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Acute intermittent hypoxia with concurrent hypercapnia evokes P2X and TRPV1 receptor-dependent sensory long-term facilitation in naïve carotid bodies

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Key points

- Activity-dependent plasticity can be induced in carotid body (CB) chemosensory afferents without chronic intermittent hypoxia (CIH) preconditioning by acute intermittent hypoxia coincident with bouts of hypercapnia (AIH-Hc).
- Several properties of this acute plasticity are shared with CIH-dependent sensory long-term facilitation (LTF) in that induction is dependent on 5-HT, angiotensin II, protein kinase C and reactive oxygen species.
- Several properties differ from CIH-dependent sensory LTF; H_2O_2 appears to play no part in induction, whereas maintenance requires purinergic P2X2/3 receptor activation and is dependent on transient receptor potential vanilloid type 1 (TRPV1) receptor sensitization.
- Because P2X2/3 and TRPV1 receptors are located in carotid sinus nerve (CSN) terminals but not presynaptic glomus cells, a primary site of the acute AIH-Hc induced sensory LTF appears to be postsynaptic.
- Our results obtained *in vivo* suggest a role for TRPV1-dependent CB activity in acute sympathetic LTF. We propose that P2X-TRPV1-receptor-dependent sensory LTF may constitute an important early mechanism linking sleep apnoea with hypertension and/or cardiovascular disease.

Abstract Apnoeas constitute an acute existential threat to neonates and adults. In large part, this threat is detected by the carotid bodies, which are the primary peripheral chemoreceptors, and is combatted by arousal and acute cardiorespiratory responses, including increased sympathetic output. Similar responses occur with repeated apnoeas but they continue beyond the last apnoea and can persist for hours [i.e. ventilatory and sympathetic long-term facilitation (LTF)]. These long-term effects may be adaptive during acute episodic apnoea, although they may prolong hypertension causing chronic cardiovascular impairment. We report a novel mechanism of acute carotid body (CB) plasticity (sensory LTF) induced by repeated apnoea-like stimuli [i.e. acute intermittent hypoxia coincident with bouts of hypercapnia (AIH-Hc)]. This plasticity did not require chronic intermittent hypoxia preconditioning, was dependent on P2X receptors and protein kinase C, and involved heat-sensitive transient receptor potential vanilloid type 1 (TRPV1) receptors. Reactive oxygen species ($O_2\cdot^-$) were involved in initiating plasticity only; no evidence was found for H_2O_2 involvement. Angiotensin II and 5-HT receptor antagonists, losartan and ketanserin, severely reduced CB responses to individual hypoxic-hypercapnic challenges and prevented the induction of sensory LTF but, if applied after AIH-Hc, failed to reduce plasticity-associated activity. Conversely, TRPV1 receptor antagonism had no effect on responses to individual hypoxic-hypercapnic challenges but reduced plasticity-associated activity by ∼50%. Further, TRPV1 receptor antagonism *in vivo* reduced sympathetic LTF caused by AIH-Hc, although only

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if the CBs were functional. These data demonstrate a new mechanism of CB plasticity and suggest P2X-TRPV1-dependent sensory LTF as a novel target for pharmacological intervention in some forms of neurogenic hypertension associated with recurrent apnoeas.

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Introduction

The carotid bodies (CBs) are the primary detectors of apnoea, responding vigorously to bouts of hypoxic-hypercapnia (as occurs during prolonged apnoea) or bouts of hypoxia against a hypercapnic background (as occurs in most sleep apnoea patients). Vigorous activity of the CB caused by hypoxia with concurrent hypercapnia, in turn, initiates life-saving cardiorespiratory and arousal reflexes (Mohan & Duffin, 1997; Duffin, 2010). However, compelling human data suggest that upregulation of CB activity and/or sensitivity is important to the aetiology of cardiorespiratory diseases, including hypertension and heart failure associated with sleep apnoea (Mansukhani *et al.* 2015). For example, sympathetic and ventilatory responses to hypoxia and hypercapnia are enhanced in sleep apnoea and/or borderline hypertensive patients compared to controls (Trzebski *et al.* 1982; Somers *et al.* 1988; Spicuzza *et al.* 2006; Imadojemu *et al.* 2007), whereas glomectomy or inactivation of the CB with excess oxygen (hyperoxia) mitigate sleep apnoea-associated increases in sympathetic outflow and blood pressure (Somers & Abboud, 1993; Narkiewicz *et al.* 1998). Moreover, 20–30 min of intermittent hypoxia in healthy humans enhances sympathetic activity, including sympathetic sensitivity to hypoxia (Cutler *et al.* 2004; Leuenberger *et al.* 2007).

In accordance with the findings in humans, CB denervation prevents increases in sympathetic nerve activity (SNA) and/or increased blood pressure in animal models of sleep apnoea (chronic intermittent hypoxia) (Fletcher, 2000; Prabhakar *et al.* 2005; McBryde *et al.* 2013). The CBs from these disease models demonstrate functional changes. Peng *et al.* (2003) demonstrated that intact and isolated superfused CB preparations from rats exposed to 10 days of chronic intermittent hypoxia (CIH) undergo sensory long-term facilitation (LTF), as characterized by a persistent increase in baseline afferent activity and augmented sensitivity to hypoxia, in response to 10 bouts of acute intermittent hypoxia (AIH). (Peng *et al.* 2003). Other studies report that rats exposed to AIH (e.g. ten 45 s periods of 10% O_2 interspersed with 100% O_2) have an enhanced chemoreflex response to hypoxia and/or demonstrate respiratory and/or sympathetic LTF without CIH preconditioning (Xie *et al.* 2000; Dick *et al.* 2007; Mahamad & Mitchell, 2007; Xing & Pilowsky, 2010), and we found qualitatively similar, although weaker, acute stimulus-dependent responses in isolated perfused rat CBs (Cummings & Wilson 2005). Taken together, these studies suggest that the sensitivity of the CB might be enhanced during recurrent apnoeas, resulting in increased sympathetic activity and hypertension. Consequently, the CB has gained renewed attention as a possible target organ for the treatment of sleep apnoea and associated hypertension (DelRio *et al.* 2015).

The mechanism(s) responsible for the induction and maintenance phases of CB sensory LTF are not fully resolved. When sensory LTF is induced by a combination of CIH preconditioning and AIH, maintenance is probably contingent on a presynaptic (glomus cell) reactive oxygen species (ROS)-dependent mechanism involving 5-HT, NADPH oxidase/ROS signalling (Peng *et al.* 2009) and a T-type voltage-gated calcium channel, type 3.2 (CaV3.2) (Makarenko *et al.* 2016). However, sensory LTF induced by acute stimuli in the absence of CIH preconditioning may be mediated by a different mechanism.

Previously, we demonstrated that transient receptor potential vanilloid type 1 (TRPV1) receptors in CB chemosensory afferents are largely responsible for the anandamide and exquisite heat sensitivity of the CB (Roy *et al.* 2012). Because TRPV1 plays an important role in neuronal plasticity in the hippocampus and several sensory systems, and because, in some of these systems, ATP activation of P2X receptor leads to TRPV1 phosphorylation, we hypothesized that TRPV1 receptors contribute to CB sensory LTF (Bhave *et al.* 2003; Marsch *et al.* 2007; Li *et al.* 2008; Hasegawa *et al.* 2009; Shen *et al.* 2012; Lin *et al.* 2013; Saloman *et al.* 2013; Ruan *et al.* 2014; Yang *et al.* 2016).

In the present study, we report robust sensory LTF (increased baseline and augmented hypoxic responses) induced by acute recurrent apnoea-like stimuli (10 bouts of acute hypoxia with a hypercapnic background, AIH-SHc; or 10 bouts of acute intermittent hypoxia coincident with bouts of hypercapnia; AIH-Hc) in perfused CBs isolated from naïve rats. We demonstrate that ATP, the primary neurotransmitter released from glomus cells and acting via P2X2/3 receptors, is necessary for the induction and maintenance of sensory LTF following AIH-Hc; however, we also demonstrate that

50% of sensory LTF is mediated by TRPV1 receptors located on afferent fibres (postsynaptic to glomus cells). Unlike P2X2/3, TRPV1 plays no role in the responses to individual hypoxic-hypercapnic challenges prior to sensory LTF induction, suggesting that TRPV1 is a primary effector of CB chemosensory plasticity.

Methods

Ethical approval

CB experiments were performed in accordance with The Canadian Council of Animal Care guidelines and were approved by the Animal Care Committee of the Cumming School of Medicine, University of Calgary, Canada. Sympathetic experiments were performed in accordance with Macquarie University Animal Care Committees and were approved by The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All animal procedures conform with the principles and regulations as described by Grundy (2015).

Experimental animals

CB *ex vivo* experiments were conducted on 126 male adult Sprague–Dawley Albino rats (150–250 g) purchased from Charles River (Quebec, Canada) and *in vivo* sympathetic experiments were conducted on 29 adult male Sprague–Dawley rats (350–500 g; Animal Resource Centre, Perth, Australia). Animals were housed conventionally in temperature and humidity controlled facilities with food and water available *ad libitum*.

Solutions and chemicals

Physiological buffer for dissection and perfusion comprised (in mM): 115 NaCl, 4 KCl, 24 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 10 D-glucose and 12 sucrose (Sigma-Aldrich, Oakville, Ontario, Canada). The chemicals used were: ATP, ketanserin and polyethylene glycol (PEG)-catalase (Sigma-Aldrich); suramin, 2,4,6-trinitrophenol (TNP)-ATP, AMG9810 [(2*E*)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-3-(4-[1,1-dimethylethyl]phenyl)-2-propenamide], GF109203X (bisindolylm-aleimide) and AIP (autocamtide-2-related inhibitory peptide) (Tocris Bioscience, Bristol, UK); losartan (Cayman Chemical Company, Ann Arbor, MI, USA); and MnTMPyP [5,10,15,20-Tetrakis(1-methylpyridinium-4-yl)-21H,23H-porphyrin manganese(III) pentachloride] (Calbiochem, San Diego, CA, USA).

Ex vivo carotid body-carotid sinus nerve (CB-CSN) preparation

This preparation was used to characterize and determine the mechanism responsible for CB sensory LTF induced by AIH with concurrent hypercapnia. Rats were heavily anaesthetized with 5% isoflurane by inhalation in air and then decapitated (lower cervical level). Immediately after killing the animal, the carotid bifurcation, including the CB, CSN and superior cervical ganglion, was quickly removed *en bloc* for *in vitro* arterial perfusion as described previously (Roy *et al.* 2012). The carotid bifurcation was then transferred to physiological buffer equilibrated with 95% O_2 and 5% CO_2. After incubating for a minimum of 20 min in ice-cold buffer, the preparation was transferred *en bloc* to a temperature-regulated recording chamber, the common carotid artery was immediately cannulated for luminal perfusion with physiological buffer equilibrated with 100 Torr P_{O_2} and 35 Torr P_{CO_2} (balanced N_2) and the CSN was freed and de-sheathed. A peristatic pump and heat exchanger were used to supply physiological saline via the cannula at a flow rate of ∼15 ml min^{-1} (the pressure at the tip of the cannula was ∼100 mmHg) and to maintain the temperature at 37 ± 0.5°C. We established that sensory LTF occurred whether or not the effluent was recirculated (see Results); therefore, in most experiments, the effluent was recirculated for convenience. Perfusate was equilibrated with computer-controlled gas mixtures. Mixtures were monitored using precision CO_2 and O_2 gas analysers (models CA-2A and PA1B, respectively; Sable Systems, Las Vegas, NV, USA) and balanced with N_2. Unless otherwise stated, 100 Torr P_{O_2} (normoxia) and 35 Torr P_{CO_2} (normocapnia; yielding pH ∼7.4) were used. Chemosensory afferent activity was recorded extracellularly from the CSN (multifibre) using a platinum-hook electrode and a differential AC amplifier (Model 1700; AM Systems Inc., Sequim, WA, USA). The neural activity was amplified, filtered (300 Hz low cut-off, 5 kHz high cut-off) and displayed on an oscilloscope (model 2230; Tektronix, Beaverton, OR, USA); raw, rectified and integrated (200 ms time constant; MA-821; CWE, Inc., Ardmore, PA, USA) data were stored on a computer using an A/D board (Digidata 1322A; Axon Instruments, Axon Instruments, Foster City, CA, USA) and data acquisition software (Axoscope, version 9.0; Molecular Devices, Sunnyvale, CA, USA) at 5 kHz.

Experimental protocols (*ex vivo* preparation)

Once stable recordings had been achieved, preparations were exposed to a brief hypoxic challenge (4 min, P_{O_2} 60 Torr); only preparations showing a clear-cut increase in activity were used. Preparations were perfused for an additional 60 min with normoxic-normocapnic buffer before initiation of the experimental protocol.

Exposure to AIH-Hc or AIH-SHc. AIH-Hc experiments were performed on naive rats, not having prior exposure to CIH. The AIH-Hc paradigm consisted of ten 1 min bouts

of hypoxia-hypercapnia (P_{O_2} = 40 Torr; P_{CO_2} = 60 Torr) interspersed with 5 min of normoxia-normocapnia (P_{O_2} = 100 Torr; P_{CO_2} = 35 Torr). The AIH-SHc paradigm consisted of ten 1 min bouts of hypoxia (P_{O_2} = 60 Torr) interspersed with 5 min of normoxia (P_{O_2} = 100 Torr) with a hypercapnic background (P_{CO_2} = 50 Torr). The preparation was perfused with normoxic-normocapnic buffer immediately after the last stimulus. Sensory LTF was defined as the difference between CSN activity at baseline (pre-stimulus) and 60 min after completion of the AIH-Hc or AIH-SHc protocols. Time control experiments were performed by challenging the CB with 10 episodes of intermittent normoxia-normocapnia.

Exposures to AIH or AIHc. Separate experiments were performed in naive rats to determine whether sensory LTF can also be evoked by stimuli other than AIH-Hc. AIH or AIHc paradigms consisted of ten 1 min bouts of hypoxia (P_{O_2} = 40 Torr) or hypercapnia (P_{CO_2} = 60 Torr). Each bout was separated by 5 min of normoxia-normocapnia.

Exposure to ACH-Hc. Separate experiments were performed to examine the cumulative effects of hypoxia-hypercapnia to determine the importance of stimulus pattern. The ACH-Hc paradigm consisted of 10 min of continuous hypoxia-hypercapnia (P_{O_2} = 40 Torr; P_{CO_2} = 60 Torr).

Data analysis (*ex vivo* preparation)

Data were analysed offline using custom software (written by RJAW). CSN activity was divided into 60 s time bins and the activity in each bin was rectified and summed (expressed as integrated neural discharge). The neural responses for different conditions in the protocol were normalized to the baseline (normoxic) condition. All data were expressed as the mean ± SEM. One-way ANOVA with Holm–Šidák *post hoc* tests were used to analyse the data (SigmaStat, version 2.03; Systat Software Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

In vivo anaesthetized rat preparation

This preparation was used to determine the role of the CB and TRPV1 receptors in sympathetic LTF induced by AIH-Hc. Experiments were conducted on adult male Sprague–Dawley rats (350–500 g; Animal Resource Centre). Rats (n = 29) were anaesthetized with urethane (1.3 g kg^{-1} in a 10% solution I.P.). Complete details of the surgical preparation and data acquisition methods are provided elsewhere (Farnham *et al.* 2008). Briefly, the core temperature was maintained at 37 ± 0.5°C. The right carotid artery and jugular vein were cannulated for measurement of mean arterial pressure (MAP) and the administration of drugs and fluids, respectively. Tracheostomy was performed to permit artificial ventilation. The left greater splanchnic sympathetic nerve and left phrenic nerve were isolated and activity was recorded using silver hook electrodes. Data were acquired using a CED 1401 ADC system and Spike 2 acquisition and analysis software, version 7.12 (Cambridge Electronic Design, Cambridge, UK). All animals were bilaterally vagotomized, ventilated with either room air (n = 14) or hyperoxia (n = 15) and paralysed (0.8 mg kg^{-1} pancuronium bromide I.V., followed by an infusion of 0.8 mg kg^{-1} h^{-1} of pancuronium in 0.9% saline at a rate of 2 ml h^{-1}; Astra Zeneca, Australia). Supplemental anaesthetic (0.2–0.4 ml of 10% urethane) was administered (I.V.), if necessary, to maintain a surgical plane of anaesthesia, which was assessed by monitoring if there was a change in blood pressure >10 mmHg in response to a tail or paw pinch.

Experimental protocols (*in vivo* preparation)

Recordings were allowed to stabilize until 30 min of stable nerve activity was recorded. All rats had 0.2 ml of arterial blood withdrawn for respiratory blood gas (O_2 and CO_2) and pH analysis (electrolyte and blood gas analyser; IDEXX Laboratories, Lenexa, KS, USA) 10 min prior to AIH-Hc. The AIH-Hc protocol consisted of 10 bouts (1 min each) of ventilation with a gas mixture of 8% CO_2 and 5.4% O_2 in N_2, interspersed at intervals of 5 min. This was performed in two groups of animals, against a backdrop of either normoxic or hyperoxic conditions (Table 1). Arterial blood gas was analysed at 30 min, 60 min and 90 min following the 10th hypoxia-hypercapnia bout (Table 1). At 60 min post-AIH-Hc, a single hypoxia-hypercapnia bout was administered with 5 min of recovery. At this point, the vehicle control solution [dimethylsulphoxide (DMSO)] or AMG9810 (100 mg kg^{-1}) was administered I.P. and recordings were maintained for another 30 min. A final arterial blood gas analysis and hypoxia-hypercapnia stimulus was performed before killing the rats with 0.5 ml of 3 M KCl.

Data analysis (*in vivo* preparation)

Mean arterial pressure, heart rate and SNA, were analysed from 1 min blocs taken 1 min prior to and also 30 min, 60 min and 90 min after AIH-Hc. Statistical analysis was conducted using Prism, version 5.01 (GraphPad Software Inc., San Diego, CA, USA). The changes in responses from 60 min to 90 min after AIH-Hc were compared between vehicle control and AMG9810 using a one-way ANOVA with *post hoc* Holm–Šidák tests.

Table 1. P_{aO_2}, O_2 saturation, P_{aCO_2}, pH and MAP measurements pre, post-AIH-Hc and during drug treatment

Background O_2	Treatment time	P_{aO_2} (Torr)	O_2 saturation (%)	P_{aCO_2} (Torr)	pH	MAP (mmHg)
Normoxia	Pre-AIH-Hc	86.8 ± 7.1	94.3 ± 2.0	39.7 ± 2.1	7.48 ± 0.02	99 ± 3
	30 min	83.5 ± 4.1	93.0 ± 1.4	38.8 ± 1.6	7.49 ± 0.02	107 ± 6
	60 min	81.7 ± 4.4	92.0 ± 1.3	39.3 ± 1.9	7.48 ± 0.01	102 ± 3
	90 min DMSO	79.3 ± 4.4	92.0 ± 1.5	39.7 ± 1.7	7.46 ± 0.01	106 ± 3
	Pre-AIH-Hc	81.2 ± 5.1	92.4 ± 1.6	41.5 ± 1.8	7.48 ± 0.02	90 ± 3
	30 min	79.0 ± 5.8	91.4 ± 2.4	41.8 ± 1.5	7.52 ± 0.04	93 ± 2
	60 min	80.3 ± 3.8	92.8 ± 0.8	40.6 ± 1.0	7.48 ± 0.01	96 ± 3
	90 min AMG9810	79.3 ± 4.4	92.0 ± 1.5	39.7 ± 1.7	7.47 ± 0.01	94 ± 3
Hyperoxia	Pre-AIH-Hc	496.8 ± 10.8	100 ± 0	42.3 ± 1.9	7.42 ± 0.01	113 ± 6
	30 min	455.8 ± 36.6	100 ± 0	41.0 ± 2.3	7.41 ± 0.01	108 ± 8
	60 min	469.5 ± 21.3	100 ± 0	40.3 ± 1.2	7.41 ± 0.01	105 ± 6
	90 min DMSO	469.8 ± 24.8	100 ± 0	43.7 ± 2.8	7.39 ± 0.02	112 ± 8
	Pre-AIH-Hc	491.0 ± 7.1	100 ± 0	40.7 ± 1.3	7.43 ± 0.01	110 ± 3
	30 min	467.0 ± 13.9	100 ± 0	41.2 ± 1.2	7.43 ± 0.01	119 ± 4
	60 min	483.0 ± 18.2	100 ± 0	41.0 ± 1.1	7.43 ± 0.01	117 ± 4
	90 min AMG9810	492.3 ± 10.6	100 ± 0	40.2 ± 1.3	7.42 ± 0.01	113 ± 3

Results

AIH with concurrent hypercapnia induces CB sensory LTF

The effects of AIH-Hc on chemosensory activity were examined in *ex vivo* perfused CBs from six naïve rats (without prior conditioning to CIH exposures). CSN activity increased progressively with each successive hypoxia-hypercapnia stimulus (Fig. 1*A*). Terminating each AIH-Hc bout with normoxia-normocapnia promptly returned the CSN activity to baseline; the baseline incremented only slightly between bouts. After the last (10th) AIH-Hc bout, baseline CSN activity gradually increased. This increase in baseline (i.e. sensory LTF) lasted at least 60 min (pre AIH-Hc baseline *vs.* baseline 60th minute post AIH-Hc: 1.00 ± 0.01 *vs.* 1.82 ± 0.13; $P < 0.001$; $n = 6$) (Fig. 2*A*). There was no difference in the magnitude of sensory LTF regardless of whether the effluent was recirculated or not ($n = 6$) (1.82 ± 0.13 *vs.* 1.71 ± 0.09; $P = 0.73$) (Fig. 1*B* and Fig. 2*F*). No sensory LTF was evident in time controls ($n = 5$) (Fig. 2*A*). During the course of our investigation into the mechanisms involved in the induction and maintenance of sensory LTF, 63 experiments were conducted using AIH-Hc in which we could assess the induction of sensory LTF without drug intervention; of these, six failed to show sensory LTF (10.5%) (Fig. 1*D*). All failures either had a progressive decline or initial progressive increase followed by a decline in peak CSN activity with each AIH-Hc exposure (Fig. 1*C*). Overall, however, there was no significant relationship between the change in CSN response from the first to last bout of AIH-Hc and the magnitude of sensory LTF 60 min after the last bout ($r^2 = 0.02$; $P = 0.19$) (Fig. 1*E*).

To determine whether sensory LTF can be induced by stimulating the CB with moderate AIH and sustained hypercapnia, i.e. AIH-SHc, naive CBs were challenged with 10 episodes of moderate AIH ($P_{O_2} = 60$ Torr) with a hypercapnia background ($P_{CO_2} = 50$ Torr). CSN activity increased progressively with each bout of AIH. During induction, baseline activity remained elevated after termination of each AIH challenge as a result of the background hypercapnia (Fig. 2*B*). Activity levels 60 min after the last challenge with the preparation receiving normoxia-normocapnia perfusate demonstrated that the combination of moderate AIH with sustained hypercapnia also resulted in sensory LTF (pre AIH baseline 1.00 ± 0.02 *vs.* baseline 60th minute post AIH 1.62 ± 0.12, $P < 0.05$, $n = 6$) (Fig. 2*B*).

To determine whether sensory LTF can also be induced by stimulating the CBs with either AIH or AIHc alone; normal CBs were subjected to ten episodes of AIH ($P_{O_2} = 40$ Torr) or AIHc ($P_{CO_2} = 60$ Torr). Each bout of hypoxia or hypercapnia augmented the CSN activity; however, the characteristic progressive increase in CSN activity seen following AIH-Hc was absent (AIH: 1.01 ± 0.02 *vs.* 0.99 ± 0.01; $P = 0.32$; $n = 6$; AIHc: 0.99 ± 0.01 *vs.* 0.98 ± 0.01; $P = 0.53$; $n = 6$) (Fig. 2*C* and *D*).

To determine whether the temporal pattern of the stimuli is critical for inducing sensory LTF, we tested the effects of an ACH-Hc, equivalent to the cumulative dose during AIH-Hc. ACH-Hc elicited a robust increase in CSN activity followed by post stimuli depression on returning to normoxia-normocapnia. At 60 min after ACH-Hc, sensory LTF was minimal (baseline pre-stimulus *vs.* 60th minute post stimulus: 1.01 ± 0.01 *vs.* 1.15 ± 0.02; $n = 6$; $P < 0.05$) (Fig. 2*E*) and only a fraction of that induced by AIH-Hc (1.15 ± 0.02 *vs.* 1.82 ± 0.13; $n = 6$;

$P < 0.05$) or AIH with a background of hypercapnia (1.15 ± 0.02 *vs.* 1.62 ± 0.12; $n = 6$; $P < 0.05$). The above observations confirm and extend our previous findings (Cummings & Wilson, 2005) suggesting that induction of sensory LTF is possible without CIH pre-conditioning and depends on a diverse stimulus patterning and strength (Fig. 2*F*).

Sensory LTF increases CB sensitivity to hypoxia and temperature

To determine whether sensory LTF increases the sensitivity of the CB to hypoxia, *ex vivo* CBs were subjected to brief (3 min) hypoxic challenges ($P_{O_2} = 60$ Torr) pre AIH-Hc and 60 min after the 10th AIH-Hc bout. The data shown in Fig. 3(*A* and *B*) show a marked increase in CSN activity to acute hypoxia during the maintenance phase of sensory LTF (ΔHx pre-sensory LTF *vs.* ΔHx during sensory LTF: 0.56 ± 0.06 *vs.* 0.85 ± 0.07; $P < 0.001$; $n = 5$). In another set of experiments, we assessed the temperature sensitivity of CBs subjected to AIH-Hc treatment (Fig. 3*C* and *D*). At 1 h after the 10th AIH-Hc bout, the CB response to an increase in basal perfusate temperature from 37°C to 39°C for 1 min was enhanced (ΔT pre-sensory LTF *vs.* ΔT during sensory LTF: 0.30 ± 0.04 *vs.* 0.52 ± 0.05; $P < 0.001$; $n = 5$).

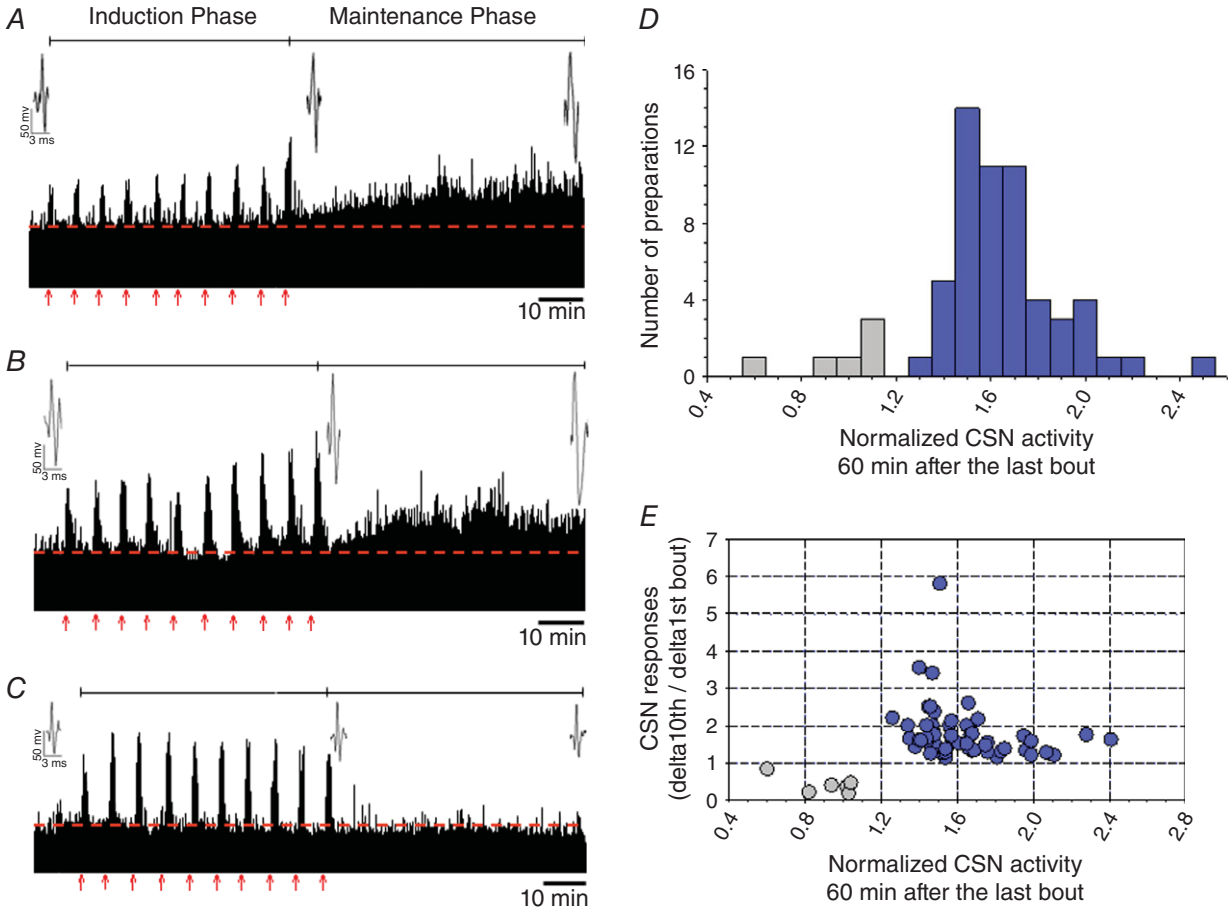

Figure 1. AIH-Hc causes sensory LTF in *ex vivo* CB from naïve rat
Examples of rectified CB sensory activity (*A–B*) from three preparations exposed to ten 1 min bouts of hypoxia-hypercapnia (red arrows; each interspersed with 5 min of normoxia-normocapnia). Insets represent action potentials from a single unit. During AIH-Hc (i.e. the induction phase), most preparations had augmenting responses with each bout (*A*, recirculating effluent; *B*, non-recirculating effluent). We observed a small number of preparations with decrementing responses, *C*, after AIH-Hc (i.e. during the maintenance phase), CSN activity increased in (*A*) and (*B*) (indicative of sensory LTF) but not in (*C*) (no sensory LTF). *D*, magnitude of CSN activity 60 min after the last hypoxia-hypercapnia bout ($n = 63$). In total, 57 preparations demonstrated sensory LTF (blue); in the remaining six, sensory LTF was absent (grey). *E*, scatter plot showing the relationship between the change in CSN activity between the first and last (10th) bout of hypoxia-hypercapnia compared to the CSN activity at 60 min after the last bout. Only preparations in which responses increased with bouts demonstrated sensory LTF (blue). However, the degree of augmentation did not predict sensory LTF magnitude ($P = 0.06$; $r^2 = 0.02$). Note that CSN activity is normalized to the baseline preceding the first bout (dashed line). [Colour figure can be viewed at wileyonlinelibrary.com]

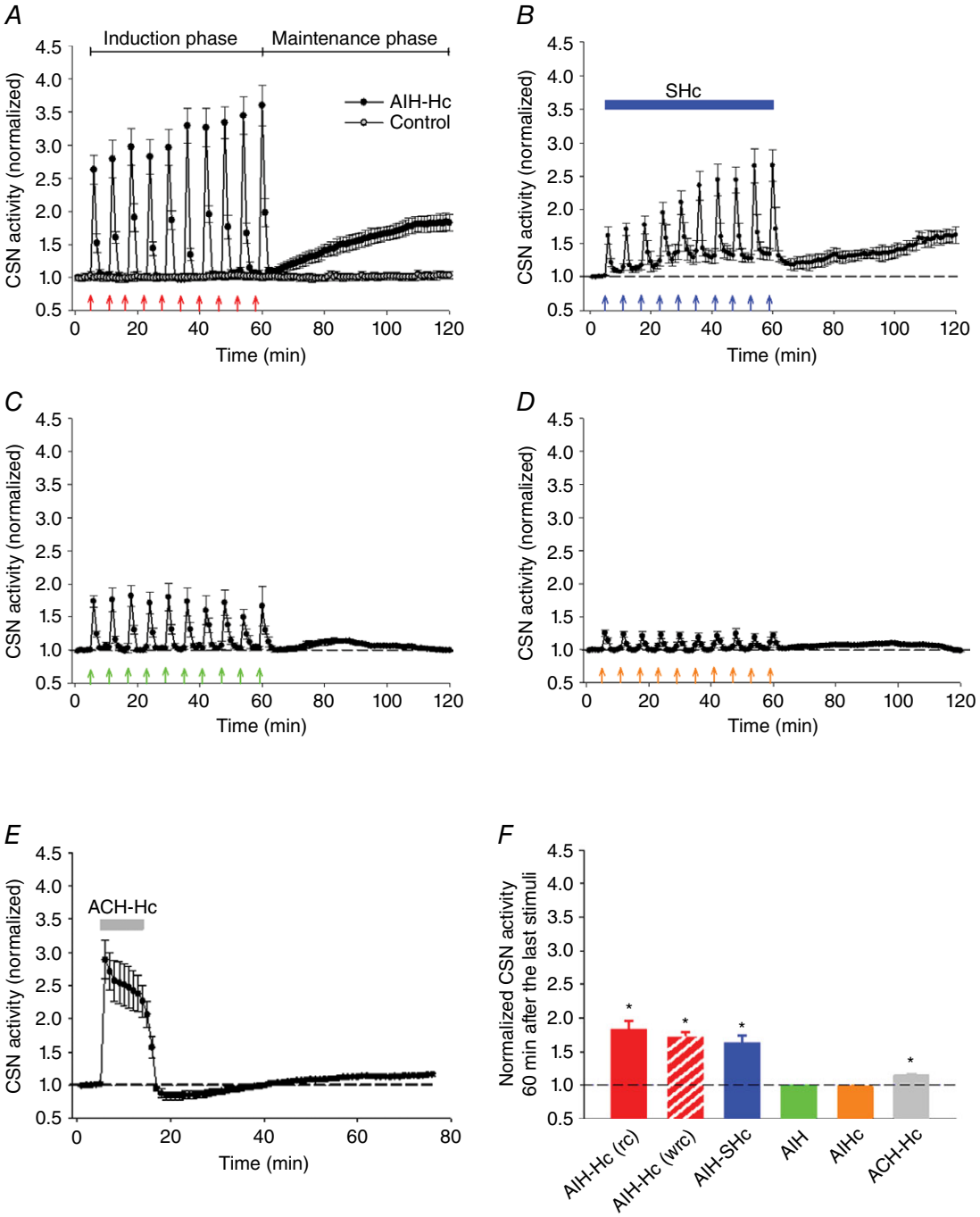

Figure 2. Sensory LTF in *ex vivo* CBs from naïve rats depends on stimulus pattern and strength
Average integrated CSN activity in response to: (*A*) no gaseous challenge (control; open circles) or AIH-Hc (red arrows; closed circles); (*B*) 10 bouts of hypoxia (P_{O_2} = 60 Torr; blue arrows) with concurrent sustained Hc (SHc) (P_{CO_2} = 50 Torr; blue horizontal bar); (*C*) 10 bouts of hypoxia (P_{O_2} = 40 Torr; green arrows); (*D*) 10 bouts of Hc (P_{CO_2} = 60 Torr; *orange arrows*); and (*E*) 10 min of acute continuous hypoxia-hypercapnia (ACH-Hc) (P_{O_2} = 40 Torr & P_{CO_2} = 60 Torr; grey bar). *F*, summary of CSN activity 60 min after the last stimuli. Note that concurrent hypercapnia, which enhances the acute response of the CB to hypoxia, was necessary to induce sensory LTF. $^*P < 0.05$ compared to baseline (dashed line) (one-way ANOVA). All data are the mean ± SEM (n = 6 per group). rc, recirculated effluent; wrc, without recirculated effluent.

Maintenance of CB sensory LTF involves ionotropic P2X receptors

Because ATP and P2X2/3 purinergic receptors play important roles in hypoxic and hypercapnic responses at the level of the CB (Prasad *et al.* 2001; Zhang *et al.* 2000) and because non-selective P2X receptor antagonist PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid) reduces phrenic LTF 60 min post AIH (Sibigtroth & Mitchell, 2011), we predicted the involvement of ATP and P2X receptors in sensory LTF. As shown in Fig. 4*A* suramin (a broad spectrum P2X receptor blocker) (100 μM) applied 60 min post AIH-Hc during the maintenance phase of sensory LTF, abolished sensory LTF within 10 min (sensory LTF pre suramin *vs.* sensory LTF post suramin: 1.60 ± 0.11 *vs.* 1.03 ± 0.02; $P < 0.001$; $n = 5$). To determine the subtypes of P2X receptors involved in the maintenance of sensory LTF, experiments were carried out using specific P2X receptor blocker TNP-ATP, which selectively inhibits P2X2/3. TNP-ATP (10 μM) abolished sensory LTF (sensory LTF pre TNP-ATP *vs.* sensory LTF post TNP-ATP: 1.55 ± 0.10 *vs.* 0.94 ± 0.03; $n = 5$; $P < 0.001$) (Fig. 4*B*). Overall, these data suggest that ATP release and activation of P2X2/3 are critical for the maintenance of sensory LTF induced by AIH-Hc.

To determine whether maintenance of sensory LTF is a result of presynaptic (glomus cells) or postsynaptic (CSN terminals) effects, we used hyperoxia ($P_{O_2} = 500$ Torr) to silence glomus cell activity and tested the postsynaptic responses to exogenous ATP challenges. As expected, hyperoxia before AIH-Hc reduced CSN activity close to zero; however, after AIH-Hc, hyperoxia reduced but failed to abolish CSN activity (before AIH-Hc *vs.* after AIH-Hc; 0.61 ± 0.05 *vs.* 1.08 ± 0.07; $P = 0.003$; $n = 5$). In both cases and as expected, adding a 1 ml bolus of ATP (100 μM at 1 ml min^{-1}) during hyperoxia to activate postsynaptic purinergic receptors

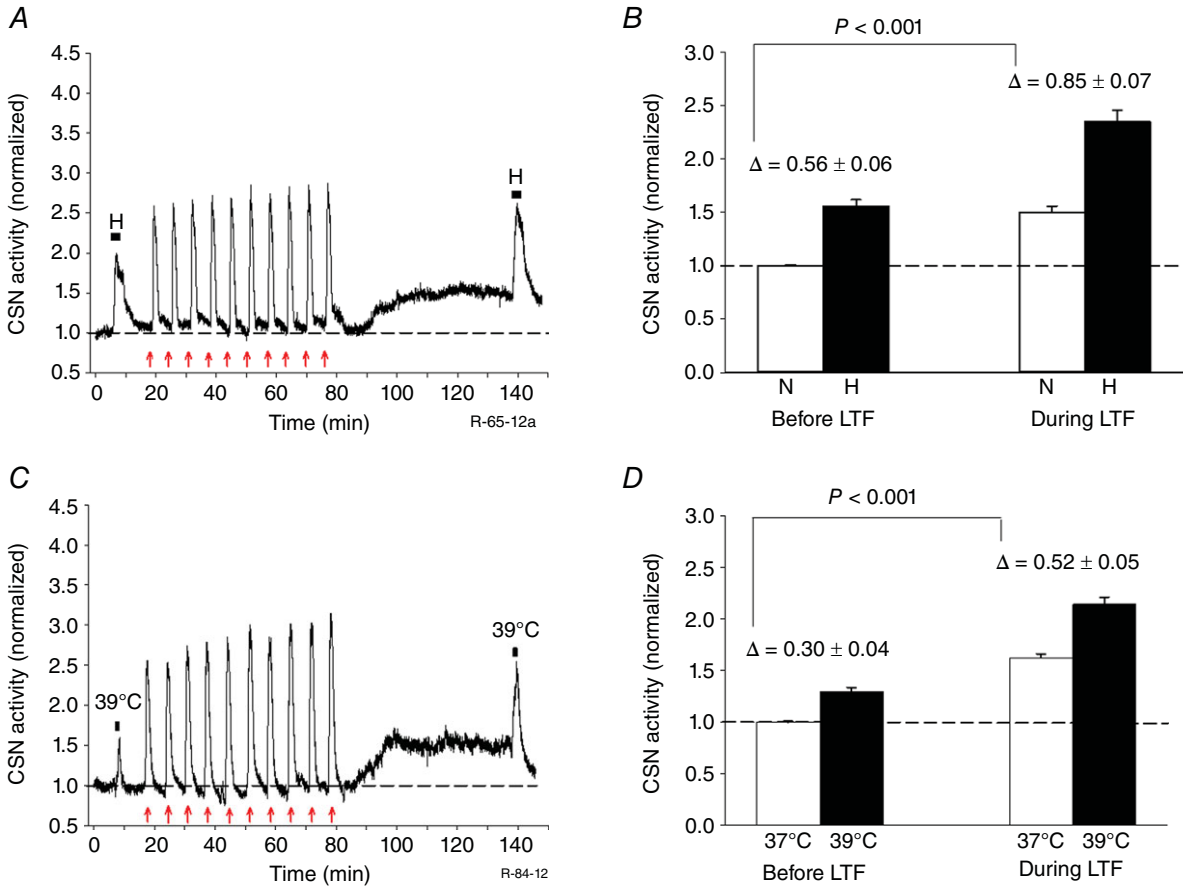

Figure 3. AIH-Hc induced sensory LTF sensitizes responses to hypoxia and heat of CBs from naïve rats
A and *C*, examples of integrated CSN responses to acute hypoxia ($P_{O_2} = 60$ Torr for 3 min; black horizontal bars) and temperature challenge (37°C to 39°C for 1 min; black horizontal bars), respectively, before and 60 min after 10 episodes of AIH-Hc (red arrows). Note the increased hypoxic and temperature responses during the maintenance phase of sensory LTF compared to control (pre-AIH-Hc). *B* and *D*, summary of the effects of hypoxia and temperature challenges, respectively, before and during sensory LTF. Data are the mean ± SEM ($n = 5$ per group). Comparison between delta changes before and during sensory LTF. N, normoxia; H, hypoxia.

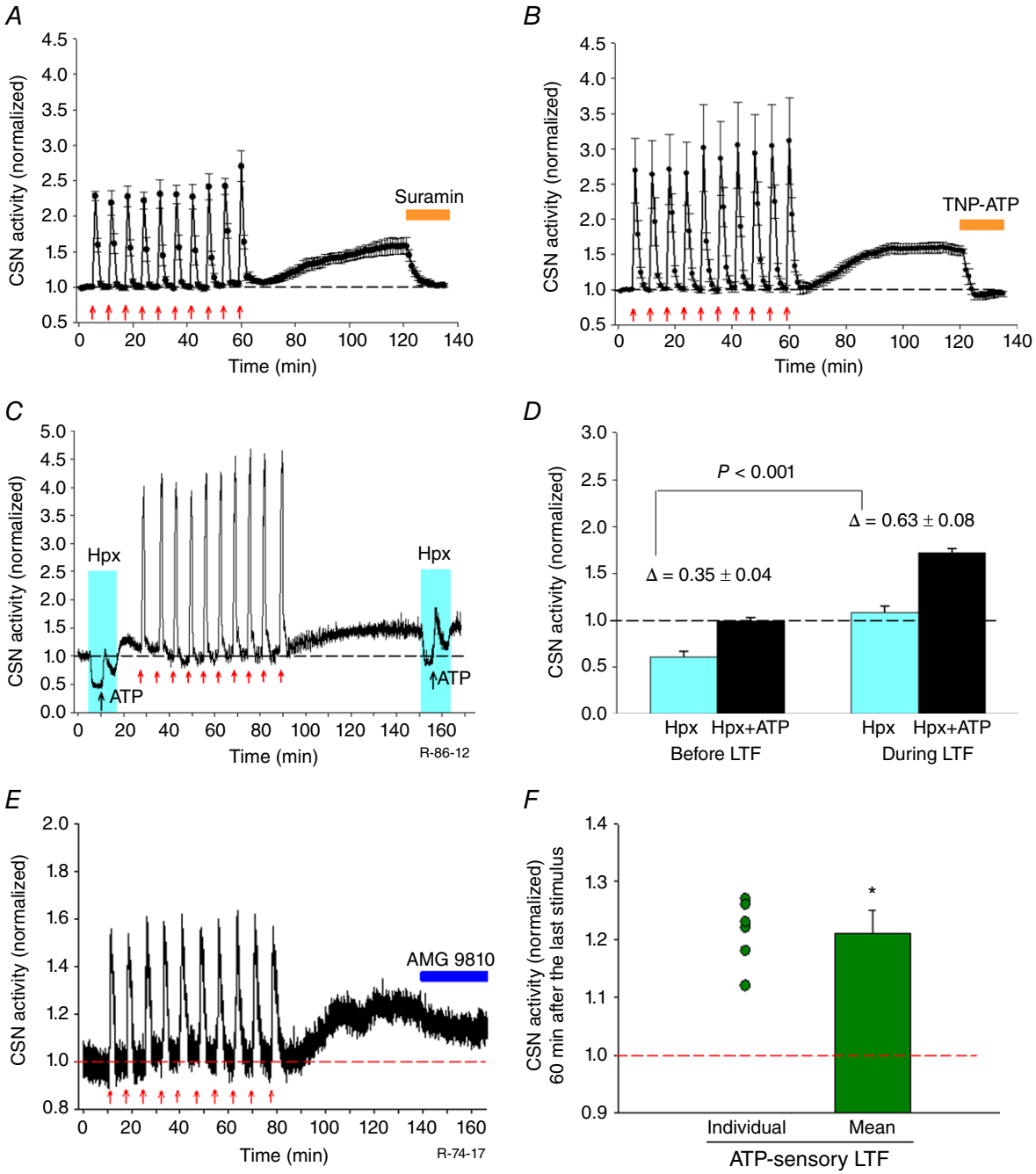

Figure 4. AIH-Hc induced sensory LTF involves P2X receptors and increases sensitivity to ATP
A and *B*, average integrated CSN activity showing sensory LTF induced by AIH-Hc is eliminated by broad spectrum purinergic P2X receptor antagonist suramin and P2X 2/3 receptor-specific antagonist TNP-ATP, respectively (orange bars; mean ± SEM; $n = 6$). *C*, integrated CSN activity from one preparation showing the response to a bolus of ATP (1 ml of 100 μM ATP for 1 min; black arrows) in hyperoxia (P_{O_2} ~ 500 Torr; blue areas) before and during sensory LTF. Prior to AIH-Hc, hyperoxia, by silencing the CB, possibly eliminates presynaptic ATP release from glomus cells (their primary neurotransmitter) and all but abolishes CSN activity; this is transiently reversed by exogenous ATP, presumably acting on postsynaptic P2X receptors. After AIH-Hc, hyperoxia reduces but does not abolish CSN activity and the ATP response is enhanced. *D*, summary of CSN activity in hyperoxia and with ATP challenges, before and during sensory LTF (mean ± SEM; $n = 5$); Comparison between delta changes with ATP, before and during sensory LTF. Hpx, hyperoxia. *E*, CB sensory responses to repetitive applications of 100 μM ATP (ten 1 min boluses at 5 min intervals; as indicated by the red arrows) produced mild sLTF; application of AMG 9810 (10 μM) partially suppressed sLTF (Fig. 5). *F*, individual scatter plot ($n = 6$) and average data of sensory LTF 60 min after the last ATP pulse (mean ± SEM; $n = 6$; $^*P < 0.05$ compared to baeline). Hpx, hyperoxia.

transiently stimulated CSN activity. Of note, however, the ATP challenge during the LTF maintenance phase was significantly more efficacious (ΔATP *vs.* ΔATP + sensory LTF: 0.35 ± 0.04 *vs.* 0.63 ± 0.08; $P < 0.001$; $n = 5$) (Fig. 4C and D). These data suggest that a postsynaptic mechanism participates in the CB plasticity evoked by AIH-Hc.

If postsynaptic P2X receptor activation is critical for AIH-Hc induced sensory LTF, spaced application of exogenous ATP should evoke sensory LTF of the CB. Accordingly, 10 repeated applications of a 1 ml bolus of ATP (100 μM at 1 ml min^{-1} without recirculation of effluent) induced sensory LTF (0.99 ± 0.01 *vs.* 1.21 ± 0.04; $P < 0.05$; $n = 6$) (Fig. 4*E* and *F*). However, the magnitude of sensory LTF was significantly less than the sensory LTF seen with AIH-Hc (1.21 ± 0.04 *vs.* 1.82 ± 0.13; $P < 0.001$; $n = 6$).

Maintenance of CB sensory LTF involves TRPV1 receptors

To assess the role of TRPV1 receptors in AIH-Hc evoked sensory LTF, we used the TRPV1 antagonist AMG9810 (10 μM) (Gavva *et al.* 2005; Roy *et al.* 2012) 60 min after the 10th AIH-Hc bout during the maintenance phase of sensory LTF. Figure 5*A* shows that 30 min of AMG9810 application suppressed CB sensory LTF by ~50% and the remaining was suppressed by suramin. Thus, AMG 9810 reduces sensory LTF (sensory LTF *vs.* sensory LTF + AMG9810: 1.86 ± 0.20 *vs.* 1.48 ± 0.12; $P = 0.017$; $n = 5$; Fig. 5*B*) but does not reduce it to baseline (1.48 ± 0.12 *vs.* 1.00 ± 0.01; $P = 0.004$; $n = 5$) (Fig. 5*B*). This partial blockade was not related to the dose of AMG 9810 because, in preliminary studies, increasing the dose of the antagonist did not improve the suppressive effect. In another set of experiments, AMG 9810 (10 μM) application failed to produce any effect on the augmented CSN activity induced by 10 min of ACH-Hc (ACH-Hc *vs.* ACH-Hc + AMG9810: 2.77 ± 0.5 *vs.* 2.77 ± 0.5; $P = 0.97$; $n = 6$) (Fig. 5*C* and *D*), suggesting that sustained stimulus has no postsynaptic influence on TRPV1 receptors. Overall, the results indicate that both P2X and TRPV1 receptor activation is involved in sensory LTF.

To further establish the involvement of TRPV1 receptors in AIH-Hc induced sensitization of the CB, we exploited the temperature sensitivity of the TRPV1 channel (Caterina *et al.* 1997; Roy *et al.* 2012) and tested whether the increase in temperature sensitivity (Fig. 3*C*) during the maintenance phase of sensory LTF was mediated via TRPV1 receptor sensitization. AMG 9810 (10 μM) reduced sensory LTF (as above) and blocked almost half of the CSN response to a temperature challenge from 37° to 39°C (sensory LTF ΔT *vs.* sensory LTF ΔT+AMG9810: 0.44 ± 0.05 *vs.* 0.13 ± 0.06; $P < 0.001$; $n = 5$) (Fig. 5*E* and *F*).

Maintenance of CB sensory LTF involves TRPV1 receptors *in vivo*

To assess the role of TRPV1 receptors in AIH-Hc evoked sensory LTF *in vivo*, we used the TRPV1 antagonist AMG9810 (100 mg kg^{-1}) (Gavva *et al.* 2005) 60 min after the 10th AIH-Hc bout, during the maintenance phase of sympathetic LTF. Under both normoxic and hyperoxic conditions, P_{aCO_2} and pH were maintained within physiologically normal levels (Table 1). AIH-Hc caused an increase in splanchnic SNA under both hyperoxic ($64.1 \pm 12\%$) and normoxic ($60.7 \pm 9.4\%$) conditions at 60 min after the 10th AIH-Hc (Fig. 6*C*). Vehicle control (100% DMSO; 1 ml kg^{-1}) or AMG9810 (100 mg kg^{-1}) was administered I.P. and recordings were maintained for another 30 min. Animals that received the vehicle control showed a continuing increase in splanchnic SNA, whereas the animals treated with AMG9810 showed a reduction but only under normoxic conditions (Fig. 6*A*, *B* and *D*). Compared to the activity present at 60 min following the 10th AIH-Hc, the DMSO treatment group increased activity by a further ~50% under both normoxic and hyperoxic conditions (Fig. 6*E*). The SNA of the hyperoxic AMG9810 group also increased by a further $30.7 \pm 18.3\%$, whereas the SNA of the AMG9810 treated normoxic group was significantly decreased by $32.0 \pm 10.1\%$ ($P = 0.01$; $n = 6$) (Fig. 6*E*). Thus, under the *in vivo* condition, TRPV1 receptors contribute to sympathetic LTF only when the CB is active.

Effects of anti-hypertensive drugs ketanserin and losartan on the AIH-Hc evoked sensory LTF

Previously, 5-HT2 and angiotensin (Ang) type 1 (AT1) receptors were shown to be required for sensory LTF induced by AIH in CIH preconditioned animals (Peng *et al.* 2006, 2011). Therefore, we examined whether 5-HT$_2$ and AT1 receptors also contribute to CB sensory LTF induced by AIH-Hc in naïve animals. 5-HT$_2$ receptor antagonist ketanserin (1 μM) applied throughout the experiment (i.e. during the induction and maintenance phases of sensory LTF) caused a progressive decline in CSN activity with each AIH-Hc bout and abolished manifestation of sensory LTF as indicated by a reduction in CSN activity (pre AIH-Hc *vs.* 60 min post AIH-Hc: 1.01 ± 0.01 *vs.* 0.88 ± 0.03; $P = 0.002$; $n = 5$) (Fig. 7*A*). However, when applied 60 min after the 10th AIH-Hc challenge, during the sensory LTF maintenance phase, LTF was unaffected (sensory LTF *vs.* ketanserin + sensory LTF: 1.56 ± 0.08 *vs.* 1.54 ± 0.10; $P = 0.48$; $n = 5$) (Fig. 7*B*). Losartan (3 μM), an AT1 receptor antagonist, had similar effects (Fig. 7*C* and *D*). Thus, it appears that both 5-HT$_2$ and AT1 receptor activation is necessary to induce but not to maintain sensory LTF.

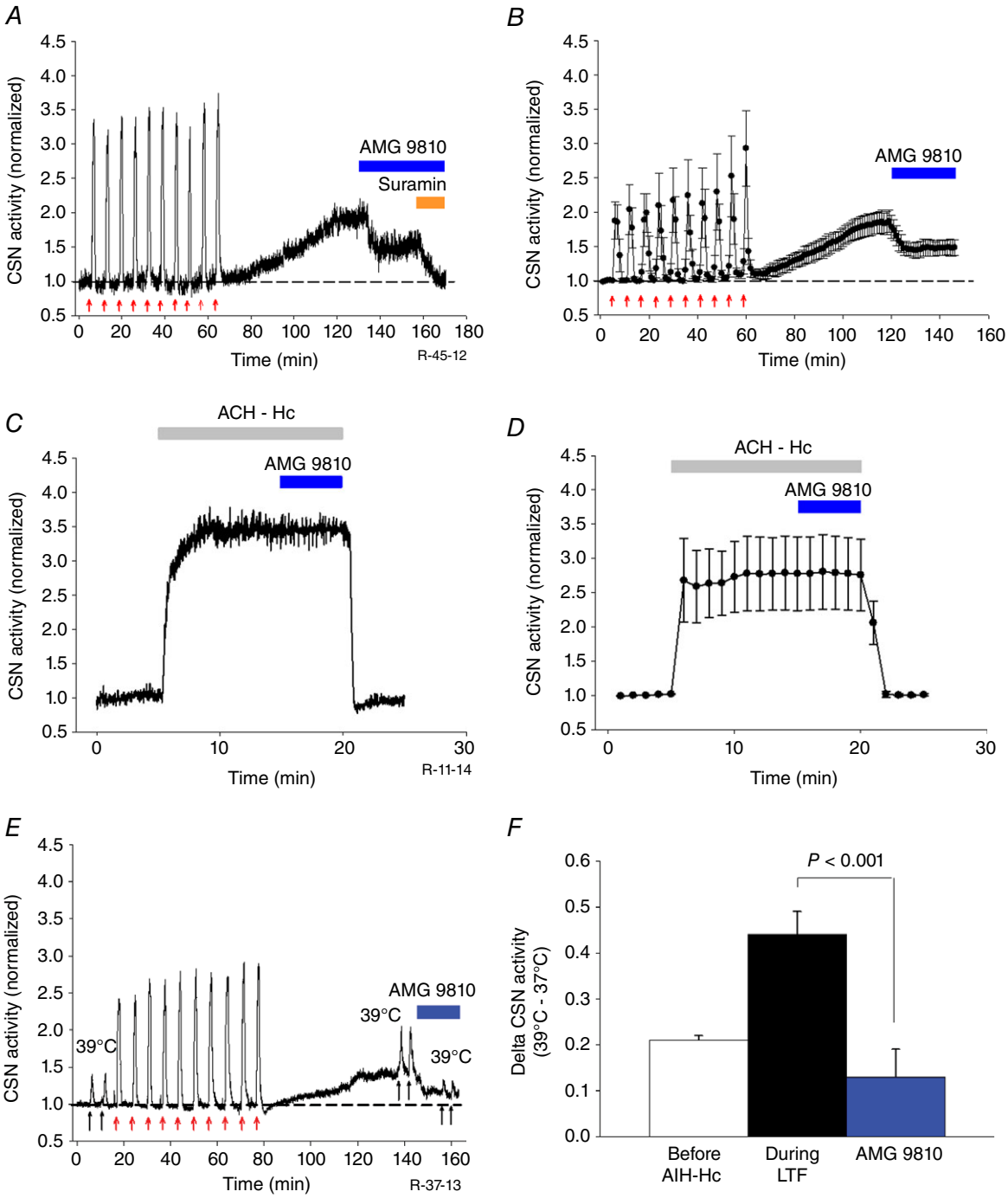

Figure 5. AIH-Hc induced sensory LTF involves heat-sensitive TRPV1 receptors and increases CB heat sensitivity

A, integrated CSN activity from one preparation showing ~50% of sensory LTF induced by AIH-Hc is inhibited by TRPV1 receptor antagonist AMG 9810 (10 μM; blue bar). The facilitated activity that remained was completely suppressed by suramin (100 μM; orange bar). *B*, average integrated CSN activity showing the effect of AMG9810 on sensory LTF (mean ± SEM; $n = 5$). *C* and *D*, integrated CSN activity from one preparation and average data (mean ± SEM; $n = 6$; $P = 0.97$) showing that AMG 9810 had no effect on the response to 10 min of acute continuous hypoxia-hypercapnia (ACH-Hc) (grey bar). *E*, integrated CSN activity from one preparation showing responses to temperature challenges (37 to 39°C for 1 min each; black arrows) before, 60 min after 10 episodes of AIH-Hc (red arrows) and, subsequently, with partial inhibition of sensory LTF with TRPV1 antagonist AMG9810 (10 μM). *F*, summary of CSN responses following temperature challenge. Data are the mean ± SEM; $n = 5$.

AIH-Hc induced sensory LTF involves protein kinase C (PKC) activation

To determine whether sensory LTF induced by AIH-Hc involves increased phosphorylation through PKC activation, the effects of GF109203X (10 μM), a potent and selective inhibitor of PKC (Toullec *et al.* 1991) during the induction and maintenance of sensory LTF were examined. As shown in Fig. 7*E*, GF 109203X applied throughout the experiment completely prevented sensory LTF, with a prominent decrease in baseline activity (pre AIH-Hc *vs.* 60 min post AIH-Hc: 1.01 ± 0.02 *vs.* 0.83 ± 0.02; $P < 0.001$; $n = 5$). To establish whether or not increased phosphorylation was essential to sustain sensory LTF, GF 109203X was applied 60 min after AIH-Hc during the maintenance phase of sensory LTF. As shown in Fig. 7*F*, GF 109203X blocked sensory LTF, demonstrating that ongoing phosphorylation is required to maintain sensory LTF (sensory LTF *vs.* sensory LTF + GF109203X: 1.51 ± 0.12 *vs.* 1.08 ± 0.07; $P < 0.001$; $n = 5$). Because CaMKII has been implicated in phosphorylation of TRPV1 receptor (Jung *et al.* 2004) and is present in the CB (Pokorski *et al.* 2012), we tested the effects of the CaMKII inhibitor AIP (2 μM) in three preparations, although it had no apparent effect on the maintenance phase of sensory LTF (sensory LTF *vs.* sensory LTF + AIP: 1.72 ± 0.12 *vs.* 1.73 ± 0.13; $P = 0.95$; $n = 3$). Thus, phosphorylation mediated by PKC is essential to induce and maintain sensory LTF evoked by AIH-Hc in naïve CB. Overall, the data indicate that the anti-hypertensive drugs ketanserin and losartan cannot prevent sensory LTF once it is established, although PKC mediated phosphorylation is essential for the development and maintenance of sensory LTF (Fig. 7*G*).

ROS scavenger does not block sensory LTF when applied post AIH-Hc

To determine whether ROS ($O_2^{\cdot-}$) are involved in induction and maintenance of sensory LTF, we

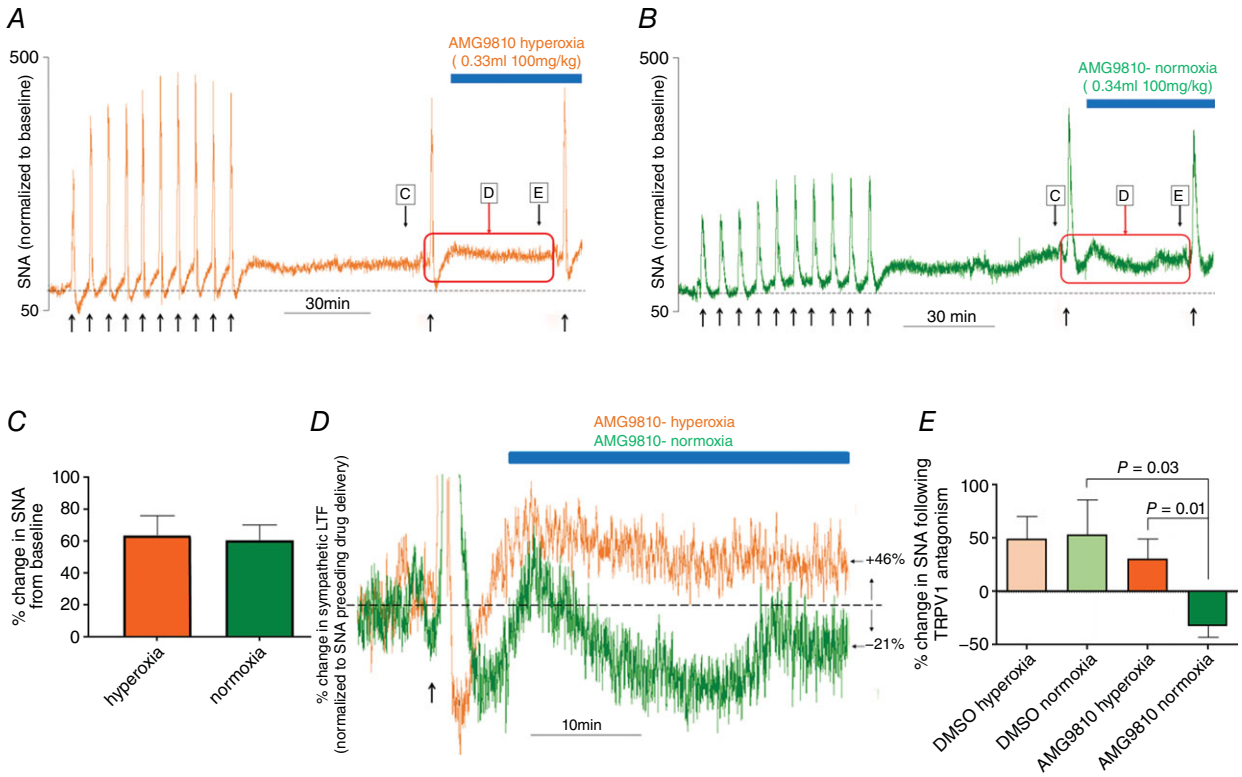

Figure 6. Effect of TRPV1 antagonism on AIH-Hc-induced sympathetic LTF in naïve rats *in vivo*
A and *B*, integrated sympathetic responses to AIH-Hc from two preparations with hyperoxic (orange trace) and normoxic (green trace) backgrounds, respectively. Note the sustained increase in sympathetic activity (indicative of sympathetic LTF) following the last bout of hypoxia-hypercapnia. *C*, the magnitude of sympathetic LTF 60 min post-AIH-Hc was not different between preparations given normoxia or hyperoxia backgrounds. *D*, TRPV1 antagonist AMG9810 reduced sympathetic LTF, in preparations with a normoxic background (green) when the CB was functional but not in preparations with a hyperoxic background (orange). *E*, summary data showing percentage change in sympathetic activity following DMSO or AMG9810 administration in preparations with normoxic and hyperoxic backgrounds. AMG9810 significantly decreased sympathetic LTF under normoxic conditions but had no effect under hyperoxic conditions. Data are the mean ± SEM ($n = 6$).

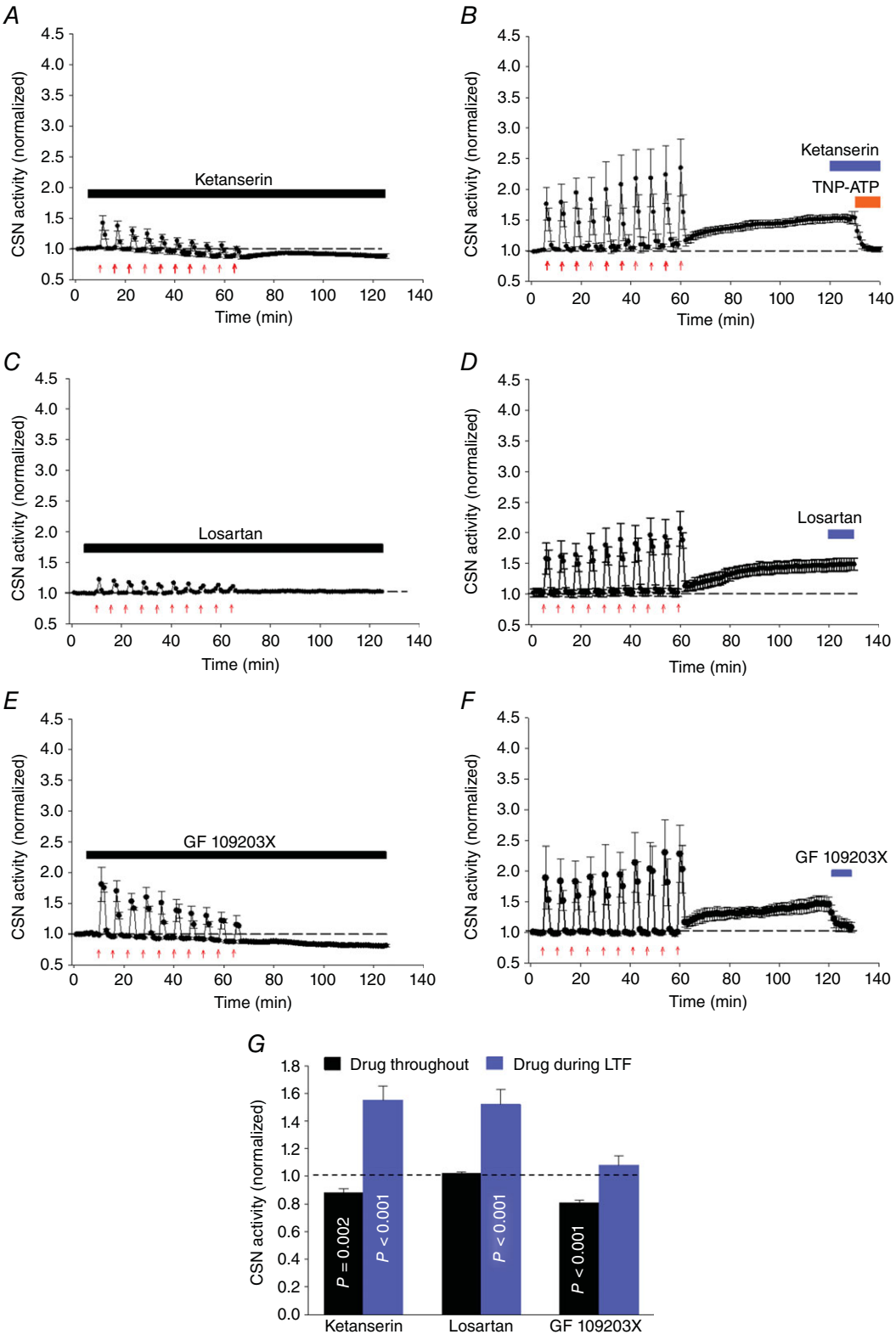

Figure 7. Involvement of 5-HT$_2$ and AT1 receptors, and PKC activation in AIH-Hc induced sensory LTF in CBs from naïve rats

Average integrated CSN activity (mean ± SEM; n = 5) showing that the anti-hypertensive drugs ketanserin (*A*, 1 μM; 5-HT$_2$ receptor antagonist) and losartan (*C*, 3 μM; AT1 receptor antagonist) applied throughout the experiment diminished responses to hypoxia-hypercapnia bouts (red arrows) and prevented sensory LTF. *B* and

D, ketanserin and losartan, respectively, had no effect during the maintenance phase (i.e. once sensory LTF was established), whereas TNP-ATP (P2X 2/3 receptor-specific antagonist) completely abolished sensory LTF (as shown in Fig. 4*B*). *E*, PKC inhibitor (GF 109203X; 10 μM) applied throughout the experiment also diminished responses to hypoxia-hypercapnia bouts and prevented sensory LTF. *F*, when applied during the maintenance phase, GF 109203X potently inhibited sensory LTF (mean ± SEM; $n = 5$). *G*, summary data; *P* values were determined by comparing CSN activities with drugs applied throughout/after sensory LTF and the basal responses before AIH-Hc (dashed line). Data are the mean ± SEM; $n = 5$).

used MnTMPyP (25 μM), a membrane permeable O_2^- scavenger. When applied throughout the entire experiment, the CSN responses after the first bout of AIH-Hc were not incremental but rather indicated a decline in activity with subsequent bouts. Moreover, manifestation of sensory LTF 60 min after the last bout was significantly reduced (baseline 1.00 ± 0.03 *vs.* sensory LTF + MnTMPyP 1.10 ± 0.05; $P = 0.008$; $n = 5$) (Fig. 8*A*). ROS therefore probably did not play an important role in the acute response of the CB to hypoxia-hypercapnia but are essential for sensory LTF induction. These findings are consistent with the role of ROS described for CIH-dependent CB sensory LTF (Peng *et al.* 2009). However, when MnTMPyP was applied 60 min after the 10th AIH-Hc episode, during the maintenance phase of sensory LTF, LTF was not diminished; indeed, CSN activity was stimulated (sensory LTF *vs.* sensory LTF+ MnTMPyP: 1.52 ±0.12 *vs.* 1.82 ± 0.20; $P = 0.03$; $n = 5$) (Fig. 8*B*). These data suggest that ROS ($O_2^{\cdot -}$) are not essential for sensory LTF maintenance and may limit the sensory LTF magnitude (Fig. 8*E*).

H_2O_2 is not involved in sensory LTF induced by AIH-Hc

PEG-catalase, a membrane permeant enzyme that breaks down H_2O_2 releasing O_2 (George, 1947) is reported to reverse sensory LTF induced by AIH in CIH pre-conditioned animals (Peng *et al.* 2009). However, when tested in naïve CBs exposed to AIH with concurrent hypercapnia, PEG-catalase (200 U ml^{-1}) had no effect whatsoever on sensory LTF if applied during the entire experiment (i.e. during both the induction and maintenance phases; baseline *vs.* sensory LTF + PEG-catalase: 1.00 ± 0.01 *vs.* 1.69 ± 0.16; $P < 0.05$; $n = 5$) (Fig. 8*C*) and caused only a transient decrease in activity when applied during the maintenance phase (sensory LTF *vs.* sensory LTF+ PEG-catalase: 1.52 ± 0.10 *vs.* 1.50 ± 0.11; $P = 0.51$, $n = 5$) (Fig. 8*D*). These data suggest that H_2O_2 is not involved in this CB plasticity mechanism (Fig. 8*E*).

Discussion

The primary novel findings of the present study are that a robust and long-lasting activity-dependent plasticity in CB can be induced by AIH-Hc without CIH-preconditioning. This sensory LTF requires P2X 2/3 receptor activation and is dependent on TRPV1 receptors. As P2X 2/3 and TRPV1 receptors are located in petrosal sensory afferents (not O_2-sensitive glomus cells) (Prasad *et al.* 2001) and repeated pulses of ATP induced mild sensory LTF, one of the main sites of plasticity appears to be postsynaptic. Our data suggest that several features of this plasticity are shared by CIH-dependent sensory LTF because the induction of plasticity is dependent on 5-HT, Ang II, PKC and ROS signalling (Peng *et al.* 2003, 2006, 2009, 2011). However, H_2O_2 appears to play no part in sensory LTF induction and 5-HT, Ang II, ROS and H_2O_2 are not required for sensory LTF maintenance (Fig. 9). Because our *in vivo* experiments highlight a role for TRPV1-dependent CB activity in sympathetic LTF, we suggest that TRPV1-dependent sensory LTF constitutes an important mechanistic link contributing to the well-documented association between sleep apnoea, sympathoexcitation, hypertension and cardiovascular disease.

Role of hypercapnia in sensory LTF in the naïve CB

Peng *et al.* (2003) reported that only CIH-preconditioned CBs manifest sensory LTF in response to AIH *in vitro* (i.e. the superfused CB). This phenomenon was not evident in CBs extracted from naïve animals. Our demonstration of robust sensory LTF induced without preconditioning may be the consequence of preparation differences (we used a perfused CB preparation in which arterial P_{O_2} and P_{CO_2} were precisely controlled and could be changed quickly) or our choice of acute stimuli. Notably, we could induce robust sensory LTF with either AIH against a background of hypercapnia or with AIH-Hc against a background of normoxic normocapnia. However, we could not induce robust sensory LTF with AIH alone, AIHc alone or following ACH-Hc. These results demonstrate the need to combine AIH with hypercapnia in the naïve CB and also show that the composition, pattern and duration of gaseous stimuli are all important for the induction of sensory LTF (Cummings & Wilson, 2005). These CB data parallel our *in vivo* data demonstrating the involvement of the CB in induction of sympathetic LTF with AIH-Hc.

Nonetheless, the importance of hypoxia *vs.* hypercapnia in inducing sympathetic LTF warrants further study. In the clinical setting, chronic and severe hypercapnia is common in sleep apnoea, and the sizable decrease in P_{aO_2} that occurs with each apnoea is accompanied by

increased P_{aCO_2} (Lanphier & Rahn, 1963; Chin *et al.* 1997). In healthy humans, ventilatory and sympathetic LTF may be enhanced and/or only evident when AIH is presented with an elevated baseline level of CO_2 (Morgan *et al.* 1995; Harris *et al.* 2006). However, in rats, AIH and CIH alone can induce sympathetic LTF. With CIH, where sympathetic LTF is accompanied by hypertension, Fletcher (2000) reports that adding CO_2 to CIH does not further elevate

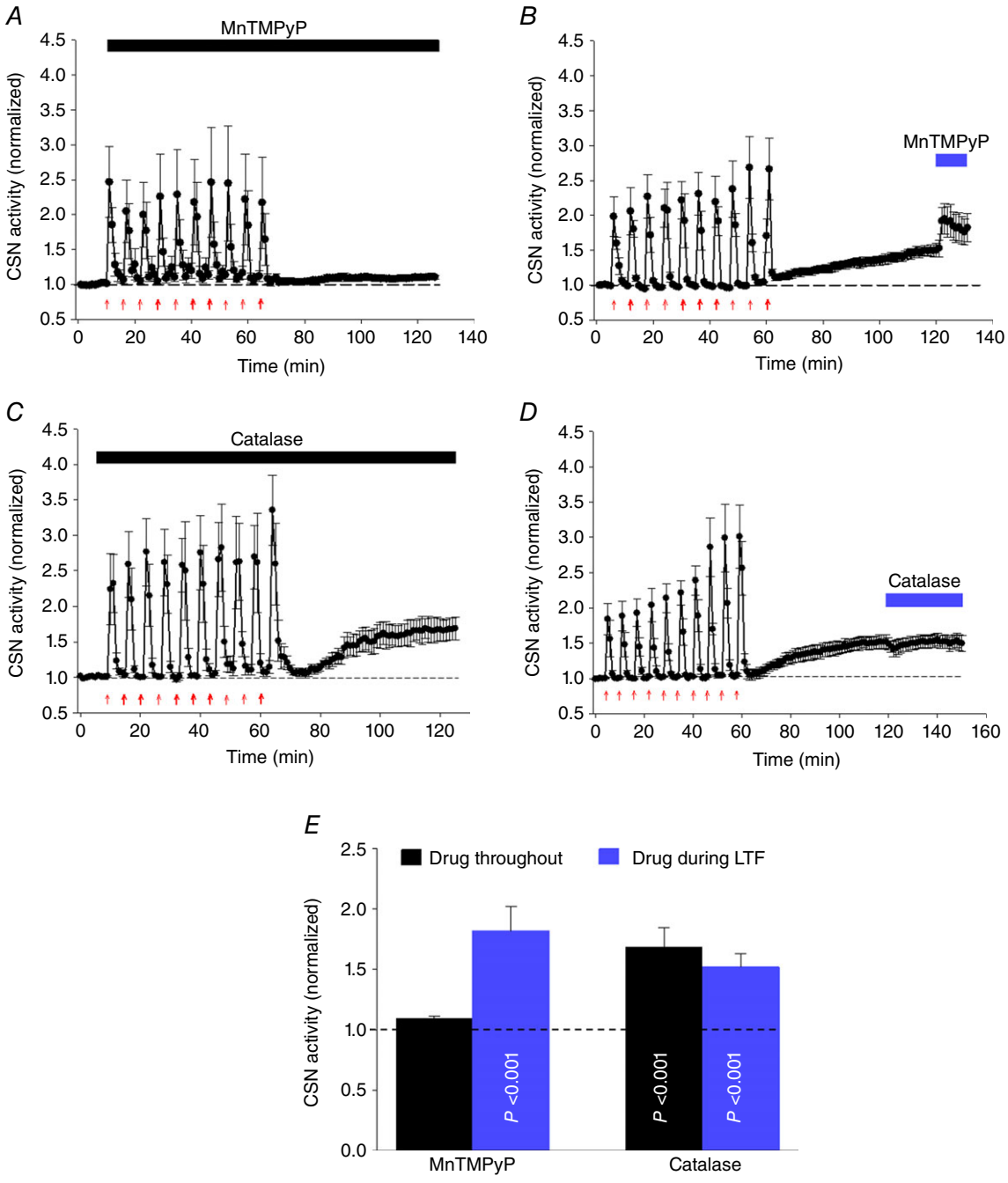

Figure 8. ROS ($O_2\cdot^-$), but not H_2O_2, is involved in sensory LTF induction evoked by AIH-Hc in naïve CB
A and *B*, average integrated CSN activity (mean ± SEM; $n = 5$) showing that ROS scavenger MnTMPyP (25 μM) applied throughout (red arrows) prevents sensory LTF but failed to suppress sensory LTF 60 min after the last hypoxia-hypercapnia bout. *C* and *D*, average integrated CSN activity (mean ± SEM; $n = 5$) demonstrating the H_2O_2 scavenger PEG-catalase (200 U ml^{-1}) had no effect when applied either the induction or maintenance phase of sensory LTF. *E*, summary data of MnTMPyP and catalase applied throughout and after sensory LTF induction. *P* values were determined by comparing CSN activities with basal responses (dashed line) before AIH-Hc stimuli. Data are the mean ± SEM; $n = 5$.

sympathetic LTF or blood pressure (Bao *et al.* 1997; Lesske *et al.* 1997; Dick *et al.* 2007; Xing & Pilowsky 2010). These results are somewhat unexpected because hypercapnia might be expected to increase sympathetic LTF based on: (i) hypercapnia increasing the CB and ventilatory responses to hypoxia (Lahiri & Delaney, 1975; Wilson & Teppema, 2016) and (ii) Fletcher (2000) and coworkers (Bao *et al.* 1997) showing that the addition of inspired CO_2 decreases the P_{aO_2} caused by inspired hypoxia and augments the effects of each hypoxic bout on sympathetic activity and blood pressure (Bao *et al.* 1997). Because acute intermittent electrical stimulation of the CSN nerve in naïve animals also causes respiratory (and presumably sympathetic) LTF (Hayashi *et al.* 1993) and hypercapnia is required for AIH-induced sensory LTF in naïve animals (present study), we propose that sympathetic LTF results from central and/or CB plasticity (with CB plasticity requiring CIH precondition or concurrent hypercapnia), with sympathetic LTF having an upper cap that limits sympathetic LTF magnitude and gives rise to CB/central redundancy. The concept of competing sites of plasticity is reminiscent of that proposed for respiratory LTF (Hayashi *et al.* 1993) and is discussed further below in the context of our *in vivo* data.

In the present study, we show that CB sensory LTF comprises two distinct phases: induction and maintenance

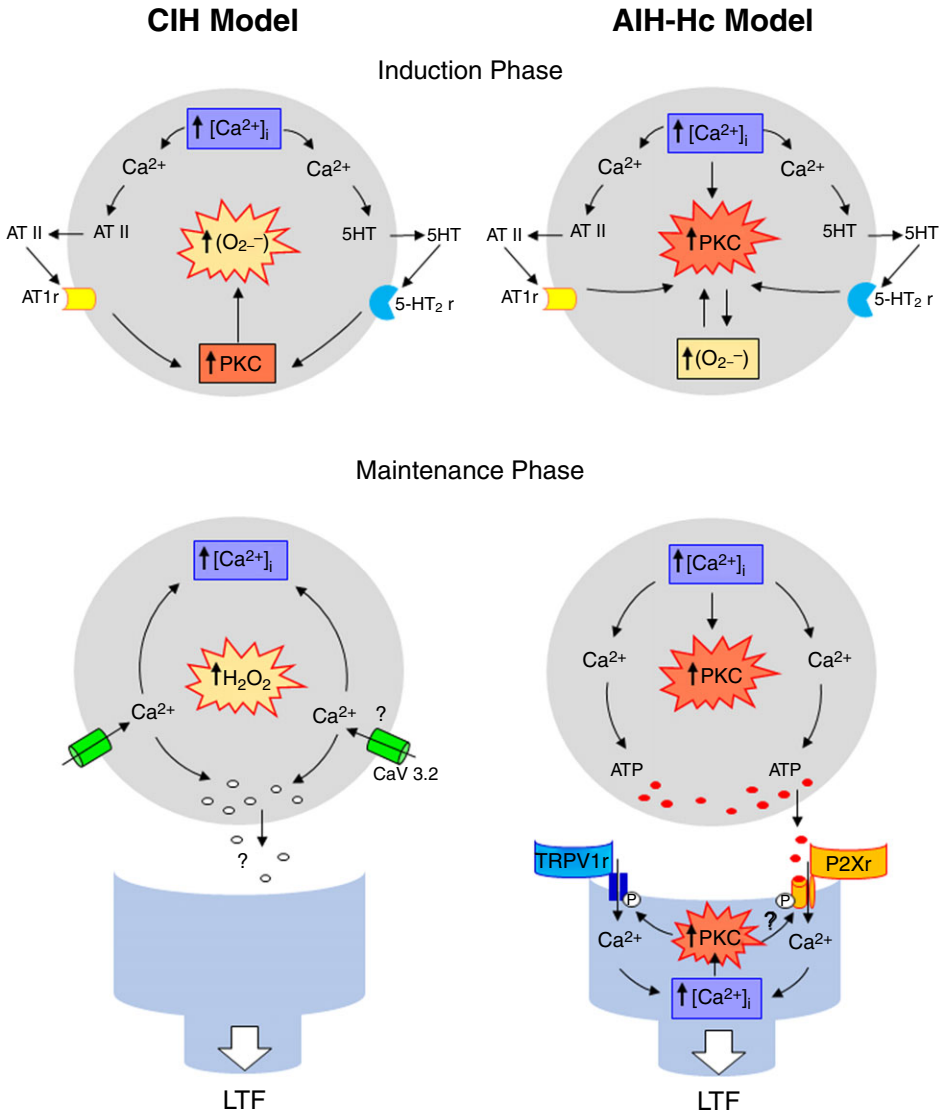

Figure 9. Schematic diagram comparing mechanisms of sensory LTF induction and maintenance by AIH requiring CIH pre-conditioned and AIH-Hc in CB from naïve rats
Although sensory LTF requiring CIH pre-conditioning is largely dependent on $O_2.^-$ and subsequent production of H_2O_2, sensory LTF resulting from AIH with concurrent hypercapnia is largely dependent on PKC. Moreover, our novel data suggest that sensory LTF resulting from AIH with concurrent hypercapnia is P2X-dependent, involves TRPV1 and is predominantly postsynaptic.

that require different cellular processes. This is reminiscent of the role of 5-HT and adenosine receptors in the maintenance of phrenic LTF, for which 5-HT and/or adenosine receptor activation is necessary to initiate but not maintain phrenic LTF following episodic hypoxia (Fuller *et al.* 2001; Fields & Mitchell, 2015). Also, activation of spinal orexin receptors is critical for enhancing chemoreflex phrenic responses to hypoxia after AIH (Kim *et al.* 2016*b*).

Mechanism of induction

Our data demonstrate that the induction of sensory LTF in naïve CBs bears some resemblance to that proposed for sensory LTF requiring CIH preconditioning (Peng *et al.* 2003). Peng *et al.* (2006, 2011) demonstrated that sensory LTF mirroring that requiring CIH could be induced by repetitive applications of 5-HT and Ang II in the naïve CB. In our model, 5-HT_2 and Ang II receptor antagonists caused a profound decrease in the response magnitude to AIH-Hc stimuli and completely abolished sensory LTF (Fig. 7*A* and *C*). The importance of endogenous 5-HT_2 and Ang II receptor activation in sensory LTF suggests that the induction of sensory LTF is dependent on ongoing PKC-mediated phosphorylation (Peng *et al.* 2006). Indeed, the PKC blocker GF109203X decreased the response magnitude to consecutive stimuli in our preparation and abolished the induction of sensory LTF in both naïve and CIH-preconditioned CBs. Using the ROS scavenger (MnTMPyP), Peng *et al.* (2003, 2009) also demonstrated that ROS ($O_2^{\cdot-}$) are necessary for the induction of CIH-preconditioned sensory LTF. Similarly, we show that MnTMPyP reduces consecutive responses to stimulus and prevents the induction of sensory LTF (Fig. 8*A*). Thus, 5-HT_2, Ang II, PKC and $O_2\cdot^-$ mediated-signalling influences the magnitude of response to individual stimuli and strongly influences the induction of both forms of sensory LTF. We therefore suggest that the mechanism of sensory LTF induction in CIH- preconditioned and naïve rats is similar, sharing a dependence on PKC-modulation of stimulus amplitude (Fig. 9, induction phase). However, PEG-catalase had no effect on induction of sensory LTF in the present study and, as such, our data do not support a role for H_2O_2, proposed as the mechanistic lynch pin between $O_2^{\cdot-}$ and glomus cell Cav3.2 modification in CIH-preconditioned sensory LTF (Makarenko *et al.* 2016).

Maintenance of sensory LTF

The release of ATP from glomus cells resulting in activation of postsynaptic P2X receptors on the terminals of chemosensory afferents plays an essential role in the acute hypoxic response of the CB (Prasad *et al.* 2001; Rong *et al.* 2003). We show that activation of P2X receptors is also important for sensory LTF maintenance because sensory LTF could be almost completely reversed with suramin. The effects of P2X receptor antagonism may reflect presynaptic plasticity resulting in an increased release of ATP and/or a postsynaptic mechanism causing an increased responsiveness to ATP. Although we cannot exclude a presynaptic mechanism, three lines of evidence suggest plasticity occurs postsynaptically. (i) The sensory response to exogenous test pulse of ATP, presumably caused by activation of P2X receptors on sensory afferents and not P2Y receptor on Type II cells (Murali and Nurse, 2016), was increased following sensory LTF induction (Fig. 4*C* and *D*). This may be a consequence of modification of the expression or function of P2X receptors or it may reflect plasticity downstream. (ii) Sensory LTF could be induced by replacing bouts of gaseous stimuli with exogenous pulses of ATP (Fig. 4*E* and *F*). We note that the magnitude of sLTF induced with pulses of ATP was less than that with AIH-Hc, probably reflecting differences in the temporal pattern and magnitude of ATP within the synaptic cleft. Consistent with this explanation, Peng *et al.* (2011) were unable to demonstrate this phenomenon in their *en bloc* preparation, possibly because they used fewer pulses of ATP (5 bouts compared to 10 bouts) and there were also increased wash in and washout times associated with superfusion compared to perfusion. (iii) Perhaps the strongest argument for postsynaptic plasticity within the CB is the involvement of TRPV1 receptors that are located exclusively on postsynaptic sensory afferents (Roy *et al.* 2012). Indeed, blocking TRPV1 during the maintenance phase with AMG9810 reduced sensory LTF by half and, unlike P2X receptors, TRPV1 plays no significant role in acute hypoxic-hypercapnic responses (Fig. 5*C* and *D*). This suggests that AIH-Hc leads to postsynaptic TRPV1 receptor modification and persistent activation. In other systems where P2X and TRPV1 receptors co-localize, the activation of purinoceptors by ATP augments ionic currents induced by activation of TRPV1 receptors, presumably via TRPV1 phosphorylation (Bhave *et al.* 2003; Saloman *et al.* 2013). Consistent with the possibility that TRPV1 phosphorylation is a critical step in sensory LTF, we demonstrate that both the induction and maintenance phases of sensory LTF could be reversed by the general PKC inhibitor, GF 109203X (Fig. 7*E* and *F*).

Interestingly, PKC activation can lead to generation of ROS ($O_2\cdot^-$ and H_2O_2) (Lee *et al.* 2004) and $O_2\cdot^-$ and H_2O_2 can activate P2X and TRPV1 receptors (Shen *et al.* 2012; Lin *et al.* 2013; Ruan *et al.* 2014). Moreover, the explanation proposed by Peng *et al.* (2009) for the changing role of $O_2\cdot^-$ in sensory LTF (MnTMPyP being important for the initiation but not maintenance of sensory LTF requiring CIH preconditioning) is that $O_2\cdot^-$ becomes ineffective when dismutated to H_2O_2, and H_2O_2 is responsible for maintaining sensory LTF. Thus, PEG-catalase, a H_2O_2 scavenger, applied 15 min after the onset of sensory LTF, completely prevented sensory

LTF requiring CIH preconditioning (Peng *et al.* 2009). Motivated by these observations, we tested the importance of ROS in TRPV1-dependent sensory LTF. Although we also found that $O_2\cdot^-$ contributed to the induction phase but not the maintenance phase, we found no evidence for a role for H_2O_2 during the induction phase. PEG-catalase had no effect whatsoever during induction and, during the maintenance phase, it caused only a transient decrease in CSN (Fig. 8*C* and *D*), consistent with the brief oxygen burst probably occurring with catalase application (George, 1947) and a concomitant brief inhibition of glomus cells. We therefore conclude that the role of ROS in sensory LTF induced by AIH with concurrent hypercapnia is limited to a role for $O_2\cdot^-$ in regulating the response magnitude during the induction phase, participating only indirectly in the plasticity mechanism (Fig. 9).

Sensory LTF increases the activity and sensitivity of the CB

During the maintenance phase of sensory LTF, in addition to increased tonic CSN activity, we observed an increased responsiveness to acute hypoxia (Fig. 3*A* and *B*), heat (change from 37^0 to 39°C) (Fig. 3*C* and *D*) and ATP (Fig. 4*C* and *D*) challenges. This is reminiscent of the increased sensory responsiveness to hypoxia reported during sensory LTF in CIH-preconditioned CBs, suggesting identical functional outcomes between the forms of sensory LTF induced by AIH with concurrent hypercapnia and CIH (Peng *et al.* 2003). With regard to breathing, increasing CB sensitivity is expected not only to increase the ability of an animal to respond to a single apnoea, but also to destabilize breathing, making apnoeas more probable; this may be partially compensated by the increased CB baseline activity that would be expected to increase the CB CO_2 reserve (Smith *et al.* 2003). However, as our *in vivo* data suggest, TRPV1-dependent CB sensory LTF can contribute to sympathetic LTF. In a clinical setting, a long-lasting increase in sympathetic tone would probably lead to target organ damage (vessels, heart, brain and kidney in particular), chronic metabolic acidosis, obesity, insulin resistance and neurogenic hypertension.

Multiple sites of plasticity involved in sympathetic LTF

Our *in vivo* data demonstrate that sympathetic LTF is only sensitive to AMG9810 under conditions that support sensory LTF (i.e. with CBs in a background of normoxia); AMG9810 was without effect on sympathetic LTF when CB activity between and after bouts of AIH-Hc was suppressed (i.e. with CBs in a background of hyperoxia) (Fig. 6). These data are entirely consistent with a direct role for CB TRPV1-mediated sensory LTF in sympathetic LTF when the CBs are fully functional. Moreover, the persistence of sympathetic LTF when sensory LTF is suppressed corroborates the concept discussed above in that there are additional presumed central sites capable of plasticity, located downstream of the CB (Xing *et al.* 2014; Yamamoto *et al.* 2015; Kim *et al.* 2016*a*, *b*).

Because the magnitude of sympathetic LTF is similar with or without the direct contribution of CBs to plasticity (Fig. 6*C*–*E*), we propose that sympathetic LTF, regardless of the loci of plasticity, is capped. Accordingly, with sensory LTF suppressed, this upper cap is reached entirely by central plasticity but, when CBs are fully functional, plasticity in the form of sensory LTF contributes substantially.

With regard to the sympathetic LTF capping mechanism, this may be mediated entirely centrally. However, we note that sympathetic LTF has no effect on blood pressure in the anaesthetized *in vivo* preparation (Dick *et al.* 2007; Xing & Pilowsky 2010; present study). This leads us to speculate that the capping mechanism is baroreflex-related, imposing the maximum long-term (i.e. tens of minutes) increase in sympathetic activity possible without a corresponding increase in blood pressure. Accordingly, should a sustained increase in sympathetic activity above the cap occur, the resulting sustained increase in blood pressure is expected to trigger feedback inhibition, thus returning long-term sympathetic activity to or below the cap and long term blood pressure back to resting.

Conclusions

The present study demonstrates plasticity in CB chemoafferent activity induced by AIH with concurrent hypercapnia, without CIH pre-conditioning. Several features of this plasticity are shared with CIH-dependent sensory LTF; however, other features are unique. Sensory LTF induced by AIH with concurrent hypercapnia is dependent on activation/sensitization of P2X and TRPV1 receptors, which are located in CB chemo-afferent terminals, suggesting that one of the main sites of plasticity is postsynaptic. Sensory LTF probably has adaptive and maladaptive effects on the ability to respond to repeated apnoeas: over the short term, sensory LTF may be beneficial for mounting responses to hypoxia, whereas, over the long term, it probably destabilizes breathing and paves the way for neurogenic hypertension. As such, the present study provides a possible mechanistic role of the CB in cardiovascular disease and indicates P2X-TRPV1 receptors as being novel targets for non-surgical intervention in humans with uncontrolled hypertension.

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Additional information

Competing interests

The authors declare that they have no competing financial interests.

Author contributions

AR and RJAW conceived and designed the experiments. AR performed the *en bloc* experiments and analysis. FD performed the analysis. RJAW wrote the software to analyse CSN activity. MJF performed the *in vivo* experiments and analysis. AR, MJF and RJAW prepared the figures. AR, MJF and RJAW wrote the first draft of the manuscript. PP provided important edits. All experiments were performed in the laboratories of RJAW and PP. The final version of the manuscript submitted for publication was approved by all of the authors. All authors agree to be accountable for all aspects of the work with respect to ensuring that the questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors qualify for authorship, and all those who qualify for authorship are listed.

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