

Long-term potentiation is impaired in CD200-deficient mice: a role for Toll-like receptor activation.

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Running title: Increased TLR enhances susceptibility in CD200^{-/-} mice

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ABSTRACT

The membrane glycoprotein CD200 is expressed on several cell types including neurons whereas expression of its receptor, CD200R, is restricted principally to cells of the myeloid lineage, including microglia. The interaction between CD200 and CD200R maintains microglia and macrophages in a quiescent state, therefore CD200-deficient mice express an inflammatory phenotype exhibiting increased macrophage or microglial activation in models of arthritis, encephalitis and uveoretinitis. Here, we report that lipopolysaccharide (LPS) and Pam₃CysSerLys₄ (Pam₃Csk₄) exerted more profound effects on release of the proinflammatory cytokines, interleukin (IL)-1 β , IL-6 and tumour necrosis factor (TNF)- α in glia prepared from CD200^{-/-}, compared with wildtype, mice. This effect is explained by the loss of CD200 on astrocytes which modulates microglial activation. Expression of Toll-like receptors (TLR)-4 and -2 was increased in glia prepared from CD200^{-/-} mice and the evidence indicates that microglial activation, assessed by the increased numbers of CD11b⁺ cells which stained positively for both MHCII and CD40, was enhanced in CD200^{-/-}, compared with wildtype, mice. These neuroinflammatory changes were associated with impaired long-term potentiation (LTP) in CA1 of hippocampal slices prepared from CD200^{-/-} mice. One possible explanation for this is the increase in TNF α in hippocampal tissue prepared from CD200^{-/-} mice, since TNF α application inhibited LTP in CA1. Significantly, LPS and Pam₃Csk₄, at concentrations which did not affect LTP in wildtype mice, inhibited LTP in slices prepared from CD200^{-/-} mice, likely due to the accompanying increase in TLR2 and TLR4. Thus the neuroinflammatory changes which result from CD200 deficiency have a negative impact on synaptic plasticity.

INTRODUCTION

CD200 is a type-1 membrane glycoprotein which has been identified as an immunosuppressive molecule, consistent with its expression on cells of the immune system including dendritic cells, T and B cells, endothelial and epithelial cells (1). Diverse immuno-

modulatory roles for CD200 have been reported; these include antigen-specific T cell responses, suppression of regulatory T cells (2), cytotoxic T cell-mediated tumour suppression (3), graft survival (4) and apoptosis-associated immune tolerance (5).

In the brain, CD200 is expressed on neurons (6) and oligodendrocytes (7) but not on microglia (8). A recent report has indicated that CD200 is expressed on reactive astrocytes in lesions from postmortem multiple sclerosis brains (7). Expression of CD200R is mainly restricted to cells of the myeloid lineage and therefore, in the brain, has been identified on microglia (6,7) but not on neurons (8). The complementary expression of ligand and receptor on neurons and microglia respectively suggests that their interaction may play a role in modulating microglial activation and recent evidence has supported this contention. The LPS-induced increases in expression of cell surface markers of microglial activation and inflammatory cytokine production were inhibited by addition of neurons and this attenuating effect of neurons was blocked by an anti-CD200 antibody (8). This finding suggests that interaction of CD200 with its receptor has the capacity to modulate microglial activation.

In CD200-deficient mice, increased microglial and/or macrophage activation has been described in several models of inflammation, for example facial nerve transection, experimental autoimmune encephalomyelitis (EAE), an animal model of arthritis (9) and experimental autoimmune uveoretinitis (10). Conversely, administration of a CD200 fusion protein ameliorates the inflammatory changes observed in collagen-induced arthritis (11,12), while the decrease in EAE-like symptoms in *Wld^s* mice has been attributed to increased expression of CD200 on spinal cord neurons (13).

Reduced expression of CD200 is coupled with increased microglial activation in hippocampus of aged and β -amyloid (A β)-treated rats (8,14), and synaptic plasticity, specifically long-term potentiation (LTP), is impaired when microglial activation is increased (15,16). Therefore we predicted that glia prepared from CD200-deficient mice would respond more profoundly to LPS and that this would be coupled with evidence of impaired LTP. The data show that LPS and

Pam₃Csk₄ exert a greater effect on glia prepared from CD200^{-/-} mice, presumably due to the observed increase in expression of TLR4 and TLR2 on these cells. In addition, LTP was markedly reduced at CA1 synapses of hippocampal slices prepared from CD200^{-/-}, compared with wildtype, mice. LPS and Pam₃Csk₄ further attenuated LTP in slices prepared from CD200^{-/-} mice. The data provide further evidence for an important immunomodulatory role for CD200, and couples the loss of CD200 with a deficit in synaptic function and with increased expression of TLR 2 and 4.

EXPERIMENTAL PROCEDURES

Animals

1 day-old and 2-6 month-old C57BL/6 or CD200^{-/-} mice were used for preparation of glial cultures or for preparation of hippocampal slices respectively. Tissue from 2-6 month-old mice was also used for analysis of expression of TLR2 and 4 and HMGB1. All experiments were performed under licence (Department of Health and Children, Ireland) and with ethical approval (BioResources, Trinity College, Dublin) in accordance with local guidelines. Animals were housed under controlled conditions (20-22°C, food and water *ad lib*) and maintained under veterinary supervision.

Preparation and treatment of primary glial cultures.

Mixed glial cultures were prepared from 1-day-old C57BL/6 mice or CD200^{-/-} mice as previously described (8). These cultures contained approximately 70% astrocytes and 30% microglia as assessed by expression of CD11b using FACS. We used mixed glia because CD200 is expressed on astrocytes, but not microglia. This means that knocking out CD200 will have no impact on microglia unless they are in culture with astrocytes and, in this case, the effect can be attributed to the loss of signaling through CD200R. In the context of this study, isolated microglia prepared from wildtype and CD200^{-/-} are essentially the same.

In one series of experiments, cells were harvested for flow cytometric analysis to evaluate expression of cell surface markers of microglial activation, GLAST to identify astrocytes, or for PCR to evaluate expression of TLRs 2 and 4. In a second series of experiments, cells were incubated in the presence or absence of LPS (100ng/ml;

Alexis Biochemical; US) or Pam₃Csk₄ (100ng/ml; InvivoGen, US) and, 24h later, supernatant was collected and assessed for concentration of IL-1 β , IL-6 and TNF α .

Purified astrocytes were prepared as described previously, using the shaking method to remove microglia (17), and membranes were isolated using a subcellular protein fractionation kit (Thermo Scientific, USA). Cells were incubated in trypsin-EDTA (1ml, 15 min, 37°C), centrifuged (500 x g, 5 min), washed with ice-cold PBS, resuspended in PBS and centrifuged (500 x g, 5 min). The pellet was re-suspended in ice-cold Cytoplasmic Extraction Buffer containing protease inhibitors (Thermo Scientific, USA), incubated (4°C, 10 min) and centrifuged (3,000 x g, 5 min); the supernatant provided the cytosolic fraction, while the pellet, which contained the membrane fraction, was resuspended in ice-cold Membrane Extraction Buffer containing protease inhibitors (Thermo Scientific, USA), incubated (4°C, 10 min) and centrifuged (3,000 x g, 5 min). The resultant supernatant provided the membrane fraction.

To prepare microglia, cells were initially seeded onto 25cm² flasks and, after 24 hours, media was replaced with cDMEM containing GM-CSF (20ng/ml) and M-CSF (5ng/ml). After 10-14 days in culture, non-adherent microglia were harvested by shaking (110rpm, 2 hours, room temperature), tapping and centrifuging (2000rpm, 5 min). The pellet was resuspended in cDMEM, the microglia were plated onto 24-well plates at a density of 1 x 10⁵ cells/ml and maintained at 37°C in a 5% CO₂ humidified atmosphere for up to 3 days prior to treatment.

Flow cytometry.

Glial cells were trypsinized (0.25% Trypsin-EDTA, Sigma, UK), washed 3 times in FACS buffer (2% FBS, 0.1% NaN₃ in PBS). Whole brain tissue was harvested and passed through a cell strainer (70 μ m) and centrifuged (170 x g, 10 min). The pellet was resuspended in PBS containing collagenase D (1mg/ml) and DNase I (200 μ g/ml), incubated at 37°C for 30 min and centrifuged (170 x g, 5 min). Pellets were resuspended in 1.088g/ml Percoll (9ml), underlaid with 1.122g/ml Percoll (5ml), and overlaid with 1.072g/ml and 1.030g/ml (9ml each) Percoll and PBS (9ml) and centrifuged

(1250 x g, 45 min). The mononuclear cells (between the 1.088:1.072 and 1.072:1.030) were centrifuged and the pellets were washed. All cells were blocked for 15 min at room temperature in FACS block (Mouse BD Fc Block (BD Pharmingen, UK); 1:500 in FACS buffer). Cells were incubated with PE-Cy7- or APC-rat anti-mouse CD11b (BD Biosciences, UK), FITC-rat anti-mouse CD40 (BD Biosciences, UK), PE-rat anti-mouse MHCII (BD Biosciences, UK), APC-rat anti-mouse CD200 (BD Biosciences, UK), PE-rat anti-mouse CD200R (Serotec, UK), FITC-rat anti-mouse TLR2 (Cambridge Biosciences, UK), FITC-rat anti-mouse TLR4 (Cambridge Biosciences, UK), PE-Cy7-anti-mouse CD45 (BD Biosciences, UK) and APC-rat anti-mouse GLAST (BD Biosciences, UK). Antibodies were diluted (1:400) in FACS buffer. Immunofluorescence analysis was performed on a DAKO Cyan ADP 7 colour flow cytometer (DAKO Cytomation, UK) with Summit v4.3 software.

Real-time PCR analysis of CD11b, CD40, TLR2 and TLR4.

Total RNA was extracted from snap-frozen hippocampal tissue and harvested mixed glial cells using a NucleoSpin® RNAII isolation kit (Macherey-Nagel Inc., Germany) and cDNA synthesis was performed on 1 µg total RNA using a High Capacity cDNA RT kit (Applied Biosystems, Germany); the protocols used were according to the manufacturer's instructions. Real-time PCR was performed as described previously (8) using an ABI Prism 7300 instrument (Applied Biosystems, Germany). The primers IDs were as follows CD11b: Mm01271265_m1, CD40: Mm00441895_m1, TLR2: Mm00442346_m1 and TLR4: Mm00445273_m1 (Applied Biosystems, Germany). Samples were assayed in duplicate and gene expression was calculated relative to the endogenous control samples (β-actin) to give an RQ value (2^{-DDCt} , where CT is the threshold cycle).

Analysis of IL-1β, IL-6 and TNFα.

The concentrations of IL-1β, IL-6 and TNFα were analyzed in triplicate by ELISA in samples of supernatant obtained from *in vitro* experiments as previously described (8).

Analysis of CD200, GFAP, pIkBα, IL-1α, IL-1β and TNFα by Western immunoblotting

Hippocampal lysate was assessed for expression of IL-1α, IL-1β and TNFα, glial cell lysate was evaluated for expression of pIkBα, and membrane and cytosolic preparations obtained from purified astrocytes were evaluated for expression of CD200 and GFAP using standard Western immunoblotting methods (8,18). Primary antibodies directed against CD200 (anti-goat; 1:500; Santa Cruz Biotechnology, US), GFAP (anti-rabbit; 1:1000; Invitrogen, US), pIkBα (ser32) (anti-rabbit; 1:1000; Cell Signaling, US), IL-1α (anti-goat; 1:1000; R & D Systems, US), IL-1β (anti-goat; 1:500; Santa Cruz Biotechnology, US) and TNFα (anti-rabbit; 1:500; Cell Signaling, US) were incubated overnight at 4°C. The secondary antibodies were conjugated to horseradish peroxidase (1:5000; Jackson ImmunoResearch, US) and after a 2 hour incubation step, membranes were washed and protein complexes visualised (Immobilon Western chemiluminescent substrate, Millipore, US). Membrane were stripped and probed for β-actin (to confirm equal loading) as previously described (8,18). Images were captured using the Fujifilm LAS-3000 imager, and densitometric analysis was used to quantify expression of the proteins. Values are presented as mean ± SEM, normalised to β-actin.

Hippocampal slice preparation and LTP recording

Acute hippocampal slices (400µm) from C57BL/6 and CD200^{-/-} mice were prepared using a McIlwain tissue chopper as previously described (18), and maintained in oxygenated artificial cerebrospinal fluid (aCSF; composition in mM: 125 NaCl, 1.25 KCl, 2 CaCl₂, 1.5 MgCl₂, 1.25 KH₂PO₄, 25 NaHCO₃, and 10 D-glucose), at room temperature (21-23°C) in a holding chamber for a minimum of 1 hour before being transferred to a submersion recording chamber. Slices were continuously perfused (2-3ml/min) with oxygenated aCSF at room temperature (21-23°C). The Schaffer collateral-commissural pathway was stimulated at 0.033Hz (0.1ms duration; 30-50% of maximal EPSP amplitude) using a bipolar tungsten stimulation electrode (Advent Materials). Stable

field excitatory postsynaptic potentials (EPSPs) were recorded from the CA1 stratum radiatum using a monopolar glass recording electrode filled with aCSF. We assessed input-output response and paired pulse facilitation to evaluate neurotransmission at CA1 synapses, and found that there were no differences between slices obtained from wildtype and CD200^{-/-} mice. In addition there was no evidence of epileptiform activity in any slice. Stable baseline EPSPs were recorded for 20 min prior to application of theta-burst stimulation [TBS; 10 trains (4 pulses at 100 Hz) repeated at 5 Hz, (18)]. In some experiments, LPS (Alexis Biochemicals, Switzerland) or Pam₃Csk₄ (InvivoGen, US) was added to the perfusate (10 µg/ml x 20 min, or 20 µg/ml x 60 min) prior to TBS. In an additional set of experiments, slices were perfused with mouse recombinant TNFα (R & D Systems, UK; 3 ng/ml in 0.002% BSA) or vehicle alone (0.002% BSA), for 20 min prior to LTP induction. This concentration of TNFα is less than that previously demonstrated to impair LTP in hippocampal slices prepared from rat (19,20). Data were acquired using WinWCP v4.0.7 software (Dr J. Dempster, Strathclyde, UK). Evoked EPSPs were normalised to the slope recorded in the 5 min period prior to LTP induction. The level of LTP was evaluated as the mean % EPSP slope during the last 5 min of recording and data are presented as mean % EPSP slope ± SEM.

Hippocampal slices not used for electrophysiology were prepared for western immunoblot analysis of IL-1α, IL-1β and TNFα expression (described above). These slices were incubated as described for a minimum of 1 hour following the slicing procedure, plus a further incubation period equivalent to the duration of LTP recording.

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 with standard errors.

RESULTS

Loss of CD200 has been associated with evidence of increased inflammatory changes in hippocampal tissue prepared from aged animals as well as LPS- and Aβ-treated animals and LPS (8,21). In this study, the effect of the TLR4 and TLR2 agonists, LPS and Pam₃Csk₄, were assessed on cytokine production in mixed glia prepared from wildtype and CD200^{-/-} mice. The data indicate that incubation of cells prepared from wildtype mice in the presence of LPS (100 ng/ml) increased release of the proinflammatory cytokines, IL-1β, IL-6 and TNFα and the effect was significant in the case of IL-6 and TNFα (**p < 0.001; ANOVA; Figure 1a-c). The effect of LPS was significantly greater in cells prepared from CD200^{-/-} mice (⁺⁺⁺p < 0.001; ANOVA; Figure 1). Incubation of mixed glia prepared from wildtype mice in the presence of Pam₃Csk₄ (100 ng/ml) also significantly increased release of inflammatory cytokines (**p < 0.01; ⁺⁺⁺p < 0.001; ANOVA; Figure 1d-f). The effect of Pam₃Csk₄ was greater in mixed glia prepared from CD200^{-/-} mice and this was statistically significant in the case of IL-6 and TNFα (⁺⁺⁺p < 0.001; wildtype vs CD200^{-/-}; ANOVA). Both LPS and Pam₃Csk₄ also increased mRNA expression of these inflammatory cytokines and the effect was greater in cells prepared from CD200^{-/-} mice (data not shown). These data indicate that tonic activation by CD200 modulates cytokine release from glia. Analysis of the effect of LPS on cytokine release prepared from purified microglia obtained from wildtype and CD200^{-/-} mice revealed no genotype-related change for IL-1β (99.64 ± 26.52 pg/ml vs 62.89 ± 6.47 pg/ml for wildtype and CD200^{-/-} cells respectively), IL-6 (3837 ± 171.8 vs 3875 ± 144.8) and TNFα (1559 ± 88.31 vs 1533 ± 204.5). This is consistent with the view that isolated microglia prepared from CD200^{-/-} mice are unaffected, whereas when cultured with astrocytes which are deficient in CD200, an activated phenotype is evident.

Using CD11b as a marker of microglia, we show that the number of CD11b⁺MHCII⁺ cells and CD11b⁺ CD40⁺ cells was increased in a mixed glial population prepared from CD200^{-/-}, compared with wildtype, mice (Figure 2). These data suggest that CD200 contributes to maintenance of microglia (in a mixed glial preparation) in a quiescent state and therefore suggests that CD200 is expressed on astrocytes. To date, its expression

on astrocytes has been reported only on reactive astrocytes in lesions from postmortem brains of individuals with multiple sclerosis (7). Here, flow cytometry was used to evaluate CD200 expression on GLAST⁺ cells in a purified culture of astrocytes prepared from wildtype and CD200^{-/-} mice (Figure 3a,b). Whereas CD200 expression was evident on GLAST⁺ cells prepared from wildtype mice, expression was absent on GLAST⁺ cells prepared from CD200^{-/-} mice. To confirm astrocytic expression of CD200, purified astrocytes were used to prepare membrane and cytosolic fractions for analysis by Western immunoblotting. CD200 was evident in membrane, but not cytosolic, fractions (Figure 3c) whereas GFAP expression was, predictably, largely confined to the cytosolic fractions.

A possible explanation for the increase in responsiveness of cells from CD200^{-/-} mice to LPS and Pam₃Csk₄ is the significant increase in expression of both TLR2 and TLR4 mRNA in mixed glia prepared from CD200^{-/-}, compared with wildtype, mice (*p < 0.05; student's t-test for independent means; Figure 4a and d). Flow cytometric analysis demonstrated that cell surface expression of both receptors was increased on CD11b⁺ cells obtained from CD200^{-/-}, compared with wildtype, mice, but the increase was significant only in the case of TLR2 (**p < 0.01; student's t-test for independent means; Figure 4b,c,e,f). The significant increase in phosphorylated IκBα in cells prepared from CD200^{-/-}, compared with wildtype, mice (*p < 0.05; student's t-test for independent means; Figure 4g) indicates that signalling through TLR is upregulated in cells prepared from CD200^{-/-} mice.

CD200 deficiency is accompanied by inflammatory changes (9,10) and, in the brain, microglial activation is coupled with decreased CD200 in brains of aged animals and also in LPS-treated and Aβ-treated animals (8,21). To investigate this correlation further, we evaluated expression of surface markers of microglial activation on cells prepared from CD200^{-/-} and wildtype mice using PCR and flow cytometry, and show that CD40 mRNA, although not CD11b mRNA, was significantly increased in tissue prepared from CD200^{-/-}, compared with wildtype, mice (*p < 0.05; student's t-test for independent means; Figure 5a,b). Analysis by flow cytometry indicated that there was no genotype-related

change in CD11b⁺ cells (Figure 5c) but the percentage of CD11b⁺ cells which were positive for MHCII and CD40 was significantly increased (*p < 0.05; ***p < 0.001; student's t-test for independent means; Figure 5d-g).

CD45 has been used as a means of discriminating between macrophages (which express high levels of CD45) and microglial (which express low levels of CD45 (22)). Flow cytometric analysis revealed that the numbers of CD11b⁺ CD45^{low} cells were significantly increased in hippocampus of CD200^{-/-}, compared with wildtype, mice (***p < 0.001; student's t-test for independent means; Figure 6a) and that CD200R expression (b), CD40 (c), TLR2 (d) and TLR4 (f) on these cells was greater in tissue prepared from CD200^{-/-}, compared with wildtype, mice. The numbers of macrophages in the brain (i.e. CD11b⁺ CD45^{high} cells) were negligible in CD200^{-/-} and wildtype mice. Analysis of expression of TLR in hippocampus revealed that both TLR2 and 4 were increased in CD200^{-/-}, compared with wildtype, mice (*p < 0.05; **p < 0.01; student's t-test for independent means; Figure 6e,g). These changes indicate that microglial activation occurs in brain tissue of CD200^{-/-} mice and therefore the changes *in vitro* are reflected *in vivo*, although the increase in expression of TLR2 mRNA in hippocampus is markedly greater than the change observed in cultured cells. Significantly, this was accompanied by a deficit in LTP in CA1 synapses where the response, 60 min following application of TBS, was markedly reduced in slices prepared from CD200^{-/-} mice (12 slices from 7 mice) compared with wildtype mice (15 slices from 11 mice; p < 0.001; unpaired Student's t-test; Figure 6h). Although a number of inflammatory cytokines released from activated microglia might exert this effect (17-21), here we show that whereas expression of IL-1α and IL-1β were similar in hippocampal tissue prepared from wildtype and CD200^{-/-} mice (Figure 7a,b), TNFα was increased (p < 0.05; student's t-test for independent means; Figure 7c). As previously demonstrated in hippocampal slices prepared from rats (19,20,23), application of TNFα (3 ng/ml) to mouse hippocampal slices significantly impaired LTP relative to vehicle controls (p < 0.05; unpaired Student's t-test; 3 slices from 2 mice; Figure 7d).

Since cells prepared from CD200^{-/-} mice showed increased susceptibility to LPS, we

predicted that concentrations of LPS which exerted no effect on LTP in wildtype mice may attenuate it in CD200^{-/-} mice. Application of LPS (20 µg/ml) to hippocampal slices from wildtype mice for 60 min prior to TBS inhibited LTP (5 slices from 5 mice) compared with controls (15 slices from 11 mice; $p < 0.001$; Figure 8a). In contrast, a lower concentration of LPS (10 µg/ml; 20 min pre-treatment) which exerted no effect on LTP in slices prepared from wildtype mice (7 slices from 6 mice; Figure 8b) significantly decreased LTP in slices from CD200^{-/-} mice (13 slices from 9 mice) relative to control (12 slices from 7 mice; $p < 0.05$; Figure 8c).

Like LPS, Pam₃Csk₄ exerted a greater effect on inflammatory markers in cells prepared from CD200^{-/-} mice, and therefore we predicted that its effect on LTP would be genotype-specific. Application of Pam₃Csk₄ (20 µg/ml) to hippocampal slices prepared from wildtype mice for 60 min prior to TBS inhibited LTP (3 slices from 3 mice) compared with untreated controls (15 slices from 11 mice; $p < 0.001$; Figure 9a). A lower concentration of Pam₃Csk₄ (10 µg/ml), applied for 20 min prior to TBS, did not affect LTP in slices prepared from wildtype mice (4 slices from 3 mice; Figure 9b), but significantly reduced LTP in slices prepared from CD200^{-/-} mice (6 slices from 5 mice) compared with control (12 slices from 7 mice; $p < 0.05$; Figure 9c).

DISCUSSION

The loss of CD200 has a significant impact on activation of microglia in response to inflammatory stimuli, probably because of increased expression of TLR4 and TLR2 *in vitro* and *in vivo*. Whereas LTP in Schaffer collateral-CA1 synapses was markedly impaired in slices prepared from CD200-deficient mice under control conditions, activation of TLR4 and TLR2, by LPS and Pam₃Csk₄ respectively, exerted a more profound effect on LTP in slices prepared from CD200^{-/-} mice. We propose that the increased expression of TLR4 and TLR2 provides a plausible explanation for the increased responsiveness of CD200^{-/-} mice to inflammatory stimuli.

LPS and Pam₃Csk₄ increased the release of proinflammatory cytokines, IL-1β, IL-6 and TNFα

from mixed glial cultures, confirming previously-described effects of TLR4 and TLR2 (21,24,25). Both agonists exerted a greater effect on release of proinflammatory cytokines in mixed glia prepared from CD200^{-/-} mice, compared with wildtype mice. Thus tonic activation of CD200 receptor by CD200 is required to modulate inflammatory cytokine production. This concurs with data indicating that the interaction of neurons and microglia by means of CD200 receptor engagement by CD200 decreased microglial activation and production of IL-1β (8). In the current study in which a mixed glial preparation was used, we propose that the modulating effect is a consequence of the interaction between microglia and astrocytes, which we demonstrate express CD200. It is known that CD200 is widely expressed on numerous cell types although, in the case of astrocytes, expression to date has been reported only on reactive astrocytes in lesions from postmortem brains of individuals with multiple sclerosis (7). An interesting possibility is that the relatively activated state of microglia in a purified microglial culture may be a consequence of the loss of the CD200-controlled modulating effect of astrocytes.

The present findings in glia mirror those observed in peritoneal macrophages; thus stimulation with LPS and peptidoglycan, and also poly I:C, increased release of TNFα and IL-6 to a greater extent in macrophages prepared from CD200^{-/-} mice compared with wildtype mice (26). Similarly alveolar macrophages prepared from CD200^{-/-} mice, when stimulated *ex vivo* with LPS or IFNγ, expressed more MHCII and released more inflammatory cytokines than macrophages from wildtype mice (27). It has been known for many years that astrocytes are capable of modulating microglial/macrophage function. They have been shown to modulate LPS-induced changes in inducible nitric oxide synthase and NO production (28,29) and expression of MHCII (30); effects which have been attributed to astrocytic release of soluble factors like transforming growth factor (TGF)β. The present findings uncover another mechanism by which astrocytes can modulate microglial activation.

Several studies have established that responses to insults which induce inflammatory changes are exacerbated in CD200^{-/-} mice. Thus the symptoms and inflammation associated with experimental

autoimmune encephalomyelitis, *Toxoplasma* encephalitis, experimental autoimmune uveoretinitis, collagen-induced arthritis and facial nerve transection and are more profound in CD200-deficient mice (9,10,31). In addition, the response to an influenza dose of haemagglutination was much more severe (inducing some fatalities) in CD200-deficient, compared with wildtype, mice (27). Although it has been shown that CD200R activation by a CD200Fc ameliorates the symptoms associated with these conditions, and although CD200R-mediated regulation of macrophages relies on the binding of dok2 to the PTB binding motif in the cytoplasmic region of CD200R and the subsequent recruitment and activation of RasGAP (32), the mechanism by which these changes lead to dampening the activation of macrophage/microglia remains to be fully explained. In this study we show that increased expression of both TLR4 and TLR2 was observed in glia prepared from CD200^{-/-} mice and this may, at least in part, provide an explanation for the susceptibility of CD200^{-/-} mice to inflammatory stimuli. Both TLR2 and TLR4 ultimately lead to activation of NFκB and, in this study, the increased receptor expression in glia prepared from CD200^{-/-} mice is coupled with increased expression of phosphorylated IκB, which is indicative of NFκB activation. These changes clearly provide one possible explanation for the increased responsiveness of these cells to LPS and Pam₃Csk₄ in the present study, and perhaps also in other models.

Loss of CD200 increases expression of markers of microglial activation in mixed glial cultures; CD200 deficiency was associated with enhanced expression of both CD40 and CD68 mRNA, although not CD11b mRNA. In parallel, flow cytometry revealed that these markers, and also MHCII, were increased on CD11b-positive cells prepared from CD200^{-/-} mice. Previous studies have highlighted the importance of the interaction between CD200 and CD200R in maintaining the quiescent state of microglia, and have revealed that the age-related and Aβ-induced increases in microglial activation are coupled with decreased CD200 expression on neurons (8,14,21). The present observations also concur with the findings that under resting conditions, spinal cord microglia adopt an inflammatory morphology expressing more CD11b (9) and the number of CD45⁺CD11b⁺

cells prepared from retina of CD200^{-/-} mice was increased (10).

In the past decade it has become clear that neuroinflammatory changes, coupled with increased microglial activation, negatively affect synaptic plasticity in aged, LPS-treated and Aβ-treated rats (15,33-35). These observations are corroborated in this study where we directly associate the loss of CD200 with microglial activation and a deficit in LTP. The evidence indicates that slices prepared from CD200^{-/-} mice do not display LTP to the same degree as slices prepared from wildtype mice. One possible explanation for this is that TNFα, which is increased in hippocampal tissue prepared from these mice, is released from activated microglia and inhibits LTP. We demonstrate that TNFα inhibits TBS-induced LTP in mouse Schaffer collateral-CA1 synapses, which concurs with previous evidence indicating that it exerts a similar effect on tetanus-induced LTP in rats, *in vitro* and *in vivo* (17,19,23).

In addition to the decrease in LTP observed in untreated slices prepared from CD200^{-/-} mice, the data indicate that a subthreshold concentration of LPS or Pam₃Csk₄, which exerts minimal effects on LTP in wildtype mice, markedly impairs LTP in slices prepared from CD200^{-/-} mice. These findings show for the first time that activation of TLR2 leads to inhibition of LTP and further emphasizes the protective effect of CD200-CD200R interaction such that a deficit in CD200 leads to increased susceptibility to inflammatory stimuli. At this point it is unclear whether the effects of LPS or Pam₃Csk₄ on LTP are secondary to changes in glia, or are a consequence of a direct effect on neuronal TLR4 and TLR2. In this regard it is important to note that while some groups have reported neuronal expression of most TLRs both *in vitro* and *in vivo* (36),(37), others have been unable to demonstrate expression of TLR2 on neurons (38). The implication of this finding for the present study is that the mechanism underlying the Pam₃Csk₄-induced depression in LTP may result from its ability to release IL-1β, IL-6 and TNFα from glia; each of these inflammatory cytokines has been shown to inhibit LTP (17,39,40).

While there is an accumulating body of evidence indicating that CD200 deficiency is associated with increased inflammatory changes in several tissues including the brain, the effect on

neuronal function is relatively unexplored. Here we report that activation of TLR4 and 2 exacerbates neuroinflammatory changes in the absence of CD200 and, importantly, demonstrate that CD200 deficiency also exerts a negative effect on LTP. A key factor underlying these changes is

increased expression of these receptors. The findings highlight the importance of CD200 as a potential therapeutic target in disorders which are characterised by neuroinflammatory changes, coupled with loss of synaptic function.

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FIGURE LEGENDS

Figure 1. TLR2- and TLR4-induced increases in inflammatory cytokines are enhanced in glia prepared from CD200^{-/-} mice.

Incubation of mixed glia prepared from wildtype mice in the presence of LPS (100 ng/ml; a-c) or Pam₃Csk₄ (Pam; 100 ng/ml; d-f) increased supernatant concentrations of IL-1 β , IL-6 and TNF α (a-c; ***p < 0.001;

ANOVA; $n=6-8$) and the effect of LPS and Pam₃Csk₄ was significantly greater in cells prepared from CD200^{-/-} mice ($^{+++}p < 0.001$; ANOVA; $n = 4$).

Figure 2. MHCII⁺ CD11b⁺ and CD40⁺ CD11b⁺ cells are increased in glia prepared from CD200^{-/-} mice. The mean percentage CD11b⁺ cells which also stained positively for MHCII⁺ (top panels) and CD40 (lower panels). Data is presented as target proteins versus side scatter (SSC). Right hand panels illustrate representative overlays.

Figure 3. CD200 is expressed on astrocytes.

CD200 expression was observed on GLAST⁺ cells from purified astrocytic cultures obtained from wildtype (a) but not CD200^{-/-} (b) mice. CD200 was observed in membrane, but not cytosolic, fractions prepared from purified astrocytes obtained from wildtype mice. GFAP expression was observed in the cytosolic fraction (c).

Figure 4. Expression of TLR2 and TLR4 is increased in glia prepared from CD200^{-/-} mice.

TLR4 mRNA (a) and TLR2 mRNA (d) and the number of CD11b⁺ cells which stained positively for TLR4 (b,c) and TLR2 (e,f) were increased in glia prepared from CD200^{-/-}, compared with wildtype, mice ($*p < 0.05$; student's t-test for independent means; $n = 5$). (g) A sample immunoblot and mean data from densitometric analysis reveal that phosphorylated I κ B α is increased in cells prepared from CD200^{-/-}, compared with wildtype, mice ($*p < 0.05$; student's t-test for independent means; $n = 4-6$).

Figure 5. Markers of microglial activation are increased in cells prepared from CD200^{-/-} mice.

(a,b) Expression of CD40 mRNA, but not CD11b mRNA, was significantly greater in mixed glia prepared from CD200^{-/-}, compared with wildtype, mice ($*p < 0.05$; student's t-test for independent means; $n=4-5$). (c-f) Flow cytometric analysis revealed that the percentage of CD11b⁺ cells was similar in wildtype and CD200^{-/-} (c) but the percentage of CD11b⁺ cells which also stained positively for CD40 (d,e) and MHCII (f,g) was significantly greater in mixed glia obtained from CD200^{-/-}, compared with wildtype, mice ($*p < 0.05$; $^{***}p < 0.001$; student's t-test for independent means; $n=4-8$).

Figure 6. The increase in hippocampal expression of TLR2 and TLR4 in CD200^{-/-} mice is coupled with a deficit in LTP.

(a) Greater numbers of CD11b⁺CD45^{low} cells were found in hippocampus of CD200^{-/-}, compared with wildtype, mice ($^{***}p < 0.001$; student's t-test for independent means) and expression of CD200R expression (b) and CD40 (c) was greater in tissue prepared from CD200^{-/-}, compared with wildtype, mice. TLR2 (d) and TLR4 (f) expression on CD11b⁺CD45^{low} cells was greater in tissue prepared from CD200^{-/-}, compared with wildtype, mice, while TLR2 mRNA (e) and TLR4 mRNA (g) expression were significantly increased in hippocampal tissue prepared from CD200^{-/-}, compared with wildtype, mice ($*p < 0.05$; $**p < 0.01$; student's t-test for independent means; $n = 5$). (h) Theta-burst stimulation (TBS; arrow) induced LTP in CA1 synapses of hippocampal slices prepared from wildtype mice (15 slices from 11 mice). LTP, measured as mean % EPSP slope in the last 5 min of the experiment, was significantly reduced in slices prepared from CD200^{-/-} mice relative to wildtype mice ($p < 0.001$; 12 slices from 7 mice). Sample recordings immediately before, and 60 min following TBS are shown for wildtype and CD200^{-/-} mice (scale bars: 1mV/20ms).

Figure 7. Increased hippocampal expression of TNF α in CD200^{-/-} mice may underlie the associated deficit in LTP.

IL-1 α and IL-1 β were similar in tissue prepared from wildtype and CD200^{-/-} mice (a,b) but TNF α was significantly increased ($p < 0.05$; student's t-test for independent means; c) as revealed by sample immunoblots and analysis of densitometric data. Application of TNF α (3 ng/ml) to hippocampal slices significantly impaired LTP relative to vehicle controls ($p < 0.05$; unpaired Student's t-test; 3 slices from 2 mice; d). Sample EPSP traces immediately prior to, and 60 min following TBS are presented (scale bars: 1mV/20ms).

Figure 8. LTP is attenuated by LPS in CD200^{-/-} mice

(a) Perfusion of LPS (20 $\mu\text{g/ml}$) for 60 min prior to TBS (arrow) decreased LTP in slices prepared from wildtype mice (5 slices from 5 mice) and the mean % EPSP slope in the last 5 min of the experiment was significantly decreased compared with control slices ($p < 0.0001$; 15 slices from 11 mice). (b and c) LTP in slices prepared from wildtype mice was unaffected by perfusion of 10 $\mu\text{g/ml}$ LPS for 20 min prior to TBS (b; 7 slices from 6 mice relative to control 15 slices from 11 mice), but LTP was attenuated in slices from CD200^{-/-} mice (c; $p < 0.05$; 13 slices from 9 mice relative to control 12 slices from 7 mice). Sample EPSP traces immediately prior to, and 60 min following TBS are presented (scale bars: 1mV/20ms).

Figure 9. LTP is attenuated by Pam₃Csk₄ in CD200^{-/-} mice

(a) Perfusion of Pam₃Csk₄ (20 $\mu\text{g/ml}$) for 60 min prior to TBS (arrow) decreased LTP in slices prepared from wildtype mice (3 slices from 3 mice) and the mean % EPSP slope in the last 5 min of the experiment was significantly decreased compared with control slices (15 slices from 11 mice; $p < 0.001$). (b and c) LTP in slices prepared from wildtype mice was unaffected by perfusion of 10 $\mu\text{g/ml}$ Pam₃Csk₄ for 20 min prior to TBS (b; 4 slices from 3 mice relative to control 15 slices from 11 mice). However, LTP was attenuated in slices from CD200^{-/-} mice following treatment with 10 $\mu\text{g/ml}$ Pam₃Csk₄ (c; 6 slices from 5 mice relative to control 12 slices from 7 mice; $p < 0.05$). Sample EPSP traces immediately prior to, and 60 min following TBS are presented (scale bars: 1mV/20ms).

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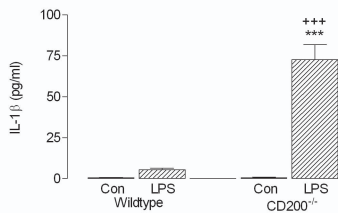
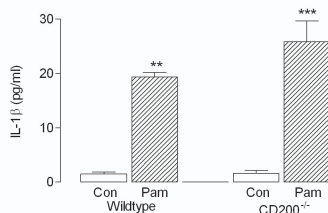
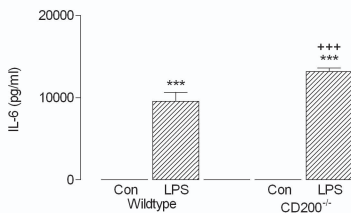
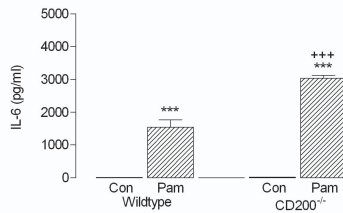
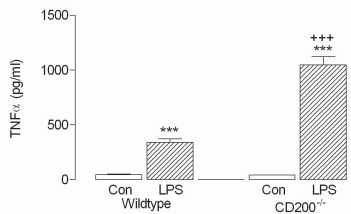
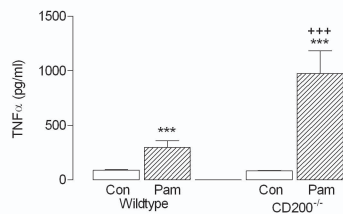
FIGURE1**(a)****(d)****(b)****(e)****(c)****(f)**

FIGURE2

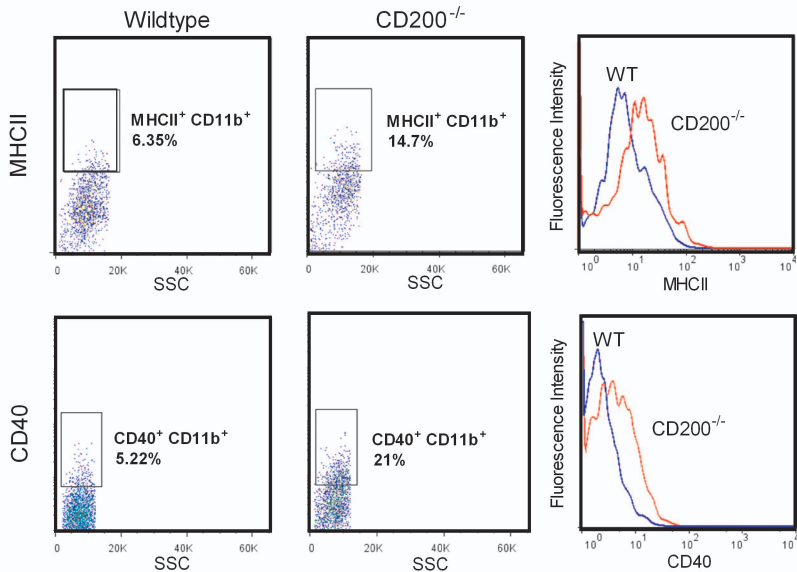
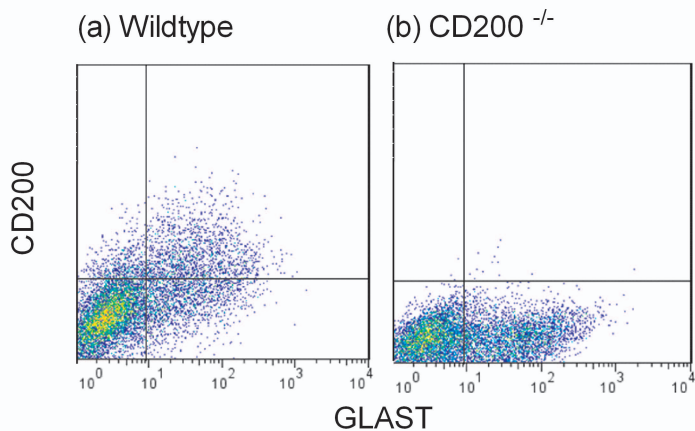


FIGURE 3



(c) Cultured astrocytes

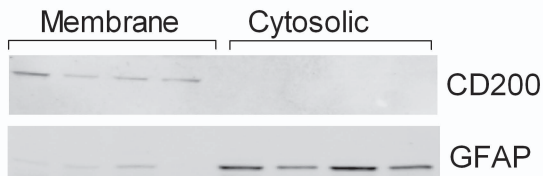


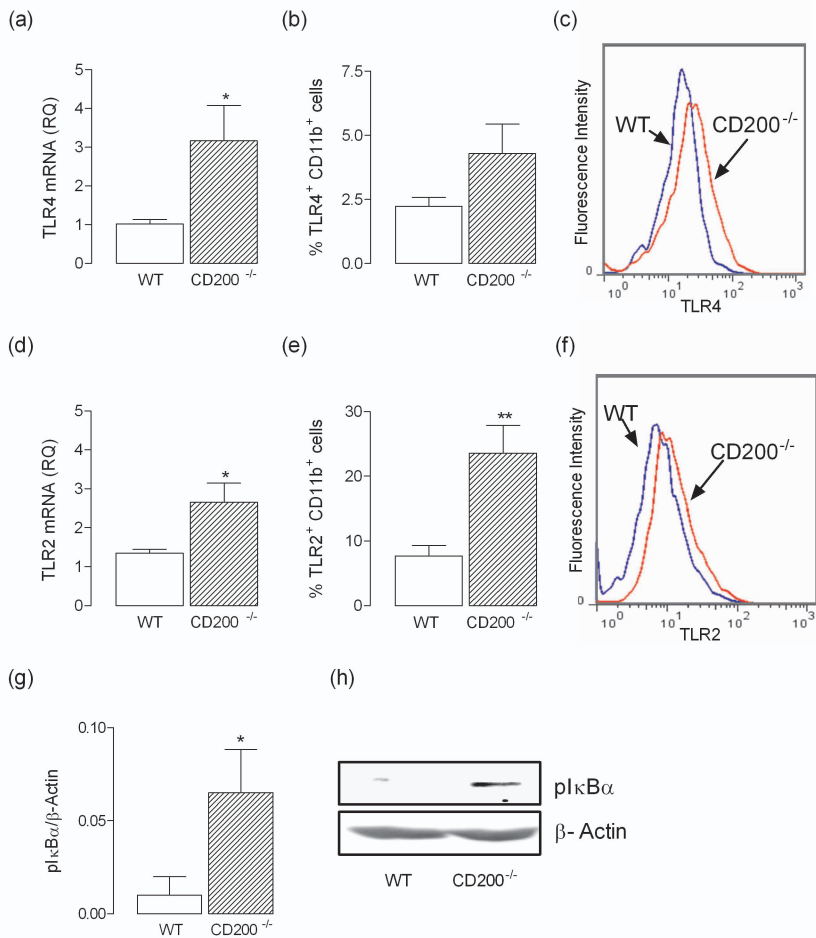
FIGURE 4

FIGURE 5

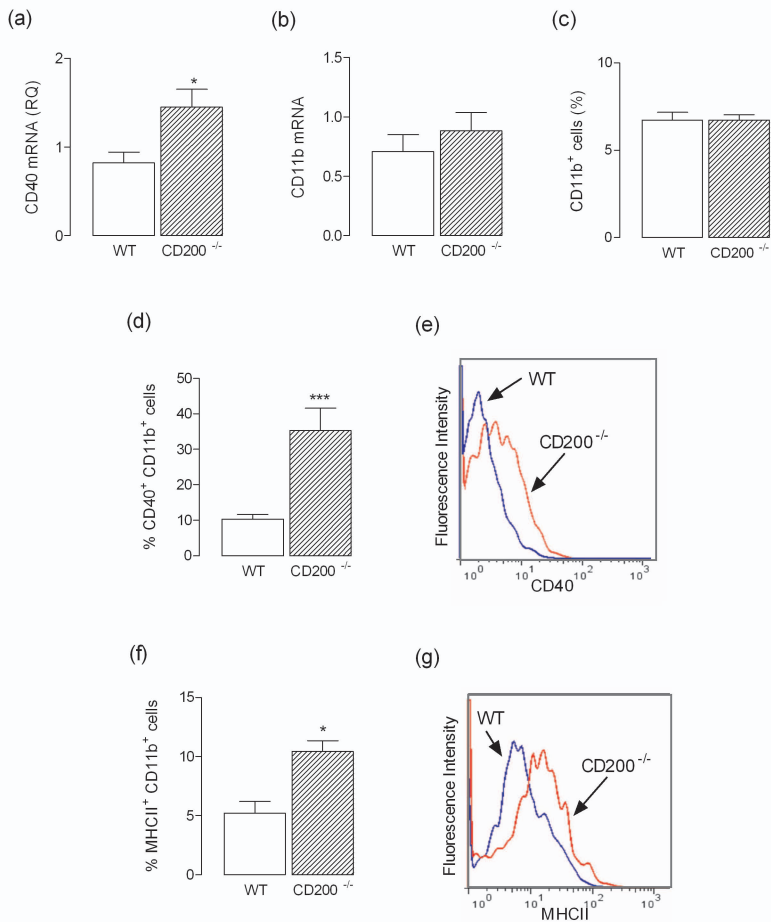


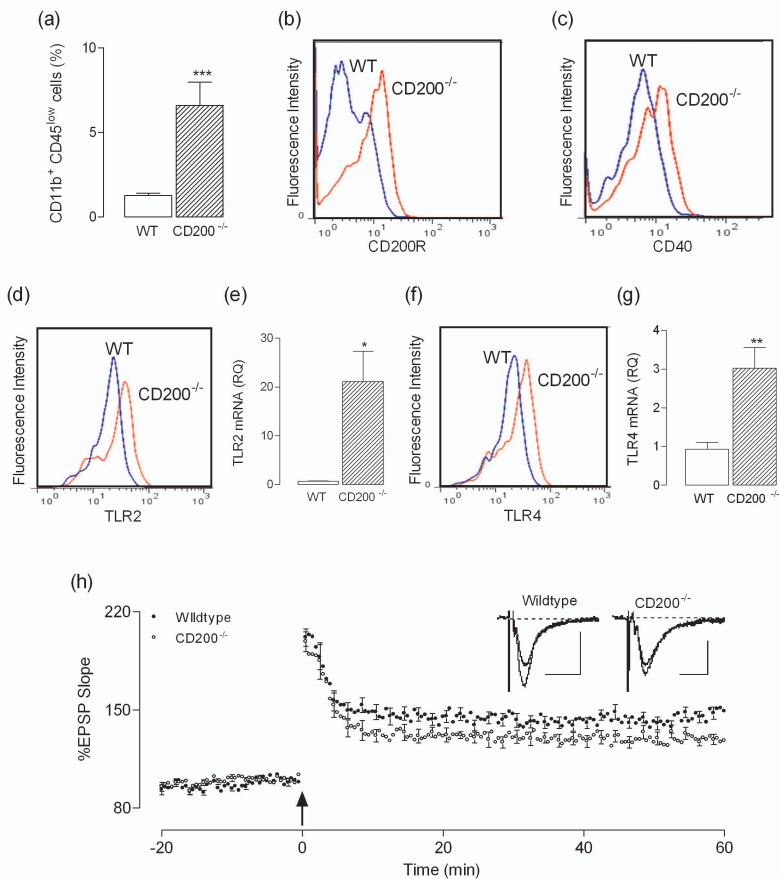
FIGURE 6

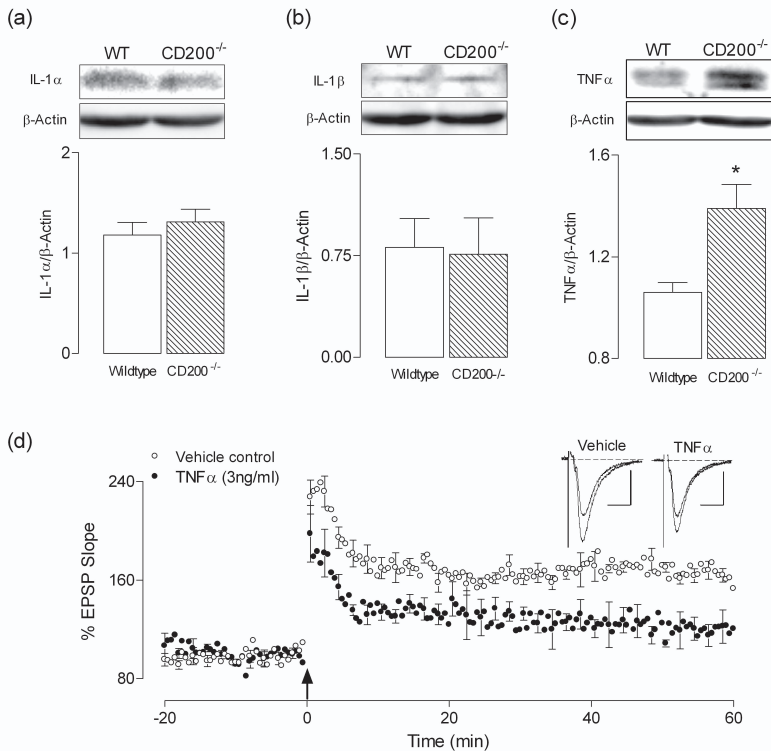
FIGURE 7

FIGURE 8

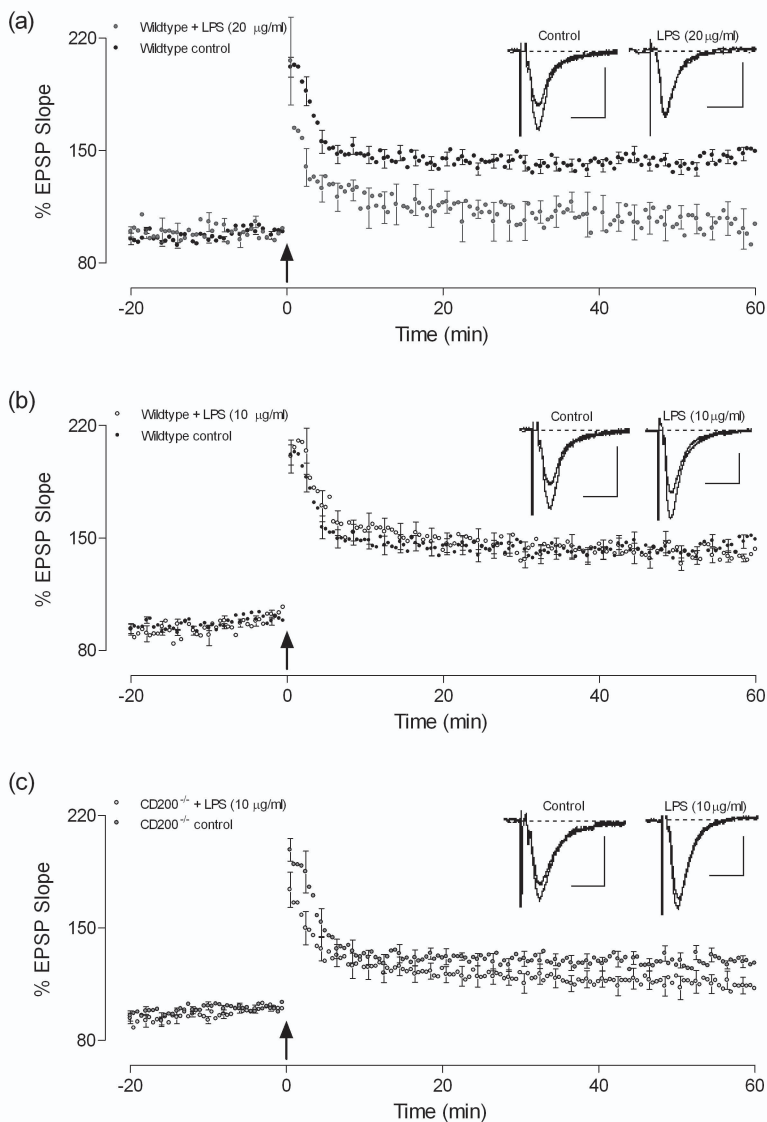


FIGURE 9

