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SPINAL nNOS REGULATES PHRENIC MOTOR FACILITATION BY A 5-HT2B RECEPTOR- AND NADPH OXIDASE-DEPENDENT MECHANISM

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Abstract—Acute intermittent hypoxia (AIH) induces phrenic long-term facilitation (pLTF) by a mechanism that requires spinal serotonin (5-HT) receptor activation and NADPH oxidase (NOX) activity. Here, we investigated whether: (1) spinal nitric oxide synthase (NOS) activity is necessary for AIH-induced pLTF; (2) episodic exogenous nitric oxide (NO) is sufficient to elicit phrenic motor facilitation (pMF) without AIH (i.e. pharmacologically); and (3) NO-induced pMF requires spinal 5-HT2B receptor and NOX activation. In anesthetized, mechanically ventilated adult male rats, AIH (3 × 5-min episodes; 10% O_2; 5 min) elicited a progressive increase in the amplitude of integrated phrenic nerve bursts (i.e. pLTF), which lasted 60 min post-AIH (45.1 ± 8.6% baseline). Pre-treatment with intrathecal (i.t.) injections of a neuronal NOS inhibitor (nNOS-inhibitor-1) near the phrenic motor nucleus attenuated pLTF (14.7 ± 2.5%), whereas an inducible NOS (iNOS) inhibitor (1400 W) had no effect (56.3 ± 8.0%). Episodic i.t. injections (3 × 5 μl volume; 5 min) of a NO donor (sodium nitroprusside; SNP) elicited pMF similar in time-course and magnitude (40.4 ± 6.0%, 60 min post-injection) to AIH-induced pLTF. SNP-induced pMF was blocked by a 5-HT2B receptor antagonist (SB206553), a superoxide dismutase mimetic (MnTMPyP), and two NOX inhibitors (apocynin and DPI). Neither pLTF nor pMF was affected by pre-treatment with a protein kinase G (PKG) inhibitor (KT-5823). Thus, spinal nNOS activity is necessary for AIH-induced pLTF, and episodic spinal NO is sufficient to elicit pMF by a mechanism that requires 5-HT2B receptor activation and NOX-derived ROS formation, which indicates AIH (and NO) elicits spinal respiratory plasticity by a nitrergic-serotonergic mechanism. Published by Elsevier Ltd. on behalf of IBRO.

Key words: intermittent hypoxia, nitric oxide, respiratory plasticity.

INTRODUCTION

Nitric oxide (NO) is critical for many forms of neuroplasticity, including hippocampal long-term potentiation (LTP, Bliss and Collingridge, 1993), cerebellar long-term depression (LTD, Shibuki and Okada, 1991) and *Aplysia* long-term sensory motor facilitation (Antonov et al., 2007). NO also plays complex, but poorly understood roles in the neural control of breathing. For example, NO inhibits carotid body chemoreceptor responses to hypoxia (Prabhakar et al., 1993; Chugh et al., 1994; Summers et al., 1999), but excites neurons in the nucleus of the solitary tract where those chemoafferent neurons terminate (Torres et al., 1997; Gozal and Gozal, 1999; Gozal et al., 2000). However, little is known concerning the role of NO in hypoxia-induced respiratory plasticity. Thus, we tested the hypothesis that NO is necessary for phrenic long-term facilitation (pLTF), a form of serotonin (5-HT)-dependent respiratory motor plasticity induced by acute intermittent hypoxia (AIH) (Bach and Mitchell, 1996; Mitchell et al., 2001; Mahamed and Mitchell, 2007; MacFarlane et al., 2008).

Key steps in the mechanism of pLTF include: spinal 5-HT receptor activation (Bach and Mitchell, 1996; Fuller et al., 2001; Baker-Herman and Mitchell, 2002; MacFarlane et al., 2011), new synthesis of brain-derived neurotrophic factor (BDNF) and activation of its high-affinity receptor, TrkB (Baker-Herman et al., 2004), followed by ERK MAP kinase signaling (Hoffman et al., 2012; Fig. 7). Other molecules regulate pLTF, including reactive Oxygen species (ROS, MacFarlane and Mitchell, 2008) NADPH oxidase (NOX; MacFarlane et al., 2008, 2009) and serine-threonine protein phosphatases (MacFarlane et al., 2008; Wilkerson et al., 2008). These molecules constitute a ''regulatory cassette'' for pLTF (Dale-Nagle et al., 2010).

Pre-conditioning with chronic intermittent hypoxia (CIH) enhances phrenic (Ling et al., 2001) and

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Abbreviations: 5-HT, serotonin; aCSF, artificial cerebral spinal fluid; AIH, acute intermittent hypoxia; CIH, chronic intermittent hypoxia; CtB, cholera toxin B fragment; DMSO, dimethylsulfoxide; iNOS, inducible NOS; LTD, long-term depression; LTP, long-term potentiation; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase; nNOS, neuronal NOS; NO, nitric oxide; NMDA, N-methyl-D-aspartate; NOS, nitric oxide synthase; NOX, NADPH oxidase; PKA, protein kinase-A; PKC, protein kinase-C; pLTF, phrenic long-term facilitation; pMF, phrenic motor facilitation; PKG, protein kinase G; ROS, reactive oxygen species; SNP, sodium nitroprusside; TC, time control; vLTF, ventilatory LTF; XII, hypoglossal.

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ventilatory LTF (vLTF; McGuire et al., 2004) by a 5-HT-dependent mechanism; however, it is not known if enhanced pLTF results from central vs peripheral mechanisms. CIH reveals a novel form of carotid chemosensory long-term facilitation (Peng et al., 2003), amplifies central neural integration of chemoafferent inputs (Ling et al., 2001) and strengthens spinal synaptic pathways to phrenic motor neurons (Fuller et al., 2003). Thus CIH preconditioning elicits both peripheral chemosensory and central neural plasticity. Episodic 5-HT receptor activation elicits chemosensory LTF by a NOX-dependent mechanism (Peng et al., 2006). Similarly, episodic spinal 5-HT receptor activation (particularly 2B receptors) elicits phrenic motor facilitation (pMF) by a NOX-dependent mechanism (MacFarlane et al., 2009, 2011). Thus, carotid chemosensory and spinal respiratory plasticity result from similar cellular mechanisms after CIH pre-conditioning.

CIH decreases carotid body neuronal nitric oxide synthase (nNOS) expression (Marcus et al., 2010), and AIH-induced vLTF is attenuated in nNOS knock-out mice (Kline et al., 2002). Further, NO triggers 5-HT release in the central nervous system (Harkin et al., 2003; Inan et al., 2004; Bryan-Lluka et al., 2004). Thus, NO may be a critical regulator of AIH-induced pLTF. To determine the role of NO in pLTF, we tested the hypotheses that: (1) spinal nNOS activity is required for pLTF; (2) episodic NO release (via sodium nitroprusside; SNP) is sufficient to elicit pMF without AIH; and (3) that SNP-induced pMF requires spinal 5-HT2B receptor activation and NOX activity.

EXPERIMENTAL PROCEDURES

Experiments were performed on 3–4-month-old male Sprague–Dawley rats (Harlan, colony 218A). All experiments were approved by The Animal Care and Use Committee at the School of Veterinary Medicine, University of Wisconsin-Madison.

Surgical preparation

Rats were briefly anesthetized with isoflurane, tracheotomized and pump ventilated (normalized for each rat to 7.5 ml/kg tidal volume and 70 breaths/min ventilator frequency; Rodent Ventilator, model 683; Harvard Apparatus, South Natick, MA, USA). Isoflurane anesthesia was maintained (3.5% in 50% O_2, balance N_2) for the duration of the surgical procedure, followed by conversion to urethane anesthesia (1.8 mg/kg) via a tail vein catheter. The adequacy of anesthesia depth was confirmed by the lack of response to a toe-pinch given prior to paralysis via an injection of pancuronium bromide. Surgery was performed on a custom-made temperature-controlled stainless-steel surgical table connected to an adjustable water bath (Isotemp 1006S, Fisher Scientific, Pittsburgh, PA, USA) to maintain body temperature constant. Rectal temperature was monitored continuously with a temperature probe (Fisher Scientific, Pittsburgh, PA, USA), and maintained constant by adjusting the temperature of the water bath. The concentration of inspired O_2 was monitored throughout experiments using a fuel-cell O_2 sensor (TED 60T, Teledyne Analytical Instruments, CA, USA). A tail vein catheter (24 gauge, Surflo, Elkton, MD, USA) was inserted to allow delivery (1.5–2 ml/h; Cole-Palmer, Vernon Hills, IL, USA) of fluids consisting of a 1:1 lactated Ringers:hetastarch solution to assist in maintenance of blood pressure (6% Hetastarch; Hospira Inc., IL, USA) and base excess (Lactated Ringers, Baxter, IL, USA). A small amount (1:20) of sodium bicarbonate (8.4% Hospira Inc., IL, USA) was also added to the infusion solution. Rats received an initial 1-ml intravenous injection of lactated ringers over a 5-min period to minimize early changes in base excess.

Rats were vagotomized bilaterally and a polyethylene catheter (PE50 ID: 0.58 mm, OD: 0.965 mm; Intramedic, MD, USA) was inserted into the right femoral artery to monitor blood pressure (Gould Pressure Transducer, P23, USA). Blood samples were analyzed for partial pressure of O_2 (PO_2) and CO_2 (PCO_2) and pH with a blood gas analyzer (ABL 800, Radiometer, Copenhagen, Denmark); base excess, calculated by the analyzer, was used as an indicator of metabolic acid–base status.

The left phrenic and hypoglossal (XII) nerves were dissected and exposed via a dorsal approach, cut distally, and de-sheathed. Nerves were submerged in mineral oil and placed on bipolar silver recording electrodes. Once electrical signals were detected, the rats were assessed for adequate depth of anesthesia by checking for transient increases in blood pressure and/or respiratory neural output following toe-pinch. Rats were then paralyzed with pancuronium bromide (~1.2 ml i.v., 1 mg/ml). The rats received another toe-pinch to test for anesthetic depth immediately prior to and at the end of the experimental protocol. We did not observe increased blood pressure or respiratory nerve activity in any of the rats, consistent with previous studies demonstrating the efficacy of urethane anesthesia for many hours longer than the duration of our experimental protocols (Maggi and Meli, 1986). End tidal CO_2 was monitored using a flow-through capnograph (Novametrix, model 1265, Wallingford, CT, USA) and maintained ~40–45 mmHg for 1hr to allow stabilization of the preparation and nerve signals. Nerve activity was amplified (gain, 10,000; A-M systems, Everett, WA, USA), bandpass-filtered (100 Hz to 10 kHz), rectified and integrated (CWE 821 filter; Paynter, Ardmore, PA, USA; time constant, 50msec). The signal was then digitized and recorded using WINDAQ data acquisition system (DATAQ Instruments, Akron, OH, USA). Data analysis was performed using custom designed software based on a Labview platform (LabVIEW, National Instruments, Austin, TX, USA).

Depending on the experiment, one or two silicone intrathecal catheters enabled localized injections of various pharmacological agents (see below) into the CSF of the cervical spinal cord. In brief, after dorsal C_2 laminectomy an incision was made in the dura and catheter(s) (2 French, ID: 0.3 mm, OD: 0.6 mm; Access