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Tracheal occlusion conditioning causes stress, anxiety and neural state changes in conscious rats

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Abstract

Evidence from human and animal studies indicates that mechanical loads to breathing are stressful stimuli and evoke compensatory behaviours. Conditioning of stressful stimuli is known to cause changes in basal stress levels and behaviour. Individuals with respiratory obstructive diseases repeatedly experience bouts of airway obstruction, which may act as a form of conditioning, and often have affective disorders, such as anxiety and depression. It is unknown whether the development of affective disorders in these individuals results from the unexpected recurring respiratory perturbations. To investigate this possibility, we developed a model to elicit tracheal occlusion (TO) in conscious rats and exposed them to 10 days of TO conditioning. We hypothesized that healthy, conscious animals exposed to TO conditioning would develop stress and anxiety and would have modulated neural activity in respiratory, stress, discriminative and affective neural regions. Following TO conditioning, rats had increased basal corticosterone levels, greater adrenal weights and elevated anxiety levels compared with animals not receiving TO. Significant increases in cytochrome oxidase staining were found in brain-stem respiratory nuclei, periaqueductal grey, dorsal raphe, thalamus and insular cortex. These results suggest that healthy animals develop stress and anxiety responses to respiratory load conditioning via inescapable tracheal occlusions, which may be mediated through state changes in specific brain nuclei.

Breathing is vital to survival, so perturbations to normal breathing pattern that threaten survival need to be avoided or escaped. A major motivating force to restore respiration may be the sensations (i.e. discomfort) that the disruption of breathing causes (von Leupoldt & Dahme, 2005b), and escaping those sensations is likely to involve activation of stress responses via the hypothalamic–pituitary–adrenocortical (HPA) axis. Prolonged stress due to an inescapable stimulus may evoke anxiety responses (Naqvi *et al.* 2012). Interestingly, individuals with chronic obstructive pulmonary disease (COPD) who experience repeated bouts of severe mechanical loading often present with affective disorders, such as anxiety and panic (Brenes, 2003; Wagena *et al.* 2005). Whether the development of affective disorders is a direct result of repeated mechanical loading alone in these individuals remains unknown.

Challenging the respiratory system in animals mechanically evokes the vagus-dependent respiratory load compensation response (Zechman *et al.* 1976); however, consciousness and

behaviour play a major role in modulating load-compensated breathing and may alter brainstem reflex responses. In addition, the initial response of a conscious animal to a respiratory stimulus may change as a result of repeated experiences with the stimulus. Indeed, respiratory responses can be classically conditioned in animals and may be evoked by discrete, non-respiratory (Gallego & Perruchet, 1991) or contextual cues (Orem, 1987; Nsegbe *et al.* 1997). Respiration, learning and behaviour appear to be tightly linked. However, it remains unclear whether respiratory conditioning using inescapable mechanical loading will cause affective responses in healthy animals and what neural mechanisms may underlie those responses.

Our current understanding of the neural pathways mediating respiratory load-compensation responses in animals has been determined using immunohistochemical and electrophysiological methods (Ambalavanar *et al.* 1999; Malakhova & Davenport, 2001; Zhang *et al.* 2009). c-Fos, a protein expressed in neurons recently activated, has been used as a metabolic marker to determine which areas of the brain are responding to a recent stimulus (Dragunow & Faull, 1989), including respiratory stimuli (Pate & Davenport, 2011). Although c-Fos can be a useful indicator of neural activity, many control groups are needed, and c-Fos expression cannot be used as an indication of inhibition or as a measure of persistent changes in neural activity. Alternatively, cytochrome oxidase (CO), an enzyme involved in oxidative metabolism in the electron transport chain, has been used to indicate both excitatory and inhibitory changes in the basal state activity levels in brain nuclei in response to repeated stimuli (Wong-Riley, 1979; Hevner *et al.* 1995). Additionally, CO is useful for identifying regions of the brain that undergo changes in activity as a result of learning or conditioning (Puga *et al.* 2007). Given that neural adaptation appears to be important in mediating the response to inspiratory resistive loading in humans (Gozal *et al.* 1995) and that learning and respiration are tightly linked (Nsegbe *et al.* 1998), CO may be beneficial in revealing adaptation within brain nuclei in response to conditioned respiratory stimuli.

Respiratory afferents activated by resistive loads to breathing project to subcortical neural areas, discriminative (Davenport *et al.* 1991; Davenport & Hutchison, 2002) and affective sensory regions (von Leupoldt & Dahme, 2005b; Schon *et al.* 2008) that may contribute to both physical and psychological stress. Stress-related neural pathways converge upon the paraventricular nucleus of the hypothalamus (Herman *et al.* 2003), which initiates a cascade of events mediated by several hormones and ending with the release of corticosterone (Cort; cortisol in humans) by the adrenal cortex. Cort plays a role in normal neuroendocrine function and assists an animal in adapting to stressors. In addition, individuals diagnosed with affective disorders are more likely to have elevated Cort levels (Vreeburg *et al.* 2010). Thus, measurement of basal Cort is one way to determine the extent of HPA activation. Previous studies have indicated that respiratory stimuli, such as hypoxia, have stimulatory effects on the HPA axis in humans and animals (Guilland *et al.* 1984; Jacobson & Dallman, 1989), while intermittent hypoxia leads to dysregulation of normal HPA activity (Ma *et al.* 2008). It remains unclear what effect inescapable respiratory obstructions may have on HPA activation in healthy, conscious animals and whether the stimuli are sufficient to cause affective responses following conditioning.

Researchers in our laboratory have developed a method to study the load-compensation reflex in conscious rats via intrinsic, transient tracheal occlusion (TO). An occlusion is an infinite resistive load to breathing, and we have previously shown that resistive loads of large magnitude are stressful stimuli (Alexander-Miller & Davenport, 2010). TO is evoked by inflating a cuff around the trachea with enough pressure to close the lumen of the trachea reversibly. Deflation of the cuff restores the trachea to its original state, with no evidence of residual damage, determined through pilot studies. To quantify stress responses, basal plasma Cort can be measured, and adrenal glands, which become enlarged due to sustained increased Cort production (Márquez *et al.* 2004), can be weighed. Psychological state can be evaluated using the elevated plus maze (EPM), a standard behavioural test for anxiety in rodents (Pellow *et al.* 1985). It was hypothesized that after 10 days of TO conditioning, rats would have elevated basal plasma Cort, increased adrenal weights, increased anxiety-like behaviour, measured via the EPM, and state changes in brain nuclei, determined by CO histochemistry, in areas involved in respiratory stress responses and discriminative and affective respiratory information processing.

Methods

Ethical approval

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida. These experiments were performed on 25 male Sprague–Dawley rats (374.0 ± 55.0 g). Two animals were housed per cage in the University of Florida Animal Care Facility and were exposed to a 12 h light–12 h dark cycle.

Surgical procedures

Animals were initially anaesthetized using isoflurane gas (2–5% in O₂) administered in a whole-body gas chamber. Anaesthetic depth was verified by the absence of a withdrawal reflex from a rear paw pinch. Buprenorphine ($0.01\text{--}0.05$ mg (kg body weight)^{−1}, Sigma-Aldrich, St. Louis, MO, USA) and carprofen (5 mg (kg body weight)^{−1}, Sigma-Aldrich) were administered preoperatively via subcutaneous injection. Incision sites were shaved and sterilized with povidone-iodine topical antiseptic solution. Body temperature was maintained at 38°C by a heating pad, and anaesthesia was maintained by isoflurane gas administered via a nose cone.

The animal was placed in a supine position and the trachea exposed by a ventral neck incision. The trachea was freed from surrounding tissue, and a saline-filled inflatable cuff (Fine Science Tools, Foster City, CA, USA) was sutured around the trachea, two cartilage rings caudal to the larynx. The actuator tube of the cuff was plugged with a blunt needle, tunnelled subcutaneously and externalized through an incision between the scapulae. The skin at the dorsal incision was sutured closed, and the tube was secured in place by tying the ends of the suture around a bead on the tube. The neck tissue exposed by the ventral incision was pulled over the cuff and the skin sutured closed. The rat was then administered subcutaneously warm normal saline ($0.01\text{--}0.02$ ml (g body weight)^{−1}), and isoflurane anaesthesia was gradually reduced. The animal was placed in a recovery cage on a heating pad and returned to the Animal Care Facility once fully mobile. Postoperative analgesia was

provided for 2–3 days using buprenorphine ($0.01\text{--}0.05\text{ mg (kg body weight)}^{-1}$) and carprofen ($5\text{ mg (kg body weight)}^{-1}$) administered every 24 h. Animals were allowed a full week of recovery before experiments began.

Protocol

The animals were retrieved from the Animal Care Facility on the morning of experimental day 1 and were taken to the laboratory. Each animal was placed on the EPM in a sound-proofroom. The animal remained on the EPM for 5 min, where it was allowed to explore the two open arms and two closed arms freely and undisturbed. The animal's movement was tracked with a video recording device, and details of its movements were analysed with the EPM software (ANY-maze version 4.30; Stoelting, Wood Dale, IL, USA). At the end of the 5 min, the animal was removed from the EPM, and the EPM was cleaned with alcohol wipes. After all animals completed the maze trial they were brought to the testing laboratory and placed in one of two recording chambers placed side by side and separated by a visual barrier. During this acclimation period lasting 15 min, the externalized actuator tube of the tracheal cuff from each animal was connected to a saline-filled syringe outside the chamber, but no pressure was applied to the syringe. At the end of the 15 min protocol, animals were returned to their home cages, and the chambers were cleaned with alcohol wipes. Once all animals had completed the acclimation protocol, they were returned to the Animal Care Facility.

Tracheal occlusion conditioning—The animals were retrieved from the Animal Care Facility on the morning of experimental day 2 and were brought to the testing laboratory. They were divided into the following two groups: those receiving TO conditioning (TO; $n = 13$); and unoccluded control animals (Ctrl; $n = 12$). One TO and one Ctrl animal were placed individually in the two recording chambers, and the actuator tube was connected to a saline-filled syringe. The TO animal was allowed to rest undisturbed in the chamber for 2.5 min, followed by a series of cuff inflations. The syringe and cuff were pressure calibrated so that a known amount of fluid movement in the syringe would result in pressure required to compress and occlude the trachea fully. Removal of the pressure allowed for full recovery of the trachea with no interference to breathing. The occlusion pressure was calibrated with post-mortem, freshly excised tracheas. The cuff of the TO animal was inflated for 3–6 s, with at least 15 s separating the occlusions, for ~35 trials in a 15 min period. The TO trials were applied in a random intertrial interval time pattern. After the final TO trial, the animal was allowed to rest undisturbed in the recording chamber for 2.5 min. The Ctrl animal remained undisturbed for the duration of the 20 min protocol. At the end of the 20 min, both animals were removed from the chambers and returned to their cages. The recording chambers were cleaned with alcohol wipes between animals. The next Ctrl and TO animals were placed in the recording chambers, and the 20 min protocol was repeated. This was continued for all animals. All animals were returned to the Animal Care Facility at the end of experimental day 2. This procedure was repeated daily for experimental days 3–11. Before the protocol on experimental day 11, all animals completed the EPM test for the second time.

The animals were brought to the laboratory on experimental day 12, and they were allowed to remain undisturbed for 2 h. In a random order, each animal was removed from its cage and placed in a chamber with isoflurane gas (5% in O₂). The animal was removed from the chamber in an anaesthetized state, within 1.5 min, and killed by decapitation. Trunk blood was collected immediately. Brains were removed ($n = 5$ per group), blocked into three pieces via coronal cuts, and placed in 4% paraformaldehyde. Adrenal glands were removed and weighed. Clotted blood was centrifuged at 4°C for 15 min, and plasma was extracted and stored at -80°C. Plasma Cort (in nanograms per millilitre) was measured using a radioimmunoassay (RIA) kit (rat Cort 125I; MP Biomedicals), generously provided by Dr Deborah Scheuer at the University of Florida.

Cytochrome oxidase—The blocked brains remained in the paraformaldehyde solution for 3 days, and then were transferred into a 30% sucrose solution for 2 days. Each brain block was individually frozen and secured to the stage of a microtome to provide coronal sections. Frozen sections of the brains were cut with the microtome at a thickness of 40 μ m, and the tissue was systematically placed in 24-well plates. Each well was filled with PBS (pH 7.4), and the tissue remained in the PBS for 5 days. One full series (column) of tissue per animal brain was used for the staining protocol, adapted from Wong-Riley (1979). Briefly, the CO solution, 600 ml PBS, 60 g sucrose, 300 mg cytochrome C (Sigma; C2506), 200 mg catalase (Sigma; C9322) and 150 mg diaminobenzidine (Sigma; D5905) was prepared and applied to the tissue. All plates with the CO solution were put on a shaker and covered to prevent light exposure. They remained on the shaker overnight and were removed 17 h later and transferred into PBS. The tissue was then mounted on glass microscope slides, dehydrated with ethanol, cleared with xylene and coverslipped with Eukitt (Sigma-Aldrich). The slides were stored in light-proof cases until analysed.

Imaging

Slides of brain tissue were viewed using light microscopy (Zeiss Axioplan2). Images were captured using a computer software system (ImagePro Plus), converted to eight-bit mono images, and stored on the computer. During each capture session, the image of a blank slide was also captured using the same settings. A standard optical density calibration was created by setting black levels to zero and using the image of the blank slide as the incident light reference. The standard calibration was applied to all images before any measurements were made. Three densitometric readings were taken from within a nucleus, defined by stereotaxic co-ordinates (Paxinos & Watson, 1997), for at least one slide per nucleus per animal (Hevner *et al.* 1995). This resulted in at least three readings per nucleus per animal. These values were averaged, and the intensity of staining in each nucleus of interest was determined for each animal, normalized to the average intensity of staining in an area of white matter (optic tract) for that animal to control for variability between staining batches.

Statistics

Normalized values of CO staining intensity were combined according to group and were used to conduct a Student's unpaired *t* test for each nucleus analysed, with the conditions for the parametric *t* test having been met. Adrenal gland weight was normalized by dividing adrenal weight by the animal's body weight on the day when the adrenals were removed.

Adrenal weights and blood Cort levels were compared between groups using Student's unpaired *t* test. Total time spent in the open, closed and centre regions of the EPM during the test were obtained for each animal on experimental days 1 and 11. If data were not usable for an animal on either of the two exposures to the EPM (i.e. if the animal fell off the platform), the data for that animal were not used in statistical analyses. Times were analysed (Pellow *et al.* 1985; Rodgers & Dalvi, 1997; Korte & De Boer, 2003) for each maze region using a oneway repeated-measures analysis of variance (RMANOVA), comparing group (TO *versus* Ctrl) and day (day 1 *versus* day 11). *Post hoc* comparisons were performed using Tukey's HSD. Statistical significance was determined when $P < 0.05$. All data are reported as means $\pm$ SEM.

Results

Tracheal occlusion conditioning causes anxiety

During the 5 min of the initial EPM, there was no difference in time spent on each part of the maze between the Ctrl and TO groups (Fig. 1), with all animals spending a majority of time on the closed arms. Following the 10 days of treatment, the EPM was utilized a second time in all animals. The Ctrl animals spent the same amount of time in each section of the maze as they did on the first exposure. However, following TO conditioning the animals spent significantly less time on the open arms during a second exposure to the EPM compared with the initial trial ($P < 0.05$; Fig. 1A). There were no pre-post differences in the TO group for time spent on the closed arms (Fig. 1B) or centre region of the maze.

Increased HPA activation following TO conditioning

Basal plasma Cort levels were significantly lower in the Ctrl compared with the TO animals after the 10 day protocol (2.9 ± 1.2 *versus* 5.2 ± 1.5 $\mu\text{g dl}^{-1}$, $P < 0.02$; Fig. 2A). Adrenal weight normalized to body weight was significantly greater in the TO animals that underwent conditioning compared with their unobstructed counterparts (0.14 ± 0.009 *versus* 0.17 ± 0.006 , $P < 0.03$), which was not due to differences in body weight (Fig. 2B).

Tracheal occlusion conditioning leads to enhanced basal neural activation

Cytochrome oxidase staining was observed and quantified in tissue sections through the brainstem, midbrain and suprapontine brain centres for both Ctrl and TO animals (Table 1). Staining in the rostral nucleus of the solitary tract (NTS) and rostral ventral respiratory group (VRG) was significantly greater in TO animals compared with Ctrl animals ($P < 0.01$ and $P < 0.02$, respectively). However, there were no significant differences in staining between groups in the caudal NTS, caudal VRG, area postrema (AP), lateral parabrachial nucleus (LPBN) or locus coeruleus (LC). There was significantly elevated CO staining throughout the periaqueductal grey (PAG) in animals that underwent TO conditioning compared with unobstructed Ctrl animals. The most significant between-group difference in staining within PAG subnuclei was seen in the caudal ventral PAG (VPAG; Fig. 3), with an 18% increase in activity in TO animals ($P < 0.004$). Significantly elevated CO staining was also observed for TO animals in the dorsal raphe (DR; $P < 0.05$). In suprapontine brain centres, CO staining was significantly elevated in TO compared with Ctrl animals in the ventroposteromedial thalamic nucleus (VPM; $P < 0.02$) and the anterior insular cortex (AI; P

< 0.02). There were no group differences in CO staining in the arcuate (Arc), medial parvocellular paraventricular hypothalamus (PaMP), centromedial thalamus (CM), ventroposterolateral thalamus (VPL), cingulate cortex (Cg1) or central nucleus of the amygdala (CeA). Interestingly, there were no nuclei in which a significant decrease in CO staining was observed in TO *versus* Ctrl animals (Table 1).

Discussion

Ten days of TO conditioning in healthy rats caused behavioural changes, elevated resting HPA activation, and increased neural activity within the rostral NTS, rostral VRG, PAG, DR, VPM and AI. The results of this study suggest that repeated, inescapable airway obstruction can cause stress and anxiety in conscious animals after 10 days of conditioning, which may be mediated by increased activity of specific neural regions. The link between respiratory stimuli (specifically, perturbations to breathing) and the development of stress, anxiety and perhaps even depression, must be explored further if we hope to understand how these disorders manifest in the human population and ultimately provide alternative treatments to affected individuals. Given that these types of experiments cannot be carried out in humans, this model may continue to be a useful tool.

Other methods of respiratory loading in conscious animals have included tracheal banding (Greenberg *et al.* 1995), restricting airflow to a head chamber (Farré *et al.* 2007), applying loads via a tracheostomy (Davenport & Hutchison, 2002) or facemask (Davenport & Hutchison, 2002), and a tracheal balloon implant (Schoorlemmer *et al.* 2011). Tracheal banding provides a sustained increase in airway resistance that leads to compensation from both mechanical and chemical activation. Restriction of airflow via a head chamber, tracheostomy or facemask provides an extrinsic airway resistance, and these techniques are primarily implemented acutely. Furthermore, the head chamber model would restrict movement of the animal owing to the placement of the apparatus around the head and neck. The tracheal balloon implant model is an effective method for evoking intrinsic mechanical loading, but it can result in air accumulation in the subcutaneous space around the throat and respiratory infections, because implantation of the balloon requires cutting into the trachea (Schoorlemmer *et al.* 2011). The TO model used by our laboratory is unique in that it allows for intrinsic respiratory obstructions to be applied reversibly to conscious, freely moving animals, with the ability to control and modulate the onset, offset and duration of each occlusion. Additionally, implantation of the occluder requires minimal surgery and results in few postoperative complications. Thus, although the TO model does not precisely mimic a specific respiratory disease *per se*, it may be a valuable tool for studying aspects of multiple respiratory diseases.

Following TO conditioning, we observed basal stress and anxiety in our animals. Stress was determined by measuring blood Cort the morning after the last TO, when the levels were lowest (Windle *et al.* 1998), and evaluating adrenal gland wet weight. The combined results indicate that TO conditioning augments basal HPA activation. Uncontrollable stress causes an increase in HPA activity (Levine, 2000), and sustained HPA activation is linked with affective disorders, such as anxiety and depression (Stenzel-Poore *et al.* 1994; Maier & Watkins, 2005). We observed anxiety in our animals following TO conditioning, which was

evaluated using the EPM. In general, one trial on the EPM is accepted as an indicator of anxiety levels resulting from treatment differences, while behaviour during a second exposure to the EPM is thought by some to originate from other factors such as the animal's fear of heights, and is resistant to anxiolytics during a short but not long test duration (File, 1993; File *et al.* 1993). However, according to Treit *et al.* (1993), open arm avoidance during the EPM test was not related to a fear of heights. This group also found that animals do not habituate to repeated exposures to the EPM, even after forced exploration of the open arms. In the present study, a two-exposure protocol was used in conjunction with the appropriate controls. Hence, the decreased open arm time after TO conditioning in these animals supports our hypothesis that repeated exposure to inescapable TO produces state changes in conscious animals, characterized by increased stress and anxiety.

The DR, an area of the midbrain responsible for releasing serotonin (5-HT), is linked with stress circuitry (Jorgensen, 2007; Valentino *et al.* 2010). After 10 days of TO conditioning, the DR had elevated steady-state activity, suggesting a potential role in the adaptive response to repeated, inescapable, life-threatening respiratory stimuli. According to Graeff *et al.* (1996), stress activates the DR, which releases 5-HT into the PAG and amygdala, causing immediate escape behaviours, which over time may lead to anxiety. Researchers in our laboratory have previously found alterations in 5-HT receptor gene expression in thalamic neural areas after one TO (Bernhardt *et al.* 2008) and also after 10 days of TO (Bernhardt *et al.* 2010). It is possible that 5-HT signalling is modulated in multiple neural regions in response to TO conditioning, and these changes may be related to 5-HT-dependent processes, such as respiratory control (Hodges *et al.* 2009) and respiratory neural plasticity (Feldman *et al.* 2003; Doi & Ramirez, 2008).

After TO conditioning, we also found enhanced activity in the PAG, more so in the caudal compared with the rostral region, which is consistent with the results of previous studies (Zhang *et al.* 2007). The PAG plays a role in respiratory activation (Zhang *et al.* 2005) and modulation of load-compensation responses (Zhang *et al.* 2009), with the DPAG and VPAG being implicated in panic and anxiety, respectively (Brandao *et al.* 1994; Vianna *et al.* 2001). Additionally, the VPAG appears to be involved in conditioned fear responses (Vianna *et al.* 2001). Following 10 days of TO, the DPAG showed significant increases in steady-state activity, while there was a trend for increases in the VPAG. These are likely to be key neural substrates involved in causing anxiety in the TO animals in this study.

Increases in steady-state neural activity after TO conditioning were seen in the TO animals compared with the control rats in the rostral NTS and rostral VRG but not in the caudal brainstem respiratory nuclei, such as the caudal VRG or caudal NTS. The caudal NTS is the primary nucleus targeted by cardiorespiratory and peripheral chemoreceptor afferents, is involved in respiratory control (Kubin *et al.* 2006), and was therefore expected to exhibit increased basal activity in animals following TO conditioning. Likewise, there was not a robust effect of TO conditioning on basal activity levels of the paraventricular subnucleus (PaMP). Considering that there was only a modest, albeit significant, increase in blood Cort in the occluded animals at the end of the experiment, it may be that steady-state activation of this neural region habituated over the course of the 10 day TO protocol. Alternatively, the observations may be related to the beginnings of a pathological HPA response to TO as a

chronic, intermittent stressor (Ma *et al.* 2008). Indeed, others have observed complex HPA responses in stress disorders and respiratory diseases (Schuetz *et al.* 2008; van Liempt *et al.* 2012). It will be important in future studies to define the HPA response to a single exposure of TO, as well as the immediate response to TO following multiple days of conditioning, in order to confirm or disprove these hypotheses.

These results do not preclude the participation of CeA, AP, LPBN or LC in the immediate response to TO, and may suggest that these neural regions either do not undergo changes in basal activity in response to TO, or that their responses habituate over time. Interestingly, Gozal *et al.* (1995) reported that inspiratory loading in humans caused neural activation of the LC, which decreased following the second presentation of the load. Neural adaptation in response to repeated stimuli may allow the central nervous system to respond to only the most relevant, and perhaps novel, stimuli.

Subcortical neural networks are essential for maintaining normal cardiorespiratory function, generating reflexes, and adapting to recurring or prolonged stimuli. However, some components of these networks can be modulated in conscious animals via cortical input. An awake animal has two defined systems involved in sensory processing (Davenport & Vovk, 2009). The discriminative system encodes stimulus details such as intensity, timing and location of the stimulus, whereas the affective system integrates the qualitative emotional aspects associated with the stimulus. Both pathways are involved in respiratory sensory processing in humans (von Leupoldt & Dahme, 2005a,b; von Leupoldt *et al.* 2008; Davenport & Vovk, 2009). Dyspnoea, the adverse sensation of respiratory discomfort (O'Donnell *et al.* 2007), is a common symptom of respiratory obstructive diseases (von Leupoldt & Dahme, 2005a; O'Donnell *et al.* 2007) and includes both a discriminative and an affective component (von Leupoldt & Dahme, 2005b). The discriminative component is relayed to the somatosensory brain network, which is processed in a similar fashion in both humans and animals (Davenport *et al.* 1991; Davenport & Hutchison, 2002). The affective component is relayed to parts of the limbic neural network and shares similarities with the affective component of pain via processing in the insular and anterior cingulate cortices (Aleksandrov *et al.* 2000; von Leupoldt & Dahme, 2005a). With TO, we have found significantly increased basal activity in the VPM thalamus, which is considered to be part of the discriminative system, as well as the AI, a component of the affective system. Tracheal afferents activated upon cuff inflation may send projections to the VPM via polysynaptic pathways (Ambalavanar *et al.* 1999). Cechetto and Saper found that the VPM projects visceral sensory information to the AI, which responds to normal respiration (Cechetto & Saper, 1987) and voluntary breath-holding manoeuvres (McKay *et al.* 2008), is involved in the neural processing of dyspnoea (von Leupoldt & Dahme, 2005a; von Leupoldt *et al.* 2008), and produces excitatory and inhibitory effects on breathing when stimulated (Aleksandrov *et al.* 2000). The VPM and AI may be activated by the sensations associated with TO and may play a significant role in modulating the load-compensation responses to TO conditioning in conscious animals. In addition, although dyspnoea cannot be assessed in animals as it is in humans, the neural correlates that mediate dyspnoea are similar. It is therefore plausible that respiratory obstructions in animals cause intense sensations of discomfort, leading to experienced negative affect. Repeated experiences with negative affect may be related to anxiety, something commonly observed in individuals with COPD

and dyspnoea (Brenes, 2003; Wagena *et al.* 2005). Dyspnoea is a highly aversive sensation, and humans as well as animals appear to modify behaviour in order to adapt to or avoid experiencing the sensation.

In order to understand the neural mechanisms involved in load-compensation responses to respiratory stimuli in human subjects, non-invasive methods such as functional magnetic resonance imaging and cortical evoked potentials are most often used (Gozal *et al.* 1995; von Leupoldt *et al.* 2008). Functional magnetic resonance imaging produces information about both inhibition and activation, but with poor temporal resolution, and cortical evoked potentials indicate temporal activity patterns with poor spatial resolution. Histochemistry can be used with invasive experiments in animals to determine specific neural changes in response to stimuli. For studies of neural adaptation or changes in basal neural activity, CO is a histochemical method that can show both excitatory and inhibitory state changes in brain activity, but CO does not provide information on acute neural activity. One potential difficulty with using CO lies in the fact that brain nuclei have inherent differences in metabolic rates. Certain areas are always less active than others and will have less intense CO staining (Hevner *et al.* 1995). The determination of group differences in metabolic activity for these nuclei can be difficult when the changes are small. While CO histochemistry remains a valuable tool for determining changes in neural activity as a result of learning, the results should be interpreted with care.

Summary

Tracheal occlusion conditioning performed once a day for 10 days led to elevated basal HPA activation, anxiety, and increases in basal activity in the NTS, VRG, PAG, DR, VPM and AI. These areas include important respiratory nuclei, nuclei involved in an animal's stress response, and nuclei mediating discriminative and affective sensory processing. The results suggest a conditioned state modulation of sensory brain regions, which is especially relevant to pulmonary diseases, such as asthma and COPD, which are associated with repeated bouts of unexpected increases in airway resistance. Patients with these pulmonary diseases have a high incidence of stress and affective disorders. Chronic experience with one type of stress can make an individual more reactive to new types of stress; patients with asthma and COPD often have additional physiological and psychological complications that could be worsened by airway obstruction-dependent HPA activation. Determination of the links between respiratory sensations, stress and psychological state is extremely important work and needs to be continued in order to deepen our understanding of respiratory diseases and rehabilitation. The model used in the present study may be an important tool for elucidating these mechanisms in the future.

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New Findings

What is the central question of this study?

Airway obstructions appear to evoke stress and to cause compensatory behaviours; individuals with respiratory obstructive diseases experience affective disorders. Respiratory patterns can be conditioned, but it is unknown whether healthy animals develop stress or anxiety following respiratory occlusion conditioning and whether persistent neural changes are involved in that development.

What is the main finding and its importance?

Respiratory occlusion conditioning caused both stress and anxiety in healthy rats, which were accompanied by increased basal neural activity in important respiratory, stress and discriminative and affective sensory processing centres. Affective disorders may follow the development of a respiratory obstructive disease through the modulation of basal activity in specific neural regions.

Figure 1. Anxiety responses to tracheal occlusion (TO) conditioning

Total time spent in the open (A) and closed sections (B) of the elevated plus maze (EPM) during one 5 min trial before (day 1) and one trial after (day 11) 10 days of TO conditioning (TO) or handling without TO (Ctrl). The time spent on the open arms of the EPM after conditioning was significantly less than time spent on the open arms of the EPM before conditioning in occluded animals (* $P < 0.05$, A).

Figure 2. Physiological stress responses to TO conditioning

A, basal plasma corticosterone (Cort) levels (in micrograms per decilitre) after 10 days of TO conditioning (TO) or handling without tracheal occlusions (Ctrl). The Ctrl animals had significantly lower resting Cort levels compared with animals receiving TO conditioning ($*P < 0.02$). **B**, adrenal weight normalized to animal body weights after 10 days of TO conditioning (TO) or handling without tracheal occlusions (Ctrl). The adrenal glands of TO animals weighed significantly more than those of Ctrl animals ($*P < 0.03$). Differences were not due to changes in body weight in either group of animals.

Figure 3. Cytochrome oxidase staining in the ventral periaqueductal grey

Cytochrome oxidase staining in the ventral periaqueductal grey in animals treated with 10 days of handling without tracheal occlusions (Ctrl; *A*) or 10 days of TO conditioning (TO; *B*). The extent of cytochrome oxidase staining was evaluated densitometrically. Animals undergoing TO conditioning had significantly greater cytochrome oxidase staining in the ventral periaqueductal grey than control animals ($P = 0.004$).

Table 1
Cytochrome oxidase (CO) staining in brain nuclei after 10 days of tracheal occlusion (TO)
conditioning or handling without tracheal occlusions (Ctrl)

Nucleus	Ctrl	TO	Percent Change	<i>P</i> value
Caudal NTS	2.46 (0.05)	2.66 (0.06)	+8	0.31
Rostral NTS	2.12 (0.06)	2.87 (0.10)	+35	0.01 *
Caudal VRG	2.86 (0.05)	3.04 (0.05)	+6	0.4
Rostral VRG	2.43 (0.06)	3.08 (0.06)	+27	0.02 *
AP	2.85 (0.15)	2.76 (0.10)	-3	0.82
LPBN	1.93 (0.07)	2.07 (0.03)	+7	0.57
LC	1.85 (0.05)	2.25 (0.04)	+22	0.08
Caudal DPAG	1.74 (0.04)	2.11 (0.04)	+21	0.02 *
Caudal VPAG	1.9 (0.02)	2.25 (0.03)	+18	0.004 *
Rostral DPAG	1.7 (0.04)	1.98 (0.04)	+16	0.06
DR	1.8 (0.04)	2.11 (0.03)	+17	0.05 *
Arc	2.16 (0.09)	2.22 (0.06)	+3	0.79
PaMP	2.12 (0.12)	2.09 (0.06)	-1	0.93
CM	1.47 (0.05)	1.74 (0.03)	+18	0.09
VPL	2.27 (0.05)	2.45 (0.06)	+8	0.37
VPM	2.11 (0.05)	2.48 (0.04)	+18	0.02 *
AI	2.43 (0.04)	2.69 (0.04)	+11	0.02 *
Cg1	2.32 (0.03)	2.6 (0.03)	+12	0.19
CeA	1.45 (0.04)	1.34 (0.03)	-8	0.39

Reported values are CO staining intensity within each nucleus normalized to unstained white matter from the same animal and staining batch ($\pm$ SEM).

Asterisks indicate significance ($P < 0.05$).

Abbreviations: AI, anterior insular cortex; AP, area postrema; Arc, arcuate; CeA, central nucleus of the amygdala; Cg1, cingulate cortex; CM, centromedial thalamus; DPAG, dorsal periaqueductal grey; DR, dorsal raphe; LC, locus coeruleus; LPBN, lateral parabrachial nucleus; NTS, nucleus of the solitary tract; PaMP, medial parvocellular paraventricular hypothalamus; VPAG, ventral periaqueductal grey; VPL, ventroposterolateral thalamus; VPM, ventroposteromedial thalamus; and VRG, ventral respiratory group.