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ORIGINAL ARTICLE

Hepcidin attenuates amyloid beta-induced inflammatory and pro-oxidant responses in astrocytes and microglia

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Abstract

Alzheimer's disease (AD) is characterized by extracellular senile plaques, intracellular neurofibrillary tangles, and neuronal death. Aggregated amyloid-β (Aβ) induces inflammation and oxidative stress, which have pivotal roles in the pathogenesis of AD. Hepcidin is a key regulator of systemic iron homeostasis. Recently, an anti-inflammatory response to hepcidin was reported in macrophages. Under the hypothesis that hepcidin mediates anti-inflammatory response in the brain, in this study, we evaluated the putative anti-inflammatory role of hepcidin on Aβ-activated astrocytes and microglia. Primary culture of astrocytes and microglia were treated with Aβ, with or without hepcidin, and cytokine levels were then evaluated. In addition, the toxicity of Aβ-treated astrocyte- or microglia-conditioned media was tested on neurons, evaluating cellular death and oxidative stress generation. Finally, mice were injected in the right lateral ventricle with Aβ, with or without hepcidin, and hippocampus glial activation and oxidative stress were evaluated. Pre-treatment with hepcidin reduced the expression and secretion of TNF-α and IL-6 in astrocytes and microglia treated with Aβ. Hepcidin also reduced neurotoxicity and oxidative damage triggered by conditioned media obtained from astrocytes and microglia treated with Aβ. Stereotaxic intracerebral injection of hepcidin reduced glial activation and oxidative damage triggered by Aβ injection in mice. Overall, these results are consistent with the hypothesis that in astrocytes and microglia hepcidin down-regulates the inflammatory and pro-oxidant processes induced by Aβ, thus protecting neighboring neurons. This is a newly described property of hepcidin in the central nervous system, which may be relevant for the development of strategies to prevent the neurodegenerative process associated with AD.

Keywords: astrocytes, hepcidin, inflammation, microglia, oxidative damage, β-amyloid.

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Alzheimer's disease is the most common cause of dementia worldwide. Classically, AD has been viewed as a neurodegenerative disease of the elderly, characterized by extracellular deposition of misfolded Aβ peptides and intracellular formation of neurofibrillary tangles (Glenner and Wong 1984; Masters *et al.* 1985; Braak and Braak 1998; Ballatore *et al.* 2007). The current hypothesis suggests that the deposition of Aβ peptides marks the first detectable stage of the disease (Hardy and Selkoe 2002; Masters and Beyreuther 2006; Hardy 2009).

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Abbreviations used: AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, amyloid-β; DAB, 3,3′-diaminobenzidine; DMSO, dimethyl sulfoxide; FPN1, ferroportin 1; GFAP, glial fibrillary acidic protein; HNE, hydroxynonenal; ICV, intracerebroventricular; IL, interleukin; LPS, lipopolysaccharide; NO, nitric oxide; PBS, phosphate-buffered saline; RAGE, receptors for advanced glycation endproduct; ROS, reactive oxygen species; RRID, research resource identifier; TLRs, Toll-like receptors; TNF, tumor necrosis factor.

Recently, neuroinflammation has emerged as an important component of AD pathology (McGeer and McGeer 2013). The sustained formation and deposition of Aβ aggregates are proposed to be the cause of chronic activation of the immune system and disturbance of microglial clearance functions (Heneka *et al.* 2015). Receptors that sense pathogen-associated molecular patterns, such as bacterial lipopolysaccharide (LPS), are also activated by Aβ aggregates. Specifically, microglia bind Aβ via cell surface receptors, including CD36, CD14, SCARA1, α6β1 integrin, CD47, and Toll-like receptors (TLR2, TLR4, TLR6 and TLR9) (Paresce *et al.* 1996; Bamberger *et al.* 2003; Liu *et al.* 2005; Udan *et al.* 2008; Stewart *et al.* 2010). This triggers microglial activation, as well as the production of the pro-inflammatory cytokines IL-6, tumor necrosis factor (TNF)-α, and IL-1β, chemokines, nitric oxide, and reactive oxygen species (Lue *et al.* 2001; El Khoury *et al.* 2003; Stewart *et al.* 2010; Schilling and Eder 2011; Sheedy *et al.* 2013; Part *et al.* 2015; Taneo *et al.* 2015). In the proximity of activated microglia, hypertrophic reactive astrocytes accumulate and surround senile plaques, which are often seen in post-mortem tissue of patients with AD (Olabarria *et al.* 2010; Medeiros and LaFerla 2013). Reactive astrocytes are characterized by increased expression of glial fibrillary acidic protein (GFAP) and signs of functional impairment (Kamphuis *et al.* 2014; Rodriguez-Vieitez *et al.* 2015). Similar to microglia, astrocytes release cytokines, nitric oxide, reactive oxygen species, and other potentially cytotoxic molecules after exposure to Aβ (Hu *et al.* 1998; Wang *et al.* 2013).

Hepcidin is a cationic peptide secreted by the liver in response to elevated iron plasma levels (Nicolas *et al.* 2002; Nemeth *et al.* 2003, 2004a; Lee *et al.* 2004, 2005; Dzikaite *et al.* 2006; Matak *et al.* 2009). The principal function of hepcidin is to reduce levels of the iron exporter ferroportin 1 (FPN1) by triggering its lysosomal degradation, which results in iron accumulation in the cell (Nemeth *et al.* 2004b; De Domenico *et al.* 2009). Hepcidin expression is induced by inflammatory stimuli (Nicolas *et al.* 2002; Nemeth *et al.* 2004a; Lee *et al.* 2005; Dzikaite *et al.* 2006). A single injection of turpentine to mice induced a sixfold increase in liver hepcidin mRNA levels, iron accumulation in reticuloendothelial cells, and a twofold decrease in serum iron, all of which underlie low serum iron levels observed in the anemia of chronic diseases (Nicolas *et al.* 2002).

Recently, a new function of hepcidin in the modulation of the inflammatory response has been described (De Domenico *et al.* 2009, 2010; Pagani *et al.* 2011; Huang *et al.* 2012). The binding of hepcidin to FPN1 activates a signal transduction pathway that reduces the response to LPS, decreasing the production and secretion of IL-6 and TNF-α and reducing the mortality associated with high LPS doses (De Domenico *et al.* 2009, 2010; Pagani *et al.* 2011; Huang *et al.* 2012).

In the central nervous system, inflammatory stimuli induce the expression of hepcidin in astrocytes, microglia, and epithelial cells (Zechel *et al.* 2006; Wang *et al.* 2008, 2010; Marques *et al.* 2009). Interestingly, the expression of hepcidin and its receptor FPN1 are reduced in AD brains, a situation that is replicated in the brains of amyloid precursor protein transgenic mice (Raha *et al.* 2013; Crespo *et al.* 2014).

In this study, we evaluated the role of hepcidin in the inflammatory response induced by Aβ deposition. For this purpose, we analyzed the role of hepcidin in the expression of IL-6 and TNF-α from microglia and astrocytes activated by Aβ *in vitro*, and we evaluated the effect of Aβ-treated microglia- and astrocyte-conditioned media on hippocampal neurons. Finally, we evaluated the *in vivo* effect of hepcidin on glial activation following Aβ peptide injection into the right lateral ventricle.

Materials and methods

Animals

For *in vivo* experiments, 12-week-old male C57BL/6J mice, 25–30 g, were obtained from Janvier Breeding Center (Le Genest St Isle, France). Mice were kept in a temperature-controlled room (23°C ± 1°C) under a 12-h light/dark cycle and had *ad libitum* access to food and water. Animal handling was carried out according to ethical regulations and guidelines (Guide for the Care and Use of Laboratory Animals, NIH publication no. 85–23, revised 1985) and the European Communities Council Directive 86/609/EEC. For *in vitro* experiments, hippocampal neuron cells were prepared from Sprague Dawley rat embryos at gestational age 18–19 days, whereas primary glial cells were obtained from the cerebral cortex of 1- to 2-day-old Sprague Dawley newborn rats, as described previously (Urrutia *et al.* 2013). Rats were obtained from the animal facilities of Pontificia Universidad Católica de Chile. Protocols for rat handling were carried out in strict accordance with the recommendations of the Assessor Committee in Bioethics guidelines from the National Fund for Scientific and Technological Development (FONDECYT, Chile) and approved by the Bioethics Committee of Faculty of Sciences, Universidad de Chile.

Antibodies

Antibodies and dilutions used in this study were mouse anti-GFAP 1 : 500 (C9205, Sigma-Aldrich, St. Louis, MO, USA, RRID: AB_476889), mouse anti-Tuj1 1 : 1000 (G7121, Promega, Madison, WI, USA, RRID:AB_430874), rabbit anti-4-hydroxynonenal 1 : 500 (HNE11-S, Alpha Diagnostics, Owings Mills, MD, USA,, RRID:AB_2629282), and rabbit anti-Iba1 1 : 500 (019-19741, WAKO, RRID:AB_839504).

RT-PCR

Semi-quantitative PCR was performed as described previously (Urrutia *et al.* 2013) using the following primers: IL-6: 5′-CAAGAGACTTCCAGCCAGTTGC-3′ (forward), 5′-TGGCCGAG TAGACCTCATAGTGACC-3′ (reverse) reported in Morimoto *et al.* (1999); TNF-α: 5′-CACCACGCTCTTCTGTCTACTGAAC-3′ (forward), 5′-CCGGACTGCGTGATGTCTAAGTACT-3′

(reverse) reported in Inoue and Aramaki (2007); β-actin: 5′-TACAGCTTCACCACCACAGC-3′ (forward), 5′-AAGGAAGGC TGGAAAAGAGC-3′ (reverse).

Preparation of Aβ aggregates

Aβ peptides 1–42 ($A\beta_{1–42}$) and 42–1 ($A\beta_{42–1}$) (BACHEM H-6466 and H-3976) were dissolved in hexafluoroisopropanol, aliquoted into sterile microcentrifuge tubes, dried in a speed-vacuum centrifuge, and store at −80°C. Prior to use, the lyophilized peptides were suspended in dimethyl sulfoxide at a final concentration of 5 mM. Subsequently, the peptides were diluted in sterile phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) at a final concentration of 1 mg/mL. The solution was then allowed to aggregate for 72 h at 37°C (Braak and Braak 1998; Walter *et al.* 2007; Ding *et al.* 2011; Ruiz-Munoz *et al.* 2011).

Neurons, astrocytes, and microglia cultures and treatments

Primary culture of hippocampal neurons, cortical astrocytes, and cortical microglia were performed as described previously (Urrutia *et al.* 2013). Astrocytes or microglia were treated with 2 μM $A\beta_{1–42}$ or $A\beta_{42–1}$. When stated, cells were pre-treated with 1 μg/mL hepcidin 2 h prior to Aβ treatment. For PCR analysis, cells were treated with Aβ aggregates for 8 h preceding RNA extraction.

Astrocyte- and microglia-conditioned media

Conditioned media from astrocytes or microglia were obtained after 24 h of treatment with either $A\beta_{1–42}$ or $A\beta_{42–1}$. When stated, cells were pre-treated for 2 h with hepcidin. Conditioned media was centrifuged to remove cell debris and was used fresh.

IL-6 and TNF-α detection

Detection of IL-6 and TNF-α in the conditioned media was performed using ELISA kits (KRC0061 and KRC3011, respectively; Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Cell viability

Cell viability was assessed by the calcein–propidium iodide assay (Coccini *et al.* 2010). Cells with an intact membrane ('live cells') were distinguished by a homogeneous green fluorescence generated upon incubation with calcein AM (20 min, 1 μM). Cells with damaged membrane integrity ('death cells') were distinguished by a bright red fluorescence resulting from propidium iodide binding to nucleic acids (1 min, 2 μM). The fluorescence intensity was measured in a fluorescence plate reader (BioTek Instruments, Winooski, VT, USA). The fluorescence emissions were acquired separately, calcein at 530 ± 12.5 nm and PI at 645 ± 20 nm. The percentage of live cells was calculated from the fluorescence readings defined as: % Live Cells = [(F(530)sample − F(530)min)/(F(530)max − F(530)min)] × 100%. The percentage of dead cells was calculated as: % Dead Cells = [(F(645)sample − F(645)min)/(F(645)max − F(645)min)] × 100%.

Stereotaxic injections, study design, and sample size estimation

Stereotaxic injections were performed in the right lateral ventricle (−0.22 AP; 1 ML; 2.5 DV) of C57Bl/6J male mice. Mice were first injected with 2 μL of 5 μg/μL hepcidin or PBS based on Wang *et al.* (2010). After 2 h, mice were re-injected with 2 μL of $A\beta_{1–42}$ or $A\beta_{42–1}$ (1 μg/μL) as described previously (Ding *et al.* 2011). To perform the procedure, animals were anesthetized prior to the hepcidin injection with 100 mg/kg ketamine (Virbac Animal Health, La Seyne-sur-Mer, France) and 10 mg/kg xylazine (Bayer, Lyon, France), and prior to the Aβ injection with 1% isoflurane (Virbac Animal Health). Animals were killed by transcardiac perfusion 72 h after the procedure. In accordance with the 3 Rs principle for animal welfare, the experiment were designed in a way to assure significant information using the lowest group size possible. Based on the resource equation method (Festing and Altman 2002; Charan and Kantharia 2013), a sample size of 24 animals was used for the six experiments described in Figs 5–7. For quantification purposes, samples were number coded and evaluated by an independent operator.

Hippocampus immunohistochemistry

Hippocampus immunohistochemistry was modified from Salazar *et al.* (2008). Briefly, mouse brains were fixed by transcardiac perfusion of 4% paraformaldehyde in PBS, dissected, and post-fixed for 24 h in the same medium. Coronal slices 20-μm thick were obtained using a freezing microtome. Free-floating sections were permeabilized, blocked for nonspecific binding, and incubated with primary antibody. Immunolabeling was visualized using Alexa 488- and Alexa 532-conjugated secondary antibodies (Invitrogen) or horseradish peroxidase (HRP)-conjugated secondary antibodies and 3,3′-diaminobenzidine staining (Vector Laboratories, Burlingame, CA, USA). The labeled cells were observed with a Zeiss LSM 510 Meta Confocal Microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). The intensity of fluorescence was determined using the Quantity One program from Bio-Rad Laboratories, Hercules, CA, USA.

Immunofluorescence

Immunofluorescence was performed in cells grown in cover slips or coronal slices. Cells were fixed with 4% paraformaldehyde/4% sucrose in PBS, permeabilized with 0.2% Triton-X-100 in PBS, incubated overnight at 4°C with anti-4-HNE (1 : 500), anti-GFAP (astrocyte marker, 1 : 500), anti-CD11b (microglia marker, 1 : 100), or anti-TujI (neuron marker, 1 : 1000) followed by incubation with Alexa-546-conjugated goat anti-mouse IgG, Alexa-488-conjugated goat anti-rabbit IgG, and TOPRO-3 (Thermo Fisher Scientific, Waltham, MA, USA) for nuclei identification. The labeled cells were observed with a Zeiss LSM 510 Meta Confocal Microscope. The intensity of fluorescence was determined using the ImageJ program.

Statistical analysis

The data presented are representative of at least three independent experiments. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Statistical significance was determined using one-way ANOVA with Tukey *post hoc* test. Values were expressed as mean ± SEM. The significance threshold was $p < 0.05$.

Results

Hepcidin reduces the expression and secretion of IL-6 and TNF-α from astrocytes or microglia treated with Aβ aggregates

In order to evaluate a putative anti-inflammatory effect of hepcidin, primary cultures of cortical astrocytes and microglia were performed. The morphology and purity of cultured neurons, astrocytes, and microglia were confirmed by antibody labeling using established markers: Tuj1 (neurons), GFAP (astrocytes), and CD11b (microglia) (Figure S1). The purity of primary cultures of astrocytes and microglia were > 95% and > 90%, respectively. Primary cultures of cortical astrocytes were pre-treated with hepcidin for 2 h and then with $A\beta_{1-42}$ for 8 h. The expression of IL-6 and TNF-α was analyzed by semi-quantitative RT-PCR (Fig. 1a). The inverted sequence peptide $A\beta_{42-1}$ was used as an additional control. Treatment with $A\beta_{1-42}$ induced significant increments in the expression of IL-6 (3.45 pM) (Fig. 1b) and TNF-α (0.29 pM) (Fig. 1c), but pre-treatment with hepcidin suppressed these increments. To assess whether the increased expression of IL-6 or TNF-α results in increased protein levels, secretion of IL-6 and TNF-α in the culture medium was evaluated. Treatment with $A\beta_{1-42}$ induced a significant increase in the secretion of IL-6 (Fig. 1d) and TNF-α (Fig. 1e). Consistent with hepcidin-induced reduction in IL-6 and TNF-α mRNA expression, pre-treatment with hepcidin reduced $A\beta_{1-42}$-induced secretion of IL-6 (Fig. 1d) and TNF-α (Fig. 1e).

The hepcidin anti-inflammatory effect was also evaluated in primary culture of cortical microglial cells. For this purpose, microglia were either pre-treated with hepcidin or not, followed by treatment with $A\beta_{1-42}$ for 8 h. The expression of the cytokines, IL-6, and TNF-α was analyzed by semi-quantitative RT-PCR (Fig. 2a and b). In addition, the secretion of these cytokines was analyzed after 24 h of treatment (Fig. 2d and e). Similar to the results observed in astrocytes, treatment with $A\beta_{1-42}$ increased both the expression and the secretion of TNF-α (0.59 pM) and IL-6 (10.29 pM) in microglial cells. As expected, pre-treatment

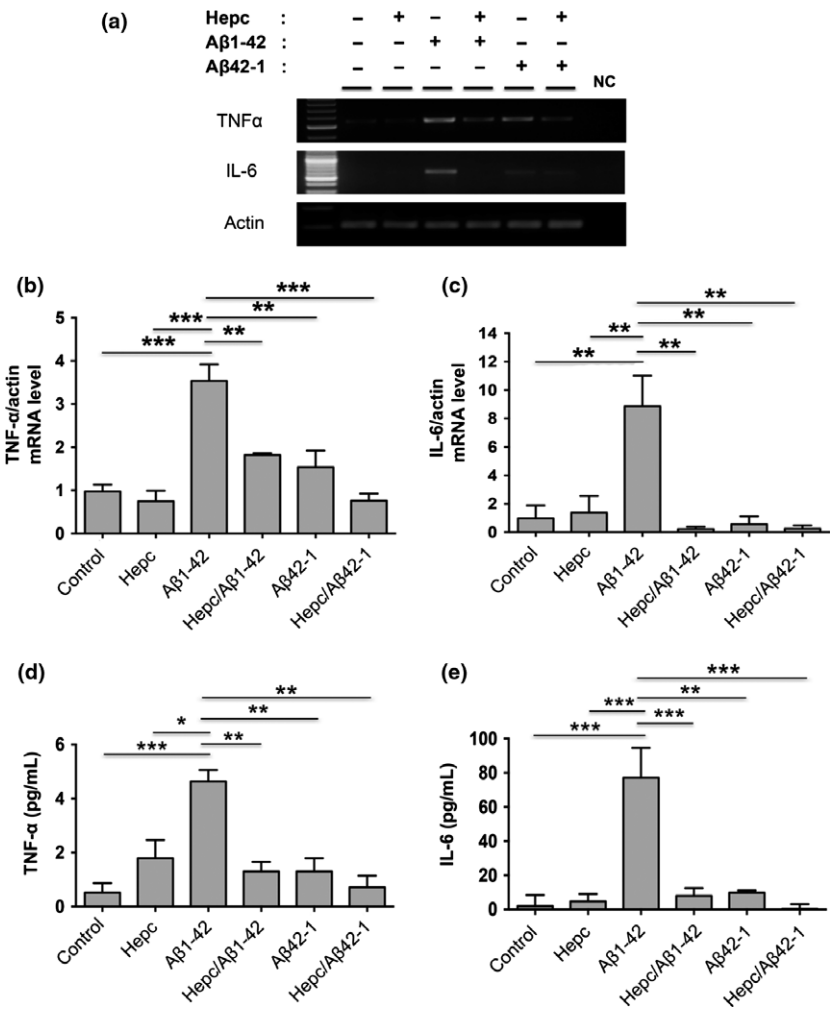


Fig. 1 Hepcidin reduces TNF-α and IL-6 expression from amyloid β-stimulated astrocytes. Cortical astrocytes were pre-treated with 1 μg/mL hepcidin for 2 h and then treated with 9 μg/mL $A\beta_{1-42}$ or $A\beta_{42-1}$ peptides for 8 h to analyze mRNA by RT-PCR or for 24 h to analyze TNF-α and IL-6 secretion by ELISA. (a) Representative image of RT-PCR for TNF-α and IL-6. Band density quantifications for TNF-α (b) and IL-6 (c) mRNA. ELISA quantification of TNF-α (c) and IL-6 (d) secretion. Values represent mean ± SEM (N = 3 independent experiments). *p < 0.05; **p < 0.01; ***p < 0.001.

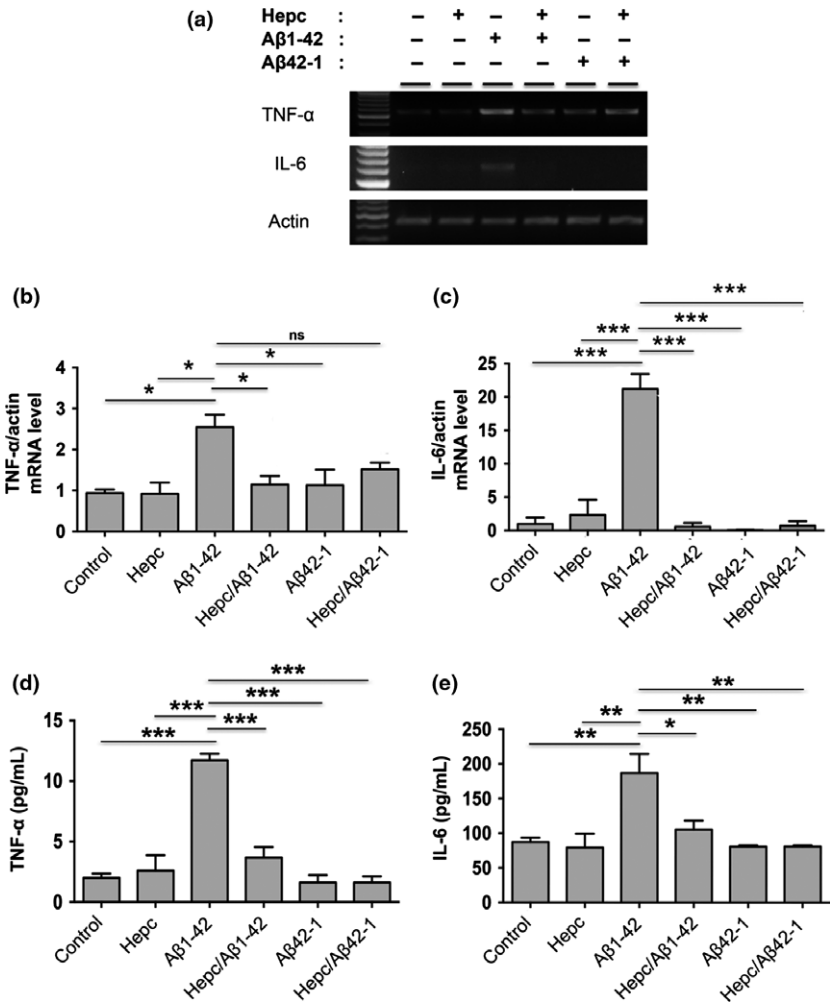


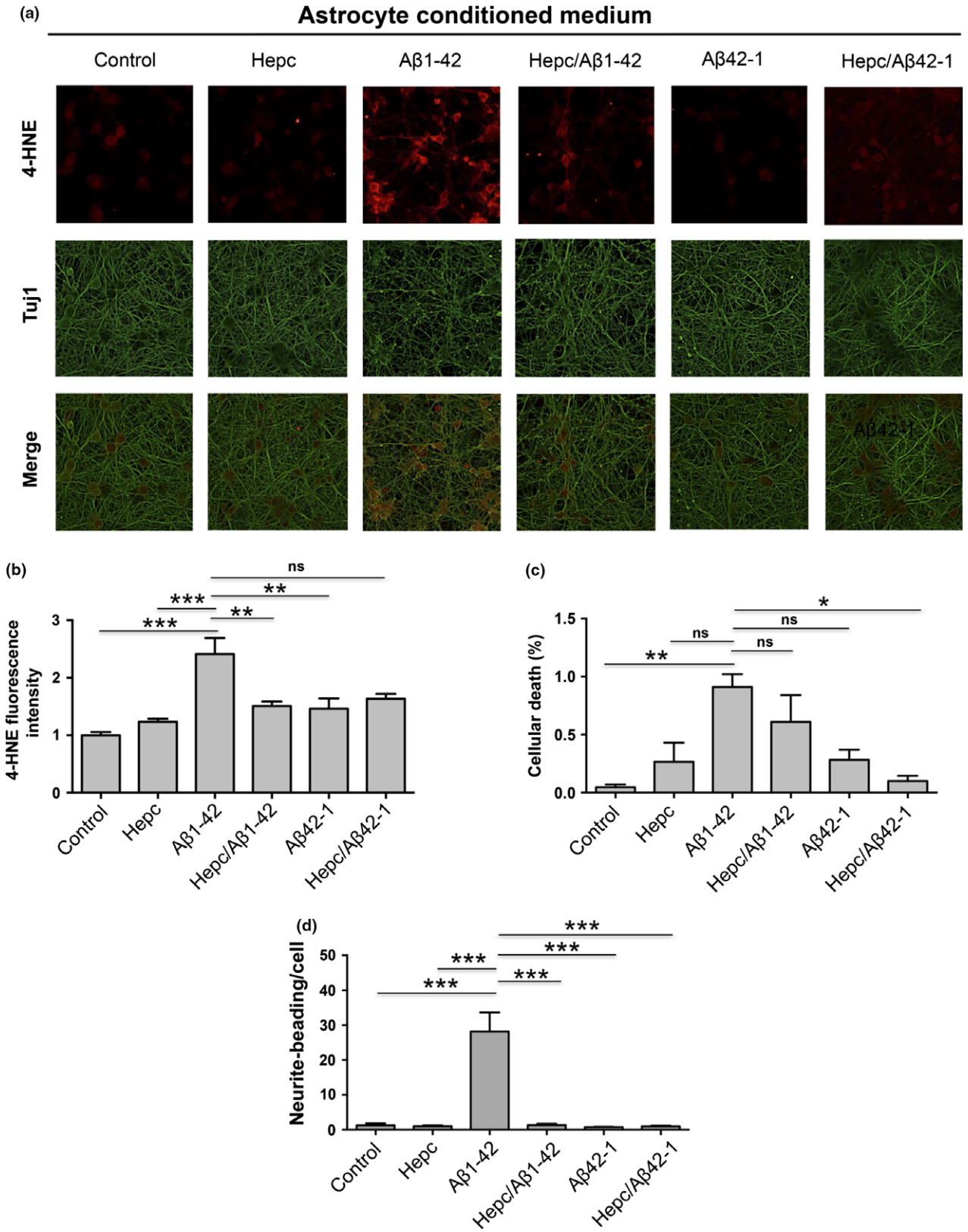
Fig. 2 Hepcidin reduces TNF-α and IL-6 expression in Aβ-stimulated microglia. Cortical microglial cells were pre-treated with 1 μg/mL hepcidin for 2 h and then treated for 8 h with 9 μg/mL of $A\beta_{1–42}$ prior to analysis of mRNA by RT-PCR or for 24 h prior to analysis of TNF-α and IL-6 secretion by ELISA. (a) Representative image of RT-PCR for TNF-α and IL-6 mRNA. Band density quantifications for TNF-α mRNA (b) and IL-6 mRNA (c). Values represent mean ± SEM ($N = 3$ independent experiments). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

with hepcidin abrogated the increased expression and secretion of TNF-α and IL-6 induced by $A\beta_{1–42}$ (Fig. 2b–e).

Hepcidin reduces the effect of conditioned media from astrocytes or from microglia treated with Aβ on hippocampal neurons

Previous reports have indicated that activated microglia and astrocytes are important mediators of Aβ-induced neurotoxicity through the secretion of toxic oxidizing species and inflammatory cytokines (von Bernhardi and Eugenin 2004; Floden *et al.* 2005; Garwood *et al.* 2011). Accordingly, we evaluated the effect of hepcidin in the generation of putatively toxic conditioned media derived from astrocytes and microglia treated with $A\beta_{1–42}$ aggregates (Fig. 3). The conditioned media obtained from astrocytes treated with $A\beta_{1–42}$ induced increased immunoreactivity against 4-HNE in hippocampal neurons compared to control or $A\beta_{1–42}$ treatment (Fig. 3a and b). Inversely, neurons treated with conditioned media obtained from astrocytes pre-treated with hepcidin did not show increased 4-HNE immunoreactivity. In initial experiments, we determined that $A\beta_{1–42}$ by itself did not show apparent neurotoxicity as demonstrated by the null formation of 4-HNE adducts (Figure S2). Conditioned media obtained from astrocytes treated with $A\beta_{1–42}$ induced a minor decrease in viability compared to control or $A\beta_{42–1}$ treatment (Fig. 3c). Pre-treatment with hepcidin did not

Fig. 3 Hepcidin reduces the oxidative damage to hippocampal neurons treated with conditioned media from amyloid β-stimulated astrocytes. Cortical astrocytes were pre-treated with 1 μg/mL hepcidin for 2 h and then treated with 9 μg/mL Aβ peptide for 24 h. The culture media was centrifuged and 300 μL of the supernatant was placed into a hippocampal neuron culture for 48 h. The production of 4-HNE adducts was analyzed by immunofluorescence to assess oxidative damage after treatment. (a) Representative images of immunofluorescence against 4-HNE (red) and Tuj1 (neuron marker, green). (b) Quantification of 4-HNE fluorescence intensity. Size bars: 50 μm. (c) Cell death determined by the calcein–propidium iodide assay. (d) Neurite beading per cell quantified with the ImageJ program. Values represent mean ± SEM ($N = 3$ independent experiments). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.


(a)
Astrocyte conditioned medium
Control
Hepc
Aβ1-42
Hepc/Aβ1-42
Aβ42-1
Hepc/Aβ42-1
4-HNE
Tuj1
Merge
Aβ42-1
(b)
4-HNE fluorescence intensity
ns
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***
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Control
Hepc
Aβ1-42
Hepc/Aβ1-42
Aβ42-1
Hepc/Aβ42-1
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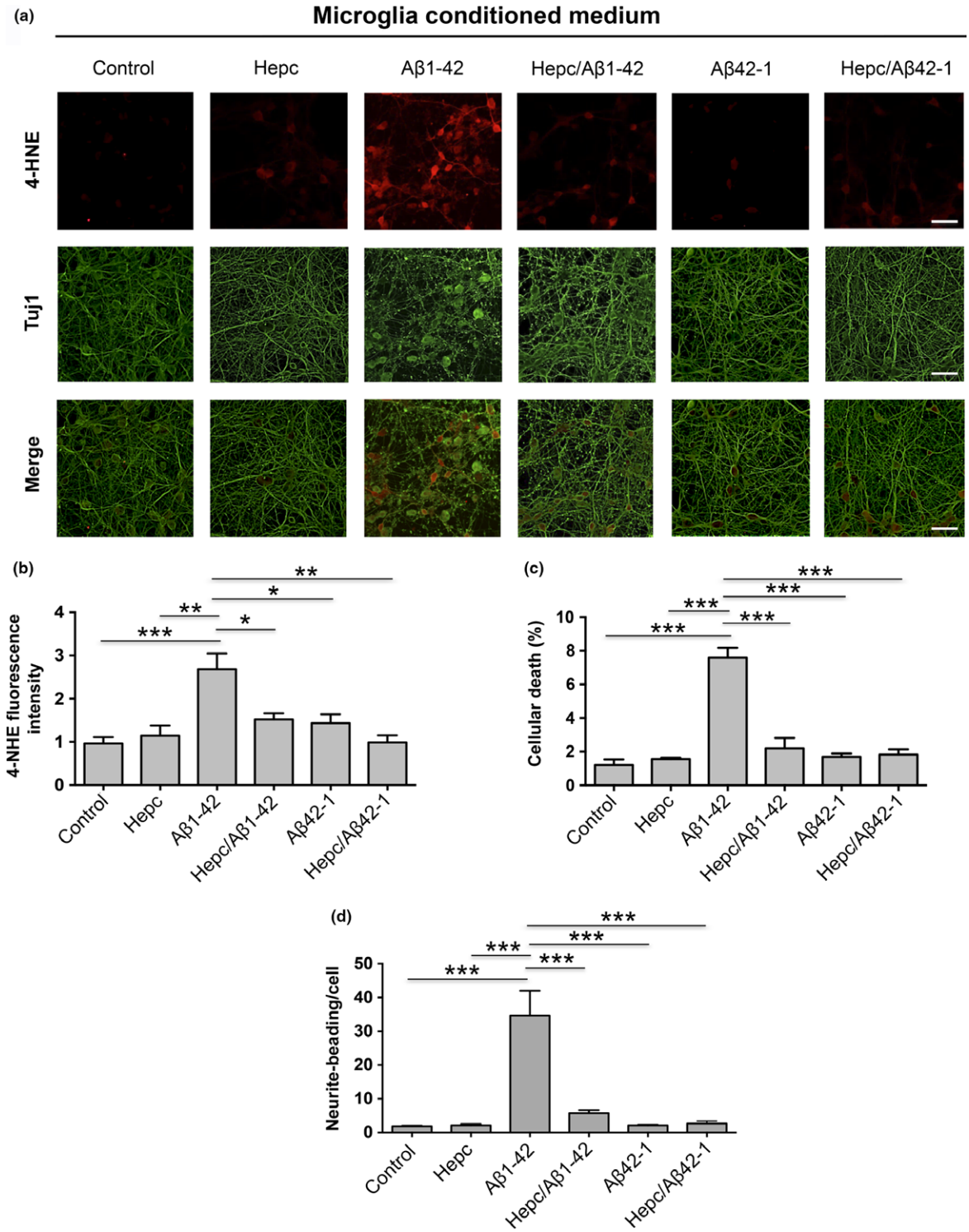

Fig. 4 Hepcidin reduces the oxidative damage to hippocampal neurons treated with conditioned media from amyloid β-stimulated microglia. Cortical microglial cells were treated with hepcidin (1 μg/mL), Aβ peptide (9 μg/mL), or pre-treated with hepcidin for 2 h and then treated with Aβ peptide for 24 h. The culture media was centrifuged and the supernatant was used as conditioned medium, which was placed on hippocampal neuron cultures for 48 h. The production of 4-HNE adducts was analyzed by immunofluorescence to assess oxidative damage after treatment. (a) Representative images of 4-HNE immunofluorescence (red) and Tuj1 immunofluorescence (green). Size bars: 50 μm. (b) Quantification of 4-HNE immunofluorescence intensity. (c) Cell death determined by the calcein–propidium iodide assay. (d) Neurite beading per cell quantified with the ImageJ program. Values represent mean ± SEM ($N = 3$ independent experiments). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

reverse this effect. In addition, $A\beta_{42-1}$ treatment induced a marked neurite beading, which was completely abolished by hepcidin co-treatment (Fig. 3a and d).

Similar effects were observed with conditioned media obtained from microglial cultures (Fig. 4). Conditioned media obtained from microglia treated with $A\beta_{1-42}$ increased the immunoreactivity against 4-HNE in hippocampal neurons by 2.5 times (Fig. 4a and b), and increased neurite beading compared to control or $A\beta_{42-1}$ treatment (Fig. 4d). In contrast, conditioned media obtained from microglia pre-treated with hepcidin, followed by $A\beta_{1-42}$ did not show an increase in 4-HNE immunoreactivity and neurite beading.

Conditioned media obtained from microglia treated with $A\beta_{1-42}$ induced 8% neuronal death compared to control or $A\beta_{42-1}$ treatments, while conditioned media from microglia pre-treated with hepcidin did not induce such increase (Fig. 4c).

To ascertain whether the toxic effect of $A\beta_{1-42}$ aggregates on neurons is due solely to TNF-α and IL-6 secretion, media obtained from untreated astrocytes or microglia were supplemented with TNF-α and IL-6 at the same concentrations found in the conditioned media of cultures treated with $A\beta_{1-42}$ previously determined by ELISA. Both astrocyte or microglia media supplemented with TNF-α and IL-6 induced an increment in neuronal 4-HNE immunoreactivity, albeit at lower levels than those induced by media obtained from microglia or astrocytes treated with $A\beta_{1-42}$ (Figure S3). This result strongly suggests that secreted compounds other than TNF-α and IL-6 synergistically contribute to $A\beta_{1-42}$ toxicity.

Hepcidin reduces astrocyte and microglial activation induced by intracerebroventricular (ICV) injection of $A\beta_{1-42}$ aggregates in mice

The above *in vitro* studies suggest anti-inflammatory and antioxidant effects of hepcidin in cells of the central nervous system. Thus, we further analyzed the effect of hepcidin in mice, by studying the effect of injected $A\beta_{1-42}$ aggregates in the presence or absence of hepcidin. GFAP and Iba1 immunostaining were performed to evaluate generation of reactive astrocytes and microglia, respectively.

Immunofluorescence was quantified in three regions of the hippocampus: the CA2 region (U1), the hippocampal fissure (U2), and the dentate gyrus (U3) (Fig. 5a). Intracerebroventricular (ICV) injection of $A\beta_{1-42}$ resulted in increased immunoreactivity against GFAP in the three regions (Fig. 5b and c). Hepcidin injection also resulted in increased GFAP immunoreactivity. However, $A\beta_{1-42}$ induced a larger increase in the U2 region than hepcidin, and this increase was averted by pre-treatment with hepcidin (Fig. 5b and c). Hence, although hepcidin increases astrocyte GFAP expression, pre-treatment with hepcidin diminishes the increase in GFAP expression induced by $A\beta_{1-42}$.

Iba1 staining in the U2 region revealed substantial changes in the morphology of microglia in mice injected with $A\beta_{1-42}$, with microglia exhibiting amoeboid structures typical of activated microglia (Fig. 6a). These changes were not observed in mice injected with hepcidin, $A\beta_{42-1}$ or (Fig. 6a). Quantification of size changes revealed that $A\beta_{1-42}$ induced a significant increment ($p < 0.001$) in mean microglia area, which was abolished by hepcidin co-treatment (Fig. 6b). Accordingly, hepcidin suppresses microglial activation induced by $A\beta_{1-42}$.

Hepcidin reduces the oxidative damage induced by ICV injection of the Aβ peptide in mice

We analyzed the effect of Aβ injection on oxidative stress in the U2 region of hippocampus. For this purpose, the formation of 4-HNE-protein adducts was evaluated by immunofluorescence. $A\beta_{1-42}$-injected mice showed a significant increase in immunoreactivity against 4-HNE compared to mice injected with vehicle, $A\beta_{42-1}$ or hepcidin (Fig. 7a and b). Importantly, pre-treatment with hepcidin suppressed the $A\beta_{1-42}$-induced increment in 4-HNE immunoreactivity. Therefore, the presence of hepcidin reduces oxidative damage to the hippocampus induced by the ICV injection of $A\beta_{1-42}$ aggregates.

Discussion

Inflammation and oxidative stress are common features of neurodegenerative diseases of the central nervous system, such as AD and Parkinson's disease (Fischer and Maier 2015; Leszek *et al.* 2016). The inflammatory phenotype of AD is characterized by the presence of amoeboid microglia and reactive astrocytes surrounding senile plaques, as well as elevated levels of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α, and other mediators (McGeer and McGeer 2013). Toll-like receptors, which detect bacterial compounds like LPS, also triggers Aβ-mediated glial activation. Several epidemiological studies show that non-steroidal anti-inflammatory drugs have a beneficial impact on the onset of AD-associated dementia, reducing AD risk (Stewart *et al.* 1997; in t' Veld *et al.* 2001). However, randomized controlled clinical trials failed to show any beneficial effect (McGeer and McGeer 2007), suggesting that the role of inflammation in AD is a complex phenomenon. The recruitment of peripheral macrophages around Aβ plaques seems to lead to the clearance of these plaques (El Khoury *et al.* 2007; Baruch *et al.* 2015, 2016). Therefore, certain aspects of the immune response might be deleterious (secretion of neurotoxic factors), whereas other aspects might actually protect against the cytotoxic properties of Aβ (e.g. clearance of Aβ) (Naert and Rivest 2013).

As mentioned earlier, in peripheral macrophages the binding of hepcidin to FPN1 activates a Jak2/Stat3/SOCS3 signal transduction pathway, resulting in the suppression of increased IL-6 and TNF-α secretion induced by LPS (De Domenico *et al.* 2010). In the central nervous system, the knowledge about the role of hepcidin is scarce, focusing mainly on its modulatory role of iron homeostasis. In

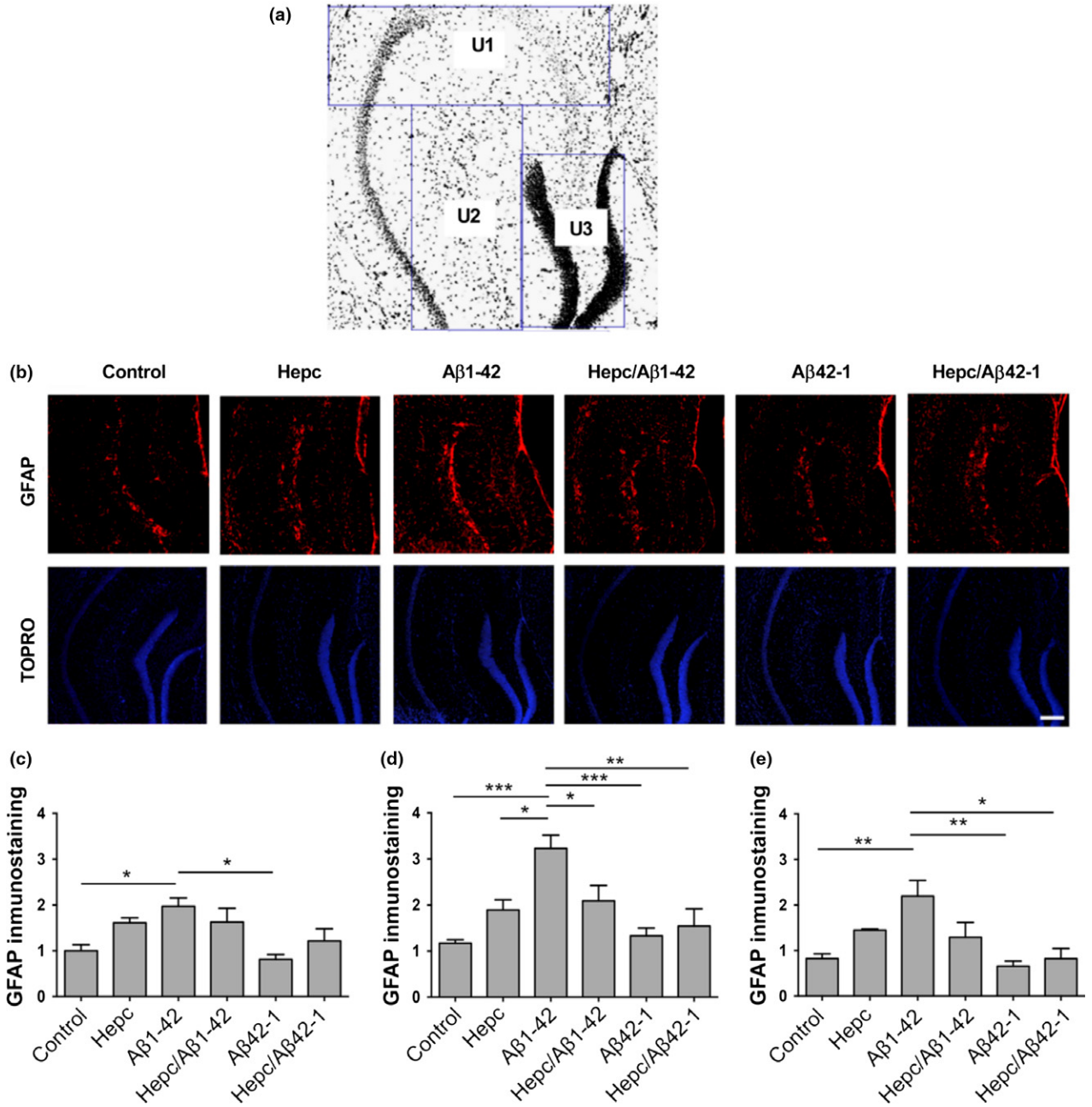

Fig. 5 Hepcidin diminishes amyloid β-induced glial fibrillary acidic protein (GFAP) expression in mice. Mice were ICV-injected with 2 μg $A\beta_{1-42}$ with or without 10 μg hepcidin in saline. Subsequently, immunofluorescence against GFAP was performed. (a) Topology of the three areas selected (U1, U2, U3) for quantification of fluorescence staining. (b) Representative images of immunofluorescence against GFAP (red) and TOPRO (blue). Size bars: 200 μm. (c) Quantification of fluorescence intensity for GFAP in the U1 area. (d) Quantification of fluorescence intensity for GFAP in the U2 area. (d) Quantification of fluorescence intensity for GFAP in the U3 area. Data represent mean ± SEM ($n = 4$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

neurons, astrocytes and microglia hepcidin decreases FPN1 levels (Wang *et al.* 2010; Li *et al.* 2011; Urrutia *et al.* 2013). Given its putative anti-inflammatory capacity, we hypothesized a possible protective effect of hepcidin in an Aβ-mediated inflammatory model. We found that hepcidin blocked $A\beta_{1-42}$-induced IL-6 and TNF-α expression and secretion, both in cultured astrocytes and in microglia. We also found that hepcidin diminished oxidative damage and cell death in neurons exposed to conditioned media of $A\beta_{1-42}$-treated astrocytes or microglia. The neuroprotective effect of hepcidin may be mediated by either its anti-inflammatory effect on glial cells or a direct neuroprotective effect against

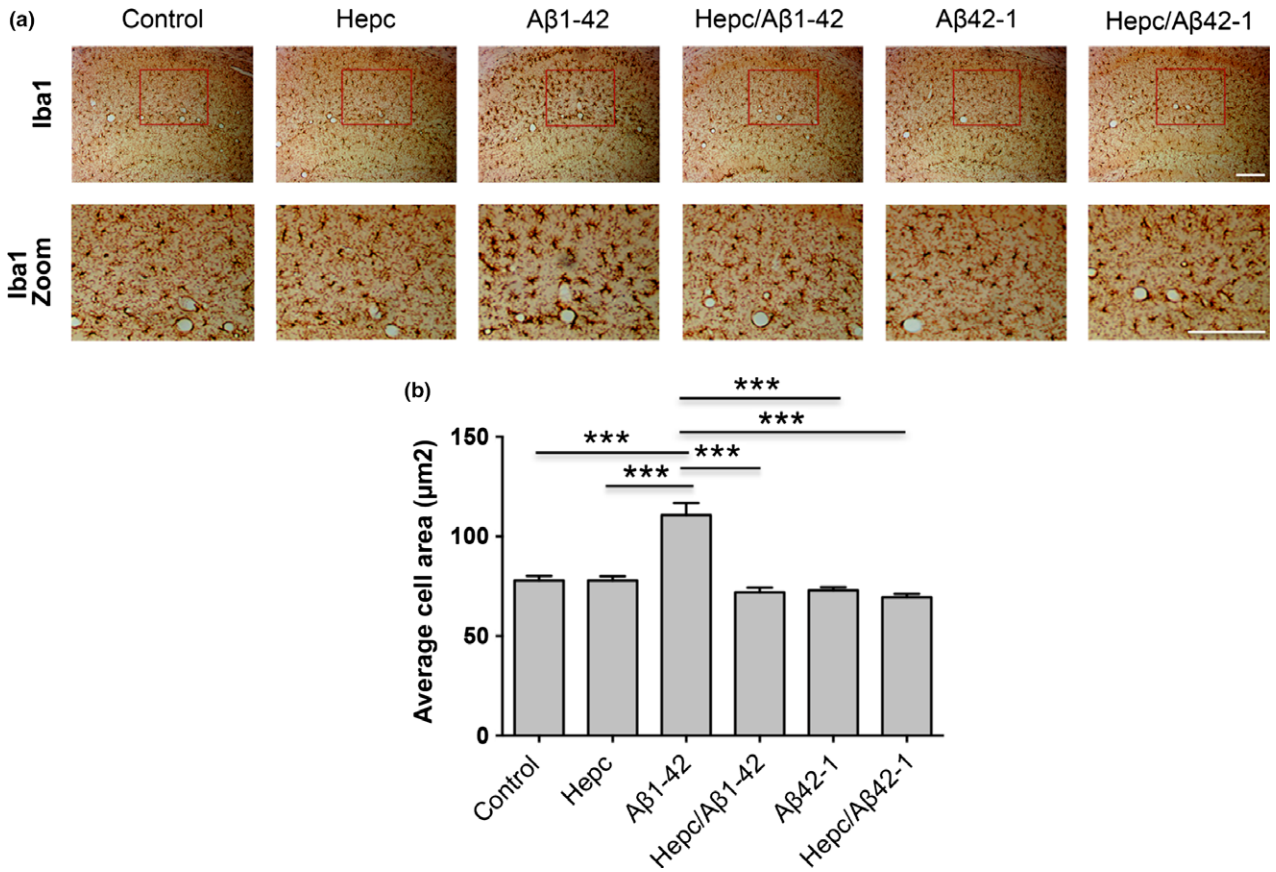

Fig. 6 Hepcidin diminishes Aβ-induced microglial activation in mice. Mice were stereotaxically injected in the right lateral ventricle with $A\beta_{1-42}$ (2 μg) with or without hepcidin (10 μg) in a saline solution. Mice were sacrificed 72 h afterwards and immunohistochemistry against the microglia marker Iba1 was performed. Size bars: 80 μm. (b) Quantification of Iba1-positive cells area determined by the ImageJ program. Values represent mean ± SEM ($n = 4$; 45–52 cells per condition). ***$p < 0.001$.

Aβ-induced toxicity in neurons. A direct neuroprotective effect of the hepcidin present in the conditioned media can be fairly discarded on the basis of published evidence. Through a degradative endocytic process, hepcidin is almost completely degraded 24 h after its addition to a cell culture (Preza *et al.* 2013). Indeed, our previous results indicate that in neurons, astrocytes, and microglia hepcidin induces the degradation of FPN1 up to 6 h post-treatment, returning FPN1 to near basal levels after 24 h of incubation (Urrutia *et al.* 2013). These data support the notion that hepcidin is continuously removed from the culture media, with little of it remaining after a 24-h incubation.

In addition, we found that ICV administration of hepcidin decreased Aβ-induced astrocyte and microglia activation. Overall, the *in vitro* and *in vivo* data are consistent with a mechanism by which hepcidin pre-treatment decreases the inflammatory response of astrocytes and microglia to $A\beta_{1-42}$, thus decreasing IL-6 and TNF-α secretion.

As astrocytes have been associated with the regulation of brain iron homeostasis, specifically by buffering extracellular iron changes at the synaptic cleft (Codazzi *et al.* 2015), the observed increase in astrocyte immunoreactivity could be a physiological response of astrocytes to increased intracellular iron levels induced by hepcidin down-regulation of FPN1.

As mentioned earlier, inflammatory stimuli induce the expression of hepcidin in astrocytes and microglia. In addition, here we report that exogenous hepcidin repressed the inflammatory effect stimulated by $A\beta_{1-42}$. It would be of interest to evaluate whether or not Aβ stimulates expression of hepcidin through its pro-inflammatory activity. If this was the case, a negative feedback mechanism could be in place in which Aβ-induced hepcidin expression may result in a homeostatic anti-inflammatory response.

$A\beta_{1-42}$ treatment resulted in increased microglial activation and oxidative damage

Binding of $A\beta_{1-42}$ to a number of microglia and astrocyte receptors, including the receptors for advanced glycation endproduct, CD14/TLR2 and CD14/TLR4, results in the

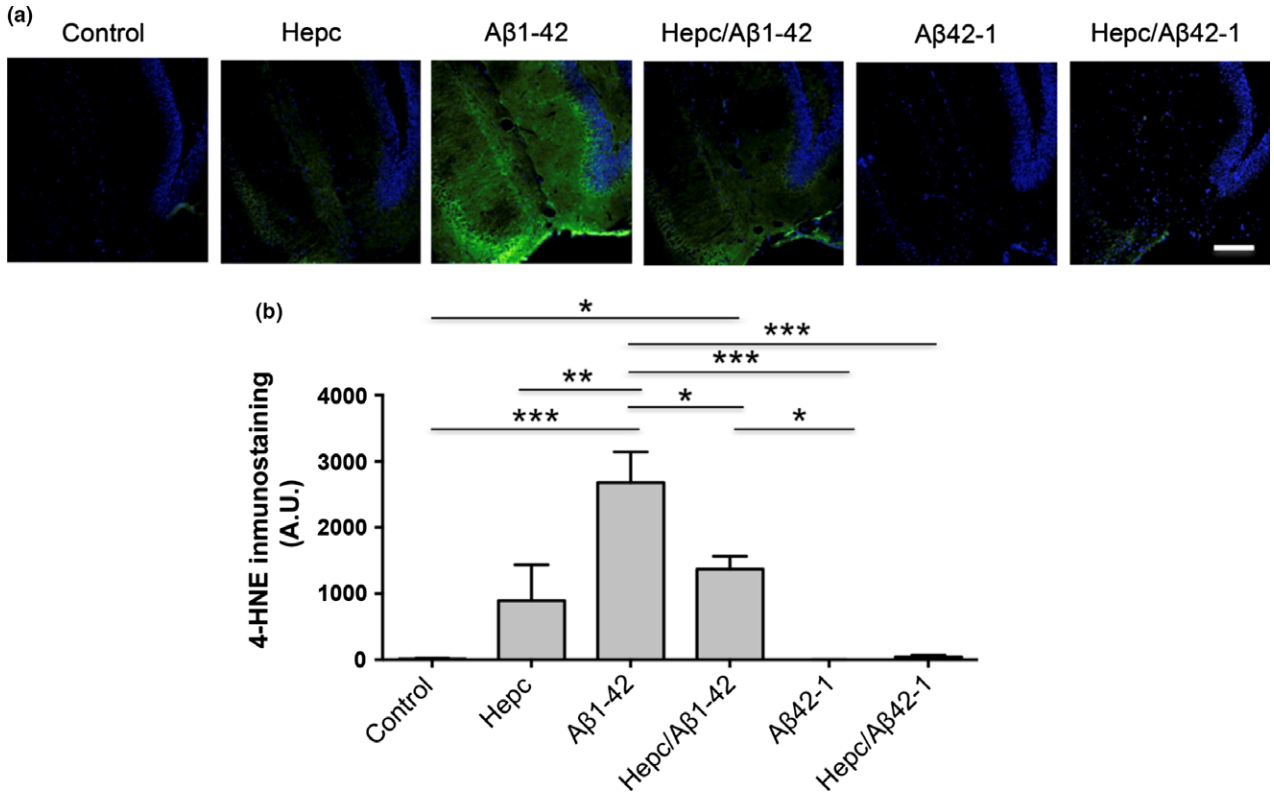

Fig. 7 Hepcidin reduces amyloid β-induced oxidative modifications in hippocampus. Mice were stereotaxically injected in the right lateral ventricle with Aβ (2 μg) with or without hepcidin (10 μg) in a saline solution. Mice were killed 72 h afterwards and 4-HNE immunofluorescence was performed in 20-μm thick hippocampal slices. (a) A representative image of a hippocampal slices immunostained for 4-HNE (green) and TOPRO (nucleus, blue). (b) Quantification of anti-HNE fluorescence intensity. Values represent mean ± SEM ($n = 4$). *$p < 0.05$; **$p < 0.05$; ***$p < 0.001$. Differences between the Hepc, $A\beta_{42-1}$, Hepc/$A\beta_{42-1}$ and control conditions were not significant.

production of inflammatory cytokines (Walter *et al.* 2007; Balistreri *et al.* 2008; Yu and Ye 2015). Aβ-induced microglial activation and oxidative damage was prevented by hepcidin pre-treatment. Furthermore, hepcidin suppressed oxidative damage in the hippocampus of Aβ-injected mice.

Taken together, these results suggest that hepcidin triggers an antioxidant response in astrocytes and microglia. It remains to be determined if this response is mediated by the Jak2/STAT3/SOCS3 signal transduction pathway.

In summary, we provide evidence indicating that hepcidin suppresses the inflammatory response triggered by $A\beta_{1-42}$ aggregates in astrocytes and microglia, reducing the expression and secretion of the pro-inflammatory cytokines, IL-6 and TNF-α, thus decreasing the oxidative damage of hippocampal neurons. In addition, stereotaxic injection of hepcidin suppressed astrocyte and microglial activation as well as induction of oxidative stress markers in neurons. These results are consistent with the hypothesis that hepcidin is an anti-inflammatory agent that blocks the pro-inflammatory and pro-oxidant actions of the Aβ peptide in the brain.

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All experiments were conducted in compliance with the ARRIVE guidelines.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Immunocytochemistry of primary culture of neurons, astrocytes and microglia.

Figure S2. $A\beta_{1-42}$ *per se* does not induce oxidative damage to neurons.

Figure S3. TNF-α and IL-6 contribute partially to Aβ-induced oxidative damage in neurons.

References

Balistreri C. R., Grimaldi M. P., Chiappelli M. *et al.* (2008) Association between the polymorphisms of TLR4 and CD14 genes and Alzheimer's disease. *Curr. Pharm. Des.* **14**, 2672–2677.

Ballatore C., Lee V. M. and Trojanowski J. Q. (2007) Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. *Nat. Rev. Neurosci.* **8**, 663–672.

Bamberger M. E., Harris M. E., McDonald D. R., Husemann J. and Landreth G. E. (2003) A cell surface receptor complex for fibrillar beta-amyloid mediates microglial activation. *J. Neurosci.* **23**, 2665–2674.

Baruch K., Kertser A., Porat Z. and Schwartz M. (2015) Cerebral nitric oxide represses choroid plexus NFkappaB-dependent gateway activity for leukocyte trafficking. *EMBO J.* **34**, 1816–1828.

Baruch K., Deczkowska A., Rosenzweig N. *et al.* (2016) PD-1 immune checkpoint blockade reduces pathology and improves memory in mouse models of Alzheimer's disease. *Nat. Med.* **22**, 135–137.

von Bernhardi R. and Eugenin J. (2004) Microglial reactivity to beta-amyloid is modulated by astrocytes and proinflammatory factors. *Brain Res.* **1025**, 186–193.

Braak H. and Braak E. (1998) Evolution of neuronal changes in the course of Alzheimer's disease. *J. Neural Transm. Suppl.* **53**, 127–140.

Charan J. and Kantharia N. D. (2013) How to calculate sample size in animal studies? *J. Pharmacol. Pharmacother* **4**, 303–306.

Coccini T., Roda E., Sarigiannis D. A., Mustarelli P., Quartarone E., Profumo A. and Manzo L. (2010) Effects of water-soluble functionalized multi-walled carbon nanotubes examined by different cytotoxicity methods in human astrocyte D384 and lung A549 cells. *Toxicology* **269**, 41–53.

Codazzi F., Pelizzoni I., Zacchetti D. and Grohovaz F. (2015) Iron entry in neurons and astrocytes: a link with synaptic activity. *Front. Mol. Neurosci.* **8**, 18.

Crespo A. C., Silva B., Marques L. *et al.* (2014) Genetic and biochemical markers in patients with Alzheimer's disease support a concerted systemic iron homeostasis dysregulation. *Neurobiol. Aging* **35**, 777–785.

De Domenico I., Lo E., Ward D. M. and Kaplan J. (2009) Hepcidin-induced internalization of ferroportin requires binding and cooperative interaction with Jak2. *Proc. Natl Acad. Sci. USA* **106**, 3800–3805.

De Domenico I., Zhang T. Y., Koening C. L. *et al.* (2010) Hepcidin mediates transcriptional changes that modulate acute cytokine-induced inflammatory responses in mice. *J. Clin. Invest.* **120**, 2395–2405.

Ding B. J., Ma W. W., He L. L., Zhou X., Yuan L. H., Yu H. L., Feng J. F. and Xiao R. (2011) Soybean isoflavone alleviates beta-amyloid 1-42 induced inflammatory response to improve learning and memory ability by down regulation of Toll-like receptor 4 expression and nuclear factor-kappaB activity in rats. *Int. J. Dev. Neurosci.* **29**, 537–542.

Dzikaite V., Holmstrom P., Stal P., Eckes K., Hagen K., Eggertsen G., Gafvels M., Melefors O. and Hultcrantz R. (2006) Regulatory effects of tumor necrosis factor-alpha and interleukin-6 on HAMP expression in iron loaded rat hepatocytes. *J. Hepatol.* **44**, 544–551.

El Khoury J. B., Moore K. J., Means T. K., Leung J., Terada K., Toft M., Freeman M. W. and Luster A. D. (2003) CD36 mediates the innate host response to beta-amyloid. *J. Exp. Med.* **197**, 1657–1666.

El Khoury J., Toft M., Hickman S. E., Means T. K., Terada K., Geula C. and Luster A. D. (2007) Ccr2 deficiency impairs microglial accumulation and accelerates progression of Alzheimer-like disease. *Nat. Med.* **13**, 432–438.

Festing M. F. and Altman D. G. (2002) Guidelines for the design and statistical analysis of experiments using laboratory animals. *ILAR J.* **43**, 244–258.

Fischer R. and Maier O. (2015) Interrelation of oxidative stress and inflammation in neurodegenerative disease: role of TNF. *Oxid. Med. Cell Longev.* **2015**, 610813.

Floden A. M., Li S. and Combs C. K. (2005) Beta-amyloid-stimulated microglia induce neuron death via synergistic stimulation of tumor necrosis factor alpha and NMDA receptors. *J. Neurosci.* **25**, 2566–2575.

Garwood C. J., Pooler A. M., Atherton J., Hanger D. P. and Noble W. (2011) Astrocytes are important mediators of Abeta-induced neurotoxicity and tau phosphorylation in primary culture. *Cell Death Dis.* **2**, e167.

Glenner G. G. and Wong C. W. (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* **120**, 885–890.

Hardy J. (2009) The amyloid hypothesis for Alzheimer's disease: a critical reappraisal. *J. Neurochem.* **110**, 1129–1134.

Hardy J. and Selkoe D. J. (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **297**, 353–356.

Heneka M. T., Golenbock D. T. and Latz E. (2015) Innate immunity in Alzheimer's disease. *Nat. Immunol.* **16**, 229–236.

Hu J., Akama K. T., Krafft G. A., Chromy B. A. and Van Eldik L. J. (1998) Amyloid-beta peptide activates cultured astrocytes: morphological alterations, cytokine induction and nitric oxide release. *Brain Res.* **785**, 195–206.

Huang Y. H., Yang Y. L., Tiao M. M., Kuo H. C., Huang L. T. and Chuang J. H. (2012) Hepcidin protects against lipopolysaccharide-induced liver injury in a mouse model of obstructive jaundice. *Peptides* **35**, 212–217.

Inoue J. and Aramaki Y. (2007) Suppression of skin lesions by transdermal application of CpG-oligodeoxynucleotides in NC/Nga mice, a model of human atopic dermatitis. *J. Immunol.* **178**, 584–591.

Kamphuis W., Middeldorp J., Kooijman L., Sluijs J. A., Kooi E. J., Moeton M., Freriks M., Mizee M. R. and Hol E. M. (2014) Glial fibrillary acidic protein isoform expression in plaque related astrogliosis in Alzheimer's disease. *Neurobiol. Aging* **35**, 492–510.

Lee P., Peng H., Gelbart T. and Beutler E. (2004) The IL-6- and lipopolysaccharide-induced transcription of hepcidin in HFE-, transferrin receptor 2-, and beta 2-microglobulin-deficient hepatocytes. *Proc. Natl Acad. Sci. USA* **101**, 9263–9265.

Lee P., Peng H., Gelbart T., Wang L. and Beutler E. (2005) Regulation of hepcidin transcription by interleukin-1 and interleukin-6. *Proc. Natl Acad. Sci. USA* **102**, 1906–1910.

Leszek J., Barreto G. E., Gasiorowski K., Koutsouraki E., Avila-Rodrigues M. and Aliev G. (2016) Inflammatory mechanisms and oxidative stress as key factors responsible for progression of neurodegeneration: role of brain innate immune system. *CNS Neurol. Disord. Drug Targets* **15**, 329–336.

Li L., Holscher C., Chen B. B., Zhang Z. F. and Liu Y. Z. (2011) Hepcidin treatment modulates the expression of divalent metal transporter-1, ceruloplasmin, and ferroportin-1 in the rat cerebral cortex and hippocampus. *Biol. Trace Elem. Res.* **143**, 1581–1593.

Liu Y., Walter S., Stagi M. *et al.* (2005) LPS receptor (CD14): a receptor for phagocytosis of Alzheimer's amyloid peptide. *Brain* **128**, 1778–1789.

Lue L. F., Rydel R., Brigham E. F. *et al.* (2001) Inflammatory repertoire of Alzheimer's disease and nondemented elderly microglia in vitro. *Glia* **35**, 72–79.

Marques F., Falcao A. M., Sousa J. C., Coppola G., Geschwind D., Sousa N., Correia-Neves M. and Palha J. A. (2009) Altered iron metabolism is part of the choroid plexus response to peripheral inflammation. *Endocrinology* **150**, 2822–2828.

Masters C. L. and Beyreuther K. (2006) Alzheimer's centennial legacy: prospects for rational therapeutic intervention targeting the Abeta amyloid pathway. *Brain* **129**, 2823–2839.

Masters C. L., Simms G., Weinman N. A., Multhaup G., McDonald B. L. and Beyreuther K. (1985) Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc. Natl Acad. Sci. USA* **82**, 4245–4249.

Matak P., Chaston T. B., Chung B., Srai S. K., McKie A. T. and Sharp P. A. (2009) Activated macrophages induce hepcidin expression in HuH7 hepatoma cells. *Haematologica* **94**, 773–780.

McGeer P. L. and McGeer E. G. (2007) NSAIDs and Alzheimer disease: epidemiological, animal model and clinical studies. *Neurobiol. Aging* **28**, 639–647.

McGeer P. L. and McGeer E. G. (2013) The amyloid cascade-inflammatory hypothesis of Alzheimer disease: implications for therapy. *Acta Neuropathol.* **126**, 479–497.

Medeiros R. and LaFerla F. M. (2013) Astrocytes: conductors of the Alzheimer disease neuroinflammatory symphony. *Exp. Neurol.* **239**, 133–138.

Morimoto Y., Tsuda T., Hori H., Yamato H., Ohgami A., Higashi T., Nagata N., Kido M. and Tanaka I. (1999) Combined effect of cigarette smoke and mineral fibers on the gene expression of cytokine mRNA. *Environ. Health Perspect.* **107**, 495–500.

Naert G. and Rivest S. (2013) A deficiency in CCR2+ monocytes: the hidden side of Alzheimer's disease. *J. Mol. Cell Biol.* **5**, 284–293.

Nemeth E., Valore E. V., Territo M., Schiller G., Lichtenstein A. and Ganz T. (2003) Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. *Blood* **101**, 2461–2463.

Nemeth E., Rivera S., Gabayan V., Keller C., Taudorf S., Pedersen B. K. and Ganz T. (2004a) IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J. Clin. Invest.* **113**, 1271–1276.

Nemeth E., Tuttle M. S., Powelson J., Vaughn M. B., Donovan A., Ward D. M., Ganz T. and Kaplan J. (2004b) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* **306**, 2090–2093.

Nicolas G., Chauvet C., Viatte L., Danan J. L., Bigard X., Devaux I., Beaumont C., Kahn A. and Vaulont S. (2002) The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *J. Clin. Investig.* **110**, 1037–1044.

Olabarria M., Noristani H. N., Verkhratsky A. and Rodriguez J. J. (2010) Concomitant astroglial atrophy and astrogliosis in a triple transgenic animal model of Alzheimer's disease. *Glia* **58**, 831–838.

Pagani A., Nai A., Corna G., Bosurgi L., Rovere-Querini P., Camaschella C. and Silvestri L. (2011) Low hepcidin accounts for the proinflammatory status associated with iron deficiency. *Blood* **118**, 736–746.

Paresce D. M., Ghosh R. N. and Maxfield F. R. (1996) Microglial cells internalize aggregates of the Alzheimer's disease amyloid beta-protein via a scavenger receptor. *Neuron* **17**, 553–565.

Part K., Kunnis-Beres K., Poska H., Land T., Shimmo R. and Zetterstrom Fernaeus S. (2015) Amyloid beta25-35 induced ROS-burst through NADPH oxidase is sensitive to iron chelation in microglial Bv2 cells. *Brain Res.* **1629**, 282–290.

Preza G. C., Pinon R., Ganz T. and Nemeth E. (2013) Cellular catabolism of the iron-regulatory peptide hormone hepcidin. *PLoS ONE* **8**, e58934.

Raha A. A., Vaishnav R. A., Friedland R. P., Bomford A. and Raha-Chowdhury R. (2013) The systemic iron-regulatory proteins hepcidin and ferroportin are reduced in the brain in Alzheimer's disease. *Acta Neuropathol. Commun.* **1**, 55.

Rodriguez-Vieitez E., Ni R., Gulyas B., Toth M., Haggkvist J., Halldin C., Voytenko L., Marutle A. and Nordberg A. (2015) Astrocytosis precedes amyloid plaque deposition in Alzheimer APPswe transgenic mouse brain: a correlative positron emission tomography and in vitro imaging study. *Eur. J. Nucl. Med. Mol. Imaging* **42**, 1119–1132.

Ruiz-Munoz A. M., Nieto-Escamez F. A., Aznar S., Colomina M. T. and Sanchez-Santed F. (2011) Cognitive and histological disturbances after chlorpyrifos exposure and chronic Abeta(1-42) infusions in Wistar rats. *Neurotoxicology* **32**, 836–844.

Salazar J., Mena N., Hunot S. *et al.* (2008) Divalent metal transporter 1 (DMT1) contributes to neurodegeneration in animal models of Parkinson's disease. *Proc. Natl Acad. Sci. USA* **105**, 18578–18583.

Schilling T. and Eder C. (2011) Amyloid-beta-induced reactive oxygen species production and priming are differentially regulated by ion channels in microglia. *J. Cell. Physiol.* **226**, 3295–3302.

Sheedy F. J., Grebe A., Rayner K. J. *et al.* (2013) CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation. *Nat. Immunol.* **14**, 812–820.

Stewart W. F., Kawas C., Corrada M. and Metter E. J. (1997) Risk of Alzheimer's disease and duration of NSAID use. *Neurology* **48**, 626–632.

Stewart C. R., Stuart L. M., Wilkinson K. *et al.* (2010) CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. *Nat. Immunol.* **11**, 155–161.

Taneo J., Adachi T., Yoshida A., Takayasu K., Takahara K. and Inaba K. (2015) Amyloid beta oligomers induce interleukin-1beta production in primary microglia in a cathepsin B- and reactive oxygen species-dependent manner. *Biochem. Biophys. Res. Commun.* **458**, 561–567.

Udan M. L., Ajit D., Crouse N. R. and Nichols M. R. (2008) Toll-like receptors 2 and 4 mediate Abeta(1-42) activation of the innate immune response in a human monocytic cell line. *J. Neurochem.* **104**, 524–533.

Urrutia P., Aguirre P., Esparza A., Tapia V., Mena N. P., Arredondo M., Gonzalez-Billault C. and Nunez M. T. (2013) Inflammation alters the expression of DMT1, FPN1 and hepcidin, and it causes iron accumulation in central nervous system cells. *J. Neurochem.* **126**, 541–549.

in t' Veld B. A., Ruitenberg A., Hofman A., Launer L. J., vanDuijn C. M., Stijnen T., Breteler M. M. and Stricker B. H. (2001) Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease. *N. Engl. J. Med.*, **345**, 1515–1521.

Walter S., Letiembre M., Liu Y. *et al.* (2007) Role of the toll-like receptor 4 in neuroinflammation in Alzheimer's disease. *Cell. Physiol. Biochem.* **20**, 947–956.

Wang Q., Du F., Qian Z. M., Ge X. H., Zhu L., Yung W. H., Yang L. and Ke Y. (2008) Lipopolysaccharide induces a significant increase in expression of iron regulatory hormone hepcidin in the cortex and substantia nigra in rat brain. *Endocrinology* **149**, 3920–3925.

Wang S. M., Fu L. J., Duan X. L. *et al.* (2010) Role of hepcidin in murine brain iron metabolism. *Cell. Mol. Life Sci.* **67**, 123–133.

Wang H. M., Zhang T., Huang J. K. and Sun X. J. (2013) 3-N-butylphthalide (NBP) attenuates the amyloid-beta-induced inflammatory responses in cultured astrocytes via the nuclear factor-kappaB signaling pathway. *Cell. Physiol. Biochem.* **32**, 235–242.

Yu Y. and Ye R. D. (2015) Microglial Abeta receptors in Alzheimer's disease. *Cell. Mol. Neurobiol.* **35**, 71–83.

Zechel S., Huber-Wittmer K. and von Bohlen und Halbach O. (2006) Distribution of the iron-regulating protein hepcidin in the murine central nervous system. *J. Neurosci. Res.* **84**, 790–800.