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Spinal vascular endothelial growth factor (VEGF) and erythropoietin (EPO) induced phrenic motor facilitation after repetitive acute intermittent hypoxia

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Abstract

Vascular endothelial growth factor (VEGF) and erythropoietin (EPO) exert neurotrophic and neuroprotective effects in the CNS. We recently demonstrated that VEGF, EPO and their receptors (VEGF-R2, EPO-R) are expressed in phrenic motor neurons, and that cervical spinal VEGF-R2 and EPO-R activation elicit long-lasting phrenic motor facilitation (pMF). Since VEGF, VEGF-R, EPO, and EPO-R are hypoxia-regulated genes, and repetitive exposure to acute intermittent hypoxia (rAIH) up-regulates these molecules in phrenic motor neurons, we tested the hypothesis that 4 weeks of rAIH (10 episodes per day, 3 days per week) enhances VEGF- or EPO- induced pMF. We confirm that cervical spinal VEGF and EPO injections elicit pMF. However, neither VEGF- nor EPO-induced pMF was affected by rAIH pre-conditioning (4 wks). Although our data confirm that spinal VEGF and EPO may play an important role in respiratory plasticity, we provide no evidence that rAIH amplifies their impact. Further experiments with more robust protocols are warranted.

Keywords

VEGF; EPO; phrenic motor facilitation; intermittent hypoxia; respiratory plasticity; spinal cord

1. INTRODUCTION

The respiratory control network must adapt to short and long-term perturbations. Feedback (e.g. chemoreceptor feedback), feed-forward (e.g. exercise) and adaptive control strategies (i.e. plasticity) all contribute to maintaining homeostasis. The role of respiratory plasticity has only recently been appreciated for its contributions to the control of breathing in long time domains (Mitchell and Johnson, 2003).

Growth/trophic factors play key roles in multiple forms of respiratory plasticity (Mitchell and Johnson, 2003; Golder, 2008; Spedding and Gressens, 2008). For example, new synthesis of brain derived neurotrophic factor (BDNF) is necessary for phrenic long-term facilitation (pLTF) induced by acute intermittent hypoxia (Baker-Herman et al., 2004).

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Indeed, cervical spinal BDNF injections are sufficient to elicit long-lasting phrenic motor facilitation (pMF; Baker-Herman et al., 2004).

We have recently come to understand that multiple distinct cell-signaling pathways associated with different growth/trophic factors give rise to distinct forms of pMF with a similar appearance (Dale-Nagle et al., 2010a). For example, the growth/trophic factors vascular endothelial growth factor (VEGF; Forsythe et al., 1996; Liu et al., 1995) and erythropoietin (EPO; Wang et al., 1995; Wang and Semenza, 1995) both elicit pMF when injected into the cervical spinal cord (Dale-Nagle et al., 2011, Dale et al., 2012). These growth factors (and their cognate receptors) are hypoxia sensitive, regulated by the transcription factors HIF-1 and (possibly) HIF-2 (Yeo et al., 2008). Although VEGF was originally known as an angiogenic and cell-permeabilizing factor (Connolly et al., 1989; Senger et al., 1986), and EPO was discovered for its hematopoietic properties (Bert, 1882; Bert 1878; Jourdanet, 1875; Stohlman, 1959), both growth factors are now known to be expressed in neurons of the central nervous system where they exert neurotrophic and neuroprotective effects (Bernaudin et al., 1999; Brines et al., 2000; Digicaylioglu et al., 1995; Gora-Kupilas and Josko, 2005; Marti and Risau 1998; Morishita et al., 1997; Storkebaum et al., 2004; Zachary, 2005). VEGF and its high affinity receptor, VEGFR-2 (Dale-Nagle et al., 2011), as well as EPO and its high affinity receptor, EPO-R (Dale et al., 2012), are expressed in phrenic motor neurons. Further, cervical spinal VEGFR-2 and EPO-R activation elicit pMF by ERK MAP kinase and Akt-dependent mechanisms (Dale-Nagle et al., 2011; Dale et al., 2012).

Similar to other neural systems, the respiratory system exhibits meta-plasticity, or “plastic plasticity” (Byrne et al., 1997; Mitchell and Johnson, 2003;). For example, chronic intermittent hypoxia (CIH; 8-12 hours per day for days to weeks) enhances AIH-induced pLTF (Ling et al., 2001; McGuire et al., 2003) and reveals a unique form of sensory long-term facilitation in carotid chemo-afferent neurons, an effect not expressed in naïve rats (Peng and Prabhakar, 2004; Peng et al., 2003). Such metaplasticity may arise via increased carotid chemoreceptor activity (Peng et al., 2003) or amplification of CNS integration of chemoafferent neurons (Fuller et al., 2003; Ling et al., 2001).

Unfortunately, in addition to eliciting plasticity, CIH also causes adverse effects, including hypertension (Fletcher et al., 1992), impaired baroreflex control of heart rate (Gu et al., 2007), neuro-cognitive deficits (Row, 2007) and metabolic syndrome (Tasali and Ip, 2008). In an attempt to harness the intrinsic capacity for intermittent hypoxia induced respiratory plasticity without adverse effects (Mitchell, 2007), we developed more modest protocols of repetitive acute intermittent hypoxia (AIH), such as daily AIH (10, 5 min episodes; 7 days; Wilkerson and Mitchell, 2009; Lovett-Barr et al., 2012) or AIH three times per week (3xwAIH) for 10 weeks (Satriotomo et al., 2012).

VEGF (Forsythe et al., 1996; Liu et al., 1995; Satriotomo et al., 2012), VEGFR-2 (Elvert et al., 2003; Marti et al., 2000; Satriotomo et al., 2012; Stowe et al., 2008), and EPO (Wang et al., 1995; Wang and Semenza, 1995) are hypoxia-sensitive genes. EPO-R may also be hypoxia sensitive (Giatromanolaki et al., 2009; Lam et al., 2009; Shein et al., 2005; Theus et al., 2008), though there are conflicting literature reports (Alnaeeli et al., 2012; Digicaylioglu et al., 1995). Since these molecules are all up-regulated by 3xwAIH (10 wks) in phrenic motor neurons (Satriotomo et al., 2012; Dale, Satriotomo and Mitchell, unpublished observations), we hypothesized that 3xwAIH would enhance pMF induced by spinal VEGF and/or EPO receptor activation.

2. METHODS

2.1 Experimental Animals

Adult male Sprague-Dawley rats (2-5 months old; colony 218A, Harlan; Indianapolis, IN) were used for all experiments. Rats were double housed with food and water *ad libitum* in a light and temperature controlled environment (12h light/dark cycle, daily humidity and temperature monitoring). All protocols were approved by The Institutional Animal Care and Use Committee at the University of Wisconsin.

2.2 Repetitive acute intermittent hypoxia (3xwAIH) protocol

Rats were exposed to intermittent hypoxia in Plexiglas chambers (1 rat per chamber; 12in $\times$ 4.5in $\times$ 4.5in) flushed with gases at 4L/min to assure adequate response time and to minimize CO₂ accumulation. Rats were placed in the chambers three times per week for 4 weeks, and allowed to acclimate under normal oxygen conditions (FIO₂ = 0.21) for 20 min. The rats were then exposed to AIH, which consisted of 10, 5-min hypoxic episodes (FIO₂ = 0.11) interspersed with 5-min normoxic intervals. Gas composition was controlled by mixing O₂ and N₂ *via* mass-flow controllers and a custom-made computer control system. Chamber oxygen levels were continuously monitored (S3A Oxygen Analyzer, Applied Electrochemistry, Inc.; Sunnyvale, CA). Intended oxygen levels were achieved within 60 $\pm$ 10s of switching gases. Control rats received the same treatment, but without hypoxia.

2.3 Neurophysiology experiments

Anesthesia was induced in a closed chamber and maintained for a short duration via nose cone (3.5% isoflurane in 50% O₂). Rats were tracheotomized and pump ventilated for the duration of experiments (2.5 ml, Rodent Ventilator, model 683; Harvard Apparatus; South Natick, MA, USA). After surgery, rats were slowly converted to urethane anesthesia (1.8 g/kg) via a tail vein catheter. In order to eliminate pulmonary stretch-receptor feedback and entrainment of respiratory activity with the ventilator, rats were bilaterally vagotomized. The right femoral artery was catheterized to allow blood gas analysis throughout the experiment. After C₂ laminectomy and durotomy, an intrathecal silicone catheter (2 French; Access Technologies, Skokie, IL) was placed on the dorsal surface of the C₄ spinal segment to enable drug delivery. The left phrenic nerve was dorsally isolated, cut distally, de-sheathed, submerged in mineral oil and placed on bipolar silver electrodes. After ensuring adequate anesthesia (no purposeful movements or cardio-respiratory responses to toe pinch) rats were paralyzed with pancuronium bromide (2.5 mg/kg, i.v.). Body temperature was measured with a rectal probe (Traceable™, Fisher Scientific; Pittsburgh, PA, USA) and maintained within $\pm$ 0.3°C of baseline via a custom temperature-controlled surgical table. End-tidal CO₂ was obtained via a flow-through capnograph with sufficient response time to measure end-tidal CO₂ in rats (Capnogard, Novamatrix; Wallingford, CT; USA). Blood samples were collected into a heparinized plastic capillary tube (Radiometer Medical Aps, Copenhagen, Denmark; 250 $\times$ 125 μ l cut in half) via the femoral artery catheter to monitor arterial blood gases (PaO₂ and PaCO₂), pH and base excess (ABL 800Flex, Radiometer; Copenhagen, Denmark). Intravenous fluid infusions (1:1.7:5 by volume of NaHCO₃/Lactated Ringer s/Hetastarch; 1.5-2.2 ml/hr) were initiated to maintain blood pressure and acid/base balance.

Phrenic nerve activity was amplified ($\times$ 10,000: A-M Systems, Everett, WA), bandpass filtered (100Hz to 10kHz), rectified, and processed with a moving averager (CWE 821 filter; Paynter, Ardmore, PA; time constant 50 ms). The digitized, integrated signal was analyzed using a WINDAQ data-acquisition system (DATAQ Instruments, Akron, OH). Peak integrated phrenic burst frequency and amplitude, and mean arterial blood pressure (MAP) were analyzed in 60 sec bins directly before blood samples were taken. Data were included only if PaCO₂ was maintained within $\pm$ 1.5 mmHg of baseline, base excess was within $\pm$ 5

mEq/L of baseline, and MAP decreased less than 30 mmHg from baseline to the end of an experiment. Frequency data are reported as bursts per minute and nerve burst amplitudes are expressed as a percentage change from baseline.

The apneic threshold (the point at which phrenic bursting ceases) was determined at least one hour after conversion to urethane anesthesia by reducing inspired CO₂ and/or increasing ventilator frequency. Baseline was established by maintaining end-tidal PCO₂ within 1-2 mmHg above the recruitment threshold, the PaCO₂ at which respiratory activity resumes (Bach and Mitchell, 1996). After 25 min of stable nerve recordings, blood was drawn to establish a point of comparison for subsequent blood gas measurements. Rats then received one of three intrathecal treatments (outlined below); arterial blood samples were taken at 15, 30, 60 and 90 min after injections. All electrophysiological data were analyzed with a repeated measures, 2-way ANOVA (SigmaStat 2.03).

2.4 Neurophysiology protocols and drug administration

Six protocols were used in the electrophysiological study: 1) 3xwAIH+10µl of VEGF (recombinant human; 100ng; R&D Systems; Minneapolis, MN) dissolved in 0.1% bovine serum albumin (BSA) and artificial cerebrospinal fluid (aCSF; 120nM NaCl, 3mM KCl, 2mM CaCl₂, 2mM MgCl₂, 23 mM NaHCO₃, 10mM glucose bubbled with CO₂); 2) 3xwAIH +10µl of Erythropoietin (recombinant human; tissue culture grade; 20 international units; R&D Systems; Minneapolis, MN) dissolved in 0.1% BSA and aCSF; 3) 3xwAIH+10µl vehicle control (0.1% BSA in aCSF); 4) Normoxia+VEGF; 5) Normoxia+EPO and 6) Normoxia + vehicle. All intrathecal drug injections were made over two min. To prevent VEGF or EPO binding, all syringes, vials and catheters were incubated beforehand with vehicle. The optimal VEGF and EPO doses were chosen based on previous studies (Dale-Nagle et al., 2011; Dale et al., 2012).

3. RESULTS

3.1 Regulation of physiological variables

Physiological variables measured during protocols are shown in Table 1. There were no differences between groups in body temperature, pH, PaCO₂ or PaO₂ values ($p>0.05$). PaCO₂ was maintained within ± 1.4 mmHg and PaO₂ remained above 250 mmHg; thus, any changes in phrenic nerve activity cannot be attributed to changes in chemoreceptor feedback.

As in other studies using this experimental preparation (Baker-Herman and Mitchell, 2008), mean arterial pressures decrease throughout a protocol. However, rats were excluded from analysis if the decrease was more than 30 mmHg from baseline values. Regardless, arterial pressure decreased over time in this study (Table 1; $p<0.05$), but to a similar degree across groups. Similarly, there was a small but significant decrease in base excess over time in 2 groups (Table 1; $p<0.05$); although not significant in other groups, this same trend was evident.

3.2 3xwAIH does *not* enhance VEGF-induced phrenic motor facilitation

Representative traces of phrenic nerve activity are shown in Figure 1A after: 1) 3xwAIH followed by intrathecal VEGF (10µl, 100ng); 2) Normoxia followed by intrathecal VEGF (10µl, 100ng); 3) 3xwAIH followed by intrathecal vehicle (10µl, aCSF+0.01% BSA); and 4) Sham Normoxia followed by intrathecal vehicle (10µl, aCSF+0.01% BSA). The VEGF dose chosen restricts protein delivery to spinal segments containing the phrenic motor nucleus, minimizing unwanted brainstem migration. This was confirmed by the absence of VEGF-induced facilitation in XII motor output or nerve burst frequency (dose from Dale-Nagle et.

al., 2011). Mean responses to 100ng VEGF are shown in Figure 1B. In rats pre-treated with 4 weeks of 3xwAIH, VEGF significantly increased phrenic burst amplitude from baseline at 30 min, and all time points thereafter (all $p < 0.003$; $n = 5$), confirming pMF. At the 60 min post-injection, increases in phrenic burst amplitude in rats receiving 3xwAIH+VEGF were significantly greater than vehicle control rats (i.e. 3xwAIH+aCSF, $n = 6$; Nx+aCSF, $n = 5$; all $p < 0.04$). At the 60 min time point, rats pre-treated with 4 weeks of normoxia also demonstrated increased phrenic motor output after intrathecal VEGF (10 μ l, 100ng; $p < 0.003$), confirming VEGF-induced pMF; pMF in 3xwAIH + VEGF rats was greater than in Nx+aCSF rats ($p < 0.04$), but not the 3xwAIH+aCSF control group ($p > 0.05$). There were no significant differences within VEGF treated rats (i.e. 3xwAIH vs. Nx groups; all $p > 0.05$), suggesting that 3xwAIH had no significant effect on VEGF-induced pMF. Vehicle control rats did not exhibit significant changes in phrenic burst amplitude at any time (all $p > 0.05$).

3.3 Hypoglossal (XII) activity and burst frequency are unaffected by VEGF

A representative trace of a XII nerve recording is shown in Figure 2A. Mean values for XII activity in all treatment groups are shown in Figure 2B. Neither intrathecal VEGF nor aCSF elicited XII motor facilitation after pre-treatment with either 3xwAIH or normoxia. There were no significant differences in XII motor output vs. baseline within treatment groups (all $p > 0.05$), indicating that brainstem VEGF migration was minimal or absent. There were no significant differences between or within groups in nerve burst frequency (all $p > 0.05$; Figure 2C), consistent with the interpretation that drug migration to brainstem respiratory centers was minimal or absent.

3.4 3xwAIH does *not* enhance EPO-induced phrenic motor facilitation

Representative traces of phrenic nerve activity are shown in Figure 3A, including: 1) 3xwAIH followed by intrathecal EPO (10 μ l, 20IU); 2) Normoxia followed by intrathecal EPO (10 μ l, 20IU); 3) 3xwAIH followed by intrathecal vehicle (10 μ l, aCSF+0.01% BSA); or 4) Normoxia followed by intrathecal vehicle (10 μ l, aCSF+0.01% BSA). The final EPO dose was chosen to restrict drug distribution to cervical spinal regions encompassing the phrenic motor nucleus while avoiding rostral (brainstem) migration (i.e. no facilitation in XII motor output or nerve burst frequency; based on Dale et. al., 2012). Mean responses to 20IU are shown in Figure 3B. In rats pre-treated with 4 weeks of 3xwAIH, phrenic burst amplitude after intrathecal EPO significantly increased from baseline at 30 min post-injection, and at all time points thereafter ($n = 5$; all $p < 0.02$), thus confirming EPO-induced pMF. At the 60 min time point, 3xwAIH+EPO rats exhibited increased phrenic motor output versus vehicle controls (i.e. 3xwAIH+aCSF, $n = 6$; Nx+aCSF, $n = 5$; all $p < 0.02$). Normoxic rats treated with EPO also exhibited increases in phrenic motor output that were significantly greater than baseline at 30 min post-injection and beyond ($n = 5$; all $p < 0.02$). At the 60 min time point, the Nx+EPO group significantly increased phrenic burst amplitude vs. the Nx+aCSF and 3xwAIH+aCSF control groups (all $p < 0.001$). There were no significant differences within EPO treated rats (i.e. 3xwAIH vs. Nx; all $p > 0.05$), thus demonstrating that 3xwAIH had no significant effect on EPO-induced pMF in this study. Vehicle control rats did not exhibit significant changes in phrenic burst amplitude at any time (all $p > 0.05$).

3.5 Hypoglossal (XII) burst amplitude and frequency unaffected by cervical EPO

Mean values for XII activity in all treatment groups are shown in Figure 4A. Neither pre-treatment (i.e. 3xwAIH and Nx) nor intrathecal EPO or aCSF injection elicited XII motor facilitation. There were no significant differences in XII motor output vs. baseline or within treatment groups (all $p > 0.05$), suggesting minimal brainstem drug migration. Nerve burst frequency was also unaffected by either pre-treatment or injection (Figure 4B). There were no significant differences between or within groups in XII burst frequency (all $p > 0.05$), confirming that brainstem drug migration was minimal or absent.

3.6 3xwAIH does *not* change baseline activity or the apneic threshold

To determine if baseline respiratory drive was affected by 3xwAIH, we analyzed the amplitude of integrated nerve bursts (volts) during baseline conditions. Mean values (both phrenic and XII) are shown in Figure 5A. There was no significant difference in voltage between rats treated with 3xwAIH (n=18) vs. normoxia treated rats (n=16; $p>0.05$). There was no significant difference in integrated XII voltage between rats treated with 3xwAIH (n=18) vs. normoxia treated rats (n=16; $p>0.05$). In agreement, there were no significant differences in baseline nerve burst frequency comparing rats pre-treated with 3xwAIH (n=18) vs. Nx (n=16; $p>0.05$; Figure 5B). Finally, there were no significant differences in the CO₂ apneic threshold between 3xwAIH (n=18) or Nx rats (n=16; $p>0.05$; Figure 5C). Collectively, these data provide no evidence for differences in baseline respiratory drive after 3xwAIH.

4. DISCUSSION

Here we confirmed that cervical spinal VEGF and EPO receptor activation elicit long-lasting phrenic motor facilitation in rats. However, although 3xwAIH (10 weeks) up-regulates VEGF and VEGFR-2 protein levels in phrenic motor neurons (Satriotomo et al., 2012), and EPO/EPO-R are hypoxia-regulated genes (with preliminary evidence suggesting upregulation in phrenic motor neurons), 4 weeks of 3xwAIH had no significant effect on the magnitude of VEGF or EPO-induced pMF. Conclusions are constrained by several considerations. For example, it is possible that 4 weeks of 3xwAIH was not sufficient to adequately increase VEGF and/or EPO receptor expression in phrenic motor neurons. Alternately, the doses of intrathecal VEGF and EPO used may have elicited near maximal levels of pMF, leaving little room for further enhancement. These possibilities remain to be explored.

4.1 Growth factors and phrenic motor facilitation

It has recently come to light that multiple, distinct cellular mechanisms give rise to pMF (Dale-Nagle et al., 2010a). Growth/trophic factors or their receptors appear to play major roles in these distinct mechanisms (Dale-Nagle et al., 2010a; Golder, 2008; Mitchell and Johnson, 2003; Spedding and Gressens, 2008), including brain derived neurotrophic factor (BDNF; Baker-Herman et al., 2004), TrkB isoforms (Golder et al., 2008), VEGF (Dale-Nagle et al., 2011) and EPO (Dale et al., 2012). Both VEGF and EPO (and their receptors) are expressed in phrenic motor neurons, and acute intrathecal administration of these growth factors elicits pMF through spinal mechanisms that require ERK MAP kinase and Akt activation (Dale-Nagle et al., 2011; Dale et al., 2012).

4.2 HIF-1 regulated transcription of VEGF and EPO

VEGF and EPO are both regulated by Hypoxia Inducible Factor 1 and/or 2 (Semenza, 2009; Wang et al., 1995; Wang and Semenza, 1993; Yeo et al., 2008). This transcription factor is often regarded as the ‘master regulator’ of oxygen homeostasis since: 1) it is activated by low oxygen tension (Semenza, 2009), and 2) its gene products are largely involved in aiding oxygen delivery (i.e. new vessel growth, increased red blood cell density, etc.).

Ten weeks of 3xwAIH upregulates VEGF and VEGFR-2 in putative phrenic motor neurons (Satriotomo et al., 2012), and we have observed similar EPO and EPO-R upregulation (Dale, Satriotomo and Mitchell, unpublished observations). Thus, VEGF and/or EPO may contribute to forms of respiratory motor plasticity induced by repetitive or prolonged intermittent hypoxia (Lovett-Barr et al., 2012; Mahamed and Mitchell, 2007; Mitchell and Johnson, 2003; Wilkerson and Mitchell, 2009). pMF elicited by VEGF and EPO may

represent another example of HIF-dependent protection of tissue oxygenation by ensuring adequate ventilation.

4.3 VEGF and the neural control of breathing

The role for VEGF in the neural control of breathing has only recently been established (Dale-Nagle et al., 2011). Though structural alterations to the carotid bodies after chronic sustained hypoxia have been attributed, in part, to activation of carotid body VEGF receptors (Chen et al., 2003), there are currently no direct links between VEGF and functional plasticity after hypoxia. There is, however, evidence that VEGF plays a role in spinal respiratory motor plasticity. In rats, cervical spinal VEGF elicits: 1) increased ERK and Akt phosphorylation in identified phrenic motor neurons; and 2) a long-lasting pMF that is both Akt and ERK-dependent (Dale-Nagle et al., 2011). Although we confirm these findings here, and 3xwAIH for ten weeks increased VEGF receptor expression in the phrenic motor nucleus (Satriotomo et al., 2012), four weeks of 3xwAIH pre-treatment had no effect on VEGF or EPO induced pMF. Perhaps 4 versus 10 weeks of 3xwAIH was not sufficient to increase VEGF or EPO receptor expression adequately. Alternatively, since the selected doses of VEGF and EPO elicited larger pMF relative to our previous study, enhanced pMF may not be expressed due to a “ceiling effect” caused by reaching maximal phrenic motor output. Finally, the possibility remains that 3xwAIH induces as yet undetected forms of respiratory motor plasticity associated with VEGF and/or EPO.

4.4 EPO and the neural control of breathing

EPO plays important roles in the neural control of breathing, both at the levels of the brainstem and the peripheral chemoreceptors. For example, in a mouse line over-expressing EPO exclusively in brain (Tg21), severe acute (6% O₂) and chronic hypoxic exposures (3 days at 10% O₂) elicited greater ventilatory responses versus wild-type mice (Soliz et al., 2005). After chemo-denervation, both Tg21 and wild-type mice were exposed to severe hypoxia. Tg21 mice were able to sustain ventilation while the wild-type mice experienced life-threatening apneas, demonstrating that EPO modulates breathing during hypoxia (Soliz et al., 2005). Immunohistochemical analyses revealed EPO-R expression in the pre-Bötzinger complex and the nucleus tractus solitarius (Soliz et al., 2005), suggesting roles in rhythm generation and chemoafferent integration. Downregulation of soluble EPO-R in brain is necessary for ventilatory acclimatization to chronic hypoxia, demonstrating a role in at least one form of respiratory plasticity (Soliz et al., 2007). Collectively, there is substantial evidence that EPO modulates breathing during hypoxia via peripheral and central neural mechanisms (Soliz et al., 2007; 2005).

Here we confirm that EPO is also involved in spinal respiratory motor plasticity since cervical spinal EPO elicits robust pMF, an effect that lasts > 90 min (Dale et al., 2012). EPO-induced pMF is also ERK MAP kinase and Akt-dependent, similar to VEGF-induced pMF (Dale-Nagle et al., 2011). Although our current experiments do not confirm a direct connection between intermittent hypoxia induced respiratory plasticity and EPO-induced pMF, the possibility of such a connection warrants further exploration.

4.5 Possible significance

By exploring mechanisms of respiratory plasticity, we may eventually harness spinal plasticity to treat cases of ventilatory compromise, such as following cervical spinal cord injury (Dale-Nagle et al., 2010b; Lovett-Barr et al., 2012; Mitchell, 2007; Vinit and Kastner, 2009; Vinit et al., 2009) or amyotrophic lateral sclerosis (Storkebaum et al., 2004). Although the present studies were aimed at enhancing (already) robust pMF induced by cervical spinal VEGF or EPO, no such effect was detected, even though neurochemical plasticity in these growth factors and their receptors has been observed in phrenic motor neurons following

longer repetitive AIH protocols (Dale et al., 2012; Dale-Nagle et al., 2011; Satriotomo et al., 2012). Further studies may elucidate the roles of VEGF and EPO in respiratory neuroplasticity during or following hypoxia.

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References

- Alnaeli M, Wang L, Piknova B, Rogers H, Li X, Noguchi CT. Erythropoietin in brain development and beyond. *Anat Res Int*. 2012 Epub 2012 Feb 26.
- Bach KB, Mitchell GS. Hypoxia-induced long-term facilitation of respiratory activity is serotonin dependent. *Respir Physiol*. 1996; 104(2-3):251–60. [PubMed: 8893371]
- Baker-Herman TL, Fuller DD, Bavis RW, Zabka AG, Golder FJ, Doperalski NJ, Johnson RA, Watters JJ, Mitchell GS. BDNF is necessary and sufficient for spinal respiratory plasticity following intermittent hypoxia. *Nat Neurosci*. 2004; 7:48–55. [PubMed: 14699417]
- Baker-Herman TL, Mitchell GS. Determinants of frequency long-term facilitation following acute intermittent hypoxia in vagotomized rats. *Respir Physiol Neurobiol*. 2008; 162:8–17. [PubMed: 18450525]
- Bernaudin M, Marti HH, Roussel S, Divoux D, Nouvelot A, MacKenzie ET, Petit E. A potential role for erythropoietin in focal permanent cerebral ischemia in mice. *J Cereb Blood Flow Metab*. 1999; 19:643–51. [PubMed: 10366194]
- Bert, P. La Pression Barométrique: Recherches de Physiologie Expérimentale. Paris: Masson; 1878.
- Bert P. Sur la richesse en hemoglobine du sang des animaux vivant sur les hauts lieux. *CR Acad Sci Paris*. 1882; 94:805–807.
- Brines ML, Ghezzi P, Keenan S, Agnello D, de Lanoerolle NC, Cerami C, Itri LM, Cerami A. Erythropoietin crosses the bloodbrain barrier to protect against experimental brain injury. *Proc Natl Acad Sci U S A*. 2000; 97:10526–31. [PubMed: 10984541]
- Chen J, Dinger B, Jyung R, Stensaas L, Fidone S. Altered expression of vascular endothelial growth factor and FLK-1 receptor in chronically hypoxic rat carotid body. *Adv Exp Med Biol*. 2003; 536:583–91. [PubMed: 14635716]
- Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J Clin Invest*. 1989; 84:1470–1478. [PubMed: 2478587]
- Byrne JH. Plastic plasticity. *Nature*. 1997; 389:791–792. [PubMed: 9349805]
- Dale EA, Satriotomo I, Mitchell GS. Cervical spinal erythropoietin induces phrenic motor facilitation via ERK and Akt signaling. *J Neurosci*. 2012; 32(17):5973–83. [PubMed: 22539857]
- Dale-Nagle EA, Satriotomo I, Mitchell GS. Spinal vascular endothelial growth factor induces phrenic motor facilitation via ERK and Akt signaling. *J Neurosci*. 2011; 31(21):7682–90. [PubMed: 21613481]
- Dale-Nagle EA, Hoffman MS, Macfarlane PM, Mitchell GS. Multiple pathways to long-lasting phrenic motor facilitation. *Adv Exp Med Biol*. 2010a; 669:225–30. [PubMed: 20217354]
- Dale-Nagle EA, Hoffman MS, MacFarlane PM, Satriotomo I, Lovett-Barr MR, Vinit S, Mitchell GS. Spinal plasticity following intermittent hypoxia: Implications for Spinal Injury. *Ann NY Acad Sci*. 2010b; 1198:252–9. [PubMed: 20536940]
- Digicaylioglu M, Bichet S, Marti HH, Wenger RH, Rivas LA, Bauer C, Gassmann M. Localization of specific erythropoietin binding sites in defined areas of the mouse brain. *Proc Natl Acad Sci U S A*. 1995; 92:3717–20. [PubMed: 7731971]
- Elvert G, Kappel A, Heidenreich R, Englmeier U, Lanz S, Acker T, Rauter M, Plate K, Sieweke M, Breier G, Flamme I. Cooperative interaction of hypoxia-inducible factor-2alpha (HIF-2alpha) and

- Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1). *J Biol Chem.* 2003; 278(9):7520–30. [PubMed: 12464608]
- Fletcher EC, Lesske J, Qian W, et al. Repetitive, episodic hypoxia causes diurnal elevation of blood pressure in rats. *Hypertension.* 1992; 19:555–561. [PubMed: 1592450]
- Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol.* 1996; 16:L4604–4613.
- Fuller DD, Johnson SM, Olson EB Jr, Mitchell GS. Synaptic pathways to phrenic motoneurons are enhanced by chronic intermittent hypoxia after cervical spinal injury. *J Neurosci.* 2003; 23(7): 2993–3000. [PubMed: 12684486]
- Giatromanolaki A, Fiska A, Pitsiava D, Kartalis G, Koukourakis MI, Sivridis E. Erythropoietin receptors in endometrial carcinoma as related to HIF1{alpha} and VEGF expression. *In Vivo.* 2009; 23(5):699–703. [PubMed: 19779103]
- Golder FJ. Receptor tyrosine kinases and respiratory motor plasticity. *Respir Physiol Neurobiol.* 2008; 164:242–251. [PubMed: 18634908]
- Gora-Kupilas K, Josko J. The neuroprotective function of vascular endothelial growth factor (VEGF). *Folia Neuropathol.* 2005; 43:31–39. [PubMed: 15827888]
- Gu H, Lin M, Liu J, et al. Selective impairment of central mediation of baroreflex in anesthetized young adult Fischer 344 rats after chronic intermittent hypoxia. *Am J Physiol Heart Circ Physiol.* 2007; 293:H2809–18. [PubMed: 17693540]
- Jourdanet, D. *Influence de la Pression de L'air sur la Vie de L'homme.* Paris: Masson; 1875.
- Lam SY, Tipoe GL, Fung ML. Upregulation of erythropoietin and its receptor expression in the rat carotid body during chronic and intermittent hypoxia. *Adv Exp Med Biol.* 2009; 648:207–14. [PubMed: 19536483]
- Ling L, Fuller DD, Bach KB, Kinkead R, Olson EB Jr, Mitchell GS. Chronic intermittent hypoxia elicits serotonin-dependent plasticity in the central neural control of breathing. *J Neurosci.* 2001; 21(14):5381–8. [PubMed: 11438615]
- Liu Y, Cox SR, Morita T, Kourembanas S. Hypoxia regulates vascular endothelial factor gene expression in endothelial cells. Identification of a 5' enhancer. *Circ Res.* 1995; 77(3):638–43. [PubMed: 7641334]
- Mahamed S, Mitchell GS. Is there a link between intermittent hypoxia induced respiratory plasticity and obstructive sleep apnoea? *Exp Physiol.* 2007; 92(1):27–37. [PubMed: 17099064]
- Marti HJ, Bernaudin M, Bellail A, Schoch H, Euler M, Petit E, Risau W. Hypoxia induced vascular endothelial growth factor expression precedes neovascularization after cerebral ischemia. *Am J Pathol.* 2000; 156(3):965–76. [PubMed: 10702412]
- Marti HH, Risau W. Systemic hypoxia changes the organ-specific distribution of vascular endothelial growth factor and its receptors. *Proc Natl Acad Sci USA.* 1998; 95(26):15809–14. [PubMed: 9861052]
- Marti HH, Wenger RH, Rivas LA, Straumann U, Digicaylioglu M, Henn V, Yonekawa Y, Bauer C, Gassmann M. Erythropoietin gene expression in human, monkey and murine brain. *Eur J Neurosci.* 1996; 8(4):666–76. [PubMed: 9081618]
- McGuire M, Zhang Y, White DP, et al. Chronic intermittent hypoxia enhances ventilatory long-term facilitation in awake rats. *J Physiol.* 2003; 95:1499–1508.
- Mitchell, GS. Respiratory plasticity following intermittent hypoxia: a guide for novel therapeutic approaches to ventilatory control disorders. In: Gaultier, C., editor. *Genetic Basis for Respiratory Control Disorders.* New York: Springer Publishing Company; 2007.
- Mitchell GS, Johnson SM. Neuroplasticity in respiratory motor control. *J Appl Physiol.* 2003; 94:358–374. [PubMed: 12486024]
- Mitchell GS, Baker TL, Nanda SA, Fuller DD, Zabka AG, Hodgeman BA, Bavis RW, Mack KJ, Olson EB Jr. Invited review: Intermittent hypoxia and respiratory plasticity. *J Appl Physiol.* 2001; 90:2466–2475. [PubMed: 11356815]
- Morishita E, Masuda S, Nagao M, Yasuda Y, Sasaki R. Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. *Neuroscience.* 1997; 76:105–16. [PubMed: 8971763]

- Peng YJ, Prabhakar NR. Effect of two paradigms of chronic intermittent hypoxia on carotid body sensory activity. *J Applied Physiol.* 2004; 96:1236–42. [PubMed: 14660510]
- Peng YJ, Overholt JL, Klin D, et al. Induction of sensory long-term facilitation in the carotid body by intermittent hypoxia: implications for recurrent apneas. *Proc Natl Acad Sci USA.* 2003; 100(17): 10073–8. [PubMed: 12907705]
- Row BW. Intermittent hypoxia and cognitive function: implications from chronic animal models. *Adv Exp Med Biol.* 2007; 618:51–67. [PubMed: 18269188]
- Satriotomo I, Dale EA, Dahlberg JM, Mitchell GS. Repetitive acute intermittent hypoxia increases expression of proteins associated with plasticity in the phrenic motor nucleus. *J Exp Neurol.* 2012 in press.
- Semenza GL. Regulation of Oxygen Homeostasis by Hypoxia-inducible factor 1. *Physiol.* 2009; 24:97–106.
- Senger DR, Perruzzi CA, Feder J, Dvorak HF. A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. *Cancer Res.* 1986; 46:5629–5632. [PubMed: 3756910]
- Shein NA, Horowitz M, Alexandrovich AG, Tsenter J, Shohami E. Heat acclimation increases hypoxia-inducible factor 1 α and erythropoietin receptor expression: implication for neuroprotection after closed head injury in mice. *J Cereb Blood Flow Metab.* 2005; 25(11):1456–65. [PubMed: 15902197]
- Soliz J, Joseph V, Soulage C, Becskei C, Vogel J, Pequignot JM, Ogunshola O, Gassmann M. Erythropoietin regulates hypoxic ventilation in mice by interacting with brainstem and carotid bodies. *J Physiol.* 2005; 568:559–571. [PubMed: 16051624]
- Soliz J, Gassmann M, Joseph V. Downregulation of soluble erythropoietin receptor in the mouse brain is required for the ventilatory acclimatization to hypoxia. *J Physiol.* 2007; 583:329–336. [PubMed: 17584830]
- Spedding M, Gressens P. Neurotrophins and cytokines in neuronal plasticity. *Novartis Found Symp.* 2008; 289:222–233. discussion 233–240. [PubMed: 18497106]
- Stohlman F Jr. Observations on the physiology of erythropoietin and its role in the regulation of red cell production. *Ann N Y Acad Sci.* 1959; 77:710–24. [PubMed: 13834786]
- Storkebaum E, Lambrechts D, Carmeliet P. VEGF: once regarded as a specific angiogenic factor, now implicated in neuroprotection. *Bioessays.* 2004; 26:943–954. [PubMed: 15351965]
- Stowe AM, Plautz EJ, Nguyen P, Frost SB, Eisner-Janowicz I, Barbay S, Dancause N, Sensarma A, Taylor MD, Zoubina EV, Nudo RJ. Neuronal HIF-1 α protein and VEGFR-2 immunoreactivity in functionally related motor areas following a focal M1 infarct. *J Cereb Blood Flow Metab.* 2008; 28(3):612–20. [PubMed: 17895908]
- Tasali E, Ip MS. Obstructive sleep apnea and metabolic syndrome: alterations in glucose metabolism and inflammation. *Proc Am Thorac Soc.* 2008; 5(2):207–17. [PubMed: 18250214]
- Theus MH, Wei L, Cui L, Francis K, Hu X, Keogh C, Yu SP. In vitro hypoxic preconditioning of embryonic stem cells as a strategy of promoting cell survival and functional benefits after transplantation into the ischemic rat brain. *Exp Neurol.* 2008; 210(2):656–70. [PubMed: 18279854]
- Vinit S, Kastner A. Descending bulbospinal pathways and recovery of respiratory motor function following spinal cord injury. *Respir Physiol Neurobiol.* 2009; 169(2):115–22. [PubMed: 19682608]
- Vinit S, Lovett-Barr MR, Mitchell GS. Intermittent hypoxia induces functional recovery following cervical spinal injury. *Respir Physiol Neurobiol.* 2009; 169(2):210–7. [PubMed: 19651247]
- Wang GL, Semenza GL. Purification and characterization of hypoxia inducible factor 1. *J Biol Chem.* 1995; 270(3):1230–7. [PubMed: 7836384]
- Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci USA.* 1995; 92:5510–5514. [PubMed: 7539918]
- Yamashita S. Heat-induced antigen retrieval: mechanisms and application to histochemistry. *Prog Histo Cyto.* 2007; 41:141–200.

- Yeo E-J, Cho Y-S, Kim M-S, Park J-W. Contribution of HIF-1 or HIF 2 to erythropoietin expression: in vivo evidence based on chromatin immunoprecipitation. *Ann Hematol.* 2008; 87:11–17. [PubMed: 17712557]
- Zachary I. Neuroprotective role of vascular endothelial growth factor: signaling mechanisms, biological function, and therapeutic potential. *Neurosignals.* 2005; 14:207–221. [PubMed: 16301836]

Abbreviations

AKT	protein kinase B
BDNF	Brain derived neurotrophic factor
CIH	chronic intermittent hypoxia
EPO	erythropoietin
EPOR	erythropoietin receptor
ERK MAP kinase	extracellular regulated kinase mitogen activated protein kinase
HIF	hypoxia inducible factor
pLTF	phrenic long-term facilitation
pMF	phrenic motor facilitation
rAIH	repetitive acute intermittent hypoxia
3xwAIH	thrice weekly acute intermittent hypoxia
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor 2

Highlights

- We confirm that cervical spinal vascular endothelial growth factor (VEGF) elicits phrenic motor facilitation.
- We confirm that cervical spinal erythropoietin (EPO) elicits phrenic motor facilitation.
- Four weeks of repetitive acute intermittent hypoxia does not enhance VEGF or EPO induced phrenic motor facilitation.

Figure 1.

3xwAIH (4 wks) does not enhance VEGF-induced phrenic motor facilitation (pMF). A) Representative compressed phrenic neurograms showing VEGF-induced pMF after 3xwAIH (upper trace), VEGF-induced pMF after 4 weeks of sham, normoxia (upper-middle), (sham) time controls after 3xwAIH with aCSF injection (lower middle), or normoxia and aCSF injection (bottom trace). Arrow and vertical grey line indicates intrathecal VEGF or vehicle injection. B) The amplitude of integrated phrenic bursts increases above baseline post-injection of 10μl (100ng) VEGF in 3xwAIH (n=5; solid diamonds) and Nx (n=6; closed circles) pre-treated rats. Both curves are also significantly greater than normoxia treated vehicle (artificial cerebrospinal fluid + 0.1% BSA) controls (Nx+aCSF: n=5, open circles) at 60 min post-injection. Rats treated with Nx+aCSF were not significantly different from 3xwAIH pre-treated controls (3xwAIH+aCSF: n=6, open diamonds). All values expressed as percent change from baseline. Mean values ± S.E.M. †: significantly different from respective baselines (all p<0.003); *significantly different from Nx+aCSF vehicle at the same time point (all p<0.04); #significantly different from 3xwAIH+aCSF at the same time point (p<0.04).

Figure 2.

Hypoglossal (XII) motor output and nerve burst frequency are not affected by cervical spinal VEGF injections. A) Representative compressed neurogram showing no facilitation in XII motor output after (any) treatment. Arrow indicates time of intrathecal injections (VEGF or vehicle), with or without 3xwAIH or sham normoxia pre-treatment (4 wks). B) There were no significant differences between groups (3xwAIH+VEGF, n=5; Nx+VEGF, n=5; 3xwAIH+aCSF, n=6; Nx+aCSF, n=5; all $p>0.05$) or versus baseline (all $p>0.05$) in XII motor output at any time point. All values expressed as percent change in XII burst amplitude from baseline. Mean values $\pm$ S.E.M. C) There were no significant differences in nerve burst frequency between groups (all $p>0.05$) or vs. baseline (all $p>0.05$) at any time point. All values expressed as percent change in nerve burst frequency from baseline. Mean values $\pm$ S.E.M.

Figure 3.

3xwAIH (4 wks) does not enhance EPO-induced phrenic motor facilitation (pMF). A) Representative compressed phrenic neurograms showing EPO-induced pMF after 3xwAIH (upper trace), EPO-induced pMF after 4 weeks of normoxia (upper middle), no facilitation after 3xwAIH and acute aCSF injection (lower middle), and no facilitation after normoxia and acute aCSF injection (bottom). Arrow and vertical grey line indicate time of intrathecal EPO (or vehicle) injection. B) The amplitude of integrated phrenic bursts increases above baseline after EPO injection (10 μ l, 20IU) in both 3xwAIH (n=6; solid diamonds) and sham, Nx (n=5; closed circles) treated rats. Both are significantly different from 3xwAIH and Nx pre-treated vehicle (artificial cerebrospinal fluid + 0.1% BSA) controls (3xwAIH+aCSF: n=6, open diamonds; Nx+aCSF: n=5, open circles) at 60 min post-injection. All values expressed as percent change in phrenic burst amplitude from baseline. Mean values $\pm$ S.E.M. †: significantly different from respective baselines (all $p < 0.02$); *: significantly different from Nx+aCSF vehicle and 3xwAIH+aCSF at the same time point (all $p < 0.02$).

Figure 4.

Hypoglossal (XII) motor output and nerve burst frequency are not affected by cervical spinal EPO injections. A) There were no significant differences between groups (3xwAIH+EPO, Nx+EPO, 3xwAIH+aCSF, Nx+aCSF; all $p>0.05$) or vs baseline (all $p>0.05$) in XII motor output at any point. All values expressed as percent change in XII burst amplitude from baseline. Mean values $\pm$ S.E.M. C) There were no significant differences in nerve burst frequency between groups (all $p>0.05$) or vs baseline (all $p>0.05$) at any point. All values expressed as percent change in nerve burst frequency expressed from baseline. Mean values $\pm$ S.E.M.

Figure 5.

3xwAIH (4 wks) does not affect baseline voltage of the integrated phrenic neurogram, frequency or apneic threshold. A) There were no significant differences in baseline phrenic voltage between 3xwAIH pre-treated rats ($n=18$; left-most column) and normoxia (Nx) controls ($n=16$; 2nd column from left; $p>0.05$). There were no significant differences in the voltage of integrated baseline hypoglossal (XII) burst amplitude between 3xwAIH ($n=18$; 2nd column from right) and Nx pre-treated rats ($n=16$; right-most column; $p>0.05$). All values expressed as voltage of the integrated neurogram, mean values $\pm$ S.E.M. B) There were no significant differences in baseline burst frequency between 3xwAIH ($n=18$) and Nx ($n=16$; $p>0.05$) pre-treated rats (bursts per minute); mean values $\pm$ S.E.M. C) There were no significant differences in the CO₂ apneic threshold between 3xwAIH ($n=18$) and Nx ($n=16$; $p>0.05$) pre-treated rats; apneic threshold is the end-tidal PCO₂ at which nerve activity ceases, mean values $\pm$ S.E.M.

Table 1

Physiological variables during experimental protocols in each treatment group, including: body temperature Tb, blood gas values (PaO₂ and PaCO₂), pH, mean arterial pressure (MAP), and standardized base excess (SBE).

Group	Tb (°C)	PaO ₂ (mmH)	PaCO ₂ (mmH)	pH	MAP(mm)	SBE
Baseline						
3xwAIH+ VEGF	37.0±0.	320.2±7.1	43.6±1.2	7.368±0.28	120.7±6.3	-0.08±1.46
3xwAIH+EPO	37.3±0.	325.7±15.1	43.5±0.5	7.349±0.01	125.7±3.9	-1.55±0.76
3xwAIH+aCSF	37.2±0.	319.0±17.8	44.4±1.4	7.333±0.01	122.0±4.0	-2.11±0.54
Nx+VEGF	37.0±0.	328.7±14.5	46.6±1.8	7.346±0.02	117.9±5.5	-0.21±0.54
Nx+EPO	36.9±0.	314.8±8.3	44.2±0.7	7.357±0.01	115.6±3.5	-0.54±0.55
Nx+aCSF	37.1±0.	325.2±13.5	41.6±1.6	7.378±0.01	119.3±4.1	-0.52±1.09
15 min						
3xwAIH+ VEGF	37.3±0.	314.6±7.9	43.4±1.1	7.350±0.02	111.9±7.7	-1.52±0.90 *
3xwAIH+EPO	37.3±0.	310.0±7.1	43.8±0.4	7.352±0.01	122.4±3.7	-1.15±0.64
3xwAIH+aCSF	37.1±0.	310.0±8.7	45.1±1.3	7.340±0.01	116.0±5.9	-1.32±0.48
Nx+VEGF	37.3±0.	314.2±12.8	46.7±1.9	7.350±0.02	108.0±6.9	0.12±0.79
Nx+EPO	36.9±0.	382.2±32.2	44.4±0.9	7.359±0.01	108.7±5.4	-0.36±0.55
Nx+aCSF	37.1±0.	316.0±10.8	41.8±1.6	7.371±0.01	114.1±7.8	-0.88±1.09
30 min						
3xwAIH+ VEGF	37.2±0.	313.6±7.5	44.0±1.3	7.343±0.02	104.8±9.4	-1.68±0.81 *
3xwAIH+EPO	37.3±0.	312.7±5.9	43.6±0.8	7.352±0.01	116.4±4.7	-1.25±0.69
3xwAIH+aCSF	36.9±0.	308.5±8.8	44.1±1.3	7.348±0.02	114.7±6.3	-1.28±0.50
Nx+VEGF	37.0±0.	311.8±12.9	46.5±1.7	7.350±0.02	107.9±6.2	0.03±0.79
Nx+EPO	37.1±0.	305.6±10.6	44.9±0.6	7.357±0.01	105.2±6.9	-0.24±0.58
Nx+aCSF	37.1±0.	330.4±16.3	41.3±1.5	7.376±0.01	108.3±5.1	-0.86±0.92
60 min						
3xwAIH+ VEGF	36.9±0.	313.8±5.1	43.8±1.2	7.345±0.02	108.2±4.4	-1.56±0.85 *
3xwAIH+EPO	37.4±0.	327.8±13.1	43.6±0.4	7.352±0.01	116.1±4.0	-1.27±0.61
3xwAIH+aCSF	36.9±0.	311.3±8.9	44.4±1.4	7.353±0.01	108.7±8.1	-0.77±0.62 *
Nx+VEGF	37.3±0.	304.5±6.9	46.5±1.8	7.349±0.02	107.9±6.2	-0.02±0.81
Nx+EPO	37.2±0.	306.5±9.3	44.8±1.1	7.355±0.01	104.7±5.2	-0.68±0.66

Group	Tb (°C)	PaO ₂ (mmHg)	PaCO ₂ (mmHg)	pH	MAP(mm)	SBE
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Nx+aCSF	36.9±0.	314.0±10.1	41.2±1.4	7.376±1.4	110.2±5.9	-0.84±0.88
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All values expressed as means ± 1 SEM

* significantly different from respective baseline values (p<0.05).