Spinal 5-HT₇ receptors induce phrenic motor facilitation via \(\text{EPAC-mTORC1} \) signaling

**D. P. Fields,¹ S. R. Springborn,¹** and **G. S. Mitchell¹,²**

¹Department of Comparative Biosciences, University of Wisconsin, Madison, Wisconsin; and ²Department of Physical Therapy and McKnight Brain Institute, University of Florida, Gainesville, Florida

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**SERO**

Serotonin plays a key role in important forms of sensory-motor plasticity, including sensitization of the gill withdrawal reflex in *Aplysia* (reviewed in Kandel 2012). For example, episodic serotonin presentations enhance sensory motor synaptic transmission, giving rise to the gill withdrawal reflex (Brunelli et al. 1976). This well-studied form of plasticity in an invertebrate model system relies on multiple serotonin receptor subtypes, each activating unique kinase signaling cascades (Barbas et al. 2003).

In ways similar to sensory motor facilitation in *Aplysia*, episodic serotonin receptor activation is necessary and sufficient for important forms of spinal respiratory motor plasticity, such as long-lasting (>90 min) phrenic motor facilitation (pMF) following acute intermittent hypoxia (AIH; reviewed by Hoffman and Mitchell 2011; MacFarlane et al. 2009, 2011). Indeed, multiple serotonin receptor subtypes elicit pMF via mechanistically distinct signaling cascades named for the G proteins most often coupled with the initiating metabotropic serotonin receptor (Dale-Nagle et al. 2010). Specifically, \(G_s\) protein-coupled serotonin type 7 (5-HT₇) receptors give rise to pMF through the “S pathway” (Dale-Nagle et al. 2010; Hoffman and Mitchell 2011), whereas \(G_q\) protein-coupled 5-HT₂ receptors induce the “Q pathway” to pMF (Dale-Nagle et al. 2010; MacFarlane et al. 2011).

While the S and Q pathways elicit pMF through distinct signaling cascades, they interact via mutual “cross-talk inhibition” (Dale-Nagle et al. 2010; Devinney et al. 2013; Hoffman and Mitchell 2013). One manifestation of cross-talk inhibition is the bell-shaped dose-response curve of pMF in response to intermittent intrathecal serotonin injections (MacFarlane and Mitchell 2009). Although low serotonin doses elicit pMF by activating spinal 5-HT₂ receptors (Fuller et al. 2001; MacFarlane et al. 2011; MacFarlane and Mitchell 2009), high doses do not unless spinal 5-HT₇ receptors are inhibited. Thus, when activated alone, 5-HT₂ (MacFarlane et al. 2011) and 5-HT₇ (Hoffman and Mitchell 2011) receptors each give rise to distinct forms of pMF. When activated concurrently, they effectively cancel each other because of balanced cross-talk inhibition (Devinney et al. 2013; Hoffman and Mitchell 2013; MacFarlane and Mitchell 2009). Cross-talk inhibition from 5-HT₇ receptors to Q pathway-induced pMF is mediated by protein kinase A (PKA) activity (Hoffman and Mitchell 2013); however, it is not known how 5-HT₇ receptor activation initiates pMF when acting alone (Hoffman and Mitchell 2011).

An alternative, but only recently appreciated, cAMP-dependent signaling cascade operating through exchange protein activated by cAMP (EPAC) signaling. Anesthetized, paralyzed, and ventilated rats receiving intrathecal \((C_{S})\) 5-HT₂ receptor agonist (AS-19) injections expressed pMF for >90 min, an effect abolished by pretreatment with a selective EPAC inhibitor (ESI-05) but not a selective PKA inhibitor (KT-5720). Furthermore, intrathecal injections of a selective EPAC activator (8-pCPT-2‘-Me-cAMP) were sufficient to elicit pMF. Finally, spinal mammalian target of rapamycin complex-1 (mTORC1) inhibition via intrathecal rapamycin abolished 5-HT₂ receptor- and EPAC-induced pMF, demonstrating that spinal 5-HT₂ receptors elicit pMF by an EPAC-mTORC1 signaling pathway. Thus 5-HT₂ receptors elicit and constrain spinal phrenic motor plasticity via distinct signaling mechanisms that diverge at cAMP (EPAC vs. PKA). Selective manipulation of these molecules may enable refined regulation of serotonin-dependent spinal motor plasticity for therapeutic advantage.

motor neuron; phrenic nerve; spinal cord; respiratory plasticity; neuroplasticity; 5-HT₇; receptor; exchange protein activated by cAMP; protein kinase A; rapamycin; mTOR

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Address for reprint requests and other correspondence: G. S. Mitchell, Dept. of Physical Therapy, College of Public Health and Health Professions, Univ. of Florida, 1225 Center Dr., PO Box 100154, Gainesville, FL, 32610 (e-mail: gsmitch@phhp.ufl.edu).
Thus we tested the hypothesis that spinal 5-HT\textsubscript{7} receptors give rise to pMF through an EPAC-mTORC1 signaling pathway. We found that spinal EPAC activation is necessary and sufficient for 5-HT\textsubscript{7} receptor-induced pMF and that these effects require mTORC1 signaling. We confirm that PKA is not necessary for 5-HT\textsubscript{7} receptor-induced pMF and conclude that divergent cAMP signaling pathways underlie the distinct functions of 5-HT\textsubscript{7} receptors as they elicit (EPAC) and constrain (PKA) spinal serotonin-dependent respiratory motor plasticity.

**METHODS**

Experiments were performed on anesthetized 300- to 400-g male Lewis rats (Colony 202c; Harlan, Indianapolis, IN). The Animal Care and Use Committee at the University of Wisconsin, Madison approved all procedures.

**Immunohistochemistry.** Although PKA constrains 5-HT\textsubscript{7} receptor-dependent pMF through cross-talk inhibition (Hoffman and Mitchell 2013), several lines of evidence suggest that EPAC (not PKA) mediates 5-HT\textsubscript{7} receptor-induced pMF. However, no study has attempted to localize these proteins in spinal respiratory neurons in general, or phrenic motor neurons (PMNs) in specific. Thus we investigated 5-HT\textsubscript{7}, receptor, PKA, and EPAC protein expression within PMNs.

To identify PMNs, four untreated rats received intrapleural injections of the retrograde tracer cholera toxin B fragment (CtB; List Biological Laboratories, Campbell, CA; Mantilla et al. 2009). Briefly, each rat was anesthetized with isoflurane (1–1.5\% in 100\% O\textsubscript{2}), and a 50-\mu l Hamilton syringe with a sterile, slightly beveled 27-gauge needle was used to inject 12.5 \mu l of 0.2\% CtB in sterile saline. Bilateral injections were made at the fifth intercostal space (25 \mu l of 0.2\% CtB total). After injection rats were carefully monitored for signs of respiratory distress associated with potential pneumothorax as isoflurane was discontinued (15 min). Seven days after injection rats were again anesthetized with isoflurane, euthanized, and transcardially perfused with 0.1 M phosphate-buffered saline (PBS; pH 7.4) washes prior to incubation with secondary antibodies (Alexa 488 anti-rabbit 1:1,000 for PKA; Invitrogen) for 2 h. Afterwards, tissues were costained for CtB:EPAC, CtB:PKA, or CtB:5-HT\textsubscript{7} with the following antibodies: goat anti-CtB (1:5,000; Calbiochem), rabbit anti-EPAC (1:100; Santa Cruz), rabbit anti-PKA (1:100; Abcam), and rabbit anti 5-HT\textsubscript{7} (1:100; Abcam). Unbound primary antibodies were removed with several PBS (0.1 M, pH 7.4) washes prior to incubation with secondary antibodies (Alexa 594 anti-goat 1:1,000 for CtB, Alexa 488 anti-rabbit 1:500 for EPAC and 5-HT\textsubscript{7}, Alexa 488 anti-rabbit 1:1,000 for PKA; Invitrogen) for 2 h at room temperature. Afterwards, sections were washed in PBS and mounted on charged slides with an antifade solution (Prolong Gold antifade reagent; Invitrogen). Control staining with each primary antibody (without secondary antibodies) and all secondary antibodies (without primary antibodies) ruled out nonspecific, off-target staining (see Fig. 1).

Slides were examined with a confocal microscope (Eclipse TE 2000-U, Nikon) using EZ-C1 software (Nikon). Z stacks were taken (26 \mu m thick, 2-\mu m steps) with a \times 100 magnification objective. Images were rendered and finished with EZ-C1 software.

**Experimental preparation.** Rat anesthesia was induced with isoflurane (3.5\%) before placement on a heated stainless steel surgical table. The inspired oxygen concentration was continuously monitored (TED 60T, Teledyne Analytical Instruments), with adjustments made as necessary by changing nitrogen and/or oxygen flow rates. A tail vein catheter (24 gauge; Surflo, Ellkton, MD) was inserted, and an infusion pump (Cole-Palmer, Vernon Hills, IL) was used to deliver intravenous fluids throughout the experiment; the solution used was 9:1 lactated Ringer solution (Baxter)-sodium bicarbonate solution (8.4\%; Hospira; 1.5–2 ml/h). Rats were tracheotomized and bilaterally vagotomized through a ventral midline incision. A polyethylene catheter (PE50, ID/OD 0.58 mm/0.965 mm; Intramedic) was inserted into the femoral artery to monitor blood pressure (Gould Pressure Transducer, P23) and allow for arterial blood sampling (ABL 500; Radiometer, Copenhagen, Denmark). Dorsal laminectomy and durotomy (C1/C2) were performed to enable intrathecal drug delivery. Muscles overlying the cervical spinal cord were separated to expose the C1-C2 cervical vertebrae; after C2 laminectomy, a silicone catheter (OD 0.6 mm; Access Technologies; primed with drug/vehicle) was inserted through a small hole in the dura and advanced caudally (3 mm), resting just over spinal regions C3–C4. This catheter was not placed in position until the end of surgical preparations to minimize unintended drug diffusion from the catheter. The left phrenic and hypoglossal (XII) nerves were dissected via a dorsal approach, desheathed, and covered with saline-soaked cotton to prevent desiccation. The rat was then slowly converted to urethane anesthesia (1.8 g/kg) while simultaneously being weaned from isoflurane (over 15–20 min). During anesthesia, the rat was allowed at least 1 h for stabilization before experimental protocols started.

During the stabilization period the intrathecal catheter was inserted while both nerves were placed on bipolar silver recording electrodes and submerged in mineral oil. Once adequacy of anesthesia was confirmed (absent blood pressure spike in response to toe pinch) and respiratory nerve activity was detected, the rat was paralyzed with pancuronium bromide (3 mg/kg; Siorc Pharmaceuticals); 20 min was allowed for further stabilization. Nerve activity was amplified (gain, 10,000; A-M Systems, Everett, WA), band-pass filtered (100 Hz to 10 kHz), and integrated (CWE 821 filter; Paynter, Ardmore, PA; time constant 50 ms). The signal was digitized and recorded with a WINDAQ data acquisition system (DATAQ Instruments, Akron, OH). The resulting signal was analyzed with custom-designed software on a LabVIEW platform.

Throughout surgery and experimental protocols rats were ventilated with 60% inspired oxygen. End-tidal CO\textsubscript{2} was monitored with a flow-through capnoguard with sufficient response time to detect end-tidal CO\textsubscript{2} levels in a rat (Novametrix, Wallingford, CT). Rats were ventilated at a frequency of 70 breaths/min and a tidal volume of 2.5 ml or less (rodent ventilator model 683; Harvard Apparatus, South Natick, MA). This ventilation level caused hypocapnia (reduced arterial partial pressure of CO\textsubscript{2} to baseline values throughout the experiment by adjusting the temperature of the surgical table. After conversion to urethane anesthesia, the rat was allowed at least 1 h for stabilization before experimental protocols started.

Arterial partial pressure of CO\textsubscript{2} was maintained within 1 mmHg of baseline values throughout the experiment by manipulating inspired CO\textsubscript{2} either by manipulator or by adjusting inspired CO\textsubscript{2} levels in a rat (Novametrix, Wallingford, CT). Rats were ventilated at a frequency of 70 breaths/min and a tidal volume of 2.5 ml or less (rodent ventilator model 683; Harvard Apparatus, South Natick, MA). This ventilation level caused hypocapnia (reduced arterial partial pressure of CO\textsubscript{2} to baseline values throughout the experiment by adjusting the temperature of the surgical table. After conversion to urethane anesthesia, the rat was allowed at least 1 h for stabilization before experimental protocols started.

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prior to blood samples in 1-min bins. At the end of experiments rats were euthanized by urethane overdose.

Physiological variables for respective groups are summarized in Table 1. While several groups showed a significant increase in firing frequency, this was mild relative to phrenic nerve amplitude changes. Because of the inconsistency and faintness of this difference we did not pursue this further.

**Drugs and vehicles.** The following drugs were obtained from Santa Cruz (Dallas, TX): AS-19 (5-HT7 receptor agonist), 8-pCPT-2′-Me-cAMP [8-pCPT; EPAC-selective activator (EPACa)], and KT-5720 [PKA-selective inhibitor (PKAi)]. Rapamycin (mTORC1 inhibitor) was obtained from Thermo-Fisher (Waltham, MA), while ESI-05 [EPAC-selective inhibitor (EPACi)] was obtained from BioLog Life Science Institute (Germany). All drugs were initially dissolved in dimethyl sulfoxide (DMSO) before dilution with vehicle (20% DMSO in sterile saline) before use. Aliquots of stock solutions remained [EPAC-selective inhibitor (EPACi)] was obtained from BioLog Life Science Institute (Germany). All drugs were initially dissolved in dimethyl sulfoxide (DMSO) before dilution with vehicle (20% DMSO in sterile saline) before use. Aliquots of stock solutions remained [EPAC-selective inhibitor (EPACi)] was obtained from BioLog Life Science Institute (Germany). All drugs were initially dissolved in dimethyl sulfoxide (DMSO) before dilution with vehicle (20% DMSO in sterile saline) before use. Aliquots of stock solutions remained.

Additional control groups were completed for vehicle (n = 6), PKAi (n = 5), and rapamycin (n = 4) in which the drug was given followed by 3 × 5-μl injections of vehicle 15 min later. None of the control groups affected phrenic nerve activity, and they were not significantly different from each other; thus these groups were assembled into a single control group (n = 20; P > 0.26).

**Statistical analyses.** Peak integrated amplitude and frequency of phrenic and XII nerves were averaged in 1-min bins at baseline (preinjection) and then 30, 60, and 90 min after intrathecal injections. Amplitude values are expressed as percent change from baseline, while frequency is expressed as change from baseline in bursts per minute. Statistical comparisons were made for control and experimental groups with a two-way repeated-measures ANOVA; a Tukey post hoc test was used to identify pairwise differences. Significance was accepted as P < 0.05. All values are expressed as means ± SE.

### Results

**Phrenic motor neuron expression of EPAC, PKA, and 5-HT7 receptors.** PMNs were identified via intrapleural CtB injections. Antibodies targeting EPAC2, PKA, and 5-HT7 receptors all revealed immunofluorescence within/near CtB-identified PMNs. EPAC2 immunoreactivity was cytoplasmic, with the densest staining in the perinuclear region (Fig. 1, A–C). PKA immunoreactivity was observed within the cytoplasm of CtB-colabeled PMNs (Fig. 1, D–F). Immunofluorescence for 5-HT7 receptors was localized to the neuropil, with relatively limited

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<th>5-HT7 Agonist</th>
<th>EPACi + 5-HT7 Agonist</th>
<th>Rapamycin + 5-HT7 Agonist</th>
<th>PKAi + 5-HT7 Agonist</th>
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Values are expressed as means ± SE. EPAC, exchange protein activated by cAMP; PKA, protein kinase A; EPACi, EPAC-selective inhibitor; PKAi, PKA-selective inhibitor; EPACa, EPAC-selective activator; PaCO2, arterial partial pressure of CO2; PaO2, arterial partial pressure of O2; MAP, mean arterial pressure. *Significantly different from baseline within same group (P ≤ 0.05).
cytoplasmic or nuclear staining (Fig. 1, G–I). Thus PMNs can directly respond to 5-HT7 receptor agonists via EPAC and/or PKA signaling, although we cannot rule out additional contributions to pMF from surrounding cells: glia, presynaptic neurons, or interneurons.

EPAC, but not PKA, is necessary for 5-HT7-induced pMF. Intermittent intrathecal injections of 5-HT7 receptor agonist (3 × 5 μl, 100 μM) AS-19 elicited long-lasting pMF expressed as a progressive enhancement in phrenic nerve amplitude (>90 min: 88.8 ± 7.2%; Fig. 2, A and Bi). The dose/selectivity of this same 5-HT7 receptor agonist was determined in a previous study using the same experimental protocol (Hoffman and Mitchell 2011).

Pretreatment with an intrathecal EPACi (10 μl, 2 mM), ESI-05, abolished 5-HT7 receptor-induced pMF (90 min: 15.1 ± 10.5%, P = 0.0002; Fig. 2, A and Bi).) demonstrating EPAC as necessary for 5-HT7 receptor-induced pMF. In contrast, pretreating with a PKAi (10 μl, 100 μM), KT-5720, did not significantly reduce pMF relative to the vehicle-pretreated 5-HT7 receptor agonist group (81.8 ± 10.0%, P = 0.61; Fig. 2,
Fig. 2. 5-HT7 receptor-induced phrenic motor facilitation (pMF) requires EPAC but not PKA activity. A: average change in phrenic burst amplitude from baseline to 90 min after injections. Lines are fitted with rats with intrathecal injections of the 5-HT7 receptor agonist (AS-19; 3 × 5 μl, 100 μM) with saline (n = 7; 10 μl), EPAC-selective inhibitor (EPAci, n = 7; 10 μl, 2 mM), or PKA-selective inhibitor (PKAi, n = 5; 10 μl, 100 μM) pretreatment or control rats that did not receive the 5-HT7 receptor agonist (n = 20; 10 μl). Data represent mean ± SE values. Significant differences from baseline, control, or *PKAi + 5-HT7 agonist: all P ≤ 0.05. B: representative traces of phrenic neurograms are shown before, during, and after intrathecal injections of vehicle + 5-HT7 receptor agonist, EPAC + 5-HT7 receptor agonist, or PKAI + 5-HT7 receptor agonist. First arrow (below trace iv) represents the pretreatment injection; 2nd arrow represents the first of 3 5-HT7 receptor agonist injections. Data from control and 5-HT7 agonist groups are repeated within multiple figures.

A and B(iii). The KT-5720 dose is based on previous work using the same in vivo protocol (Hoffman and Mitchell 2013) and PKA cell culture activity assays (Davies et al. 2000). Cervical spinal injections of the 5-HT7 receptor agonist did not affect hypoglossal nerve (XII) activity (n = 4; 90 min: -0.1 ± 13.1%, P = 0.95), suggesting that 5-HT7 receptor agonist-induced pMF requires activation of spinal (vs. brain stem) receptors (Baker-Herman and Mitchell 2002; MacFarlane and Mitchell 2009).

Spinal EPAC activation elicits pMF. Intrathecal injection of an EPACi (10 μl, 100 μM), 8-pCPT, elicited robust pMF lasting at least 90 min (104.4 ± 7.8%, P = 0.000040; Fig. 3, A and B(ii)). Pretreatment with an intrathecal EPACi (10 μl, 2 mM), ESI-05, significantly attenuated EPACa-induced pMF (90 min: 24.5 ± 7.8%, P = 0.00064; Fig. 3, A and B(iii)), confirming the selectivity of EPACa. Although cervical spinal 5-HT7 receptor agonist injections did not cause XII facilitation, similar EPACa injections did elicit significant XII burst amplitude facilitation (data not shown) at 10 μM (n = 2; 90 min: 59.5 ± 8.2%) and 100 μM (n = 4; 90 min: 77.2 ± 23.1%); this effect was blocked by intrathecal EPACi pretreatment (P = 0.00064). These differential 5-HT7 vs. EPAC effects on XII activity are difficult to explain, and we did not pursue this issue further in the present study.

mTORC1 is necessary for 5-HT7- and EPAC-induced pMF. Pretreatment with the mTORC1 inhibitor rapamycin (10 μl; 100 μM) blocked both 5-HT7 receptor (11.2 ± 9.2%, P = 0.00007) and EPACa (9.9 ± 10.0%, P = 0.00039)-induced pMF (Fig. 4, A and B, respectively), demonstrating that mTORC1 is necessary for both processes.

DISCUSSION

Although cervical spinal 5-HT7 receptor activation elicits pMF, it also constrains 5-HT2 receptor-induced pMF via cross-
talk inhibition (Hoffman and Mitchell 2013; MacFarlane and Mitchell 2009). Here we show that these diverse actions result from divergent cAMP signaling. Specifically, 5-HT7 receptors constrain 5-HT7 receptor-induced pMF through cAMP/PKA signaling (Hoffman and Mitchell 2013) but elicit pMF via a mechanistically distinct cAMP/EPAC pathway. Since 5-HT7 receptor- and EPAC-induced pMF are rapamycin sensitive, this pathway requires mTORC1 signaling. We conclude that serotonin elicits spinal plasticity via diverse mechanisms. First, serotonin elicits phrenic motor plasticity via unique mechanisms associated with distinct serotonin receptor subtypes (e.g., 5-HT7 vs. 5-HT2 receptors). Second, 5-HT7 receptors elicit heterogeneous effects (presumably) within the same cell via divergent cAMP signaling (PKA and EPAC). Understanding distinct serotonin and cAMP functions may be critical as we attempt to harness serotonin-dependent plasticity as a treatment for severe neuro-motor disorders that compromise breathing, such as spinal injury and amyotrophic lateral sclerosis (Dale et al. 2014; Mitchell 2007).

While EPAC activation is necessary and sufficient for some forms of cAMP-dependent plasticity, selective PKA activation (EPAC independent) also gives rise to plasticity in some model systems (Castellucci et al. 1980). Thus EPAC and PKA may give rise to alternate, parallel signaling cascades for cAMP-dependent plasticity. Although PKA-dependent plasticity has been well studied, considerably less is known concerning how EPAC activation elicits plasticity. Here we demonstrate that EPAC-dependent, 5-HT7 receptor-induced pMF is mechanistically distinct from the serotonin-induced PKA-ERK-brain-derived neurotrophic factor (BDNF) signaling pathway described in invertebrate model systems (for review see Kandel 2012). As shown by the available evidence concerning 5-HT7 receptor-induced pMF, 5-HT7 receptors elicit pMF via EPAC-Akt-mTORC1 signaling, resulting in new synthesis of an immature TrkB isoform (Golder et al. 2008; Hoffman and Mitchell 2011). This mechanism is independent of PKA, ERK signaling, or new BDNF synthesis (Hoffman and Mitchell 2011).

We are not aware of any previous studies connecting EPAC or mTORC1 signaling with serotonin-induced plasticity. By showing that the canonical pathway of PKA-ERK-BDNF does not play a role in 5-HT7 receptor-induced spinal respiratory motor plasticity we have uncovered an interesting question: what purpose do parallel plasticity pathways operating downstream from a single receptor serve? We suggest that mechanistic heterogeneity in spinal motor plasticity signaling is important to maintain appropriate network activity throughout maturation, during changing environmental conditions, and during severe disease states (Dale et al. 2014). Although PKA signaling does not contribute to 5-HT7-induced pMF, it may serve as a “reserve” pathway when EPAC-dependent signaling is impaired. For example, while wild-type mice express EPAC-dependent (PKA-independent) mossy fiber plasticity, EPAC2−/− knockout mice retain certain forms of mossy fiber plasticity by shifting to PKA-dependent signaling (Fernandes et al. 2015). Thus in some conditions PKA activity is sufficient to induce plasticity during compromised EPAC signaling.

Finally, since PKA does not normally contribute to 5-HT7 receptor-induced pMF our findings suggest that reduced PKA signaling may prevent cross-talk inhibition without compromising 5-HT7 receptor-induced pMF. By minimizing cross-talk inhibition with PKA-selective inhibitors it may be possible for both 5-HT2 and 5-HT7 receptors to independently contribute to an enhanced form of pMF (i.e., metaplasticity; Fields and Mitchell 2015). In agreement, whereas PKA mediates cross-talk inhibition (Hoffman and Mitchell 2013), EPAC enables concurrent activation of signaling pathways operating downstream from 5-HT2 and 5-HT7 receptors (Johnson-Farley et al. 2005). The respective contributions of reduced PKA and/or enhanced EPAC activity to enhanced A1H-induced pMF following intermittent hypoxia preconditioning (Fields and Mitchell 2015) remain to be explored.

Although we are only beginning to understand interpathway interactions in serotonin receptor-induced spinal motor plasticity, the ability of nonessential accessory signaling pathways to regulate plasticity has considerable significance, from both a biological and a therapeutic perspective.

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