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REPETITIVE ACUTE INTERMITTENT HYPOXIA INCREASES GROWTH/NEUROTROPHIC FACTOR EXPRESSION IN NON-RESPIRATORY MOTOR NEURONS

I Satriotomo[*,§], **N. L. Nichols**[†], **E. A. Dale**[‡], **A. T. Emery**, **J. M. Dahlberg**, and **G. S. Mitchell**[§]

Department of Comparative Biosciences, University of Wisconsin, Madison, WI 53706, USA

Abstract

Repetitive acute intermittent hypoxia (rAIH) increases growth/trophic factor expression in respiratory motor neurons, thereby eliciting spinal respiratory motor plasticity and/or neuroprotection. Here we demonstrate that rAIH effects are not unique to respiratory motor neurons, but are also expressed in non-respiratory, spinal alpha motor neurons and upper motor neurons of the motor cortex. In specific, we used immunohistochemistry and immunofluorescence to assess growth/trophic factor protein expression in spinal sections from rats exposed to AIH three times per week for 10 weeks (3 × wAIH). 3 × wAIH increased brain-derived neurotrophic factor (BDNF), its high-affinity receptor, tropomyosin receptor kinase B (TrkB), and phosphorylated TrkB (pTrkB) immunoreactivity in putative alpha motor neurons of spinal cervical 7 (C_7) and lumbar 3 (L_3) segments, as well as in upper motor neurons of the primary motor cortex (M_1). 3 × wAIH also increased immunoreactivity of vascular endothelial growth factor A (VEGFA), the high-affinity VEGFA receptor (VEGFR-2) and an important VEGF gene regulator, hypoxia-inducible factor-1α (HIF-1α). Thus, rAIH effects on growth/trophic factors are characteristic of non-respiratory as well as respiratory motor neurons. rAIH may be a useful tool in the treatment of disorders causing paralysis, such as spinal injury and motor neuron disease, as a pretreatment to enhance motor neuron survival during disease, or as preconditioning for cell-transplant therapies.

Keywords

intermittent hypoxia; BDNF; VEGF; TrkB; HIF-1; motor neuron

INTRODUCTION

System and cellular adaptations to hypoxia are crucial in many physiological and pathophysiological states. At the systems level, intermittent hypoxia (IH) elicits respiratory plasticity, potentially minimizing future recurrence of IH (Mitchell et al., 2001; Feldman et al., 2003; Mitchell and Johnson, 2003; Mahamed and Mitchell, 2007; Devinney et al., 2013).

[*]Corresponding author. Address: Department of Physical Therapy, University of Florida, 330 Center Drive, Gainesville, FL 32610, USA. Tel: 352-273-5972. satriotomo@ufl.edu (I. Satriotomo).

[†]Current address: Department of Biomedical Sciences, University of Missouri, Columbia, MO 65211, USA.

[‡]Current address: Department of Integrative Biology and Physiology, UCLA, Los Angeles, CA 90095, USA.

[§]Current address: Department of Physical Therapy and McKnight Brain Institute, University of Florida, Gainesville, FL 32610, USA.

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IH-induced plasticity occurs at multiple sites in the neural system controlling breathing, including peripheral chemoreceptors (Prabhakar, 2001, 2011), brainstem integrating neurons (Ling et al., 2001; Kline et al., 2007; Kline, 2010) and respiratory motor nuclei (Baker-Herman and Mitchell, 2002; Baker-Herman et al., 2004).

On a cellular level, IH alters the expression of key molecules associated with both respiratory plasticity and neuroprotection. The most widely studied model of IH-induced respiratory plasticity, phrenic long-term facilitation (pLTF) following acute intermittent hypoxia (AIH), requires spinal serotonin receptor activation (Bach and Mitchell, 1996; Baker-Herman and Mitchell, 2002) and serotonin-dependent synthesis of brain-derived neurotrophic factor (BDNF; Baker-Herman et al., 2004). Repetitive AIH (rAIH) elicits long-lasting increases in the expression of many molecules necessary for pLTF within the phrenic motor nucleus, including BDNF and its high-affinity receptor, tropomyosin receptor kinase B (TrkB) (Wilkerson and Mitchell, 2009; Lovett-Barr et al., 2012; Satriotomo et al., 2012). Apart from its key role in neuroplasticity, BDNF is neuroprotective for neurons stressed by ischemia (Duncan et al., 2004; Ferrer et al., 2004). The transcription factor hypoxia inducible factor 1α (HIF-1α; Semenza, 2007) regulates expression of other growth/trophic factors, such as vascular endothelial growth factor (VEGF) and its high-affinity receptor, VEGFR-2 (Calvani et al., 2012). VEGF and VEGFR-2 are expressed in motor neurons (Yang et al., 2003), elicit respiratory motor plasticity (Dale-Nagle et al., 2011), and are neuroprotective against ischemic injury (van Bruggen et al., 1999; Jin et al., 2000). Thus, BDNF and VEGF are hypoxia-regulated genes that elicit both spinal plasticity and neuroprotection.

IH elicits plasticity in neural systems not directly linked to breathing. For example, a single presentation of AIH elicits transient increases in sympathetic nerve activity (Dick et al., 2007; Xing and Pilowsky, 2010) and daily AIH (dAIH) for one week elicits prolonged improvement in forelimb function of rats with cervical spinal injuries, an effect that lasts weeks following treatment (Lovett-Barr et al., 2012; Prosser-Loose et al., 2015). A single AIH exposure (15, 1-min hypoxic episodes, 9% inspired O_2; 1-min intervals) improves leg strength in persons with chronic spinal injuries (Trumbower et al., 2012), and dAIH (15, 1.5 min episodes per day, 9% O_2; 1.5 min intervals) and dAIH paired with 30-min of overground walking practice improved walking speed and endurance in patients with chronic incomplete spinal cord injuries (Hayes et al., 2014). Thus, IH may elicit similar plasticity in respiratory and non-respiratory motor systems. Fundamental mechanisms giving rise to such similar functional plasticity have not been adequately explored.

We previously demonstrated that a distinct protocol of repetitive AIH consisting of AIH (10, 5-min episodes of 10.5% O_2 per day; 5-min normoxic intervals) three times per week for 10 weeks (3 × wAIH) elicits neurochemical plasticity in phrenic motor neurons (Satriotomo et al., 2012). Here, we tested the hypothesis that 3 × wAIH also increases the BDNF, TrkB, p-TrkB, HIF-1α, VEGF and VEGFR-2 expression in non-respiratory motor neurons. Immunohistochemical techniques were utilized to localize the expression of these growth/trophic factors and their main receptors in alpha motor neurons innervating upper and lower limbs in C_7 and L_3 ventral gray matter, and in the primary motor cortex (M_1).

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An understanding of rAIH-induced growth/trophic factor expression may be useful as we develop therapeutic strategies to treat motor deficits in patients, including those with cervical spinal injuries or motor neuron disease (Dale et al., 2014; Navarrete-Opazo and Mitchell, 2014).

EXPERIMENTAL PROCEDURES

Animals and experimental treatments

Twenty male adult Sprague–Dawley (SD) rats weighing 300–330 g were randomly exposed to normoxia ($n = 10$) or 3 × wAIH for 10 weeks ($n = 10$). AIH was accomplished by placing unrestrained rats in Plexiglass chambers (one rat per chamber, dimensions 12 in × 4.5 in × 4.5 in) while gases flushing through the chambers (4 L/min) were alternated between 21% and 10.5% O_2 at 5-min intervals. One day prior to treatment onset, rats were acclimated to the exposure chambers before beginning the 3 × wAIH protocol: 10, 5-min hypoxic episodes ($FIO_2 = 0.105$), separated by 5-min normoxic intervals ($FIO_2 = 0.21$), three times per week for 10 weeks as described previously (Satriotomo et al., 2012). Sham rats were in chambers for an equivalent period of time, but did not receive hypoxia. Chamber oxygen levels were continuously monitored (AX300-1, Teledyne Analytical Instruments, City of Industry, CA, USA). Both 3 × wAIH and normoxia-treated rats rested quietly or slept during exposure periods. All procedures in this study were carried out in accordance with the National Institutes of Health (NIH) guidelines for care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at the School of Veterinary Medicine, University of Wisconsin-Madison.

Immunohistochemistry—All rats treated with normoxia or 3 × wAIH were euthanized and perfused transcardially with cold 0.01 M phosphate-buffered saline (PBS, pH 7.4), followed by 4% buffered paraformaldehyde. The brain and spinal cords were immediately removed, and cryoprotected in 30% sucrose at 4 °C until they sank. Transverse sections of the cortical area of the primary motor cortex (M_1), cervical spinal (C_7) and lumbar spinal segments (L_3) were processed for immunohistochemistry. Transverse sections (40 μm) were cut using a freezing microtome (Leica SM 200R, Germany). For immunostaining, free-floating sections were washed in 0.1 M Tris-buffered saline with 0.1% Triton-X100 (TBS-Tx; 3 × 5 min) and incubated (30 min) in TBS containing 1% H_2O_2. After washing (3 × 5 min) in TBS-Tx, tissues were blocked (60 min) with 5% of normal goat serum or normal rabbit serum and then tissue was incubated at 4 °C overnight in primary antibodies: rabbit polyclonal anti-BDNF (N-20, 1/1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit polyclonal anti-TrkB (1/500, Santa Cruz Biotechnology, Santa Cruz, CA); rabbit serum anti phospo-TrkB (1/1000, courtesy of Dr. Moses Chao, NYU); rabbit polyclonal anti-VEGF (A-20, 1/1000, Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal anti-VEGFR-2 or KDR (Kinase insert Domain Receptor) (V3003, 1/500, Sigma–Aldrich, St. Louis, MO, USA) and rabbit polyclonal anti-HIF-1α (1/500, Santa Cruz Biotechnology, Santa Cruz, CA). Following overnight incubation, sections were washed and incubated in either biotinylated secondary goat anti-rabbit antibody (1:1,000, Vector Laboratories, Burlingame, CA, USA) for BDNF, TrkB, phospho-TrkB, and VEGF, or biotinylated secondary goat anti-mouse antibody for VEGFR-2 (1:1000, Vector Laboratories,

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Burlingame, CA). Conjugation with avidin–biotin complex (Vecstatin Elite ABC kit, Vector Laboratories, Burlingame, CA) was followed by visualization with 3,′-diaminobenzidine-hydrogen peroxidase (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Sections were then washed in TBS, placed in gelatin-coated slides, dried, dehydrated in a graded alcohol series, and then cleared with xylenes and mounted with Eukitt mounting medium (Electron microscope sciences, Hatfield, PA, USA).

All images were captured and analyzed with a digital camera (SPOT II; Diagnostic Instruments, Sterling Heights, MI, USA). Final photomicrographs were created with Adobe Photoshop software (Adobe System, San Jose, CA, USA). All images received equivalent adjustments to tone scale, gamma and sharpness. Sections incubated without primary or secondary antibodies served as negative controls. In addition we pre-absorbed the primary BDNF and VEGF antibodies with a fivefold (by concentration) excess of specific blocking peptides (sc-546 P and sc-152 P; both from Santa Cruz Biotechnology). A parallel set of TrkB-stained sections was subjected to cresyl-violet staining to identify the morphology of the cells.

Immunofluorescence—To identify the expression of the neurotrophic/growth- and transcription-factor proteins in somatic motor neurons, tissues were incubated at 4 °C overnight with either rabbit polyclonal anti-BDNF (N-20; 1/500, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-VEGF (A-20, 1/200, Sigma– Aldrich, St. Louis, MO) or rabbit polyclonal anti-HIF-1α (1/200, Santa Cruz Biotechnology, Santa Cruz, CA), and a second primary antibody for the neuronal marker mouse monoclonal anti-NeuN (1/200, Chemicon, Temecula, CA). After washing with TBS-Tx (3 × 5 min), tissues were incubated in a mixture of conjugated goat anti-rabbit red fluorescent Alexa 495 and conjugated goat anti-mouse green fluorescent Alexa 488 (1:200, Molecular Probes, Eugene, Oregon) at room temperature for 60 min. Stained tissues were mounted on glass using anti-fade solution (Prolong Gold anti fade reagent, Invitrogen, Oregon) and examined using an epifluorescence microscope (Nikon, Japan).

Quantification and statistical analysis—The expression of BDNF, VEGF and HIF-1α in lumbar L_3 spinal segments was quantified as previously described (Satriotomo et al., 2012). The expression of growth/neurotrophic factors in C_7 spinal segment and M_1 upper motor neurons was not quantified because the C_7 segment may contribute to intercostal muscle (C_7–T_{13}s segments) but the L_3 segment does not innervate the abdominal respiratory motor neuron (i.e. T_8–L_2 segment; Giraudin et al., 2008). Sections of the L_3 spinal segment were numbered sequentially, and every 8th section was selected for immunohistochemistry using, 30-diaminobenzidine-hydrogen peroxidase (DAB) methods. Approximately five sections from each segmental level were used in this study. Putative alpha motor neurons were identified as large (> ~295 μm^2) NeuN-positive cells (Friese et al., 2009) in lamina IX of the lumbar L_3 ventral horn (Paxinos and Watson, 1997); the investigator was blinded to the experimental treatment in all analyses.

Analysis of BDNF, VEGF and HIF-1α immunoreactive alpha motor neurons was performed on images taken at 40× (SPOT II; Diagnostic Instruments, Sterling Heights, MI). Raw images were analyzed using Image J software (NIH, Bethesda, MD; http://rsb.info.nih.gov/

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ij). All images were converted to eight-bit resolution, and threshold was set between 120 and 160 during all analyses; a threshold was chosen for each group in which all motor neurons were visible but not saturated in ImageJ (i.e. both normoxia and 3 × wAIH images were treated identically within each group). Densitometry was performed by circumscribing individual motor neurons in lamina IX and the fractional area occupied by BDNF, VEGF or HIF-1α label was computed by Image J (i.e. the percentage of immuno-positive pixels within each motor neuron that was above threshold). Area fraction for individual motor neurons were then averaged for each section, and then further averaged per animal and group for each treatment. Data were compared between the 3 × wAIH and normoxia-treated groups for BDNF ($n = 7$ for normoxia and $n = 10$ for 3 × wAIH), VEGF (each $n = 6$) and HIF-1α (each $n = 4$) using a *T*-test. Differences were considered significant if $p < 0.05$. All values are expressed as mean±1 SEM.

RESULTS

3 × wAIH increases BDNF, TrkB and p-TrkB immunoreactivity in motor neurons

The primary motor cortex (M_1), including layers I–V (Miller, 1987; Fig. 1A), sends long axons down the spinal cord that synapse on spinal interneurons and alpha motor neurons. Photomicrographs of coronal sections of the cerebral cortex showed only faint BDNF staining in any layer in normoxic rats (Fig. 1A). 3 × wAIH enhanced BDNF immunoreactivity in M_1 motor cortex, especially in Betz cells of layer V as revealed at higher magnification (Fig. 1B).

3 × wAIH also increases BDNF immunostaining in somatic motor neurons of the C_7 and L_3 ventral horns (Fig. 1C–F); increased BDNF immunoreactivity was observed in putative alpha motor neurons (larger group of neurons in lamina IX, arrows) and presumptive interneurons (group of small neurons in lamina VII and VIII, arrow head) in C_7 (Fig. 1C, D) and L_3 (Fig. 1E, F) ventral horns, respectively. Immunofluorescence also revealed increased BDNF expression after 3 × wAIH in L_3 putative alpha-motor neurons (larger cells stained with NeuN; Fig. 1G–J); increased BDNF following 3 × wAIH was confirmed by densitometry in L_3 ventral horn (83.8±2.5 vs. 54.1±11.8, 3 × wAIH vs. normoxia respectively, $p < 0.05$; Fig. 1K).

In normoxic rats, TrkB immunoreactivity in layer V of motor cortex was faint in neurons and axonal fibers (Fig. 2A). 3 × wAIH increased TrkB immunostaining in pyramidal neurons (Fig. 2B). Increased TrkB expression was observed in the cell soma, and surrounding regions that presumably reflect labeling in neuronal processes. 3 × wAIH also increased TrkB immunostaining within non-respiratory motor neurons of the C_7 ventral horn (Fig. 2C, D). 3 × wAIH also increased phospho-TrkB immunoreactivity in neurons of layer V of cortical M_1, and in the C_7 spinal ventral horn (Fig. 2E–H). Increased TrkB phosphorylation suggests greater receptor activation following 3 × wAIH.

VEGF and VEGFR-2 immunoreactivity are upregulated by 3 × wAIH

As shown in Fig. 3(A–C), VEGF expression in cortical M_1, and C_7 and L_3 spinal ventral horns is low in normoxic rats. However, 3 × wAIH induced VEGF immunoreactivity in

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neurons within each region, including putative alpha motor neurons and inter-neurons (Fig. 3D–F). When visualized with immunofluorescence, VEGF protein was localized in presumptive alpha motor neurons (large NeuN-positive cells); 3 × wAIH enhanced VEGF expression in these cells (Fig. 3G–J). Using densitometry, 3 × wAIH increased VEGF expression in the L_3 ventral horn (88.6±1.1 vs. 62.3±5.6; 3 × wAIH vs. normoxia respectively; $p < 0.001$; Fig. 3K).

VEGFR-2 protein is also expressed in neurons of cortical M_1 and in the C_7 and L_3 spinal ventral horns (Fig. 4A–C); 3 × wAIH increased VEGFR-2 expression in each of these regions (Fig. 4D–F).

HIF1α is upregulated following 3 × wAIH

Because of its role in regulating gene expression in response to hypoxia, we evaluated HIF-1α expression after 3 × wAIH. In normoxia, HIF-1α is expressed in neurons of M_1 cortex, and in the C_7 and L_3 spinal ventral horns (Fig. 5A–C). 3 × wAIH increased HIF-1α immunostaining versus normoxia (Fig. 5D–F). Increased HIF-1α was observed in the cytoplasm (arrow) and cell nucleus (arrow head). With immunofluorescence, HIF-1α immunostaining in the C_7 spinal ventral horn is faint in normoxia, and predominantly in the cytoplasm (Fig. 5G, H). After 3 × wAIH, cytoplasmic staining increased, and was now also evident in the cell nucleus (arrow head; Fig. 5I, J). Densitometry confirmed that HIF-1α protein expression increased after 3 × wAIH (65.9±13.1 vs. 17.5±5.4; 3 × wAIH vs. normoxia respectively; $p < 0.05$; Fig. 5K). Thus, HIF-1α may induce transcriptional regulation of hypoxia-sensitive genes in spinal motor neurons following 3 × wAIH.

DISCUSSION

Here, we demonstrate increased expression of growth/trophic factors known to mediate neuroprotection and neuroplasticity in non-respiratory spinal motor neurons and the primary motor cortex following a modest protocol of repetitive AIH. In specific, we demonstrate that 3 × wAIH increases BDNF and VEGF protein levels (and their receptors) in putative alpha motor neurons of the C_7 and L_3 spinal ventral horn, and in neurons of layer V of the motor cortex.

AIH and rAIH elicit motor plasticity in non-respiratory motor systems following spinal injury (Lovett-Barr et al., 2012; Trumbower et al., 2012; Hayes et al., 2014; Prosser-Loose et al., 2015), and we demonstrated previously increased growth/trophic factor expression associated with dAIH-induced motor plasticity in both respiratory (C_4) and non-respiratory (C_7) motor nuclei (Lovett-Barr et al., 2012). We now demonstrate similar changes in key growth/trophic factors at other CNS sites associated with non-respiratory motor behaviors, including the motor cortex and non-respiratory (C_7 and L_3) spinal motor neurons. Each of these changes may contribute to motor plasticity (Dale et al., 2014).

Previous attempts to harness exogenous growth/trophic factor delivery for therapeutic benefit have met with limited success, at least in part because their high-affinity receptors down regulate (Wada et al., 2006; Jia et al., 2008), undermining therapeutic efficacy. Repetitive AIH may represent a safe, simple and effective means of endogenous growth

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factor ‘‘delivery” to the CNS since it increases expression of growth/trophic factors and their high-affinity receptors.

rAIH regulation of BDNF and TrkB expression

New cervical spinal BDNF synthesis is necessary and sufficient for AIH-induced spinal respiratory motor plasticity (Baker-Herman et al., 2004). Following AIH, new BDNF synthesis requires spinal serotonin receptor activation (Baker-Herman et al., 2004), suggesting that BDNF synthesis is regulated by intermittent serotonin receptor activation versus hypoxia *per se* (Baker-Herman and Mitchell, 2002; MacFarlane and Mitchell, 2009; MacFarlane et al., 2011). In this short time domain, new BDNF synthesis most likely involves translational regulation from existing mRNA (Baker-Herman and Mitchell, 2002).

Although we do not yet know if rAIH-induced BDNF expression in non-respiratory motor neurons is serotonin-dependent, we speculate that similar, serotonin-dependent mechanisms increase BDNF synthesis following AIH in respiratory and nonrespiratory motor neurons (Lovett-Barr et al., 2012). The most likely cellular mechanism of BDNF upregulation is referred as the “Q pathway” to phrenic motor facilitation (pMF; Dale-Nagle et al., 2010a,b; Dale et al., 2014), since multiple metabotropic Gq protein-coupled receptors elicit pMF by a similar BDNF synthesis-dependent mechanism. Metabotropic receptors known to elicit the Q pathway to pMF include serotonin 2A and 2B (MacFarlane and Mitchell, 2009; MacFarlane et al., 2011), and α1-adrenergic receptors (Huxtable et al., 2014).

With multi-day, repetitive AIH exposures, additional mechanisms involving hypoxia-induced transcriptional regulation are likely involved. Our observation that 3 × wAIH upregulates HIF-1α in motor neurons is consistent with a role in transcriptional regulation of BDNF and TrkB since both are either directly or indirectly regulated by HIF-1α (Martens et al., 2007). Although the BDNF gene is not reported to have a hypoxia response element in its promoter (i.e. the promoter site binding HIF-1α), hypoxia response elements are found on the promoter regions of other transcription factors that directly regulate BDNF (e.g. DEC1/BHLHB2; Miyazaki et al., 2002; Jiang et al., 2008). Further, Neurotrophic Tyrosine Kinase Receptor 2 (NTRK2), which regulates TrkB, is in turn regulated by HIF-1α (Martens et al., 2007). The respective roles of serotonin versus HIF-1α-dependent BDNF and TrkB regulation after 3 × wAIH require further investigation.

BDNF and TrkB roles in rAIH-induced motor plasticity

Modest rAIH protocols induce respiratory motor plasticity and metaplasticity (Wilkerson and Mitchell, 2009; Vinit et al., 2010; Lovett-Barr et al., 2012). For example, dAIH (10 hypoxic episodes per day, 7 days) increases ventral spinal BDNF (Wilkerson and Mitchell, 2009), enhances phrenic motor facilitation after a single (three episode) AIH exposure (Wilkerson and Mitchell, 2009), and improves breathing capacity in rats with chronic cervical spinal injuries (Lovett-Barr et al., 2012). Similarly, 3 × wAIH (4 or 10 weeks) enhances phrenic motor facilitation (Vinit et al., 2010) and increases BDNF protein expression in the ventral C_4 spinal cord (Satriotomo et al., 2012). Although dAIH elicits a lesser increase in BDNF expression versus 3 × wAIH, there is no apparent difference in BDNF upregulation with 3 × wAIH for 4 versus 10 weeks (Satriotomo et al., 2010, 2012).

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In addition to its effects on respiratory function, dAIH improves forelimb function in rats with chronic cervical spinal injuries (Lovett-Barr et al., 2012). Even a single AIH exposure increases leg strength (plantar flexion torque) (Trumbower et al., 2012), and daily AIH for 5 days improves walking ability in patients with chronic, incomplete spinal injuries (Hayes et al., 2014). Thus, rAIH represents a novel therapeutic approach to improve non-respiratory somatic motor function following chronic spinal injury. These findings also suggest that common mechanisms may be at play, with the specific motor enhancement arising from the same cellular mechanisms arising in different motor neuron pools (Dale et al., 2014).

BDNF is a powerful modulator of neuronal excitability and synaptic transmission (Causing et al., 1997; Lu and Figurov, 1997; Kafitz et al., 1999). Physical activity also increases brain and spinal BDNF expression (Neeper et al., 1995; Gomez-Pinilla et al., 2001) and, in turn, spinal BDNF enhances locomotion (Jakeman et al., 1998). Repetitive AIH represents a novel means of inducing spinal BDNF and TrkB function to trigger motor plasticity without the difficulties of over-ground walking in paralyzed individuals, complications due to exogenous BDNF administration (Jia et al., 2008; Zhang et al., 2011), or deleterious effects characteristic of severe/prolonged intermittent hypoxia protocols (e.g. hypertension, hippocampal inflammation or neuronal cell death; for review see Navarrete-Opazo and Mitchell, 2014). Thus, BDNF (and TrkB) may play important roles in non-respiratory motor plasticity following intermittent hypoxia (Dale-Nagle et al., 2010a,b; Lovett-Barr et al., 2012; Trumbower et al., 2012; Dale et al., 2014; Hayes et al., 2014; Navarrete-Opazo and Mitchell, 2014).

VEGF and VEGF receptor 2 expression following rAIH

VEGF and VEGF receptors are expressed in phrenic motor neurons (Dale-Nagle et al., 2011) and are upregulated by 3 × wAIH (Satriotomo et al., 2012; Dale and Mitchell, 2013). Here we confirm that VEGF and VEGF receptor 2 are expressed in non-respiratory spinal motor neurons (Sato et al., 2012), and demonstrate that they are also upregulated by 3 × wAIH. Mechanisms increasing VEGF and VEGFR-2 expression are unknown, but likely result from HIF1 transcriptional regulation.

Many long-term cellular adaptations to hypoxia are mediated by HIF-1 (Semenza, 2007; Sharp et al., 2001; Yamakawa et al., 2003; Zhou et al., 2003). During normoxia, HIF-1α subunits are rapidly degraded in the proteasome, but are stabilized by hypoxia (Maxwell et al., 1999); HIF-1α then translocates to the cell nucleus where it binds with HIF-1β subunits, and initiates transcriptional activity. HIF-1 is a major regulator of the VEGF gene (Yamakawa et al., 2003). On other hand, HIF-2α shares 80% sequence homology to HIF-1α and interacts with HIF-1β (Ema et al., 1997). Some HIF-1 target genes, including VEGF, EPO, GLUT1 and EGLN3, can be activated either by HIF-1 or by HIF-2 (Elvidge et al., 2006). Nanduri et al. (2009) demonstrated that HIF-1α was up-regulated and HIF-2α was down-regulated by chronic intermittent hypoxia. An understanding of the complex interactions between these HIF isoforms and their respective contributions to growth/trophic factor regulation in somatic motor neurons awaits further investigation.

BDNF also regulates VEGF by a TrkB and HIF-1-dependent mechanism (Nakamura et al., 2006). Conversely, transcriptional regulation of the NTRK2 gene, which encodes the TrkB

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receptor tyrosine kinase, increases during hypoxia by a HIF-1 dependent mechanism (Martens et al., 2007). Thus, rAIH may increase VEGF and VEGF receptor expression indirectly via HIF-1 effects on the BDNF/TrkB system.

Roles of VEGF and VEGF receptor 2 in rAIH-induced motor plasticity

Although originally known for its roles in angiogenesis and cell permeability, VEGF is also an important trophic factor that is neuroprotective in motor neurons (Storkebaum et al., 2004; Zachary, 2005). Cervical spinal VEGF injections elicit phrenic motor plasticity, similar to BDNF (Dale-Nagle et al., 2011; Dale and Mitchell, 2013). Although 3 × wAIH upregulates VEGF and VEGF receptor 2 expression in phrenic motor neurons, we found no clear evidence that this upregulation affects the magnitude of VEGF-induced phrenic motor facilitation (Dale and Mitchell, 2013). It is not currently known if VEGF and VEGF receptor activation elicit similar motor plasticity in non-respiratory motor neurons. Thus, additional work is needed to determine what role VEGF upregulation following 3 × wAIH plays in intermittent hypoxia-induced plasticity.

Significance

Increased expression of growth/trophic factors that confer neuroprotection and neuroplasticity may have considerable advantage to an animal faced with repetitive exposure to intermittent hypoxia. These responses to “low-dose” intermittent hypoxia (well below those experienced during sleep apnea) may represent a form of pre-conditioning, protecting against future (and more severe) hypoxic insults. The ability of these same growth factors to elicit respiratory and non-respiratory motor plasticity is also of considerable interest, and may present selective advantages by: (1) stabilizing breathing, thereby the recurrence of the hypoxic events; and/or (2) enhancing non-respiratory somatic motor function, enabling movement away from regions of environmental hypoxia, at least in aquatic organisms (Dale et al., 2014).

From another perspective, our findings may have considerable clinical significance, presenting a novel means of increasing endogenous levels of growth/trophic factors that elicit motor plasticity in respiratory and non-respiratory motor neurons. For example, in disorders such as spinal injury and motor neuron disease that cause paralysis, rAIH may be a simple, safe and effective means of restoring lost motor function (Mitchell, 2007). Proof of principle that these concepts apply to non-respiratory (limb) motor systems has already been provided in rodent models (Lovett-Barr et al., 2012) and humans with chronic incomplete spinal injuries (Trumbower et al., 2012; Hayes et al., 2014), as well as in rodent models of ALS (Nichols et al., 2013). “Low-dose” intermittent hypoxia may be used in combination with other therapeutic strategies, including preconditioning for stem cell therapies (Nichols et al., 2013), or traditional physical rehabilitation.

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Abbreviations

AIH	acute intermittent hypoxia
BDNF	brain-derived neurotrophic factor
dAIH	daily AIH
HIF-1α	hypoxia-inducible factor-1α
IH	intermittent hypoxia
NTRK2	Neurotrophic Tyrosine Kinase Receptor 2
pLTF	phrenic long-term facilitation
pMF	phrenic motor facilitation
pTrkB	phosphorylated TrkB
rAIH	repetitive acute intermittent hypoxia
TrkB	tropomyosin receptor kinase B
VEGF	vascular endothelial growth factor.

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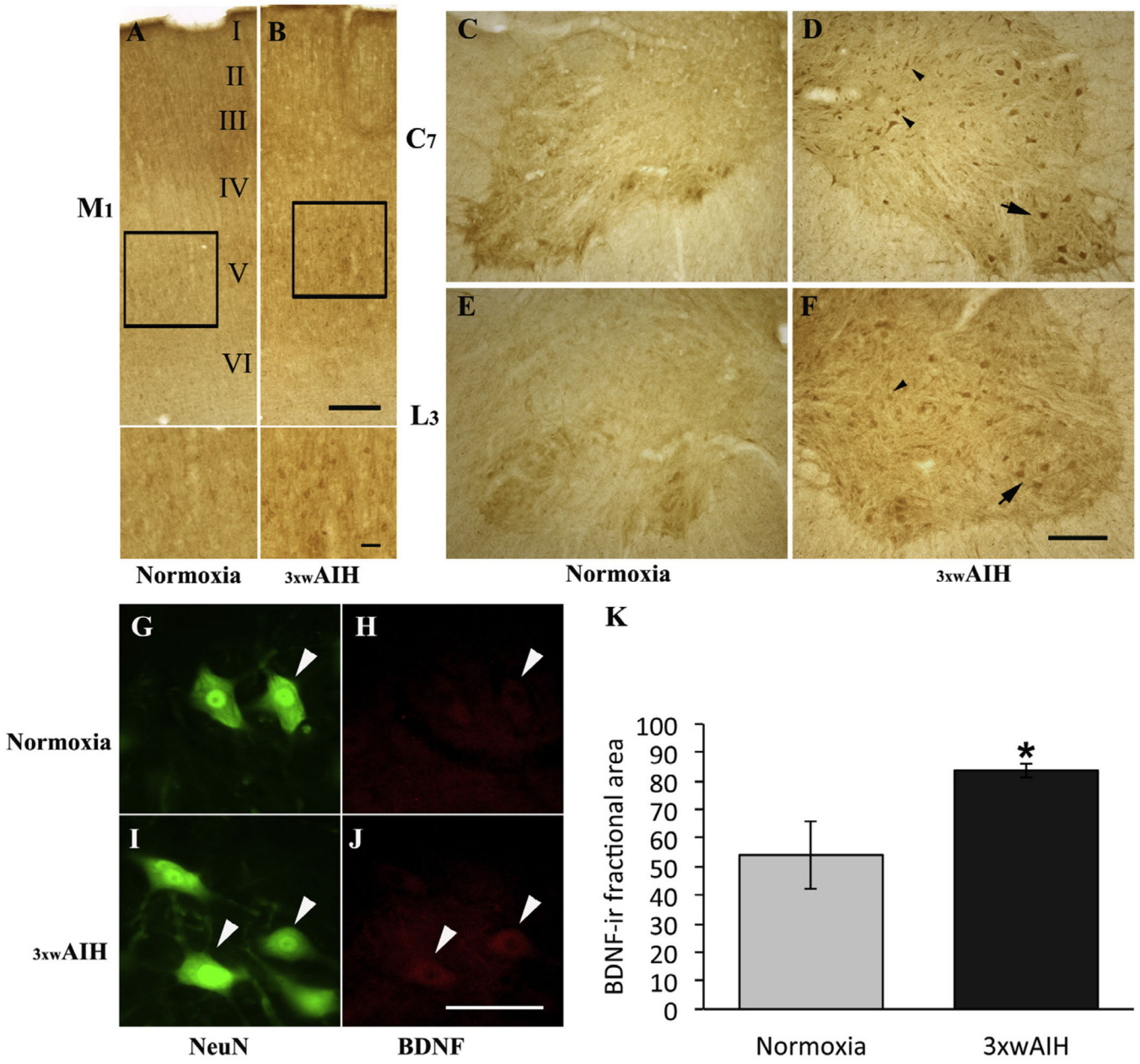

Fig. 1.

Representative images of BDNF immunostaining in primary motor cortex (M_1), cervical (C_7) and lumbar (L_3) ventral horn spinal cord. 3 × wAIH increased BDNF protein expression in upper motor neurons in M_1, especially in layer V (Boxes) (B) versus normoxic control (A). Higher magnification images from layer V are in the bottom of the images. Immunostaining also confirmed BDNF expression in presumptive alpha motor neurons (big arrow) and interneurons (small arrow heads) in cervical (C_7) and lumbar (L_3) segments, and its immunoreactivity is enhanced following 3 × wAIH (C–F). BDNF and NeuN (Neuronal marker)-positive neurons (white arrow head) in L_3 in lamina nine ventral horn, where BDNF immunoreactivity was increased by 3 × wAIH (G–J), as confirmed by densitometry (K).

Scale bars: A, B 400 μm, higher-magnification on figures A, B is 100 μm, C–F is 200 μm, G–J is 100 μm. Data are mean±1 SEM. *$p < 0.05$ versus normoxia.

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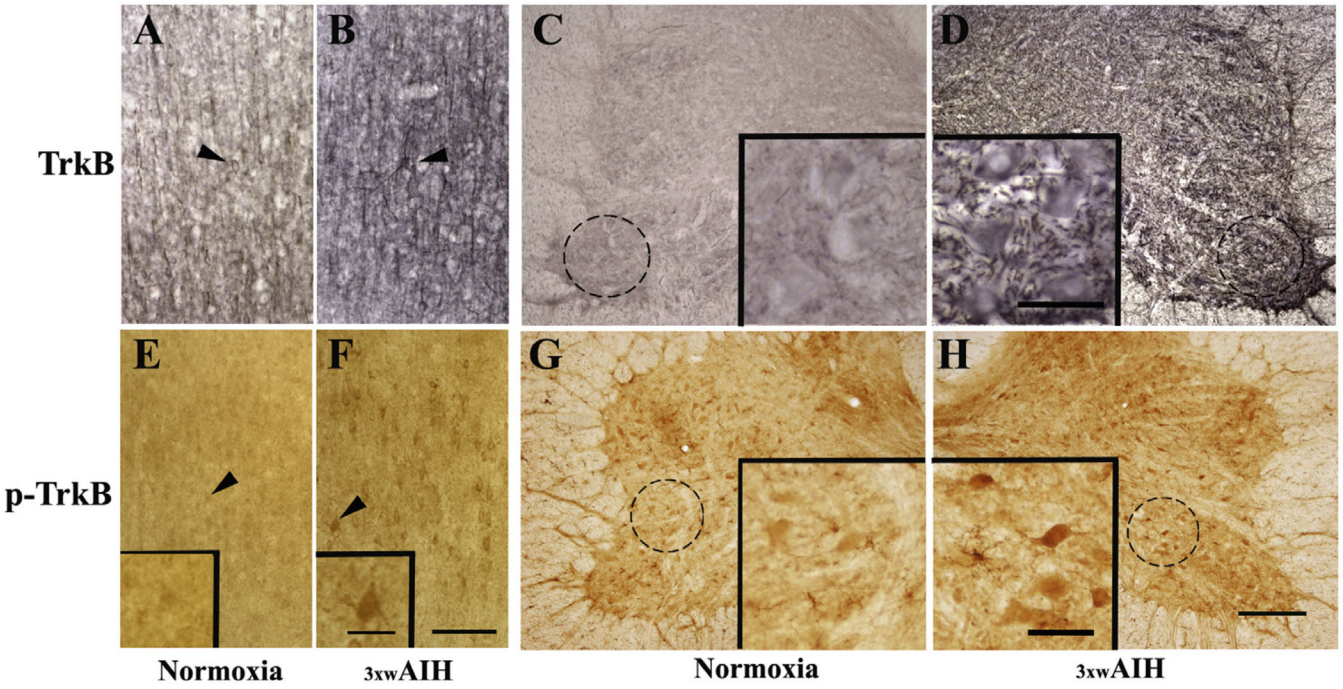

Fig. 2.

Photomicrographs of TrkB (A–D) and phospho-TrkB (E, F) in primary motor cortex (layer V of M_1) and C_7 ventral horn. TrkB immunoreactivity (dark brown) was counterstained with cresyl-violet (blue) to determine the localization of Betz cells in layer V of the primary motor cortex (A, B) and in alpha motoneurons of C_7 ventral horn (C, D). TrkB receptor protein expression and phospho-TrkB were upregulated in motor neurons of M_1 and C_7 following 3 × wAIH treatment (B, D, F, H) compared to normoxic controls (A, C, E, G). Higher-magnification images (100 μm; insets) from C_7 (C, D, G, H) and M_1 (E, F) are shown in enlarged boxes. Scale bars: A–F 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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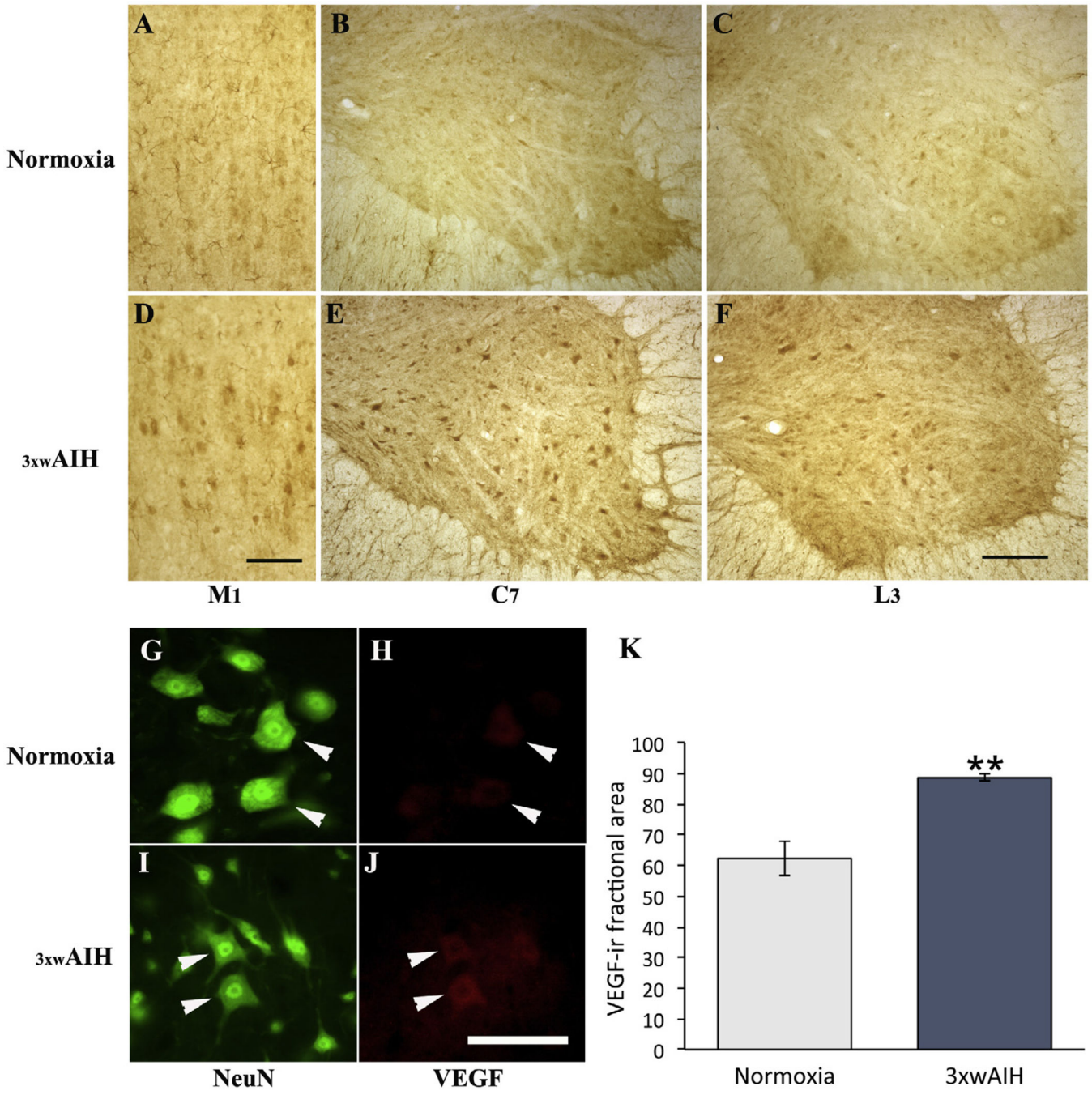

Fig. 3.

Representative images of VEGF immunostaining in layer V of M_1 and cervical (C_7) and lumbar (L_3) ventral horn. VEGF immunostaining is increased in M_1, C_7 and L_3 ventral horn after 3 × wAIH treatment (D–F) versus normoxic control (A–C). VEGF and NeuN-positive neurons (white arrow head), where VEGF immunoreactivity was increased following 3 × wAIH (G–J), as confirmed by densitometry (K). Scale bar for A–F: 200 μm; G–J is 100 μm. Data are mean±1 SEM. $^{**}p < 0.001$ versus normoxia.

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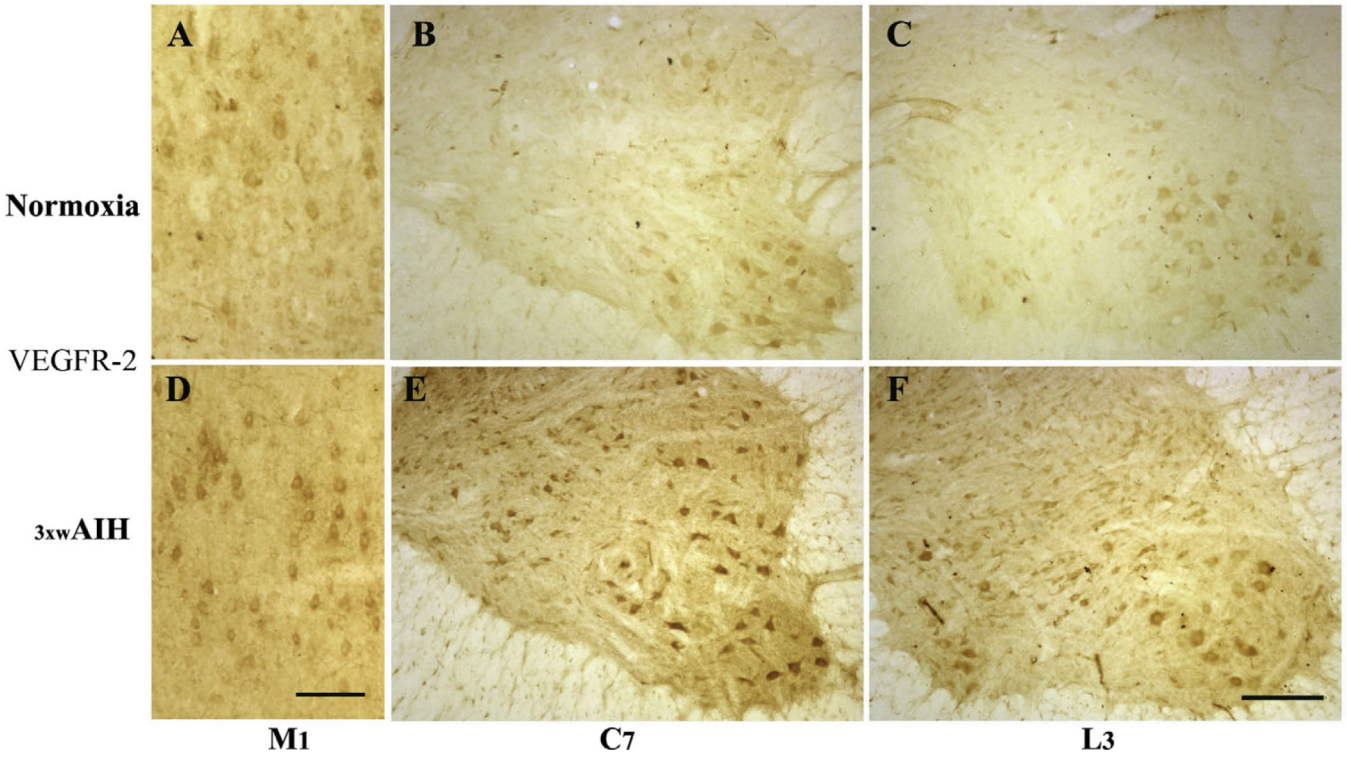

Fig. 4.

Photomicrographs of VEGF receptor-2 (VEGFR-2/KDR) immunostaining in layer V of M_1 and in C_7 and L_3 ventral horn. 3 × wAIH increased VEGF receptor protein expression in layer V of primary motor cortex (M_1) and in alpha-motor neurons of C_7 and L_3 ventral horn (D–F) versus normoxic control (A–C). Scale bars for A–F: 200 μm.

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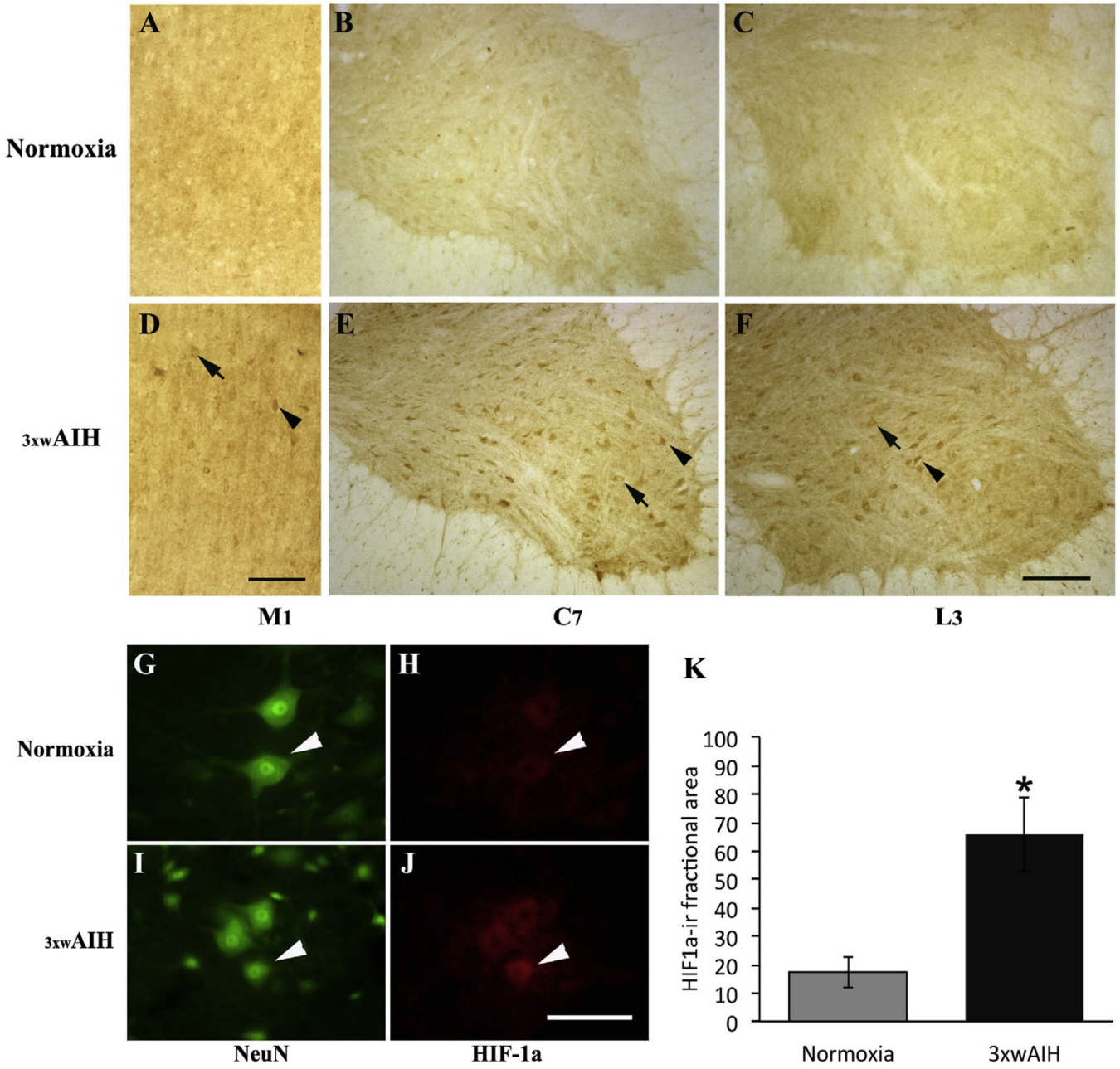

Fig. 5.

Photomicrographs of HIF-1α immunostaining in layer V of cortical M_1 and in C_7 and L_3 ventral horns. Repetitive AIH increased HIF-1α protein expression in motor neurons in layer V of the primary motor cortex (M_1) and in alpha motor neurons of C_7 and L_3 ventral horns (D–F) versus normoxic control (A–C). HIF-1α and NeuN-positive neurons (white arrow head), where HIF-1α immunoreactivity was increased following 3 × wAIH (G–J), as confirmed by densitometry (K). Scale bars for A–F: 200 μm; G–J is 100 μm. Data are mean ±1 SEM. *$p < 0.05$ versus normoxia.