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A role for prolyl hydroxylase domain proteins in hippocampal synaptic plasticity

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Abstract

Hypoxia-inducible factors (HIFs) are key transcriptional regulators that play a major role in oxygen homeostasis. HIF activity is tightly regulated by oxygen-dependent hydroxylases, which additionally require iron and 2-oxoglutarate as co-factors. Inhibition of these enzymes has become a novel target to modulate the hypoxic response for therapeutic benefit. Inhibition of prolyl-4-hydroxylase domains (PHDs) have been shown to delay neuronal cell death and protect against ischemic injury in the hippocampus. In this study we have examined the effects of prolyl hydroxylase inhibition on synaptic transmission and plasticity in the hippocampus. Field excitatory postsynaptic potentials (fEPSPs) and excitatory postsynaptic currents (EPSCs) were elicited by stimulation of the Schaffer collateral pathway in the CA1 region of the hippocampus. Treatment of rat hippocampal slices with low concentrations (10 μ M) of the iron chelator deferoxamine (DFO) or the 2-oxoglutarate analogue dimethyloxalyl glycine (DMOG) had no effect on fEPSP. In contrast, application of 1 mM DMOG resulted in a significant decrease in fEPSP slope. Antagonism of the NMDA receptor attenuated the effects of DMOG on baseline synaptic signalling. In rat hippocampal slices pre-treated with DMOG and DFO the induction of long-term potentiation (LTP) by tetanic stimulation was strongly impaired. Similarly, neuronal knockout of the single PHD family member PHD2 prevented murine hippocampal LTP. Pre-conditioning of PHD2 deficient hippocampi with either DMOG, DFO or the PHD specific inhibitor JNJ-42041935, did not further decrease LTP suggesting that DMOG and DFO influences synaptic plasticity primarily by inhibiting PHDs rather than unspecific effects. These findings provide striking evidence for a modulatory role of PHD proteins on synaptic plasticity in the hippocampus.

Introduction

The cellular response to hypoxia is regulated by hypoxia-inducible factors (HIFs), which promote the transcription of numerous genes required for the adaptation to low oxygen tensions (Wang and Semenza, 1993; Forsythe et al., 1996; Palmer et al., 1998). HIFs are heterodimeric proteins composed of

an α (1 α , 2 α or 3 α) and a β subunit. Under normoxic conditions, the α subunit is hydroxylated on conserved prolyl residues targeting HIF- α for proteasomal degradation (Kaelin and Ratcliffe, 2008). In addition, HIF-1 α is hydroxylated on a single conserved asparaginyl residue, which blocks its interaction with the transcriptional coactivators CBP/p300 required for the full transcriptional activity of HIF-1. Prolyl and asparaginyl hydroxylation is mediated through prolyl-4-hydroxylase domain proteins (PHDs) and factor-inhibiting HIF-1 (FIH-1), respectively by using molecular oxygen. Under hypoxic/ischemic conditions, where oxygen is limited, PHDs as well as FIH-1 are less active and thus hypo-hydroxylated HIF- α becomes stabilized resulting in transcription of HIF target genes. In addition to oxygen both PHDs and FIH-1 require iron (Fe²⁺) and 2-oxoglutarate (2-OG) as co-factors for hydroxylation (Kaelin and Ratcliffe, 2008). Pharmacological inhibition of these enzymes can be achieved by substituting 2-OG with dimethyloxalyl glycine (DMOG) or the iron chelator deferoxamine (DFO) (Ivan et al. 2001; Jaakkola et al. 2001; Siddiq et al. 2005).

Pharmacological inhibition of PHDs has become a novel therapeutic target for preconditioning against ischemic attacks and intervention following ischemia (Siddiq et al., 2005; Nagel et al., 2011). This could be of special interest for application in the central nervous system as ischemic stroke is one of the leading causes of death worldwide and a major cause for long-term disability (Rothwell, 2001). Ischemic stroke occurs when a thrombus or embolism blocks a cerebral blood vessel. Reduced blood flow to the brain causes massive neuronal cell death in the infarct core area and malfunctioning but still viable neural tissue in the adjacent penumbra region due to insufficient oxygen and glucose supply. At least in rodents pharmacological inhibition of PHDs has been shown to increase regional blood flow and decrease infarct volume following permanent or transient middle cerebral artery occlusion, delay neuronal injury in response to growth factor deprivation and increase cell survival in the hippocampus in response to an excitotoxic glutamate insult (Lomb et al., 2007, 2009; Batti et al., 2010; Rabie et al., 2011; Nagel et al., 2011). Post-ischemic intervention with PHD inhibitors decreases neuronal damage and attenuates behavioural deficits associated with ischemia (Ogle et al., 2012). Previously, we have demonstrated an acute depressing effect of DMOG on baseline synaptic transmission in the CA1 region of the hippocampus which is independent of the adenosine mediated decrease in synaptic signalling associated with hypoxia (Wu and Saggau, 1994; Scholz and Miller, 1996; Batti et al., 2010). Of note, pathologically elevated synaptic activity is a common symptom of many neurological diseases including ischemic stroke. Thus, in the present study, we investigated the impact of PHD inhibition on hippocampal long-term potentiation (LTP) by using pharmacological and genetic approaches.

Materials & Methods

Preparation of acute hippocampal slices

3-week-old male Wistar rats (P21-26) were used in these experiments. The Animal Research Ethics Committee of the Biomedical Facility at University College Dublin approved all experimental procedures, in accordance with European legislation. Animals were anaesthetized using 5% isoflurane and

decapitated using a guillotine. The brain was removed and placed in ice-cold artificial cerebrospinal fluid (aCSF) consisting of 120 mM NaCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.5 mM KCl, 10 mM Glucose, 1.2 mM MgSO₄ and 2 mM CaCl₂. 350 μ m transverse hippocampal slices were prepared using a Leica VT 1000S Vibroslice. Slices were left to recover for 1 hr at room temperature while supplied with 95%O₂/5%CO₂. Hippocampal slices were transferred to a submerged recording chamber continuously perfused with aCSF from a 100ml reservoir at a flow rate of 4 ml/min. Previously in our laboratory we have used fluorescence-quenching oxymetry (OXILITE™) to obtain oxygen profiles within the hippocampal tissue in control and hypoxic conditions (Batti et al., 2010). In hypoxic conditions (95%N₂/5%CO₂) pO₂ levels at the surface of the slice reduce to 40-45 mm Hg and reduce to approximately 8 mm Hg, 100 μ m below the surface of the slice where recording electrode tips are located. The temperature in the bath was maintained at 32-34°C. Temperatures lower than those found *in vivo* (37°C) are required for all *in vitro* brain slice preparations so that stable recordings can be made for over 4 hr. Neuron specific PHD2 knockout mice were generated as described previously (Kunze et al., 2012). Hippocampal slices were prepared from conventional and conditional Phd2^{flox/flox} mice as described above for Wistar rats. During dissection mice hippocampi were chilled with 0°C Ringer throughout.

Electrophysiology - Field recording

Field excitatory postsynaptic potentials (fEPSPs) were evoked by stimulation of the Schaffer-collateral pathway of the CA1 region of the hippocampus using aCSF-filled monopolar glass electrodes. fEPSPs were elicited at a frequency of 0.033 Hz and recorded from the dendritic field of the CA1 pyramidal neurons using aCSF-filled monopolar glass electrodes. The stimulating electrode was connected to a S48 Stimulator (Grass Instrument; Massachusetts, USA) via Grass SIU5 stimulus isolation unit. The fEPSPs were recorded from the dendritic field of CA1 pyramidal neurons. The recording electrode was connected to an Axopatch 1D via a CV-4 head stage, which amplified evoked responses 1000-fold. fEPSPs were acquired at 20 kHz and filtered at 5 kHz. Stimulus strength was adjusted to give 50% maximal response, determined by an input/output curve. Paired-pulse facilitation was elicited by stimulation of the Schaffer collateral pathway twice at 50 ms intervals. Paired-pulse ratio (PPR) was quantified as the ratio of fEPSP2/fEPSP1 slope. Long-term potentiation was elicited by high frequency stimulation (HFS), 3 trains at 100 Hz (1 s duration) separated by 20 s intervals. Recordings were acquired and analyzed using the software package WinWCP (J. Dempster, Strathclyde).

Whole-cell patch clamp recording

Whole-cell patch-clamp recordings were made from CA1 pyramidal neurons, at 32-34 °C and visualized using an upright microscope (Olympus BX51 WI, Middlesex, UK) with infrared differential interference contrast optics (IR-DIC). Patch pipettes were filled with intracellular solution containing (in mM) 130 KMeSO₄, 10 KCl, 0.2 EGTA, 10 HEPES, 20 phosphocreatine, 2 Mg₂ATP, 0.3 NaGTP (pH 7.3, 290-300 mOsm). Cells were voltage clamped at -60 mV and HFS was delivered in current clamp mode. Recordings were made using a Multiclamp 700B (Molecular Devices, Foster City, CA). Signals were

filtered at 5 kHz using a 4-pole Bessel filter and were digitized at 10 kHz using a Digidata 1440 analogue-digital interface (Molecular Devices). Data were acquired and analysed using PClamp 10 and Clampfit (Molecular Devices).

Primary hippocampal cell culture

Primary hippocampal cells were isolated from embryonic Wistar rats (E18). Cells were maintained in Neurobasal media with B-27, L-glutamine and penicillin/streptomycin. Experiments were performed at 14 days *in vitro*.

Immunoblotting

Primary hippocampal cells were treated with DMOG (10 μ M and 1 mM), DFO (10 μ M) and JNJ-42041935 (10 μ M) for 1 hr. Cells were washed in ice-cold phosphate buffered saline (PBS) before being scrapped with RIPA buffer supplemented with protease cocktail inhibitor, phosphatase inhibitor cocktail II and phosphatase inhibitor cocktail III. Hippocampal slices were homogenised in the same lysis buffer. 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. Proteins (20 μ g) were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with primary antibodies against HIF-1 α (1:500, Novus Biologicals), anti- α tubulin (1:1000, Sigma-Aldrich), anti-PHD2 (1:500, Novus Biologicals) and secondary anti mouse-HRP linked antibody (Cell Signalling).

Drugs

Dimethylallyl glycine (DMOG) and deferoxamine (DFO) were obtained from Sigma-Aldrich; D-2-amino-5-phosphonopentanoic acid (D-AP5) and 5,7-dichlorokynurenic acid (DCKA) were obtained from Tocris Bioscience. JNJ-42041935 was obtained from Johnson & Johnson. All agents were dissolved in dimethyl sulfoxide (DMSO) with a final concentration of less than 0.05% when diluted in aCSF.

Data analysis

All fEPSP slope and EPSC amplitude measurements are presented as a percentage of the baseline recordings. Baseline recordings were determined by the average of fEPSP slope or EPSC amplitude over 20 minutes prior to drug application. Data are presented as mean \pm SEM. Statistical significance was determined using either the non-parametric paired Wilcoxon matched t-test or the Mann-Whitney unpaired t-test.

Results

Pharmacological prolyl hydroxylase inhibition increases HIF-1 α stability and reduces baseline synaptic transmission

Acute PHD inhibition by application of 10 and 1000 μ M DMOG and 10 μ M DFO in cultured primary neurons caused a stabilization of HIF-1 α compared to control cells (Figure 1A). The effect of prolyl hydroxylase inhibition by DMOG on synaptic transmission was examined in the Schaffer collateral pathway. Application of 10 μ M DMOG caused no significant change in fEPSP slope 60 minutes after application (101.4 \pm 7.9%) compared to baseline (100.8 \pm 2.1%, n = 7, P > 0.05, Figure 1B). 1 mM DMOG caused a significant decrease in fEPSP slope 60 minutes after application (76.6 \pm 12.2%, n = 8, P < 0.01, Figure 1C). This effect has previously been shown by our laboratory to be reversible and mediated

through the postsynaptic NMDA receptor (Batti et al., 2010). Application of 10 μ M DFO had no significant effect on fEPSP slope after 60 minutes ($96.5 \pm 10.4\%$) compared to baseline ($99.2 \pm 6.1\%$, $n = 7$, $P > 0.05$, Figure 1D). Similar effects of DMOG on synaptic transmission were observed in whole-cell patch clamp recordings from CA1 pyramidal neurons, where acute application of DMOG (0.5 mM) depressed EPSC amplitude to $75.0 \pm 6.0\%$ of baseline ($n = 5$, Figure 1E).

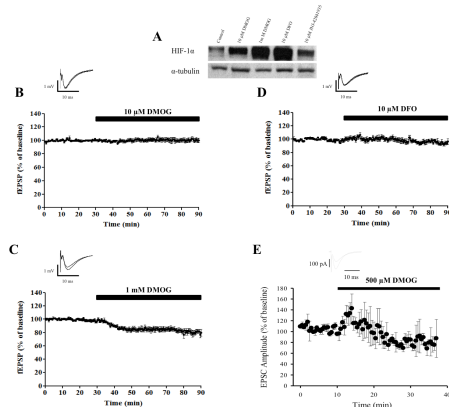


Figure 1
Pharmacological PHD inhibition increases HIF-1 α stability in hippocampal neurons and reduces baseline synaptic transmission in the CA1 region. (A) 1 hr treatment with DMOG (10 μ M and 1 mM), DFO (10 μ M) and JNJ-42041935 (10 μ M) stabilised HIF-1 α in cultured primary hippocampal neurons. (B) Normalised CA1 fEPSP slope shows no significant change 60 minutes after application of 10 μ M DMOG. Insets are representative traces showing no change in fEPSP before and following 10 μ M DMOG treatment. (C) Normalised CA1 fEPSP slope is significantly decreased following application of 1 mM DMOG ($76.62 \pm 12.2\%$, $n = 8$, $P < 0.01$ at 60 min). Insets are representative traces showing the change in fEPSP before and following 1 mM DMOG treatment. (D) Normalised CA1 fEPSP slope is not affected by 10 μ M DFO applied for 60 minutes. Insets are representative traces showing the change in fEPSP before and following 10 μ M DFO treatment. (E) The time-course for normalised CA1 neuronal EPSC amplitude over a 45 min period is shown. 500 μ M DMOG perfused for 30 min significantly depressed the EPSC amplitude to $75 \pm 6\%$ of baseline ($n = 5$).

Analysis of paired-pulse ratio (PPR) showed no significant difference following application of 10 μ M DMOG (1.53 ± 0.06), 1 mM DMOG (1.55 ± 0.27) or 10 μ M DFO (1.52 ± 0.03) compared to controls (1.51 ± 0.21). This finding on PPR was in contrast to our previously published work using field recordings from the CA1 region of the hippocampus, where 1 mM DMOG was shown to have a small but significant effect on PPF. We therefore carried out whole cell patch clamp analysis of PPF in CA1 pyramidal cells. DMOG had no effect on paired-pulse facilitation (1.48 ± 0.04 in control and 1.52 ± 0.1 in DMOG, $P > 0.05$, $n = 5$, data not shown).

A role for NMDARs in the inhibitory effects of DMOG on synaptic signalling

Previously, we have described that the acute effects of DMOG on fEPSP slope also decrease the isolated NMDA-mediated postsynaptic potential (Batti et al., 2010). Here we have further investigated the role of NMDA receptors in this inhibitory effect of DMOG. Inhibition of NMDARs with 50 μ M D-AP5 had no significant effect on fEPSP slope ($102.2 \pm 2.5\%$, $n = 6$, $P > 0.05$), and attenuated the suppressant effect of 1 mM DMOG on fEPSP slope ($101.7 \pm 6.6\%$, $n = 6$, $P > 0.05$, Figure 2). We postulated that the effects of DMOG may be mediated via the glycine binding site on the NMDAR. Specific inhibition of the glycine binding site by application of 5 μ M DCKA alone, resulted in a small but significant decrease in fEPSP

slope after 30 minutes. The baseline was normalised again in the presence of DCKA. Additional application of 1 mM DMOG caused no further significant change in fEPSP slope. ($89.9 \pm 5.6\%$, $n = 7$, $P > 0.05$, Figure 2).

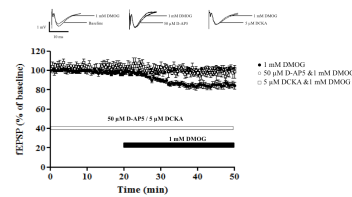


Figure 2
NMDA receptor antagonism reverses the inhibitory effects of DMOG on synaptic signalling. Inhibition of the NMDA receptor with 50 μ M D-AP5 followed by addition of 1 mM DMOG (open circles) resulted in no significant change in fEPSP slope ($n = 6$, $P > 0.05$). Addition of 1 mM DMOG had no significant effect on fEPSP slope in the presence of DCKA (5 μ M; $89.9 \pm 5.6\%$, $n = 7$, $P > 0.05$, open squares). Insets are representative traces showing the change in fEPSP slope during DMOG application, D-AP5 & DMOG application and DCKA & DMOG application.

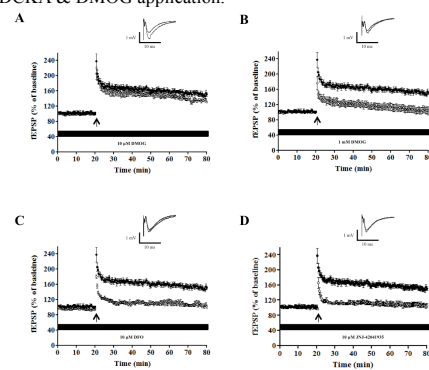


Figure 3
DMOG impairs LTP in the CA1 region. (A) Application of 10 μ M DMOG for 60 min prior to LTP induction has no effect on LTP. Shown is normalized fEPSP slope for controls (filled circles) or in the presence of 10 μ M DMOG (open circles) for 80 minutes. HFS (indicated by arrow) caused significant post tetanic potentiation in both controls and DMOG treated slices. DMOG treated slices maintained significant LTP for 60 minutes ($141.1 \pm 6.8\%$, $n = 5$, $P < 0.01$), which was not significantly different to controls ($150.8 \pm 12.5\%$, $n = 7$, $P > 0.05$). Insets are representative traces from DMOG treated slices showing the change in fEPSP 10 min before and 60 min following 10 μ M DMOG treatment. (B) Slices were treated with 1 mM DMOG (open circles) causing a significant decrease in LTP (Figure 1B). fEPSP slope was normalized for the final 20 minutes of treatment before HFS (indicated by arrow). DMOG treated slices show impaired PTP and failed to generate LTP after 60 minutes ($105.2 \pm 18.5\%$, $n = 8$, $P > 0.05$) which was significantly lower than control slices ($150.8 \pm 12.5\%$, $n = 7$, $P < 0.001$). Insets are representative traces from DMOG treated slices showing the change in fEPSP 10 min before and 60 min following 10 μ M DMOG treatment. (C) Normalized fEPSP slope in the presence of DFO (open circles) for 60 minutes before HFS (indicated by arrow). HFS failed to induce LTP after 60 minutes, which was significantly impaired, compared to control slices ($101.6 \pm 10.7\%$, $n = 7$, $P < 0.001$). Insets are representative traces from DFO treated slices showing the change in fEPSP 10 min before and 60 min following 10 μ M DFO treatment. (D) Normalised fEPSP slope in JNJ-42041935-treated slices (open circles). HFS failed to generate LTP 60 minutes after induction ($106.57 \pm 5.66\%$, $n = 6$, $P < 0.001$). Insets are representative traces from JNJ-42041935 treated slices showing the change in fEPSP 10 min before and 60 min following treatment.

Effect of pharmacological prolyl hydroxylase inhibition on synaptic plasticity

Slices were treated with DMOG (10 μ M and 1 mM), DFO (10 μ M) and JNJ-42041935 (10 μ M) for one hr.

The final 20 minutes of the fEPSP slopes were normalised to 100 % before inducing LTP in the Schaffer collateral pathway. Treatment with 10 μ M DMOG caused no significant change in post-tetanic potentiation or LTP after 60 minutes (141.1 ± 6.8 %, $n = 5$, $P > 0.05$) compared to controls (150.9 ± 13.8 %, $n = 7$, Figure 3A). Application of 1 mM DMOG caused a significant decrease in fEPSP slope 5 minutes after HFS compared to controls. DMOG (1 mM) treated slices failed to generate LTP after 60 minutes (105.2 ± 18.5 %, $n = 8$, $P < 0.001$) compared to baseline (100.3 ± 5.5 %). Potentiation after 60 minutes was also significantly impaired compared to control slices (150.9 ± 13.8 %, $n = 7$, $P < 0.001$, Figure 3B). Application of DFO caused a significant impairment in post-tetanic potentiation compared to controls 5 minutes after HFS. Potentiation remained significantly lower than control slices 60 minutes after HFS (101.4 ± 11.2 %, $n = 7$, $P < 0.001$, Figure 3C). Application of the novel PHD inhibitor JNJ-42041935 had no effect on baseline transmission or isolated NMDA fEPSPs (data not shown). JNJ-42041935 treated slices failed to generate LTP 60 minutes after HFS (106.57 ± 5.66 %, $n = 6$, $P < 0.001$, Figure 3D). DMOG also inhibited LTP in whole-cell patch clamp recordings in slices pre-incubated with 0.5 mM DMOG for 30 min. In control recordings HFS potentiated EPSCs to 132 ± 7 % of baseline amplitude ($n = 5$, Figure 4A). In DMOG-treated slices HFS failed to induce potentiation. In fact EPSCs were depressed although not significantly to 79.0 ± 10.1 % of baseline amplitude at 60 min after HFS ($n = 9$, Figure 4B, C).

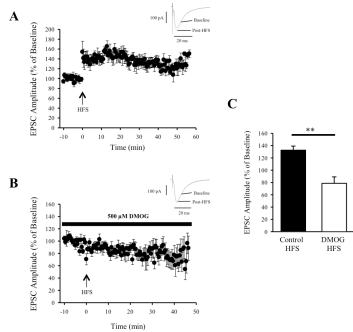


Figure 4
Effect of DMOG on LTP in whole cell recordings from CA1 pyramidal cells (A) In control slices HFS, indicated by arrow, gave rise to robust LTP monitored for over 60 min (potentiated EPSC amplitude was 132 ± 7 % of baseline amplitude at 60 min, $n = 5$). Insets are representative traces in control slices showing the change in EPSC amplitude 10 min before and 60 min following HFS. (B) DMOG-treated slices failed to induce potentiation of the EPSC (79 ± 10 % of baseline amplitude at 60 min after HFS; $n = 9$). Insets are representative traces from the DMOG treated slices showing the change in EPSC 10 min before and 60 min following HFS. (C) Summary of data from figures A and B at 60 min after HFS (** $P < 0.01$).

Neuron-specific inactivation of PHD2 impairs long-term potentiation

Next, we aimed to analyse whether the effects of the pan-hydroxylase inhibitors DMOG, DFO and JNJ-42041935 on LTP are primarily mediated through PHD inhibition. We used transgenic mice with specific neuronal PHD2 loss as PHD2 is probably the most abundant PHD isoform in the brain of adult mice, and plays a key role in regulating steady-state levels of HIF-1 α under normoxic conditions (Kunze et al., 2012). Mice with conditional knockout of

neuronal PHD2 (nPhd2 Δ/Δ) showed elevated levels of HIF-1 α and decreased amounts of PHD2 in hippocampal slices as compared to control mice (Phd2 $^{flox/flox}$) ($n = 4$, Figure 5 A). We examined the effect of conditional knockout of neuronal PHD2 had on synaptic transmission and kinetics. We first examined the effect of nPhd2 Δ/Δ on the sensitivity of the synaptic response to an increased stimulus input. As the presynaptic fibre volley increases, the postsynaptic fEPSP slope increases in a linear fashion. There is no significant difference at any input-output responses between Phd2 $^{flox/flox}$ and nPhd2 Δ/Δ (Figure 5B). Analysis of baseline PPR show no significant difference between Phd2 $^{flox/flox}$ (1.49 ± 0.03 , $n = 15$) and nPhd2 Δ/Δ (1.45 ± 0.04 , $n = 14$, $P > 0.05$) (Figure 5C). nPhd2 Δ/Δ slices were able to generate LTP in CA1 pyramidal neurons 60 minutes after HFS (122.5 ± 6.7 % of baseline, $n = 9$, $P < 0.01$). However, this was significantly lower than in Phd2 $^{flox/flox}$ mice, which were able to sustain 152.2 ± 8.0 % of baseline potentiation ($n = 9$, $P < 0.05$) (Figure 5D).

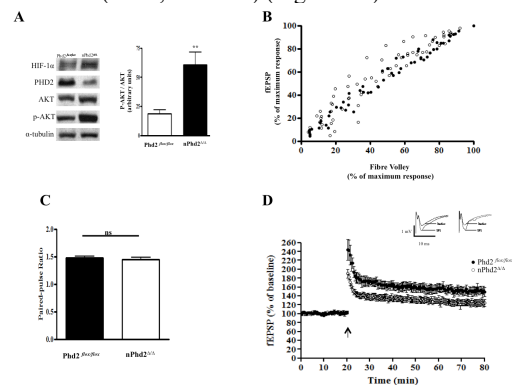


Figure 5
Loss of neuronal PHD2 impairs LTP in the CA1 region (A) Immunoblotting for HIF-1 α (top row) shows elevated HIF-1 α expression (row 1) and reduced PHD2 (row 2) in nPhd2 Δ/Δ hippocampal slices compared to Phd2 $^{flox/flox}$. α -tubulin expression confirms equal loading (5th row). p-AKT levels are also elevated in these mice (4th row and right hand column) (B) Basal synaptic transmission was measured in conventional and conditional Phd2 $^{flox/flox}$ mice. The slope of the input-output curve was similar in both mice. Fibre volley (x-axis) is graphed against fEPSP slope (y-axis) (C) Paired pulse facilitation is not altered in conditional Phd2 $^{flox/flox}$ mice. PPF ratio was measured using a 50 ms interstimulus interval with the second fEPSP divided by the first. (D) In Phd2 $^{flox/flox}$ slices (black circles), HFS gave rise to robust LTP monitored for over 60 min (potentiated fEPSP amplitude was 155.24 ± 8.01 % of baseline amplitude at 60 min, $n = 9$). In nPhd2 Δ/Δ slices (open circles), HFS induced LTP, which was sustained for 60 minutes but was significantly lower than in Phd2 $^{flox/flox}$ slices (122.51 ± 6.7 % of baseline, $n = 9$, $P < 0.05$). Insets are representative traces in showing the change in fEPSP amplitude 10 min before and 60 min following HFS.

Phd2 $^{flox/flox}$ slices treated with 1 mM DMOG significantly impaired LTP following HFS (106.9 ± 7.0 % of baseline, $n = 8$, $P < 0.001$) compared to controls (Figure 6A). In nPhd2 Δ/Δ slices, 1 mM DMOG application resulted in significant LTP which is not significantly different to untreated nPhd2 Δ/Δ slices (116.2 ± 6.4 % of baseline, $n = 8$, $P > 0.05$) (Figure 6B). Application of 10 μ M DFO significantly impaired LTP in Phd2 $^{flox/flox}$ slices following HFS (103.0 ± 7.7 % of baseline, $n = 7$, $P < 0.001$) compared to controls (Figure 6C). In nPhd2 Δ/Δ slices, 10 μ M DFO application fails to impair LTP resulting in robust LTP 60 minutes after HFS (121.5 ± 10.0 % of baseline, $n = 7$) which was not significantly different to control nPhd2 Δ/Δ slices (Figure 6D, $P > 0.05$). Application of 10 μ M JNJ-42041935, a specific

PHD inhibitor, significantly impaired LTP following HFS in Phd2^{lox/lox} slices (124.2 ± 2.9 % of baseline, $n = 8$, $P < 0.05$) compared to controls (Figure 7A). In nPhd2^{Δ/Δ} slices treated with JNJ-42041935 for 60 minutes, HFS induced a robust LTP 60 minutes after HFS which is not significantly different to non treated nPhd2^{Δ/Δ} slices (138.5 ± 3.7 % of baseline, $n = 6$, $P > 0.05$) (Figure 7B).

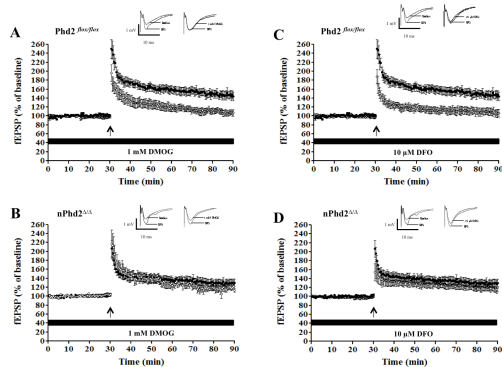


Figure 6
Effects of DMOG and DFO on LTP in the PHD2 deficient hippocampus

(A) In Phd2^{lox/lox} slices, DMOG (open circles) significantly impaired LTP following HFS (106.88 ± 7.04 % of baseline, $n = 8$, $P < 0.001$) compared to controls (black circles). (B) In nPhd2^{Δ/Δ} slices, DMOG application results in significant LTP which is not significantly different to control nPhd2^{Δ/Δ} slices (116.19 ± 6.41 % of baseline, $n = 8$, $P > 0.05$). (C) In Phd2^{lox/lox} slices, DFO (open circles) significantly impaired LTP following HFS (102.98 ± 7.73 % of baseline, $n = 7$, $P < 0.001$) compared to controls (black circles). (D) In nPhd2^{Δ/Δ} slices, DFO application fails to impair LTP (121.48 ± 9.96 % of baseline, $n = 7$) which was not significantly different to control nPhd2^{Δ/Δ} slices (black circles, $P > 0.05$). Insets are representative traces showing the change in fEPSP amplitude 10 min before and 60 min following HFS.

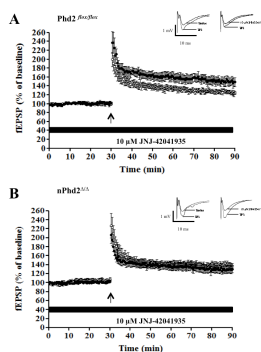


Figure 7
Effects of JNJ-42041935 on LTP in the PHD2 deficient hippocampus
(A) 10 μ M JNJ-42041935 (open circles) significantly impaired LTP following HFS in Phd2^{lox/lox} slices (124.23 ± 2.85 % of baseline, $n = 8$, $P < 0.05$) compared to controls (black circles). (B) In nPhd2^{Δ/Δ} slices, JNJ-42041935 application results in significant LTP (open circles) which is not significantly different to control nPhd2^{Δ/Δ} slices (138.45 ± 3.65 % of baseline, $n = 6$, $P > 0.05$).

Discussion

Since their discovery and subsequent characterisation, PHDs have become recognized as cellular oxygen sensors. They suppress the HIF pathway mediating an adaptive cellular response to hypoxia/ischemia by destabilization of the HIF- α subunits in an oxygen-dependent manner (Willam et al., 2004). PHD inhibition has become a novel target for preconditioning systems for ischemic as well as therapeutic intervention following ischemic attacks (Miyata et al., 2011; Ogle et al., 2012). The benefits of such preconditioning have been overwhelmingly positive both *in vitro* (Lomb et al., 2007, 2009; Batti

et al., 2010) and *in vivo* (Kasiganesan et al., 2007; Chen et al., 2008; Nagel et al., 2011). In this study, we observed that acute treatment with the pan-hydroxylase inhibitors DMOG, DFO and JNJ-42041935, at concentrations widely used in the literature significantly decreases long-term potentiation in the CA1 region of the murine hippocampus. In addition we provide evidence of a role for PHD2 mediating synaptic plasticity and the LTP impairments associated with PHD inhibition.

We have previously reported that application of DMOG causes a reversible inhibition of synaptic signalling and also reduces the isolated NMDA fEPSP in rat hippocampus (Batti et al., 2010). In this work we also reported a small but significant effect of DMOG on PPR using fEPSP recordings. Here we show, using whole-cell recordings, that EPSC PPR is unchanged in the presence of DMOG. The lack of change in paired-pulse ratio in response to DMOG application, carried out under voltage clamp conditions, may indicate an effect that is tied to postsynaptic actions. It is possible that DMOG may have a low affinity for postsynaptic NMDARs resulting in partial antagonism of the receptor through the glycine-binding site. In our experiments both D-AP5 and DCKA, the NMDAR glycine-binding site antagonist reversed the effects of DMOG on baseline synaptic transmission.

We used 3 pan-hydroxylase inhibitors to determine a role for PHDs on synaptic plasticity. It has been previously reported that DFO impairs synaptic signalling and plasticity in a NMDA-dependent manner (Munoz et al., 2011). However, this was achieved at a much higher concentration than the one used in this study. Our results confirm DFO impairs LTP even at low concentrations. Low concentrations of DMOG (10 μ M) was sufficient to stabilize HIF-1 α but had no effect on baseline transmission or LTP suggesting the effects of PHD inhibition on synaptic plasticity is independent of HIF-1 α activity. Higher concentrations of DMOG (1 mM) caused a significant reduction in LTP, which may be mediated by the potential antagonism of NMDARs, similar to DFO. It has also been reported that the concentrations of DFO and DMOG used is enough to decrease cell viability (Milosevic et al., 2009). However, we noticed no toxic effects during the acute treatment used in this study. We also examined the effects of a specific PHD inhibitor, JNJ-42041935, on synaptic signalling and plasticity. JNJ-42021935 has much greater affinity for PHDs compared to DFO and DMOG although is not specific for any particular isoform (Barrett et al., 2011). JNJ-42041935 had no significant effect on synaptic signalling or isolated NMDA-mediated fEPSPs but significantly reduced LTP after 1 hr treatment. Whilst DMOG and DFO may affect NMDAR function, the results of JNJ-42041935 inhibition suggest a potential role for PHDs mediating synaptic plasticity in the CA1 region of the hippocampus.

In these experiments neuronal ablation of the single PHD family member PHD2 prevented murine hippocampal LTP but did not affect baseline synaptic transmission or neuronal sensitivity. Interestingly, the inhibitory potential of DMOG, DFO or JNJ-42041935 on LTP in PHD2 deficient hippocampi was lower compared to that in hippocampal tissue derived from floxed PHD2 mice implying that neuronal PHD2 ablation per se might lower synaptic plasticity, but simultaneously induce an unknown compensatory

mechanism promoting LTP, which cannot be impaired by the pan-hydroxylase inhibitors. Assuming that pan-hydroxylase inhibitors influence synaptic plasticity primarily by inhibiting PHDs this compensatory response might not involve the other isoforms, PHD1 and PHD3 (see figure 8). Previously our immunohistochemical analyses have revealed that the majority of PHD2 expressing cells within cortex, striatum and hippocampus, represent

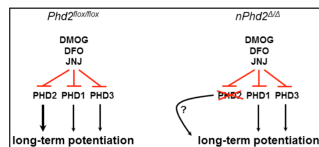


Figure 8
Prolyl hydroxylase domain 2 plays a role in the modulation of long-term potentiation. Under normal oxygen conditions PHD2 plays a major role in the recycling of AMPA receptors during LTP (thicker arrow). During hypoxia and the inactivation of PHD2, LTP may be impaired. The process by which this occurs is still to be determined (?). A role for PHD1 and 3 has yet to be elucidated.

neurons (Kunze et al., 2012). Moreover, neuron-specific ablation of PHD2 in the forebrain resulted in 90% reduction in protein level in brain hemispheres. This finding was surprising as glial and endothelial cells outnumber neuronal cells by at least 10-fold and further indicates that neurons are the main cerebral cell type expressing PHD2 (Kunze et al., 2012). However, it remains open whether inhibition of astroglial and endothelial PHD enzymes upon treatment with DMOG, DFO or JNJ contributes to impairment of hippocampal LTP.

In brains of PHD2 deficient mice we did not find altered expression of the other isoforms PHD1 and PHD3 (Kunze et al., 2012), but we cannot exclude that PHD2 deficient hippocampal neurons exhibit altered enzymatic activity of PHD1 and/or PHD3 that may account for the LTP phenotype observed in the present study. As DMOG, DFO and possibly also JNJ unselectively inhibit all PHD isoforms, the pharmacological approach does not allow us to determine if PHD1 and/or PHD3 inactivation contributes to the impairment of hippocampal LTP.

PHD2 deficiency significantly increased the protein abundance of HIF-1 α in hippocampal neurons. HIF-1 α promotes the transcription of numerous target genes including VEGF that was recently shown to interfere with synaptic plasticity (McCloskey et al., 2005; Huang et al., 2010; Licht et al., 2011). There are also an increasing number of alternative PHD targets such as I κ B kinase β (IKK β), regulating nuclear factor- κ B (NF- κ B) activity that may play a role in modulating LTP in PHD2 deficient mice (Cummins et al., 2006; Fu et al., 2010). They also showed that treatment of cells with DMOG or siRNA against PHD1 or PHD2 led to NF- κ B activation (Cummins et al., 2006). In a previous study we have demonstrated that NF- κ B activity is modulated during LTP (Sheridan et al.,

2007). Moreover, mice deficient for c-Rel, a NF- κ B family member had normal baseline synaptic transmission but exhibited reduced LTP at Schaffer collateral synapses (Ahn et al., 2008). However, it remains to be seen if the loss of PHD2 influences synaptic plasticity by transcriptional (HIF/NF- κ B-dependent or -independent) or non-transcriptional mechanisms.

LTP suppression caused by one hr pre-treatment with the pan-hydroxylase inhibitors argues against a transcriptional mechanism and suggests a rapid non-transcriptional regulation of synaptic plasticity. In addition to HIF- α and IKK β , a limited number of proteins targeted by PHDs has been identified such as LIN-10, transient receptor potential cation channel A1 (TRPA1), activating transcription factor 4 (ATF4), paired box gene 2 (Pax-2), sprouty homolog 2 (Spry2), large subunit of RNA polymerase II (Rpb1), β 2-adrenergic receptor (β 2-AR) and phosphodiesterase 4D (PDE4D; Wong et al., 2013). Among them LIN-10 and PDE4D could be potential targets linking PHD activity to hippocampal LTP. A recent study in the nematode *Caenorhabditis elegans* demonstrated that egg laying defective (Egl)-9E (a PHD homolog in the nematode) interacts with LIN-10, a protein that is homologous to mammalian Mint proteins promoting the recycling of endocytosed AMPA receptor subunit glutamate receptor (GLR)-1 to the plasma membrane. Egl-9E ensures the endosomal localization of LIN-10 facilitating GLR-1 trafficking to synapses (Park et al., 2012; Wong et al., 2013). PDE4D hydrolyzes intracellular cyclic adenosine monophosphate (cAMP) and thereby prevents the cAMP/protein kinase A (PKA)/cAMP-responsive element binding protein (CREB) pathway that is crucial for LTP and transcription of LTP-related genes. Accordingly, PDE4D deficient mice displayed enhanced early hippocampal LTP following HFS (Rutten et al., 2008). In cardiomyocytes PHD2 controls intracellular cAMP level by inhibiting PDE4D suggesting that a similar mechanism could also be present in hippocampal neurons (Huo et al., 2012). Further work will be required to elucidate signaling pathways downstream of the PHD enzymes affecting hippocampal long-term potentiation.

Hippocampal LTP is widely accepted as one of the main cellular mechanisms underlying memory formation and learning. However, to elucidate if impaired LTP in PHD2 deficient hippocampi causes altered cognitive function *in vivo*, multiple behavioral testing is required. Neuronal cell death by over-excitation (excitotoxicity) is a characteristic of several neurodegenerative diseases such as ischemic stroke. As LTP and excitotoxicity share the activation of postsynaptic NMDAR by glutamate released from the presynaptic compartment, one might suppose that PHD2 deficient neurons are at least partly protected from excitotoxicity. Accordingly, we recently demonstrated that PHD2 knockout animals display a significantly reduced cell death of excitotoxicity-sensitive hippocampal CA1 neurons upon acute ischemic stroke (Kunze et al., 2012). PHD2 plays an important role in oxygen homeostasis and cell survival and conditional knockouts have highlighted the role for PHD2 in mediating this protection in response to ischemic injury (Hölscher et al., 2011; Chen et al., 2012; Kunze et al., 2012; Franke et al., 2013).

It is important to note that when isolating hippocampal slices we are producing an ischemic state within the slice and this may affect our attempts to

alter the PHD/HIF system during electrophysiological recording. Under our *in vitro* conditions, metabolic changes are likely to occur and alter the redox state of the neurons and may influence PHD activity and HIF accumulation. Also, recording neurotransmission from the hippocampus requires incubation in a hyperoxic state and may further alter the HIF pathway. However, the changes in oxygen tension or PHD inhibition in this paradigm results in a hypoxic physiological state (Batti et al., 2010; Batti & O'Connor, 2010). Further work, including *in vivo* electrophysiological recording in wild type and PHD2 deficient mice as well as animals treated with pan-hydroxylase inhibitors would eliminate the limitations associated with *in vitro* field recording. There is no doubt that there are significant differences in oxygen availability between slices and the intact brain during neuronal activity. For a detailed review of some of these findings, see Turner et al., (2007).

Finally Aragonés et al. (2008) demonstrated that general loss of PHD1 lowers oxygen consumption in skeletal muscle by reprogramming glucose metabolism from oxidative to more anaerobic ATP production through up-regulation of pyruvate dehydrogenase kinase (PDK) 1 and 4. PDKs restrict the entry of glycolytic intermediates into the TCA cycle by inhibiting the activity of pyruvate dehydrogenase complex (PDC) required for the conversion of pyruvate to acetyl CoA (Aragonés et al., 2008). Of note, PDK1 is described as HIF-1 target gene (Kim et al., 2012). Notably, under normoxic conditions the expression of glycolysis-related enzymes (including PDK1) in brains of neuron-specific PHD2 knockout mice was not different in comparison to that in wild type littermates (Kunze et al., 2012). However, we cannot fully exclude that any non-transcriptional mechanisms promote the switch from oxidative to anaerobic glucose metabolism causing reduced ATP production in PHD2-deficient (hippocampal) neurons, which may contribute to the decreased hippocampal LTP.

In conclusion this study provides novel evidence for a role for PHD2 in long-term potentiation in the hippocampal CA1 region.

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