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Stimulating the human midbrain to reveal the link between pain and blood pressure

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In 1884 William James suggested that pain sensations are at least partly due to autonomic reactions changing local blood flow and blood pressure (James, 1884). For treatment of chronic neuropathic pain, we implanted deep brain-stimulating electrodes into the periaqueductal grey (PAG).

Thirteen male and 3 female patients (mean age 52 years)were entered into this study. In awake, non-sedated patients, we continuously measured finger arterial pressure and ECG during periods of PAG stimulation 'on' or 'off'. Stimulation parameters during 'on' sessions were those that provided optimal analgesia. Patients provided a visual analogue score (VAS) of their pain during each session using a scale of 0 to 100 (0 = no pain, 100 = worst pain imaginable). To compare long-term analgesia, on-year McGill's pain questionnaire (MPQ) scores were also compared to blood pressure changes. The MPQ score consists of a list of words that describe the pain, that are selected by the patient. We used the scoring system devised by Melzack (Melzack 1975).

Linear regression analysis of improvement in VAS and blood pressure showed a significant correlation between reduction in pain score and reduction in blood pressure (r2 =0.62, p=0.0105, n=16), reduction in dP/dt (r2= 0.62, p<0.008, n=16) but only a weak correlation with reduction in pulse pressure (r2=0.48, n=16). These results are summarized in Fig. 1. Autoregressive power spectral analysis of systolic blood pressure variability (Fig. 2) revealed that in the group with decreased blood pressure, there was a significant reduction (p<0.001, n=7) in the logarithm of the low frequency component (0.023-0.4Hz). The converse was true for increases in blood pressure (p<0.001, n=4). Long-term MPQ scores showed similar results. Electrodes that reduced blood pressure were ventral to those that increased it and there was a significant better analgesia in patients with ventral electrodes (p=0.036, Wilcoxon, n=16).

The analgesic effect of stimulation of human PAG is associated with changes in blood pressure. The underlying mechanism appears to be an alteration in sympathetic activity, as indicated by power spectral analysis of the blood pressure variability and the change in dP/dt which is related to contractility of the myocardium (Brinton et al. 1997).

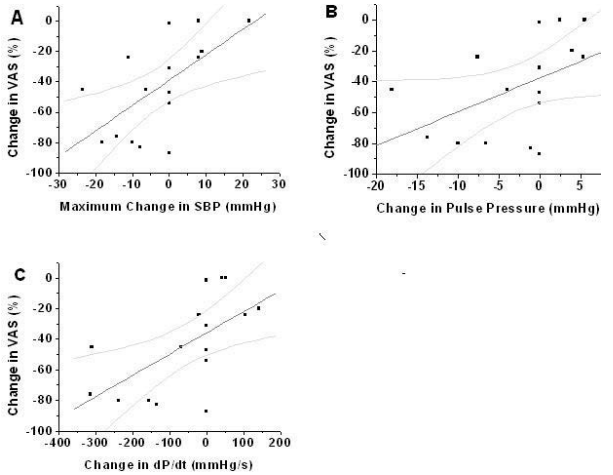

Figure 1. Comparison of visual analogue scores with cardiovascular variables. A, Average changes in VAS (%) significantly correlated to systolic blood pressure (A, r2=0.62. p=0.01, n=16) and dP/dt changes (C, r2=0.62. p=0.01, n=16), but weakly associated with pulse pressure changes with stimulation (B, r2= 0.48, p=0.06, n=16). Black lines, linear regression; outer grey lines, upper and lower 95% confidence intervals. VAS, visual analogue score; SBP, systolic blood pressure.

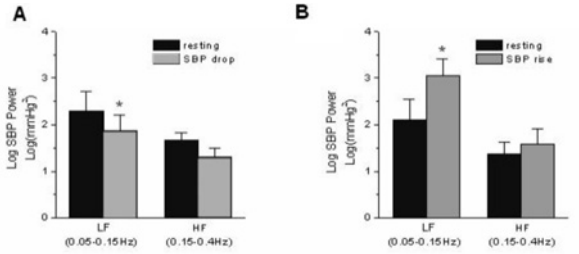

Figure 2. Power spectral analysis of systolic blood pressure A) in those patients with a significant reduction in systolic blood pressure with stimulation, there was a significant decrease (p<0.001) in the low frequency component (0.05 to 0.15Hz) of systolic blood pressure variability. B) Similar results for patients with increases in systolic blood pressure (p<0.001). Error bars indicate one standard deviation of the mean. n=7 for drop and n=4 for rise group. *Statistically significant.

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Modulation of respiratory-related sensory-evoked potentials in humans by voluntary breathing

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The cortical processing of respiratory-related sensory information can be investigated using transient occlusions of inspiration. This afferent stimulation results in respiratory related sensory evoked potentials (RREPs) which exhibit short latency components (Nf, P1) occurring within 100ms of the stimulus onset and longer latency components (N1, P2, P3) that occur 100-350ms after stimulation (Davenport *et al.* 2002). It is known that somatosensory evoked potentials from the limbs can be modulated by voluntary movement (Lee & White, 1974; Wasaka *et al.* 2005). From this we hypothesized that RREPs will also be modulated by voluntary breathing movements.

Surface EEG electrodes were placed to record RREPs at frontal, central and parietal cortical sites. RREPs were elicited by transient inspiratory occlusions lasting 0.3s and were recorded in 15 healthy subjects, in control and voluntary breathing conditions. The amplitude of the early component, P1, was significantly greater ($p=0.046$, ANOVA) during voluntary breathing (2.80 ± 0.55 μV; mean ± SEM) compared to control (1.28 ± 0.55 μV). The later component, P2, was also significantly increased ($p=0.006$) during voluntary breathing (8.40 ± 1.00 μV) compared to control (4.07 ± 0.80 μV). No effects of voluntary breathing were observed on other amplitude or latency component of the RREP.

These results show that there is a modulation of respiratory-related sensory information by voluntary breathing. The present study cannot distinguish at which level(s) this modulation occurs, but it may reflect input from the primary motor cortex and supplementary motor areas to the primary and secondary somatosensory areas or the thalamus.

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C90

Neuronal nitric oxide contributes to the renal sympatho-inhibition of volume expansion in a model of heart hypertrophy

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Heart failure is associated with a sympatho-excitation and a suppression of baroreceptor mediated reflexes in response to the decreased arterial pressure and cardiac output. Previously we reported that nitric oxide (NO) mediated, in part, the attenuation of the renal sympatho-inhibition arising from stimulation of the cardiopulmonary reflex in a model of heart hypertrophy induced by caffeine/isoprenaline [1]. The aim of this study was to determine the isoform of nitric oxide synthase (NOS) that might be involved. To that end, the heart hypertrophy model was used and the impact of a relatively selective neuronal NOS (nNOS) blocker on the cardiopulmonary reflex mediated inhibition of RSNA was examined.

Groups of male Wistar rats (n=6) were maintained on a normal diet and tap water or on caffeine water (61.54mg/l) and isoprenaline subcutaneous injections (5mg/kg) for 2 weeks to induce cardiac damage [2]. Following anaesthesia, (1ml chloralose/urethane, 16.5/250mg/ml, i.p.), the femoral artery and femoral vein were cannulated for measurement of blood pressure (BP) and heart rate (HR) and saline infusion. The left kidney was exposed by a flank incision and the renal nerves placed on recording electrodes. All animals were subjected to two periods of volume expansion (VE) at a rate of 0.25% of body weight per minute for 30 min. Following the first period of VE, 30 min was allowed after which the nNOS inhibitor S-methyl thiocitrulline (SMTC) was administered at 10mg/min/kg for 40 min. Thereafter, the animals were subjected to a second period of VE. Data were presented as a mean value over each 5 min ± S.E.M and subjected to ANOVA. Significance was taken at $P<0.05$.

Heart rate, at 6±0.51 Hz, was decreased ($P<0.01$) by 8 and 9% in the normal and heart hypertrophy groups during the first VE, and by 6 and 4% in the second VE. Blood pressure, at 90±8 mmHg, was unchanged in the normal group throughout both VEs, while it was decreased ($P<0.01$) by 9 and 7% in the heart hypertrophy group during the first and second VE, respectively. VE in the normal group resulted in a 49% decrease ($P<0.001$) in RSNA at 30 min, but by contrast, RSNA did not change in the heart hypertrophy group. During the second VE, RSNA was decreased by 83 and 61% in the normal and heart hypertrophy group, respectively, which were responses larger (both $P<0.001$) than those obtained in the absence of SMTC. The RSNA suppression during VE illustrates the cardiopulmonary reflex and its absence in the heart hypertrophy group implies a deficit in the reflex. Inhibition of nNOS with SMTC enhanced the reflex RSNA suppression to VE in the normal rats and re-established the VE induced renal sympatho-inhibition in the heart hypertrophy rats. These observations suggest that nNOS contributes to the generation of NO which attenuates the cardiopulmonary reflex.

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C91

Excitatory glutamatergic projections from the central nucleus of the amygdala to the rostral ventrolateral medulla

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The central nucleus amygdala (CeA), a limbic forebrain structure, has an important role in the integration of emotional and

cardiovascular responses (Iwata et al. 1987; Dampney, 1994). We have earlier confirmed that there is a projection from the CeA that forms GABAergic synapses with neurons in the nucleus of the solitary tract (Saha et al. 2000), the medullary site of the first synapse for afferent fibers from baroreceptors, chemoreceptors and cardiac receptors, and have recently demonstrated that CeA neurons project directly to blood pressure regulating neurons in the rostral ventrolateral medulla (RVLM, Saha et al. 2005). The RVLM contains sympathetic premotor neurones and plays an important role in blood pressure regulation through its projections to preganglionic sympathetic neurons in the spinal cord. The present study was designed to investigate the neurochemical nature of CeA terminals in the RVLM by using anterograde tracing with vesicular glutamate transporter (VGLUT1, VGLUT2) immunocytochemistry.

All experiments were performed on 280-300g rats anaesthetised with halothane (5% in O_2). The CeA terminals in the medulla were labelled by microinjecting an anterograde tracer, biotin dextran amine (BDA, 0.2 µl of 10% in saline) stereotaxically into the CeA. Following 7-10 days survival time, with buprenorphine analgesia as required, the rats were re-anaesthetised and perfused with 4% paraformaldehyde ± 0.05% glutaraldehyde fixative, and vibratome sections of the brain stem were processed for confocal and electron microscopy. For confocal microscopy, BDA-labelled terminals were visualised with streptavidin Alexa488 (green fluorescence) and VGLUTs were visualised by incubating sections in antibodies to VGLUT1 or VGLUT2 raised in rabbit (gift of Dr J D Erickson; Varoqui et al. 2002) and then in anti-rabbit IgG conjugated to Cy3 (red fluorescence). For electron microscopy, BDA-labelled terminals were visualised by incubating sections in avidin-biotin complex and reacting with diaminobenzidine and hydrogen peroxide. The glutamate transporters were visualised with 1nm gold particles conjugated to second antibodies.

Injections of BDA into the CeA resulted in anterogradely labelled axons and axon terminals in the RVLM. At the confocal microscopic level, CeA terminals in the RVLM contained VGLUT2 but not VGLUT1 immunoreactivity. At the electron microscopic level, many of the axon terminals were found to make asymmetric synapses and contain VGLUT2 immunoreactivity. The results suggest that neural projections from the CeA to the RVLM are predominantly excitatory in nature. The CeA may influence the sympathetic outflow during stressful situation via these direct excitatory projections to RVLM neurons.

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SA45

Hypothalamic-brainstem mechanisms co-ordinating cardiorespiratory regulation

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Co-ordinated changes in cardiovascular and respiratory (cardiorespiratory) function are an essential part of many behavioural responses, such as those associated with exercise or defensive behaviour. Various homeostatic reflexes, such as that evoked by a hypoxic challenge, also include co-ordinated cardiorespiratory responses. Understanding the mechanisms within the brainstem (and forebrain) that produce appropriate co-ordinated cardiorespiratory responses, either reflexly evoked or as part of more generalized behavioural responses, has long been a major challenge to physiologists. There are two general hypotheses concerning the organization of the central pathways that can explain how such co-ordinated responses are produced. According to the first hypothesis, brainstem respiratory neurons that control the motor outputs to the respiratory muscles also have outputs to brainstem neurons controlling the sympathetic outflow to the heart and blood vessels. Thus, according to this hypothesis, the increased activity of sympathetic premotor neurons innervating the heart and blood vessels that occurs during certain behaviours or reflex responses is a consequence of the excitation of such neurons by inputs from brainstem respiratory neurons. An alternative hypothesis, however, is that both central respiratory and sympathetic premotor neurons receive inputs from a common set of neurons (i.e. 'command neurons'), so that increased drive from these command neurons will lead to increased respiratory and sympathetic activity in parallel.

This presentation will first briefly review the results of studies from our laboratory as well as other laboratories, on the functional organization of the central pathways subserving the chemoreceptor reflex, as an example of a reflexly-evoked integrated cardiorespiratory response. The presentation will then focus on the alerting or defence response, as an example of an integrated cardiorespiratory response associated with a more generalized behavioural response. With regard to the latter, it is generally accepted that the dorsomedial hypothalamus (DMH) contains neurons that are an essential part of the pathways producing stress-evoked cardiorespiratory responses (diMicco et al. 2002; Dampney et al. 2005). Activation of these neurons results in increased sympathetic vasomotor activity, heart rate and respiratory activity as well as other effects including increased ACTH release (diMicco et al. 2002). It has been shown that sympathetic premotor neurons in the rostral ventrolateral medulla (RVLM) mediate the increased sympathetic vasomotor activity evoked from the DMH, whereas the increase in heart rate is mediated by neurons in the raphe pallidus in the midline medulla (diMicco et al. 2002; Cao et al. 2004; Horiuchi et al. 2004). In addition, activation of DMH neurons also resets the baroreceptor reflex, such that it remains effective, without any decrease in sensitivity, over a higher range of operating pressure (McDowall et al. 2006). It is proposed that this baroreflex re-setting is mediated by a pathway from the DMH to the nucleus tracti solitarii (NTS) in the medulla.

The role of the DMH in respiratory regulation has not been studied extensively. We have recently found, however, that DMH activation also increases the rate and amplitude of phrenic nerve activity, in parallel with increases in heart rate and sympathetic vasomotor activity.

In reviewing studies on the central mechanisms subserving cardiorespiratory responses reflexly evoked by stimulation of peripheral chemoreceptors, or those evoked from the forebrain, the question will be considered as to what extent the observations of these studies support the hypothesis of 'command neurons' with collateral outputs that simultaneously regulate central cardiac, vasomotor and respiratory neurons.

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Developmental basis of respiratory rhythm generation: a role for Hox genes

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The ability to produce rhythmic motor behaviours linked to a respiratory function is a property of the brainstem reticular formation, which has been remarkably conserved during the evolution of vertebrate. The functional scaffold of brainstem neuronal circuits is first set in the embryonic neural tube, when the hindbrain is partitioned along the anterior-posterior axis into polyclonal developmental compartments called rhombomeres (r). In the hindbrain, the Hox genes provide cells with positional information and rhombomeric identity along the antero-posterior axis of the embryonic body. Hox expression is activated in the hindbrain neuroepithelium before segmentation, in response to inductive signals such as retinoic acid, and maintained through later developmental stages in neuronal progenitors and some postmitotic neurons. Analysis of loss– and gain–of–function mutations in mouse and chick embryos revealed an important role for Hox genes in the establishment of rhombomeric territories, the assignment of segmental identities, and rhombomere-specific neuronal patterns eventually required for a normal breathing behaviour at birth.

The presentation will address whether and how the rhombomeric organisation of the hindbrain neural tube influences later function of respiratory control networks in chicks and mice. This involves "gene to behaviour" strategies combining studies in embryos and analysis of transgenic models. Life-threatening deficiency of a central respiratory rhythm promoting system has been first described in Krox-20-/- and Hoxa1-/- mice in which progenitor cells are mis-specified during early development [2]. In the absence of Hoxa1, some neural precursors at the presumptive r3–r4 levels fail to activate or properly maintain their

appropriate molecular programs and many cells deriving from r4 are eliminated. The Krox20 gene product in r3 acts as a direct transcriptional inhibitor of r4-related Hox and participates in the formation of r3 and the r3/r4 boundary. Interestingly, r3 displays a marked delay compared with r4, in the timing of neuronal differentiation and axonal outgrowth. Therefore, heterochrony of neurogenic processes allows neuronal differentiation in r4 and continuing expression of segmental genes such as Krox20 in r3 at the same developmental stage. Given that rhombomeric heterochrony is found in both chick and mouse, we hypothesize that there are likely to be conserved signaling interactions by which the expression of Krox20 in r3 may influence neural circuits developing in r4. We are currently using chick embryological approach to investigate these signaling interactions [1,3].

Respiratory consequences of the mis-specification of r3 and r4 in mutant mice showed that Krox20 influence on r4 is of vital physiological significance in mice during the first days after birth. An "anti-apneic" neuronal system has been located in the r4-derived ("para-facial") caudal pontine reticular formation, ventral to the facial motor nucleus (another r4-derived structure) [2]. In vivo, neonatal mice with impaired anti-apneic (para-facial) function show an abnormally low respiratory frequency and apnoeas lasting 10-times longer than normal. Most of the animals die during the first two days after birth. Rhombomere r3 is important as a source of Krox20, that is crucial to initiate parafacial development [3]. Current studies with calcium imaging of rhythm generators in mice also show that the parafacial control is embryologically distinct from the post-otic (pre-Bötzinger) respiratory generator originating caudal to r54. Finally, genetic abnormalities affecting rhombomeres rostral to r3 can lead to pontine defects, in which the respiratory frequency is not significantly affected. Altogether, data in mutant mice therefore identify a dual (parafacial and post-otic) brainstem control of the breathing rhythm.

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Purinergic signalling in central and peripheral chemosensory transduction

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Arterial PO_2 and PCO_2 are maintained at constant levels by neural activity that controls breathing. Central respiratory drive is sensitive to changes in arterial PO_2 and PCO_2 which are monitored by the peripheral and central chemoreceptors. The role of purinergic signalling in central and peripheral mechanisms of chemosensitivity has been studied extensively in our laboratories over the last several years. Results obtained allow us to propose a unifying hypothesis of central (within the ventrolateral medulla of the brainstem) and peripheral (within the carotid body) chemosensory transduction, which involves ATP as a common mediator.

Indeed, P2 receptors for ATP are expressed within the ventrolateral medulla (in particular by the respiratory neurones) as well as by the peripheral chemosensory afferent neurones which relay information to the brainstem. Blockade of the P2 receptors within the ventrolateral medulla attenuates the CO_2-induced increase in respiration while blockade of the P2X receptors in the carotid body (or their elimination in the knockout mice) greatly diminishes the ventilatory response to hypoxia and impairs carotid body function. ATP is released from the ventral surface of the medulla during hypercapnia and from the carotid body during hypoxia. Finally, exogenous ATP applied on the ventral surface of the medulla evokes rapid increase in respiration, while ATP applied to the carotid body evokes marked excitation of the carotid sinus nerve chemoafferents.

We suggest that in the ventrolateral medulla ATP is produced following CO_2/H^+-induced activation of central chemosensors (neuronal and/or glial) and acts within the respiratory network to produce physiologically relevant changes in ventilation. In the carotid body, ATP contributes in a significant manner to the transmission of the sensitivity of the carotid body to changes in arterial PO_2 and may be considered as a key transmitter released by chemoreceptor cells to activate endings of the sinus nerve afferent fibres.

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Retrotrapezoid nucleus and central chemoreception

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Central respiratory chemoreception (CRC) is the mechanism by which brain pCO2 regulates breathing (Feldman et al. 2003). Central chemoreception clearly relies on changes in brain extracellular fluid pH but the molecular, cellular and network basis of CRC is far from understood. The very existence of bona fide central chemoreceptors can even be legitimately questioned. Indeed, given the widespread effect of pH on brainstem neurons in vitro, one can still persuasively argue that CRC may be an emergent property of the central respiratory pattern generators (CPGs) caused by the cumulative effects of pH on most if not all of its component neurons. The contrary and more prevalent view is that CRC relies on one or more clusters of neurons that are primarily responsible for the exquisite sensitivity of the respiratory network to CO2 (Feldman et al. 2003). The importance of these clusters may have to do with the dynamic range of their discharge in response to changes in ECF pH and/or to some par-

ticular combination of connectivity and transmitter effectiveness. None of these characteristics absolutely requires that respiratory chemoreceptor neurones possess unique pH-sensitive conductances and, in fact, no such conductance has been identified so far. Research started in the 1960s suggests that a group of central chemoreceptors that regulates breathing and cardiovascular function resides at the ventral medullary surface (VMS) under the facial motor nucleus (Loeschcke, 1982). In this talk I shall consider whether these elusive VMS chemoreceptors reside in the brain region recently identified as the retrotrapezoid nucleus (RTN).

RTN neurons are glutamatergic propriobulbar interneurons that innervate the entire ventral respiratory column and selected regions of the dorsolateral pons and nucleus of the solitary tract (NTS)(Mulkey et al. 2004). These neurons are located very close to ventral surface and have extensive dendrites within the marginal layer of the brainstem (Mulkey et al. 2004). They respond vigorously to CO2 in vivo and their response is unaffected by procedures that silence the CPGs such as the administration of opiate agonists or antagonists of ionotropic glutamatergic receptors (Mulkey et al. 2004). RTN neurons display numerous types of respiratory-related patterns (Guyenet et al. 2005). These patterns are caused by inputs from multiple types of CPG neurons, the majority of which are probably inhibitory. In the absence of CPG activity and peripheral chemoreceptor input, RTN neurons discharge tonically at a rate that is inversely proportional to arterial pH (average threshold: pH 7.5) (Guyenet et al. 2005). However, RTN neurons also receive excitatory inputs from peripheral chemoreceptors via a direct pathway from the NTS that bypasses the CPGs. Thus RTN neurons have the properties of a chemosensory integrative center that is independent of the CPGs. In rat and mouse slices, RTN neurons are silent at pH 7.5, they are vigorously excited by acidification and their discharge pattern is tonic. When examined at physiological temperature, the dynamic range of their response to pH is similar in vitro and in vivo (Guyenet et al. 2005). Under conditions of reduced synaptic activity (tetrodotoxin), acidification produces an inward current in RTN neurons which is attributable to the closure of a resting potassium conductance, currently unidentified (Mulkey et al. 2004). The presence of purinergic receptor antagonists (PPADS and others) does not modify the pH sensitivity of RTN neurons in slices (Guyenet et al. 2005) but these cells are activated by the P2Y-selective agonist UTP (Mulkey, Guyenet and Bayliss, unpublished).

In conclusion, RTN neurons detect brain pCO2 at least in part by virtue of an intrinsic pH sensitivity that involves an unknown resting potassium conductance. We found no evidence that a purinergic paracrine mechanism contributes to their chemosensitivity in vitro. However, these neurons probably express P2Y receptors and therefore, their activity could be up-regulated by ATP in vivo as proposed by Gourine and others (Gourine et al. 2005). RTN neurones also respond to arterial blood gases via a very direct excitatory pathway from carotid peripheral chemoreceptors and they receive a feed-back from the CPG. These characteristics are consistent with the view that RTN is a chemosensory integrating center. The exact role of RTN neurones is not fully clarified by our experiments however. Given their projections and location, it is conceivable that RTN neurons could be encoding some of the chemical drive to breathe but they could also play a role in cardiorespiratory coupling.

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