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Episodic spinal serotonin receptor activation elicits long-lasting phrenic motor facilitation by an NADPH oxidase-dependent mechanism

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Phrenic long-term facilitation (pLTF) is a serotonin (5-HT)-dependent augmentation of phrenic motor output induced by acute intermittent hypoxia (AIH). AIH-induced pLTF requires spinal NADPH oxidase activity and reactive oxygen species (ROS) formation. Since 5-HT receptor activation stimulates NADPH oxidase activity in some cell types, we tested the hypothesis that episodic spinal 5-HT receptor activation (without AIH) is *sufficient* to elicit an NADPH oxidase-dependent facilitation of phrenic motor output (pMF). In anaesthetised, artificially ventilated adult male rats, episodic intrathecal 5-HT injections (3 × 6 μl injections at 5 min intervals) into the cerebrospinal fluid (CSF) near cervical spinal segments containing the phrenic motor nucleus elicited a progressive increase in integrated phrenic nerve burst amplitude (i.e. pMF) lasting at least 60 min post-5-HT administration. Hypoglossal (XII) nerve activity was unaffected, suggesting that effective doses of 5-HT did not reach the brainstem. A single 5-HT injection was without effect. 5-HT-induced pMF was dose dependent, but exhibited a bell-shaped dose–response curve. Activation of different 5-HT receptor subtypes, specifically 5-HT_2 *versus* 5-HT_7 receptors, may underlie the bell-shaped dose–response curve via a mechanism of 'cross-talk' inhibition. Pre-treatment with NADPH oxidase inhibitors, apocynin or diphenylenodium (DPI), blocked 5-HT induced pMF. Thus, episodic spinal 5-HT receptor activation is *sufficient* to elicit pMF by an NADPH oxidase-dependent mechanism, suggesting common mechanisms of ROS formation with AIH-induced pLTF. An understanding of the mechanisms giving rise to AIH-induced pLTF and 5-HT induced pMF may inspire novel therapeutic strategies for respiratory insufficiency in diverse conditions, such as sleep apnoea, cervical spinal injury or amyotrophic lateral sclerosis.

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Abbreviations AIH, acute intermittent hypoxia; CSF, cerebrospinal fluid; DPI, diphenylenodium; MAP, mean arterial pressure; ROS, reactive oxygen species; XII, hypoglossal; pLTF, phrenic long-term facilitation; pMF, phrenic motor facilitation.

Introduction

Acute intermittent hypoxia (AIH) elicits a form of serotonin-dependent respiratory plasticity known as long-term facilitation (LTF; Bach & Mitchell, 1996; Feldman *et al.* 2003; MacFarlane *et al.* 2008). Phrenic (pLTF) and hypoglossal (XII) LTF are expressed as a progressive and sustained increase in respiratory burst amplitude that persists for hours following AIH. Serotonin (5-HT) is released within the phrenic motor nucleus during each hypoxic episode (Kinkead *et al.* 2001), activating 5-HT_2 receptors on or near phrenic motor neurons and initiating intracellular signalling cascades that give rise to sustained increases in motor neuron activity (Kinkead *et al.* 1998; Fuller *et al.* 2001*b*; Baker-Herman & Mitchell, 2002). Episodic bath application of 5-HT (or 5-HT_2 receptor agonist) to neonatal phrenic (Lovett-Barr *et al.* 2006) or XII (Bocchiaro & Feldman, 2004) motor neurons is sufficient to elicit LTF *in vitro*, demonstrating that 5-HT receptor activation is both necessary and sufficient for LTF in at least some conditions. Although considerable progress has been made towards an understanding of cellular mechanisms giving rise to pLTF (Feldman *et al.* 2003; Baker-Herman

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et al. 2004; Mahamed & Mitchell, 2007; MacFarlane *et al.* 2008), we still lack a detailed understanding of the role played by 5-HT, particularly in adults.

Spinal reactive oxygen species (ROS) formation is necessary for AIH-induced pLTF (MacFarlane & Mitchell, 2008*a*), and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex is a critical source of relevant ROS (MacFarlane *et al.* 2009). Both hypoxia (Dinger *et al.* 2007; Rathore *et al.* 2008) and re-oxygenation (Abramov *et al.* 2007) are capable of increasing NADPH oxidase activity and ROS formation. Thus, AIH has the requisite characteristics to induce NADPH oxidase activity. On the other hand, 5-HT2 receptor activation stimulates NADPH oxidase activity and ROS formation without changes in oxygenation in renal mesangial cells, suggesting that serotonergic signalling also activates NADPH oxidase in some cell types (Grewal *et al.* 1999). Episodic (but not continuous) bath application of 5-HT induces long-lasting facilitation of chemo-sensory discharge in *ex vivo* carotid bodies by a mechanism that requires 5-HT_2 receptor activation and NADPH oxidase activity (Peng *et al.* 2006, 2009). Since hypoxia stimulates raphe serotonergic neurons (Erickson & Millhorn, 1991, 1994; Teppema *et al.* 1997) and releases 5-HT in the phrenic motor nucleus (Kinkead *et al.* 2001), 5-HT receptor activation (*vs.* hypoxia/re-oxygenation) may induce the NADPH oxidase activity necessary for AIH-induced pLTF.

Here we tested the hypotheses that episodic spinal 5-HT receptor activation (without hypoxia/re-oxygenation) induces a long lasting increase in the amplitude of integrated phrenic nerve discharge (phrenic motor facilitation, pMF) in adult rats *in vivo*, and that it requires spinal NADPH oxidase activity. We demonstrate that episodic spinal 5-HT (without AIH) is *sufficient* to elicit pMF, although these effects exhibit a bell-shaped dose–response curve. Maximal pMF was observed at 5-HT concentrations below the threshold for the induction of tonic motor neuron activity, suggesting that pMF results from mechanisms distinct from 'classical' serotonergic modulation of motor neurons (e.g. Lindsay & Feldman, 1993; Bayliss *et al.* 1995; McCrimmon *et al.* 1995; Jacobs & Fornal, 1997; Rekling *et al.* 2000; Schwarzacher *et al.* 2002; Hodges & Richerson, 2008). Finally, 5-HT-induced pMF requires NADPH oxidase activity, suggesting that 5-HT receptor activation *per se* may be the relevant stimulus to NADPH oxidase activity necessary for AIH-induced pLTF in adult rats.

Methods

Ethical approval

Experiments were performed on 3- to 4-month-old male Sprague–Dawley rats (Colony 236, Harlan, Indianapolis, IN, USA). All experiments were approved by the Animal Care and Use Committee at the School of Veterinary Medicine, University of Wisconsin–Madison and are in compliance with the policies and regulations outlined by *The Journal of Physiology* (Drummond, 2009).

Experimental preparation

All surgical procedures were performed under isoflurane anaesthesia (~3.5% in 50% O_2, balance N_2). Rectal temperature was continuously monitored (Fisher Scientific, USA) and regulated with a temperature controlled surgical table. An O_2 sensor (TED 60T, Teledyne Analytical Instruments, City of Industry, CA, USA) was used to monitor inspired O_2 concentration throughout the experiment; inspired gas levels for O_2 and CO_2 were varied using flow meters. Following induction of anaesthesia with isoflurane, the tail vein was catheterised (24 gauge, Surflo, Elkton, MD, USA) and the rat was given an injection of 3 ml kg^{-1} of lactated Ringer solution (Baxter, Deerfield, IL, USA) to maintain fluid balance and minimise deviations in the base excess. To maintain base-excess levels throughout surgery and experimental protocols, an infusion pump (Cole-Palmer, Vernon Hills, IL, USA) provided a slow continuous intravenous infusion (~4 ml kg^{-1} h^{-1}) of a solution containing lactated Ringer, Hetastarch (6%; Hospira Inc., Lake Forest, IL, USA) and sodium bicarbonate (8.4%; Hospira), mixed in a 10 : 10 : 1 ratio (respectively).

Following tests to ensure adequate anaesthesia (toe-pinch, corneal reflexes), rats were tracheotomised for artificial ventilation (tidal volume, 2.5 ml; Rodent Ventilator, model 683; Harvard Apparatus, South Natick, MA, USA) and bilaterally vagotomised through a mid-line ventral neck incision. A catheter was inserted into the right femoral artery and connected to a pressure transducer (Gould Pressure Transducer, P23, USA) to monitor blood pressure and sample blood for gas analyses. Blood samples taken at various time points were analysed for arterial partial pressures of O_2 (PaO_2) and CO_2 ($PaCO_2$), as well as pH and base-excess using a blood gas analyser (ABL 500, Radiometer, Copenhagen, Denmark). The left phrenic and XII nerves were dissected using a dorsal approach, cut distally and de-sheathed. Once the nerves were isolated, they were covered with saline-soaked cotton wool for the duration of the remainder of surgical preparation (i.e. during placement of intrathecal catheters) to prevent desiccation. To enable placement of the intrathecal catheters, a 1-segment laminectomy was performed at C2 and a silicone catheter (outer diameter, 0.6 mm; Access Technologies, Skokie, IL, USA) was inserted through a limited durotomy. The catheter was advanced ~2 mm caudally so that its tip was positioned at the anterior edge of C4 as described previously (Baker-Herman & Mitchell, 2002; MacFarlane

et al. 2009). The catheter was primed with serotonin (or vehicle) solutions prior to placement. During dissection of the nerves, the rat was slowly converted to urethane anaesthesia (~1.8 g kg^{-1}) while at the same time, the isoflurane was slowly withdrawn.

When the surgical preparation was complete, the nerves were submerged in mineral oil and placed on bipolar silver recording electrodes. Once nerve signals were observed, the rats were subject to neuromuscular blockade with an intravenous injection of pancuronium bromide (1 mg kg^{-1}; Sicor Pharmaceuticals, Irvine, CA, USA) and allowed ~1 h for electroneurograms and blood pressure to stabilise. Nerve activity was amplified (gain, 10,000; A-M systems, Everett, WA, USA), bandpass-filtered (100 Hz to 10 kHz), and integrated (CWE 821 filter; Paynter, Ardmore, PA, USA; time constant, 50 ms). The integrated signal was digitised and recorded using the WINDAQ data acquisition system (DATAQ Instruments, Akron, OH, USA) and subsequently analysed using custom-designed software on a LabView platform (National Instruments, Austin, TX, USA). At the end of each experiment, the rat was humanely killed by urethane overdose in accordance with our animal protocol approval.

Drugs and vehicles

Serotonin hydrochloride (Sigma-Aldrich, St Louis, MO, USA) was dissolved in artificial cerebral spinal fluid (aCSF), which had been bubbled with a gas mixture consisting of 95% O_2 and 5% CO_2. A small amount of the NADPH oxidase inhibitor, apocynin (Sigma-Aldrich), was weighed, dissolved in dimethylsulphoxide (DMSO) and diluted in bubbled aCSF (1 : 1000, DMSO : aCSF) for a final concentration of 600 μM. Diphenylenodium (DPI; Sigma-Aldrich, MO, USA) was prepared in a similar manner, but with a 1 : 100 mixing ratio of DMSO : aCSF to achieve a final concentration of 1 mM. Methysergide maleate (Sigma-Aldrich), a broad spectrum 5-HT receptor antagonist, was dissolved in saline and administered intravenously (4 mg kg^{-1}); 10 min after injection, the apnoeic and recruitment thresholds were determined, followed by a further 30 min of baseline nerve recording prior to AIH. The 5-HT$_7$ antagonist, SB269970 (Sigma-Aldrich) was dissolved in DMSO and frozen in 100 μl aliquots; on the day of the experiment, the aliquot was thawed and mixed with 400 μl aCSF at a final concentration of 5 mM. aCSF consisted of NaCl (120 mM), KCl (2.9 mM), $CaCl_2.2H_2O$ (2 mM), $MgCl_2.6H_2O$ hexahydrate (2 mM), $NaHCO_3$ (23 mM) and glucose (10 mM) dissolved in double-distilled water.

Experimental protocols

To establish baseline nerve activity, rats were ventilated with 50% O_2 to establish P_{aO_2} of ~250 mmHg; CO_2 was added to the inspired gas as necessary to regulate arterial P_{aCO_2}. After a stabilisation period of 1 h, the apnoeic CO_2 threshold was determined by progressively lowering inspired CO_2 until rhythmic phrenic activity ceased; the CO_2 recruitment threshold was then determined by progressively raising the inspired CO_2 until rhythmic activity resumed. The end- tidal P_{CO_2} was then set 2–3 mmHg above the CO_2 recruitment threshold (see Bach & Mitchell, 1996). End-tidal P_{CO_2} was monitored throughout the experiment using a flow-through capnograph (Novametrix, Wallingford, CT, USA) placed in the ex-current section of the end-otracheal Y-tube. P_{aCO_2} was maintained within 1.5 mmHg of baseline throughout the experiment by manipulating inspired CO_2. Blood gases and arterial blood pressure were monitored and corrected as necessary. Negative base excess values less than -3 mEq l^{-1} were corrected with intravenous injections of sodium bicarbonate, and progressive reductions in blood pressure were offset by constant intravenous infusion, or less frequently supplemented with intravenous bolus injections of lactated Ringer solution. Phrenic and XII nerve activity were recorded continuously. Blood samples (0.3 ml in a heparinised glass syringe) were collected once baseline nerve recordings had stabilised, and then again at 30 and 60 min post-episodic 5-HT injections. Measurements of phrenic and XII burst amplitude and frequency were evaluated in 1 min periods immediately prior to each blood sample.

Initial experiments were designed to determine dose-dependent effects of episodic intrathecal injections of 5-HT on phrenic nerve activity (i.e. pMF). Three consecutive intrathecal injections of 5-HT (6 μl per injection at 5 min intervals) were administered immediately after the baseline blood sample and nerve signals had stabilised. The following concentrations were used: 50–100 μM ($n = 6$), 500 μM ($n = 6$), 1 mM ($n = 9$), 10 mM ($n = 5$) and 100 mM ($n = 7$). Since 1 mM 5-HT was the most effective concentration for episodic injections, additional rats were used with this same concentration to test if 5-HT-induced pMF (1) required patterned application of 5-HT, (2) was the result of 5-HT receptor activation, (3) required NADPH oxidase activity and (4) required sustained NADPH oxidase activity for maintenance of pMF. To confirm that 5-HT-induced pMF required patterned application, rats received a single intrathecal injection (1 mM, 6 μl; $n = 5$) of 5-HT (Fig. 1*B*). To confirm that 5-HT-induced pMF resulted from spinal 5-HT receptor activation, rats ($n = 5$) were pre-treated with an intravenous injection of methysergide maleate (4 mg kg^{-1}) ~40 min prior to episodic 5-HT. The methysergide dose was chosen because it was previously shown to inhibit AIH-induced pLTF (Bach & Mitchell, 1996). To test if 5-HT-induced pMF was NADPH oxidase dependent, two rat groups received either a single bolus injection of apocynin (600 μM, 15 μl; $n = 6$) or DPI (1 mM, 15 μl, $n = 5$) *via* a second intrathecal catheter

~20 min prior to episodic 1 mM 5-HT. DPI was chosen because its mechanism of action on NADPH oxidase activity is distinct from apocynin (Hancock & Jones, 1987; O'Donnell *et al.* 1993; Heumüller *et al.* 2008); the same drug doses used in the current study were previously shown to inhibit AIH-induced pLTF (MacFarlane *et al.* 2009). To test if NADPH oxidase activity is necessary to maintain (*versus* initiate) 5-HT-induced pMF, one group of rats ($n = 5$) was injected with apocynin (600 μM, 15 μl) 5 min after episodic 5-HT injections.

The magnitude of 5-HT-induced pMF was concentration dependent, but exhibited a bell-shaped concentration–response curve with a constant injection volume (see Fig. 2*A*); thus, we tested the hypothesis that the inability of high concentrations (i.e. 100 mM) of 5-HT to elicit pMF resulted from the activation of different 5-HT receptors. We reasoned that antagonism of such receptors, particularly of the 5-HT_7 subtype, should restore 5-HT-induced pMF. To test this hypothesis we injected the highest concentration of 5-HT (100 mM; which consistently failed to elicit pMF; Fig. 2*A* and also 4*A*) into rats ($n = 5$) pre-treated with an intrathecal injection of the 5-HT_7 antagonist, SB269970 (5 mM; 12 μl injection volume; 10 min prior to episodic 5-HT). This SB269970 concentration was chosen because it enhances AIH-induced pLTF, suggesting that 5-HT_7 receptor activation during AIH constrains mechanisms of pLTF (Hoffman MS and Mitchell GS, unpublished observations).

Time-matched control experiments were performed in rats that received episodic aCSF (3×6 μl, $n = 2$), apocynin (600 μM, 15 μl) or DPI (1 mM, 15 μl) + episodic aCSF ($n = 3$), SB269970 (5 mM, 15 μl) + aCSF ($n = 4$) or methysergide (4 mg kg^{-1}) + episodic aCSF ($n = 2$), i.e. without episodic 5-HT. In these experiments, nerve activity was monitored for the duration of a normal protocol. None of these 'sham treatments' elicited detectable pMF, although methysergide caused a

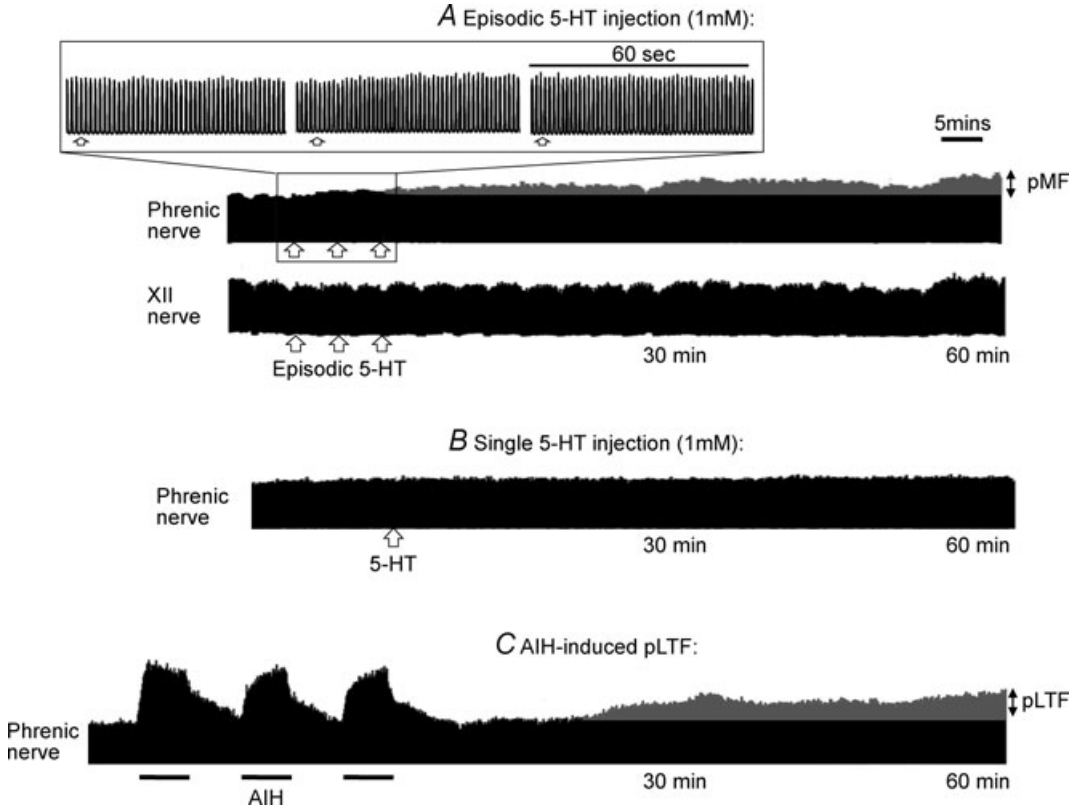

Figure 1. Representative neurograms from rats that received intrathecal injections of 1 mM 5-HT
A, episodic 5-HT (3×6 μl, 5 min intervals) elicited a significant increase in integrated phrenic (i.e. phrenic motor facilitation, pMF), but not hypoglossal (XII) nerve burst amplitude for at least 60 min post-injections. Inset: expanded 60 s traces of neural activity during and following each injection. Note that there was no effect of 5-HT injections on rhythmic or tonic phrenic motor activity. *B*, single intrathecal 5-HT injections (6 μl; 1 mM) were not sufficient to elicit pMF. An open arrow indicates 5-HT injections. *C*, for comparison, a neurogram illustrating phrenic long-term facilitation (pLTF) following acute intermittent hypoxia (AIH); AIH consisted of 3×5 min hypoxic episodes with 5 min intervals (10% O_2; black bars). Note pLTF following AIH is comparable in magnitude and time-course to pMF following episodic 1 mM 5-HT-injections. Arterial blood (see also Table 1) was sampled and assessed for P_{O_2}, P_{CO_2} and pH at baseline and 30 and 60 min after episodic 5-HT or AIH.

significant and sustained increase in phrenic nerve burst amplitude as reported previously (Bach & Mitchell, 1996; Baker-Herman & Mitchell, 2002). Because there were no differences among time-control groups, these rats were pooled into a single time control (TC) group for further analyses.

Statistical analysis

Peak integrated nerve burst amplitude and frequency (bursts per minute) of phrenic and XII nerve activity were averaged in 1 min bins at each recorded data point (baseline, 30 and 60 min post-episodic serotonin). Values were expressed as a percentage change from baseline. For changes in phrenic and XII nerve activity, within-rat comparisons were made with baseline; comparisons were also made with TC rats at all time points. Statistical comparisons were made for time and drug treatment using two-way, repeated measures ANOVA with Bonferroni's *post hoc* test to make individual comparisons. Differences were considered significant at $P < 0.05$. All values are expressed as means ± 1 S.E.M.

Results

Values for body temperature (T_b), arterial blood gases (P_{O_2} and P_{CO_2}) and pH are provided in Table 1 for various treatment groups. Body temperature, P_{aCO_2} and blood pH were kept constant throughout the experiment for each group. However, there was a small, but significant, decrease in P_{aO_2} and mean arterial pressure (MAP) for each treatment group, including time control rats. We don't anticipate that these changes in P_{aO_2} and MAP contribute to the magnitude of pMF because they were small, they occurred in time-control animals and in rats that did not express pMF. We also observed a small tendency for an upward drift in respiratory frequency in virtually all treatment groups, although it was more pronounced in animals that received the highest concentration (100 mM) of 5-HT. Nevertheless, it is unlikely that spinally applied 5-HT reached brainstem regions where it could affect respiratory frequency because: (1) there was no effect on XII nerve activity (Figs 1*A* and 2*B*), and (2) time control rats (which did not receive 5-HT) also tended to increase frequency.

Episodic 5-HT is sufficient to induce pMF

Serotonin (5-HT) applied episodically (3 injections, 6 μl each, 5 min intervals) into the cerebrospinal fluid (CSF) of the cervical spinal cord caused a concentration-dependent, progressive and sustained increase in the amplitude of integrated phrenic nerve burst activity (i.e. pMF). When observed, this effect lasted at least 60 min following the final 5-HT injection (Figs 1*A* and 2*A*). The magnitude of pMF (quantified at 60 min post-injection) was larger at progressively higher 5-HT concentrations up to 1 mM (43 ± 9%, $n = 9$, Figs 1*A* and 2*A*). However, at higher concentrations, pMF magnitude declined, demonstrating a bell-shaped dose–response curve. In general, concentrations effective at inducing pMF did not cause acute changes in phrenic nerve activity immediately following injection; higher concentrations that failed to elicit pMF frequently coincided with increased tonic phrenic activity which is typically regarded as a hallmark of serotonergic modulation of respiratory motor neurons (McCrimmon *et al.* 1995; Rekling *et al.* 2000).

A representative experimental trace illustrating the effects of episodic 1 mM 5-HT on phrenic nerve activity is shown in Fig. 1*A*. The magnitude and time course of 5-HT-induced pMF (~45%) is similar to AIH-induced long-term facilitation (Fig. 1*C*); indeed, although there are strain and sub-strain differences in the magnitude of AIH-induced LTF (Fuller *et al.* 2001*a*), the most recent

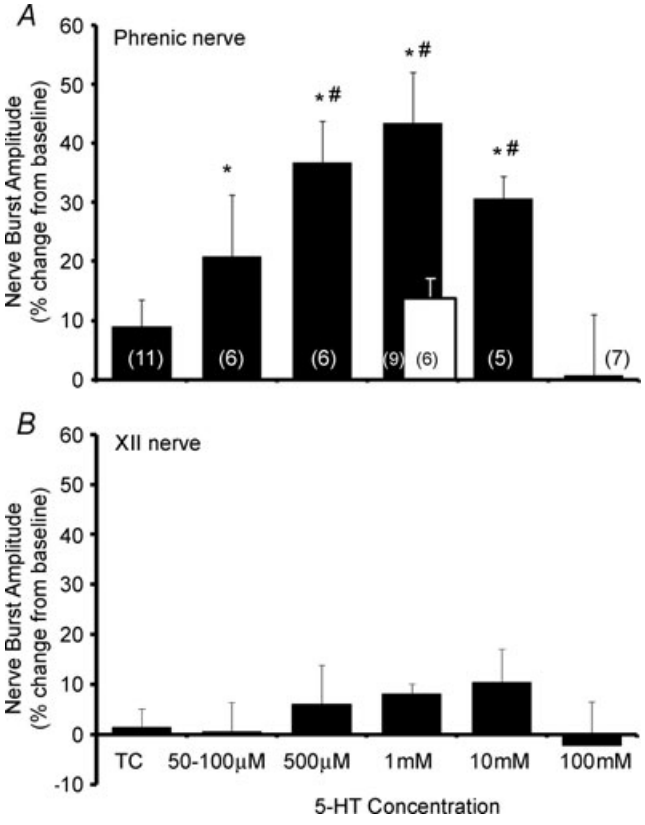

Figure 2. Concentration-dependent effects of episodic intrathecal 5-HT injections (3 × 6 μl) on integrated phrenic (*A*) and XII (*B*) nerve amplitude at 60 min post-injection
At progressively higher 5-HT concentrations, pMF increased, reaching a peak at 1 mM. At concentrations higher than 1 mM, pMF decreased and was no longer significant at 100 mM, thus revealing a bell-shaped dose–response curve. Open bar in *A* indicates the average effect of a single 1 mM injection of 5-HT. There was no effect of intrathecal 5-HT on XII motor activity at any concentration (see also Fig. 1*A*). Values are mean ± 1 S.E.M.; *n* values for each treatment group are indicated in parentheses. *Significant difference from baseline; #significant difference from time control (TC) group ($P < 0.05$).

Table 1. Body temperature (T_b), blood gas values and respiratory frequency (f_R) for various experimental treatment groups before (i.e. baseline) and 60 min after episodic 5-HT

	Group	BW (g)	T_b (°C)	pH	P_{aCO_2} (mmHg)	P_{aO_2} (mmHg)	MAP (mmHg)	f_R (breaths min^{-1})
Baseline	TC	359 ± 9	37.7 ± 0.1	7.350 ± 0.005	44.4 ± 0.5	288.7 ± 2.4	107.8 ± 5.1	46.1 ± 1.3
	5-HT (1 mM)	373 ± 4	38.1 ± 0.2	7.374 ± 0.011	42.9 ± 0.7	273.2 ± 6.0	107.8 ± 4.7	44.3 ± 2.5
	+ apocynin (pre)	373 ± 8	38.1 ± 0.2	7.337 ± 0.005	43.3 ± 0.9	280.6 ± 4.0	113.2 ± 4.2	46.3 ± 1.0
	+ DPI	368 ± 8	37.4 ± 0.1	7.345 ± 0.013	46.4 ± 2.2	285.0 ± 8.9	114.5 ± 3.9	42.5 ± 1.7
	+ methysergide	393 ± 7	38.1 ± 0.2	7.337 ± 0.013	45.9 ± 1.5	285.7 ± 9.0	120.8 ± 8.7	47.1 ± 2.8
	+ apocynin (post)	377 ± 13	38.0 ± 0.1	7.321 ± 0.018	48.9 ± 0.8	288.7 ± 6.0	102.6 ± 11.3	45.4 ± 2.2
	5-HT (100 mM)	364 ± 10	38.2 ± 0.3	7.326 ± 0.012	43.3 ± 0.7	272.9 ± 5.3	120.8 ± 5.2	40.4 ± 1.4
	+ SB269970	368 ± 14	37.6 ± 0.2	7.358 ± 0.008	45.5 ± 1.3	291.4 ± 6.9	98.8 ± 4.0	43.7 ± 1.1
60 min	TC	—	37.9 ± 0.1	7.350 ± 0.006	44.4 ± 0.4	281.0 ± 2.1	99.6 ± 5.4	48.8 ± 0.6
	5-HT (1 mM)	—	38.0 ± 0.2	7.368 ± 0.009	43.3 ± 0.9	263.1 ± 4.8	96.8 ± 4.1	50.9 ± 2.0
	+ apocynin (pre)	—	38.0 ± 0.2	7.341 ± 0.006	43.3 ± 1.0	271.6 ± 2.4	100.6 ± 6.6	50.1 ± 0.9
	+ DPI	—	37.3 ± 0.2	7.356 ± 0.012	46.4 ± 2.4	276.6 ± 7.0	101.1 ± 3.5	45.0 ± 1.6
	+ methysergide	—	38.3 ± 0.2	7.338 ± 0.013	46.0 ± 2.0	273.0 ± 6.7	108.8 ± 6.4	50.5 ± 1.5
	+ apocynin (post)	—	37.9 ± 0.1	7.324 ± 0.023	48.7 ± 0.8	289.2 ± 4.8	93.1 ± 6.8	47.9 ± 3.1
	5-HT (100 mM)	—	38.0 ± 0.3	7.313 ± 0.016	43.6 ± 0.9	272.4 ± 5.4	104.6 ± 5.1	47.1 ± 1.1
	+ SB269970	—	37.8 ± 0.1	7.358 ± 0.010	45.5 ± 1.3	280.5 ± 6.4	86.7 ± 5.9	51.7 ± 1.3
Δ(60 min − baseline)	TC	—	0.1	0.001	0.0	−7.7*	−8.2*	2.4*
	5-HT (1 mM)	—	−0.1	−0.006	0.4	−10.2*	−11.0*	6.2*
	+ apocynin (pre)	—	−0.1	0.004	−0.1	−9.0*	−12.6*	3.4*
	+ DPI	—	−0.1	0.011	0.0	−8.3	−13.4*	2.4
	+ methysergide	—	0.2	0.001	0.2	−12.6*	−12.0*	3.4*
	+ apocynin (post)	—	−0.1	0.004	−0.3	0.5	−9.6	2.5
	5-HT (100 mM)	—	−0.2	−0.013	0.3	−0.5	−16.1*	6.7*
	+ SB269970	—	0.1	0.001	0.0	−11*	−12.0*	8.0*

Values are means ± 1 S.E.M. Note, included in the table are the groups that were pre- and also post-treated with apocynin. Drugs: apocynin, 600 μM; DPI, 1 mM; methysergide, 4 mg kg^{-1} (intravenously); SB269970, 5 mM. Also provided is the calculated mean difference between baseline and the 60 min time point for each treatment group. *Significant difference between 60 min and baseline.

report by us using this same strain (Sprague–Dawley) indicated 46% pLTF following AIH (MacFarlane *et al.* 2009). At concentrations higher than 1 mM 5-HT, pMF magnitude progressively decreased, completely disappearing at 100 mM (0.7 ± 10.3%, $n = 7$, Fig. 2*A*). There was no significant effect of episodic spinal 5-HT on XII nerve activity at any concentration used (Figs 1*A* and 2*B*), suggesting that 5-HT had not spread to the brainstem in sufficient quantity to elicit XII motor facilitation. Thus, we believe that intrathecal 5-HT injections reached effective concentrations only in spinal regions associated with the phrenic motor nucleus.

A single intrathecal injection of 5-HT (1 mM, 6 μl) also failed to elicit pMF (Fig. 1*B*). Episodic 5-HT-induced pMF was blocked by pre-treatment with an intravenous injection of methysergide (Fig. 3).

Serotonin-induced pMF requires NADPH oxidase activity for initiation and maintenance

Pre-treatment with intrathecal apocynin (600 μM, 12 μl) or DPI (1 mM, 12 μl) blocked episodic 5-HT (1 mM) induced pMF (9.5 ± 3.7%, $n = 6$; and 11.0 ± 8.2%, $n = 5$, respectively; Fig. 4*A*–*C*). Neither apocynin nor DPI had any effect on baseline phrenic nerve activity, nor did they elicit an apparent facilitation in time-control experiments (Fig. 4*A*). When apocynin was administered 5 min after the third 5-HT injection, pMF was observed 30 min post-injection (20.2 ± 6.4%, $n = 5$), with a magnitude similar to 5-HT-treated rats at this same time point (26.7 ± 4.5%, $n = 9$, Fig. 4*A*). However, 60 min following episodic 5-HT, pMF was significantly blocked (17.6 ± 6.4%; $P < 0.05$), suggesting that continued NADPH oxidase activity is necessary to maintain pMF.

Bell-shaped dose–response results from co-activation of multiple receptor subtypes

Since 5-HT concentrations above 1 mM (6 μl) decreased pMF magnitude, we investigated whether this effect may relate to co-activation of opposing 5-HT receptor subtypes. While there are multiple candidate receptor subtypes, we hypothesised that the 5-HT$_7$ receptor mediates this inhibitory effect because these receptors constrain the magnitude of AIH-induced

pLTF (Hoffman MS and Mitchell GS, unpublished observations). Thus, we pre-treated rats with a 5-HT_7 receptor antagonist and injected episodic 5-HT at a concentration of 100 mM (6 μl). Pre-treatment with the 5-HT_7 antagonist SB269970 (5 mM) restored pMF following episodic 100 mM 5-HT injections (39.7 $\pm$ 3.4%, $n = 5$, Fig. 5*A* and *B*) to levels comparable with the peak concentration of 1 mM (43.3 $\pm$ 8.7%, Fig. 2*A*). SB269970 by itself had no effect on phrenic nerve activity in time-control ($n = 4$) experiments (Fig. 5*A* and *C*).

Neuromodulatory effects of episodic 5-HT on phrenic nerve activity

5-HT concentrations that elicited pMF (up to 1 mM) had no immediate effects on spontaneous phrenic nerve activity (i.e. 'classical' neuromodulation; McCrimmon *et al.* 1995). However, at higher concentrations, 5-HT had variable effects on tonic phrenic activity. On many occasions (3 of 7), 100 mM 5-HT caused a rapid (seconds) and robust increase in phrenic nerve activity that lasted many minutes (Fig. 5*D* and *E*); the response frequently (4 of 7), though not always (e.g. Fig. 5*D*), exhibited increased tonic phrenic activity during the expiratory phase of the respiratory cycle (Fig. 5*E*). When observed, tonic phrenic activity was accompanied by a small, but sustained, decrease in arterial blood pressure (data not shown). In rats pre-treated with the 5-HT7 antagonist (SB269970), the effects of 100 mM 5-HT on tonic phrenic activity were attenuated (Fig. 5*B*). Thus, we observed a negative correlation between the extent of neuromodulatory activity on phrenic nerve activity and the magnitude of pMF; specifically, pMF was not associated with the short-term, neuromodulatory effects of 5-HT.

Discussion

Here, we demonstrate that spinal 5-HT receptor activation (without hypoxia/re-oxygenation) is *sufficient* to elicit long-lasting phrenic motor facilitation (pMF) in adult rats. This form of phrenic motor facilitation requires episodic 5-HT receptor activation and exhibits a bell-shaped dose–response curve. The down-slope of the dose–response curve most likely results from co-activation of multiple serotonin receptor subtypes that interact via 'cross-talk inhibition;' we suggest that the relevant serotonin receptor subtypes are 5-HT_2 and 5-HT_7 receptors. Serotonin-induced pMF does not require 'classical' neuromodulatory actions of 5-HT on spontaneous phrenic nerve activity, but requires spinal NADPH oxidase activity for its induction and maintenance. These findings advance our understanding of phrenic long-term facilitation, a frequently studied model of spinal respiratory plasticity induced by acute intermittent hypoxia (Mitchell *et al.* 2001; Feldman *et al.* 2003; Mahamed & Mitchell, 2007). Such an understanding may aid in the development of novel therapies to treat diverse ventilatory control disorders, including obstructive sleep apnoea, spinal injury and motor neuron disease.

Effective 5-HT concentration following intrathecal injections

Although 5-HT concentrations used to elicit pMF are reported as the concentration of injected solutions (with a constant 6 μl volume), the actual concentration in the ventral spinal cord at the level of phrenic motor neurons is expected to be considerably lower. Brumley and colleagues (2007) utilised 5-HT-sensitive micro-electrodes placed in lamina VIII of the lumbar spinal cord in anaesthetised rats to determine changes in 5-HT concentration in response to dorsal spinal 5-HT administration. Although they detected a sharp rise in intra-spinal 5-HT concentration within 30 s of serotonin administration, the peak intra-spinal concentration reached values of only 0.001% (i.e. $\sim$10 nM) of the exogenously applied level (1 mM). At concentrations lower than 1 mM, a rapid return of 5-HT to baseline levels was observed, even during sustained applications, presumably due to the efficiency of intrinsic 5-HT re-uptake mechanisms. Thus, considering the small intrathecal injection volumes (6 μl) and the limited range of 5-HT concentrations used in our study, we anticipate that the actual concentration within the phrenic motor nucleus is orders of magnitude lower than the concentrations delivered, both due to dilution in the spinal extracellular fluid and the avid re-uptake of 5-HT by serotonergic terminals and astro-glia (Brumley *et al.*

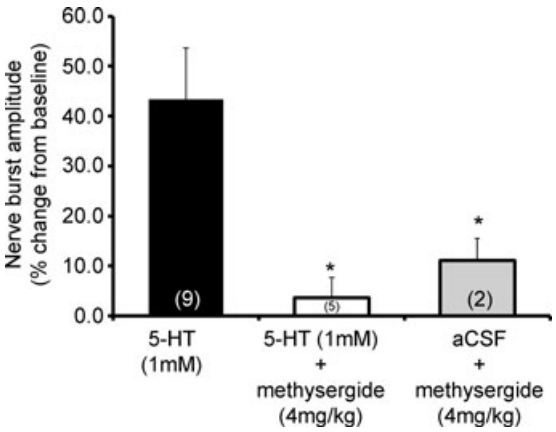

Figure 3. Magnitude of pMF following episodic 5-HT (3 × 6 μl; 1 mM) or vehicle (aCSF) and the effects of pre-treatment with an intravenous injection of the broad spectrum 5-HT receptor antagonist methysergide maleate (4 mg kg^{-1})
Values are means $\pm$ 1 S.E.M. for 60 min post-episodic 5-HT; *n* values for each treatment group are indicated in parentheses. *Significant difference from 1 mM 5-HT group ($P < 0.05$).

2007). Since available evidence suggests that extracellular 5-HT concentrations in the phrenic motor nucleus in the present study were low, and that intrathecal injections caused a pulse of 5-HT deep within the spinal cord, we believe that episodic 5-HT injections caused episodic (*versus* continuous or incrementing) 5-HT receptor activation.

Episodic spinal 5-HT receptor activation is sufficient to induce pMF

Episodic spinal 5-HT injections at concentrations less than 100 mM elicited pMF lasting at least 60 min post-injection, an effect similar in magnitude and time course to AIH-induced phrenic long-term facilitation (pLTF; Fig. 1). On the other hand, XII nerve activity was unaffected by spinal injections, suggesting that persistent changes in phrenic nerve activity resulted from spinal 5-HT receptor activation, and that effective serotonin concentrations did not reach the brainstem. XII activity has been used previously as an 'internal control' to test for unintended drug distribution (Baker-Herman & Mitchell, 2002; MacFarlane *et al.* 2009).

Single 5-HT injections did not elicit pMF, indicating that repetitive 5-HT receptor activation is a requirement, consistent with the pattern sensitivity of AIH-induced pLTF (Baker & Mitchell, 2000; Baker *et al.* 2001). These data are also consistent with reported effects of episodic 5-HT on facilitation of integrated phrenic (Lovett-Barr *et al.* 2006) and XII (Bocchiaro & Feldman, 2004) nerve activity using *in vitro* experimental preparations from neonatal rats, and 5-HT induced sensory-motor long-term facilitation in *Aplysia* (Mauelshagen *et al.* 1998).

5-HT-induced pMF requires spinal NADPH oxidase activity for induction and maintenance

Pre-treatment with DPI or apocynin attenuated 5-HT induced pMF, demonstrating that spinal NADPH oxidase activity is necessary in its underlying mechanism. These

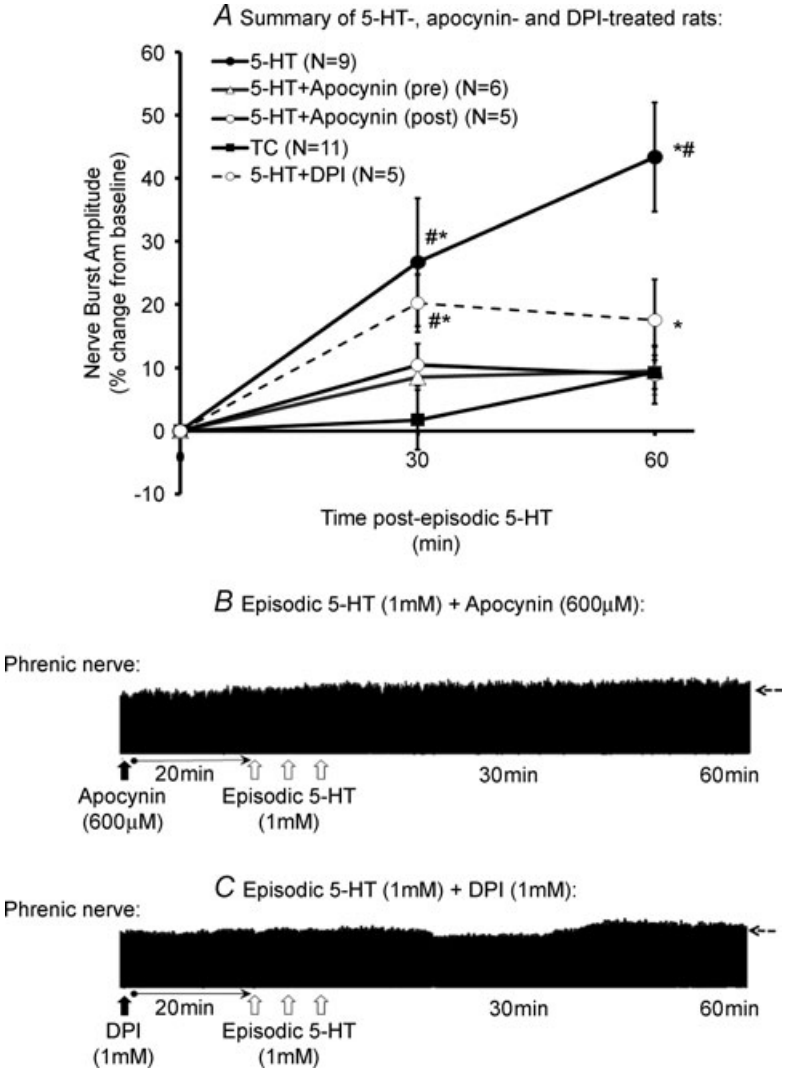

Figure 4. Effects of NADPH oxidase inhibitors, apocynin and DPI, on 5-HT-induced pMF
A, episodic 5-HT-induced pMF (3 × 6 μl; 1 mM; filled symbols) in rats that were pre-treated with an intrathecal injection of the NADPH oxidase inhibitors, apocynin (12 μl; 600 μM) or DPI (12 μl; 1 mM). Note, apocynin and DPI blocked episodic 5-HT-induced pMF. In a separate group, rats received intrathecal apocynin 5 min post-episodic 5-HT, which significantly attenuated pMF at 60 min. Values are means, ± 1 S.E.M. Note, the time control group (TC) in *A* consists of pooled data for all control groups (see methods) *Significant difference from baseline values; #significant difference from apocynin and DPI-treated groups ($P < 0.05$). *B* and *C*, also shown are two representative phrenic neurograms in rats that were pre-treated with either apocynin (*B*) or DPI (*C*) prior to episodic 1 mM 5-HT. In *B* and *C*, open arrows indicate an injection of 1 mM 5-HT (6 μl); filled arrow indicates injection of apocynin (*B*) or DPI (*C*). Blood samples (and nerve activity) were assessed at 30 and 60 min post-episodic 5-HT, as indicated. Dashed horizontal arrows at 60 min extrapolate the baseline nerve burst amplitude.

findings are similar to AIH-induced pLTF, which also requires spinal 5-HT receptor activation (Fuller *et al.* 2001*b*; Baker-Herman & Mitchell, 2002) and ROS formation (MacFarlane *et al.* 2008) via NADPH oxidase activity (MacFarlane *et al.* 2009). Indeed, key subunits of the NADPH oxidase complex are localised in putative phrenic motor neurons (MacFarlane *et al.* 2009), and the NADPH oxidase complex has been implicated in

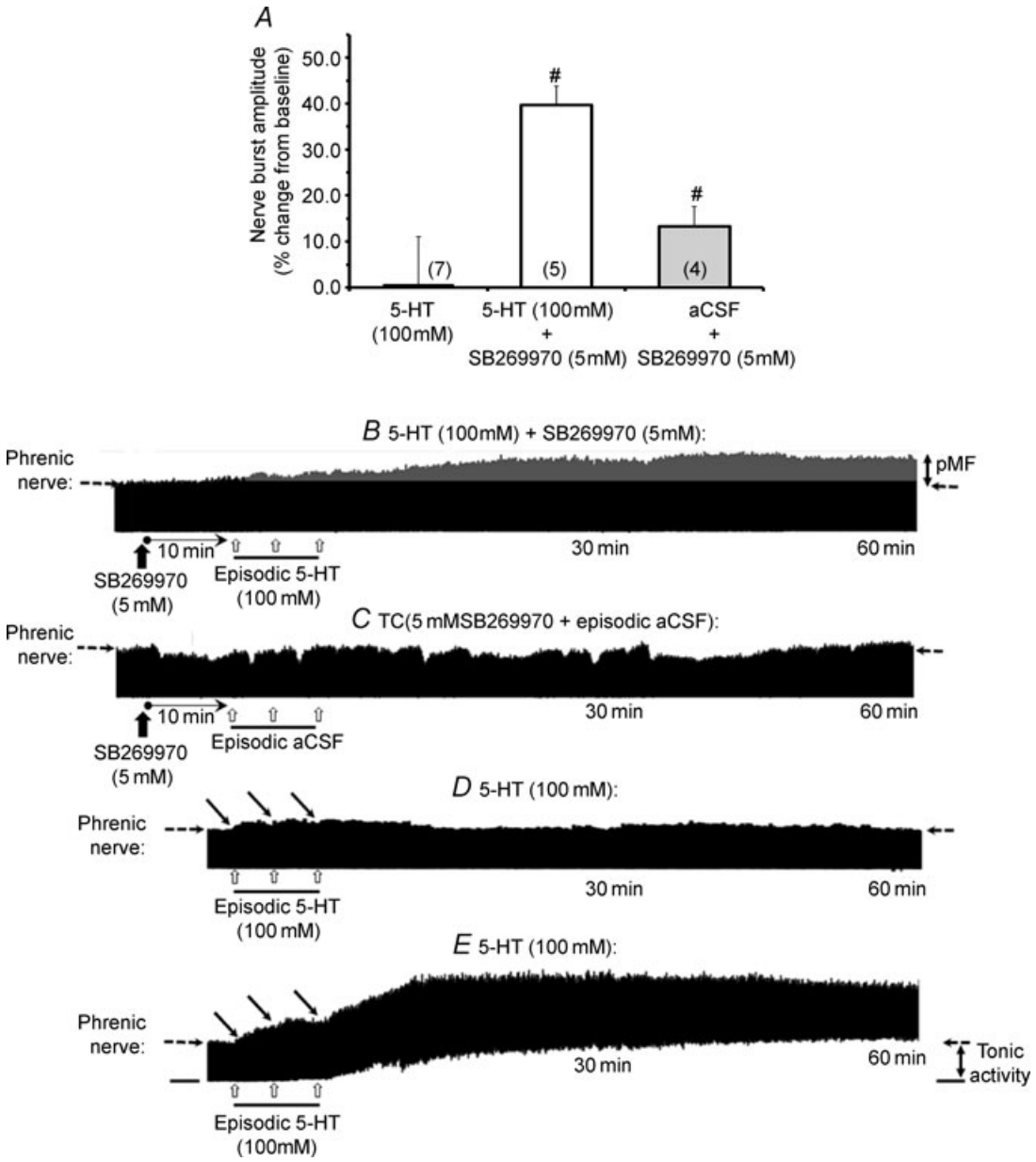

Figure 5. Effects of episodic intrathecal 100 mM 5-HT injections (3 × 6 μl, 5 min intervals) on pMF with or without pre-treatment with the 5-HT$_7$ antagonist, SB269970 (5 mM; 12 μl)

A, although 100 mM 5-HT did not elicit pMF, SB269970 pre-treatment fully restored 5-HT induced pMF. Values are means ± 1 S.E.M. at 60 min following episodic 5-HT; #significant difference from 100 mM 5-HT treated animals ($P < 0.05$); *n* values for each treatment group are given in parentheses. Representative phrenic neurograms are also provided from a rat in which pMF was restored by pre-treatment with SB269970 prior to episodic 100 mM 5-HT (*B*) and a time control (TC) rat treated with SB269970 prior to aCSF (*C*). Additional neurograms illustrate variable neuromodulatory effects immediately following 100 mM 5-HT injections on phrenic nerve activity (*D* and *E*). Note, in some (3 of 7) cases (*D*), each 5-HT injection rapidly increased integrated phrenic nerve activity (open arrows), followed by a return to baseline levels within minutes; on the other hand, increased phrenic nerve activity was sometimes (4 of 7) associated with a profound increase in tonic expiratory activity that lasted >60 min post-injections. Dashed horizontal arrows at the 60 min time point extrapolates baseline nerve burst amplitude. pMF was never observed in rats where 100 mM 5-HT injections caused short-term neuromodulatory effects on phrenic nerve activity.

multiple forms of neuroplasticity, including hippocampal LTP (Kishida *et al.* 2006; see Kishida & Klann, 2007) and sensory facilitation of the carotid body (Peng *et al.* 2006, 2009). Since pMF elicited by episodic spinal 5-HT receptor activation (without hypoxia/re-oxygenation) shares common features with AIH-induced pLTF, 5-HT receptor activation during AIH is most likely the relevant stimulus to pLTF *versus* repetitive hypoxia/re-oxygenation *per se*, at least in the experimental conditions most frequently studied (Mitchell *et al.* 2001; Baker-Herman & Mitchell, 2008; MacFarlane *et al.* 2008).

NADPH oxidase activity is necessary for pMF maintenance as demonstrated by the reversal of pMF when apocynin is delivered to the intrathecal space after episodic 5-HT administration. When apocynin was administered 5 min post-episodic 5-HT, significant pMF was observed at 30 but not 60 min post-injection. Reversal of pMF by apocynin post-treatment likely results from a combination of factors, including the diffusion dynamics of apocynin to the target region, or a 'slow' action of apocynin on NADPH oxidase. Thus, signalling cascades mediating 5-HT induced pMF appear to require persistent NADPH oxidase activity to maintain the plasticity. This conclusion contrasts with the role of 5-HT receptor activation *per se*, which is necessary to initiate, but not maintain, AIH-induced pLTF (Fuller *et al.* 2001*b*). The requirement for persistent NADPH oxidase activity to maintain AIH-induced pLTF has not been investigated. Nevertheless, because of similarities between 5-HT induced pMF and AIH-induced pLTF, we hypothesise that 5-HT receptor activation stimulates the NADPH oxidase activity necessary for AIH-induced pLTF, and that persistent NADPH oxidase activity is necessary to maintain AIH-induced pLTF.

The processes leading to persistent NADPH oxidase activity are not known, but could involve serotonin-dependent phosphorylation of the cytosolic NADPH oxidase subunits (such as $p47^{phox}$ and $p67^{phox}$); phosphorylation of these subunits is necessary for activation of the NADPH oxidase complex (Valko *et al.* 2007). We propose that persistent ROS formation is necessary to inhibit the activity of serine/threonine protein phosphatases that constrain, and possibly reverse, AIH-induced pLTF (Wilkerson *et al.* 2007; MacFarlane *et al.* 2008). Apocynin is thought to interfere with translocation of phosphorylated $p47^{phox}$ subunits to the membrane, where it normally binds to and activates membrane-bound subunits, subsequently increasing ROS formation (Valko *et al.* 2007). By inhibiting NADPH oxidase activity with apocynin following episodic 5-HT receptor activation, impaired ROS formation presumably restores the phosphatase constraint, thereby preventing further development of, or even reversing, 5-HT induced pMF or AIH-induced pLTF (MacFarlane *et al.* 2008).

Dose-response of 5-HT induced pMF

We hypothesise that the bell shaped dose–response curve of 5-HT induced pMF results from co-activation of different receptor subtypes, leading to 'cross-talk inhibition' between their respective cellular cascades. The 5-HT_2 receptor family is relevant to pLTF/pMF initiation since episodic intrathecal injections of 5-HT_2 receptor agonists elicit pMF (MacFarlane & Mitchell, 2008*b*) and 5-HT_2 receptor activation is necessary for AIH-induced pLTF (Kinkead *et al.* 1998; Fuller *et al.* 2001*b*; Baker-Herman & Mitchell, 2002). Furthermore, 5-HT_2 receptors are abundant in respiratory motor nuclei, including the XII (Kubin & Volgin, 2008) and phrenic motor pools (Basura *et al.* 2001). At higher concentrations of 5-HT (>1 mM), however, other serotonergic receptors may play progressively greater roles in determining the expression of pMF, including G_i ($5\text{-HT}_{1/5}$) and/or G_s-coupled ($5\text{-HT}_{4/6/7}$) metabotropic receptors (Hannon & Hoyer, 2008). These receptors may initiate processes that converge on and inhibit 5-HT_2-dependent pMF. This 'cross-talk inhibition' between G-protein coupled receptors and their downstream signalling processes has been described in the literature (Robinson-White & Stratakis, 2002), and has been proposed to be an important mechanism regulating AIH-induced pLTF (Hoffman & Mitchell, 2008). Since pre-treatment with a 5-HT7 receptor antagonist enhances AIH-induced pLTF, these receptors constrain pLTF, most likely by a PKA-dependent mechanism (Hoffman & Mitchell, 2008). Thus, the declining phase of the bell-shaped concentration–response curve may involve progressively greater 5-HT7 receptor activation. Similar concentration-dependent effects of 5-HT on synaptic plasticity occur in sensory inputs to the spinal dorsal horn. At low concentrations of 5-HT, ineffective (i.e. 'silent') glutamatergic synapses in the spinal dorsal horn became functional, whereas higher concentrations were inhibitory due to co-activation of $5\text{-HT}_{1A/7}$ receptors (Li & Zhuo, 1998).

Neuromodulatory effects of 5-HT are not necessary for pMF

The observation that 5-HT induced pMF was greatest at concentrations too low to elicit the 'classical' neuromodulatory actions of 5-HT on phrenic motor neurons (McCrimmon *et al.* 1995; Rekling *et al.* 2000) is of interest in several important respects. Although this dissociation is difficult to explain, it does suggest that 'classical neuromodulation' and pMF arise from distinct cellular mechanisms. The observation may indicate that different 5-HT receptor subtypes underlie pMF and classical neuromodulation, respectively. On the other hand, it may be that low levels of 5-HT_2 receptor activation elicit pMF,

whereas greater activation elicits neuromodulation and, in turn, inhibits subsequent development of pMF. These competing hypotheses remain to be explored. Regardless, the dissociation between pMF and the tonic, neuromodulatory effects of 5-HT suggest that future studies of 5-HT induced facilitation must carefully consider the concentration of 5-HT (or agonists) used, and the potential impact of the tonic, neuromodulatory actions of 5-HT receptor activation (e.g. Monteau *et al.* 1990; Berger *et al.* 1992; Bocchiaro & Feldman 2004; Peng *et al.* 2006; Brandes *et al.* 2006).

Although we did not investigate which 5-HT receptor subtype caused tonic phrenic activity, others have implicated 5-$HT_{1A/2C}$ (Kubin *et al.* 1992) or 5-HT_{2A} receptors (Lindsay & Feldman, 1993; DiPasqual *et al.* 1997). Here, we observed that the 5-HT_7 receptor antagonist SB269970 attenuated the tonic activity, suggesting a prominent role for this receptor type in mediating phrenic neuromodulation. Overall, the variability in the responses to 5-HT (or agonists) could depend on several factors including the concentrations used, the selectivity of agonists for the target receptors, species differences, the neural populations under investigation, and the experimental preparation (*in vitro versus in vivo*, developmental age, etc.).

Conclusions

We provide the first *in vivo* evidence that spinal serotonin receptor activation is sufficient to elicit long-lasting phrenic motor facilitation in adult rats via an NADPH oxidase (presumably ROS)-dependent mechanism. These data are consistent with previous reports concerning the role of spinal serotonin receptors (Fuller *et al.* 2001*b*; Baker-Herman & Mitchell, 2002) and NADPH oxidase (MacFarlane *et al.* 2009) in AIH-induced pLTF, suggesting that both forms of plasticity share common mechanisms. Thus, since hypoxia stimulates raphe serotonergic neurons (Erickson & Millhorn, 1994) and 5-HT release in respiratory motor nuclei (Kinkead *et al.* 2001), we suggest that the relevant stimulus to AIH-induced pLTF is repetitive serotonin receptor activation *versus* hypoxia/re-oxygenation *per se*.

The bell-shaped dose–response curve indicates that 5-HT-induced pMF depends on the relative activation of different 5-HT receptor subtypes, possibly invoking 'cross-talk inhibition'. Furthermore, since pMF is not observed if the 5-HT concentration used induced tonic, neuromodulatory effects on phrenic nerve activity, we suggest that mechanisms of pMF and classical neuromodulation in respiratory motor neurons are distinct. The diversity of serotonin receptor subtypes and the ways in which they determine different motor neuron behaviours (e.g. neuromodulation *versus* plasticity; Mitchell & Johnson, 2003) could be important, enabling the respiratory neural networks to mount appropriate responses when confronted with varied challenges.

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Author contributions

Both authors 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.

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