse revealed a high Heph expression in the small intestine and colon, and a low level of expression in other tissues [Vulpe et al., 1999]. The diminished transport of iron from the mucosal cell to the circulation in *sla* mice implies that Heph has a role in the physiological iron absorption in the small intestine. However, Heph contains only one predicted carboxy-terminal transmembrane domain, therefore, it is unlikely that Heph itself is a membrane iron transporter [Vulpe et al., 1999; Anderson et al., 2002]. It might interact with ferroportin1 (or Ireg1/MTP1) in the process of iron transport across the basolateral membrane of enterocytes [Anderson et al., 2002]. Iron in the intestinal lumen might first be reduced by duodenal cytochrome b and then cross the apical membrane by the divalent metal transporter 1 [Gunshin et al., 1997]. Once inside the cells, iron might either be stored in ferritin or transported across the basolateral membrane by ferroportin1 [Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000]. The form of iron that is exported by ferroportin1 might be Fe2+ [Donovan et al., 2000]. Heph might oxidize Fe2+ to Fe3+ and then load iron (Fe3+) onto transferrin in general circulation [Sargent et al., 2005; Wessling-Resnick, 2006].

The process that occurred in the enterocytes is very similar to that of iron transport across

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the blood-brain barrier (BBB) cells [Qian and Shen, 2001; Qian et al., 2002]. In the BBB, iron first crosses the apical membrane of endothelium, probably by a transferrin receptor-mediated pathway, and then possibly also in the form of Fe2+, crosses the basolateral membrane and enters into the brain [Bradbury, 1997; Moos and Morgan, 1998; Qian and Shen, 2001]. The relevant mechanism remains a mystery. However, based on the similarity of the two processes and the proposed function of Heph (and ferroportin1) in the enterocytes, it is possible that Heph might play a similar role in iron export from the BBB cells as it works in the enterocytes [Qian and Shen, 2001; Ke and Qian, 2003]. It is also possible that Heph might have a function in iron efflux from other brain cells because ceruloplasmin, the only known iron 'release' protein, might not play such a role in these cells due to its unique location and expression form in the brain. Recent studies have demonstrated the existence of ferroportin 1 in the brain [Burdo et al., 2001; Jiang et al., 2002; Jeong and David, 2003; Wu et al., 2004; Chang et al., 2006]. Localization of Heph in Muller glia and retinal pigment epithelium (a blood-brain barrier) has been reported [Hahn et al., 2004]. A pathological study using retinas from mice deficient in Heph (and ceruloplasmin) indicated that Heph is critical for CNS iron homeostasis and that the loss of Heph in mice would lead to an age-dependent retinal neurodegeneration [Hahn et al., 2004]. In this study, we investigated the effects of iron and age on Heph expression in the cortex, hippocampus, striatum and substantia nigra in male Sprague–Dawley rats. The results showed that expression of Heph is probably regulated at the transcriptional level by both age and iron and that the effects of iron status are regionally specific.

MATERIALS AND METHODS

Materials and Animals

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co., St. Louis, MO. Agarose, Hepes, SDS, acrylamide, bisacrylamide Ethidium Bromide and prestained protein marker were purchased from Bio-Rad Laboratories, Hercules, CA and Trizol® Reagent nd Taq DNA polymerase was from Invitrogen, Carlsbad, CA. ExpressHyb hybridization solution was obtained from Clontech, Palo Alto, CA, and Prime-a-Gene labeling system was from Promega, WI. Advantage™ RT-for-PCR kit from Clontech and RNeasy Mini kit and QIAEX II gel extraction kit were obtained from QIAGEN Valencia, CA. Micro Spin G-50 column, [α-^{32}P]dCTP and ECL Western blotting analysis system kit were purchased from Amersham Biosciences, England, and the antibodies against Heph were from Alpha Diagnostic, San Antonio, TX. Male Sprague–Dawley (SD) rats were supplied by the Centralized Animal Facilities of The Hong Kong Polytechnic University. The Health Department of the Hong Kong Government and Animal Ethics Committee of The Hong Kong Polytechnic University approved the use of animals for this study. All the animals were housed in pairs in stainless steel cages at $21 \pm 2^{\circ}$C with relative humidity of 60–65% and alternating 12-hour periods of light (7:00–19:00) and darkness (19:00–7:00).

Experimental Design

To investigate the effect of the variations in dietary iron on Heph expression, male SD rats (21 days of age) in the control ($n = 7$), the high iron ($n = 7$) and the low iron ($n = 7$) groups were fed with the Basal Purified Diet (containing 60 mg Fe/kg diet) (PMI, Catalog #7024), the Basal Purified Diet supplied with 2.5% carbonyl iron (PMI, Catalog #43784) and the Low Iron Purified Diet containing no added iron (PMI, Catalog #7444) (residue of 10 mg Fe/kg diet); respectively [Ke et al., 2003]. The diets were given ad libitum to the rats for 8 weeks. For the determination of Heph expression in the different brain regions of the developing rat, male SD rats at postnatal weeks (PNW) 1, 3, 9, and 28 (All $n = 7$) were used. All animals were provided free access to food (the Laboratory Rodent Diet, PMI, Brentwood, MO, Cat#5001) and distilled water at all times. At the end of the period or different ages, the animals were anesthetized with 1% pentobarbital sodium (40 mg/kg body weight, i.p.) and decapitated. Blood samples were then collected into heparinized syringes and aliquots were taken immediately for the determination of hemoglobin (Hb) concentration and hematocrit (Hct). The serum samples were analyzed for serum iron, total iron-binding capacity (TIBC) and transferrin saturation (serum iron/TIBC). After perfusion with ice-cold phosphate-buffered saline (PBS, Milli-Q water prepared and DEPC

treated, pH 7.4) through the left ventricle, the brain was rapidly removed and immediately dissected into four brain regions: cortex, hippocampus, striatum and substantia nigra. Iron measurements, RT-PCR or Northern blot (Heph mRMA) and Western blot analysis (protein) were then conducted.

RT-PCR Amplification and Sequence Analysis

Total RNA was isolated from the different brain regions of rats of different ages by the RNeasy Mini Kit according to the manufacturer's instructions. The relative purity of the isolated RNA was assessed spectrophotometrically and the ratio of A260–A280 nm exceeded 1.9 for all preparations. The total RNA (1 μg) was reversely transcribed in a 20 μl reaction using the Advantage RT-for-PCR kit. The forward primer sequence of Heph was 5′-AAGGCAGAGGATGGAATCAG-3′ (3,659–3,678 nt) and the reverse primer sequence was 5′-CGAACATGGAGAGGACACTC-3′ (4,182–4,201 nt). Amplification was performed with an initial denaturation at 94°C for 3 min, followed by 28 cycles at 94°C (45 s), 60°C (45 s) and 72°C (2 min), and a single final extension at 72°C for 7 min using the GeneAmp® PCR System 9700. The reaction mixture lacking reverse transcriptase was used as a negative control and β-actin cDNA (forward primer 5′-GGTCACCCACACT-GTGCCCATCTA-3′, reverse primer 5′-GACCGT-CAGGCAGCTCACATAGCTCT-3′) was amplified simultaneously as the internal control. The PCR products were analyzed on a 1.8% agarose gel using LumiAnalyst Image Analysis software (Roche, Mannheim, Germany). Gene expression values were normalized for β-actin expression and expressed in units relative to the controls. The products were confirmed by sequencing using a fluorescent-tagged dideoxy chain termination method, and then analyzed with an ABI Prism™ 310 Genetic Analyzer (PE Applied Biosystems, Inc., Foster City, CA).

Northern Blot Assay

Trizol® Reagent was used to isolate the total RNA from the different brain regions of rats pretreated with different iron diets according to the manufacturer's instructions. The relative purity of the isolated RNA was assessed spectrophotometrically and the ratio of A260–A280 nm exceeded 1.9 for all preparations. All Heph and β-actin cDNAs used as probes were obtained by cloning the RT-PCR products of the total RNA prepared from the brain of the rats, and their identities were confirmed by sequencing. The sequence numbers used to amplify Heph were 3,659–4,201 (Genbank, AF246120). The RNA samples (30 μg) were electrophoresed on 1.2% formaldehyde-agarose gels. They were then transferred to the Hybond-N membranes and then immobilized using an UV cross-linker (Fisher). The blots were prehybridized at 65°C in the ExpressHyb hybridization solution for 1 h, and then hybridized overnight at 65°C in the same solution which contained a ^{32}P-labeled probe using the Prime-a-Gene labeling system (Promega). After three 10-min washes with 2× standard sodium chloride-sodium citrate (SSC) containing 0.05% sodium dodecyl sulfate (SDS) at room temperature, the blots were washed in 0.1× SSC containing 0.1% SDS with continuous shaking at 50–60°C for 10 min for 3–4 times. Radioactivity was then detected by a phosphorimager and quantified using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). For normalization, the blot was stripped and re-probed with a β-actin probe corresponding to the position 474–736 of the rat β-actin (Genbank, NM031144). The results were expressed as the ratio to β-actin.

Western Blot Analysis

Brain tissues were washed and homogenized in a RIPA buffer containing 1% Triton X-100 and protease inhibitors (pepstatin 1 μg/ml, aprotinin 1 μg/ml, leupeptin 1 μg/ml). After centrifugation at 10,000*g* for 15 min at 4°C, the supernatant was collected and the protein content assayed. The proteins (40 μg) were diluted in 2× sample buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% β-mercaptoethanol) and heated for 5 min at 95°C before SDS–PAGE on 10% gel and subsequently transferred to a pure nitrocellulose membrane (Bio-Rad). Then, the membrane was blocked with a 5% blocking reagent (Amersham Biosciences) in TBS containing 0.1% Tween-20 for 2 h at room temperature. The membrane was rinsed in three changes of TBS-T, incubated once for 15 min and twice for 5 min in a fresh washing buffer, and then incubated with a primary antibody (rabbit anti-mouse Heph polyclonal antibody) at 1:5,000 for 2 h at room temperature. After three washes in the washing buffer, the membrane was incubated for 2 h in a horseradish peroxidase-conjugated anti-rabbit second antibody (1:5,000) and developed using enhanced chemiluminescence (ECL

western blotting analysis system kit). The blots were detected by the Lumi-imager F1 workstation (Roche Molecular Biochemical). The intensity of the specific bands was determined by densitometry with the use of the LumiAnalyst 3.1 software (Roche Molecular Biochemical). To ensure even loading of the samples, the same membrane was probed with the rabbit anti-human β-actin polyclonal antibody (Sigma-Aldrich) at a 1:5,000 dilution.

Statistical Analysis

Total iron in the different brain regions was determined using a graphite furnace atomic absorption spectrophotometer (Perkin Elmer, A100). The results were expressed as the Means ± SEM. Difference between the means was determined by one-way ANOVA followed by a Student-Newman-Keuls test for multiple comparisons. A probability value of $P < 0.05$ was taken to be statistically significant.

RESULTS

Effect of Age and Dietary Iron on Iron Content in Different Brain Regions

The findings from rats of different ages showed that the development has a significant effect on the brain iron content (Table I). This was similar to the observations made by other investigators [Erikson et al., 1997; Pinero et al., 2000]. Total iron concentrations in the cortex, striatum and substantia nigra were significantly higher in the PNW3 rats than in the PNW1 rats, while in the hippocampus, a significant increase in the iron content was found in the PNW9 rats as compared with the PNW1 rats. However, there were no significant differences in the total iron levels in the cortex or hippocampus between the rats of PNW9 and 28, and in the substantia nigra among the rats of PNW3, 9, and 28, although the iron content in these regions increased with the increase of age after PNW3.

The rats that were fed with a low-iron diet for 8 weeks had significantly lower Hb, Hct, transferrin saturation, TIBC, serum iron and non-heme iron in the liver as compared to the controls. In rats that were treated with a high-iron diet, all these indicators were significantly higher than those of the control rats (data not shown). The total iron concentrations measured in all four regions were significantly lower in the low-iron rats and higher in the high-iron rats than those in the control animals (all $P < 0.05$ or 0.01; Table II). The results were in good agreement with our previous studies [Ke et al., 2005; Chang et al., 2005] and the findings reported by Pinero et al. [2000]. They also reconfirmed that treatment with a low- or high-iron diet for a given period under our experimental conditions could alter the brain iron level.

Effect of Age on Expression of Heph in the Cortex, Hippocampus, Striatum, and Substantia Nigra

Expression of Heph in the different brain regions of the PNW1, 3, 9, and 28 rats was investigated using RT-PCR (mRNA) and Western blot (protein) analysis respectively. The results showed that all the four regions we examined have the ability to express Heph mRNA and protein and that the expression is significantly affected by age (Figs. 1 and 2). The changes in Heph mRNA expression that were induced by age displayed a very similar tendency in all brain regions including the cortex, hippocampus, striatum and substantia nigra (Fig. 1). The lowest expression of Heph mRNA in all the four brain regions examined was all found in the rats of PNW1. The mRNA expression in the rats of PNW3, 9, and 28 was significantly higher than that in the rats of PNW1 and the highest expression of Heph

TABLE I. Iron Content in Different Brain Regions of Developing Rats

	PNW1 (n = 7)	PNW3 (n = 7)	PNW9 (n = 7)	PNW28 (n = 7)
Cortex	179.1 ± 7.93	252.2 ± 9.32**	291.6 ± 7.84***,##	312.1 ± 7.11***,#
Hippocampus	221.1 ± 6.34	214.1 ± 6.22	297.5 ± 7.36**,##	298.8 ± 4.88**,#
Striatum	232.4 ± 5.89	283.1 ± 9.21**	290.4 ± 7.67**	313.3 ± 7.69***,#,$
Substantia nigra	281.9 ± 7.55	333.1 ± 9.28**	342.2 ± 8.41**	346.1 ± 8.81**

Data were Means ± SEM nmol/g wet weight of brain tissue.
**$P < 0.01$.
***$P < 0.001$ versus PNW1 (postnatal week 1).
#$P < 0.05$.
##$P < 0.01$ versus PNW3.
$$P < 0.05$ versus PNW9.

TABLE II. Regional Iron Concentrations in the Rats Fed With Low-Iron (LF), Normal Iron (Control) or High-Iron (HF) Diet for 8 Weeks

	Control (n = 7)	LF (n = 7)	HF (n = 7)
Cortex	283.2 ± 7.68	251.1 ± 6.44**	332.2 ± 6.67**
Hippocampus	291.8 ± 5.51	228.2 ± 6.52**	347.7 ± 5.31**
Striatum	282.2 ± 8.77	243.3 ± 5.44*	372.6 ± 7.18**
Substantia nigra	331.6 ± 9.21	242.5 ± 6.55**	388.7 ± 11.14**

Data were presented as Mean ± SEM nmol/g wet weight of brain tissue.
*$P < 0.05$.
**$P < 0.01$ versus the control.

mRNA was found in all the brain regions of the rats of PNW9.

The results also revealed that changes in the contents of Heph protein in all brain regions corresponded to changes of its mRNA level in the rats aged PNW1, 3, 9, and 28. Increase in the Heph protein expression was in parallel with increase in Heph mRNA in all four regions during the period from PNW1 to 9 (Fig. 2). The highest expression of Heph protein was also found in all the brain regions of the PNW 9 rats. Both the expression of Heph mRNA and protein were relatively lower in the rats of PNW28 than in those of PNW9. The similar regulation

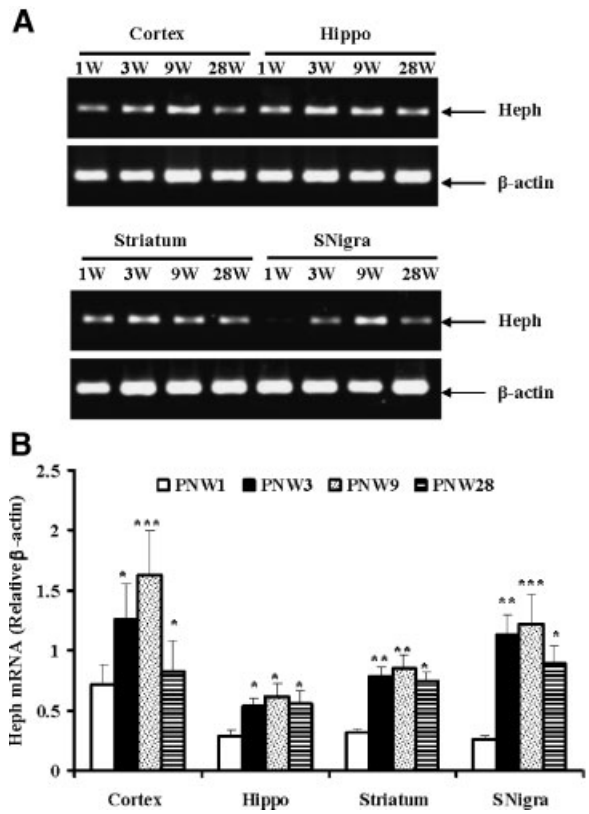

Fig. 1. Expression of hephaestin (Heph) mRNA in the cortex, hippocampus (Hippo), striatum and substantia nigra (SNigra) of rats at the ages of postnatal weeks (PNW)1, 3, 9, and 28. Total RNA (1 μg) was reversely transcribed and amplification was performed as described in Materials and Methods. The PCR products were analyzed on a 1.6% agarose gel using LumiAnalyst Image Analysis software. **A**: A representative experiment of RT-PCR products of Heph and β-actin mRNA. **B**: Summarized data of intensity. Data were the Mean ± SEM (n = 7). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus PNW1.

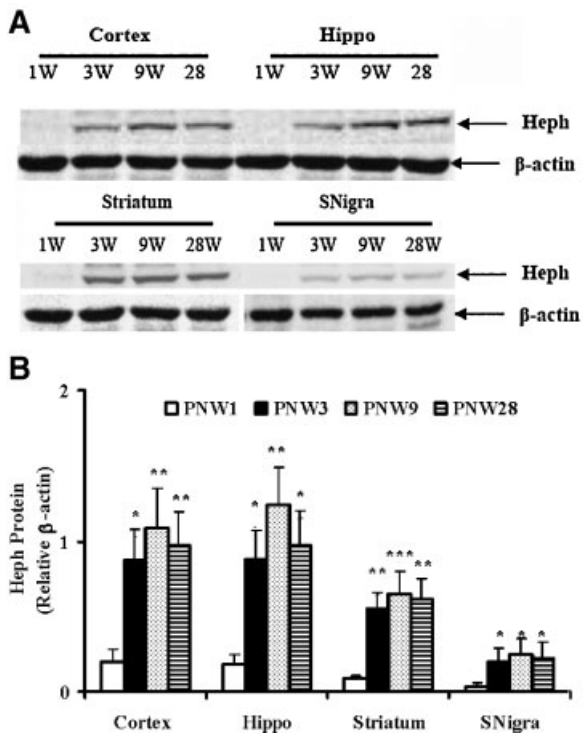

Fig. 2. Expression of hephaestin (Heph) protein in the cortex, hippocampus (Hippo), striatum and substantia nigra (SNigra) at the ages of postnatal week (PNW)1, 3, 9, and 28. Brain tissues were washed, homogenized and centrifuged, and forty micrograms of protein were diluted, heated and subsequently transferred to a pure nitrocellulose membrane. The membrane was blocked, rinsed and then incubated with primary antibody and secondary antibody as detailed in Materials and Methods. **A**: A representative experiment of Western blots of the Heph protein. **B**: Summarized data of intensity. Data were the Mean ± SEM (n = 7). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus PNW1.

patterns between the expression of Heph mRNA and protein suggested the existence of an age-dependent transcriptional regulation.

Effects of Dietary Iron on Hephaestin Expression in the Cortex, Hippocampus, Striatum, and Substantia Nigra

Expression of Heph mRNA and protein in rats fed with the normal, low- or high-iron diets were investigated using Northern blot and Western blot analysis respectively. After the rats were fed with the different iron diets for 8 weeks, total iron in the cortex, hippocampus, striatem, and substantia nigra was significantly changed (Table II). A significant effect of iron status on the expression of Heph mRNA and protein in the different brain regions was also observed (Figs. 3 and 4). Representative Northern blot showing the expression of Heph was presented in Figure 3A. One mRNA band with molecular mass ~5 kb was detected with a randomly primed ^{32}P-labeled probe for Heph. This molecular weight was in agreement with those observed in the mice intestinal tissue and

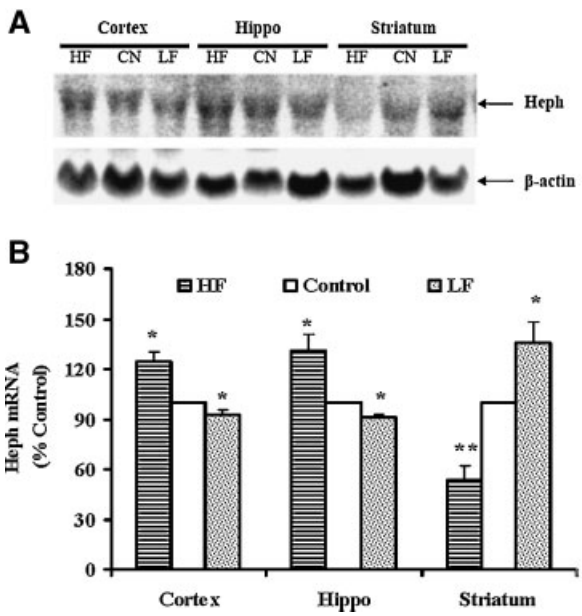

Fig. 3. Effect of iron status on the expression of hephaestin (Heph) mRNA in the cortex, hippocampus (Hippo), striatum and substantia nigra (SNigra) of rats fed with a low-iron (LF), normal iron (Control) or high-iron (HF) diet for 8 weeks. Total RNA was isolated from different brain regions of the rats pre-treated with different iron diets and Northern blot assay was conducted as detailed in Materials and Methods. **A**: A representative experiment of Northern blot analysis of Heph and β-actin mRNA. **B**: Summarized data of intensity. Data were the Mean ± SEM (% control, n = 7). $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control.

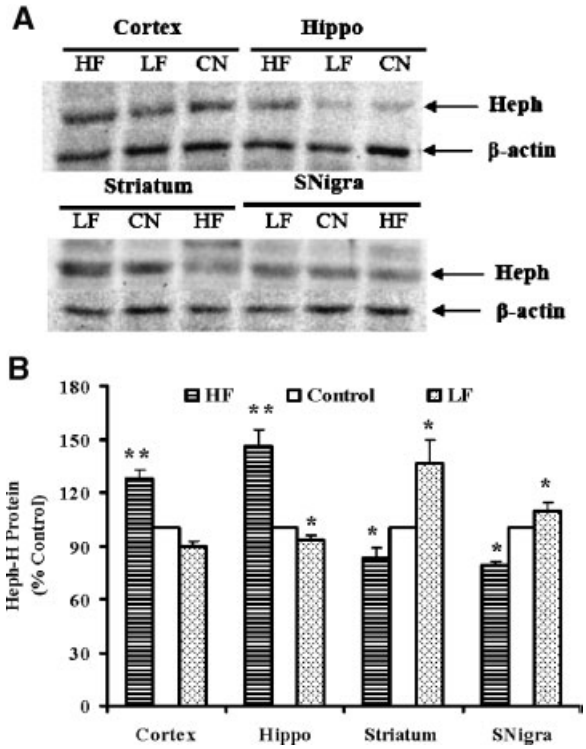

Fig. 4. Effect of brain iron status on hephaestin (Heph) protein synthesis in the cortex, hippocampus (Hippo), striatum and substantia nigra (SNigra) of rats fed with a low-iron (LF), normal iron (Control) or high-iron (HF) diet for 8 weeks. **A**: A representative experiment of Western blots of the Heph protein and β-actin protein. **B**: Summarized data of intensity. Data were the Means ± SEM (% control, n = 7). $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control.

Caco-2 cells [Vulpe et al., 1999; Han and Wessling-Resnick, 2002].

In rats fed with a high-iron diet, the levels of Heph mRNA significantly increased in the cortex and hippocampus but dramatically decreased in the striatum as compared to the controls (Fig. 3). Also, expression of Heph protein significantly increased in the cortex and hippocampus and decreased in the striatum (Fig. 3). The effect of iron status on the expression of Heph protein in the substantia nigra was similar to that in the striatum (Fig. 3). Heph mRNA expression in the substantia nigra was not measured because not enough tissue samples could be obtained. In contrast, in rats fed with the low-iron diet, Heph mRNA was significantly down-regulated in the cortex and hippocampus but up-regulated in the striatum ($P < 0.05$ or 0.01, Fig. 3), and expression of Heph protein decreased in the cortex and hippocampus but significantly increased in the

striatum and substantia nigra (both $P < 0.05$, Fig. 3). These findings showed that the effects of iron status on Heph expression in the cortex and hippocampus were different from those in the striatum and substantia nigra. The reasons for the regionally specific effects of iron status on Heph g expression are unknown. However, Heph protein expression almost corresponded to its mRNA level in all regions examined although the effects were regionally specific. The similar regulation patterns between Heph mRNA and protein expression suggested that the regulation of Heph expression by iron might also occur at the transcriptional level.

DISCUSSION

Intracellular iron balance is dependent not only upon the amount of iron uptake by but also the amount of iron released from cells. The excessive accumulation of iron in the brain found in some neurodegenerative diseases may result from an increased uptake by as well as a decreased release from cells [Qian and Wang, 1998]. Little is known about the molecules involved in the iron efflux from brain cells under physiological conditions. Although ceruloplasmin is widely believed to have a role in the iron release from the cells [Harris et al., 1999], the expression of ceruloplasmin in the brain is only observed in those astrocytes predominantly surrounding the microvasculature, but not in all glial cells and other brain cells [Klomp et al., 1996]. Also, the GPI-anchored ceruloplasmin expressed by astrocytes is the predominant form of this protein in the brain [Patel and David, 1997; Patel et al., 2000]. The unique location and expression form of this protein in the brain suggest that after Fe2+ crosses the abluminal membrane of the BBB endothelial cells probably via ferroportin 1 [Jeong and David, 2003], ceruloplasmin might be necessary for Fe2+ to be oxidized to Fe3+ to enable the latter to bind to the transport carriers [Patel et al., 2000, 2002; Qian and Ke, 2001; Qian and Shen, 2001]. Also, these characteristics of ceruloplasmin, together with some recent studies in vitro on the effect of ceruloplasmin on iron transport in the brain glomia cells [Qian et al., 2001; Xie et al., 2002] and other types of cells [Mukhopadhyay et al., 1998; Attieh et al., 1999], imply the impossibility for ceruloplasmin to play any function in the iron efflux from other brain cells except for the BBB cells and astrocytes. Therefore, there is probably other molecule(s) that has a ceruloplasmin-like role in the brain, playing a role in the iron release from the relevant brain cells.

In the present study, we investigated expression of Heph in the brain and demonstrated that all the four regions we examined, the cortex, hippocampus, striatum, and substantia nigra, have the ability to express Heph mRNA and protein. The data also demonstrated that the development has a significant effect on iron contents as well as the expression of Heph mRNA and protein and that changes in the brain iron status induced by variations in dietary iron can significantly affect the expression of Heph mRNA as well as protein in all of these regions. These findings, together with the data on the functions of Heph in the release of iron from other types of cells, imply that Heph might be one of the molecules that have a ceruloplasmin-like role, playing a role in the iron export from the relevant cells in the brain. Iron (Fe2+) might first be transported across the membrane of these cells by ferroportin 1. Heph, acting as a ferroxidase [Griffiths et al., 2005; Petrak and Vyoral 2005], might oxidize Fe2+ to Fe3+ and subsequently load iron (Fe3+) onto transferrin or other iron carriers in the brain. The existence of ferroportin 1 in the brain is well-documented by some recent studies [Burdo et al., 2001; Jiang et al., 2002; Jeong and David, 2003; Wu et al., 2004; Chang et al., 2006] and this is in favor of this hypothesis, although further studies are still absolutely necessary.

It was found that the responses of Heph expression to age in different brain regions were very similar and also the trends in age-induced changes were similar between Heph mRNA and protein expression in all four regions. The similarity of regulation patterns or responses of Heph mRNA and protein suggested the existence of an age-dependent transcriptional regulation in these brain regions. We also found that the tendency in the iron-induced changes of Heph mRNA expression was very similar to that of Heph protein expression in all brain regions. The similar regulation patterns suggest that the regulation of Heph expression by iron might also occur at the transcriptional level. Studies in the duodenum also demonstrated that the regulation of Heph protein in response to variations in iron status is in general similar to that of the mRNA [Anderson et al., 2002].

These data suggest that Heph is not strongly regulated at the translational level in the duodenum and the brain. Unlike mRNAs for Ferroportin 1 and some other iron metabolism proteins, the message for Heph does not harbor an iron-responsive element (IRE). IREs are posttranscriptional regulators of gene expression [Han and Wessling-Resnick, 2002]. It might be the cause for Heph not to be strongly regulated at the translational level. Currently, little is known about the mechanisms involved in the iron induced-transcriptional regulation of Heph in the brain. Further work is necessary to understand this aspect.

In all the brain regions we examined, iron status showed a significant effect on Heph mRNA expression as well as protein. This finding is different from that found in the duodenum where variations in iron status can only induce a small but non-significant effect on Heph mRNA expression [Frazer et al., 2001; Sakakibara and Aoyama, 2002]. A study using the C57BL/6J mice showed that the enterocyte iron level (local signal of iron status) has no significant effect on Heph expression [Chen et al., 2003]. The lower response of Heph to iron in the duodenum suggests that Heph might not play a primary regulatory role and is not rate limiting for intestinal iron transport although Heph plays a critical role in iron absorption [Anderson et al., 2002]. It is unknown whether the higher response of Heph to iron in the brain implies that Heph has a key role in cellular iron balance and is essential for the iron release from the brain cells. An unexpected finding in this study was that the effects of iron status on Heph gene expression in the cortex and hippocampus were different from those in the striatum and substantia nigra. It is also unknown whether this is attributable to the regional differences in the capacity to manage iron and oxidative stress, and related to the uneven distribution of iron in the brain. The precise reason for the regionally specific effects of iron status on Heph expression also needs to be clarified.

In summary, in the present study, we demonstrated for the first time that all the four regions we examined, the cortex, hippocampus, striatum, and substantia nigra, have the ability to express Heph mRNA and protein. The findings also showed that both the development and iron status have a significant effect on Heph expression and that the trends in development and iron status-induced changes were similar between Heph mRNA and protein expression in all four regions, suggesting the existence of a development and iron status-dependent transcriptional regulation in these regions.

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