

HIF1 α IS NECESSARY FOR EXERCISE-INDUCED NEUROPROTECTION WHILE HIF2 α IS NEEDED FOR DOPAMINERGIC NEURON SURVIVAL IN THE SUBSTANTIA NIGRA PARS COMPACTA

M. SMEYNE,^a P. SLADEN,^a Y. JIAO,^a
I. DRAGATSIS^b AND R. J. SMEYNE^{a*}

^a Department of Developmental Neurobiology, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, United States

^b Department of Physiology, The University of Tennessee Health Science Center, 894 Union Avenue, Memphis, TN 38163, United States

Abstract—Exercise reduces the risk of developing a number of neurological disorders and increases the efficiency of cellular energy production. However, overly strenuous exercise produces oxidative stress. Proper oxygenation is crucial for the health of all tissues, and tight regulation of cellular oxygen is critical to balance O₂ levels and redox homeostasis in the brain. Hypoxia Inducible Factor (HIF)1 α and HIF2 α are transcription factors regulated by cellular oxygen concentration that initiate gene regulation of vascular development, redox homeostasis, and cell cycle control. HIF1 α and HIF2 α contribute to important adaptive mechanisms that occur when oxygen and ROS homeostasis become unbalanced. It has been shown that preconditioning by exposure to a stressor prior to a hypoxic event reduces damage that would otherwise occur. Previously we reported that 3 months of exercise protects SNpc dopaminergic (DA) neurons from toxicity caused by Complex I inhibition. Here, we identify the cells in the SNpc that express HIF1 α and HIF2 α and show that running exercise produces hypoxia in SNpc DA neurons, and alters the expression of HIF1 α and HIF2 α . In mice carrying a conditional knockout of *Hif1 α* in postnatal neurons we observe that exercise alone produces SNpc TH+ DA neuron loss. Loss of HIF1 α also abolishes exercise-induced neuroprotection. In mice lacking *Hif2 α* in postnatal neurons, the number of TH+ DA neurons in the adult SNpc is diminished, but 3 months of exercise rescues this loss. We conclude that HIF1 α is necessary for exercise-induced neuroprotection and both HIF1 α and HIF2 α are necessary for the survival and function of adult SNpc DA neurons. © 2015 The Authors. Published by Elsevier Ltd. on behalf of IBRO. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

*Corresponding author. Tel: +1-901-595-2830.

E-mail address: Richard.smeyne@stjude.org (R. J. Smeyne).

Abbreviations: DA, dopaminergic; EF-5, 2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide; *egl1n1*, *egl nine homologue 1*; GFAP, Glial Fibrillary Acidic Acid; HIF, Hypoxia Inducible Factor; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PHDs, prolyl hydroxylases; ROS, reactive oxygen species; SNpc, substantia nigra pars compacta; *vegfa*, vascular endothelial growth factor A.

Key words: hypoxia, exercise, substantia nigra, neuroprotection, preconditioning, oxidative stress.

INTRODUCTION

Exercise is beneficial for cognitive health and reduces the risk of developing a number neurological disorders including stroke (Curry et al., 2010; Liebelt et al., 2010), Alzheimer's (Briones, 2006; Stranahan et al., 2012) and Parkinson's disease (Petzinger et al., 2013; Murray et al., 2014) but the factors that underlie this neuroprotection are not entirely clear. Cellular hypoxia that occurs during aerobic exercise may induce responses that precondition the cell and are later protective during periods of oxidative stress. The activity of Hypoxia Inducible Factor (HIF) is modulated by the relative levels of antioxidant and reactive oxygen species (ROS) in the microenvironment as well as the O₂ environment of the cells (Wang et al., 1995; Chandel et al., 1998; Tajima et al., 2009; Radak et al., 2013); each of these are factors modulated by exercise (Radak et al., 2008, 2013).

Oxidative phosphorylation, the production of ATP, and oxygen homeostasis are critical to cell survival. Within the cell, molecular oxygen is tightly regulated, and a key component of this oxygen-sensing pathway is the transcription factor HIF (Wang and Semenza, 1993, 1995). HIF is important for the adaptation of the cell during periods of reduced oxygen availability and its activation promotes the expression of numerous genes, including those involved in erythropoiesis (Semenza and Wang, 1992), vascular development and angiogenesis (Carmeliet et al., 1998; Iyer et al., 1998), and glycolysis (Semenza et al., 1994). HIF is a heterodimer consisting of two subunits, a constitutively present HIF1 β (Aryl Hydrocarbon Nuclear Receptor Translocator, ARNT) (Wang et al., 1995), and HIF1 α , a subunit that is expressed in hypoxic conditions (Wang and Semenza, 1993). In the presence of O₂, iron and 2-oxoglutarate, HIF1 α is hydroxylated by prolyl hydroxylases (PHDs) for proteasomal destruction (Epstein et al., 2001). However, when cells become hypoxic, PHD's are inhibited and HIF1 α is stabilized (Bruck and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001). Although much has been published concerning the stabilization of HIF protein, HIF biological activity and expression is known to be regulated at multiple levels including mRNA expression, nuclear localization, and

<http://dx.doi.org/10.1016/j.neuroscience.2015.03.015>

0306-4522/© 2015 The Authors. Published by Elsevier Ltd. on behalf of IBRO.

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

transactivation as well as protein stabilization (Wang et al., 1995; Semenza, 2000; Tajima et al., 2009). The induction and accumulation of HIF1 α require an intact mitochondrial respiratory chain (Chandel et al., 1998; Agani et al., 2000, 2002). It has also been shown that ROS and antioxidant levels in the microenvironment modulate Hif mRNA expression (Chandel et al., 1998).

HIF2 α , encoded by *Epas1*, is also a member of the bHLH-PAS subfamily, binds to HIF1 β , is regulated by O₂, and has 48% sequence similarity to HIF1 α (Tian et al., 1997). Although they share biochemical characteristics, the expression patterns of HIF1 α and HIF2 α do not completely overlap (Tian et al., 1997; Jain et al., 1998) and it has been shown that HIF1 α and HIF2 α have unique and sometimes antagonistic functions (Yuan et al., 2013). HIF2 α is also involved in catecholamine synthesis and the regulation of cardiac function (Tian et al., 1998). Previous studies have also shown that complete loss of *hif1 α* (Iyer et al., 1998) or *epas1* (Peng et al., 2000; Scortegagna et al., 2003) in mice results in embryonic/perinatal lethality.

Here we identify the cells in the substantia nigra pars compacta (SNpc) that express HIF1 α and HIF2 α and show that exercise induces hypoxia in DA neurons of the SNpc, and modulates HIF expression in the SN. Reduction of neuronal Hif1 α results in loss of DA neurons with exercise, while Hif2 α is necessary for the survival of DA neurons in standard conditions. Therefore, while HIF1 α and HIF2 α are both necessary for DA neuron survival, they play different roles in the subsistence of these neurons.

EXPERIMENTAL PROCEDURES

Animals

All the experimental animal procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the St. Jude Children's Research Hospital IACUC (protocol 364). Experiments were carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments. Mice were maintained on a 12:12 light:dark cycle and with food and water *ad libitum*. Equal numbers of male and female mice were used in each condition in all the experiments.

Hif1 α and *Epas1*-CKO mice were generated by mating floxed *Hif1 α* (B6.129-Hif1^{atm3Rsj0}/J; stock # 007561) or *Hif2 α* (*Epas1*^{tm1Mcs}/J; stock # 008407) (Jackson Laboratories, Bar Harbor, ME, USA) with a transgenic mouse expressing Cre Recombinase driven by a CaMKII promoter (L7ag#13cre) (Dragatsis and Zeitlin, 2000; Baranova et al., 2007) backcrossed to a C57BL/6 background. Control mice were *hif1 α* or *epas1* non-recombined floxed mice (referred to as WT) or C57BL/6J stock mice (Stock number 000664, Jackson Laboratories).

Voluntary exercise: wheel running

At 3 months of age, mice were placed into individual monitored running cages (Lafayette Instruments, Cat#80820, Lafayette, IN, USA) and allowed

unrestricted access to the running wheel. Wheel revolutions were recorded using Animal Wheel Monitor (AWM) software (Lafayette Instruments, Lafayette, IN, Cat# 86065). Littermate mice were used in standard housing (SH) conditions.

Genotyping

Genotyping of transgenic mice was performed using PCR primers and protocols suggested by the Jackson Laboratories. Primers used to identify *hif1 α* transgenic mice were: Primer 1, 5' to 3':TGCTCATCAGTTGCCACTT and Primer 2, 5' to 3':GTTGGGGCAGTACTGG-AAAG; *epas1*: 5' to 3':GAGAGCAGCTTCTCCTGGAA and 5' to 3':TGTAGGCAAGGAAACCAAGG. L7ag#13cre were genotyped using 5' to 3':CTGCCACGACCAAGT GACAGC and 5' to 3':CTTCTCTACACCTGCGGT GCT.

PCR products were run on 3% (*hif1 α* and *epas1*) or 2% (L7ag#13cre) agarose gels.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment

MPTP is a xenobiotic that is metabolized by MAO-B to MPP⁺, which readily crosses the blood–brain barrier, enters DA neurons through the dopamine transporter and blocks Complex I (Smeyne and Jackson-Lewis, 2005). Here, mice were injected using the acute MPTP protocol that is 4 i.p. injections of 20 mg/kg MPTP-HCl spaced 2 h apart (Hamre et al., 1999). After 7 days, some mice were transcardially perfused with 1 \times phosphate-buffered saline (PBS) followed by 2% paraformaldehyde (PFA) for anatomical analysis.

β -Galactosidase histochemical staining

To detect cells within the SNpc that expressed CaMKII, the L7ag#13 CRE mouse was crossed with a ROSA26-CRE reporter mouse. Adult mice from this cross were sectioned at 40 μ m through the SN and stained for β -galactosidase histochemistry as previously described (Oberdick et al., 1994).

Immunocytochemistry

Mice were transcardially perfused with 2% PFA after clearing with PBS. Brains were removed from the calvaria and postfixed in 2% PFA overnight. After postfixation, brains were placed in 30% sucrose/PBS until they were no longer floating. Once cryoprotected, the brains were blocked and rapidly frozen in TFMTM Tissue Freezing Medium (Fisher), maintained at -18°C and sectioned at 40 μ m. Serial sections were collected in 1 \times TBS and stored at 4 $^{\circ}\text{C}$ until processed.

Sections containing the SN (Paxinos and Franklin, 2001) were selected and treated for antigen retrieval by incubation in 2 N HCl for 10 min (37–39 $^{\circ}\text{C}$) and then in 0.5 M boric acid for 10 min at RT. Sections were rinsed in 1 \times PBS, blocked and permeabilized in 10% goat serum, 0.3% Triton X-100 for 60 min. at RT and incubated in primary antibodies to HIF1 α (H-206, 1:100, Santa Cruz, Dallas, TX, USA) or Hif2 α (GTX30114, 1:100, GeneTex,

Irvine, CA, USA) overnight at 4 °C. On day 2 sections were rinsed thoroughly in 1× PBS and double labeled for tyrosine hydroxylase (TH, Sigma #T1299, 1:200, St. Louis, MO, USA), Glial Fibrillary Acidic Acid (GFAP, Sigma #G3893, 1:500), CD31 (BD#550274, BD Bioscience, San Jose, CA, USA), or EAAC1, Millipore #AB1520 1:2000, Temecula, CA, USA) for 1 h at RT. Sections were rinsed and secondary antibodies (AlexaFluor™, Life Technologies, Carlsbad, CA, USA) were applied for 1 h at RT in the dark. *NOTE: When sections were treated with more than 1 antibody secondary antibodies were applied in succession.* After secondary antibody application, sections were rinsed with 1× PBS and mounted with Vectashield mounting media for fluorescence (Vector Labs, Burlingame, CA, USA) or Vectashield plus DAPI mounting media. Sections used for immunofluorescence were alternate sections from the same mice used for TH cell counts and were processed 7 days after the administration of MPTP, a time when the toxin-induced cell death was maximal (Boyd et al., 2007). Confocal images were collected on a Zeiss LSM 510 microscope and analyzed with LSM Image Browser software (Carl Zeiss, Germany). HIF1 α or HIF2 α positive or negative immunostaining illustrated by confocal images in each cell type were seen in SN sections from at least 3–5 mice in each condition. Following processing, the 40- μ m sections shrink to approximately 22 μ m. Sections were scanned at 1 μ m through the entire z-plane, and images were collected.

Nissl immunohistochemistry

Sections previously stained for TH+ immunoreactivity were rehydrated and incubated in Cresyl Violet stain for 2 h at RT. Sections were then rinsed in water, dehydrated to 95% ethanol, differentiated with glacial acetic acid in 95% ethanol, fully dehydrated in 100% ethanol, cleared in xylene and coverslipped with Permount™.

2-(2-Nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide (EF-5) visualization

Three-month-old C57Bl/6N mice (Harlan, Indianapolis, IN, USA) were placed into running or SH cages. One to two hours into the second evening, mice were removed, lightly anesthetized with 2,2,2-tribromoethanol (Avertin) and tail veins injected (following manufacturers suggested protocol or 10 mM in 1% volume of body mass) with EF-5 solution (Hypoxia Imaging, Philadelphia, PA, USA) (Bergeron et al., 1999) or 0.9% saline. Once mice recovered from the anesthesia, they were returned to their home cages for an additional 3 h, after which they were deeply anesthetized with Avertin and decapitated. Brains were rapidly removed and sliced while chilled on dry ice, and a tissue block containing the midbrain was rapidly frozen in cryoprotective media and stored at –80 °C until sectioned. Twenty micron sections were cut on a cryostat and fixed in 3% PFA on polyionic slides for 1 h in the dark. Sections were then processed for detection of both EF-5 using an ELK3-51 cy3-conjugated antibody (Bergeron et al., 1999) and TH to identify

dopaminergic (DA) neurons (Baquet et al., 2009). Confocal images were collected on a Zeiss LSM 510 microscope and analyzed with LSM Image Browser software. Individual mice were scored as positive or negative for ELK3-51 antibody staining after 2–3 sections were examined using the confocal microscope to focus through the full z plane of the SN in each section. Images shown in Fig. 4 are generated from a single 1- μ m z plane.

Removal of SN for mRNA and protein samples

Mice were anesthetized with Avertin and brains were removed and placed in a brain matrix (Model BS-AL-5000C, Braintree Scientific, Braintree, MA, USA), chilled on dry ice, ventral side up. Two-millimeter coronal slices including the SN were isolated using anatomical landmarks for the midbrain/SN (Jang et al., 2012) and placed immediately in Eppendorf tubes in dry ice. Samples were stored in dry ice until transfer to –80 °C. All exercise samples and SH cohorts were collected during the active running period of the mice, while MPTP samples were collected 2 h after the final injection of MPTP. This time point was chosen based upon the peak of oxidative stress after MPTP as indicated by changes in expression of GSTpi (Smeyne et al., 2007).

Reverse-transcriptase quantitative PCR

mRNA was isolated from individual SN using a MELT™ kit (Invitrogen, Life Technologies, Carlsbad, CA, USA), following manufacturers suggested protocol. Taqman Gene Expression assays (Life Technologies, Carlsbad, CA, USA) following manufacturers suggested protocol were used for Hif1a (#Mm00468875_m1), Epas1 (#Mm01236112_m1), Vegfa (#Mm01281449_m1), EglN1 (#Mm00459770_m1), and RPS18S (#Mm02601778_g1) ($n = 5–8$ for each exercise condition, $n = 18$ for SH). Samples were processed on an Eppendorf RealPlex2 Mastercycler and analyzed using included Realplex software (Fisher Scientific, Pittsburgh, PA, USA). Statistical analysis of differences between conditions was analyzed by analysis of variance (ANOVA) followed by Fisher's LSD post hoc analysis (Prism V.6, GraphPad Software, LaJolla, CA, USA).

Analysis of HIF1 α and HIF2 α protein levels in SN

Western blot analysis of HIF1 α and HIF2 α were performed using protein extracts from individual SN. Protein was isolated by mechanical dissociation using RIPA buffer supplemented with protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) at 4 °C, incubated for 1 h at 4 °C, and separated by centrifugation at 13 kG for 30 min at 4 °C. Protein concentration was determined by Lowry assay with Dc™ protein assay kit (BioRad, Hercules, CA, USA). Lysates were aliquoted and stored at –80 °C until use. Electrophoresis was performed using standard protocol and Tris–HCl precast gels (7.5%, BioRad), wells were loaded with 20 μ g per lane, from individual SN samples ($n = 4$ per condition). Proteins were transferred to a

polyvinylidene fluoride (PVDF) membrane, rinsed, incubated in blocking buffer and incubated overnight at 4 °C in primary antibody: mouse anti-HIF1 α (1:500, R&D Systems, Minneapolis, MN, USA) or goat anti-HIF2 α (1:300, R&D Systems). Blots were rinsed and incubated with peroxidase linked secondary antibody (HIF1 α ; ECL Amersham, GE Healthcare, Pittsburgh, PA, USA; HIF2 α ; Santa Cruz, Dallas, TX, USA) 1 h RT, rinsed and reacted with DuraWest Super Signal (ECL Pierce, ThermoFisher, Rockford, IL, USA), and visualized with an Odyssey Fc system. Blots were then stripped (Restore buffer, Pierce), rinsed, and incubated with rabbit anti- β -actin (Abcam, Cambridge, MA, USA) for 1 h RT, rinsed and incubated with peroxidase-labeled anti-Rabbit IgG (ECL Amersham, GE Healthcare Biosciences, Pittsburgh, PA, USA), and processed as above. To reduce experimental variability, the electrophoresis and transfer of each set of HIF1 α and HIF2 α immunoblots were run concurrently with the same protein samples. Western blots were analyzed with Image StudioLite software (LI-COR Biotechnology, Lincoln, NE, USA) and compared to SH condition after normalization to β -actin. COS-7 cell extracts (in normoxic and hypoxic conditions) (Novus Biologicals, Littleton, CO, USA), and HIF2 α recombinant protein (Novus Biologicals) were loaded as controls. Bands corresponding to approximately 120 kD (the expected MW of antibody-labeled HIF1 α and HIF2 α) and the hypoxia induced COS7 band (HIF1 α and HIF2 α) and HIF2 α recombinant protein (HIF2 α) were used for analysis. We saw no cross-reaction of the HIF1 α antibodies with the HIF2 α recombinant protein (results not shown). Statistical analysis of differences between conditions was analyzed by unpaired non-parametric comparisons (Kolmogorov–Smirnov analysis, Prism V.6, GraphPad Software, LaJolla, CA, USA).

Stereology of TH+ SNpc neurons and Nissl-stained cells

Forty micron floating sections spaced 200 μ m apart were immunostained for tyrosine hydroxylase followed by a Cresyl Violet counterstain. The number of these TH+ neurons was estimated using optical fractionator stereological methods as described in Baquet et al. (2009). Similar parameters were used for estimating the number of Cresyl Violet-stained cells in the SN.

Since daily wheel running activity varies between (Lightfoot et al., 2004) and within (Coletti et al., 2013) mouse strains, to determine whether running is protective for DA neurons, mice running an average of 16 k or greater revolutions per day over 3 months were used in this analysis, since this amount of exercise has been previously shown to be neuroprotective in C57BL/6 mice (Gerecke et al., 2010).

RESULTS

Cellular localization of HIF1 α and HIF2 α in the SNpc

Although the substantia nigra (SN) contains a number of cell types including DA, glutamatergic, and GABAergic

neurons, astrocytes, microglia, and endothelial cells of the vasculature, it is the DA neurons of the SNpc that are highly sensitive to oxidative stress and are lost during the progression of Parkinson's disease. The presence of HIF1 α and HIF2 α and their cell specificity in the SNpc was investigated to explore the possible role of HIF during exercise-induced neuroprotection and oxidative stress generated by the administration of MPTP. In the WT SNpc, HIF1 α is expressed at barely detectable levels in DA neurons (Fig. 1A–C) and more abundantly in endothelial cells (Fig. 2A–C). HIF1 α does not appear to be expressed in astrocytes or glutamatergic neurons (Fig. 2D–F and not shown). HIF2 α is expressed in DA neurons (Fig. 1D–F), astrocytes, endothelial cells and glutamatergic neurons (Fig. 3A–I), and in all conditions is more consistently discernable than HIF1 α (Fig. 1D–F, J–L). Exercise enhances HIF immunostaining of DA neurons in the SNpc (Fig. 1G–L).

Exercise produces hypoxic conditions in SN DA neurons

Numerous forms of exercise in humans and rodents have been used to demonstrate the benefits of exercise, and both moderate and more intensive exercise regimes have been shown to be effective (Cotman and Berchtold, 2002; Zigmond and Smeyne, 2014). Neuroprotection from oxidative stress produced by MPTP treatment in mice, that is afforded by wheel running, requires 3 months of running at daily averages greater than 12 k wheel revolutions (Gerecke et al., 2010). Given the substantial nature of this exercise, we hypothesize that it generates aerobic conditions in the brain and induces the activity of hypoxia-sensitive factors. To assess the state of oxygenation of neurons during exercise, C57BL/6N mice were injected with the compound EF-5 during the period of active running, as were cohorts of mice housed in standard cages (SH). The immunovisualization of EF-5 by the ELK3-51 antibody has been used to label hypoxic cells (Bergeron et al., 1999) in the brain. EF-5 is a pentafluorinated derivative of the drug etanidazole and is used to detect reduced oxygenation in tissues (Koch et al., 1995). Additionally, when the cell is hypoxic EF-5 is reduced by a redox-dependent mechanism, and forms stable adducts with cellular macromolecules by covalent binding to protein thiols (Evans et al., 1995; Marques de Olivera et al., 2010); an indication that hypoxic cells may be experiencing oxidative stress (Chandel and Budinger, 2007). EF-5 binding is inhibited as oxygen concentration rises and increases as oxygen concentration is lowered (Koch et al., 1995; Chavez and LaManna, 2002). The monoclonal antibody, Elk3-51 binds specifically to these adducts and may be used to detect and localize low oxygenation at the cellular level (Koch et al., 1995). Here, C57BL/6J mice were exposed to 2 days of voluntary exercise (9000–39,000 running wheel revolutions daily, with 7/10 mice averaging greater than 20,000 wheel revolutions daily) or standard housing (SH), during which they were injected with EF-5 or saline via the tail vein. Of the cohorts injected with EF-5, ELK3-51+ DA neurons were

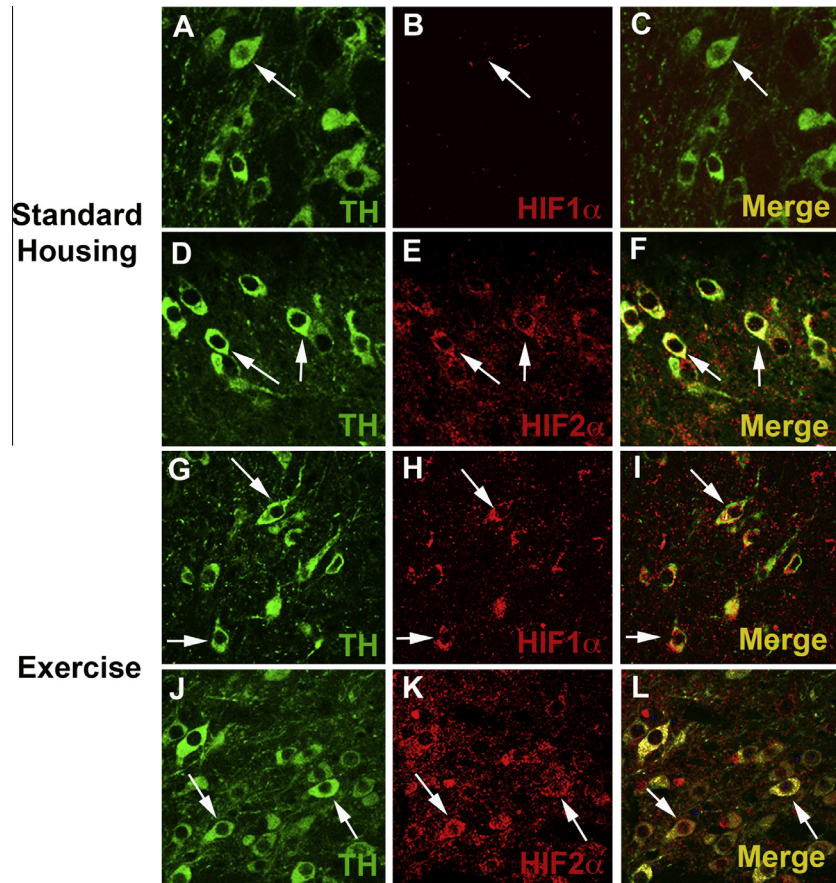


Fig. 1. Exercise enhances HIF1 α and HIF2 α immunostaining of dopaminergic cells. (A–F): SNpc from WT mice in standard housing conditions. An arrow identifies a TH+ dopaminergic (DA) neuron (A, C, green). (B, C) HIF1 α (red) is barely detectable in these neurons. (D) TH+ dopaminergic neurons (arrows) are also labeled with HIF2 α antibody (E, F). (G–L): SNpc from WT mice housed in wheel cages and allowed voluntary access to running for 3 months. Exercise does not alter the appearance of TH+ neurons in the SNpc (G, J) (arrows), but HIF1 α (H, I) and HIF2 α (K, L) immunostaining is more evident in SNpc DA neurons (arrows). Each image is taken from a single 1 μ m z-plane to insure that any co-localization of immunofluorescence is from the same cell.

observed in none of the mice that were housed in standard conditions (0/5) (Fig. 4A, B, E) while ELK3-51+ DA neurons were observed in the SNpc of all the mice in exercise cages (5/5) (Fig. 4C–E). Mice exposed to exercise ($n = 5$) or SH ($n = 3$) conditions injected with saline showed absent (6/8) or very low (2/8) ELK3-51+ staining.

HIF expression in the SN is modulated with voluntary wheel running

Previous studies have shown that HIF1 α is induced in the brain during initial exposure to hypoxia, although with chronic hypoxia HIF1 α levels decline unless another hypoxic challenge occurs (Chavez et al., 2000). To determine whether exercise produces changes in the expression of hypoxia-sensitive molecules in the SN, *hif1 α* , *epas1*, *egl nine homologue1* (*egln1*), and *vascular endothelial growth factor A* (*vegfa*) levels were measured. mRNA was isolated from the SN of C57BL/6J mice housed in SH conditions or after 1,3,5,7, or 14 days of voluntary exercise (during the active period) in cages with monitored running wheels. Exercise induces a significant reduction in *hif1 α* expression ($p \leq 0.01$) at 3 days, is

elevated with 5 days of exercise ($p \leq 0.05$), and returns to SH levels at 7 and 14 days of exercise (Fig. 5A). *Epas1* expression follows a pattern similar to *hif1 α* ($p \leq 0.05$ at 3 and 5 days) but remains somewhat elevated through 7 days of exercise before decreasing to approximate SH levels at 14 days of exercise (Fig. 5B). The expression of *egln1*, the gene that encodes the PHD2 protein that functions in the hydroxylation and degradation of HIF1 α (Epstein et al., 2001), follows an expression pattern similar to *hif1 α* levels after exercise, with 5 days of exercise promoting a significant increase in *egln1* expression ($p \leq 0.01$), compared to all other conditions (Fig. 5C). *Vegfa* expression decreases after 3 days of exercise ($p \leq 0.01$) but returns to baseline levels after 5 days of exercise (Fig. 5D).

The effect of exercise on the accumulation of HIF protein was measured in SN collected from C57BL/6J mice during their active periods at 1,3,7,14, and 21 days. HIF1 α protein increases an average of 20–30% after 3 days of running ($p \leq 0.05$), but by day 7 returns to levels similar to mice in SH (Fig. 5E, F). Following MPTP administration, mice housed in SH conditions show no significant change in HIF1 α compared to the control mice. In mice allowed 3 days of

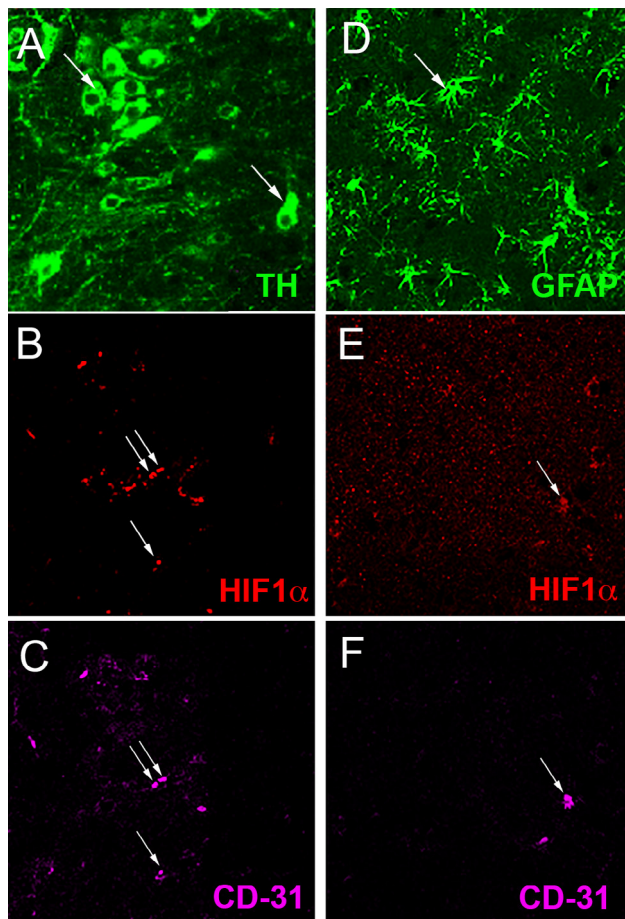


Fig. 2. Cellular localization of HIF1 α in the SNpc. (A–C): A section immunostained with TH (A, arrows), HIF1 α (B, arrows) and the endothelial cell marker CD-31 (C, arrows) shows HIF1 α present in endothelial cells but absent in TH+ DA neurons. GFAP+ astrocytes (D, arrow) are not labeled with HIF1 α (E, arrow) that colocalizes with the endothelial marker CD31 (F, arrow).

exercise, little change in HIF1 α expression was observed, however, after 21 days of exercise MPTP treatment results in a significant increase in HIF1 α protein in the SN, ($p \leq 0.05$) compared to mice in SH conditions (Fig. 5E, F). The HIF1 α increase seen in mice allowed 21 days of exercise and treated with MPTP is similar to HIF1 α levels in mice after 3 days of exercise, and greater than HIF1 α after 21 days of exercise. The MPTP induced increase in HIF1 α protein after 3 weeks of exercise may involve processes similar to the rebound effect of HIF expression reported in other brain areas (Chavez et al., 2000). Lysate from COS7 cells exposed to normoxic and hypoxic conditions are used as negative and positive controls for comparison and analysis of the HIF1 α response to exercise and MPTP (Fig. 5F). The presence of differentially labeled bands at approximately 120 kD when comparing normoxic and hypoxic COS7 lysates were used to identify the analytes.

Immunoblot results of HIF2 α levels in the SN reveal no significant differences between any of the observed conditions. HIF2 α bands corresponding to COS7 cell hypoxic response and HIF2 α recombinant protein are

barely detectable, however, there is a trend where HIF2 α rises early in a running regime (3 days), then falls (7–14 days), before returning to approximate baseline levels (21 days). MPTP does not appear to affect HIF2 α levels in mice housed in SH conditions, or in animals allowed exercise (Fig. 5G, H). As in the HIF1 α blots, COS7 cell lysate (normoxic and hypoxic) and HIF2 α recombinant protein were used for comparison and analysis of HIF2 α response to exercise and MPTP (Fig. 5H).

After 3 months of running, examination of mRNA levels shows no significant differences in the expression of *hif1 α* , *egln1*, or *vegfa* from SN samples obtained from WT mice in SH, MPTP treatment, 3 months of exercise, or 3 months of exercise plus MPTP treatment conditions (Fig. 6A, C, D). Exercise increases *epas1* expression in WT mice (124%, $p \leq 0.02$), but when MPTP is administered after 3 months of exercise this increase is not seen (Fig. 6B).

Conditional deletion of HIF1 α and HIF2 α reduces their expression in postnatal SN DA neurons

To examine the role of *hif1 α* and *hif2 α* during oxidative stress in the SN, we administered MPTP to mice in SH or exercise conditions carrying a conditional deletion of *hif1 α* or *hif2 α* based on the expression of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) using the L7ag#13 CRE driver mouse. Targeting of postnatal neurons for *hif1 α* and *hif2 α* deletion was chosen to examine the reduction of these factors in this specific population of cells. CaMKII is expressed in subsets of postnatal neurons in cerebellum, hippocampus, cortex, and basal ganglia (Dragatsis and Zeitlin, 2000). Using a ROSA26-CRE reporter, we empirically determined that CaMKII is expressed in the DA neurons of the SN (Fig. 7A), but not in GFAP-positive astrocytes (Fig. 7B). Mice carrying the deletion of *hif1 α* in postnatal neurons are referred to as *hif1 α* CKO and *epas1* (*hif2 α*) deletion from postnatal neurons as *epas1*-CKO.

Although we did not expect to see HIF1 α -immunopositive SNpc DA neurons in the *hif1 α* CKO from SH (Fig. 8A–C), the veracity of the deletion is shown by our observations of no HIF1 α -immunopositive SNpc DA neurons following treatment with MPTP, exercise, or exercise followed by MPTP treatment (compare Fig. 8G, H, I exercise plus MPTP with Fig. 1G–I). Loss of *hif1 α* does not appear to affect HIF2 α staining as DA neurons are labeled with HIF2 α in both SH (Fig. 8D–F) and exercise plus MPTP conditions (Fig. 8J–L).

We also examined HIF1 α - and HIF2 α -immunostaining in the SN of *epas1*-CKO mice. Sections through the SN from *epas1*-CKO mice labeled with antibodies to HIF1 α revealed little HIF1 α in the SH condition (Fig. 9A–C) and slightly more HIF1 α labeling in the exercise plus MPTP condition (Fig. 9G–I). HIF2 α labeling in the SN from *epas1*-CKO mice shows robust staining of cells other than TH-positive neurons in both SH (Figs. 9D–F, and 10A–C) and exercise plus MPTP conditions (Fig. 9J–L).

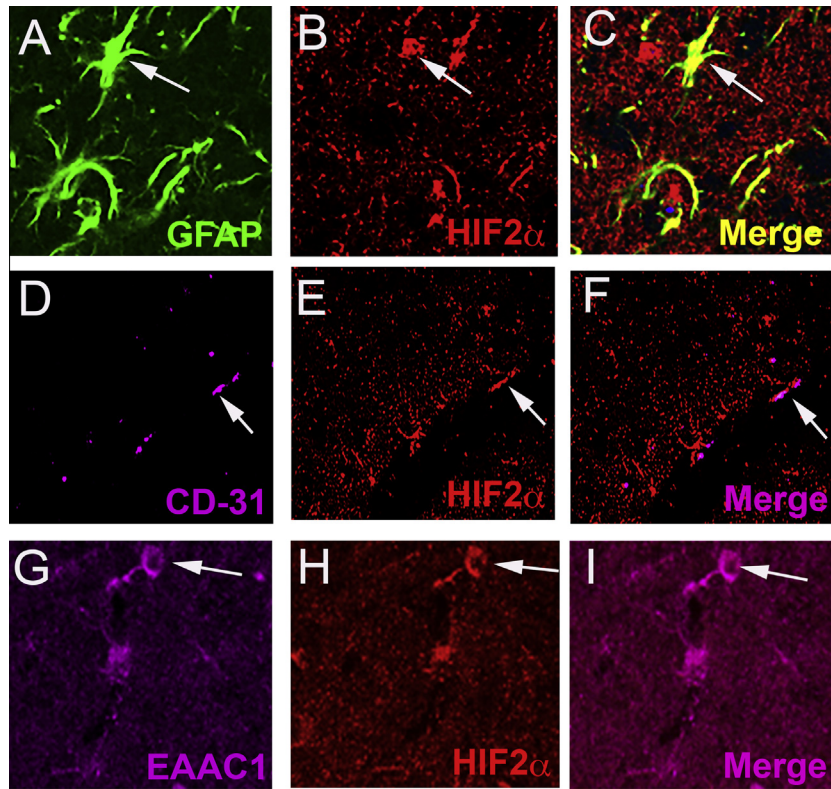


Fig. 3. HIF2 α is present in several cell types in addition to DA neurons in the SN. HIF2 α antibody (B, C, E, F, H, I, red) colocalizes with astrocytes (GFAP, A, C, green), endothelial cells (CD31, D, F, magenta), and glutamatergic neurons (EAAC1, G, I, magenta).

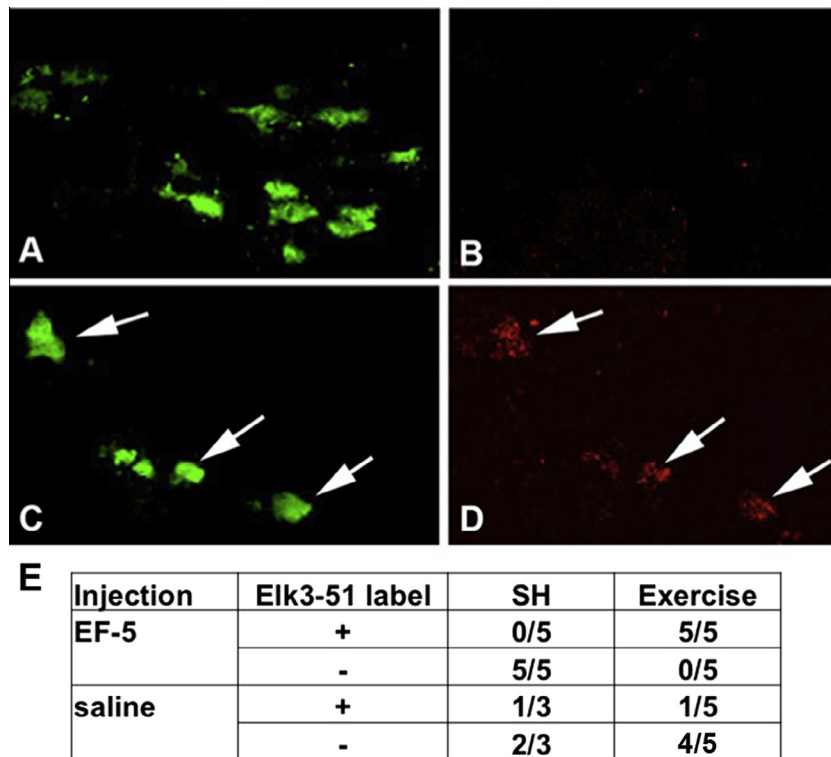


Fig. 4. Exercise induces hypoxia in SNpc DA neurons. (A, B) C57BL/6 mice in the SH condition taken during the evening active period show DA neurons labeled with TH (A) but not Elk3-51 (B). (C, D) SNpc from C57BL/6 mice in wheel cages taken during day 2 in the active period (running) show DA neurons co labeled with TH (C) and Elk3-51 (D). (E) All the mice in exercise conditions injected with EF-5 (5/5) show Elk3-51 labeling of DA neurons, while none of the mice in SH injected with EF-5 (0/5) show Elk3-51+ DA neurons.

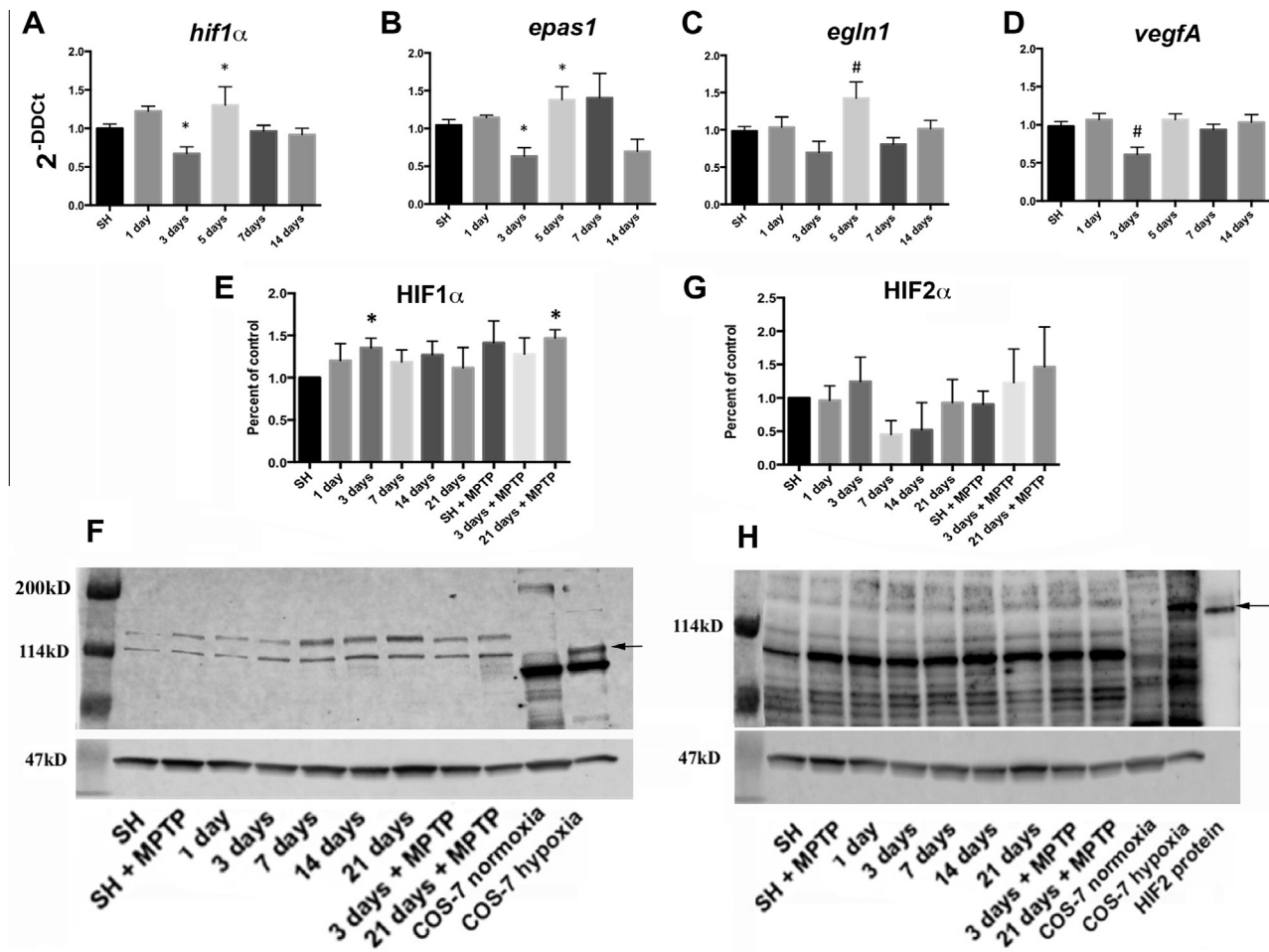


Fig. 5. Expression of hypoxia-sensitive molecules in the SN is modulated by wheel running exercise. *hif1α* (A), *epas1* (B), *eglN1* (C), and *vegfA* (D) expression from SN mRNA isolated at 1, 3, 5, 7, and 14 days of running show a significant reduction at 3 days (A, B, D) and an increase at 5 days (A–C) of running compared to controls in SH. * $p < 0.05$, # $p < 0.01$. $n = 17$ – 18 for SH, 5–8 for each exercise condition. HIF1 α protein from the SN (E, F) is significantly increased at running day 3 and after 21 days of running plus MPTP administration (SN taken at 2 h after the last MPTP injection). * $p < 0.05$. SN HIF2 α protein (G, H) shows no statistically significant change with running. Error bars = \pm SEM. Arrows indicate 114 kD, expected bands for these HIF1 α and HIF2 α antibodies are \sim 120 kD. Lysates from COS-7 cells in normoxic and hypoxic conditions are used as negative and positive controls (F, H), and HIF2 α human recombinant protein (H) as a positive control (\sim 120 kD). Values are expressed as percent of control relative to SH after normalization to β -actin (F, H, \sim 47 kD) for individual samples. $n = 4$ SN for each condition.

Loss of HIF alters TH cell number, sensitivity to MPTP, and neuroprotection from oxidative stress

There is no difference in the number of DA neurons in the SNpc of adult WT and *hif1α*-CKO mice in SH conditions (Fig. 11A). Following administration of acute dosing of MPTP, the number of DA neurons is significantly reduced in the WT strain (25% cell loss, $p \leq 0.0001$) and in *hif1α*-CKO mice as well ($p \leq 0.05$).

To determine if loss of *hif1α* affected exercise-induced neuroprotection, we examined these mice after 3 months of exercise and after 3 months of exercise followed by MPTP treatment. Previously we have shown that the number of SNpc DA neurons in WT mice is not affected by 3 months of running wheel exercise and that 3 months of exercise protects SNpc DA neurons from MPTP toxicity (Gerecke et al., 2010, 2012). These findings in WT mice are replicated here (Fig. 11A). Unlike WT mice, *hif1α*-CKO allowed 3 months of exercise demonstrate a decrease in DA neuron number compared

to both WT and *hif1α*-CKO in SH conditions ($p < 0.01$, Fig. 11A). *Hif1α*-CKO mice exercised for 3 months and administered MPTP also show a loss of DA neurons compared to WT SH and *hif1α*-CKO SH conditions ($p < 0.01$) (Fig. 11A).

Wheel running was monitored for individual mice in exercise and exercise followed by MPTP treatment conditions for these WT, *hif1α*-CKO, and *epas1*-CKO cohorts. We have previously determined that 3 months of unrestricted wheel running (12–18 k average daily revolutions) is necessary for exercise-induced neuroprotection, while 2 months of unrestricted running provides partial protection (Gerecke et al., 2010). Daily wheel running varies between (Lerman et al., 2002; Lightfoot et al., 2004) and within (Coletti et al., 2013) mouse strains, so to determine whether running is protective for DA neurons, only mice running an average of 16 k or greater revolutions per day for 3 months were used in this analysis. Average daily wheel revolutions (range = 16,196–33,766 revolutions per day) were

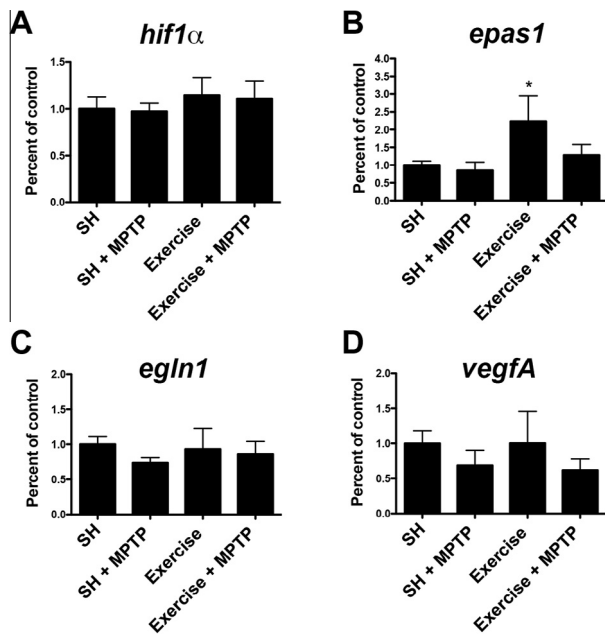


Fig. 6. *Epas1* (*Hif2α*) is increased in the SN with 3 months of wheel running exercise. The expression of *hif1α* (A), *epas1* (B), *eglN1* (C), and *vegfA* (D) in the SN from WT mice after 3 months of exercise or SH was measured by qPCR. *Epas1* (B) in the SN of exercised mice is significantly elevated ($*p \leq 0.02$). mRNA levels are normalized to 18S ribosomal RNA and compared to expression in SH. *Epas1* expression from mice in SH + MPTP and exercise + MPTP showed no changes, neither did *hif1α*, *eglN1*, or *vegfA* expression in any condition.

calculated and correlated to the number of TH+ neurons in the SNpc after 3 months of exercise. A negative correlation between daily wheel running and TH+ cell number was found for *hif1α*-CKO mice ($r^2 = 0.70$, $p \leq 0.03$, Fig. 11B) in the exercise only condition. This is in agreement with the finding that after 3 months of exercise, *hif1α*-CKO mice have a reduced number of DA neurons, a decrease similar to WT and *hif1α*-CKO mice in SH conditions that are treated with MPTP. No linear relationship for average daily wheel revolutions and TH cell number was observed for WT and *Epas1*-CKO mice (not shown).

In contrast to *hif1α*-CKO mice, baseline DA neuron number in the SNpc of *epas1*-CKO mice is significantly lower than WT mice ($p \leq 0.02$) (Fig. 12A, Table 1). Another disparity between *hif1α*-CKO and *epas1*-CKO mice is the lack of a significant TH+ cell loss with MPTP treatment in *epas1*-CKO SNpc (Fig. 12A). *Epas1*-CKO mice in exercise conditions have TH+ cell number similar to, and slightly higher than WT mice in both SH and exercise conditions, and a significantly higher number of TH+ cells compared to either *epas1*-CKO mice in SH ($p \leq 0.005$) or WT mice in MPTP treatment conditions ($p \leq 0.0004$) (Fig. 12A). Exercise is protective for both WT and *epas1*-CKO mice in exercise followed by MPTP treatment conditions, as each have TH+ cell numbers similar to WT mice in SH conditions.

It is possible that the loss of TH+ SNpc DA neurons in the *epas1*-CKO is not a true cell loss but results from a loss of its TH phenotype since *epas1* (Brown et al.,

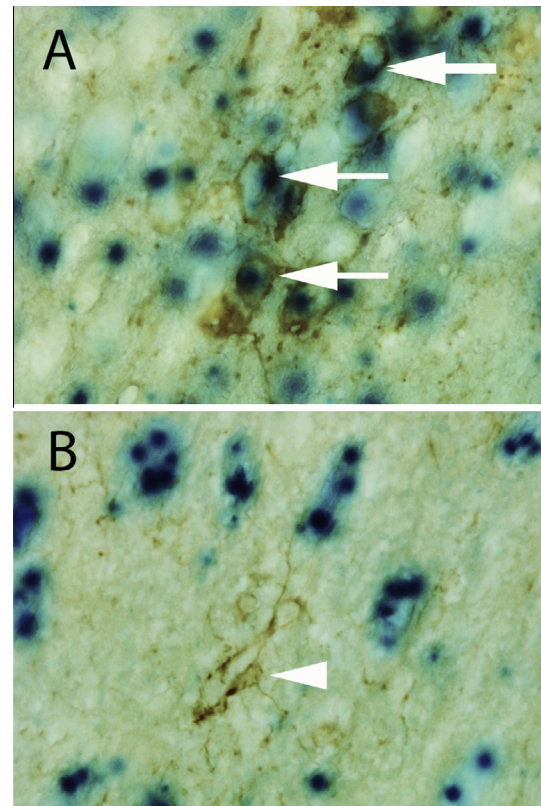


Fig. 7. CaMKII is expressed in DA neurons in the SN. Using a ROSA26 reporter mouse, the pattern of CaMKII expression is identified by expression of β -galactosidase (blue cells). These are colocalized with (A) TH+ positive cells in the SN (arrows) but not in (B) GFAP+ astrocytes (arrowhead).

2009) but not *hif1α* (Gammella et al., 2010) has been shown to contribute to the expression of tyrosine hydroxylase in catecholamine synthesis. To examine this possibility, we counterstained the sections used to determine TH+ number with a Nissl stain, Cresyl Violet (Fig. 12B, D). If the reduction in TH+ cell number in the *epas1*-CKO is due to the physical loss of these cells, then the Nissl cell number will be reduced compared to WT by an amount equal to that seen in TH+ counts (Nissl counts – TH counts will be equal) (Table 1). If the reduction in TH+ is due to a loss of phenotype, then the Nissl cell counts will be identical (Nissl counts – TH counts will be unequal). As previously noted, the number of SNpc TH+ DA neurons in the *epas1*-CKO mouse was reduced by 32% (11,294 compared to 7681, a difference of 3613 neurons, Table 1). Counts of Nissl-stained cells between WT and *epas1*-CKO mice showed a similar cell reduction and there were no differences in Nissl – TH+ cell numbers between these groups of mice (Fig. 12C, Table 1). This suggests that the reduction in TH+ DA neurons in the SNpc is a true cell loss and is not due to a loss of TH cell phenotype.

We also noted that WT mice allowed exercise had a significant increase in the total cell number compared to WT SH, WT SH + MPTP, *epas1*-CKO SH, *epas1* CKO SH + MPTP, and WT Exercise + MPTP ($p < 0.05$) (Fig. 12B, C). This increase is likely due to proliferation

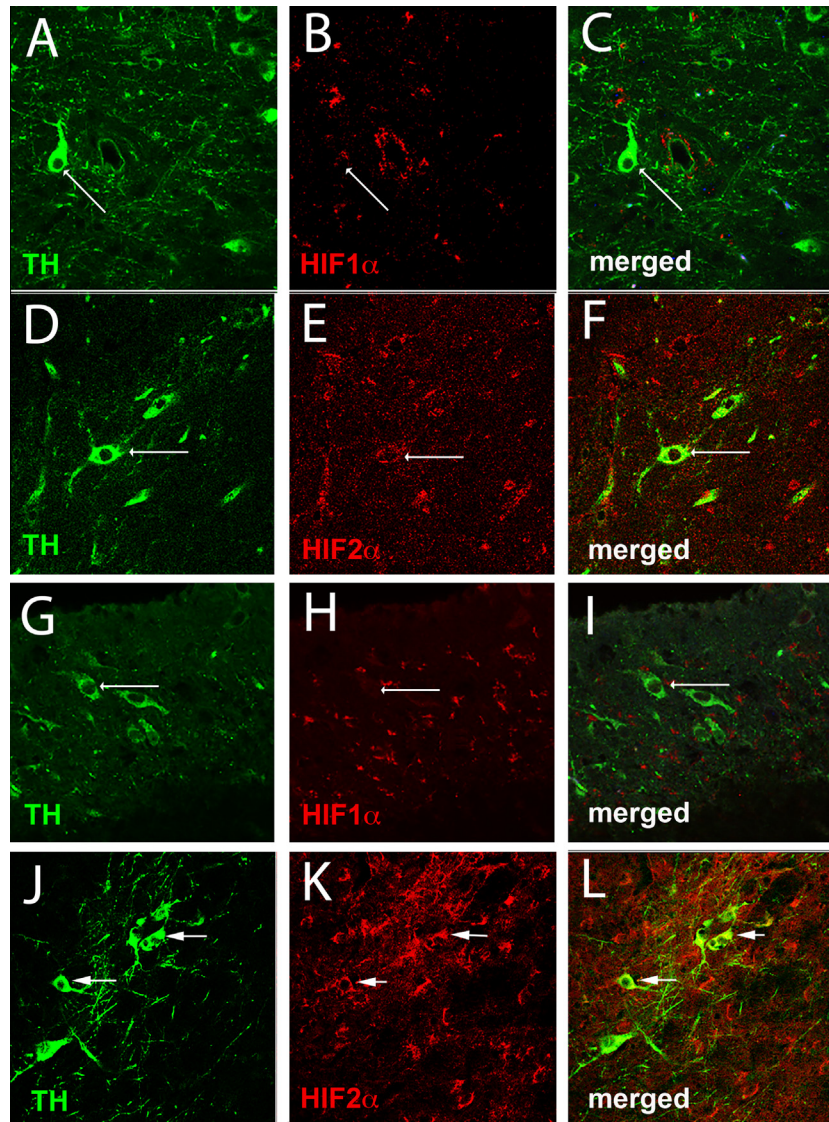


Fig. 8. HIF in the SNpc of *Hif1α*-CKO mice. SNpc sections from *Hif1α*-CKO mice in SH condition (A–F) or 3 months of exercise followed by MPTP administration (G–L) were labeled with antibodies to TH, HIF1 α , and HIF2 α . HIF1 α (B, C, H, I) is not expressed in SNpc TH+ DA neurons (A, C, G, I), while HIF2 α (E, F, K, L) is expressed in SNpc TH+ DA neurons (D, F, J, L). Arrows mark TH+ cells.

of astrocytes in the exercised mice (Li et al., 2005; Ferreira et al., 2011) since we (Gerecke et al., 2010) and other labs (Smith et al., 2011; Wu et al., 2011) have reported no evidence of neuron increases in the SNpc after exercise in the brain.

DISCUSSION

It is likely that the interaction of numerous factors contribute to the neuroprotection seen in the complex environment of the CNS. Here, we have investigated two transcription factors, Hif1 α and Hif2 α that are induced with an exercise regime intense enough to produce hypoxia in neurons. We show that neuronal HIF1 α , but not HIF2 α , is necessary for exercise-induced neuroprotection.

Hif1α and *hif2α*, two related but unique transcription factors, function to activate a number of genes that

enable cells to survive under a variety of unfavorable circumstances (Semenza, 2000; Scortegagna et al., 2003). For example, conditions that increase cellular oxidative stress or alter ROS homeostasis activate *hif1α* (Brunelle et al., 2005), while antioxidants and reducing agents reduce *hif1α* activity (Tajima et al., 2009). *Hif1α* has been shown to induce genes in a number of pathways involved in protecting cells from oxidative stress. These include proteins in the glycolytic pathway (Semenza et al., 1994) the production of erythropoietin (Wang and Semenza, 1993), VEGF (Levy et al., 1995) and VEGF receptor (Okuyama et al., 2006); each of which has been shown to have neuroprotective effects (Yasuhara et al., 2004). *Hif2α* (*epas1*) is necessary for mitochondrial homeostasis, control of glycemic and lactic acid levels, regulation of ROS, redox signaling, expression of antioxidant enzymes (Lando et al., 2000; Scortegagna et al., 2003; Peng et al., 2011), control of TH expression

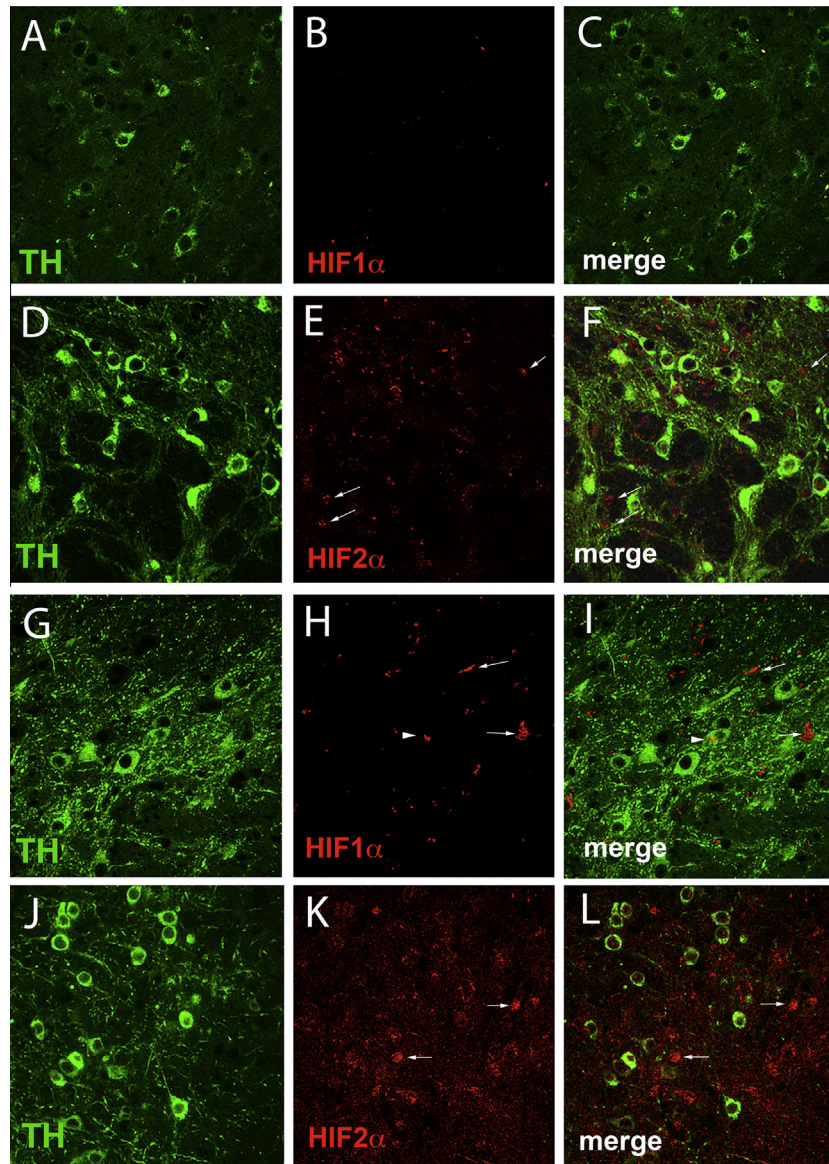


Fig. 9. HIF in the SNpc of *Epas1*-CKO mice. Co-expression of TH, (A, C, D, F, G, I, J, L) HIF1 α (B, C, H, I) and HIF2 α (E, F, K, L) in *Epas1*-CKO SNpc in SH (A–F) and after 3 months of exercise + MPTP (G–L). Merged images are shown in C, F, I, L. While HIF1 α is absent in SH conditions (B, C), it appears more abundant with MPTP administration after 3 months of exercise (H, I). HIF2 α is present in cells other than DA neurons in SH (E, F) also with MPTP after 3 months of exercise (K, L). Arrows point to HIF1 α - or HIF2 α -labeled cells. Arrowhead identifies HIF1 α -TH+ neuron (H, I).

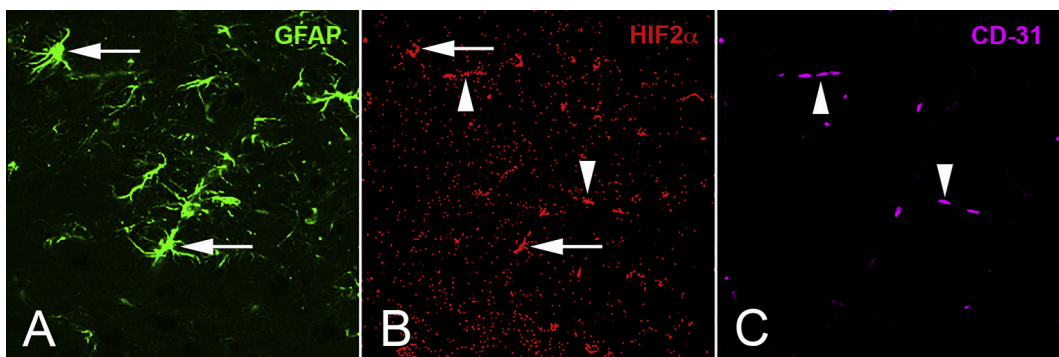


Fig. 10. Localization of HIF2 α in the SN of *Epas1*-CKO mice. While absent from neurons, HIF2 α (B) is found in (A) GFAP+ astrocytes (arrows) and (C) CD31+ endothelial cells in the SN. Arrowheads mark HIF2 α and CD-31 regions of co-localization.

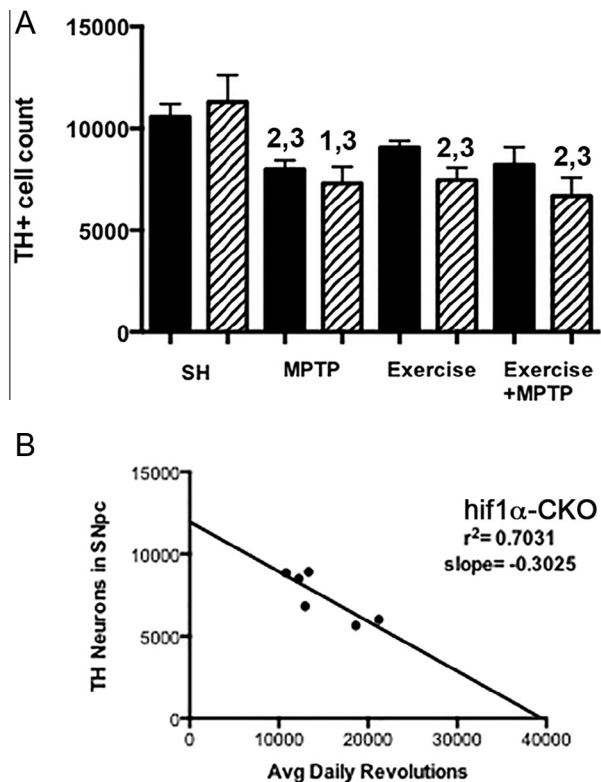


Fig. 11. Reduced TH+ cell number in the SNpc of *Hif1α*-CKO mice with oxidative stress. TH+ neuron number is not different in the SNpc of WT and *Hif1α*-CKO mice in SH (A). However, *Hif1α*-CKO results in significantly reduced TH+ cell number with MPTP, exercise, or exercise + MPTP. TH+ cell number is also reduced in WT SNpc after MPTP administration. 1 = $p < 0.05$ vs. WT SH, 2 = $p < 0.01$ vs. WT SH, 3 = $p < 0.01$ vs. *Hif1α*-CKO SH. Error bars = \pm SEM (B) SNpc TH+ cell number is inversely related to daily exercise in *Hif1α*-CKO mice. Solid bars are WT mice, Hatched bars are *Hif1α*-CKO mice.

(Schnell et al., 2003) and vascular remodeling (Peng et al., 2000).

Previous reports in the literature suggest that HIF1 α is expressed in virtually all cells, while HIF2 α has a more

limited expression (Chavez et al., 2006; Vangeison et al., 2008). Our studies show HIF1 α is expressed in SNpc DA neurons and endothelial cells, while HIF2 α is expressed in DA and glutamatergic neurons, astrocytes, and endothelial cells of the SN. The identification of the cells that express HIF1 α or HIF2 α is important to interpret the studies that use conditional gene deletion based on expression of CaMKII. CaMKII-cre is expressed in postnatal neurons in the SN. Thus, any effects of *hif1α* or *hif2α* deletion would be due to effects on this specific population, without direct effects from non-neuronal (astrocytes, microglia, vascular) cells. Given that the neurons in the SN account for approximately half of the total number of SN cells (Table 1 and Smeyne et al., 2005), it is noteworthy that we observe any significant changes in overall HIF1 α and HIF2 α protein and mRNA levels in the SN. In addition, although the reduction of HIF1 α in the SN of the *hif1α*-CKO is less than 15%, this reduction is critical to the survival of the DA neurons during the hypoxia induced by exercise or generation of oxidative stress by the administration of MPTP.

A major finding in this study is that there is differential loss of SNpc DA neurons in *hif1α* and *epas1* conditional knockouts, with and without MPTP; and this suggests that these genes serve different biological processes in neurons of the SN. Loss of *hif1α* from postnatal SN neurons in standard conditions appears to have little effect, a not unexpected finding since its expression in the brain with normal O₂ levels is low. However, when mice exercise at a level that can induce neuroprotection (Gerecke et al., 2010), these same neurons, as shown by visualization of EF-5, become hypoxic. In non-transgenic mice, this induction of hypoxia has no effect on cell survival. However, in exercised *hif1α*-CKO mice, we observe a reduction in TH+ SNpc DAergic neurons; at a level similar to mice treated with the Complex I inhibitor, MPTP. We also find no additional loss when these two treatments are combined. Both hypoxia (Chandel and Budinger, 2007) and MPTP (Jackson-Lewis and Smeyne, 2005) have been shown to induce ROS formation. Lack of a combinatorial loss of SNpc DA neurons

Table 1. Quantitative assessment of TH+ and Nissl-stained cells in the SNpc of wild-type and *Epas1*-CKO mice. Cell numbers were estimated using Optical Fractionator methods within StereoInvestigator (MBF Biosciences, Williston, VT, USA). The cell number estimates of total Nissl cells minus TH+ cells shows that the loss of TH+ neurons is due to actual cell loss and not a loss of the TH phenotype

	Mean total Nissl cell number	SEM	Statistics	Mean TH+ cell number	SEM	Statistics	Mean Nissl-TH+ cell number	<i>n</i>	Statistics
WT SH	22,693	1100	4	11,294	755	–	11,399	15	4
WT SH + MPTP	20,865	921	4	7999	707	1,5	12,866	16	4
WT Exercise	27,037	1523	–	10,117	1342	–	16,920	5	–
WT Exercise + MPTP	20,831	1680	4	9607	774	–	11,224	7	4
<i>Epas1</i> CKO SH	19,854	1877	4	7681	1285	1,5	12,173	4	4
<i>Epas1</i> CKO SH + MPTP	20,449	3845	4	8697	1758	5	11,753	3	4
<i>Epas1</i> CKO Exercise	24,341	1819	–	12,937	1134	–	11,405	4	4
<i>Epas1</i> CKO Exercise + MPTP	24,979	1422	–	10,068	1301	–	14,911	4	–

Statistics:

1 = $p \leq 0.05$ compared to WT SH; 2 = $p \leq 0.01$ compared to WT SH; 3 = $p \leq 0.01$ compared to CKO SH; 4 = $p \leq 0.05$ compared to WT Exercise; 5 = $p \leq 0.05$ compared to *Epas1*-CKO Exercise.

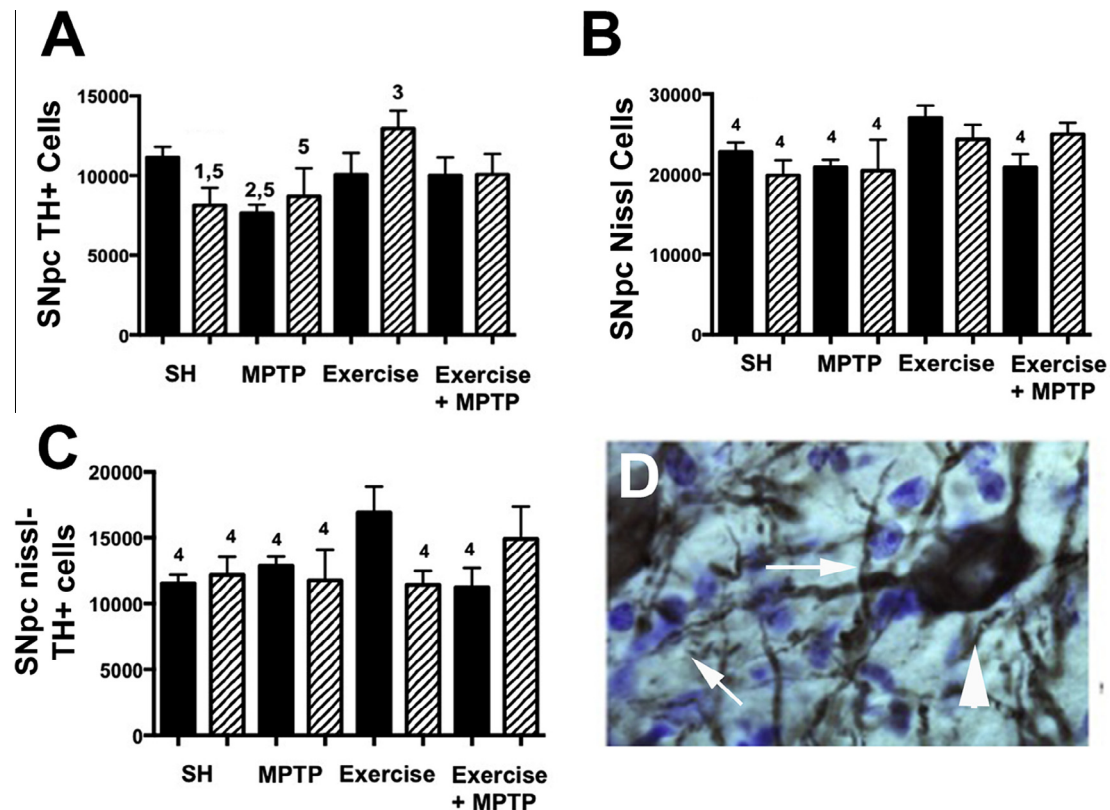


Fig. 12. TH and Nissl cell count reveals DA neuron loss in the SNpc of *Epas1*-CKO mice. (A) TH+ cells are reduced in *Epas1*-CKO SH ($p \leq 0.05$) and WT SH + MPTP ($p \leq 0.01$) conditions compared to WT SH. TH+ cell number is rescued in the *Epas1*-CKO exercise condition, and is reduced in the *Epas1*-CKO SH + MPTP ($p \leq 0.05$) condition when compared to the *Epas1*-CKO exercise condition. (B) Nissl cell count is significantly lower in the WT and *Epas1*-CKO SH and SH + MPTP conditions as well as the WT exercise + MPTP condition when compared to the WT Exercise condition ($p \leq 0.05$). (C) SNpc Nissl minus TH cell count reveals significantly less cells in the WT and *Epas1*-CKO SH and SH + MPTP conditions, *Epas1*-CKO exercise, and WT exercise + MPTP conditions compared to the WT exercise condition ($p \leq 0.05$). (D) Appearance of Nissl cells (arrows) and TH+ cell (arrowhead) in the SNpc. Statistics: 1 = $p \leq 0.05$ compared to WT SH, 2 = $p \leq 0.01$ compared to WT SH, 3 = $p \leq 0.01$ compared to *Epas1*-CKO SH, 4 = $p \leq 0.05$ compared to WT Exercise, 5 = $p \leq 0.05$ compared to *Epas1*-CKO Exercise. Error bars = \pm SEM. Solid bars are WT mice, Hatched bars are *epas1*-CKO mice.

may be explained by the observation that different tiers of the SNpc DA neurons are particularly sensitive to oxidative stress (German et al., 1996); and that exercise-induced hypoxic effects and MPTP act upon the same population of cells. The importance of HIF1 α in exercise-induced SNpc DA neuroprotection is supported by our findings that TH+ cell number in the *hif1* α -CKO mouse is inversely related to the total amount of exercise performed.

The loss of DA neurons in the SNpc of *epas1*-CKO mice housed in standard conditions suggests that HIF2 α plays a different role than HIF1 α in regard to development and maintenance of the SN. In standard conditions, adult *epas1*-CKO mice have lower SNpc TH+ DA cell numbers; however this reduction is rescued by 3 months of exercise. Additionally, a significant loss of TH+ neurons following the administration of MPTP in *epas1*-CKO mice is not seen. The low TH+ neuron number in standard conditions has two potential explanations. First, in the absence of postnatal neuronal *hif2* α , there is an increase in the naturally occurring cell death that takes place during the early postnatal period. This hypothesis is supported by studies showing that loss of *hif2* α , but not *hif1* α , leads

to increases in cell death based on downregulation of members of the IAP family (Dong et al., 2001; Ko et al., 2011). The second explanation for the lower number of TH+ neurons, is that we are observing only a loss of the DA neuron phenotype without true neuronal loss. Loss of *hif2* α leads to a deficiency in catecholamine production (Tian et al., 1998), likely via indirect control of transcription of the von Hippel–Lindau tumor suppressor protein (Schnell et al., 2003), whose expression has been shown to be inversely related to TH production (Czyzyk-Krzeska et al., 2003). The reduction in total Nissl-stained cells in SNpc from *epas1*-CKO mice in SH (compared to WT SH), which vanishes when the TH+ population is subtracted from the Nissl-stained number, supports the first explanation for reduced TH+ cell number, that is true loss of DA neurons when *epas1* is absent. In regard to the exercise-induced rescue of TH+ neurons, we suggest two possibilities: first, exercise upregulates *hif2* α in non-neuronal cells and is subsequently protective to neurons, perhaps by increased antioxidant or trophic factor activity. Second, it is also known that exercise (Tajiri et al., 2010; Lau et al., 2011), and specifically astrocytes, provide trophic (e.g., GDNF, BDNF, VEGF) and antioxidant (glutathione and its constitutive amino acids) (Smeyne and

Smeyne, 2013) factors. The increased Nissl cell number in the SNpc of exercised WT mice likely reflects an increase in astrocyte population. Thus, *epas1* may directly (Takeda et al., 2004) or indirectly function as a modulator for the protection of DA neurons in exercised *epas1*-CKO mice that may bestow protection before the inhibition of Complex I by MPTP.

Transient sublethal hypoxia has been shown to induce a phenomenon called preconditioning; and this is a well-recognized cellular process that is protective against later insult in a number of physiological systems (Chavez et al., 2000; Semenza, 2011; Dornbos et al., 2013). In this study, we suggest that regular and sustained exercise produces a recurring and intermittent hypoxia that induces members of the HIF family. This family of transcription factors that are responsible for the induction of numerous adaptive pathways involved in cell metabolism and survival may activate processes that have long-lasting effects. Recurrent oxidative stress produces more efficient energy use (Mason et al., 2007; Semenza, 2011; Dornbos et al., 2013). Endurance training increases the oxidative capacity in skeletal muscle, a phenomenon that is reproduced in Hif1 α null muscle without training (Mason et al., 2007). Low tissue oxygenation also initiates angiogenesis through HIF and VEGF-A induction, factors that initially rise then return to prehypoxic levels, while vascular changes remain for several weeks (LaManna et al., 2004). The intensity and period of exercise necessary for neuroprotection in the SN may reflect the initial induction of *hif* and the subsequent expression of adaptive genes leading to long-term changes such as increased vascularity and oxidative capacity that are advantageous in the event of ischemic or xenobiotic trauma. *Hif1 α* loss in postnatal DA neurons of the SNpc eliminates exercise-induced neuroprotection and provides clues to the pathways whose activation is critical to initiate this process. Future investigation to determine if *hif1 α* or *epas1* can compensate for one another, the role that exercise and Hif induction play in the generation of or adaptation to ROS in the SN, and to discover which downstream signaling pathways are affected by the reduction of neuronal HIFs may further our understanding of how exercise benefits neuron vitality.

Acknowledgments—The authors thank Jarad Hopper, Yannan Ouyang and Rolanda Peterson for technical assistance. This work is supported by RO1-NS070825 and ALSAC.

REFERENCES

- Agani FH, Pichiule P, Chavez JC, LaManna JC (2000) The role of mitochondria in the regulation of hypoxia-inducible factor 1 expression during hypoxia. *J Biol Chem* 275:35863–35867.
- Agani FH, Puchowicz M, Chavez JC, Pichiule P, LaManna J (2002) Role of nitric oxide in the regulation of HIF-1 α expression during hypoxia. *Am J Physiol Cell Physiol* 283:C178–C186.
- Baquet ZC, Williams D, Brody J, Smeyne RJ (2009) A comparison of model-based (2D) and design-based (3D) stereological methods for estimating cell number in the substantia nigra pars compacta (SNpc) of the C57BL/6J mouse. *Neuroscience* 161:1082–1090.
- Baranova O, Miranda LF, Pichiule P, Dragatsis I, Johnson RS, Chavez JC (2007) Neuron-specific inactivation of the hypoxia inducible factor 1 alpha increases brain injury in a mouse model of transient focal cerebral ischemia. *J Neurosci* 27:6320–6332.
- Bergeron M, Evans SM, Sharp FR, Koch CJ, Lord EM, Ferriero DM (1999) Detection of hypoxic cells with the 2-nitroimidazole, EF5, correlates with early redox changes in rat brain after perinatal hypoxia-ischemia. *Neuroscience* 89:1357–1366.
- Boyd JD, Jang H, Shepherd KR, Faherty C, Slack S, Jiao Y, Smeyne RJ (2007) Response to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) differs in mouse strains and reveals a divergence in JNK signaling and COX-2 induction prior to loss of neurons in the substantia nigra pars compacta. *Brain Res* 1175:107–116.
- Brones TL (2006) Environment, physical activity, and neurogenesis: implications for prevention and treatment of Alzheimer's disease. *Curr Alzheimer Res* 3:49–54.
- Brown ST, Kelly KF, Daniel JM, Nurse CA (2009) Hypoxia inducible factor (HIF)-2 alpha is required for the development of the catecholaminergic phenotype of sympathoadrenal cells. *J Neurochem* 110:622–630.
- Bruick RK, McKnight SL (2001) A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 294:1337–1340.
- Brunelle JK, Bell EL, Quesada NM, Vercauteren K, Tiranti V, Zeviani M, Scarpulla RC, Chandel NS (2005) Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation. *Cell Metab* 1:409–414.
- Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshert E (1998) Role of HIF-1 α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* 394:485–490.
- Chandel NS, Budinger GR (2007) The cellular basis for diverse responses to oxygen. *Free Radic Biol Med* 42:165–174.
- Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT (1998) Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proc Natl Acad Sci U S A* 95:11715–11720.
- Chavez JC, LaManna JC (2002) Activation of hypoxia-inducible factor-1 in the rat cerebral cortex after transient global ischemia: potential role of insulin-like growth factor-1. *J Neurosci* 22:8922–8931.
- Chavez JC, Agani F, Pichiule P, LaManna JC (2000) Expression of hypoxia-inducible factor-1 α in the brain of rats during chronic hypoxia. *J Appl Physiol* 89:1937–1942.
- Chavez JC, Baranova O, Lin J, Pichiule P (2006) The transcriptional activator hypoxia inducible factor 2 (HIF-2/EPAS-1) regulates the oxygen-dependent expression of erythropoietin in cortical astrocytes. *J Neurosci* 26:9471–9481.
- Coletti D, Berardi E, Aulino P, Rossi E, Moresi V, Li Z, Adamo S (2013) Substrains of inbred mice differ in their physical activity as a behavior. *ScientificWorldJournal* 2013:237260.
- Cotman CW, Berchtold NC (2002) Exercise: a behavioral intervention to enhance brain health and plasticity. *Trends Neurosci* 25:295–301.
- Curry A, Guo M, Patel R, Liebelt B, Sprague S, Lai Q, Zwagerman N, Cao FX, Jimenez D, Ding Y (2010) Exercise pre-conditioning reduces brain inflammation in stroke via tumor necrosis factor-alpha, extracellular signal-regulated kinase 1/2 and matrix metalloproteinase-9 activity. *Neurol Res* 32:756–762.
- Czyzyk-Krzeska MF, Schnell PO, Bauer AL, Streit JB, Nash JA, Kuznetsova AV, Hui AS (2003) Regulation of tyrosine hydroxylase gene expression by hypoxia in neuroendocrine cells. New York: Marcel Dekker, Inc.
- Dong Z, Venkatachalam MA, Wang J, Patel Y, Saikumar P, Semenza GL, Force T, Nishiyama J (2001) Up-regulation of apoptosis inhibitory protein IAP-2 by hypoxia. Hif-1-independent mechanisms. *J Biol Chem* 276:18702–18709.
- Dornbos 3rd D, Zwagerman N, Guo M, Ding JY, Peng C, Esmail F, Sikharam C, Geng X, Guthikonda M, Ding Y (2013) Preischemic exercise reduces brain damage by ameliorating metabolic

- disorder in ischemia/reperfusion injury. *J Neurosci Res* 91:818–827.
- Dragatsis I, Zeitlin S (2000) CaMKIIalpha-Cre transgene expression and recombination patterns in the mouse brain. *Genesis* 26:133–135.
- Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ (2001) *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107:43–54.
- Evans SM, Joiner B, Jenkins WT, Laughlin KM, Lord EM, Koch CJ (1995) Identification of hypoxia in cells and tissues of epigastric 9L rat glioma using EF5 [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide]. *Br J Cancer* 72:875–882.
- Ferreira AF, Real CC, Rodrigues AC, Alves AS, Britto LR (2011) Short-term, moderate exercise is capable of inducing structural, BDNF-independent hippocampal plasticity. *Brain Res* 1425: 111–122.
- Gammella E, Cairo G, Tacchini L (2010) Adenosine A(2)A receptor but not HIF-1 mediates tyrosine hydroxylase induction in hypoxic PC12 cells. *J Neurosci Res* 88:2007–2016.
- Gerecke KM, Jiao Y, Pani A, Pagala V, Smeyne RJ (2010) Exercise protects against MPTP-induced neurotoxicity in mice. *Brain Res*.
- Gerecke KM, Jiao Y, Pagala V, Smeyne RJ (2012) Exercise does not protect against MPTP-induced neurotoxicity in BDNF haploinsufficient mice. *PLoS One* 7:e43250.
- German DC, Nelson EL, Liang CL, Speciale SG, Sinton CM, Sonsalla PK (1996) The neurotoxin MPTP causes degeneration of specific nucleus A8, A9 and A10 dopaminergic neurons in the mouse. *Neurodegeneration* 5:299–312.
- Hamre K, Tharp R, Poon K, Xiong X, Smeyne RJ (1999) Differential strain susceptibility following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration acts in an autosomal dominant fashion: quantitative analysis in seven strains of *Mus musculus*. *Brain Res* 828:91–103.
- Ivan M, Kondo K, Yang H, Kim W, Valiano J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin Jr WG (2001) HIF1alpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science* 292:464–468.
- Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, Semenza GL (1998) Cellular and developmental control of O₂ homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev* 12:149–162.
- Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, von Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ (2001) Targeting of HIF-1alpha to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science* 292:468–472.
- Jackson-Lewis V, Smeyne RJ (2005) MPTP and SNpc DA neuronal vulnerability: role of dopamine, superoxide and nitric oxide in neurotoxicity. *Minireview. Neurotox Res* 7:193–202.
- Jain S, Maltepe E, Lu MM, Simon C, Bradfield CA (1998) Expression of ARNT, ARNT2, HIF1 alpha, HIF2 alpha and Ah receptor mRNAs in the developing mouse. *Mech Dev* 73:117–123.
- Jang H, Boltz D, McClaren J, Pani AK, Smeyne M, Korff A, Webster R, Smeyne RJ (2012) Inflammatory effects of highly pathogenic H5N1 influenza virus infection in the CNS of mice. *J Neurosci* 32:1545–1559.
- Ko CY, Tsai MY, Tseng WF, Cheng CH, Huang CR, Wu JS, Chung HY, Hsieh CS, Sun CK, Hwang SP, Yuh CH, Huang CJ, Pai TW, Tzou WS, Hu CH (2011) Integration of CNS survival and differentiation by HIF2alpha. *Cell Death Differ* 18:1757–1770.
- Koch CJ, Evans SM, Lord EM (1995) Oxygen dependence of cellular uptake of EF5 [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide]: analysis of drug adducts by fluorescent antibodies vs bound radioactivity. *Br J Cancer* 72:869–874.
- LaManna JC, Chavez JC, Pichiule P (2004) Structural and functional adaptation to hypoxia in the rat brain. *J Exp Biol* 207:3163–3169.
- Lando D, Pongratz I, Poellinger L, Whitelaw ML (2000) A redox mechanism controls differential DNA binding activities of hypoxia-inducible factor (HIF) 1alpha and the HIF-like factor. *J Biol Chem* 275:4618–4627.
- Lau YS, Patki G, Das-Panja K, Le WD, Ahmad SO (2011) Neuroprotective effects and mechanisms of exercise in a chronic mouse model of Parkinson's disease with moderate neurodegeneration. *Eur J Neurosci* 33:1264–1274.
- Lerman I, Harrison BC, Freeman K, Hewett TE, Allen DL, Robbins J, Leinwand LA (2002) Genetic variability in forced and voluntary endurance exercise performance in seven inbred mouse strains. *J Appl Physiol* 92:2245–2255.
- Levy AP, Levy NS, Wegner S, Goldberg MA (1995) Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *J Biol Chem* 270:13333–13340.
- Li J, Ding YH, Rafols JA, Lai Q, McAllister 2nd JP, Ding Y (2005) Increased astrocyte proliferation in rats after running exercise. *Neurosci Lett* 386:160–164.
- Liebelt B, Papapetrou P, Ali A, Guo M, Ji X, Peng C, Rogers R, Curry A, Jimenez D, Ding Y (2010) Exercise preconditioning reduces neuronal apoptosis in stroke by up-regulating heat shock protein-70 (heat shock protein-72) and extracellular-signal-regulated-kinase 1/2. *Neuroscience* 166:1091–1100.
- Lightfoot JT, Turner MJ, Daves M, Vordermark A, Kleeberger SR (2004) Genetic influence on daily wheel running activity level. *Physiol Genomics* 19:270–276.
- Marques de Olivera M, Bonatto D, Pegas Henriques JA (2010) Nitroreductases: enzymes with environmental, biotechnological and clinical importance. In: Mendez-Vilas A, editor. *Current research technology and education topics in applied microbiology and microbial biotechnology*, vol. 2. Spain: Formatex Research Center. p. 1008–1019.
- Mason SD, Rundqvist H, Papandreou I, Duh R, McNulty WJ, Howlett RA, Olfert IM, Sundberg CJ, Denko NC, Poellinger L, Johnson RS (2007) HIF-1alpha in endurance training: suppression of oxidative metabolism. *Am J Physiol Regul Integr Comp Physiol* 293:R2059–R2069.
- Murray DK, Sacheli MA, Eng JJ, Stoessl AJ (2014) The effects of exercise on cognition in Parkinson's disease: a systematic review. *Transl Neurodegener* 3:5.
- Oberdick J, Wallace JD, Lewin A, Smeyne RJ (1994) Transgenic expression to monitor dynamic organization of neuronal development: use of the *E. coli* lacZ gene product, beta-galactosidase. *Neuroprotocols* 5:54–62.
- Okuyama H, Krishnamachary B, Zhou YF, Nagasawa H, Bosch-Marce M, Semenza GL (2006) Expression of vascular endothelial growth factor receptor 1 in bone marrow-derived mesenchymal cells is dependent on hypoxia-inducible factor 1. *J Biol Chem* 281:15554–15563.
- Paxinos G, Franklin KBJ (2001) *The mouse brain in stereotaxic coordinates*. San Diego: Academic Press.
- Peng J, Zhang L, Drysdale L, Fong GH (2000) The transcription factor EPAS-1/hypoxia-inducible factor 2alpha plays an important role in vascular remodeling. *Proc Natl Acad Sci U S A* 97:8386–8391.
- Peng YJ, Nanduri J, Khan SA, Yuan G, Wang N, Kinsman B, Vaddi DR, Kumar GK, Garcia JA, Semenza GL, Prabhakar NR (2011) Hypoxia-inducible factor 2alpha (HIF-2alpha) heterozygous-null mice exhibit exaggerated carotid body sensitivity to hypoxia, breathing instability, and hypertension. *Proc Natl Acad Sci U S A* 108:3065–3070.
- Petzinger GM, Fisher BE, McEwen S, Beeler JA, Walsh JP, Jakowec MW (2013) Exercise-enhanced neuroplasticity targeting motor and cognitive circuitry in Parkinson's disease. *Lancet Neurol* 12:716–726.
- Radak Z, Chung HY, Koltai E, Taylor AW, Goto S (2008) Exercise, oxidative stress and hormones. *Ageing Res Rev* 7:34–42.
- Radak Z, Zhao Z, Koltai E, Ohno H, Atalay M (2013) Oxygen consumption and usage during physical exercise: the balance between oxidative stress and ROS-dependent adaptive signaling. *Antioxid Redox Signal* 18:1208–1246.

- Schnell PO, Ignacak ML, Bauer AL, Striet JB, Paulding WR, Czyzyk-Krzeska MF (2003) Regulation of tyrosine hydroxylase promoter activity by the von Hippel–Lindau tumor suppressor protein and hypoxia-inducible transcription factors. *J Neurochem* 85:483–491.
- Scortegagna M, Ding K, Oktay Y, Gaur A, Thurmond F, Yan LJ, Marck BT, Matsumoto AM, Shelton JM, Richardson JA, Bennett MJ, Garcia JA (2003) Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in *Epas1*^{−/−} mice. *Nat Genet* 35:331–340.
- Semenza GL (2000) HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J Appl Physiol* 88:1474–1480.
- Semenza GL (2011) Hypoxia-inducible factor 1: regulator of mitochondrial metabolism and mediator of ischemic preconditioning. *Biochim Biophys Acta* 1813:1263–1268.
- Semenza GL, Wang GL (1992) A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol Cell Biol* 12:5447–5454.
- Semenza GL, Roth PH, Fang HM, Wang GL (1994) Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J Biol Chem* 269:23757–23763.
- Smeyne RJ, Jackson-Lewis V (2005) The MPTP model of Parkinson's disease. *Mol Brain Res* 134:57–66.
- Smeyne M, Smeyne RJ (2013) Glutathione metabolism and Parkinson's disease. *Free Radic Biol Med* 62:13–25.
- Smeyne M, Jiao Y, Shepherd KR, Smeyne RJ (2005) Glia cell number modulates sensitivity to MPTP in mice. *Glia* 52:144–152.
- Smeyne M, Boyd J, Raviie Shepherd K, Jiao Y, Pond BB, Hatler M, Wolf R, Henderson C, Smeyne RJ (2007) GSTpi expression mediates dopaminergic neuron sensitivity in experimental Parkinsonism. *Proc Natl Acad Sci U S A* 104:1977–1982.
- Smith BA, Goldberg NR, Meshul CK (2011) Effects of treadmill exercise on behavioral recovery and neural changes in the substantia nigra and striatum of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned mouse. *Brain Res* 1386:70–80.
- Stranahan AM, Martin B, Maudsley S (2012) Anti-inflammatory effects of physical activity in relationship to improved cognitive status in humans and mouse models of Alzheimer's disease. *Curr Alzheimer Res* 9:86–92.
- Tajima M, Kurashima Y, Sugiyama K, Ogura T, Sakagami H (2009) The redox state of glutathione regulates the hypoxic induction of HIF-1. *Eur J Pharmacol* 606:45–49.
- Tajiri N, Yasuhara T, Shingo T, Kondo A, Yuan W, Kadota T, Wang F, Baba T, Tayra JT, Morimoto T, Jing M, Kikuchi Y, Kuramoto S, Agari T, Miyoshi Y, Fujino H, Obata F, Takeda I, Furuta T, Date I (2010) Exercise exerts neuroprotective effects on Parkinson's disease model of rats. *Brain Res* 1310:200–207.
- Takeda N, Maemura K, Imai Y, Harada T, Kawanami D, Nojiri T, Manabe I, Nagai R (2004) Endothelial PAS domain protein 1 gene promotes angiogenesis through the transactivation of both vascular endothelial growth factor and its receptor, Flt-1. *Circ Res* 95:146–153.
- Tian H, McKnight SL, Russell DW (1997) Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev* 11:72–82.
- Tian H, Hammer RE, Matsumoto AM, Russell DW, McKnight SL (1998) The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. *Genes Dev* 12:3320–3324.
- Vangeison G, Carr D, Federoff HJ, Rempel DA (2008) The good, the bad, and the cell type-specific roles of hypoxia inducible factor-1 alpha in neurons and astrocytes. *J Neurosci* 28:1988–1993.
- Wang GL, Semenza GL (1993) General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc Natl Acad Sci U S A* 90:4304–4308.
- Wang GL, Semenza GL (1995) Purification and characterization of hypoxia-inducible factor 1. *J Biol Chem* 270:1230–1237.
- Wang GL, Jiang BH, Rue EA, Semenza GL (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci U S A* 92:5510–5514.
- Wu SY, Wang TF, Yu L, Jen CJ, Chuang JI, Wu FS, Wu CW, Kuo YM (2011) Running exercise protects the substantia nigra dopaminergic neurons against inflammation-induced degeneration via the activation of BDNF signaling pathway. *Brain Behav Immun* 25:135–146.
- Yasuhara T, Shingo T, Kobayashi K, Takeuchi A, Yano A, Muraoka K, Matsui T, Miyoshi Y, Hamada H, Date I (2004) Neuroprotective effects of vascular endothelial growth factor (VEGF) upon dopaminergic neurons in a rat model of Parkinson's disease. *Eur J Neurosci* 19:1494–1504.
- Yuan G, Peng YJ, Reddy VD, Makarenko VV, Nanduri J, Khan SA, Garcia JA, Kumar GK, Semenza GL, Prabhakar NR (2013) Mutual antagonism between hypoxia-inducible factors 1alpha and 2alpha regulates oxygen sensing and cardio-respiratory homeostasis. *Proc Natl Acad Sci U S A* 110:E1788–E1796.
- Zigmond MJ, Smeyne RJ (2014) Exercise: is it a neuroprotective and if so, how does it work? *Parkinsonism Relat Disord* 20(Suppl. 1):S123–S127.