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IL-1 α and HMGB1 mediate hippocampal dysfunction in SIGIRR-deficient mice

Abbreviated title: **SIGIRR deficiency leads to hippocampal dysfunction**

Derek A. Costello^{1,§}, Melanie B. Watson^{1,*}, Thelma R. Cowley¹, Niamh Murphy¹, Ciarán Murphy Royal¹, Cecilia Garlanda² and Marina A. Lynch¹.

1. Trinity College Institute of Neuroscience, Department of Physiology,

Trinity College Dublin, Dublin 2, Ireland. 2. Istituto Clinico Humanitas, IRCCS, via Manzoni 113, 20089 Rozzano (Milan) Italy.

*Present address: Department of Neurology and Neurobiology, The David Geffen School of Medicine at UCLA, 710 Westwood Plaza, Los Angeles, CA 90095-1769, USA

§Corresponding author.

Tel.: 353-1-896-8476; fax: 353-1-679-3545. E-mail address: derek.costello@tcd.ie

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Abstract

Single-Ig-interleukin-1 related receptor (SIGIRR) is a member of the interleukin (IL)-1/Toll-like receptor (TLR) family. It negatively regulates inflammation, rendering SIGIRR^{-/-} mice more susceptible to inflammatory challenge. This susceptibility extends to the brain, where increased responsiveness to lipopolysaccharide (LPS) has been observed in SIGIRR-deficient mice. While this is likely due to enhanced TLR4-mediated signalling, the functional consequences of these changes have not yet been described. In the current study, we have investigated the impact of SIGIRR deficiency on hippocampal function, and show that novel object recognition, spatial reference memory and long-term potentiation (LTP) were impaired in SIGIRR^{-/-} mice. These changes were accompanied by increased expression of IL-1RI and TLR4, and up-regulation of their downstream signalling events, namely IRAK1, JNK and NFκB. The deficit in LTP was attenuated by the endogenous IL-1 receptor antagonist (IL-1ra) and an anti-TLR4 antibody, and also by inhibition of JNK and NFκB. We propose that IL-1RI is activated by IL-1α and TLR4 is activated by the endogenous agonist, high mobility group box 1 (HMGB1), as we identified enhanced expression of both cytokines in the hippocampus of SIGIRR^{-/-} mice. Additionally, application of HMGB1 increased the activation of JNK and NFκB and was found to be

detrimental to LTP in a TLR4-dependent manner. These findings highlight the functional role of SIGIRR in regulating inflammatory-mediated synaptic and cognitive decline, and describe evidence of the key role of HMGB1 in this process.

Introduction

The ageing process, along with neurodegenerative disorders including Alzheimer's disease (AD), are characterized by an underlying neuroinflammation (Tuppo and Arias, 2005; Glass et al., 2010), which is associated with deficits in cognitive function and hippocampal synaptic plasticity, such as long-term potentiation (LTP). Investigations into the mechanisms underlying these inflammatory processes have illustrated that pro-inflammatory cytokines induce an impairment in hippocampal-dependent memory (Barrientos et al., 2002; Hein et al., 2010) and LTP (Vereker et al., 2000; Curran and O'Connor, 2001). Additionally, inflammatory challenge associated with age, β -amyloid, or lipopolysaccharide (LPS) elicit similar deficits in LTP through activation of stress-activated protein kinases (O'Donnell et al., 2000; Kelly et al., 2003; Minogue et al., 2003; Costello and Herron, 2004; Barry et al., 2005).

Single-Ig-interleukin-1 related receptor (SIGIRR) is a member of the TLR/IL-1R family (Thomassen et al., 1999). It is abundant in peripheral tissues including lung, kidneys and the gastrointestinal tract (Polentarutti et al., 2003; Wald et al., 2003), where it performs an anti-inflammatory role (Garlanda et al., 2009). Specifically, SIGIRR-deficient mice exhibit exaggerated symptoms of inflammatory conditions such as colitis-associated cancer (Garlanda et al., 2007), experimental autoimmune encephalitis (Gulen et al., 2010) and rheumatoid arthritis (Drexler et al., 2010). Furthermore, SIGIRR^{-/-} mice

demonstrate pronounced susceptibility to the inflammatory challenge posed by the pathogen LPS (Wald et al., 2003; Garlanda et al., 2004; Lech et al., 2007). A recent report from this laboratory provides the first evidence that these effects are extended to the brain, demonstrating exaggerated microglial activation and inflammatory regulation in hippocampus of SIGIRR^{-/-} mice in response to LPS (Watson et al., 2010).

In addition to analysis in SIGIRR-deficient mice, over-expression of SIGIRR has proven beneficial to our understanding of how its anti-inflammatory effects may be mediated (Wald et al., 2003; Qin et al., 2005; Zhang et al., 2010). Evidence suggests that SIGIRR interacts with both IL-1RI and TLR4 to decrease the impact of pro-inflammatory stimuli (Qin et al., 2005; Huang et al., 2006; Gulen et al., 2010). SIGIRR over-expression reduces the production of pro-inflammatory cytokines and NFκB activation, while kidney epithelial cells from SIGIRR^{-/-} mice exhibit prolonged phosphorylation of the stress-activated protein kinase, JNK, leading to activation of NFκB (Wald et al., 2003). Furthermore, Qin and colleagues (2005) propose that SIGIRR interaction with IL-1R and TLR4 interferes with recruitment of the adaptor molecule myeloid differentiation factor 88 (MyD88) to down-regulate the pro-inflammatory response (Qin et al., 2005).

In the current study, the impact of SIGIRR deficiency on hippocampal function was assessed. We demonstrate a cognitive deficit and an impairment of LTP at CA1 synapses, associated with up-regulation of IL-1R- and TLR4-mediated signalling in SIGIRR^{-/-} mice. We propose that the augmented expression of the endogenous TLR ligand, high mobility group-box 1 (HMGB1) in SIGIRR^{-/-} mice is pivotal to the deficit in hippocampal function.

Methods and Materials

Animals

Male and female C57BL/6 mice (2-6 months; Harlan UK), SIGIRR^{-/-}/TIR8^{-/-} mice (2-6 months; a gift from Professor A Mantovani, Istituto Clinico Humanitas IRCCS, Milan; (Garlanda et al., 2004)) and TLR4^{-/-} mice (4-6 months; a gift from Professor P Fallon, School of Medicine, Trinity College Dublin) were maintained in the Bioresources Unit, Trinity College Dublin. All experiments were performed under licence from the Department of Health and Children (Ireland) and with local ethical approval.

Behavioural analysis

The novel object recognition task is based on the innate tendency of rodents to differentially explore novel objects over familiar ones. The apparatus consisted of a white circular arena (diameter 200cm, height 50cm) placed in a dimly-lit room. Mice were habituated to the arena for 20 min in the absence of objects each day for 3 days before the experiment was performed. In the training trial the mice were presented with a pair of identical objects (A and B) positioned in the centre of the arena. Mice were placed into the arena at random entry points for 3 × 5 min trials with an inter-trial rest period of 5 min and the time spent exploring each object was recorded using stopwatches. Objects were thoroughly cleaned between trials to ensure the absence of olfactory cues. In the testing trial, performed 24 hours later, one of the familiar objects was exchanged for a novel object (C), placed at exactly the same position, and mice were reintroduced into the arena for a single 5 min trial; the time spent exploring each object was recorded as before. The time spent exploring each object (in seconds) is expressed as a percentage of

the total exploration time. The criteria for exploration were strictly based on active exploration, in which mice had to be touching the object with their nose or head. The discrimination index for the novel object is the proportion of time mice spent exploring the novel object minus the proportion spent exploring the familiar one in the testing period.

Male and female wildtype and SIGIRR^{-/-} mice (3-5 months old) were assessed for hippocampal-dependent spatial learning using the Y-maze task (Deacon et al., 2008; Cunningham et al., 2009). Mice were placed into one arm of a clear, perspex Y-maze containing water (2cm deep; 20-22°C). Mice were encouraged to escape through a burrowing tube at the distal end of another arm, and returned to their home cages. Burrowing tubes were placed at the end of the remaining arms with plugged exits, to ensure all arms appeared identical. Prominent visual cues were placed throughout the room surrounding the maze. The location of the exit was fixed for each animal and mice were placed in either one of the two possible start arms for 10 trials (15 min inter-trial interval). The groups were counterbalanced with respect to the escape arm. Escape latency was measured as time taken for each mouse to enter the exit tube, and mean values were calculated across trials 1-4, 5-7 and 8-10. Male and female mice did not demonstrate behavioural differences, and results were therefore pooled. Statistical differences between wildtype and SIGIRR^{-/-} mice across trials were determined using 2-way ANOVA and post-hoc Bonferroni tests.

Activity and anxiety were evaluated in wildtype and SIGIRR^{-/-} mice by assessing their behaviour in a hole-board arena (60cm width×60cm length×35cm height), divided into 25 equal-sized squares (16 border and 8 central squares). Mice were placed on the

outer corner of a square in the arena and activity was recorded using a video camera and advanced motion-recognition software package (Mediacruise Software, Canopus Corporation, UK) for 2 minutes. The distance covered and the time spent in the border and central squares was recorded.

Hippocampal slice preparation and LTP

Female C57BL/6 and SIGIRR^{-/-}/TIR8^{-/-} mice, and male C57BL/6 and TLR4^{-/-} mice were decapitated under isoflurane anaesthesia (Merial, Harlow, UK), brains were rapidly removed and hippocampi dissected in ice-cold, oxygenated (95%O₂/5%CO₂) artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 1.25 KCl, 1 CaCl₂, 1.5 MgCl₂, 1.25 KH₂PO₄, 25 NaHCO₃, and 10 D-glucose. Hippocampal slices (400µm) were prepared using a McIlwain tissue chopper, and incubated in a submerged chamber at room temperature for at least 1 hour prior to experimentation. Slices were transferred to a submersion recording chamber and continually perfused (2-3ml/min) with oxygenated aCSF containing (in mM): 125 NaCl, 1.25 KCl, 2 CaCl₂, 1.5 MgCl₂, 1.25 KH₂PO₄, 25 NaHCO₃, and 10 D-glucose, at room temperature (22-23°C).

The Schaffer collateral-commissural pathway was stimulated at 0.033Hz (0.1ms duration) using a bipolar tungsten stimulation electrode (Advent Materials, UK). Field excitatory postsynaptic potentials (EPSPs) were recorded from the CA1 stratum radiatum using a monopolar recording electrode. Recording electrodes (~2 MΩ) were pulled from borosilicate glass capillary tubes (Harvard Apparatus, US) and filled with aCSF. The stimulus intensity was adjusted to produce a response ~50% of maximal EPSP amplitude as determined from an input-output curve for each experiment. A stable baseline of at

least 10-20 min was recorded prior to application of theta-burst stimulation (TBS), which consisted of 10 trains (4 pulses at 100 Hz) repeated at 5 Hz. To assess the effect of pharmacological agents on LTP, IL-1ra (2 μ g/ml), HMGB1 (10ng/ml; a gift from Professor K Mills, School of Biochemistry and Immunology, TCD (Figure 6); Sigma-Aldrich, UK (Figure 7)), D-JNKi1 (2 μ M), wedelolactone (30 μ M; Enzo Life Sciences, UK), anti-TLR4 antibody (2.5 μ g/ml; Hycult Biotechnology, Netherlands or Santa Cruz Biotechnology, USA) or isotype control IgG (2.5 μ g/ml; Santa Cruz Biotechnology, USA) was applied to the perfusate 20-30 min prior to TBS, and maintained for the duration of each experiment. Data were acquired using WinWCP v4.0.7 software (Dr J Dempster, Strathclyde, UK) and evoked EPSPs were normalised to the slope recorded in the 5 min period prior to LTP induction. LTP was measured as a mean value of the final 5 min of recording (55-60 min post-TBS). Data are presented as mean percentage EPSP slope \pm SEM. Sample EPSP traces represent an average of 4 consecutive EPSPs, taken immediately prior to TBS, and 60 min following LTP induction.

Western immunoblotting

Hippocampal slices not used for electrophysiological recording were placed into the recording chamber and perfused with aCSF, in the presence or absence of pharmacological agents (20-30 min) as described above. Following perfusion, tissue was harvested and stored in lysis buffer (100 μ l; composition in mM: Tris-HCl 10, NaCl 50, Na₄P₂O₇.H₂O 10, NaF 50, 1% Igepal, phosphatase inhibitor cocktail I and II, protease inhibitor cocktail; Sigma, U.K.) at -80°C. For analysis, samples were added to 2x SDS sample buffer (composition: Tris-HCl 100mM, pH 6.8, 4% SDS, 2% bromophenol blue,

20% glycerol; Sigma, UK) and heated to 70°C for 10 min. Samples (20µg) were separated on 7, 10 or 12% standard SDS gels. Proteins were transferred to nitrocellulose membrane (Schleicher and Schuell, Germany) and blocked for 1 hour in Tris-buffered-saline-0.05% Tween 20 (TBS-T) and 5% non-fat dried milk/TBS-T at room temperature. Membranes were incubated overnight at 4°C with anti-IL-1α (1:500; R & D Systems, US), anti-IL-1β and anti-IL-1RI (1:500; Santa Cruz Biotechnology, US), anti-IRAK1 (1:1000), anti-p-IκBα (ser32) (1:1000) or anti-p-JNK (1:1000; Cell Signaling, US), anti-TLR4 (1:1000) or anti-HMGB1 (1:250; Abcam, UK) antibody in 2% non-fat dried milk/TBS-T, washed, and incubated with a secondary anti-rabbit (1:2500) or anti-goat (1:5000; Jackson ImmunoResearch, US) antibody in 2% non-fat dried milk/TBS-T for 1 hour. Immunoreactive bands were detected using Immobilon Western chemiluminescent substrate (Millipore, US), and blots were stripped (Re-blot Plus; Chemicon, US) and reprobed using anti-β-actin (1:10,000; Sigma, UK) in 2% non-fat dried milk/TBS-T and a peroxidase-conjugated secondary anti-mouse antibody (1:5000; Jackson ImmunoResearch, US) in 2% non-fat dried milk/TBS-T. Images were captured using the Fujifilm LAS-3000.

One objective of this study was to assess whether HMGB1 interacted with TLR4, and to do so, hippocampal samples were equalized to provide 500µg protein. Samples were incubated overnight at 4°C in the presence of anti-TLR4 antibody (5µg; Hycult Biotechnology, Netherlands). A/G protein agarose beads (50µl; Santa Cruz Biotechnology, US) were added, and samples were incubated for 2 hours, washed in PBS containing NP-40 (0.01%) and centrifuged. Loading buffer was added to each sample and samples were heated to 70°C. Proteins were separated by gel electrophoresis as described above and

HMGB1 was visualised by incubating the membrane in the presence of anti-HMGB1 antibody (as above).

Statistical analysis

Data were assessed using two-tailed Student's t-tests (paired or unpaired as appropriate), one-way analysis of variance (ANOVA) followed by post-hoc Student Newman-Keuls test to determine significant differences between multiple groups, or two-way ANOVA with post-hoc Bonferroni test to assess progressive changes between groups.

Results

SIGIRR deficiency has been associated with increased inflammation in several experimental models (Drexler et al., 2010; Gulen et al., 2010), and recent data indicate that LPS induces a more profound effect on proinflammatory cytokine production in SIGIRR^{-/-}, compared with wildtype mice *in vitro* and *in vivo* (Watson et al., 2010). These changes are paralleled by, and probably due to, increased microglial activation in SIGIRR^{-/-} mice (Watson et al., 2010).

Here we demonstrate that hippocampal function is impaired in SIGIRR^{-/-} mice. Using an object recognition task we found that, while wildtype mice spend a significantly greater amount of time exploring a novel object relative to a familiar object (***p < 0.001; ANOVA; Figure 1A), the time spent by SIGIRR^{-/-} mice exploring both objects was not significantly different. Accordingly, the discrimination index illustrates that the difference in exploration time between the familiar and novel objects was significantly

lower in SIGIRR^{-/-} mice than wildtypes (*p < 0.05; unpaired Student's t-test; Figure 1A). This suggests that the deficit identified in SIGIRR^{-/-} mice is due to a cognitive impairment, and not simply a consequence of reduced exploratory behaviour. There were no significant differences between wildtype and SIGIRR^{-/-} mice in terms of activity; in a hole board arena (60x60cm), the mean total distance travelled (1831cm ± 195 SEM) and 2049 cm ± 114.4 SEM) and the mean velocity (18.1cm/s ± 1.5 SEM and 20.5cm/s ± 1.2) were similar in wildtype and SIGIRR^{-/-} mice. The mean percentage time spent in the outer zone was significantly greater in SIGIRR^{-/-} mice compared with wildtype mice (81.5% ± 2.0 SEM and 63.1% ± 4.6 SEM; p < 0.01) and SIGIRR^{-/-} mice stayed closer to the outer perimeter of the arena than wildtype mice (10.06cm ± 0.25 SEM and 15.18cm ± 1.01 SEM respectively; p < 0.01). These behaviours are indicative of anxiety. Therefore we conclude that, although SIGIRR^{-/-} mice appear more anxious than wildtype mice, their activity levels are similar.

To further assess cognitive function in wildtype and SIGIRR^{-/-} mice, animals were tested for cognitive performance in the Y-maze, a task which is known to evaluate hippocampal-dependent spatial reference memory (Deacon et al., 2008; Cunningham et al., 2009). Escape latency was measured for each animal, across 10 trials, and mean values were calculated for trials 1-4, 5-7 and 8-10. Two-way ANOVA revealed that SIGIRR^{-/-} mice were significantly impaired in acquiring the task, compared with wildtypes (Figure 1B). Significant effects across trials (p < 0.0001) and mouse strains (p < 0.05) were identified, along with a significant interaction between between both (p < 0.05). Post-hoc analysis using Bonferroni test also revealed a significant difference in escape latency between wildtype and SIGIRR^{-/-} mice during trials 5-7 (**p < 0.01; Figure

1B). Similarly, the total number of arm entered by SIGIRR^{-/-} mice prior to escape was also significantly greater than wildtype mice during the same period (data not shown). Intracerebroventricular injection of LPS or IL-1 β exerted no significant effect on any of these measures in wildtype or SIGIRR^{-/-} mice (data not shown). In light of these findings, we assessed LTP of CA1 synapses in hippocampal slices prepared from wildtype and SIGIRR^{-/-} mice. TBS reliably induced robust and reproducible LTP in wildtype mice, but the mean percentage change in EPSP slope was markedly reduced (Figure 1C) and significantly attenuated in slices from SIGIRR^{-/-} mice when assessed 60 min following induction ($p < 0.0001$; unpaired Student's t-test).

To identify the potential mechanisms responsible for these impairments in hippocampal function, we first investigated the expression of the IL-1RI-mediated signal in hippocampal tissue from wildtype and SIGIRR^{-/-} mice. As SIGIRR is known to modulate this pathway through interaction with IL-1RI (Wald et al., 2003; Qin et al., 2005), it seemed likely that this may be affected as a result of SIGIRR deficiency. We identified a significant increase in expression of IL-1 α (** $p < 0.01$; unpaired Student's t-test; Figure 2A) and IL-1RI in SIGIRR^{-/-} mice relative to wildtype mice (* $p < 0.05$; unpaired Student's t-test; Figure 2B). Interestingly, hippocampal expression of IL-1 β was not altered (Figure 2A). Furthermore, expression of the downstream kinase, IL-1R-associated kinase 1 (IRAK1), was also significantly enhanced in tissue from SIGIRR^{-/-} mice (* $p < 0.05$; unpaired Student's t-test; Figure 2B). As these findings suggest that the IL-1RI mediated signalling is up-regulated in SIGIRR^{-/-} mice, we used the endogenous IL-1R antagonist (IL-1ra) to investigate whether this pathway may underlie the impairment in LTP. Application of IL-1ra (2 μ g/ml; 20 min) to slices prepared from

SIGIRR^{-/-} mice reversed the deficit in LTP, producing a response to TBS which was similar to that in slices prepared from wildtype mice ($p < 0.001$; unpaired Student's t-test; Figure 2C). It has been demonstrated that IL-1ra can prove detrimental to hippocampal LTP (Loscher et al., 2003; Ross et al., 2003; Schmid et al., 2009), and the present data confirm that extracellular application of IL-1ra (2 μ g/ml) for 20 min significantly attenuated TBS-induced LTP in hippocampal slices of wildtype mice ($p < 0.05$, unpaired Student's t-test; Figure 2C).

One downstream consequence of IL-1RI activation is phosphorylation of the stress-activated protein kinase, JNK. Here we demonstrate that hippocampal tissue from SIGIRR^{-/-} mice exhibit significantly higher levels of phosphorylated JNK (p46; pJNK) compared to wildtypes (* $p < 0.05$, unpaired Student's t-test; Figure 3A). Interestingly, treatment with IL-1ra attenuated the level of pJNK in tissue prepared from SIGIRR^{-/-} mice (* $p < 0.05$, paired Student's t-test; Figure 3B), but not wildtypes (data not shown). Importantly, incubation with D-JNKi1 (2 μ M), a specific inhibitor of pJNK, significantly alleviated the impairment in LTP observed in SIGIRR^{-/-} mice ($p < 0.01$; unpaired Student's t-test; Figure 3C), thus restoring it to levels observed in wildtype slices. The magnitude of LTP recorded in hippocampal slices of wildtype mice was not significantly altered by the presence of D-JNKi1 (data not shown).

A previous report from our laboratory identified that SIGIRR deficiency was associated with increased expression of TLR4 mRNA and CD14 mRNA, coupled with NF κ B activation (Watson et al., 2010). Here we demonstrate a significant increase in TLR4 protein in hippocampal tissue of SIGIRR^{-/-} relative to wildtype mice (** $p < 0.01$; unpaired Student's t-test; Figure 4A), and an associated increase in pI κ B α (** $p < 0.01$;

unpaired Student's t-test; Figure 4B). To assess the role of TLR4 activation on the LTP impairment observed in SIGIRR^{-/-} mice, either an anti-TLR4 antibody or isotype control IgG (2.5µg/ml) was applied to slices from wildtype and SIGIRR^{-/-} mice. Anti-TLR4 antibody did not alter LTP recorded from wildtype slices, although a brief impairment in short-term plasticity was observed, immediately following TBS application (Figure 4C). However, the presence of an anti-TLR4 antibody significantly increased the level of LTP recorded in SIGIRR^{-/-} mice, relative to values obtained with control IgG ($p < 0.01$; unpaired Student's t-test; Figure 4D). While SIGIRR^{-/-} LTP was enhanced by the anti-TLR4 antibody, this was still significantly less than values obtained in wildtype slices ($p < 0.05$; Newman-Keuls test). These findings suggest that activation of TLR4 mediates, at least partially, the deficit in synaptic plasticity in SIGIRR^{-/-} mice. Using wedelolactone, an inhibitor of IκB phosphorylation, we also assessed whether activation of NFκB was associated with this LTP impairment. Wedelolactone (30µM), applied 20 min prior to LTP induction, markedly increased the level of LTP recorded in SIGIRR^{-/-} mice ($p < 0.0001$; unpaired Student's t-test), but did not significantly alter LTP in wildtype slices (Figure 4E). Interestingly, the enhanced LTP recorded in SIGIRR^{-/-} mice in the presence of wedelolactone was also significantly greater than LTP observed in control experiments from wildtype mice ($p < 0.001$; Newman-Keuls test; Figure 4E).

HMGB1 is a ubiquitous nuclear protein, with known affinity for TLR2 and 4 (Park et al., 2006). It is released by a number of cell types, including monocytes, macrophages and neurons, in response to inflammatory stimuli, pro-inflammatory cytokines (Wang et al., 1999; O'Connor et al., 2003; Qiu et al., 2008) and during necrosis (Scaffidi et al., 2002). Additionally, HMGB1 is known to augment the activity of pro-

inflammatory stimuli such as IL-1 (Sha et al., 2008; Hreggvidsdottir et al., 2009). Here we show that expression of HMGB1 is significantly higher in hippocampal tissue from SIGIRR^{-/-} mice compared with wildtype mice (*p < 0.05; unpaired Student's t-test; Figure 5A), suggesting that it may be responsible for the up-regulated TLR4-mediated signalling and elevated IL-RI-mediated response identified in SIGIRR^{-/-} mice. An interaction between HMGB1 and TLR4 has been reported in peripheral blood mononuclear cells (Hreggvidsdottir et al., 2009). We aimed to establish whether a similar interaction occurred in hippocampal tissue, and therefore samples were immunoprecipitated with anti-TLR4 and probed with an anti-HMGB1 antibody. The findings provide clear evidence of an interaction (*p < 0.05; unpaired Student's t-test; Figure 5B). The data suggest that a greater interaction occurs in samples from SIGIRR^{-/-}, compared with wildtype mice, a likely consequence of increased HMGB1 and TLR4 in hippocampus of SIGIRR^{-/-} mice. We therefore investigated the effects of acute HMGB1 application on LTP in hippocampal slices of wildtype mice, and demonstrate that recombinant HMGB1 (10ng/ml), applied 20 min prior to TBS, substantially impaired LTP of CA1 synapses (p < 0.01; unpaired Student's t-test; Figure 6A). Additionally, HMGB1 increased pJNK (*p < 0.05; paired Student's t-test; Figure 6b) and pIκBα (**p < 0.01; paired Student's t-test; Figure 6C) in hippocampal tissue.

To further investigate the role of TLR4 in the HMGB1-mediated impairment of LTP, we carried out additional experiments on wildtype and TLR4^{-/-} mice. Using hippocampal slices from male wildtype mice, we confirmed our previous finding in females (Figure 6A) that HMGB1 (10ng/ml) can significantly attenuate LTP (p < 0.05; Student's t-test; Figure 7Ai). As before, treatment of hippocampal tissue with HMGB1

(10ng/ml; 30 min) was coupled with a significant increase in expression of pI κ B α in wildtype mice (* $p < 0.05$; Student's t-test; Figure 7Aii), indicative of NF κ B activation. The level of LTP evoked in slices from TLR4^{-/-} mice under control conditions was not different from that recorded in wildtype mice. However, there was no significant effect of HMGB1 application in TLR4^{-/-} slices when compared with control values (Student's t-test; Figure 7Bi). Similarly, treatment of hippocampal tissue from TLR4^{-/-} mice with HMGB1 (10ng/ml; 30 min) was not associated with increased pI κ B α expression (Student's t-test; Figure 7Bii).

Discussion

The current study demonstrates that SIGIRR expression in brain is essential for optimal cognitive and synaptic function. The impaired synaptic function in its absence is due to increased signalling through IL-1RI and TLR4 and the evidence indicates that increased expression of IL-1 α and the TLR ligand, HMGB1, may be the primary cause of the deficit.

LTP is widely used as an indicator of healthy brain function and, accordingly, it is impaired in a number of neurodegenerative disease models which are associated with inflammatory changes. Thus deficits in LTP, accompanied by cognitive dysfunction, have been reported in models of AD where there is an over-expression of human presenilin 1 and/or human β -amyloid (Chapman et al., 1999; Puolivali et al., 2002; Auffret et al., 2009; Townsend et al., 2010). The present data show that both LTP and hippocampal-dependent cognition were markedly decreased in SIGIRR^{-/-} mice providing

further evidence of the negative impact of inflammation on synaptic plasticity, and identifying a specific role for SIGIRR in maintenance of optimal cognitive and network function. Novel object recognition and Y-maze performances are known to be coupled with changes in LTP at hippocampal synapses (Wang et al., 2004; Clarke et al., 2010). Furthermore, impaired novel object recognition, associated with inflammatory responses, has been reported in a number of experimental models including transgenic mice expressing AD pathology (Feng et al., 2004; Heneka et al., 2006; Jardanhazi-Kurutz et al., 2010) and aged animals (Pitsikas et al., 2005; Garelick et al., 2009).

A recent report demonstrated that LPS exerted an enhanced response in glial cells and hippocampal tissue from SIGIRR^{-/-}, compared with wildtype mice (Watson et al., 2010). In addition, intraperitoneal injection of LPS decreased exploratory behaviour to a greater extent in SIGIRR^{-/-}, compared with wildtype, mice (Watson et al., 2010). The present data indicate that, in the absence of any external inflammatory stimulus, the cognitive and synaptic deficits observed in SIGIRR^{-/-} mice are associated with upregulation of IL-1RI- and TLR4-mediated signal transduction in hippocampus. While SIGIRR is known to regulate inflammation through interaction with both IL-1RI and TLR4 (Qin et al., 2005; Huang et al., 2006; Gulen et al., 2010), we now show that SIGIRR deficiency is associated with increased expression of these receptors as well as signalling mediated by receptor activation. Thus SIGIRR^{-/-} mice exhibit increased expression of IL-1 α , IL-1RI and IRAK relative to their wildtype counterparts. The key role of IL-1RI activation in mediating the decrease in LTP in SIGIRR^{-/-} mice is demonstrated by the findings that LTP is restored by IL-1ra. It is important to point out that application of IL-1ra attenuates LTP under control conditions, as previously

described by us (Loscher et al., 2003; Schmid et al., 2009) and others (Ross et al., 2003). The data also highlight a role for TLR4 activation in mediating the decrease in LTP in SIGIRR^{-/-} mice since LTP was maintained in slices prepared from SIGIRR^{-/-} mice that were perfused with an anti-TLR4 antibody. Since both IL-1ra and anti-TLR4 antibody restored LTP, it must be concluded that either TLR4 activation increases cytokine release which act in a paracrine or autocrine fashion, or that there is a convergence at key signalling events downstream of receptor activation. Consistent with the latter, the data show that activation of stress-associated signals, JNK and NFκB, which are both upregulated downstream of IL-1RI and TLR4 activation (Ninomiya-Tsuji et al., 1999), was increased in hippocampal tissue prepared from SIGIRR^{-/-} mice. Targeted inhibition of JNK and NFκB restored LTP in these mice suggesting a pivotal role for these signalling events in the impaired synaptic function. At present, we must conclude that both TLR4 and IL-1RI activation combine to increase activation of JNK and NFκB in SIGIRR-deficient mice. Enhanced activation of both have been reported in aged animals, and following LPS or Aβ administration, and is associated with impaired LTP (O'Donnell et al., 2000; Vereker et al., 2000; Kelly et al., 2003; Minogue et al., 2003; Costello and Herron, 2004; Barry et al., 2005). Similarly, inhibition of JNK or NFκB alleviates the depression of LTP observed in response to LPS and/or Aβ (Kelly et al., 2003; Minogue et al., 2003; Costello and Herron, 2004; Barry et al., 2005). Interestingly, the evidence indicates that wedelolactone, which inhibits NFκB activation (Kobori et al., 2004), increases LTP above control levels. One possible explanation for this is that by inhibiting NFκB, wedelolactone also decreases production of cytokines like IL-1β, IL-6 and TNFα, which are known to exert an inhibitory effect on LTP (Tancredi et al.,

1992; Tancredi et al., 2000; Vereker et al., 2000; Ross et al., 2003). While it has been known for some time that TLR4 activation plays a significant role in modulating hippocampal function (Barry et al., 2005; Cunningham et al., 2009; Czapski et al., 2010), it has recently been reported that hippocampal-dependent memory, accompanied by neurogenesis, was enhanced in TLR3-deficient mice (Okun et al., 2010).

Increased expression of TLR4 mRNA has been reported to accompany inflammation in a mouse model of AD, and brain tissue from AD patients (Walter et al., 2007), as well as in age-related conditions (Letiembre et al., 2007; Balistreri et al., 2009). Additionally, the therapeutic benefits of targeting IL-1 receptor for treatment of inflammatory diseases have been well described (Halle et al., 2008). SIGIRR deficiency is accompanied by increased expression of HMGB1 in hippocampus, a putative endogenous TLR4 ligand. HMGB1 was first described as a nuclear DNA binding protein, but more recently has been identified as a ligand for TLR2, TLR4 (Park et al., 2006) and receptor for advanced glycation end-products (Kokkola et al., 2005). In addition to its release in response to damage and inflammation (Scaffidi et al., 2002; O'Connor et al., 2003; Qiu et al., 2008; Maroso et al., 2010), HMGB1 acts as a pro-inflammatory cytokine within the CNS (O'Connor et al., 2003; Qiu et al., 2008). The capacity of HMGB1 to augment the activity of pro-inflammatory stimuli, such as IL-1 and LPS, has also been reported (Sha et al., 2008; Hreggvidsdottir et al., 2009). Accordingly, we show that HMGB1 attenuates hippocampal LTP and, consistent with its ability to activate TLR4, leads to phosphorylation of both JNK and I κ B. These data suggest that the combined increases in expression of HMGB1 and TLR4 in hippocampus of SIGIRR^{-/-} mice, and their enhanced association, may be responsible for the impairment in synaptic plasticity.

A recent study by Maroso and colleagues (2010) proposed a role for HMGB1-TLR4 interaction in facilitating epileptiform activity in experimental models of seizure. These authors attribute this effect to targeting of the NR2B subunit of NMDA receptors (Maroso et al., 2010), an effect similar to that previously reported following IL-1RI activation (Viviani et al., 2003). Since activity-dependent regulation of the NR2A and NR2B subunits modulates the threshold for induction of synaptic plasticity at hippocampal CA1 synapses (Xu et al., 2009), it is plausible that the HMGB1-mediated reduction in LTP is a result of modulation of NMDA receptor function. However it is acknowledged that TLR4 is expressed on glial cells (Olson and Miller, 2004; Jack et al., 2005) as well as neurons (Tang et al., 2007; Maroso et al., 2010) but the cell on which HMGB1 exerts its primary effect in this study is unknown. Similarly, while HMGB1 is released from macrophages and neurons following damage or insult (Scaffidi et al., 2002; O'Connor et al., 2003; Qiu et al., 2008), the cell source of the increased HMGB1 in hippocampus of SIGIRR^{-/-} mice remains to be determined. It is worth noting that HMGB1 is described as a late mediator of inflammation (Wang et al., 1999) and therefore it is possible that its effects are self-perpetuating.

We conclude that SIGIRR impacts upon synaptic function by negatively regulating signalling through IL-1RI and TLR4. HMGB1 is likely to be the endogenous activator of TLR4 since its expression is increased and heavily associated with the receptor in SIGIRR^{-/-} mice. A similar increase in expression of TLR4 and HMGB1 in other tissues may explain the increased inflammation reported in EAE, colitis and arthritis in SIGIRR^{-/-} mice, suggesting that pharmacological targeting of HMGB1 may be advantageous to the treatment of systemic inflammatory conditions (Yang et al., 2002). Of particular

importance is that similar pharmacological targeting may be appropriate to attenuate the loss of cognitive function associated with neurodegenerative diseases and neuroinflammatory disorders, particularly if future studies demonstrate disruption of SIGIRR or SIGIRR-regulated signalling in these conditions.

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FIGURE LEGENDS

Figure 1. Impaired cognition and long-term potentiation in SIGIRR^{-/-} mice. (A; i) Wildtype and SIGIRR^{-/-} mice were exposed to two identical objects (A and B) for 5 min periods, over 3 trials with 5 min intervals. When tested 24 hours later, wildtype mice spent a significantly greater amount of time exploring a novel object (object C), relative to a familiar object (object A; ***p < 0.001, n = 6). However, during the testing period, SIGIRR^{-/-} mice spent equal amounts of time exploring both the familiar and novel objects (n = 7). (ii) The same experimental data represented as the discrimination index illustrates that SIGIRR^{-/-} mice displayed significantly less discrimination between the familiar and novel objects compared with wildtypes, relative to the total exploration time (*p < 0.05, n = 6-7). (B) Wildtype and SIGIRR^{-/-} mice were assessed in the Y-maze. Mean escape latency was calculated for trials 1-4, 5-7 and 8-10. SIGIRR^{-/-} mice (n = 7) were impaired in acquiring the task with respect to wildtype mice (n = 8). Two-way ANOVA revealed significant effects across trials (p < 0.0001) and mouse strains (p < 0.05), and a significant interaction was identified between both (p < 0.05). Post-hoc analysis revealed a significant difference in escape latency between wildtype and SIGIRR^{-/-} mice during trials 5-7 (**p < 0.01). (C) Theta-burst stimulation induced a consistent LTP in CA1 synapses of hippocampal slices taken from wildtype mice, which persisted for a minimum of 60 min following induction (n = 9, N = 7). However, the LTP achieved in slices from SIGIRR^{-/-} mice, under the same recording conditions, was significantly reduced (p < 0.0001, n = 11, N = 8) compared with wildtype mice. Arrow indicates application of theta-burst stimulation. Inset displays sample EPSP traces taken from a

single experiment immediately prior to, and 60 min following LTP induction (scale bars: 1mV/20ms).

Figure 2. Up-regulation of IL-1RI-mediated signalling impairs LTP in SIGIRR^{-/-} mice.

(A) Western immunoblot analysis revealed a significant increase in the expression of IL-1 α (i; **p < 0.01, n = 3-4) but not IL-1 β (ii; n = 3-4) in hippocampal tissue from SIGIRR^{-/-} mice relative to wildtype mice. (B) A similar increase was identified in the expression of IL-1RI (i; *p < 0.05, n = 6) and IL-1R activating kinase 1 (IRAK1), both in its non-phosphorylated (ii; IRAK1) and phosphorylated (iii; pIRAK1) forms (*p < 0.05; n = 6) in hippocampus of SIGIRR^{-/-} mice. All target proteins were normalised to β -actin. Insets illustrate representative blots for IL-1 α , IL-1 β , IL-1RI, IRAK and pIRAK, along with respective β -actin for each blot. (C) IL-1 receptor antagonist (IL-1ra; 2 μ g/ml), applied for 20 min prior to LTP induction, significantly attenuated LTP in wildtype mice (p < 0.05, n = 5, N = 3). In the presence of IL-1ra, slices from SIGIRR^{-/-} mice produced significantly greater LTP than that observed under control conditions (p < 0.001, n = 5, N = 4), of similar magnitude to LTP recorded in wildtype mice. Arrow indicates application of theta-burst stimulation. Inset displays sample EPSP traces taken from a single experiment immediately prior to, and 60 min following LTP induction (scale bars: 1mV/20ms).

Figure 3. Inhibition of JNK rescues the deficit in LTP recorded in SIGIRR^{-/-} mice.

(A) Expression of phosphorylated JNK (p46; pJNK) was significantly increased in hippocampal tissue from SIGIRR^{-/-} mice relative to wildtype mice (*p < 0.05, n = 5-6).

(B) Interestingly, 20 min application of IL-1ra significantly reduced the level of pJNK in hippocampus of SIGIRR^{-/-} mice, relative to untreated tissue (*p < 0.05, n = 6). Overlay illustrates individual values for pJNK obtained by Western immunoblot in hippocampal tissue from SIGIRR^{-/-} mice under control conditions, and following IL-1ra treatment. Values for pJNK expression were normalised to β-actin. Insets illustrate representative blots of pJNK in wildtype and SIGIRR^{-/-} tissue (i) and in control and IL-1ra-treated tissue from SIGIRR^{-/-} mice (ii), with respective β-actin blots. (C) Application of D-JNKi1 (2μM) for 20 min prior to TBS, significantly attenuated the deficit in LTP recorded in slices from SIGIRR^{-/-} mice (p < 0.01, n = 4, N = 3), relative to un-treated SIGIRR^{-/-} slices. The level of LTP obtained was similar to that observed in wildtype control experiments. Arrow indicates application of theta-burst stimulation. Inset displays sample EPSP traces taken from a single experiment immediately prior to, and 60 min following LTP induction (scale bars: 1mV/20ms).

Figure 4. LTP impairment in SIGIRR^{-/-} mice is associated with increased hippocampal expression of TLR4, pIκBβ and NFκB activation. Western immunoblot revealed an increase in expression of (A) TLR4 and (B) phosphorylated IκBα (pIκBα) in hippocampal tissue prepared from SIGIRR^{-/-} mice, relative to wildtype mice (**p < 0.01, n = 5-6). (C) anti-TLR4 antibody did not alter LTP in wildtype slices, compared with an isotype control IgG (2.5μg/ml; n = 3, N = 2). (D) LTP recorded in SIGIRR-deficient slices (n = 3, N = 3) was significantly enhanced relative to values obtained in the presence of a control IgG (p < 0.01, n = 4, N = 3). (E) Application of the pIκBα inhibitor, wedelolactone (wedelo.; 30μM) for 20 min prior to TBS, did not significantly alter LTP

recorded in hippocampal slices from wildtype mice ($n = 4$, $N = 3$). However, the level of LTP recorded from SIGIRR^{-/-} mice in the presence of wedelolactone was significantly increased ($p < 0.0001$, $n = 4$, $N = 3$), relative to un-treated SIGIRR^{-/-} slices and indeed un-treated wildtype slices ($p < 0.005$). Arrow indicates application of theta-burst stimulation. Inset displays sample EPSP traces taken from a single experiment immediately prior to, and 60 min following LTP induction (scale bars: 1mV/20ms). Expression levels of target proteins are normalised to β -actin. Insets illustrate representative blots of (A) TLR4 and (B) pI κ B α , along with respective β -actin blots.

Figure 5. SIGIRR deficiency is associated with enhanced expression of HMGB1.

(A) Levels of HMGB1 were increased in hippocampus of SIGIRR^{-/-} mice relative to hippocampal tissue from wildtype mice ($*p < 0.05$, $n = 6$). Expression levels of HMGB1 are normalised to β -actin. (B) Hippocampal samples (equalized for protein; 500 μ g), immunoprecipitated with anti-TLR4 antibody and probed for HMGB1, revealed an association between HMGB1 with TLR4 which was enhanced in tissue from SIGIRR^{-/-} mice ($*p < 0.05$; $n = 4$); a sample immunoblot (upper blot (TLR4 IP)) is presented. The lower immunoblot indicates that HMGB1 expression in tissue lysate (ie in which no immunoprecipitation was undertaken) was greater in tissue from SIGIRR^{-/-} mice.

Figure 6. Acute application of HMGB1 impairs LTP and increases hippocampal expression of pJNK and pI κ B α . (A) Application of HMGB1 (10ng/ml) for 20 min substantially reduced the level of LTP obtained 60 min following induction ($p < 0.01$, $n = 9$, $N = 6$) relative to untreated controls ($n = 8$, $N = 6$). Arrow indicates application of

theta-burst stimulation. Inset displays sample EPSP traces taken from a single experiment immediately prior to, and 60 min following LTP induction (scale bars: 1mV/20ms). Treatment of hippocampal slices with HMGB1 (10ng/ml) for 20 min lead to a significant increase in pJNK (B; * $p < 0.05$, $n = 5-6$) and pI κ B α (C; ** $p < 0.01$, $n = 5-6$), relative to tissue incubated in the absence of HMGB1. Protein expression was determined by Western immunoblot, and values were normalised to β -actin. Insets illustrate representative blots of (B) pJNK and (C) pI κ B α , along with respective β -actin blots.

Figure 7. HMGB1 does not alter LTP or pI κ B α expression in hippocampus of TLR4^{-/-} mice. (A; i) Application of HMGB1 (10ng/ml) for 30 min attenuated the level of LTP obtained in male wildtype mice 60 min following induction ($p < 0.05$, $n = 4$, $N = 4$) relative to untreated controls ($n = 4$, $N = 4$). Treatment of hippocampal slices with HMGB1 (10ng/ml) for 30 min significantly increased pI κ B α expression (ii; * $p < 0.05$, $n = 4$), relative to tissue incubated in the absence of HMGB1. (B; i) LTP recorded in hippocampal slices from male TLR4^{-/-} mice was not significantly altered following HMGB1 application (10ng/ml; 30 min; $n = 4$, $N = 4$) compared with control values obtained from TLR4^{-/-} slices ($n = 4$, $N = 4$). (B; ii) Hippocampal slices from TLR4^{-/-} mice treated with HMGB1 did not show altered expression of pI κ B α when compared with untreated tissue from TLR4^{-/-} mice ($n = 4$, $N = 4$). (i) Arrows indicate application of theta-burst stimulation. Insets display sample EPSP traces taken from a single experiment immediately prior to, and 60 min following LTP induction (scale bars: 1mV/20ms). (ii) Protein expression was determined by Western immunoblot, and values were normalised

to β -actin. Insets illustrate representative blots of $pI\kappa B\alpha$, along with respective β -actin blots.

FIGURE 1

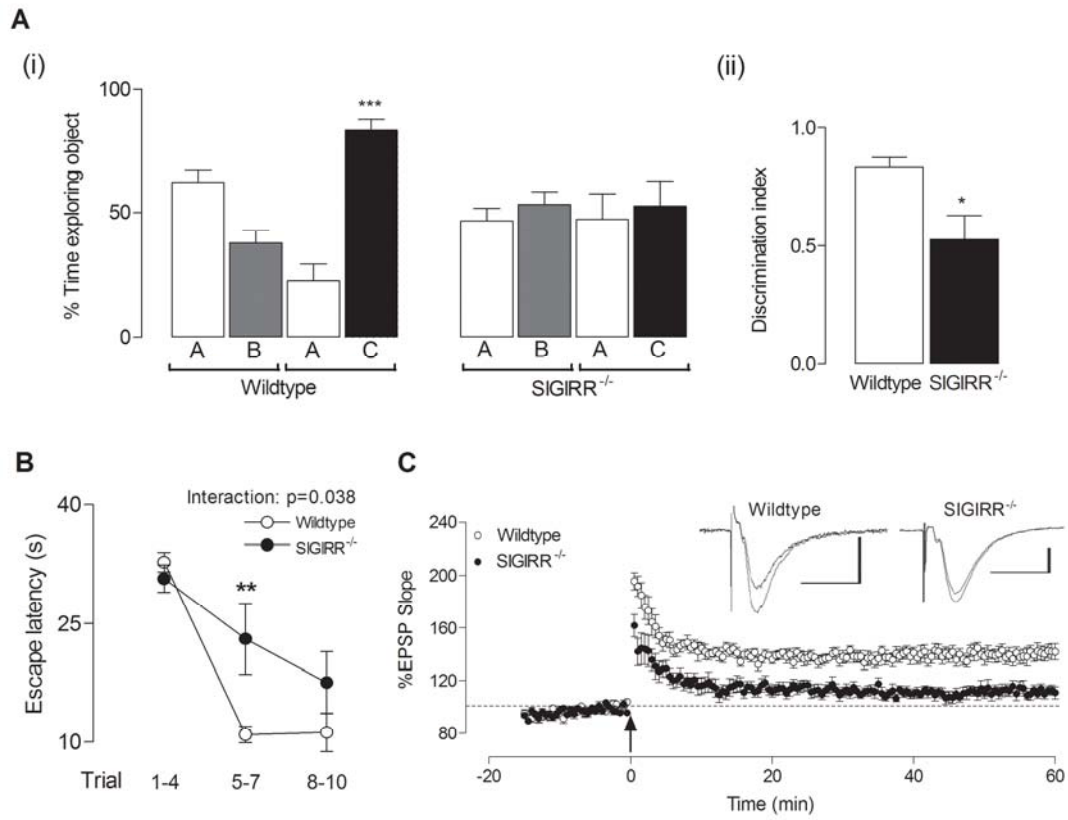


FIGURE 2

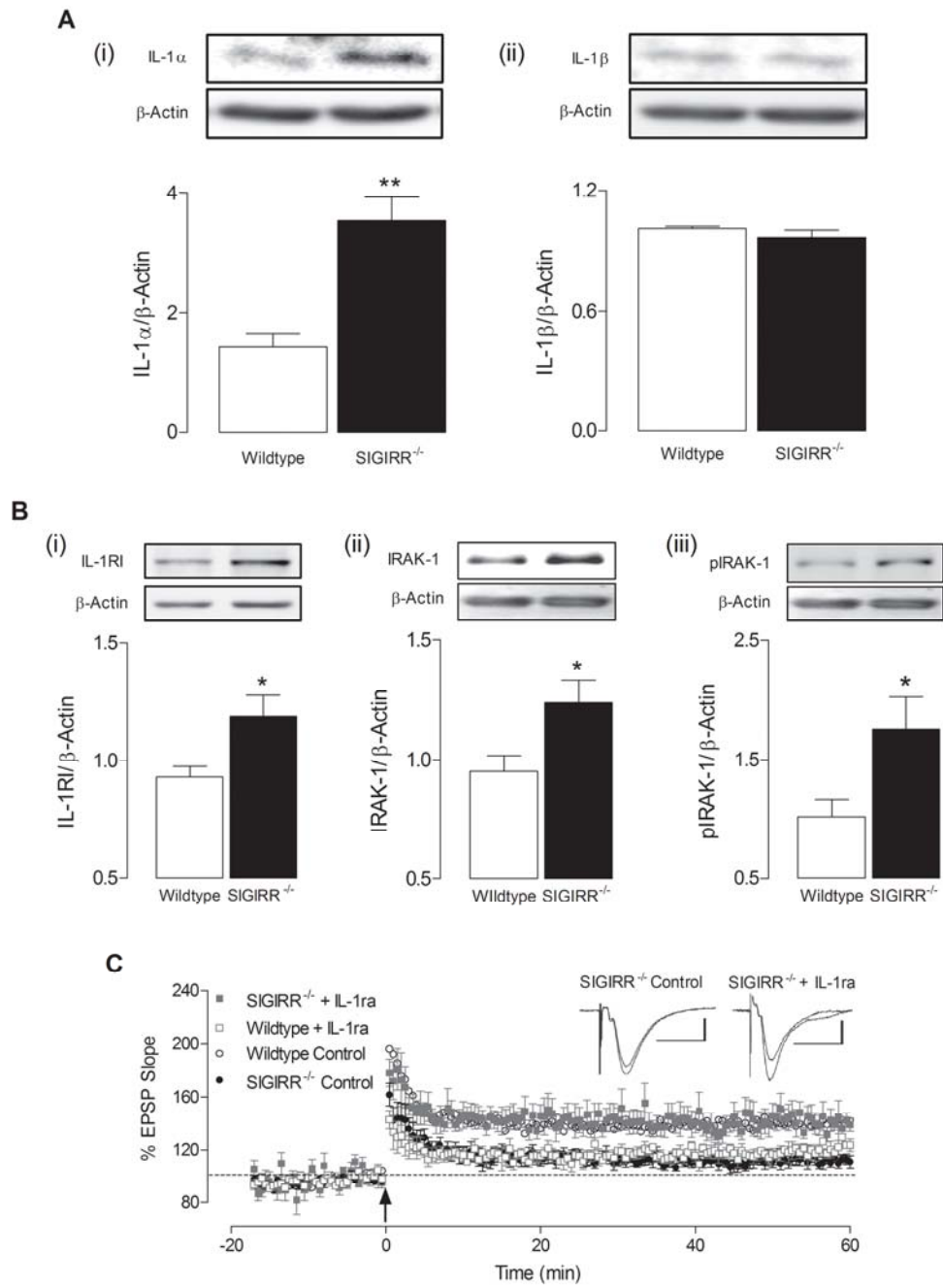


FIGURE 3

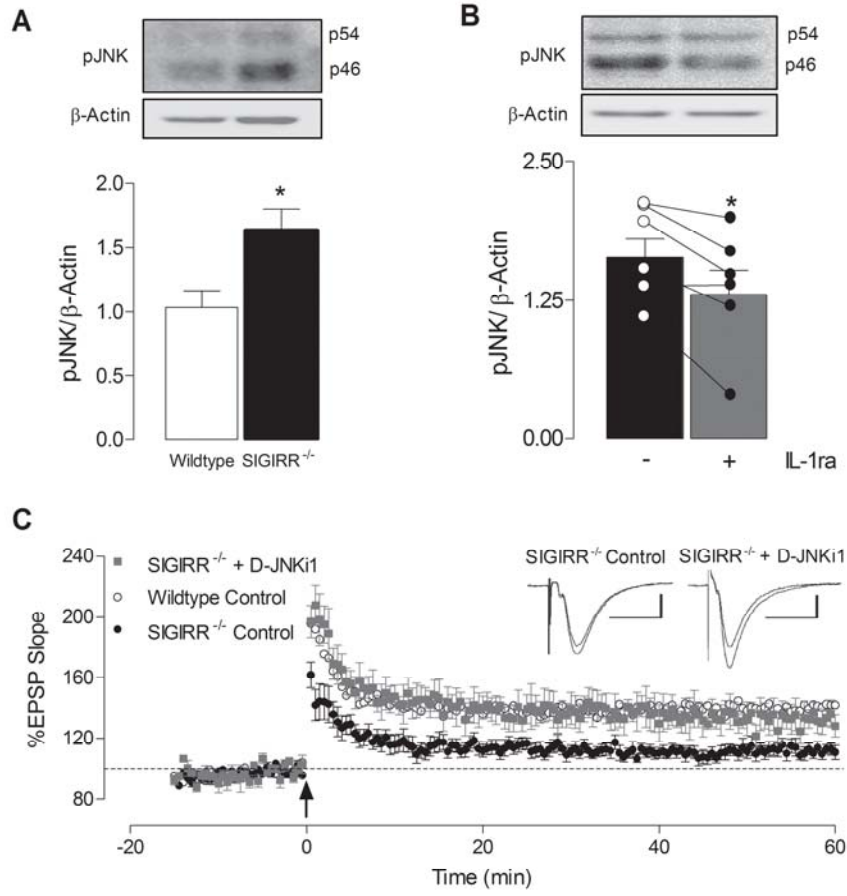


FIGURE 4

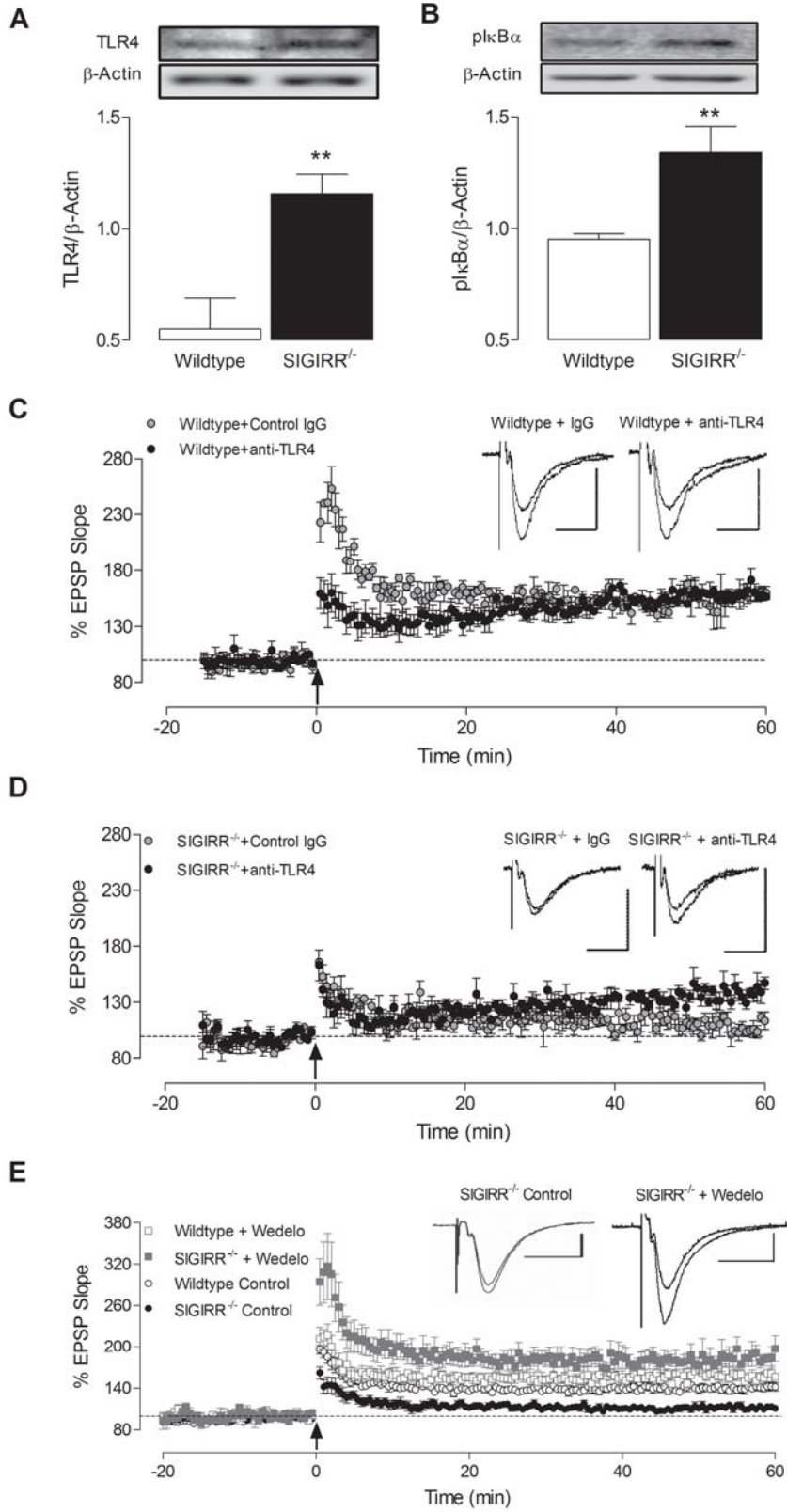


FIGURE 5

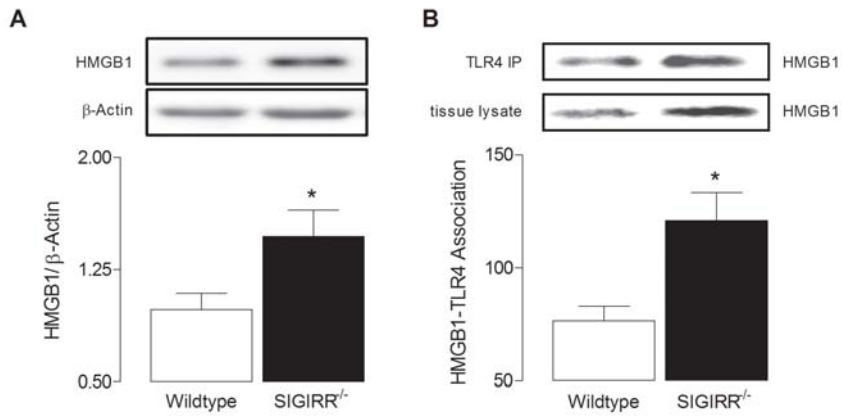


FIGURE 6

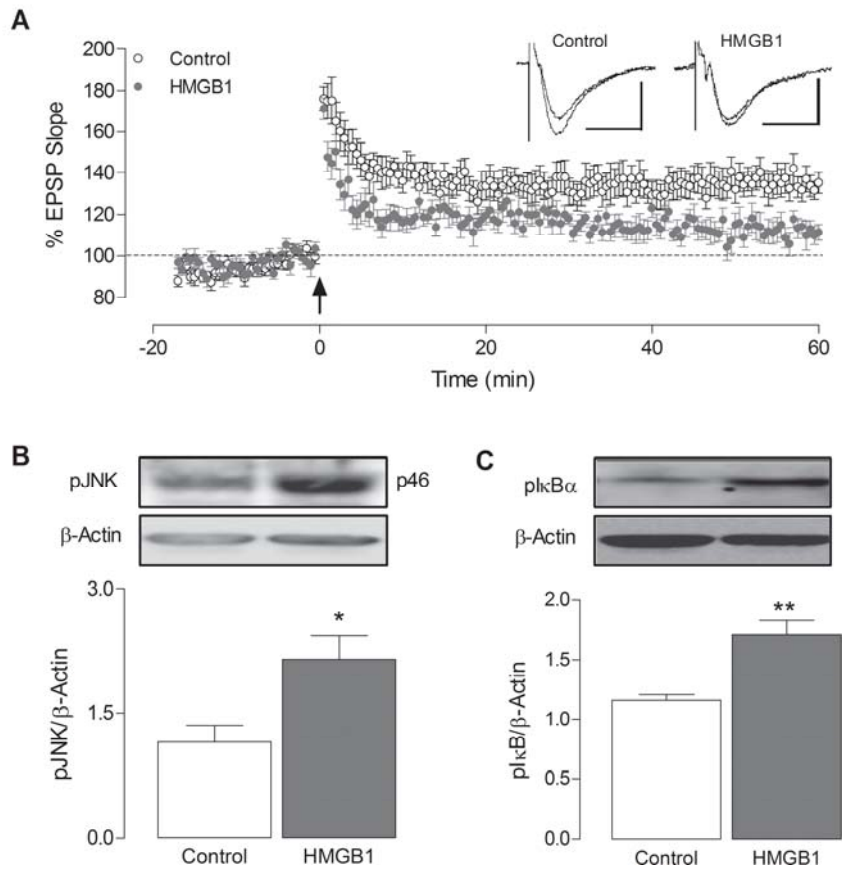


FIGURE 7

