SUB-CORTICAL AND BRAINSTEM SITES ASSOCIATED WITH CHEMO-STIMULATED INCREASES IN VENTILATION IN HUMANS

Leanne C. McKay[1*], Hugo D. Critchley[2,3], Kevin Murphy[1], Richard S.J. Frackowiak[2,5] & Douglas R. Corfield[1,2,4]

[1]Department of Respiratory Medicine, National Heart and Lung Institute, Imperial College School of Medicine, Charing Cross Hospital, London, UK;

[2]Wellcome Trust Centre for Neuroimaging, UCL, London, UK;

[3]Brighton and Sussex Medical School, University of Sussex, Brighton, UK;

[4]Institute of Science & Technology in Medicine, Keele University, Staffordshore, UK;

[5]Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland, CH

* present address : Neuroscience & Molecular Pharmacology, University of Glasgow.

Corresponding author (and to whom requests for reprints should be addressed)

Douglas R. Corfield, Institute of Science & Technology in Medicine, Huxley Building, Keele University, Staffordshire, U.K. ST5 5BG.

E-mail: d.corfield@keele.ac.uk

Tel: +44 (0) 1782 734654

Fax: +44 (0) 1782 734637

ABSTRACT

We investigated the neural basis for spontaneous chemostimulated increases in ventilation in awake, healthy humans. Blood oxygen level dependent (BOLD) functional MRI was performed in nine healthy subjects using T2* weighted echo planar imaging. Brain volumes (52 transverse slices, cortex to high spinal cord) were acquired every 3.9 s. The 30 minute paradigm consisted of six, five-minute cycles, each cycle comprising 45 seconds of hypoxic-isocapnia, 45 seconds of isooxic-hypercapnia and 45 seconds of hypoxic-hypercapnia, with 55 seconds of non-stimulatory hyperoxic-isocapnia (control) separating each stimulus period. Ventilation was significantly ($p < 0.001$) increased during hypoxic-isocapnia, isooxic-hypercapnia and hypoxic-hypercapnia (17.0, 13.8, 24.9 L/min respectively) *vs.* control (8.4 L/min) and was associated with significant ($p < 0.05$, corrected for multiple comparisons) signal increases within a bilateral network that included the basal ganglia, thalamus, red nucleus, cerebellum, parietal cortex, cingulate and superior mid pons. The neuroanatomical structures identified provide evidence for the spontaneous control of breathing to be mediated by higher brain centres, as well as respiratory nuclei in the brainstem.

INTRODUCTION

In humans, surprisingly little is known of the neural processes that underlie spontaneous breathing. Clinical observations have long identified the functional significance of the brainstem in the generation of breathing; for example, life-threatening deficits in respiratory control, particularly during sleep, are manifest in patients with medullary damage (Plum, 1992; Plum and Leigh, 1981) or lesions of the bulbospinal pathway (Nathan, 1963; Severinghaus and Mitchell, 1962). These clinical studies, however, do not shed light on the specific brainstem nuclei involved in generating spontaneous breathing, a greater understanding of this is provided by animal models. In rodents and goats, an essential component of the brainstem respiratory rhythm-generating circuitry is the preBötzinger Complex (preBötC), a cluster of neurokinin 1 receptor (NK1R) expressing neurones within the ventrolateral medulla (Gray et al., 2001; McKay et al., 2005; Smith et al., 1991; Wenninger et al., 2004). Recent studies of NK1R expression in human postmortem tissue have anatomically localized the presumptive human preBötC to a bilateral cluster within the reticular formation of the caudal brainstem (Lavezzi and Matturri, 2008; Schwarzacher et al., 2007); the functional significance of this anatomical landmark with respect to the afore mentioned clinical studies is unknown.

More recently, brain imaging techniques have provided greater insight into the neural basis of breathing in humans, specifically volitional control of breathing. We (McKay et al., 2008; McKay et al., 2003), and others (Colebatch et al., 1991; Evans et al., 1999; Ramsay et al., 1993), have shown that volitional control of breathing involves a bilateral network composed of the primary motor and sensory cortices, basal ganglia, thalamus, limbic system, cerebellum and brainstem. Less well characterised is the neural basis of involuntary control, e.g., chemo-stimulated breathing (i.e changes in breathing mediated by hypoxic or hypercapnic stimuli). Positron emission tomography (PET) imaging studies of CO_2-stimulated changes in breathing highlight a role for the limbic

system and basal ganglia (Brannan et al., 2001; Corfield et al., 1995a); however, in these studies the chemo-stimulation was associated with an awareness of the stimulus (dyspnoea), which in itself activates higher centres (Evans et al., 2002). At present, we are unaware of any studies that have investigated the neural basis of hypoxia-induced increases in ventilation in humans.

Functional brain imaging techniques, including BOLD and PET, measure changes in tissue perfusion and oxygenation consequent upon changes in neuronal activity. Brain blood flow and oxygenation are sensitive to non-specific factors, e.g., hypercapnia and hypoxia; thus, the interpretation of functional imaging studies of chemo-stimulated breathing is problematic because direct effects of the chemo-stimulation produce vascular changes across the whole brain that overshadow the identification of regional signals related to neural activity. Existing respiratory related imaging studies have attempted to account for this major confound (Brannan et al., 2001; Corfield et al., 1995a; Harper et al., 2005; Peiffer et al., 2001). The tight temporal association between the chemical stimulus, the local neural response and the confounding whole brain signal requires particular attention. For the present study we evolved our existing approach (McKay et al., 2008) to overcome such confounding effects by using hypoxia, hypercapnia and a combination of both stimuli in order to dissociate the time courses of neuronal and non-neuronally induced changes in BOLD signal, whilst achieving the physiological aim of spontaneously increasing ventilation (this methodological issue is addressed in greater detail within the supplementary online information). The aim of the present study was to elucidate the sensorimotor mechanisms underlying spontaneous, chemo-stimulated (hypercapnia and hypoxia) increases in ventilation, in the absence of any conscious awareness of the stimuli, using BOLD fMRI. We hypothesised that the neural substrates underlying increased ventilation resulting from changes in chemosensation in the awake human, are likely to be mediated via brainstem nuclei, engendered through descending cortico-limbic and striatal pathways that are in common with behavioural respiratory control.

METHODS

Subjects

All experimental studies conformed to the standards set by the declaration of Helsinki. All eleven healthy right-handed volunteers (aged 20 – 37 yrs; 7 female) gave fully informed, written consent and were studied with ethical approval from the Riverside Ethics Committee (Imperial College London) and the Joint Ethical Committees of the Institute of Neurology and National Hospital for Neurology and Neurosurgery (University College London). Due to large head movements, data from two subjects were discarded. The data of nine subjects (six female) are presented in this manuscript.

Experimental paradigm

Subjects performed a continuous 30-minute breathing paradigm that consisted of six, five-minute cycles. Each cycle comprised of 45 seconds of hypoxic-isocapnia, 45 seconds of isooxic-hypercapnia and 45 seconds of hypoxic-hypercapnia, with 55 seconds of hyperoxic-isocapnia (control condition) separating each 45-second stimulus period. The periods of hypoxic-isocapnia and isooxic-hypercapnia were designed to increase ventilation by approximately twofold and hypoxic-hypercapnia by approximately fourfold. The aim was to spontaneously increase ventilation using varying stimuli and not to quantify the changes in ventilation in relation to specific stimuli. The rationale for the protocol design and the use of various gas mixtures is described in detail within the supplementary online information. The order of stimulatory periods within each cycle was randomised within and between subjects. A customised breathing apparatus (adapted from (Banzett et al., 2000; McKay et al., 2003)) enabled accurate delivery and control of respired gases for each subject.

A visual stimulus was presented to the subjects during the scanning period (alternating periods of 5 scans/19.5 seconds of grey screen with 5 scans/19.5 seconds of flashing checkerboard screen). The

visual stimulus enabled testing for an interaction between neural and whole brain BOLD signal changes, manipulation of the whole brain BOLD signal being necessary to dissociate neurally-induced BOLD signal changes from confounding vascular changes elicited non-specifically by hypercapnia and hypoxia (see supplementary online information for full details).

Physiological monitoring

Subjects were required to breathe on an apparatus designed to maintain the partial pressure of end tidal CO_2 ($P_{ET}CO_2$) to ±2 mmHg of isocapnia (adapted from (Banzett et al., 2000; McKay et al., 2003). Specialised gas blenders controlled the composition of inspired gas that was delivered to the subject via two gas lines that fed the inspiratory reservoir bag. Line one delivered a constant flow of 30% O_2/balanced N_2 except during periods of hypoxic-isocapnia and hypoxic-hypercapnia when 100% N_2 was blended with the 30% O_2 mixture. Line two delivered CO_2 during periods of isooxic-hypercapnia and hypoxic-hypercapnia, during these periods $P_{ET}CO_2$ was increased by approximately 7–8 mmHg. Flow rate was adjusted to suit each subject. Combining hypoxia with hypercapnia induced a three to fourfold increase in ventilation. The purpose of combining the two stimuli was to produce a third condition that would further dissociate the whole brain BOLD signal from the regional BOLD signal. As a safety precaution, a third gas line was attached to the circuit close to the mouthpiece, which if required delivered 100% O_2 at a high flow rate to the subject.

Respired gases were sampled via a probe inserted into the mouthpiece and determined by a quadrupole respiratory mass spectrometer (MGA 900, Case Medical). Changes in respiratory frequency and tidal volume (V_T) were measured by a pneumotachograph (Collins, INC, MA, USA) that was positioned in the expiratory line of the circuit and connected to a differential pressure transducer (Validyne, CA, USA). Airway pressure was determined via a probe inserted into the mouthpiece, which was connected to a differential pressure transducer (Validyne, CA, USA). All

signals were recorded on a personal computer (DELL Optiplex GX, DELL computer corporation) via an analogue to digital interface (1401 Plus, Cambridge Electronic Design Limited, Cambridge, UK). Subjects were required to wear a nose clip throughout the experiment.

Prior to the scanning day, subjects visited the physiology lab in Charing Cross hospital in order to become familiar with the equipment and procedures, and for the investigators to determine the appropriate levels of hypercapnic and hypoxic stimulation for each subject. These parameters were then used when carrying out the protocol in the fMRI scanner. Oxygen saturation was continuously monitored. During periods of hypoxia, oxygen saturation was maintained above 75% to ensure subject safety and to keep in line with ethical guidelines. Subjects were informed that they may experience an increase in ventilation comparable to light exercise but that the experiment was not designed to cause discomfort or extreme breathlessness.

fMRI data acquisition

T2* weighted echoplanar images were acquired at 1.5 Tesla (Siemens SONATA) during the breathing paradigm. The time series comprised of 450 brain volumes, each whole brain volume was acquired in 3.9 seconds and consisted of 52 transverse slices with an isotropic voxel resolution of 3mm. For each subject an anatomical T1 weighted image was also obtained (resolution of 1 x 1 x 1.5 mm) for anatomical co-registration.

External padding was used to hold the head in place and subjects were attached to the breathing circuit via a personalised mouthpiece, which was securely attached to the head coil using customised support. The subjects wore earplugs that were connected to a microphone in the control room that enabled the subjects to hear verbal instructions.

De-briefing

Immediately after the scanning session subjects were encouraged to volunteer information before being asked a standard set of questions (see supplementary information appendix 1). The aim of the debriefing was to assess the level of awareness of changes to breathing pattern, discomfort, feelings of air hunger or sensations experienced during the scanning session.

Data analysis

Data from 9 subjects (6 female) were included in the analysis. In two subjects only three cycles of the experiment were available for analysis due to fMRI data collection problems. Ventilatory data were analysed on a breath-by-breath basis (Spike 2, CED, Cambridge UK) to calculate frequency and tidal volume and are expressed as mean±S.E.M. Statistical analyses were performed using paired t-tests (SigmaStat, Systat software, CA, USA), differences were regarded as significant if $p<0.05$. The fMRI time series data were analysed using SPM software (Wellcome Trust Centre for Neuroimaging; http://www.fil.ion.ucl.ac.uk/spm). Images for each subject were corrected for head movement, normalised into standard stereotaxic Montreal Neurological Institute (MNI) space, resampled at a resolution of 2 x 2 x 2 mm, and spatially smoothed (filter size, full width, half-maximum = 4 mm). The time series data underwent a fixed effects multiple linear regression analysis on a voxel by voxel basis, to determine the changes in the BOLD signal related to the following main effects: 1) ventilation, 2) visual stimulation 3) whole brain changes in BOLD signal and 4) interaction between visual stimulation and ventilation. For this model, the on/off periods of visual stimuli were modelled as a binary 'box car' function, which was convolved with the haemodynamic response function to represent the relationship between neural activity and cerebral blood flow changes (Friston et al., 1994). Breath-by-breath minute ventilation values were translated into scan domain i.e. a minute ventilation value was assigned to each image in the fMRI time series using linear interpolation. To account for delay in the local cerebrovascular response to increased ventilation, the ventilation regressor was convolved with the haemodynamic response function. To account for whole brain BOLD signal intensity changes resulting from periods of

hypercapnia and periods of hypoxia, the average whole brain signal intensity for each image in the time series was included as a regressor. To determine the presence of any interaction between the visual stimulus and ventilation (e.g., increased BOLD signal intensity when flashing checkerboard and increased ventilation occurred together), a regressor representing the interaction of these two factors (visual stimulus∗ventilation) was included within the model. The data were temporally smoothed by applying a high pass filter (cut-off set at 78 seconds, twice the experimental period for the visual stimulus, the highest frequency component). A separate analysis to investigate and characterise the presence of an interaction between local neuronal changes induced by the visual stimulus with hypercapnia and hypoxia-induced changes in whole brain cerebral blood flow is described in detail within the supplementary online information.

A statistical threshold of $p<0.05$ (corrected for multiple comparisons using a family-wise error correction based on the random fields theory, $T > 5$) was set to determine voxel-wise statistical significance for each condition. *A priori* hypothesis that increased ventilation is associated with increased neuronal activity within the brainstem, permitted small volume corrections for multiple comparisons (Worsley et al., 1996) to be performed within the pons (centred 0 -26 -36; 15mm radius) and the medulla (0 –37 –56; 10mm radius). To determine the anatomical location of each maximum, statistical maps were superimposed onto structural images and identified with reference to standard anatomical atlases (Duvernoy, 1995; Duvernoy et al., 1999; Haines, 1991; Talairach and Tournoux, 1988).

RESULTS

Ventilatory responses

During each chemo-stimulatory period, ventilation was significantly increased compared to the control state (individual example: Fig 1; group mean data: Table 1, $p < 0.002$). Each period of isooxic-hypercapnia induced a twofold increase in peak ventilation. Hypoxic-isocapnia stimulation

induced a slightly smaller increase in peak ventilation. Periods of hypoxic-hypercapnia induced a three fold increase in peak ventilation. Changes in ventilation were accompanied by positively correlated changes in heart rate in 8 individuals (correlation coefficient (r) ranged from 0.2 – 0.6, mean 0.47; Table 1 and Fig. 1).

De-correlating ventilation from whole brain BOLD signal changes

The experimental paradigm successfully dissociated the time-course of the regional BOLD signal related to ventilation from the whole brain BOLD signal related to non-neuronal factors (Fig. 2). Periods of isooxic-hypercapnia were associated with increased whole brain BOLD signal intensity and periods of hypoxic-isocapnia with decreased whole brain BOLD signal intensity. During periods of hypoxic-hypercapnia the whole brain BOLD signal fluctuated around an arbitrary baseline (Fig. 2). Ventilation increased during each stimulus period; thus, the timeline of the ventilation variable does not correlate with the timeline of the whole brain BOLD signal intensity variable (correlation coefficient (r) ranged from 0.25 to – 0.19, mean -0.02). Changes in the whole brain BOLD signal followed changes in PCO_2 and PO_2 by approximately 4 scans (15.6 seconds) and 2 scans (7.8 seconds) respectively, reflecting the delay in cerebrovascular blood flow response to the chemo-stimuli.

Respiratory sensations

Of the nine subjects included in the analysis, eight reported an increased awareness of breathing for short, transient periods during the scanning session, describing their breathing as increasing in volume or becoming deeper, which was associated with feeling slightly uncomfortable but none reported feeling distressed or that they had deliberately modulated their breathing in response to the sensations. Of these eight subjects, two reported feeling the need for more air but when asked to give detailed descriptions it transpired that for one subject the mouth piece was a source of irritation and exacerbated the feeling of not receiving enough air; the second subject had a high sensitivity to

the taste of the gases. By contrast, the ninth subject was completely unaware of any change in breathing throughout the scanning session. One of the subjects, who was excluded from the study due to excessive head movement, reported an awareness of breathing, anxiousness and a need for more air throughout the study, and described the scanner setting as being more intimidating than the lab setting.

Activations associated with chemo-stimulated ventilation

Across the group, the main effect of chemo-stimulated breathing was associated with bilateral activation of predominantly sub-cortical structures including the brainstem (Table 2). Activity increases were observed within the basal ganglia, specifically posterior and anterior regions of putamen and caudate (Fig. 3a), and within ventrolateral thalamic nuclei. Distinct clusters of increased activity were also present bilaterally within the red nucleus (Fig. 3b), and within inferior occipito-temporal (visual) cortices. Activation within the cerebellum was distributed superiorly within the cerebellar cortex and in discrete clusters dispersed throughout the cerebellar lobes (Fig. 3c).

Two clusters of activity were observed within the cingulate cortex. Firstly, increased ventilation was associated with enhanced activity of the posterior cingulate gyrus (Fig 3d), extending superiorly into the medial parietal cortex (precuneus, Fig 3e) and anteriorly to the mid-cingulate gyrus. Secondly, there was enhanced activation of the left dorsal anterior cingulate gyrus (Fig. 3f). Chemo-stimulated breathing was not associated with activity within the motor cortex, confirmed using a bilateral small volume correction centred on voxels (-22, -20 74) and (22, -20, 74) (co-ordinates determined from significant foci associated with voluntary hyperventilation (McKay et al., 2003)) within which no significant activation of motor cortex was observed ($p > 0.05$).

The *a priori* hypothesis that chemo-stimulated ventilation would be associated with an engagement of respiratory centres within the brainstem was tested using small volume corrections within the pons (centre 0, -26, -36; 15mm radius sphere) and the medulla (centre 0, -37, -56; 10mm radius sphere). These co-ordinates were determined from significant maxima identified in our previous respiratory-related imaging studies (McKay et al., 2008; McKay et al., 2003). A discrete cluster of activity was identified within the superior mid pons extending bilaterally from the midline (Fig. 4). There were no significant clusters surviving a small volume correction within the medulla; however, at a lower statistical threshold ($p<0.001$, uncorrected for multiple comparisons) a small cluster of activity was identified within the superior dorsal medulla.

*Visual stimulus, whole brain BOLD signal and visual*ventilation interaction*

As intended, the flashing checkerboard visual stimulus produced a robust pattern of activity (corrected for multiple comparisons, $p < 0.05$) within primary and adjoining visual cortices within the occipital cortex. This visual stimulus was included within the paradigm for mathematical and psychological reasons; first, so that the interaction between neural activity and whole brain BOLD fluctuations could be characterised, and second, to serve as a psychological distraction. A widespread pattern of BOLD signal intensity changes was associated with the whole brain fluctuations in cerebral blood flow. Importantly, there was no significant interaction between the visual stimulation and ventilation confirming that presentation of the flashing checkerboard stimulus did not inadvertently influence breathing or that the ventilatory changes did not affect the visual response (see supplementary online information for further details).

DISCUSSION

In the present study, novel experimental design enabled the identification of a distributed system of brain centres engaged during chemo-stimulated increases in ventilation. Changes in breathing elicited by hypoxia, hypercapnia, and a combination of these stimuli, were associated with increases

in neural activity within regions of the brainstem, thalamus, striatum, cingulate cortex and cerebellum, implicating these centres as mediators in the neural control of "spontaneous" changes in breathing that underlie chemosensation in the awake human.

The experimentally induced chemo-stimulatory challenges produced increases in ventilation with minimal subject discomfort or awareness. In contrast, a number of earlier neuro-imaging studies have used qualitatively similar stimuli but of a magnitude sufficient to elicit subjective feelings of dyspnoea, breathlessness and respiratory discomfort (Banzett et al., 2000; Brannan et al., 2001; Evans et al., 2002). These aversive feelings are associated with predominately limbic and paralimbic structures including the right anterior insular cortex, a putative substrate for conscious dyspnoeic experience (Evans et al., 2002). The present study did not elicit such patterns of activity and although some subjects reported an awareness of increased breathing, they did report lasting respiratory discomfort; thus, the results of this study are unlikely to be related to unpleasant sensations.

Activity in sub-cortical nuclei and the cerebellum

While activation of a discrete set of subcortical brain centres and cerebellum was observed, the temporal constraints of fMRI do not permit further dissection of the precise functional interaction of these nuclei. Nevertheless, within the basal ganglia, a region associated with programmed motor control (Mink, 1996), activity changes observed bilaterally within the anterior and posterior putamen are likely to reflect respiratory motor facilitation, as suggested by previous studies of inspiratory loading (Isaev et al., 2002), CO_2-stimulated breathing (Brannan et al., 2001) and volitional breathing (Evans et al., 1999). The concurrent activation of motor regions of the thalamus, a target of striatal projections, is consistent with engagement of a specific cortico-striato-thalamo-cortical functional circuit dedicated to adaptive respiratory control (Chen et al., 1992; Evans et al., 2002; Fink et al., 1995; Ramsay et al., 1993), in a manner similar to that

implicated in skeletomotor control (Middleton and Strick, 1997). The link to respiratory control during chemo-stimulation is further highlighted by animal studies reporting that electrical stimulation of the mediodorsal thalamus directly inhibits phasic respiratory neurons in the medulla during hypoxic conditions (Akopyan et al., 2000), while lesions of the posteromedial thalamus eliminates respiratory responses to hypoxia and adenosine during fetal development (Koos et al., 2000). In the present study, activity changes within the thalamus were maximal bilaterally within the ventrolateral nucleus, a region anatomically connected with the deep cerebellar nuclei and the ventral respiratory group in the medulla (Gaytan and Pasaro, 1998). The ventrolateral thalamus has recently been implicated in CO_2-stimulated breathing in humans and diffusion tractography has highlighted connectivity between this nucleus and the primary and supplementary motor areas (Pattinson et al., 2009).

Cerebellar cortices are implicated in motor co-ordination and motor learning (Schmahmann and Pandya, 1997), and evidence from animal literature highlights the contributions of the vermis and deep cerebellar nuclei to cardiorespiratory control, including the ventilatory response to hypoxia and hypercapnia (Xu and Frazier, 2002). Respiratory-related neurons within the cerebellar nuclei and cortices receive inputs from the medullary ventral respiratory group (Gaytan and Pasaro, 1998). Neuroimaging studies of congenital central hypoventilation syndrome (CCHS) patients, in whom diminished or absent chemoreception is a symptom, report deficient activity within the cerebellar nuclei in response to hypercapnic and hypoxic challenge (Harper et al., 2005; Kumar et al., 2005; Macey et al., 2005). Consistent with a role in shaping respiratory responses, activity changes within the cerebellum are reported across many respiratory-related imaging studies, including during volitional breathing (Colebatch et al., 1991; McKay et al., 2003; Ramsay et al., 1993), exercise (Fink et al., 1995) and CO_2-stimulated breathing (Corfield et al., 1995a), suggesting a generic role for the cerebellum in the modulation of respiratory patterns in response to chemo-stimulation or volitional demand. Afferents from the cerebellum converge onto the red nucleus (Middleton and

Strick, 1997), which was engaged in this study and has been previously linked to loaded breathing (Isaev et al., 2002) and modulation of ventilation during hypoxia (Ackland et al., 1997), probably via a pontine relay.

Brainstem control of chemo-stimulated ventilation

Activity within the superior dorsal pons, here in the context of the ventilatory response to hypercapnic/hypoxic challenge, has been observed previously with CO_2-stimulated breathing (Pattinson et al., 2009) and with volitional breath holding in humans (McKay et al., 2008). It is well established that neurons with respiratory-related firing patterns are present within the dorsolateral pons (Bertrand et al., 1973; Chamberlin, 2004; Dick et al., 1994); however, the pontine role in respiratory modulation is complex because of the many respiratory patterns e.g., tachypnea, bradypnea, apneusis and apnea, that result from dorsolateral pontine stimulation (Chamberlin, 2004; Chamberlin and Saper, 1998). Neurones of the dorsolateral pons have reciprocal connections with respiratory neurones of the ventrolateral medulla (Herbert et al., 1990; Smith et al., 1989), the nucleus tractus solitarius (Herbert et al., 1990) and phrenic motoneurones (Chamberlin, 2004; Yokota et al., 2001). The pons also serves as a relay station for neural pathways to and from the cerebellum (Middleton and Strick, 1997) and as a relay for inteorceptive sensory information from the spinal cord to supra brainstem structures (Cechetto, 1995). We consider the pons to have a role in integrating sensory information from the lungs and chemoreceptors (central and peripheral), with descending drives from the supra-brainstem and cerebellum, which in turn influences the dynamic output of the medullary respiratory centres. While the BOLD activity within the medulla was less robust than elsewhere (perhaps reflecting decreased signal-to-noise at the periphery of the field of view), the location of the cluster was similar to that observed in association with voluntary hyperventilation (McKay et al., 2003). As in the voluntary hyperventilation study, proprioceptive input was not controlled for in this study; therefore we accept that the increases in BOLD signal within the brainstem may be explained by excitation or inhibition of sensory afferents. Sensory

vagal afferents from the lungs, airways and chest wall project to and terminate onto respiratory neurones within the nucleus of the solitary tract (NTS), where they have an excitatory or inhibitory effect (Bianchi et al., 1995; Kubin and Davies, 1988). This study did not highlight activity within the hypothalamus, amygdala and insula which receive projections from the NTS and ventrolateral medulla (Gaytan and Pasaro, 1998). There is also evidence for intercostal nerve afferents projecting to the cerebral cortex (Gandevia and Macefield, 1989) but it is not known whether this projection is via the brainstem.

Cortical activity

The cortical regions associated with chemo-stimulated ventilation included posterior cingulate/precuneus and the anterior cingulate cortex, which we have highlighted in our previous imaging studies of volitional breathing (McKay et al., 2003) and breath holding (McKay et al., 2008).

The normal physiological response to chemo-stimulation includes both ventilatory and heart rate changes (arterial chemoreceptor inputs and brain stem respiratory centre outputs, directly affect brain stem cardiovascular centres) so it is not possible to experimentally separate these two effects and it is difficult to interpret one of these variables without considering the other. Critchley et al., (Critchley et al., 2000) have previously associated cardiovascular arousal (increased heart rate and blood pressure) with activity specifically within the anterior cingulate. Their changes in cardiovascular arousal were more marked than the present study, where the heart rate changes associated with chemostimulation were modest. Thus, for the present study, cardiomotor changes likely account for a small portion of the neurally related signal changes.

Previous respiratory-related imaging studies attribute activity within the lateral and inferior parietal regions, including the supramarginal gyrus (Isaev et al., 2002; McKay et al., 2003), to an increased

awareness of breathing. In neuroimaging literature, activity within the posterior cingulate and precuneus is typically ascribed to default states of introspective self-awareness (Kjaer et al., 2001). The precuneus has reciprocal connections with the posterior cingulate cortex (Cavanna and Trimble, 2006), and the latter region is activated during CO_2-stimulated breathing (Corfield et al., 1995a) and load-induced dyspnea (Peiffer et al., 2001), suggesting a role in affective/cognitive processing of the dyspnea sensation (Peiffer et al., 2001); however, in the present study, dyspnoeic sensations were rarely present during chemo-stimulated breathing. We speculate that the dorsal cingulate, along with the precuneus, may contribute to self-referential perception of spontaneous changes in breathing pattern in the awake, resting state. These data are interpreted with caution (see methodological issues); a greater understanding of these cortical areas will require further study.

Conscious drive

It was beyond the scope of this study to investigate the significant changes in respiratory control that accompany the sleep/wake cycle, such as sleep-related reductions in the ventilatory responses to hypercapnia and hypoxia (Douglas et al., 1982a; Douglas et al., 1982b). For instance, hypocapnia, induced through mechanical hyperventilation, consistently produces apnoea during non-REM sleep (Skatrud and Dempsey, 1983) but not during wakefulness (Corfield et al., 1995b), unless brain injury is present (Heywood et al., 1996; Plum and Leigh, 1981). Correspondingly, in patients with CCHS, breathing during wakefulness appears normal, but the ventilatory responses to hypercapnia and hypoxia are reduced or absent (Shea, 1997), and during sleep, breathing is characterised by frequent hypopnoea and apnoea. Together, these physiological and clinical observations indicate that there is a “wakefulness drive to breathe” (Fink, 1961), absent during sleep, that appears to be linked to chemosensation. While the functional brain processes we describe may underlie the “wakefulness drive to breathe”, to validate this association in full, the same methodology needs to be carried out in sleeping individuals in order to characterise changes in the chemoresponsive network during different stages of sleep.

Methodological issues

Neurally-derived BOLD signal changes are indirect measures of neuronal activity; focal increases in neuronal activity are associated with focal increases in blood flow that exceeds the increased oxygen extraction required for local metabolism. These changes, together with an increase in venous blood volume result in focal increases in the BOLD signal that reflect increased neural activity. By its nature, the BOLD signal is sensitive to non-neural factors such as PCO_2 and PO_2 that directly alter blood flow or blood oxygenation 'globally' across the brain. Any stimulus that induces a neurally related BOLD signal and at the same time induces a non-specific systemic change in the BOLD signal is problematic; with such a stimulus it is not possible to ascribe the change in BOLD signal exclusively to either the neural or non-neural component. In the context of ventilatory control studies, a hypercapnic stimulus alone would result in a strong temporal correlation between the changes in ventilation and changes in whole-brain BOLD signal (increasing ventilation being associated with increasing BOLD signal). For hypoxia, there would be concomitant changes in ventilation and whole-brain BOLD signal (ventilation increasing as BOLD decreases). The present study was designed to address this directly. The stimulus combinations of hypoxia, hypercapnia and both stimuli combined, ensured that the temporal correlation between the whole-brain BOLD signal and the local neurally-related BOLD signal (reflecting the changes in ventilation), was substantially dissociated. In principle, the effects of hypercapnia and hypoxia would be best modelled using the grey matter signal as the whole-brain covariate, rather than the whole-brain BOLD signal intensity. This would require an accurate segmentation of the BOLD image into the separate tissue types (i.e. grey matter, white matter and cerebrospinal fluid). Although this can be done reliably for high resolution T1 structural images, we are unaware of this process being performed successfully on BOLD images. In particular, the inherently lower resolution of the T2* image would lead to significant partial volume effects and problematic segmentation. We believe that by using the approach described above, we have successfully

reported activity related to the chemical control of ventilation in humans that has fully accounted for this confound. One limitation of this approach is that it can only identify the neural activity associated with the overall changes in ventilation; it cannot identify, separately, the neural activity associated uniquely with either hypoxia or hypercapnia.

For the purpose of the present study, it is necessary to treat the global and local BOLD signal changes as being independent and additive within the general linear model (Friston et al., 1990). Our data suggest that there is some small interaction between the local and global signals; however, this interaction is not of a magnitude that would substantially affect the model or its outcomes. This issue and related literature are discussed in further detail within the online supplementary information.

A fixed effects analysis was performed because the cohort was not of sufficient size to perform a random effects analysis but we do not consider this a major limitation on the interpretation of the study. This is an investigation of normal physiology rather than pathophysiology and the results for this study and our previous respiratory-related fMRI studies have shown that there is very little inter-subject variability in responses, e.g., in this study the putamen, thalamus, cerebellum and red nucleus were bilaterally significant in half of the individual analyses at $p<0.05$ (corrected for multiple comparisons) and in all individuals when the threshold was lowered to $p<0.01$ (uncorrected for multiple comparisons). In addition, respiratory-related fMRI studies conducted by us and others have been replicated in a general population subject cohort and have been shown to be reproducible (Evans et al., 1999; McKay et al., 2008; McKay et al., 2003; Pattinson et al., 2009).

BOLD fMRI is highly is susceptible to motion induced signal changes, particularly along the midline at the border of grey matter with CSF, which is where we identified BOLD signal increases within the precuneus (Fig 3e) and posterior cingluate (Fig 3d). We have previously identified

increased BOLD signal intensity within the area of the precuneus associated with breath holding during hypercapnia (unpublished data). An angiography to compare the anatomical location of the venous sinuses with the pattern of BOLD signal intensity and detailed analyses of these data indicated that the BOLD signal increases were not movement related or due to inadequate modeling of hypercapnia but possibly due to BOLD signal within the venous sinuses rather than grey matter. The intensity threshold applied within SPM determines which voxels within each scan are to be included in the analysis. Voxels with an intensity value below the mean, usually non brain areas, e.g., venous sinuses, are rejected from the analysis. The most likely explanation for the inclusion of the sinuses in the analysis is the CO_2 –induced increase in blood flow and blood oxygenation within the venous system. The elevated level of oxygenation within the sinuses will be associated with an increase in BOLD signal intensity; thus, pushing the venous sinuses above the mean threshold level so that the voxels are included in the statistical analysis. Thus some caution must be exercised in concluding that the ventilation-related BOLD signal changes in the precuneus and posterior cingulate are of neural origin.

Conclusions

The bilateral pattern of activity in sub-cortical structures and in the cingulate, cerebellum and pons, provides evidence for the non-volitional control of breathing in humans to be mediated by higher brain centres, as well as respiratory nuclei in the brainstem. We propose that the neuroanatomical circuitry identified in this study mediates the neural control of “spontaneous” changes in breathing that underlie chemosensation.

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Table 1: Summary of group (n = 9) ventilatory data.

	Control (hyperoxic isocapnia)	Isooxic hypercapnia	Hypoxic isocapnia	Hypoxic hypercapnia
Ventilation (L/min)	8.4 (0.7)	17.0 (1.1) p<0.001	13.8 (1.3) p<0.002	24.9 (2.0) p<0.001
$P_{ET}CO_2$ (mmHg)	37.0 (0.9)	47.6 (1.1)	36.6 (0.2)	46.6 (1.0)
$P_{ET}O_2$ (mmHg)	167.9 (2.6)	175 (1.1)	54.2 (1.4)	55.6 (1.4)
SaO_2 (%)	98 (0.2)	98 (0.6)	85 (1.0)	85 (1.1)
Mouth pressure max (cmH2O)	2.3 (0.6)	3.7 (1.2)	4.6 (0.4)	8.1 (0.4)
Mouth pressure min (cmH2O)	-2.6 (0.2)	-3.2 (1.2)	-3.9 (0.5)	-7 (0.7)
Heart rate (beats/min)	71 (3.3)	77 (3.6)	81 (4.0)	81 (3.4)

Table 2: Co-ordinates of significant local maxima associated with chemo-stimulated increases in ventilation for the group (n = 9).

Activated Brain Areas	**Left Side** x y z	**T-Score**	**Right Side** x y z	**T-Score**
Parietal Cortex				
Precuneus	-2 -66 56	9.91	12 -84 42	6.90
Cingulate Gyrus				
Posterior Cingulate	0 -40 26	8.50	2 -56 20	8.97
Mid Cingulate	-12 -28 42	6.43	2 -26 28	7.27
Anterior Cingulate	-4 14 42	5.95		
Basal Ganglia				
Posterior Putamen	-30 -16 0	8.40	30 -12 4	6.75
Anterior Putamen	-24 4 -2	7.49	24 4 2	7.71
Mid Brain				
Red Nucleus	-6 -24 -6	7.29	6 -24 -4	6.40
Occipital Lobe				
Inferior Occipital Gyrus	-6 -98 -6	8.38	12 -100 -4	6.63
Fusiform Gyrus	-26 -70 -16	7.12	22 -66 -14	6.40
Cerebellum				
Superior Cerebellum	-20 -54 -28	5.77	4 -52 -24	5.61
Frontal Cortex				
Operculum	-54 18 -4	5.43		
Thalamus				
Ventrolateral Thalamus	-12 -16 8	5.17	10 -14 12	7.17
Pons				
Superior mid-pons	-4 -24 -24	4.35	4 -24 -24	4.26
Medulla (uncorrected $p < 0.001$)				
Superior medulla	-4 -40 -48	3.55		

FIGURE AND TABLE LEGENDS

Table 1

Summary of group (n = 9) ventilatory data. The table shows the peak (Mean ± S.E.M.) change for each variable during each stimulatory period compared to the control period. Ventilation during the stimulatory periods is significantly greater compared to ventilation during the control (hyperoxic-isocapnia) period ($p < 0.002$). Ventilation (L/min); $P_{ET}CO_2$ (mmHg): partial pressure of endtidal CO_2; $P_{ET}O_2$(mmHg): partial pressure of endtidal; SaO_2 (%): percentage of O_2 saturation; Mouth pressure max (cmH_2O): maximum pressure at mouth; Mouth pressure min (cmH_2O): minimum pressure at mouth; Heat rate (beats per minute).

Table 2

Co-ordinates of significant local maxima associated with chemo-stimulated increases in ventilation for the group (n = 9). Co-ordinates are in mm, x co-ordinate is distance right (+) or left (-) of mid-sagittal line, y co-ordinate is distance anterior (+) or posterior (-) to a vertical plane through the anterior commissure, z co-ordinate is distance above (+) or below (-) the inter-commissural (AC-PC) line. T-score is the significance statistic.

Figure 1

A period of ventilatory data collected during the scanning session from one individual representative of the group. The dashed vertical lines indicate one experimental cycle, comprising of one period of isooxic-hypercapnia, one period of hypoxic-hypercapnia and one period of hypoxic-isocapnia. These stimulus periods are most clearly seen on the PO_2 and PCO_2 traces. A control period of hyperoxic-isocapnia (55 seconds) separates each stimulus period. Ventilation, inferred from changes in tidal volume and respiratory frequency, increased in response to all stimuli, this is clearly seen on the expired V_T tracing. Hr – Heart rate (beats/min); SaO_2 – Oxygen saturation (%); Pmouth – Pressure at mouth (cmH_2O); PO_2 – Partial pressure of O_2 (mmHg); PCO_2

– Partial pressure of CO_2 (mmHg); V_T – Tidal Volume (L). Interference from the MRI scanner resulted in noisy heart rate and oxygen saturation tracings.

Figure 2

Data from one individual representative of the group data are illustrated. For each scan, indicated by the x-axis, an average value for the following variables was plotted: a) PCO_2 (mmHg), b) PO_2 (mmHg), c) ventilation (L/min), d) whole brain BOLD signal intensity (arbitrary units). Changes in whole brain BOLD signal intensity (d) lagged changes in PCO_2 (a) and PO_2 (b) by 4 scan durations (15.6 seconds) and 2 scan durations (7.8 seconds) respectively. Ventilation (c) increased during each stimulus period. The time line of the ventilation variable does not match the timeline for the whole brain BOLD signal intensity variable. The dark gray band highlights a period of hypoxia and hypercapnia combined; the intermediate coloured band highlights a period of hypoxia alone, the light gray band highlights a period of hypercapnia alone.

Figure 3

Statistical images of significant activity within cortical and sub-cortical structures associated with chemo-stimulated increases in ventilation for the group (n=9), superimposed onto a group mean structural brain image. The illustrated maxima survived a correction for multiple comparisons ($p<0.05$). The blue crosshairs are centred on each maximum illustrated in the coronal and transverse planes: a) precuneus b) posterior cingulate c) cerebellum d) posterior putamen e) red nucleus f) anterior cingulate. A: anterior, R: Right.

Figure 4

Statistical images of significant activity within the pons associated with chemo-stimulated increases in ventilation for the group (n=9), superimposed onto a group mean structural brain image. The blue crosshairs are centred on a maximum (sagittal and transverse planes) within the superior mid pons that survived a small volume correction for multiple comparisons ($p < 0.05$).