

Tumor necrosis factor- α potentiates long-term potentiation in the rat dentate gyrus after acute hypoxia

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ABSTRACT

An inadequate supply of oxygen in the brain may lead to the introduction of an inflammatory response through neuronal and glial cells that can result in neuronal damage. Tumor necrosis factor alpha (TNF- α) is a pro-inflammatory cytokine that is released during acute hypoxia and can have neurotoxic or neuroprotective effects in the brain. TNF- α has been shown by a number of research groups to alter synaptic scaling and also to inhibit long-term potentiation (LTP) in the hippocampus when induced by specific high frequency stimulation protocols. In this study we have examined the effects of TNF- α on synaptic transmission and plasticity in hippocampal slices after acute hypoxia using two high frequency stimulation protocols. Field excitatory postsynaptic potentials were elicited in the medial perforant pathway of the dentate gyrus. Exogenous TNF- α (5 ng/ml) attenuated LTP induced by theta burst stimulation but had no effect on LTP induced by a more prolonged high frequency stimulation (HFS). Pre-treatment with lipopolysaccharide (100 ng/ml) or TNF- α but not IL-1 β (4 ng/ml) prior to a 30 min hypoxic insult resulted in a significant enhancement of LTP post hypoxia when induced by the HFS. Anti-TNF, 3,6 dithiothalidomide (a TNF- α synthesis inhibitor), SB203580 (a p38 MAPK inhibitor) and NMDA receptor block significantly reduced this effect. Application of the hypoxic mimetic, dimethylxalylglycine a prolyl hydroxylase inhibitor, did not enhance LTP in the presence of TNF- α . These results demonstrate an important modulatory role for elevated TNF- α levels on LTP in the hippocampus after an acute hypoxic event.

Key words:

Tumor Necrosis Factor- α ; Interleukin-1 β ; Lipopolysaccharide; Long term potentiation; Dentate Gyrus; Hypoxia; High frequency stimulation; Theta burst stimulation; 3,6' Dithiothalidomide; 1,6'-Dithioevlimid; CNTO1081; Prolyl hydroxylase inhibition.

Abbreviations

α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA); artificial cerebrospinal fluid (aCSF); cAMP response element-binding protein (CREB); Dimethylxalylglycine (DMOG); 3,6'-Dithiothalidomide (3,6'DT); 1,6'-Dithioevlimid (1,6'DR); 8-cyclopentyl-1,3-dipropylxanthine (DPCPX); 4-[5-(4-Fluorophenyl)-

2-[4-(methylsulphonyl)phenyl]-1H-imidazol-4-yl]pyridine hydrochloride (SB203580); field excitatory postsynaptic potentials (fEPSPs); High frequency stimulation (HFS); Interleukin-1 β (IL-1 β); Lipopolysaccharide (LPS); Long term potentiation (LTP); p38 Mitogen activated kinase (p38 MAPK); Prolyl hydroxylase (PHD); Theta burst stimulation (TBS); Tumor necrosis factor- α (TNF- α).

INTRODUCTION:

It is well established that a continuous supply of oxygen is crucial for the brain to maintain normal function. A short period of hypoxia leads to a cascade of events resulting in neuronal damage and death. An insufficient supply of oxygen to the brain results in oxidative stress followed by the release of pro-inflammatory mediators such as tumor necrosis factor alpha (TNF- α) and interleukin 1 β (IL-1 β , Batti et al., 2010; Row et al., 2003; Marchetti et al., 2004). TNF- α is a well-known pro-inflammatory cytokine released from both neuronal and glial cells and has been associated with the pathophysiology of various diseases and processes such as neuroprotection and synaptic signaling (Baune et al., 2012; Pickering et al., 2005). The modulation of synaptic signaling by TNF- α is thought to be via the activation of TNF R1 and R2 receptors (TNFR1 and TNFR2) on both the pre- and post-synaptic membranes of neurons and glia (Marchetti et al., 2004; Belarbi et al., 2012). TNFR1 activation has been shown to be associated with either hippocampal cell death or cell survival whereas TNFR2 activation may lead to neuroprotection (He et al 2012; Baune et al., 2012). It has been postulated that the neuronal expression ratio of TNFR1: TNFR2 post TNF- α binding may influence synaptic signaling observed in the CNS (Marchetti et al., 2004). Also the synthesis inhibition of TNF- α can restore neuronal function as well as reverse cognitive deficits through the attenuation of inflammation (Belarbi et al., 2012; Tweedie et al., 2012).

It has been observed that hypoxic exposure in the hippocampus leads to cognitive deficits due to impairment in synaptic plasticity, specifically long-term potentiation (LTP; Row et al., 2003; Furling et al., 2000; Belarbi et al., 2012). Previous work has shown that there is differential susceptibility to hypoxia in the hippocampus with the CA1 region more vulnerable than the dentate gyrus region (Kreisman et al., 2000). We have previously shown that chronic intermittent hypoxia and dimethylxalylglycine (DMOG, a prolyl hydroxylase inhibitor) treatment leads to LTP impairment in the CA1 but not the dentate gyrus (Wall et al., 2014). It has also been shown that TNF- α and IL-1 β do not affect low frequency synaptic transmission but impair LTP within the CA1 (Tancredi et al., 1992; Bellinger et al., 1993) and dentate gyrus (Cunningham et al., 1996; Butler et al., 2004; Batti et al., 2010; Cumiskey et al., 2007).

The modulation of inflammation in the CNS not only leads to increases in pro-inflammatory cytokines like TNF- α but also glutamate from glial cells (Stellwagen et al., 2005; Belarbi et al., 2012). This increased concentration of glutamate release onto post synaptic AMPA receptors (AMPA receptors) may be due to changes in TNF- α expression (Baune et al., 2012). Surface expression of AMPARs has been shown to be increased on the post-synaptic membrane of neurons in the presence of TNF- α through activation of

TNFR1 (Stellwagen & Malenka, 2006; Stellwagen et al., 2005). Other work has shown that the elevation in both glutamate and TNF- α after a transient ischemic attack provides protection against an ischemic insult possibly through TNFR1 (Wang et al., 2007; Watters & O'Connor, 2011; Watters et al., 2012). The induction of LTP in the dentate gyrus has been associated with an increase in the concentration of glutamate in the synaptic cleft and the interaction between TNF- α and glutamate receptors is thought to play a role in both learning and memory (Richter-Levin et al., 1995; Baune et al., 2012). Also as a result of hypoxia, an increase in extracellular adenosine has been observed that can exert a neuroprotective and anti-inflammatory effect (Dale et al., 2000). This increased adenosine causes a depression of synaptic transmission, which is primarily mediated through the adenosine A₁ receptor (A1R).

Our laboratory has recently shown that the addition of exogenous TNF- α during an acute hypoxic event can impair the recovery of synaptic transmission post hypoxia in the CA1 region (Batti & O'Connor, 2010). This effect was reversed with pre-treatment of the p38 MAP kinase inhibitor, SB3203580. More recently Zhang et al., (2014) have shown that the addition of LPS (which may elevate TNF- α levels) to the hippocampus during hypoxia can induce an LTD of synaptic transmission. Since the functional role of TNF- α in hypoxia and indeed ischemia is divergent we have investigated if the addition of TNF- α during hypoxia will have modulatory effects on synaptic plasticity post hypoxia. We have performed our experiments in the medial perforant path of the dentate gyrus (MPP) to be able to compare this and previous work to that in the CA1 region (Butler et al., 2004; Coogan et al., 1999; Curran et al., 2003; Wall et al., 2014). Using external field recording techniques in the dentate gyrus region of the hippocampal slice preparation we have investigated these effects and the role that p38 MAPK and adenosine receptor inhibition may play in them. Previous studies investigating the effects of TNF- α on synaptic plasticity have used a wide range of stimulation protocols to induce LTP. In the present study we have used both a theta burst (TBS) and a high frequency stimulation (HFS) protocol to elicit LTP in the presence or absence of TNF- α . We report that by using the HFS protocol but not the TBS, a significant enhancement of LTP post hypoxia occurs when exogenous TNF is applied.

MATERIALS AND METHODS:

Hippocampal Slice Preparation

The Animal Research Ethics Committee of the Biomedical Facility of University College Dublin approved the experimental procedures carried out in these experiments. Three to four-week old male Wistar rats (P21-28) were anaesthetized with 5% Isoflurane and decapitated using a guillotine. The brains were immediately removed and placed in ice-cold cutting solution comprising 120mM NaCl, 26mM NaHCO₃, 1.25mM NaH₂PO₄, 2.5mM KCl, 10mM glucose, 2mM MgSO₄ and 2mM CaCl₂. The solution was kept bubbling with 95%O₂/ 5%CO₂ on ice. The forebrain and cerebellum of the extracted brain were removed with a blade and 350 μ m thick transverse hippocampal sections were sliced using a vibratome (LEICA VT1000S) in ice-cold cutting solution. The separated slices were transferred to a holding chamber containing 150mL ice-cold cutting solution perfused with 95%O₂/5%CO₂ for 1 hour at room temperature to recover. Hippocampal slices were placed in a submerged recording chamber connected to a 50mL

reservoir of aCSF comprised of 120mM NaCl, 26mM NaHCO₃, 1.25mM NaH₂PO₄, 2.5mM KCl, 10mM glucose, 1.2mM MgSO₄ and 2mM CaCl₂, bubbling at 95%O₂/5%CO₂ at a maintained temperature of 32-33°C with a flow rate of 5mL/min. The slice was allowed to recover in the recording chamber for a minimum of 20 min prior to any recordings. Previous work in our laboratory has used fluorescence-quenching oxymetry to obtain oxygen levels on the surface and within the hippocampal slices in control and during hypoxic conditions (see Batti et al., 2010). The hypoxic condition (95%N₂/5%CO₂) was administered for 30 min followed by recovery through re-oxygenation (95%O₂/5%CO₂) of hippocampal slices. The temperature in the bath was maintained at 32-33°C throughout.

Electrophysiology field recordings

Monopolar glass electrodes (GC150F-10 Harvard Apparatus Ltd) used for recording and stimulating were pulled with a micropipette puller (AVANTEC Stutter Instrument Co.), and filled with aCSF. Field excitatory postsynaptic potentials (fEPSPs) were elicited at a frequency of 0.033 Hz in the medial perforant pathway of the dentate gyrus. Responses were increased 1000-fold using a DAM50 differential amplifier (World Precision Instruments) low pass filter 0.1Hz, high pass filter 10KHz and recorded onto a PC using WCP 3.10 software (J Dempster, Strathclyde). When paired stimuli are applied to this pathway, paired pulse depression is often observed in contrast to stimulation of the lateral perforant pathway (LPP). Also by placing recording and stimulating electrodes closer (<50 μ m) to the cell bodies of the granule cells, this PPD is observed (O'Leary et al., 1997).

Stimulation protocols

Prior to any recording from hippocampal slices, input/output curves were carried out to determine the stimulus voltage required to evoke 50% and 70% of the maximal measured EPSP amplitude. For LTP to be observed in the dentate gyrus, picrotoxin was added to the perfusing solution. The slices were stimulated to acquire a baseline recording at 50% maximal EPSP amplitude for a minimum of 20 min. In some experiments paired pulse stimuli were applied at an interval of 50 ms in order to observe paired pulse depression (see O'Leary et al., 1997). Long-term potentiation was elicited under two conditions 1) by theta burst stimulation (TBS), 5 trains of 10 stimuli at 100 Hz each separated by 200 ms (at 50% maximal fEPSP amplitude) or 2) by high frequency stimulation (HFS) 3 trains of 100 stimuli at 100Hz each separated by 20 s (at 70% maximal fEPSP amplitude). All recordings were analyzed using Strathclyde electrophysiology software, WCP version 3.10 (J. Dempster, Strathclyde).

Drugs

((2R)-amino-5-phosphonovaleric acid; (2R)-amino-5-phosphonopentanoate) (AP-5), Picrotoxin, 4-[5-(4-Fluorophenyl)-2-[4-(methylsulphonyl)phenyl]-1H-imidazol-4-yl]pyridine hydrochloride (SB203580) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were obtained from Sigma-Aldrich Co. Ltd., UK. Picrotoxin and DPCPX were dissolved in dimethyl sulfoxide (DMSO) with a final concentration in the aCSF reservoir of 100 μ M and 100 nM respectively. SB203580, AP-5 and LPS were dissolved in sterile filtered PBS and added to the aCSF reservoir to give a final concentration of 1 μ M, 50 μ M and 100 ng/ml respectively. DMSO controls were carried out at the same dilution (0.025% V/V). Thalidomide analogues 3,6'-

dithiothaldomide (3,6'-DT) and 1,6'-dithiorevlimid (1,6'-DR), were synthesized (Zhu et al., 2003; Luo et al., 2008) and chemical characterization verified the structure and demonstrated purity of greater than 99% for each. The final concentration in the aCSF reservoir was 20 μ M. Anti-rat TNF antibody, CNTO1081 (anti-TNF) was a gift from Johnson & Johnson was used at a final concentration of 50 μ g/ml. Recombinant rat TNF- α (Biosciences, UK) was periodically tested for potency using routine NFkB reporter assays. Application of 5 ng/ml TNF- α typically gave rise to a one hundred-fold increase in luciferase activity for NFkB (data not shown). All inhibitors were added to the perfusing medium through a 1.2 mM aCSF reservoir. Following stable baseline recordings dimethylxaloylglycine (DMOG) was added to perfusate for 30 min then washed out. All other drugs, once added, were present for the entire duration of the experiment. Control data was gathered on different days/weeks throughout the time course of these experiments. For the timeline of all the hippocampal slice experiments see Figure 1A.

Primary Hippocampal Cell Cultures

P1 Wister rat pups were decapitated and the heads were immediately placed in a 140 mm petri dish containing ice cold Hanks Balanced Salt Solution (Sigma). Under a light microscope, the brains and cerebellum were removed and the hemispheres were separated using a fine tipped tweezers. The meninges were removed along the septum, hypothalamus and thalamus; the hippocampus was visualized and removed. All hippocampi were placed in a 15 ml sterile Greiner and brought to a volume of 5 ml with buffered Hanks solution. 100 μ l of 2.5% trypsin (Sigma Aldrich) was added to the cells, they were left to incubate at 37°C for 10-15 minutes in a water bath. Under a laminar flow hood, the HBSS and trypsin solution was removed as to not disturb the pellet. 1ml of cortical plating medium (CPM) with 5% fetal bovine serum (FBS) was added to the pellet, which terminated the enzymatic reaction. CPM contained neurobasal medium (NBM, Gibco), 2% B27+ supplement (Gibco), 0.5mM l-glutamine, 2.5 μ g amphotericin B (Sigma), 100U/ml penicillin and 0.1mg/ml streptomycin (Sigma Aldrich). Using a sterile pipette and sterile flame polished glass pipette the tissue was titrated to dissociate the cells. The cells were then spun at 1000rpm for 5 minutes, supernatant was removed, and the pellet was re-suspended in 4ml warm CPM. 15 μ l of the cell suspension was added to 15 μ l 0.4% Trypan Blue (Sigma Aldrich), cells were counted using a hemocytometer under a light microscope. The cells were added to poly-lysine coated glass coverslips in sterile 12 well plates at a high density, 152,000 cells/ml. The cells were incubated in a 95%O₂/5%CO₂ sterile environment at 37°C. After 4 DIV and every 3 days until treatment, 50% of the CPM was replaced with warm maintenance media, which had the same composition of CPM but had a 2% B27-AO supplement instead of 2% B27+AO.

A lactate dehydrogenase assay was carried out at 9 DIV. A concentration of 0.5 ng/ml, 5 ng/ml and 50 ng/ml of TNF- α were applied for 2½ hr in triplicate to 12 well plates with the control samples containing no drugs. Samples of medium were taken pre-treatment, immediately after treatment and 24 hr after treatment. A lactate dehydrogenase assay was carried to using a TOX-7 assay kit (Sigma). Absorbance of samples which corresponded to levels of LDH released before treatment, immediately after treatment and 24 hr after treatment were read at absorbance of 490 nm using a Walla

Victor 2 plate reader. Results are given as a percentage of the total number of cell lysed.

Statistical Analysis

Bar chart values in Figure 1 were analysed using Students t-test. For time course data in figures 2 to 8, 5 min readings (10 recordings) were taken from critical time points during the experiments, that is the baseline, the end of acute hypoxic exposure and also from 55 to 60 min post LTP induction. These values were analysed using one way ANOVA with Tukey post-test analysis. In figures 6, 7 and 8, two way ANOVA with post Bonferroni test was carried out to compare the different sets of data. All data was expressed as means \pm standard error mean. P values of P<0.05 or greater were considered to be statistically significant.

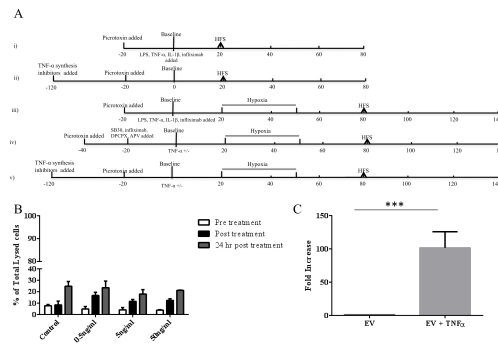


Figure 1

Timeline of experiments and effects of TNF- α on primary hippocampal cells

A i) fEPSP amplitude was recorded for 20 min in the presence of 100 μ M picrotoxin before LPS (100ng/ml), TNF- α (5 ng/ml), IL-1 β (4 ng/ml) or anti-TNF (12 ng/ml) were added to the bath. After a 20 min stable baseline recording theta burst or HFS was applied and fEPSP amplitude recorded for 1 hr. ii) 120 min before baseline recordings began the TNF- α synthesis inhibitors 3,6'DT (20 μ M) and 1,6'DR (20 μ M) were added to the bath. The protocol then continued as in i). iii) fEPSP amplitude was recorded for 20 min in the presence of 100 μ M picrotoxin before LPS (100 ng/ml), TNF- α (5 ng/ml), IL-1 β (4 ng/ml) or anti-TNF (12 ng/ml) were added to the bath. After a 20 min stable baseline recording, an acute hypoxic episode (95%N₂/5%CO₂) was administered for 30 min followed by recovery through re-oxygenation (95%O₂/5%CO₂) of hippocampal slices for 30 min. Theta burst or HFS was then applied and fEPSP amplitude was recorded for 1 hr. iv) As protocol iii) but with SB203580 (1 μ M), anti-TNF (12 ng/ml), DPCPX (200 nM) or APV (50 μ M) added to the bath. Experiments were carried out for each of the drug treatments in the presence or absence of TNF- α (5 ng/ml). v) 120 min before baseline recordings began the TNF- α synthesis inhibitors 3,6'DT (20 μ M) or 1,6'DR (20 μ M) were added to the bath. The protocol then continued as in iv). B) LDH analysis of the effects of TNF- α treatment on primary hippocampal cells. TNF- α concentrations of 0.5 ng/ml, 5 ng/ml and 50 ng/ml were applied to the cells and samples taken pre treatment (white), 2.5 hr after application (black) and 24 hr after TNF- α treatment (grey; n=4 for all bars). All data is expressed as mean \pm sem.

RESULTS:

Effect of TNF- α on primary hippocampal cultured cells

In order to establish that TNF- α (5ng/ml; 300pM) was non-toxic over the time course of electrophysiological recordings, an LDH assay was performed. TNF- α at concentrations of 0.5, 5 and 50 ng/ml applied to primary hippocampal cell cultures had no significant effect on LDH levels compared to controls when taken immediately after treatment or 24 hr later indicating that the TNF- α at the concentrations used in our experiments was non-toxic to the cells (Figure 1B).

TNF- α and IL-1 β inhibit LTP following theta burst stimulation.

It has been suggested that theta wave activity can influence memory formation and TBS has previously been shown to be a robust protocol for inducing LTP (Larson et al., 1986). Therefore the effects of acute application of pro inflammatory cytokines on the induction of LTP by TBS was determined. Theta burst stimulation was used to induce LTP in controls and brain slices perfused with TNF- α (5 ng/ml) or IL-1 β (4 ng/ml) following a 20 min stable baseline (Figure 2A, B). Robust LTP was induced in controls following theta burst stimulation (white bar; 131.9 \pm 2.6%, n=5). Both TNF- α (black bar) and IL-1 β (hatched bar) gave rise to a significant impairment of LTP at 55-60 min post induction (107.3 \pm 5.5% and 97.9 \pm 7%, respectively; n=5 for both; Figure 2 C).

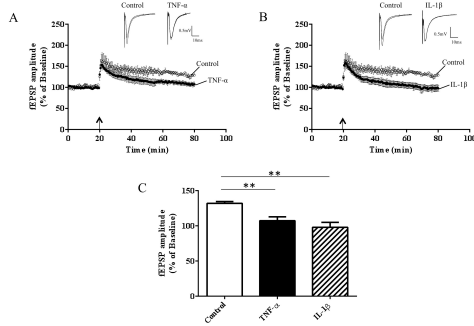


Figure 2
Time course showing changes in fEPSP amplitude after induction of LTP using the theta burst stimulation protocol. A) Effects of TNF- α (5 ng/ml; closed circles) on LTP. Baseline fEPSP amplitude was recorded for 20 min followed by theta burst stimulation (indicated by the arrow). LTP was monitored for 1 hr post induction. Insets show sample fEPSP traces, 60 min following theta burst stimulation superimposed over a representative fEPSP 10 min prior to theta burst stimulation for controls (left) and TNF- α treated slices (right). B) Effects of IL-1 β (4 ng/ml; closed circles) on theta burst induced LTP. Baseline fEPSP amplitude was recorded for 20 min followed by theta burst stimulation (indicated by the arrow). LTP was monitored for 1 hr post induction. Insets show sample fEPSP traces, 60 min following theta burst stimulation superimposed over a representative fEPSP 10 min prior to theta burst stimulation for controls (left) and IL-1 β treated slices (right). C) Summary data showing fEPSP amplitude 55-60 min following theta burst stimulation, for controls (white bar), TNF- α (black bar) and IL-1 β (hatched bar) treated groups. All data is expressed as mean \pm SEM %, n=5; ** P<0.01 compared to controls.

LPS, TNF- α and IL-1 β do not inhibit LTP following high frequency stimulation.

Previous studies have shown conflicting results on the effects of pro inflammatory cytokines on synaptic plasticity. While some have found the presence of TNF- α or IL-1 β caused impairments in LTP induction, others have found that they do not have significant effects on LTP (Cunningham et al., 1996; Bellinger et al., 1993; Tancredi et al., 1992; Stellwagen et al., 2006). HFS (3 trains of 1s at 100Hz) is one such protocol that has been successfully used as an induction protocol for LTP previously (Stellwagen et al., 2006). LTP induced by HFS gave rise to a robust LTP that was not significantly different from theta burst induced LTP (132.8 \pm 7.1%, n=10). Application of LPS (100 ng/ml), TNF- α (5 ng/ml) or IL-1 β (4 ng/ml) had no significant effect on LTP measured at 55-60 min compared to controls (137.6 \pm 4.9%, 128.6 \pm 8.4% and 132.5 \pm 3.4%, respectively, n=5 for all; Figure 3A-D). Additionally none of the TNF- α

inhibitors used in these experiments had a significant effect on HFS induced LTP (anti-TNF, 131.9 \pm 2.6%; 3,6' DT, 134.7 \pm 12.8%; 1,6' DR, 144.8 \pm 19%; n=5 for all groups; Figure 3E-G). A summary of these results 55-60 min post induction of LTP is shown in Figure 3H.

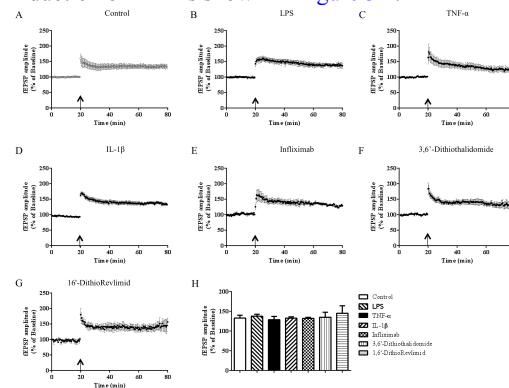


Figure 3
Time course showing changes in fEPSP amplitude after induction of LTP using the high frequency stimulation protocol in the dentate gyrus region. Baseline fEPSP amplitude was recorded for 20 min followed by HFS (indicated by the arrow). LTP was monitored for 1 hr post induction. (A) Changes in fEPSP amplitude before and after HFS in control slices. B) to G) Time course showing changes in fEPSP amplitude when the indicated drug was applied to slices in the dentate gyrus region. Each of the drugs were applied 20 min prior to baseline recordings. LTP was induced by HFS (indicated by the arrow) and monitored for 1 hr post induction in all cases. H) Summary data showing fEPSP amplitude 55-60 min following HFS, for controls (white bar), LPS (hatched bar), TNF- α (black bar), IL-1 β (reverse hatched bar), anti-TNF (chequered bar), 3,6'DT (vertical striped bar) and 1,6'DR (horizontal striped bar) treated groups. No drug had a significant effect on LTP on their own. All data is expressed as mean \pm SEM %, n= 5 for all figures.

TNF- α potentiates LTP after acute hypoxia induced by HFS but not TBS

Experiments were then carried out to determine the effects of TNF- α on LTP induction in the dentate gyrus following acute hypoxia. Figure 4 shows the timecourse of events when slices were exposed to 30 min of hypoxia followed by induction of LTP. In controls during hypoxia, fEPSP amplitude was reduced to 51.7 \pm 1%, (n=6). Following re-oxygenation fEPSP amplitude recovered to 76.2 \pm 2.4% of controls (Figure 4A). Following recovery from a 30 min hypoxic episode LTP was induced in slices using HFS or theta burst stimulation. There was no significant difference in the magnitude of LTP induced following either stimulation protocol (HFS, 125.9 \pm 3.4%, n=6, versus theta burst stimulation, 112.5 \pm 2.6%, n=5 at 55-60 min post LTP). In the presence of TNF- α (5 ng/ml), the hypoxic exposure gave rise to a similar reduction in fEPSP amplitude (46.5 \pm 3.1% versus 45.6 \pm 7.5% in controls, n=5 for both) and recovery (88 \pm 2.7% versus 79.3 \pm 4.9% in controls, n=5 for both; Figure 4B). Following recovery from the 30 min hypoxic episode, LTP was induced in slices using either HFS or theta burst stimulation. In the presence of TNF- α both induction protocols induced robust LTP, however the magnitude of LTP induced following HFS (176 \pm 4.6%, n=5,) was significantly increased compared to LTP induced following theta burst stimulation (125.5 \pm 7.2%, n=5; P<0.001; Figure 4B, C).

LPS but not IL-1 β potentiates LTP after acute hypoxia induced by HFS

In the presence of LPS (100 ng/ml), and IL-1 β (5 ng/ml), hypoxic exposure for 30 min gave rise to a similar

reduction in fEPSP amplitude compared to controls (controls $51.7 \pm 1\%$, LPS $58.6 \pm 7.6\%$, IL-1 β $65 \pm 6.6\%$, $n=6$). The magnitude of recovery following hypoxia was also similar in all groups (controls, $76.2 \pm 2.4\%$, LPS, $79.7 \pm 8.3\%$, IL-1 β , $85 \pm 4.6\%$, $n=5-6$; **Figure 5**). Since HFS induced similar LTP in controls and slices treated with LPS, TNF- α and IL-1 β in the absence of hypoxia (see also **Figure 3**), we

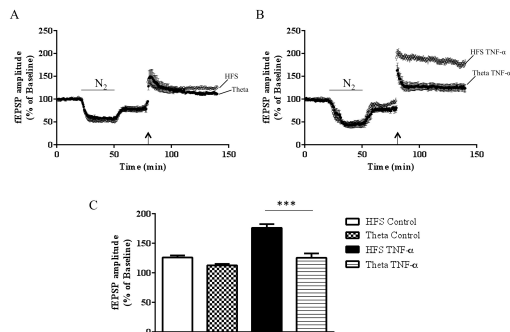


Figure 4

Effects of prior exposure to hypoxia and TNF- α on LTP in the dentate gyrus.

A) Time course showing the effect of 30-min hypoxia on fEPSP amplitude and subsequent induction of LTP. Following a 20 min stable recording, hypoxia was induced by switching from 95% O₂/5% CO₂ to 95% N₂/5%CO₂ for 30 min. Upon re-oxygenation the fEPSP amplitude recovered over a period of 5 to 10 min to approximately 80%. HFS (open circles) or theta burst stimulation (closed circles) was applied 30 min after re-oxygenation and LTP was monitored for 1 hr. B) Time course showing the effect of 30-min hypoxia on fEPSP amplitude in the dentate gyrus region for TNF- α treated slices. Following a 20 min stable recording in the presence of TNF- α , hypoxia was induced by switching from 95% O₂/5% CO₂ to 95% N₂/5%CO₂ for 30 min (indicated by grey line). Upon re-oxygenation the fEPSP amplitude recovered over a period of 5 to 10 min. HFS (open circles) or theta burst stimulation (closed circles) was applied 30 min after re-oxygenation. In the presence of TNF- α HFS induced LTP was significantly increased compared to that induced by theta burst stimulation. C) Summary data showing fEPSP amplitude 55-60 min post LTP induction, for control HFS (white bar), control TBS (hatched bar), TNF- α HFS post hypoxia (black bar) and TNF- α theta burst stimulation post hypoxia (striped bar). All data is expressed as mean \pm SEM %; $n=5$ for all bars; *** $P < 0.01$.

therefore used this protocol to induce LTP following the 30 min of hypoxia for the remainder of the experiments. LTP was significantly enhanced in slices perfused with LPS (166.3 ± 4.8 ; $n=6$; $P < 0.001$) when compared to controls ($125.9 \pm 3.4\%$, $n=6$; **Figure 5A, C**). The magnitude of LTP was similarly enhanced in slices treated with TNF- α following a 30 min acute hypoxic episode ($176 \pm 4.6\%$, $n=5$, $P < 0.001$; see **Figure 4B**). However, there was no difference in the magnitude of control LTP and slices treated with IL-1 β ($122.8 \pm 8\%$ versus $125.9 \pm 3.4\%$, $n=6$; **Figure 5B, C**). Anti-rat TNF antibody, CNTO1081 was used to determine if the TNF- α effect on enhanced LTP was TNF- α specific. Application of anti-TNF, prior to the 30 min hypoxia significantly reversed the effects of TNF- α on LTP post hypoxia ($126.7 \pm 8.3\%$, $n=4$; **Figure 6A**, $P < 0.001$ compared to TNF- α alone). Anti-TNF on its own had no effect on control LTP post hypoxia ($138.7 \pm 7.6\%$, $n=4$).

Effect of AP-5, anti-TNF and A₁R antagonism on the effect of TNF- α on LTP post hypoxia

In order to investigate a potential role for NMDA receptors in the enhanced effect of TNF- α on LTP post hypoxia, we

applied AP-5 (50 μ m; a selective NMDA receptor antagonist) to the bath in the presence of TNF- α . A similar reduction ($55.9 \pm 5.7\%$, $n=5$) and recovery ($79.9 \pm 6.8\%$, $n=5$) of fEPSP amplitude during hypoxia and re-oxygenation was observed in the presence of AP-5+TNF- α treated slices. The presence of AP-5 significantly impaired the induction of LTP even when co applied with TNF- α ($96 \pm 3\%$, $n=5$, $P < 0.001$; **Figure 6A**).

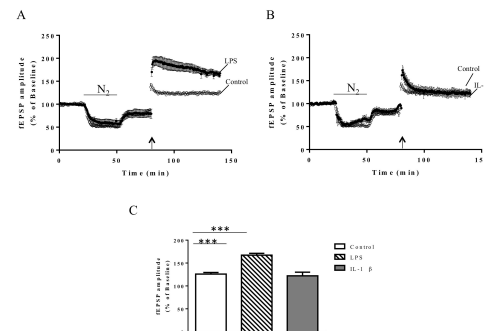


Figure 5

Effects of LPS and IL-1 β on HFS induced LTP post hypoxia

A) Time course showing the effect of 30 min hypoxia and subsequent LTP on fEPSP amplitude in the dentate gyrus region for control (open circles) and LPS (grey circles) treated groups. Following a 20 min stable recording, hypoxia was induced by switching from 95% O₂/5% CO₂ to 95% N₂/5%CO₂ for 30 min (indicated by black line). Upon re-oxygenation the fEPSP amplitude recovered over a period of 5 to 10 min. HFS was applied 30 min after re-oxygenation.

B) Time course showing the effect of 30-min hypoxia and subsequent LTP on fEPSP amplitude in the dentate gyrus region for control (open circles) and IL-1 β (closed circles) treated groups. Following a 20 min stable recording, hypoxia was induced by switching from 95% O₂/5% CO₂ to 95% N₂/5%CO₂ for 30 min (indicated by grey line). Upon re-oxygenation the fEPSP amplitude recovered over a period of 5 to 10 min. HFS was applied 30 min after re-oxygenation.

C) Summary data showing fEPSP amplitude 55-60 min post HFS, for controls (white bar), LPS (hatched bar) and IL-1 β (grey bar) treated groups. All data is expressed as mean \pm SEM %; *** $P < 0.01$; $n=4-5$ for all bars.

Next we perfused slices with DPCPX (200 nM), an adenosine A₁ receptor antagonist. As has previously been demonstrated DPCPX significantly attenuated the reduction in fEPSP amplitude during hypoxia ($73.2 \pm 4.2\%$, $n=4$, $P < 0.05$ compared to controls; **Figure 6B**). The concentration of DPCPX used in these studies did not affect the magnitude of LTP post hypoxia when compared to controls ($112.3 \pm 4.9\%$ versus control of $125.9 \pm 3.4\%$, $n=6$; **Figure 6B**). Experiments were repeated in the presence of DPCPX and TNF- α . Stable fEPSPs were generated for 20 min in the presence of DPCPX before application of TNF- α to the perfusion. fEPSP depression during hypoxia was significantly reduced compared to controls (88.1 ± 4.2 , $n=4$, $P < 0.01$). However, similar enhanced LTP observed with TNF- α alone treatment, was observed in slices treated with DPCPX + TNF- α (161.3 ± 3.3 , $n=4$, $P < 0.001$; **Figure 6B**). Using a 2 way ANOVA with post hoc Bonferroni (control, anti-TNF and DPCPX before and after TNF- α), over all TNF- α had a marked potentiating effect on LTP post hypoxia [$F(1,2) = 42.1$, $P < 0.001$] but there was a significant interaction term [$F(1,2) = 20.5$, $P < 0.0001$]. Post Bonferroni analysis showed there was no significant difference between TNF- α and DPCPX + TNF- α ($P > 0.05$). A summary of the

effects of control, TNF- α , anti-TNF alone and anti-TNF + TNF- α , 55 to 60 min post LTP are shown in [Figure 6C](#)

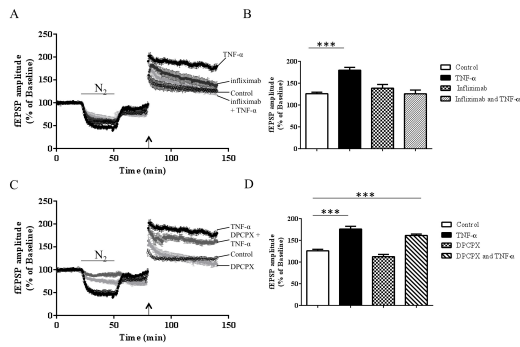


Figure 6
Effects of AP-5, anti-TNF and adenosine A₁ receptor inhibition on HFS induced LTP post hypoxia

A) Time course showing the effect of 30-min hypoxia and subsequent LTP on fEPSP amplitude in the dentate gyrus region for control (open circles), TNF- α (closed circles), anti-TNF (dark grey circles), anti-TNF + TNF- α (grey circles) and AP-5 (light grey circles) treated groups. Following a 20 min stable recording, hypoxia was induced by switching from 95% O₂/5% CO₂ to 95% N₂/5%CO₂ for 30 min (indicated by grey line). Upon re-oxygenation the fEPSP amplitude recovered over a period of 5 to 10 min. HFS was applied 30 min after re-oxygenation. LTP was monitored for 1 hr post induction.

B) Time course showing the effect of 30-min hypoxia and subsequent LTP on fEPSP amplitude in the dentate gyrus region for control (open circles), TNF- α (closed circles), DPCPX (light grey circles), DPCPX + TNF- α (dark grey circles) treated groups. Following a 20 min stable recording, hypoxia was induced by switching from 95% O₂/5% CO₂ to 95% N₂/5%CO₂ for 30 min (indicated by grey line). Upon re-oxygenation the fEPSP amplitude recovered over a period of 5 to 10 min. HFS was applied 30 min after re-oxygenation. LTP was monitored for 1 hr post induction.

C) Summary data showing fEPSP amplitude 55-60 min following HFS in the absence (white bars) and presence (black bars) of TNF- α . Slices were either untreated or treated with TNF- α in the presence of vehicle (control), anti-TNF or DPCPX. All data is expressed as mean \pm SEM %; *** P<0.01; n=4-6 for all bars.

Effect of TNF- α synthesis inhibitors on the effect of TNF- α on LTP post hypoxia

Slices were perfused for 2 hrs with 3,6'DT and 1,6'DR, both TNF- α synthesis inhibitors, before baseline recordings were initiated. These inhibitors had no effect on baseline fEPSP amplitude on their own (results not shown). Whilst these inhibitors had no effect on control LTP (see [Figure 3F, G](#)), LTP was significantly impaired, in slices treated with 3,6'DT following a 30 min acute hypoxic episode (106.6 \pm 8.8%, versus control of 125.9 \pm 3.4; n=5; one way ANOVA, P<0.05; [Figure 7A](#)). This impairment in LTP post hypoxia was not seen in slices treated with 1,6'DR (115.7 \pm 6.8%, n=5; [Figure 7B](#)). Application of exogenous TNF- α (5 ng/ml) to slices pre treated with 3,6'DT increased the magnitude of LTP when compared to pre treatment with 3,6'DT alone (143.3 \pm 4.8%, P<0.05; n=5; [Figure 7A](#)). We also applied exogenous TNF- α to slices treated with 1,6'DR. A significant increase in LTP compared to slices pre treated with 1,6'DR alone was also observed (153.1 \pm 10.1, n=5; P<0.01; [Figure 7B](#)). To further interpret these data we carried out a 2 way ANOVA with post Bonferroni analysis (control, 3,6'DT and 1,6'DR before and after TNF- α). Overall TNF- α had a marked potentiating effect on LTP post hypoxia in all treated slices [F(1,2)=62.3, P<0.001] and there was no overall significant interaction term [F(1,2) = 0.96, P=0.39]. This may have been due to the fact that post

Bonferroni analysis did not show a significant effect between control LTP post hypoxia and in the presence of 3,6'DT. Also analysis indicated a significant difference between TNF- α LTP post hypoxia and TNF- α LTP in the presence of 3,6'DT (P<0.01; [Figure 7C](#)).

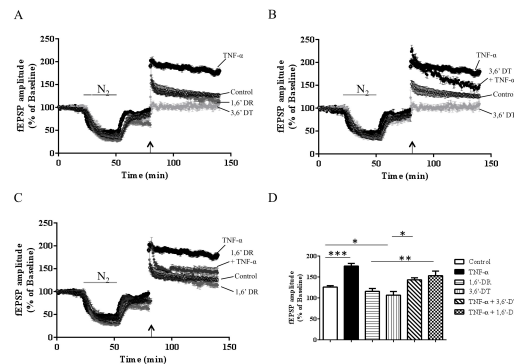


Figure 7
Effects of TNF- α synthesis inhibition on HFS induced LTP post hypoxia

A) Time course showing the effect of 30 min hypoxia and subsequent LTP on fEPSP amplitude in the dentate gyrus region for control (open circles), TNF- α (closed circles), 3,6'DT (light grey circles) and 3,6'DT+TNF- α (dark grey circles) treated groups. Following a 20 min stable recording, hypoxia was induced by switching from 95% O₂/5% CO₂ to 95% N₂/5%CO₂ for 30 min (indicated by grey line). Upon re-oxygenation the fEPSP amplitude recovered over a period of 5 to 10 min. HFS was applied 30 min after re-oxygenation. LTP was monitored for 1 hr.

B) Time course showing the effect of 30 min hypoxia and subsequent LTP on fEPSP amplitude in the dentate gyrus region for control (open circles), TNF- α (closed circles), 1,6'DR (grey circles) and 1,6'DR+TNF- α (dark grey circles) treated groups. Following a 20 min stable recording, hypoxia was induced by switching from 95% O₂/5% CO₂ to 95% N₂/5%CO₂ for 30 min (indicated by grey line). Upon re-oxygenation the fEPSP amplitude recovered over a period of 5 to 10 min. HFS was applied 30 min after re-oxygenation. LTP was monitored for 1 hr.

C) Summary data showing fEPSP amplitude 55-60 min following HFS in the absence (white bars) and presence (black bars) of TNF- α . Slices were either untreated or treated with TNF- α in the presence of vehicle (control), 3,6'DT or 1,6'DR. All data is expressed as mean \pm SEM %; *** P<0.01; **P<0.01; *P<0.05; n=5 for all bars.

Effect of p38 MAPK inhibition and DMOG application on the effect of TNF- α on LTP

Next slices were treated with SB203580 (1 μ M) a p38 MAPK inhibitor, for the duration of the experiment. SB203580 alone had no effect on baseline fEPSP amplitude, the reduction in fEPSP amplitude during hypoxia (59.6 \pm 10.4, n=4), recovery of fEPSP amplitude upon re-oxygenation (70.8 \pm 7.7%, n=4) or LTP post hypoxia (114.6 \pm 2.1, n=4) when compared to controls. However application of SB203580 significantly reduced the effects of TNF- α on LTP post hypoxia (111.3 \pm 1.7, n=4; [Figure 8A](#)). A role for PHD enzymes in glutamate receptor trafficking and synaptic plasticity has recently been proposed (Park et al., 2012; Corcoran et al., 2013). Therefore experiments were also carried out to determine a possible interaction with TNF- α and PHD enzymes that may explain the enhanced LTP following hypoxia. DMOG (500 μ M), a PHD inhibitor, was applied to slices for 30 min at a concentration which does not inhibit LTP (see Corcoran et al., 2013) and LTP induced in the presence or absence of TNF- α . LTP was not significantly enhanced after application of TNF- α in the presence of DMOG (122.3 \pm 1.3%, DMOG, versus 134.5 \pm 5.0% DMOG+TNF- α , n=4; [Figure 8B](#)). Using a 2

way ANOVA with post hoc Bonferroni (control, SB203580 and DMOG before and after TNF- α), TNF- α had an overall marked potentiating effect on LTP post hypoxia [$F(1,2) = 36.01$, $P < 0.001$] and there was a significant interaction term [$F(1,2) = 29.9$, $P < 0.001$] which may be attributed to an apparent lack of change in the SB203580 and DMOG group ($P > 0.05$). A summary of the effects of control, TNF- α , SB203580 and DMOG, 55 to 60 min post LTP are shown in Figure 8C.

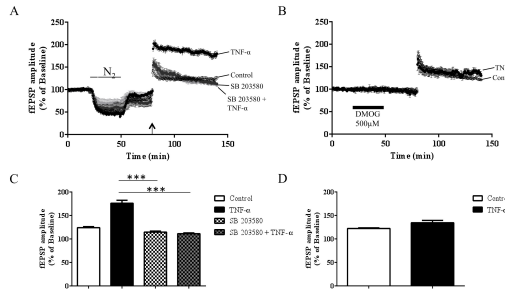


Figure 8
Effects of p38 MAPK and PHD inhibition on HFS induced LTP post hypoxia

A) Time course showing the effect of 30 min hypoxia and subsequent LTP on fEPSP amplitude in the dentate gyrus region for control (open circles), TNF- α (closed circles), SB203580 (dark grey circles) and SB203580+TNF- α (light grey circles) treated groups. Following a 20 min stable recording, hypoxia was induced by switching from 95% O₂/5% CO₂ to 95% N₂/5%CO₂ for 30 min (indicated by grey line). Upon re-oxygenation the fEPSP amplitude recovered over a period of 5 to 10 min. HFS was applied 30 min after re-oxygenation. LTP was monitored for 1 hr.

B) Time course showing the effect of 30 min DMOG treatment and subsequent LTP on fEPSP amplitude in the dentate gyrus region for control (open circles), and TNF- α treated slices (closed circles). Following 20 min stable recording, slices were perfused with DMOG (500 μ M) for 30 min (indicated by black line). HFS was applied 30 min after wash out of DMOG. LTP was monitored for 1 hr. DMOG treatment did not effect LTP induction in either treatment group.

C) Summary data showing fEPSP amplitude 55-60 min following HFS in the absence (white bars) and presence (black bars) of TNF- α . Slices were either untreated or treated with TNF- α in the presence of vehicle (control), SB203580 or DMOG. All data is expressed as mean \pm SEM %; *** $P < 0.01$; n=5 for all bars.

DISCUSSION

Our data provides novel evidence of a role for pro-inflammatory cytokines in the modulation of synaptic plasticity following acute hypoxia. In this study we used two different stimulation protocols to investigate the effects of pro-inflammatory cytokines and cytokine inhibitors on the induction of LTP in the dentate gyrus. The protocols differed in the frequency and strength of stimulation applied, namely a theta burst (TBS) and a more robust high frequency induced stimulation protocol (HFS). Following exogenous application of cytokines, LTP was inhibited using the TBS protocol. Exogenous application of cytokines or cytokine inhibitors had no effect on LTP induction when the HFS protocol was used. There have been many studies that have provided insight into the role of pro-inflammatory cytokines such as IL-1 β and TNF- α in synaptic transmission and plasticity. Previous work has detailed the inhibitory effects of IL-1 β on LTP in the CA1 (Bellinger et al., 1993; Ross et al., 2003) and dentate gyrus (Cunningham et al., 1996; Coogan et al., 1999; Coogan & O'Connor, 1999;

Curran et al., 2003). Other studies have shown an inhibitory effect of TNF- α on LTP in the CA1 (Tancredi et al., 1992) and dentate gyrus (Butler et al., 2004), while others have provided evidence that exogenous application of TNF- α affects homeostatic plasticity (synaptic scaling) rather than synaptic plasticity (Stellwagen & Malenka, 2006) and glutamatergic gliotransmission (Santello et al., 2011). These reports all demonstrate the importance of variable parameters such as region of brain, age of animal, and concentration of cytokine applied when investigating these modulating effects. HFS would be considered to be a more robust induction protocol than TBS, potentially releasing more glutamate and for longer periods. We consider it may also activate different intracellular signals, possible different CREB phosphorylation and differential activation of other kinase pathways. It is also possible that TBS is a more physiological induction protocol and that for the effects of TNF- α on LTP post hypoxia to be observed there is a requirement of a minimum amount of either pre-synaptic glutamate release or post-synaptic depolarisation which may or may not be supplied under TBS stimulation. Therefore it seems the choice of stimulation protocol to induce LTP is very important when investigating the actions of pathophysiological levels of TNF- α .

Most strikingly our data provides novel evidence of a role for exogenous pro-inflammatory cytokines in modulating synaptic plasticity following an acute hypoxic episode. Application of LPS or TNF- α but not IL-1 β to hippocampal slices for 20 min followed by 30 min hypoxic exposure and 30 min recovery resulted in a significant increase in LTP compared to controls. APV, a NMDA receptor antagonist and, anti-TNF, a monoclonal antibody that binds to TNF- α and prevents it from binding to its receptors (Elliot et al., 1993), were able to reverse this effect of exogenous TNF- α on LTP. Previously we have demonstrated a decrease in the recovery of synaptic transmission in the CA1 region of the hippocampus after a 2 hr hypoxic event in the presence of TNF- α (Batti and O'Connor, 2010). This effect was independent of adenosine receptor activation but dependent on p38 MAPK activity. In the present studies in the dentate gyrus we used a shorter hypoxic exposure and did not see the decreased recovery from hypoxia in the presence of TNF- α . Zhang et al., (2014) have very recently demonstrated that perfusion with LPS during hypoxia can induce a long-term depression that requires microglial CR3, activation of NADPH oxidase and AMPAR internalization but not NMDARs or mGluRs. Our difference in severity of hypoxic insult, region of hippocampus and the induction of LTP, may explain some of the differences seen in our two previous studies and that of Zhang et al., (2014).

Previous work in our laboratory has also looked at the effect of anti-TNF administration in chronic intermittent hypoxia (CIH) treated animals. We found that *in vivo* anti-TNF treatment alone impaired synaptic plasticity in the CA1 region of Wistar rats, an impairment that was not seen in the dentate gyrus of the same animals. Surprisingly animals treated with CIH and the TNF- α inhibitor *in vivo*, still exhibited impaired LTP in the CA1 region and again no change in LTP in the dentate gyrus (unpublished data). These results may indicate an important role for TNF- α in enhancing memory mechanisms after an acute hypoxic event in the brain.

There is now evidence for a cross-talk among hypoxic and inflammatory pathways (Scholz & Taylor, 2013). However

few papers have looked at the effect of acute hypoxia and inflammatory mediators on synaptic transmission (Batti et al., 2010; O'Connor, 2013). Investigations have looked at the effect of inflammation on the induction of motor plasticity and long-term facilitation, using acute intermittent hypoxia. In the phrenic nerve, long-term facilitation can be induced using an acute hypoxic insult of three 5 min hypoxic episodes in quick succession. LPS induced inflammation has been shown to inhibit LTF in Lewis and Sprague Dawley rats (Vinit et al., 2011; Huxtable et al., 2013). Acute intermittent hypoxia is a milder hypoxic insult than that of this study, and it is possible the effects on plasticity we report are due to the presence of pro inflammatory mediators throughout the 30 min hypoxic episode.

Other studies have shown that glia released TNF- α is required for synaptic scaling through AMPAR trafficking to the membrane (Beattie et al., 2002; Stellwagen et al., 2005; Stellwagen & Malenka, 2006). These studies found that the increase in AMPAR expression on the cell surface is mediated through the P13 kinase pathway and the AMPAR trafficked were lacking the GLR-2 subunit. Since LTP is dependent on synaptic glutamate it is also interesting to note that TNF- α has been shown to increase glutamate release from astrocytes (Vesce et al., 2007), can block glutamate transporters (Korn et al., 2005) and also that it may have a modulatory effect on the expression of GLT-1 and GLT-2. These combined effects may result in increased glutamate concentrations in the synaptic cleft (Boycott et al., 2008; Carmen et al., 2009), which may influence the magnitude of LTP in the region we have been investigating. Alternatively Pribiag & Stellwagen (2013) have shown that acute application of TNF- α induces a rapid and persistent decrease of inhibitory synaptic strength and down-regulation of cell-surface levels of GABA_ARs containing α 1, α 2, β 2/3, and γ 2 subunits. This effect was mediated specifically by neuronally expressed TNFR1 and required activation of p38 MAPK, phosphatidylinositol 3-kinase, protein phosphatase 1 (PP1), and dynamin GTPase. These effects on GABA_ARs during hypoxia may also be a contributing factor to the change in magnitude of LTP observed post hypoxia.

Recently a new relationship between oxygen sensing molecules and synaptic transmission in *C. elegans* (Park et al., 2012) has been detected. The authors showed an important role for prolyl hydroxylases (PHDs) in the regulation of the AMPA subunit (GLR-1), through the integration of a PHD isoform Egl-9E with LIN-10, a protein involved in GLR trafficking. This interaction, under normoxic conditions, leads to an increase in GLR-1 recycling to the synaptic membrane. During hypoxia, the decreased interaction between Egl-9E and LIN-10 allows phosphorylation of the Ser and Thr residues on LIN-10 by CD5. This action results in GLR-1 being trafficked to endosomes rather than the synaptic site. PHDs, which regulate levels of HIF-1 α , have been shown to be positive regulators of LPS induced inflammation in hypoxic cells (Fujita et al., 2012). In these cells application of TNF- α and IL-1 β increased PHD3 mRNA expression in less than 4 hr. Our laboratory has also studied the relationship between PHD inhibition and synaptic plasticity. In vitro application of DMOG to acute slices significantly impaired synaptic plasticity in the CA1 of rat hippocampal slices (Corcoran et al., 2013). In the present experiments application of a concentration of DMOG, which does not significantly impair LTP (500 μ M), and in the presence of TNF- α did not give rise to an increase in LTP. This may indicate that PHD

inhibition does not play a role in the enhanced LTP observed following hypoxia and TNF- α .

In the present work we have also shown that thalidomide derivatives may influence the magnitude of LTP post hypoxia in the absence and presence of TNF- α . These analogs have been shown to have a number of effects on TNF- α synthesis (Greig et al., 2004), lowering its generation in cellular studies as well as both systemically and within the brain of rodents (Zhu et al., 2003; Tweedie et al., 2012; Yoon et al., 2013). 3,6'-dithiothalidomide (3,6'-DT) has previously been shown to restore neuronal function and alleviate cognitive deficits from chronic neuroinflammation and minimal traumatic brain injury (Belarbi et al., 2012 and Baratz et al., 2011). They have also been shown to attenuate inflammatory markers, Alzheimer pathology and behavioral deficits (Tweedie et al., 2012). Our data showed that application of 3,6'DT caused a moderate attenuation of LTP in the dentate gyrus post hypoxia. Application of exogenous TNF- α to slices only partially reversed this effect of 3,6'DT to TNF- α alone levels. The addition of the second analogue 1,6'-DR did not alter LTP induction or maintenance post hypoxia when applied on its own. Interestingly perfusion of exogenous TNF- α in the presence of 1,6'DR increased LTP post hypoxia to levels similar to that seen with TNF- α alone. This may be due to the fact that 1,6'DR is not as potent as 3,6'DT in hippocampal slices (Zhu et al., 2003). In recent studies, we have lately used 3,6'DT to effectively mitigate neuroinflammation and apoptosis within the penumbra of focal ischemic stroke in mice, substantially lowering stroke-induced TNF- α levels (Yoon et al., 2013), as well as to lower TNF- α and cerebral aneurysm formation and progression to rupture in mice (Starke et al., 2014; Ali et al., 2013). The specific effects of 3,6'DT observed in these studies may suggest an important role for TNF- α in the modulation of synaptic plasticity following an acute hypoxic episode.

We and many others have observed regional differences in the hippocampus in response to hypoxia. For example the CA1 is more susceptible to hypoxia than certain parts of the dentate gyrus (Kreisman et al., 2000). In addition, the CA1 has been shown to be more susceptible to synaptic transmission failure from shorter periods of hypoxic exposure (Kass & Lipton, 1986). One possible explanation for the regional differences in response to hypoxia may be due to CREB phosphorylation. The importance of CREB in neuronal excitability and LTP has been previously reviewed by Benito & Barco (2010). Recently it has been reported that transgenic mice with constitutively active CREB showed increased LTP *in vivo* in pyramidal neurons which may be due to increased excitability of these neurons (Gruart et al., 2012). Interestingly the dentate gyrus does not show a decrease but an increase in CREB phosphorylation following hypoxia and this increase continues in a time dependent manner (Hu et al., 1999). A modulatory role for CREB induction via hypoxia and inflammatory cytokines, such as TNF- α , and alternative pathways has been investigated (Lin et al., 2001). An increase in CREB phosphorylation within the granule cells of the dentate gyrus following acute hypoxia exacerbated by inflammatory mediated CREB phosphorylation may be another mechanism for the increased synaptic plasticity we observed. Previous work found increased CREB phosphorylation 20 min following mild hypoxia in PC12 cells (Beitner-Johnson & Millhorn, 1998). Previously we have shown in our

hippocampal slices an increase in CREB phosphorylation following chronic intermittent hypoxia (Wall et al., 2013).

Application of TNF- α causes an increase in p38 MAPK phosphorylation in the dentate gyrus (Coogan et al., 1999). In the presence of the p38 MAPK inhibitor, SB203580, exogenous addition of TNF- α to slices treated with hypoxia, did not demonstrate the enhanced LTP seen with TNF and hypoxia alone. Therefore this effect of hypoxia and TNF- α on LTP may also involve the activation of p38 MAPK. Previously in our laboratory we have examined the role of p38 MAPK in ameliorating the deleterious effects of TNF- α on synaptic transmission in the CA1 region (Batti & O'Connor, 2010) where it was able to reverse this effect. P38MAP kinase is activated in our brain tissue within 15-20 min of TNF- α application (Butler et al., 2004). TNF- α has been shown to induce NMDAR1 subunit phosphorylation and clustering, which was spongomyelinase-2 dependent and shown to increase NMDA-evoked calcium flux as well as enhanced EPSCs' (Wheeler et al., 2009). Therefore calcium influx following HFS via NMDARs and subsequent activation of MAPK (see also review by Haddad, 2005) may be involved in the potentiated LTP seen in the presence of TNF- α after acute hypoxia.

Conclusion

In conclusion we have demonstrated an enhanced synaptic plasticity in the dentate gyrus in the presence of exogenous TNF- α following an acute hypoxic episode. Our data show that whilst this effect is independent of adenosine A₁ receptor activation, there is a role for NMDA receptors and the p38 MAPK pathway in this effect of TNF- α on LTP post hypoxia. Finally the inhibition of PHDs in the presence of TNF- α did not give rise to an enhanced LTP post hypoxia. Further studies will be required to understand the importance of this enhanced plasticity and to discover if it is observed in other brain regions.

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