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Determination of the human brainstem respiratory control network and its cortical connections *in vivo* using functional and structural imaging

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ABSTRACT

This study combined functional and structural magnetic resonance imaging techniques, optimized for the human brainstem, to investigate activity in brainstem respiratory control centres in a group of 12 healthy human volunteers. We stimulated respiration with carbon dioxide (CO_2), and utilized novel methodology to separate its vascular from its neuronal effects upon the blood oxygen level dependent (BOLD) signal. In the brainstem we observed activity in the dorsal rostral pons (representing the Kölliker-Fuse/parabrachial (KF/PB) nuclei and locus coeruleus), the inferior ventral pons and the dorsal and lateral medulla. These areas of activation correspond to respiratory nuclei identified in recent rodent studies. Our results also reveal functional participation of the anteroventral (AV), ventral posterolateral (VPL) ventrolateral thalamic nuclei, and the posterior putamen in the response to CO_2 stimulation, suggesting that these centres may play a role in gating respiratory information to the cortex. As the functional imaging plane was limited to the brainstem and adjacent subcortical areas, we employed diffusion tractography to further investigate cortical connectivity of the thalamic activations. This revealed distinct connectivity profiles of these thalamic activations suggesting subdivision of the thalamus with regards to respiratory control. From these results we speculate that the thalamus plays an important role in integrating respiratory signals to and from the brainstem respiratory centres.

Introduction

Rodent brainstem models have significantly furthered the understanding of respiratory control, addressing functional and structural mechanisms of rhythm generation (Feldman and Del Negro, 2006; Paton et al., 2006; St-John and Paton, 2004), chemoreception (i.e. the response to changes in pH and hypoxia) (Kang et al., 2007; Lahiri et al., 2006; Nattie, 2000), and connectivity of the brainstem respiratory control network (Bianchi et al., 1995; Rosin et al., 2006). In humans, the structural and functional neuroanatomy of the respiratory control system is less well understood due to the ethical and technical constraints that limit invasive studies. Brainstem activity has been observed in some functional magnetic resonance imaging (FMRI) studies of human respiration, relating to dyspnoea or volitional control of breathing (McKay et al., 2003, 2008; Peiffer et al., 2001). There are, however no human studies specifically investigating brainstem activity relating to the automatic or unconscious control of respiration, a fundamental function that is essential for life.

In this study we examined responses to chemically stimulated breathing in healthy human volunteers with FMRI. As activity in chemoreceptive brainstem respiratory control centres is stimulated by CO_2 (Feldman et al., 2003), we hypothesized that we would identify pontine and medullary activity in response to CO_2 stimulation. We also expected to observe activity in subcortical centres previously identified in response to CO_2 stimulation. To maximize resolution within the brainstem, FMRI was limited to a narrow field of view focused on the brainstem.

Although post mortem studies (Zec and Kinney, 2003) shed some light on the structural organisation of the human brainstem respiratory network, they are unable to demonstrate function. In the second part of this study, we used diffusion tractography to investigate how activations in the thalamus connect with higher centres in the cortex in order to differentiate their potential contributions to respiratory control.

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Methodological issues

Imaging studies of the respiratory system are challenging because changes in arterial CO_2 ($PaCO_2$) levels cause confounding effects on the blood oxygen level dependent (BOLD) signal. In this study we used novel methodology to dissociate the vasodilatory effects of CO_2 from its neuronal, respiratory stimulant effects.

Carbon dioxide is a potent cerebral vasodilator, and spontaneous fluctuations in $PaCO_2$ at rest are a significant source of low-frequency variations in the BOLD signal (Wise et al., 2004). The basis of the CO_2 dissociation technique used in the present study was to compare the difference between signal changes from external administration of CO_2 with the signal changes correlated with the natural, resting state fluctuations in CO_2. Resting-state CO_2-related fluctuations have recently been used as an FMRI scaling factor by Kannurpatti and Biswal (2008) to minimize the neural stimulation that can potentially be caused by CO_2 challenges. In the present study we are interested in identifying this CO_2 induced neural stimulation. Our work is therefore an extension of their technique.

We hypothesized that in the non-respiratory areas of the brain, the BOLD response to spontaneous resting state fluctuations in CO_2 would represent a direct effect of CO_2 on the cerebral vasculature, and that the approximately linear relationship between BOLD and $PaCO_2$ would remain constant with administration of CO_2 challenges.

In brain areas in which CO_2 causes neuronal activation, we hypothesized that CO_2 challenges would cause the relationship between BOLD and $PaCO_2$ to become much stronger, due to an additional contribution to BOLD from neural activation. As resting fluctuations in CO_2 are also correlated with fluctuations in breathing (Van den Aardweg and Karemaker, 2002), the additional BOLD response related to CO_2-induced neural activation would represent neural activity above the normal baseline level. Direct recordings of gated activation of the respiratory network during hypercapnia (Chen et al., 1991, 1992), which is absent at normal CO_2 levels, gives physiological support to our hypothesis. We therefore sought areas in the brain with increased BOLD sensitivity to CO_2 during externally delivered CO_2 challenges compared with the baseline "resting state".

Methods

12 right-handed healthy volunteers, age 32 (SD(±5)) years (3 female) participated in this study after giving written informed consent in accordance with the Oxfordshire Clinical Research Ethics Committee.

Respiratory protocols

Subjects wore a tight fitting facemask (Hans Rudolph, Kansas City, MO, USA) attached to a breathing system, which delivered mixtures of air, O_2 and CO_2. A minimum of 10 min was allowed to adapt to the mask. Continuous recordings were made of tidal CO_2 and O_2 (CD-3A and S-3A; AEI Technologies, Pittsburgh, PA, USA), respiratory volume (VMM-400, Interface Associates, Laguna Niguel, CA, USA) and oxygen saturations (9500 Multigas Monitor, MR Equipment Corp., NY, USA). The subjects were asked to keep their eyes open throughout the experiment.

For the first half of the study, the baseline resting breathing experiment, the subjects were asked to perform no particular task other than to remain awake, breathing air, and were monitored as above. This part of the study provided information on how natural, resting-state fluctuations in end-tidal CO_2 ($P_{ET}CO_2$) correlate with changes in BOLD, and provided a baseline for the second half of the experiment, where signal changes from administration of CO_2 were measured.

In the second half of the experiment, we delivered intermittent CO_2 challenges to stimulate breathing. The CO_2 challenges were delivered via a computer controlled gas mixing system (dynamic end tidal forcing) (Wise et al., 2007). The CO_2 challenges were designed to raise the subject's $P_{ET}CO_2$ by either 2 or 4 mmHg above a baseline level maintained at 1 mmHg above the subject's natural $P_{ET}CO_2$ (Fig. 1). The raised baseline was essential for the gas delivery system to function correctly. We chose the levels of CO_2 stimulation (2 and 4 mmHg above the baseline) based on pilot data that indicated these would be the lowest levels of CO_2 stimulation to give a reliable range of increases in minute ventilation. We wished to minimize the increase in baseline $PaCO_2$ to minimize changes in behaviour (or non-linearities) of the BOLD response that may be seen during hypercapnia (Cohen et al., 2004; Corfield et al., 2001; Posse et al., 2001). The CO_2 challenges lasted between 11 and 120 s. This methodology gave an expanded range of $P_{ET}CO_2$ values for comparison with the resting-state data from the first half of the experiment. During this part of the experiment end-tidal oxygen ($P_{ET}CO_2$) was maintained at 200 mmHg, independent of changes in breathing, a value that is mildly above normal.

BOLD imaging

MRI scan parameters: Two thousand seven hundred T_2^* weighted echo planar image (EPI) volumes were acquired on a Siemens Trio 3T

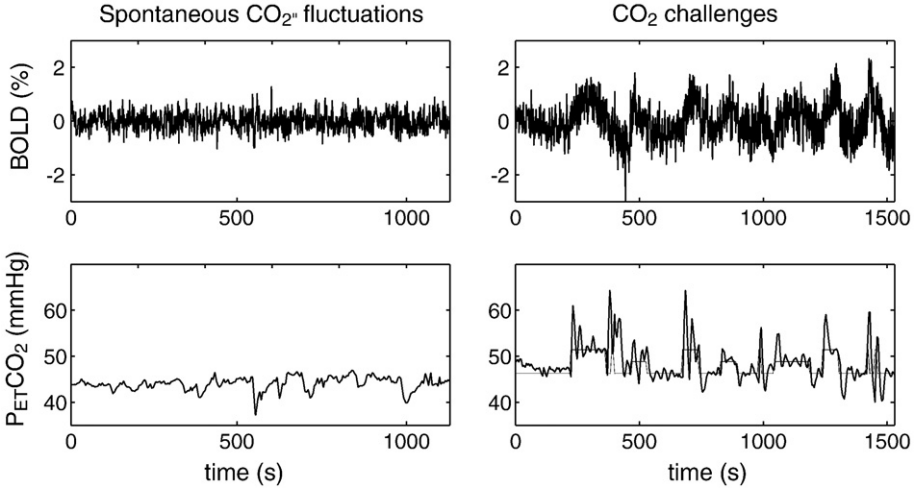

Fig. 1. Example of changes in $P_{ET}CO_2$ and BOLD in one representative subject in one activated region of interest (dorsal pons). On the left are the traces from the resting study and on the right from the CO_2 challenges. The square wave in the lower right figure (CO_2 challenges) represents the desired $P_{ET}CO_2$ levels programmed into the gas control system.

scanner. The field of view (Fig. 4) comprised 16 coronal oblique slices of the brainstem (sequence parameters: TE = 30 ms, TR = 1 s, voxel size 2.5 × 2.5 × 3 mm, flip angle 70°). In pilot studies this coronal-oblique sequence gave less distortions than axial acquisitions. This sequence gave reliable images of the whole brainstem extending rostrally to the putamen, and thalamus. We were unable to include the limited cortical area above the corpus callosum in our analysis for two reasons: Firstly, there was inconsistent overlap in cortical areas between subjects (therefore the remaining area was small) and secondly there was some image contamination from brainstem related MRI wrap. The experiment was divided into two stages, although scanning was continuous: The first 1130 images (18 min 50 s) comprised the baseline experiment, the duration based upon Wise et al. (2004) but lengthened to compensate for the poorer signal to noise ratio in the brainstem. The final 1530 images (25 min 30 s) comprised the CO_2 stimulation experiment. The duration was determined by adaptation of a similar CO_2 challenge protocol (Pedersen et al., 1999) for use in the MRI scanner. We also acquired a single volume whole head echo planar image taken with the same resolution and orientation as the brainstem scans to aid with registration to each subject's structural MRI scan. We acquired field maps to help correct distortions in the EPI images. We also acquired a high resolution T_1 weighted structural scan (voxel size 1 × 1 × 1 mm) to aid registration to common stereotactic space.

FMRI analysis

Preprocessing

Image preprocessing was carried out by using the Oxford Centre for Functional Magnetic Resonance Imaging of the Brain Software Library (FMRIB, Oxford, UK, FSL version 4.0 (http://www.fmrib.ox.ac.uk/fsl/)). The following prestatistics processing was applied: removal of non brain structures (i.e. skull and surrounding tissues) (Smith, 2002), spatial smoothing by using a Gaussian kernel of 3.5 mm FWHM, mean-based intensity normalisation of each 4-dimensional dataset by the same factor (so that the grand mean within the brain for each subject, averaged across all timepoints was the same), and high pass temporal filtering (Gaussian-weighted least-squares straight line fitting).

Motion and physiological noise correction

The brainstem is particularly susceptible to respiratory (Van de Moortele et al., 2002) and cardiac (Dagli et al., 1999) noise due to varying effects of blood and cerebrospinal fluid flow during the cardiac cycle (Friese et al., 2004), and due to magnetic field changes caused by the varying volume of the lungs during the respiratory cycle (Windischberger et al., 2002). Therefore, in addition to standard motion correction techniques (Jenkinson et al., 2002), we also employed a modified version of a noise correction technique, RETROICOR (Brooks et al., 2008; Glover et al., 2000; Harvey et al., in press). RETROICOR involves fitting low-order Fourier series to the EPI data based on the time of each image acquisition relative to the phase of the cardiac and respiratory cycles.

Statistical analysis

Time-series statistical analysis was carried out using FILM with local autocorrelation correction (Woolrich et al., 2001). For the first level analysis we used a general linear model where the regressor of interest was $P_{ET}CO_2$. Six motion correction parameters (Jenkinson et al., 2002) were included as regressors of no-interest. We assumed a 6 second hemodynamic delay but included the temporal derivative of the CO_2 regressor to account for variation around this value. Voxel-wise statistical analysis was extended to a second (group) level in a mixed effects analysis using FLAME (Woolrich et al., 2004). Z statistical images were thresholded using clusters determined by $Z > 2.3$ and a (corrected) cluster significance threshold of $P = 0.05$.

We assessed the responsiveness of BOLD signal to hypercapnia, defined as the BOLD signal change per unit change in $P_{ET}CO_2$. Paired t-tests were performed within FEAT (http://www.fmrib.ox.ac.uk/fsl/) to compare the CO_2 response between those derived from the spontaneous "resting state" fluctuations in $P_{ET}CO_2$ and those derived from CO_2 challenges, by contrasting the mean and difference of the first level analyses. We were particularly interested in identifying brain regions which demonstrated the strongest increase in BOLD CO_2 sensitivity during external CO_2 administration.

Image registration

After preprocessing, the functional scans were registered to the MNI152 standard space (average T_1 brain image constructed from 152 normal subjects at the Montreal Neurological institute, Montreal, QC, Canada) using linear registration with FLIRT (Jenkinson et al., 2002; Jenkinson and Smith, 2001). Correction for B_0 distortion in the EPI images was performed with FUGUE (Jenkinson, 2003). Registration of the functional images to the T_1 structural images was specifically optimized for the brainstem as follows:

- Registration of brainstem EPI to wholehead EPI with 6 degrees of freedom and an input weighting mask comprising the whole field of view from the brainstem EPI.
- Registration of wholehead EPI to T_1 structural, again with FLIRT, but with a reference weighting mask that comprised the brainstem and cerebellum.
- Registration of the subjects' T_1 structural to standard space using affine transformation (12 degrees of freedom) and a standard space brainstem weighting mask.

Diffusion-weighted imaging

In a separate scanning session diffusion weighted data (3 acquisitions of 60 directions with 5 non-diffusion weighted images, b-value 1000 s mm^{-2}, voxel size 1.5 × 1.5 × 1.5 mm, 100 slices) were acquired on a Siemens Trio 3T scanner in the same group of 12 subjects who underwent the first part of this study. Cardiac gating was used to minimize artifacts from pulsatile flow of the cerebrospinal fluid. Preprocessing included extraction of non-brain tissue with BET (Smith, 2002) and the resulting brain images were registered to standard space using methods described above. The data from the three acquisitions were averaged to improve the signal to noise ratio.

Probabilistic modelling of diffusion parameters and tractography were carried out using previously described methods (Behrens et al., 2003, 2007) with FDT (http://www.fmrib.ox.ac.uk/fsl/) with 5000 samples per voxel. The resulting images that were obtained in each subject were subsequently summed across the subjects and overlaid onto the standard brain image. To quantify connectivity between the thalamic activations and the cortex, target masks were defined from the functional activations in the thalamus (thresholded at $Z > 2.6$). Seed masks were chosen from cortical areas that have been identified in previous imaging studies of respiration (Banzett et al., 2000; Corfield et al., 1995; Evans et al., 2002; McKay et al., 2003, 2008; Peiffer et al., 2001; von Leupoldt et al., 2008). The areas chosen were as follows: prefrontal cortex, amygdala, anterior cingulate cortex (ACC), anterior insula, postcentral gyrus, precentral gyrus. For each voxel in the seed masks, the number of samples reaching a particular thalamic target was recorded.

The cortical seed masks were defined from the Harvard Oxford Cortical and Subcortical Structural Atlas (part of FSLView version 3.0 (http://www.fmrib.ox.ac.uk/fsl/)) which is a probabilistic population-based atlas. Regions of interest were thresholded so that only voxels estimated at greater than 35% of probability of being in that structure were included in the mask. This ensured a conservative definition of each anatomical region. To determine whether these thalamic target

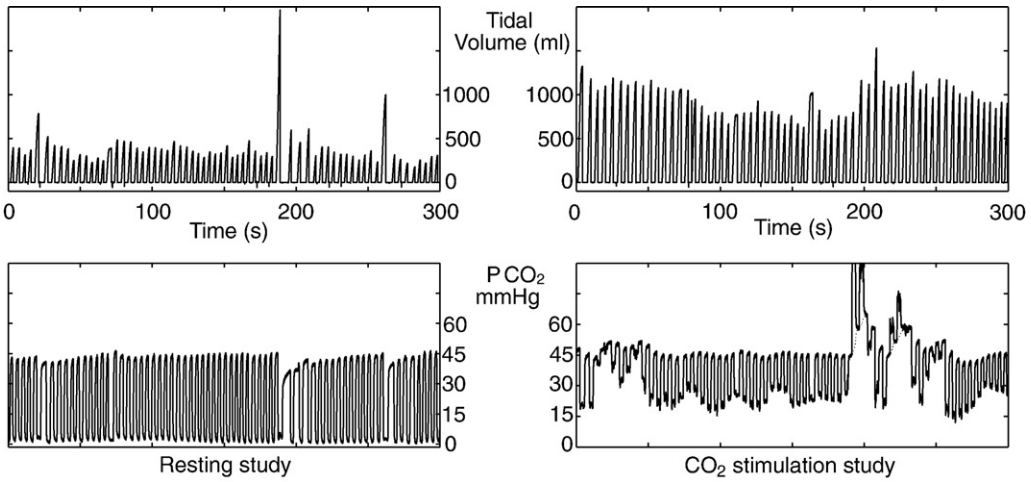

Fig. 2. Example of respiratory (inspiratory tidal volume) and tidal CO_2 traces in a 300 second portion of each part of the experiment in one subject. The expiratory volume trace is not shown but is similar to the inspiratory trace. For the analysis the $P_{ET}CO_2$ was derived from the value measured at the end of expiration, measured by the respiratory turbine, rather than the peak CO_2 value. This avoided measurement errors when inspiratory PCO_2 was greater than $P_{ET}CO_2$ (as seen at about 200 s in the lower right trace).

areas could be classified according to their connectivity to cortical areas previously identified in respiratory FMRI studies, we carried out unpaired *t*-tests between the connection profiles.

Results

Respiratory changes

The main effect of the CO_2 challenges on breathing was to increase minute ventilation from (mean (±SD)) 5.4 (±1.5) l min^{-1} during quiet breathing to 9.6 (±3.4) l min^{-1} $P<0.01$). The respiratory rate also rose from 12.9 (±3.3) to 14.0 (±3.6) per min ($P>0.05$) and the mean tidal volume from 460 (±230) to 730 (±360) ml ($P<0.001$). The mean $P_{ET}CO_2$ rose from 44.4 (±1.1) mmHg to 47.7 (±2.0) mmHg ($P<0.01$). End tidal oxygen levels were 105 (±4) mmHg during quiet respiration and 209 (±1) mmHg during CO_2 challenges ($P<0.001$). A representative trace of the respiratory recordings and changes in tidal CO_2 are illustrated in Fig. 2. Mean heart rate was 67 (±13) beats per min during quiet breathing and remained the same (67 (±13) beats per min) during CO_2 challenges.

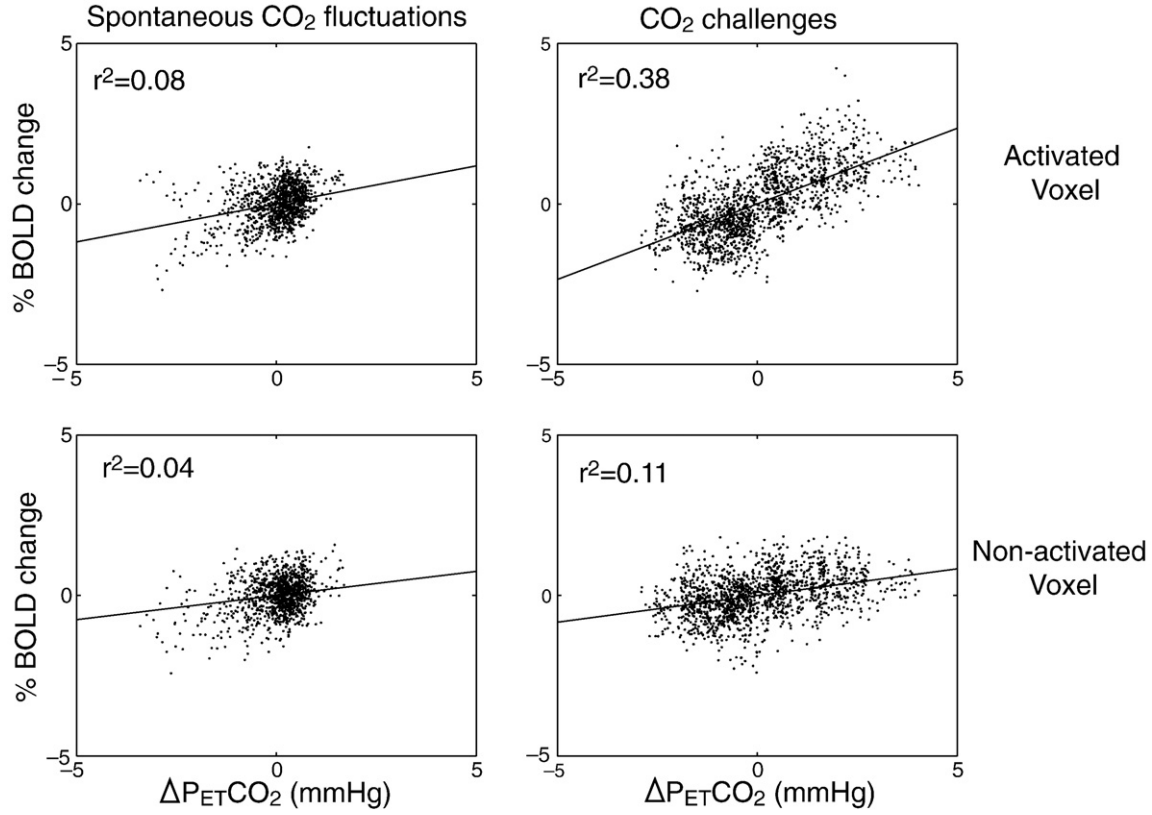

Fig. 3. BOLD vs $P_{ET}CO_2$ plot for one subject, in a non-activated (lower) and activated (upper) voxel, in the thalamus, for spontaneous CO_2 fluctuations (left) and CO_2 challenges (right). Each point represents one time point in the voxel. The FMRI analysis was based upon detecting a difference in the slope of the BOLD-$P_{ET}CO_2$ relationship between the two experimental conditions.

BOLD imaging

By comparing the signal changes from the CO_2 challenges with those correlated with the natural resting-state fluctuations in CO_2, we identified brain areas that demonstrated an increase in BOLD sensitivity to CO_2. A representative plot of the BOLD to $P_{ET}CO_2$ relationship, for the two parts of the experiment is illustrated in Fig. 3. The areas with this stronger response during external CO_2 challenges were as follows: bilateral anterior thalamus and ventral posterior lateral (VPL) nucleus of the thalamus, the right posterior putamen, the left ventrolateral (VL) nucleus of the thalamus, and in the midline in the rostral dorsal pons, the inferior ventral pons (Figs. 4 and 5), and in the dorsal and ventrolateral medulla. There were no areas of significantly greater signal changes during resting fluctuations compared with CO_2 challenges.

Diffusion tractography

For every voxel in the activations observed in the thalamus, the automated tractography algorithm was used to define connectivity strength to each cortical target (Behrens et al., 2003). As the connectivity of the putamen and the VL nuclei were similar to that of the VPL, we have not described the results further. Similarly the connectivity profile of the right AV was similar to that of the left AV, and therefore we have performed more detailed analysis on the connectivity profiles of the left AV and the left VPL thalamic nuclei. The activations in the anterior thalamus demonstrate strong connections to the supplementary motor area, the premotor cortex, the frontal and prefrontal cortex, and the anterior cingulate (Fig. 6). The VPL, VL and putamen demonstrate connectivity to the primary and supplementary motor areas. The anterior insula had a similar connectivity profile for the AV and VPL nuclei.

Discussion

In this study we have determined areas in the brainstem, thalamus and putamen that respond to CO_2 stimulation. We have then investigated the connections between the thalamic areas and higher cortical centres with diffusion tractography. The main findings in the brainstem were signal increases in the inferior ventral pons and the rostral dorsal pons (Kölliker-Fuse, parabrachial nuclei and locus coeruleus) and the dorsal and lateral medulla. We observed signal increases in the left VPL, left VL and bilateral AV nuclei of the thalamus and in the right posterior putamen. The anterior thalamic activations had strongest connectivity with brain areas that have been shown in other FMRI studies to mediate the affective components of respiration (particularly the amygdala, the frontal cortex and the ACC) whereas the activations in the VPL, VL and putamen were more strongly associated with motor and somatosensory cortices. The AV and VPL had similar connectivity profiles to the anterior insula.

There are relatively few human functional neuroimaging studies of respiratory control. Structure–functional relationships, as explored in this study with diffusion tractography, have not been investigated. Two studies have specifically examined responses to chemically stimulated breathing: With positron emission tomography (PET) Corfield et al. (1995) revealed activation in the limbic system and a variety of cortical areas in response to CO_2 stimulation, but did not specifically examine the brainstem, as in the present study. In a study by Gozal et al. (1994) the brainstem responses to CO_2 stimulation were examined using a steady-state free precession MRI technique; however the findings are rather difficult to interpret as activations were reported in many surface areas of the brainstem that are particularly susceptible to physiological noise artifacts (Harvey et al., in press). Specific

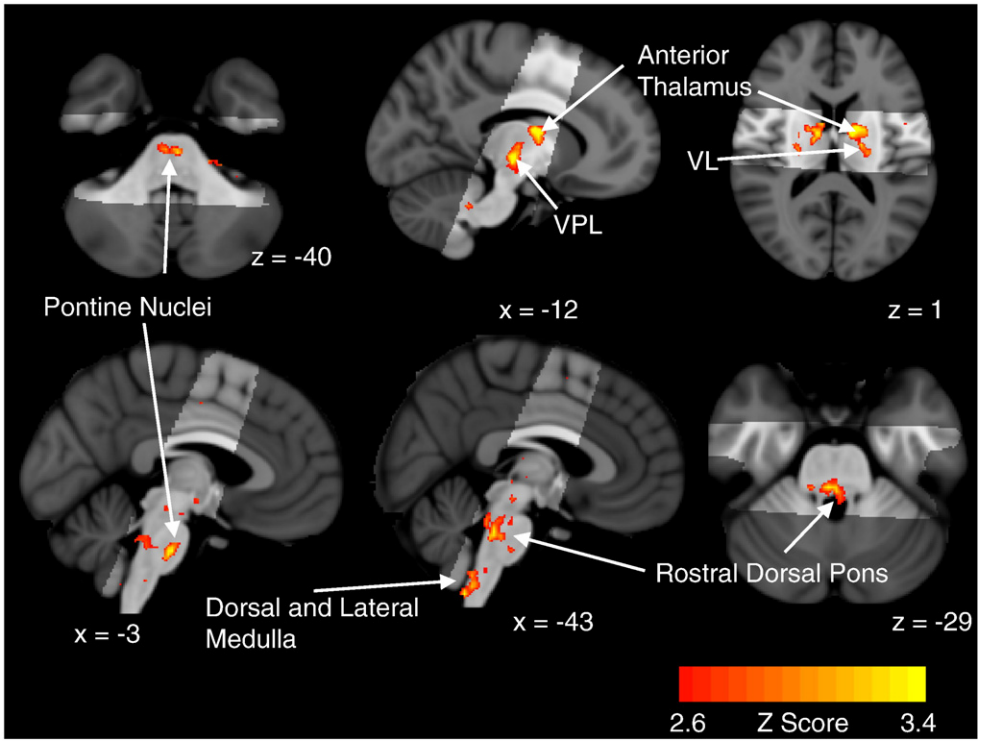

Fig. 4. Group map showing brain areas with a stronger BOLD sensitivity to CO_2 stimulation than to baseline "resting-state" spontaneous fluctuations in $P_{ET}CO_2$. Significant regions are displayed with a threshold of $Z>2.6$, with a cluster probability threshold of $P<0.05$. The area scanned is shown in lighter grey scale, and superimposed on the subjects' mean high resolution image transformed to MNI standard space (darker grey). Abbreviations: AV anteroventral nucleus of thalamus, VPL ventral posterior lateral nucleus of the thalamus, VL ventrolateral nucleus of thalamus.

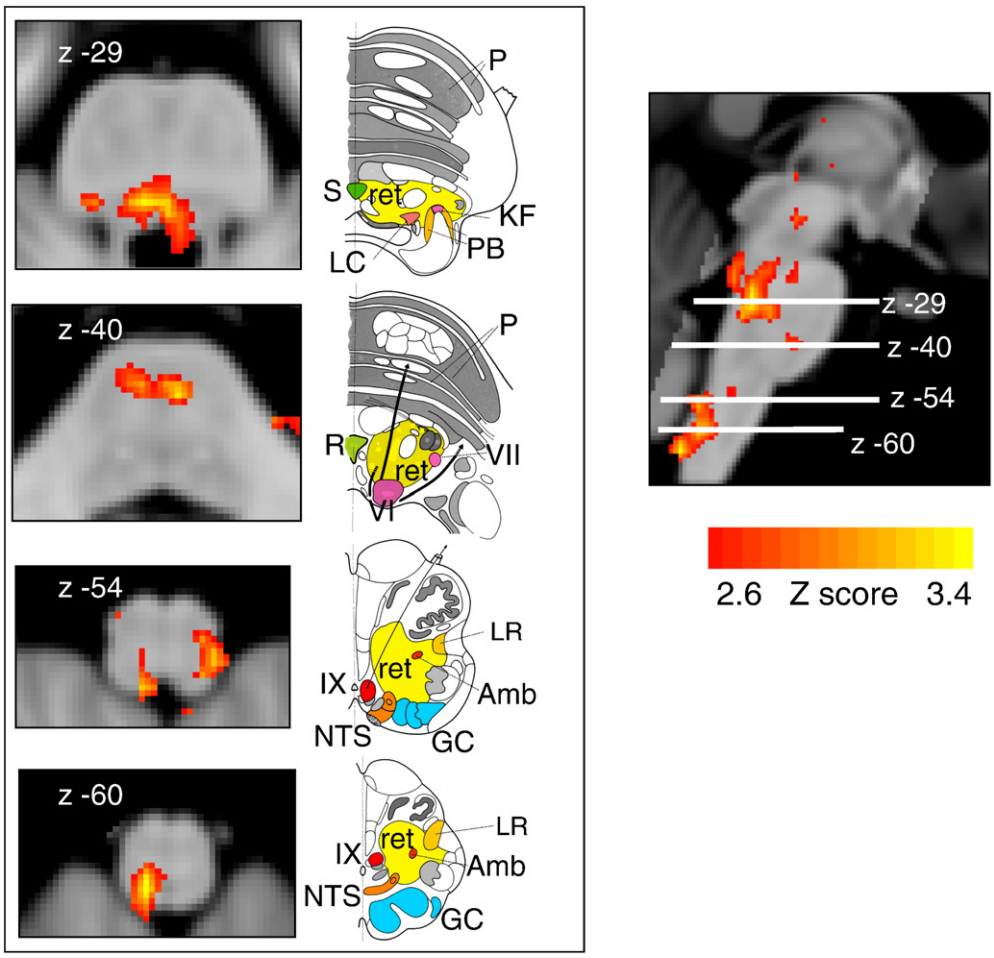

Fig. 5. Group map (in MNI standard space) showing areas in the pons and medulla with a stronger BOLD sensitivity to CO_2 stimulation than to baseline "resting-state" spontaneous fluctuations in $P_{ET}CO_2$. Significant regions are displayed with a threshold of $Z>2.6$, with a cluster probability threshold of $P<0.05$. Abbreviations: P pontine nuclei, S nucleus reticularis centralis superior, KF Kölliker-Fuse nucleus, PB parabrachial complex, LC locus coeruleus, R raphe nuclei (serotoninergic), ret nuclei reticularis including adrenergic and noradrenergic centres, VI abducent nucleus, VII Facial nucleus, Amb nucleus ambiguus, IX glossopharyngeal nucleus, NTS nucleus tractus solitarius, GC gracile (medial) and cuneate (lateral) nuclei (in blue). Line drawing adapted from Duvernoy (Duvernoy, 1995).

anatomical localization of discrete nuclei was not achieved. Two more recent studies of voluntary respiration revealed activity in brainstem areas that correspond to those identified in the present study. Breath holding (McKay et al., 2008) was associated with activity in rostral dorsal and inferior ventral pons. Voluntary hyperpnea (McKay et al., 2003) was associated with activity in the dorsal medulla. The findings of the present study suggest that common brainstem areas integrate respiratory control, whether mediated by conscious (breath hold, voluntary hyperpnea) or unconscious (chemical stimulation) mechanisms.

Although hypercapnia causes cardiovascular stimulation that is, in part, mediated by various nuclei in the brainstem (Polson et al., 2007), in this study we were careful to limit such effects by choosing a relatively mild hypercapnic stimulus. We observed no significant changes in heart rate between the two parts of the experiment (67± 13 beats per min for both parts of the experiment). Although we did not measure blood pressure in this study, sustained hypercapnia of 5 mmHg above normal (Ainslie et al., 2005) had more profound effects on heart rate than on mean arterial pressure (heart rate: 60 beats per min at baseline to 65 beats per min during hypercapnia ($P<0.05$), mean arterial pressure: 89 mmHg at baseline to 93.3 mmHg with hypercapnia). Therefore we feel that it is unlikely that our subjects experienced significant cardiovascular stimulation that would contribute greatly to our results.

Brainstem, rostral dorsal pons

The activation in dorsal rostral pons is likely to be analogous to activity in the Kölliker-Fuse (KF) and the parabrachial (PB) nuclei, and/or the locus coeruleus. These nuclei are physically close to each other, and therefore are impossible to distinguish with the resolution of FMRI. The KF and PB are closely apposed to each other, and are often considered together as the KF/PB complex. The KF has only recently been defined in humans (Lavezzi et al., 2004).

The importance of the KF/PB nuclei in respiratory control has been thoroughly investigated in rats: These nuclei are the major targets of the nucleus tractus solitarii (Loewy and Burton, 1978; Ricardo and Koh, 1978), which receive afferents from the vagus (pulmonary stretch receptors) and glossopharyngeal (peripheral chemoreception) nerves. They are integral for sensory processing of respiratory signals and are responsible for gating mechanisms in primary sensory nuclei. The KF/PB complex is responsible for motor co-ordination of respiratory related activity (e.g. control of laryngeal reflexes), particularly functioning as a post-inspiratory off-switch (Dutschmann and Herbert, 2006). In rats the KF/PB has been shown to have strong connections with the limbic system (Fulwiler and Saper, 1984; Saper and Loewy, 1980), including the thalamus, amygdala and insula, but also with the respiratory column in the ventral lateral medulla (Herbert et al., 1990).

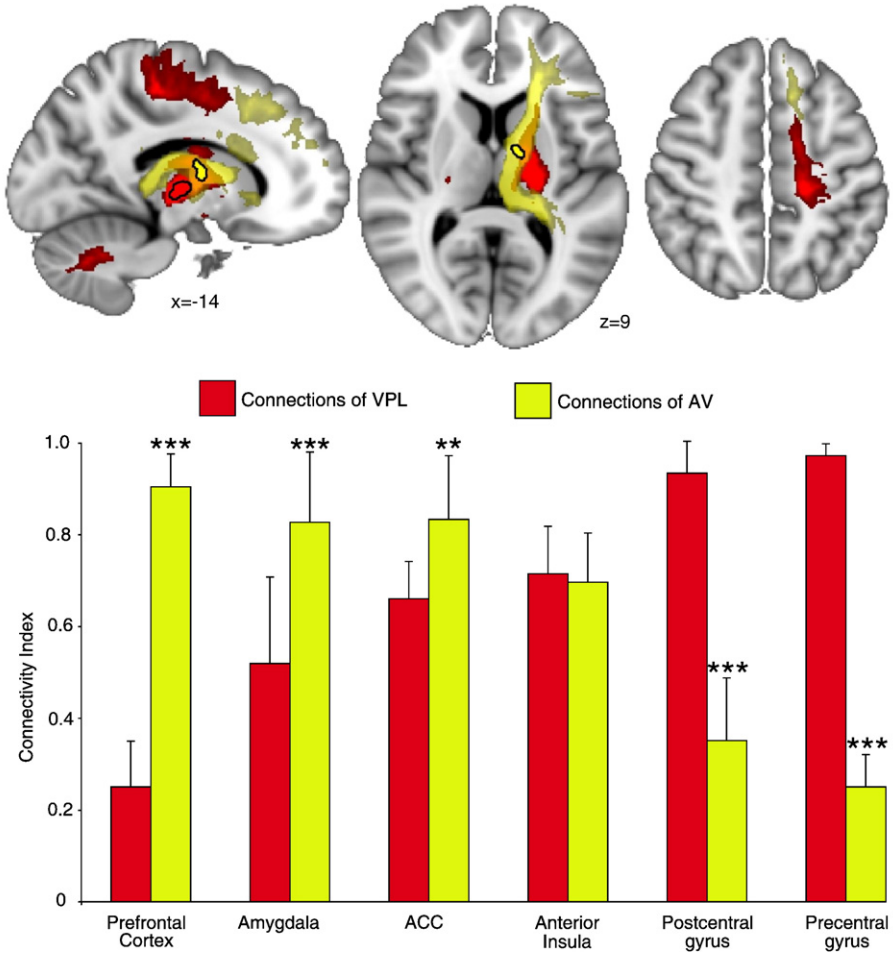

Fig. 6. Upper image: Cortical connections of the AV thalamic nucleus (yellow, FMRI activation seed region yellow box) and the VPL thalamic nucleus (red), using diffusion tractography. Seed regions based upon functional activation from BOLD imaging. The MR image is the group mean of the tracts from all 12 subjects, thresholded to show the top 99% tracts and superimposed on an MNI standard brain. Lower image: This graph displays the connectivity between the two seed regions (AV and VPL) and the cortical targets, demonstrating the different cortical connection profile of the two seed regions. *P* values are unpaired *t*-tests, $^{**}P<0.01$, $^{***}P<0.001$.

The locus coeruleus is strongly chemosensitive, is the main adrenergic nucleus of the brain (Berridge and Waterhouse, 2003), and plays an important role in mediating the ventilatory response to hypercapnia (Biancardi et al., 2008; Filosa et al., 2002; Oyamada et al., 1998; Pineda and Aghajanian, 1997). Similar to the KF/PB nuclei, the locus coeruleus projects to the nucleus tractus solitarius, the vagus motor nucleus and the nucleus ambiguus (McBride and Sutin, 1976; Oyamada et al., 1998).

Activity in this region of the dorsal rostral pons has been identified in two human FMRI studies, one of inspiratory loading (Gozal et al., 1995), and the other in breath holding (McKay et al., 2008), both of which may represent altered respiratory-related signalling within the brainstem. We suggest that the activity demonstrated in the dorsal rostral pons in the present study represents both direct local stimulation of chemoreceptors and also afferent inputs from the lower brainstem including the ventral lateral medulla and the NTS in the dorsal medulla (Herbert et al., 1990; McBride and Sutin, 1976; Oyamada et al., 1998).

Brainstem, inferior ventral pons

In the lower pons we observed bilateral BOLD activations in the pontine nuclei, at the same level as the facial nuclei (Fig. 4). Activation in a similar location (McKay et al., 2008) was reported in a BOLD FMRI study of breath holding, in which activity was hypothesized to be related to inhibitory cortical effects on the brainstem respiratory control network. In the present study, however, we stimulated respiration (without intending to recruit cortical centres) and therefore, we feel that in this case the activations represent increased activity in the rostral part of the ventral respiratory group. The exact location of the ventral respiratory group has not yet been confirmed in adult humans, but is likely to be located in the upper medulla and the lower pons, extending caudally beyond the areas activated in this study.

Brainstem, dorsal and lateral medulla

The activations in the dorsal and lateral medulla (Fig. 4) correspond with known locations of chemoreceptive and integrative sites for respiration. The dorsal medullary activation seen in Fig. 4, slice z −60 is likely to represent the nucleus tractus solitarius (NTS) which is a major relay of homeostatic information from the respiratory, cardiovascular and gastrointestinal systems (Bailey et al., 2007). With regards to respiration, it is chemosensitive and receives afferents from lung mechanosensors, and from peripheral chemoreceptors in the carotid body (St-John and Paton, 2004). Projections from the NTS have been demonstrated to connect with the KF/PB nuclei in the dorsal pons (Loewy and Burton, 1978; Ricardo and Koh, 1978) and with the ventral lateral medulla (Rosin et al., 2006). The activity observed in the NTS in the present study is therefore likely to be related to a number of converging inputs that all have excitatory influences on the brainstem respiratory network. These include

increased afferent input from pulmonary mechanosensors in response to increased lung distension, as well as chemoreceptive activation from direct stimulation and from the peripheral chemoreceptors in the carotid bodies that sense hypercapnia.

The lateral activation observed in slice z −54, Fig. 4 is likely to represent respiratory related activity in the ventral respiratory column, and probably reflects activation of chemosensitive and rhythm generating structures that include the nucleus ambiguus, the pre-Bötzinger complex and the retrotrapezoid nucleus. As these nuclei are in close proximity to each other we interpret the activations in the present study to be reflective of increased motor activity and a direct action of hypercapnia on the chemosensitive cells. FMRI does not, however, have the spatial resolution to distinguish between these particular nuclei. No other FMRI studies of respiration have observed discrete areas of activity in the lateral medulla.

The pre-Bötzinger complex has recently been identified (in rodent models) as an essential structure for the generation of the respiratory rhythm (Feldman and Del Negro, 2006). Experimental lesions of the pre-Bötzinger complex disrupt respiration, especially during sleep (McKay et al., 2005; McKay and Feldman, 2008) and if complete, lead to death through respiratory failure. Although not formally identified in humans, one preliminary report (in abstract form) (Schwarzacher et al., 2006) suggests that the pre-Bötzinger complex may be located in the vicinity of the lateral reticular formation in the medulla and therefore exactly at the position of this FMRI activation. Although in neonatal rats, the pre-Bötzinger complex is described as being located in the ventral lateral medulla (Feldman and Del Negro, 2006), it appears that from the limited evidence in humans (Schwarzacher et al., 2006), "lateral medulla" would be a more correct terminology. The retrotrapezoid nucleus has recently been proposed as another important respiratory control centre of the ventral respiratory column. In neonatal rats it is located adjacent to the pre-Bötzinger complex and the nucleus ambiguus. It has not yet been identified in man, highlighting the gap between the understanding of respiratory control in rodents and man.

Activations in the dorsal medulla were observed in an FMRI study of voluntary hyperpnea by McKay et al. (2003). The authors concluded that this represented activity in the NTS, and was related to descending cortical input or afferents from pulmonary mechanoreceptors. The present study extends their findings by showing similarly located activations, but additional activations in the lateral medulla. In our study, increased respiration was due to chemical stimulation of brainstem centres, whereas in McKay et al. (2003) voluntary hyperventilation could have "bypassed" some of the medullary respiratory control centres, hence some differences were observed in the pattern of activation. In interpreting the differences between McKay et al. (2003) and the present study, we speculate that the lateral medullary activity may represent increased "automatic" CO_2-driven network activity.

We consider that the unilateral activations observed in the medulla may relate to technical aspects of imaging the lower brainstem, as we cannot find evidence for laterality of respiratory control in animal studies.

AV thalamic nucleus

The activity in the AV thalamic nucleus implicates its role in mediating sensory and affective components of respiration. Our data is supported by two studies, one of self-paced breathing (McKay et al., 2003) and another of breath holding (McKay et al., 2008). In a study of air hunger and CO_2 stimulation, where sensory feedback was controlled, anterior thalamic activation was not seen (Evans et al., 2002). Anterior thalamic activity was not observed in the two PET studies of CO_2 stimulated breathing (Corfield et al., 1995; Liotti et al., 2001), that may be due to the differing methods of dealing with CO_2 related confounds in PET. Our diffusion tractography results suggest that the activity observed in the AV thalamic nucleus is more strongly connected with the frontal cortex, the amygdala and the anterior cingulate than the VPL. The activation in the AV thalamic nucleus demonstrates a similar connectivity profile to the anterior insula as the VPL. Areas in the frontal cortex are associated with decision making motor planning, vigilance and attention (Marklund et al., 2007; Pardo et al., 1991) and have been identified in neuroimaging studies of voluntary breathing, (McKay et al., 2003) loaded breathing (Isaev et al., 2002), and dyspnoea (Evans et al., 2002). The ACC is important for emotional, attentional and premotor processing (Turken and Swick, 1999), is involved in pain processing, (Bantick et al., 2002; Tracey and Mantyh, 2007) and has been identified in previous imaging studies of air hunger (Evans et al., 2002; von Leupoldt et al., 2008) and CO_2 stimulated breathing (Liotti et al., 2001). Anterior insular activation is a consistent feature of many imaging studies of dyspnoea (Banzett et al., 2000; Peiffer et al., 2008; Peiffer et al., 2001; von Leupoldt et al., 2008). Although these structures in the cortex were not imaged in the FMRI part of this study, evidence from the literature strongly suggests their functional participation and this is supported by the connectivity profiles of the thalamic activations seen in the AV nucleus in the present study.

VL and VPL nuclei of thalamus and putamen

We observed signal increases in the left VL, the left VPL nuclei of the thalamus and in the right putamen. Activations in these areas were observed in BOLD FMRI studies of breath holding (McKay et al., 2008), and with dyspnoea (Evans et al., 2002). Thalamic activation has also been observed in a PET study of CO_2 stimulated breathing (Corfield et al., 1995), but subdivision within the thalamus is limited by the poorer spatial and temporal resolution of PET.

Our diffusion tractography data show similar cortical connectivity of the VL, VPL and putamen, but a clear distinction between these nuclei and the anterior thalamic nucleus. We therefore have only shown the connections for the VPL in Fig. 6. These similar connectivity profiles of the VL, VPL and putamen could be due to limited spatial resolution or to partial voluming in the regions of interest because different connectivity profiles of these nuclei have been demonstrated by Behrens et al. (2003) when compared using a wider range of cortical targets. The aim of the tractography analysis employed in this study was to differentiate connectivity based upon previously identified cortical areas that specifically mediate respiration, and in this regard the anterior thalamus has a clearly different connection profile to the VPL.

The primary motor cortex has been implicated in imaging studies of voluntary breathing (Evans et al., 1999; McKay et al., 2003), but not with CO_2 stimulated breathing (Brannan et al., 2001; Corfield et al., 1995) where minute volume was quadrupled above baseline (whereas in this study minute volume approximately doubled). We therefore do not have any reason to hypothesize that there was significant motor cortical involvement in this study.

The observed thalamic activity is supported by animal studies that implicate it as an important relay for respiratory sensations: Direct neural recordings in the midbrain (Chen et al., 1991) and the thalamus (Bernhardt et al., 2008; Chen et al., 1992) of vagotomised decerebrate cats stimulated with CO_2 show a silent response being activated. The fast acting peripheral chemoreceptors in the carotid bodies and pulmonary stretch receptors are likely to be responsible for sensing these changes, transmitted through pathways described below. Clinical evidence for thalamic involvement in respiration is limited to one case report (Hermann et al., 2007), describing sleep disordered breathing in patients suffering strokes affecting the thalamus.

The ability to image respiratory related activity in the brainstem is an important step towards understanding and treating diseases that may affect respiratory control in the brainstem. Altered chemoreception during sleep has a profound effect upon respiration (Li et al., 1999;

Nattie and Li, 2001, 2002a,b), and may be a mechanistic factor in sleep disordered breathing that is seen in obstructive sleep apnoea (Solin et al., 2000; Wang et al., 2007), Ondine's curse (Harper et al., 2005; Macey et al., 2004; Paton et al., 1989), and multiple systems' atrophy (Benarroch et al., 2007; Shimohata et al., 2007). Patients with obstructive sleep apnoea have altered ventilatory responses to hypercapnia and hypoxia (Spicuzza et al., 2006) that return towards normal with treatment (continuous positive airway pressure), and the mechanisms and effects of these changes could be explored using a similar methodology to that presented in this paper.

Discussion of method for dissociating neuronal from vascular CO_2 related effects on the BOLD signal

Our method for dissociating neuronal from vascular CO_2 effects has identified areas of neuronal activation caused by CO_2 stimulation in the brainstem, thalamus and putamen. The results are supported by findings from animal literature and the emerging body of literature on respiratory FMRI in humans.

The cerebral circulation is exquisitely sensitive to small changes in $PaCO_2$. Hypercapnia (raised $PaCO_2$) dilates the cerebral vasculature (Hutchinson et al., 2006) and increases cerebral blood flow (CBF) (Ide et al., 2003; Rostrup et al., 2000). Hypercapnia causes an increase in the baseline BOLD signal that is approximately linear over the normal to mild hypercapnia range used here (Corfield et al., 2001), but flattens off at higher $PaCO_2$ levels (Posse et al., 2001). The brainstem respiratory network is also exquisitely sensitive to changes in $PaCO_2$ (Feldman et al., 2003). Hypercapnia causes an increase in respiration through stimulation of neural activity in chemoreceptors (Feldman et al., 2003; Pedersen et al., 1999).

We hypothesized that stimulation with CO_2 would cause a generalised increase in BOLD throughout the brain related to its vascular effect, but additionally there would be further localized vasodilatation in the vicinity of CO_2 sensitive neurones as a hemodynamic response to neural activity. In these regions there would be an additional BOLD response to CO_2.

Spontaneous fluctuations in $PaCO_2$ occur in humans at rest (Crosby and Robbins, 2003; Modarreszadeh and Bruce, 1994; Van den Aardweg and Karemaker, 2002). These fluctuations are in the order of approximately 1–2 mmHg, and are correlated with significant changes in cerebral blood flow velocity (Mitsis et al., 2004; Panerai et al., 2000), and BOLD (Wise et al., 2004). Spontaneous fluctuations in CO_2 have been used to map regional differences in the BOLD responsiveness to CO_2 throughout the brain (Wise et al., 2004). With this approach BOLD responsiveness to CO_2 can be measured over a normal range, and thus activation of CO_2 sensitive neuronal systems is minimized as much as possible. The main disadvantage of this technique is poor signal-to-noise necessitating relatively long recording times.

In the present study we hypothesized that the change in BOLD signal to "resting state" spontaneous fluctuations in CO_2 would largely reflect vascular tone, whereas the change in BOLD signal to externally delivered CO_2 challenges would also be dependent upon whether the brain region is activated by CO_2. In areas not activated, the change in BOLD would remain proportional to the change in $PaCO_2$, whereas in activated brain areas we would expect a significantly greater increase in BOLD. In our FMRI analysis, we aimed to identify those areas with a disproportionate increase in BOLD sensitivity to externally delivered CO_2 as being part of the brainstem respiratory network. This hypothesis is supported by work in decerebrate cats, where supra-linear neuronal response to CO_2 stimulation was observed in the midbrain and thalamus (Chen et al., 1991, 1992) with direct neuronal recordings.

Although mean oxygen and CO_2 levels were significantly greater in the CO_2 stimulation study we note this may potentially dampen BOLD responses (Bandettini and Wong, 1997; Bulte et al., 2007; Cohen et al., 2002), therefore our ability to detect a significant difference is even more compelling. The difference in mean $P_{ET}CO_2$ was unavoidable, but by delivering relatively mild CO_2 challenges we attempted to avoid saturation of the stimulus-evoked BOLD response that is observed with greater levels of hypercapnia (Bandettini and Wong, 1997; Cohen et al., 2002). This hypercapnic depression of BOLD responsiveness is likely to be less profound at lower levels of hypercapnia. A study by Corfield et al. (2001) found that mild hypercapnia (such as employed in this study) had no effect upon the BOLD response to visual stimulation. For each 1 mmHg increase or decrease in $PaCO_2$ over the range of 20–60 mmHg, there is a corresponding CBF change in the same direction of approximately 1–2 ml/100 g/min, or 2.5% (Ide et al., 2003; Poulin et al., 1996). We recorded a rise in mean $P_{ET}CO_2$ of approximately 3.4 mmHg, that would therefore increase mean CBF by approximately 7 to 16%. This change in CBF could potentially dampen the BOLD response to CO_2 induced neuronal activation, with the effect of making our estimates of respiratory related brain activity more rather than less conservative.

By focusing the imaging upon the brainstem at relatively high spatial (2.5×2.5×3 mm) and temporal resolution (TR 1 s), we have been able to separate activity between various discrete areas of brainstem and subcortical areas. The fast image acquisition permitted effective physiological noise correction (Harvey et al., in press) and thus we were able to detect small differences in the BOLD response to CO_2. Although cortical areas were not imaged (e.g. insula, amygdala, ACC) these have been identified in previous imaging studies (Corfield et al., 1995).

Limitations of the study

1. As the aim of this study was to measure chemically stimulated breathing, we did not take subjective measurements of respiration during the study, as we felt that asking subjects to think about their breathing during the experiment may have modulatory effects on respiration (Han et al., 1997). Based on findings from a pilot study, we used a relatively mild CO_2 stimulus that was designed to increase minute ventilation no greater than a level that subjects just notice (West et al., 1975). Considerably higher levels of CO_2 stimulation (i.e. inspired CO_2 of up to 35%) are administered to intentionally induce anxiety (Battaglia et al., 2007). In this study there were no spontaneous reports of anxiety although a formal debrief was not performed.
2. Our assumption in dissociating the direct vascular from the neuronal-induced CO_2-related BOLD activity is that the vascular component of the blood flow response remains relatively linear, whereas localized neural activation increases BOLD more strongly. As spontaneous fluctuations in $PaCO_2$ are a source of variability in respiration, it is possible that we have not completely dissociated all the neural and vascular effects of CO_2. It is much more likely that we have identified CO_2-related neuronal activity above a "normal" baseline. If our *a priori* hypothesis of dissociating neural and vascular effects is correct, then we may expect to see greater changes in BOLD where there is a "gated" response to hypercapnia (i.e. a "threshold" response above a certain $PaCO_2$, as seen in the thalamus and midbrain in animal studies (Bernhardt et al., 2008; Chen et al., 1991, 1992)). Synaptic amplification of brainstem neuronal activity has been demonstrated during hypercapnia (Su et al., 2007; Yang et al., 2008) which may explain the change in the ratio of neuronal-induced to vascular-induced BOLD. However, some brain areas that are similarly responsive to CO_2 across the whole range of $PaCO_2$ (e.g. potentially in some chemoreceptive areas) may not display such a profound change in BOLD responsiveness.
3. Diffusion tractography has given us some insights into the spatial organisation of the respiratory network, and how centres in the thalamus may connect with higher cortical centres that have been

identified in other published studies of respiration. Importantly, it does not determine the direction of connections, nor whether such connections were actually "activated" in this experimental paradigm. Although we were interested in using diffusion tractography to determine connectivity from the brainstem activations, in practice we found that the presence of large white matter tracts "passing through" the brainstem made it impossible to distinguish tracts with confidence. Therefore we have only presented the connection profiles of the thalamic activations.

Conclusions

In summary, we have shown, for the first time in humans that stimulation with CO_2 activates a network of brainstem areas that include the KF/PB nuclei and locus coeruleus in the rostral dorsal pons and nuclei in the inferior ventral pons and ventrolateral medulla. We suggest that afferents from these brainstem centres connect with nuclei in the thalamus and putamen, with synaptic connections to higher cortical centres. This is the first human study to describe the thalamus in detail with regards to respiratory control, and supports data from animal studies that the thalamus may "gate" respiratory sensations between the cortex and the brainstem. Our novel findings in the brainstem are supported by a growing body of literature from invasive studies in rodents, and open the door to further investigation of factors affecting the control of breathing in humans.

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