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An acute exposure to intermittent negative airway pressure elicits respiratory long-term facilitation in awake humans

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ABSTRACT

Background: A sustained elevation in respiratory drive following removal of the inducing stimulus is known as respiratory long-term facilitation (rLTF). We investigated whether an acute exposure to intermittent negative airway pressure (INAP) elicits rLTF in humans.

Method: 13 healthy males (20.9 ± 2.8 years) undertook two trials (INAP and Control). In the INAP trial participants were exposed to one hour of 30-second episodes of breathing against negative pressure (-10 cmH_2O) interspersed by 60-second intervals of breathing at atmospheric pressure. In the Control trial participants breathed at atmospheric pressure for one hour. Ventilation following INAP (recovery phase) was compared to that during baseline.

Results: Ventilation increased from baseline to recovery in the INAP trial (14.9 ± 0.9 vs 19.1 ± 0.7 L/min, $P = 0.002$). This increase was significantly greater than the equivalent during the Control trial ($P = 0.019$). Data shown as mean ± SEM.

Conclusion: In this study INAP elicited rLTF in awake, healthy humans. Further research is required to investigate the responsible mechanisms.

1. Introduction

It is now well known that the neural network responsible for respiratory control is capable of significant adaptation known as neuroplasticity (Mitchell and Johnson, 2003). The most documented form of respiratory neuroplasticity is respiratory long-term facilitation (rLTF). rLTF is a sustained augmentation in respiratory motor output that has been demonstrated following an acute exposure to either intermittent carotid sinus nerve stimulation or intermittent hypoxia (IH) (Mitchell and Johnson, 2003). Specifically, rLTF following IH has been extensively demonstrated in both humans and various other animals and the neurological mechanisms that are involved in its development are well described (Mateika and Sandhu, 2011). rLTF has also been shown in anaesthetised rodents following an acute exposure of the upper airway to intermittent negative airway pressure (INAP) (Ryan and Nolan, 2009b), but this has never been investigated in humans.

In humans and other animals, receptors that respond to negative airway pressure are located throughout the upper airway and to a lesser extent in the sub-glottic airways (Horner et al., 1991b; Hwang et al., 1984; Mathew et al., 1982a; van Lunteren et al., 1984). Stimulation of these receptors elicits a neural reflex that produces a coordinated contraction of various muscles within the upper airway that help to maintain airway patency (Akahoshi et al., 2001; Doherty et al., 2008; Mortimore and Douglas, 1997; Horner et al., 1991a,1991b). The precise location of these receptors within the airway wall and their form remains to be fully determined. However, significant attenuation in the negative pressure reflex in humans following anaesthetic blockade of the internal branches of the superior laryngeal nerves (Horner et al., 1991b) and denervation in rabbits and dogs (Mathew et al., 1982b; Curran et al., 1997) suggests the superior laryngeal nerve is an important nerve afferent in this reflex. However, the reflex is attenuated not abolished by superior laryngeal nerve blockade or denervation suggesting other nerve afferents also play a role (Horner et al., 1991b; Curran et al., 1997).

In a study on anaesthetised rats performed by Ryan and Nolan (2009b), repetitive exposure of these receptors with negative pressure elicited rLTF. This was evidenced by an elevation in diaphragm EMG activity and increased breathing frequency (BF) that lasted during the

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entire one-hour recovery period under atmospheric pressure conditions.

Although this was the first study to demonstrate that INAP can cause rLTF, there is extensive evidence to show that IH elicits rLTF in animal preparations and in humans when the arterial levels of carbon dioxide are slightly elevated (Gerst et al., 2011; Harris et al., 2006; Lee et al., 2009; Mateika and Sandhu, 2011; Wadhwa et al., 2008; Griffin et al., 2012). Raising carbon dioxide seems to be a requirement for the expression of rLTF because raising carbon dioxide ensures that ventilation is controlled by chemoreceptors rather than the 'wakefulness drive' that is governed by arousal and/or behavioural stimuli which otherwise masks the expression of rLTF (Harris et al., 2006; Mateika and Sandhu, 2011). Ryan and Nolan (2009b) showed that the magnitude of rLTF following INAP was comparable to that following the same duration of IH in the same anaesthetised, spontaneously breathing rats (increased diaphragm EMG activity of ˜31% vs ˜42%, respectively) (Ryan and Nolan, 2009a). Furthermore, they showed that combining IH and INAP did not increase the magnitude of rLTF further than the increase that was caused by INAP alone (Ryan and Nolan, 2009b). It is therefore tempting to speculate that the responsible mechanism for rLTF following these two different stimuli could be the same. The possibility of a shared mechanistic pathway could have important implications considering that animal research has shown that the mechanism responsible for IH-induced rLTF is the same as that responsible for improved somatic motor function in rodents with incomplete spinal cord damage (Lovett-Barr et al., 2012). This is an intriguing finding as recent evidence has shown IH also causes a lasting enhancement in somatic motor function in humans with incomplete spinal cord damage as demonstrated by increased calf muscle strength and walking speed (Hayes et al., 2014; Trumbower et al., 2012).

Furthermore, enhancing our understanding of the control of breathing and identifying potential novel stimuli such as INAP for augmenting ventilation (i.e. rLTF) could have implications for the care of patients suffering from both acute and chronic hypoventilation disorders (e.g. respiratory muscle weakness, obesity, motor-neurone disease etc).

However, to date we are not aware of any research performed on humans to have investigated the effects of INAP on rLTF. Therefore, we designed and undertook a novel study to determine whether INAP can elicit rLTF in awake humans. Given the evidence in the existing literature on animal research we hypothesise that application of INAP will also elicit rLTF in humans.

2. Methods

2.1. Participants

Thirteen healthy male students participated in the study. All participants were non-smokers, had no history of cardio-vascular, respiratory or metabolic disease and were not taking any medication. Furthermore, the existence of obstructive or central sleep apnoea that would suggest an upper airway or respiratory control abnormality was excluded by performing a domiciliary sleep study. All participants received detailed information describing the procedures and risks involved and all provided written consent to take part. The study was performed according to the latest revision of the *Declaration of Helsinki* and was approved by the local ethics committee (University of Birmingham Ethical Review Committee – ERN 14–1087).

2.2. Protocol

Participants visited the laboratory for two experimental trials (INAP and Control) separated by one to two weeks. Prior to undertaking experimental trials participants also undertook a preliminary visit to become familiarised with the instrumentation and all experimental procedures. In both experimental trials participants were asked to refrain from consuming alcohol and undertaking moderate to vigorous exercise 24 h, and from caffeine intake for 12 h prior to laboratory visits. Experimental trials were performed at least 3 h after food consumption. Furthermore, participants were asked to record what they ate prior to the first experimental trial and replicate this prior to the second experimental trial. Both experimental trials were performed at the same time of day and performed in a randomised order.

2.3. Preliminary visit and domiciliary sleep study

Having given consent to participate in the study all participants were asked to undertake a single night domiciliary sleep study (ApneaLink, Resmed, USA). The equipment continuously measured nasal flow via a nasal cannula and SpO_2 % via pulse oximetry from a finger. If the apnoea-hypopnoea index (AHI) was < 5/hour participants were deemed to be free from sleep apnoea and were thus invited to continue with the study.

During the preliminary visit participants positioned themselves comfortably on a bed in the semi-supine position where they breathed room air for 10 min. This period was used to establish a good baseline for each participant's normal end-tidal partial pressure of carbon dioxide (P_{ETCO_2}). This was followed by a 10-minute period during which P_{ETCO_2} was raised by 3–4 mmHg above normal. Finally, participants were exposed to 10 min of INAP at the same level and pattern as would be experienced in the experimental trial. The instrumentation for this visit was identical to experimental trials as described later in "*Instrumentation*".

2.4. INAP trial

Once positioned comfortably on the bed participants breathed room air for 15 min in order to establish their normal P_{ETCO_2}. Subsequently, P_{ETCO_2} was elevated by 3–4 mmHg above their normal level for a period lasting 20 min. During the elevation in P_{ETCO_2} the end-tidal partial pressure of oxygen (P_{ETO_2}) was maintained at a normoxic level (100 mmHg). Participants were then instrumented for the intervention period which involved a one-hour exposure to INAP whilst P_{ETCO_2} and P_{ETO_2} were no longer controlled (i.e. inspired gas was of room air composition). INAP consisted of breathing room air against a negative pressure of -10 cmH_2O for 30 s, interspersed with one-minute periods of breathing at atmospheric pressure. Following the final exposure to negative pressure the P_{ETCO_2} was elevated to the same absolute level as achieved during the baseline period and $P_{ETO_2}$was again controlled at 100 mmHg. This was maintained for one hour which formed the recovery period.

2.5. Control trial

The Control trial was identical to the INAP trial except that the one-hour intervention period involved breathing room air at atmospheric pressure.

2.6. Instrumentation

2.6.1. Baseline and recovery periods

To measure ventilation during all baseline and recovery periods, participants wore a comfortable nasal mask (a modified TrueBlue, Philips Respironics, USA). A good seal was ensured at all times by careful observation of gas waveforms on the computer monitor. Respiratory gases were sampled and analysed continuously via a catheter connected to oxygen and carbon dioxide analysers (CD-3A and S-3A/I, AEI Technologies, USA). P_{ETCO_2} and P_{ETO_2} were controlled using a computerised dynamic end-tidal forcing system, described in detail elsewhere (Robbins et al., 1982). Inspired gases were heated and humidified. Respiratory volumes were measured by use of a rotating vane spirometer (VMM-400, Interface Associates, USA) and ventilation is reported in BTPS. The stability of gas analysers and the rotary vane

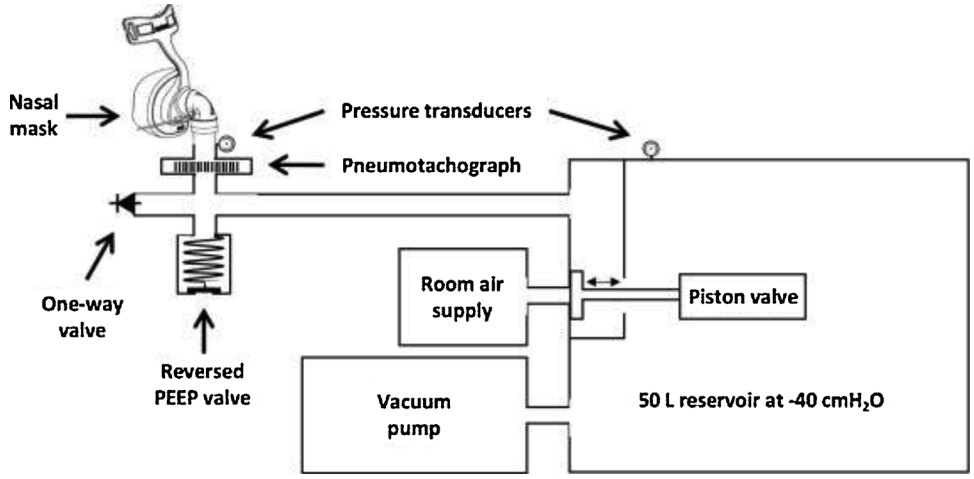

Fig. 1. Schematic showing the experimental setup used to induce INAP.

spirometer were confirmed by verifying with calibration gas and a three-litre volume syringe immediately before and after each experimental trial.

2.6.2. Intervention period

During the one-hour intervention period participants wore the same nasal mask as during the baseline and recovery periods but they were connected to a circuit of variable breathing pressure (Fig. 1). When breathing at atmospheric pressure, participants inspired from a continuous flow of room air supplied by a pump (REMstar Pro M series CPAP, Respironics, USA). A one-way valve allowed excess and expired air to be expelled from the breathing circuit, preventing re-breathing (5800 Giant, Hands Rudolph Inc, USA). When breathing against negative pressure, participants breathed from a 50-litre reservoir that was maintained at -40 cmH_2O by a vacuum pump. A spring-loaded 'reversed' positive end-expiratory pressure (PEEP) valve positioned close to the nasal mask allowed air to leak into the circuit and it could be adjusted to ensure that upper airway pressure was controlled at -10 cmH_2O. A pressure transducer (40PC, Honeywell, USA) attached to the nasal mask allowed us to monitor the adjustment of the PEEP valve and served as an indirect measurement of upper airway pressure. Switching between negative and atmospheric pressures was a fully automated procedure that used a computer-based signal acquisition system (Spike2; CED, Cambridge UK) to operate a piston solenoid valve which allowed a rapid switch between the two conditions. The design of this system ensured that a change of pressure was felt at the airway in less than 10 ms every time the breathing circuit changed from atmospheric to negative pressure and vice versa. The activation of the piston was controlled using the waveform of respiration through a pneumotachograph (4813, Hands Rudolph, USA). After each minute of breathing at atmospheric pressure the piston was activated as soon as flow through a pneumotachograph became positive (i.e. the start of inspiration). After 30 s of breathing against negative pressure the piston would again be activated as soon as flow through the pneumotachograph became negative (i.e. the start of expiration) returning the breathing circuit to atmospheric pressure.

2.7. Measuring the negative pressure reflex

One participant visited the laboratory for an additional trial in order to confirm the 30 s exposures to -10 cmH_2O did indeed stimulate pressure receptors within the airways and activate the negative pressure reflex. The genioglossus muscle is responsible for protruding the tongue and acts as one of the primary dilator muscles of the upper airway. Several studies have shown an elevation in genioglossus EMG activity to occur within 30–50 milliseconds following exposure to negative pressure in humans, consistent with other short latency neural reflexes (Horner et al., 1991a, Horner et al., 1991b; 1994; Innes et al., 1995; Shea et al., 1999; Wheatley et al., 1993). As such, continuous measurements of genioglossus activity were made before, during and after 30-second exposures to -10 cmH_2O using a non-invasive surface EMG. A custom made gum shield was constructed in order to hold the surface electrodes in contact with the mucosa of the floor of the mouth (directly above the genioglossus) as previously described by (O'Connor et al., 2007). To this end, dental impression material was applied to the bottom teeth and the floor of the mouth. A gum shield was then made from the impression and two silver coated wire electrodes were sewn into the underlying surface. A ground electrode was placed on the participant's forehead to filter background noise. The genioglossus surface EMG signal was then amplified and filtered (amplification: 10,000, filtered: 1–450 Hz) using a pre-amplifier (Bagnoli-2, Delsys, USA) positioned close to the mouth. Signals were recorded at 4000 Hz using a computer-based signal acquisition system (Spike2; CED, Cambridge, UK) for offline analysis.

2.8. Ventilatory analyses

In all experimental trials minute ventilation ($\dot{V}_E$), breathing frequency (BF), tidal volume (V_T), P_{ETCO_2} and P_{ETO_2} were recorded continuously and will be referred to collectively as ventilatory measurements from here on. Ventilatory measurements were made during baseline and recovery by averaging the last five minutes of each period (i.e. 15–20 minutes of baseline and 55–60 minutes recovery).

2.9. Statistical analysis

A two-way analysis of variance (ANOVA) with repeated measures in conjunction with a post hoc Bonferroni adjustment was used to assess whether ventilatory measurements during recovery were significantly different from baseline within each trial and whether there were significant differences between trials. Differences were considered significant if $P \leq 0.05$. Data is expressed as mean ± SEM except demographic data which is mean ± SD.

3. Results

3.1. Participants

Thirteen healthy male participants completed the experiment, age 20.9 ± 2.8 years, weight 77.2 ± 8.6 kg and height 1.80 ± 0.07 m. The mean body mass index (BMI) was 23.8 ± 2.3.

Table 1
Averaged P_{ETCO_2}, P_{ETO_2}, V_T and BF measurements during baseline and recovery.

	INAP		Control	
	Baseline	Recovery	Baseline	Recovery
$PETCO_2$ (mmHg)	43.4 ± 0.4	43.4 ± 0.4	43.9 ± 0.4	43.9 ± 0.5
$PETO_2$ (mmHg)	100.09 ± 0.1	100.03 ± 0.0	100.03 ± 0.0	100.04 ± 0.0
VT (ml)	1072 ± 84	1132 ± 86	1145 ± 119	1163 ± 117
BF (bpm)	14.8 ± 1.2	17.9 ± 0.8 [a,b]	14.6 ± 1.3	16.0 ± 1.1[a]

[a] Indicates significantly greater than baseline of the same trial.
[b] Indicates significantly greater than the corresponding time point of the Control trial. "bpm" stands for "breaths per minute".

3.2. Gas control

The dynamic end-tidal forcing system is capable of controlling end-tidal gases with impressive precision indicated by the small variability shown below. In both trials $P_{ETCO_2}$was elevated by 3–4 mmHg above resting conditions to induce mild hypercapnia and was tightly matched between baseline and recovery periods (INAP: 43.4 ± 0.4 vs 43.4 ± 0.4 and Control: 43.9 ± 0.4 vs 43.9 ± 0.5 mmHg). Furthermore, normoxia was maintained throughout baseline and recovery periods in both trials (INAP: 100.09 ± 0.1 vs 100.03 ± 0.0 and Control: 100.03 ± 0.0 vs 100.04 ± 0.0 mmHg). See Table 1.

3.3. Ventilation

Fig. 2 shows average $\dot{V}_E$ during each minute of the INAP and Control trials. Fig. 3 shows ventilation averaged over the last five minutes of the baseline and recovery periods. As expected $\dot{V}_E$ increased rapidly in response to hypercapnia (P_{ETCO_2} elevated by 3–4 mmHg) during the 20-minute baseline period and to the same level in the two trials (P = 0.806). During the first 20 min of recovery, $\dot{V}_E$ also rapidly increased in both trials in response to the re-initiation of hypercapnia, to a comparable level to that of the 20-minute baseline period. In the control trial, $\dot{V}_E$ then continued to increase albeit much more gradually over the remaining 40 min of the recovery hour and as such, during the final five minutes of recovery it was significantly greater than baseline by 2.16 ± 0.83 L/min (P = 0.023). However, in the INAP trial the change in $\dot{V}_E$ between baseline and the end of the recovery period was significantly greater than the Control trial (P = 0.019). Indeed, $\dot{V}_E$ during the end of recovery had increased by 4.22 ± 1.10 L/min from baseline (P = 0.002) and was significantly greater than the comparable time point in the Control trial (P = 0.009). See Fig. 3.

This increase in $\dot{V}_E$ in the INAP trial was predominately a product of increased BF. Indeed, BF during recovery increased by 3.09 ± 0.88 bpm from baseline (P = 0.004). In the Control trial BF also significantly increased, but by a more modest degree of 1.43 ± 0.59 bpm (P = 0.033). As such, BF during recovery was significantly greater in the INAP trial (P = 0.034). In contrast V_T did not significantly increase in either trial (INAP: P = 0.240, Control: P = 0.610). See Table 1.

3.4. Genioglossus EMG

Fig. 4 is a raw trace of genioglossus EMG activity recorded from one participant during a 30 s exposure to -10 cmH_2O, which was identical to those performed in the INAP trial. The figure clearly shows an elevation in EMG activity throughout the 30 s exposure to negative pressure with a rapid return to baseline levels following resumption of atmospheric pressure.

4. Discussion

The primary finding of this study was that exposure to one hour of INAP induces a sustained elevation in ventilation in awake, healthy humans. To the best of our knowledge, this is the first study to investigate and demonstrate INAP-induced rLTF in humans.

Although this study was not designed to specifically investigate the responsible mechanism(s) we speculated that they could be the same spinal serotonin-dependent mechanism shown to be responsible for IH-induced rLTF and sLTF in animal studies. However, the significant increase in BF rather than V_T in our study suggests an alternative

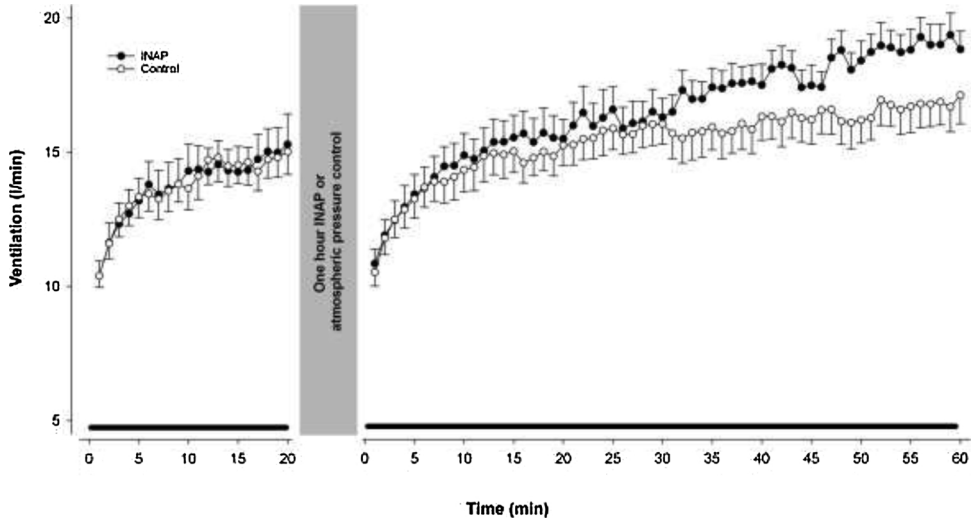

Fig. 2. $\dot{V}_E$ averaged for all participants for each minute of the INAP trial (closed circles) and the Control trial (open circles). Shaded area represents the intervention period [INAP or atmospheric pressure (Control trial)] where P_{ETCO_2} was not elevated. Black bars represent periods during which P_{ETCO_2} was elevated 3–4 mmHg above normal.

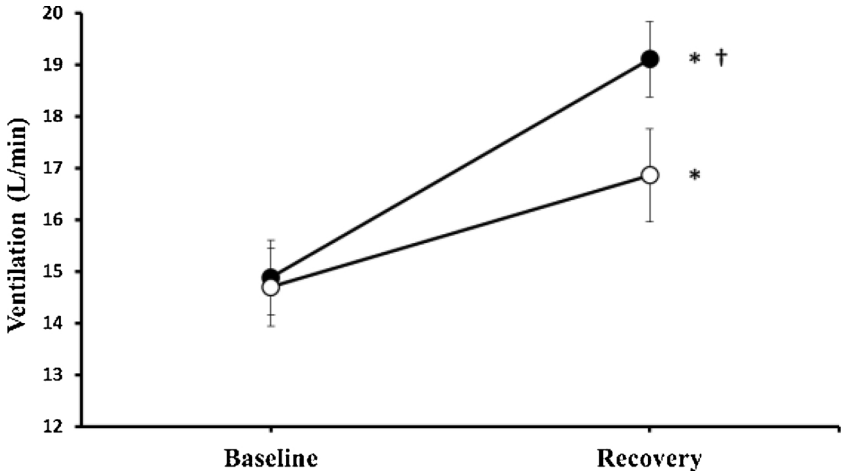

Fig. 3. Change in $\dot{V}_E$ from baseline to recovery during the INAP trial (closed markers) and Control trial (open markers). * indicates significantly greater than baseline of the same trial, † indicates significantly greater than the corresponding time point of the Control trial.

mechanism may be responsible. Neural plasticity within the spinal cord would have been expected to have manifested as an enhancement in V_T as discussed in more detail below.

Although INAP-induced rLTF has only been investigated and demonstrated once in rats (Ryan and Nolan, 2009b), there exist several decades of research demonstrating IH-induced rLTF in both humans and other species (Mateika and Sandhu, 2011). In animal studies IH-induced rLTF following IH has been identified as a form of neural plasticity within the spinal cord that augments diaphragm activity and thus V_T (Baker-Herman and Mitchell, 2002; MacFarlane and Mitchell, 2009; Mahamed and Mitchell, 2007). More specifically, IH causes the release of serotonin in the spinal cord (Baker-Herman and Mitchell, 2002) which activates serotonin receptors (5-HT_2) on respiratory motoneurons (Fuller et al., 2001; Kinkead and Mitchell, 1999).

Recent animal studies using similar methodologies have shown the same serotonin-dependent mechanism to be responsible for somatic motor activity (sLTF) following IH (Lovett-Barr et al., 2012).

In studies of humans, the rLTF following IH is a product of both a significant increase in V_T and BF (Gerst et al., 2011; Harris et al., 2006; Lee et al., 2009; Wadhwa et al., 2008; Griffin et al., 2012). However, the requirement for invasive, unsafe techniques has meant the spinal serotonin-dependent mechanism shown to be responsible for the increased V_T in animal studies cannot be confirmed or refuted in humans. Furthermore, the responsible mechanism(s) for the enhanced BF remains to be determined. Nonetheless, having identified spinal neural plasticity as the responsible mechanism for IH-induced elevations in rLTF and sLTF in animal studies, researchers have investigated whether this phenomenon could benefit patients with incomplete spinal cord damage. Results have thus far been promising, as IH has been shown to increase calf muscle strength and walking speed in these patients (Hayes et al., 2014; Trumbower et al., 2012) which supports but doesn't confirm the hypothesis that IH elicits a spinal serotonin dependent neuroplasticity.

However, there remains some apprehension about its use as a clinical therapy in light of previous evidence to suggest chronic exposure to IH can have health consequences such as, increased BP, decreased insulin sensitivity and increased risk of cardiovascular events (Foster et al., 2007; Levy et al., 2008; Lavie and Lavie, 2009).We were therefore, attracted to investigating whether INAP could elicit rLTF because we considered that it might be a safer stimulus than IH and that the study by Ryan and Nolan (2009b) presented results that suggested INAP might share the same spinal serotonin-dependant mechanism as IH-induced LTF. That study, however, was not designed to specifically investigate the responsible mechanisms of INAP-induced rTLF and we are not aware of any further research to have done so. Nonetheless, they showed that the magnitude of rLTF following INAP was comparable to

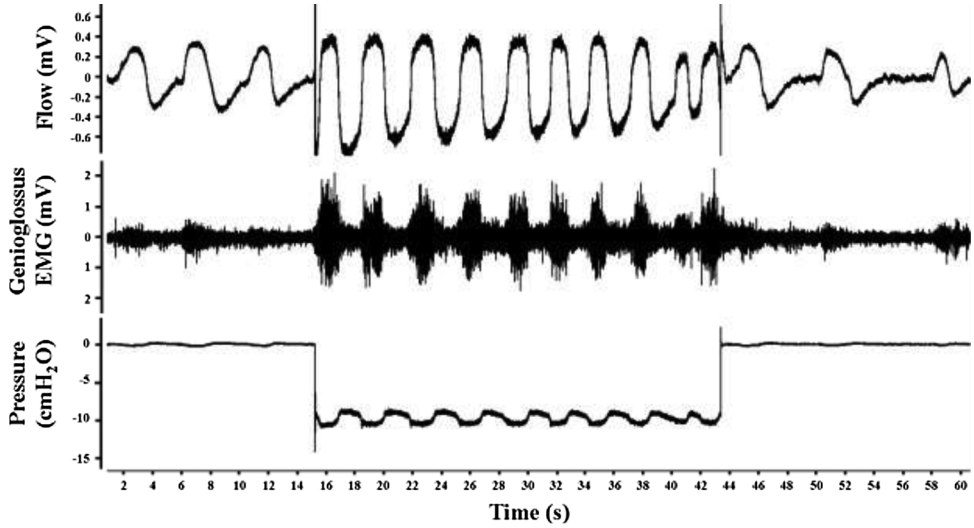

Fig. 4. Raw traces from one participant of uncalibrated flow through the pneumotach (top panel), genioglossus EMG activity (middle panel) and mask pressure (bottom panel).

that occurring after IH using the same rat model. Furthermore, they showed that combining IH with INAP does not increase the magnitude of rLTF elicited by INAP alone, which might have been expected if the responsible mechanistic pathways of the two stimuli were different (Ryan and Nolan, 2009b).

In contrast to previous studies investigating IH-induced rLTF in humans, our study investigating the effects of INAP did not show a significant increase in V_T whereas BF was significantly increased (Gerst et al., 2011; Harris et al., 2006; Lee et al., 2009; Wadhwa et al., 2008; Griffin et al., 2012). In contrast to changes in V_T which are thought to reflect modifications in spinal motor neuronal activity, changes in BF are thought to be mediated at the medullary level. Although purely speculative in the absence of any mechanistic research we would like to suggest a possible alternative 5-HT dependent mechanism for INAP-induced rLTF. It is plausible that repeated activation of the negative pressure reflex and subsequent stimulation of the superior laryngeal nerve could activate 5-HT receptors in the nucleus tractus solitarii and enhance inspiratory drive. In young infants but not adults, inspiratory drive is attenuated during liquid exposure in the larynx due to the laryngeal chemoreflex (Downing and Lee, 1975; Fagenholz et al., 1979). Afferents mediating the laryngeal chemoreflex are located predominately in the superior laryngeal nerve, and this is also true for afferents mediating the negative pressure reflex as shown by studies that have attenuated both reflexes by bilateral sectioning of the superior laryngeal nerve (Mathew and Farber, 1983; Ryan et al., 2001). In contrast to the attenuating effect of the laryngeal chemoreflex on breathing, recent research in rodents has shown it also stimulates afferent pathways in the superior laryngeal nerve that activate 5-HT_3 receptors within the nucleus tractus solitarii and stimulate breathing (Donnelly et al., 2016). This recent finding may be of significance when it is considered that the negative pressure reflex also has neural connections to respiratory control centers in the brain as exposure to negative pressure causes a reflex attenuation in inspiratory drive (Mathew and Farber, 1983; Mathew et al., 1982b; Ryan and Nolan, 2009b; Ryan et al., 2001). Although speculative, if the negative pressure reflex also causes the release of 5-HT in the nucleus tractus solitarii (NTS) then the repeated excitation of 5-HT receptors during INAP could in theory mediate the rLTF shown in our study.

Although INAP was easily tolerated by healthy volunteers in this study, patients with acute or chronic hypoventilation disorders (e.g. respiratory muscle weakness, obesity, motor-neurone disease etc) are less likely to endure INAP exposures and the potential risks may outweigh any benefits. However, a clear understanding of the responsible mechanisms could theoretically be used to develop an indirect pharmaceutical method of invoking the same sustained elevation in ventilation. It should be noted that even if a pharmaceutical agent could be identified there would be significant challenges in localising its actions to the desired site, such as the NTS. The invasive techniques required to investigate the proposed superior laryngeal nerve mechanism (sectioning of the nerve) and also the previously discussed spinal serotonin-dependent mechanism (spinal cord injections of serotonin or 5-HT_2 inhibition) cannot currently be replicated in humans. As such, animal studies may be required to investigate the potential mechanisms for INAP-induced rLTF.

4.1. Hypercapnia and rLTF

In our study we were able to demonstrate a sustained elevation in ventilation under mildly hypercapnic conditions following a one-hour exposure to INAP. The importance of increasing carbon dioxide levels in the expression of rLTF has been well investigated in studies using IH. Despite numerous attempts, IH-induced rLTF was not demonstrated in awake humans until more than two decades after the first published account in animals (Mateika and Sandhu, 2011). Harris et al. (2006) first reported the existence of rLTF in awake humans and provided compelling evidence to show P_{ETCO_2} needed to be elevated in order to unmask its appearance. The authors suggest the absence of rLTF in awake humans in previous studies was due to carbon dioxide levels being below the threshold for the central and peripheral chemoreflex. As such, ventilation may have been predominantly driven by arousal and/or behavioural stimuli, termed the 'wakefulness drive'. Indeed, our group and others have since been able to demonstrate IH-induced rLTF in awake humans only when P_{ETCO_2} has been raised above resting levels (Gerst et al., 2011; Harris et al., 2006; Lee et al., 2009; Mateika and Sandhu, 2011; Wadhwa et al., 2008; Griffin et al., 2012).

We have previously shown that during hypercapnic exposures there is a rapid increase in $\dot{V}_E$ for ~15 min followed by a more gradual increase that lasts a further ~25 min (Griffin et al., 2012). A similar gradual rise in $\dot{V}_E$ under hypercapnic conditions has also been shown by some (Gerst et al., 2011) but not all (Harris et al., 2006). The change in $\dot{V}_E$ in the Control trial in the present study follows a similar pattern, although no clear plateau is reached during the one-hour recovery period. The mechanism(s) responsible for the gradual rise in $\dot{V}_E$ under stable P_{ETCO_2} conditions remains to be determined, although previous data by our group have shown it is not mediated by arterial chemoreceptors (Griffin et al., 2012). A potential explanation may be that because the permeability of the blood-brain barrier to H^+ is poor, during hypercapnic exposures there is lag between a rapid rise in H^+ in arterial blood (stimulating arterial chemoreceptors) and a delayed rise in the brain (stimulating medullary chemoreceptors). Indeed, it has previously been shown in dogs that during intravenous H^+ infusions, an almost immediate elevation in ventilation occurs simultaneously with increased arterial H^+. Further elevations in ventilation do not occur until > 3 h later when levels of H^+ begin to rise in the cerebrospinal fluid (Bureau et al., 1979).

4.2. Future research to optimise INAP-induced rLTF

Several decades of animal and human research have been undertaken in order to optimise the rLTF induced by exposure to IH (Mateika and Sandhu, 2011). In contrast, to the best of our knowledge we are the first to investigate whether INAP can induce rLTF in humans and as such, had limited evidence to guide the design of our protocol. It is therefore possible that INAP could elicit a substantially greater magnitude of rLTF by changing specific features of our study design.

We chose to lower the pressure to -10 cmH_2O during the INAP intervention to match the level shown by Ryan and colleagues used to induce rLTF in rats. Furthermore, -10 cmH_2O has previously been shown to elicit a neural reflex that causes contraction of upper airway dilator muscles in humans (Akahoshi et al., 2001; Doherty et al., 2008; Mortimore and Douglas, 1997; Horner et al., 1991a,b). We confirmed that -10 cmH_2O was sufficient to activate the reflex in our experimental setup by recording EMG activity of the genioglossus muscle during INAP (see Fig. 4). However, Horner et al. (1991a) have previously demonstrated that the magnitude of the reflex does not plateau until the pressure is as low as -35 cmH_2O. Nonetheless, we chose not to expose participants to pressures of more negative than - 10 cmH_20 because we felt an hour exposure to this more negative INAP could elicit respiratory muscle fatigue and attenuate any rLTF. Future studies should now investigate what pressure is required to induce the greatest magnitude of rLTF.

Finally, it is well known that IH-induced rLTF is greatly affected by both the frequency and duration of hypoxic episodes (Mateika and Sandhu, 2011). Therefore, future studies should consider investigating whether the magnitude of INAP-induced rLTF is also affected by these parameters.

In contrast to the previously discussed potential to increase the magnitude of rLTF it is important to consider factors that might attenuate the response. It is interesting to note that animal and human studies suggest rLTF following IH is attenuated by increased age and is also reduced in females (Mateika and Sandhu, 2011). In our study,

healthy, young male participants were tested (mean age 20.9 ± 2.8 years) because we have previously shown this population is capable of demonstrating IH-induced rLTF and are predominately free from confounding factors such as, sleep apnoea and cardiovascular disease. Future studies should now investigate whether similar results are demonstrated in different populations including the elderly and pre and post-menopausal women. This research may be important when considering whether INAP-induced rLTF or indirect activation of the mechanistic pathway could be used to benefits patients with respiratory insufficiency as these patient groups are often of increased age.

5. Conclusions

In conclusion, we have shown that an acute exposure of INAP elicits rLTF in awake, healthy humans as previously shown in anaesthetised rats. Further research is required to identify the responsible mechanisms as this may help in determining whether INAP-induced rLTF could be utilised to benefit patients with acute or chronic hypoventilation disorders.

References

Akahoshi, T., White, D.P., Edwards, J.K., Beauregard, J., Shea, S.A., 2001. Phasic mechanoreceptor stimuli can induce phasic activation of upper airway muscles in humans. Journal of Physiology-London 531, 677–691.

Baker-Herman, T.L., Mitchell, G.S., 2002. Phrenic long-term facilitation requires spinal **serotonin** receptor activation and protein synthesis. J. Neurosci. 22, 6239–6246.

Bureau, M.A., Ouellet, G., Begin, R., Gagnon, N., Geoffroy, L., Berthiaume, Y., 1979. Dynamics of the control of ventilation during metabolic acidosis and its correction. Am. Rev. Respir. Dis. 119, 933–939.

Curran, A.K., Eastwood, P.R., Harms, C.A., Smith, C.A., Dempsey, J.A., 1997. Superior laryngeal nerve section alters responses to upper airway distortion in sleeping dogs. J. Appl. Physiol. 83, 768–775.

Doherty, L.S., Cullen, J.P., Nolan, P., Mcnicholas, W.T., 2008. The human genioglossus response to negative airway pressure: stimulus timing and route of delivery. Exp. Physiol. 93, 288–295.

Donnelly, W.T., Bartlett Jr., D., Leiter, J.C., 2016. Serotonin in the solitary tract nucleus shortens the laryngeal chemoreflex in anaesthetized neonatal rats. Exp. Physiol. 101, 946–961.

Downing, S.E., Lee, J.C., 1975. Laryngeal chemosensitivity: a possible mechanism for sudden infant death. Pediatrics 55, 640–649.

Fagenholz, S.A., Lee, J.C., Downing, S.E., 1979. Laryngeal reflex apnea in the chemodenervated newborn piglet. Am. J. Physiol. 237, R10–4.

Foster, G.E., Poulin, M.J., Hanly, P.J., 2007. Intermittent hypoxia and vascular function: implications for obstructive sleep apnoea. Exp. Physiol. 92, 51–65.

Fuller, D.D., Zabka, A.G., Baker, T.L., Mitchell, G.S., 2001. Physiological and genomic consequences of intermittent hypoxia - selected contribution: phrenic long-term facilitation requires 5-HT receptor activation during but not following episodic hypoxia. J. Appl. Physiol. 90, 2001–2006.

Gerst, D.G., Yokhana, S.S., Carney, L.M., Lee, D.S., Badr, M.S., Qureshi, T., Anthouard, M.N., Mateika, J.H., 2011. The hypoxic ventilatory response and ventilatory long-term facilitation are altered by time of day and repeated daily exposure to intermittent hypoxia. J. Appl. Physiol. 110, 15–28.

Griffin, H.S., Pugh, K., Kumar, P., Balanos, G.M., 2012. Long-term facilitation of ventilation following acute continuous hypoxia in awake humans during sustained hypercapnia. J. Physiol. London 590, 5151–5165.

Harris, D.P., Balasubramaniam, A., Badr, M.S., Mateika, J.H., 2006. Long-term facilitation of ventilation and genioglossus muscle activity is evident in the presence of elevated levels of carbon dioxide in awake humans. Am. J. Physiol. Renal. Physiol. 291, R1111–R1119.

Hayes, H.B., Jayaraman, A., Herrmann, M., Mitchell, G.S., Rymer, W.Z., Trumbower, R.D., 2014. Daily intermittent hypoxia enhances walking after chronic spinal cord injury A randomized trial. Neurology 82, 104–113.

Horner, R.L., Innes, J.A., Murphy, K., Guz, A., 1991a. Evidence for reflex upper airway dilator muscle activation by sudden negative airway pressure in man. J Physiol 436, 15–29.

Horner, R.L., Innes, J.A., Holden, H.B., Guz, A., 1991b. Afferent pathway(s) for pharyngeal dilator reflex to negative-pressure in man: a study using upper airway anesthesia. J. Physiol. London 436, 31–44.

Horner, R.L., Innes, J.A., Morrell, M.J., Shea, S.A., Guz, A., 1994. The effect of sleep on reflex genioglossus muscle activation by stimuli of negative airway pressure in humans. J. Physiol. (Paris) 476, 141–151.

Hwang, J.C., Stjohn, W.M., Bartlett Jr, D., 1984. Afferent pathways for hypoglossal and phrenic responses to changes in upper airway pressure. Respir. Physiol. 55, 341–354.

Innes, J.A., Morrell, M.J., Kobayashi, I., Hamilton, R.D., Guz, A., 1995. Central and reflex neural control of genioglossus in subjects who underwent laryngectomy. J. Appl. Physiol. 78 (1985), 2180–2186.

Kinkead, R., Mitchell, G.S., 1999. Time-dependent hypoxic ventilatory responses in rats: effects of ketanserin and 5-carboxamidotryptamine. Am. J. Physiol. Renal. Physiol. 277, R658–R666.

Lavie, L., Lavie, P., 2009. Molecular mechanisms of cardiovascular disease in OSAHS: the oxidative stress link. Eur. Respir. J. 33, 1467–1484.

Lee, D.S., Badr, M.S., Mateika, J.H., 2009. Progressive augmentation and ventilatory long-term facilitation are enhanced in sleep apnoea patients and are mitigated by antioxidant administration. J.Physiol. London 587, 5451–5467.

Levy, P., Pepin, J.L., Arnaud, C., Tamisier, R., Borel, J.C., Dematteis, M., Godin-Ribuot, D., Ribuot, C., 2008. Intermittent hypoxia and sleep-disordered breathing: current concepts and perspectives. Eur. Respir. J. 32, 1082–1095.

Lovett-Barr, M.R., Satriotomo, I., Muir, G.D., Wilkerson, J.E.R., Hoffman, M.S., Vinit, S., Mitchell, G.S., 2012. Repetitive intermittent hypoxia induces respiratory and somatic motor recovery after chronic cervical spinal injury. J. Neurosci. 32, 3591–3600.

Macfarlane, P.M., Mitchell, G.S., 2009. Episodic spinal serotonin receptor activation elicits long-lasting phrenic motor facilitation by an NADPH oxidase-dependent mechanism. J.Physiol. London 587, 5469–5481.

Mahamed, S., Mitchell, G.S., 2007. Is there a link between intermittent hypoxia-induced respiratory plasticity and obstructive sleep apnoea? Exp. Physiol. 92, 27–37.

Mateika, J.H., Sandhu, K.S., 2011. Experimental protocols and preparations to study respiratory long term facilitation. Respir. Physiol. Neurobiol. 176, 1–11.

Mathew, O.P., Farber, J.P., 1983. Effect of upper airway negative pressure on respiratory timing. Respir. Physiol. 54, 259–268.

Mathew, O.P., Abu-Osba, Y.K., Thach, B.T., 1982a. Genioglossus muscle responses to upper airway pressure changes: afferent pathways. J. Appl. Physiol. Respir. Environ. Exerc. Physiol. 52, 445–450.

Mathew, O.P., Abu-Osba, Y.K., Thach, B.T., 1982b. Influence of upper airway pressure changes on respiratory frequency. Respir. Physiol. 49, 223–233.

Mitchell, G.S., Johnson, S.M., 2003. Neuroplasticity in respiratory motor control. J Appl Physiol. 94, 358–374.

Mortimore, I.L., Douglas, N.J., 1997. Palatal muscle EMG response to negative pressure in awake sleep apneic and control subjects. Am. J. Respir. Crit. Care Med. 156, 867–873.

O'connor, C.M., Lowery, M.M., Doherty, L.S., Mchugh, M., O'muircheartaigh, C., Cullen, J., Nolan, P., Mcnicholas, W.T., O'malley, M.J., 2007. Improved surface EMG electrode for measuring genioglossus muscle activity. Respir. Physiol. Neurobiol. 159, 55–67.

Robbins, P.A., Swanson, G.D., Howson, M.G., 1982. A prediction-correction scheme for forcing alveolar gases along certain time courses. J. Appl. Physiol. 52, 1353–1357.

Ryan, S., Nolan, P., 2009a. Episodic hypoxia induces long-term facilitation of upper airway muscle activity in spontaneously breathing anaesthetized rats. J. Physiol. London 587, 3329–3342.

Ryan, S., Nolan, P., 2009b. Long-term facilitation of upper airway muscle activity induced by episodic upper airway negative pressure and hypoxia in spontaneously breathing anaesthetized rats. J. Physiol. London 587, 3343–3353.

Ryan, S., Mcnicholas, W.T., O'regan, R.G., Nolan, P., 2001. Reflex respiratory response to changes in upper airway pressure in the anaesthetized rat. J. Physiol. London 537, 251–265.

Shea, S.A., Edwards, J.K., White, D.P., 1999. Effect of wake-sleep transitions and rapid eye movement sleep on pharyngeal muscle response to negative pressure in humans. J. Physiol. 520 (Pt 3), 897–908.

Trumbower, R.D., Jayaraman, A., Mitchell, G.S., Rymer, W.Z., 2012. Exposure to acute intermittent hypoxia augments somatic motor function in humans with incomplete spinal cord injury. Neurorehabil. Neural Repair 26, 163–172.

Van Lunteren, E., Van de Graaff, W.B., Parker, D.M., Mitra, J., Haxhiu, M.A., Strohl, K.P., Cherniack, N.S., 1984. Nasal and laryngeal reflex responses to negative upper airway pressure. J. Appl. Physiol. Respir. Environ. Exerc. Physiol. 56, 746–752.

Wadhwa, H., Gradinaru, C., Gates, G.J., Badr, M.S., Mateika, J.H., 2008. Impact of intermittent hypoxia on long-term facilitation of minute ventilation and heart rate variability in men and women: do sex differences exist? J. Appl. Physiol. 104, 1625–1633.

Wheatley, J.R., Mezzanotte, W.S., Tangel, D.J., White, D.P., 1993. Influence of sleep on genioglossus muscle activation by negative pressure in normal men. Am. Rev. Respir. Dis. 148, 597–605.