

Role of hepcidin in murine brain iron metabolism

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Abstract Brain iron homeostasis is maintained by a balance of both iron uptake and release, and accumulating evidence has revealed that brain iron concentrations increase with aging. Hepcidin, an iron regulatory hormone produced by hepatocytes in response to inflammatory stimuli, iron, and hypoxia, has been shown to be the long-sought hormone responsible for the regulation of body iron balance and recycling in mammals. In this study, we report that hepcidin is widely expressed in the murine brain. In cerebral cortex, hippocampus and striatum, hepcidin mRNA levels increased with aging. Injection of hepcidin into the lateral cerebral ventricle resulted in decreased Fpn1 protein levels in cerebral cortex, hippocampus, and striatum. Additionally, treatment of primary cultured neurons with hepcidin caused decreased neuronal iron release and Fpn1 protein levels. Together, our data provide further evidence that hepcidin may be involved in the regulation of brain iron metabolism.

Keywords Hepcidin · Ferroportin1 ·
Brain iron metabolism · Aging · Murine

Introduction

It is known that the brain accumulates iron progressively in normal aging [1, 2], which suggests that the import of iron into the brain exceeds its export during the course of life. Elevations in brain iron during aging are complex, and the mechanisms involved remain unclear. Although management of intracellular iron is critical in all cells, free iron is also toxic to cells as it catalyzes the generation of hydroxyl radicals, which can react with lipid membranes and DNA causing damage and premature cell death [3]. Abnormal levels of brain iron have been reported in a number of age-related neurodegenerative disorders including Alzheimer's disease (AD) [4] and Parkinson's disease (PD) [5].

In order to maintain iron levels in the brain, an array of proteins regulate cellular iron by modulating iron uptake, storage, and export. Recent reports on the role of divalent metal transporter 1 (DMT1), ceruloplasmin (CP), hephaestin (HP), and ferroportin1 (Fpn1) in brain iron transport represent important advances in our understanding of the physiology and pathophysiology of brain iron metabolism. A recent study reported that the treatment of C6 glioma cells with different concentrations of L-DOPA significantly increased the expression of DMT1 (-IRE) mRNA and protein, and enhanced uptake of ferrous iron [6]. These findings suggest that increased DMT1 expression might be associated with the neurotoxicity of L-DOPA. Early studies established that CP functions to promote iron release from storage cells to plasma in vivo [7]. However, some in vitro studies have reported that CP might play a role in iron uptake in addition to iron release [8–11]. Fpn1 is the first

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and only member of the SLC40 family of transporters that has been shown to mediate the efflux of iron from cells. Recent findings in our laboratory suggest that Fpn1 might play a role in iron export from PC12 cells [12]. In addition, there is a correlation between Fpn1 expression levels and the rate of cellular iron efflux [13]. Accordingly, disruption in Fpn1 protein expression and the associated decline in the iron release from brain cells might ultimately lead to increased brain iron levels. However, the factors regulating the expression and activity of iron transport proteins in the brain remain to be identified.

Hepcidin, also known as ‘liver expressed antimicrobial peptide’ (LEAP), is an iron regulatory hormone mainly produced by hepatocytes in response to inflammatory stimuli, iron, and hypoxia [14–17]. The hepcidin peptides, of 25, 22, and 20 amino acids in size, are derived from an 84 amino acid precursor peptide (pro-hepcidin). The human hepcidin gene (HAMP) contains two introns and three exons, and homologous genes have been found in pig, rat, mouse, flounder, and the long-jawed mudsucker [14]. There is only one copy of hepcidin gene in human genome, while the mouse genome contains two highly similar hepcidin genes, *hepc1* and *hepc2* [18]. However, Lou et al. [19] described the severe iron-deficient anemia phenotype in transgenic mice overexpressing *hepc1* in the liver, while none of the seven founder hepcidin2-transgenic animals suffered from anemia. Functional studies demonstrated that hepcidin binds to Fpn1 in cultured HEK 293 cells, leading to internalization of this complex and degradation of Fpn1, thereby reducing cellular iron efflux [20]. This mechanism is sufficient to explain the regulation of the balance of iron absorption and recycling by circulating hepcidin, which appears to be involved in the maintenance of body iron status by regulating the Fpn1 concentration on the surface of enterocytes and macrophages.

A recent study [21], and the results presented here, show that hepcidin is widely expressed in mouse brain. Localization and functional characterization of hepcidin in neural tissues suggest that this peptide might play a role in the maintenance of brain iron homeostasis. In early-onset restless legs syndrome (RLS) patients, pro-hepcidin levels in cerebrospinal fluid (CSF) were significantly decreased as compared to controls, while the concentration of pro-hepcidin was significantly increased as compared to controls in the neuromelanin cells, as well as in the homogenates of substantia nigra and putamen [22]. Since hepcidin negatively regulates Fpn1 protein levels [20, 23], we speculate that disruption of hepcidin expression in brain may be involved in brain iron disorders.

In the present study, we report that brain hepcidin1 mRNA expression in the mouse varies with the stage of aging. Furthermore, Fpn1 protein expression was down-regulated in cerebral cortex, hippocampus and striatum

following cerebral ventricular injection of synthetic hepcidin. Finally, total iron release and Fpn1 protein expression were diminished in primary cultured neurons treated with hepcidin. Our results provide compelling evidence implicating the role of hepcidin in regulation of brain iron metabolism.

Materials and methods

Animals and experimental design

For analysis of brain-regional hepcidin expression, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from three 9-week-old BALB/c mice according to the manufacturer’s instructions. Whole brain was dissected after perfusion with ice-cold RNase-free 0.9% saline. Next, the cerebral cortex, hippocampus, striatum, diencephalon, midbrain, cerebellum, and pons of each mouse were dissected and used for analysis. Kidney was used as the positive control.

To investigate hepcidin1 mRNA expression in the brains of mice of different ages, three each of BALB/c mice at postnatal 1, 3, 9, 28, and 72 weeks were used. After perfusion, the brain was rapidly removed and dissected into cerebral cortex, hippocampus, and striatum.

Nine-week-old BALB/c mice were used for the lateral cerebral ventricle injection and divided randomly into four groups. The control group was injected the 0.9% saline, and the other groups were injected with 5 μ l of synthetic hepcidin at concentrations of 0.2, 1, or 5 μ g/ μ l. Brains of three mice from each group were dissected into cerebral cortex, hippocampus, and striatum, and used for western blotting. Three additional mice were used for immunohistochemistry from the control and 5 μ g/ μ l hepcidin-injected groups. All experiments were approved by the Animal Care and Use Committee of Hebei Science and Technical Bureau in the PRC.

Quantitative Real-Time PCR

The relative purity of isolated total RNA was assessed spectrophotometrically and the ratio of A260/A280 nm exceeded 1.9 for all preparations. Total RNA (1 μ g) was reverse transcribed in a 20- μ l reaction using RT kit (Takara, Dalian, China) according to the manufacturer’s instructions. Primers designed with the Primer Express software and 1 μ l cDNA were then used as template for real-time PCR with SYBR Premix Ex TaqTM (Takara). PCR amplification was performed with the ABI PRISM 7000 with the following cycling parameters: 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and then 60°C for 31 s. Expression of target gene was determined by

normalizing to the respective β -actin levels. Each amplification was repeated three times from different RT reactions, and the data were averaged. The following primers were utilized for PCR amplification: for the hepcidin1 (accession no. AF503444): forward 5'-TTGCGA TACCAATGCAGAAGAG-3' and reverse 5'-AATTGTTA CAGCATTACAGCAGAAGA-3'; for the β -actin (accession no. M12481): forward 5'-AGGCCAGAGCAA GAGAGGTA-3' and reverse 5'-TCTCCATGTCGTCCC AGTTG-3'.

Peptide synthesis and hepcidin western blot

Hepcidin (mouse sequence, 25 amino acids) was synthesized by Genemedsyn, USA according to published methods [14]. The biological activity of chemically synthesized hepcidin (hep25) on *Fpn1* distribution has been reported [20, 24, 25]. We also compared the synthetic peptide with liver hepcidin, by 13% glycerol gel system (Fig. 1). We found that the synthetic peptide and the liver hepcidin were immunoreactive with antibody raised against murine hepcidin (1:1,000) (Fig. 1). The antibody was purchased from Alpha Diagnostic International (San Antonio, TX, USA). The specificity of the antibody (Hepc-25) has been demonstrated in mouse by Peyssonnaud C et al. [26], and in rat by Wang Q et al. [27] and Dallalio G et al. [28]. We concluded that our synthetic peptide is of the correct molecular weight for hepcidin.

Lateral cerebral ventricle injection

Before surgery, the animals were anesthetized with pentobarbital (1 mg/kg) i.p. Mice were placed in a stereotactic device and 5 μ l of synthetic hepcidin (0.2, 1 and 5 μ g/ μ l) or vehicle (sterile 0.9% saline) were infused into the right

lateral cerebral ventricle (0.5 mm posterior, 1.0 mm lateral, and 2.0 mm ventral to bregma) [29]. The animals maintained for 12 h after surgery. All experiments were repeated three times, and data from each experiment were combined.

Immunohistochemistry

The animals were transcardially perfused, under anesthesia with Nembutal, with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M PB. The brains were removed, postfixed for 1.5–4 h and then stored overnight in 20% sucrose in 0.1 M PB. Serial coronal sections were cut at 10 μ m on a freezing microtome, and mounted onto a slide covered with APES (Beijing ZhongShan Biotechnology, Beijing, China). The same coronal level sections on one slide were selected to do the immunohistochemistry analysis in the control and hepcidin-treated groups. The slices were washed with 0.01 M PBS before incubating in 0.6% H_2O_2 for 20 min to quench the endogenous peroxidase activity. Antigen retrieval was performed in an autoclave at 95°C for 10 min in 10 mM citrate buffer, pH 6.0 [30]. After blocking with normal goat serum prepared in PBS for 1 h, the slices were incubated overnight at 4°C with the rabbit anti-Fpn1 polyclonal antibody (1:500; Alpha Diagnostic International). The specificity of the antibody for murine [31] and rat Fpn1 [32, 33] has been described. The slides were then washed three times with PBS for 10 min. Biotinylated goat anti-rabbit secondary antibody (Zymed Laboratories, San Francisco, CA, USA) was used at a dilution of 1:200 in 60-min incubation at 37°C. After washing, the sections were incubated with streptavidin-horseradish peroxidase conjugate (1:200 dilution; Zymed Laboratories) for 60 min at 37°C. The slides were then washed four times with PBS for 5 min and the staining reaction was carried out using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as the chromogen. Negative controls were processed by replacing the primary antibody with diluent rabbit serum (1:500). The sections were dehydrated in ethanol, cleared in xylene, and cover-slipped with neutral balsam. Finally, slices were photographed with an Olympus DP70 microscope with DPController set to the same parameters (sensitivity: ISO 200; exposure time: 1/4.0 s; spot: 30%; accumulation mode: average; objective: $\times 10$; field diaphragm: 0.75). The same areas were selected in cortex (pyramidal layer cells of ectorhinal cortex) and in hippocampus (cells of polymorph layer and granular layers in dentate gyrus) between control and hepcidin injected groups to take photographs.

The photographs were analyzed with the Image-Pro Plus software, and the AOI was set as the whole image. Firstly, the intensity was calibrated with standard optical density (OD). Secondly, we chose manual color select (in the

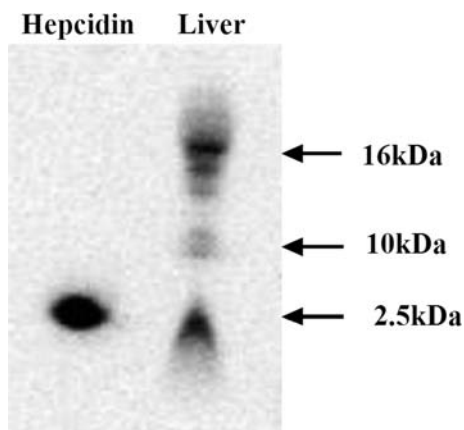


Fig. 1 Analysis of synthetic hepcidin peptide by western blot. The standard is 1 μ g of hepcidin produced synthetically. Liver protein serves as the control, containing pro-hepcidin (9.2 kDa) and hepcidin (2.5 kDa)

count/size column) and set the background gray level at 150 in all slices of the control and hepcidin groups, and count 150–255 (max.) signal in the histogram. Thirdly, the positive area and density (mean) were selected. The range of area was set from 2,000 pixels in order to filter the non-cellular structure signal in the background. Finally, we counted the positive area to get the mean density. The data are shown in Fig. 6 (see below).

Western blot analysis

Primary cultured neurons (see below) or brain tissues including cerebral cortex, hippocampus, and striatum were washed and homogenized in RIPA buffer containing 1% Triton X-100. After centrifugation at 10,000g for 30 min at 4°C, the supernatant was collected and protein concentration measured. Aliquots of the extract containing about 35 µg of protein were separated by reducing 10% SDS-PAGE and electroblotted onto PVDF membranes for 45 min at room temperature. The membranes were blocked in 5% non-fat milk containing 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20 (TBS-T) for 2 h at room temperature, and then incubated with rabbit anti-mouse Fpn1 (1:5,000) (Alpha Diagnostic International) or rabbit anti-mouse ferritin (H-chain) (1:2,000) (Alpha Diagnostic International) for overnight at 4°C. After washing with TBS-T three times, the membranes were incubated in anti-rabbit secondary antibody conjugated horseradish peroxidase (1:5,000) (Amersham, UK) for 2 h at room temperature. Immunoreactive proteins were detected by using the enhanced chemiluminescence method (ECL kit; Amersham), and quantified by transmittance densitometry using volume integration with Gel-Pro software. To ensure even loading of the samples, the same membrane was probed with rabbit anti-human β -actin antibody (1:5,000) (Sigma-Aldrich, St. Louis, MO, USA). Fpn1 or ferritin protein levels in each specimen were normalized to β -actin.

Primary neuronal culture and treatment

Primary cultured rat neurons were prepared by the modified method of Nagasawa [34]. Briefly, the cortical and hippocampal tissue of newborn SD rats were dissected in ice-cold Krebs' solution. Blood vessels and membranes were thoroughly removed from cortex and hippocampus, and then the tissues were digested in 10 ml 0.05% trypsin solution and 37°C for 10 min. After digestion, trypsin inhibitor was added to the mixture, and the sample was mechanically dissociated with a pipette. The cell suspension was centrifuged, followed by re-suspension in an appropriate volume of DMEM culture medium. The cells obtained were seeded into poly-L-Lysine-coated 6-well plates, and the medium was changed to B-27 Supplement

(Invitrogen) supplemented DMEM medium after 24 h. Subsequently, the medium was changed every 2 days, and cultures were used for experiments following 6 days of culture. With this procedure, at least 90% of the cells in culture were neuronal, as judged immunohistochemistry for neuron-specific enolase (NSE).

After culture for 6 days, the neurons were washed three times with PBS, and 1 ml DMEM medium (serum-free) was added to every well of the 6-well plate. The neurons were divided into two groups randomly, and added synthesized hepcidin (2.5 µg/ml) or the vehicle solution (sterile 0.9% saline) into the wells, respectively. The neurons were then incubated at 37°C for 12 h and used for iron release analysis or western blot.

Measurement of iron release

Iron-55 solution was prepared by mixing $^{55}\text{FeCl}_3$ with FeSO_4 in a molar ratio of 1:10 followed by a 50-fold molar excess of 2-mercaptoethanol and 0.32 M sucrose as described previously [12]. After three washes with PBS, primary neurons were incubated with $^{55}\text{FeCl}_3$ solution (1 mM) for 60 min at 37°C and then washed three times with cold PBS. Next, 1 ml buffered saline was added, and incubation was continued for 30 min at 37°C. Radiolabeled iron released into the medium was measured, as well as intracellular iron following centrifugation of the cells. Equation: ^{55}Fe release rate (%) = (cpm in supernatant)/(cpm in supernatant + cpm in cells) \times 100%.

Statistical analysis

Statistical analysis was performed using SPSS 12.0 (SPSS, Chicago, IL, USA). Results are expressed as mean \pm SEM. The difference between means was determined by one-way ANOVA followed by a Student-Newman-Keuls test for multiple comparisons (Figs. 3, 4, and 5) and *t* test (the other data). A probability value of $P < 0.05$ was taken to be statistically significant.

Results

Hepcidin 1 mRNA expression varies in different brain regions

Hepcidin 1 mRNA and protein levels are reported to be widely expressed in different brain regions [21]. We used real-time PCR to quantify hepcidin 1 mRNA in cerebral cortex, hippocampus, striatum, diencephalon, midbrain, cerebellum, and pons of BALB/c mouse brains. Hepcidin 1 mRNA expression level in kidney [35] was used as a positive control (Fig. 2). Hepcidin 1 mRNA levels in

kidney, cerebral cortex, cerebellum, midbrain, and pons were significantly higher than those of hippocampus.

Hepcidin 1 mRNA levels in the mouse brain increase with aging

To determine the influence of developmental stage on brain hepcidin 1 gene expression, brain-regional hepcidin1 mRNA levels in cerebral cortex, striatum, and hippocampus of mice of postnatal 1, 3, 9, 28, and 72 weeks were investigated using real-time PCR. Our results show that the hepcidin 1 mRNA expression was lowest at 1 week, and increased gradually with aging, reaching the highest level at 72 weeks in these regions (Fig. 3).

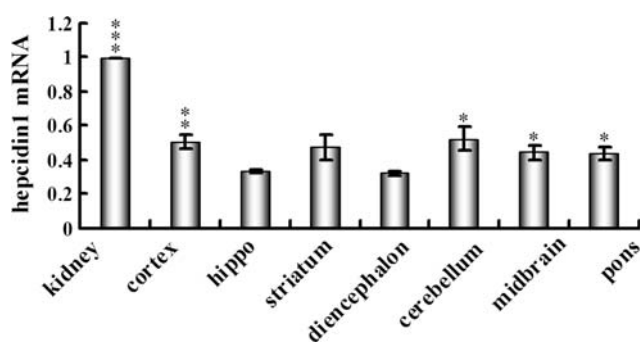


Fig. 2 Hepcidin 1 mRNA expression in different brain regions. Quantification of hepcidin-1 gene expression in the kidney (positive control) and different brain areas (including cerebral cortex, hippocampus, striatum, diencephalon, cerebellum, midbrain, and pons) in the mice from the BALB/c background, by real-time RT-PCR. Data are means \pm SEM, $n = 3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus hippocampus

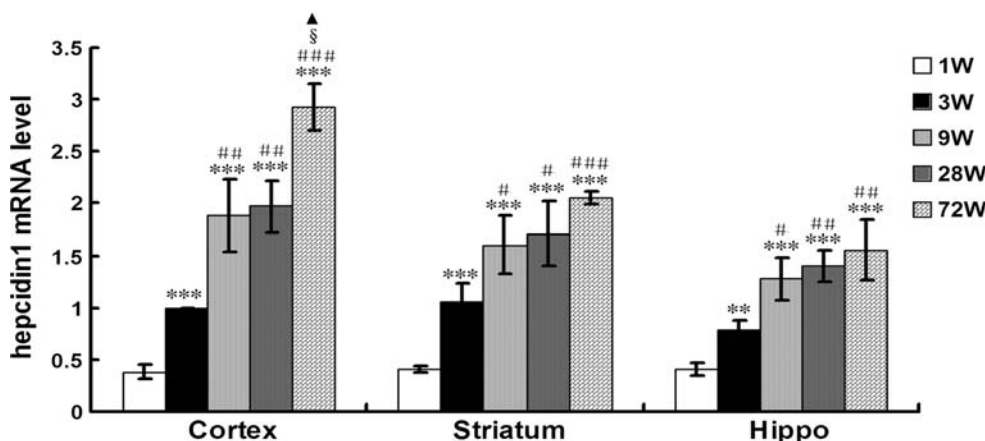


Fig. 3 Hepcidin 1 mRNA levels in the mouse brain increase with aging. Quantification of hepcidin1 gene expression in different brain regions (including cerebral cortex, striatum and hippocampus) in mice of different ages by real-time RT-PCR. Hepcidin 1 mRNA expression was lowest at 1 week, and increased gradually with age, reaching the

Hepcidin injection decreases Fpn1 and increases ferritin expression in the mouse brain

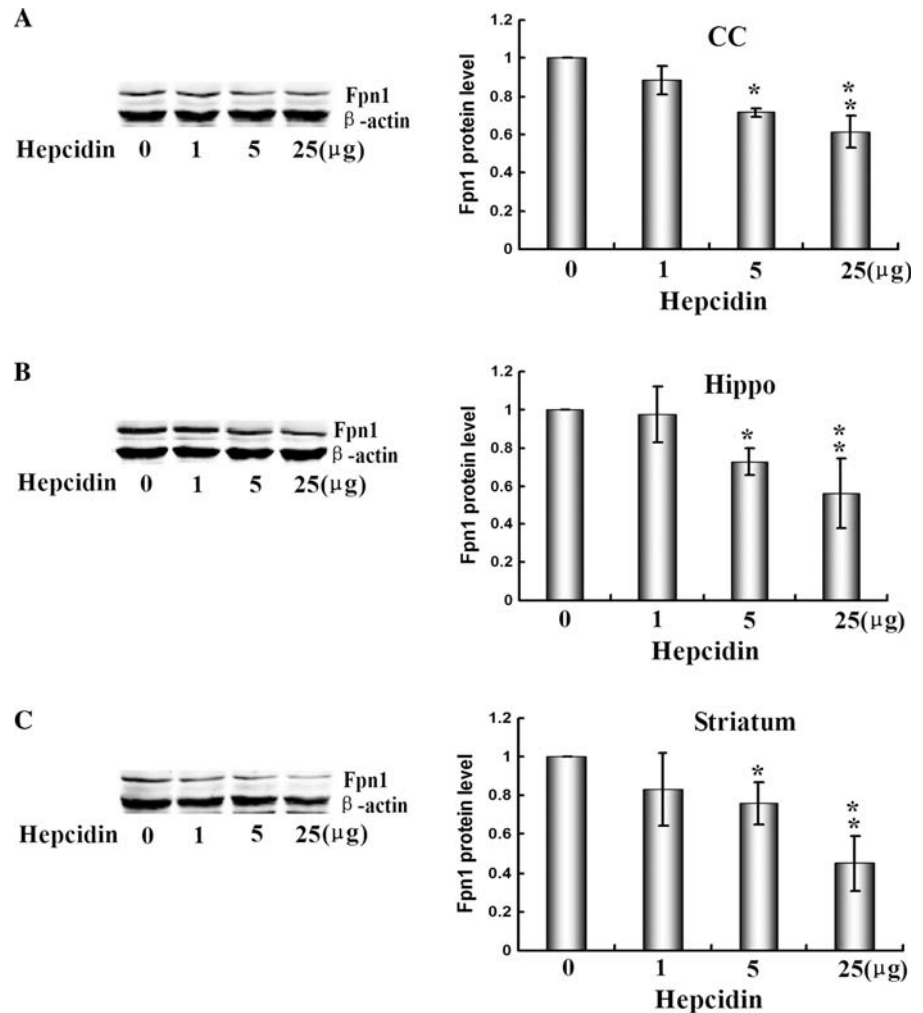
To investigate the possible role of hepcidin on regulation of Fpn1 expression in brain, Fpn1 protein levels were measured in cerebral cortex, hippocampus, and striatum after injecting synthetic hepcidin in the lateral cerebral ventricle (Fig. 4). Our data show that the Fpn1 protein levels were significantly down-regulated by hepcidin treatment (5 and 25 μ g), while no significant differences were observed in the 1 μ g hepcidin-treated group in any brain region.

Ferritin is the main form of tissue non-heme iron stores [36, 37], and the ferritin levels might reflect tissue iron levels. We performed western blot analyses to determine ferritin protein levels in hepcidin-treated mice. H-ferritin levels in cerebral cortex and striatum were significantly increased after treatment with the high dose of hepcidin (Fig. 5; 25 μ g hepcidin). No significant change was observed following treatment with lower doses of hepcidin (1 and 5 μ g) in cerebral cortex, hippocampus, and striatum, or following high dose treatment of hepcidin in hippocampus (Fig. 5).

The regulation of hepcidin on Fpn1 protein in the cerebral cortex, hippocampus, and striatum was further documented by immunohistochemistry. Expression of Fpn1 protein in mouse brain tissue was examined by immunohistochemistry after lateral cerebral ventricle injection of hepcidin and saline. Positive staining was observed in neuronal cells and glial cells. Fpn1 positive staining of cerebral cortex (CC), hippocampus (Hippo) and caudate putamen (CPu) in the hepcidin injected groups (Fig. 6b, d, f) is visible weak reaction than the control (Fig. 6a, c, e). The mean density of different brain region

highest level at 72 weeks in these regions. Data are means \pm SEM, $n = 3$. ** $P < 0.01$, *** $P < 0.001$ versus postnatal 1 week mouse. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ versus postnatal 3 week mouse. § $P < 0.05$ versus postnatal 9 week mouse. ^ $P < 0.05$ versus postnatal 28 week mouse

Fig. 4 Injection of hepcidin in the lateral cerebral ventricle leads to a decrease in Fpn1 protein levels. Fpn1 protein levels quantification and a representative western blot of Fpn1 protein and β -actin (control). **a** Cerebral cortex (CC), **b** hippocampus (*Hippo*), **c** *Striatum*. Expression levels were normalized to β -actin and expressed as the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ versus control



images of hepcidin treated group was significantly lower than the control, respectively (Fig. 6g).

Hepcidin decreases iron release from cultured primary neurons

Hepcidin regulates iron efflux by binding to Fpn1 and inducing its internalization [19]. To investigate the possible role of hepcidin in the regulation of iron efflux in neuronal cells, iron release was measured in the primary cultured neurons after incubation with synthetic hepcidin for 12 h. The rate of cellular iron release decreased markedly after hepcidin treatment ($n = 6$, $P < 0.05$) (Fig. 7).

Fpn1 levels are decreased in cultured primary neurons treated with hepcidin. Given the previously established correlation between Fpn1 expression levels and iron efflux in SH-SY5Y and hippocampal cells [13], Fpn1 protein expression level in primary neuronal cultures was examined after incubation with synthetic hepcidin for 12 h. Western blot analysis revealed that Fpn1 protein

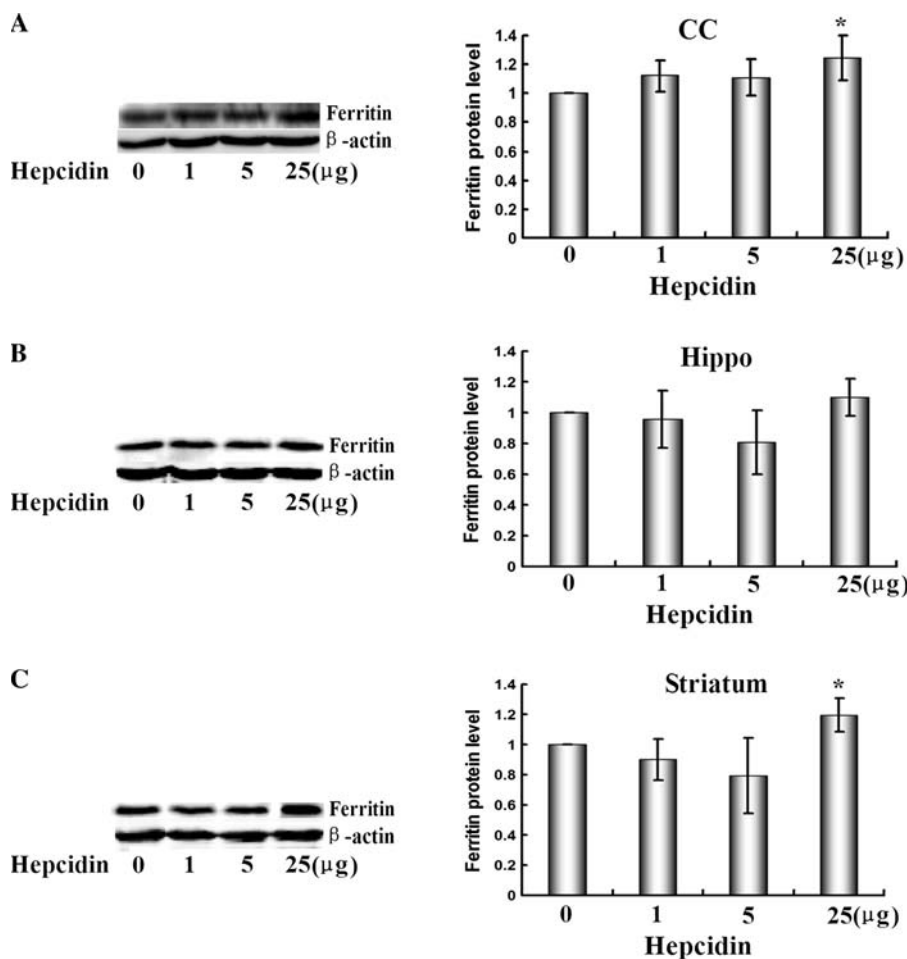
expression declined significantly during hepcidin treatment ($n = 6$, $P < 0.01$) (Fig. 8a, b).

Discussion

Iron is an essential trace metal required during brain development. The brain accumulates iron progressively during normal aging, and there is evidence that misregulation of brain iron metabolism might be a cause of neuronal death in some neurodegenerative disorders [38]. Disruption in the expression of iron metabolism proteins could lead to iron imbalance in the brain, contributing to oxidative stress and neuronal cell death. The aim of this study was to investigate the role of hepcidin in brain iron metabolism by analyzing its mRNA levels in brain with aging and measuring Fpn1 and ferritin protein levels after cerebral ventricular injection of synthetic hepcidin.

In this study, we report that hepcidin mRNA expression levels increase in the mouse brain with aging. Following

Fig. 5 Expression of ferritin protein levels in the cerebral cortex (CC), hippocampus (Hippo) and striatum of mouse after hepcidin injection in the lateral cerebral ventricle. Ferritin protein levels and a representative western blot of the H-ferritin protein and β -actin (control). **a** Cerebral cortex (CC), **b** hippocampus (Hippo), **c** Striatum. Expression levels were normalized for β -actin and expressed as the mean \pm SEM. * $P < 0.05$ versus control

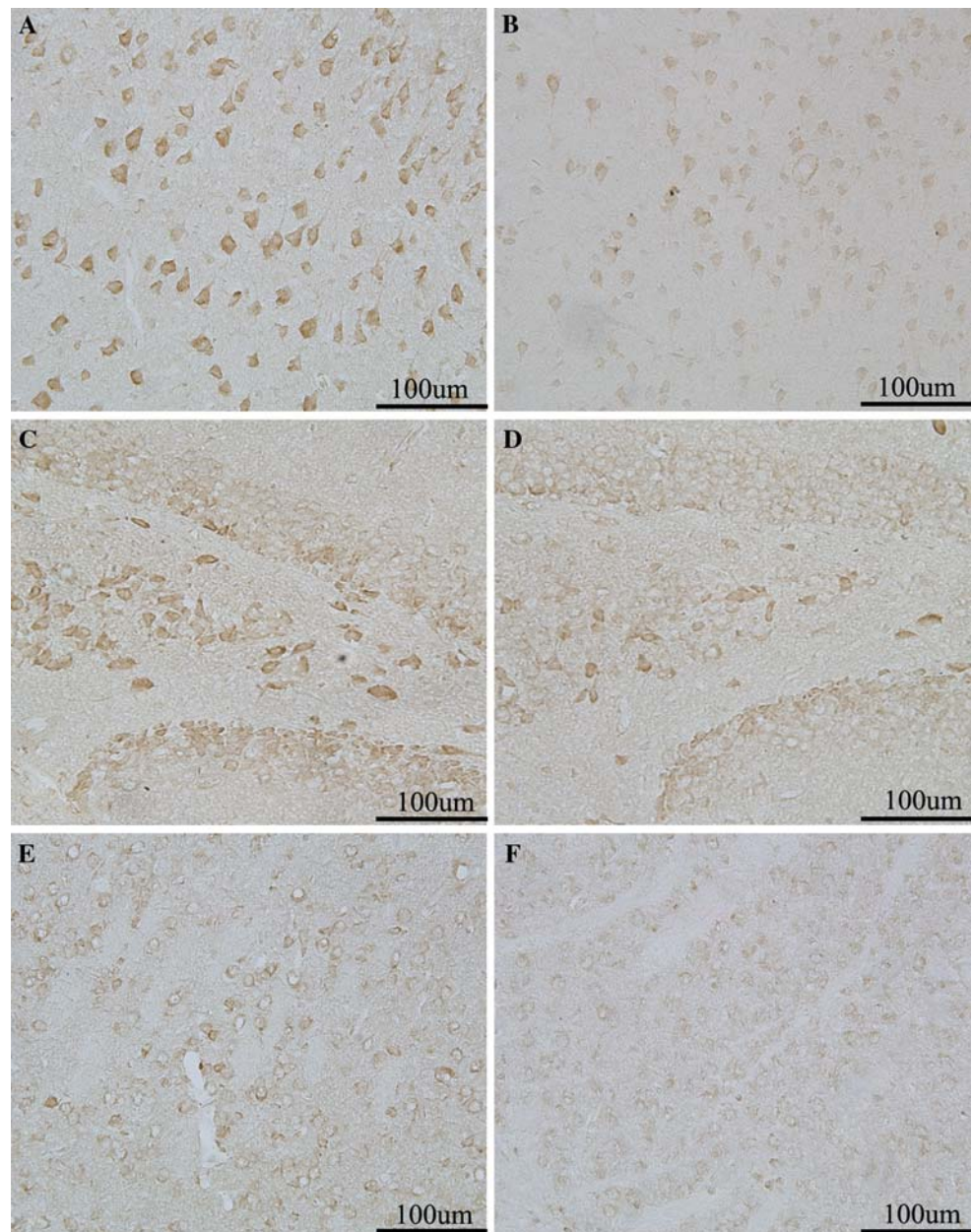


cerebral ventricular injection of synthetic hepcidin, Fpn1 protein levels were down-regulated in cerebral cortex, hippocampus, and striatum, suggesting that brain iron metabolism might be influenced by hepcidin. The possibility of a role for hepcidin in the control of brain iron efflux was substantiated by the observation of decreased iron release and Fpn1 protein expression in primary cultured neurons during treatment with hepcidin. Together, these results suggest that regulation of brain iron efflux by hepcidin could play a role in the control of brain iron metabolism.

Several proteins could mediate neuronal iron uptake including transferrin receptor, lactoferrin receptor, p97 (melanotransferrin, human melanoma tumor-associated antigen), and DMT1 [38]. Little is known about the components that mediate neuronal iron efflux. Fpn1, also called Slc40a1, metal transport protein 1 (MTP1) or iron-regulated transporter 1 (Ireg1), is found in a number of cell types in the body, including duodenal enterocytes, placental trophoblast, macrophages, and hepatocytes [39–41]. However, Jiang et al. [42] provided evidence for the existence of Fpn1 protein in the cortex, hippocampus,

striatum, and substantia nigra of rat brain. Using in situ hybridization and immunohistochemistry, Wu et al. [43] discovered that Fpn1 is expressed in the endothelial cells of the blood–brain barrier, neurons, oligodendrocytes, astrocytes, choroid plexus, and ependymal cells. Studies have also suggested a role for CP in iron efflux from brain cells. De Domenico et al. [44] suggested that the coordinated actions of GPI-anchored CP and Fpn1 may be required for iron efflux from neural cells, and that disruption of this balance could lead to iron accumulation and neurodegeneration in the central nervous system. Jeong and David [45] demonstrated that Fpn1 co-localizes with CP on the surface of astrocytes and is physically associated with CP. In addition, our recent findings suggest that Fpn1 might have a role in iron export from PC12 cells [12]. Aguirre et al. [13] showed that iron accumulation killed a large proportion of cells (SH-SY5Y and hippocampal cells), but a sub-population became resistant to iron. The surviving cells evoked an adaptive response consisting of increased synthesis of the iron-storage protein ferritin and iron export transporter Fpn1, and decreased synthesis of the iron import protein DMT1. These studies support the hypothesis

Fig. 6 Fpn1 expression shown by immunohistochemistry of different brain regions after hepcidin injection from lateral cerebral ventricle. **a, c, e** Fpn1 positive staining of cerebral cortex (CC), hippocampus (Hippo) and caudate putamen (CPu) in the control groups, respectively. Positive staining is evident in cell bodies. **b, d, f** Fpn1 positive staining of cerebral cortex (CC), hippocampus (Hippo) and caudate putamen (CPu) in the hepcidin injected groups. There is a weaker reactivity in the hepcidin injected groups than the control. **g** The mean density of positive staining measured by the Image-Pro Plus software. Data are presented as means \pm SEM. ** $P < 0.01$ versus control, *** $P < 0.001$



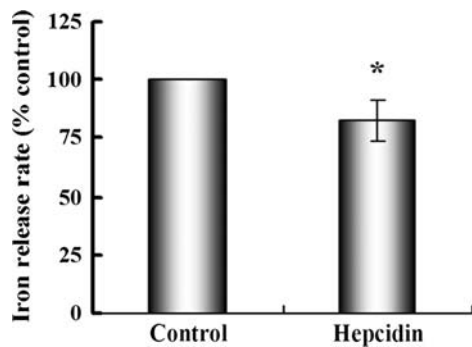


Fig. 7 Hepcidin treatment decreases iron release from primary cultured neurons. Radiolabeled iron released into the medium and intracellular iron were measured. The data were presented as means \pm SEM. (% control, $n = 6$). * $P < 0.05$ versus control

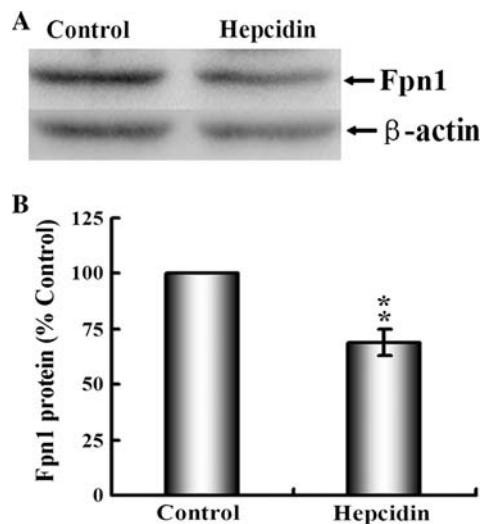


Fig. 8 Expression of Fpn1 protein is decreased in primary cultured neurons following synthetic hepcidin incubation for 24 h. **a** Fpn1 protein and **b** a representative western blot of Fpn1 and β -actin. Expression values were normalized to β -actin and expressed as the mean \pm SEM (% control). ** $P < 0.01$ versus control (**b**)

that Fpn1 might also be required for iron release from brain cells, thus playing an important role in brain iron homeostasis [10, 43, 46].

While hepcidin is known to be a key regulator of peripheral iron metabolism [17, 23], the function of hepcidin in the brain remains an open question. A recent study [21] and our results show that hepcidin 1 mRNA is widely expressed in mouse brain. In this study, we have demonstrated that the aging has a significant effect on brain hepcidin 1 mRNA expression. In addition, our data show that exogenous synthetic hepcidin can regulate Fpn1 protein expression in vivo, as well as neuronal iron release and Fpn1 protein expression in vitro. This study provides further evidence for a role of hepcidin in regulating brain iron efflux in the postnatal developing brain.

Hepcidin is widely distributed in different brain regions, including the olfactory bulb, cortex, hippocampus, amygdala, thalamus, hypothalamus, mesencephalon, cerebellum, pons, and spinal cord [21]. To compare hepcidin expression levels in different brain regions, we used real-time PCR to quantify hepcidin 1 mRNA levels in different brain regions in BALB/c mice. Our data show that the hepcidin 1 mRNA levels in cerebral cortex, cerebellum, midbrain, and pons are higher than that of hippocampus (Fig. 2). This suggests that endogenous hepcidin gene expression may be brain-region or cell type-specific. Indeed, in one study, hepcidin immunoreactivity was detected in both neurons and GFAP-positive glial cells [21].

It is generally accepted that iron accumulates in the brain during aging; however, little is known about the mechanism underlying this phenomenon [47]. Brain iron homeostasis depends on cellular iron uptake, release, storage, and regulation. From postnatal day 17, iron and ferritin levels increase throughout the lifetime of the rat [48]. The iron uptake proteins transferrin receptor [49] and DMT1 [2] increase up to postnatal day 21 and then decreased, which can be explained with IRE-IRP regulatory mechanism. However, Fpn1 protein is at its highest level at 9 weeks, and shows a slight subsequent decrease in cortex, hippocampus, and striatum at 28 weeks [42]. Our results suggest a possible mechanism to explain the regulation of Fpn1 expression in the aging brain, as hepcidin 1 mRNA levels increased with age in the cerebral cortex, hippocampus, and striatum. Since hepcidin can bind and induce degradation of Fpn1 [24], increased hepcidin expression would lead to decreased presence of Fpn1 on the plasma membrane despite continued accumulation of iron within the aging brain. These data provide a possible mechanism to explain the iron accumulation observed in the aging brain.

The trends in hepcidin expression reported here are similar to those observed in the mouse liver, where hepcidin expression was observed at low levels during most of embryonic and early postnatal life, but displayed significant up-regulation by PND 56 [23]. We speculate the hepcidin could be responsive to stimuli found in brain as well as in the peripheral organs [17]. However, several groups have proposed that hepcidin may play a distinct role in the brain iron metabolism [21, 22]. Clardy et al. examined the relationship between hepcidin and RLS (a neurological disorder characterized by a strong urge to move the legs), and found that pro-hepcidin levels in the CSF were significantly decreased in early-onset RLS patient samples, but not in late-onset RLS patients, when compared to controls. And the autopsy [50] and MRI [51] data show that ferritin has a consistent decrease in CSF in the early-onset RLS. Our work demonstrates that Fpn1

can be down-regulated in the cortex, hippocampus, and striatum following intra-ventricular injection of synthetic hepcidin. Furthermore, we demonstrated that treatment of primary cultured neurons with hepcidin decreased both Fpn1 protein abundance and cellular iron efflux. Although Clardy et al. were unable to determine whether the lower levels of pro-hepcidin in the CSF represent a compensatory response to the decreased levels of iron in the brain or a defective signaling mechanism in RLS, nonetheless their data and our experiments support the evidence that abnormal hepcidin levels in the brain may be associated with disorders of brain iron homeostasis. Liu et al. [52] examined the role of endogenously synthesized hepcidin in the regulation of Fpn1 expression in a COS1 cell model, and their results indicate that local production of hepcidin may be more important than hepcidin provided in culture medium in regulation of Fpn1 expression in COS1 cells. Together, these findings imply that hepcidin may be an important regulator of Fpn1 expression in brain.

Fpn1 is likely regulated transcriptionally by iron and inflammation, post-transcriptionally by iron- and inflammation-induced changes in IRE/IRP interactions [12, 53], and post-translationally via interactions with hepcidin. Although hepcidin can regulate Fpn1 expression in the brain, the detailed mechanisms underlying this regulation are not clear. Moreover, the relationship between hepcidin-mediated control of Fpn1 expression and other potential iron regulatory mechanisms in the brain is also unclear. Thus, further studies directed at characterizing the role of hepcidin in mediating brain Fpn1 expression and cellular iron homeostasis are clearly merited. Such efforts might offer new therapeutic strategies for treatment of RLS and other neurodegenerative disorders.

Conclusion

The present work demonstrates that developmental age has a significant effect on hepcidin1 mRNA expression levels in the mouse brain. Furthermore, synthetic hepcidin down-regulated Fpn1 protein levels in vivo and in vitro, and decreased cellular iron release in vitro. Further studies are needed to elucidate the molecular mechanisms of hepcidin action in the brain. One of the key questions is whether endogenous hepcidin acts by itself or interacts with specific receptors. Another is whether circulating hepcidin participates in the regulation of brain iron metabolism.

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