

RESEARCH PAPER

Interleukin-1-induced interleukin-6 synthesis is mediated by the neutral sphingomyelinase/Src kinase pathway in neurones

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Background and purpose: Interleukin (IL)-1 is a key mediator of inflammatory and host defence responses and its effects in the brain are mediated primarily via effects on glia. IL-1 induces release of inflammatory mediators such as IL-6 from glia via the type-1 receptor (IL-1R1) and established signalling mechanisms including mitogen-activated protein kinases and nuclear factor kappa-B. IL-1 also modifies physiological functions via actions on neurones, through activation of the neutral sphingomyelinase (nSMase)/Src kinase signalling pathway, although the mechanism of IL-1-induced IL-6 synthesis in neurones remains unknown.

Experimental approach: Primary mouse neuronal cell cultures, ELISA, Western blot and immunocytochemistry techniques were used.

Key results: We show here that IL-1 β induces the synthesis of IL-6 in primary mouse neuronal cultures, and this is dependent on the activation of IL-1R1, nSMase and Src kinase. We demonstrate that IL-1 β -induced Src kinase activation triggers the phosphorylation of the NMDA receptor NR2B subunit, leading to activation of Ca²⁺/calmodulin-dependent protein kinase II (CamKII) and the nuclear transcription factor CREB. We also show that NR2B, CamKII and CREB are essential signalling elements involved in IL-1 β -induced IL-6 synthesis in neurones.

Conclusions and implications: These results demonstrate that IL-1 interacts with the same receptors on neurones and glia to elicit IL-6 release, but does so via distinct signalling pathways. The mechanism by which IL-1 β induces IL-6 synthesis in neurones could be critical in both physiological and pathophysiological actions of IL-1 β , and may provide a new therapeutic target for the treatment of acute CNS injury.

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Abbreviations: 3OMS, 3-O-methyl-sphingomyelin; CamKII, calmodulin-dependent protein kinase II; CREB, cAMP response element-binding protein; ERK, extracellular signal-regulated kinase; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; nSMase, neutral sphingomyelinase

Introduction

Interleukin (IL)-1 is a prototypic proinflammatory cytokine involved in the immune and host defence response to infection and injury, and is a primary mediator of inflammatory responses to acute and chronic CNS disorders. Brain IL-1 expression is dramatically increased in experimental and clinical CNS injury, whereas inhibiting its actions improves neurological outcome (see Allan *et al.*, 1998; Allan and Rothwell 2003; Rothwell 2003 for review). Some central actions of IL-1 may contribute to the regulation of

physiological functions such as sleep, memory and long-term potentiation (Schneider *et al.*, 1998; Rachal *et al.*, 2001; Ross *et al.*, 2003), as well as host defence responses, such as fever and sickness behaviour (Takahashi *et al.*, 1996; Dantzer *et al.*, 1998; Schneider *et al.*, 1998; Rachal *et al.*, 2001; Watkins *et al.*, 2001; Ross *et al.*, 2003; Bilbo *et al.*, 2005).

IL-1 is produced in the brain primarily by microglia and has pleiotropic actions on all resident cell types of the CNS, including astrocytes, microglia, oligodendrocytes, endothelial cells and neurones (Giulian *et al.*, 1988; Mason *et al.*, 2001; Vela *et al.*, 2002; Basu *et al.*, 2002, 2004; John *et al.*, 2004; Konsman *et al.*, 2007). In glial cells, IL-1 triggers cell proliferation and activation (that is astrogliosis), and the production of key inflammatory mediators, such as IL-6, nitric oxide, prostaglandin E₂ and chemokines (Laflamme

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et al., 1999; Basu *et al.*, 2004; John *et al.*, 2005). These effects are all dependent on activation of the classical IL-1 signalling pathways, including the mitogen-activated protein kinases (MAPKs) and nuclear factor- κ B pathways (Parker *et al.*, 2002).

In neuronal cells, IL-1 triggers cellular responses that are associated with physiological functions, including changes in the electrophysiological state and excitability of neurones (for example GABAergic inhibition, cell depolarization and rapid changes in membrane ion currents) (Katsuki *et al.*, 1990; Zeise *et al.*, 1992; Murray *et al.*, 1997; Schneider *et al.*, 1998; Kelly *et al.*, 2001; Desson and Ferguson 2003; Viviani *et al.*, 2003; Shu *et al.*, 2007). IL-1 induces changes in neuronal function via distinct pathways, including sphingomyelinase/ceramide and Src kinase activation, which can activate NMDA receptors via phosphorylation of the NMDA receptor 2A/NMDA receptor 2B (NR2A/NR2B) subunits, thus increasing receptor currents and Ca^{2+} influx (Salter and Kalia 2004; Viviani *et al.*, 2006). Src kinase-induced NMDA receptor activation is believed to be involved in physiological functions, such as plasticity and gene expression, and ischaemic neuronal injury (Salter and Kalia 2004).

Previous studies have shown that IL-1 also induces the production of IL-6 in neurones (Murphy *et al.*, 1995; Ringheim *et al.*, 1995), but the signalling mechanisms of IL-1-induced IL-6 synthesis in neurones remain unknown. We report for the first time that the neutral sphingomyelinase (nSMase)/Src kinase signalling pathway mediates the synthesis of IL-6 in neurones, and we further demonstrate that IL-1 β -induced IL-6 synthesis in neurones is dependent on the activation of the NR2B subunit, the Ca^{2+} /calmodulin-dependent protein kinase II (CamKII) (and possibly CamKIV) and cAMP response element-binding protein (CREB). Thus, IL-1 induces the key inflammatory mediator, IL-6, by totally distinct and separate pathways in neurones and glia.

Methods

Animals

Animal care and experimental procedures were conducted in accordance with the guidelines set by the European Council directives (86/609/EEC) and the UK Home Office, Animals (Scientific Procedures) Act 1986. All studies were performed using cell cultures prepared from wild-type mice (C57/BL6x129sv, Charles River, UK).

Reagents and drugs

General laboratory reagents were obtained from Sigma (Poole, UK) or BDH Ltd (Lutterworth, UK). Tissue culture reagents were supplied by Invitrogen. Rat recombinant IL-1 β and human recombinant IL-1RA were provided by Dr Steve Poole from the National Institute of Biological Standards and Controls (NIBSC, Potters Bar, UK).

Primary cortical neuronal cell culture and treatments

Primary, neuronal cell cultures were prepared from embryonic (day 16) mice as described previously (Moore *et al.*, 2002).

The cultures were used on days 12–14. Cell-specific immunostaining revealed that all cultures were highly enriched in neurones with no glial contamination (data not shown).

To assess the effect of IL-1 β on the synthesis of IL-6 in neurones, primary cultures were treated with vehicle (0.1%, low-endotoxin, BSA, in sterile 0.9% NaCl) or with rat recombinant IL-1 β (from 0.03 to 30 U ml $^{-1}$ diluted in vehicle) for 7 h (optimum time of treatment for IL-6 synthesis (Tsakiri *et al.*, 2007)). To investigate the effect of IL-1 β on the activation of MAPKs (extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38), Src kinase, CamKII and CREB, on the phosphorylation of NR2B, and on the synthesis of IL-6, primary cultures were incubated with vehicle or IL-1 β (0.3 U ml $^{-1}$), in the absence or presence of the Src kinase inhibitor PP2 (10 μ M, Calbiochem, Nottingham, UK), the nSMase inhibitor 3-O-methyl-sphingomyelin (3OMS) (15, 25 μ M, Biomol, USA), the NMDA receptor inhibitors MK-801 (20 μ M), AP5 (50 μ M) or ifenprodil (20 μ M) (Tocris, UK), the Ca^{2+} /CamKII/IV inhibitor KN-62 (10 μ M) (Calbiochem, UK) or interleukin-1 receptor antagonist (IL-1RA) (50 ng ml $^{-1}$) at various time points from 5 min to 7 h.

Immunocytochemistry

Cultures grown on poly-D-lysine-treated glass coverslips were washed twice with ice-cold phosphate-buffered saline, and then fixed in 4% paraformaldehyde and 4% sucrose for 20 min at room temperature. The cells were then permeabilized in 0.2% Triton X-100 for 15 min, and nonspecific sites were blocked by incubation in 10% foetal calf serum for 1 h at room temperature. The cells were then incubated for 1 h at room temperature with primary antibodies diluted in 0.3% BSA: rabbit anti-phosphorylated-CREB (1:100; Cell Signalling, Danvers, USA) and mouse anti-neuronal nuclei (1:400; Chemicon, Chandlers Ford, UK). The cells were then washed three times with 0.1% BSA and incubated for 1 h with appropriate secondary antibodies diluted in 3% BSA: Texas-red horse anti-mouse (1:600; Vector Laboratories, Burlingame, UK) and Alexa Fluor 488 goat anti-rabbit (1:2000; Invitrogen, Paisley, UK). Finally, the cells were washed three times with phosphate-buffered saline and mounted on glass slides with Prolong gold antifade reagent with 4,6-diamidino-2-phenylindole (Invitrogen, UK). Negative control staining was performed with an unrelated primary antibody (rabbit anti-GFAP). Images were obtained using an Olympus fluorescent microscope attached to a digital camera with MetaVue software.

IL-6 synthesis and release

Immunoreactive IL-6 in neuronal cell culture supernatant and cell lysates was assayed using a validated mouse-specific ELISA (DuoSet) purchased from R&D Systems (Abingdon, UK). IL-6 standards were assayed in triplicate and samples (100 μ l) in duplicate. The absorbance was then measured using a plate reader (MRX, Dynatech, UK), and results were calculated from the standard curve. The detection limit of the assay was 3 pg ml $^{-1}$.

Western blot analysis

Western blot analysis for Src kinase, CamKII and CREB activation was carried out as described previously (Parker *et al.*, 2002), using primary antibodies diluted in phosphate-buffered saline containing 0.1% Tween-20 and 0.1% BSA; anti-phosphorylated-ERK, anti-phosphorylated-JNK, anti-phosphorylated-p38, anti-total-p38 anti-phosphorylated-Src, anti-total-Src, anti-phosphorylated-CREB, anti-total-CREB, anti-phosphorylated-CamKII, anti-total-CamKII (all at 1:1000 dilution; Cell Signalling); anti-phospho-NR2B or anti-total-NR2B (1:1000; R&D Systems); anti-total-ERK or anti-total JNK (both at 1:5000 dilution; SantaCruz Biotech., Calne, UK); and subsequent incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2000 dilution in 5% milk/phosphate-buffered saline/0.1% Tween-20) (Dako, Ely, UK). The secondary antibody was detected using enhanced chemiluminescence using western blotting detection reagent (Amersham, Little Chalfont, UK), and films were analysed densitometrically with Northern Eclipse software.

Data analysis

Data were analysed with GraphPad Prism 4.0 software and expressed as the mean of at least three independent experiments \pm s.d. Differences between treated and control groups were analysed by one-way ANOVA with Dunnett's multiple comparisons *post hoc* test. Differences between IL-1 β alone and IL-1 β in the presence of inhibitors were analysed by one-way ANOVA with Bonferroni's *post hoc* test, and differences between cell lysates and supernatants of the same treatment were analysed by unpaired Student's *t*-test. For all analyses, a value of $P < 0.05$ was considered statistically significant.

Results

IL-1 β -induced IL-6 production in primary neurones is dependent on Src kinase and nSMase activation

IL-1 β (0.03, 0.3 and 3 U ml⁻¹) induced significant synthesis of IL-6 protein in primary neurones, which was mainly

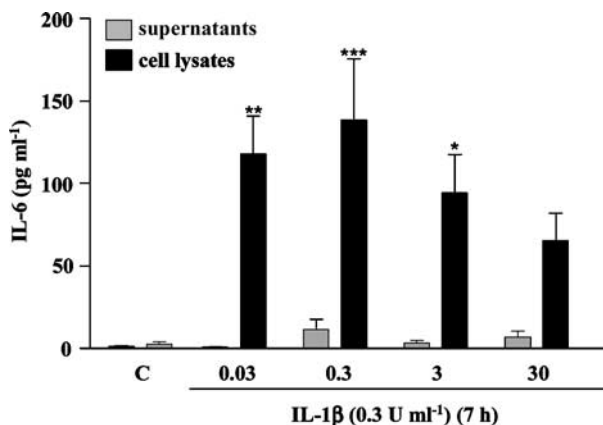


Figure 1 IL-1 β -induced IL-6 synthesis in primary cortical neurones. Cultures were treated with vehicle (C) or IL-1 β (0.03–30 U ml⁻¹) for 7 h, and cell lysates and supernatants were collected and assayed for IL-6 using a mouse-specific ELISA. Data are presented as the mean \pm s.d. of seven independent experiments * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, IL-1 β vs control. IL, interleukin.

detected in the intracellular compartment (Figure 1). The dose that optimally induced the production of IL-6 was 0.3 U ml⁻¹ and was therefore selected for further studies.

To identify the signalling pathways through which IL-1 β induces IL-6 expression, cell lysates of IL-1 β -treated neurones (0.3 U ml⁻¹ for 5, 15, 30 or 60 min) were assayed for ERK, JNK and p38 activation by western blot analysis. These MAPKs mediate IL-1 β -induced IL-6 synthesis in glial cells (Parker *et al.*, 2002). IL-1 β failed to activate any of these MAPKs at any time point tested (Figure 2a). In contrast, IL-1 β induced a significant 2.5-fold activation of Src kinase after 5 and 15 min of treatment (Figure 2b). IL-1 β -induced Src kinase activation was significantly reduced in the presence of excess IL-1RA (Figure 3a), the specific Src kinase inhibitor PP2 (Figure 3b) or the specific nSMase inhibitor 3OMS (Figure 3c), whereas the inhibitors alone had no effects (data not shown).

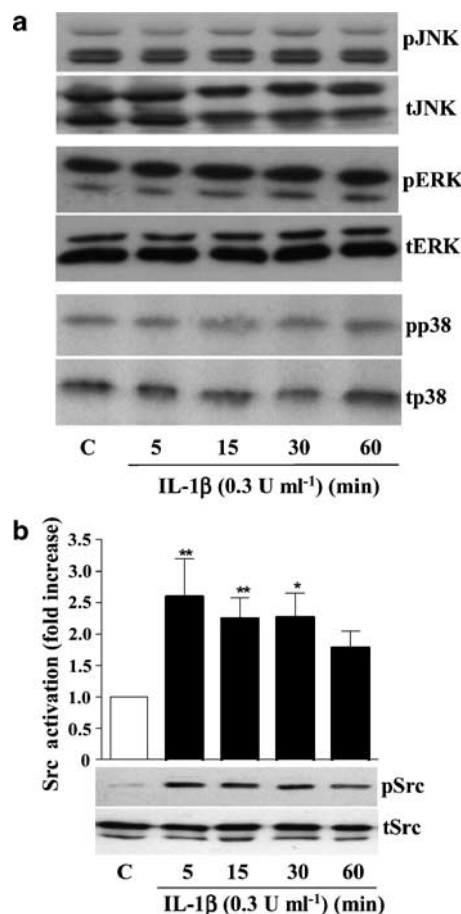


Figure 2 Effect of IL-1 β on MAPKs and Src kinase activity in primary cortical neurones. Cultures were treated with vehicle (C) or IL-1 β (0.3 U ml⁻¹) for 5, 15, 30 or 60 min, and cell lysates were assayed by western blot analysis for (a) ERK, JNK or p38 activation, or (b) Src kinase activation, using specific antibodies against total (t) or phosphorylated (p) isoforms of ERK, JNK, p38 or Src kinase. Images of blots in (a) are from a single experiment representative of three independent experiments carried out on separate cultures. Data in (b) are presented as the mean \pm s.d. of three independent experiments carried out on separate cultures. * $P < 0.05$, ** $P < 0.01$, IL-1 β vs control. ERK, extracellular signal-regulated kinase; IL, interleukin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

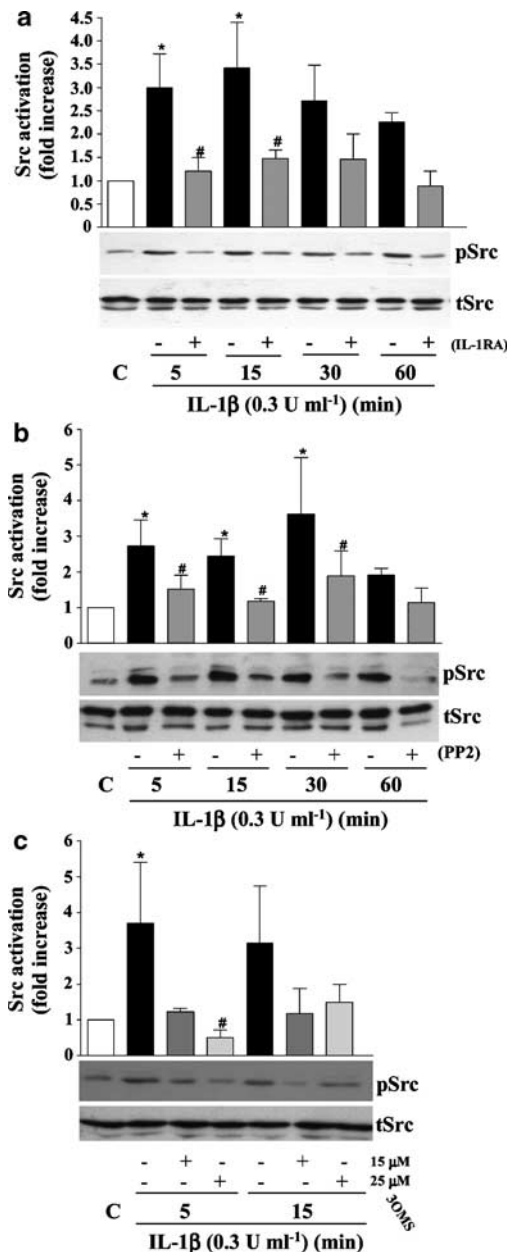


Figure 3 Effect of IL-1RA, PP2 or 3OMS on IL-1 β -induced Src kinase in primary cortical neurones. Cultures were treated with vehicle (C) or IL-1 β (0.3 U ml⁻¹) for 5, 15, 30 or 60 min, in the absence or presence of (a) IL-1RA (50 ng ml⁻¹), (b) PP2 (10 μ M) or (c) 3OMS (15, 25 μ M). Cell lysates were assayed by western blot analysis for Src kinase activation using specific antibodies against total or phosphorylated isoforms of Src kinase. Data are presented as the mean \pm s.d. of three independent experiments carried out on separate cultures. * P < 0.05, IL-1 β vs control, # P < 0.05, IL-1 β + IL-1RA/PP2/3OMS vs IL-1 β alone. IL, interleukin; 3OMS, 3-O-methyl-sphingomyelin.

To investigate whether Src kinase and nSMase are involved in IL-1 β -induced IL-6 synthesis in neurones, supernatants and cell lysates from cultures treated with IL-1 β (0.3 U ml⁻¹) for 7 h, in the presence or absence of PP2, IL-1RA or 3OMS, were assayed for IL-6 by ELISA. The levels of IL-6 detected in cell lysates in response to IL-1 β were significantly decreased by pretreatment with PP2 (30%) or IL-1RA (80%) and were comparable to control levels in the presence of 3OMS (25 μ M)

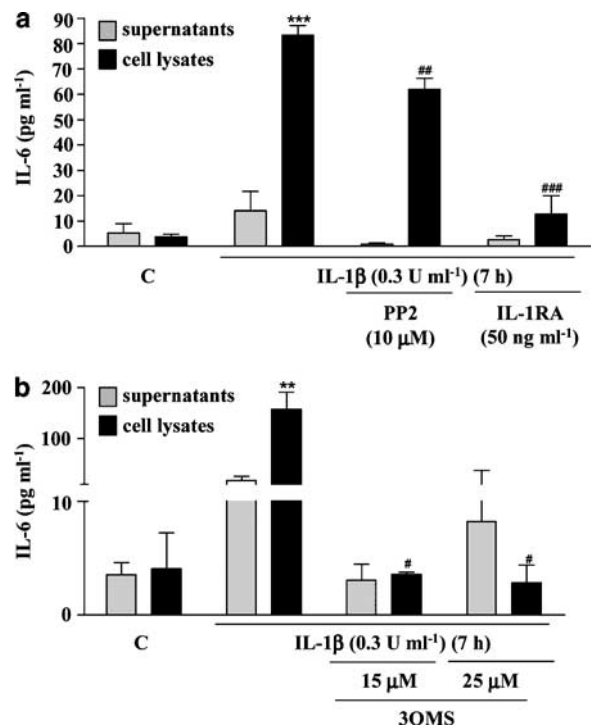


Figure 4 Effect of IL-1RA, PP2 or 3OMS on IL-1 β -induced IL-6 synthesis in primary cortical neurones. Cultures were treated with IL-1 β (0.3 U ml⁻¹) for 7 h in the absence or presence of (a) PP2 (10 μ M) or IL-1RA (50 ng ml⁻¹), or (b) 3OMS (15, 25 μ M). Cell lysates and supernatants were assayed for IL-6 using a specific mouse ELISA. Data are presented as the mean \pm s.d. of three different experiments carried out on separate cultures. ** P < 0.01, *** P < 0.001, IL-1 β vs control, # P < 0.05, ## P < 0.01, ### P < 0.001, IL-1 β + PP2/IL-1RA/3OMS vs IL-1 β alone. IL, interleukin; 3OMS, 3-O-methyl-sphingomyelin.

(Figures 4a and b). The inhibitors alone had no significant effect on IL-6 levels (data not shown).

IL-1 β -induced IL-6 production in primary neurones is dependent on NMDA receptor activation

Activation of Src kinase is linked to NMDA receptor activation, mainly through phosphorylation of NR2 subunits (Salter and Kalia 2004). To test the hypothesis that NMDA receptor activation is involved in the signalling mechanisms of IL-1 β -induced IL-6 synthesis, cytoplasmic membrane preparations from primary neuronal cultures treated with IL-1 β (0.3 U ml⁻¹) for 10 min, in the absence or presence of PP2, 3OMS or IL-1RA, were assessed for NR2B phosphorylation by western blot. IL-1 β induced the phosphorylation of NR2B, which was prevented by co-incubation with PP2, 3OMS or IL-1RA (Figure 5a). Furthermore, three NMDA receptor inhibitors, MK-801, AP5 and ifenprodil, reduced IL-1 β -induced IL-6 synthesis, but only MK-801 induced a statistically significant decrease (95%) (Figure 5b), suggesting that the effects of IL-1 β on IL-6 production are dependent on NMDA receptor activation.

IL-6 synthesis induced by IL-1 β in primary neurones is mediated by CamKII and CREB

As activation of NMDA receptors leads to Ca²⁺ influx, we next investigated the effect of IL-1 β on Ca²⁺-dependent

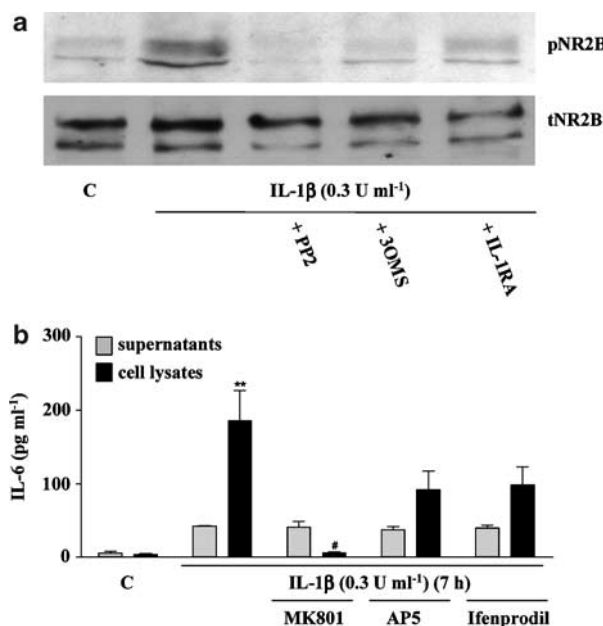


Figure 5 IL-1 β -induced NR2B phosphorylation and effect of NMDA inhibitors on IL-1 β -induced IL-6 synthesis in primary cortical neurones. (a) Cultures were treated with vehicle (C) or IL-1 β (0.3 U ml $^{-1}$) for 10 min, in the absence or presence of the Src kinase inhibitor PP2 (10 μ M), 3OMS (25 μ M) or IL-1RA (50 ng ml $^{-1}$). Cytoplasmic membrane extracts were assayed for NR2B phosphorylation by western blot analysis using specific antibodies against total (t) or phosphorylated (p) isoforms of the NR2B subunit of the receptor. (b) Cultures were treated with vehicle (C) or IL-1 β (0.3 U ml $^{-1}$) for 7 h in the absence or presence of the NMDA receptor blocker MK-801 (20 μ M), the competitive inhibitor AP5 (50 μ M), and the specific inhibitor of NR2B-containing NMDA receptors, ifenprodil (20 μ M). Cell lysates and supernatants were assayed for IL-6 using a specific mouse ELISA. Data are presented as the mean \pm s.d. of three independent experiments carried out on separate cultures. ** P < 0.05, IL-1 β vs control, # P < 0.05, IL-1 β + MK-801 vs IL-1 β alone. IL, interleukin; 3OMS, 3-O-methyl-sphingomyelin.

CamKII activation. Neurones treated with IL-1 β (0.3 U ml $^{-1}$) in the absence or presence of the selective inhibitor of Ca $^{2+}$ /CamKII/IV, KN-62 or inhibitors of identified upstream signalling elements (PP2, 3OMS, IL-1RA or AP5) were assayed for CamKII activity by western blot. IL-1 β induced significant activation of CamKII (Figure 6a), which was completely blocked by all the inhibitors tested, with significant inhibition produced by IL-1RA, AP-5 and KN-62. In addition, KN-62 significantly reduced (58%) IL-1 β -induced IL-6 synthesis (Figure 6b), further implicating CamKII in the signalling mechanism of IL-1 β actions in neurones. We also found that IL-1 β induced activation of the Ca $^{2+}$ -dependent transcription factor CREB, after 20 and 45 min of treatment (Figure 7a). Western blot analysis of the nuclear extracts of neurones treated with IL-1 β for 20 min confirmed strong activation of CREB, which was totally abolished by IL-1RA, PP2, 3OMS, KN-62 or AP5 (Figure 7b).

Discussion and conclusions

Proinflammatory actions of IL-1 β are mediated in the CNS primarily by glial cells, but direct actions of this cytokine on

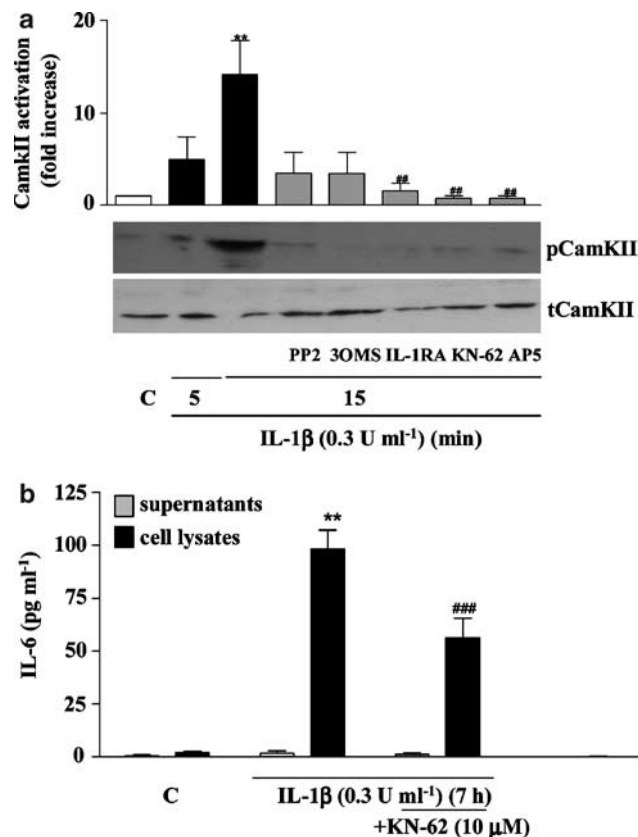


Figure 6 IL-1 β -induced CamKII activation and effect of KN-62 on IL-1 β -induced IL-6 synthesis in primary cortical neurones. (a) Western blot analysis of CamKII activation in total cell extracts of cultures treated with vehicle (C) or IL-1 β (0.3 U ml $^{-1}$) for 5 or 15 min, in the absence or presence of IL-1RA (50 ng ml $^{-1}$), 3OMS (25 μ M), PP2 (10 μ M), KN-62 (CamkII/IV inhibitor, 10 μ M) or AP5 (50 μ M). Data are presented as the mean \pm s.d. of five different experiments in separate cultures, ** P < 0.01, IL-1 β vs control, ## P < 0.01, IL-1 β + inhibitor vs IL-1 β alone. (b) Cultures were treated with IL-1 β (0.3 U ml $^{-1}$) for 7 h in the absence or presence of KN-62 (10 μ M). Cell lysates and supernatants were assayed for IL-6 using a specific mouse ELISA. Data are presented as the mean \pm s.d. of six different experiments in separate cultures. ** P < 0.01 IL-1 β vs control, ### P < 0.001, IL-1 β + KN-62 vs IL-1 β alone. IL, interleukin; 3OMS, 3-O-methyl-sphingomyelin.

neurones have been identified and mediate important physiological functions via fast activation of the nSMase/ Src kinase pathway (Sanchez-Alavez *et al.*, 2006). IL-1 β also induces IL-6 expression in neurones (Murphy *et al.*, 1995; Ringheim *et al.*, 1995), but the signalling mechanisms that mediate this response were unknown. In the present study, we identified signalling pathways involved in the mechanisms of IL-6 synthesis induced by IL-1 β in primary cortical neurones and show that IL-1 induces IL-6 synthesis by totally distinct signalling pathways in glia and in neurones.

Firstly, we demonstrated that IL-1 β does not activate the classical IL-1 signalling pathways (that is MAPKs) in cortical neurones, but activates nSMase and Src kinase in an IL-1R1-dependent manner, which confirms previous findings obtained on hypothalamic and hippocampal neurones (Viviani *et al.*, 2003; Davis *et al.*, 2006). In our study, IL-1 β -induced Src kinase activation was blocked by the nSMase-specific inhibitor 3OMS, providing evidence that nSMase

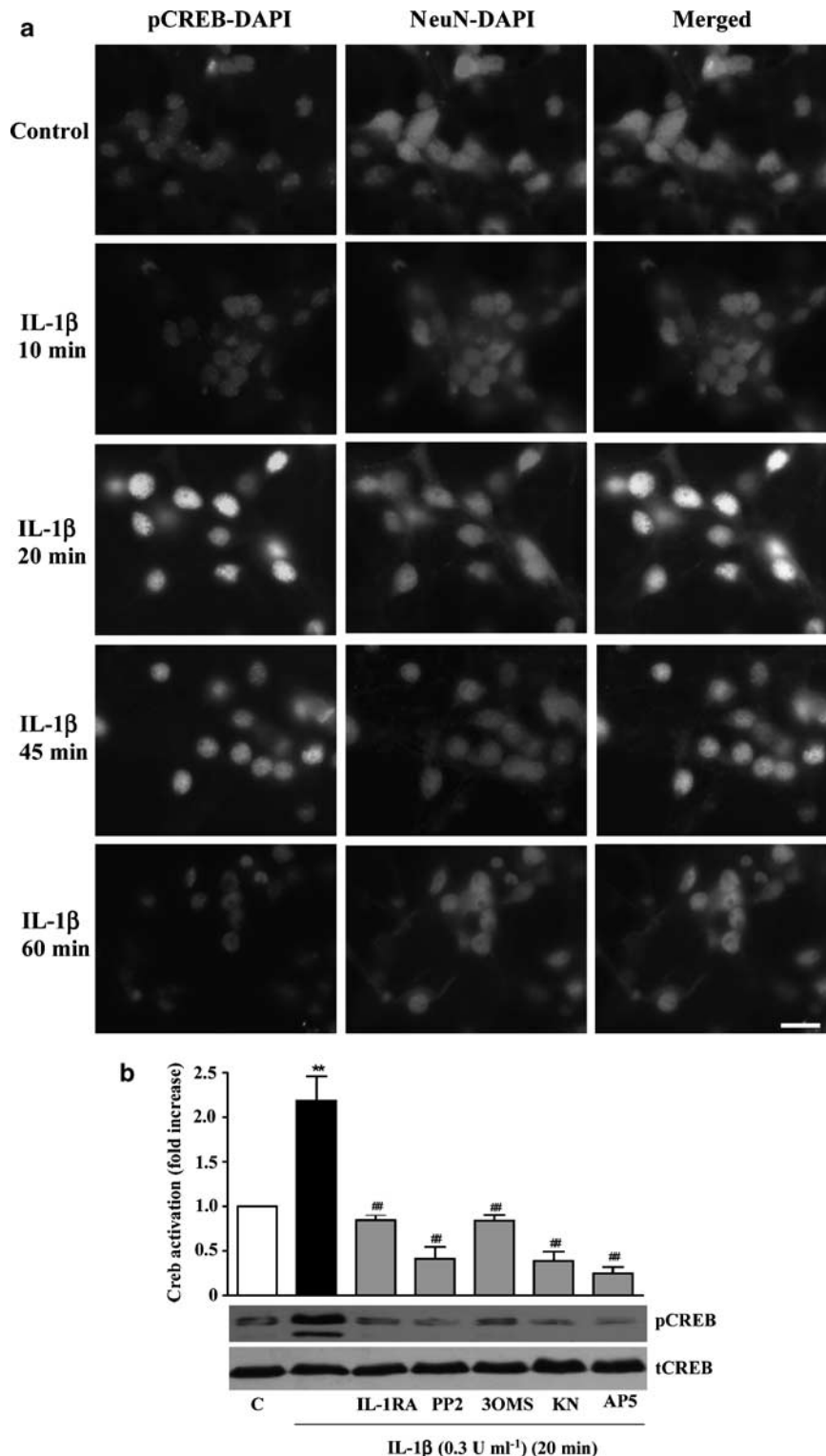


Figure 7 Effect of IL-1 β on CREB activation and effect of IL-1RA, PP2, 3OMS and AP5 on IL-1 β -induced CREB activation in primary cortical neurones. (a) Cultures were treated with vehicle (C) or IL-1 β (0.3 U ml⁻¹) for 10, 20, 45 or 60 min, fixed and stained for pCREB, NeuN (neuronal nuclei) and DAPI (all nuclei). Scale bar 5 μ m. (b) Primary neurones were treated with vehicle (C) or IL-1 β (0.3 U ml⁻¹) for 20 min, in the absence or presence of PP2 (10 μ M), 3OMS (25 μ M), IL-1RA (50 ng ml⁻¹), KN-62 (10 μ M) or AP5 (50 μ M). Nuclear extracts were assayed for CREB activity by western blot analysis using specific antibodies against total (tCREB) or phosphorylated CREB (pCREB). Data are presented as the mean \pm s.d. of four independent experiments carried out on separate cultures. ** P < 0.01, IL-1 β vs control, ## P < 0.01 IL-1 β + inhibitor vs IL-1 β alone. CREB, cAMP response element-binding protein; DAPI, 4,6-diamidino-2-phenylindole; IL, interleukin; NeuN, anti-neuronal nuclei; 3OMS, 3-O-methyl-sphingomyelin.

activity is upstream of Src kinase in this pathway, which also confirms the findings of Viviani *et al.* (2003) and Davis *et al.* (2006). We also showed that IL-1 β induces the phosphorylation of the NMDA receptor NR2B subunit, which in turn leads to the activation of CamKII and CREB, and we demonstrated, using pharmacological approaches, that this series of signalling events occurs downstream of nSMase/Src kinase activation, and are all IL-1R1 dependent. This neuronal-specific IL-1 signalling pathway (nSMase/Src/NR2B/CamKII/CREB), which is known to mediate the fast electrophysiological actions of IL-1 on neurones, also causes IL-6 protein synthesis in these cells, as IL-1RA, 3OMS, PP2, KN-62 and MK-801 all significantly inhibited IL-1 β -induced IL-6 synthesis in primary neurones. In contrast, we found that IL-1 β failed to activate Src kinase activation in glia (data not shown), whereas IL-1 β -induced IL-6 synthesis in glia is dependent on the activation of the classical IL-1 signalling pathway (that is MAPKs and nuclear factor- κ B) (Parker *et al.*, 2002).

These observations demonstrate that IL-1 can trigger, in two different brain cell types, the synthesis of the same inflammatory mediator (that is IL-6) via two different cell-specific signalling mechanisms. In addition, IL-1 β induces optimal IL-6 expression at a much lower concentration (0.3 U ml⁻¹) in neurones than those required for optimal actions on glia (30 U ml⁻¹). These discoveries suggest that the IL-1 receptor complexes may differ between glia and neurones and suggest new targets to differentiate cell specific effects of IL-1.

In the normal brain, IL-1 and IL-6 are known to modulate neuronal functions and physiological events, such as long-term potentiation, sleep, memory, pain and fever (Takahashi *et al.*, 1996; Schneider *et al.*, 1998; Rachal *et al.*, 2001;

Watkins *et al.*, 2001; Ross *et al.*, 2003; Bilbo *et al.*, 2005). Src kinase and its ability to modify NMDA receptors has also been linked with several physiological functions, such as memory, long-term potentiation and pain (Salter and Kalia 2004). Further *in vitro* studies showed that NMDA receptor activation triggered by cell depolarization leads to IL-6 expression in neurones (Ali *et al.*, 2000; Sallmann *et al.*, 2000). These observations combined with our data suggest that the mechanism of IL-1 β -induced IL-6 synthesis that is mediated by the nSMase/Src kinase pathway could play a very important role in the physiological actions of these two cytokines in the brain. IL-1 and IL-6 are also key mediators of neuroinflammation (Gallo *et al.*, 1989; Yamasaki *et al.*, 1995; Bagetta *et al.*, 1999; Vezzani *et al.*, 2000; Allan and Rothwell 2001) and the effect of IL-1 β on neuronal cells in CNS injury could profoundly influence the inflammatory response and cell death. Indeed, it has been shown that Src kinase activity is increased after experimentally induced cerebral ischaemia, whereas its inhibition improves neurological outcome (Pei *et al.*, 2000; Paul *et al.*, 2001; Ardizzone *et al.*, 2007). Furthermore, NMDA receptor activation induces cytokine expression after ischaemia (Acarin *et al.*, 2000), whereas IL-6 expression is suppressed by inhibition of Ca²⁺ influx in cerebral ischaemia (Suzuki *et al.*, 2000). We have shown that IL-1 β induces NR2B subunit phosphorylation and this could be a critical control mechanism to modulate the susceptibility of neurones to injury.

In summary, we have elucidated a major signalling pathway through which IL-1 β acts on cortical neurones to induce the synthesis of IL-6, and these signalling mechanisms are summarized in Figure 8. In cortical neurones, IL-1 β action through nSMase/Src kinase/NMDA receptor activation triggers the synthesis of an inflammatory mediator (IL-6)

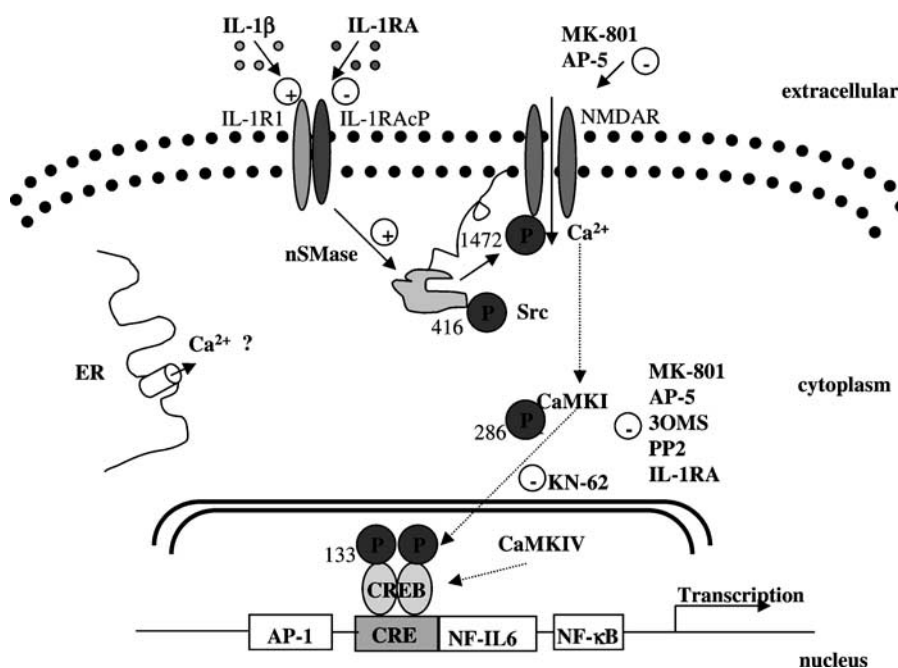


Figure 8 Schematic diagram of proposed mechanism of the IL-1 β -induced signalling pathway involved in the synthesis of IL-6 in primary cortical neurones. IL-1 β binds to IL-1R1 in primary neurones to triggers downstream activation of nSMase and Src kinase, which in turn activates NMDA receptors. This leads to an increase in Ca²⁺ influx in the cells, which triggers activation of CamKII and CREB, leading to the ultimate expression of IL-6 gene and synthesis of the protein. CREB, cAMP response element-binding protein; IL, interleukin.

via CamKII/CREB activation, which links neuronal activity with gene expression. This mechanism could be critical in both physiological and pathological actions of IL-1 β , and could be a new therapeutic target for the treatment of acute CNS injury.

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Conflict of interest

The authors state no conflict of interest.

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