Fractalkine activates NRF2/NFE2L2 and heme oxygenase 1 to restrain tauopathy-induced microgliosis

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The chemokine fractalkine modulates microglial responses in neurodegenerative diseases, including tauopathies, but the mechanistic processes and their relevance in human brain pathologies is not yet known. Here, we show that hippocampal HT22 cells expressing human TAU P301L mutant protein produce fractalkine, which in microglia activates AKT, inhibits glycogen synthase kinase-3β and upregulates the transcription factor NRF2/NFE2L2 and its target genes including heme oxygenase 1. In a mouse model of tauopathy based on stereotaxic delivery in hippocampus of an adeno-associated viral vector for expression of TAUP301L, we confirmed that tau-injured neurons express fractalkine. NRF2- and fractalkine receptor-knockout mice did not express heme oxygenase 1 in microglia and exhibited increased microgliosis and astrogliosis in response to neuronal TAU P301L expression, demonstrating a crucial role of the fractalkine/NRF2/heme oxygenase 1 pathway in attenuation of the pro-inflammatory phenotype. The hippocampus of patients with Alzheimer’s disease also exhibits increased expression of fractalkine in TAU-injured neurons that recruit microglia. These events correlated with increased levels of NRF2 and heme oxygenase 1 proteins, suggesting an attempt of the diseased brain to limit microgliosis. Our combined results indicate that fractalkine mobilizes NRF2 to limit over-activation of microglia and identify this new target to control unremitting neuroinflammation in tauopathies.

Keywords: adeno-associated virus; oxidative stress; Alzheimer’s disease; neuroimmunology

Abbreviations: ARE = antioxidant response element; HO-1 = heme oxygenase 1; NRF2 = nuclear factor (erythroid-derived 2)-like 2
Introduction

Tauopathies are a group of >20 different dementias and movement disorders that have in common the intracellular accumulation of insoluble hyperphosphorylated microtubule-associated protein TAU (Bouchard and Suchowsky, 2011). In these diseases, there is strong evidence that low-grade chronic inflammation, mainly elicited by microglia, aggravates the pathological course by exacerbated production of reactive oxygen species, chemotactic molecules, metalloproteases and pro-inflammatory cytokines (Galimberti and Scarpini, 2011; Jaworski et al., 2011). Crucial events in the development of these diseases are the recruitment of microglia into the proximity of neurons that suffer TAU-related proteotoxic damage, and the modulation of microglial dynamics between the classical pro-inflammatory and the alternative wound-healing phenotypes (Lee et al., 2013). Based on these facts, we aimed to determine in this study the role of the chemokine fractalkine (CX3CL1) in mediating the communication between TAU-injured neurons and microglia, and to define the signalling pathways that modulate microglial activity.

Neurons express CX3CL1 (Harrison et al., 1998), which exists in both membrane-bound and soluble forms (Hatori et al., 2002). Other cell types such as vascular endothelial cells and astrocytes also express this chemokine (Yoshida et al., 2001; Imaizumi et al., 2004) and may contribute to the soluble pool of CX3CL1 in brain. The membrane-bound CX3CL1 can serve as an adhesion molecule for leukocytes (Imai et al., 1997) whereas soluble CX3CL1, which is released by cathepsin S or ADAM-mediated cleavage (Clark et al., 2009; Hurst et al., 2012), can function as chemo-attractant for lymphocytes and monocytes (Chapman et al., 2000; Desforges et al., 2012). It is not clear how CX3CL1 modulates the neurotoxic or neuroprotective profiles of microglia but several studies in different models of tauopathy suggest that CX3CL1 participates in prevention of disproportionate inflammation. Thus, mice deficient in CX3CR1 receptor (Cx3cr1<sup>+/−</sup>), which in the brain is expressed exclusively in microglia, exhibit exacerbated inflammation in the tauopathy model to be used here, based on stereotoxic delivery of AAV-TAU<sup>ator</sup> to the hippocampus (Jaworski et al., 2011). In addition, CX3CL1 over-expression using adeno-associated viral vectors significantly reduced TAU pathology in the rTg4510 mice, attenuated microglial activation and prevented neurodegeneration normally found in this model (Nash et al., 2013). The neurotoxic effects of lipopolysaccharide-induced microglial activation (Noda et al., 2011) as well as hyperphosphorylation and aggregation of mouse TAU and transgenic human TAU (Bhaskar et al., 2010) are further aggravated in mice lacking microglial CX3CR1. These reports show a connection between CX3CL1 and modulation of microglia, but little is known about the relevance of this chemokine in human tauopathies such as Alzheimer’s disease or progressive supranuclear palsy.

The signalling pathway used by the pair CX3CL1–CX3CR1 to modulate the inflammatory phenotype of microglia is not known either. Oxidative stress participates in persistent up-regulated inflammation and is an invariant hallmark of tauopathy in patients and animal models (Koudinov et al., 2009). We therefore analysed whether CX3CL1 targets signalling pathways that upregulate the transcription factor NRF2 [nuclear factor (erythroid-derived 2)-like 2, currently known as NFE2L2], which is the master regulator of redox homeostasis that modulates inflammation. NRF2 regulates the expression of a battery of cytoprotective genes that share a cis-acting enhancer sequence termed ‘antioxidant response element’ (ARE) (Innamorato et al., 2009; Jazwa and Cuadrado, 2010). These genes include those coding the antioxidant enzymes heme oxygenase 1 (HO-1), NADP(H) quinone oxidoreductase (NQO1), enzymes of glutathione metabolism and protein degradation through the proteasome and autophagy routes (Joshi and Johnson, 2012). Therefore, the NRF2-ARE pathway provides microglia with a reductive environment that attenuates NF-κB-dependent pro-inflammatory responses by mechanisms that are being investigated (Wakabayashi et al., 2010; Brigelius-Flohé and Flohe, 2011; Aw Yeang et al., 2012).

We and others have shown that NRF2 modulates macrophage activation in peripheral tissues and microglia in the CNS. Previous studies have demonstrated that NRF2 attenuates macrophage activation in the lung in response to cigarette smoke extracts and to lipopolysaccharide (Rangasamy et al., 2004, 2005; Thimmulappa et al., 2006). We have further extended those observations to demonstrate that NRF2 also participates in modulation of microglia under conditions of acute lipopolysaccharide-induced neuroinflammation as well as low-grade neuroinflammation induced by chronic administration of the parkinsonian toxin MPTP or by over-expression of α-synuclein (Innamorato et al., 2008, 2009; Lastres-Becker et al., 2012). At least one of the NRF2-ARE genes, Hmox1 coding HO-1, appears to be of particular relevance in the anti-inflammatory functions of NRF2 (Cuadrado and Rojo, 2008). HO-1 cleaves heme to release biliverdin-IXa, carbon monoxide and iron. Both biliverdin-IXa and carbon monoxide have been reported to have anti-inflammatory functions in multiple scenarios such as immunomodulation of macrophages (Chora et al., 2007; Gozzelino et al., 2010). However, despite preliminary evidence showing that CX3CL1 activates JNK signalling to induce nuclear translocation of the transcription factor NRF2 and expression of HO-1 (Noda et al., 2011) the functional consequence of activation of the CX3CL1/NRF2 axis and its downstream target genes, including HO-1, in modulation of TAU pathology has not been investigated.

In this study, we report that CX3CL1, acting on the CX3CR1 receptor of microglia, activates NRF2-ARE and attenuates the TAU<sup>ator</sup>-induced microgliosis. Together our results indicate that NRF2 is a relevant CX3CL1 target that limits overactivation of microglia and provide a rationale to control unremitting neuroinflammation of tauopathies by targeting NRF2.

Materials and methods

Cell culture

HT22 immortalized mouse hippocampal neuronal cells were maintained in Dulbecco’s modified Eagle medium with 10% foetal bovine serum, 1% penicillin/streptomycin and 2 mM l-glutamine supplement, in 5% CO₂ at 37°C, 50% relative humidity. BV-2 microglial cells were cultured in RPMI 1640 medium supplemented with 10% foetal calf...
serum and 80 μg/ml gentamicin. Cells were changed to serum-free RPMI without antibiotics 16 h before addition of recombinant rat CX3CL1 (PeproTech). For primary microglial cell cultures, neonatal (post-natal Days 0–2) cortex from Nrf2<sup>−/−</sup> and Nrf2<sup>−/+</sup> mice were mechanically dissociated and the cells were seeded into 75 cm<sup>2</sup> flasks in Dulbecco’s modified Eagle medium supplemented with 10% foetal calf serum and 1% penicillin/streptomycin. After 2 weeks in culture, flasks were trypsinized and separated using CD11b MicroBeads for magnetic cell sorting (MACS Miltenyi Biotec). Microglial and astroglial cultures were at least 99% pure, as judged by immunocytochemical criteria. Medium was changed to serum-free Dulbecco’s modified Eagle medium without antibiotics 16 h before treatment. Cell cultures were then treated with recombinant CX3CL1 as indicated.

**TAU<sup>P301L</sup>** lentivirus construct

Human 4-repeat mutant TAU<sup>P301L</sup> lentiviral construct was kindly provided by Dr Charbel E.-H. Moussa (Khandelwal et al., 2012). Lentiviral vector stocks were generated in HEK293T cells by calcium phosphate-mediated transfection of three plasmids: the transfer plasmid (Lenti6-D-TOPO-TAU<sup>P301L</sup>), the packaging plasmid psPAX2, and the VSV-G envelope protein-coding plasmid pMD2G. After transfection for 24 and 48 h, the cellular supernatant was removed, centrifuged at 700 g for 10 min at 4°C, passed through 45-μm pore size filters and concentrated by centrifugation at 50,000 g for 2 h at 4°C using Beckman tubes. The pellet was carefully resuspended in Hank’s Balanced Salt Solution (Invitrogen). The titres of lentiviral stocks were in the range of 3–5 x 10<sup>8</sup>IU/ml as determined by immunocytochemical analysis of HEK293T-infected cells.

**Human brain extract**

Frozen post-mortem brain tissues were obtained from four control subjects (aged 78 and 90 years), four patients with Alzheimer’s disease (age range 73–90 years) and four patients with progressive supranuclear palsy within a 5 h post-mortem interval, according to the standardized procedures of Banco de Tejidos de la Fundación CIEN (Madrid, Spain). Information on patient samples, including APOE genotype, is provided in Supplementary Table 1. Hippocampal protein lysate (50 μg) was processed as described below for immunoblotting.

**Immunoblotting**

Microglial cells were washed with cold PBS, pH 7.4, lysed in RIPA buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1 mM EGTA, 1% IGEPAL<sup>®</sup>, 1% sodium deoxycholate, 0.1% SDS, 1 mM PSMF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 1 μg/ml aprotinin and 1 μg/ml leupeptin), and sonicated on ice for 10 s. Brain tissues were processed in the same buffer. Lysates were precleared by centrifugation and 25 μg of protein was mixed with 2 x Laemmli buffer, boiled for 2 min, resolved in SDS-PAGE and transferred to Immobilon-P membranes (Millipore Iberica). These membranes were analysed using the primary antibodies indicated above and appropriate peroxidase-conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence (Amersham).

**Preparation of nuclear and cytosolic extracts**

Microglial BV-2 cells were seeded in p100 plates (2 x 10<sup>6</sup> cells/plate). BV-2 cells were treated with CX3CL1 (100 nM) for different times. Cytosolic and nuclear fractions were prepared as described previously (Rojo et al., 2004). Briefly, cells were washed with cold PBS and harvested by centrifugation at 110 g for 10 min. The cell pellet was re-suspended in three pellet volumes of cold buffer A (20 mM HEPES, pH 7.0, 0.15 mM EDTA, 0.015 mM EGTA, 10 mM KCl, 1% Nonidet<sup>®</sup>P-40, 1 mM phenylmethylsulphonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin) and incubated on ice for 30 min. The homogenate was centrifuged at 500 g for 5 min. The supernatants were taken as the cytosolic fraction. The nuclear pellet was re-suspended in five volumes of cold buffer B (10 mM HEPES, pH 8.0, 0.1 mM EDTA, 0.1 mM NaCl, 25% glycerol, 1 mM phenylmethylsulphonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin). After centrifugation in the same conditions indicated above, the nuclei were re-suspended in loading buffer containing 0.5% SDS. The cytosolic and nuclear fractions were resolved in SDS-PAGE and immunoblotted with the indicated antibodies.

**Analysis of messenger RNA levels by quantitative real-time polymerase chain reaction**

Total RNA from HT22 cells, BV-2 cells and microglial primary cultures was extracted using TRIzol<sup>®</sup> reagent according to the manufacturer’s instructions (Invitrogen). One microgram of RNA from each experimental condition was treated with DNase (Invitrogen) and reverse-transcribed using 4 μl high capacity RNA-to-cDNA Master Mix (Applied Biosystem). For real-time PCR analysis, we performed the method previously described (Rojo et al., 2010; Jazwa et al., 2011). Primer sequences are shown in Supplementary Table 2. To ensure that equal amounts of complementary DNA were added to the PCR, the actin, beta housekeeping gene was amplified. Data analysis was based on the ΔΔCt method with normalization of the raw data to housekeeping genes as described in the manufacturer’s manual (Applied Biosystem). All PCRs were performed in triplicate.

**Measurement of soluble levels of CX3CL1**

The concentration of soluble CX3CL1 was measured using an ELISA kit (R&D Systems) in accordance with the manufacturer’s protocol in HT22 cell medium after TAU<sup>P301L</sup> lentiviral exposure for 24 h. All samples were stored at −80°C before use.

**Animals and treatments**

Colonies of Nrf2<sup>−/−</sup> mice and Nrf2<sup>−/+</sup> littermates were established from founders kindly provided by Dr. Masayuki Yamamoto (Tohoku University Graduate School of Medicine, Sendai, Japan) (Itoh et al., 1997). Cx3cr1<sup>−/−</sup> mice (B6.129 P-Cx3cr1<sup>tm1Itjt/J</sup>) and their wild-type
controls were obtained from Jackson Laboratory (Jung et al., 2000). Each experimental group comprised four to eight animals.

Recombinant adenov-associated virus vectors of hybrid serotype 1/2 to express mutant TAU P301L under control of the human synapsin I gene promoter were used as described (Iaworski et al., 2009). Surgical procedures and unilateral intracerebral injection of viral particles into the right hemisphere were performed as described. In brief, 2 μl viral suspension containing 10^8 t.u. was injected at the stereotaxic coordinates 1.94 mm posterior, 1.4 mm lateral, and 2.2 mm ventral relative to bregma. All experiments were performed with certified researchers conforming to regional, national, and European regulations concerning animal welfare and animal experimentation, and were authorized and supervised by the University Animal Welfare Commission (Ethische Commissie Dierenwelzijn, Katholieke Universiteit Leuven) and the Ethical Committee for Research of the Autonomous University of Madrid following institutional, Spanish and European guidelines.

**Immunohistochemistry on mouse tissues**

Immunohistochemistry in mice was performed on 30-μm thick coronal brain sections with a standard avidin-biotin immunohistochemical protocol as previously described (Rojo et al., 2010). Primary antibody was monoclonal anti-human phospho-TAU (1:100, HT7, Thermo Scientific). Secondary biotinylated secondary antisera (Vector Labs Inc) was developed using diaminobenzidine.

**Immunofluorescence of mouse tissues**

The protocol was previously described (Rojo et al., 2010). Primary antibodies included: rabbit anti-IBA-1 (1:100, Wako Chemicals), rabbit anti-GFAP that recognizes the mouse protein (1:500, Dako Diagnostics), monoclonal anti-human phospho-TAU (1:100, HT7, Thermo Scientific), rabbit anti-HO-1 (1:200, Millipore), anti-NeuN (1:200, clone A60, MA8377, Millipore), and goat anti-CX3CL1 (1:100, R&D Systems). Secondary antibodies were: Alexa Fluor® 546 goat anti-mouse, Alexa Fluor®546 goat anti-rabbit and Alexa Fluor® 488 goat anti-mouse (1:500, Life technologies). Control sections were treated following identical protocols but omitting the primary antibody.

**Post-mortem tissues of human brain**

The control subject had no background of neuropsychiatric disease and a full neuropathological examination excluded relevant brain pathology. Alzheimer’s disease diagnosis was confirmed by HT100 staining on frozen tissue sections from the same cases used in the immunofluorescence studies. All cases with Alzheimer’s disease were categorized within Braak stages 2–4.

**Statistical analyses**

Data are presented as mean ± SEM. To determine the statistical test to be used, we used GraphPad Instat 3, which includes the analysis of the data to normal distribution by Kolmogorov-Smirnov test. In addition, statistical assessments of differences between groups were analysed (GraphPad Prism 5) by unpaired Student’s t-tests when normal distribution and equal variances were fulfilled, or by the non-parametric Mann–Whitney test. One and two-way ANOVA with post hoc Newman-Keuls or Bonferroni’s test were used, as appropriate.

**Results**

**CX3CL1 released by TAU-injured neurons activates NRF2 in microglia**

To determine if TAU expression leads to CX3CL1 release, mouse hippocampal HT22 cells were infected with a lentiviral vector expressing human mutant TAU P301L. After 48h, we observed ectopic expression of messenger RNA of TAU P301L by quantitative real-time PCR (Fig. 1A) and overexpression of total TAU protein by immunoblot with an antibody that recognizes both mouse and human TAU (Fig. 1B). Immunofluorescence analysis evidenced that TAU P301L induced a predominantly pyramidal shape in HT22 cells (Fig. 1C). TAU-overexpressing neurons exhibited increased production of CX3CL1 as assessed by ELISA on the culture medium (Fig. 1D) and quantitative real-time PCR on cell lysates (Fig. 1E). These results confirmed previous observations indicating that TAU-stressed neurons release CX3CL1 (Bhaskar et al., 2010).

We further analysed the impact of TAU P301L overexpression on several stress response systems, including proteasome, autophagy, oxidative stress and endoplasmic reticulum stress. The messenger RNA and protein levels of PSMB7, a prototypic β-subunit of the catalytic core 20S proteasome, were significantly increased after TAU P301L expression (Supplementary Fig. 1A, E and F), indicating that TAU induces proteasomal alterations. Regarding macroautophagy, we observed a small but statistically significant reduction in the messenger RNA and protein levels of p62 (Supplementary Fig. 1B, E and G) suggesting that elevated levels of TAU P301L induced a moderated autophagy response. When we measured the messenger RNA and protein levels of HO-1, as an oxidative stress marker (Supplementary Fig. 1C, E and H), and BIP/Grp78 as an endoplasmic reticulum stress marker (Supplementary Fig. 1D, E and I), we could not detect significant differences. These results indicate that TAU P301L elicits a defensive response in neurons that is most obvious on the proteasome and autophagy degradation pathways.

We reported previously the regulation of NRF2 by glycogen synthase kinase 3 (GSK3) (Rada et al., 2011, 2012), and here we analysed the regulation of this kinase by CX3CL1 signalling in the microglial cell line BV-2. Cells were maintained under serum-free conditions for 16 h and then stimulated with CX3CL1 (100 nM). The Ser/Thr protein kinase AKT, the upstream regulator of GSK-3β, was activated after 30 and 60 min of CX3CL1 stimulation as determined by increased phosphorylation at S473 (Fig. 2A and B), paralleling similar kinetics of inactivating phosphorylation of GSK-3βSer9 (Fig. 2A and C).

We analysed the expression of NRF2 and NRF2-regulated genes in response to CX3CL1. In BV-2 mouse microglial cells, CX3CL1 did not induce any significant changes in Nrf2 messenger RNA levels (Fig. 2D). However, CX3CL1 induced an increase in NRF2 protein levels that was most obvious in the nuclear fraction (Fig. 2E). These results are consistent with a CX3CL1-induced upregulation of NRF2, most likely due to increased protein stability. Moreover, four genes encoding HO-1 (HMOX1), GPx (GPX1), GCLC and GCLM were upregulated after 4, 8 and 24 h incubation with recombinant CX3CL1 (Fig. 2F–I, respectively) as determined...
by quantitative real-time PCR. This increase was further validated at the protein level for HO-1 and GCLC with maximal induction at 24 h (Fig. 2J–L). The relevance of NRF2 in this response was confirmed in primary cultures of microglia from NRF2-knockout (Nrf2−/−) mice and wild-type (Nrf2+/+) littermates as control. As shown in Fig. 3A–D, CX3CL1 activated the antioxidant profile of primary microglia from Nrf2+/+ mice, but not from Nrf2−/− mice, therefore demonstrating the participation of NRF2 in this process. As a control, we observed that microglia from both genotypes exhibited similar messenger RNA levels of CX3CL1 normalized by ACTB (β-Actin) levels. (E) Quantitative real-time PCR determination of messenger RNA levels of CX3CL1 normalized by ELISA. (F) Determination of soluble CX3CL1 levels by ELISA. (G) Immunofluorescence analysis of TAU (Mapt) messenger RNA by quantitative real-time PCR (Fig. 4A) and of phosphorylated TAU (P301L) expressing cells: DAPI (blue), TAU (red) as evidenced with anti-TAU antibody. (H) Determination of soluble CX3CL1 levels by ELISA. (I) Immunoblot analysis in whole cell lysates of human TAU protein levels (top), ACTB (β-Actin) used as a protein loading control (bottom). (J) Immunofluorescence analysis of NRF2 protein expression in control and TAU expressing cells: DAPI (blue), TAU (red) as evidenced with anti-TAU antibody. (K) Determination of soluble CX3CL1 levels by ELISA.

**TAU expression aggravates gliosis and inflammation at the hippocampus of Nrf2−/− mice**

The role of NRF2-ARE in TAU-induced microglial dynamics was further analysed in a mouse model of tauopathy based on stereotaxic delivery to the hippocampus of an adeno-associated vector expressing human TAU directly under the control of the human synapsin 1 gene promoter. The pathological and neuroinflammatory outcomes of this model have been studied in wild-type mice (Jaworski et al., 2009, 2011). A control adeno-associated virus vector expressing green fluorescence protein did not elicit significant changes in inflammation or gliosis (data not shown). Nrf2+/+ and Nrf2−/− mice were injected with AAV-TAU expression in the right hippocampus (ipsilateral side) and the left hippocampus was used as control (contralateral side). Three weeks post-injection, we observed similar levels of TAU (Mapt) messenger RNA by quantitative real-time PCR (Fig. 4A) and of phosphorylated TAU expressing cells in both genotypes. Immunofluorescence showed microglial (Iba1) and astroglial (GFAP) markers to be higher in Nrf2−/− than in Nrf2+/+ mice (Supplementary Fig. 3A) as expected (Innamorato et al., 2008; Rojo et al., 2010).

Most importantly, the brain regions with TAU expression also exhibited increased abundance of microglia and astrocytes, which was most evident in the Nrf2−/− mice. Quantitative real-time PCR for Iba1 and Gfap messenger RNA, as respective markers of microglia and astroglia, further confirmed that Nrf2−/− mice exhibited exacerbated gliosis in response to over-expression of TAU (Supplementary Fig. 3B and C).

Messenger RNA levels of HO-1 (Hmox1) (Fig. 4C) and Gclc (Fig. 4D) were increased in Nrf2+/+ hippocampus in response to TAU, but there was no significant induction in Nrf2−/− mice. The pro-inflammatory cytokines Tnf (Fig. 4E) and Il6 (Fig. 4F) were also induced but in this case Nrf2−/− mice exhibited the strongest response, consistent with the immunomodulator role of NRF2.

Next we sought to identify which brain cells were activating NRF2 in response to TAU expression. We could not analyse directly NRF2 expression by immunofluorescence because of the poor quality of available antibodies. Instead, we performed double immunofluorescence with anti-HO-1 and either anti-TAU, anti-Iba1 or anti-GFAP antibodies. As shown in Fig. 4G, HO-1 was barely detectable in Nrf2−/− hippocampus despite TAU expression, as expected. By contrast, in Nrf2+/+ mice we
CX3CL1 induces the NRF2-ARE response in BV-2 microglia. Cells were incubated in the presence of recombinant CX3CL1 (100 nM) for 5, 15, 30 and 60 min as indicated. (A) Immunoblot analysis in whole cell lysates of phospho-AKT Ser473 and total AKT, and phospho-GSK3β Ser9 and total GSK3. (B and C) Densitometric quantification of representative blots from A. (D) Cells were incubated in the presence of recombinant CX3CL1 (100 nM) for 4 and 8 h and quantitative real-time PCR determination of messenger RNA for NRF2 was analysed and normalized by Actb (β-Actin) messenger RNA levels. (E) Cells were incubated in the presence of CX3CL1 for 1, 2, 4 and 8 h, and analysed by subcellular fractionation in immunoblots: NRF2 levels (top); GAPDH levels used as cytosol protein loading control. (continued)
observed increased HO-1 expression in cells that did not co-localize with TAU P301L expression. In fact, most of HO-1+ cells were IBA-1+ (microglia) (Fig. 4H) or GFAP+ (astroglia) (Fig. 4I). These results suggest that neurons expressing TAU P301L induce in the brain parenchyma the activation of glial cells that participate in pro-inflammatory and anti-inflammatory responses, HO-1 being a marker of the NRF2-ARE dependent anti-inflammatory response.

**TAUP301L** expression increases the levels of CX3CL1 and CX3CR1

We performed double immunofluorescence with anti-phospho-TAU and anti-CX3CL1 antibodies in 30-μm thick coronal sections of hippocampus from Nrf2+/+ and Nrf2−/− mice. CX3CL1 expression was detected specifically in pyramidal neurons that were also expressing TAU P301L in both Nrf2+/+ (Fig. 5A) and Nrf2−/− (Fig. 5B) mice, demonstrating that TAU-injured neurons express CX3CL1 in vivo as observed in vitro. Furthermore, CX3CL1 expression was barely detectable in the control contralateral hemisphere. In support of the immunohistochemistry results, quantitative real-time PCR for Cx3cl1 (Fig. 5C) and Cx3cr1 (Fig. 5D) messenger RNA indicated that both ligand and receptor were overexpressed to a similar degree in the hippocampus of TAU P301L-expressing Nrf2+/+ and Nrf2−/− mice. Taken together, these results indicate that TAUP301L increased the expression of the CX3CL1/CX3CR1 pair, which is involved in

*Figure 3* The transcription factor NRF2 is required by CX3CL1 to induce the NRF2-ARE response in microglia. Primary cultures of microglia from control wild-type mice (*Nrf2*+/+, empty bars) and Nrf2-knockout mice (*Nrf2*−/−, filled bars) were incubated with recombinant CX3CL1 (100 nM, 24 h). (A–D) Quantitative real-time PCR determination of messenger RNA levels of NRF2-regulated genes coding HO-1, GPx, GCLC and GCLM, respectively, normalized by Actb (β-Actin) messenger RNA levels. (E–H) Quantitative real-time PCR determination of messenger RNA levels for the CX3CL1 receptor (*Cx3cr1*), *Mfge8*, *Il6* and *Il4* normalized to Actb (β-Actin) messenger RNA levels. Two-way ANOVA followed by Bonferroni post-test was used to assess significant differences among groups. Asterisks denote significant differences *P* < 0.05, **P* < 0.01 comparing the indicated groups and ***P* < 0.001 respect to the basal Nrf2+/+ group.

*Figure 2* Continued

(middle); LAMIN B level was used as nuclear protein loading control (bottom). (F–I) Quantitative real-time PCR determination of messenger RNA levels of NRF2-regulated genes coding HO-1, GPx, GCLC and GCLM, respectively, normalized by β-actin messenger RNA levels. (E) Immunoblot analysis in whole cell lysates of protein levels of HO-1, GCLC and β-actin as loading control. (F and G) Densitometric quantification of representative blots from E normalized for β-actin levels. Bars indicate mean of three samples ± SEM. Asterisks denote significant differences *P* < 0.05, **P* < 0.01, comparing the indicated groups with the basal condition according to a one-way ANOVA followed by Newman-Keuls post-test.
the inflammatory process but that NRF2 does not participate in this effect.

To determine whether the induction of NRF2-ARE genes could be attributed to CX3CL1 signalling, we used knockout-mice lacking its receptor (Cx3cr1^{-/-}) and compared them with wild-type mice (Cx3cr1^{+/+}). In Cx3cr1^{-/-} mice, the gene encoding the receptor has been knocked-in with the coding sequence of green fluorescence protein (GFP) and therefore cells lacking CX3CR1 can be precisely monitored because they express membrane-bound GFP instead (Jung et al., 2000). In fact, double immunofluorescence with anti-GFP and anti-IBA-1 antibodies demonstrated that the GFP signal strictly co-localized with the IBA-1 marker in microglia (Supplementary Fig. 4). TAU P301L expression evoked a stronger microglial reaction in Cx3cr1^{-/-} mice versus control Cx3cr1^{+/+} mice (Fig. 6A and B, respectively) as expected (Fig. 6C) (Jaworski et al., 2011). But more importantly, Cx3cr1^{-/-} microglia did not express HO-1 in either the control or the TAU P301L-expressing hippocampus. In Cx3cr1^{+/+}

![Figure 4](image-url)
mice, the number of HO-1+ microglial cells considerably increased in the TAU P301L expressing side (Fig. 6A and D). Furthermore, Cx3cr1−/− mice showed increased HO-1 expression in cells that were neither microglia (Fig. 6B) nor neurons (Supplementary Fig. 5), as deduced by the lack of overlapping staining. We further corroborated that these HO-1+ cells were astrocytes, by double immunofluorescence with anti-GFAP and anti-HO-1 antibodies (Supplementary Fig. 6). These results strongly suggest that CX3CL1 signalling is responsible of induction of the NRF2/HO-1 antioxidant and immunomodulation responses in microglia.

Hyperphosphorylated TAU correlates with activation of the CX3CL1/NRF2/ 
HO-1 pathway in post-mortem biopsies of patients with Alzheimer’s disease

To determine the relevance of the CX3CL1/NRF2 axis in modulation of inflammation in human TAU pathology we analysed post-mortem biopsies of hippocampus from four control asymptomatic donors and four patients with Alzheimer’s disease with neurofibrillary tangles demonstrated by TAU hyperphosphorylation. We first compared phospho-TAU (AT100 antibody) and CX3CL1 expression and found that, consistently with the in vitro cell culture data and in the TAU P301L-expressing mice, expression of CX3CL1 was restricted to neurons containing phospho-TAU (Fig. 7A). Moreover, double immunofluorescence with anti-IBA-1 and anti-HO-1 antibodies indicated that microglia from this affected region expressed high levels of HO-1 protein (Fig. 7B). Of note, microglia was recruited to the phospho-TAU and CX3CL1 expressing neurons (Fig. 7C). We further quantified these results by immunoblot analysis of the protein levels of CX3CL1 (Fig. 8C), HO-1 (Fig. 8D), NRF2 (Fig. 8E) and IBA-1 (Fig. 8F) in Alzheimer’s disease (Fig. 8A) and other tauopathies clinically diagnosed as progressive supranuclear palsy (Fig. 8B). Despite the expected variability in disease progression among these subjects, the four proteins were increased in patients with Alzheimer’s disease and progressive supranuclear palsy compared with the four asymptomatic controls suggesting that, in agreement with our results in the cellular and animal models, there is a causal connection between CX3CL1 expression in TAU-injured neurons and modulation of microglia through the CX3CR1/NRF2 signalling pathway and activation of the NRF2-ARE genes including HO-1 expression.

Figure 5 Continued

(C and D) Quantitative real-time PCR determination of messenger RNA levels for Cx3cl1 and its receptor Cx3cr1 normalized by Actb (β-Actin) messenger RNA levels. Note that ectopic expression of TAU P301L induces the expression of both ligand and receptor but there is not a significant difference between Nrf2+/+ and Nrf2−/− genotypes. Two-way ANOVA followed by Bonferroni post-test was used to assess significant differences among groups. Asterisks denote significant differences **P < 0.01, ***P < 0.001 comparing the indicated groups.
Discussion

Considering that neuroinflammation is increasingly being recognized as a major contributing factor in the initiation and progression of neurodegenerative diseases, much attention has been placed on the mechanisms of communication between neurons and microglia, the main inflammatory cells in the brain. Here, we found that neurons expressing TAU<sup>P301L</sup> release CX3CL1. This could be interpreted as a 'help me' signal released by damaged neurons (Hochreiter-Hufford and Ravichandran, 2013). In fact, the use of CX3CL1-deficient mice (Morganti et al., 2012) as well as viral expression of this chemokine (Nash et al., 2013) confirm its role in neuroprotection. Several studies suggested that CX3CL1 might modulate microglial activation, from the classical pro-inflammatory M1 phenotype towards a pro-resolution M2 phenotype characterized by migration to the site of lesion, increased phagocytic capacity and reduced release of pro-inflammatory cytokines (Desforges et al., 2012). Moreover, plasma and CSF levels of soluble CX3CL1 have been shown to be elevated in individuals with mild cognitive impairment and Alzheimer’s disease (Kim et al., 2008; Shi et al., 2011) further highlighting the importance of this chemokine in brain pathologies. Considering that NRF2, the master regulator of redox homeostasis, seems to play a similar role in conversion of peripheral macrophages and

Figure 6 The CX3CR1 receptor is required to induce HO-1 in microglia in response to TAU<sup>P301L</sup> expression. (A) Wild-type mice (Cx3cr<sup>1<sup>+/+</sup></sup>) exhibit increased microglial activation (Iba-1, green) and microglial HO-1 expression (red) in the hippocampus expressing TAU<sup>P301L</sup>. White arrows in merge indicate microglia expressing HO-1. (B) Microglia from Cx3cr<sup>1<sup>−/−</sup></sup> mice, stained with anti-GFP antibody (green) see (Supplementary Fig. 4) do not express HO-1 (red) in response to TAU<sup>P301L</sup>. (C) Stereological quantification of the number of microglial cells in the control side and the TAU<sup>P301L</sup> expressing side of Cx3cr<sup>1<sup>+/+</sup></sup> versus Cx3cr<sup>1<sup>−/−</sup></sup> mice. Two-way ANOVA followed by Bonferroni post-test was used to assess significant differences among groups. Asterisks denote significant differences *P < 0.05, **P < 0.01 comparing the indicated groups. (D) Stereological quantification of the number HO-1<sup>+</sup> microglia in the control side and the TAU<sup>P301L</sup> expressing side of Cx3cr<sup>1<sup>+/+</sup></sup> versus Cx3cr<sup>1<sup>−/−</sup></sup> mice. Asterisk denotes significant differences *P < 0.05 according to a Student’s t-test.
Figure 8  CX3CL1, HO-1, NRF2 and IBA-1 protein levels are upregulated in the hippocampus of patients with Alzheimer’s disease (AD) with tauopathy. (A and B) Immunoblot analysis of CX3CL1, HO-1, NRF2 and IBA-1 as indicated for asymptomatic controls without tauopathy and four patients with Alzheimer’s disease with neurofibrillary tangles (A) and four patients with progressive supranuclear palsy (PSP) (B). (C–F) Densitometric quantification of each protein from representative immunoblots as in A and B. Bars indicate mean (n = 4 for controls; n = 4 for patients with Alzheimer’s disease; n = 4 for progressive supranuclear palsy) ± SEM. Asterisks denote significant differences *P < 0.05, **P < 0.01 and ***P < 0.001 according to a Student’s t-test.
microglia from the ‘less M1’ to the ‘more M2’ phenotype (Innamorato et al., 2009; Rojo et al., 2010; Boche et al., 2013), we analysed here if CX3CL1 and NRF2 are mechanistically and functionally interconnected to modulate inflammation.

Several important findings connect NRF2 with neuroprotection against the amyloid component of Alzheimer’s disease pathology in transgenic APP/P51 mice, showing that boosting NRF2 activity reduces amyloid load and neuronal damage (Kanninen et al., 2008, 2009) but those studies did not analyse microgliosis or the inflammatory component of the disease. Considering that we have found that CX3CL1 attenuates microglial activation through NRF2 induction, our results would predict a diminished capacity of the diseased brain to remove amyloid plaque. Indeed recent studies suggest that CX3CL1 might attenuate amyloid clearance (Fuhrmann et al., 2010; Lee et al., 2010) in which case, the CX3CL1/NRF2 axis would be beneficial against tauopathy but not amyloidopathy.

Our study describes for the first time the relevance of NRF2 in modulation of TAU-induced neuroinflammation. In the hippocampus of mice expressing human TAU protein and in the hippocampus of patients with Alzheimer’s disease with tauopathy we found that TAU-injured neurons express CX3CL1. Most likely this chemokine is produced in both membrane-bound and soluble forms because we could detect it by immunofluorescence on the neurons of mouse and human hippocampus as well as by ELISA in the culture medium of HT22 cells. These results are consistent with the notion that CX3CL1 participates in both recruitment to the site of injury and also modulation of microglial phenotypes (Ransohoff et al., 2007; Desforges et al., 2012).

In addition, we report that CX3CL1 activates NRF2-ARE in microglia. To examine the relevance of this observation, we used mouse strains that lack either NRF2 or CX3CR1. We found that both knockout mice exhibit a similar alteration in the microglial response, characterized by an exacerbated increase in the number of IBA-1+ microglia in areas of TAU-injured neurons. The mechanistic connection between CX3CL1 and NRF2 was investigated in BV-2 microglial cell cultures submitted to recombinant CX3CL1, where we found that this chemokine increases the expression of NRF2-ARE genes. Moreover, the NRF2-ARE induction was further related to NRF2 expression because primary microglia from Nfr2−/− mice did not induce NRF2-ARE genes in response to CX3CL1. In search for the signalling pathway used by CX3CL1 to activate NRF2, we found that recombinant CX3CL1 activates AKT (phospho-AKTSer473), which leads to inhibition of its downstream target GSK3B by phosphorylation of Ser9 (phospho-GSK3βSer9). Moreover, inhibition of GSK3B correlated with stabilization of NRF2 protein levels, suggesting that inhibition of this kinase prevents NRF2 degradation. NRF2 is principally controlled through regulation of protein turnover by two E3 ligase adapters: KEAP1 (Kelch-like ECH-associated protein 1) (Tong et al., 2006; Villeneuve et al., 2010) and β-TrCP (beta-transducin repeat containing protein, BTRC) (Rada et al., 2011, 2012). KEAP1 is a redox sensor that links NRF2 stability to stress signals and has been studied extensively but KEAP1 protein levels were not altered in response to CX3CL1 (data not shown). On the other hand, we reported that GSK3 phosphorylates specific residues in the Neh6 domain of NRF2 and creates a degradation domain that is then recognized by β-TrCP (Rada et al., 2011, 2012). This mechanism links NRF2 stability with signalling pathways that regulate this kinase. Therefore, our findings suggest that lack of CX3CR1/PI3K/AKT signalling leaves GSK3 in a non-phosphorylated active conformation that leads to strong downregulation of NRF2 through the GSK3β/β-TrCP pathway. In agreement with this, in Cx3cr1−/− microglia we could barely detect expression the NRF2-ARE reporter HO-1.

In patients with Alzheimer’s disease with tauopathy we found that some clusters of TAU-injured neurons were surrounded by IBA-1+ cells. These cells may be recruited as resident microglia or may result from infiltration of peripheral macrophages and most likely represent an attempt to re-establish tissue homeostasis (Rezai-Zadeh et al., 2011). However, full tissue recovery also requires the inflammatory phase to be resolved. Our findings in the Cx3cr1−/− mice, evidencing exacerbated recruiting of microglia but lack of NRF2-ARE induction, suggest that in the absence of activation of the CX3CL1/NRF2 axis, microglia lose that capacity to transit towards a pro-resolution phenotype and worsens disease progression. This could happen in patients with tauopathy whose main risk factor is ageing, because GSK3B activity augments (Jimenez et al., 2011; Tomobe et al., 2012) and NRF2 activity declines with ageing (Suh et al., 2004; Shenvi et al., 2012).

Another relevant issue regarding tauopathies and other neurodegenerative disorders is the integrity of the blood–brain barrier. Blood–brain barrier dysfunction is associated with accumulation of neurotoxic molecules within the brain parenchyma (Zlokovic, 2011; Bell et al., 2012; Sengillo et al., 2013). This problem is reproduced in transgenic mice expressing human TAU (Forman et al., 2005). Interestingly, enhancing expression of NRF2 in brain tissue and microvessels has been shown to reduce loss of endothelial cell markers and tight junction proteins and preserve the blood–brain barrier (Zhao et al., 2007; Dash et al., 2009). Given that blood–brain barrier dysfunction is becoming increasingly recognized as a contributor to the pathogenesis of several neurodegenerative disorders, NRF2 might be targeted to preserve blood–brain barrier integrity.

Importantly, NRF2 activity may be restored pharmacologically in old animals by targeting the KEAP1/NRF2 axis (Suh et al., 2004) and we have reported a beneficial effect of the potent NRF2 inducer sulforaphane in the MPTP model of Parkinson’s disease in old animals. Therefore, pharmacological activation of the NRF2 pathway with drugs that exhibit CX3CL1-like activity and activate the NRF2/ARE pathway may provide disease modifying therapy for tauopathies.

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Supplementary material

Supplementary material is available at Brain online.

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