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ORIGINAL ARTICLE

Inflammation alters the expression of DMT1, FPN1 and hepcidin, and it causes iron accumulation in central nervous system cells

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Abstract

Inflammation and iron accumulation are present in a variety of neurodegenerative diseases that include Alzheimer's disease and Parkinson's disease. The study of the putative association between inflammation and iron accumulation in central nervous system cells is relevant to understand the contribution of these processes to the progression of neuronal death. In this study, we analyzed the effects of the inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) and of lipopolysaccharide on total cell iron content and on the expression and abundance of the iron transporters divalent metal transporter 1 (DMT1) and Ferroportin 1 (FPN1) in neurons, astrocytes and microglia obtained from rat brain. Considering previous reports indicating that inflammatory stimuli induce the systemic synthesis of the master iron regulator hepcidin, we identified brain cells that produce hepcidin in response to inflammatory stimuli, as well as hepcidin-target cells. We found that inflammatory stimuli increased the expression of DMT1 in neurons, astrocytes, and microglia. Inflammatory stimuli also induced the expression of hepcidin in astrocytes and microglia, but not in neurons. Incubation with hepcidin decreased the expression of FPN1 in the three cell types. The net result of these changes was increased iron accumulation in neurons and microglia but not in astrocytes. The data presented here establish for the first time a causal association between inflammation and iron accumulation in brain cells, probably promoted by changes in DMT1 and FPN1 expression and mediated in part by hepcidin. This connection may potentially contribute to the progression of neurodegenerative diseases by enhancing iron-induced oxidative damage.

Keywords: DMT1, FPN1, hepcidin, inflammation, iron accumulation, neurodegeneration.

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Active inflammatory processes and iron accumulation are hallmarks of a variety of neurodegenerative diseases that include Parkinson's disease (PD) and Alzheimer's disease (AD) (Hunot and Hirsch 2003; Ong and Farooqui 2005; Cahill *et al.* 2009; Sian-Hulsmann *et al.* 2011; von Bernhardi and Eugenin 2012). Inflammation is mediated by reactive microglia and astrocytes that become neurotoxic through the secretion of inflammatory cytokines such as IL-1, IL-6, and TNF-α (Kaushal and Schlichter 2008; Lee *et al.* 2010; Smith *et al.* 2012). Indeed, the brains of PD patients contain elevated levels of cytokines, including IL1-β, IL2, IL4, IL-6, and TNF-α (Mogi *et al.* 1994a, b, 1996), while in AD microglia surrounding Aβ plaques show increased secretion of

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Abbreviations used: CD11b, Cluster of differentiation molecule 11B; CMF-HBSS, Calcium- and magnesium-free Hank's buffered salt solution; CNS, Central nervous system; DIV, Days *in vitro*; DMT1, Divalent metal transporter 1; FBS, Fetal bovine serum; FPN1, Ferroportin 1; GFAP, Glial fibrillary acidic protein; IL-6, Interleukin 6; INF-γ, Interferon-gamma; LPS, Lipopolysaccharide; MEM, Minimum essential medium; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; NTA, Nitrilotriacetic acid; RT-PCR, Reverse transcriptase polymerase chain reaction; TGF-β1, Transforming growth factor beta 1; TNF-α, Tumor necrosis factor alpha; Tuj1, Neuron-specific class III beta-tubulin.

IL-1, IL-6, and TNF-α (Rogers and Lue 2001; Lee *et al.* 2010).

Iron is an intrinsic producer of reactive oxygen species through connected processes including hydroxyl radical formation, glutathione consumption, protein aggregation, lipid peroxidation, and nucleic acid modification (Jomova and Valko 2011; Núñez *et al.* 2012). Cellular iron homeostasis is regulated by the iron transporters DMT1 and FPN1 and by the iron storage protein ferritin. The main iron uptake transporter is DMT1, while FPN1 functions essentially as an iron efflux transporter (Anderson and Vulpe 2009). In multiple cell types, diverse cytokines modify DMT1 expression. As an example, INF-γ and TNF-α up-regulate the expression of DMT1 and increase iron uptake into bronchial epithelial cells (Wang *et al.* 2005). Similarly, DMT1 expression is transcriptionally enhanced by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Paradkar and Roth 2006), which is activated downstream of many cytokine receptors including the receptors for TNF-α, lipopolysaccharide (LPS), and IL-1 (Hanke and Kielian 2011; Wajant and Scheurich 2011).

Hepcidin, a small polypeptide synthesized and secreted by the liver, is considered a master regulator of body iron homeostasis. The physiological function of hepcidin is to decrease circulating iron levels through down-regulation of FPN1 in macrophages (Nemeth *et al.* 2004). The action of hepcidin is tissue-specific: in enterocytes, hepcidin does not change FPN1 levels but decreases DMT1 expression (Chaston *et al.* 2008; Mena *et al.* 2008; Brasse-Lagnel *et al.* 2011). Hepcidin-induced FPN1 degradation is transient reaching a maximal effect at 2–6 h, with FPN1 levels returning to control values at 24 h (Yeh *et al.* 2004; Chaston *et al.* 2008; Mena *et al.* 2008; Ge *et al.* 2009; Du *et al.* 2012). Hepcidin synthesis is stimulated by increased plasma iron levels and by inflammatory cytokines under conditions of infection by pathogens (Lee *et al.* 2005; Wrighting and Andrews 2006; Gnana-Prakasam *et al.* 2008; Frazier *et al.* 2011).

Recent studies show that hepcidin is also expressed in the central nervous system (CNS) (Zechel *et al.* 2006; Wang *et al.* 2010) and that its expression increases in response to systemic administration of LPS (Wang *et al.* 2008). Hepcidin may provide a link between inflammation and iron accumulation in the CNS. Early studies showed increased activation of NF-κB and secretion of proinflammatory cytokines in microglia cultured with excess iron (Saleppico *et al.* 1996), and that toxic concentrations of IFN-γ and TNF-α are lower in iron-loaded oligodendrocytes (Zhang *et al.* 2005). These data suggest that iron accumulation enhances the toxic effects of inflammatory cytokines. Which are the sequence of events leading to neurodegeneration, inflammation, or iron accumulation remains an open question (Zivadinov *et al.* 2011). Stereotaxic injections of LPS in the striatum increase microglia activation, ferritin expression, and total nigral iron content in aged rats (Hunter *et al.* 2008), an indication that inflammation drives iron accumulation. Nevertheless, cellular inflammation is not necessarily dependent on iron deposition as demonstrated by microglia activation without iron accumulation in an animal model of multiple sclerosis (Williams *et al.* 2011). Recently, it was shown that TNF-α and TGF-β1 induce opposite changes in the expression of DMT1, FPN1, and ceruloplasmin in astrocytes and microglia. TNF-α treatment results in increased iron retention by both astrocytes and microglia; in contrast, the anti-inflammatory, anti-apoptotic cytokine TGF-β1 causes iron retention in microglia but promotes iron efflux from astrocytes (Rathore *et al.* 2012).

In this report, we tested the hypothesis that inflammation drives iron accumulation in CNS cells by modifying the expression of iron transporters. Considering that hepcidin could be a link between inflammation and iron accumulation in the CNS, we also determined the cellular sources of hepcidin and its cellular targets in response to inflammatory stimuli. Our results strongly suggest that inflammation induces iron accumulation in neurons and microglia, promoted by changes in DMT1 and FPN1 expression and partly mediated by hepcidin.

Methods

Cells

Primary cultures of embryonic rat hippocampal neurons were obtained as described (Kaech and Banker 2006). The cells were seeded onto poly-L-lysine-coated six-well plates, and the plating medium was changed after 2 h to supplemented neurobasal B-27 medium. Three days after plating, 4 μM 1-b-D-arabinofuranosylcytosine was added to prevent glial proliferation. Rat cortical glial cells were obtained as described (Viviani 2006) with modifications. Briefly, cerebral cortex from 1- to 2-day-old Sprague–Dawley either male or female rat pups were dissected in CMF-HBSS and digested using 0.25% trypsin supplemented with 0.5 mg/mL DNase I. The mixed astrocytes/microglia culture was incubated in minimum essential medium (MEM)-F12 containing 10% fetal bovine serum (FBS). After 13 days in culture cells were vigorously stirred on an orbital shaker for 16 h at 260 rpm to detach microglial cells. The microglial cells were plated in MEM-F12 10% FBS. After 20 min of incubation at 37°C non-adherent cells (mostly astrocytes) were removed with a change in medium. The remnant cells in the flask were shaken again for 72 h to remove remaining microglia. After shaking, astrocytes were trypsinized, replated into 35-mm plates, and grown until confluence prior to the treatments.

Rats were obtained from the animal facilities of Pontificia Universidad Catolica de Chile. The protocol for the handling of animals was approved by the Ethics Committee for the Handling of Live Species and Biosafety of Faculty of Sciences, Universidad de Chile.

Cytokines treatment

Neurons at DIV 7 cultured in fresh neurobasal B27 medium were treated for 18 h with 50 ng/mL TNF-α (R&D Systems, Minneapo-

lis, MN, USA), 50 ng/mL IL-6 (R&D Systems), 1 μg/mL LPS from *Escherichia coli* 026:B6 1 (Sigma-Aldrich: St. Louis, MO, USA), or 40 μM Fe-NTA (1 : 2.2, mol/mol). Cortical astrocytes or microglial cells cultured in high glucose MEM medium supplemented with 1% FBS and 7 μM iron were similarly treated for 18 h with 50 ng/mL TNF-α, 50 ng/mL IL-6, or 1 μg/mL LPS.

Hepcidin treatment

Hepcidin treatments were done at DIV 7. Cells were incubated for 6 h with 350 nM human hepcidin (Peptide International: Louisville, KY, USA) prior to western blot analysis. Preliminary experiments indicated that treatment of hippocampal neurons for 6 h with 350 nM hepcidin resulted in a 50% decrease in FPN1 levels (Figure S1a).

RT-PCR

Semi-quantitative PCRs for DMT1, FPN1, or hepcidin were performed as described (Mena *et al.* 2008). The primers used are described in Table S1. The primers used for DMT1 recognize an internal segment of its mRNA common to all DMT1 isoforms, which in the case of brain cells correspond to isoforms 1B+IRE and 1B-IRE (Hubert and Hentze 2002).

Antibodies

The antibodies used in this study were as follows: rabbit polyclonal anti-panDMT1 (Aguirre *et al.* 2012); rabbit polyclonal anti-FPN1 (Alpha Diagnostics, San Antonio, TX, USA); rabbit polyclonal anti-hepcidin (Alpha Diagnostics); mouse monoclonal anti-tubulin and mouse monoclonal anti- GFAP (Sigma-Aldrich); mouse monoclonal anti-cluster of differentiation molecule 11B (CD11b) and mouse anti- Tuj1 (Promega, Madison, WI, USA).

Western blot analysis

Extracts were prepared by lysing cells with lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, and protease inhibitor cocktail) for 15 min on ice and centrifugation for 15 min at 12 000 *g*. Protein concentrations were determined using bicinchoninic acid protein assay (Thermo Scientic-Pierce, Rockford, IL, USA). For western blot analysis, 20 μg of proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes and blocked for 1 h at 25°C with 5% nonfat dry milk in Tris-buffered saline/Tween-20 buffer (20 mM Tris, 0.5 M NaCl, 0.1% Tween-20). Membranes were incubated overnight a 4°C with rabbit anti-DMT1 (1 : 1000), rabbit anti-FPN1 (1 : 1000), or mouse anti-tubulin (1 : 10 000), followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody for 2 h at 25°C. The immunoreactive bands were developed with a peroxidase-based SuperSignal chemiluminescence assay kit (Thermo Scientific-Pierce) and quantified with the Quantity One software (Bio-Rad, Hercules, CA, USA).

Immunofluorescence

Cells grown in cover slips were fixed with 4% paraformaldehyde/4% sucrose in phosphate-buffered saline, permeabilized with 0.2% Triton-X-100 in phosphate-buffered saline, incubated overnight at 4°C with anti-FPN1 (1 : 100), anti-DMT1 (1 : 500), anti-GFAP (astrocyte marker, 1 : 100), anti-CD11b (microglia marker, 1 : 100), anti-TujI (neuron marker, 1 : 1000) or anti-hepcidin (1 : 100) followed by incubation with Alexa-546-conjugated goat anti-mouse IgG, Alexa-488-conjugated goat anti-rabbit IgG and TOPRO for nuclei identification. The labeled cells were observed with a Zeiss LSM 510 Meta confocal laser-scanning microscope (Carl Zeiss Microscopy GmbH: Jena, Germany).

Total iron measurement

Total iron content was determined by atomic absorption spectroscopy (AAS) as described (Aguirre *et al.* 2005). Briefly, cell extracts were mixed with 0.5 mL of sub-boiling ultrapure nitric acid and incubated at 25°C for 48 h. Total cellular iron analysis was determined by total reflection X-ray fluorescence using a Seifert EXTRA-II Rich spectrometer (Seifert & Co: Ahrensburg, Germany).

Statistical analysis

Differences between means were analyzed by one-way ANOVA. Dunnett's *post-hoc* test was used for comparison using InStat, GraphPad Software (GraphPad Software Inc, La Jolla, CA, USA) p-values < 0.05 were considered significant.

Results

To ascertain if inflammatory treatments resulted in iron accumulation, we evaluated total iron content in neurons, astrocytes, and microglia treated with the pro-inflammatory cytokines TNF-α or IL-6, or with LPS, a Toll-like receptor 4 agonist. After 18 h treatment, total iron content increased significantly ($p < 0.01$) in neurons and microglia, whereas iron content in astrocytes remained unchanged (Fig. 1a). After treatments, iron content in microglia reached values close to 400 ng of Fe per mg of protein; in neurons, iron content increased from 60 to 100–120 ng of Fe per mg of protein and in astrocytes it remained in the range of 45–50 ng of Fe per mg of protein.

We evaluated next if the increase in iron content produced by inflammatory stimuli associated with changes in the levels of iron transporters. Rat hippocampal neurons treated for 18 h with TNF-α, IL-6, or LPS presented a significant 50–67% increase in DMT1 protein content and 45–65% decrease in FPN1 protein levels when compared to control (Fig. 2a). Treatment of astrocytes with TNF-α, IL-6 or LPS also induced a significant increase in DMT1 protein content (Fig. 2b), while treatment with LPS also induced a small but significant decrease in FPN1 protein content when compared to control (Fig. 2b). Treatment of microglia with TNF-α, IL-6, or LPS resulted in increased DMT1 levels without significant changes in FPN1 (Fig. 2c). Figure S2 shows images of DMT1 and FPN1 fluorescence immunostaining in neurons, astrocytes, and microglia. An increase in DMT1 immunostaining in neurons and microglia, and a decrease in FPN1 immunostaining in neurons is evident.

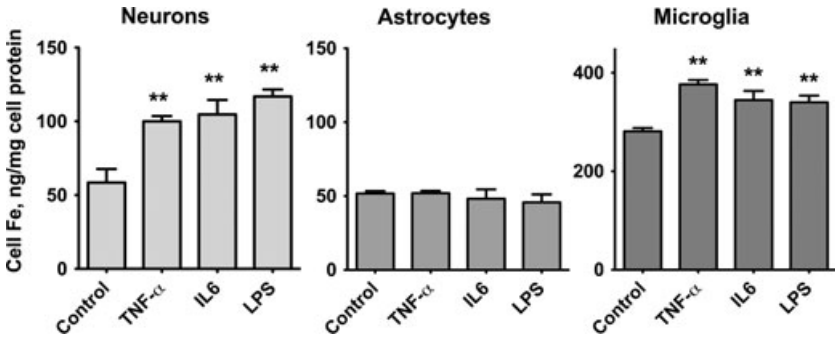

Fig. 1 Changes in iron content induced by inflammatory stimuli. Hippocampal neurons, cortical astrocytes, and cortical microglia were treated for 18 h with tumor necrosis factor alpha (TNF-α) (50 ng/mL), IL-6 (50 ng/mL), or lipopolysaccharide (LPS) (1 μg/mL) after which the iron content was determined. Values represent mean ± SEM ($n = 3$). $^{**}p < 0.01$ as compared to the corresponding control.

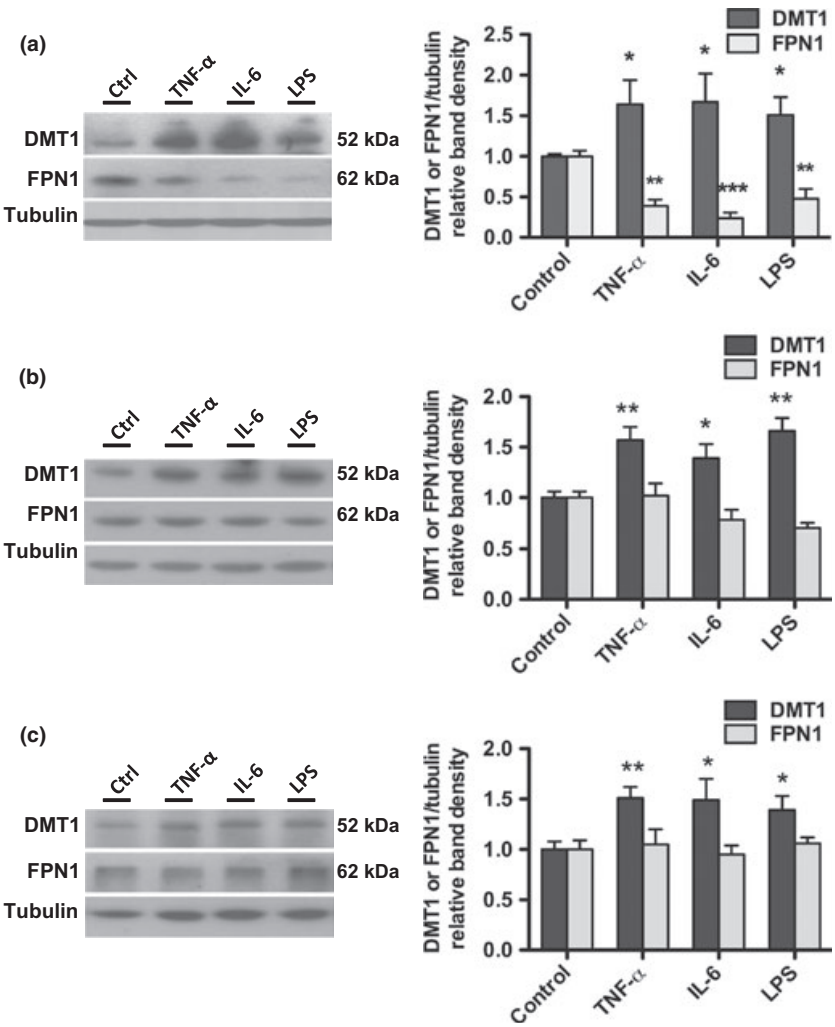

Fig. 2 Tumor necrosis factor alpha (TNF-α), IL-6, and lipopolysaccharide (LPS) induce changes in the protein levels of the iron transporters divalent metal transporter 1 (DMT1) and ferroportin 1 (FPN1) in CNS-derived cells. Hippocampal neurons (a), cortical astrocytes (b), and cortical microglia (c) were treated for 18 h with TNF-α (50 ng/mL), IL-6 (50 ng/mL), or LPS (1 μg/mL) and changes in DMT1 and FPN1 expression were analyzed by western blot. Left panels show representative blots and right panels show the quantification of band density. Values represent mean ± SEM ($n = 6$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, compared to control.

To ascertain if changes in protein levels were associated with changes in mRNA levels, RT-PCR was performed using specific primers for DMT1 and FPN1 in neurons, astrocytes, and microglia upon treatment for 18 h with TNF-α, IL-6, or LPS (Fig. 3). In neurons, all three agents increased DMT1 mRNA levels without change in FPN1 expression (Fig. 3a). Astrocytes presented significant increases in DMT1 mRNA, while FPN1 mRNA decreased significantly only with LPS (Fig. 3b). TNF-α increased significantly DMT1 mRNA in microglia while significant decrease in FPN1 mRNA was observed following TNF-α and LPS treatment (Fig. 3c).

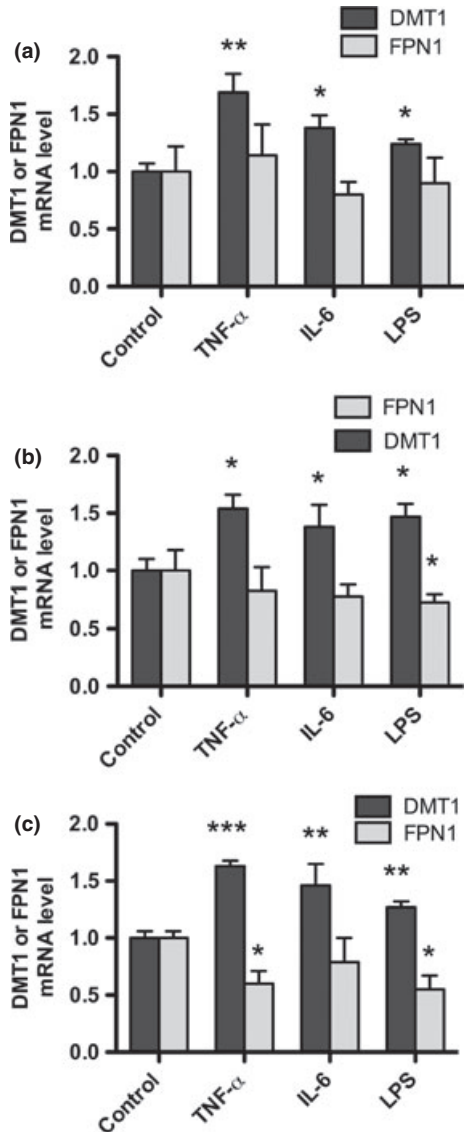

Fig. 3 Tumor necrosis factor alpha (TNF-α), IL-6, and lipopolysaccharide (LPS) induce changes in the mRNA levels of the iron transporters divalent metal transporter 1 (DMT1) and ferroportin 1 (FPN1) in CNS-derived cells. Hippocampal neurons (a), cortical astrocytes (b), and cortical microglia (c) were treated with TNF-α (50 ng/mL), IL-6 (50 ng/mL), or LPS (1 μl/mL) for 18 h. Changes in DMT1 and FPN1 mRNA levels were analyzed by RT-PCR. Panels show quantification of band density relative to control. Values represent mean ± SEM ($n = 4$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ as compared to control.

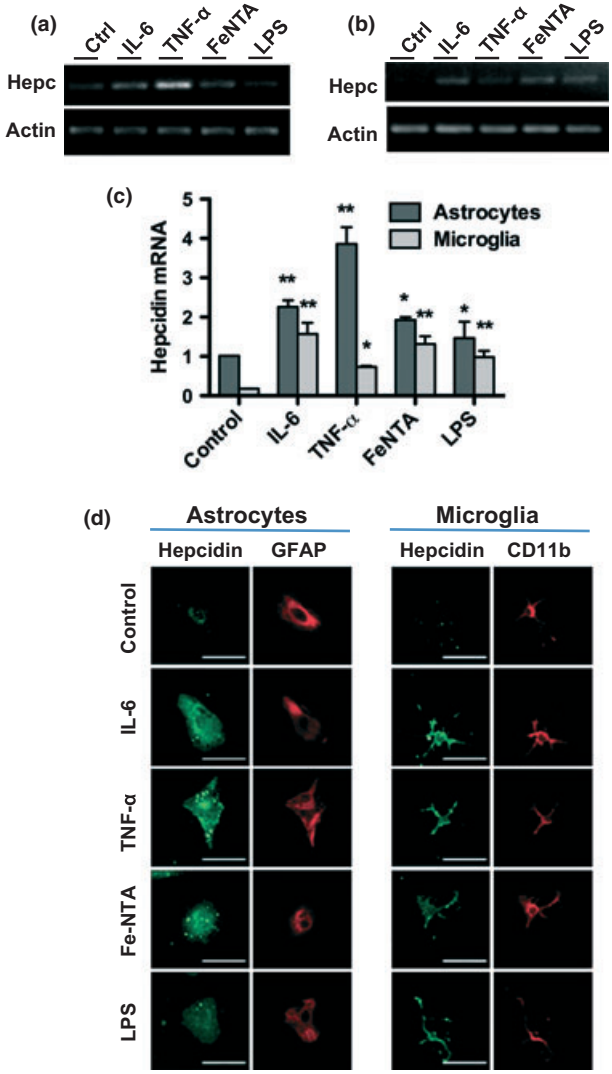

Fig. 4 Inflammatory stimuli induce the expression of hepcidin in astrocytes and microglia. Expression of mRNA for hepcidin in astrocytes (a) and microglia (b) after treatment for 2 h with tumor necrosis factor alpha (TNF-α) (50 ng/mL), IL-6 (50 ng/mL), Fe-NTA (40 μM), or lipopolysaccharide (LPS) (1 μg/mL). (c) Quantification of band density of hepcidin mRNA levels relative to untreated astrocytes. Values represent mean ± SEM ($n = 3$). *$p < 0.05$; **$p < 0.01$ as compared to control. (d) Hepcidin immunofluorescence (green) in astrocytes and microglia prior (Control) and after treatment for 2 h with TNF-α (50 ng/mL), IL-6 (50 ng/mL), Fe-NTA (40 μM), or LPS (1 μg/mL). GFAP and CD11b co-staining (red) was used for positive identification of astrocytes and microglia, respectively.

As stated above, hepcidin is a possible link between inflammatory stimuli and iron homeostasis. Thus, it was of interest to assess the effect of inflammatory stimuli on the putative expression of hepcidin by neurons, astrocytes and microglia. In addition, we tested if iron accumulation *per se* could induce hepcidin expression (Fig. 4). Astrocytes (Fig. 4a) and microglia (Fig. 4b) subjected for 2 h to high iron or inflammatory stimuli presented increased levels of hepcidin mRNA. Both basal and stimulated hepcidin mRNA levels were higher in astrocytes than microglia; in all conditions tested, the increase in hepcidin mRNA levels was significant (Fig. 4c). Hepcidin mRNA in neurons was undetectable under basal conditions and expression was not induced by TNF-α, IL-6, LPS, or Fe treatment (not shown).

Treatment of astrocytes and microglia with TNF-α, IL-6, LPS, or Fe also increased hepcidin protein content, as revealed by immunodetection of hepcidin in cell cultures (Fig. 4d). In agreement with the absence of hepcidin mRNA, neuronal cultures did not show detectable hepcidin immunostaining (not shown).

We next evaluated the effect of hepcidin on the levels of DMT1 and FPN1 in neurons astrocytes and microglia. A 6 h period of treatment was chosen because in preliminary experiments we determined that the effect of hepcidin on FPN1 protein levels was maximal at 6 h of treatment decreasing thereafter (Figure S1b). Consistent with its known action in systemic cells, treatment with hepcidin decreased FPN1 protein levels in neurons (Fig. 5a), astrocytes (Fig. 5b), and microglia (Fig. 5c). In addition, hepcidin treatment resulted in a significant increase in DMT1 only in microglia (Fig. 5c). Figure S3 shows immunofluorescence images of the variation in the cellular content of DMT1 and FPN1 in neurons, astrocytes, and microglia after hepcidin treatment. A decrease in FPN1 immunolabeling in neurons and astrocytes is noticeable, together with increased DMT1 levels in microglia.

Discussion

Inflammation and iron accumulation are amply recognized as pathognomonic signs of several neurodegenerative diseases that include AD and PD. However, knowledge on how inflammation and iron accumulation cross-talk is, at its best, incipient. The results presented here show that the inflammatory cytokines IL-6 and TNF-α and the Toll-like receptor 4 agonist LPS exercise profound effects on iron homeostasis, inducing DMT1 expression and iron accumulation in neurons and microglia. Moreover, we ascertained that astrocytes and microglia increase hepcidin expression in response to inflammatory stimuli. We also observed that 6 h treatment with hepcidin decreased the levels of FPN1 in neurons, astrocytes and microglia. Figure 6 presents a scheme summarizing the finding reported here.

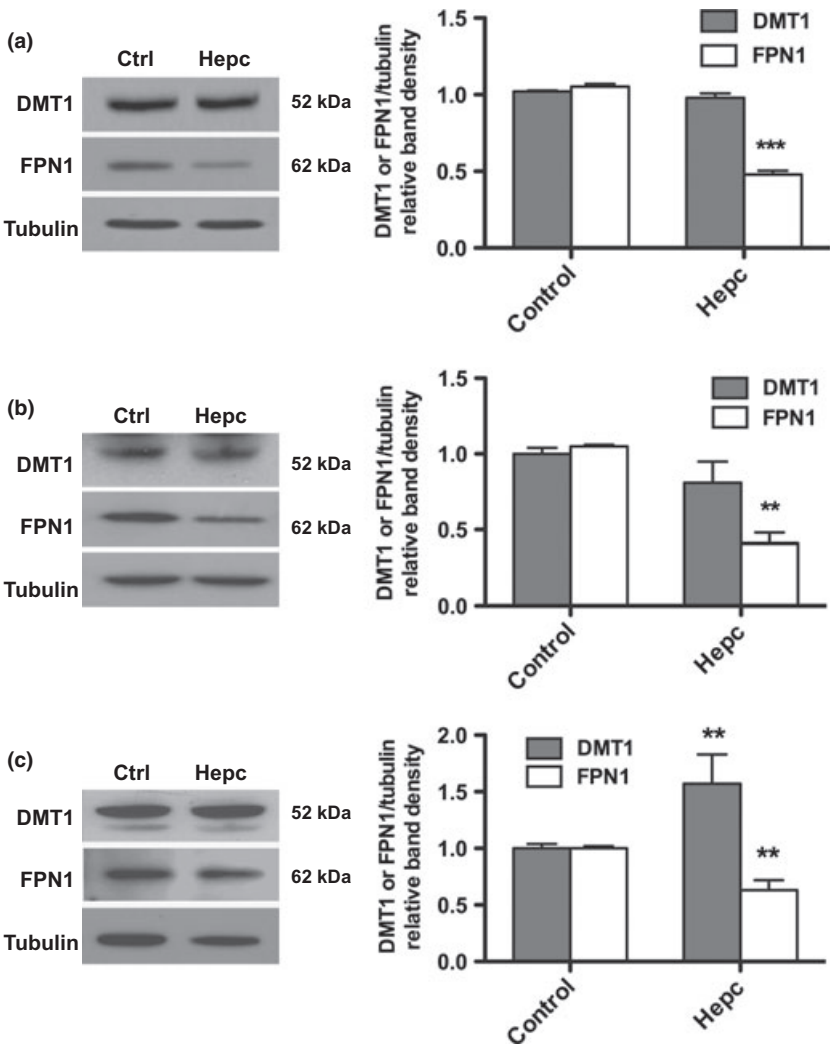

Fig. 5 Hepcidin induces changes in ferroportin 1 (FPN1) in CNS cells. Hippocampal neurons (a), cortical astrocytes (b), and cortical microglia (c) were treated with 350 nM hepcidin for 6 h after which divalent metal transporter 1 (DMT1) and FPN1 expression were analyzed by western blot. Left panels show representative blots and right panels show the quantification of band density. Values represent mean ± SEM ($n = 6$). $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to control.

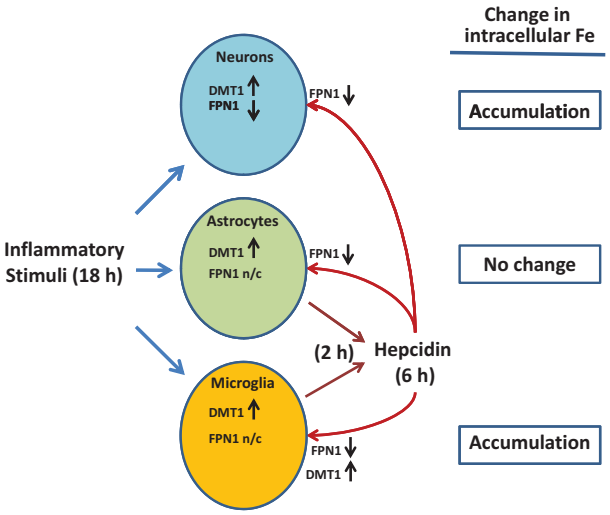

Fig. 6 Schematic representation depicting the changes in iron homeostasis of CNS cells subjected to inflammatory stimuli. Inflammatory stimuli (TNF-α, IL-6, LPS) for 18 h result in increased expression of divalent metal transporter 1 (DMT1) in neurons, astrocytes, and microglia, together with decreased expression of ferroportin 1 (FPN1) in neurons. Hepcidin, secreted by astrocytes and microglia in response to inflammatory stimuli, induces after 6 h a transient decrease in FPN1 in the 3 cell types and an increase in DMT1 in microglia. The net result of inflammatory stimuli and hepcidin mediation is iron accumulation in neurons and microglia but not in astrocytes.

Neurons

In neurons, TNF-α, IL-6, and LPS treatment increased DMT1 and decreased FPN1 protein levels. Probably, the increase in DMT1 was a consequence of the activation of signaling pathways that induced DMT1 mRNA expression. NF-κB, a transcription factor activated by both TNF-α and LPS signaling pathways (Hanke and Kielian 2011; Wajant and Scheurich 2011), is a likely effector since NF-κB induces DMT1 expression in P19 embryonic carcinoma cells (Paradkar and Roth 2006). The cause for the observed decrease in neuronal FPN1 is unknown, but it could be because of a post-transcriptional control of FPN1 expression by inflammatory stimuli as described for splenic macrophages (Liu *et al.* 2005).

As described for macrophages and other cell types (Ganz and Nemeth 2012), hepcidin induced a canonical down-regulation of FPN1 protein in neurons. The decrease in FPN1 produced by hepcidin and the increase in DMT1 as a result of inflammatory stimuli were most probably instrumental to the observed iron accumulation. Indeed, cellular iron content doubled after 18 h treatment with TNF-α, IL-6, or LPS, a condition that should result in an increased labile iron pool, with the consequent increase in oxidative stress and damage (Kruszewski 2003; Núñez *et al.* 2012).

Astrocytes

In astrocytes, treatment with TNF-α, IL-6, or LPS resulted in increased levels of DMT1, while decreased levels of FPN1 were significant only after LPS treatment. As discussed elsewhere in the article, the increase in DMT1 was probably a direct consequence of the activation of DMT1 transcription by TNF-α and LPS signaling pathways. These results are in general agreement with a recent study, which reported that in astrocytes TNF-α induced an increase in DMT1 expression coupled to decreased FPN1 expression (Rathore *et al.* 2012). Nevertheless, the authors reported that iron efflux from astrocytes, albeit larger than from microglia, did not change after TNF-α treatment, which would indicate that transport-active FPN1 levels did not change.

Treatment for 2 h with inflammatory agents and with 40 μM Fe-NTA resulted in increased levels of hepcidin mRNA in both astrocytes and microglia. Although the effect of inflammatory stimuli on hepcidin expression is widely acknowledged, the iron effect reported here is novel. In a previous work, we determined that the exposure of hippocampus neurons for 0 to 20 min to 100 μM Fe-NTA produce a fast increase in the labile iron pool and in reactive oxygen species (ROS) (Muñoz *et al.* 2011). Since ROS induce hepcidin synthesis (Millonig *et al.* 2012), it is possible that ROS generated by an increase in the labile iron pool may mediate the observed increase in hepcidin mRNA levels.

Hepcidin treatment resulted in decreased levels of FPN1 in astrocytes. This change was not maintained in time since after 18 h of treatment with inflammatory factors, which induce hepcidin expression in astrocytes, FPN1 approached control values (compare Figs 2b and 5b). The transient nature of the hepcidin effect on FPN1 levels has been described in other cell systems (Yeh *et al.* 2004; Chaston *et al.* 2008; Mena *et al.* 2008; Ge *et al.* 2009; Du *et al.* 2012). We found that treatment for 18 h with TNF-α, IL-6, or LPS resulted in no significant changes in cellular iron content. The conservation of the iron export capacity is probably the main reason why no iron accumulation was observed as a result of inflammatory stimuli. This conclusion is in line with the observation that iron efflux from astrocytes, *per se* larger than from microglia, did not change after TNF-α treatment despite a decrease in FPN1 levels (Rathore *et al.* 2012).

Microglia

After 18 h of treatment with inflammatory stimuli, microglia presented increased levels of DMT1 protein and mRNA accompanied by a 30% increase in iron content as compared to control. As discussed above, the increase in DMT1 mRNA was probably consequence of NFκB activation by the inflammatory stimuli.

As with neurons and astrocytes, hepcidin treatment resulted in decreased FPN1 levels, probably a consequence

of hepcidin-induced FPN1 degradation. Unexpectedly, hepcidin treatment resulted in a significant increase in DMT1 protein. One precedent in the literature for this result is the report that stereotaxic injection of hepcidin into the left lateral ventricle in rats resulted in increased DMT1 mRNA in cerebral cortex and hippocampus (Li *et al.* 2011). It can be envisioned that after 2 h of treatment, inflammatory stimuli induce hepcidin expression, which in turn at 6 h induces changes in iron transporters leading to iron accumulation. At 18 h of treatment, homeostatic mechanisms would bring FPN1 near to control levels but iron accumulation persists because of increased DMT1 levels. Microglia were the cell type with the largest content of iron. By sequestering extracellular iron in response to hepcidin, microglia may have an important protective role on neuronal viability.

The findings reported here provide a partial response to the question of what comes first, neuroinflammation or iron accumulation: in neurons and microglia, inflammatory stimuli induce iron accumulation. The induction of hepcidin expression by iron could produce a positive feedback loop of increased iron accumulation.

In summary, inflammatory stimuli induced iron accumulation in neurons and microglia, accumulation that was probably associated with changes in the levels of iron transporters. Inflammatory stimuli also induced the expression of hepcidin in astrocytes and microglia, being astrocytes the largest producers. In turn, hepcidin decreased the expression of FPN1 in neurons, astrocytes, and microglia. The data presented here establish for the first time a causal relationship between inflammation and iron accumulation in neurons and microglia. Considering that iron accumulation results in hydroxyl radical production and extensive cellular damage, this process may potentially contribute to the progression of neuronal death by enhancing iron-mediated oxidative damage.

Acknowledgements

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Time- and concentration-dependency of hepcidin-mediated FPN1 decrease in CNS cells.

Figure S2. Expression of DMT1 and FPN1 in CNS-derived cells treated with TNF-α, IL-6, and LPS visualized by immunocytochemistry.

Figure S3. DMT1 and FPN1 immunodetection in cells treated with hepcidin.

Table S1. List of PCR primers used in this study.

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