

Spinal adenosine A_{2A} receptor inhibition enhances phrenic long term facilitation following acute intermittent hypoxia

M. S. Hoffman, F. J. Golder, S. Mahamed and G. S. Mitchell

Department of Comparative Biosciences, School of Veterinary Medicine, University of Wisconsin, 2015 Linden Drive, Madison, WI 53706, USA

Phrenic long term facilitation (pLTF) is a form of respiratory plasticity induced by acute intermittent hypoxia. pLTF requires spinal serotonin receptor activation, new BDNF synthesis and TrkB receptor activation. Spinal adenosine 2A (A_{2A}) receptor activation also elicits phrenic motor facilitation, but by a distinct mechanism involving new TrkB synthesis. Because extracellular adenosine increases during hypoxia, we hypothesized that A_{2A} receptor activation contributes to acute intermittent hypoxia (AIH)-induced pLTF. A selective A_{2A} receptor antagonist (MSX-3, 8 μg kg⁻¹, 12 μl) was administered intrathecally (C4) to anaesthetized, vagotomized and ventilated male Sprague–Dawley rats before AIH (three 5 min episodes, 11% O₂). Contrary to our hypothesis, pLTF was greater in MSX-3 versus vehicle (aCSF) treated rats (97 ± 6% vs. 49 ± 4% at 60 min post-AIH, respectively; *P* < 0.05). MSX-3 and aCSF treated rats did not exhibit facilitation without AIH (time controls; 7 ± 5% and 9 ± 9%, respectively; *P* > 0.05). A second A_{2A} receptor antagonist (ZM2412385, 7 μg kg⁻¹, 7 μl) enhanced pLTF (85 ± 11%, *P* < 0.05), but an adenosine A₁ receptor antagonist (DPCPX, 3 μg kg⁻¹, 10 μl) had no effect (51% ± 8%, *P* > 0.05), indicating specific A_{2A} receptor effects. Intrathecal methysergide (306 μg kg⁻¹, 15 μl) blocked AIH-induced pLTF in both MSX-3 and aCSF treated rats, confirming that enhanced pLTF is serotonin dependent. Intravenous MSX-3 (140 μg kg⁻¹, 1 ml) enhanced both phrenic (104 ± 7% vs. 57 ± 5%, *P* < 0.05) and hypoglossal LTF (46 ± 13% vs. 28 ± 10%; *P* < 0.05). In conclusion, A_{2A} receptors constrain the expression of serotonin-dependent phrenic and hypoglossal LTF following AIH. A_{2A} receptor antagonists (such as caffeine) may exert beneficial therapeutic effects by enhancing the capacity for AIH-induced respiratory plasticity.

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Corresponding author G. S. Mitchell: Department of Comparative Biosciences, 2015 Linden Drive, Madison, WI 53706-1102, USA. Email: mitchell@svm.vetmed.wisc.edu

Abbreviations A_{2A}, adenosine 2A; AIH, acute intermittent hypoxia; PMF, phrenic motor facilitation; ROS, reactive oxygen species; pLTF, phrenic long-term facilitation; XII, hypoglossal.

Introduction

Plasticity is a fundamental property of the respiratory control system (Mitchell & Johnson, 2003). One frequently studied model of respiratory plasticity is phrenic long-term facilitation (pLTF) following acute intermittent hypoxia (AIH; Bach & Mitchell, 1996; Baker & Mitchell, 2000). Although considerable recent progress has been made towards an understanding of cellular/synaptic mechanisms giving rise to pLTF (Feldman *et al.* 2003; Mitchell & Johnson, 2003), our understanding remains incomplete.

Current evidence supports a mechanism whereby spinal serotonin receptor activation initiates (Baker-Herman & Mitchell, 2002), but does not maintain, AIH-induced

pLTF (Fuller *et al.* 2001*b*). Specifically, Gq protein-coupled metabotropic serotonin 2 (5-HT₂) receptor activation on phrenic motor neurons is believed to activate downstream kinases (e.g. PKC), leading to new protein synthesis, including new synthesis of brain derived neurotrophic factor (BDNF) (Baker-Herman & Mitchell, 2002; Feldman *et al.* 2003; Baker-Herman *et al.* 2004; Feldman *et al.* 2005). Spinal BDNF subsequently elicits pLTF by activation of its high affinity receptor, TrkB (Baker-Herman *et al.* 2004).

Although serotonergic signalling initiates cellular events underlying pLTF, we recently reported an alternative mechanism of long-lasting phrenic motor facilitation that involves TrkB trans-activation (Golder *et al.* 2008). Specifically, activation of spinal G_s protein-coupled metabotropic adenosine 2A (A_{2A}) receptors stimulates

new synthesis and phosphorylation of an immature TrkB isoform within phrenic motor neurons, thereby inducing phrenic motor facilitation (Golder *et al.* 2008).

Since moderate hypoxia increases extracellular adenosine levels *via* active transport and ATP degradation (Winn *et al.* 1981; Gourine *et al.* 2002), we hypothesized that adenosine release (and subsequent A_{2A} receptor activation) contributes to AIH-induced pLTF expression. To test this hypothesis, we delivered intrathecal, cervical injections of adenosine receptor antagonists to localize effective drug concentrations to the vicinity of phrenic motor nuclei. A similar approach has been used extensively to study spinal mechanisms of pain perception (Yaksh & Rudy, 1977) and AIH-induced pLTF (Baker-Herman & Mitchell, 2002; Macfarlane & Mitchell, 2007; Wilkerson *et al.* 2008). An experimental advantage of this approach is the ability to bathe both phrenic motor nuclei (which span four spinal segments; Goshgarian & Rafols, 1981) with drug, while preventing effective drug concentrations elsewhere in the central nervous system. Simultaneous recordings of phrenic and hypoglossal nerve activity provide an internal control for unintended drug distribution (Baker-Herman & Mitchell, 2002).

Contrary to our hypothesis, we discovered a novel mechanism whereby spinal adenosine A_{2A} receptors constrain (*versus* contribute to) AIH-induced pLTF. A_{2A} receptor modulation of pLTF may provide unique insights concerning fundamental cellular/synaptic mechanisms regulating AIH-induced respiratory plasticity.

Methods

Animals

All experiments were performed on adult (3–6 months) male Sprague–Dawley rats (303–390 g, colonies 236/217/218a; Harlan Inc., Indianapolis, IN, USA). The University of Wisconsin Animal Care and Use committee approved all experimental protocols performed in this study.

In the first series of experiments we tested the hypothesis that spinal adenosine A_{2A} receptors contributed to AIH-induced pLTF by using spinal application of a selective receptor antagonist. The second series was designed to confirm that A_{2A} receptor inhibition *per se* was responsible for enhanced pLTF. Therefore, a distinct A_{2A} receptor antagonist was used to confirm our results, and contrasted with spinal administration of an A_1 receptor antagonist. The third experimental series tested whether spinal A_{2A} receptor antagonist-enhanced pLTF required spinal serotonin receptor activity, confirming that the mechanism is similar to pLTF in normal animals. In the final experimental series, we tested whether systemic (intravenous) A_{2A} receptor antagonist administration enhanced both phrenic and hypoglossal LTF.

Surgical preparation

Anaesthesia was induced with isoflurane (2.5–3.5% in 50% O_2 , balance N_2), and isoflurane was continued during surgical preparations. Once surgical procedures (described below) had been completed, rats were converted to urethane anaesthesia over 15 min *via* intravenous injection (1.75 g kg^{-1}). Adequate anaesthetic depth was tested by lack of any pressor or respiratory neural response to toe pinch with a haemostat. After conversion to urethane anaesthesia, a continuous intravenous infusion ($4\text{--}6.5 \text{ ml kg}^{-1} \text{ h}^{-1}$) of a 1:4 mixture of 6% hetastarch and lactated Ringer solution was implemented to maintain fluid balance and acid–base status. A tracheal cannula was placed in the neck to enable artificial ventilation (Rodent Respirator, model 683, Harvard Apparatus, Holliston, MA, USA; tidal volume = 2.5 ml). A rapidly responding flow-through carbon dioxide analyser (Capnogard, Novamatrix, Wallingford, CT, USA) was placed on the expired limb of a Y-tube connected to the tracheal cannula to enable measurements of end-tidal CO_2 partial pressures (P_{ETCO_2}). The vagus nerves were cut in the mid-cervical region to prevent entrainment of respiratory neural activity with the ventilator. During ventilation, rats were subjected to neuromuscular blockade with pancuronium bromide (2.5 mg kg^{-1} , i.v.). A polyethylene catheter (PE50, Intra-medical) was placed in the right femoral artery and blood pressure was monitored with a pressure transducer (Gould, P23ID). A three-way stop-cock, attached to the arterial catheter, was used to withdraw blood samples (0.2–0.4 ml) for blood gas analysis (ABL-500, Radiometer; Copenhagen, Denmark); during an experiment, blood gas measurements were made during baseline, during the first hypoxic episode, and at 15, 30 and 60 min post-intermittent hypoxia. Body temperature was monitored with a rectal thermometer (Fisher Scientific, Pittsburg, PA, USA) and maintained ($37.5 \pm 1^\circ\text{C}$) with a heated surgical table.

The left phrenic and hypoglossal nerves were isolated using a dorsal approach, cut distally, desheathed and placed on bipolar silver electrodes to record respiratory neural activity. Phrenic and XII nerve signals were amplified ($100\,000\times$), band-pass filtered (300–10 000 Hz, Model 1800, A-M Systems, Carlsborg, WA, USA), rectified and integrated (Paynter filter; time constant, 50 ms, MA-821, CWE Inc., Ardmore, PA, USA). The resulting integrated nerve bursts were digitized (8 kHz) and analysed using a WINDAQ data acquisition system (DATAQ Instruments, Akron, OH, USA).

In some rats, the spinal column was exposed dorsally, followed by laminectomy at cervical level 2 (C2), where a small incision was made in dura. A soft silicone catheter (2 French; Access Technologies, Skokie, IL, USA) was inserted caudally through the incision until tip was located at

approximately C4. The catheter was attached to a 50 μ l Hamilton syringe filled with adenosinergic drug solutions or vehicle to allow localized drug injections into the cervical spinal cord. In experiments using a serotonin receptor antagonist, a second catheter was placed to allow drug administration at different times. The final surgical step was placement of the intrathecal catheter. Rats were then converted to urethane anaesthesia and allowed a minimum of 1 h to stabilize before beginning experimental protocol. Upon completion of experimental protocols, all animals were killed by urethane overdose.

Experimental protocol

The experimental phase began at least 1 h after conversion to urethane anaesthesia when apnoeic and recruitment thresholds were determined by lowering P_{ETCO_2} and increasing ventilation until rhythmic nerve bursts could no longer be detected (apnoeic threshold). After approximately 1 min, the ventilator rate was slowly decreased or inspired CO_2 was slowly increased until rhythmic nerve bursts resumed (i.e. recruitment threshold). Baseline conditions were then established by holding P_{ETCO_2} 2 mmHg above the recruitment threshold and allowing for stable neural activity (≥ 15 min). At this point an arterial blood sample was taken to document baseline blood gas levels. Throughout the protocol, arterial P_{CO_2} was maintained within ± 1.5 mmHg of baseline levels by manipulation of inspired CO_2 and/or ventilation rate. Subsets of rats (see Spinal treatment groups for details of n values) received intrathecal injections of vehicle solution (artificial cerebral spinal fluid (aCSF; mM): 120 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 23 NaHCO₃, 10 glucose, pH 7.3–7.45), 10% saline or drug (see below) slowly injected over 1 min, 5 min prior to first hypoxic episode (or equivalent time for control groups). Acute hypoxic exposures consisted of three 5 min episodes of isocapnic (± 1.5 mmHg) hypoxia (11% inspired O_2 , $P_{\text{aO}_2} = 35\text{--}45$ mmHg), separated by 5 min intervals of baseline oxygen levels (51% inspired O_2 , $P_{\text{aO}_2} \geq 150$ mmHg). After the third hypoxic episode, rats were returned to baseline inspired oxygen levels and maintained for the duration of an experiment. To test the hypothesis that spinal $\text{A}_{2\text{A}}$ receptors contributed to AIH-induced pLTF, a selective $\text{A}_{2\text{A}}$ receptor antagonist (MSX-3, Sigma-Aldrich) was injected *via* an intrathecal catheter over the cervical spinal cord (C3–5). In this series of experiments, MSX-3 was dissolved in aCSF (10% saline) to an effective concentration determined from preliminary experiments (12 μ l, 490 μM ; 8 $\mu\text{g kg}^{-1}$), and delivered 5 min prior to AIH. To provide additional support for $\text{A}_{2\text{A}}$ receptor inhibition enhancing phrenic LTF, a distinct $\text{A}_{2\text{A}}$ receptor antagonist (ZM241385, 7 μ l, 1 mM; 10% DMSO/aCSF, 7 $\mu\text{g kg}^{-1}$; Tocris) and an adenosine A_1 receptor antagonist (8-cyclopentyl-1,3-dipropylxanthine

(DPCPX), 10 μ l, 330 μM , 10% DMSO/aCSF, 3 $\mu\text{g kg}^{-1}$; Tocris Bioscience, Ellisville, MO, USA) were administered identically to MSX-3 and/or vehicle (aCSF). To test whether serotonin receptor activity was required for enhanced pLTF, a subset of rats were exposed to AIH following pretreatment with the broad-spectrum serotonin receptor antagonist methysergide maleate (15 μ l, 20 mM, 300 $\mu\text{g kg}^{-1}$; Sigma-Aldrich), dissolved in aCSF (Baker-Herman & Mitchell, 2002); methysergide was administered 20 min prior to MSX-3 or vehicle injections. Control rats received either vehicle (aCSF, 12 μ l) or drug (MSX-3) 5 min prior to initiating an experimental protocol without AIH. The final series of experiments tested whether systemic MSX-3 injections could augment both phrenic and hypoglossal (XII) LTF; thus, rats received MSX-3 dissolved in saline (140 $\mu\text{g kg}^{-1}$, 1 ml) infused intravenously over 1 min 5 min prior to hypoxic exposure. Again, control rats received MSX-3, in the absence of hypoxia, 5 min prior to initiating a protocol. The following treatment groups were studied.

Spinal treatment groups: (1) vehicle (aCSF) + AIH ($n = 7$); (2) vehicle, no AIH ($n = 8$); (3) MSX-3 + AIH ($n = 8$); (4) MSX-3, no AIH ($n = 7$); (5) ZM241385 + AIH ($n = 5$); (6) DPCPX + AIH ($n = 5$). A second intrathecal catheter was placed in rats pretreated with methysergide; thus, a subset of control rats also received dual-catheter placement: (1) vehicle/MSX-3 + AIH ($n = 3$); (2) methysergide/MSX-3 + AIH ($n = 5$); (3) vehicle/vehicle + AIH ($n = 5$); (4) methysergide/vehicle + AIH ($n = 5$); (5) methysergide/MSX-3, no AIH ($n = 6$).

Systemic treatment groups: (1) vehicle (saline) + AIH ($n = 9$); (2) MSX-3 + AIH ($n = 12$); (3) MSX-3, no AIH ($n = 9$).

Data analysis

Integrated phrenic and hypoglossal nerve burst amplitudes and burst frequencies were averaged over 1 min bins at each experimental time point (baseline, 15, 30 and 60 min). Nerve burst amplitudes were normalized by subtracting from the baseline value, then dividing by the baseline and reported as percentage change from baseline. Burst frequencies were also normalized to baseline, but expressed as an absolute difference in bursts per minute. All statistical comparisons between treatment groups for nerve amplitude (15, 30 and 60 min time points) were made using a two-way ANOVA with a repeated measures design. Since no differences were detected between hypoxic exposures within groups (data not shown), comparisons were made using two-way ANOVA of phrenic burst amplitude during the fifth minute of hypoxic episodes averaged from all three episodes. Nerve bursts from rats treated with methysergide (serotonin receptor antagonist), ZM241385 ($\text{A}_{2\text{A}}$ antagonist) and DPCPX

Table 1. Measurements of P_{aCO_2} , P_{aO_2} and mean arterial pressure (MAP) during baseline, hypoxia and 60 min post-hypoxia

	P_{aCO_2} (mmHg)			P_{aO_2} (mmHg)			MAP (mmHg)		
	baseline	hypoxia	60 min	baseline	hypoxia	60 min	baseline	hypoxia	60 min
Spinal Groups									
vehicle	41.4 ± 1.0	43.1 ± 0.8	41.2 ± 1.1	260 ± 7	35 ± 2†‡	234 ± 15	118 ± 7	97 ± 8	106 ± 8
vehicle (-AIH)	44.6 ± 1.2	45.5 ± 1.2	44.9 ± 1.1	274 ± 9	270 ± 7	272 ± 7	95 ± 4	101 ± 3	103 ± 4
MSX-3	41.9 ± 0.9	42.0 ± 1.0	41.5 ± 0.8	255 ± 8	38 ± 2†‡	247 ± 9	117 ± 7	80 ± 13†	103 ± 8
MSX3 (-AIH)	44.0 ± 1.6	42.8 ± 1.5	43.8 ± 1.3	260 ± 14	254 ± 15	260 ± 10	112 ± 5	103 ± 6	99 ± 6
DPCPX	43.8 ± 1.4	43.7 ± 1.1	44.1 ± 1.3	269 ± 2	40 ± 0†‡	266 ± 2	137 ± 8‡	108 ± 11†	118 ± 7
ZM241385	43.1 ± 1.5	43.3 ± 2.2	43.2 ± 1.5	264 ± 1	37 ± 0†‡	244 ± 2	108 ± 8	83 ± 19	93 ± 7
vehicle+vehicle	43.6 ± 1.2	44.2 ± 1.2	43.5 ± 1.0	272 ± 5	37 ± 2†	272 ± 9	116 ± 3	85 ± 10	101 ± 4
methy+vehicle	42.5 ± 1.1	45.3 ± 0.7	42.8 ± 1.2	279 ± 6	37 ± 1†	273 ± 7	124 ± 9	109 ± 10	118 ± 8
MSX-3+vehicle	44.5 ± 1.6	45.1 ± 1.6	44.7 ± 1.3	287 ± 8	37 ± 3	273 ± 6	108 ± 4	96 ± 10	92 ± 9
MSX-3+methy	42.1 ± 1.3	42.1 ± 1.5	43.4 ± 1.3	279 ± 12	43 ± 2†	266 ± 7	126 ± 9	110 ± 15	119 ± 9
Systemic Groups									
vehicle	43.0 ± 0.6	42.9 ± 1.0	43.4 ± 0.6	270 ± 5	36 ± 1†‡	256 ± 7	113 ± 6	110 ± 5	107 ± 8
MSX-3	43.8 ± 0.5	42.5 ± 0.9	44.2 ± 0.5	258 ± 7	35 ± 1†‡	242 ± 9	120 ± 4	98 ± 8†	103 ± 6†
vehicle (-AIH)	43.4 ± 0.5	42.2 ± 1.2	43.4 ± 0.5	257 ± 12	272 ± 7	265 ± 15	117 ± 5	113 ± 4	117 ± 5

Values are means ± S.E.M. MAP, mean arterial pressure. †Significant different than baseline; ‡significant different than vehicle (-AIH).

(A_1 antagonist) were compared at the 60 min post-AIH time point using a two-way ANOVA. Comparisons for mean arterial pressures, P_{aCO_2} and P_{aO_2} were made at baseline, hypoxia 1 and 60 min post-AIH using two-way ANOVA. All individual comparisons were made using the Student–Neuman–Keuls (SNK) *post hoc* test (Sigma-Stat version 2.03; Systat Software Inc., San Jose, CA, USA). Differences between groups were considered significant if $P < 0.05$. All values are expressed as means ± S.E.M.

Results

Blood gases and mean arterial pressures

Measurements of arterial P_{CO_2} and P_{O_2} during baseline conditions were similar in all experimental groups (see Table 1). Furthermore, in all groups, arterial P_{CO_2} was regulated successfully within 1.5 mmHg of its baseline value and arterial P_{O_2} remained above 150 mmHg (Table 1). Mean arterial pressure (MAP) was similar between all treatment groups at baseline, during hypoxia and 60 min post-AIH (Table 1). Thus P_{aCO_2} , P_{aO_2} and MAP were stable throughout the experimental protocol in all groups.

Short-term hypoxic responses

Spinally treated rats. Spinal injection of an A_{2A} receptor antagonist (MSX-3, 12 μ l) had no effect on baseline frequency or amplitude of peak integrated inspiratory phrenic or hypoglossal nerve bursts (data not shown), thus assuring that pLTF comparisons relative to baseline were appropriate. Typical phrenic neurograms

during experimental protocols for each treatment group are illustrated in Fig. 1. Hypoxia elicited a rapid increase in phrenic and hypoglossal burst amplitude in vehicle ($133 \pm 10\%$, $n = 7$; $180 \pm 48\%$, $n = 5$; respectively; $P < 0.001$) and MSX-3 treated rats ($184 \pm 12\%$, $n = 9$; $176\% \pm 16\%$, $n = 6$; respectively; $P < 0.001$; Fig. 2). Phrenic burst amplitude from MSX-3 treated rats was significantly greater than in vehicle-treated rats ($184 \pm 12\%$ versus $133 \pm 10\%$, respectively; $P = 0.007$); however, there was no difference in hypoglossal burst amplitude ($180 \pm 28\%$ versus $176 \pm 16\%$, respectively, $P = 0.816$; Fig. 2).

Systemically treated rats. Exposure to hypoxia elicited a rapid increase in phrenic and hypoglossal burst amplitudes in rats receiving intravenous vehicle (saline: $157 \pm 17\%$, $n = 9$; $151 \pm 20\%$, $n = 5$; respectively; $P < 0.001$) and MSX-3 ($177 \pm 23\%$, $n = 12$; $270 \pm 80\%$, $n = 4$; respectively; $P < 0.001$). However, in contrast to intrathecal drug administration, short-term hypoxic responses were not different between experimental groups for either nerve ($P > 0.15$; Fig. 2).

Spinal A_{2A} , but not A_1 receptors constrain AIH-induced pLTF

AIH-induced pLTF in vehicle treated rats (artificial CSF, 12 μ l; Figs 2 and 3A) is expressed as an increase in phrenic burst amplitude ($49 \pm 4\%$, 60 min post-AIH; $P < 0.001$; $n = 7$). Phrenic burst amplitude was also significantly greater in vehicle treated rats receiving AIH versus those

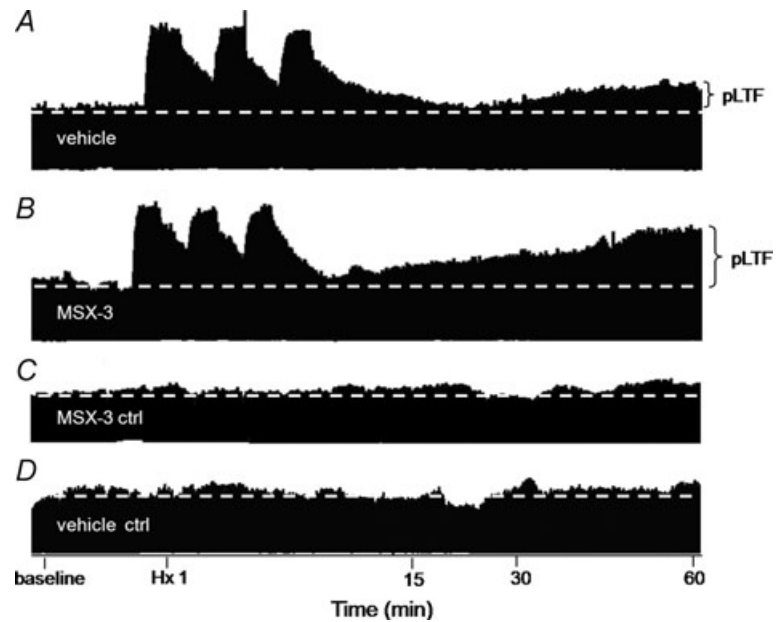


Figure 1. Representative phrenic neurograms depicting experimental protocols in rats from different treatment groups

A, intrathecal vehicle (aCSF) prior to AIH. *B*, intrathecal A_{2A} receptor antagonist (MSX-3) prior to AIH. *C*, time control receiving intrathecal MSX-3 without AIH. *D*, time control receiving intrathecal vehicle without AIH. MSX-3 pretreatment enhanced pLTF relative to AIH exposed rats given control, vehicle injections. Neither time control experiment exhibited pLTF.

not exposed to AIH (i.e. time controls: $9 \pm 5\%$, $P < 0.001$, $n = 8$; Fig. 3A) at 60 min post-AIH.

To test whether spinal G_s protein-coupled A_{2A} receptors contribute to pLTF, two distinct antagonists, MSX-3 ($12 \mu\text{l}$, $8 \mu\text{g kg}^{-1}$) and ZM241385 ($7 \mu\text{l}$, $7 \mu\text{g kg}^{-1}$), were administered to the intrathecal space of the cervical spinal cord (C3–5). Contrary to our original hypothesis, phrenic burst amplitude was increased above baseline levels at 60 min post-AIH (MSX-3: $97 \pm 10\%$, $n = 8$; Fig. 3A; ZM241385: $85 \pm 15\%$, $n = 5$, $P < 0.001$; Fig. 4A) and were significantly greater than vehicle-treated rats receiving AIH ($49 \pm 4\%$, $P < 0.001$; Fig. 3A) or controls not exposed to AIH (vehicle: $9 \pm 5\%$, $n = 8$; $P < 0.001$; MSX-3: $10 \pm 6\%$, $n = 7$; $P < 0.001$; Fig. 3A). However, no difference in phrenic burst amplitude was detected between MSX-3 and ZM241385 treatment at the 60 min time point ($P = 0.458$, Fig. 4A).

To address possible concerns that spinal A_{2A} receptor inhibition alone induces pLTF, rats were treated with intrathecal MSX-3 but without AIH ($n = 7$); in these rats, no significant increase in phrenic burst amplitude was observed at any time post-AIH (15 min: $13 \pm 8\%$, $P = 0.353$; 30 min: $13 \pm 8\%$, $P = 0.242$; 60 min: $10 \pm 6\%$, $P = 0.24$; Fig. 3A).

Since adenosinergic A_1 receptor inhibition increases synaptic transmission, reportedly via presynaptic disinhibition of neurotransmitter release (Bellingham & Berger, 1994; Dong & Feldman, 1995), we tested whether unintended effects of MSX-3 on A_1 receptors could account for enhanced pLTF. In rats pre-treated with an A_1 receptor antagonist (DPCPX, $10 \mu\text{l}$, $3 \mu\text{g kg}^{-1}$), phrenic burst amplitude was above baseline at 60 min post-AIH ($51 \pm 8\%$, $n = 6$; $P = 0.005$; Fig. 4A) and greater than in control rats receiving vehicle or MSX-3 without AIH

($9 \pm 5\%$, $n = 8$; $10 \pm 6\%$, $n = 7$; respectively; $P < 0.001$). Phrenic burst amplitude after A_1 receptor inhibition (DPCPX) was not different from vehicle ($49 \pm 4\%$, $P = 0.820$, $n = 7$) treatment at 60 min post-AIH, but was significantly lower than in A_{2A} antagonist treated rats

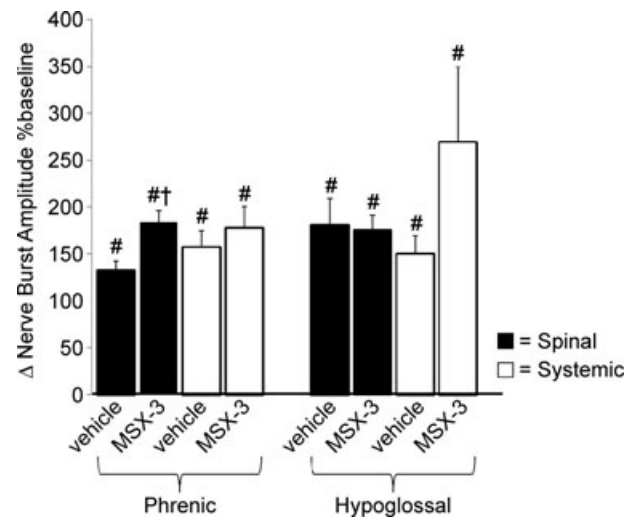


Figure 2. Hypoxic phrenic and hypoglossal responses during hypoxic episodes (i.e. short-term hypoxic response)

Changes in phrenic and hypoglossal (XII) burst amplitudes during final minute of hypoxic exposures (average of 3) from spinal (■) vehicle (aCSF; phr, $n = 7$; XII, $n = 5$) and A_{2A} receptor antagonist (MSX-3; phr, $n = 8$; XII, $n = 6$); systemic (□) vehicle (saline; phr, $n = 9$; XII, $n = 5$) and MSX-3 (Phr, $n = 12$; XII, $n = 4$) treated rats. Phrenic burst amplitudes from spinal MSX-3 treated rats were increased versus vehicle treated rats, but not in rats with systemic MSX-3 administration. Values are means \pm s.e.m. #Different from baseline; †different from vehicle, ANOVA, $P < 0.05$.

following AIH (MSX-3: $97 \pm 6\%$, $n = 8$; and ZM241385: $85 \pm 15\%$, $n = 5$; $P < 0.001$; Fig. 4A). Together, these results confirm that A_{2A} , and not A_1 , receptors are the relevant receptor subtype for enhanced pLTF. Thus, spinal injections of A_{2A} receptor antagonists enhance AIH-induced pLTF, suggesting that endogenous spinal A_{2A} receptors actually constrain rather than contribute to AIH-induced pLTF.

Enhanced pLTF requires spinal serotonin receptor activation

Since activation of spinal serotonin receptors is necessary for AIH-induced pLTF in normal rats (Baker-Herman & Mitchell, 2002), we hypothesized that spinal MSX-3 enhanced pLTF results from the same, serotonin-dependent mechanism. To test this hypothesis, a serotonin receptor antagonist (methysergide-maleate, $15 \mu\text{l}$, $300 \mu\text{g kg}^{-1}$) was applied prior to MSX-3 administration and exposure to AIH. Because methysergide injections required placement of

a second catheter into the intrathecal space, we repeated a subset of experiments (see Methods: Spinal treatment groups) to confirm that placement of two intrathecal catheters did not influence our results. Combined intrathecal methysergide and MSX-3 in the absence of hypoxia had no significant effect on frequency or amplitude of phrenic and hypoglossal nerve bursts (data not shown), and thus pLTF comparisons were made relative to baseline. pLTF after artificial CSF in one catheter and MSX-3 in the other (i.e. dual catheter placement) was significantly greater than in dual-vehicle injections at 60 min post-AIH

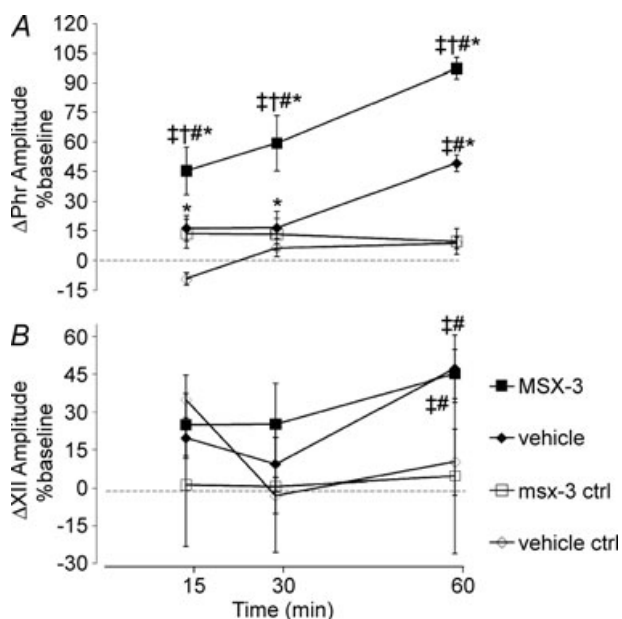


Figure 3. Time course of phrenic and hypoglossal (XII) burst amplitude changes following spinal drug (or vehicle) injections with or without (ctrl) AIH

Changes in phrenic (A) and XII (B) burst amplitudes (percentage change from baseline) in rats receiving vehicle + AIH (\blacklozenge , $n = 7$), MSX-3 + AIH (\blacksquare , $n = 8$), vehicle - AIH (\diamond , $n = 8$) and MSX-3 - AIH (\square , $n = 7$). Spinal MSX-3 augmented phrenic burst amplitude responses following AIH, indicative of enhanced pLTF. However, similar enhancement of XII LTF was not observed, suggesting that drug distribution was restricted to the spinal cord. Values are means \pm S.E.M. #Different from baseline; †different from vehicle at the same time point; ‡different from MSX-3 ctrl at the same time point; *different from vehicle ctrl at the same time point; RMANOVA; $P < 0.05$.

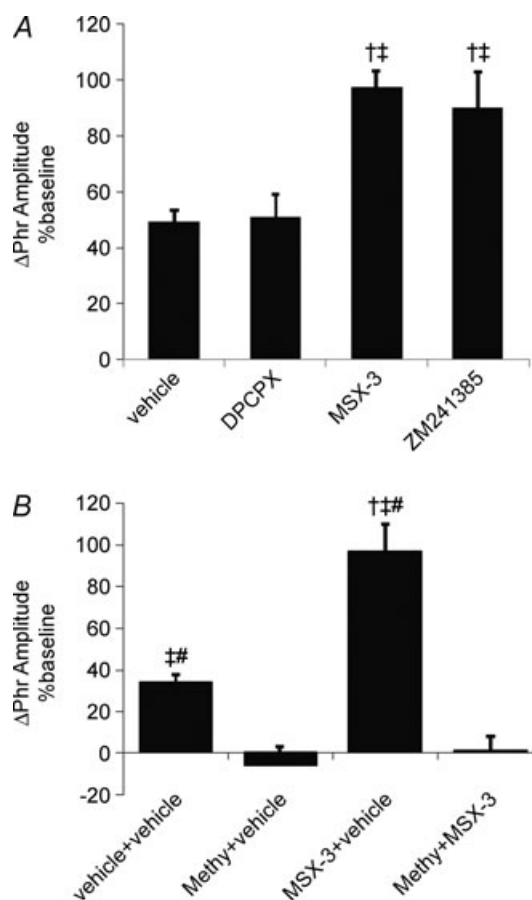


Figure 4. Effect of spinal adenosine A_1 , A_{2A} and serotonin receptor antagonists on pLTF

A, changes in phrenic burst amplitudes (% baseline) at 60 min post-AIH are depicted in rats receiving intrathecal injections of vehicle (aCSF), spinal adenosine A_1 receptor antagonist (DPCPX, $n = 6$) and two A_{2A} receptor antagonists (MSX-3, $n = 8$; and ZM241385, $n = 5$). †Different from vehicle; ‡different from DPCPX. B, with dual catheter placement, vehicle + vehicle with AIH ($n = 4$), methysergide + vehicle with AIH ($n = 5$), MSX-3 + vehicle with AIH ($n = 4$) and methysergide + MSX-3 with AIH. pLTF enhancement is not observed following spinal A_1 receptor inhibition, in contrasted to A_{2A} receptor antagonists. Enhanced pLTF following MSX-3 is effectively blocked by methysergide. Values are means \pm S.E.M. †Different from vehicle + vehicle; ‡different from methysergide + vehicle; #different from methysergide + MSX-3; ANOVA; $P < 0.05$.

($97 \pm 13\%$, $n = 4$; $34 \pm 4\%$, $n = 4$; respectively; $P < 0.001$; Fig. 4B), demonstrating enhanced pLTF. When pre-treated with methysergide, pLTF was abolished in vehicle ($-6 \pm 9\%$, $P < 0.001$, $n = 5$) and MSX-3 treated rats at 60 min post-AIH ($-5 \pm 7\%$, $P < 0.001$, $n = 5$; Fig. 4B), confirming that enhanced pLTF is serotonin-dependent.

Spinal MSX-3 does not affect hypoglossal LTF

Our goal was to localize the effects of MSX-3 to the spinal cord, avoiding effects on brainstem respiratory neurons. Thus, recordings of hypoglossal burst activity were made to assure that similar effects (MSX-3 enhancement) were not observed in these cranial motor neurons. Rats receiving either intrathecal vehicle or MSX-3 injections exhibited significant hypoglossal LTF at 60 min post-AIH ($47 \pm 15\%$, $P = 0.001$, $n = 5$; $45 \pm 8\%$, $P < 0.001$, $n = 6$, respectively; Fig. 3B), and these effects did not differ from one another ($P = 0.884$; Fig. 3B). In control rats receiving spinal MSX-3 without hypoxia, hypoglossal burst amplitude was not different from baseline at any time point (15 min: $0 \pm 7\%$, $P = 0.982$; 30 min: $-10 \pm 8\%$, $P = 0.707$; 60 min: $-4 \pm 10\%$, $P = 0.928$, $n = 7$; Fig. 3B). Therefore, although spinal A_{2A} receptor inhibition augments pLTF, it has no effect on hypoglossal LTF, suggesting that drug distribution was restricted to the region of interest (i.e. cervical spinal cord).

Intravenous MSX-3 enhances hypoglossal and phrenic LTF

Although the primary purpose of this study was to test whether spinal A_{2A} receptors contribute to AIH-induced pLTF, we also sought to know whether intravenous MSX-3, distributing to the entire CNS, would have similar effects on phrenic and hypoglossal LTF. Since MSX-3 is a water-soluble drug capable of crossing the blood–brain barrier (Schindler *et al.* 2005), we predicted that systemic MSX-3 would enhance both phrenic and hypoglossal LTF. In rats receiving intravenous vehicle (saline) injection, hypoglossal nerve burst amplitude was not significantly different from baseline at 60 min post-AIH ($28 \pm 10\%$, $P = 0.349$, $n = 4$; Fig. 5B). Thus, hypoglossal LTF is weak in the specific rats used in this experimental series, a finding previously reported in certain sub-strains of Sprague–Dawley rats (Fuller *et al.* 2001a). However, in rats pre-treated with MSX-3, robust hypoglossal LTF was observed 60 min post-AIH ($46 \pm 13\%$, $P = 0.008$, $n = 4$), an effect significantly greater than in rats receiving MSX-3 without AIH ($1 \pm 6\%$, $P = 0.01$; Fig. 5B).

Similar to spinally treated rats, phrenic burst amplitude from vehicle (saline) treated rats was above baseline at 60 min post-AIH ($57 \pm 6\%$, $P < 0.001$, $n = 9$). Systemic MSX-3 significantly enhanced pLTF 60 min post-AIH

($107 \pm 9\%$, $P < 0.001$, $n = 12$; Fig. 5A). Following intravenous MSX-3 administration without AIH, phrenic burst amplitude was significantly above baseline levels at the 60 min time point ($19 \pm 8\%$, $P = 0.024$, $n = 9$; Fig. 5A), suggesting that certain A_{2A} receptor sites may influence respiratory motor activity following systemic, but not spinal, MSX-3 administration. Nevertheless, phrenic burst amplitude after vehicle (saline) and MSX-3 treatment was significantly greater than MSX-3 without AIH ($P < 0.001$, respectively; Fig. 5A). Collectively, these results demonstrate that systemic A_{2A} antagonists enhance AIH-induced respiratory plasticity in both hypoglossal and phrenic motor output.

Respiratory frequency facilitation

AIH-induced frequency LTF in this experimental preparation is generally small and inconsistent (Baker-Herman & Mitchell, 2008). In spinally treated rats receiving AIH, phrenic burst frequency increased over baseline in vehicle (7 ± 3 bursts min^{-1} ,

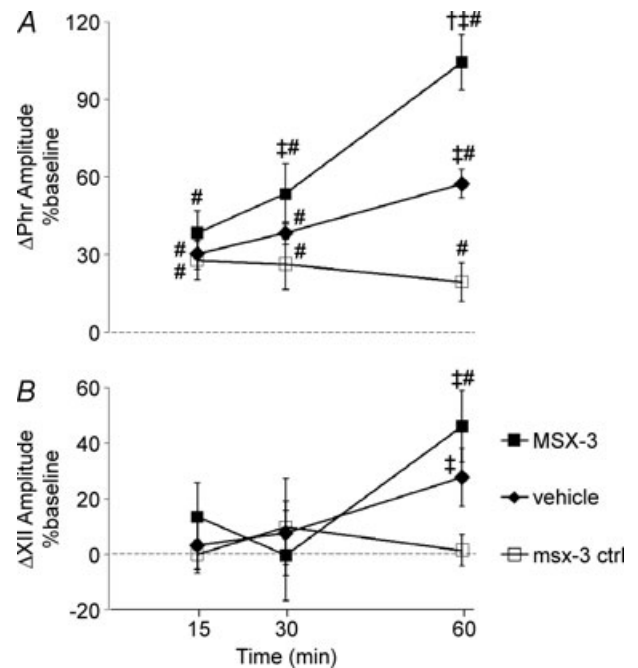


Figure 5. Time course of phrenic and hypoglossal (XII) burst amplitude changes following systemic drug injections

Changes in integrated phrenic (A) and hypoglossal (XII) (B) burst amplitudes from rats receiving intravenous vehicle with AIH (saline, ◆ phrenic $n = 9$, XII $n = 6$), MSX-3 with AIH (■; phrenic $n = 12$, XII $n = 5$), and control (ctrl) MSX-3 without AIH (□; phrenic $n = 9$, XII $n = 4$). Systemic MSX-3 enhanced both phrenic and XII LTF. Values are means \pm s.e.m. #Different from baseline; †different from vehicle at the same time point; ‡different from MSX-3 ctrl at the same time point; RMANOVA; $P < 0.05$.

$P = 0.03$, $n = 7$) and MSX-3 treated rats at 60 min post-AIH (9 ± 2 bursts min^{-1} , $P = 0.002$, $n = 8$; Fig. 6A). However, no burst frequency differences were detected between vehicle and MSX-3 treated rats ($P = 0.20$). Phrenic burst frequency following spinal MSX-3 alone was not different from baseline at any measured time point (15 min: 1 ± 1 bursts min^{-1} , $P = 0.996$; 30 min: 1 ± 1 bursts min^{-1} , $P = 0.903$; 60 min: 1 ± 2 bursts min^{-1} , $P = 0.982$; $n = 7$), and was significantly less than in vehicle ($P = 0.011$) and MSX-3 treated rats ($P < 0.001$) 60 min post-AIH. Burst frequency responses in vehicle and MSX-3 treated rats without AIH did not differ ($P = 0.287$; Fig. 6A).

Phrenic burst frequency in rats receiving intravenous vehicle (saline) or MSX-3 was increased over baseline at 60 min post-AIH (11 ± 2 bursts min^{-1} , $P < 0.001$, $n = 12$; 12 ± 1 bursts min^{-1} , $P < 0.001$, $n = 9$; respectively; Fig. 6B), but were not different from one another. Phrenic burst frequency following spinal MSX-3 treatment alone was not different from baseline at the 60 min time point (-2 ± 2 , $P = 0.333$;

$n = 9$), but was significantly decreased from MSX-3 and vehicle ($P < 0.001$, respectively) treated rats 60 min post-AIH (Fig. 6B). Therefore neither spinal nor systemic MSX-3 appears to augment AIH-induced burst frequency facilitation.

Discussion

Here we demonstrate that spinal A_{2A} receptor activation constrains AIH-induced pLTF in rats, refuting our original hypothesis that these receptors contribute to pLTF. This is the first evidence that A_{2A} receptor signalling modulates respiratory plasticity following acute intermittent hypoxia, and is difficult to reconcile with our previous report that A_{2A} receptor activation without hypoxia elicits long-lasting phrenic motor facilitation (Golder *et al.* 2008). We now postulate that AIH-induced pLTF (G_q protein-mediated) and A_{2A} receptor induced phrenic motor facilitation (G_s protein-mediated) are distinct mechanisms that interact *via* cross-talk inhibition. In this context, we propose that both serotonin receptors associated with phrenic LTF and A_{2A} receptors are activated during AIH, but that serotonin-dependent mechanisms predominate. Thus, instead of inducing phrenic motor facilitation, cross-talk inhibition from the A_{2A} receptor-dependent pathway constrains serotonin-dependent pLTF during AIH.

Since systemic MSX-3 augments both hypoglossal and phrenic LTF, whereas spinal MSX-3 augments pLTF alone, we suggest that the A_{2A} receptor constraint to AIH-induced LTF occurs within these respective motor nuclei. Elucidation of cellular/synaptic signalling events underlying MSX-3 enhanced pLTF may provide unique insights concerning regulatory mechanisms that govern respiratory LTF expression. Moreover, small molecules that cross the blood–brain barrier, targeting respiratory motor neurons, hold promise as a novel therapeutic strategy to treat respiratory insufficiency during lung, airway or neural disease (Mahamed & Mitchell, 2007; Mitchell, 2007).

Enhanced pLTF is serotonin dependent

pLTF is a form of respiratory plasticity induced by serotonin receptor activation (Feldman *et al.* 2003; Mitchell & Johnson, 2003). Our working model of AIH-induced pLTF is that episodic hypoxia activates raphe serotonergic neurons that project to phrenic motor nuclei. Spinal serotonin release during hypoxic episodes subsequently activates G_q protein-coupled 5-HT₂ receptors on or near phrenic motor neurons, and initiates intracellular cascades that underlie pLTF.

A_{2A} receptors in the nucleus tractus solitarius (NTS) augment electrically evoked serotonin release in

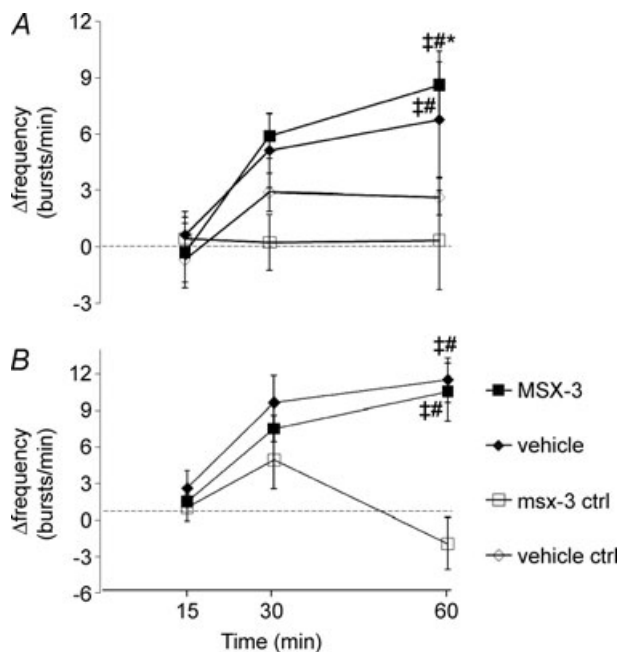


Figure 6. Time course of changes in respiratory nerve burst frequency in experimental groups

Changes in phrenic nerve burst frequency from baseline (Δ frequency, bursts min^{-1}) in rats receiving spinal (A) and intravenous (B) treatments: vehicle with AIH (◆ aCSF or saline, respectively), MSX-3 with AIH (■), MSX-3 time-control without AIH (□) and vehicle control without AIH (◇). Phrenic burst frequency in rats receiving AIH increased from baseline at 60 min post-AIH, indicative of frequency LTF. However, this response was unaffected by MSX-3. #Different from baseline; ‡different from MSX-3 ctrl, *different from vehicle ctrl; RMANOVA; $P < 0.05$.

superfused rat medullary slices *via* presynaptic mechanisms (Barraco *et al.* 1996). Additionally, selective A_{2A} receptor agonists (e.g. PD125944, cgs21680) increase extracellular serotonin levels in the hippocampus, although only in the presence of A_1 receptor antagonists (Okada *et al.* 1997). Hence, spinal A_{2A} receptors may augment available serotonin *via* increased release and/or decreased re-uptake. This study provides no evidence for such an effect during AIH since, contrary to predictions, A_{2A} receptor antagonists enhanced pLTF.

Other experimental interventions also induce serotonin receptor-dependent enhancement of pLTF, such as pre-conditioning with chronic intermittent hypoxia or chronic cervical dorsal rhizotomy (Kinkead *et al.* 1998; Ling *et al.* 2001). Ling and colleagues reported that chronic intermittent hypoxia enhanced the short-term hypoxic phrenic response and pLTF (Ling *et al.* 2001). The enhanced pLTF was methysergide sensitive, and of a similar magnitude to MSX-3 enhanced phrenic LTF (Ling *et al.* 2001). Cervical dorsal rhizotomy increases serotonergic innervation of the phrenic motor nucleus and enhances AIH-induced pLTF (Kinkead *et al.* 1998). Moreover cervical dorsal rhizotomy increases spinal BDNF and NT-3 levels in ventral cervical segments that encompass the phrenic motor nucleus (Johnson *et al.* 2000). MSX-3 enhanced pLTF may involve similar signalling molecules to these experimental treatments, but this hypothesis remains to be tested. Since both chronic intermittent hypoxia and cervical dorsal rhizotomy are lasting pre-conditions that elicit respiratory meta-plasticity (i.e. enhanced phrenic LTF; Mitchell & Johnson, 2003), MSX-3 is unique as an acute pharmacological intervention that produces the same experimental result. A more complete understanding of cellular mechanisms giving rise to A_{2A} receptor antagonist enhancement of pLTF may also increase our understanding of signalling events that underlie 'normal' AIH-induced pLTF.

Potential cellular mechanism of MSX-3 enhanced pLTF

There is an abundance of literature demonstrating 'cross-talk' between G_q and G_s protein-coupled receptor pathways (Meszaros *et al.* 2000; Roy *et al.* 2006; Ryzhov *et al.* 2006). For example, PKC activation (e.g. 5-HT₂ receptors) modulates G_s protein-coupled receptor (G_s PCR) pathways (e.g. A_{2A}), possibly through adenylyl cyclase or regulators of G-proteins (RGS; Zimmermann & Taussig, 1996; Lai *et al.* 1997; Cunningham *et al.* 2001; Roy *et al.* 2006). A_{2A} receptor activation of adenylyl cyclase is inhibited by PKC phosphorylation, which prevents second messenger transduction through G-proteins (Zimmermann & Taussig, 1996; Lai *et al.* 1997). Moreover PKC is known to phosphorylate RGS proteins which, in turn, modulate activity of multiple G-proteins, including

those involved in G_s PCR signalling (e.g. $G_{\alpha s}$; Cunningham *et al.* 2001). Although speculative, we propose that, during AIH, G_q PCR mediated signalling (i.e. AIH-induced pLTF) predominates, and suppresses G_s PCR mediated signalling *via* 'cross-talk' inhibition.

Conversely, PKA may mediate inhibitory 'cross-talk' to G_q PCR signalling *via* modulation of cellular reactive oxygen species (ROS) production. Hypoxia followed by re-oxygenation generates ROS by several cellular mechanisms (Zulueta *et al.* 1997; Li & Jackson, 2002; Abramov *et al.* 2007). Scavenging superoxide anions with a superoxide dismutase mimetic blocks AIH-induced pLTF, demonstrating that superoxide anions are necessary in its underlying mechanism (Macfarlane & Mitchell, 2007). Relevant ROS appear to be derived from spinal NADPH oxidase activity since spinal administration of the NADPH oxidase inhibitors apocynin and diphenyleioidonium chloride (DP) abolish pLTF (Macfarlane *et al.* 2008; MacFarlane *et al.* 2009). In our working cellular/synaptic model of pLTF, ROS generated during AIH inhibit protein phosphatases that normally constrain the cellular cascade leading to pLTF (Macfarlane *et al.* 2008; Wilkerson *et al.* 2008). NADPH oxidase-derived ROS have been implicated in other forms of respiratory plasticity, including carotid sensory LTF in rats pre-treated with chronic intermittent hypoxia (Peng & Prabhakar, 2003; Peng *et al.* 2006). We suggest that modulatory influences of A_{2A} receptors on pLTF (i.e. G_q PCR-induced PMF) are indirectly mediated *via* regulation of NADPH oxidase activity as described above. Thus, MSX-3 may remove an endogenous adenosine A_{2A} receptor/PKA-dependent constraint on transient ROS production necessary for AIH-induced pLTF. A_{2A} receptor (and subsequent PKA) activation during AIH may inhibit NADPH-dependent ROS formation, maintaining suboptimal ROS levels for cellular mechanisms that normally gives rise to pLTF. Spinal A_{2A} receptor antagonists may relieve this limitation, increasing ROS formation and enhancing pLTF.

Experimental Considerations

Drug localization. AIH-induced pLTF results from mechanisms on or near phrenic motor neurons (Mitchell *et al.* 2001). For example, pLTF is abolished after cervical spinal application of serotonin receptor antagonists or protein synthesis inhibitors (Baker-Herman & Mitchell, 2002), suggesting that pLTF results from plasticity in or near the phrenic motor nucleus. Contrary to our original hypothesis that spinal A_{2A} receptors contribute to AIH-induced pLTF, A_{2A} receptors appear to constrain these mechanisms.

MSX-3 applied to the cervical spinal cord augmented phrenic, but not hypoglossal, LTF. Since intravenous MSX-3 administration enhances both phrenic and hypoglossal LTF, hypoglossal motor output is capable of

expressing enhanced LTF. Collectively, these results suggest that effective MSX-3 concentrations following intrathecal administration were restricted to spinal regions associated within the phrenic motor nucleus, and did not reach brainstem XII motor neurons.

In time-control rats receiving systemic MSX-3 without AIH, modest drift in phrenic burst amplitude was observed (Fig. 5A). A similar response was not observed in spinally treated rats (Fig. 3A). This effect may result from MSX-3 acting at the level of carotid chemoreceptors since A_{2A} receptors are expressed by carotid body type I cells and inhibit their activity (Kobayashi *et al.* 2000; Conde *et al.* 2006). This hypothesis remains to be tested.

Significance. AIH induced pLTF is one of the most frequently studied models of respiratory plasticity (Mahamed & Mitchell, 2007). Our understanding of cellular/synaptic mechanisms giving rise to pLTF has increased substantially, yet critical details of the signalling mechanism are not yet known (Mahamed & Mitchell, 2007). Elucidation of cellular mechanisms whereby A_{2A} receptors modulate pLTF expression may yield novel insights concerning fundamental mechanisms of hypoxia-induced respiratory plasticity. MSX-3 enhanced pLTF may 'amplify' existing signalling pathways to pLTF, possibly increasing our ability to discriminate these signalling events.

From a different perspective, multiple ventilatory control disorders share a common therapeutic goal: amplification of synaptic inputs onto respiratory motor neurons (Mitchell, 2007). A_{2A} receptor antagonists represent an interesting class of small molecules that cross the blood-brain barrier (Sauer *et al.* 2000) and have the capacity to (indirectly) augment respiratory motor neuron function, at least in concert with AIH. Caffeine, an adenosine (A_{2A}) receptor antagonist, is commonly used in neonatal intensive care units as a respiratory stimulant for premature newborn infants with respiratory instability (Finer *et al.* 2006). While, the localization of caffeine effects (central nervous system and/or peripheral chemoreceptors) is not known, current clinical use suggests that further development of selective adenosine receptor antagonists may be useful in augmenting respiratory motor output. Thus, A_{2A} receptor antagonists may be of clinical interest in the treatment of respiratory insufficiency after spinal cord injury, during neurodegenerative diseases and, possibly, apnoea of prematurity and/or obstructive sleep apnoea (Mitchell, 2007).

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Author contributions

M.H., F.G., S.M., and G.M., contributed to the conception, design, and analysis of experiments including interpretation of data and drafting/revising the final version

of the article approved for publication. All experiments were carried out at University of Wisconsin, Madison, Wisconsin, USA.

S. Mahamed: Department of Physiology, School of Medicine and Public Health, University of Wisconsin, 1300 University Avenue, Madison, WI 53706, USA

Authors' present addresses

F. J. Golder: Department of Clinical Studies – Philadelphia, University of Pennsylvania, United States 3900 Delancey Street, Philadelphia, PA 19104, USA

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