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Fractalkine-induced activation of the phosphatidylinositol-3 kinase pathway attentuates microglial activation *in vivo* and *in vitro*

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Abstract

Several neurodegenerative disorders are associated with evidence of inflammation, one feature of which is increased activation of microglia, the most likely cellular source of inflammatory cytokines like interleukin-1β. It is now recognized that interaction of microglia with other cells contributes to maintenance of microglia in a quiescent state and the complementary distribution of the chemokine, fractalkine (CX_3CL1) on neurons and its receptor (CX_3CR1) on microglia, suggests that this interaction may play a role in modulating microglial activation. Here we demonstrate that both soluble and membrane-bound fractalkine attenuate lipopolysaccharide-induced microglial activation *in vitro*. We also show that fractalkine expression is reduced in the brain of aged rats and this is accompanied by an age-related increase in microglial activation. Treatment of aged rats with fractalkine attenuates the age-related increase in microglial activation and the evidence indicates that fractalkine-induced activation of the phosphatidylinositol-3 kinase pathway is required to maintain microglia in a quiescent state both *in vivo* and *in vitro*.

Keywords: cytokines, fractalkine, microglia, neuroinflammation, phosphatidylinositol-3 kinase signaling and aging.

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Neuroinflammation, with the characteristic activation of microglia, is a feature common to several neurodegenerative disorders (Block and Hong 2005; Zipp and Aktas 2006; Skaper 2007; Rojo *et al.* 2008) and, although there is considerable debate regarding the potential benefit of controlled microglial activation, it is generally agreed that prolonged activation is damaging. This debate highlights the need to understand the mechanisms which contribute to modulation of microglial activation and to recognize that several activation states of microglia (Schwab and McGeer 2008), like macrophages (Gordon 2003), probably exist.

Neuroinflammatory changes have also been observed in normal aging, with evidence of age-related increases in microglial activation as demonstrated by evidence of an up-regulation in cell surface markers of activation, accompanied by increased expression of the proinflammatory cytokines like interleukin-1β (IL-1β) and IL-6 (Sheng *et al.* 1998; Godbout and Johnson 2004; Lynch 2004; Griffin *et al.* 2006). Additionally, a number of studies using microarray analysis have revealed that several genes, which are indicative of inflammatory change, are up-regulated with age (Godbout *et al.* 2005).

The trigger leading to the age-related increase in microglial activation and the subsequent increased expression of proinflammatory mediators is not known. One possibility is that interferon-γ, which is among the most potent activators of microglia (Nguyen and Benveniste 2000; Lyons *et al.* 2007b) may initiate microglial activation, but it is becoming increasingly clear that interaction of microglia with other cell types can also modulate their activation state. Recent evidence from this laboratory has demonstrated that the interaction of CD200, which is expressed on neurons, with its receptor CD200R, which is expressed on microglia, contributes to the maintenance of microglia in a quiescent state (Lyons *et al.* 2007a). It was further shown that the age-

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Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

related increase in microglial activation directly correlated with a decrease in neuronal expression of CD200. However, the interaction between these two cell surface markers is unlikely to be the only cell–cell interaction which plays a role in modulating microglial activity, and interaction of fractalkine with its receptor may also have a similar modulatory effect (Cardona *et al.* 2006).

Fractalkine (also known as CX_3CL1) is a member of the chemokine family of proteins but is the sole member of the CX_3C subfamily (Bazan *et al.* 1997). Both fractalkine and its receptor are expressed in the brain (Mizuno *et al.* 2003; Re and Przedborski 2006) with fractalkine localized principally on neurons (Harrison *et al.* 1998; Maciejewski-Lenoir *et al.* 1999), where it expressed constituitively (Lauro *et al.* 2008). Conversely, the consensus is that the receptor is expressed chiefly on microglial cells (Harrison *et al.* 1998). Although there are reports indicating expression of the ligand on glial cells (Maciejewski-Lenoir *et al.* 1999) and the receptor on neurons (Hughes *et al.* 2002), the largely complimentary distribution pattern suggests that fractalkine may play a role in signaling between neurons and microglia.

Here we set out to examine the role of fractalkine in modulating microglial activation and report that both soluble and membrane-bound fractalkine attenuate lipopolysaccharide (LPS)-induced microglial activation. Consistent with this observation, we demonstrate that the age-related increase in microglial activation is accompanied by a decrease in fractalkine expression, and that treatment of aged rats with fractalkine attenuates the age-related increase in microglial activation.

Materials and methods

Animals

Male Wistar rats (Bantham and Kingman, Hull, UK) aged 3 months (250–350 g) or 22 months (550–650 g) were housed under a 12 h light/dark schedule and at an ambient temperature of between 22–23°C. Rats were maintained in the BioResources Unit at Trinity College, Dublin, Ireland under veterinary supervision throughout the study, and experiments were performed under a license issued by the Department of Health (Ireland) and in accordance with the guidelines laid down by the local ethical committee. In the first series of experiments, young and aged rats were killed by cervical dislocation, the brain was rapidly removed and the hippocampus was dissected free. One portion of the hippocampus was flash-frozen in liquid nitrogen for later extraction of mRNA and the second portion was cross-chopped into tissue prisms (350 μm × 350 μm), rinsed and stored in Krebs solution containing 10% dimethylsulfoxide at −80°C (Martin *et al.* 2002) until required for analysis.

In the second series of experiments, young and aged animals were randomly assigned to control groups which received an intracerebroventricular injection of saline (5 μL), or treatment groups which received an intracerebroventricular injection of fractalkine (1 μg/5 μL rat recombinant fractalkine, chemokine domain (amino acids 25–100); Calbiochem, Nottingham, UK) and 4 h after injection rats were killed by cervical dislocation. This time was chosen because previous experiments have indicated that IL-4 and IL-10 exerted anti-inflammatory effects in this timeframe (Lynch *et al.* 2004; Barry *et al.* 2005; Nolan *et al.* 2005). The brain was hemisected; one half of the brain was coated with OCT compound (Sakura Tissue-Tek, Brussels, Belgium), immersed in isopentane at −30°C and stored at −80°C until sections were prepared. Cryostat sections (10 μm) were mounted on gelatine-coated slides, air-dried for 30 min and stored at −20°C until used for immunohistochemical analysis. The hippocampus was dissected free from the second half of the brain, divided in two, with one half flash-frozen in liquid nitrogen for later extraction of mRNA, while the other used for protein analysis.

Preparation and treatment of primary glial and neuronal cultures

Mixed glia were prepared from the cortices of 1-day-old Wistar rats (Trinity College, Dublin, Ireland) by dissecting cortical tissue free. Tissue was cross-chopped and incubated for 25 min at 37°C in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Paisley, UK) supplemented with 10% foetal bovine serum (FBS), and plated (2.5×10^5 cells/mL) as previously described (Nolan *et al.* 2004). In this study, cells prepared from each rat was treated separately. After 12 days, cells were pre-treated with fractalkine (0–200 ng/mL) for 20 min and incubated in the presence or absence of lipopolysaccharide for 24 h (LPS; 100 ng/mL; 3000 endotoxin units/mL; Sigma, Gillingham, UK). Supernatant was collected and assessed for IL-1β, while cells were harvested for analysis of MHC Class II expression by flow cytometry. In one series of experiments, in which IL-1β was assessed, the effect of addition of an anti-fractalkine receptor antibody (5 μg/mL; Millipore, Ireland) was assessed. We used mixed glia rather than isolated microglia in these experiments because previous experiments indicated that isolated microglia exhibited morphology consistent with an activated state (few processes and large cell bodies) and increased expression of CD40.

In a second series of experiments, glial cells and neurons were prepared. Briefly, neurons were prepared from cortices of neonatal rats. Tissue was cross-chopped and incubated in phosphate-buffered saline (PBS) with trypsin (0.25 μg/mL, Sigma) for 25 min at 37°C. Tissue was triturated in PBS containing soybean trypsin inhibitor (0.2 μg/mL, Sigma) and DNase (0.2 mg/mL, Sigma) and gently passed through a sterile mesh filter (40 μm). The suspension was centrifuged at $2000 \times g$ for 3 min at 20°C, and the pellet was resuspended in warm NeuroBasal Media (Invitrogen), supplemented with 10% FBS, penicillin (100 units/mL, Invitrogen). After 12 days glial cells were ready for treatment. In relevant experiments, glial cells were pre-treated with anti-fractalkine receptor antibody (5 μg/mL, Abcam, Cambridge, UK) for 30 min prior to the treatment with or without neurons; [a suspension of primary neuronal cells in 10% FBS/DMEM (1 : 8 neurons:glia)]. Following the addition of neurons, LPS (100 ng/mL) was immediately added to the relevant treatment groups for 24 h. Supernatants were subsequently taken and assessed for IL-1β expression.

In another series of experiments, designed to identify the signaling events induced by fractalkine, mixed glia were washed and serum-starved for 4 h. Cells were incubated in the presence of fractalkine (200 ng/mL, R&D Systems, Abingdon, UK) for 20, 40 or 60 min or 24 h in 2% FBS/DMEM and expression of

phosphorylated Akt (pAkt, Cell Signaling Technology, Danvers, MA, USA) was assessed by western immunoblotting. Cells were also pre-treated with the PI-3 kinase pathway inhibitor, LY294002 (10 μM; Calbiochem) for 30 min prior to the addition of fractalkine (200 ng/mL) for 24 h. In another experiment, mixed glial cells were incubated in the presence and absence of LPS (100 ng/mL), fractalkine (200 ng/mL) and LY294002 (10 μM). Cells were again pre-treated with LY294002 for 30 min prior to the addition of fractalkine and/or LPS for 24 h; IL-1β concentration was assessed in samples of supernatant and cells were harvested for analysis of MHCII mRNA by PCR.

Isolated microglia were prepared from the cortices of 1-day-old Wistar rats as previously described (Downer *et al.* 2008) For preparation of microglia, dissected cortices were chopped in 10% FBS/DMEM, passed through a sterile mesh filter (40 μm) and centrifuged (2000 × *g* for 3 min at 20°C). The pellet was resuspended in DMEM and plated onto T25 flasks. Cells were grown at 37°C in a humidified environment (5% CO_2:95% air). DMEM containing macrophage colony stimulating factor (M-CSF) (20 ng/mL; R&D Systems) and granulocyte macrophage colony stimulating factor (GM-CSF) (50 ng/mL; R&D Systems) was changed after 1, 5 and 8 days. At day 14, non-adherent cells (microglia) were isolated by shaking, cells were centrifuged (2000 × *g* for 5 min at 20°C), and the microglia-enriched pellet was resuspended in DMEM. Microglia were plated (1×10^5 cells/mL) on 10-mm diameter coverslips coated with poly-l-lysine (Sigma). After a further 2 days, isolated microglia were pre-treated with fractalkine (0–200 ng/mL) for 24 h and incubated in the presence or absence of LPS (100 ng/mL) for 24 h. Supernatants were collected and stored at −80°C for later analysis of IL-1β.

Analysis of IL-1β and fractalkine by ELISA

The concentrations of IL-1β and fractalkine were assessed in supernatant obtained from glial cultures and in hippocampal homogenates. Analysis was carried out by ELISA (R&D Systems) as previously described (Lyons *et al.* 2007b). Absorbance was read at 450 nm, values were expressed as pg/mL (supernatant) and in the case of homogenates, values were corrected for protein concentration and expressed as pg/mg protein.

Analysis of phosphorylated Akt (pAkt)

Expression of pAkt was assessed in samples of cell lysate obtained from cultured mixed glia and in homogenate prepared from hippocampus of fractalkine-treated aged and young rats. Samples were equalized for protein concentration and diluted to a final protein concentration of 1 mg/mL. Aliquots (10 μL) were added to NuPAGE–LDL sample buffer (Invitrogen) containing NuPAGE reducing agent, heated at 70°C for 10 min and loaded onto 10% Nu-polyacrylamide gel electrophoresis-low density lipoprotein Novex Bis–Tris gels (Invitrogen). Samples were separated by application of a constant current (30 mA, 25–30 min), transferred onto nitrocellulose strips (225 mA, 75 min) and incubated for 60 min in blocking buffer [5% milk in Tris-buffered saline (TBS) containing Tween-20]. Phosphorylated Akt expression was assessed by incubating membranes overnight with a pAkt (Ser473) monoclonal IgG1 antibody (1 : 1000 in TBS/T containing 2% milk; Cell Signaling Technology). Membranes were washed, incubated for 1 h at room temperature in the presence of the appropriate horseradish peroxidase-conjugated secondary antibody (1 : 1000 in TBS containing 5% milk) and washed again. Immunoreactive bands were detected using enhanced chemiluminescence (Amersham, Little Chalfont, UK). Following western immunoblotting for pAkt, blots were stripped with an antibody stripping solution (1 : 10 dilution in dH_2O; ReBlot Plus Strong Antibody Stripping Solution; Millipore) and reprobed for β-actin expression to confirm equal loading of protein. Bands were quantified by densitometry (Labworks v4.5, MediaCybernetics, Bethesda, MD, USA). Values were normalized for protein loading using the actin protein expression values.

Fluorescent staining of fractalkine and MHCII

Cryostat sections (10 μm) were prepared and assessed for MHCII expression as previously described (Lyons *et al.* 2007a). Neuronal fractalkine expression was determined in sections using rabbit anti-rat fractalkine (1 : 300; Torrey Pines Biolabs Inc, East Orange, NJ, USA). Sections were washed in PBS and incubated with fluorescently-tagged anti-rabbit IgG antibody (1 : 1500, Alexa 488, Molecular Probes, Invitrogen) for 2 h at room temperature. Double-immunostaining was carried out with the neuronal marker NeuN (mouse monoclonal anti-rat nuclear protein antibody; 1 : 300; Millipore) and in the presence of fluorescently-tagged anti-mouse Alexa 546 (1 : 1500, Invitrogen). Sections were mounted in aqueous mountant (Vectashield; Vector, UK) and viewed with a Zeiss 510 Meta confocal laser microscope with an Axiovert 200 M inverted microscope, at × 40 and × 63 magnification. Hoescht [4′-6-diamidino-2-phenylindole (DAPI)] staining of nuclei was visualized using the 543 nm helium neon laser. Negative control experiments were performed by replacing the primary antibody with normal IgG antibodies and using equal gain settings during acquisition and analysis.

Real-time PCR analysis of MHCII, CD40, IL-1β, fractalkine and fractalkine receptor mRNA expression

Total RNA was extracted from snap-frozen hippocampal tissue and harvested mixed glial cells using a NucleoSpin® RNAII isolation kit (Macherey-Nagel Inc., Düren, Germany) as per the manufacturer's instructions. Denaturing agarose gel electrophoresis was used to assess the RNA integrity. Total RNA concentrations were determined by spectrophotometry, samples were equalized and stored at −80°C until required for cDNA synthesis. cDNA synthesis was performed on 1 μg total RNA using a High Capacity cDNA RT kit (Applied Biosystems, Warrington, UK) as per the manufacturer's instructions. Real-time PCR was performed using Taqman Gene Expression Assays (Applied Biosystems) which contain forward and reverse primers, and a FAM™-labeled MGB Taqman probe for each gene of interest. The assay IDs for the genes examined in this study were as follows: MHCII (Rn01768597_m1), CD40 (Rn00584362_m1), IL-1β (Rn00580432_m1), fractalkine (Rn00593186_m1) and fractalkine receptor (Rn00591798_m1). All real-time PCR was conducted using an ABI Prism 7300 instrument (Applied Biosystems). A 20 μL volume was added to each well (9 μL of diluted cDNA, 1 μL of primer and 10 μL of Taqman® Universal PCR Master Mix). Samples were assayed in duplicate in one run (40 cycles), which consisted of three stages, 95°C for 10 min, 95°C for 15 s for each cycle (denaturation) and finally the transcription step at 60°C for 1 min. β-actin was used as endogenous control to normalize gene expression data, and β-actin expression was conducted using a gene expression assay containing

forward and reverse primers (primer limited) and a VIC-labeled MGB Taqman probe from Applied Biosystems (Assay ID: 4352341E). Gene expression was calculated relative to the endogenous control samples and to the control sample giving an RQ value ($2^{-\Delta\Delta Ct}$, where Ct is the threshold cycle).

Statistical analysis

Data were analyzed using either Student's *t*-test for independent means, or analysis of variance (ANOVA) followed by *post hoc* Student–Newman–Keuls test to determine which conditions were significantly different from each other. Data are expressed as means ± standard errors, where the *n* values represent the number of animals per treatment group.

Results

Fractalkine modulates glial cell activation

Fractalkine mRNA and fractalkine concentration were significantly decreased in hippocampal tissue prepared from aged, compared with young, rats ($^{*}p < 0.05$; Fig. 1a and b), although the data indicate that there was a significant increase in fractalkine receptor mRNA ($^{*}p < 0.05$; Fig. 1c). The age-related decreases in fractalkine mRNA and protein were accompanied by evidence of glial cell activation as indicated by increases in several markers of activation, MHCII mRNA, CD40 mRNA and IL-1β concentration ($^{*}p < 0.05$; $^{**}p < 0.01$; Fig. 1d–f).

The inverse relationship between fractalkine expression and glial cell activation supports the proposal that fractalkine modulates microglial activation (Zujovic *et al.* 2000; Mizuno *et al.* 2003) and in an effort to further investigate this, we evaluated the effect of fractalkine on LPS-induced changes in cultured glial cells. Incubation of isolated microglia or mixed glial cells in the presence of LPS (100 ng/mL) significantly increased the concentration of IL-1β in supernatant ($^{***}p < 0.001$, relative to control; Fig. 2a and b, for isolated microglia and mixed glial cells, respectively) and treatment with fractalkine attenuated the LPS-induced increase in a concentration-dependent manner so that at the highest concentration (200 ng/mL), fractalkine significantly reduced the effect of LPS ($^{+}p < 0.05$; $^{+++}p < 0.001$; compared with LPS alone, in isolated microglia and mixed glial cultures respectively). Analysis of cell surface expression of MHCII by flow cytometry revealed a robust LPS-induced increase on CD11b positive cells, which was attenuated in a concentration-dependent manner by fractalkine (data not shown). As astrocytes do not express the fractalkine receptor, and isolated microglia exhibit increased background activation (data not shown), it was decided to use mixed glial cells for further fractalkine analysis and as an indicator of change in microglia.

The modulatory role of fractalkine on microglial activation depends on interaction with the fractalkine receptor

These data demonstrate that soluble fractalkine modulates the activation state of microglia. To confirm that this was dependent on interaction with the fractalkine receptor, we re-examined the effect of fractalkine on LPS-induced release of IL-1β in mixed glial cells but, on this occasion, assessed changes in the presence and absence of an anti-fractalkine receptor antibody. As described above, the significant LPS-induced increase in IL-1β ($^{***}p < 0.001$, compared with control; Fig. 3a) was significantly inhibited by fractalkine (200 ng/mL) ($^{++}p < 0.01$; compared with LPS alone). However, while incubation in the presence of a blocking anti-fractalkine receptor antibody (5 μg/mL) did not affect IL-1β, it completely reversed the modulatory effect of fractalkine ($^{§§}p < 0.01$ compared with LPS + fractalkine; Fig. 3a).

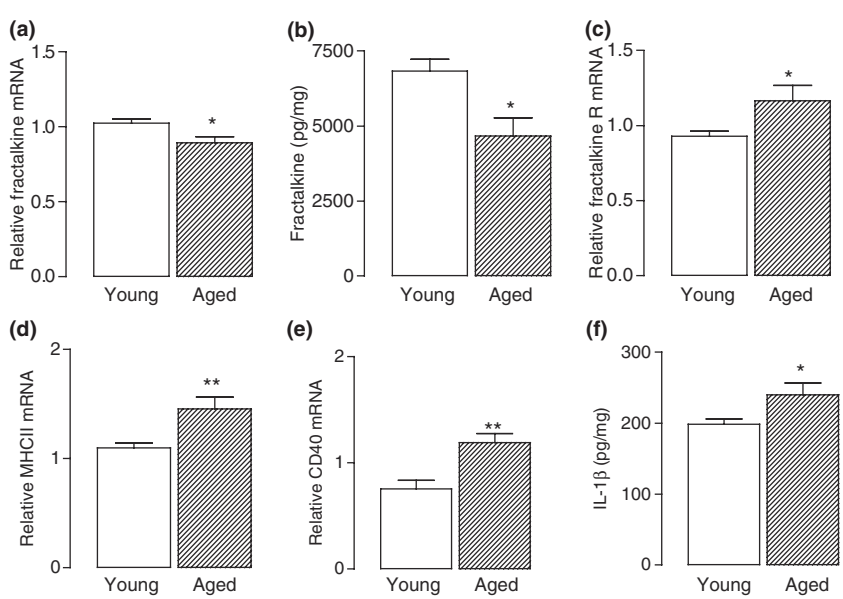

Fig. 1 Age-related decreases in fractalkine mRNA and protein are accompanied by evidence of microglial activation. (a) Fractalkine mRNA and (b) protein were significantly decreased in hippocampal tissue prepared from aged, compared with young rats ($^{*}p < 0.05$; Student's *t*-test for independent means; $n = 12$), whereas (c) fractalkine receptor mRNA was significantly increased ($^{*}p < 0.05$; Student's *t*-test for independent means; $n = 12$). (d) MHCII mRNA, (e) CD40 mRNA and (f) IL-1β concentration were significantly increased in hippocampal tissue prepared from aged, compared with young rats ($^{*}p < 0.05$, $^{**}p < 0.01$; Student's *t*-test for independent means; $n = 12$). Data are expressed as means ± SEM.

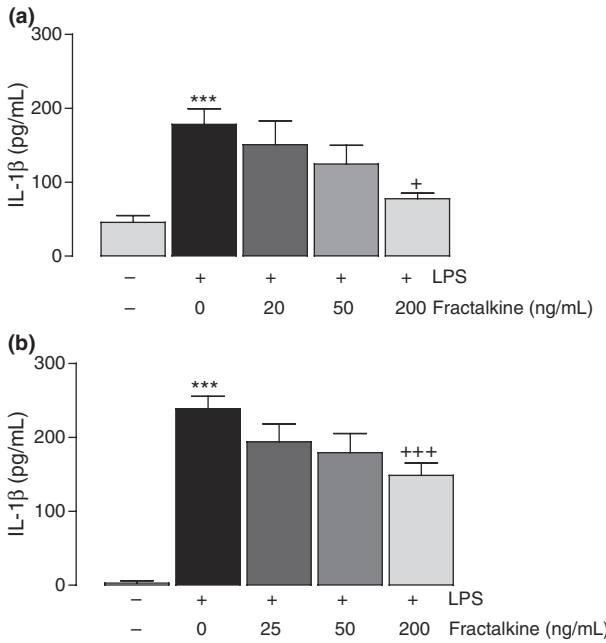

Fig. 2 Increasing concentrations of fractalkine inhibits LPS-induced activation of isolated microglia and mixed glial cells. LPS (100 ng/mL) significantly increases IL-1β concentration released from (a) microglia and (b), mixed glia (***$p < 0.001$, ANOVA; $n = 8$) and fractalkine (200 ng/mL) significantly inhibits the LPS-induced release [$^{+}p < 0.05$; $^{+++}p < 0.001$ ANOVA; $n = 8$ (where n refers to the number of animals per treatment group and also the number of individual treatments)]. Data are expressed as means ± SEM.

Is the effect of soluble fractalkine mimicked by membrane-associated fractalkine?

It appears that both soluble and membrane-associated fractalkine bind to fractalkine receptors (Bazan *et al.* 1997; Vitale *et al.* 2004) and one objective of this study was to assess whether membrane-associated fractalkine exerted effects which are similar to those induced by soluble fractalkine. Consistent with previous observations, we demonstrate that fractalkine is expressed on neurons. Double-fluorescent immunostaining was performed on frozen rat brain sections and we show that fractalkine co-localizes with the neuronal marker NeuN, suggesting neuron-specific localization (Fig. 3b–d), with evidence of cell surface expression. The data demonstrate that the effects of soluble fractalkine are mimicked by membrane-associated fractalkine. The ability of fractalkine to inhibit the LPS-induced IL-1β release was mimicked when neurons were added to glia in the presence of LPS ($^{++}p < 0.01$, LPS compared with LPS + neurons; Fig. 3e). In order to show that the inhibition of glial cell activation was specific to fractalkine-fractalkine receptor interaction, a blocking antibody was used to disrupt binding. This resulted in a significant reversal in the ability of membrane-bound fractalkine to maintain glial cells in a quiescent state ($^{§§§}p < 0.001$ compared with LPS + neurons; Fig. 3e).

Fractalkine treatment modulates age-related microglial activation

Others have demonstrated that fractalkine down-regulates microglial activation (Mizuno *et al.* 2003) and we have previously shown that microglial activation is increased with age (Griffin *et al.* 2006; Lyons *et al.* 2007b); in this study, we investigated whether fractalkine treatment might exert a beneficial effect in aged rats. We prepared cryostat sections from brains of young and aged, control-treated and fractalkine-treated rats and assessed them for MHCII immunostaining; the findings are presented in Fig. 4. While there was minimal MHCII positive staining in sections of hippocampus prepared from young rats (Fig. 4a), marked staining was observed in sections prepared from aged rats. Fractalkine treatment exerted no discernible effect on MHCII staining in tissue prepared from young rats but markedly reduced the staining observed in aged animals. We also investigated hippocampal expression of MHCII mRNA and CD40 mRNA as further indicators of microglial activation; the data indicate significant age-related increases in both markers (**$p < 0.01$, aged compared with young animals; Fig. 4b and c) and demonstrate that fractalkine treatment attenuated these changes, although the attenuation was statistically significant only in the case of CD40 mRNA ($^{+}p < 0.05$; aged control-treated versus aged fractalkine-treated samples; Fig. 4c).

The action of fractalkine is dependent on Akt activation

We set out to examine the mechanism by which fractalkine exerts the effects described here, cognizant of the findings that suggested a possible role for Akt in mediating fractalkine-induced modulation of neurotoxicity caused by the HIV envelope protein gp120 (Meucci *et al.* 2000) and excitotoxicity induced by glutamate (Limatola *et al.* 2005), and because we have previously coupled a decrease in PI-3 kinase with the age-related reduction in synaptic function (Kelly and Lynch 2000). In this study, we asked whether fractalkine attenuated IL-1β production by LPS-stimulated glia in an Akt-dependent manner. First we demonstrate that fractalkine increases phosphorylation of Akt (pAkt) in mixed glia and that this effect was initially transient but significantly greater and more sustained after 24 h (***$p < 0.001$, compared with control; Fig. 5a). Co-incubation in the presence of LY294002 (10μM) completely blocked the fractalkine-induced change ($^{+++}p < 0.001$; compared with 24 h fractalkine-treated). In support of the data described in Fig. 2, we show that fractalkine significantly inhibited the LPS-induced increases in MHCII mRNA and IL-1β ($^{+}p < 0.05$; $^{+++}p < 0.001$, compared with LPS-treated; Fig. 5b and c) and that the ability of fractalkine to inhibit the LPS-induced increase in IL-1β, but not MHCII mRNA, was significantly attenuated by LY294002 ($^{§§}p < 0.001$, compared with LPS + fractalkine; Fig. 5c).

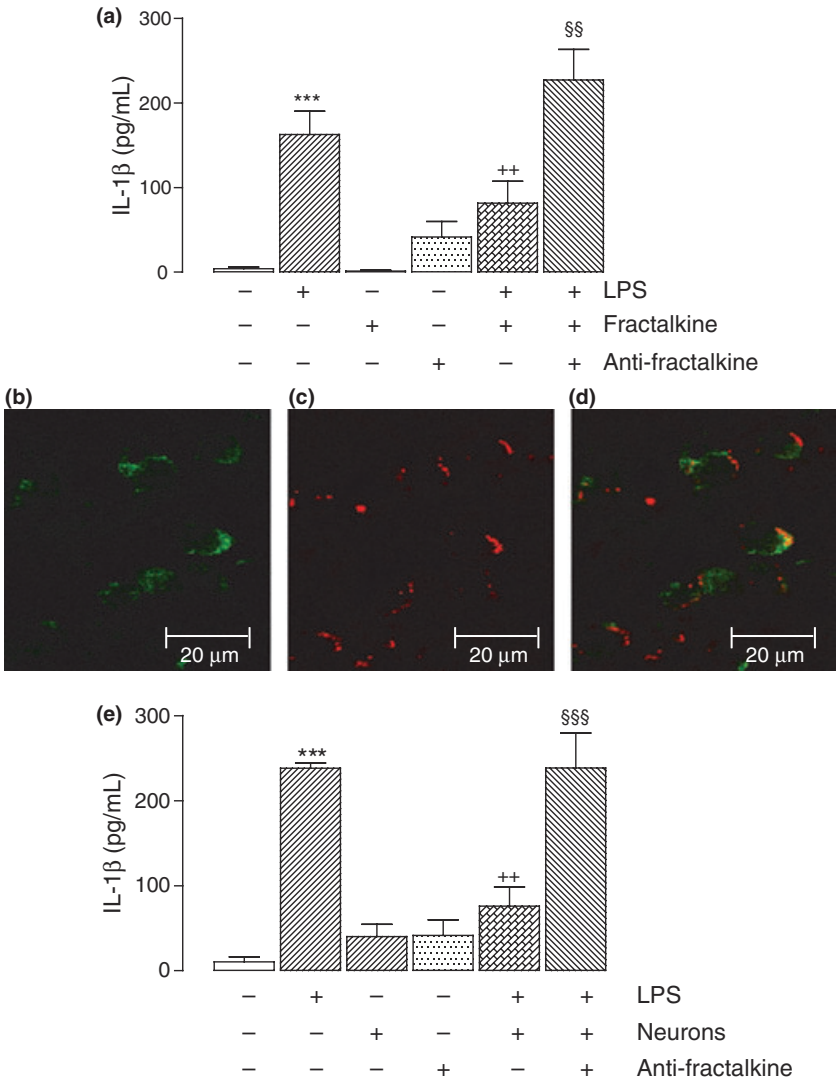

Fig. 3 Soluble and membrane-associated fractalkine attenuate LPS-induced increase in IL-1β. (a) LPS (100 ng/mL) significantly increased IL-1β in supernatant prepared from mixed glia (***$p < 0.001$; ANOVA; $n = 6$) and this was attenuated by addition of fractalkine (200 ng/mL). Addition of anti-fractalkine receptor antibody significantly abrogated the effect of fractalkine ($^{§§}p < 0.01$; ANOVA; $n = 6$). (b) Neurons were immunostained with anti-NeuN antibody which was bound to anti-mouse Alexa 488 (green), (c) fractalkine was immunostained with anti-fractalkine antibody which was bound to anti-rabbit Alexa 546 (red) and (d) the overlap of the combined staining (yellow/red) revealed membrane localization. Scale bar: 20 μm. (e) LPS (100 ng/mL) significantly increased IL-1β in supernatant prepared from mixed glia (***$p < 0.001$; ANOVA; $n = 6$) and this was attenuated by addition of neurons, as a source of membrane-bound fractalkine ($^{++}p < 0.01$, ANOVA; $n = 6$). Again, addition of an anti-fractalkine receptor antibody significantly abrogated the effect of neuronal membrane-bound fractalkine ($^{§§§}p < 0.001$; ANOVA; $n = 6$ (where n refers to the number of animals per treatment group and also the number of individual treatments)). Data are expressed as means ± SEM.

In vivo, we demonstrate that Akt phosphorylation was significantly decreased in the hippocampus of aged rats ($^{+}p < 0.05$, compared with young and aged animals treated with fractalkine; Fig. 6). Consistent with a role for Akt in fractalkine-induced changes, there is increased Akt phosphorylation in hippocampal tissue prepared from both young and aged rats following intracerebroventricular injection of fractalkine (*$p < 0.05$, compared with young control animals; Fig. 6).

Discussion

We set out to investigate the modulatory effect of fractalkine on microglial activation. Here we demonstrate that fractalkine attenuates the LPS-induced increase in microglial activation *in vitro* and the age-associated increase *in vivo*. The evidence from *in vitro* studies suggests that this effect is shared by both soluble and membrane-associated forms of fractalkine. Significantly, we report that fractalkine attenuates the increase in microglial activation observed in hippocampus of aged rats and the effects of fractalkine are mediated through phosphorylation of Akt and activation of the PI-3 kinase pathway.

We demonstrate that hippocampal fractalkine mRNA expression and fractalkine concentration were both significantly decreased with age and that these changes correlate with age-related increases in CD40 mRNA and MHCII mRNA and IL-1β concentration. This is consistent with reports that there is a decrease in the number of fractalkine-positive cells in hippocampus and cortex of 9-month-old mice which overexpress human amyloid precursor protein (Tg2576), compared with wild-type, mice (Duan *et al.* 2008); interestingly there is also evidence of microglial activation in these animals (Frautschy *et al.* 1998). The underlying cause of the age-related decrease in fractalkine is not clear and may be associated with a decrease in

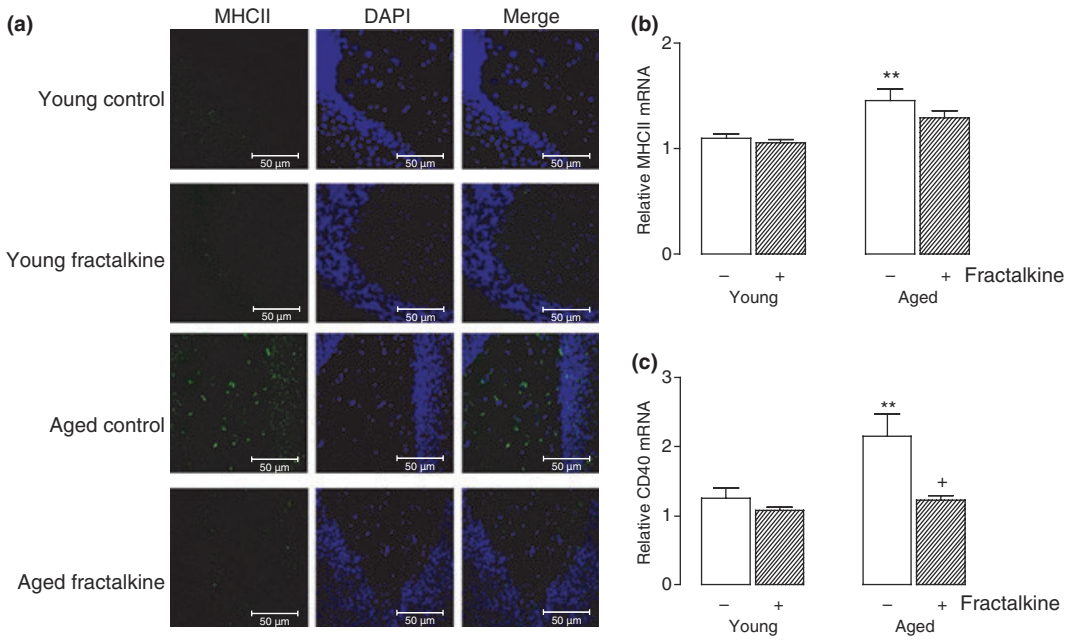

Fig. 4 Fractalkine inhibits age-related changes in microglial cell activation. (a) Fluorescent images of MHCII in the hippocampus of young and aged animals treated with or without fractalkine revealed that the age-related increase in MHCII was attenuated by fractalkine. Scale bars: 50 μm. The significant age-related increase in hippocampal expression of (b) MHCII mRNA and (c) CD40 mRNA (**$p < 0.01$; ANOVA; $n = 8$) were attenuated in aged rats treated with fractalkine; this effect was statistically significant in the case of CD40 mRNA [$^{+}p < 0.05$; ANOVA; $n = 8$ (where n refers to the number of animals per treatment group)]. Data are expressed as means ± SEM.

transcription and translation, which has been described in the hippocampus of aged rats (Mullany and Lynch 1997), or a loss of fractalkine-producing cells.

Analysis of the effect of soluble fractalkine *in vitro* revealed that it attenuated the LPS-induced release of IL-1β from isolated microglia and mixed glial cultures; a similar, though less robust, effect was observed when IL-6 was assessed (data not shown). The concentration required to modulate LPS-induced changes is high; this may reflect that other mechanisms, for example, the interaction between CD200 and its receptor (Lyons *et al.* 2007a; Downer *et al.* 2008) also play in modulating microglial activation. LPS transiently decreases fractalkine receptor on microglia and this may also contribute to the changes described here (Boddeke *et al.* 1999). Fractalkine has previously been shown to partially attenuate the LPS-induced increase in TNFα (Zujovic *et al.* 2000) and IL-6, as well as nitric oxide production and iNOS expression in isolated microglia (Mizuno *et al.* 2003). Consistently LPS-triggered microglial activation was demonstrated to be exaggerated in fractalkine receptor-deficient mice compared with wild-type mice providing further evidence of a role for fractalkine in modulating microglial activation (Cardona *et al.* 2006).

It has previously been shown that fractalkine is principally localized on neurons (Harrison *et al.* 1998; Maciejewski-Lenoir *et al.* 1999; Lauro *et al.* 2008). Here we confirm that fractalkine is expressed on neurons and, having established that soluble fractalkine attenuated LPS-induced glial cell activation, we set out to assess whether membrane-associated fractalkine on neurons exerted a similar effect. To do so, we assessed the effect of neurons on LPS-treated glia and show that the LPS-induced increase in IL-1β production was attenuated by the addition of neurons. Importantly, this effect of neurons, like the effect of soluble fractalkine, was blocked by an anti-fractalkine receptor antibody indicating that the effect was dependent on the binding of fractalkine on neurons to its receptor on glial cells.

In recent years it has become evident that microglial activation increases with age and that strategies which reduce activation improve synaptic plasticity (Griffin *et al.* 2006; Lyons *et al.* 2007b; Clarke *et al.* 2008). In light of the modulatory effect of fractalkine, we assessed the effect of an intracerebroventricular injection of fractalkine in aged and young rats. The data demonstrate that the age-related increase in MHCII staining was significantly increased in hippocampal sections prepared from aged, compared with young rats; here we examined the changes in dentate gyrus but similar age-related changes in MHCII immunoreactivity are also observed in areas CA1 and CA3 (Downer *et al.*, unpublished). The age-related increase in MCHII immunoreactivity in dentate gyrus was paralleled by an increase in MHCII mRNA and another marker of microglial activation, CD40 mRNA. These findings confirm previous data which have suggest that microglial activation increases with age

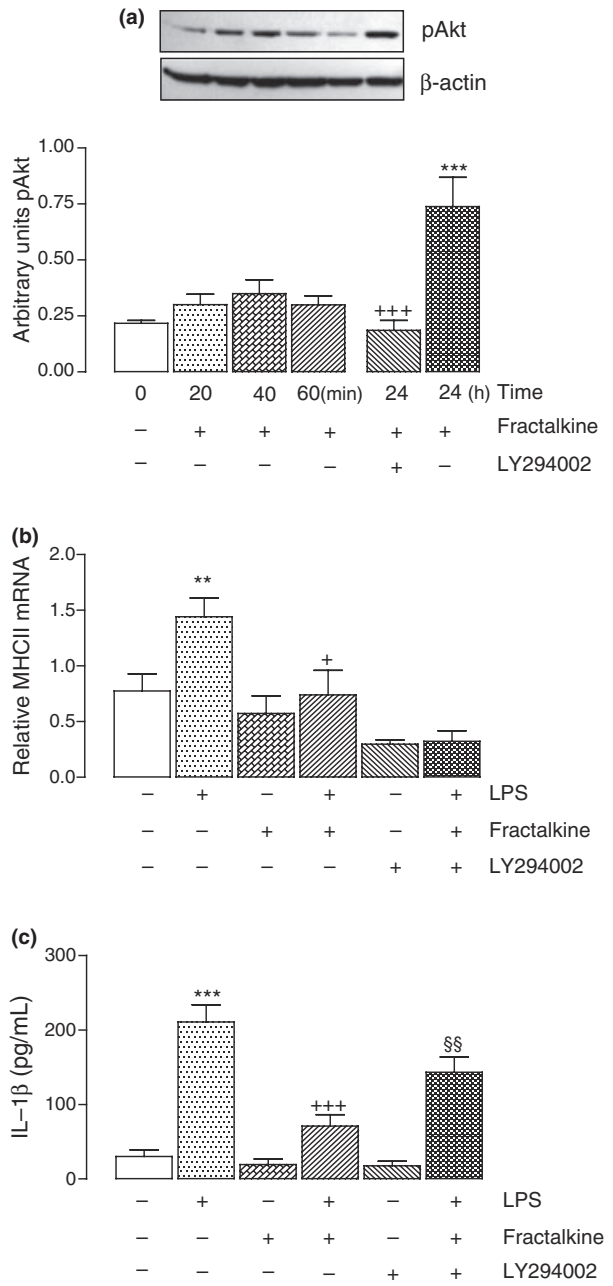

Fig. 5 Fractalkine-induced Akt phosphorylation blocks LPS-mediated glial cell activation. (a) Incubation of mixed glia with fractalkine for 20, 40 and 60 min increased phosphorylation of Akt but the effect was statistically significant after incubation for 24 h (***$p < 0.001$; ANOVA; $n = 5$). Co-incubation in the presence of LY294002 (10 μM) completely blocked the fractalkine-induced change ($^{+++}p < 0.001$; ANOVA; $n = 5$). The significant LPS-induced (100 ng/mL) increases in (b) MHCII mRNA and (C), IL-1β (**$p < 0.01$; ***$p < 0.001$; ANOVA; $n = 10$) were attenuated by fractalkine ($^{+}p < 0.05$; $^{+++}p < 0.001$, respectively; ANOVA; $n = 10$). Addition of LY294002 significantly abrogated the effect of fractalkine on LPS-induced IL-1β, but not MHCII mRNA [$^{§§}p < 0.01$; ANOVA; $n = 10$ (where n refers to the number of animals per treatment group]. Data are expressed as means ± SEM.

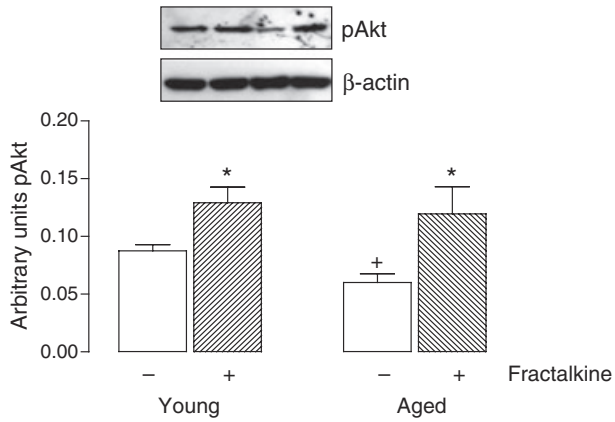

Fig. 6 Fractalkine increases Akt phosphorylation *in vivo*. Akt phosphorylation was significantly decreased in hippocampal tissue prepared from aged, compared with young rats ($^{+}p < 0.05$; Student's *t*-test for independent means; $n = 12$). Fractalkine significantly increased Akt phosphorylation in hippocampal tissue prepared from young and aged rats relative to controls [*$p < 0.05$; ANOVA; $n = 8$ (where n refers to the number of animals per treatment group and also the number of individual treatments)]. Data are expressed as means ± SEM.

(Lyons *et al.* 2007a; Clarke *et al.* 2008), although is it accepted that microglia, like macrophages (Gordon 2003), can adopt alternative activation states (Schwab and McGeer 2008). Importantly, fractalkine treatment effectively blocked the age-related increase in MHCII staining, and although it did not significantly affect the age-related increase in MHCII mRNA, it completely attenuated the increase in CD40 mRNA. Thus fractalkine appears to exert differential effects on different markers of microglial activation. With respect to the differential effects on MHCII mRNA and protein, it might be suggested that the effect of fractalkine is targeted at the translational level or alternatively that it exerts an effect on recycling of MHCII (Harding *et al.* 1990; Walseng *et al.* 2008). Targeting the fractalkine receptor as a potential therapeutic in Alzheimer's Disease has been suggested (Streit *et al.* 2005); the present data support this potential.

We next sought to examine the mechanism by which fractalkine exerts its effects. Here we asked whether its modulatory effects on LPS-induced IL-1β production in glia was dependent on activation of the PI-3 kinase pathway. We have previously shown that there is a decrease in PI-3 kinase pathway activation in the aged brain (Maher *et al.* 2004), therefore we decided to investigate whether this decrease may be coupled with the reduced fractalkine observed here. The data show that incubation of glia in the presence of fractalkine significantly increases Akt phosphorylation and reveal that the modulatory effect of fractalkine on LPS-induced IL-1β production was sensitive to the presence of the PI-3 kinase inhibitor, LY294002. Similarly, analysis of Akt phosphorylation in hippocampal tissue revealed a significant decrease in the aged brain relative to young. When young

and aged rats were treated with fractalkine there was a significant increase in Akt phosphorylation in samples prepared from young and aged rats injected with fractalkine compared with samples prepared from control-treated animals. These findings, which suggest a role for Akt activation in mediating fractalkine-induced changes, mirror a previous observation which shows that the neurotoxicity induced by the HIV envelope protein gp120 was attenuated by fractalkine receptor activation (Meucci *et al.* 2000); in that study it was shown that the neurotoxicity was blocked by inhibition of PI-3 kinase and mimicked by a phospholipid activator of Akt. Similarly, in hippocampal neurons, the ability of fractalkine to attenuate glutamate-induced excitotoxicity has been attributed to activation of ERK and PI-3 kinase (Limatola *et al.* 2005; Lauro *et al.* 2008). The signaling cascades in which Akt plays a role are complex and the upstream and downstream changes associated with fractalkine-induced Akt activation remain to be identified. In addition, it must be recognized that signaling cascades other than PI-3 kinase may play a role in mediating the effects of fractalkine.

Although the present findings and earlier observations have highlighted the potential beneficial effects of fractalkine in modulating microglial activation, ischaemic insult resulted in a better outcome in fractalkine-deficient, compared with wild-type, mice (Soriano *et al.* 2002; Denes *et al.* 2008); infarct size was smaller, functional recovery and mortality was improved and there was no evidence of increased numbers of IL-1β-positive microglia. These findings indicate that the effects of fractalkine receptor activation depend on the experimental conditions and may be region-specific in the brain.

It is becoming increasingly clear that interaction of microglia with other cell types determines their activation state. We have recently highlighted the importance of CD200-CD200 receptor activation in maintaining microglia in a quiescent state (Lyons *et al.* 2007a; Downer *et al.* 2008) while here the evidence obtained, from assessing the effect of fractalkine on LPS-induced changes *in vitro*, suggests that activation of the fractalkine receptor on microglia by soluble fractalkine or the membrane-associated form which is localized to neurons, exerts a similar modulatory effect on microglial activation. It is possible that other similar mechanisms also contribute to modulating microglial activation for example, the interaction between CD47 and CD172a (Barclay *et al.* 2002). Analysis of changes in young and aged rats, and the fractalkine-induced changes in these animals, permits us to propose that the age-related decrease in fractalkine may contribute the increase in microglial activation in aged rats.

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