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Effects of prolyl-hydroxylase inhibition and chronic intermittent hypoxia on synaptic transmission and plasticity in the rat CA1 and dentate gyrus

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Abbreviations (footnote page 1)
2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA), AMP-activated protein kinase (AMPK), artificial cerebrospinal fluid (aCSF), brain derived neurotrophic factor (BDNF), cyclic adenosine monophosphate (cAMP), chronic intermittent hypoxia (CIH), cornu ammonis 1 (CA1), cAMP response element-binding protein (CREB), dimethyloxaloylglycine (DMOG), dimethyl sulfoxide (DMSO), erythropoietin (EPO), field excitatory postsynaptic potential (fEPSP), high frequency stimulation (HFS), hypoxia inducible factor (HIF), long-term potentiation (LTP), middle cerebral artery occlusion (MCAO), obstrutive sleep apnoea (OSA), phosphodiesterase 4D (PDE4D), prolyl hydroxylase domain (PHD), reactive oxygen species (ROS).

Abstract
Chronic intermittent hypoxia (CIH) is an underlying component of obstructive sleep apnoea and has been shown to have deleterious and damaging effects on central neurons and to impair synaptic plasticity in the CA1 region of the rat hippocampus. CIH has previously been shown to impair synaptic plasticity and working memory. CIH is a potent inducer of hypoxia inducible factor (HIF), a key regulator in a cell's adaptation to hypoxia that plays an important role in the fate of neurons during ischemia. Levels of HIF-1α are regulated by the activity of a group of enzymes called HIF-prolyl 4-hydroxylases (PHDs) and these have become potential pharmalogical targets for preconditioning against ischemia. However little is known about the effects of prolyl hydroxylase inhibition and CIH on synaptic transmission and plasticity in sub-regions of the hippocampus. Male Wistar rats were treated for 7-days with either saline, CIH or PHD inhibition (dimethyloxaloylglycine, DMOG; 50mg/kg, i.p.). At the end of treatment all three groups showed no change in synaptic excitability using paired pulse paradigms. However long-term potentiation (LTP) was impaired in the CA1 region of the hippocampus in both CIH and DMOG treated animals. LTP induced in the dentate gyrus was not significantly affected by either CIH or DMOG treatment. We also investigated the effect of 7-day CIH and DMOG treatment on the recovery of synaptic transmission following an acute 30 min hypoxic insult. CIH treated animals showed an improved rate of recovery of synaptic transmission following re-oxygenation in both the CA1 and dentate gyrus. These results suggest that LTP induction in the CA1 region is more sensitive to both CIH and DMOG treatment than the dentate gyrus.

Key Words: Chronic intermittent hypoxia (CIH), long term potentiation (LTP), Cornu Ammons 1 (CA1), dentate gyrus (dentate gyrus), dimethyloxaloylglycine (DMOG), prolyl hydroxylase domain (PHD), hypoxia inducible factor 1 alpha.

Introduction
Chronic intermittent hypoxia (CIH) is a characteristic feature of sleep apnoea which can lead to significant memory deficits, as well as to cortical and hippocampal apoptosis (Gozal *et al*., 2001). It has been shown to cause neurocognitive deficits such as spatial learning impairments with increased cell death and structural changes to hippocampal and cortical regions (Gozal *et al*., 2001, Row *et al*., 2002, Row *et al*., 2003, Klein *et al*., 2003, Cai *et al*., 2010, Nair *et al*., 2011, Gozal *et al*., 2013). There is also evidence that these effects are correlated with impairments in synaptic plasticity, namely long-term potentiation (LTP) in the rodent hippocampus (Payne *et al*., 2004; Xie *et al*., 2010; Xie & Yung, 2012). Payne *et al*., (2004) showed that 3- and 7-day CIH treatment impaired population spike LTP (PS-LTP) in the CA1 region of rat hippocampal slices. Deficits in LTP due to CIH treatment were reversed with acute application of BDNF and 7-day *in vivo* treatment of BDNF in mice (Xie *et al* 2010). Although these studies have shown CIH-induced impairments in synaptic plasticity in the hippocampal CA1 region no research has been carried out on the effects of CIH and other chronic hypoxic treatments on synaptic transmission and plasticity in granule cells of the dentate gyrus. Previous work has demonstrated that the dentate gyrus is also susceptible to hypoxia but that certain blades of the dentate gyrus are more resistant to a decrease in oxygen availability than for example the CA1 region (Kreisman *et al* 2000). Previous work has also shown differential susceptibility of the CA1 and CA3 regions of the hippocampus to intermittent hypoxia (Gozal *et al*., 2002).

CIH is a potent inducer of HIF-1α, a key regulator of the hypoxic response which promotes the transcription of numerous genes required for adaptation to decreased oxygen tension (Wang and Semenza, 1993; Forsythe *et al*., 1996). Under normoxic conditions, HIF-1α is hydroxylated on specific proline residues which targets HIF for proteosomal degradation (Jaakkola *et al*., 2001; Kaelin and Ratcliffe, 2008). This prolyl hydroxylation is mediated by three prolyl-4-hydroxylase domain proteins, PHD1, 2 and 3 during normoxia. During hypoxia, the loss of the co-factor, oxygen, inhibits PHD-mediated hydroxylation resulting in stabilization of HIF-1α. The discovery of PHDs as cellular regulators of the

hypoxic response has led to a resurgence of hypoxic preconditioning as a therapeutic strategy (Siddiq *et al*., 2005). Pharmacological inhibition of PHDs prior to middle cerebral artery occlusion increases cerebral blood flow, delays neuronal injury and decreases infarct volume (Nagel *et al*., 2011, Kunze *et al*., 2013). Additionally, post-ischemic intervention with PHD inhibitors decreases neuronal damage and attenuates behavioural deficits associated with ischemia (Ogle *et al*., 2012). We have recently shown in isolated hippocampal slices that acute PHD inhibition using dimethyloxaloylglycine (DMOG) and other specific PHD inhibitors, can impair synaptic transmission and plasticity in the rat CA1 region (Batti *et al*., 2010; Corcoran *et al*., 2013). Furthermore these effects were shown to be mediated by the PHD2 isofom of the hydroxylase (Corcoran *et al*., 2013). It is therefore important to investigate the effects of chronic treatment with PHD inhibitors on synaptic plasticity.

In the present study we have compared the effects of 7-day CIH and DMOG treatment on synaptic transmission and plasticity in two regions of the rat hippocampus, namely by stimulation of the stratum radiatum of the CA1 region and stimulation of the medial perforant path of the dorsal dentate gyrus (suprapyramidal (upper) blade). We have investigated whether the effect of a hypoxic mimetic would have similar impairments on LTP as has previously been reported for CIH treated animals in the CA1 region (Payne *et al*., 2004; Xie *et al*., 2010). Since hypoxic preconditioning has been shown to alleviate neuronal damage and that especially associated with cerebral ischemia (see reviews by Dirnagl *et al*., 2009 and Eltzschig & Eckle, 2011), we also explored a putative preconditioning effect of CIH and PHD inhibition on the recovery of synaptic transmission in the dentate gyrus and CA1 regions following an acute hypoxic insult in isolated hippocampal slices.

Methods and Materials

Animals

Male Wistar rats (50-100g) were used in these experiments. All experimental procedures were approved by the Animal Research Ethics Committee of the Biomedical Facility of University College Dublin, Ireland. Animals were grouped as either sham, CIH, DMOG (50mg/kg; i.p.) or CIH+DMOG (50mg/kg, i.p.) treated, with each group consisting of 5 animals. After 7 days of treatment animals were anesthetised using 5% isoflurane and decapitated by guillotine. Upon decapitation blood was taken and a triplicate haematocrit performed. The brains were quickly removed and placed in oxygenated, chilled artificial cerebrospinal fluid (aCSF) composed of (mM): NaCl 120; $NaHCO_3$ 26; NaH_2PO_4 1.25; KCl 2.5; Glucose 10; $MgSO_4$ 2; $CaCl_2$ 2. 350 μm transverse hippocampal slices were prepared from the dorsal hippocampus using a vibratome. Hippocampal slices were transferred to a holding chamber which contained oxygenated aCSF ($95\%O_2/5\%CO_2$) for 1 hr at room temperature. Hippocampal slices were transferred to a submerged recording chamber continuously perfused from a 100 ml reservoir with oxygenated aCSF at a flow rate of 4 ml/min and maintained at 30-32 °C. All drugs were added via the 100 ml aCSF reservoir.

Electrophysiology

Field excitatory postsynaptic potentials (fEPSP's) were elicited in the medial perforant pathway of the dentate gryus region and the Schaffer collateral pathway in the CA1 using aCSF-filled monopolar glass electrodes at a frequency of 0.033 Hz. Recordings were obtained 50-100 μm adjacent to the granule cells and in the stratum radiatum respectively. Stimulation intensity was then adjusted to give 50% maximal response, determined by an input/output curve. Paired-pulse responses were elicited with two stimuli every 30 s separated by 50 ms. The paired-pulse ratio (PPR) was quantified as the ratio of fEPSP2/fEPSP1 amplitude. This typically resulted in potentiation of the second fEPSP amplitude in the CA1 region and depression of the second fEPSP amplitude in the detate gyrus (see O'Leary & O'Connor, 1997). Long-term potentiation was elicited by high frequency stimulation (HFS), 3 trains of 1 s at 100 Hz separated by 20 s intervals. In the dentate gyrus picrotoxin (100 μm) was added to the bath to ensure robust LTP was achieved. Recordings were acquired and analysed using the software package WinWCP (J. Dempster, Strathclyde).

Delivery of 30 min acute hypoxia and 7-day chronic hypoxic exposures

During the isolated brain slice experiments, acute hypoxia was induced by switching the 95% O_2/5% CO_2 gas that aerated the 50 ml reservoir of aCSF to 95% N_2/5% CO_2. Re-oxygenation was achieved by switching 95% N_2/5% CO_2 back to 95% O_2/5% CO_2 at the flow rates indicated previously. This protocol produced O_2 levels of 30-40 mmHg (4-5%) at the surface of the slice and O_2 levels of 0-5 mmHg (0-0.7%) 100 μm inside the slice, where the recording electrode was positioned (see figure 1 of Batti *et al*., 2010). For chronic gas treatments, animals were housed in their normal cage environment placed in commercial environmental chambers (Biospherix Oxycycler^{TM}) allowing precise control of ambient oxygen concentration. The CIH protocol consisted of 90 s cycles of normoxia (21% O_2) alternated with 90 s cycles of hypoxia (5% O_2) during the light cycle (Figure 1A). CIH treatment lasted 8 hr a day for 7 days. Sham rats were held at constant normoxia in identical chambers.

Immunoblotting

Forebrain tissue samples from sham, DMOG, CIH and CIH+DMOG treated animals were weighed and homogenised in ice-cold RIPA buffer supplemented with protease cocktail inhibitor, phosphatase inhibitor cocktail II and phosphatase inhibitor cocktail III. Samples were then centrifuged for 10 min at 10,000 rpm at 4 °C. The pellet was discarded and supernatant used for protein concentration assay (BCA Protein Assay Reagent, Pierce) and loading sample preparation. Equal protein concentrations (20 µg) were separated by 10 % SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with primary antibodies against HIF-1α (1:500, Novus Biologicals), anti-EPO (1:100, Novus Biologicals), anti-α tubulin (1:1000, Novus Biologicals), anti-CREB (1:1000, Novus Biologicals), anti-CREB [P Ser 133] (1:1000, Novus Biologicals) and HRP-conjugated anti-mouse secondary antibody (Cell Signalling). Optical densities were measured using ImageJ and expressed as the fold change compared to controls.

Drugs

Picrotoxin was obtained from Sigma-Aldrich Co. Ltd, UK and dissolved in dimethyl sulfoxide (DMSO) with a final concentration of 0.05 %. DMSO solvent controls were carried out at this concentration to show that baseline measurements were not affected. DMOG was obtained from Caymen, US and was dissolved in saline. Saline was delivered by intra peritoneal injection to sham and CIH treatment groups. DMOG (50mg/kg) was delivered by intra-peritoneal (i.p.) injection to our DMOG and DMOG+CIH groups. This included one loading dose prior to starting treatment and then one injection every 2 days over the 7 day treatment period.

Data Analysis

All fEPSP amplitudes are given as a percentage of the initial mean baseline peak. This baseline measurement is an average of the fEPSP amplitude recorded 20 min prior to any drug application or induction of hypoxia. 5 min readings (10 recordings) were taken from critical time points during the experiment, the baseline, end of acute hypoxic exposure, 7 min after reoxygenation and 30 min after reoxygenation and also from 55 to 60 min post LTP in figures 3, 4 and 5). These values were analysed using one way ANOVA with Bonferroni post-test analysis. Input-output curves were determined by increasing input voltage and measuring the peak amplitude of the fibre volley and associated fEPSP (figure 2). Both fibre volley and fEPSPs are expressed as a percentage of the maximum response achieved. Pearson correlation coefficient values were obtained for data in figure 2 using Graph Pad Prism, version 5.0. In figures 6 and 7 two way ANOVA with post Bonferroni was carried out to compare groups at the given time points. All results are expressed as mean ± SEM. P values < 0.05 were considered significant

Results

7-day CIH and DMOG treatment increases hematocrit levels and alters the expression of HIF-1α, EPO and CREB

Haematocrit levels were significantly elevated in both CIH (black bar, 41.8±1.9%, n=5) and DMOG (hatched bar, 41.0±0.7%, n=5) treated groups when compared to shams (white bar, 29.8±1.2%, n=5; P<0.01) indicating that hypoxia was induced in both treatment groups (Figure 1B). Western blot analysis of total forebrain protein showed that HIF-1α, and EPO protein expression in both CIH (black bars) and DMOG (hatched bars, 50mg/kg, i.p.) treated groups was significantly increased compared to control shams (white bars). Levels of HIF-1 α increased 6.2±0.5 fold in CIH treated animals and by 5.8±0.1 fold in DMOG treated animals (n=3; P<0.01, for both compared to sham; Figure 1C i, ii). Levels of EPO increased 2.8±0.1 fold in CIH treated animals and by 2.7±0.05 fold in DMOG treated animals (n=3; P<0.01, for both compared to sham; Figure 1C i, iii). However CREB phosphorylation at Ser 133 was decreased in both CIH and DMOG treated animals (CIH, 0.38±0.08 and DMOG, 0.37±0.14; n=3; P<0.01 for both compared to sham; Figure 1C i, iv)

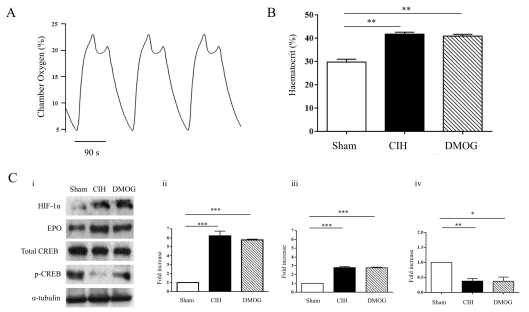

Figure 1

7-day CIH and DMOG treatmented increases hematocrit levels and alters HIF-1α, EPO and CREB protein expression

A) The chronic intermittent hypoxia profile consisted of 90 s of 5% O_2 followed by 90 s 21% for 8 hours a day for 7 days, 20 cycles per hour. B) 7-day CIH (black bar) and DMOG (hatched bar, 50mg/kg, i.p.) treated animals showed a significantly increased hematocrit level compared to the sham group (white bar). C i) Representative Western blots for HIF-1α, EPO, CREB, CREB [Ser 133}] and α-tubulin. C ii) Quantified Western Blot analysis showing that HIF-1α expression is increased in CIH (black bar) and DMOG (hatched bar) treated groups compared to sham controls (white bar). C iii) Quantified Western Blot analysis showing that EPO expression is increased in CIH (black bar) and DMOG (hatched bar) treated groups compared to sham controls (white bar). C iv) Analysis showing a decrease of CREB phosphorylation at Ser 133 in both CIH (black bar) and DMOG (hatched bar) treated groups compared to sham controls (white bar). All data points in B and C are mean±SEM; n=5 for all data points in B and n=3 for all data points in C (*P<0.05; ** P<0.01; ***P<0.001).

7-day CIH and DMOG treatment does not effect cell excitability and synaptic transmission in the CA1 and dentate gyrus

At the beginning of experiments in both the CA1 and dentate gyrus regions stimulation with increased intensity was carried out and the resulting increase in fEPSP amplitude and field presynaptic fibre volley amplitude were measured. fEPSP amplitude was graphed against the corresponding amplitude of the presynaptic fibre volley (input/output curves). Pearson correlation coefficient values were obtained for all treatment groups. There was no significant difference in cell excitability between sham and CIH treatment groups in the CA1 (Figure 2Ai; sham, open circles, r = 0.95 vs. CIH, closed circles, r = 0.95 and in the dentate gyrus (Figure 2Aii; sham, open circles, r = 0.97 vs. CIH, closed circles, r = 0.98). Similar results were observed in the DMOG treated animals in the CA1 (Figure 2Bi; sham, open circles, r = 0.97 vs. DMOG, closed circles, r = 0.96) and the dentate gyrus (Figure 2Bii; sham, open circles, r = 0.97 vs. DMOG, closed circles, r = 0.98).

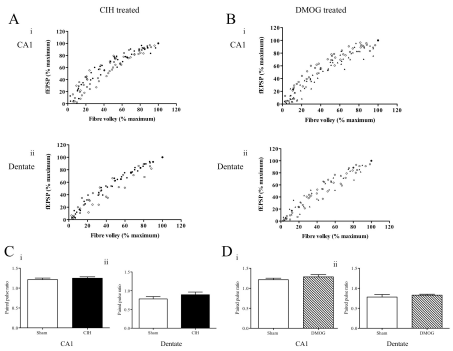

Figure 2
7-day CIH and DMOG treatment does not affect cell excitability or baseline synaptic transmission in CA1 or dentate gyrus.

A) Input / output (I/O) curves of the fEPSP amplitude (% maximum) vs fibre volley amplitude (% maximum) in the CA1 (Ai) and dentate gyrus (Aii) regions of sham (open circles) and CIH treated animals (closed circles). For CA1 sham, r = 0.95 vs. CIH, r = 0.95; for dentate gyrus, sham, r = 0.97 vs. CIH. r = 0.98. B) Input / output (I/O) curves of the fEPSP amplitude (% maximum) vs fibre volley amplitude (% maximum) in the CA1 (Bi) and dentate gyrus (Bii) regions of sham (open circles) and DMOG treated animals (closed circles). For CA1 sham, r = 0.97 vs. DMOG, r = 0.96; for dentate gyrus, sham, r = 0.97 vs. DMOG, r = 0.98. C) Paired pulse facilitation when recorded in the CA1 (Ci) and paired pulse depression when recorded in the dentate gyrus (Cii) were not significantly different in CIH treated animals (black bar) compared to sham controls (white bars). D) Paired pulse facilitation when recorded in the CA1 (Di) and paired pulse depression recorded in the dentate gyrus (Dii) were not significantly different in DMOG treated animals (hatched bar) compared to sham controls (white bars). All data is expessed as mean±SEM %, n=5.

Paired pulse stimulation (interstimulus interval 50 ms) in the CA1 region typically gave rise to facilitation of the second fEPSP amplitude. The magnitude of this facilitation was not affected in both CIH (black bars) and DMOG (hatched bars) treatment groups when compared to shams (white bars; Figure 2Ci and 2Di). Paired pulse stimulation (interstimulus interval 50 ms) in the dentate gyrus (medial path) region typically gave rise to depression of the second fEPSP amplitude. The magnitude of the paired pulse depression was not affected in both CIH (black bars) and DMOG (hatched bars) treatment groups in the dentate gyrus when compared to shams (white bars; Figure 2Cii and 2Dii).

Effects of 7-day CIH treatment on synaptic plasticity in rat hippocampal slices

HFS was used to induce LTP in acute hippocampal slices derived from sham and 7-day CIH treated animals. In the CA1 region following 20 minutes of stable baseline recordings, HFS induced robust LTP in shams (open circles/white bar, 150.4±.5.5%, n=8) whilst LTP was impaired in the CIH group (filled circles/black bar) at 60 min post HFS (111.1±10.6%, n=5, **P<0.01; Figure 3A, B). In the dentate gyrus LTP was induced in the presence of 100 μm picrotoxin in order to induce robust LTP that could be measured for longer than 60 min. HFS induced significant LTP, albeit smaller than that observed in the CA1, with no

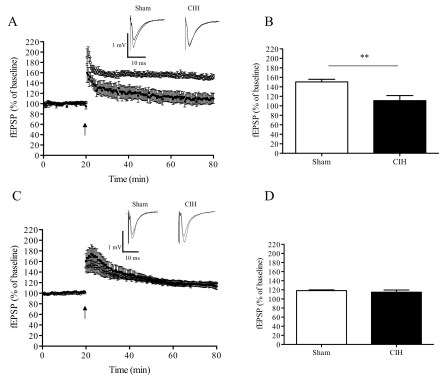

Figure 3
7-day CIH treatment impairs LTP in the CA1 but not dentate gyrus

A) Time course showing changes in fEPSP amplitude of sham treated animals (open circles) and 7-day CIH treated animals (closed circles) in the CA1 region. Baseline fEPSP amplitude was recorded for 20 minutes followed by HFS (indicated by the arrow). LTP was monitored for 1 hr post induction. Insets show sample fEPSP traces, 60 min following HFS superimposed over a representative fEPSP 10 min prior to HFS for sham (left) and 7-day CIH (right) treated animals. B) Summary data showing fEPSP amplitude 60 min following HFS, for sham (white bar) and 7-day CIH treated groups (black bar). C) Time course showing changes in fEPSP slope of sham treated animals (open circles) and 7-day CIH treated animals (closed circles) in the dentate gyrus. Baseline fEPSP amplitude was recorded for 20 minutes followed by HFS (indicated by arrow). LTP was recorded for 1 hour post induction. Insets show sample fEPSP traces, 60 min following HFS superimposed over a representative fEPSP 10 min prior to HFS for sham (left) and 7-day CIH (right) treated animals. D) Summary data showing fEPSP amplitude 60 min following HFS, for sham (white bar) and 7-day CIH (black bar) treated groups. All data is expessed as mean±SEM %, n=5; ** P<0.01.

significant difference between sham (open circles/white bar) and the CIH (filled circles/black bar) treatment groups (118.4±1.7%, vs. 114.9±4.6%, n=5, respectively; Figure 3C, D).

Effects of 7-day DMOG treatment on synaptic plasticity in rat hippocampal slices

In the CA1 region LTP was significantly impaired in slices from the 7-day DMOG treated group (closed circles/hatched bar, 113.4±7.1%, vs. shams, open circles/white bar, 150.4±.5.5%, n=8; P<0.01; Figure 4A, B). In the dentate gyrus there was no significant difference in the magnitude of LTP recorded 60 min post HFS between the sham (open circles/white bar, 118.4±1.7%, n=5) and DMOG (filled circles/hatched bar, 125.2±7.2%, n=5) treated groups (Figure 4C, D).

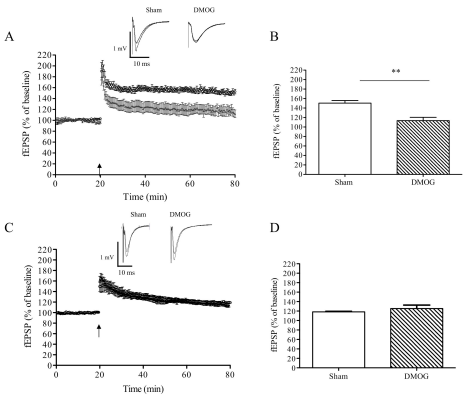

Figure 4
7-day DMOG treatment impairs LTP in the CA1 but not dentate gyrus

A) Time course showing changes in fEPSP amplitude of sham treated animals (open circles) and 7-day DMOG treated animals (closed circles) in the CA1 region. Baseline fEPSP amplitude was recorded for 20 minutes followed by HFS (indicated by the arrow). LTP was monitored for 1 hr post induction. Insets show sample fEPSP traces, 60 min following HFS superimposed over a representative fEPSP 10 min prior to HFS for sham (left) and 7-day CIH (right) treated animals. B) Summary data showing fEPSP amplitude 60 min following HFS, for sham (white bar) and 7-day DMOG (hatched bar) treated groups. C) Time course showing changes in fEPSP amplitude of sham treated animals (open circles) and 7-day DMOG (closed circles) treated animals in the dentate gyrus. Baseline fEPSP amplitude was recorded for 20 minutes followed by HFS (indicated by the arrow). LTP was recorded for 1 hour post induction. Insets show sample fEPSP traces, 60 min following HFS superimposed over a representative fEPSP 10 min prior to HFS for sham (left) and 7-day DMOG (right) treated animals. D) Summary data showing fEPSP amplitude 60 min following HFS, for sham (white bar) and 7-day DMOG (hatched bar) treated groups. All data is expessed as mean±SEM %, n=5; ** P<0.01.

Effect of combined 7-day CIH and DMOG treatment on synaptic plasticity in rat hippocampal slices

HFS was used to induce LTP in hippocampal slices derived from 7-day CIH treated animals who had also been treated simultaneously with DMOG for 7 days. In the CA1 LTP was significantly impaired in the CIH+DMOG treated group (grey circles/grey bar, 88.1±4.5%, n=7, P<0.05) compared to controls (Figure 5 and B). This reduction in LTP was greater than that observed in CIH treated animals alone (filled circles/black bar, 111.1±10.6%, n=5, P<0.05). In the dentate gyrus there was no impairment in LTP recorded 60 min post HFS in CIH+DMOG (grey circles/grey bar, 125.7±7.2%, n=4) when compared to CIH alone (filled circles/black bar, 114.9±4.6%, n=5; Figure 5C and D).

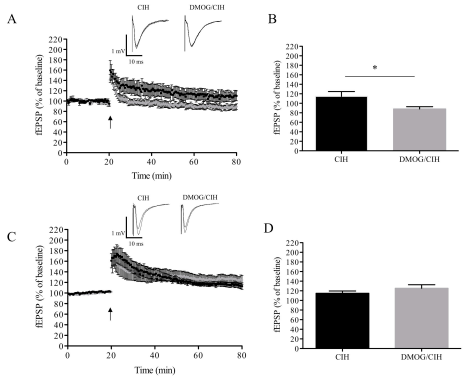

Figure 5
Effects of combined treatment with DMOG and CIH on LTP

A) Time course showing changes in fEPSP amplitude of CIH treated animals (filled circles) and 7-day DMOG combined with CIH (grey circles) in the CA1 region. Baseline fEPSP amplitude was recorded for 20 minutes followed by HFS (indicated by the arrow). LTP was monitored for 1 hr. Insets show sample fEPSP traces, 60 min following HFS superimposed over a representative fEPSP 10 min prior to HFS for CIH (left) and DMOG+CIH (right) treated animals. B) Summary data showing fEPSP amplitude 60 min following HFS, for CIH (black bar) and DMOG + CIH (grey bar) treated groups. C) Time course showing changes in fEPSP amplitude of CIH treated animals (closed circles) and DMOG+CIH (grey circles) treated animals in the dentate gyrus. Baseline fEPSP slope was recorded for 20 minutes followed by HFS (indicated by the arrow). LTP was recorded for 1 hour. Insets show sample fEPSP traces, 60 min following HFS superimposed over a representative fEPSP 10 min prior to HFS for CIH (left) and DMOG+CIH (right) treated animals. D) Summary data showing fEPSP amplitude 60 min following HFS, for CIH (black bar) and DMOG+CIH (grey bar) treated groups.All data is expessed as mean±SEM %, n=5; * P<0.05.

Effect of 7-day CIH treatment on the recovery of the fEPSP from 30 min acute hypoxia

In the CA1 and dentate gyrus, following a 20 min stable baseline recording, oxygenation was switched from 95% O_2/5% CO_2 to 95%N_2/5%CO_2 for 30 min (Figure 6A, C). In the CA1 region during hypoxic exposure the fEPSP amplitude was reduced to less than 20% of baseline for both CIH (filled circles, 19.37±5.7%, n=5) and sham treated animals (open circles, 11.14±1.5%, n=5, Figure A and B). Following the hypoxic insult in CIH treated animals, the fEPSP showed a greater rate of recovery compared to sham treated animals. Two way ANOVA showed a significant interaction between CIH treated and sham animals in the CA1 (F = 2.21, P<0.001). 7 min post re-oxygenation indicated a recovery of fEPSP amplitude to 96.1±11.5% (n=5) in CIH treated animals (black bars) compared to sham treated (white bars) groups

of 45.6±11.2% (n=5, 8 slices; P<0.05; Figure 6A and B).

In the dentate gyrus following 30 min of hypoxia the fEPSP amplitude was reduced to 65.7±3.9% (n=5) and 53.4±12.3% (n=5) of baseline in CIH (filled circles) and sham (open circles) treated animals respectively. Two way ANOVA also showed a significant interaction between CIH treated and sham animals in the dentate gyrus (F = 1.77, P<0.001). Post hoc analysis showed there was a significant difference between both groups in the amount of recovery of fEPSP amplitude following re-oxygenation at 30 min only (CIH, black bars, 108.4±7.2%, n=5 vs. sham, white bars, 80.9±7.6%, at 30 min; n=5; *P<0.05, Figure C and D).

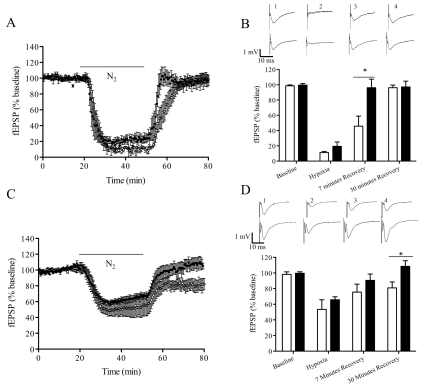

Figure 6
Effect of 7-day CIH treatment on the recovery of the fEPSP from 30-min acute hypoxia

A) Time course showing the effect of 30-min hypoxia on fEPSP amplitude in the CA1 region for sham (open circles) and CIH (closed circles) treated groups. Following 20 min stable recording, hypoxia was induced by switching from 95% O_2/5% CO_2 to 95% $N_2$5%CO_2 for 30 min (indicated by black line). Upon re-oxygentation the fEPSP amplitude recovered over a period of 5 to 10 min. B) Summary data showing fEPSP amplitude recorded at 10 min (baseline), 30 min after hypoxia induction (hypoxia), 7 min after the return to normoxia (7 min recovery), 30 min after the return to normoxia (30 min recovery) for both sham (white bars) and 7-day CIH (black bars) treated animals. Insets show sample traces from sham (upper traces) and CIH (lower traces) treated groups at 1) baseline 2) end of acute hypoxia 3) 7 min following re-oxygenation 4) 30 min following re-oxygenation. C) Time course showing the effect of 30-min hypoxia on fEPSP amplitude in the dentate gyrus for sham (open circles) and CIH (closed circles) treated groups. Following a 20 min stable recording, hypoxia was induced by switching from 95% O_2/5% CO_2 to 95% $N_2$5%CO_2 for 30 min (indicated by black line). Upon re-oxygentation the fEPSP amplitude recovered over a period of 5 to 10 min. B) Summary data showing fEPSP amplitude recorded at 10 min (baseline), 30 min after hypoxia induction (hypoxia), 7 min after the return to normoxia (7 min recovery), 30 min after the return to normoxia (30 min recovery) for both sham (white bars) and 7-day CIH (black bars) treated animals. Insets show sample traces from sham (upper traces) and CIH (lower traces) treated groups at 1) baseline 2) end of acute hypoxia 3) 7 min following re-oxygenation 4) 30 min following re-oxygenation. All data is expessed as mean±SEM %, n=5; * P<0.05.

Effect of chronic DMOG treatment on the recovery of fEPSP amplitude from 30 min acute hypoxia

In the CA1 and dentate gyrus, following a 20 min of stable baseline recording, oxygenation was switched from 95% O_2/5% CO_2 to 95%N_2/5%CO_2 for 30 min (Figure 7A and C). In the CA1 region sham (open circles) and chronic DMOG (filled circles) treated groups had similar maximum reduction in fEPSP amplitude during the 30 min hypoxic exposure (11.1±1.5%, n=6 vs. 11.7±1.5%, n=5; respectively). Following the 30 min hypoxic exposure in DMOG (hatched bars) treated animals, the fEPSP amplitude showed a greater rate of recovery compared to sham (white bars) treated animals. Two way ANOVA did not show a significant interaction between CIH treated and sham animals in the CA1 (F = 0.93, P = 0.73). This may have been due to the fact that analysis at 7 min post re-oxygenation indicated a recovery of fEPSP amplitude to 80.5±6.3% (n=4) compared to sham treated groups of 45.8±13.2% (n=6; P<0.05; Figure 7A and B), although this recovery of fEPSP amplitude was not maintained in the CA1 area.

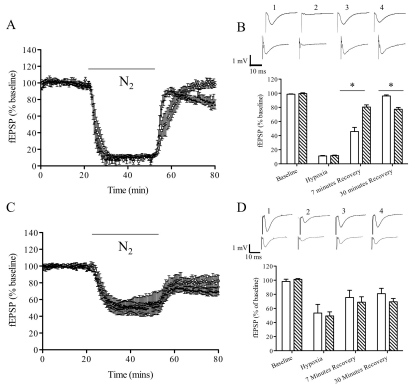

Figure 7
Effect of Chronic DMOG treatment on recovery from acute hypoxia

A) Time course showing the effect of 30-min hypoxia on fEPSP amplitude in the CA1 region for sham (open circles) and DMOG (closed circles) treated groups. Following 20 min stable recording, hypoxia was induced by switching from 95% O_2/5% CO_2 to 95% $N_2$5%CO_2 for 30 min (indicated by black line). Upon re-oxygentation the fEPSP amplitude recovered over a period of 5 to 10 min. B) Summary data showing fEPSP amplitude recorded at 10 min (baseline), 30 min after hypoxia induction (hypoxia), 7 min after the return to normoxia (7 min recovery), 30 min after the return to normoxia (30 min recovery) for both sham (white bars) and 7-day DMOG (hatched bars) treated animals. Insets show sample traces from sham (upper traces) and CIH (lower traces) treated groups at 1) baseline 2) end of acute hypoxia 3) 7 min following re-oxygenation 4) 30 min following re-oxygenation. C) Time course showing the effect of 30-min hypoxia on fEPSP amplitude in the dentate gyrus for sham (open circles) and DMOG treated groups (closed circles). Following a 20 min stable recording, hypoxia was induced by switching from 95% O_2/5% CO_2 to 95% $N_2$5%CO_2 for 30 min (indicated by black line). Upon re-oxygentation the fEPSP amplitude recovered over a period of 5 to 10 min. B) Summary data showing fEPSP amplitude recorded at 10 min (baseline), 30 min after hypoxia induction (hypoxia), 7 min after the return to normoxia (7 min recovery), 30 min after the return to normoxia (30 min recovery) for both sham (white bars) and 7-day DMOG treated animals (hatched bars). Insets show sample traces from sham (upper traces) and DMOG (lower traces) treated groups at 1) baseline 2) end of acute hypoxia 3) 7 min following re-oxygenation 4) 30 min following re-oxygenation. All data is expessed as mean±SEM %, n=5; * P<0.05.

That is after 30 min of re-oxygenation fEPSP amplitude was significantly reduced in the DMOG treatment group compared to shams (77.4±5.2%, n=6 vs 96.2±3.5%, n=5; respectively, $P<0.05$; Figure 7B).

In the dentate gyrus the maximum reduction in fEPSP amplitude in the sham (open circles) and DMOG (filled circles) treatment groups was not significantly different (53.4±12.3%, n=5 vs. 49.4±5.4% control, n=5; respectively; Figure 7C and D). Two way ANOVA did not show a significant interaction between DMOG treated and sham animals in the dentate ($F = 0.30$, $P = 0.99$). There was no significant difference between sham (white bars) and DMOG (hatched bars) treated groups in the rate or amount of recovery of fEPSP following re-oxygenation at both 7 min (75.6±10.2%, n=5 vs. 68.8±7.6%, n=4) and 30 min (80.9±7.6%, n=5 vs 69.5±4.6%, n=5; respectively; Figure 7C and D).

Discussion

Our data provide evidence of a significant impairment of synaptic plasticity in the CA1 but not dentate gyrus region of the hippocampus in CIH and DMOG treated animals. This was associated with a decreased activation of CREB possibly due to reduced cyclic adenosine monophosphate (cAMP), availability. CIH and DMOG treated animals showed elevated haematocrit percentages indicated by increased red blood cell counts. This was associated with up-regulation of EPO expression in CIH and DMOG treated animals. EPO is directly responsible for the production of red blood cells and is a recognised target of HIF-1α (Jelkmann, 2011). Also in our experiments both treatments significantly stabilized HIF-1α expression in the brain.

Our study confirms the effects of CIH on synaptic plasticity in the CA1 region of the rodent hippocampus (Payne *et al.*, 2004; Xie *et al.*, 2010). We were interested in determining the effects of CIH and DMOG treatment in the CA1 region and the dentate gyrus due to the differences in the response of each region to hypoxia and the fact that the effects of CIH on dentate gyrus function have yet to be reported. It has previously been shown that certain regions of the dentate gyrus (including the region investigated in this work) are more resistant to a decrease in oxygen availability than in the CA1 region (Kreisman *et al* 2000). Certainly in younger rat studies, such as these experiments in the dorsal hippoampus, the decrease in fEPSP amplitude is significantly less in the dentate gyrus than the CA1 region in response to acute hypoxia. It is also well reported that the dorsal hippocampus plays a crucial role in spatial memory formation and consolidation (Lee *et al.,* 2009). The ventral hippocampus would seem to be more involved in emotional and motivated behaviors (Kheirbek & Hen, 2011). Previous studies indicate that CIH impairs water maze performance and we were interested in determining if this correlated with decreased plasticity in the dentate gyrus (Goldbert et al., 2003). Certainly it will be interesting in the future to compare our results with those of the ventral hippocampus.

Interestingly, 7-day CIH treatment did not significantly affect LTP in the dentate gyrus. Of note, 7-day CIH treatment did not produce any changes in pyramidal or granular neuron excitability indicating that the presynaptic neurons and neurotransmitter release machinery may not be affected by CIH treatment. Xie *et al*. (2010) demonstrated that 7-day and 14-day CIH treatment resulted in significantly impaired LTP in the CA1 region of the mouse hippocampus which was associated with decreased expression of brain-derived neurotrophic factor (BDNF). BDNF activity has been implicated in the maintenance of LTP and the regulation of AMPA receptor subunits (Jourdi and Kabbaj, 2013; Lu *et al*., 2008). Both acute and chronic administration of BDNF rescued LTP deficits in CIH treated animals. Interestingly, BDNF expression was significantly down-regulated after 3-day CIH but did not produce LTP deficits. Given the importance of CREB phosphorylation in mediating synaptic plasticity we examined the effect of CIH on CREB activity. 7-day CIH treatment has been shown to be associated with impaired spatial learning (Goldbart *et al.*, 2003). Consistent with previous findings, 7-day CIH treatment significantly impaired phosphorylation of CREB (Goldbart *et al.*, 2003). The noted impairment in CREB phosphorylation began at day 1, 1 hr after CIH and remains depressed after 30 days CIH. The observed modulation of CREB may account for the decreased LTP expression we observed and the reduced Morris water maze performance observed by Goldbart *et al* (2003).

We have also provided evidence that chronic administration of DMOG gives rise to significant impairments in LTP in the CA1 region of the rat hippocampus. This impairment is associated with decreased activation of CREB in the hippocampus. Chronic inhibition of PHDs has become an attractive therapeutic target for the treatment and preconditioning of ischemic events including stroke. Chronic inhibition of PHDs with the pan-hydroxylase inhibitor DMOG 24 hours before middle cerebral artery occlusion (MCAO), significantly decreased infarct volume of the affected region and increased regional blood flow (Nagel *et al*., 2011). Although preconditioning is an unviable treatment option for primary ischemic attacks, research highlights the role of HIF stabilization and the neuroprotective properties associated with transcriptional activation of HIF. To determine the therapeutic effect of PHD inhibition following an ischemic event, Ogle *et al*. (2012) treated rats with DMOG 30 and 60 min after MCAO. Both treatment options significantly decreased infarct volume and decreased activation of apoptotic related caspase 3 (Ogle *et al*., 2012).

Despite the neuroprotective effects of PHD inhibition, acute application of DMOG has been shown to impair synaptic signalling and plasticity in the CA1 region of the rat hippocampus (Batti *et al*., 2010; Corcoran *et al*., 2013; Corcoran & O'Connor, 2013). Furthermore, given the depressant effects of acute DMOG application on synaptic signalling, DMOG administration may affect learning, memory and the behaviour of the treated rats, and further studies are required to address this current gap in our knowledge.

Inhibition of PHD2 also significantly decreases the availability of cAMP in the cytoplasm by increased expression of phosphodiesterase 4D (PDE4D; Huo *et al.*, 2012). Chronic PHD inhibition results in impaired CREB activation which is crucial for expression of LTP. Additionally PHD inhibition alters the trafficking of 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA) receptors in *Caenorhabditis elegans* which could potentially regulate LTP expression in mammals (Park *et al*., 2012). However it remains unknown if PHD inhibition regulates AMPA expression or trafficking in mammals.

In both treatment groups we noted a significant increased recovery rate or increased maximum fEPSP response following an acute hypoxic insult compared to sham animals. Induction of hypoxia results in a failure to maintain energy supply to the cells. Accordingly, neuronal cells decrease synaptic transmission in a protective manner to conserve energy for vital cellular maintenance. Evidence suggests that hypoxia is not the detrimental factor during ischemic injury but rather it is the re-oxygenation phase (Abramov *et al*., 2007). During normoxic conditions, individual mitochondria undergo spontaneous bursts of superoxide generation termed "superoxide flashes" (Wang *et al.*, 2008). Re-oxygenation following hypoxia leads to increase superoxide flash generation and contributes to increased oxidative stress during hypoxic insults. The generation of reactive oxygen species (ROS) during the re-oxygenation phase results in increased expression and activation of caspase-3 and associated apoptosis of neuronal cells. Accordingly, treatment with antioxidants significantly improves cell viability during oxidative stress associated with hypoxia-reoxygenation injury as well as oxygen glucose deprevation (Yao *et al.*, 2011; Massaad *et al*., 2011; Kim *et al.*, 2012). Importantly, preconditioning cells with acute hypoxia-reoxygenation insults (15 min hypoxia/15 min normoxia) improves cell viability to subsequent oxidative stress (Yao *et al.*, 2011). Recently, PHD inhibition has also been shown to decrease cortical neuron cell death induced by oxidative stress (Niatsetskaya *et al.*, 2010). The neuroprotective properties of hypoxic pre-treatment and PHD inhibition may be responsible for the improved recovery rates we observed in the CIH and DMOG treated animals. CIH and DMOG treatment may reduce the oxidative stress associated with reoxygenation from acute hypoxia and allow the neurons to return to maximum function at an increased rate (Massaad *et al.,* 2011). Although the hypoxic insult we have used is comparatively short in duration, previous work from our laboratory shows that exposure of neurons to hypoxia of up to 2 hr does not significantly alter neuronal recovery upon re-oxygenation (Batti & O'Connor, 2010). However, during severe ischemic conditions such as oxygen-glucose deprivation (OGD), synaptic signalling is irreversibly depressed even after 5-10 min (Pugliese et al. 2006). Since recent work highlights a neuroprotective role for DMOG preconditioning during cerebral ischemia (Nagel *et al.,* 2011), future work will help determine if CIH or DMOG preconditioning may confer neuroprotection to OGD *in vitro* or ischemic injuries *in vivo*.

It was also of interest to note that the recovery of fEPSP amplitude post acute hypoxia in 7-day DMOG treated animals was not maintained and showed a decline from peak recovery (figure 7A & B). We have attempted to use concentrations of DMOG that do not have a direct effect on synaptic transmission (see Batti et al., 2010). It is unlikely there are concentrations of DMOG remaining in the tissue that might be having a direct effect on synaptic transmission. Since no decline was observed on baseline in 7-day DMOG treated rats over an hr, it is more likely that the hypoxic event has triggered this decline. This will have to be investigated further to identify if it is an interaction of significance.

Hypoxia leads to anaerobic respiration within the affected cells and ATP production is reduced. This leads to an increase in AMP and adenosine in the cells which is secreted into the synapse to decrease synaptic transmission through adenosine A_1 autoreceptors. Maintenance of ATP degradation and AMP production is regulated by AMP-activated protein kinase (AMPK; Choi *et al*., 2001). AMPK is an intracellular warning system for decreased energy and is active during high AMP:ATP states such as hypoxia (Ahn *et al.*, 2012). Fluctuations of the AMP:ATP ratio are sensed by AMPK which compensates for decreased ATP by increasing glucose transport into the cells. Ischemic injury leads to the activation of AMPK and thus regulation of cellular homeostasis. Hypoxic cells show increased activation of AMPK to compensate for the hypoxic environment (Zhdanov *et al*., 2013). It is possible that AMPK may regulate the acute response to hypoxia in hippocampal cells. AMPK provides a neuroprotective effect during ischemia (McCullough *et al*., 2005; Kuramoto *et al.*, 2007; Venna *et al*., 2011). Up-regulation of AMPK during CIH or DMOG treatment may increase the sensitivity of hippocampal neurons to hypoxic stress and allow to cells to compensate/adapt to the threat at an enhanced rate to recover synaptic

function. Recently it has been shown that hypoxia may trigger AMPK activation through ROS-mediated activation of calcium release-activated calcium channels (Mungai *et al*., 2011). Further work will need to be performed to determine the mechanism underlying the improved time to recovery in CIH and DMOG treated animals.

Conclusion

In conclusion our study has shown that 7-day CIH impairs LTP in the CA1, but not the dentate gyrus, of rat hippocampal slices. Additionally, we provide novel evidence suggesting that chronic PHD inhibition (DMOG) impairs LTP in the CA1 region, but not the dentate gyrus, of the rat hippocampus. The impairments in LTP are not associated with any changes in synaptic excitability but rather with decreased CREB activity in the hippocampus. Despite CIH and DMOG treatment causing inhibitory effects on synaptic plasticity in the hippocampus, both treatments significantly increase the recovery rate of pyramidal neurons to an acute hypoxic insult suggesting adaptation to intermittent hypoxia or chronic pseudohypoxia.

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