Development/Plasticity/Repair

Sustained Hypoxia Elicits Competing Spinal Mechanisms of Phrenic Motor Facilitation

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Acute intermittent hypoxia (AIH) induces phrenic long-term facilitation (pLTF), a form of spinal motor plasticity. Competing mechanisms give rise to phrenic motor facilitation (pMF; a general term including pLTF) depending on the severity of hypoxia within episodes. In contrast, moderate acute sustained hypoxia (mASH) does not elicit pMF. By varying the severity of ASH and targeting competing mechanisms of pMF, we sought to illustrate why moderate AIH (mAIH) elicits pMF but mASH does not. Although mAIH elicits serotonin-dependent pLTF, mASH does not; thus, mAIH-induced pLTF is pattern sensitive. In contrast, severe AIH (sAIH) elicits pLTF through adenosine-dependent mechanisms, likely from greater extracellular adenosine accumulation. Because serotonin- and adenosine-dependent pMF interact via cross talk inhibition, we hypothesized that pMF is obscured because the competing mechanisms of pMF are balanced and offsetting during mASH. Here, we demonstrate the following: (1) blocking spinal A_{2A} receptors with MSX-3 reveals mASH-induced pMF; and (2) sASH elicits A_{2A}-dependent pMF. In anesthetized rats pretreated with intrathecal A_{2A} receptor antagonist injections before mASH ($PaO_2 = 40–54$ mmHg) or sASH ($PaO_2 = 25–36$ mmHg), (1) mASH induced a serotonin-dependent pMF and (2) sASH induced an adenosine-dependent pMF, which was enhanced by spinal serotonin receptor inhibition. Thus, competing adenosine- and serotonin-dependent mechanisms contribute differentially to pMF depending on the pattern/severity of hypoxia. Understanding interactions between these mechanisms has clinical relevance as we develop therapies to treat severe neuromuscular disorders that compromise somatic motor behaviors, including breathing. Moreover, these results demonstrate how competing mechanisms of plasticity can give rise to pattern sensitivity in pLTF.

Key words: hypoxia; long-term facilitation; motor control; pattern sensitivity; phrenic; spinal cord

Significance Statement

Intermittent hypoxia elicits pattern-sensitive spinal plasticity and improves motor function after spinal injury or during neuromuscular disease. Specific mechanisms of pattern sensitivity in this form of plasticity are unknown. We provide evidence that competing mechanisms of phrenic motor facilitation mediated by adenosine 2A and serotonin 2 receptors are differentially expressed, depending on the pattern/severity of hypoxia. Understanding how these distinct mechanisms interact during hypoxic exposures differing in severity and duration will help explain interesting properties of plasticity, such as pattern sensitivity, and may help optimize therapies to restore motor function in patients with neuromuscular disorders that compromise movement.

Introduction

The neural system controlling breathing (including spinal respiratory motor neurons) undergoes considerable plasticity when animals experience intermittent periods of low oxygen or intermittent hypoxia (Mitchell and Johnson, 2003; Devinney et al., 2013). Intermittent hypoxia-induced respiratory plasticity may be a key respiratory control strategy beyond simple chemofeedback, conferring more robust and effective homeostatic regulation of breathing (Mitchell and Johnson, 2003).

Although hypoxia triggers plasticity in both respiratory and nonrespiratory motor systems (EA Dale et al., 2014; Gonzalez-Rothi et al., 2015), which arise from multiple, discrete cellular mechanisms (Dale-Nagle et al., 2010; Devinney et al., 2013), we

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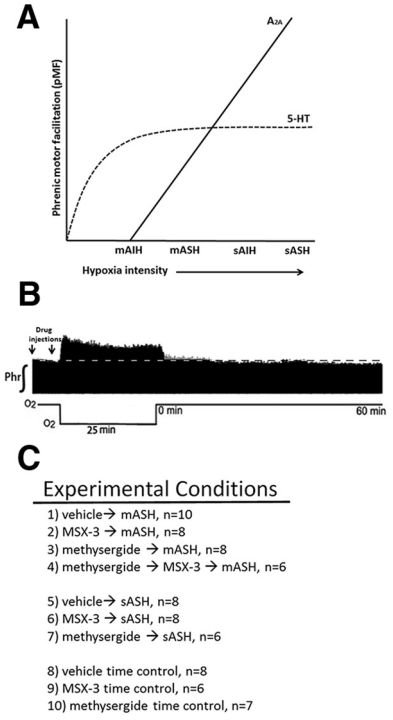

Figure 1. Hypotheses providing the rationale for experimental design. ***A***, The cumulative stimulus driving pMF mechanisms during hypoxic exposures increases, depending on pattern (sustained > intermittent) and severity (severe > moderate) of hypoxia. Hypoxia causes the following: (1) adenosine accumulation and activation of spinal A_{2A} receptors, which we illustrate here as increasing with hypoxia "intensity" (solid line); and (2) serotonin (5-HT) release from descending raphe neurons, which reaches a maximum during mASH, with fixed serotonin receptor activation during sAIH and sASH (dashed line). pMF is not observed with mASH because both mechanisms are equal and offsetting due to balanced cross talk inhibition. Overall pMF expression may depend on the relative activation of A_{2A} versus 5-HT receptors during hypoxia. ***B***, Representative phrenic nerve trace demonstrating experimental protocol. During phrenic nerve recordings, intrathecal drug injections are delivered 12–20 min before exposure to 25 min of mASH or sASH. pMF is observed 60 min after mASH or sASH. ***C***, Experimental conditions are listed for clarification.

know little concerning the functional significance of mechanistic heterogeneity, or the nature of interactions between these mechanisms. By greater understanding of interactions between mechanisms, we hope to gain insight concerning the biological significance and clinical relevance of intermittent hypoxia-induced respiratory motor plasticity. Indeed, intermittent hypoxia has been used as a therapeutic tool to restore respiratory and nonrespiratory motor function with spinal injury or neuromuscular disease (N Dale and Frenguelli, 2014; Navarrete-Opazo and Mitchell, 2014b; Gonzalez-Rothi et al., 2015).

A prominent example of intermittent hypoxia-induced respiratory motor plasticity is phrenic long-term facilitation (pLTF), a sustained increase in phrenic activity lasting hours after exposure to acute intermittent hypoxia (AIH). pLTF is one of multiple forms of phrenic motor facilitation (pMF), a more general term that refers to any long-lasting increase in phrenic motor output caused by spinal plasticity in or near the phrenic motor nucleus, regardless of the initiating stimulus. pLTF is pattern-sensitive because it is observed following moderate acute intermittent (mAIH), but not moderate acute sustained hypoxia (mASH) (Baker and Mitchell, 2000).

We have learned a great deal about cellular mechanisms giving rise to pMF in recent years (Dale-Nagle et al., 2010; Devinney et al., 2013; Fields and Mitchell, 2015). When pLTF is initiated by mAIH, it requires: intermittent activation of spinal Gq protein coupled serotonin 2 (5-HT2) receptors (Fuller et al., 2001b; Baker-Herman and Mitchell, 2002; Macfarlane and Mitchell, 2009, 2011), ERK MAP kinase activity (Hoffman et al., 2012), new brain derived neurotrophic factor synthesis (Baker-Herman et al., 2004), TrkB activation (Dale et al., 2016), and PKCθ activity (Devinney et al., 2015). In contrast, when pLTF is initiated by AIH consisting of severe hypoxic episodes (sAIH), it requires spinal adenosine 2A (A2A) receptor activation and is serotonin-independent (Nichols et al., 2012). Our working model is that sAIH-induced pLTF arises from Gs protein-coupled receptor activation, triggering a distinct mechanism that requires exchange protein activated by cAMP (EPAC) (Fields et al., 2015), Akt and mammalian target of rapamycin signaling (Dougherty et al., 2015) and new TrkB synthesis (vs BDNF) (Golder et al., 2008). These diverse mechanisms interact via cross talk inhibition (Dale-Nagle et al., 2010), with the serotonin-dependent mechanism predominant following mAIH (Hoffman et al., 2010; Navarrete-Opazo and Mitchell, 2014a), and the adenosine-dependent mechanism predominant following sAIH (Nichols et al., 2012). The shift from serotonin-dependent to adenosine-dependent pLTF with the progression from mAIH to sAIH may result from greater extracellular adenine nucleotide accumulation during severe hypoxia, shifting the balance between the competing serotonin- and adenosine-dependent mechanisms (Conde and Monteiro, 2004; Gourine et al., 2005; Dale and Frenguelli, 2009). When these mechanisms are activated equally, they may cancel one another, thereby obscuring pMF.

Here we reasoned that extracellular adenosine accumulation is greater with more severe (i.e., sAIH and sASH vs mAIH and mASH), or longer cumulative durations of hypoxia with the same severity (i.e., mASH vs mAIH; sASA vs sAIH). We hypothesized that greater A_{2A} receptor activation masks pMF following mASH and that A_{2A} receptor inhibition would reveal serotonin-dependent pMF. Further, we hypothesized that both sAIH and sASH elicit A_{2A} receptor-dependent pMF and that this pMF is enhanced by 5-HT_2 receptor inhibition. Overall, we demonstrate that competing adenosine- and serotonin-dependent mechanisms contribute differentially to pMF depending on the severity and duration of hypoxia.

Materials and Methods

Animals. Adult (12–16 weeks; 280–500 g) male Sprague Dawley rats (Harlan, Colony 211, Colony 218a) were studied. Rats from each colony were evenly distributed among the experimental groups to mitigate any impact from substrain differences on experimental results (Fuller et al., 2001a). The University of Wisconsin Animal Care and Use committee approved all experimental procedures.

Intrathecal drug injections. Treatment groups were given intrathecal injections of 10% DMSO in ACSF (vehicle, 12 μl ACSF; in mM as follows: 120 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 23 $NaHCO_3$, 10 glucose, equilibrated with 95% O_2, 5% CO_2 for 15 min), the A_{2A} receptor antagonist MSX-3 (Sigma-Aldrich, 200 μM in 12 μl of 10% DMSO in ACSF), or the broad-spectrum serotonin receptor antagonist methysergide maleate (Sigma-Aldrich, 20 mM in 12 μl ACSF). Injections were made 12 min before exposure to mASH, sASH, or sham (i.e., no hypoxia).

Experimental preparation. Rats were induced with isoflurane in a closed chamber and placed on a heated surgical table to maintain body temperature; anesthesia was maintained via nose cone (3.5% isoflurane, 50% O_2). Tracheotomy was performed and rats were pump-ventilated through a tracheal tube (Rodent Ventilator 683, Harvard Apparatus; tidal volume 2.2–2.7 ml; frequency 70–75 breaths/min) with a gas mixture of ~50% O_2, 50% N_2, and 3.5% isoflurane. End-tidal CO_2 was maintained at 40–44 mmHg by adjusting the ventilator and/or adding CO_2 to the inspired gas mixture. Tracheal pressure was continuously

Table 1. Arterial partial pressure of carbon dioxide ($PaCO_2$) and oxygen (PaO_2), mean arterial pressure (MAP), and base excess during baseline, hypoxia, and 60 min after hypoxia[a]

Treatment groups	$PaCO_2$ (mmHg)			PaO_2 (mmHg)			MAP (mmHg)			Base excess (mEq/L)		
	Baseline	Hypoxia	60 min	Baseline	Hypoxia	60 min	Baseline	Hypoxia	60 min	Baseline	Hypoxia	60 min
mASH												
Vehicle	46.0 ± 1.1*	46.2 ± 1.1	45.9 ± 1.2	313 ± 9	46.2 ± 1**,†,††	294 ± 8**	108 ± 4	81 ± 6**	100 ± 4**	−0.2 ± 0.6	−0.6 ± 0.6	1.2 ± 0.5
MSX-3	48.6 ± 0.9	48.8 ± 1.0	49.2 ± 0.9	313 ± 5	44.5 ± 1**,†,††	295 ± 5**	118 ± 9	77 ± 8**	106 ± 7**	−0.4 ± 0.5	−1.3 ± 0.5	0.7 ± 0.5
Methy	46.0 ± 1.3	45.7 ± 1.2	46.4 ± 1.3	295 ± 6	44.7 ± 1**,†,††	263 ± 11**	106 ± 4	66 ± 4**	91 ± 3**	−1.0 ± 0.5	−1.5 ± 0.5	0.0 ± 0.5
Methy + MSX-3	46.3 ± 0.5	47.0 ± 0.6	46.4 ± 0.7	290 ± 12*,†††	45.4 ± 2**,†,††	278 ± 10	97 ± 8*	68 ± 7**	94 ± 5	0.3 ± 0.7	−0.3 ± 1.0	1.6 ± 1.0
sASH												
Vehicle	45.7 ± 0.9*	44.2 ± 0.8**	46.4 ± 0.8	310 ± 5	30.4 ± 1**,***,††	291 ± 9**	110 ± 8	52 ± 9**	94 ± 9**	−0.9 ± 0.5	−8.9 ± 1.6**	0.9 ± 1.0
MSX-3	46.9 ± 1.0	46.7 ± 0.9	47.0 ± 0.9	309 ± 4	31.8 ± 1**,***,††	291 ± 6**	106 ± 6	49 ± 5**	83 ± 5**	−0.2 ± 0.3	−7.1 ± 1.1**	−0.1 ± 0.4
Methy	48.1 ± 1.3	48.2 ± 1.0	48.5 ± 1.3	262 ± 13‡	30.3 ± 1**,***,††	238 ± 9**	99 ± 6	40 ± 5**	91 ± 5	1.0 ± 0.5	−8.4 ± 0.9**	−0.5 ± 1.2
Time control												
Vehicle	46.0 ± 0.9	46.1 ± 0.8	45.3 ± 0.8	311 ± 10	310 ± 8	301 ± 7	101 ± 7*	99 ± 7	93 ± 7	−0.1 ± 0.7	0.0 ± 0.6	1.1 ± 0.6
MSX-3	48.3 ± 0.4	48.5 ± 0.5	48.9 ± 0.5	310 ± 7	306 ± 6	304 ± 8	112 ± 4	109 ± 4	100 ± 5**	0.5 ± 0.6	1.1 ± 0.3	2.5 ± 0.4
Methy	47.0 ± 0.9	47.1 ± 1.0	46.9 ± 0.8	299 ± 9	299 ± 11	276 ± 9**	103 ± 6	101 ± 6	104 ± 8	−0.2 ± 0.4	0.1 ± 0.4	1.1 ± 0.2

[a]Values are mean ± SE.

Significant difference ($p < 0.05$) from MSX3 + mASH (*), baseline (**), all mASH groups (***), all sASH groups (†), all time control groups (††), vehicle + mASH (†††), or all groups (‡).

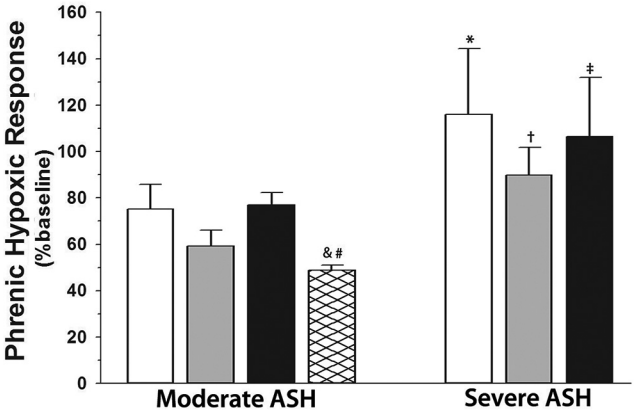

Figure 2. Short-term hypoxic phrenic responses during mASH or sASH after intrathecal injections of vehicle (open square), MSX-3 (gray square), methysergide (filled square), and methysergide + MSX-3 (square filled with diagonal crossing lines). All groups are significantly increased from time controls (no ASH, 2.5 ± 1.6%; $p \leq 0.002$). [&]Significantly decreased versus vehicle + mASH. [#]Significantly decreased versus methysergide + mASH. *Significantly increased versus vehicle + mASH. †Significantly increased versus MSX-3 + mASH. ‡Significantly increased versus methysergide + mASH. Values are mean ± SE. Significance is $p < 0.05$.

monitored. A catheter was placed in the tail or femoral vein to administer intravenous fluids (1.5–3.0 ml/h of 64%–75% lactated Ringer's solution, 4%–10% HCO_3, and 15%–30% of 6% hetastarch in 0.9% NaCl) to maintain arterial fluid and acid base balance (base excess change < ±3 mEq/L during protocol). A catheter was placed in the femoral artery to monitor blood pressure and draw arterial blood samples for analysis (PaO_2, $PaCO_2$, pH, base excess; ABL800 Radiometer).

Using a dorsal approach, the left phrenic nerve was isolated, cut, and the central end was desheathed and protected with saline-soaked cotton until nerve recordings were initiated. For intrathecal drug delivery, a C_2 laminectomy was performed and a silicone catheter (2 Fr; Access Technologies) attached to a 50 μl Hamilton syringe that was inserted through the dura and advanced until the tip was over the C_4 spinal segment. The rats were converted to urethane anesthesia by slowly withdrawing inhaled isoflurane while administering intravenous urethane (1.7–1.9 mg/kg). After ~1 h, the rat was paralyzed with pancuronium bromide (2.5 mg/kg, i.v.). Body temperature was maintained at 37.5 ± 1°C (rectal thermometer; Fisher Scientific). Blood pressure was monitored to ensure physiological stability (80–150 mmHg baseline, <30 mmHg change at 60 min). Adequate anesthesia was tested by toe pinch induced responses in blood pressure or phrenic nerve activity. Maximal CO_2 responses were elicited at the end of each experiment to verify adequate nerve responses and preparation stability. Rats with ≤30% hypoxic response during hypoxia or ≤50% maximal hypercapnic response were eliminated from analysis.

Neurophysiological measurements. At ~1 h following conversion to urethane anesthesia, the desheathed phrenic nerve was covered with mineral oil and placed on bipolar silver electrodes. Nerve activity was amplified (10,000×), bandpass filtered (300–10,000 Hz model 1800, A-M Systems), rectified and integrated with a continuous moving averager (time constant: 50 ms; CWE, MA-821 filter). The integrated signal was digitized and analyzed with a data acquisition system (WINDAQ, DATAQ Instruments). To determine the CO_2 apneic and recruitment thresholds, inspired CO_2 was lowered and/or ventilator rate increased until phrenic nerve bursting ceased; CO_2 was then slowly raised by increasing inspired CO_2 and/or decreasing ventilator rate until phrenic nerve bursting resumed. Baseline end-tidal CO_2 was set 2–3 mmHg above the recruitment threshold. Baseline nerve activity was recorded for at least 20 min.

Rats then received 12 μl intrathecal injections of vehicle (10% DMSO in ACSF), methysergide (20 mM), or MSX-3 (200 μM) at C4 delivered as 2 μl boluses every 30 s. At 12 min following MSX-3 or vehicle injections, a blood sample was drawn to assess baseline conditions. For methysergide, baseline was recorded for 15–20 min after injection, consistent with previous studies (Baker-Herman and Mitchell, 2002). In rats where both methysergide and MSX-3 were delivered, MSX-3 was delivered 5–8 min after methysergide. Rats were then exposed to mASH (12%–14% O_2; PaO_2 = 40–54 mmHg), sASH (6%–8% O_2; PaO_2 = 25–36 mmHg), or no hypoxia (i.e., time controls, ~50% O_2). Arterial PCO_2 was maintained within ±1.5 mmHg of baseline. PaO_2 was >150 mmHg at baseline and after hypoxia in all studies. Blood samples were drawn at 5 and 20 min of ASH. After ASH, baseline conditions were restored and confirmed with blood gas analysis at 15, 30, and 60 min after ASH (PaO_2 > 180 mmHg, $PaCO_2$ ± 1.5 mmHg baseline, and base excess ±3 mEq/L baseline).

Data analyses. Integrated phrenic burst amplitude and frequency were averaged in 60 s bins at baseline, during ASH, and at 15, 30, and 60 min after ASH (or similar times in time control experiments). Burst amplitudes were normalized as a percentage change from baseline in each rat. Frequency was expressed as change from baseline value (burst/min). Two-way ANOVA with a repeated-measures design was used to analyze phrenic burst amplitude, burst frequency, $PaCO_2$, PaO_2, mean arterial pressure, and base excess at baseline, during ASH, and 15, 30, and 60 min after ASH. *Post hoc* comparisons were made with Fisher's LSD (SigmaPlot version 12.0; Systat Software). In some cases, MSX-3 and methysergide time controls were grouped together as drug time control experiments because no significant differences were found between groups. For PaO_2 during hypoxia, all rats exposed to mASH or sASH were grouped together because no significant differences were found between drug treatment groups. Differences between groups were con-

sidered significant if $p < 0.05$. Listing all p values for *post hoc* comparisons was done sparingly because of the number of significant differences in individual comparisons; specific p values are given when appropriate. Values are mean ± SEM.

Experimental design. We hypothesize that, while spinal adenosine and serotonin are each capable of inducing pMF alone, they exhibit mutual inhibition during sustained hypoxia, and this balance varies with the severity of hypoxia. In Figure 1*A*, we illustrate the hypothetical adenosine 2A and serotonin receptor activation during increasingly severe and/or prolonged hypoxic episodes; implicit in this schema is the assumption that adenosine progressively rises with increasing severity and/or duration of hypoxia, whereas serotonin effects are already maximal with moderate hypoxia.

The experimental design is illustrated in Figure 1*B*, showing timing of intrathecal drug injections before mASH or sASH exposures. Experimental groups included 6–10 rats pretreated with spinal injections of vehicle, MSX-3, or methysergide, or sequential injections of methysergide followed by MSX-3 before exposure to sham (time control; no hypoxia), mASH, or sASH while recording phrenic nerves.

The experimental groups studied (with n values) were as follows: (1) vehicle before mASH (veh + mASH, $n = 10$); (2) MSX-3 before mASH (MSX-3 + mod ASH, $n = 8$); (3) methysergide (methy) before mASH (methy + mASH, $n = 8$); (4) methysergide before MSX-3 followed by mASH (methy + MSX-3 + mASH, $n = 6$); (5) vehicle before sASH (veh + sASH, $n = 8$), (6) MSX-3 before sASH (MSX-3 + sASH, $n = 8$); (7) methysergide before sASH (methy + sASH, $n = 6$); (8) vehicle without hypoxia (veh time control, $n = 8$); (9) MSX-3 without hypoxia (MSX-3 time control, $n = 6$); and (10) methysergide without hypoxia (methy time control, $n = 7$).

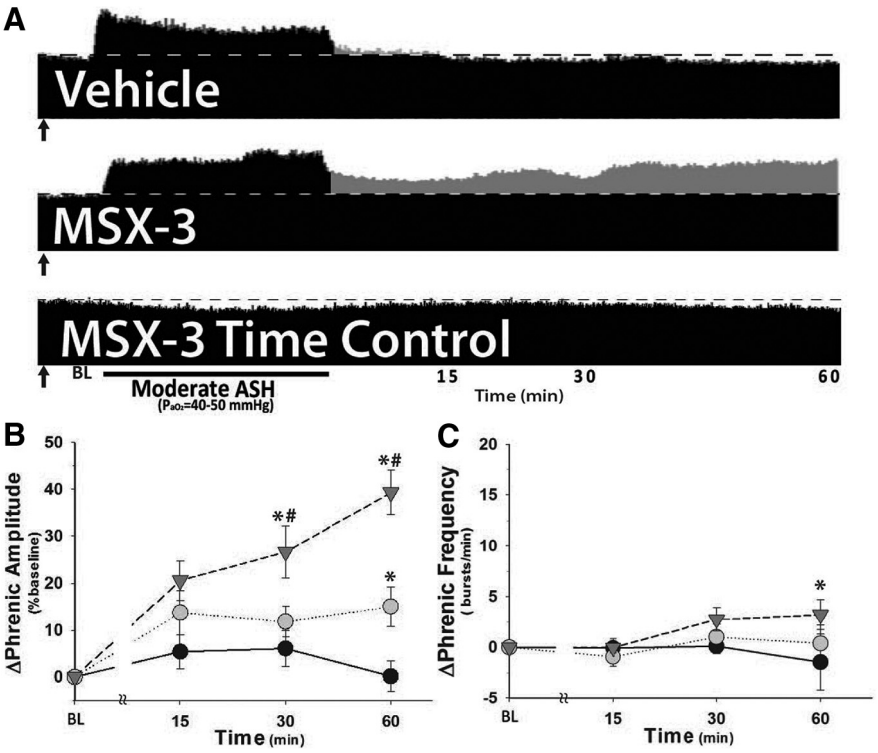

Figure 3. Pretreatment with spinal A_{2A} receptor antagonist (200 mM MSX-3) reveals pMF following mASH. ***A***, Representative phrenic neurograms after mASH ($PaO_2 = 40–50$ mmHg, 25 min) from rats given intrathecal injections of vehicle or MSX-3 (↑) compared with rats treated as time controls and not exposed to ASH after MSX-3 delivery. Dotted line indicates baseline. Gray represents pMF. ***B***, Summary data for δ phrenic amplitude following mASH exposure in rats given vehicle (inverted gray triangle) or MSX-3 (gray circle) and exposed to mASH. MSX-3 time controls are shown for comparison (no mASH, filled circle). ***C***, Summary data for Δ phrenic burst frequency following mASH exposure. $n = 10$, vehicle; $n = 8$, MSX-3; $n = 6$, MSX-3 time control (no ASH). *Significantly increased from MSX-3 time control. #Significantly increased from MSX-3 + mASH. All significant differences are $p < 0.05$.

Results

Blood gases, mean arterial pressure, and base excess

Blood gases were similar between all groups throughout the protocol, except when inspired oxygen was purposefully adjusted during ASH. Average PaO_2 was >200 mmHg during baseline and after hypoxia, demonstrating that changes in PaO_2 are not influencing phrenic activity at these times. Average $PaCO_2$ was not significantly different among groups at baseline, except for vehicle-treated rats that were to be exposed to mASH or sASH, which had slightly lower $PaCO_2$ versus rats later given MSX-3 and exposed to mASH ($p = 0.04$, $p = 0.05$, respectively; Table 1); there is no clear rationale for these differences because the rats had not yet received differential treatments. As expected, PaO_2 during ASH was significantly decreased from baseline and time controls ($p < 0.001$, Table 1). During mASH, PaO_2 was between 40 and 54 mmHg and was significantly higher than PaO_2 during sASH (26–36 mmHg; $p < 0.001$, Table 1). Rats given vehicle injections had significantly lower $PaCO_2$ values during sASH ($p = 0.002$, Table 1). However, $PaCO_2$ was not significantly different from baseline in any group at 60 min after hypoxia, demonstrating that blood gas differences cannot explain observed changes in phrenic nerve burst amplitude.

Mean arterial pressure (MAP) at baseline was the same in most groups, although, for unknown reasons, it was slightly lower in vehicle time control rats ($p = 0.045$; Table 1), and rats later injected with methysergide plus MSX-3 and exposed to mASH ($p = 0.028$; Table 1). As expected, MAP decreased significantly from baseline values during ASH ($p < 0.001$; Table 1). All rats exposed to ASH, except for rats given methysergide and MSX-3 and exposed to mASH, or methysergide and exposed to sASH, exhibited slight but significant decreases in MAP at 60 min (8–23 mmHg, $p < 0.05$; Table 1). MSX-3-injected time control rats also exhibited a slight decrease in MAP at 60 min after sham ASH (12 mmHg, $p = 0.005$; Table 1). Such time-dependent MAP changes are characteristic of this experimental preparation (Fuller et al., 2001b; Baker-Herman and Mitchell, 2008). Previously, we demonstrated that MAP changes of ~20 mmHg had minimal effect on respiratory activity in this experimental preparation (Bach and Mitchell, 1996). Overall, there is no clear evidence that changes in MAP from the beginning to the end of experiments, or among experimental groups, were sufficient to influence the basic conclusions of this study.

Base excess was held within 1.5 mEq/L of baseline throughout protocols, except during sASH, when base excess values significantly decreased ($p < 0.001$; Table 1). This decrease most likely results from transient lactic acidosis due to severe hypoxemia. However, no groups had significantly different base excess values 60 min after hypoxia versus baseline; thus, acidosis is not directly responsible for enhanced phrenic nerve activity following sASH. Each value is the average from multiple rats (vehicle, $n = 10$; MSX-3, $n = 8$; MSX-3 time control, $n = 6$).

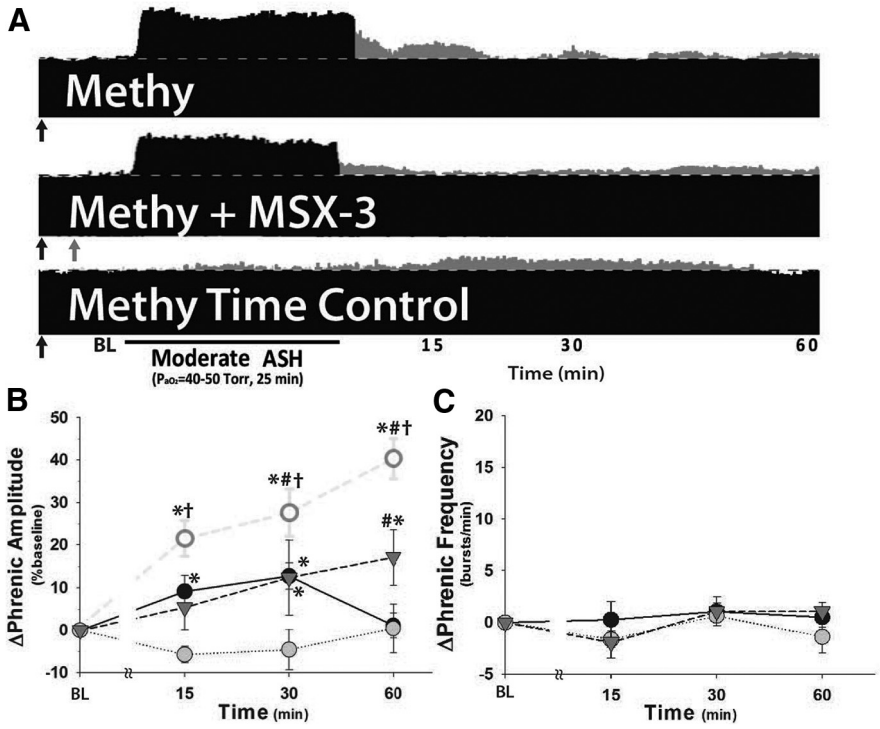

Figure 4. Pretreatment with spinal 5-HT receptor antagonist (20 mM methysergide, 20 min prior ASH; methy) prevents pMF in rats injected with spinal A_{2A} receptor antagonist (200 mM MSX-3, 12 min prior mASH) and exposed to mASH. ***A***, Representative phrenic neurograms after mASH from rats given intrathecal injections of methysergide (↑) or both methysergide and MSX-3 (↑) compared with rats treated as time controls and not exposed to mASH after methysergide delivery. The dotted line indicates baseline. Gray represents pMF. ***B***, Summary data for Δ phrenic amplitude in rats given methysergide (inverted gray triangle) or methysergide + MSX-3 (gray circle) and exposed to mASH or methysergide time controls (no ASH, filled circle). Rats given MSX-3 and exposed to mASH (from Fig. 1, open circle) are shown for comparison. ***C***, Summary data for change in phrenic burst frequency (Δ frequency) following mASH exposure in the same groups. $n = 8$, methysergide; $n = 7$, methy + MSX-3; $n = 7$, methysergide time control (no ASH). *Significant difference versus methysergide + MSX-3 + mASH. #Significant difference versus methysergide time controls (no ASH). †Significant difference versus methysergide + mASH. All significant differences are $p < 0.05$.

Short-term hypoxic responses

ASH increased phrenic nerve burst amplitude versus baseline or time controls ($p \leq 0.002$; Fig. 2). Vehicle, MSX-3, or methysergide injections had no significant effects on phrenic nerve burst amplitude during ASH ($p > 0.05$; Fig. 2). However, rats injected with methysergide plus MSX-3 exposed to mASH showed a significantly decreased phrenic burst amplitude response versus rats injected with vehicle ($p = 0.047$) or methysergide ($p = 0.044$) and exposed to mASH (Fig. 2). Phrenic responses to severe ASH were greater than responses to mASH; rats injected with vehicle, MSX-3, or methysergide and exposed to sASH exhibited significantly greater amplitude response versus the same drug groups during moderate ASH ($p \leq 0.036$; Fig. 2), similar to the short-term hypoxic phrenic response in a prior study comparing mAIH versus sAIH (Nichols et al., 2012). No significant differences in the short-term hypoxic phrenic response were found between rats injected with vehicle, MSX-3, or methysergide during sASH ($p > 0.05$; Fig. 2).

Spinal A_{2A} receptor blockade reveals pMF following moderate ASH

Compressed traces of phrenic neurograms illustrate phrenic burst amplitude before, during, and after mASH in rats pretreated with intrathecal injections of vehicle or MSX-3 (A_{2A} receptor antagonist; injections indicated in Fig. 3*A*, black arrows). In rats given vehicle injections and exposed to mASH, there was no significant pMF versus vehicle time controls (15 ± 4% vs 10 ± 3%, respectively; $p = 0.40$; Fig. 3*B*), in agreement with previous reports from our laboratory (Baker and Mitchell, 2000; Wilkerson et al., 2008). In contrast, rats given intrathecal MSX-3 exhibited significant pMF following mASH (39 ± 5%), an effect significantly greater than rats injected with vehicle and exposed to mASH (15 ± 4%, $p < 0.001$; Fig. 3*B*), or MSX-3 time controls (0 ± 3%, $p < 0.001$; Fig. 3*B*). There was a slight, but significant, increase in phrenic burst frequency (frequency LTF) at 60 min after ASH in rats injected with MSX-3 and exposed to mASH (3.2 ± 1.5 bursts/min) versus time controls (−1.4 ± 2.8 bursts/min at 60 min, $p = 0.007$; Fig. 3*C*), but not versus rats given vehicle injections and exposed to mASH (0.4 ± 1.8 bursts/min, $p = 0.06$; Fig. 3*C*). Small, significant changes in frequency following hypoxic exposures are consistent with findings of other studies on pMF/pLTF (Baker-Herman and Mitchell, 2002, 2008; Nichols et al., 2012).

Spinal serotonin receptor blockade does not reveal pMF following moderate ASH

Intrathecal methysergide injections before mASH revealed a slight but significant increase in phrenic burst amplitude (17 ± 7%) versus methysergide time controls (1 ± 3%, $p = 0.007$; Fig. 4*B*). However, rats injected with methysergide and then exposed to mASH were not significantly different from rats injected with vehicle and exposed to moderate ASH (15 ± 4%, $p = 0.688$), suggesting that spinal serotonin receptor blockade with methysergide does not reveal pMF following mASH. However, in addition to its effects on 5-HT_2 receptors, it blocks multiple other serotonin receptor subtypes that may contribute to the adenosine-dependent S pathway to pMF, such as 5-HT_7 receptors (Hoffman et al., 2011). There was no significant phrenic long-term facilitation in these groups ($p > 0.05$, Fig. 4*C*).

pMF following A_{2A} blockade and moderate ASH is serotonin-dependent

Compressed phrenic neurograms illustrate phrenic burst amplitude before, during, and after mASH in rats pretreated with intrathecal methysergide, MSX-3 or both (methysergide injections denoted by black arrows; MSX-3 injections denoted by gray arrows; Fig. 4*A*). Methysergide prevented pMF in rats given MSX-3 and mASH (0 ± 6%, $p < 0.001$ vs MSX-3 + mASH and $p > 0.05$ vs time controls; Fig. 4*B*), demonstrating that mASH-induced pMF revealed by A_{2A} receptor inhibition is serotonin-dependent. Thus, MSX-3 revealed pMF is similar to pLTF following mild or moderate AIH (Bach and Mitchell, 1996; Fuller et al., 2001b; Baker-Herman and Mitchell, 2002; Nichols et al., 2012) (PaO_2 35–55 mmHg). Methysergide also prevented frequency LTF in rats given MSX-3 plus mASH (−1.4 ± 1.6 bursts/min; $p = 0.005$ vs MSX-3 + mASH, $p > 0.05$ vs all other groups; Fig. 4*C*); this effect is consistent with previous reports concerning the impact of spinal methysergide on frequency LTF in previous studies of

mild and moderate AIH (Baker-Herman and Mitchell, 2002; Nichols et al., 2012).

Severe ASH causes pMF via an A_{2A}-dependent mechanism

Compressed phrenic neurograms illustrate phrenic burst amplitude before, during, and after sASH in rats pretreated with vehicle, MSX-3, or methysergide (injections denoted in Fig. 5*A*, black arrows). Vehicle-injected rats exhibited significant pMF following sASH (53 ± 12%) versus vehicle (10 ± 3%, $p < 0.001$) or drug time controls (MSX-3 or methysergide, 1 ± 2%; $p < 0.001$; Fig. 5*B*). In contrast to mASH, rats given MSX-3 and sASH no longer exhibit significant pMF versus vehicle (10 ± 3%, $p = 0.966$) or drug time controls (MSX-3 or methysergide, 1 ± 2%; $p = 0.394$; Fig. 5*B*). Thus, sASH-induced pMF requires spinal A_{2A} receptor activation, similar to sAIH-induced pLTF (Nichols et al., 2012). Significant frequency LTF was not observed in rats pretreated with vehicle or MSX-3 and sASH ($p > 0.05$; Fig. 5*C*), unlike one earlier study on sAIH (Nichols et al., 2012). This discrepancy might be due to pattern-sensitive effects of severe hypoxia on frequency LTF, rat strain differences, or variability between studies because frequency LTF is typically small and inconsistent (Baker-Herman and Mitchell, 2008).

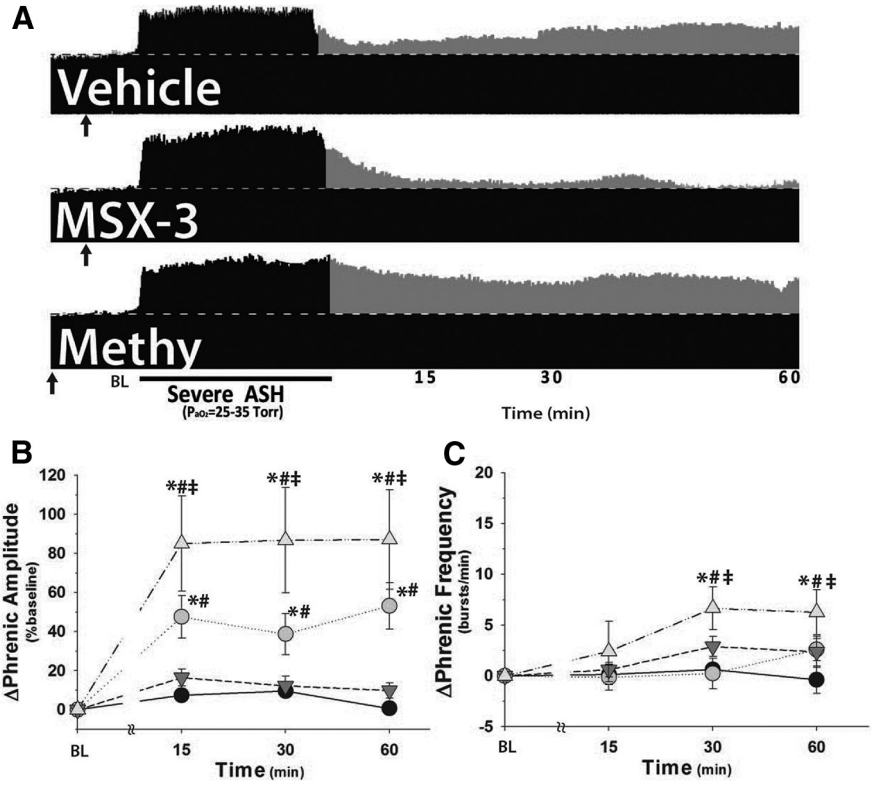

Figure 5. sASH elicits A_{2A} receptor-dependent pMF, which is enhanced by pretreatment with spinal 5-HT receptor blockade (20 mM methysergide, 20 min prior ASH; methy). ***A***, Representative phrenic neurograms after sASH (PaO_2 = 25–35 mmHg, 25 min) from rats given intrathecal injections of vehicle, MSX-3, or methysergide (↑). Dotted line indicates baseline. Gray represents pMF. ***B***, Summary data for Δ phrenic amplitude in rats given vehicle (gray circle), MSX-3 (inverted gray triangle), or methysergide (gray triangle) and exposed to sASH or no hypoxia (time controls; methysergide or MSX-3 injections, filled circle). ***C***, Summary data for change in phrenic burst frequency (Δ frequency) for the same groups. $n = 8$, vehicle; $n = 8$, MSX-3; $n = 6$, methysergide; $n = 13$, time controls. *Significant difference versus time controls. #Significant difference versus MSX-3 + sASH. ‡Significant difference versus vehicle + sASH. All significant differences are $p < 0.05$.

Spinal serotonin receptor blockade enhances sASH-induced pMF

Rats pretreated with spinal methysergide (Fig. 4*A*, black arrow) exhibited significantly higher pMF (87 ± 26%) versus vehicle-injected rats after sASH (53 ± 12%, $p = 0.011$; Fig. 5*B*). Enhanced pMF was present at each time after sASH ($p \leq 0.011$ vs vehicle + sASH), suggesting that the enhancement occurs during sASH per se. In contrast, pLTF in rats exposed to sAIH is unaffected by spinal methysergide (Nichols et al., 2012). Spinal methysergide revealed a slight, but significant, frequency LTF (6.2 ± 2.2 bursts/min) versus drug time controls (MSX-3 or methysergide, −0.4 ± 1.3 bursts/min; $p < 0.001$; Fig. 5*C*), or vehicle-injected rats exposed to sASH (2.6 ± 1.1 bursts/min, $p = 0.049$; Fig. 5*C*).

sASH elicits pMF, but moderate ASH does not

Although mASH did not elicit significant pMF (15 ± 4%) in vehicle-injected rats (vs time controls), sASH did (severe ASH: 53 ± 12%; time controls: 10 ± 3%, $p < 0.001$). Responses to mAIH and sAIH were significantly different from one another ($p < 0.001$; Fig. 6*A*), suggesting that the severity of hypoxia in addition to pattern (intermittent vs sustained) is a key factor regulating phrenic motor plasticity.

Discussion

The fundamental conclusion of this study is that both moderate and severe hypoxia evoke pMF via competing serotonin- and adenosine-dependent mechanisms. Interactions between these pathways ultimately determine pMF expression. The magnitude of adenosine and serotonin-dependent pathway activation, and their relative contributions to pMF depend on the pattern and severity of hypoxia. Our findings are summarized in a simplified model (Fig. 6). During mASH, serotonin and adenosine receptor activation are balanced, preventing pMF due to offsetting cross talk inhibition (Fig. 6*B*). Thus, by blocking spinal A_{2A} receptors during mASH, a serotonin-dependent pMF is revealed (Fig. 6*C*). This pMF revealed by MSX-3 is serotonin dependent because it was blocked by methysergide, similar to serotonin-dependent, mAIH-induced pLTF (Fig. 6*D*).

In contrast to mASH, sASH elicits A_{2A}-dependent pMF (Fig. 6*E*,*F*); because sASH induced pMF is enhanced by spinal methysergide, serotonin receptor activation inhibits A_{2A} receptor-dependent pMF (Fig. 6*G*). Together, these results suggest mutual inhibitory interactions between serotonin- and adenosine-dependent pathways to pMF, and that these inhibitory interactions modulate pMF with both mAIH and sASH. We conclude that pattern sensitivity in hypoxia-induced pMF/pLTF occurs when the cross talk inhibition is balanced and offsetting, negating the expression of either mechanism (i.e., no pMF). The offsetting effects (i.e., pattern sensitivity) can be explained by inhibitory interactions between serotonin- and adenosine-dependent pathways to pMF. In contrast, severe ASH induces pMF whether delivered as AIH or ASH, likely because adenosine receptor activation exceeds serotonin receptor activation in both cases (for clarification of this concept, see Fig. 1*A*).

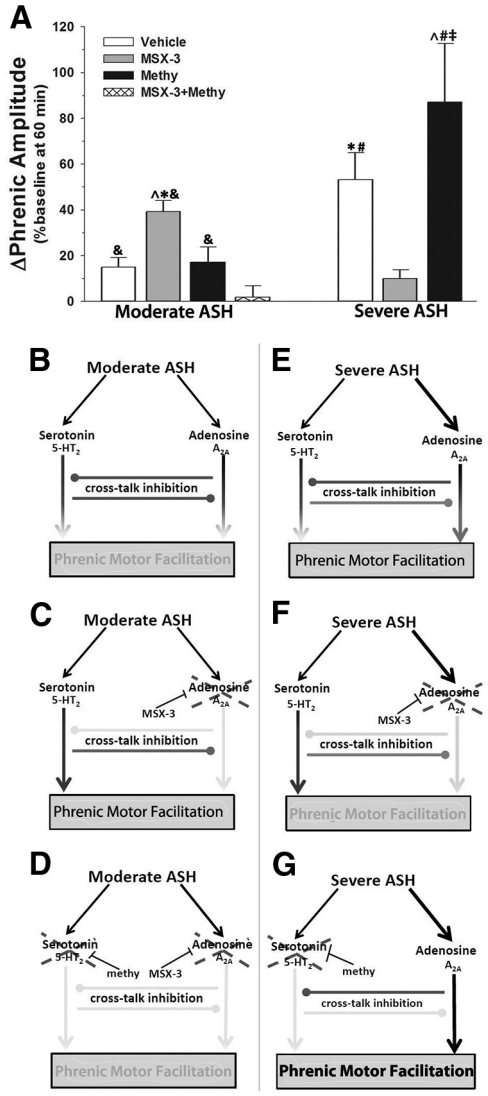

Figure 6. Competing 5-HT_2/A_{2A}-dependent mechanisms could explain pattern sensitivity of pMF induced by moderate hypoxia. ***A***, Summary of pMF following mASH or sASH in rats treated with vehicle, 200 mM MSX-3, 20 mM methysergide, or 20 mM methysergide + 200 mM MSX-3. ***B***, Proposed 5-HT_2/A_{2A} interactions predict magnitude of pMF. ↓ indicates activation. Gray line with gray circle represents inhibition. Gray line with intersecting bar represents blockade by intrathecal drug. During mASH, balanced activation of 5-HT_2- and A_{2A}-dependent pathways allows for cross talk inhibition to constrain expression of pMF. ***C***, Spinal blockade of A_{2A} pathway with MSX-3 during mASH prevents cross talk inhibition of the 5-HT_2 pathway, revealing pMF. ***D***, Blockade of serotonin receptors with methysergide (methy) before A_{2A} receptor blockade and mASH exposure prevents pMF, demonstrating that cross talk inhibition from A_{2A}-dependent pathway restrains 5-HT_2-dependent pMF during mASH. ***E***, sASH causes pMF because of greater relative A_{2A} receptor activation, overcoming cross talk inhibition. ***F***, Blockade of spinal A_{2A} receptors prevents expression of pMF following severe ASH, demonstrating that pMF following severe ASH is A_{2A}-dependent. ***G***, Blockade of spinal serotonin receptors prevents cross talk inhibition of the A_{2A} pathway during severe ASH, causing enhanced pMF. *Significant versus vehicle + mASH. ^Significant versus methysergide + mASH. &Significant versus methysergide + MSX-3 + mASH. #Significant versus MX3 + sASH. ‡Significant versus vehicle + sASH.

Serotonin receptor activation during sASH constrains A_{2A}-dependent pMF

Although several studies demonstrated that A_{2A} receptors inhibit 5-HT_2 receptor-dependent pMF following mAIH (Hoffman et al., 2010; Nichols et al., 2012; Navarrete-Opazo and Mitchell, 2014), less information is available concerning the impact of serotonin receptors on adenosine-dependent pMF with sAIH or sASH. Here we demonstrate that spinal serotonin receptor activation with sASH constrains A_{2A}-dependent pMF (Fig. 4). However, both A_{2A} (Golder et al., 2008; Hoffman et al., 2010) and 5-HT_7 receptors (Hoffman and Mitchell, 2011, 2013) activate G_s-coupled receptors (S pathway) and constrain G_q-protein coupled receptor-dependent pLTF (Q pathway) following mAIH (Dale-Nagle et al., 2010). Because methysergide is a potent inhibitor of both 5-HT_2 (G_q) and 5-HT_7 (G_s) receptors (Peroutka, 1988; Villalón et al., 1997), we may have underestimated the magnitude of serotonin (5-HT_2) receptor inhibition of severe hypoxia-induced pMF because methysergide is also expected to reduce 5-HT_7 contributions to pMF with severe hypoxia. Nevertheless, inhibitory interactions between serotonin- and adenosine-dependent pathways to pMF appear to be bidirectional, assuring that either serotonergic or adenosinergic contributions to pMF predominate, except when these cross talk interactions are balanced and offsetting, cancelling pMF.

Because methysergide did not reveal significant pMF following mASH (Fig. 5*A*), A_{2A} receptor activation alone is not sufficient to elicit pMF with mASH. However, the failure of methysergide to reveal pMF after mASH could be due to known methysergide effects on 5-HT_7 receptors (see above). These receptors signal differently from 5-HT_2 receptors that underlie mAIH-induced pLTF (Fuller et al., 2001b; Macfarlane et al., 2011) but signal more like the G_s-coupled A_{2A} receptors. Indeed, 5-HT_7 receptor activation alone elicits pMF (Hoffman and Mitchell, 2011) and constrains mAIH-induced pMF (Hoffman and Mitchell, 2013). Because A_{2A} and 5-HT_7 receptors activate similar (cAMP-dependent) signaling cascades, coactivation may be necessary for pMF following mASH. Because 5-HT_7 receptors may contribute to the A_{2A}-dependent pMF with mASH, selective 5-HT_2 receptor inhibition (e.g., ketanserin) during mASH may reveal pMF.

Cross talk interactions via downstream signaling molecules

Inhibitory coupling between A_{2A} and 5-HT_2 receptor-induced pMF likely occurs via downstream signaling cascades, such as protein kinase C and adenylate cyclase activation. Because spinal protein kinase A (PKA) attenuates, and PKA inhibition enhances mAIH-induced pLTF (Hoffman and Mitchell, 2013), PKA is essential for S (A_{2A}, 5-HT_7) to Q pathway inhibition (5-HT_2). Alternative cAMP signaling underlies the S pathway to pMF, specifically EPAC (Fields et al., 2015). In contrast, 5-HT_2-dependent pMF following mAIH requires protein kinase Cθ activity (Devinney et al., 2015). Cross talk interactions between PKC and PKA pathways are known in other model systems (e.g., *Aplysia*) where PKC constrains PKA activity and prevents sensorimotor long-term facilitation (Farah et al., 2009).

PKA also negatively regulates PKC activity via modulation of reactive oxygen species formation. In cell cultures, PKA decreases ROS production by phosphorylating the NADPH oxidase A1 subunit (Kim et al., 2007), or via downregulation of the p47 subunit (Bengis-Garber and Gruener, 1996). NADPH oxidase activity is required for pLTF induced by mAIH, most likely because reactive oxygen species inhibit constraining protein phosphatases (Wilkerson et al., 2007; Macfarlane et al., 2009). Multiple inhibitory cross talk interactions may occur between PKC-dependent (e.g., 5-HT_2) and PKA-dependent (e.g., A_{2A} and 5-HT_7 receptor) signaling, allowing for multilevel control of different cellular pathways to pMF.

Pattern sensitivity

Patterned stimuli have a profound influence on the expression of plasticity. The benefits of "spaced" training on long-term mem-

ory formation have long been appreciated and are being actively explored to refine therapeutic approaches to learning disorders (Ebbinghaus, 1913; Philips et al., 2013a). However, the cellular and molecular correlates of pattern sensitivity have seldom been explored and have been mostly studied in invertebrate model systems, such as sensorimotor long-term facilitation in *Aplysia* (Mauelshagen et al., 1998). Studies of pattern sensitivity in pLTF demonstrated that okadaic acid-sensitive serine/threonine phosphatases constrain (and prevent) serotonin-dependent pMF with mASH, suggesting that serotonin-dependent mechanisms may be activated but undermined by concurrent phosphatase activity (Wilkerson et al., 2007, 2008).

Here, we present evidence for a novel mechanism of pattern sensitivity in spinal neuroplasticity: mutual, inhibitory coupling between competing 5-HT_2 and A_{2A}-induced signaling cascades. Pattern sensitivity in *Aplysia* sensorimotor long-term facilitations occurs at multiple levels, including G-proteins (Ye et al., 2008), temporal integration of PKA and MAPK signaling (Philips et al., 2013b), and PKA inhibition from increased PKC activity (Farah et al., 2009). An emerging principle from all of these studies is that pattern sensitivity arises from inhibitory interactions between signaling molecules involved in different forms of plasticity. The present study is consistent with this principle and indicates that pattern sensitivity in hypoxia-induced pMF emerges from inhibitory coupling between G_q and G_s protein-coupled signaling cascades.

Some forms of memory and neuroplasticity lack apparent pattern sensitivity. For example, short-term memory, which lasts on the order of minutes, is not subject to spacing effects (Xia et al., 1998). Similarly, traumatic/emotional memories (e.g., flashbulb memory) are induced by a single event and form long-term memories via mechanisms distinct from long-term memories produced by spaced trials (Irvine et al., 2005, 2006; Diamond et al., 2007). Plasticity without apparent pattern sensitivity may allow storage of critical memories following a single trial of intense stimuli, such as remembering never to touch a hot stove again.

Here, sASH elicited pMF by a mechanism insensitive to the pattern of hypoxia. We postulate that sASH causes greater extracellular adenosine accumulation and, thus, greater A_{2A} receptor activation (vs 5-HT_2 receptors; Fig. 1*A*). This pattern insensitivity might elevate respiratory motor output after even a single, intense hypoxic episode, analogous to touching a hot stove. Similar to "flashbulb memories" after traumatic events, sASH-induced plasticity may permit significant adaptation in response to life-threatening events, such as transient respiratory failure, severely hypoxic environments, or end stages of respiratory disease. Pattern-insensitive plasticity may allow more rapid and complete adaptations in response to such life-threatening events.

Clinical relevance

In recent years, we have elucidated multiple mechanisms, each capable of giving rise to spinal, respiratory plasticity. We are just now beginning to appreciate how these mechanisms interact to influence the expression of plasticity after a given stimulus (including its pattern, severity, and duration). We demonstrate that pharmacological inhibition of spinal A_{2A} receptors disrupts cross talk inhibition, revealing pMF following mASH, a stimulus that does not ordinarily trigger pMF (Baker and Mitchell, 2000; Wilkerson et al., 2008). Furthermore, sASH elicits A_{2A}-dependent pMF, an effect enhanced by spinal serotonin receptor inhibition. A detailed understanding of mechanisms giving rise to pMF may guide future clinical applications based on the ability to elicit motor plasticity with hypoxia and/or small molecules that manipulate this intricate system (Mahamed and Mitchell, 2007). Such therapeutic approaches are currently in development for diverse clinical disorders that challenge respiratory (Mahamed and Mitchell, 2007; Lovett-Barr et al., 2012; Nichols et al., 2013) and nonrespiratory motor function (Trumbower et al., 2012; Hayes et al., 2014), including spinal cord injury, amyotrophic lateral sclerosis, and sleep apnea.

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