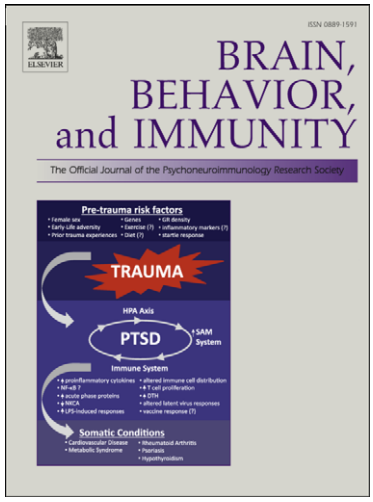

CD200 fusion protein decreases microglial activation in the hippocampus of aged rats

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CD200 fusion protein decreases microglial activation in the hippocampus of aged rats.

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Abstract

The glycoprotein, CD200, is primarily expressed on neurons and its cognate receptor CD200R is expressed principally on cells of the myeloid lineage, including microglia. The interaction of CD200 with its receptor plays a significant role in maintaining microglia in a quiescent state and therefore a decrease in CD200 expression in brain is associated with evidence of microglial activation. Conversely, activation of CD200R, for example using a CD200 fusion protein (CD200Fc), should result in a decrease in microglial activation. Here we assessed the effect of delivery of CD200Fc intrahippocampally on microglial activation and on long-term potentiation (LTP) in perforant path-granule cell synapses in young and aged rats. We hypothesized that the age-related changes in microglial activation would be attenuated by CD200Fc resulting in an improved ability of aged rats to sustain LTP. The data indicate that expression of markers of microglial activation including major histocompatibility complex Class II (MHCII) and CD40 mRNA, as well as MHCII immunoreactivity, were increased in hippocampus of aged, compared with young, rats and that these changes were associated with a deficit in LTP; these changes were attenuated in hippocampal tissue prepared from aged rats which received CD200Fc. Microglial activation and a deficit in LTP have also been reported in lipopolysaccharide (LPS)-treated rats and, here, we report that these changes were also attenuated in CD200Fc-treated animals. Thus the negative impact of microglial activation on the ability of aged and LPS-treated rats to sustain LTP is ameliorated when CD200R is activated by CD200Fc.

Key words: **Age, CD200, long-term potentiation (LTP), microglial activation, hippocampus, lipopolysaccharide (LPS)**

1. Introduction

CD200 is a type-1 membrane glycoprotein which has been identified as an immunosuppressive molecule. It is expressed on several cell types and, in the brain, CD200 is expressed on neurons (Barclay et al., 2002) and oligodendrocytes (Koning et al., 2009) but not on microglia (Lyons et al., 2007a). CD200 was reported to be expressed on reactive astrocytes in lesions from postmortem multiple sclerosis brains (Koning et al., 2009) but recent evidence from this laboratory suggests that it is also expressed on astrocytes prepared from 1 day-old mice (Costello et al., 2011). The receptor for CD200, CD200 receptor (CD200R), is also a membrane glycoprotein and has an NPXY signalling motif containing 3 tyrosine residues in its intracellular domain (Snelgrove et al., 2008; Wright et al., 2000). This contrasts with CD200, which has a short cytoplasmic domain with no signalling motifs (Barclay et al., 2002). CD200R expression is restricted primarily to cells of the myeloid lineage and therefore, in the brain, has been identified on microglia (Barclay et al., 2002; Koning et al., 2009) but not on neurons (Lyons et al., 2007a) or astrocytes (Denieffe et al., unpublished).

The complementary expression of ligand and receptor on neurons and microglia respectively, suggested that the interaction between CD200 and its receptor may play a role in modulating microglial activation and recent evidence supports this contention. Thus the lipopolysaccharide (LPS)- and amyloid-β (Aβ)-induced increase in expression of cellular markers for microglial activation was inhibited when glia were co-cultured with neurons and this effect of neurons was attributed to CD200-CD200R interaction since it was blocked by an anti-CD200 antibody (Lyons et al., 2007a; Lyons et al., 2009b). This finding, and others, suggests that interaction of CD200 with its receptor modulates microglial activation. This has been confirmed by analysis in CD200-deficient mice; thus microglial and/or macrophage activation occurs to a greater extent in these mice compared with wildtype mice in several models of inflammation, for example facial nerve transection, experimental autoimmune encephalomyelitis (EAE), an animal model of arthritis (Hoek et al., 2000) and experimental autoimmune uveoretinitis (Broderick et al., 2002). Consistently, the decrease in EAE-like symptoms in *Wld*^{s} mice has been attributed to increased expression of CD200 on spinal cord neurons (Chitnis et al., 2007). Conversely, administration of a CD200 fusion protein, containing the ectodomain of CD200 bound to a murine IgG2a module, ameliorates the inflammatory changes observed in collagen-induced arthritis (Gorczynski et al., 2002; Gorczynski et al., 2001).

One consequence of the neuroinflammatory changes which accompany microglial activation is a deficit in synaptic plasticity, specifically long-term potentiation (LTP) (Lynch et al., 2007; Nolan et al., 2005). Here we considered that if the age-related microglial activation was reduced by activating CD200R, then the ability of rats to sustain LTP may be improved and therefore we set out to investigate the effect of a CD200 fusion protein (CD200Fc) on microglial activation and LTP in aged rats. We argued that activation of CD200R by CD200Fc would also attenuate the LPS-induced microglial activation and consequently reduce the LPS-induced deficit in LTP. The data show that intrahippocampal delivery of CD200Fc ameliorated the age-related and LPS-induced activation of microglia and the accompanying deficit in LTP suggesting that CD200R activation, by modulating microglial activation, positively impacts on neuronal function.

2. Materials and Methods

2.1. Animals.

Young (3 months; 250–350g) and aged (20-22 months; 550-600g) male Wistar rats (Bantham and Kingman, UK) were housed in a controlled environment (temperature: 20-

22 °C; 12:12h light/ dark cycle) in the BioResources Unit, Trinity College, Dublin. Animals had free access to food and water and were maintained under veterinary supervision for the duration of the experiment. All experiments were carried out under licence from the Department of Health and Children (Ireland) and with ethical approval from the Trinity College Ethical Committee.

2.2. Analysis of LTP in vivo.

Rats were anaesthetized by intraperitoneal injection of urethane (1.5 g/kg) and the absence of a pedal reflex was considered to be an indicator of deep anaesthesia. In the first series of experiments, young and aged animals were subdivided into an experimental and a control group, with 6 animals per group, and all animals received a single unilateral injection. Animals were placed in a stereotaxic frame, the skull was exposed and a dental drill was used to make a small hole to allow the intrahippocampal injections to be made. The experimental group received CD200Fc intrahippocampally (2 µg/µl; 5 µl injection volume; 0.8 mm lateral and 3.5 mm dorsoventral to Bregma) and the control groups received sterile saline (5 µl). The recombinant mouse CD200Fc used here (murine myeloma cell line, NSO-derived; Cat. No. 3355-CD; R&D Systems, US) was prepared by fusing the N-terminal domain of CD200 (Gln31-Gly232) to human IgG_1); it is known to bind CD200R1 in a linear manner within the range 0.4-25ng/ml but its affinity for other CD200R family members is not known. Preliminary experiments were undertaken to assess the effect, if any, of the Ig tag on the CD200Fc construct and no differences between the ability of saline-injected and Ig-injected rats on LTP were identified. Therefore saline was used as control in all further experiments. Following injection, bore holes were made in the skull to enable placement of the electrodes; a bipolar stimulating electrode was stereotaxically positioned in the perforant path (4.4 mm lateral to Bregma) and a unipolar recording electrode was placed in the dorsal cell body region of the dentate gyrus (2.5 mm lateral and 3.9 mm posterior to Bregma). Following a period of stabilisation, test shocks were delivered at 30 second intervals and stable baseline responses were recorded for 10 minutes prior to tetanic stimulation which was delivered 1 hour after intrahippocampal injection. The tetanus consisted of 3 trains of high-frequency stimuli (250 Hz for 200 ms; 30 second inter-train interval) delivered to the perforant path and following this stimulation, recording at test shock frequency resumed for the remainder of the experiment (Martin et al., 2002). Animals were killed by cervical dislocation and this was 2 hours after intrahippocampal injection. The slope of the excitatory post-synaptic potential (EPSP) was used as a measure of excitatory synaptic transmission in the dentate gyrus.

In the second series of experiments, young animals were divided into 4 groups of 6 rats: control rats, rats which received an intraperitoneal injection of LPS (100 µg/kg; from E.coli, serotype EH100(Ra), TLR grade; Alexis Biochemicals, UK; Cat. No. ALX-581-010-L002), rats which received CD200Fc intrahippocampally (2 µg/µl; 5 µl injection volume) and rats which received both LPS and CD200Fc. All rats were placed in the stereotaxic frame, as described above, for administration of a single unilateral injection of either saline or CD200Fc, and were then removed from the frame. Ten minutes later, rats received a single intraperitoneal injection of either saline or LPS. Three 3 hours later, rats were replaced in the stereotaxic frame to enable placement of the electrodes and analysis of LTP, as described above. In this case, recording started 4 hours after LPS injection (with the tetanus delivered 10 minutes later) and animals were killed approximately 5 hours after LPS.

At the end of the period of recording, rats were killed by cervical dislocation. The lateral third of the injected side of the brain was coated with OCT compound (Sakura

Tissue-Tek, Netherlands), immersed in isopentane at –30°C and stored at -80°C until sections were prepared. Cryostat sections (10 μm) were mounted on Superfrost® Plus slides (Thermo Scientific, Germany), air-dried for 30 min and stored at -20°C until used for immunohistochemical analysis of MHCII. The remaining tissue (medial hippocampus from the injected side) was snap-frozen and used to prepare mRNA for PCR analysis.

2.3. Real-time PCR analysis of cytokines and cell surface markers.

Total RNA was extracted from snap-frozen hippocampal tissue using a NucleoSpin® RNAII isolation kit (Macherey-Nagel Inc., Germany) according to the manufacturer's instructions. RNA integrity and total RNA concentration were assessed, and cDNA synthesis was performed as described previously (Lyons et al., 2011). Real-time PCR was performed using Taqman Gene Expression Assays (Applied Biosystems, Germany) 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 (Mm00441895_m1), CD11b (Mm001271265_m1), CD68 (Rn01495631_g1), inducible nitric oxide synthase (iNOS) (Rn00561646_m1), interferon gamma-induced protein 10 (IP-10; Rn00594648_m1) and monocyte chemotactic protein-1 (MCP-1; Rn00580555_m1). All real-time PCR was conducted using an ABI Prism 7300 instrument (Applied Biosystems, Germany). 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 3 stages, 95°C for 10 min, 95°C for 15 sec for each cycle (denaturation) and finally the transcription step at 60°C for 1 min. β-actin was used as the 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 (Germany; Assay ID: 4352341E). Gene expression was calculated relative to the endogenous control samples and to the control sample giving an RQ value (2^{-DDCt}, where CT is the threshold cycle).

2.4. Staining of MHCII

Frozen cryostat sections (10 μm) were prepared as described previously (Nolan et al., 2005). Sections were fixed in ice-cold ethanol, blocked with 10% goat serum, 4% bovine serum albumin (BSA) and incubated overnight at 4°C in the presence of a mouse monoclonal MHCII antibody (OX6; 1:200; Serotec Inc, UK). Negative control experiments were performed by replacing the primary antibody with a mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Sections were washed and incubated with a biotinylated anti-mouse IgG antibody (1:200; Vector Laboratories, Peterborough, UK) for 2 hours, exposed to avidin–biotin–horseradish peroxidase solution for 1 hour (Vectastain Elite ABC kit; Vector Laboratories), and reacted with 3,3′-diaminobenzidine (DAB) using the DAB Enhanced Liquid Substrate System (Sigma, UK); in this system colour development is achieved by mixing the chromagen solution (DAB Liquid Chromagen Solution B; D6085) with H_2O_2 (DAB Liquid Buffer Solution A; D6190) according to the manufacturer's instructions. The reaction was terminated using distilled H_2O, and positive cells were viewed by light microscopy. The sections were counterstained with 1% methyl green (Sigma, UK) for 10 min, rinsed with distilled water, dehydrated through a series of graded alcohols (70%; 80%; 95%; 100%; 100%; Sigma, UK) and cleared by immersion in xylene (Sigma, UK). Coverslips were applied using DPX (RA Lamb, UK) as the mount. MHCII-immunoreactive cells were quantified by counting the number of positively stained cells in a 0.75mm^2 field between the two

blades of the dentate gyrus in 3 slides prepared from each animal (n=5-6 per treatment group).

2.5. Western immunoblotting

Hippocampal tissue was homogenized in lysis buffer as described previously (Lyons et al., 2007b). Briefly, lysates were centrifuged (20,000 x *g* for 12 min) and the supernatant prepared for gel electrophoresis. Samples (10 μg) were added to NuPAGE LDL sample buffer, heated at 70^{o}C for 10 min and separated on 4-12% gradient gels (Invitrogen, UK). Proteins were transferred to nitrocellulose membrane (Sigma, UK) and blocked for 1 hour in Tris-buffered saline-0.05% Tween-20 (TBS-T) and 5% bovine serum albumin (BSA). For analysis, membranes were incubated overnight at 4^{o}C with anti-CD200 antibody (OX 2; 1:500; Santa Cruz Biotechnology, US; R17 goat polyclonal CD200 antibody (sc-14388)), anti-CD200R antibody (D-20; 1:200; Santa Cruz Biotechnology, US; D20; goat polyclonal antibody (sc-14394); Sigma UK) or anti-synaptophysin antibody (SVP-38; 1:5,000; mouse monoclonal antibody (S5768); Sigma, UK) in TBS-T/1% BSA. Samples were washed and incubated with secondary antibody (1:1000 in 5% BSA/TBS-T (Sigma, UK) for 2 hours in the case of CD200 and synaptophysin or for 1 hour in the case of CD200R. Immunoreactive bands were detected using enhanced chemiluminescence (Amersham, UK) and blots were stripped (Re-blot Plus; Chemicon International, Temecula, CA) and re-probed using anti-β-actin (1:4000 in 5% BSA/TBS-T; Sigma, UK) and a peroxidase-conjugated secondary antibody (1:1000 in 5% BSA/TBS-T; Sigma, UK). Bands were quantified by densitometry (Labworks v4.5, MediaCybernetics, Bethesda, MD). Values were normalized for protein loading using the actin protein expression values.

2.6. Statistical analysis

Some data were analyzed using the Student's t-test for independent means. Where appropriate, a 2-way analysis of variance (ANOVA) was performed to evaluate whether or not significant interactions existed. Data are expressed as means ± standard errors.

3. Results

3.1. Synaptophysin and CD200 are decreased with age

Both synaptophysin (a) and CD200 (b) were significantly decreased in hippocampal tissue prepared from aged, compared with young, mice as indicated by western immunoblotting (*$p < 0.05$; student's t-test for independent means; Figure 1); CD200R was unchanged. The decrease in CD200 concurs with our previous findings (Lyons et al., 2007a) and the age-related decrease in synaptophysin in hippocampus is similar to that described previously in the dentate gyrus (Davies et al., 2003; Mullany and Lynch, 1997) and is consistent with the finding that CD200 is expressed at synapses (Ojo et al., 2011).

3.2. CD200Fc ameliorates the age-related increase in markers of microglial activation

Because the interaction between CD200 and CD200R is known to decrease microglial activation (Lyons et al., 2007a), we considered that injection of CD200Fc might impact on expression of markers of microglial activation which have been shown to be upregulated with age. We assessed expression of MHCII mRNA, and analysis of the data by 2-way ANOVA indicated a significant interaction of aging and CD200Fc treatment ($F_{(1,20)} = 6.92$, *$p < 0.05$; Figure 2a). There was a marked increase in MHCII immunoreactivity in sections prepared from aged, compared with young, rats (compare (iii) with (i); Figure 2b) and the age-related increase was attenuated in sections prepared from aged rats which received CD200Fc (compare (iii) with (iv). Statistical analysis of

the data indicated that there was a significant interaction of age and CD200Fc treatment ($F_{(1,20)} = 4.86$, *p < 0.05; 2-way ANOVA; Figure 2c).

Age-related increases in expression of other cell surface markers of microglial activation, CD40, CD11b and CD68 mRNA, were also observed (Figure 3); in the case of CD40 mRNA a significant interaction of aging and CD200Fc treatment was observed ($F_{(1,16)} = 19.42$, ***p < 0.001; 2-way ANOVA; Figure 3a), whereas a significant age effect was observed for CD11b ($F_{(1,19)} = 16.88$, §§§p < 0.001; 2-way ANOVA; Figure 3b) and CD68 ($F_{(1,20)} = 28.7$, §§§p < 0.001; 2-way ANOVA; Figure 3c). iNOS is also an indicator of microglial activation, and is considered to be a marker of the classical activation state (Colton, 2009). A significant interaction of aging and CD200Fc treatment was observed for iNOS mRNA ($F_{(1,19)} = 5.50$, p < 0.05; 2-way ANOVA; Figure 3d).

3.3. CD200Fc decreases the age-related increases in the chemokine, IP-10 and MCP-1

We also examined the mRNA expression of MCP-1 and IP-10, chemokines which are expressed at low levels under resting conditions but expression of which is markedly increased in inflammatory conditions (Duan et al., 2008; Zhang et al., 2006) where they function to recruit monocytes and lymphocytes. Analysis by 2-way ANOVA indicated a significant interaction of aging and CD200Fc treatment on MCP-1 ($F_{(1,21)} = 11.32$, **p < 0.01; Figure 4b) and IP-10 ($F_{(1,21)} = 7.93$, *p < 0.05; Figure 4b).

3.4. The age-related decrease in LTP in dentate gyrus is attenuated in CD200Fc-treated rats

It has been consistently reported that LTP, at least in perforant path-granule cell synapses, is markedly reduced with age and this deficit has been linked with increased microglial activation (Lynch, 2010). Here we confirm that the ability of aged rats to sustain LTP is markedly reduced compared with young rats (Figure 5a). Analysis of the change in EPSP slope in the 5 minutes immediately following tetanic stimulation and in the last 5 minutes of the experiment revealed significant age x CD200Fc treatment interactions ($F_{(1,36)} = 77.23$, ***p < 0.001 and $F_{(1,44)} = 955.6$, ***p < 0.001 respectively; Figure 5b,c).

3.5. CD200Fc attenuates the effect of LPS on MHCII mRNA and LTP

A great deal of evidence has indicated that intraperitoneal injection of LPS leads to inflammatory changes in the brain and, among these changes, is an increase in microglial activation. Since we demonstrated that CD200Fc was capable of attenuating the age-related increase in microglial activation, we asked whether or not it might similarly affect the LPS-induced change. Analysis of MHCII mRNA in hippocampal tissue revealed a significant LPS x CD200Fc interaction ($F_{(1,20)} = 7.41$, *p < 0.05; 2-way ANOVA; Figure 6a). As previously reported (Clarke et al., 2008), LPS was associated with a marked decrease in the ability of rats to sustain LTP as indicated by the decrease in mean EPSP slope following tetanic stimulation (Figure 6b). Analysis of the changes in the 5 minutes immediately following tetanic stimulation and in the last 5 minutes of the experiment revealed a significant LPS x CD200Fc interaction ($F_{(1,36)} = 15.56$, ***p < 0.001 and $F_{(1,44)} = 114.4$, ***p < 0.001 respectively; 2-way ANOVA; Figure 6c,d). It has been reported that a high dose of LPS (5 mg/kg) decreased CD200R in samples prepared from whole mouse brain {Masocha, 2009 #5704}; since such an effect would affect the interpretation of our findings, it was important to establish whether 100 µg/kg LPS exerted any effect on CD200R in rat hippocampus. Figure 6d indicates that mean CD200R was similar in hippocampus of control-treated and LPS-treated rats.

Discussion

The primary objective of this study was to establish whether CD200Fc might modulate microglial activation in hippocampus of aged rats and to assess whether any change was associated with an improvement in the ability of aged rats to sustain LTP in perforant path-granule cell synapses. The data demonstrate that the age-related microglial activation, which was accompanied by a decrease in CD200, was attenuated by CD200Fc and that LTP in CD200Fc-treated aged rats was similar to that observed in young rats. CD200Fc similarly attenuated the deficit in LTP induced by intraperitoneal injection of LPS and the associated microglial activation.

The interaction of CD200 with its receptor is known to contribute to the maintenance of macrophages and microglia in a quiescent state and, conversely, a deficit in CD200 is associated with activation of these cells (Barclay et al., 2002; Hoek et al., 2000; Shinohara et al., 2005). Here, an age-related decrease in hippocampal expression of CD200 is described and this is accompanied by increased expression of several markers of microglial activation. These findings confirm the previously-reported age-related decrease in CD200 (Lyons et al., 2007a) and suggest that the loss of synapses, indicated by decreased synaptophysin, might contribute to this change. Interestingly, CD200 has been reported to be expressed on presynaptic terminals where co-localization with synaptophysin has been observed (Ojo et al., 2011). There was no evidence of an age-related change in CD200R.

The decrease in CD200 was also associated with evidence of microglial activation as indicated by increased mRNA expression of MHCII, CD40, CD11b and CD68. These changes concur with the findings of previous studies where increased expression of one or more of these markers of microglial activation has been described in the hippocampus of aged, compared with young, rats (Cowley et al., 2010). The data also provide support for previous studies which have highlighted the importance of the interaction between CD200 and CD200R in maintaining microglia in a quiescent state; thus it has been shown that the age-related and Aβ-induced increases in microglial activation are coupled with decreased CD200 expression on neurons (Downer et al., 2009; Downer et al., 2010; Lyons et al., 2007a).

An inflammatory phenotype and/or an exaggerated response to stressors has been consistently described in CD200-deficient mice, relative to wildtype mice. Under resting conditions, spinal cord microglia in these mice adopted an inflammatory morphology expressing more CD11b than wildtype mice (Hoek et al., 2000) and the number of $CD45^{+}CD11b^{+}$ cells prepared from retina of $CD200^{-/-}$ mice was increased compared with their wildtype counterparts (Broderick et al., 2002). Microglial activation was exacerbated in $CD200^{-/-}$ mice following facial nerve transection, and a similar enhanced inflammatory response accompanied increased symptomatology in EAE in these mice (Hoek et al., 2000). Furthermore, the development of experimental autoimmune uveoretinitis was induced more rapidly in $CD200^{-/-}$ mice (Broderick et al., 2002), and this was mimicked by immunization with a blocking CD200R antibody (Banerjee and Dick, 2004). Increased microglial activation has also been described following Toxoplasma encephalitis infection in $CD200^{-/-}$ mice (Deckert et al., 2006).

We observed that expression of iNOS was also increased in hippocampal tissue prepared from aged, compared with young, rats; this is an indicator of classical activation of microglia which is considered to be triggered by IFNγ (Colton, 2009). Although IFNγ was not assessed in this study, an age-related increase in hippocampal concentration of this cytokine has been reported (Downer et al., 2009), perhaps released by the infiltrating natural killer (NK) cells which are present in the brain of aged animals (Lyons et al., 2011). Increased iNOS expression in hippocampus has been reported with age (Gavilan

et al., 2007) and also in brain tissue obtained from mouse models of Alzheimer's Disease (Moreno-Gonzalez et al., 2009; Yin et al., 2011); in these instances it has been linked with evidence of microglial activation and/or deficits in cognitive function (Yin et al., 2011). Like iNOS mRNA, the present data show that expression of MCP-1 and IP-10 were also increased with age. This confirms our recent observations which linked changes in these chemokines with increased infiltration of NK cells into the brain (Lyons et al., 2011). It is also consistent with evidence suggesting that MCP-1 and IP-10 are increased in an age-dependent manner in mouse models of Alzheimer's Disease (Duan et al., 2008; Ruan et al., 2009); interestingly it has been shown that MCP-1 is expressed by activated microglia in this case (Ruan et al., 2009).

The most significant finding presented here is that injection of CD200Fc attenuated the age-related increase in expression of several markers of microglial activation and that this was coupled with improved ability of aged rats to sustain LTP. CD200Fc has also beeen shown to effectively decrease the symptoms of EAE and the associated activation of microglia/macrophages {Liu, 2010 #6061}. The authors of this study highlighted the possibility that the effect on cell activation may be confounded by the finding that CD200Fc treatment was associated with an increase in the number of apoptotic $CD11b^{+}$ cells prepared from spinal cord of mice 30 days after induction of EAE mice. CD200Fc (100 μg/100 μl) was administered subcutaneously for 20 days so that each mouse received a total dose of 1100 μg/mouse; this contrasts sharply with the single intracerebroventricular dose of 10 μg CD200Fc given to mice in the present study. Here, we report that expression of MHCII and CD40, as well as iNOS and the chemokines, were similar in hippocampal tissue prepared from aged CD200Fc-treated rats and young rats; the effect of CD200Fc on these markers was rapid, observed only 3 hours following treatment. The mechanism underlying this remains to be established, but it is known that CD200R activation rapidly initiates a cascade of signalling events, including activation of Dok1 and Dok2 negatively regulating Ras–ERK signaling and microglial/macrophage activation in response to TLR4 activation (Shinohara et al., 2005). However, whether these signalling events are important in modulation of microglial activation by CD200Fc require investigation. Interestingly CD200Fc did not affect the age-related increases in CD68 or CD11b suggesting that its effect is mainly directed at modulating antigen presentation. However it is important to stress that we report changes in mRNA expression and that further analysis is necessary to explore this issue fully. CD68 is a lysosomal protein thought to be upregulated during phagocytosis (Sanchez-Guajardo et al., 2010) while the role of CD11b appears to be more closely coupled with cell motility and chemotaxis than with antigen presentation (Solovjov et al., 2005). In addition to microglia, it must be assumed that perivascular macrophages also express CD200R and that they will also respond to CD200Fc. Similar markers of activation are expressed on microglia and macrophages and therefore it is not possible to attribute the findings described here exclusively to microglia.

Increased expression and/or production of iNOS has been correlated with oxidative changes (Bonomini et al., 2010) and here we show that CD200Fc attenuated the age-related increase in iNOS mRNA in hippocampus. Thus activation of CD200R plays an important role in modulating microglial activation and the associated oxidative changes, supporting the evidence that CD200 acts as a neuroimmune regulatory molecule (Barclay et al., 2002). The modulatory effect of CD200Fc on microglial activation described here is broadly consistent with the evidence that it decreases release of inflammatory cytokines from mast cells, splenocytes and macrophages (Boudakov et al., 2007; Gorczynski et al., 2008; Jenmalm et al., 2006; Zhang et al., 2004). Similarly, CD200Fc has been shown to suppress macrophage and microglial accumulation and activation, and

delay the progression of EAE (Liu et al., 2010) and these findings concur with the current observations. Consistently, the progression of experimental autoimmune uveoretinitis, which is exacerbated in CD200-deficient mice, is delayed an agonist CD200R antibody (Copland et al., 2007), which parallels the action of CD200Fc in EAE {Liu, 2010 #6061} . In addition, CD200Fc ameliorated the inflammatory changes which characterize collagen-induced arthritis {Gorczynski, 2002 #242;Gorczynski, 2001 #241;Simelyte, 2008 #5708}. It also decreased inflammatory lung disease and tissue damage in influenza-infected mice without affecting viral clearance (Snelgrove et al., 2008).

The age-related increase in microglial activation described here was accompanied by a decrease in LTP which concurs with earlier reports (Cowley et al., 2010; Lynch, 2010). Importantly this deficit was attenuated by CD200Fc adding to the accumulated data which suggest that if microglial activation can be decreased in hippocampus of aged animals, then some restoration of LTP can occur (Cowley et al., 2010). The data are also consistent with our recent finding that LTP is markedly decreased in hippocampal slices prepared from $CD200^{-/-}$ mice compared with wildtype mice (Costello et al., 2011)..

To consolidate the finding that CD200Fc was capable of affecting LTP in circumstances in which microglial activation occurs, we also investigated its effect on LPS-induced impairment in LTP. As previously reported (Barry et al., 2005; Nolan et al., 2005), LPS robustly inhibited LTP and this was accompanied by an increase in MHCII mRNA reflecting the activation of microglia which has been shown before (Clarke et al., 2008; Hauss-Wegrzyniak et al., 2002; Rosi et al., 2006). The significant finding here is that CD200Fc attenuated both the LPS-induced decrease in LTP and the increase in MHCII mRNA, consolidating the findings observed in aged rats. Although a decrease in expression of CD200R has been reported in mouse brain following injection of a high concentration of LPS {Masocha, 2009 #5704}, no change was observed in the present study where a lower concentration of LPS was used. Overall, the present data associate the activation of CD200R with a decrease in microglial activation state and suggest that maintaining microglia in a quiescent state by receptor activation contributes to the preservation of LTP. Thus CD200R activation may provide an important therapeutic approach for modulating inflammatory changes in central nervous system, one consequence of which may be to counteract the impairment of function which is the hallmark of neurodegenerative conditions.

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Figure Legends

Figure 1. Expression of synaptophysin and CD200 protein in hippocampus, but not CD200R protein, is decreased with age.

Synaptophysin (a) and CD200 (b) were decreased in hippocampal tissue prepared from aged, compared with young, rats (*$p < 0.05$; student's t-test for independent means; n=5 or 6). No age-related change in CD200R was observed. The data are mean values obtained from densitometric analysis and are expressed as a ratio of the proteins to β-actin.

Figure 2. CD200Fc attenuates the age-related increase in MHCII mRNA.

(a) A significant age x treatment interaction was observed for MHCII mRNA ($F_{(1,20)}$, *$p < 0.05$; 2-way ANOVA). (b) MHCII immunoreactivity was markedly increased in hippocampal sections prepared from aged (iii), compared with young (i), rats. Immunoreactivity was similar in CD200Fc-treated and control-treated young rats (compare (i) and (ii)) but was reduced in CD200Fc-treated compared with control-treated aged rats (compare (iii) and (iv)). Images are representative of at least 3 sections obtained from each of the rats in the 4 treatment groups. (c) Analysis of $MHCII^+$ cell numbers (counted in 3 sections from each of the 5 or 6 animals in each treatment group) revealed a significant interaction of aging and CD200Fc treatment ($F_{(1,20)} = 4.86$, *$p < 0.05$; 2-way ANOVA).

Figure 3. CD200Fc attenuates the age-related increases in expression of CD40 and iNOS but not CD11b and CD68.

Age-related increases in CD40 (a), CD11b (b), CD68 (c) and iNOS (d) mRNA in hippocampal tissue prepared from aged, compared with young, control-treated, rats were observed. A significant age x treatment interaction was observed in CD40 mRNA ($F_{(1,16)} = 19.42$, ***$p < 0.001$; 2-way ANOVA) and iNOS ($F_{(1,19)} = 5.50$, *$p < 0.05$; 2-way ANOVA), whereas a significant age effect was observed for CD11b ($F_{(1,19)} = 16.88$, $^{§§§}p < 0.001$) and CD68 ($F_{(1,20)} = 28.7$, $^{§§§}p < 0.001$; 2-way ANOVA).

Figure 4. CD200Fc attenuates the age-related increase in expression in IP-10 mRNA.
Expression of MCP-1 mRNA (a) and IP-10 mRNA was assessed in hippocampal tissue prepared from control- and CD200Fc-treated young and aged rats. A significant age x treatment interaction was observed for both MCP-1 ($F_{(1,21)}$ = 11.32, **p < 0.01; 2-way ANOVA) and IP-10 ($F_{(1,21)}$ = 7.93, *p < 0.05; 2-way ANOVA).

Figure 5. CD200Fc attenuates the age-related decrease in LTP in dentate gyrus.
(a) Delivery of a high frequency train of stimuli to the perforant path (at time 0) induced an immediate and sustained increase in EPSP slope in control-treated young rats and this effect was markedly decreased in control-treated aged rats. CD200Fc exerted no effect on LTP in young rats but aged rats treated with CD200Fc sustained LTP in a manner similar to young animals. (b,c) The mean changes in EPSP slope in the 5 minutes immediately following tetanic stimulation (b) and in the last 5 minutes of the experiment (c) revealed a significant age x CD200Fc treatment interaction ($F_{(1,36)}$ = 77.23, p < 0.001 and $F_{(1,44)}$ = 955.6, p < 0.001; 2-way ANOVA).

Figure 6. CD200Fc decreased the LPS-induced changes in MHCII mRNA in hippocampus and the LPS-induced decrease in LTP.
(a) MHCII mRNA was increased in hippocampal tissue prepared from LPS-treated, compared with control-treated, rats; a significant LPS x CD200Fc interaction was observed ($F_{(1,20)}$ = 7.41, *p < 0.05; 2-way ANOVA). (b-d) LPS markedly decreased LTP in perforant path-granule synapses (b) and analysis of the mean changes in EPSP slope in the 5 minutes immediately following tetanic stimulation (c) and in the last 5 minutes of the experiment (d) revealed a significant LPS x CD200Fc interaction ($F_{(1,36)}$ = 15.56, ***p < 0.001 and $F_{(1,44)}$ = 114.4, ***p < 0.001 respectively; 2-way ANOVA). (e) CD200R was similar in hippocampal tissue prepared from control- and LPS-treated rats.

Figure 1

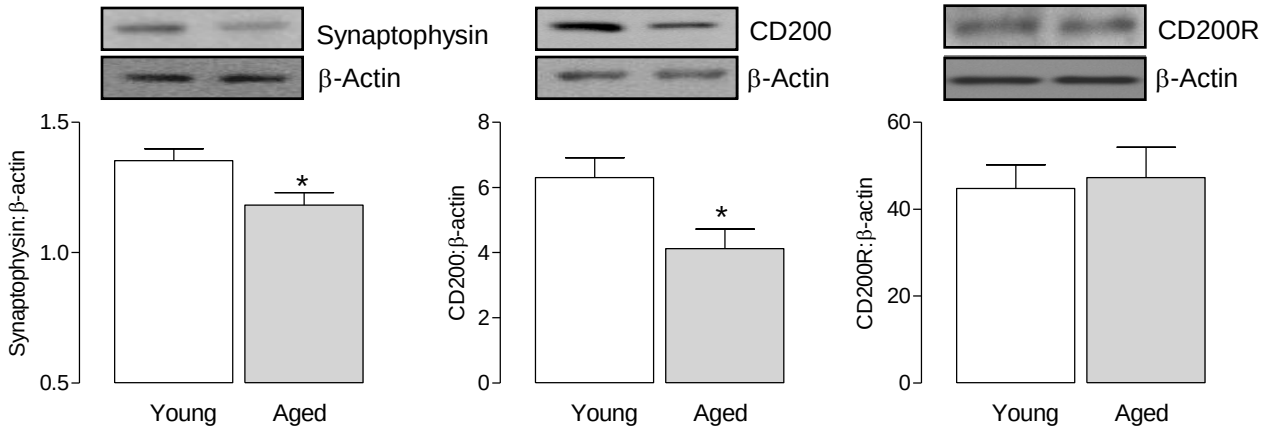

Synaptophysin
β-Actin
CD200
β-Actin
CD200R
β-Actin
Synaptophysin:β-actin
1.5
1.0
0.5
*
Young
Aged
CD200:β-actin
8
6
4
2
0
*
Young
Aged
CD200R:β-actin
60
40
20
0
Young
Aged

Figure 2

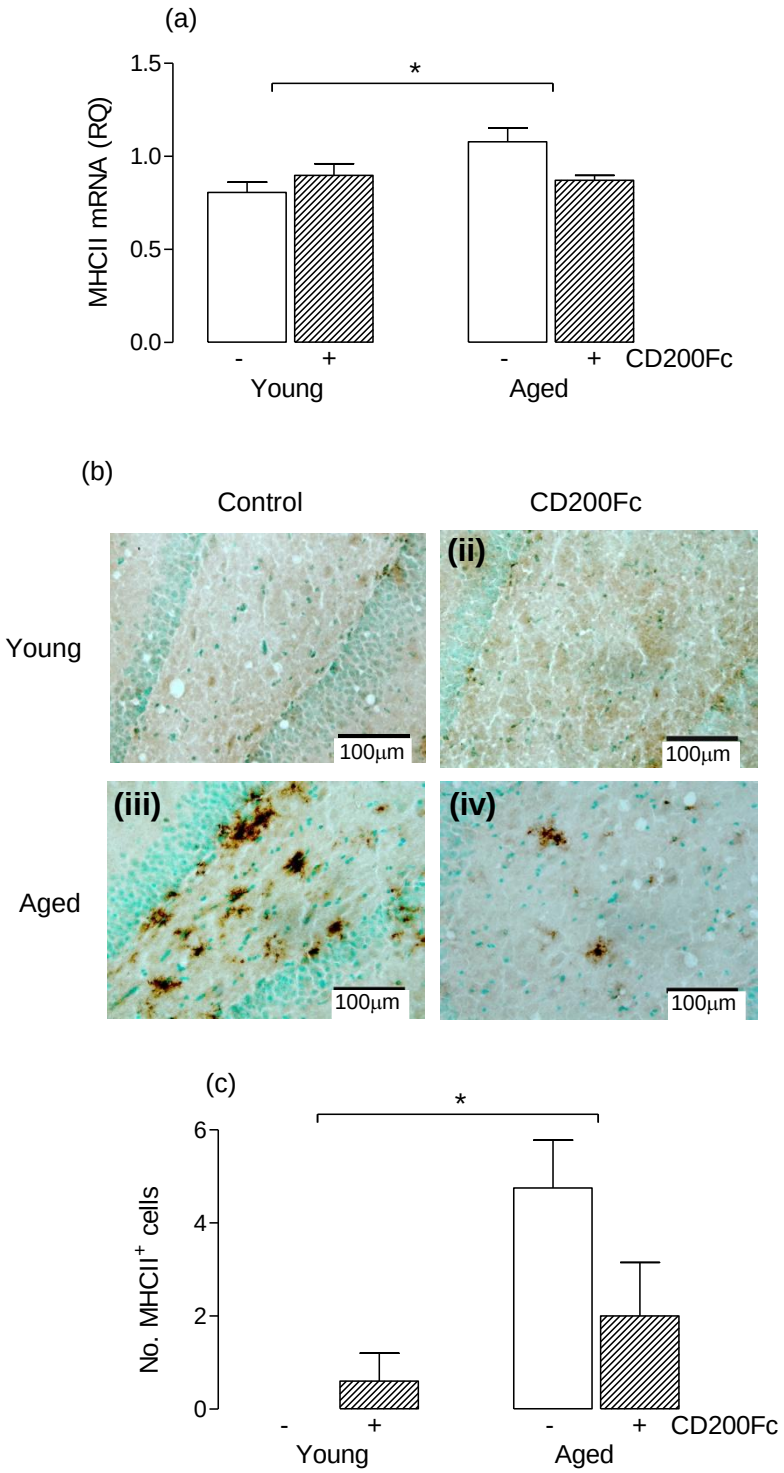

(a)
MHCII mRNA (RQ)
1.5
1.0
0.5
0.0
*
-
+
-
+
CD200Fc
Young
Aged
(b)
Control
CD200Fc
Young
Aged
(ii)
(iii)
(iv)
100μm
(c)
No. MHCII+ cells
6
4
2
0
*
-
+
-
+
CD200Fc
Young
Aged

Figure 3

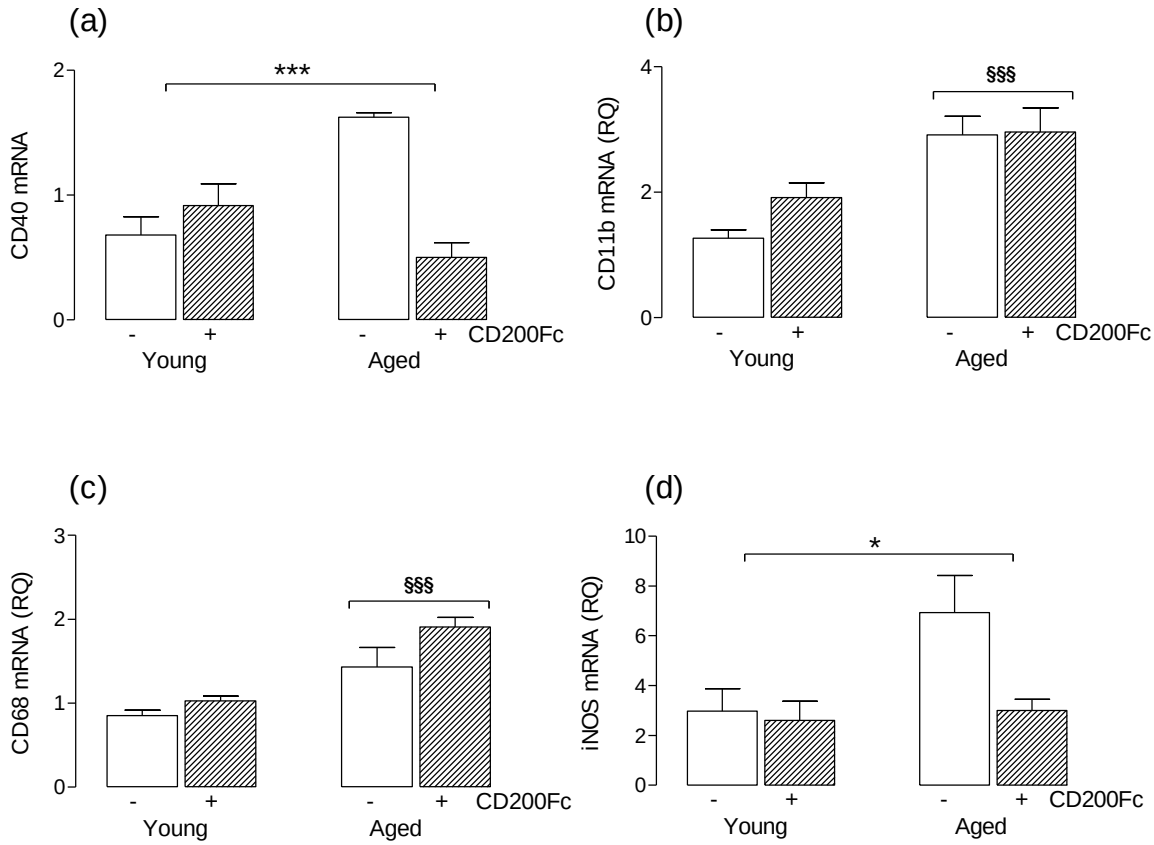

(a)
CD40 mRNA
2
1
0
***
- + - + CD200Fc
Young
Aged
(b)
CD11b mRNA (RQ)
4
2
0
§§§
- + - + CD200Fc
Young
Aged
(c)
CD68 mRNA (RQ)
3
2
1
0
§§§
- + - + CD200Fc
Young
Aged
(d)
iNOS mRNA (RQ)
10
8
6
4
2
0
*
- + - + CD200Fc
Young
Aged

Figure 4

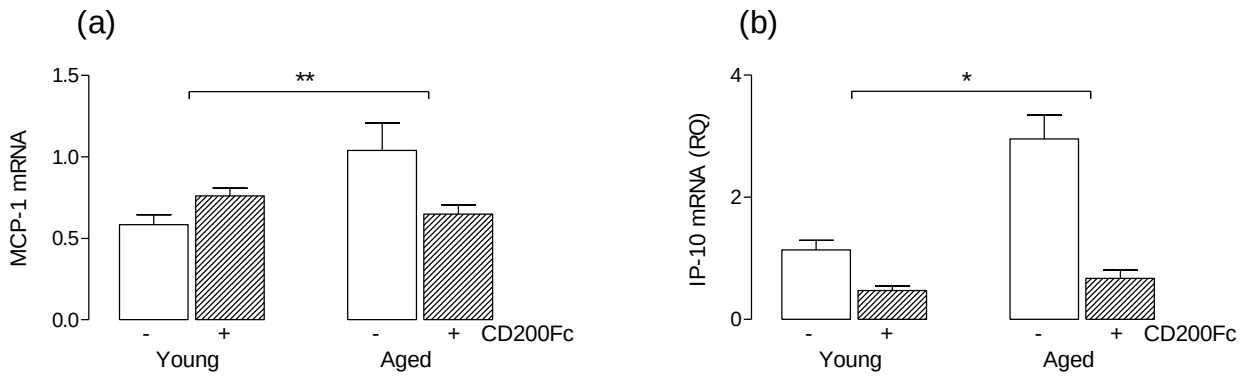

(a)
MCP-1 mRNA
1.5
1.0
0.5
0.0
**
- + - + CD200Fc
Young
Aged
(b)
IP-10 mRNA (RQ)
4
2
0
*
- + - + CD200Fc
Young
Aged

Figure 5

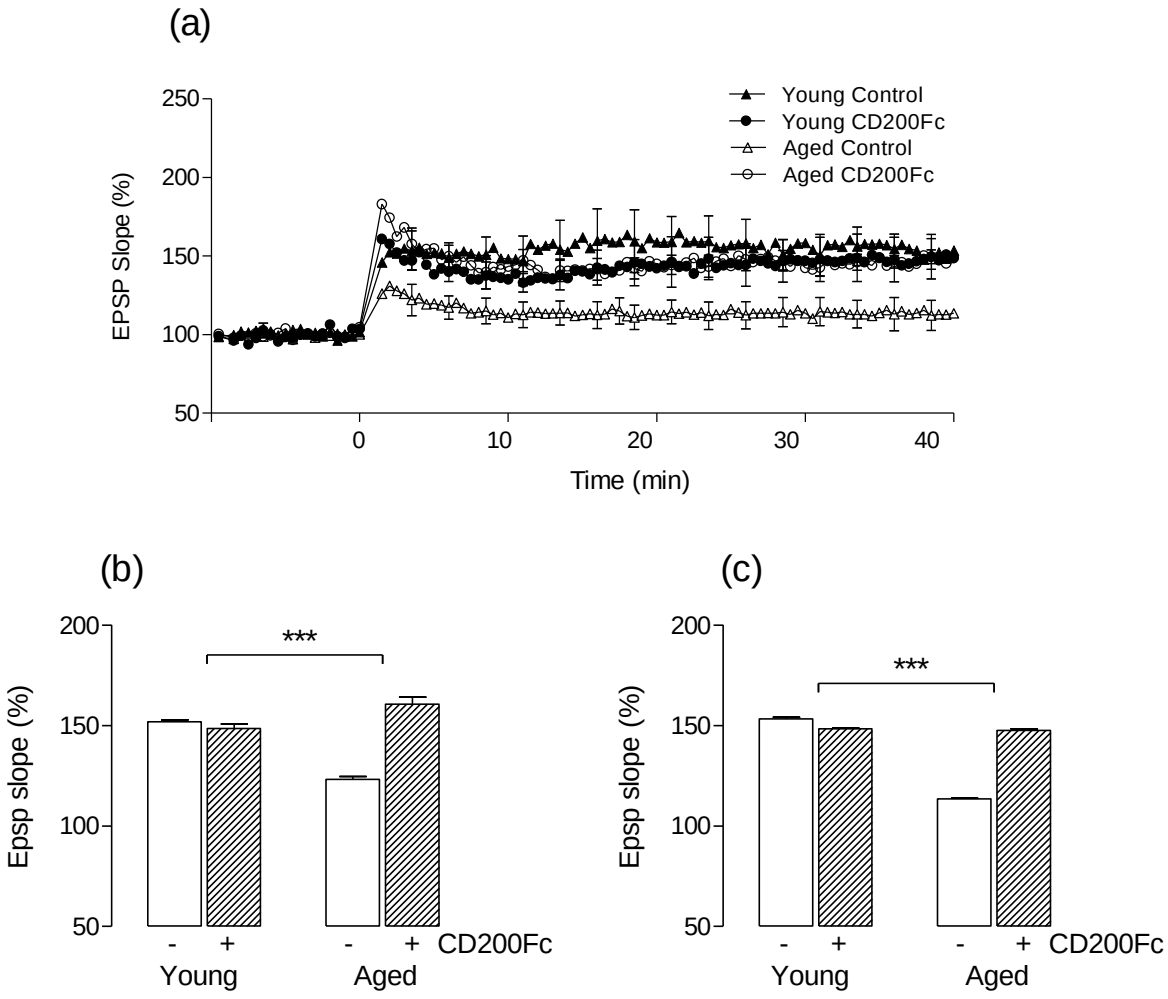
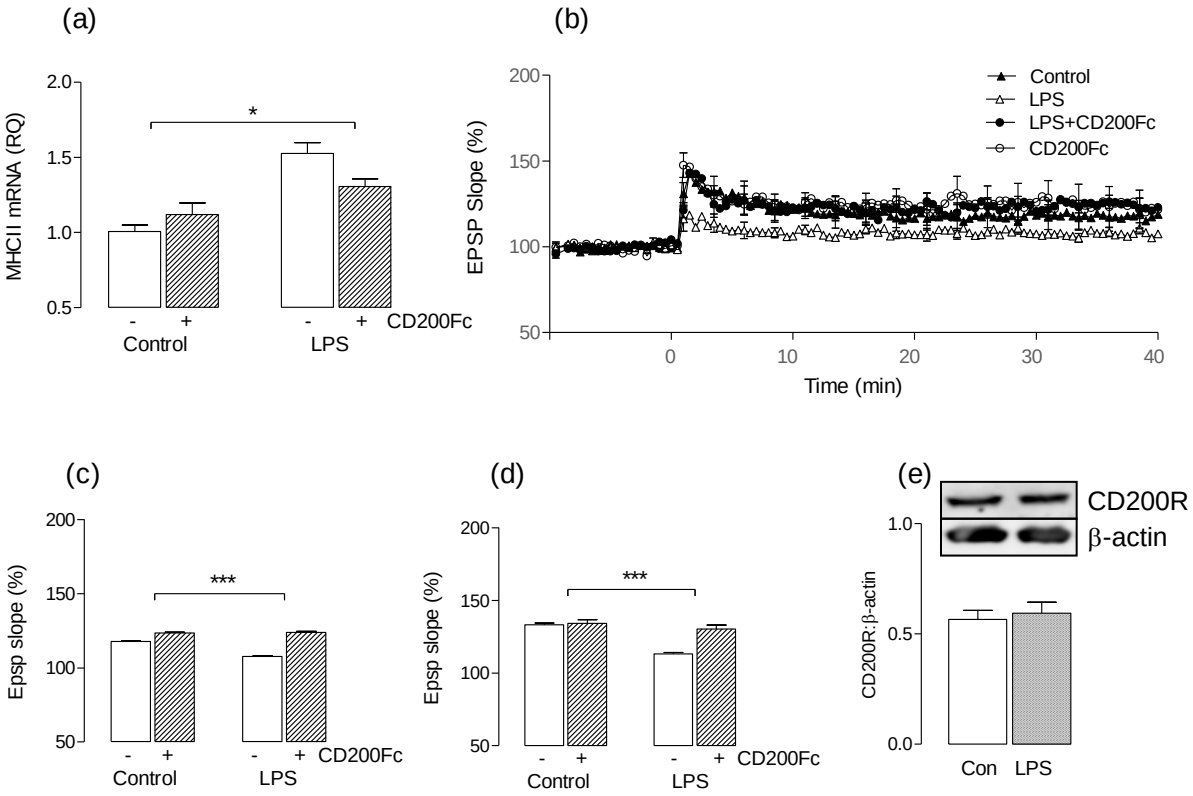

(a)
Young Control
Young CD200Fc
Aged Control
Aged CD200Fc
EPSP Slope (%)
250
200
150
100
50
0
10
20
30
40
Time (min)
(b)
Epsp slope (%)
200
150
100
50
***
- + - + CD200Fc
Young
Aged
(c)
Epsp slope (%)
200
150
100
50
***
- + - + CD200Fc
Young
Aged

Figure 6


(a)
MHCII mRNA (RQ)
2.0
1.5
1.0
0.5
*
- + - + CD200Fc
Control
LPS
(b)
EPSP Slope (%)
200
150
100
50
Control
LPS
LPS+CD200Fc
CD200Fc
0
10
20
30
40
Time (min)
(c)
Epsp slope (%)
200
150
100
50
***
- + - + CD200Fc
Control
LPS
(d)
Epsp slope (%)
200
150
100
50
***
- + - + CD200Fc
Control
LPS
(e)
CD200R
β-actin
CD200R:β-actin
1.0
0.5
0.0
Con
LPS

CD200Fc attenuates age-related changes in microglial activation and LTP suggesting that CD200R activation is key to modulating neuroinflammation and synaptic plasticity.