Hypoxia inducible factor signalling mechanisms in the central nervous system

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Abstract
In the CNS neurons are highly sensitive to the availability of oxygen. In conditions where oxygen availability is decreased neuronal function can be altered, leading to injury and cell death. Hypoxia has been implicated in a number of central nervous system pathologies including stroke, head trauma, and neurodegenerative diseases. Cellular responses to oxygen deprivation are complex and result in activation of short- and long-term mechanisms to conserve energy and protect cells. Failure of synaptic transmission can be observed within minutes following this hypoxia. The acute effects of hypoxia on synaptic transmission are primarily mediated by altering ion fluxes across membranes, presynaptic effects of adenosine and other actions at glutamatergic receptors. A more long-term feature of the response of neurons to hypoxia is the activation of transcription factors such as hypoxia inducible factor. The activation of hypoxia inducible factor is governed by a family of dioxygenases called hypoxia inducible factor prolyl 4 hydroxylases (PHDs). Under hypoxic conditions, PHD activity is inhibited, thereby allowing hypoxia inducible factor to accumulate and translocate to the nucleus, where it binds to the hypoxia-responsive element sequences of target gene promoters. Inhibition of PHD activity stabilizes hypoxia inducible factor and other proteins thus acting as a neuroprotective agent. This review will focus on the response of neuronal cells to hypoxia inducible factor and its targets, including the prolyl hydroxylases. We also present evidence for acute effects of PHD inhibition on synaptic transmission and plasticity in the hippocampus.

Keywords: Hypoxia inducible factor, prolyl hydroxylase domain, hippocampus, synaptic transmission, long-term potentiation, AMPA receptors.

Introduction

Maintenance of normal brain function depends on a continuous supply of oxygen (O_2). When oxygen demand exceeds supply cells become hypoxic. This hypoxia can be caused by a reduction in partial pressure of O_2 in the atmosphere, such as high altitude, or a failure in the transport of O_2 by the vascular system. Failures can occur by occlusion, constriction or haemorrhage of the blood vessels leading in impaired blood flow to the tissue as well as decreased availability of haemoglobin. Systemic hypoxia, hypercapnia and alterations in blood pH are detected by the carotid body and aortic body chemoreceptors (Prabhakar, 2000). During hypoxia, the chemoreceptors release neurotransmitters to afferent fibres which project to the nucleus tractus solitarius in the medulla, the central respiratory centre. Hypoxia within the central nervous system is detected by central chemoreceptors located in the medulla of the brainstem. Efferent neurons from the central medulla regulate respiration and cardiovascular tone to adjust to the hypoxic conditions. It is important to note that under normal conditions, the mammalian brain experiences 1 to 5 % O_2 (7.6–38 mmHg), low enough to be considered hypoxic in systemic organs but enough to meet the metabolic demands of the tissue (Silver and Erecinska, 1998). Thus, cells of the central nervous system are adapted to low oxygen concentrations and can respond to decreases in O_2 below 1 %. Oxygen sensing is critical for cell survival and rapid adaptation to hypoxic stresses. Oxygen sensing mechanisms have been implicated in numerous pathophysiological conditions including, stroke, cancer, Alzheimer's disease and obstructive sleep apnoea. The global response to a hypoxic stress is to increase heart rate and respiration in an attempt to increase oxygen uptake and transportation. However, this review will focus on the acute and chronic response of cells within the central nervous system.

HIF and cellular responses to hypoxia

The discovery of the hypoxia-inducible factor (HIF) family of transcription factors has provided insight into the cellular mechanisms of oxygen homeostasis and the response to hypoxia. The HIF complex consists of two subunits, HIF-α and HIF-β. HIF-β subunits are constitutively expressed nuclear proteins which are not subject to oxygen-sensitive degradation (Gu et al., 2000). HIF-1β plays an important role in midline development in the central nervous system. However, this response is dependent on HIF-1β-SIM dimerization rather than a hypoxic response (Franks and Crews, 1994). HIF-α exists as multiple isoforms, namely HIF-1α, HIF-2α and HIF-3α (Gu et al., 1998; Wang et al., 1995; Wenger, 2002). HIF-1α is a cytosolic protein, which is constitutively expressed but rapidly degraded. The stabilization and activity of HIF-1α is oxygen-sensitive. HIF-1α levels increase exponentially with a fall in PO_2 with maximal levels induced at <0.5% O_2 (Jiang et al., 1996). HIF-1α is expressed in cells throughout the central nervous system in response to hypoxia, including neurons, astrocytes, oligodendrocytes, microglia, neural progenitor cells and ependymal cells (Chávez et al., 2000; Lu et al., 2006; Roitbak et al., 2011; Ruscher et al., 1998; Yao et al., 2008). Under normoxic conditions, HIF-1α has a half-life of approximately 8 minutes (Jewell and Gassmann, 2001). However, neural progenitor cells in the hippocampal dentate gyrus constitutively express HIF-1α which is not subject to oxygen dependent proteolysis (Roitbak et al., 2011).

As well as their importance in the cellular response to hypoxia, HIF-1α and HIF-2α are both necessary for cellular and developmental homeostasis (Tian et al., 1997). This is evident by the generation of homozygous HIF-1α knockouts, which are embryonically lethal at E11. Mice with total HIF-1α ablation present with massive death of cells within the cephalic mesenchyme. The loss of supporting cells leads to aberrant formation of the neural fold. Furthermore, homozygous HIF-2α knockout mice are embryonically lethal between E9.5 and E13.5 due to abnormal vascular remodelling (Iyer et al., 1998; Peng et al., 2000). To highlight the importance of HIF-1α in neuronal development, mice with a conditional knock out of HIF-1α in neuronal cells were generated. Mice deficient in neuronal HIF-1α show a reduced neural cell count, hydrocephalus and impairments in spatial memory (Tomita et al., 2003). Additionally, mice lacking neuronal HIF-1α suffer

significantly greater tissue damage following a middle cerebral artery occlusion (MCAO) highlighting the important role of HIF-1α in tissue recovery following ischemia (Baranova et al., 2007). Conversely, early RNA interference of HIF improves the outcome after ischemia-reperfusion highlighting an important dynamic of HIF signalling during cerebral ischemia (Chen et al., 2009). Collectively, this highlights multiple roles for HIF beyond the hypoxic response.

Under normoxic conditions, HIF-α expression is tightly regulated by proteolytic degradation, mediated by an oxygen-dependent degradation. Specific proline hydroxylation of Proline 402 and Proline 564 occurs within the oxygen-dependent degradation domain to allow binding of von Hipppel-Lindau tumour suppressor protein (pVHL) (Hon et al., 2002; Huang et al., 1998; Min et al., 2002). pVHL serves as a substrate for ubiquitin E3 ligase complex which initiates proteosomal degradation of HIF-1α (Cockman et al., 2000; Maxwell et al., 1999).

During normoxia, HIF-1α hydroxylation is determined by proline and asparagine hydroxylation. Proline hydroxylation of HIF-1α has been shown to be regulated by prolyl hydroxylase domain (PHD) enzymes, members of the 2-oxoglutarate-dependent oxygenases. To date, three isoforms of PHD have been identified (PHD1-3) and shown to hydroxylate HIF-1α (Bruick and McKnight, 2001; Epstein et al., 2001). Aparaginyl hydroxylation is mediated by factor inhibiting HIF (FIH). Functional activity of both PHDs and FIH requires iron (Fe^{2+}), ascorbate, 2-oxoglutarate (2-OG) and O_2 as co-factors which produce succinate and CO_2 as products (Berra et al., 2003; Epstein et al., 2001; Hewitson et al., 2002). Succinate can also be transported from mitochondria to the cytosol where it can impair PHD activity leading to HIF-1α stabilisation and activation ('pseudohypoxia', Selak et al., 2005). It is therefore likely that different cells and different regions of the CNS could have different HIF responses due to different redox states despite the same oxygen levels.

Hydroxylation of the asparagine residue 803 in the C-terminal activation domain prevents the binding of HIF co-activators p300 and CBP rather than target HIF for proteosomal degradation. The inability to form the HIF-p300/CBP means transcription of HIF target proteins is prevented (Lando et al., 2002 but see also Freedman et al., 2002). As illustrated in Figure 1, during hypoxic conditions polyl- and asparaginyl-hydroxylases are inhibited by loss of the co-factor O_2, thereby preventing the hydroxylation of HIF-1α. Inhibition of PHDs prevents degradation and leads to a rapid accumulation of HIF-1α in the cytosol. HIF-1α dimerizes with HIF-1β and thus the inhibition of FIH allows the HIF-1α/HIF-1β dimer to bind to p300/CBP. The HIF complex binds to hypoxic response elements (HREs) on HIF target genes. This complex initiates transcription of a wide variety of genes to adapt to the hypoxic stress including proteins involved in angiogenesis (VEGF, VEGF receptor), erthropoiesis (EPO), vasomotor control (nitric oxide synthase), energy metabolism (glucose transporters) and iron metabolism (transferrin) (Ebert et al., 1995; Forsythe et al., 1996; Gerber et al., 1997; Palmer et al., 1998; Rolfs et al., 1997; Tacchini et al., 1999; Wang and Semenza, 1993).

PHD isoforms in the CNS

Although the hydroxylase function of the three PHD enzymes is identical, each subtype has different tissue distribution (Cioffi et al., 2003; Lieb et al., 2002; Menzies et al., 2004; Soilleux et al., 2005) and cellular localisation depending on cell type (Metzen et al., 2003; Soilleux et al., 2005). All three PHD isoforms are expressed in the mammalian brain. Both PHD 1 and 3 show increased expression with age in the rat brain, whereas PHD2 mRNA expression remains similar to that of a young rat. This results in a decrease in HIF-1α expression in aged rat brain (Ndubuizu et al., 2012). HIF activity regulates PHD2 & 3 expression indicating a negative feedback mechanism under hypoxic conditions (Appelhoff et al., 2004; Metzen et al., 2005; Stiehl et al., 2006).

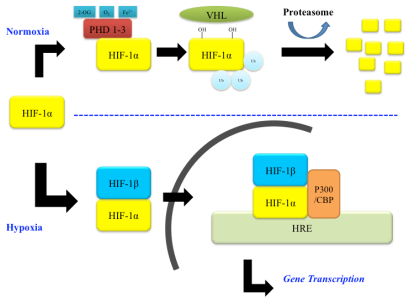

Figure 1

Cellular response to hypoxia

Under normoxic conditions, levels of HIF-1α are regulated by cellular oxygen. Prolyl hydroxylase domains (PHD1-3) require molecular oxygen (O_2), iron (Fe^{2+}) and ascorbate as co-factors to hydroxylate specific proline residues (Pro 402 and 564) on HIF to facilitate binding of VHL protein. Binding of VHL results in rapid ubiquitination and subsequent proteasomal degradation. Under hypoxic condictions PHDs are inhibited due to the loss of the rate limiting O_2 resulting in stabilization of HIF-1α. HIF-1α translocates to the nucleus and forms a hetrodimer with HIF-1β. Recruitment of P300/CBP allows the HIF complex to bind to the hypoxic response element (HRE) or the DNA and initiates gene transcription of hypoxia related proteins. CBP, CREB-binding protein: VHL, von Hippel-Lindau.

PHD1 is constitutively expressed throughout the CNS (Lieb et al., 2002) and PHD1$^{-/-}$ mice have been developed and display no phenotype associated with dysregulation of the HIF system (Aragonés et al., 2008). Mice deficient in PHD1 show increased muscle fatigue but are tolerant to acute hepatic ischemia (Aragonés et al., 2008; Schneider et al., 2010). However, these ischemic protective benefits do not apply to cerebral ischemia following middle cerebral artery occlusion. PHD1$^{-/-}$ do not display any significant neuroprotective benefits (Chen et al., 2012).

PHD2 has been highlighted as the most important isoform for HIF regulation based on its ubiquitous expression and dominant function in HIF regulation during normoxia (Berra et al., 2003). This was confirmed by two independent groups who developed homozygous PHD2 KO mice. PHD2$^{-/-}$ mice died in utero between E12.5 and 14.5. PHD1$^{-/-}$ and PHD3$^{-/-}$ mice are viable, highlighting a dominant role for PHD2 as a critical regulator of the hypoxic response during embryonic development (Takeda et al., 2006). PHD2$^{-/-}$ mice presented poor cardiac and placental phenotypes, including retardation in heart development, open intraventricular septum and enlarged atria (Minamishima et al., 2008; Takeda et al., 2006). Since then, tetracycline-induced PHD2$^{-/-}$ mice have been developed. The phenotype of the mice is typical of HIF over expression including increased erythropoiesis and angiogenesis, including cerebral blood vessels.

Mice lacking PHD2 exhibit premature mortality associated with marked venous congestion. However, PHD2 was poorly knocked down in the brains of these mice (Minamishima et al., 2008; Takeda et al., 2007). To determine the importance of PHD2 in brain tissue,

PHD2$^{+/-}$ and conditional neuronal PHD2$^{\Delta/\Delta}$ and CD68 conditional PHD2 KO mice were developed by three independent groups (Franke et al., 2012; Kunze et al., 2012; Mazzone et al., 2009). All three animal models display a phenotype typical of HIF over expression including elevated EPO and haematocrit. Interestingly, neither PHD1 or 3 expression increased to compensate for the loss of PHD2 (Franke et al., 2012; Kunze et al., 2012). However, PHD3 mRNA levels did significantly increase following a hypoxic stress. PHD2$^{+/-}$ mice exhibit improved recovery of cerebral blood flow following middle cerebral artery occlusion and improved behavioural neuroscores (Chen et al., 2012). PHD2$^{\Delta/\Delta}$ mice suffered a reduced infarct volume compared to controls and showed significantly less neuronal cell death in the peri-infarct regions (Kunze et al., 2012). Immunohistochemical analysis also revealed that the majority of PHD2 expressing cells within cortex, striatum and hippocampus, represent neurons. Surprisingly, neuron-specific ablation of PHD2 in the forebrain resulted in 90% reduction in protein level in brain hemispheres despite 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). The authors report no altered expression of PHD1 or PHD3 but did not rule out the possibility of altered enzyme activity to compensate for the loss of PHD2. The loss of PHD2 and subsequent stabilization of HIF-1α and HIF-2α confer neuroprotection to the mice after middle cerebral artery occlusion (MCAO) (Kunze et al., 2012).

PHD3 is expressed in low levels under normoxic conditions with a higher affinity to HIF-2α than to HIF-1α (Appelhoff et al., 2004). PHD3 has been shown to play an important role in neuronal apoptosis during development (Lee et al., 2005). Overexpression of PHD3 in primary sympathetic neurons results in apoptosis despite the presence of nerve growth factor (Lipscomb et al., 2001, 1999). Interestingly, knocking out PHD3 is not fatal and mice show no evidence of a haematopoietic phenotype similar to that of PHD2 KO mice (Takeda et al., 2006).

In the central nervous system, PHD3$^{-/-}$ mice show impaired recovery of regional blood flow following ischemia-reperfusion (Chen et al., 2012). PHD3$^{-/-}$ mice also exhibit an abnormal sympathetic innervations of target tissue, associated with decreased plasma levels of catecholamines (Bishop et al., 2008). This suggests, whilst there is an important role for PHD3 in the response to ischemia, there is also a physiological role for PHD3 beyond the hypoxia response. Double knockout of CD68^{+} PHD2 and PHD3 results in a majority of mice dying shortly after birth. Analysis of brain lysates shows significant upregulation of pro-angiogenic factors and pro-inflammatory cytokines, including TNF-α, IL-1α and IL-1β which is independent of HIF-1α expression but rather HIF-2α activity (Franke et al., 2012). This suggests PHD3 plays a protective role in the absence of PHD2 loss.

Effect of acute hypoxia and HIF stabilisers on neuronal signalling

Approximately 33 to 50 % of cerebral oxygen is utilised for synaptic transmission. Given the high demand for O_2, the relationship between hypoxia and synaptic signalling is very important (Astrup, 1982). HIF stabilization has become well recognised as a cellular response to hypoxia. However, neurons can alter synaptic transmission in response to hypoxic conditions within minutes suggesting a mechanism independent of PHD and FIH inhibition. During hypoxia in isolated hippocampal slices, neuronal transmission is significantly decreased. Upon reoxygenation, synaptic transmission can fully recover within minutes (Fowler, 1989; Lipton & Whittingham, 1978). This decrease in neurotransmission is suggested to protect neurons during ischemic events. The depression of synaptic transmission during hypoxia is primarily mediated by adenosine, the concentration of which is greatly increased during cerebral ischemia (Laghi Pasini et al., 2000). The release of adenosine from cells in response to reduced regional blood flow, which is not significant to induce glutamate excitotoxicity, suggests adenosine may play some role in alleviating the potential for excitotoxicity (Matsumoto et al., 1992).

Adenosine is secreted by all cells in the CNS and exerts its actions by activation of both pre-synaptic and post-synaptic A_1 receptors. Activation of pre-synaptic A_1 receptors caused inhibition of voltage-gated calcium channels, which in turn, prevents re-uptake of calcium from the synapse (Scholz and Miller, 1996; Zhu and Ikeda, 1993). Decreased calcium ultimately decreases mobilization of neurotransmitter-containing vesicles and thus decreases synaptic transmission (see Figure 2). Alternatively, activation of post-synaptic A_1 receptors results in activation of inwardly rectifying potassium channels, which causes hyperpolarization of the post-synaptic neurons (Gerber et al., 1989; Lüscher et al., 1997). Post-synaptic adenosine receptor activation also decreases conductance of NMDA receptors (De Mendonça et al., 1995; Nörenberg et al., 1997; Pamenter et al., 2008). The combined actions of adenosine release are to shut down neuronal activity and prevent excitotoxicity during hypoxia to meet metabolic demands. Specific deletion or antagonism of autonomic A_1 receptors impairs the ability of neurons to recover from the hypoxic stress (Arrigoni et al., 2005; Pearson et al., 2001; Batti & O'Connor, 2010). Inhibition of synaptic signalling can also be observed *in vivo* after occlusion of the middle cerebral artery or carotid artery (Branston and Symon, 1976; Fowler et al., 2003; Gervitz et al., 2001; van de Bor et al., 1999). Secreted adenosine also protects neighbouring astrocytes from hypoxic damage. This cytoprotection is mediated through A_1 and A_3 receptors (Björklund et al., 2008).

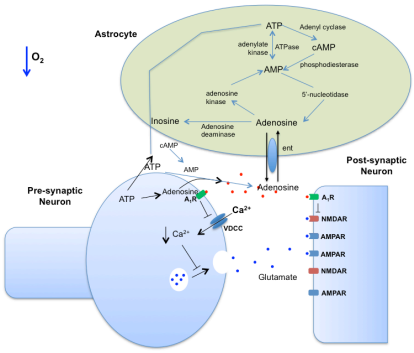

Figure 2

Schematic representation of adenosine neuro-regulation following an energy failure in the CNS

The hypoxia-triggered accumulation of adenosine (ad) and the enzymes involved in this intra-cellular and extra-cellular regulation are illustrated. Adenosine typically accumulates in the extracellular space during conditions of hypoxia and fatigue, that is, when the rate of ATP utilization exceeds the rate of ATP synthesis. Adenosine can also be increased by high levels of S-adenosyl homosysteine (SAH) or reduced by an excess of L-homocysteine (not shown). In the CNS this accumulation can be very rapid. The binding of adenosine to presynaptic A_1Rs, inhibits Ca^{2+} influx and glutamate release from the presynaptic terminal repressing activation of postsynaptic glutamate receptors. The action of adenosine on A_2R and A_3R is not shown for clarity.

It has been recently demonstrated that neuronal adenosine, and not astrocytic, may be solely responsible for mediating feedback inhibition to prevent excitotoxicity (Lovatt et al., 2012). Although the majority of research indicates a presynaptic mechanism of action, recent work suggests postsynaptic neurons may also depolarise to assist in preventing glutamate-induced excitotoxicity and release a novel retrograde messenger involving glutamate or adenosine (Nuritova and Frenguelli, 2012). Hippocampal neurons can undergo hypoxic conditions for up to two hours and regain full function upon reoxygenation (Batti and O'Connor, 2010). However, under more relevant ischemic conditions such as oxygen-glucose deprivation (OGD), synaptic transmission can be irreversibly decreased after 10 minutes (Pugliese et al., 2006). Short episodes of OGD (less than 5 minutes) can result in hyperexcitability of hippocampal neurons following reoxygenation, a form of synaptic plasticity which can last hours (Ai and Baker, 2006). This form of plasticity is pre-synaptic and independent of AMPA or NMDA receptors. This prolonged glutamate release may be associated with neuronal excitotoxicity experienced during cerebral ischemia (Choi, 1992; Lipton, 1999).

During ischemia, over activation of post-synaptic NMDA receptors leads to neuronal cell death (Arundine and Tymianski, 2003). Neurons combat this stress by decreasing NMDA subunit expression. Decreased mRNA levels of NR1, NR2A and NR2B NMDA subunits are observed after only 30 minutes OGD (Dos-Anjos et al., 2009). OGD can also lead to receptor subunit transitions from NR2B to NR2A dominant (Wise-Faberowski et al., 2009). Complete inhibition of NMDA receptors before global ischemia attenuates the damage observed (Arias et al., 1999). Specific antagonism of the glycine-binding site on NMDA receptors improves the recovery of synaptic signalling following hypoxia and OGD (Frankiewicz et al., 2000). Extrasynaptic NMDA receptors play a role in neuroprotection to a hypoxic stress and selective inhibition of synaptic NMDA receptors with memantine protected neurons against a prolonged hypoxic insult. This highlights a potential role for synaptic NMDA receptors mediating hypoxia induced excitotoxicity (Wroge et al., 2012). Such hypoxic stresses can lead to a disruption in NMDA receptor activity and potentially interrupt synaptic plasticity. Accordingly, OGD has been shown to impair the induction of long term potentiation (LTP), a form of synaptic plasticity believed to be the molecular mechanism for learning and memory (Gasparova et al., 2008).

When hippocampal slices are exposed to a pseudo-hypoxic environment via pharmacological PHD inhibition, synaptic transmission was significantly decreased by approximately 20 % within 10 minutes. Importantly, the decrease in synaptic transmission was not as pronounced as hypoxia, suggesting an alternative mechanism of action beyond the traditional cellular response to hypoxia. There was no effect on paired pulse stimulation indicating a post-synaptic effect of inhibition. The effect was also independent of A_1 receptor activity and instead, seemed to be mediated through post-synaptic NMDA receptors. Both dentate gryus and CA1 hippocampal regions seemed to be equally affected (Batti et al., 2010; Corcoran and O'Connor, 2011). The effects of iron chelation via application of DFO are even greater, decreasing transmission by approximately 50 % and inhibiting long term potentiation via disruption of NMDAR activity (Munoz et al., 2011). In these experiments primary hippocampal neurons were stimulated with NMDA and the resulting sustained cellular Ca2+ concentration was made up of an early and late component. The application of DFO inhibited the late component suggesting that iron is needed to sustain the Ca2+ increase elicited by NMDA receptor stimulation. They propose that iron is required in hippocampal neurons to generate the reactive oxygen species (ROS) needed to stimulate ryanodine-reduced receptor (RyR) mediated Ca2+ release. This RyR mediated Ca2+ release plays a central role in the postsynaptic Ca2+ signals generated in hippocampal CA1 dendritic spines during basal synaptic transmission. Preliminary work from our laboratory suggests acute and chronic inhibition of PHDs by dimethyloxalyl glycine (DMOG) and DFO impairs synaptic plasticity (long-term potentiation) in the rat and mouse hippocampus (Corcoran and O'Connor, 2011).

The adverse effects of DMOG and DFO on LTP may be attributed to NMDA receptor inhibition as previously observed (Corcoran and O'Connor, 2011; Munoz et al., 2011). However, a growing body of evidence suggests a role for PHDs in regulating proteins associated with LTP. Recent work in C. elegans highlights a role for hypoxia and PHDs in the regulation of AMPA receptor trafficking and activity and which may decrease the availability and function of AMPA receptors in the post synaptic membrane (Park et al., 2012). This might explain the inhibitory effects of acute PHD inhibition on LTP in mice (Corcoran & O'Connor, 2011). Furthermore, in the mammalian cardiomyocyte, specific knockdown of PHD2 causes a reduction in cAMP concentration within the cytosol (Huo et al., 2012). If the process is similar in central neurons, decreased cAMP could potentially regulate LTP by reduced CREB activation. Together, this evidence suggests a potential novel role of PHD enzymes in synaptic signalling and plasticity independent of HIF activity (see Figure 3).

Protective effects of prolyl hydroxylases (artificial hypoxia) in the CNS

Due to the complicated nature of cerebral ischemia and the convergence of detrimental conditions to mediate cell death, an effective therapeutic strategy has been difficult to achieve. *In vitro* experiments highlight a role for HIF-1α mediating cellular protection in response to glutamate

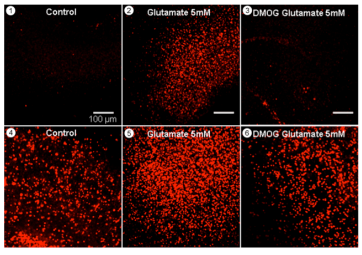

Figure 3

Prolyl hydroxylase domain isoforms play a role in the modulation of long-term potentiation.

Under normal oxygen conditions (left side) PHD2 plays a major role in the recycling of AMPA receptors during LTP (thicker arrow). During hypoxia and the inactivation of PHD2 (right side), 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.

excitotoxicity. When HIF-1α stabilization is inhibited before exposure to a hypoxic stress, astrocytes suffer increased cell death due to glutamate toxicity (Badawi et al., 2012). Conversely, up-regulation of HIF-1α is associated with protection from ischemia-reperfusion

injury in astrocytes (Du et al., 2010).

In vivo studies have previously demonstrated that application of hypoxic or sub lethal ischemic conditions prior to stroke significantly reduces infarct severity in neonates and adult rats (Bernaudin et al., 2002a; Gidday et al., 1999; Miller et al., 2001; Vannucci et al., 1998). It has been proposed that tolerance to ischemic insults by hypoxia preconditioning is due to activation of HIF and HIF target genes including VEGF, EPO, GLUT-1 and adrenomedullin (Bergeron et al., 1999; Bernaudin et al., 2002b). Since their discovery, PHDs have become a novel therapeutic target for hypoxic injuries. Pharmacological inhibition of PHDs via 2-OG competitive antagonism or iron chelation has become an attractive strategy to precondition neurons for a subsequent hypoxic stress. *In vitro*, various types of PHD inhibitors have been shown to stabilize HIF-1α, either by 2-OG antagonism (N-oxalylglycine, DMOG, 3,4-dihydroxybenzoate (DHB)), iron chelation (DFO) or heavy metal substitution of iron (CoCl) (Epstein et al., 2001; Huang et al., 2003; Siddiq et al., 2005).

The evidence for a neuroprotective role of HIF by PHD inhibition has become substantial both *in vitro* and *in vivo*. HIF stabilization by DHB, DMOG and DFO prevents oxidative glutamate toxicity in cortical neurons and is neuroprotective during OGD in organotypic hippocampal cultures (Batti et al., 2010; Siddiq et al., 2005)(see Figure 4). Whilst PHD inhibition is protective to oxidative stress, it can also improve cell viability in response to mitochondrial dysfunction, glutathione depletion and nerve growth factor withdrawal, which is dependent upon extracellular glucose and HIF-2α activity (Batti et al., 2010; Lee et al., 2009; Lomb et al., 2009, 2007; Ma et al., 2013; Niatsetskaya et al., 2010; Siddiq et al., 2005; Tjong et al., 2008). The evidence for neuroprotection *in vitro* gives promise to the potential therapeutic effects *in vivo*.

In vivo studies show PHD inhibition via DMOG or DFO confers robust neuroprotection in rodent models of stoke (Nagel et al., 2010; Prass et al., 2002; Siddiq et al., 2005; Takizawa et al., 2012). Also preconditioning neurons to the PHD inhibitor DMOG greatly reduces the excitotoxic affect of glutamate (Batti et al., 2010; Figure 4). Although preconditioning for a primary ischemic attack may be an unviable therapeutic strategy, it has yielded vital information for further use of PHD inhibitors. Treatment following ischemic stress has now become an attractive paradigm to test the value of PHD inhibitors as therapeutic agents. For example it has been shown that DFO has neuroprotective effects when administered after a middle cerebral artery occlusion in neonatal rats (Mu et al., 2005). Recently, it has been demonstrated that administration of DMOG following stroke significantly reduces ischemic brain injury (Ogle et al., 2011). Transgenic animal models of all PHD isoforms have highlighted PHD2 and PHD3 as the dominant isoforms which act to aid recovery following middle cerebral artery occlusion (MCAO). Mice with neuronal ablation or a heterozygous knockout of PHD2 and homozygous PHD3 KO mice show impaired recovery from MCAO (Chen et al., 2012; Kunze et al., 2012). PHD2 has also been shown to mediate oxygen-induced retinopathy (Duan et al., 2011). Together, the growing body of research suggests a neuroprotective role for PHDs before and after MCAO and are potentially regulated by PHD2 activity.

Conclusion

There is now evidence that the hypoxia-inducible factor prolyl hydroxylases (HIF PHDs) are important regulators of both transcriptional and non- transcriptional adaptation to hypoxia, oxidative stress, and excitotoxicity. Evidence from a number of laboratories supports the notion that HIF PHD inhibition can improve functional outcomes in ischemic and haemorrhagic stroke models. While the benefits of HIF activation through a variety of PHD inhibition mechanisms have been found, a major factor to take into consideration is that in the majority of cases, these investigations have been carried out in animal models and in a controlled environment. Although these results have indicated promising future treatments for hypoxia related disorders such as stroke, the effects of translating these strategies to human clinical trials have as yet to be determined. Short-term stabilization of HIF may also provide a novel therapeutic strategy for protecting neuronal cells under ischemic attack. In experimental studies, this has been achieved by administering either non-specific or specific pharmacological inhibitors of PHD. There is now evidence emerging for a role for PHD inhibition on synaptic transmission in the brain. DFO, DMOG and other specific PHD inhibitors have been shown to decrease synaptic transmission and impair synaptic plasticity in rat models. Whether these acute affects are HIF-dependent or independent remains to be determined. In summary HIF signalling pathway provides an important therapeutic target for protecting neuronal cells. In the future, the development of novel and specific inhibitors of PHD (or even VHL) or HIF activators may provide an innovative therapeutic strategy for treating patients with ischemic disease.

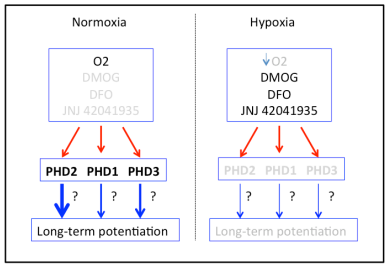

Figure 4

The effects of DMOG preconditioning on glutamate induced excitotoxicity

12 days *in vitro* hippocampal organotypic slice cultures were exposed to 24 h of glutamate (5 mM) or vehicle (1), in the absence (2) or presence of prior DMOG pre-conditioning (3), and then left to recover for a further 48 h in fresh medium with propidium iodide (PI; 2μM). DMOG (1 mM) was applied at 8 and 10 days *in vitro* cultures and removed before glutamate application. For each slice, 10X confocal images were taken before (1,2 and 3) and after (4,5 and 6) fixation and additional incubation with PI (6.7 μM) in the same area corresponding to the CA1 region. Data resulting from the staining before fixation was counted as "cell death" values, whilst those from post-fixation and further PI incubation were counted as "cell total" values. Adapted from Batti et al., 2010 (seeking permission).

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Conflict of Interest

The authors sate that there is no conflict of interest

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