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Title Page

Your input is a breath of fresh air! A chemosensory microcircuit of medullary raphe and RTN neurons

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Breathing is our first act upon birth and the last action we complete before death. The first to last breath taken, is in fact, how we define someone's life. Since it was first reported that the blood concentration of CO_2 is tightly controlled, and provides the dominant drive to breathe, the search for the cells that regulate it began. It took almost 60 years for the identification of the first central chemosensitive areas, regions within the brain that respond to specific chemical stimuli (such as CO_2 or its proxy H^+), found at the ventrolateral surface of the medulla (VLM). Since then the debate over which cells in these areas are responsible for detecting CO_2 and signalling its fluctuations to the respiratory oscillators, has been extensive and heated. Chemosensitive cells are thought to have cell bodies located in, or close to, the VLM with dendrites in close apposition to blood vessels to better detect changes in blood gases. Several candidates fulfil this criteria, including the retrotrapezoid nucleus (RTN) and medullary raphe.

The RTN gained traction as a central chemoreceptor with the discovery that it expresses the homeobox gene, Phox2b, linking it to congenital central hypoventilation syndrome (CCHS) (Stornetta *et al.*, 2006); a respiratory disorder, characterised by a complete lack of chemosensitivity and an absence of the drive to breathe during sleep. Whilst CCHS can be replicated by targeted loss of

Phox2b neurons in the RTN, respiratory responses to CO_2 partially recover by 3 months of age (Ramanantsoa *et al.*, 2011). Therefore the importance of RTN neurons to the hypercapnic ventilatory response (HVCR) diminishes with age, with other nuclei assuming this role. Contrary to the RTN, the medullary raphe become more responsive to hypercapnia with postnatal development (Cerpa *et al.*, 2017). Thus age must be considered when choosing a model for testing the chemosensitivity of these 2 nuclei (Huckstepp & Dale, 2011).

In this issue of the Journal of Physiology, *Wu et al.*, demonstrate the chemosensitive response of juvenile to young adult RTN neurons is largely dependent on serotonergic input from the medullary raphe (Wu *et al.*, 2019). Furthermore, they were able to recapitulate the *in vivo* connectivity of the raphe and RTN (Mulkey *et al.*, 2007), in a co-culture of neurons from these regions upon a bed of glia. This provides a novel reduced model for studying this important microcircuit without confounding afferents from other regions; following antagonism of serotonergic signalling the residual response to hypercapnic acidosis of RTN neurons in culture was considerably lower than that of RTN neurons in the more intact circuitry of brain slices (Wu *et al.*, 2019).

Given the response of serotonergic neurons to hypercapnic acidosis and their influence on RTN neurons (Wu *et al.*, 2019), it is not surprising that loss of serotonergic neurons leads to a 50% reduction in the HCVR of adult mice (Hodges *et al.*, 2008). Intriguingly, the HCVR of these mice can be rescued by intracerebroventricular administration of 5-HT (Hodges *et al.*, 2008). Investigating this further, *Wu et al.* show blockade of the serotonin transporter increased the response of RTN neurons to hypercapnic acidosis, whilst depletion of serotonin by the tryptophan hydroxylase inhibitor, PCPA, blunted it. Interestingly, exogenous 5-HT added to the milieu of PCPA treated neurons did not restore the hypercapnic response of RTN neurons when hyperpolarising current was added to restore membrane potential to normal resting levels. Therefore, it appears that 5-HT could increase the HVCR by 2 mechanisms; 1) signalling the increased activity of the raphe in response to H^+/CO_2, and 2) inducing a depolarising current that brings the membrane potential of neurons in other respiratory nuclei (e.g. RTN) into a more excitable range, allowing typically subthreshold currents to induce neuronal firing.

Following antagonism of both 5-HT_2 and 5-HT_7 receptors at room temperature, and 5-HT_7 at a more physiologically relevant temperature, there was a residual response to hypercapnic acidosis in RTN neurons in culture of 20% and 17% respectively (Wu *et al.*, 2019). As these antagonists were not able to fully prevent the response of RTN neurons to exogenously applied 5-HT, it may be that the remaining response to hypercapnic acidosis comes from other serotonergic pathways that do not involve these 5-HT receptor sub-types. However, depletion of serotonin by PCPA reduced the hypercapnic response of RTN neurons by a similar amount (18%) (Wu *et al.*, 2019), suggesting this is not the case.

So where might this residual chemosensitivity come from? Firstly, the medullary raphe are still able to excite RTN neurons via thyrotropin-releasing hormone and/or substance-P (Mulkey *et al.*, 2007), and both pathways remain intact throughout the experiments performed by *Wu et al*. Another potential interpretation is stimulation of RTN neurons by gliotransmitters (Huckstepp & Dale, 2011), as the RTN responds to glial-derived signalling molecules and glia are present in *Wu et al.'s* cultures. Alternatively, it may be due to an intrinsic chemosensitivity of RTN neurons. The acid-sensing channel, GPR4, was recently identified as a pH-sensor in the RTN (Kumar *et al.*, 2015). However, this

channel fails the test of a chemosensory transducer because its presence is not *sufficient* to convey chemosensitivity (Huckstepp & Dale, 2011), as removal of GPR4 in the raphe did not affect the CO_2-response of these neurons (Kumar *et al.*, 2015). Moreover, expression of GPR4 in the RTN is relatively low and it's pH-sensitive range is not conducive to driving the respiratory response to CO_2 (Hosford *et al.*, 2018), posing serious doubts over the role of this channel as a physiologically relevant CO_2/H^+ transducer. Thus, for intrinsic chemosensitivity of RTN neurons to be a convincing possibility, more conclusive evidence for GPR4 bestowing acid sensitivity to these neurons, or a new candidate chemosensory transducer, must be found.

In summary, the multiple roles of the medullary raphe in the respiratory response to hypercapnia of juvenile and young adult mice has been further illuminated by this comprehensive and compelling study by *Wu et al.* The burning question now is - what underlies the hypercapnic response of RTN neurons that persists in the absence of 5-HT_7 and 5-HT_{2a} signalling, and depletion of serotonin?

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