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Research Report

Egr2-neurons control the adult respiratory response to hypercapnia

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ABSTRACT

'The early growth response 2 transcription factor, *Egr2*, establishes a population of brainstem neurons essential for normal breathing at birth. *Egr2*-null mice die perinatally of respiratory insufficiency characterized by subnormal respiratory rate and severe apneas. Here we bypass this lethality using a noninvasive pharmacogenetic approach to inducibly perturb neuron activity postnatally, and ask if *Egr2*-neurons control respiration in adult mice. We found that the normal ventilatory increase in response to elevated tissue CO_2 was impaired, blunted by 63.1±8.7% after neuron perturbation due to deficits in both respiratory amplitude and frequency. By contrast, room-air breathing was unaffected, suggesting that the drive for baseline breathing may not require those *Egr2*-neurons manipulated here. Of the multiple brainstem sites proposed to affect ventilation in response to hypercapnia, only the retrotrapezoid nucleus, a portion of the serotonergic raphé, and a portion of the A5 nucleus have a history of *Egr2* expression. We recently showed that acute inhibition of serotonergic neurons en masse blunts the CO_2 chemoreflex in adults, causing a difference in hypercapnic response of ~50% after neuron perturbation through effects on respiratory amplitude only. The suppressed respiratory frequency upon perturbation of *Egr2*-neurons thus may stem from non-serotonergic neurons within the *Egr2* domain. Perturbation of *Egr2*-neurons did not affect body temperature, even on exposure to ambient 4 °C. These findings support a model in which *Egr2*-neurons are a critical component of the respiratory chemoreflex into adulthood. Methodologically, these results highlight how pharmacogenetic approaches allow neuron function to be queried in unanesthetized adult animals, reaching beyond the roadblocks of developmental lethality and compensation as well as the anatomical disturbances associated with invasive methods.

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1. Introduction

The neural circuitry controlling breathing is protectively redundant, matures postnatally, and is thought to differentially engage distinct types of neurons depending on age and arousal state (Carroll, 2003; Feldman et al., 2003; Nattie and Li, 2009; Nattie, 2001). Physical features further challenge its investigation, including hard-to-access locations in the brainstem, highly

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dispersed neuron populations difficult to capture for en masse manipulations such as electrolytic lesion or viral transgene delivery, and expression of neurotransmitters and signaling systems that offer only modest cell-type specificity for targeted probing pharmacologically. These formidable barriers are being surmounted in part through the application of pharmacogenetic approaches for inducible neuron perturbation in vivo. Here we apply one such pharmacogenetic approach involving the conditional inhibitory Designer Receptor Exclusively Activated by Designer Drug (DREADD) (Armbruster et al., 2007) allele called *RC::PDi* (Ray et al., 2011), to query if neurons defined by expression of the early growth response 2 (*Egr2*) transcription factor play a role in respiratory function in adult mice. *Egr2*-null mice typically die within 18 h of birth due to respiratory insufficiency (Jacquin et al., 1996), precluding the study of *Egr2*-defined neurons in adults. Moreover, *Egr2*-null neonates show substantial non-cell autonomous brainstem abnormalities (Schneider-Maunoury et al., 1997; Voiculescu et al., 2001), confounding attribution of respiratory functions specifically to *Egr2*-neurons even in neonates. Given the importance of *Egr2* defined cells in early postnatal respiratory function, we hypothesize that these same *Egr2* neurons play a critical role in adult respiratory homeostasis.

To test the hypothesis that *Egr2*-defined neurons are required for adult respiratory function, we employed *RC::PDi* (Ray et al., 2011) which is an allele engineered for Cre-dependent expression of Di, a synthetic Gi/o protein-coupled receptor that appears physiologically neutral until triggered by binding the synthetic ligand clozapine-N-oxide (CNO) (Armbruster et al., 2007). Di activation by CNO has been shown to hyperpolarize and inhibit the action potential firing of neurons cell-autonomously, likely via Gi/o gating of endogenously expressed Kir3 channels (Armbruster et al., 2007; Ferguson et al., 2011; Ray et al., 2011). Here, we use *Egr2*$^{tm2(cre)Pch}$ (Voiculescu et al., 2000), referred to as *Egr2*^{cre}, to drive *RC::PDi* recombination and thus constitutive Di expression selectively in neurons with a history of *Egr2* expression, offering the capability to inducibly inhibit these neurons following administration of CNO (Fig. 1). Relative neutrality of the Di receptor in the absence of CNO permits normal development into adulthood (Ray et al., 2011). Avoided are compensatory circuitry changes or perturbations to other brainstem regions, allowing for the direct mapping of CNO/Di-triggered physiological deficits specifically to *Egr2*-neuron function. Further, because CNO can be effectively administered via intraperitoneal injection, analyses can be performed without anesthetics, which are known to perturb autonomic function and respiratory output. Anatomical disturbances associated with cannulas and stereotaxic injections are also avoided. Thus, the *RC::PDi* allele paired with the *Egr2*^{cre} driver permit testing the hypothesis that *Egr2*-defined neurons are required for respiratory function in the adult mouse.

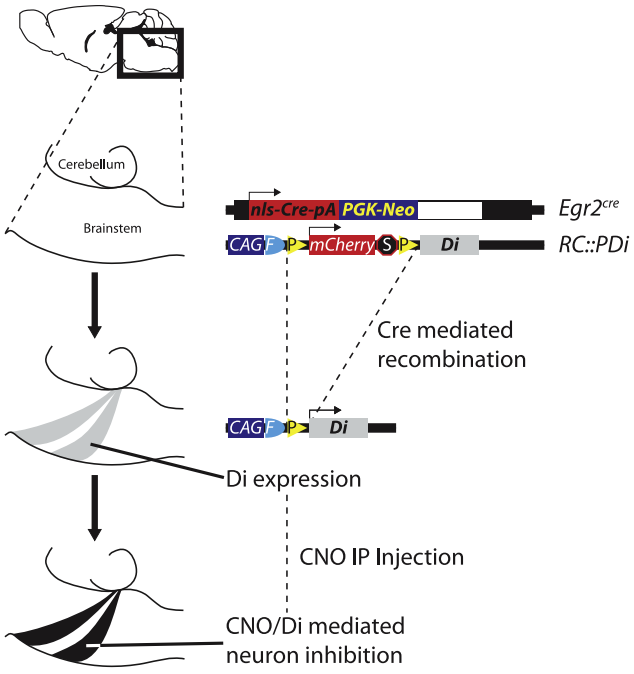

Fig. 1 – The *RC::PDi* allele (Ray et al., 2011) is combined with the *Egr2*^{cre} (Voiculescu et al., 2000) (top schema) to achieve cell specific *Di* expression in rhombomeres (r)3 and (r)5 in the brainstem and elsewhere. Cre mediated recombination of *loxP* sites removes the mCherry-stop cassette and results in *Di* expression under the control of the *CAG* and *R26* regulatory elements (gray areas represent *Egr2* defined brainstem neurons; middle schema). Intraperitoneal (I.P.) injection of Clozapine-N-Oxide (CNO) activates the Di receptor to inhibit neuron function (black area, lower schema).

Fig. 2 – (A) Brainstem schematic depicting selected hindbrain cell populations particularly relevant to respiratory function and their relative relationships to r3 and r5. Note that this cartoon does not convey the mixing of *Egr2*-derived and non-*Egr2*-derived cells even in regions of overlap, nor the considerable cell migration of some *Egr2*-derived cell populations, but is designed as a general anatomical framework. DRN, dorsal raphé nucleus; MRN, median raphé nucleus; LC, locus coeruleus; RTN, retrotrapezoid nucleus; 7N, facial nucleus; NTS, nucleus of the solitary tract; PBC, preBötzinger complex. Inset embryonic schematic shows the location of r3 and r5 in inset of B. (B) Sagittal brainstem section showing *Egr2*^{cre} dependent Tdtomato expression (red) and DAPI (blue) at postnatal day 33 (P33) and embryonic day 12.5 (e12.5, inset) in double transgenic *Egr2*^{cre};*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$. (C) Coronal section depicting *Egr2*^{cre} dependent Tdtomato expression (red) and DAPI (blue) at P33. (D) *Egr2*^{cre} dependent Tdtomato expression in the caudal raphé (red, left panel) co-stained for tryptophan hydroxylase 2 (Tph2; green, middle panel). Tdtomato, Tph2 and DAPI (blue) overlay in right panel. (E) *Egr2*^{cre} dependent Tdtomato expression below the 7 N in the RTN region (red, left panel), costained for Phox2b (green, middle panel). Tdtomato, Phox2b and DAPI (blue) overlay in right panel. Arrowheads point to Phox2b positive nuclei that co-express Tdtomato. (F) *Egr2*^{cre} dependent eGFP expression in the ventral region of the A5 nucleus (green, left panel) in double transgenic *Egr2*^{cre}; *RC::rePe* mice, co-stained for tyrosine hydroxylase (TH; red, middle panel). eGFP, TH and DAPI (blue) overlay in right panel. Arrow points to a neuron positive for GFP and TH.