A bilateral cortico-bulbar network associated with breath-holding in humans, determined by functional magnetic resonance imaging.

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ABSRACT

Few tasks are simpler to perform than a breath hold; however, the neural basis underlying this voluntary inhibitory behaviour, which must suppress spontaneous respiratory motor output, is unknown. Here, using blood oxygen level dependant functional magnetic resonance imaging (BOLD fMRI), we investigated the neural network responsible for volitional breath holding in 8 healthy humans. BOLD images of the whole brain (156 brain volumes, voxel resolution 3x3x3mm) were acquired every 5.2s. All breath holds were performed for 15 seconds at resting expiratory lung volume when respiratory musculature was presumed to be relaxed, which ensured that the protocol highlighted the inhibitory components underlying the breath hold. An experimental paradigm was designed to dissociate the time course of the whole-brain BOLD signal from the time course of the local, neural-related BOLD signal associated with the inhibitory task. We identified a bilateral network of cortical and subcortical structures including the insula, basal ganglia, frontal cortex, parietal cortex and thalamus, that are in common with response inhibition tasks, and in addition, activity within the pons. From these results we speculate that the pons has a role in integrating information from supra-brainstem structures, and in turn it exerts an inhibitory effect on medullary respiratory neurones to inhibit breathing during breath holding.

INTRODUCTION

In humans, the continuous, involuntary rhythm of breathing that is generated within the brainstem (Feldman and Del Negro, 2006), is modulated by emotion, e.g., laughter; and by volitional behaviours, e.g., speaking. Evidence from human stimulation and imaging studies has shown that these modulatory inputs originate from supra-brainstem structures e.g. the motor cortex, thalamus and cerebellum (Colebatch et al., 1991; Corfield et al., 1998; McKay et al., 2003). How these behavioural inputs modulate respiratory spinal motor neurones is unclear; possibly by a 'direct' corticospinal pathway, demonstrated both by electrophysiological studies in animals (Aminoff and Sears, 1971) and clinical observations in humans (Corfield et al., 1998; Nathan, 1963; Severinghaus and Mitchell, 1962); or by an 'indirect' pathway via the brain stem respiratory centres and their associated bulbospinal neurones (Orem and Netick, 1986).

The complexity of respiratory control is exemplified by the wilful act of breath holding, when voluntary and involuntary control mechanisms are clearly in competition. Whilst a voluntary breath hold is easy to initiate, it will eventually be terminated when strong involuntary mechanisms overwhelm the volitional inhibitory control (Godfrey and Campbell, 1968; Parkes, 2006). Numerous physiological factors, e.g., lung volume, blood gas composition and respiratory muscle contraction, that together with non-physiological factors, e.g., anxiety or willpower, determine the length and breaking point of a breath hold, which can be highly variable between individuals and even within the same individual (Godfrey and Campbell, 1968; Parkes, 2006). At present, there is no information on the neural mechanisms that either underlie the voluntary inhibition of spontaneous breathing during a breath hold or determine the breath hold breaking point.

Here, using blood oxygen level dependant functional magnetic resonance imaging (BOLD fMRI), we investigated the neural correlates of breath holding after a normal expiration at resting expiratory lung volume. Commonly, breath holding is performed at end inspiration when the lungs are full of air and there is active closure of the glottis. In this study, we aimed to highlight only the inhibitory components of breath holding by investigating short breath holds that were voluntarily terminated to remove the variability of breaking point, and were performed at the resting expiratory lung volume when the respiratory musculature is presumed to be relaxed. Previous respiratory related imaging studies have highlighted specific loci within the sensorimotor cortices and the subcortex, specifically the thalamus and basal ganglia ((Evans et al., 1999; McKay et al., 2000), that underlie volitional respiratory control; however, these studies have investigated active respiratory control rather than inhibition. Direct parallels of breath holding do not exist in other human behaviours but imaging studies of response inhibition during Go/NoGo and Stop-signal tasks (Aron and Poldrack, 2006; Liddle et al., 2001; Watanabe et al., 2002) and those related to inhibiting saccadic eye reflexes (Jueptner et al., 1996), highlight the dorsolateral prefrontal cortex, basal ganglia and thalamus as having a role in inhibiting behaviours. Within the brainstem, animal models have shown that stimulation of pontine respiratory neurones can lead to apnoea, hypernoea or apneusis (Chamberlin, 2004; Fung and St John, 1994; Mutolo et al., 1998), while ablation of the preBötzinger complex (preBötC), a small region of the ventrolateral medulla, can lead to central sleep apnoea and ataxic breathing during wakefulness (Gray et al., 2001; McKay et al., 2005) We hypothesised that breath holding would be associated with an inhibitory network of subcortical structures including the pons. We were aware that our subjects would experience secondary effects, such as interoceptive awareness and an increasing desire for, and an anticipation of, the subsequent breath; therefore, we also hypothesised activity within areas associated with respiratory awareness and air hunger, such as the insula and cingulate cortices (Banzett et al., 2000b; Evans et al., 2002; Peiffer et al., 2001).

Breath holding increases arterial PCO_2 , which consequently leads to a vasodilation of the cerebral circulation and a concomitant increase in whole-brain BOLD signal that is highly correlated with local, neurally-induced, BOLD signal changes of interest. To address this caveat, we designed a novel experimental paradigm in which PCO_2 levels were manipulated throughout the experiment to dissociate the time course of the whole-brain BOLD signal from the time course of the local, neural-related BOLD signal; this allowed local activity associated with breath holding to be determined independent of whole-brain BOLD effects.

METHODS

Subjects

Eight healthy right-handed individuals (age 20-35yrs; 6 female) were studied. All volunteers gave full informed 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).

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., 2000a). 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) 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.

On a separate occasion prior to scanning, subjects underwent training to perform the 15 second breath hold. During the training sessions, which were held within the Department of Respiratory Medicine, Charing Cross Hospital, Imperial College, ECG and diaphragmatic EMG activity were recorded, and at the termination of each breath hold, subjects were asked to rate how strongly they felt the 'urge to breathe' associated with the end of the breath hold using a finger slider bar which altered a visual analogue scale (VAS). These parameters were recorded in order to determine that the subjects were relaxed, were not experiencing adverse effects and that they were performing the breath hold at resting expiratory volume when the diaphragm is presumed to be quiescent (see *supplementary information* for more details).

Imaging

All imaging studies were carried out at the Wellcome Trust Centre for Neuroimaging, UCL. Brain images were acquired using a Siemens VISION MRI scanner operating at 2 Tesla. For each subject a T1-weighted structural scan was acquired at the beginning of the session; subsequently, functional images were acquired using BOLD echoplanar imaging. The functional sequences consisted of 156 brain volumes; each brain volume was acquired in 5.2 seconds (i.e. ~3 full brain scans per breath hold time of 15 seconds) and consisted of 56 transverse slices with an isotropic voxel resolution of 3mm and a matrix size of 64 x 64 pixels.

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.

Breathing paradigm

A short audio cue was given at the beginning of inspiration informing the subject to complete the breath as normal and to begin a relaxed breath hold at the end of the breath, following a normal expiration. All breath holds were performed with lung volume at resting expiratory level i.e. functional residual capacity. A second audio cue was given 15 seconds after the commencement of the breath hold, as a cue to immediately terminate the breath hold (i.e. all breath holds were of 15 seconds duration). Approximately 45 seconds after termination of each breath hold, CO_2 was experimentally added into the inspiratory reservoir of the circuit for 15 seconds. The experimentally added bolus of CO_2 increased $P_{ET}CO_2$ by approximately 7-8mmHg, which is equivalent to the increase in $P_{ET}CO_2$ resulting from a 15 second breath hold (measured for each subject during the training session). Presentations of the breath hold and CO_2 bolus were alternated within a single experimental run, each stimulus being repeated 9-12 times.

The inspired gas composition delivered to the subject via the reservoir bag was controlled using two blenders, which were supplied by three gases. Blender one was set to give an output of 30% O_2 by mixing medical air (21% O_2) with 100% O_2; this ensured that each subject was mildly hyperoxic throughout the study. In this way arterial O_2 saturation (SaO_2) remained on the "flat portion" of the oxygen-hemoglobin dissociation curve, essentially at 100%, and prevented any confounding changes in SaO_2 during the breath holding or hypercapnic periods. Blender two controlled the fraction of inspired CO_2 by mixing the output from blender one with a gas mixture of 30% O_2 /10% CO_2 /balanced N_2. During periods when CO_2 was not being added to the circuit, blender two was set at zero.

De-briefing

Immediately after the study subjects were encouraged to volunteer information before being asked a standard set of questions *(see supplementary information).* The aim of debriefing was to assess any difficulties with performing the task and assess feelings of air hunger or sensations associated with the breath holds.

Data analysis

$P_{ET}CO_2$ data were analysed on a breath-by-breath basis (Spike 2, Cambridge Electronic Design). The fMRI time series data were analysed using SPM99 software (Wellcome Department of Imaging Neuroscience; http://www.fil.ion.ucl.ac.uk/spm). Prior to statistical analysis, images for each subject were corrected for head movement and then 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 = 6 mm). After pre-processing, the time series data underwent a fixed effects multiple linear regression analysis, on a voxel by voxel basis. For each subject a design matrix was constructed to model each breath hold as a 15 second event convolved with a haemodynamic response function to represent the relationship between neural activity and cerebral blood flow changes (Friston et al., 1994). The data were then temporally smoothed by applying a high pass filter (cut-off set at 240 s, twice the experimental period) to remove low frequency signal changes, and a low pass filter (4 s FWHM) to remove high frequency noise. To account for the whole-brain BOLD signal intensity changes resulting from increases in arterial PCO_2 throughout the experiment (i.e. due to the breath hold and the experimentally added CO_2), the average whole-brain signal intensity for each image in the time series was included as a regressor of no interest (Corfield et al., 2001). For the whole-brain 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 statistical significance voxel by voxel. Our *a priori* hypothesis was that breath holding would be associated with increased neural activity within the pons and medulla; consequently, for each individual analysis and the group analysis, a small volume correction for multiple comparisons was performed on a spherical area within the pons (centred 0, –26, –36; 15 mm radius) and medulla (centred 0, -37, -56; 10mm radius). The positions of the local maxima are reported in MNI space. To determine the anatomical location of each maximum, statistical maps were superimposed onto a group-mean structural image and onto the appropriate individual's structural image and identified with reference to standard anatomical atlases (Duvernoy et al., 1999; Talairach and Tournoux, 1988).

RESULTS

In all subjects, preliminary studies determined that a 15 second breath hold increased alveolar PCO_2 by ~7 mmHg. During the scanning session, and subsequent to each breath hold, a bolus of CO_2 was experimentally added to the circuit to match the CO_2 rise induced by each breath hold (Fig 1 and supplementary table). $P_{ET}CO_2$ measured prior to each breath hold and prior to each bolus of CO_2 was clamped to within ± 2mmHg of isocapnia: 39.4±1.0 mmHg (mean±S.E.M) and 39.1±1.0 mmHg respectively. Recordings of diaphragmatic EMG, performed outside the scanner, indicated that electrical activity in the diaphragm was typically quiescent for the first 12 seconds of each breath hold, thereafter irregular twitches on the EMG were observed; heart rate was unchanged during breath holding time.

As illustrated in Fig 2, each period of experimentally added CO_2 produced a change in the whole-brain BOLD signal comparable to that associated with the preceding breath hold. The

protocol successfully dissociated whole-brain BOLD signal changes from local BOLD signal changes. This dissociation, together with the assumption that local and whole-brain BOLD effects are independent (Corfield et al., 2001), allowed an ANCOVA analysis with the whole-brain BOLD signal being treated as an independent regressor of no interest.

In the post study de-briefing (see supplementary information), all subjects reported feeling 'a need to take a breath' at the end of each breath holding period. The intensity of this feeling varied between breath holds within a subject and also between subjects. None of the subjects reported severe to extreme levels of air hunger, or any discomfort or adverse sensations associated with breath holding, or a need to terminate the study.

Neural Activations

Breath holding was associated with a bilateral pattern of increased activity in cortical and subcortical regions including the insula, basal ganglia, thalamus, frontal cortex, parietal cortex and pons ($p<0.05$, corrected for multiple comparisons; Table 1 & Fig 3). The most statistically significant changes in BOLD signal intensity (i.e. those with the largest T socres) were identified within the inferior, anterior insula (Fig 4b) that extended laterally into the operculum. In the posterior insula, activity was confined to the inferior portions. Within the basal ganglia, significant signal increases were restricted to the putamen and substantia nigra (Fig 4c). In the thalamus, activity was identified in the ventrolateral thalamus (Fig 4e), which on the right hand side extended into the anterior and mediodorsal thalamic nuclei.

In the frontal cortex activity was detected within the dorsolateral prefrontal cortex (Fig 4a) and extended posteriorly into the ventral aspect of the lateral pre-motor cortex. Significant but more discrete activations were identified bilaterally in the inferior frontal gyri, occipital lobes and amygdala. The cingulate was activated bilaterally, from mid to anterior cingulate and superiorly

into the pre-SMA and medial aspect of the superior frontal gyrus (Fig 4d). Within the parietal lobe, an extensive activation was identified bilaterally in the supramarginal gyrus (Fig 4f). This activation extended inferiorly and anteriorly into the inferior parietal and superior temporal gyri and on the right continued into the middle temporal gyrus. There was no activity identified within the primary motor or sensory cortices or within the cerebellum.

A cluster of significant activity was identified in the mid-superior dorsal pons that extended bilaterally from the midline (Fig 5). Anatomically, this area of the human brainstem appears to be analogous to the dorsolateral pontine group of respiratory neurones identified within the rat and cat. A second cluster was also identified anteriorly within the inferior pons (Fig 5). There was no significant maximum observed within the medulla even at a lower statistical threshold; however, a deactivation (i.e. a decrease in signal intensity) was observed within the medulla but since this condition was not explicitly defined, interpretation is limited.

Individual analyses

Each individual analysis when corrected for multiple comparisons ($p<0.05$) produced a similar pattern of activity, although less extensive, to that presented in the group result. In 6 of the 8 individuals, bilateral activity was identified in the insula and putamen and in all subjects, activity was present within the pre-frontal cortex, although the location of maxima varied between subjects. Cingulate activity was apparent in all individuals, with the right mid-anterior cingulate gyrus being the most significant. The supramarginal gyrus within the parietal lobe also appeared bilaterally significant in 5 of the 8 individuals. In the brainstem, a small volume correction for multiple comparisons was performed on an area centred in the pons (0 –26 -36) with a 15mm radius and an area centred in the medulla (0, -37, -56) with a 10mm radius. Pontine activity was identified in all individuals; in four of the individuals the co-ordinates of the pontine maximum

were very similar to that of the group result. Activity within the medulla did not survive correction.

DISCUSSION

The ability to appropriately inhibit breathing is an essential component of normal everyday life. For example, in response to noxious stimuli such as smoke inhalation, we are able to protect the respiratory system by volitionally suppressing breathing and closing off the glottis. Such a response is essential for survival if one is plunged into water, and is a necessary behaviour for recreational swimming and diving. A breath hold is easy to perform consciously until the desire to breathe becomes extreme and the breath hold is involuntarily terminated. By using BOLD functional imaging we have shown that breath holding, albeit a straightforward action is associated with a complex neural network of cortical, subcortical and brainstem structures.

Associated with breath holding, we identified activity within the insula, basal ganglia, frontal cortex, parietal cortex, thalamus and temporal cortex, areas that are commonly reported in imaging studies of response inhibition using Go/NoGo and Stop-signal paradigms (Aron et al., 2003; Liddle et al., 2001; Watanabe et al., 2002). In Go/NoGo paradigms the subject is required to inhibit a prepotent response, whereas in the Stop-signal paradigms the subject inhibits an action that has already been initiated. Although breath holding, which is the inhibition of an ongoing involuntary activity, differs from the Go/NoGo and Stop-signal paradigms, our results suggest that there is some common neural circuitry underlying these different inhibitory tasks. Here, inhibition was identified bilaterally within the inferior frontal gyrus including the dorsolateral pre-frontal cortex, an area associated with response inhibition (Aron et al., 2003; Liddle et al., 2001; Watanabe et al., 2002), and also bilaterally within nuclei of the basal ganglia, specifically the putamen and the substantia nigra, that are not typically associated with response

inhibition (Aron and Poldrack, 2006). It is suggested that the subthalamic nucleus (a subcortical region in the basal ganglia) may play a role in inhibiting initiated responses by suppressing basal ganglia-thalamocortical output via an inhibitory pathway from the inferior frontal cortex (Aron and Poldrack, 2006). We did not identify activity within the subthalamic nucleus associated with breath holding but our results support the presence of an inhibitory pathway originating in the inferior frontal cortex that projects to the substantia nigra and ventrolateral thalamus. We speculate that the cortical and subcortical structures discussed above are part of an inhibitory network that modulates central respiratory output via the pons; however, we cannot exclude the possibility that the inhibition of respiratory output is achieved by higher centres acting independently of the pons.

Since Lumsden's definition of the pneumotaxic centre in cats (Lumsden, 1923), it is well established that neurones with respiratory-related firing patterns are present within the dorsolateral pons (Alheid et al., 2004; Chamberlin, 2004; Dick et al., 1994), particularly within the Kolliker Fuse nucleus and medial parabrachial nucleus. In humans, the parabrachial nucleus is situated in the dorsolateral pontine tegmentum surrounding the superior cerebellar peduncle; the Kölliker-Fuse nucleus has yet to be defined (Lavezzi et al., 2004). The precise role of pontine neurons in respiratory modulation remains unclear since stimulation of different regions of the dorsolateral pons can result in tachypnea, bradypnea, apneusis or apnoea (Chamberlin and Saper, 1994; Fung and St John, 1994; Mutolo et al., 1998). Neurones within the dorsolateral pons have reciprocal connections, some via the ventrolateral pons (Herbert et al., 1990; Song and Poon, 2004), with respiratory neurones within the ventrolateral medulla (Ellenberger and Feldman, 1990; Herbert et al., 1990; Song and Poon, 2004), and some of these neurones project to the nucleus tractus solitarius (Herbert et al., 1990) and phrenic motoneurones (Yokota et al., 2001). Evidence for whether respiratory rhythmic output persists during breath holding is unclear. In cats, medullary neuronal activity is quiescent during behaviourally conditioned

breath holding (Orem and Netick, 1986), whereas recent evidence in humans has shown that sinus arrhythmia, indicative of central respiratory rhythmic output, may persist during volitional breath holding (Parkes, 2006), and in a clinical study of a patient with supranuclear palsy, automatic breathing persisted, albeit reduced, during a volitionally induced breath hold (Haouzi et al., 2006). These latter results suggest that central respiratory rhythmic output cannot be stopped voluntarily but that there is a behavioural influence on central respiratory control and that neuronal output is suppressed either prior to the motoneurones or at the level of the motoneurones in the spinal cord that innervate the respiratory musculature. Here, we highlight a role for the superior dorsal and inferior ventral pons in voluntary breath holding and speculate that the pons serves to integrate signals from supra-brainstem structures, and in turn it exerts an inhibitory influence on breathing at the level of the medulla, or via a direct pathway to phrenic motorneurones in the spinal cord.

The parabrachial nucleus is also a major relay for viscerosensory information from the nucleus of the solitary tract to higher brain areas such as the thalamus, amygdala and insula (Cechetto, 1987, 1995), and has recently been imaged, with respect to visceral control, using fMRI (Topolovec et al., 2004). Vagal fibres transmitting afferent information from the lungs, chest wall and respiratory muscles as a result of lung inflation, project to and terminate onto neurones within the nucleus of the solitary tract (Bianchi et al., 1995), which in turn project to the parabrachial nucleus (Cechetto, 1995; Herbert et al., 1990). Although it is possible that the pontine activity identified in this study reflects sensory feedback from the lungs, we think this is unlikely because the protocol was designed to minimise afferent signalling by performing the breath hold at resting expiratory lung volume (i.e. functional residual capacity) when the respiratory musculature is presumed to be relaxed. Consistent with this and, as we predicted, the breath hold was associated with no detectable activity within the primary motor cortex. Interestingly, breath holding was not associated with increased activity within the cerebellum;

cerebellar activity is commonly reported in human respiratory related imaging paradigms (Evans et al., 2002; McKay et al., 2003; Parsons et al., 2001) and in animals (Xu and Frazier, 2002) usually when task-related respiratory motor activity is increased.

Significant bilateral activity was identified within parts of the limbic system, including the insular cortex, extending into the operculum, the cingulate cortex and the amygdala; all these areas are associated with emotion, especially of an aversive nature (Lane et al., 1997; Morris et al., 1998; Phillips et al., 1997), and hypercapnia (Corfield et al., 1995). The anterior cingulate is associated with many tasks that involve cognitive awareness (Frith et al., 1991), more specifically cognitive behaviours that elicit autonomic arousal (Critchley et al., 2001). Likewise, the insula has been implicated in the explicit awareness of internal bodily states (Cechetto, 1987; Craig, 2002; Critchley et al., 2001) and in the perception of dyspnoea (Banzett et al., 2000b; Evans et al., 2002) and hypercapnia (Corfield et al., 1995). Our post study de-brief determined that 'a need for more air' was the most common sensation/feeling experienced during breath holding but in all subjects this sensation was mild and was limited only to a short period towards the end of the breath hold. We speculate that the limbic areas, in particular the insular cortex, are associated with respiratory interoception and specifically with the perception and monitoring of the apnoeic state that precedes the development of air hunger.

In common with Go/NoGo tasks, activity was highlighted within the supramarginal gyrus (Liddle et al., 2001), a component of the frontoparietal network that includes the lateral pre-frontal cortex, cingulate and pre-SMA. Isaev et al., (Isaev et al., 2002) using PET, identified activity within the parietal cortex associated with inspiratory loading and notably, in common with this present study, found an absence of activity within the sensoricortices, probably because afferent signalling from the lungs, airways and chest wall was insufficient (Isaev et al., 2002). With respect to these respiratory related imaging studies, the supramarginal gyrus is likely

associated with behavioural awareness of respiration, perceived as an absence of respiratory movements during a breath hold, and perceived as inspiratory restriction when breathing with an inspiratory load (Isaev et al., 2002).

The temporal resolution of the study was limited by the requirement of a long TR to achieve whole brain coverage. It is not possible to separately consider activity associated with the onset, maintenance or termination of the breath hold. Any future studies with anatomically narrower *a priori* hypotheses would allow a shorter TR, and a higher temporal resolution.

Methodological Issues

The BOLD technique has greatly enhanced our understanding of human brain function; however, the neurovascular and neurometabolic coupling that underlies the BOLD signal is not fully understood. The BOLD signal is derived from changes in oxygenation within venous blood and is an indirect measure of neuronal activity; recent studies suggest that it is the increased metabolic activity at presynaptic terminals that drives the fMRI signal (Logothetis, 2002; Viswanathan and Freeman, 2007). The BOLD signal is dependent on blood flow, blood volume and blood oxygenation and is sensitive to non specific factors acting throughout the body such as fluctuations in PCO_2 and PO_2, which means that the study of breath holding with fMRI is potentially problematic, even breath holds of a short duration (Kastrup et al., 1998; Liu et al., 2002). During a breath hold there is a gradual increase in arterial PCO_2. Carbon dioxide is a cerebral vasodilator; therefore, an elevation in PCO_2 will subsequently increase whole-brain cerebral perfusion, resulting in a decrease in the deoxyhaemoglobin concentration of the cerebral blood and ultimately a whole-brain increase in the BOLD fMRI signal (Corfield et al., 2001; Li et al., 2000). If whole-brain BOLD fluctuations are highly correlated with task-associated local BOLD signal increases, then statistical analysis will not be able to separate the two effects. To address this confound in this study, each breath hold was followed by a short bolus of CO_2

added to the inspiratory reservoir of the breathing apparatus; this led to an increase in arterial PCO_2 independent of breath holding. In this way, non-specific whole-brain BOLD signal changes occurred with both the breath hold and CO_2 bolus; however, local, neural-related BOLD signal changes were only present with the breath hold. The paradigm partly dissociated the time course of the whole-brain BOLD signal from the time course of the local BOLD signal and allowed the local BOLD signal changes associated with breath holding to be determined independent of whole-brain BOLD effects. We are not aware that this "decorrelation" approach has been used before and whilst it appears to be successful there are a number of potential caveats that should be considered. The relationship between the "local" neuronally generated signal is assumed to be independent of, and additive with, the non-neural vascular effects of CO_2; data from our own laboratory support this within a limited CO_2 range (Corfield et al., 2001). Other studies indicate that the neurovascular response may be attenuated as arterial CO_2 increases; such an effect would tend to underestimate the neural BOLD signal. Ideally, the time course of the local neural signal would be orthogonal to the time course of the CO_2 related vascular signal; in practice this is not possible as the breath hold will always be associated with an increase in CO_2; thus, the two time courses are only partially dissociated. In the analysis, this partial correlation would potentially reduce the power to detect significant activation related changes associated with the breath hold. The vascular effect of CO_2 has been modelled identically at each voxel by including a regressor based on the 'global' signal intensity. This approach can partly account for local differences in the response to CO_2 because the model will allow each voxel to have a separate scaling factor; however, other differences in the response will not be accounted for, e.g., the shape and timing of the vascular response to CO_2 may differ from brain region to brain region. We assume that the global signal, being the sum of the local signals, will be a sufficiently good model of the CO_2 effects. One possible approach to account for temporal differences in the CO_2 response would be to include the temporal derivative of the global response in the model; however, as the global effect is so robust, there is a very strong correlation

between the global regressor and the same regressor lagged, or advanced, by a physiological meaningful time period (the same effect that would be modelled by including the temporal derivative). From this, we would expect only a limited residual variance that would be modelled by inclusion of the temporal derivative.

In principle, the global effects of CO_2 would be best modelled using the grey matter (GM) signal as the global covariate, rather than the global BOLD signal intensity. This would require an accurate segmentation of the BOLD image into the separate tissue types (i.e. GM, 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.

The experimental approach also assumes that there are no activations associated with the CO_2 bolus that are in common with breath holding. Activations common to both states would be missed in the analysis. It is possible that other non-specific confounds have been overlooked, e.g., breathing movements between different states; however, no breathing related movements were observed in the rigid body realignment parameters or in the functional data. In summary, this novel approach, which dissociates the timecourse of global CO_2-induced vasodilation from the time course of the local, neurally-related BOLD signal, appears successful and offers an approach for other studies in which a global vascular confound is present.

In conclusion, the circuitry underlying the neural basis of respiratory suppression is composed of a cortico-bulbar network that likely exerts an inhibitory effect on the medullary respiratory centres. This process of respiratory inhibition requires some neural components that are common to other inhibitory behaviours. The particular role of the pons in breath-holding cannot be determined from this study; however, and in support of animal models of respiratory control,

we speculate that inhibitory motor outputs from supra-brainstem structures are integrated by the pons, which, in turn, modulates rhythmic respiratory output from the medulla. Respiratory inhibition is one component of respiratory control, of which the underlying neural mechanisms are poorly understood in humans but are of importance if we are to increase our understanding of, and to provide better prevention and therapies for many respiratory disorders such as extreme breathlessness and sleep apnoea.

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Figure Legends

Figure 1: Respiratory traces.

A period of ventilatory data from one subject collected during the scanning session. Each subject breathed through a mouthpiece that was attached to an apparatus designed to maintain end tidal CO_2 ($P_{ET}CO_2$) levels to ± 2 mmHg of isocapnia. Tidal volume (Expired V_T) and pressure (Pmouth) tracings illustrate changes in breathing pattern. The 15 second breath hold is clearly observed on the $P_{ET}CO_2$ tracing; a verbal cue (cue) was given to instruct the subjects to complete the breath as normal and begin a breath hold at the end of the breath out (begin); a verbal cue was given to end (end) the breath hold. It should be noted that the increase in alveolar PCO_2 that occurred during the breath hold is not seen on the $P_{ET}CO_2$ tracing because the subject did not expire at the end of the breath hold; additionally, the large spontaneous breath taken after the breath hold returned arterial PCO_2 to isocapnia. Approximately 45 seconds after termination of the breath hold, a CO_2 bolus was added into the circuit (outlined by the dashed lines on the tracing) to mirror the increase in $P_{ET}CO_2$ that occurred during the preceding breath hold.

Figure 2: BOLD signal changes.

Whole-brain BOLD signal changes associated with 12 breath holds (B) alternating with 12 periods of added CO_2 for one subject. The signal change associated with each period of experimentally added CO_2 is comparable to the signal change associated with the preceding breath hold.

Figure 3: SPM projections of neural activity associated with breath holding.

SPM (t) projections of statistically significant signal increases (corrected for multiple comparisons, $p<0.05$) associated with breath holding for the group (n=8), represented according to convention by Talairach and Tourneaux. A: anterior; R: right.

Figure 4: Neural activity associated with breath holding.

Statistical images of significant activity associated with breath holding for the group (n=8), 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 sagittal and coronal planes (a: dorsolateral pre-frontal cortex; b: insula; c: putamen; d: cingulate; e: ventrolateral thalamus; f: supramarginal gyrus.). A: anterior, R: Right.

Figure 5: Pontine activity associated with breath holding.

Statistical images of significant activity within the pons associated with breath holding for the group (n=8), superimposed onto a group mean structural brain image. The illustrated maxima survived a correction for multiple comparisons ($p<0.05$). A: anterior, R: Right.

Table 1: Co-ordinates of local maxima of significant signal increases associated with breath holding.

Co-ordinates are in mm, x co-ordinate is distance right (+) or left (-) of midsagittal line, y co-ordinate is distance anterior (+) or posterior (-) to a vertical plane through the anterior commisure, z co-ordinate is distance above (+) or below (-) the intercommisural (AC-PC) line. T-score is the significance statistic. “Beta” indicates the magnitude of the BOLD signal change at each maximum (for the breathhold regressor in the statistical model), expressed as a fraction of the largest value for beta determined across all maxima (i.e. normalised to that at 46, 48, 8; mean +/- se for the 8 subjects).