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Research Paper

Phrenic motor neuron TrkB expression is necessary for acute intermittent hypoxia-induced phrenic long-term facilitation

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ABSTRACT

Phrenic long-term facilitation (pLTF) is a form of hypoxia-induced spinal respiratory motor plasticity that requires new synthesis of brain derived neurotrophic factor (BDNF) and activation of its high-affinity receptor, tropomyosin receptor kinase B (TrkB). Since the cellular location of relevant TrkB receptors is not known, we utilized intrapleural siRNA injections to selectively knock down TrkB receptor protein within phrenic motor neurons. TrkB receptors within phrenic motor neurons are necessary for BDNF-dependent acute intermittent hypoxia-induced pLTF, demonstrating that phrenic motor neurons are a critical site of respiratory motor plasticity.

1. Introduction

Neuroplasticity has long been associated with conscious memory formation and motor learning in mammals (Ramón y Cajal, 1907; Hebb, 1949; Bliss and Lomo, 1973; Bailey et al., 1996), but has only recently been appreciated in the neural system controlling breathing (Mitchell and Johnson, 2003; Feldman et al., 2003). Although several examples of spinal motor neuron plasticity exist in invertebrate motor systems (Glanzman, 2009; Kandel and Tauc, 1965a, 1965b), mammalian motor neurons have traditionally been portrayed as inflexible relays between the brain and muscles actuating movements (Eccles and Sherrington, 1930).

One stimulus for eliciting respiratory plasticity is low oxygen (hypoxia), such as the carotid body plasticity observed following sustained or intermittent hypoxia (Kumar and Prabhakar, 2012), and spinal motor plasticity following acute intermittent hypoxia (AIH; Bach and Mitchell, 1996; Baker and Mitchell, 2000). Although spinal respiratory motor plasticity is suspected to arise from cellular mechanisms within respiratory motor neurons (Baker-Herman and Mitchell, 2002; Mitchell et al., 2001; Feldman et al., 2003), this hypothesis has not been adequately tested. Previous work using adeno-associated viruses to deliver TrkB protein to phrenic motor neurons demonstrated that phrenic motor neuron TrkB expression promotes recovery following C2 spinal hemisection (Mantilla et al., 2013; Gransee et al., 2013), but this study did not evaluate other, non-injury related forms of phrenic motor plasticity.

Considerable effort has been focused on understanding cellular and synaptic mechanisms of AIH-induced phrenic long-term facilitation (pLTF), and we know that it: 1) is initiated by spinal, serotonin type 2 receptor activation (5-HT2; Baker-Herman and Mitchell, 2002; Fuller et al., 2001; Kinkead et al., 1998; MacFarlane et al., 2011); 2) requires protein kinas C-θ activity within phrenic motor neurons (Devinney et al., 2015); 3) requires new synthesis of spinal brain derived neurotrophic factor (BDNF; Baker-Herman et al., 2004); and 4) requires ERK/MAP kinase signaling (Hoffman et al., 2012). Although new BDNF synthesis is necessary and sufficient for AIH-induced pLTF (Baker-Herman et al., 2004), the involvement of its high affinity receptor, tropomyosin receptor kinase B (TrkB), has not been conclusively demonstrated. Previous studies used a non-selective drug (K252a; Baker-Herman et al., 2004; Ruegg and Burgess, 1989) to block tyrosine kinase activity throughout the cervical spinal region, but the cell type hosting the relevant BDNF/TrkB signaling could not be determined. Here, we tested the hypothesis that phrenic motor neuron TrkB protein expression is necessary for

Abbreviations: 5HT, serotonin; AIH, acute intermittent hypoxia; BDNF, brain derived neurotrophic factor; CtB, choleratoxin B fragment; ERK, extracellular regulated kinase; LTP, long term potentiation; Nx, normoxia; (p)LTF, (phrenic) long-term facilitation; siNT, non-targeting siRNA; siTrkB, siRNA targeting TrkB; TrkB, tropomyosin related kinase B.

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BDNF-dependent phrenic long-term facilitation following AIH. We demonstrate that intrapleural administration of siRNAs targeting TrkB mRNA knocks down TrkB protein in the phrenic motor nucleus, but not in nearby spinal regions expected to contain pre-phrenic interneurons. Since, TrkB knock-down with intrapleural siRNAs abolishes AIH-induced pLTF, AIH-induced pLTF results from spinal motor neuron plasticity.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats (2–5 months old; colony 218A, Harlan; Indianapolis, IN) were studied. Rats were double-housed with food and water **ad libitum**, in a 12 h light/dark cycle with controlled humidity and temperature. The University of Wisconsin Institutional Animal Care and Use Committee approved all animal procedures/protocols.

2.2. siRNA and cholera toxin B fragment delivery

siRNAs were prepared per manufacturer instructions (Dharmacon Inc.). Pools of four Accell-modified siRNA (21 base pairs) duplexes targeting different mRNA sequences were used; each duplex was either non-targeting (siNT) or targeting TrkB (siTrkB). siTrkB duplexes targeted the following sequences: GCUUAAAGUUUGUGGCUUA, CUAAUGGCCAAGAAUGAAU, GUAUCAGCUAUCAAACAAC, and GCACCAACCAUCACAUUUC. Accell-modified siRNAs were used because they preferentially transfect neurons (Nakajima et al., 2012).

Each pool was suspended in Dharmacon siRNA buffer at a concentration of 5 μM, aliquoted and stored at −20 °C. Prior to intrapleural injections, 20 μl of siRNA was added to 6 μl of 5× siRNA buffer (Dharmacon), 3.2 μl of Oligofectamine Transfection Reagent (Invitrogen), 0.8 μl of RNAase free H_2O (final siRNA concentration of 3.33 μM) and mixed for 20 min prior to injection, thus enabling siRNAs to complex with the transfection reagent.

Cholera toxin B (CtB) fragment and siRNAs were injected intrapleurally similar to earlier reports to respectively back-label phrenic motor neurons (Mantilla et al., 2009) and induce RNA interference (Mantilla et al., 2013). Anesthesia was induced with 5% isoflurane in 100% O_2, and then maintained via nose cone (3.5% isoflurane, 100% O_2). 12.5 μl of CtB (2 μg/μl in sterile H_2O) was loaded into a 25 μl Hamilton syringe attached to a 6 mm sterile needle before bilateral injections (2 × 12.5 μl of 2 μg/μl total) at the 5th intercostal space anterior axillary line 7 days prior to tissue harvest. Intrapleural siRNAs were delivered daily as 30 μl injections bilaterally (2 × 30μl of 5 μM siRNA) from a 50 μl Hamilton syringe 3, 2 and 1 day prior to tissue harvest or neurophysiology experiments (outlined in Fig. 1A). This technique was adapted from previous reports using intrapleural siRNA injections to knockdown gene expression within phrenic motor neurons (Mantilla et al., 2013; Devinney et al., 2015). After injections, isoflurane was discontinued and chest movements were monitored for signs of distress and/or pneumothorax (none observed). Rats were housed and monitored daily until used for neurophysiology or immunohistochemistry (IHC) experiments.

2.3. Experimental groups

Four rat groups were studied for neurophysiology experiments: 1) siTrkB + AIH ($n = 7$); 2) siNT + AIH ($n = 5$); 3) vehicle + AIH ($n = 11$); 4) vehicle + normoxia (Nx). Normoxia control groups experienced persistent ventilation with normoxia air (50% O_2 + 2% CO_2 + 48% N_2). 3 separate rat groups were used for IHC analysis. The IHC groups received intrapleural CtB and siRNA injections (outlined above) but did not undergo the neurophysiology protocol (outlined below). The IHC groups were: 1) siTrkB ($n = 6$); 2) siNT ($n = 6$); 3) vehicle ($n = 6$). 50 total rats were used in this study.

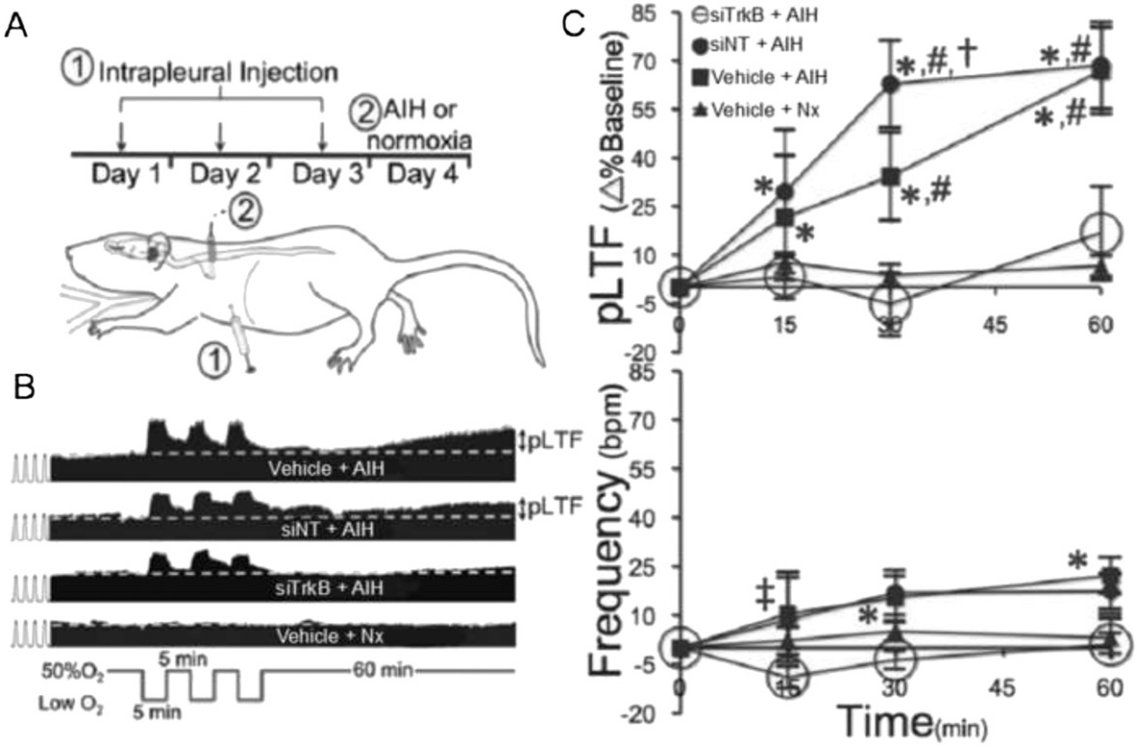

Fig. 1. Intrapleural siTrkB injections abolish pLTF. A) After siTrkB, siNT or vehicle injections (1), rats were given either Nx or AIH, and assessed for pLTF (2). B) In compressed phrenic neurograms, rats given vehicle (top) or siNT (second trace) exhibit pLTF following AIH; in contrast, rats receiving siTrkB (third trace) did not express pLTF following AIH. Control trace of rats receiving intrapleural vehicle injections without AIH (bottom) also did not exhibit pLTF. C) AIH increases phrenic burst amplitude in control (■; $n = 11$) and siNT rats (●; $n = 5$), but not siTrkB rats (○; $n = 7$), relative to Nx (▲; $n = 9$) controls 60-min post-AIH. D) AIH elicited small increases in frequency within vehicle (■) and siNT (●) rats (versus baseline), but not siTrkB rats (○). Mean values ± 1S.E.M. * vs baseline ($p < 0.001$); # vs vehicle + Nx at same time point ($p < 0.02$); † vs vehicle + AIH at same time ($p < 0.001$).