Respiration and Emotion: How and where are they linked?

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Thesis submitted for PhD (Human Physiology)

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The thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made available in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act of 1968.*

* Part of the experiment in Chapter 1 (10 animals implanted with telemetric transmitters for cardiac assessment) was performed as part of my Honours project.

The data was subsequently reanalyzed, combined with data obtained during my PhD project and submitted for publication, which was written as part of my PhD project.

I hereby certify that this thesis is in the form of a series of published papers of which I am a joint author. I have included as part of the thesis a written statement from each co-author, endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the joint publications.

The following publications are included as part of the thesis:

Bondarenko, E., Hodgson, D.M. and Nalivaiko, E. (*submitted*). Respiratory arousal is an autonomic and a behavioral index of anxiety in rats.

Bondarenko, E., Beig, M.I., Hodgson, D.M., Braga, V. and Nalivaiko, E., 2015. Blockade of the dorsomedial hypothalamus and the perifornical area inhibits respiratory responses to arousing and stressful stimuli. Am. J. Physiol. Regul. Integr. Comp. Physiol., doi: 10.1152/ajpregu.00415.2014.

Bondarenko, E., Hodgson, D.M., Nalivaiko, E., 2014. Amygdala mediates respiratory responses to sudden arousing stimuli and to restraint stress in rats. Am. J. Physiol. Regul. Integr. Comp. Physiol. 306, R951-R959, doi: 10.1152/ajpregu.00528.2013.

Bondarenko, E., Hodgson, D.M., Nalivaiko, E., 2014. Prelimbic prefrontal cortex mediates respiratory responses to mild and potent prolonged, but not brief, stressors. Respir. Physiol. Neurobiol., doi: 10.1016/j.resp.2014.07.009.

I, Eugene Nalivaiko, attest that Research Higher Degree candidate Evgeny Bondarenko performed experiments, analyzed data, interpreted results of experiments, prepared figures, drafted, edited and revised the manuscript “Respiratory arousal is an autonomic and a behavioral index of anxiety in rats.”

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CONTENTS:

ABSTRACT

The link between respiration and emotions is well documented in humans, but animal studies addressing this issue are limited. The current project aims to systematically examine respiratory responses to stressors and stimuli of various intensity and length in rats, compare and correlate these responses with cardiac responses and behavioural indices of anxiety and lastly to investigate central neuronal pathways that mediate them. In the first chapter we show that respiratory responses to brief changes in arousal are more sensitive than traditionally used cardiac responses. Furthermore, respiration during the novelty stress is highly correlated with behavioural indices of anxiety in rats. The subsequent three chapters investigate involvement of the dorsomedial hypothalamic area, the amygdala and the prelimbic prefrontal cortex in mediating respiratory responses to brief and prolonged stimuli of various intensities. This is achieved by examining the effects of inhibition of the target areas with a microinjection of $GABA_A$ agonist muscimol. Inhibition of the dorsomedial hypothalamic area abolished respiratory response to the novelty and restraint stress protocols and also significantly inhibited responses to the brief acoustic stimuli. Blockade of the amygdala significantly inhibited responses to the high-intensity stressors of both brief (70-90dB acoustic stimuli) and prolonged (restraint) duration, but had little effect on responses to the low-intensity stimuli (novelty stress and 40-70dB acoustic stimuli). Lastly, inhibition of the prelimbic prefrontal cortex significantly inhibited the respiratory responses to the prolonged stressors (restraint and novelty stress), but had no effect on responses to brief stimuli (acoustic stimuli). Overall, our findings suggest that (i) assessment of respiratory response can be used as a novel index of anxiety in rats; (ii) respiratory rate is more sensitive to changes in arousal than traditionally used heart rate, which has implications for the definition of an orienting response as it is currently defined only in terms of heart rate. Lastly, (iii) we show that the dorsomedial hypothalamic area and the amygdala have critical roles in mediating stress-induced respiratory changes.

OVERVIEW

Links between respiration and emotion were demonstrated in humans a century ago (Feleky, 1914); yet very little animal research has been done on this topic. Our recent observations suggest that respiratory rate is closely related to animals' anxiety state as it is sensitive to anxiolytic drug diazepam (Nalivaiko et al., 2011) and is elevated in animals bred for high anxiety behaviour (Carnevali et al., 2013). Yet, to date, there was no systematic investigation of respiratory changes accompanying brief and prolonged changes in physiological arousal and central neuronal structures that mediate such changes.

The aim of this thesis is to systematically investigate and document respiratory responses to stimuli and stressors of various intensities and duration and to investigate underlying central pathways that mediate these responses. In the 1st Chapter we compare and correlate such responses with cardiac responses, a traditionally used index of autonomic activation, and also with behavioural measures of anxiety as assessed by the Elevated Plus Maze, a standard behavioural test for anxiety. Our results indicate that in rats respiratory responses to brief stimuli have a lower threshold of activation than cardiac responses. This finding has implications for the definition of an orienting response (i.e. a physiological response that indicates that an animal has paid attention to a stimulus). Furthermore, respiration during novelty stress was highly significantly correlated with behavioural measures of anxiety as assessed by the Elevated Plus Maze. This finding suggests that assessment of respiration can potentially be used as a measure of anxiety in animals.

The subsequent chapters investigate central neuronal pathways that mediate such respiratory responses to various stimuli. In particular, we investigate involvement of the dorsomedial hypothalamic area (Chapter 2), the amygdala (Chapter 3) and the prelimbic prefrontal cortex (Chapter 4). Inhibition of the dorsomedial hypothalamic area almost completely abolished respiratory responses to brief acoustic stimuli and abolished an increase in respiration during prolonged stressors. Previous studies suggest that the dorsomedial hypothalamic area is the integrative centre of central autonomic information (Buijs and Van

Eden, 2000); inhibition of this area abolishes increases in heart rate and arterial pressure in response to stress (DiMicco et al., 1996). Our finding extends this previous knowledge and suggests that the dorsomedial hypothalamic area similarly integrates central respiratory command.

Both the amygdala and the prelimbic prefrontal cortex have projections to the dorsomedial hypothalamic area (Dampney et al., 2008). Inhibition of the amygdala significantly inhibited respiratory responses to the high intensity stimuli and stress, both brief and prolonged, which is in line with its well-documented role in fear and emotional processing. Interestingly, inhibition of this area did not affect respiratory responses to low-intensity stimuli and stress (i.e. those that evoke an orienting response). Inhibition of the prelimbic prefrontal cortex attenuated respiratory arousal during presentation of prolonged stressors – novelty stress and restraint, but did not affect generation of respiratory responses to brief acoustic stimuli. The exact role of the prelimbic prefrontal cortex in autonomic processing is still poorly understood; previous studies suggest that this area modulates cardiovascular changes during stress and baroreflex (Resstel and Corrêa, 2005). Our findings suggest that the prelimbic prefrontal cortex also modulates respiratory changes during stress by modulating the overall level of autonomic arousal; however, it does not affect brief changes in autonomic arousal.

The findings of the current thesis propose that respiratory activation can be used a non-invasive measure of autonomic activation in rats. Also, assessment of respiration during novelty stress can be used as a locomotion-independent measure of anxiety. This is of special merit to preclinical psychopharmacology field as all existing anxiety tests are based on animals' ambulation. The thesis further describes relative contribution of three central neuronal structures – the dorsomedial hypothalamic area, the amygdala and the prefrontal cortex – to central processing of respiratory activation in response to brief and prolonged stressors of varying intensities.

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CHAPTER 1

RESPIRATORY AROUSAL IS AN AUTONOMIC AND A BEHAVIOURAL INDEX OF ANXIETY IN RATS

INTRODUCTION TO CHAPTER 1

Stress can be defined as a state of intense psychophysiological arousal. The body reacts to stress by mobilisation of resources for a fight or flight response. Mild stress (or arousal) also results in mobilisation of resources, but for enhanced perception. In physiology the former is named a "defence response" (Hilton, 1982), while the later is called an "orienting response" (Sokolov, 1963). Both of these responses are traditionally defined in terms of cardiac changes – an increase in heart rate is an indicator of a defence reaction, while heart rate deceleration is indicative of an orienting response. However, a recent study in our laboratory demonstrated respiratory response to brief arousing stimuli in absence of any cardiac change in rats (Kabir et al., 2010), which suggests that at least in rodents respiratory responses to arousing stimuli might be more sensitive than cardiac responses.

Association between respiration and stress has been known in humans for more than a century (Feleky, 1914), but very few animal studies have investigated this relationship. A study by Dauger et al. (1998) demonstrated significant increase in respiratory rate in mice subjected to restraint stress. Also, basal respiratory rate of rats bred for low anxiety behavior was lower than that of rats bred for high anxiety behavior (Carnevali et al., 2013). Lastly, using an animal model of psychopathology we demonstrated that high anxiety caused by neonatal lipopolysacharine injection is associated with significantly higher respiratory rate responses to acoustic stimuli than controls (Sominsky et al., 2013). Therefore, there is some evidence that respiratory pattern is linked to animals' anxiety state; however this has never been systematically investigated.

The aim of chapter 1 of the current thesis is to compare and correlate respiratory responses to brief and prolonged stimuli and stressors with cardiac responses. I will investigate correlations between the two indices and thresholds of activation of each index. Also, I aim to investigate correlations between respiratory responses to various stimuli and stressors and animals' anxiety indices as determined by a traditionally used behavioural test (Elevated Plus Maze). Animals' anxiety state will be manipulated by administration of a

classical anxiolytic drug diazepam or saline prior to respiratory, cardiac and behavioural assessment.

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Respiratory arousal is an autonomic and a behavioural index of anxiety in rats

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ABSTRACT

The link between respiration and emotions is well documented in humans, but animal studies addressing this issue are limited. To fill this gap, we recorded respiratory (whole-body plethysmography) and cardiac (ECG telemetry) responses to sudden arousing stimuli (50-90 dB white noise) and to prolonged stressors (novelty and restraint) in conscious adult male Wistar rats. We also assessed sensitivity of these responses to the anxiolytic diazepam and correlated them with behavioural anxiety measures. Respiratory and heart rates progressively declined during the initial placement into the plethysmograph (novelty); both indices were also elevated by the restraint stress (n=10). Diazepam significantly inhibited tachypnoea, but not tachycardia, during novelty and restraint. Acoustic stimuli elicited transient tachypnoeic responses, with the amplitudes directly proportional and the latencies inversely proportional to the sound intensity. Cardiac responses to acoustic stimuli were tri-phasic (tachy-brady-tachycardia). Respiratory responses had a lower activation threshold compared to cardiac responses. Diazepam significantly inhibited respiratory responses, while the drug effect on the heart rate responses was contaminated by its tachycardic side action. We correlated respiratory responses to brief and prolonged stressors with behavioural indices of anxiety assessed by the elevated plus-maze. The latter were highly correlated with the respiratory rate during exposure to the novel environment. We conclude that: i) respiratory rate is a sensitive index of autonomic arousal, with substantial differences and advantages compared to heart rate; ii) respiratory response to mild stress (novelty) is potentially a very useful locomotion-independent index of anxiety in rats.

HIGHLIGHTS

- We recorded respiratory and heart rates in rats after diazepam or saline
- We presented brief and prolonged stimuli and stressors of varying intensities
- Respiratory responses were more sensitive than heart rate responses
- Diazepam inhibited respiratory responses, but had mixed effect on heart rate
- Respiration during novelty stress is highly correlated with EPM measures of anxiety

Stress and arousal evoke a pattern of behavioural and physiological changes, including changes in autonomic parameters. A term “defence response” is traditionally used to describe mobilization of bodily resources for a fight or flight response; defence response is associated with increases in cardiac and respiratory functions (Hilton and Redfern, 1986). Effects of stress on cardiovascular parameters have been studied extensively in both animals and humans; yet effects of stress on respiration have generally been neglected in the animal research. Most animal studies of respiration focused on homeostatic mechanisms and examined neural networks of respiratory rhythm-generating centres in the brainstem (see Feldman et al., 2013, Dutschmann and Dick, 2012, and Smith et al., 2013, for reviews). None of these studies have assessed respiratory responses to changes in emotional arousal. Only recently preclinical research has started investigating respiratory responses in animal models of anxiety. Kinkead and colleagues have described changes in respiratory phenotype in rats after neonatal maternal separation, such as altered respiratory responses to hypoxia (Genest et al., 2004) and hypercapnia (Genest et al., 2006). In our recent study we reported significant differences in basal respiratory rate and in sighing frequency in rats bred for high anxiety behaviour compared to rats bred for low anxiety behaviour (Carnevali et al., 2013). Taken together, these findings indicate that anxiety states may affect respiratory function in animals, similar to humans (Boiten et al., 1994).

Even less is currently known about respiratory changes that accompany mild arousal or an “orienting response”. Orienting response is a physiological response of paying attention to a stimulus; it is traditionally characterized by heart rate deceleration (Sokolov, 1963). However, we have recently reported that

presentation of alerting stimuli of various sensory modalities to conscious rats provoke vigorous respiratory responses ("sniffing") that often occur without any changes in cardiac parameters (Kabir et al., 2010; Nalivaiko et al., 2011). This is surprising, providing that brief bradycardia is a traditional feature of an "orienting response". Given the evidence of this response pattern, it is clear there is a need for a systematic description of respiratory and cardiac responses to stimuli of varying length and intensity; this is the primary aim of the present work. Comparison between the activation patterns of cardiac and respiratory parameters in response to alerting stimuli and stress may lead to the establishment of respiratory changes as a new, more sensitive index of autonomic activation and a better operational definition of the orienting and defence responses in terms of both respiratory and cardiac changes.

Autonomic and behavioural effects of stress can be neutralized pharmacologically by using anxiolytic drugs. The most commonly used anxiolytic drug is diazepam. In humans diazepam suppresses auditory-evoked potentials and startle reflex by reducing levels of alertness, arousal and fear (Al-Abduljawad et al., 2008). Diazepam also inhibits stress-induced behavioural (Liebsch et al., 1998) and cardiovascular responses to stress in rats (van den Buuse et al., 2001), but its effect on stress-induced respiratory responses was not investigated to date.

The purpose of this study is to compare respiratory responses to alerting and stressful stimuli with cardiac responses and with behavioural indices of anxiety. We aim to validate a pattern of respiratory response to alerting and stressful stimuli as both an autonomic and a behavioural index of anxiety. This is achieved by

firstly comparing respiratory and cardiac responses to alerting and stressful stimuli in the first group of rats, in which we assess cardiac and respiratory responses to well-established stressors (restraint, novel environment, brief acoustic stimuli) and sensitivity of these responses to diazepam. Rats in the second group, in addition to respiratory assessment, were also subjected to the most commonly used behavioural test of anxiety – Elevated Plus-Maze (Pellow et al., 1985). In this group we examined relationships between respiratory responses to alerting and stressful stimuli, and behavioural indices anxiety, with and without diazepam pre-treatment.

METHODS

Animals

Twenty adult male Outbred Wistar rats, weighing 400 ± 20 g, were received from the University of Newcastle Animal House facility. Ten animals were allocated to group 1. These animals were subjected to a preliminary surgery to implant ECG transmitters. The remaining ten animals were allocated to group 2. These animals were not subjected to any surgeries; however, they were subjected to behavioural anxiety testing in addition to respiratory assessment. Animals were kept on a reverse 12-12h light-dark cycle at 21±1°C with rat chow and water provided *ad libitum*. All experiments were conducted between 10am and 3pm during animals' active phase. All experimental procedures were approved by the Animal Care and Ethics Committee of the University of Newcastle and were conducted in accordance with the 8th edition of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Preliminary surgery (group 1 only)

Prior to the commencement of the experimental protocol ten rats were implanted with telemetric ECG transmitters (TA11CTA-F40, Data Sciences Int., St. Paul, MN, USA) under isoflurane anaesthesia (2% isoflurane in 100% oxygen). The electrodes were positioned according to a surgical procedure that allows high quality ECG assessment even during periods of locomotor activity (Sgoifo et al., 1996). Animals recovered from anaesthesia and were returned to their home cages, where they had daily injections of antibiotic (Baytril, 5mg/kg, s.c.) and analgesics (Carprofen, 5mg/kg, s.c., & Buprenorphine, 0.05 mg/kg, s.c.) for three days. The experimental protocol commenced after the animals recovered to baseline body weight and no earlier than one week post-surgery.

Recording apparatus and data collection

We simultaneously recorded respiratory parameters and gross motor activity in all rats. Rats were placed inside a custom-built whole-body plethysmograph (Perspex cylinder, i.d. 95 mm, length 260 mm, volume 1.8 l, wall thickness 3 mm) with a removable lead on one side and a constant flush of medical air at a rate of 3 L/min. When placed inside the recording chamber, an adult Outbred Wistar rat occupies approximately 40% of the volume inside the chamber. The chamber is large enough for an animal to freely move, groom and turn around. The output airflow from the chamber was divided into two lines using a T-connector. One line was attached to the differential pressure amplifier, while the other line was open to room air. Gross motor activity was assessed with a piezoelectric transducer (MLT1010/D, ADInstruments, Sydney, Australia) placed underneath the recording cylinder. Heart rate was also recorded in ten animals (group 1). To this end, ECG

signal from the implanted telemetric transmitter was transmitted to a receiver placed in close proximity of the plethysmographic chamber. Analogue signals were continuously recorded using LabChart (Version 7.1) and PowerLab 8s, (ADInstruments, Sydney, Australia) at a sampling rate of 1 kHz. Instantaneous respiratory and heart rates were computed online from the intervals between peaks on the respiratory signal and from ECG R-R intervals, respectively.

Recording session protocol

On the experimental days rats were individually placed inside the recording apparatus and left undisturbed for 15 minutes; in the text we refer to this period as "novelty stress". Afterwards, five acoustic tones of increasing intensities (white noise of 50, 60, 70, 80 and 90 dB intensity as assessed inside the recording chamber, 500 ms in duration) were presented from a loudspeaker. Tones were generated with ToneGen software (NCH Software, Sydney, Australia) and sent though a generic amplifier. Acoustic stimuli were presented during periods of no motor activity, and were at least 5 minutes apart. Ten minutes after presentation of the last acoustic stimulus rats were subjected to a 15-minute restraint stress. For this, animals were removed from the plethysmographic chamber, placed into a tight stainless steel mesh and returned into the plethysmograph.

Elevated Plus Maze testing (group 2 only)

Behavioural parameters in ten animals (group 2) were assessed using Elevated Plus Maze (EPM) test based on Pellow et al. (1985). The maze consisted of 4 arms (50 x 10 cm) arranged in a cross-like position and elevated by 100 cm above the floor. Two opposing arms were enclosed with walls 40 cm high. The maze was

illuminated with a dim light at 40 lux (intersection), 15 lux (ends of open arms) and 5 lux (ends of closed arms). Rats were placed inside the centre square; test duration was 5 min. Their behaviour was recorded with a video camera mounted directly above the centre square. These video records were analysed using EthoVision XT software (Noldus Information Technology, Wageningen, The Netherlands). We recorded number of entries into the open and closed arms, percentage of open arm entries (out of total number of open and closed arm entries), a ratio between the number of open and closed arm entries, and time spent in open and in closed arms. An arm entry was scored when the centre point of an animal crossed the border between an arm and a centre square.

Experimental design

On different days, at least 48h apart, animals in group 1 were subjected to the recording session twice in a counter-balanced design – once with diazepam (2.5 mg/kg i.p. in 0.5 ml Ringer; 15 minutes prior to placement into the plethysmograph) and once with saline injection (same volume). After completion of the protocol rats were euthanized with Lethabarb (2 ml/kg i.p.). On different days animals in group 2 completed both the recording session and the behavioural anxiety testing twice – once with diazepam (same dose) and once with saline pre-treatment. Behavioural tests and respiratory recordings were performed on different days in a counter-balanced design, at least 48h apart.

Data analysis

We assessed the mean and the dominant respiratory rates ($RespR_{MEAN}$ and $RespR_{DOMINANT}$) during novelty stress and restraint. Also, the mean heart rate (HR)

was assessed in group 1. These values were determined for three 5-min intervals of novelty stress, 5 minutes of baseline immediately prior to placement into restraint and three 5-min intervals of restraint. As respiratory rate in rats is highly variable and involves both slow breathing and sniffing (Kabir et al., 2010), we have also assessed the dominant respiratory rate ($RespR_{MEAN}$) using IgorPro software (Wavemetrics, USA). In brief, we computed time histograms of 5-min intervals for novelty stress, baseline and restraint; the mode of this histogram represents the dominant rate. This dominant rate, we believe, represents the baseline/"resting", locomotion-free assessment of the respiratory rate (see Bondarenko et al., 2014a and Carnevali et al., 2013 for more detailed descriptions of this procedure).

Novelty stress data were first analysed by 3x2 (3 five minute intervals of novelty stress x drug pre-treatment) within-subjects ANOVAs with subsequent LSD (Least Significant Difference) post-hoc tests when the main effect of drug or an interaction between time and drug were observed. To analyse restraint data, we have similarly performed 4x2 (baseline and 3 five minute intervals of restraint x drug pre-treatment) within-subjects ANOVAs for each index with LSD post-hoc tests when the main effect of drug or an interaction between time and drug were observed.

To assess respiratory and cardiac responses to the acoustic stimuli, we initially averaged respiratory and heart rate signals in response to each of the stimuli from all animals on saline trials to investigate general patterns of responses (an example of respiratory and cardiac responses to a 70dB stimulus is presented in Fig. 2). This preliminary analysis revealed that the respiratory response is monophasic, while

the cardiac response has three distinct phases – an initial tachycardia, a bradycardia and a secondary tachycardia. Subsequently, we manually computed each phase of the respiratory and the heart rate responses as a maximal or minimal value within the window that was determined by averaging raw traces of respiratory and cardiac signals (i.e. a window for an initial tachycardic response to the 70dB was from 300 to 800ms after the stimulus onset, see Fig. 2). In order to investigate the thresholds of activation of each of the phases, we performed 5x2 (intensity x baseline/response) within-subject ANOVAs for each phase of the respiratory and the cardiac responses on saline trials in group 1. We used the LSD post-hoc test when the 5x2 ANOVAs of respiratory or cardiac analysis indicated a significant main effect of phase or a significant interaction between stimulus intensity and phase.

Subsequently, in order to analyse the effects of diazepam on the evoked respiratory and cardiac responses, we calculated the amplitude of each phase of the response (Δ or change from baseline). The amplitudes of each phase of the respiratory and cardiac responses were analysed by 5x2 (intensity x drug) within-subjects ANOVAs with post-hoc LSD test performed when the main effect of drug or an interaction between time and drug were observed. We also assessed latencies of each phase of the respiratory and the cardiac responses to the acoustic stimuli for the 70, 80 and 90dB stimuli (we could not assess it for the 50 and 60dB stimuli due to a lack of pronounced responses to these stimuli in some animals). These data were computed manually in the LabChart software (Version 7.1, ADInstruments) by assessing time from the stimulus onset to the first detectable change in the respiratory signal and to each phase of the cardiac response to the acoustic stimuli.

These data were analysed by 3x2 (intensity x drug) within-subjects ANOVAs with post-hoc LSD test performed when the main effect of drug or an interaction between time and drug were significant.

Lastly, we performed a series of Pearson's one-tailed correlations between respiratory and cardiac parameters in group 1 and between respiratory and behavioural indices in group 2. In group 1 respiratory parameters during each phase of the protocol were correlated with corresponding heart rate parameters (i.e. respiratory rate during the first 5 minutes of novelty stress was correlated with the heart rate during this period). Also, the amplitudes of tachypnoeic responses to the acoustic stimuli were correlated with each phase of the cardiac response. In group 1 this analysis was performed separately for the saline and diazepam pre-treatment trials due to diazepam having a tachycardic, but no tachypnoeic side-effect. In group 2 one-tailed Pearson's correlations were performed between all respiratory parameters during novelty stress, presentation of acoustic stimuli and restraint and all behavioural parameters from the EPM. For this analysis values from saline and diazepam trials were collated together.

RESULTS

All respiratory data is pooled from both groups 1 and 2 (total n=20) to avoid unnecessary repetition of analysis and figures, as the data from both groups are virtually identical. Heart rate data are from group 1 only (n=10) and behavioural data are from group 2 only (n=10).

Respiratory response to the novelty stress (groups 1 & 2)

After being placed into the plethysmographic chamber, rats exhibited a mean respiratory rate ($RespR_{MEAN}$) of 208 ± 11 cpm during the first 5 minutes, which declined to 154 ± 8 cpm during the last 5-minute interval (Fig. 1B). Similarly, the dominant respiratory rate ($RespR_{DOMINANT}$) gradually declined from 111 ± 4 cpm during the first 5 minutes to 94 ± 3 cpm during the third 5-minute interval. Pre-treatment with diazepam significantly inhibited $RespR_{MEAN}$ and $RespR_{DOMINANT}$ during all three 5-min intervals of novelty stress ($RespR_{MEAN}$: all $p < .05$; $RespR_{DOMINANT}$: all $p < .01$). Figures 1B and 1C depict the mean and the dominant respiratory rates during novelty stress.

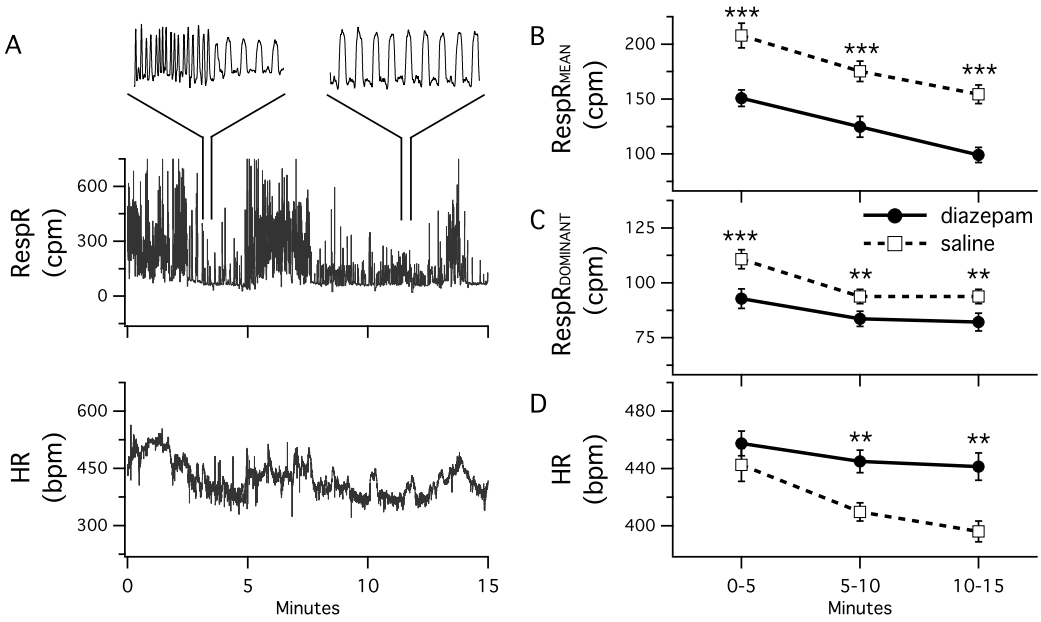

Figure 1. Respiratory and heart rates during the 15-minute novelty stress. Respiratory rate is not constant with periods of high highly variably respiratory rate (generally associated with some motor activity) intertwined with periods of relatively low and stable respiratory rate (A). Diazepam significantly inhibited the mean and the dominant respiratory rates during all three 5-minute intervals of the novelty stress (B & C; n = 20); Diazepam significantly elevated heart rate during

the second and third 5-minute intervals of the novelty stress (D; n = 10). *Note:* * - significant at $p < .05$, ** - significant at $p < .01$, *** - significant at $p < .001$.

Respiratory response to the acoustic stimuli (groups 1 & 2)

Presentation of acoustic stimuli produced transient monophasic tachypnoeic responses that were proportional to the stimulus intensity, ranging from +106 ± 28 cpm in response to the 50dB stimulus to +450 ± 30 cpm in response to the 90dB stimulus on the saline pre-treatment trials. We have assessed latencies of respiratory responses to the 70-90dB stimuli only. These latencies were inversely proportional to the stimulus intensity; responses occurred 92±11 ms after the 70dB stimulus onset, while the 90dB stimulus evoked a respiratory response 42 ± 4 ms after the stimulus onset. Figure 2A presents respiratory response to a 70dB stimulus averaged from all saline trials.

In analysing these data, we initially assessed a threshold of activation of the respiratory response to the acoustic stimuli on saline trials. To this end we performed a 5x2 within-subjects ANOVAs (stimulus intensity x phase) that compared the respiratory response with a 10s baseline immediately prior to presentation of acoustic stimuli. Presentation of acoustic stimuli of all intensities produced significant respiratory rate responses on saline trials (all $p < .001$). Fig. 3 illustrates these results. Next, we analysed the effects of diazepam pre-treatment on the magnitude (Δ) of respiratory responses. We found that diazepam pre-treatment significantly inhibited respiratory responses to all stimuli (all $p < .05$; Fig 4A) and the magnitude of respiratory responses to the acoustic stimuli was linearly dependent upon the stimulus intensity ($p < .001$; Fig. 4A). Lastly, we analysed the

effects of diazepam pre-treatment on the latency of respiratory responses to the 70-90dB stimuli. The results indicated that diazepam significantly increased latencies of respiratory responses to all three stimuli (all $p < .05$).

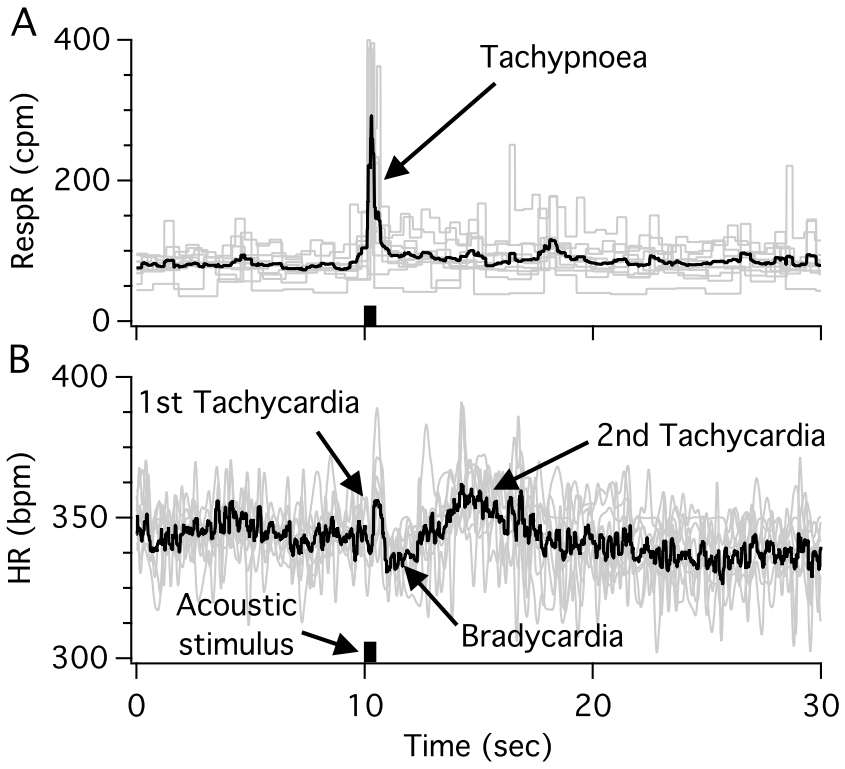

Figure 2. Respiratory rate (A) and heart rate (B) responses to a 70dB 0.5s white noise stimulus averaged from 10 rats (group 1) from trials with saline pre-treatment. Acoustic stimulus evoked a mono-phasic transient tachypnoeic response, while the heart rate response was tri-phasic: brief tachycardia, bradycardia and a secondary prolonged tachycardia. The phases of the responses are indicated with arrows. Grey lines indicate individual traces. Individual heart rate traces (grey) were offset to the same average baseline (first 10 secs) due to variability of basal heart rate.

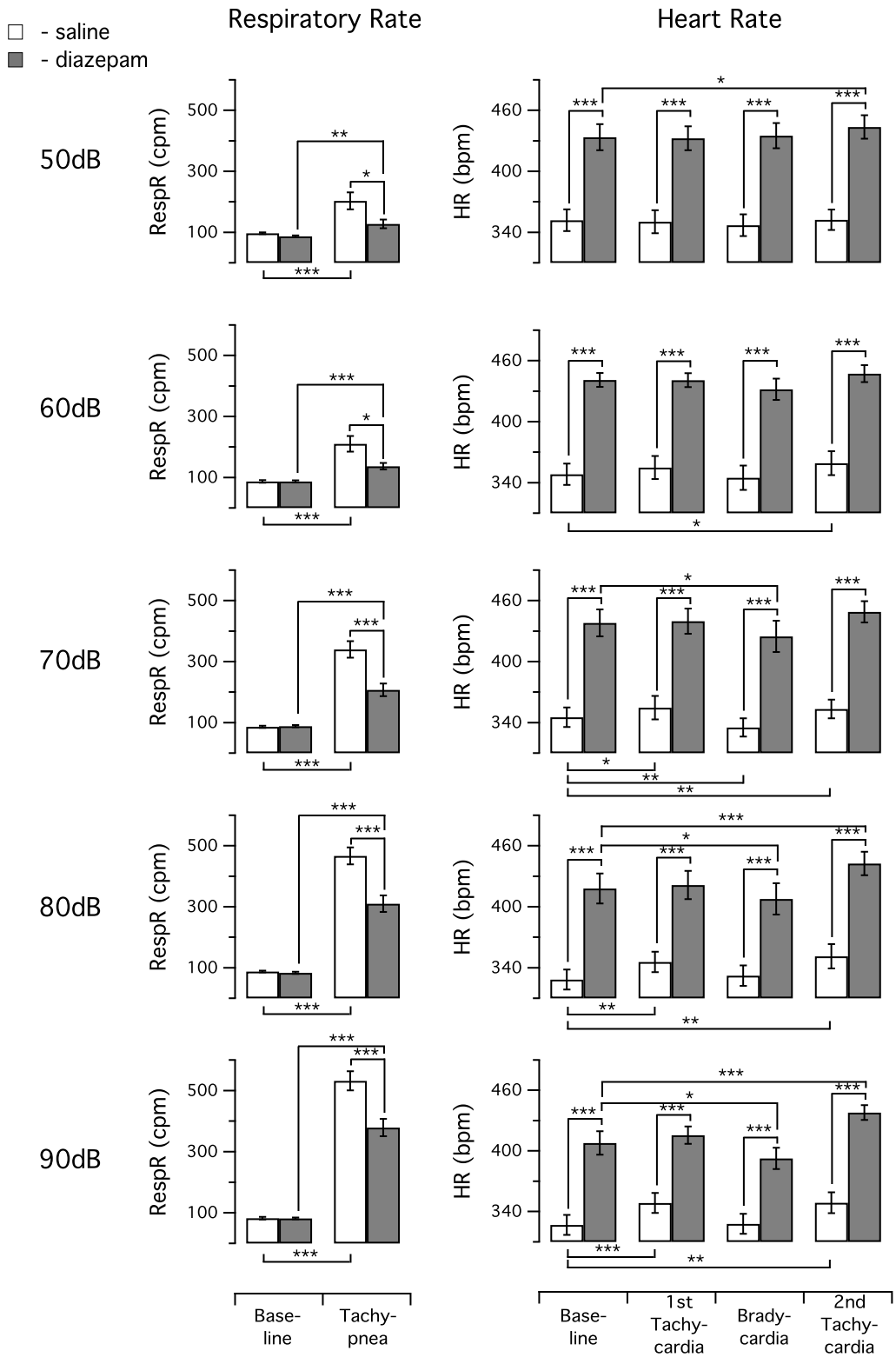

Figure 3. Baseline and response values of each phase of the respiratory and heart rate responses to the acoustic stimuli of increasing intensity (50-90dB). Respiratory responses included a monophasic brief period of tachypnoea (see Figure 2A for

illustration). All stimuli (50-90dB) evoked significant changes in respiratory rate. Diazepam significantly inhibited the magnitude of respiratory rate responses to all stimuli. Heart rate responses were three-phasic: a brief period of tachycardia, followed by a bradycardia and a secondary prolonged tachycardia (see Figure 2B for illustration). 70-90dB stimuli evoked significant initial and secondary tachycardic responses, while only the 70dB stimulus evoked a significant bradycardic response. Diazepam significantly elevated heart rate values during baseline and presentation of all stimuli. On diazepam pre-treatment trials 70-90dB stimuli evoked significant bradycardic responses and 80-90 dB evoked significant secondary tachycardic responses. No significant initial tachycardic responses were observed on these trials. See Figure 4 for these data expressed as a change from baseline (Δ). *Note:* * - significant at $p < .05$, ** - significant at $p < .01$, *** - significant at $p < .001$.

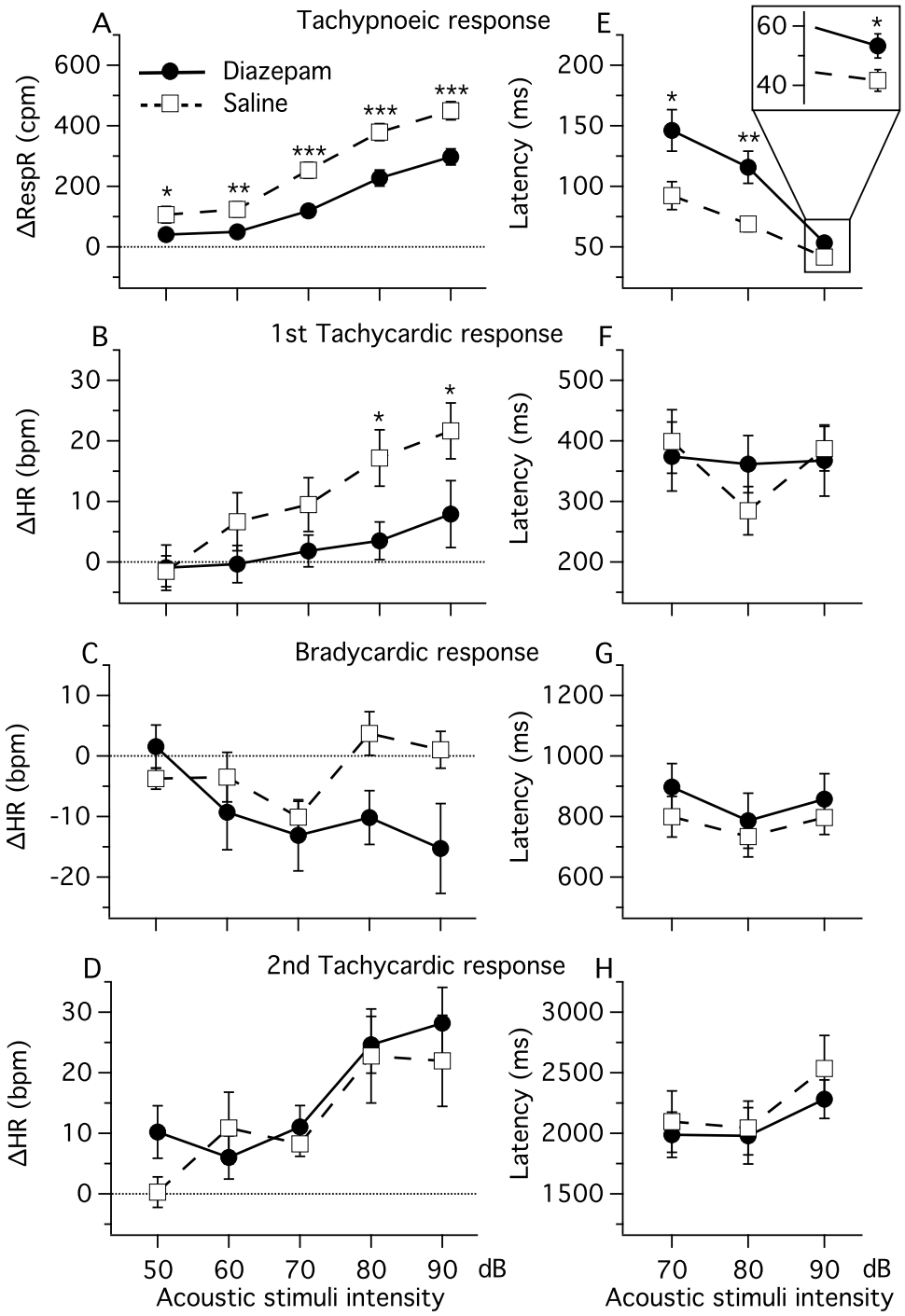

Figure 4. Amplitudes (A-D) and latencies (E-H) of respiratory and heart rate responses to the acoustic stimuli of increasing intensity (50-90dB) presented as line graphs. The magnitudes of respiratory and initial tachycardic responses were linearly dependent upon stimulus intensity. Diazepam significantly inhibited the magnitude of respiratory responses to all stimuli and initial tachycardic responses

to the 80 and 90dB stimuli as well as the latency of tachypnoeic response to the 80dB stimulus. *Note:* * - significant at $p < .05$, ** - significant at $p < .01$.

Respiratory response to the restraint stress (groups 1 & 2)

Restraint stress significantly elevated $RespR_{MEAN}$ from 93 ± 4 cpm to 179 ± 4 cpm and $RespR_{DOMINANT}$ from 79 ± 3 to 154 ± 6 cpm on saline trials (both $p < .001$); both parameters gradually declined as the restraint stress progressed (see Fig. 5A & B). Diazepam significantly inhibited the mean respiratory rate during the first and the second 5-minute intervals of restraint (both $p < .05$) and also inhibited the dominant respiratory rate during the first 5-minute interval ($p = .007$).

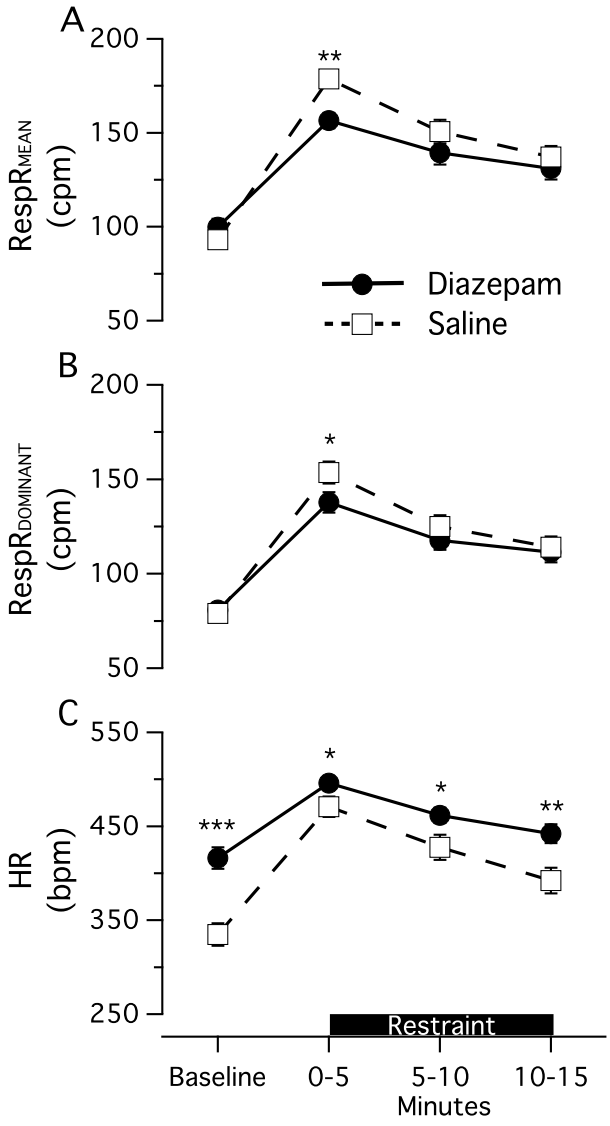

Figure 5. Respiratory rate and heart rate during restraint on diazepam and saline pre-treatment trials. Diazepam significantly inhibited the mean and the dominant respiratory rate (A&B) during the first 5-mintue interval of restraint, while it increased the heart rate during baseline and all periods of restraint (C&D). *Note:* * - significant at $p < .05$, ** - significant at $p < .01$, *** - significant at $p < .001$.

Heart rate response to the novelty stress (group 1)

When animals (n=10) were placed into the recording chamber, they exhibited a mean heart rate of 442 ± 12 bpm during the first 5 minutes, which declined to 396 ± 7 bpm during the third 5-min interval of the novelty stress. Diazepam injection significantly elevated the mean heart rate during the second and the third 5-minute intervals of the novelty stress (both $p < .01$). Data values and results of statistical analysis are summarized in Figures 2D and 2E.

Heart rate response to the acoustic stimuli (group 1)

Heart rate responses to the acoustic stimuli of 70dB and higher had three distinct phases – initial tachycardia of approximately 500 ms duration, then bradycardia of approximately 1.5 s duration followed by a prolonged secondary tachycardia of approximately 3.5 s duration. Figure 2B depicts the heart rate response to the 70dB stimulus averaged from all saline trials, with the three phases of a response labelled. Heart rate responses to the lower intensity stimuli (50 and 60 dB) were less consistent and the initial tachycardia and/or bradycardia were absent on some trials, suggesting that these components of a cardiac response have higher thresholds of activation. Compared with respiratory responses, heart rate responses had longer latencies (Figure 4). The initial tachycardic response occurred 399 ± 52 ms after the 70dB stimulus onset; the bradycardic response occurred 799 ± 67ms after the stimulus onset, while the second tachycardic response occurred 2097 ± 253ms after the onset of the 70dB stimulus.

In order to characterize cardiac responses to the acoustic stimuli we firstly assessed thresholds of activation of each phase of the cardiac response on saline trials. 5x2 ANOVAs were performed on all three phases of the cardiac response values. We

found that presentation of 70, 80 and 90dB stimuli evoked significant initial and secondary tachycardic responses (all $p < .05$), while presentation of only the 70dB stimulus evoked a significant bradycardic response ($p = .002$). Results of this analysis are summarised in Fig. 3.

Next, we analysed the effects of diazepam pre-treatment on each phase of the evoked heart rate response (determined as Δ). Diazepam significantly inhibited 1st tachycardic response to the 80 and 90 dB stimuli, $p = .002$ and .030, respectively. There was no effect of drug pre-treatment on the 2nd tachycardic response ($p = .406$; Fig. 4D). We found that the relationships between stimulus intensity and both tachycardic responses follow linear trends ($p < .001$ and $p = .002$, respectively; Fig. 4B&D). None of the main effects or post-hoc comparisons of the bradycardic response were significant (Fig.4C). Also, no significant main effects of drug or of time or interactions were observed in the latencies of cardiac responses data (Fig. 4F-H).

Heart rate response to the restraint stress (group 1)

Restraint stress elevated HR_{MEAN} from 335 ± 12 to 470 ± 11 bpm. Similar to respiratory rate, heart rate declined during the restraint stress, from 470 ± 11 to 392 ± 14 bpm (see Fig. 5A & B). Diazepam pre-treatment significantly elevated the mean heart rate during baseline and all three 5-minute intervals of restraint (all $p < .001$). Group data values of the mean heart rate during the restraint stress are presented in Figure 5C & D.

Behavioural parameters during the EPM test (group 2)

In random order, animals of group 2 (n=10) performed the EPM twice – once with saline and once with diazepam pre-treatment. Overall, rats displayed more exploratory behaviour on trials with diazepam pre-treatment than on trials with saline pre-treatment (Fig 6). Repeated measures one-way t-tests indicated that on trials with diazepam pre-treatment rats exhibited significantly more closed arm entries (p = .006) and spent significantly more time in the open arms of the maze (p = .022) than on trials with saline pre-treatment. Diazepam also increased the percentage of open arm entries out of total arm entries (p = .006) and also increased the ratio between the number of open arm entries and closed arm entries (p = .018). There were no differences in the number of open arm entries (p = .098) and time spent in closed arms (p = .128) between trials with saline and diazepam pre-treatment.

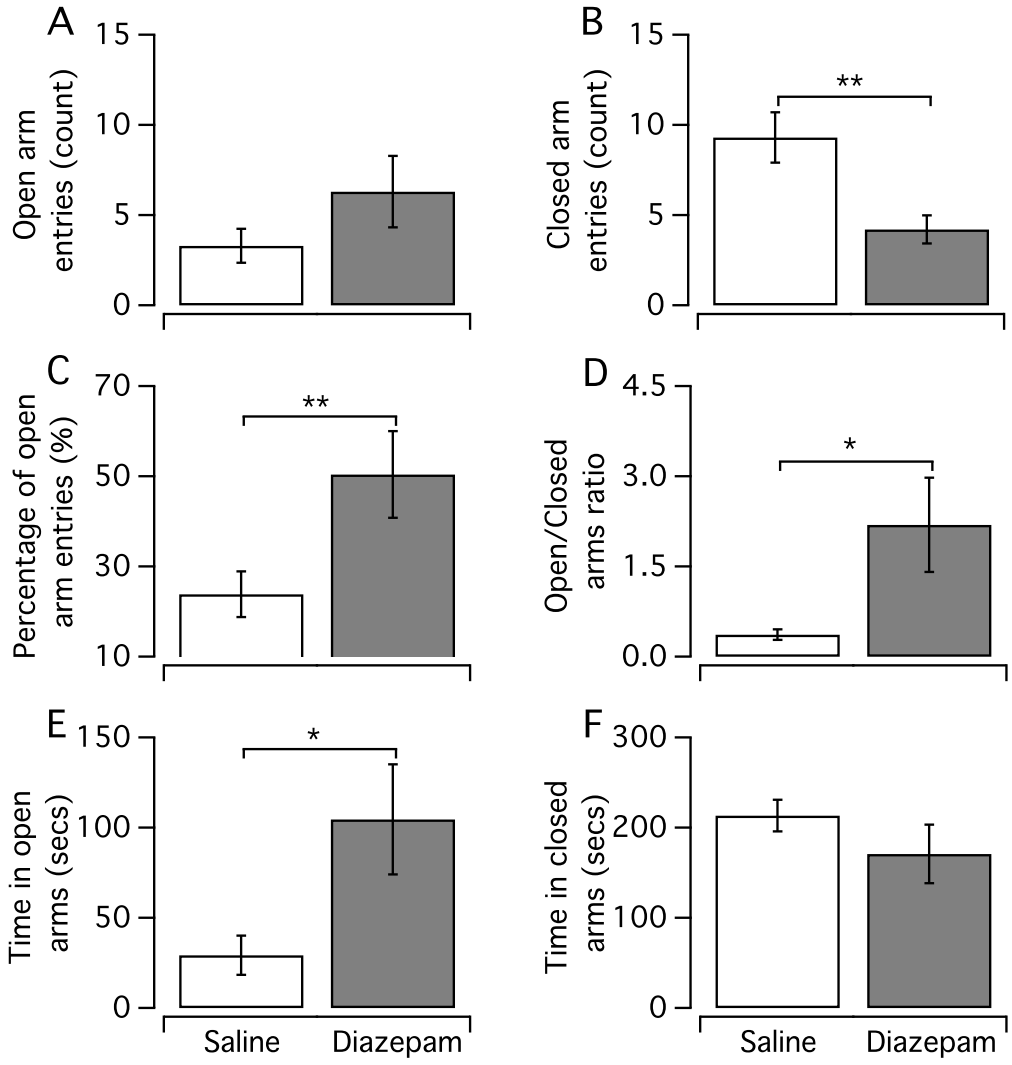

Figure 6. Results of elevated-plus maze testing. Diazepam significantly inhibited the number of closed arm entries (B), percentage of open arm entries (C), a ratio between the number of open arm entries and closed arm entries (D) and also time spent in open arms (E). Diazepam did not significantly affect the number of open arm entries (A) and time spent in closed arms (F). *Note:* * - significant at $p < .05$, ** - significant at $p < .01$, *** - significant at $p < .001$.

Correlations between respiratory and cardiac responses (group 1)

As diazepam had a significant tachycardic, but no tachypnoeic, side effect, we performed the correlations separately for the diazepam and saline trials. The sample size for each of these correlations is therefore 10 animals. We examined correlations between respiratory and cardiac responses to the acoustic stimuli as well as during novelty and restraint stresses (Table 1). The only significant correlation of the heart rate on the diazepam pre-treatment trials was with $RespR_{MEAN}$ during the baseline period ($R^2 = .346$, $p = .037$). On saline trials we found significant correlations between cardiac and respiratory parameters during all three 5-min intervals of novelty stress as well as during baseline, with slightly stronger correlations between HR and $RespR_{DOMINANT}$ (all $R^2 > .514$, all $p < .02$) than between HR and $RespR_{MEAN}$ (all $R^2 > .441$, all $p < .03$). Lastly, we found significant correlations between the magnitudes of respiratory and cardiac responses to the 60dB and the 80dB (see Table 1).

Correlations between respiratory and behavioural parameters (group 2)

For this analysis, saline and diazepam data were collated together; therefore the sample size for these correlations is 20 animals (as each of the 10 animals completed respiratory and behavioural assessment twice – once with saline and once with diazepam pre-treatment).

A number of behavioural indices of anxiety, as assessed by EPM, were positively correlated with respiratory parameters, especially respiratory parameters during novelty stress. Data values from this analysis are presented in Table 2. Dominant respiratory rates during the first, second and third 5-minute intervals of novelty stress were found to be significantly correlated with almost all behavioural parameters (see Table 2).

Secondly, we found that the number of closed arm entries was significantly correlated with the dominant respiratory rate ($RR_{DOMINANT}$) during all three 5-minute intervals of restraint (all $R^2 > .250$, all $p < .012$). See Table 2 for a summary of all correlations between respiratory and behavioural parameters.

DISCUSSION

Our study is the first systematic exploration of respiratory, cardiac and behavioural variables in conscious rats under various emotional and arousal states. The major finding of this study is the dependency of the magnitude of respiratory responses to the alerting and stressful stimuli on the intensity of these stimuli and on the anxiety state of the animal. We have found that the respiratory rate during novelty stress is significantly correlated with traditionally used behavioural indices of anxiety assessed by the Elevated Plus Maze, suggesting that assessment of respiration may potentially be used as a locomotion-independent alternative to traditional behavioural tests of anxiety. Together, these findings indicate that although respiration is traditionally considered as an autonomic parameter reflecting homeostatic control of blood gases, it shares some characteristics with behavioural indices of anxiety.

Prolonged stressors: Novelty stress & restraint stress

During the 15-minute novelty stress and during the 15-minute restraint stress there were gradual reductions in the mean and the dominant respiratory rates, similar to the findings of our previous studies (Bondarenko et al., 2014a; Carnevali et al., 2013; Kabir et al., 2010). We believe that the 5-minute period prior to placement

into the restrainer represents a baseline (or close to baseline) respiratory rate, as the heart rate of all animals during this period was within the resting range (Braga and Prabhakar, 2009); moreover, this period was at least 1 hour after animals were placed into the chamber, and the respiratory and heart rates of all animals were the lowest observed during this period. If that is the case, then we can conclude that the prolonged stressors evoked changes in respiratory and cardiac parameters in an intensity-dependent manner, as a less intense stressor – novelty stress, evoked a smaller change compared with baseline than the more intense stress – restraint.

Pre-treatment with diazepam had differential effects on the cardiac and respiratory indices during the novelty and the restraint stresses: the drug significantly reduced respiratory rate but increased HR. The latter is not surprising providing a well-known tachycardic side effect of the drug (Mailliet et al., 2001), which is presumably mediated by the suppression of cardiac vagal tone via activation of benzodiazepine receptors coupled to the GABA receptors (DiMicco, 1987). As HR is the most common indicator of physiological arousal, the increase in heart rate after diazepam treatment is a serious confounding factor in stress/arousal studies; judging by heart rate alone, we could have concluded that animals exhibited an increase in arousal. By contrast, the effects of diazepam on respiration during novelty stress were consistent with its anxiolytic properties and are indicative of a decrease in arousal. Combined, these data provide strong evidence that in rodents, similar to humans (Boiten et al., 1994), level of arousal is reflected by the respiratory rate.

Brief stressors: Acoustic stimuli

Presentation of acoustic stimuli evoked transient increases in respiratory rate and a triphasic response in the HR: an initial brief tachycardia, followed by a bradycardia and a secondary prolonged tachycardia. The lowest intensity stimulus, 50dB, evoked a respiratory response in the absence of any cardiac changes (Fig. 3, top traces), indicating that respiratory indices are more sensitive to subtle changes in arousal than cardiac changes. This observation confirms our previous findings (Kabir et al., 2010; Nalivaiko et al., 2011) and suggests that the current definition of the autonomic changes accompanying an orienting response are incomplete, as it is currently defined only in terms of bradycardia (Bradley, 2009). Animals in the current experiment did attend to the low-intensity stimuli, as they exhibited an increase in respiratory rate (sniffing behaviour), but did so in the absence of any cardiac changes. In fact, this observation makes perfect sense, as rodents rely on their olfactory input as a primary sensory channel. It is likely that rodent respiratory arousal response is equivalent to a human behaviour of orienting their head and looking for the source of the stimulus. Respiratory change is therefore much more relevant to the cognitive ("orienting" or "what is it?") processing of the stimulus than cardiac changes. Thus, it is not surprising that respiration is a more sensitive index of orienting response than heart rate, at least in rodents.

To our knowledge, the current experiment is the first to report a triphasic HR response to acoustic stimuli in rats. The triphasic cardiac response was previously shown in humans (Vila et al., 2007), but animal literature is largely inconsistent in the description of effects of acoustic stimuli on heart rate. Berntson, et al. (1997) described bradycardia in adult rats in response to 50 ms 95 dB stimuli. Kurtz and Campbell (1994) demonstrated that a 10 second 80dB acoustic stimulus evokes

bradycardia in young rats, while the same stimulus evokes tachycardia in adult rats. Blanc et al. (1997) reported bi-phasic cardiac responses (bradycardia followed by tachycardia) to 700 ms 110dB acoustic stimuli. Taylor et al. (1991) found a similar response, but in response to the air puff stress. A lack of consistency in the pattern of cardiac responses could be due to differences in methodologies between studies, such as differences in the duration and intensity of the stimuli, inter-stimulus intervals and the number of stimuli presented. For instance, Berntson et al. (1997) averaged the pattern of a response from 48 trials, with only 25-sec inter-stimulus interval; it is thus possible that some habituating components of the response were not detected. Furthermore, some earlier studies missed temporal resolution due to averaging HR at 1-s intervals (Berntson et al., 1997; Kurtz and Campbell, 1994) or only assessed maximal change from baseline (Blanc et al., 1997; Taylor et al., 1991).

Without pharmacological dissection, it is not possible to identify the mechanisms that mediated the tri-phasic HR responses; nevertheless, some suggestions could be made. The short latency and short duration of the initial tachycardic response suggest that it was likely mediated by vagal withdrawal. Indeed, in humans this initial acceleration of the heart rate is accompanied by a reduction of vagally mediated respiratory sinus arrhythmia (Reyes del Paso and Vila, 1993). The bradycardic component was most likely a consequence of vagal activation as it was shown to be sensitive to atropine (Hunt, 2004), an antagonist of the muscarinic acetylcholine receptors. In addition, human studies suggest possible involvement of the baroreflex in its origin (Reyes del Paso and Vila, 1993). The fact that 80 and 90 dB stimuli did not evoke a significant bradycardia in the current experiment

could be due to a greater vagal withdrawal (1-st phase of the HR response) evoked by these stimuli. The secondary tachycardia could be mediated mainly by sympathetic activation, as it is relatively slow and prolonged. This tachycardia can also, in part, be associated with motor activity exhibited by animals after the stimulus presentation.

Respiratory responses are more sensitive to changes in arousal than cardiac responses

Overall, the magnitude of respiratory responses observed in the current study was proportional to the stimulus intensity, for both short and prolonged stimuli. Lower intensity brief stimuli (acoustic) evoked relatively small changes, while high intensity brief stimuli (acoustic) evoked larger responses. Similarly, the changes evoked by the restraint, a high intensity prolonged stressor, were larger than those evoked by placement into the new environment. Heart rate responses to the brief stimuli were more variable than the respiratory responses, presumably due to an interplay of sympathetic and parasympathetic control. Cardiac responses to the prolonged stressors, on the other hand, were consistent, but the effects of diazepam on HR were difficult to determine due to a tachycardic side effect of the drug. Therefore, respiratory responses, at least in the methodology used in the current study, were much more consistent and informative than HR responses. In line with that, we observed correlations between respiratory and cardiac parameters only during mild stressors (novelty stress and 60dB and 80dB stimuli) and only on saline trials. This observation and the fact that heart rate responses to the acoustic stimuli had a significantly higher threshold of activation indicates that respiratory parameters were much more sensitive to changes in arousal than heart rate.

While the higher neural control mechanisms of both respiratory and cardiac activation may share some brain areas (Bondarenko et al., 2014a; Bondarenko et al., 2014b; Dampney et al., 2008; Hassan et al., 2013), the lower brainstem mechanisms are very different. Heart rate acceleration in response to environmental challenges is mediated by the sympathetic preganglionic motor neurons (Samuels et al., 2002), while heart rate deceleration is mediated by cardiac vagal motor neurones (Hunt et al., 1994). Respiratory activation, on the other hand, is mediated by premotor respiratory neurons in the ventral respiratory group and the parahypoglossal region, which has projections to both inspiratory and expiratory muscles (Tan et al., 2010; Feldman et al., 2013). Presumably, such a "monomodal" neuronal control where a single structure has both acceleratory and deceleratory influence is responsible for a higher sensitivity of respiratory rate to the arousal state and for the linear dependence from the stimulus intensity.

Correlations between respiratory and behavioural indices

Our major finding from the second group of rats is a close correlation between the established behavioural indices of anxiety and respiratory changes during novelty stress.

Anxiolytic action of diazepam that we observed in the EPM test is in full accord with the background literature. (Doremus et al., 2006; Griebel et al., 1996; Pellow et al., 1985). Overall, diazepam increased exploratory behaviour, which was evident in increases in the time spent in open arms, the percentage of open arm entries and the ratio of open to closed arm entries and also decreased the number of

closed arm entries. Such increases in exploratory behaviour are presumably due to animals' decreased anxiety state.

We observed significant strong correlations between behavioural indices of anxiety and the respiratory parameters, particularly during the mild stress (novelty). The strongest correlations were between the dominant respiratory rate during novelty and the percentage of open arm entries as well as with the ratio between the number of open and closed arm entries. The dominant respiratory rate represents a locomotion- and sniffing-unrelated or "resting" respiratory rate. Therefore, we can speculate that it is the anxiety-related component of the respiratory rate, not a locomotion-related component that is most significantly correlated with the behavioural indices of anxiety.

Interestingly, respiratory responses to acoustic stimuli or to restraint stress were generally not significantly correlated with behavioural indices of anxiety. It could be that respiration during novelty stress is correlated with the EPM indices because both these procedures involve exposure to a mildly stressful novelty situation for extended periods of time. Submission to restraint stress, on the other hand, is much more stressful and evokes a fear response, while presentation of acoustic stimuli evokes sudden changes in the anxiety level and attention. Therefore, the EPM setting is most similar to the novelty stress of all stressors used in the current experiment.

Conclusions and perspectives

Respiratory arousal response is a promising new index of autonomic activation. It is clearly different from the startle response in its longer latency and sensitivity to

low-intensity sensory stimuli (Nalivaiko et al., 2011), and thus reflects some aspects of sensory-motor processing related to selective attention. Respiratory arousal shares some characteristics with both autonomic and behavioural parameters. It is similar to autonomic parameters in that respiration is a vital physiological process that is necessary for gas exchange. It is controlled by lower brainstem mechanisms, but also has influences from the amygdala (Bondarenko et al., 2014a), the dorsomedial hypothalamus (Tanaka and McAllen, 2008) and the prefrontal cortex (Bondarenko et al., 2014b; Hassan et al., 2013). On the other hand, respiration can be reflexively or even voluntarily augmented for olfactory sampling, particularly in rodents that heavily rely on their olfactory sense. This property of respiration makes it a unique autonomic function, as other autonomic parameters do not have such an extensive conscious control. Therefore, we propose that respiration is both an autonomic and a behavioural index.

Respiration differs from a traditionally used heart rate index in that it is more sensitive to brief changes in arousal than heart rate. We have observed much stronger inhibition of respiratory rate responses than of heart rate responses by diazepam. Respiratory rate responses were easier to assess and interpret, as they did not have a complex polyphasic response pattern. Also, respiratory assessment can be used in methodologies when heart rate is adversely affected (i.e. by a side effect of a drug, as in our case with diazepam). On the other hand, respiratory arousal shares some similarities with behavioural indices of anxiety. Respiratory rate (particularly, the dominant respiratory rate) was highly and significantly correlated with anxiety-like behaviour as assessed by the elevated plus maze. In contrast to such behavioural measures, respiratory assessment does not rely on

animals' locomotor activity and thus can be used in methodologies when locomotion is adversely affected. Furthermore, animal respiratory measures are directly translatable to human studies, unlike traditional animal behavioural tests of anxiety. These suggest that assessment of respiratory responses to various arousing and alerting stimuli can be used as a viable new index of anxiety in animals as well as an index of autonomic activation.

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Table 1: Correlations between simultaneously recorded respiratory and cardiac parameters during presentation of prolonged stressors (A) and brief acoustic stimuli (B). Respiratory parameters were highly and significantly correlated with cardiac parameters on saline trials during presentation of low potency stimuli and stressors – a novelty stress and low intensity acoustic stimuli. The correlations are based on data of 10 animals; the analyses were performed separately for the saline and diazepam pre-treatment trials due to a potent tachycardic side-effect of diazepam.

A.

Correlation between:

		Resp.R_{MEAN} & HR		Resp.$R_{DOMINANT}$ & HR	
		saline	diazepam	saline	diazepam
Novelty stress	0-5min	**		*	
	5-10min	**		*	
	10-15min	*		**	
Restraint stress	baseline	**	*		
	0-5min				
	5-10min				
	10-15min				

Strength of significant correlations:

Colour	R^2 between
	0.6 - 0.7
	0.5 - 0.6
	0.4 - 0.5
	0.3 - 0.4

Significance:

** $p < .05$

* $p < .01$

B.

Correlation between:

		Respiratory response & 1st tachycardic response		Respiratory response & brachycardic response		Respiratory response & 2nd tachycardic response	
		saline	diazepam	saline	diazepam	saline	diazepam
Ampli-tude	50dB stim						
	60dB stim	**		**		**	
	70dB stim						
	80dB stim			*		*	
	90dB stim						
Latency	70dB stim						
	80dB stim						
	90dB stim						

Table 2: Correlations between respiratory responses to stimuli and stressors and behavioural parameters as assessed by the Elevated plus maze on separate days. The dominant respiratory rate during the novelty stress was highly significantly correlated with almost all behavioural indices of anxiety, except for the number of closed arm entries. The number of closed arm entries was significantly correlated with the dominant respiratory rate during the restraint stress. Diazepam and saline trials were collated together for this analysis; the analysis includes data from 20 trials.

			Elevated plus maze index					
			OAE	CAE	TOA	TCA	O/C	%O
Ampli-tude of resp. response	50dB stimulus							
	60dB stimulus							
	70dB stimulus				*	*		
	80dB stimulus			*				
	90dB stimulus		*	*			*	*
Latency of resp. response	70dB stimulus							
	80dB stimulus							
	90dB stimulus							
Novelty stress	$RespR_{MEAN}$	0-5min		**				
		5-10min					*	*
		10-15min		*			*	
	$RespR_{DOMINANT}$	0-5min	*		**	*	*	**
		5-10min	**		**	*	**	***
		10-15min	*	*	*		*	**
Restraint stress	$RespR_{MEAN}$	baseline						
		0-5min					*	*
		5-10min						
		10-15min						
	$RespR_{DOMINANT}$	baseline						
		0-5min		*				
		5-10min		**				
		10-15min		*				

Abbreviations:

OAE - Open arm entries

CAE - Closed arm entries

TOA - Time in open arms

TCA - Time in closed arms

O/C - Ratio between open and closed arm entries

%O - Percentage of open arm

Strength of significant correlations:

Colour	R^2 between
	0.5 - 0.6
	0.4 - 0.5
	0.3 - 0.4
	0.2 - 0.3
	0.1 - 0.2

Significance

*	$p < .05$
**	$p < .01$
***	$p < .001$

CHAPTER 2

BLOCKADE OF THE DORSOMEDIAL HYPOTHALAMUS AND THE PERIFORNICAL AREA INHIBITS RESPIRATORY RESPONSES TO AROUSING AND STRESSFUL STIMULI

INTRODUCTION TO CHAPTER 2

Results of Chapter 1 indicate that respiratory responses to brief arousing stimuli have a lower threshold of activation than traditionally used cardiac indices. Furthermore, respiration is closely linked with an anxiety state of an animal as it is highly correlated with behavioural indices of anxiety and is sensitive to an anxiolytic drug diazepam. Previous studies that investigated neuronal structures that mediate respiratory activation have mainly focused on homeostatic mechanisms mediated by the lower brainstem structures, while the contribution of higher neuronal structure to the respiratory arousal has never been investigated in conscious animals. The subsequent chapters of the current thesis aim to investigate involvement of different forebrain regions in mediating respiratory responses to brief and prolonged stressors of various intensities. I hypothesise that central structures that mediate such respiratory activation are similar to the ones that were shown to mediate cardiovascular activation. For this reason I aim to investigate involvement of the dorsomedial hypothalamic area, the amygdala, and the prelimbic prefrontal cortex. Consequently, the current paragraph is applicable to the Chapters 2-4 of the thesis.

The dorsomedial hypothalamic area is recognized as a crucial region for integration central "higher order" autonomic information (Buijs and Van Eden, 2000). Inhibition of this structure prevents stress-induced tachycardic and pressor responses in conscious rats (DiMicco et al., 2002). Furthermore, activation of this structure evokes an increase in heart rate, arterial pressure, thermogenesis and respiratory rate in anesthetized rats (DiMicco and Zaretsky, 2007; Tanaka and McAllen, 2008). However, the effects of inhibition of the dorsomedial hypothalamic area on respiration have never been investigated in conscious animals. The aim of Chapter 2 is to investigate effects of pharmacological inhibition of the dorsomedial hypothalamic area on respiratory responses to brief and prolonged stressors of various intensities in conscious unrestrained rats.

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Blockade of the dorsomedial hypothalamus and the perifornical area inhibits respiratory responses to arousing and stressful stimuli.

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ABSTRACT

The dorsomedial hypothalamus and the perifornical area (DMH/PeF) is one of the key regions of central autonomic processing. Previous studies have established that this region contains neurons that may be involved in respiratory processing; however, this has never been tested in conscious animals. The aim of our study was to investigate the involvement of the DMH/PeF area in mediating respiratory responses to stressors of various intensities and duration. Adult male Wistar rats (n = 8) received microinjections of $GABA_A$ agonist muscimol or saline into the DMH/PeF bilaterally and were subjected to a respiratory recording using whole body plethysmography. Presentation of acoustic stimuli (500-ms white noise) evoked transient responses in respiratory rate, proportional to the stimulus intensity, ranging from +44±27 to +329±31cpm. Blockade of the DMH/PeF almost completely abolished respiratory rate and tidal volume responses to the 40-70dB stimuli and also significantly attenuated responses to the 80-90dB stimuli. Also, it significantly attenuated respiratory rate during the acclimatization period (novel environment stress). The light stimulus (30-s 2000lux) as well as 15-min restraint stress significantly elevated respiratory rate from 95±4.0 to 236±29cpm and from 117±5.2 to 189±13cpm respectively; this response was abolished after the DMH/PeF blockade. We conclude that integrity of the DMH/PeF area is essential for generation of respiratory responses to both stressful and alerting stimuli.

Key words: Dorsomedial hypothalamus; respiratory rate; tidal volume; arousal; stress.

Respiration is a unique physiological function: unlike other autonomic parameters, respiration can be modified by higher order behavioral or conscious influences. This property of the respiratory system is due to the fact that it serves for such diverse purposes as autonomically controlled homeostasis of blood gases and more centrally mediated ventilatory adjustments necessary for non-homeostatic purposes such as olfactory sampling and vocalization. Such a dual nature of the respiratory system makes respiratory indices useful for studying not only brainstem homeostatic mechanisms but also various aspects of stress and cognition.

The respiratory pattern of rodents follows a distribution with two distinct modes – baseline respiratory rate (i.e. during rest) and respiratory rate during sniffing (17). Various stressful and arousing stimuli can modulate these modes. An increase in baseline/resting respiratory rate after presentation of prolonged stressors is a component of an integrated defense reaction (an increase in gas exchange for preparation for a fight-or-flight response) (13). Brief mild non-noxious stimuli (e.g. sudden noise), on the other hand, evoke transient increases in respiratory rate (i.e. "sniffing; Ref #28), and it is tempting to interpret this reaction as a respiratory/olfactory component of the orienting response. While in adult humans the magnitude of such tachypnea is very modest (28), it is quite substantial in infants, with about a two-fold transient increase in the respiratory rate in response to tactile stimulus (22). These respiratory arousal responses are much more prominent in rodents, where sudden stimuli often cause rapid rises in their respiratory rate from 80-100 cycles per min (cpm) to over 500 cpm (17, 24). Presumably this inter-species difference could be explained by the fact that rodents heavily rely on their olfactory input while exploring environment, so that prominent increase of their respiratory rate during sniffing is a part of their behavioral repertoire for the risk assessment.

We have recently demonstrated in a rat study that the integrity of the amygdala is essential for the generation of respiratory responses to stressors of high, but not low, intensity of both short and long duration (1). This finding is in line with the well documented role of the amygdala in autonomic and emotional processing during stress (20), and suggests that stress-induced respiratory arousal is possibly mediated by those brain areas that are also responsible for other autonomic manifestations of stress (i.e. thermogenesis, hypertension and tachycardia). In addition, our previous results raise the possibility that different central neuronal pathways mediate the defense and the orienting components of respiratory responses. In an attempt to address this question, in the current study we are focusing on the dorsomedial hypothalamus (DMH) and the adjacent perifornical area (PeF) - a crucial region within the central network of autonomic control. Our choice of this brain area was in particular based on the fact that the amygdala-induced cardiovascular arousal is mediated by the DMH (35), and we thus hypothesized that similar relation might exist for the respiratory arousal. While the DMH influences on stress-induced thermogenesis, heat conservation, arterial pressure and tachycardia are well documented (6, 9, 10), data on the DMH involvement in the respiratory function is limited to studies conducted in anesthetized animals.

Our principal aim was to determine whether normal neuronal activity in the DMH/PeF is essential for the expression of tachypnoeic responses to arousing and stressful stimuli. We thus examined the effects of chemical inhibition of the DMH/PeF region by a $GABA_A$ agonist muscimol on the respiratory responses to sudden acoustic stimuli of various intensities, which evoke brief vigorous respiratory

responses, and to prolonged stressors (novel environment and restraint), which evoke an increase in resting (i.e. locomotion-independent) respiratory rate, in conscious unrestrained rats.

METHODS

Animals

Eight male Outbred Wistar rats were received from the University of Newcastle animal house. They were individually housed at 12/12h light/dark cycle (lights on at 7am) with *ad libitum* access to food and water. All experimental procedures were performed at least 7 days after delivery of animals. All experimental procedures were approved by the Animal Care and Ethics Committee of the University of Newcastle and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

During a preliminary surgery animals were anesthetized with Isoflurane (2% in oxygen) and were implanted with bilateral guide cannulas targeting the dorsomedial hypothalamus (-3.3 mm caudal, 9 mm ventral, 0.5 mm lateral from bregma)**.** Carprofen (5 mg/kg) and enrofloxacin (10 mg/kg) were used as an analgesic and an antibiotic.

At least 7 days after the surgery and no earlier than a recovery to body weight prior to the surgery, animals were subjected to two recording sessions with at least 48 hours between them. Twenty minutes prior to each recording session animals received microinjections of either muscimol (20 nmol in 200 nl) or saline (equal volume) bilaterally into the DMH/PeF in a counter-balanced design. The injections were performed using a syringe pump (KDS200, KD Scientific, Holliston, MA) attached to the injector over at least 1 minute. The injection volume was assessed by observation

of the meniscus in a glass capillary attached to the injection cannula. After each successful injection the injector remained in place for at least 30 seconds to prevent spill of drug back into the cannula during removal of the injector. A relatively large dose of muscimol (20 nmol in 200 nl) was selected in this study as the protocol of the current study was longer than protocols of previous similar studies (such as Ref. 15) and we were aiming to inhibit both the dorsomedial hypothalamus and the perifornical area for at least 2 hours. Each animal performed the session twice – once with muscimol to the DMH/PeF and once with saline to the DMH/PeF pre-treatment. At the beginning of this study, we also attempted to perform control microinjections of muscimol dorsal to the DMH/PeF; however, such microinjections resulted in animals loosing consciousness, presumably due to blockade of the thalamus. Muscimol was purchased from Sigma-Aldrich (USA).

Experimental design

During a recording session, animals were individually placed inside an open system whole-body plethysmograph with a constant flush of air at the rate of 2 L/m (see Ref. 17). By monitoring the rate of pressure fluctuations of the outflow line, we are able to assess the respiratory rate online. Also, we are able to indirectly assess the change in tidal volume by measuring the magnitude of these pressure fluctuations. The apparatus used in this study does not allow reliable assessment of the tidal volume during prolonged periods, as this requires continuous measurements of body temperature and air humidity. However, during short periods, such as during presentation of the acoustic or light stimuli, changes in the body temperature or air humidity are assumed to be negligible and therefore an estimate of the tidal volume can be assessed more reliably. Animal bedding was placed on the bottom of the

plethysmograph. The apparatus was placed in a soundproof box, and constantly illuminated by a 20 lux LED light. Animals' gross motor activity was monitored by a piezoelectric pulse transducer (MLT1010/D, ADInstruments, Sydney, Australia) placed under the plethysmograph. The plethysmograph and the piezoelectric pulse transducer were connected to the PowerLab data acquisition system (ADInstruments, Sydney, Australia).

Each recording session consisted of a 40-minute acclimatization period followed by presentation of six acoustic stimuli (500ms white noise, 50ms rise and 50ms fall) of increasing intensity (40-90dB in 10dB steps). Stimuli were presented from a generic speaker placed underneath the plethysmograph. A 30-second light stimulus (2000 lux) was presented after the last acoustic stimulus. All stimuli were presented with 5-minute inter-stimulus interval. 10 minutes after the last stimulus, animals were removed from the chamber, placed into a tight metal mesh (restrainer) and returned into the plethysmograph for the final 15 minutes of recording.

Raw respiratory and motor signals were sampled at 1kHz rate. Respiratory rate (in cycles per minute, cpm) and the estimate of the tidal volume (in arbitrary units) were computed online using LabChart software (Version 7.1, ADInstruments, Sydney, Australia). Respiratory rate responses were quantified as a maximal change from baseline (Δ) during the first second after the stimulus onset. Similarly, responses in the estimate of the tidal volume were quantified as a maximal change from baseline (expressed as percentage of baseline). Latency of respiratory responses (expressed in ms) was computed offline as a time from the onset of stimulus to the first detectable change in the raw respiratory signal. This parameter was only computed for the 70-90dB stimuli due to lack of responses on some trials with less intense stimuli.

For characterizing respiratory pattern during acclimatization and during restraint we used 4 parameters: mean respiratory rate (Resp $Rate_{MEAN}$), dominant respiratory rate (Resp $Rate_{DOMINANT}$), standard deviation of respiratory rate (SD_{RR}), and percentage of high frequency (>250cpm) respiratory rate (%HF). These parameters were assessed for each 5-minute interval of acclimatization (8 intervals in total), a 5-minute baseline immediately prior to restraint and each 5-minute interval of restraint (3 intervals in total). Resp $Rate_{MEAN}$ and SD_{RR} were calculated in LabChart software. Resp $Rate_{DOMINANT}$ and %HF were calculated in Igor Pro software (Wavemetrics, USA) by plotting time histograms for each of the 5-minute intervals. The modes of such histograms represented Resp $Rate_{DOMINANT}$. %HF was assessed as the ratio $AUC_{250-650}/AUC_{0-650}$ (AUC = area under the curve). See Refs. 1 and 4 for more detailed descriptions of this procedure.

Acclimatization data was initially analyzed by 8x2 (interval x drug) within-subjects ANOVAs for each of the assessed parameters. If the main effect of drug or an interaction between the effects of interval and drug were significant, we performed Fisher's Least Significant Difference (LSD) post-hoc test between trials with saline and muscimol pre-treatment. To analyze restraint data we first expressed each of the parameters for each of the intervals of restraint as a change from the baseline 5-minute interval (Δ). Subsequently, we performed 3x2 (interval x drug) within-subjects ANOVAs for each of the assessed parameters with LSD post-hoc test.

We used three parameters to describe respiratory responses to the acoustic stimuli: the amplitude of respiratory rate response (ΔRespRate), the proportional change in the estimate of tidal volume (ΔTv) and latency of respiratory response. Each of the parameters was initially analysed by a 6x2 (intensity x drug) within-subjects ANOVA with subsequent post-hoc LSD test between saline and muscimol

pre-treatment trials. Respiratory response to the light stimulus was characterized by the respiratory rate and the tidal volume parameters. We assessed the mean of respiratory rate and the mean of tidal volume during the 30-second presentation of the light stimulus. Subsequently, the respiratory rate response was expressed as a change (Δ) from the 30-s baseline baseline immediately prior to the stimulus onset, while the tidal volume response was expressed as a percentage of the baseline. Then, we compared these parameters between trials with saline and muscimol pre-treatment using paired-samples t-tests.

RESULTS

DMH/PeF blockade affected respiratory pattern during acclimatisation

Upon placement into the plethymograph, animals displayed a mean respiratory rate (Resp $Rate_{MEAN}$) of 221 ± 19 and a dominant respiratory rate (Resp $Rate_{DOMINANT}$) of 127 ± 4.2 during the first 5-minute interval of acclimatization on the saline trials (Fig. 1). Both parameters gradually declined during the acclimatization and were 122 ± 11 and 96 ± 3.4 respectively during the last 5-minute interval. 8x2 within-subjects ANOVAs were performed on the Resp $Rate_{MEAN}$, the Resp $Rate_{DOMINANT}$, standard deviation of respiratory rate (SD_{RR}) and the percentage of high frequency respiratory rate (%HF). Blockade of the DMH/PeF inhibited the Resp $Rate_{MEAN}$ during all but the last 5-minute intervals of acclimatization ($p < .05$). Muscimol to the DMH/PeF also decreased the Resp $Rate_{DOMINANT}$ during the first 5-minute interval, $p = .032$, while it increased it during the seventh 5-minute interval, $p = .028$. Lastly, we found that blockade of the DMH/PeF region has significantly inhibited the standard deviation of respiratory rate and percentage of high frequency

respiratory rate during all eight 5-minute intervals of acclimatization (all $p < .05$). All data from the acclimatization period are summarized in Fig. 1.

DMH/PeF blockade inhibited respiratory responses to the short acoustic stimuli

Presentation of acoustic stimuli evoked transient responses in respiratory rate proportional to the stimulus intensity, ranging from an increase of 44 ± 27 cpm in response to the 40 dB stimulus to 329 ± 31 cpm after the 90 dB stimulus (Fig. 2A). 6x2 (intensity x drug) within-subject ANOVAs were performed on the amplitudes of respiratory rate responses (ΔRespRate) and proportional changes in the estimated tidal volume responses (ΔTv) and a 3x2 (intensity x drug) within-subjects ANOVA was performed on the latencies of respiratory response to the 70-90dB stimuli. We have found that the magnitudes of the respiratory rate and the tidal volume responses were linearly proportional to the stimulus intensity (both $p < .001$), while the latency of respiratory responses was negatively linearly related to the stimulus intensity ($p < .001$). Blockade of the DMH/PeF significantly inhibited respiratory rate responses to stimuli of all six intensities (all $p < .05$). It also significantly inhibited tidal volume responses to the 70-90dB stimuli (all $p < .05$). Lastly, muscimol microinjection to the DMH/PeF significantly prolonged latencies of respiratory response to the 70 and 80dB stimuli (both $p < .05$). See Fig. 2 for the summary of data of responses to the acoustic stimuli.

DMH/PeF blockade abolished respiratory rate response to the 30s light stimulus

Presentation of the light stimulus significantly elevated the respiratory rate on saline trials ($p = .001$; Fig. 3A), but not on muscimol pre-treatment trials ($p = .125$). Subsequently, a paired-sample t-test indicated that muscimol microinjection into the

DMH/PeF significantly attenuated the amplitude respiratory rate response to the light stimulus (p = .002; Fig. 3B). The tidal volume response was also significantly attenuated (p = .047; Fig. 3C).

DMH/PeF blockade significantly attenuated respiratory response to the restraint

Submission to the restraint stress elevated the mean (Resp $Rate_{MEAN}$) and the dominant respiratory rates (Resp $Rate_{DOMINANT}$) from 117 ± 5.2 to 189 ± 13 cpm and from 102 ± 5.3 to 154 ± 8.1 cpm respectively (Fig. 4). Both the mean and the dominant respiratory rates gradually declined as the restraint stress progressed.

4x2 (time x drug) within-subject ANOVAs were performed on the Resp $Rate_{MEAN}$, the Resp $Rate_{DOMINANT}$, the standard deviation of respiratory rate (SD_{RR}) and the percentage of high frequency respiratory rate (%HF). Inhibition of the DMH/PeF significantly inhibited the mean of respiratory rate during baseline and all three 5-min intervals of restraint (all $p < .05$). Also, it inhibited the dominant respiratory during the first and second 5-min interval of restraint (both $p < .05$). Importantly, restraint failed to evoke any significant change in the dominant respiratory rate after the muscimol microinjection into the DMH/PeF (p = .38; see Fig. 4B).

Submission to restraint stress significantly elevated the standard deviation of respiratory rate (from 32 ± 6 to 65 ± 7 cpm). Inhibition of the DMH/PeF significantly inhibited this response during the first and second 5-minute interval of restraint (both $p < .05$). There were no effects of drug or time on the percentage of high frequency respiratory rate (see Fig. 4).

An example of histologically verified microinjection and a summary diagram of injection sites in 8 animals are presented in Fig. 5.

DISCUSSION

Our main finding is that the dorsomedial and the perifornical hypothalamic region (DMH/PeF) is essential for generation of respiratory responses to both stressful (normally evoking a defence reaction) stimuli as well as to alerting (normally evoking an orienting response) stimuli. Brief stimuli, such as the acoustic stimuli used in the current study, evoke a pattern of vigorous respiratory response ("sniffing"), which is presumably associated with a brief change of arousal. Prolonged stressors, such as the novelty stress and the restraint, evoke a sustained increase in respiratory rate. Inhibition of the DMH/PeF abolished respiratory responses to both brief stimuli and prolonged stressors. In particular, it abolished responses to the light stimulus, the 40-70dB acoustic stimuli and the restraint stress. The only respiratory responses that were generated after the DMH/PeF blockade were in response to the 80-90dB acoustic stimuli and in response to placement into the recording chamber (novelty stress), but all of these responses were also significantly inhibited.

Evidence of DMH/PeF involvement in respiratory function

The current experiment is the first demonstration that integrity of the DMH/PeF region is essential for generation of respiratory responses to alerting stimuli and stress in conscious animals. This finding is supported by previous studies in anesthetized animals that described tachypneic responses to chemical stimulation of the DMH/PeF area (8, 33). Our results are also in full accord with the previous findings that describe the DMH/PeF as a key region of the central network mediating autonomic responses

to stress. Blockade of the DMH/PeF abolished cardiac and pressor responses to restraint stress in conscious animals (32). Conversely, disinhibition of the DMH by $GABA_A$ antagonist bicuculine evokes increases in the heart rate, arterial pressure and respiratory rate in anesthetized animals (21, 33), while in conscious animals it also evokes panic-like behaviour (7). Our findings show that the DMH/PeF is as important for mediating respiratory arousal, as for mediating other autonomic changes. It is generally believed that the DMH integrates information from other nuclei of the central autonomic network (6). Indeed, it was shown that blockade of the DMH abolishes increases in the arterial pressure and respiration evoked by the periaqueductal grey stimulation (14). Also, blockade of the prelimbic medial prefrontal cortex (PFC) decreased the panic-like behaviour induced by the DMH disinhibition (7). Zhang et al. (35) has shown that neurons in the DMH/PeF region that receive projections from the amygdala contain orexin. Orexin is a neurotransmitter than is almost exclusively produced by a small number of cells of the hypothalamus; however, these cells have axonal projections throughout the brain, including projections from the DMH/PeF area to the plPFC, the amygdala and the periaqueductal grey (29). Disinhibition of the amygdala by $GABA_A$ antagonist bicuculine evoked significant cardiac and respiratory responses in the wild-type mice, but not in orexin neuron ablated mice under anaesthesia (35). Similarly, disinhibition of the DMH/PeF evoked significantly smaller respiratory responses in orexin knockout mice than in wild-type mice (18), suggesting that the orexin system of the DMH/PeF could be involved in mediating respiratory responses to the arousing and stressful stimuli.

Another potential mechanism, by which inhibition of the DMH/PeF area could have affected respiration, is by inhibition of the metabolic processes. Indeed,

stimulation of the DMH was shown to elevate thermogenesis via increased heat production in the brown adipose tissue (BAT), increase in CO_2 production and subsequent increase in respiratory rate via chemoreflex mechanisms (3). Furthermore, previous studies reported increases in BAT temperature during stress (25). Inhibition of the DMH/PeF area in the current study possibly inhibited such stress-induced heat production in the BAT, leading to decreased respiratory responses. However, it is unlikely that this process alone can explain the observed inhibition of respiratory rate during any of the stimuli and stress in the current study, as increase of core temperature and subsequent increase in metabolic demand is a relatively slow process, which requires at least 10 minutes to reach peak values during the restraint stress (25) or during direct stimulation of the DMH (3). If respiratory responses to stress were secondary to increases in metabolic demand, then the maximal values would have been observed 10-15 minutes after the onset of the stressor on saline trials. As this was not the case, it must be that inhibition of respiratory rate response to restraint and novelty stress was primarily due to a blockade of the central pathway directly mediating respiratory activation in response to stress. However, inhibition of metabolic processes via BAT heat production probably did contribute to a reduction of respiratory rate on muscimol pre-treatment trials, but only at the later stages of restraint and novelty stress.

Using a methodology identical to the one employed in the current study we have previously investigated the effects of the amygdala or the prelimbic prefrontal cortex (plPFC) blockade on the respiratory responses to brief acoustic stimuli, novel environment stress and restraint. Blockade of the amygdala inhibited the magnitude, but not the latency of respiratory responses to the high-intensity acoustic stimuli (70-90dB) (1), while blockade of the plPFC had no effect on these responses (2).

Furthermore, the extent of inhibition of the respiratory responses after the amygdala blockade was very similar to the one observed in the current study after the DMH/PeF blockade. As the amygdala has direct projections to the DMH/PeF (35) it is therefore possible that the amygdala contributes emotional connotation to a response that can be evoked in absence of any input from the amygdala. Therefore, it could be contributing to the magnitude, but not the speed of these responses. In the aforementioned study blockade of the amygdala by muscimol has also decreased the respiratory rate during the 5-minutes of restraint stress and inhibited time-dependent reduction in respiratory rate during the restraint (1). Also, inhibition of the prelimbic prefrontal cortex (plPFC) decreased the respiratory rate during all three 5-minute intervals of restraint, but preserves the time-dependent reduction (2). Blockade of the DMH/PeF in the current study inhibited the respiratory rate during all three 5-minute intervals of restraint and also inhibited the time-dependent reduction in the respiratory rate. As both the plPFC and the amygdala have direct projections to the DMH/PeF region (26), it is therefore possible that this effect was due to inhibition of the two components – a fear-related respiratory response to the restraint stress that dissipates with time and is presumably mediated by the amygdala and a general arousal-related increase in respiratory rate that is presumably mediated by the prelimbic prefrontal cortex.

Data of the current study also suggest that the DMH/PeF region mediates respiratory component of the orienting response as we found significant inhibition of the respiratory rate responses to the low-intensity acoustic stimuli. We have previously shown that this response is not mediated by the amygdala (1), which is in line with previous findings that the habituation, but not the expression of the cardiac component of the orienting response is mediated by the amygdala (30). It is currently unknown which central regions trigger the respiratory component of the orienting

response. It is possible that other central structures, such as the infralimbic prefrontal cortex, are involved in initiating this response as it is activated in humans during orienting (34). Also, it is possible that this response is mediated by the lateral hypothalamic area as its electrical stimulation elicits a bradycardic and a tachypneic responses (12), which is consistent with a pattern of orienting response. Another potential candidate is the periaqueductal gray (PAG) as it has been recently demonstrated that stimulation of the PAG area evokes tachypnea in anesthetized rats and this response can be abolished by the DMH blockade (14).

We must acknowledge that our methodology does not have spatial resolution required to separate the effects of the blockade of the DMH and the immediately adjacent PeF, as a volume of a microinjection that was required for an experiment in conscious animals most likely affected both regions. Some previous studies in conscious rats have similarly investigated effects of inhibition of both regions (e.g**.** Ref. 16). Furthermore, Zhang et al. have shown that orexin neurons that mediate respiratory responses to stress are located in both the DMH and the PeF (35). However, a functional topographic investigation of not only the DMH (e.g. Ref. 33), but also of the PeF is required in order to separate influences of the both regions on the central respiratory drive.

In the current study we were unable to perform control injections of muscimol into an area other than the DMH/PeF. The guide cannula used in the experiment was 5mm long, as it ensures good accuracy of microinjections, therefore we were only able to perform injections at least 6mm ventral from the surface of the skull. Injections dorsal to this area, around the thalamus, resulted in rats losing consciousness. However, we do not believe that this is a serious limitation as we have previously performed control injections of muscimol into an area that is not part of the

central autonomic network in a methodology identical to the one used in this study and we did not see significant effects on the respiratory responses to various stressors (1).

Also, it is important to note that studies that implicated the DMH/PeF region in autonomic control have only been performed in animals. Only recently the research that implicates the involvement of the DMH/PeF in humans in autonomic processing started to emerge (19). However, involvement of the DMH/PeF area in the central command of respiration has not been shown in humans.

Connectivity of the DMH/PeF with the lower brainstem respiratory network

A recent neuroanatomical tracing study described dense projections from the perifornical area to the lateral and medial parabrachial nuclei (26). Also, there were sparse projections from the DMH to the Kölliker-Fuse nucleus as well as to the lateral and medial parabrachial nuclei (26). Kölliker-Fuse and the parabrachial nuclei form the pontine respiratory group that in turn has projections to the central respiratory pattern generating nuclei in the lower pons and medulla – the PreBötzinger complex and the retrotrapezoid nucleus (11, 31). Activation of neurons within the pontine respiratory group evokes tachypneic or apneic responses (5). The theory that projections from the DMH/PeF to the parabrachial region mediate the stress- and arousal-induced respiratory responses is supported by the findings of Mizusawa et al. (23) and Horiuchi et al. (14). Stimulation of the dorsal periaqueductal grey produces a tachypneic response; this response can be blocked by the inhibition of the parabrachial nucleus (23) or the DMH (14).

Significance and perspectives

In summary, we have demonstrated that integrity of the dorsomedial and perifornical hypothalamic region is essential for the generation of respiratory components of both defence and orienting responses. Inhibition of this region abolishes respiratory response to a restraint stress, a light stimulus and to low-intensity acoustic stimuli, while it also attenuates responses to high-intensity acoustic stimuli and to novel environment. This supports previous findings that the DMH/PeF region is critical for generation of autonomic responses. By comparing findings of the current study with experiments employing the same methodology, but targeting other structures of the central respiratory network, it is possible to elucidate contributions of different structures within the central network to expression of respiratory arousal in response to alerting and stressful stimuli.

GRANTS

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Figure 1. Changes in respiratory indices during acclimatisation period on the muscimol (solid line) and saline (dotted line) pre-treatment trials. The dorsomedial and perifornical hypothalamic area (DMH/PeF) blockade inhibited the mean respiratory rate (A) during all but the last 5-minute intervals of acclimatization, but had a less pronounced effect on the dominant (median) respiratory rate (B) as it inhibited it during the first 5-minute interval, but elevated during the seventh. Muscimol microinjection to the DMH/PeF has also inhibited the standard deviation of respiratory rate (C) and the percentage of high frequency respiratory rate during all eight intervals of acclimatization. * - significant difference between muscimol and saline pre-treatment trials, $p < .05$; ** - significant difference between muscimol and saline pre-treatment trials, $p < .01$; *** - - significant difference between muscimol and saline pre-treatment trials, $p < .001$.

Figure 2. Effects of the dorsomedial and perifornical hypothalamic region (DMH/PeF) inhibition by the muscimol microinjection on the respiratory response to 500ms white noise acoustic stimuli of 40-90dB intensity. Inhibition of the DMH/PeF significantly inhibited the respiratory rate responses to stimuli of all intensities (A). It also inhibited the tidal volume response to the 70-90dB stimuli (B) and prolonged the latency of respiratory response to the 70 and 80dB stimuli (C). *Note:* * - significant difference between muscimol and saline pre-treatment trials, $p < .05$; ** - significant difference between muscimol and saline pre-treatment trials, $p < .01$.

Figure 3. Baseline respiratory rate values before and during presentation of the 30-s light stimulus (A). Microinjection of muscimol into the dorsomedial and perifornical hypothalamic region (DMH/PeF)significantly decreased the mean of respiratory rate response (B) and the tidal volume response (C), to the light stimulus. Each data point represents a mean of 30 seconds of 8 rats after saline (dotted line in A) or muscimol (solid line in A) bilateral microinjection into the DMH/PeF. * - significant difference between muscimol and saline pre-treatment trials, $p < .05$; ** - significant difference between muscimol and saline pre-treatment trials, $p < .01$.

Figure 4. Microinjection of muscimol into the dorsomedial and perifornical hypothalamic region (DMH/PeF) significantly decreased the mean of respiratory rate during baseline and restraint (A), while it also decreased the dominant respiratory rate during the first two 5-minute intervals of restraint (B). Inhibition of the DMH/PeF inhibited the standard deviation of respiratory rate during the first and second 5-minute intervals of restraint (C), but it did not affect the percentage of high frequency respiratory rate (D). Each data point represents a mean of 30 seconds of 8 rats after saline (dotted line) or muscimol (solid line) bilateral microinjection into the prelimbic prefrontal cortex. *Note:* * - significant difference with $p < .05$; ** - significant difference between muscimol and saline pre-treatment trials, $p < .01$; *** - significant difference between muscimol and saline pre-treatment trials, $p < .001$.

Figure 5. Histological verification of microinjection sites into the dorsomedial hypothalamic area. Right side of the picture displays a coronal section of brain of one

of the rats in the current experiment; the arrowhead points to a microinjection site. The left side of the picture displays centers of successful microinjection sites (black circles) drawn on a standard coronal section diagram from the atlas of Paxinos and Watson (27). Abbreviations: ic, internal capsule; dmh, dorsomedial hypothalamus; f, fornix; mb, mamillo-thalamic tract; ot, optic tract; pef, perifornical area; III, third ventricle.

Figure 1

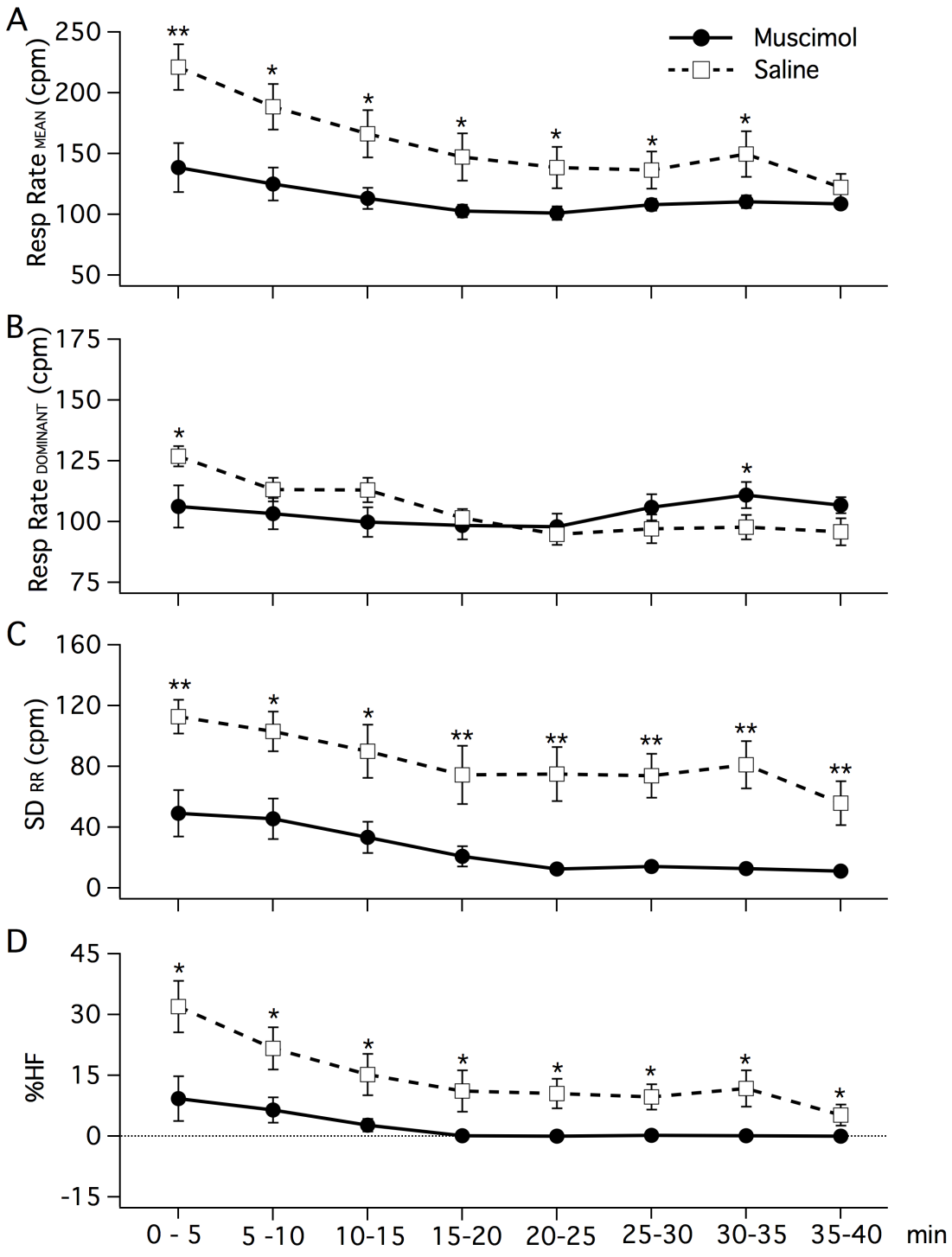

A
Resp Rate MEAN (cpm)
250
200
150
100
50
Muscimol
Saline
B
Resp Rate DOMINANT (cpm)
175
150
125
100
75
C
SD RR (cpm)
160
120
80
40
0
D
%HF
45
30
15
0
-15
0 - 5
5 -10
10-15
15-20
20-25
25-30
30-35
35-40
min

Figure 2

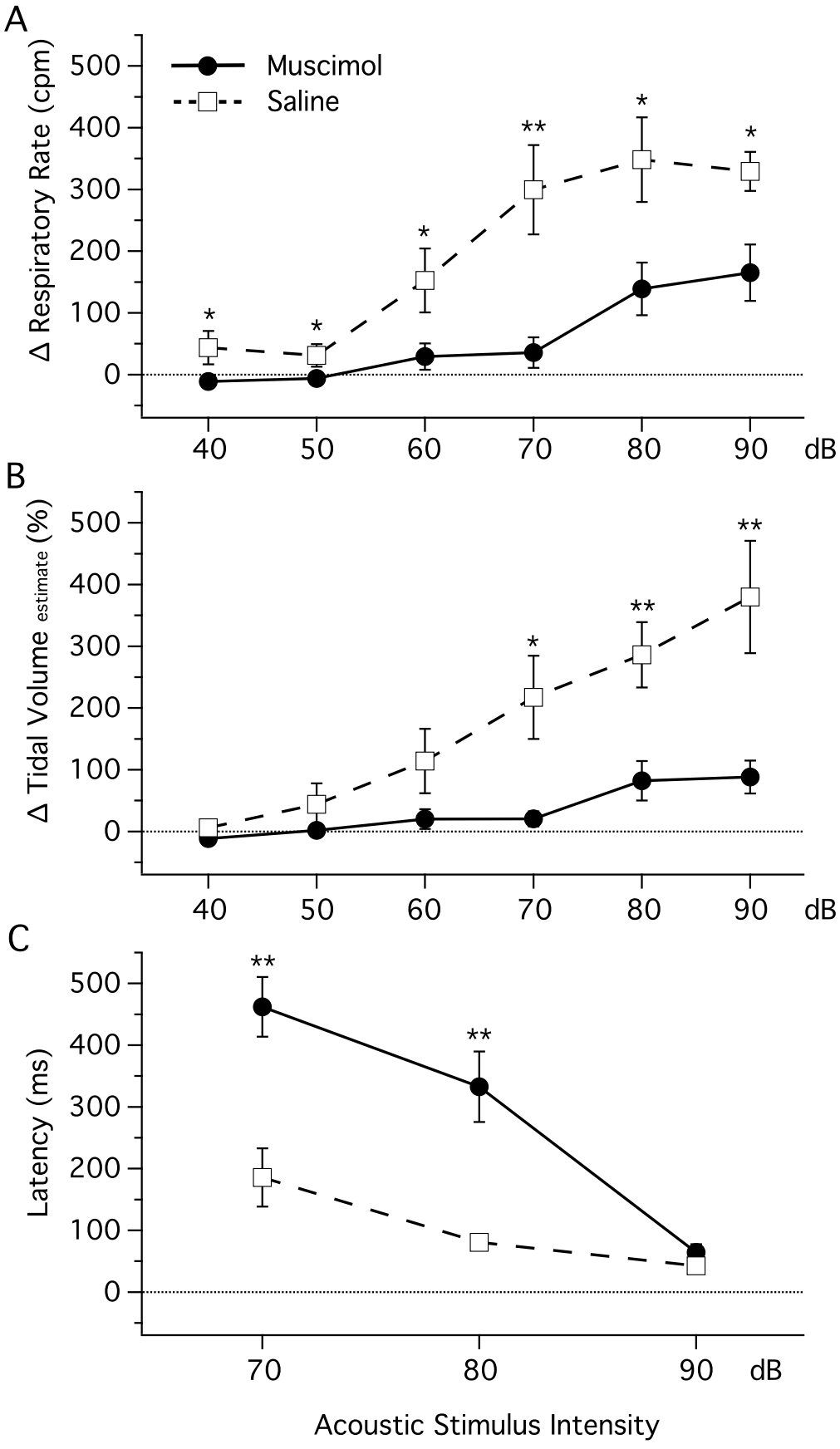

A
Muscimol
Saline
Δ Respiratory Rate (cpm)
500
400
300
200
100
0
*
*
*
**
*
*
40
50
60
70
80
90
dB
B
Δ Tidal Volume estimate (%)
500
400
300
200
100
0
*
**
**
40
50
60
70
80
90
dB
C
Latency (ms)
500
400
300
200
100
0
**
**
70
80
90
dB
Acoustic Stimulus Intensity

Figure 3.

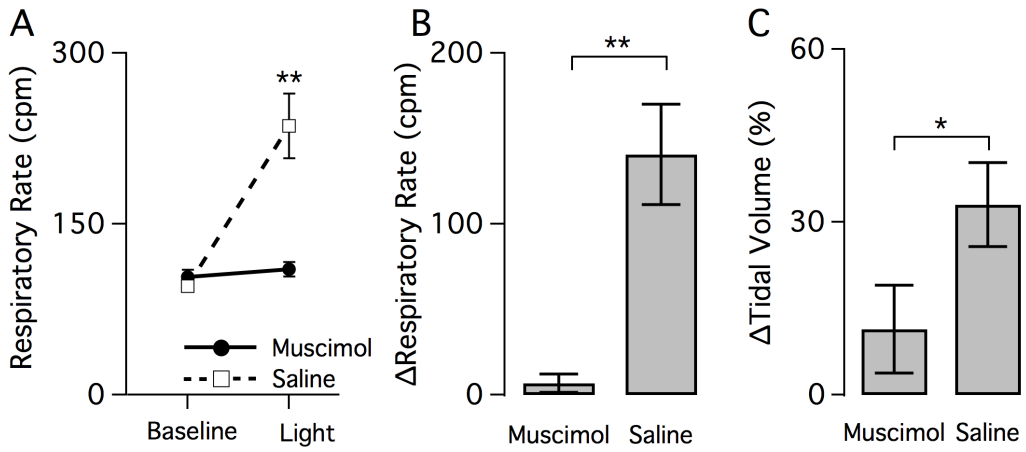

A
300
150
0
Respiratory Rate (cpm)
**
Muscimol
Saline
Baseline
Light
B
200
100
0
ΔRespiratory Rate (cpm)
**
Muscimol
Saline
C
60
30
0
ΔTidal Volume (%)
*
Muscimol
Saline

Figure 4.

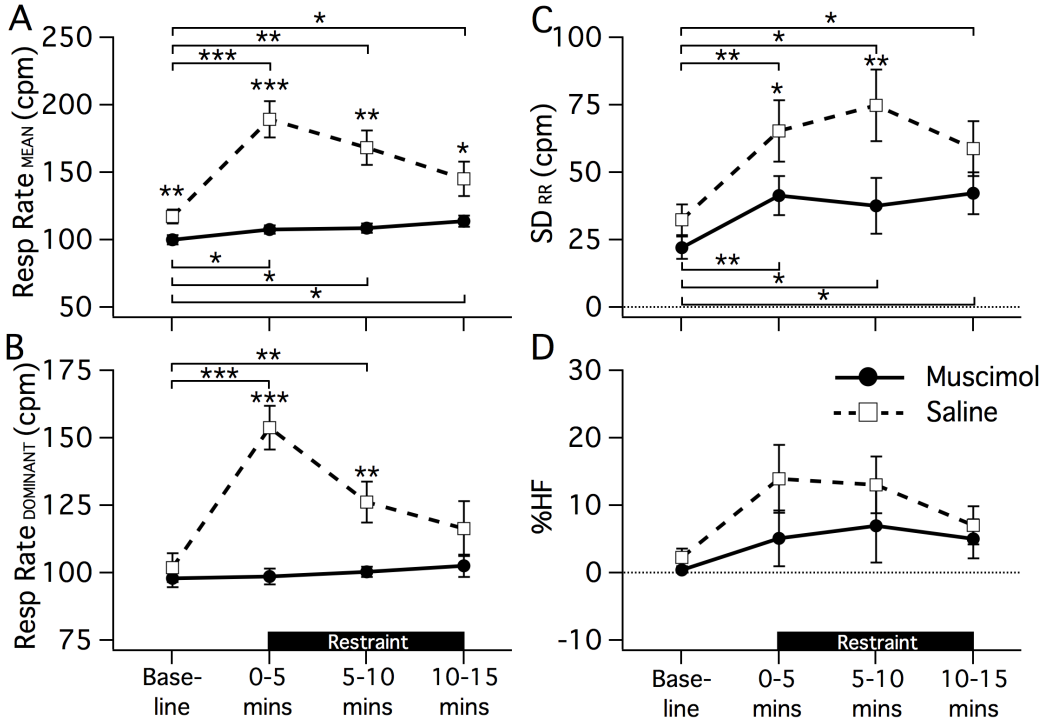
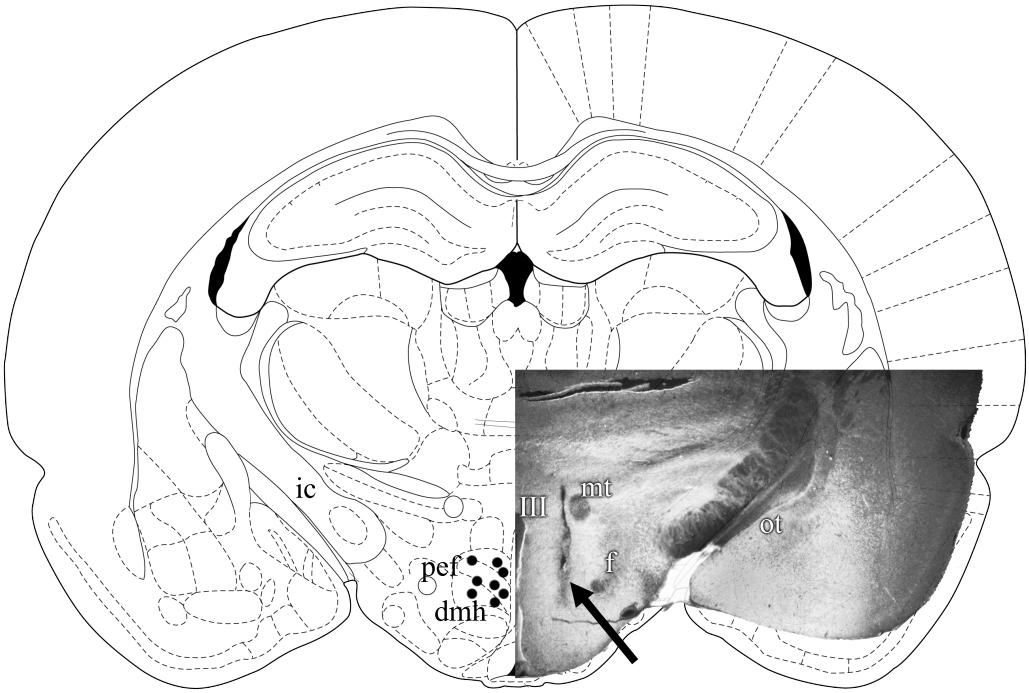

A
B
C
D
Resp Rate MEAN (cpm)
Resp Rate DOMINANT (cpm)
SD RR (cpm)
%HF
Muscimol
Saline
Restraint
Base-line
0-5 mins
5-10 mins
10-15 mins

Figure 5.



CHAPTER 3

AMYGDALA MEDIATES RESPIRATORY RESPONSES TO SUDDEN AROUSING STIMULI AND TO RESTRAINT STRESS IN RATS

INTRODUCTION TO CHAPTER 3

Chapter 3 investigates involvement of the amygdala in the central respiratory stress network. Electrical stimulation of the amygdala evokes tachycardic and pressor responses in conscious animals (Gören et al., 1996) and also tachypnoea in anesthetized animals (Zhang et al., 2009). Blockade of this region attenuates heart rate and arterial pressure responses to stress (Salomé et al., 2007). Furthermore, electrical stimulation of the amygdala was shown to elevate respiratory rate in a human patient (Masaoka and Homma, 2005). Chapter 3 investigates the effect of pharmacological blockade of the amygdala on the respiratory responses to brief and prolonged stressors of various intensities in conscious unrestrained rats.

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Amygdala mediates respiratory responses to sudden arousing stimuli and to restraint stress in rats

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Bondarenko E, Hodgson DM, Nalivaiko E. Amygdala mediates respiratory responses to sudden arousing stimuli and to restraint stress in rats. *Am J Physiol Regul Integr Comp Physiol* 306: R951–R959, 2014. First published April 16, 2014; doi:10.1152/ajpregu.00528.2013.—Both human and animal studies have demonstrated that respiratory parameters change in response to presentation of alerting stimuli, as well as during stress, yet central neuronal pathways that mediate such responses remain unknown. The aim of our study was to investigate the involvement of the amygdala in mediating respiratory responses to stressors of various intensities and duration. Adult male Wistar rats ($n = 8$) received microinjections of $GABA_A$ agonist muscimol or saline into the amygdala bilaterally and were subjected to a respiratory recording using whole body plethysmography. Presentation of acoustic stimuli (500-ms white noise, 40–90 dB) caused transient responses in respiratory rate that were proportional to the stimulus intensity, ranging from +13 ± 9 cpm to +276 ± 67 cpm for 40- and 90-dB stimuli, respectively. Inhibition of the amygdala significantly suppressed respiratory rate responses to the high-intensity stimuli (70–90 dB). Submitting rats to the restraint stress significantly elevated the mean respiratory rate (+72 ± 8 cpm) and the dominant respiratory rate (+51 ± 12 cpm), as well as the fraction of high-frequency respiratory rate (+10 ± 3%). Inhibition of the amygdala by muscimol significantly suppressed these responses. We conclude that the amygdala is one of the key structures that are essential for expression of respiratory responses to stressful or alerting stimuli in rats.

amygdala; respiratory rate; tidal volume; arousal; stress

RESPIRATION IS A UNIQUE PHYSIOLOGICAL activity. On the one hand, it is an autonomic function responsible for maintaining the homeostasis of blood gases. On the other hand, respiratory parameters can be readily modified by higher-order, behavioral, or even conscious influences. One of such influences is the influence of emotions. Numerous studies described tight links between respiration and different emotional states in humans (see Refs. 4 and 14 for reviews). Anxiety is one such emotional state; it is physiologically associated with defense mechanisms or fight-or-flight response. Human studies that have used various laboratory stressors have firmly established that prolonged states of stress and anxiety increase respiratory rate and decrease tidal volume (33, 34, 36). Respiratory disturbances are an established sign of panic disorder (see Ref. 25 for review). Sudden arousing acoustic stimuli produce rapid and dramatic increase in respiratory rate in humans (27). Paradoxically, very little is known about the link between respiration and emotion in laboratory animals, as most respiratory animal studies have focused on homeostatic pontomedullary mechanisms (9, 30).

Preclinical research has recently started investigating respiratory responses in animal models of anxiety. Early studies lacked nonintrusive and precise techniques for assessing respiratory indices in nonanesthetized animals. Among modern methods, whole body plethysmography represents a promising approach, as it is entirely noninvasive and, thus, does not introduce any confounding factors. Using this method, Kinkead and colleagues (12, 13) have recently demonstrated that neonatal maternal separation in rats provokes a respiratory phenotype in adulthood that presents many anxiety-related features. Such animals have altered respiratory responses to hypoxia (12) and hypercapnia (13), with the underlying mechanisms involving both alterations in the chemoreflex circuitry in the lower brain stem (18) and descending influences from the hypothalamus (11).

There is currently limited information on relations between arousal or emotional states and respiration in animals. In rats, sudden alerting stimuli of various sensory modalities provoke vigorous respiratory responses (sniffing) (16, 24). These responses are likely linked with animals' anxiety state, as they are sensitive to anxiolytic drug diazepam (23) and are significantly increased in rats with induced high-anxiety behavior (32). There are also substantial differences in respiratory pattern between rats bred for low-anxiety behavior compared with animals bred for high-anxiety behavior (5). The brains substrate of the anxiety-related respiratory responses is poorly understood. Our present work is focused on the amygdala, a key neuronal structure in processing fear and anxiety. There is firm evidence, both in humans and animals, that the amygdala mediates stress-induced cardiovascular responses (22). In fact, involvement of the amygdala in conditioned fear response was established by assessing cardiovascular parameters (1, 15). There is also some evidence obtained in anesthetized animals that suggests that the amygdala may mediate stress-induced respiratory response (19), but this has never been directly studied.

In contrast to well-documented involvement of the amygdala in cardiovascular responses to various stressors, only a few animal studies provide evidence for the link between the amygdala and the respiratory function. Specifically, electrical or pharmacological activation of the amygdala resulted in increases in respiratory parameters in anesthetized mice (38). Electrical stimulation of the amygdala in awake rabbits has also resulted in an increase of respiratory rate (2). Likewise, electrical stimulation of the amygdala in an epileptic patient was associated with an increase in respiration (21). All of these findings suggest that the amygdala may be involved in processing some aspect of anxiety-induced respiratory effects but

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do not directly prove its involvement. Obtaining such direct evidence in animal experiments was our primary aim. To that end, we investigated the effects of pharmacological inhibition of the amygdala by microinjections of $GABA_A$ agonist muscimol on changes in the respiratory parameters elicited by brief standardized acoustic stimuli, a visual stimulus, and by a prolonged stressor (restraint).

METHODS

Animals. Eight outbred male Wistar rats, weighing 350–400 g, were obtained from the University of Newcastle Animal Services unit. For the duration of the protocol, they were single-housed and kept on a reverse dark-light cycle (lights on at 1900). All experimental procedures were approved by the University of Newcastle Animal Care and Ethics Committee and were in accordance with Animal Research Regulation 2010 of New South Wales, Australia.

During preliminary surgery conducted under isoflurane anesthesia (2% in oxygen), guide cannulas targeting the central amygdaloid nucleus (CAm) were implanted bilaterally (−2.7 mm caudal, 7.8 mm ventral, 4.2 mm lateral from bregma). Carprofen (5 mg/kg) was used as an analgesic, and enrofloxacin (10 mg/kg) was used as an antibiotic after the surgery. Animals were allowed to recover for at least 7 days and then were subjected to three recording sessions with at least 48 h between them. Twenty minutes before each session, they received microinjections of $GABA_A$ agonist muscimol (20 nmol in 200 nl) to CAm, 200 nl of saline to the CAm or microinjection of equal volume and concentration of muscimol 3 mm dorsal to CAm (approximately in the location of dorsal parts of internal capsule or caudate nucleus) in a counter-balanced, within-subjects design. Each animal underwent the protocol with all three types of microinjection. Muscimol was purchased from Sigma Aldrich (St. Louis, MO).

Recording technique and experimental protocol. During the recording session, rats were placed inside a plethysmographic chamber (Perspex cylinder, I.D.: 95 mm, length: 260 mm, volume: 1.8 liters, wall thickness: 3 mm) with animal bedding provided on the bottom of the chamber and constant illumination of 20 lux. The chamber was fitted with a removable lid on one side and had a constant flush of compressed air at a rate of 3 l/min. The output flow line made of polyethylene tubing (O.D.: 1.45 mm, I.D.: 0.75 mm) was divided into two lines using a T-connector. One end (10 cm) was attached to the differential pressure amplifier (model 24PC01SMT, Honeywell Sensing and Control, Golden Valley, MN), while the other end (60 cm long) was open to the room air. Each respiratory cycle of a rat placed inside this system corresponded to a brief change of pressure inside a cylinder due to a difference in the temperature of inhaled and exhaled air, while the amplitude of this change was related to the depth of each breath. This apparatus allowed online assessment of respiratory rate and indirect assessment of the change in tidal volume. The chamber was located in a sound-attenuating box, and animals' behavior was observed using a video monitor. For monitoring animals' motor activity, a piezoelectric pulse transducer (MLT1010/D, ADInstruments, Sydney, Australia) was placed under the plethysmograph.

Each recording session consisted of a 40-min "acclimatization" period, followed by presentation of six brief acoustic stimuli of progressively increasing intensity [500-ms white noise; 50-ms rise, and 50-ms fall duration (40–90 dB intensity)] followed by a 30-s light stimulus (2000 lux). These stimuli were presented at 3–4-min interstimulus intervals, as shorter intervals resulted in habituation. All stimuli were presented when animals were awake and quiet and when their breathing was slow (<150 cpm) and regular (without obvious accelerations or decelerations) for at least 10 s. Ten minutes later, rats were subjected to a restraint stress with respiratory assessment. For this, they were removed from the plethysmograph, placed inside a tight metal mesh and then placed back inside the plethysmograph for the final 15 min of recording.

Data acquisition and analysis. Analog respiratory (pressure) and motor data were continuously sampled at 1 kHz and recorded using PowerLab 4SP data acquisition system (ADInstruments). Respiratory rate was computed online with subsequent off-line verification using LabChart software (version 7.1, ADInstruments). We also determined relative changes in tidal volume provoked by sensory and stressful stimuli. We were unable to assess the absolute values of tidal volume, as this required measurements of body temperature and chamber air humidity. However, we assumed that for short-term recordings, as in the case of acoustic and visual stimuli, these variables were constant, and thus, changes in chamber pressure were only determined by inspiratory and expiratory movements. Tidal volume changes were quantified as % of change from baseline.

Acclimatization and restraint. For characterizing respiratory pattern during acclimatization and restraint, we used four parameters: mean respiratory rate ($RespR_{mean}$) was computed by LabChart software from peaks in the respiratory signal, coefficient of variation, (K_{var} = S.D./$RespR_{mean}$), dominant respiratory rate ($RespR_{dom}$ − respiratory frequency at which an animal spent most of its time during recordings) and the percentage of time spent at high respiratory frequency (%HF). For the latter two measures, using IgorPro software (Wavemetrics, Tigard, OR), we first constructed time histograms for each recording with bin width equal 10 cycles/min; an example of such histogram is shown in Fig. 1*B*. This graphic representation indicates how much time (in ms) animals spent at a given respiratory frequency. The mode of such histograms represents the dominant respiratory rate ($RespR_{dom}$). Respiratory rate is relatively stable during periods of no locomotor activity, but it is highly elevated and variable during locomotion (16). Assessment of a dominant respiratory rate provides a way of assessing locomotion-free respiratory rate. %HF was computed as the ratio $AUC_{250-650}/AUC_{0-650}$ (AUC = area under the curve). We computed these for values for the following 5-min intervals: eight epochs of acclimatization, baseline before restraint, and three epochs during restraint.

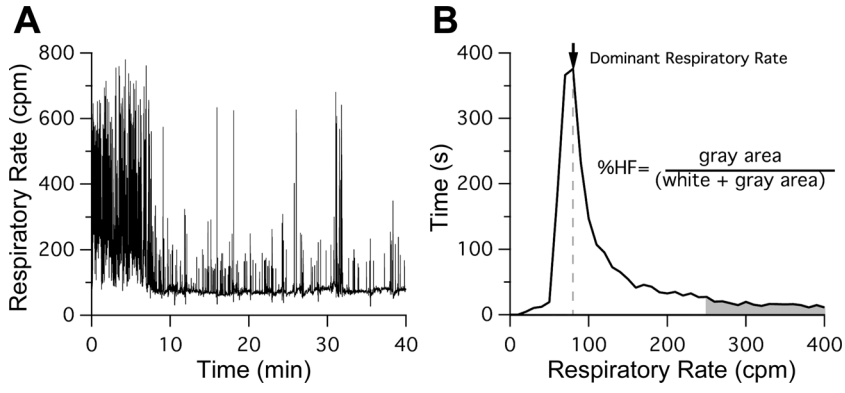

Fig. 1. Respiratory rate during acclimatization. *A*: raw trace of respiratory rate during 40 min of acclimatization of one of the rats. *B*: histogram of acclimatization period of the same rat with the *x*-axis displaying respiratory rate and the *y*-axis indicating time that was spent at a particular respiratory rate. Respiratory rate is remarkably variable, but the dominant respiratory rate (i.e., a respiratory frequency that the rat spent most time on) is stable. Percentage of high-frequency respiratory rate (%HF) was calculated as a proportion between the area under the curve between 250 and 650 cpm respiratory rates (gray area) and the total area under the curve (gray and white area).

Acclimatization data were first analyzed by 8 × 3 within-subjects ANOVAs (interval × pretreatment) for each of the four respiratory parameters measured ($RespR_{mean}$, $RespR_{dom}$, K_{var}, and %HF). If the ANOVA indicated a significant main effect of drug pretreatment, we performed pairwise comparisons of the main effects of drugs. Subsequently, post hoc least significant difference (LSD) comparisons were performed between the drugs that were shown to have significantly different main effects. If the 8 × 3 ANOVA indicated a significant interaction between the effects of time interval and drug, three 8 × 2 ANOVAs (interval × muscimol vs. saline, interval × muscimol vs. control muscimol, interval × saline vs. control muscimol) were performed. If one of these ANOVAs indicated a significant main effect of drug or a significant interaction between the time interval and drug pretreatment, post LSD tests were performed between the drug pretreatments. For the restraint data, we calculated changes from baseline for each index (Δ) and performed a 3 × 3 within-subjects ANOVA (drug pretreatment × interval) on these computed Δ indices. A similar approach to acclimatization analysis was taken for the restraint analysis. If a 3 × 3 ANOVA indicated a main effect of drug pretreatment, we performed a pairwise comparison of main effects of drugs. If one of these comparisons were significant, we performed a post hoc LSD test. If, however, a 3 × 3 ANOVA indicated a significant interaction between the effects of drug and time interval, we performed three 3 × 2 ANOVAs testing different pairs of drug pretreatments. Subsequently, we performed a post hoc LSD test for ANOVAs that indicated significant main effects of drug or significant interactions between the effects of drug and of time interval.

Acoustic stimuli. In analyzing responses to acoustic stimuli, we assessed the amplitudes of changes in respiratory rate and in tidal volume and the latencies of responses. Both respiratory rate and tidal volume responses were computed manually as a maximum change from baseline. Latency of responses was analyzed only for the 70-, 80-, and 90-dB stimuli due to a lack of pronounced responses to the less intense stimuli in some subjects. These responses (RR amplitude, T_V amplitude, and latency) were then analyzed by within-subjects ANOVAs (stimulus intensity × pretreatment). If these ANOVAs indicated a significant main effect of drug on one of the dependent variables, we performed pairwise comparisons of main effects of drugs. Subsequently, post hoc LSD test was used for comparison between drug pretreatments that were shown to have significantly different main effects. If the within-subjects 6 × 3 (3 × 3 for latency) ANOVAs indicated significant interactions between the effects of drug and stimulus intensity, three 6 × 2 (3 × 2 for latency) ANOVAs were performed. If one of these ANOVAs indicated a significant main effect of drug or a significant interaction between the stimulus intensity and drug pretreatment, post LSD tests were performed between the drug pretreatments.

Light stimulus. Mean values of respiratory rate ($RespR_{mean}$) and tidal volume (Vt) were determined for two 30-s intervals—one immediately before presentation of light and one during the light stimulus. First, we performed paired *t*-tests for these two values for saline pretreatment to describe a general pattern of response to this stimulus. Second, we computed delta respiratory rate (Δ $RespR_{mean}$) and delta tidal volume (Δ Vt) for each rat as differences between the interval during the presentation of the light stimulus and the baseline. Lastly, we performed one-way within-subjects ANOVAs for Δ $RespR_{mean}$ and Δ Vt with post hoc LSD test.

RESULTS

Respiratory pattern during acclimatization period. An 8 × 3 within-subjects ANOVA indicated a significant main effect of time on the mean respiratory rate during the acclimatization period, F (7,49) = 11.904, $P < 0.001$. After being placed into the plethysmograph, rats displayed an elevated respiratory rate of 214 ± 21 cpm in the saline trials during the first 5 min, which gradually declined to 122 ± 18 cpm during the last 5-min interval of the 40-min acclimatization period; an example from one animal is illustrated in Fig. 1*A*. 8 × 3 within-subjects ANOVA indicated a significant interaction between drug pretreatment and interval number in the percentage of high-frequency respiratory rate (%HF) index, F (14,98) = 2.293, $P = 0.009$. Three 8 × 2 (time interval × three different pairs of drug pretreatment) within-subjects ANOVAs indicated that the interaction between the effects of drug and time interval exists between the saline and muscimol pretreatment trials in %HF, F (7,49) = 3.990; $P = 0.002$. Post hoc LSD analysis revealed that muscimol microinjection into the amygdala significantly decreased %HF during the first 5-min interval from 43.2 ± 6.8% to 22.1 ± 7.8% ($P = 0.008$). Injections of muscimol dorsal to the amygdala did not produce responses that were different from those rats that received saline or muscimol into the amygdala. There were no other significant main effects or interactions in any of the ANOVAs and no differences in any other measured indices between trials with muscimol to the amygdala, saline to the amygdala, or control microinjection of muscimol. Fig. 2 depicts responses to the acclimatization after muscimol, saline, and control muscimol microinjections.

Respiratory responses to the alerting stimuli. Presentation of acoustic stimuli provoked transient tachypneic responses that were proportional to the stimulus intensity, ranging from +13 ± 9 cpm in response to the lowest intensity stimulus to +276 ± 67 cpm in response to the 90-dB stimulus. Fig. 3 illustrates an example of a respiratory signal during an acoustic stimulus presentation and a response to the 80-dB stimulus averaged from all saline trials. A 6 × 3 within-subjects ANOVA revealed a significant interaction between the drug pretreatment (saline vs. muscimol to the target area vs. control muscimol injection) and the stimulus intensity in the amplitude of respiratory rate response, F (10,70 =2.748; $P = 0.006$). Respiratory rate responses to the acoustic stimuli were linearly dependent upon stimulus intensity, F (1,7) = 24.435; $P = 0.002$. A muscimol vs. saline 6 × 2 ANOVAs (intensity × drug pretreatment) indicated a significant interaction between the effects of intensity and drug pretreatment, F (5,35) = 3.862; $P = 0.007$. Post hoc LSD test revealed that muscimol microinjection significantly decreased amplitudes of respiratory response to the 70-, 80-, and 90-dB stimuli compared with the saline microinjection ($P = 0.009$, $P = 0.021$, and $P = 0.043$, respectively; Fig. 4*A*). A muscimol vs. control muscimol 6 × 2 ANOVA (intensity × drug pretreatment) indicated a significant main effect of drug, F (1,7) = 5.859; $P = 0.046$. Post hoc LSD test revealed that muscimol to the amygdala microinjection significantly inhibited respiratory rate responses to the 70- and 90-dB intensity stimuli ($P = 0.036$ and $P = 0.043$, respectively) compared with muscimol dorsal to the amygdala microinjection. A control muscimol vs. saline 6 × 2 ANOVA (intensity × drug pretreatment) indicated no significant main effects of drug [F (1,7) = 2.047; $P = 0.196$] or a drug by intensity interaction [F (5,35) = 1.356; $P = 0.265$].

We also found a significant main effect of stimulus intensity on the tidal volume, F (10,70) = 11.590; $P < 0.001$. Tidal volume responses to the acoustic stimuli were linearly dependent upon stimulus intensity [F (1,7) = 19.882; $P = 0.003$] and were ranging from 10 ± 23% increase over baseline after a 40-dB stimulus to 186 ± 57% increase over baseline after a

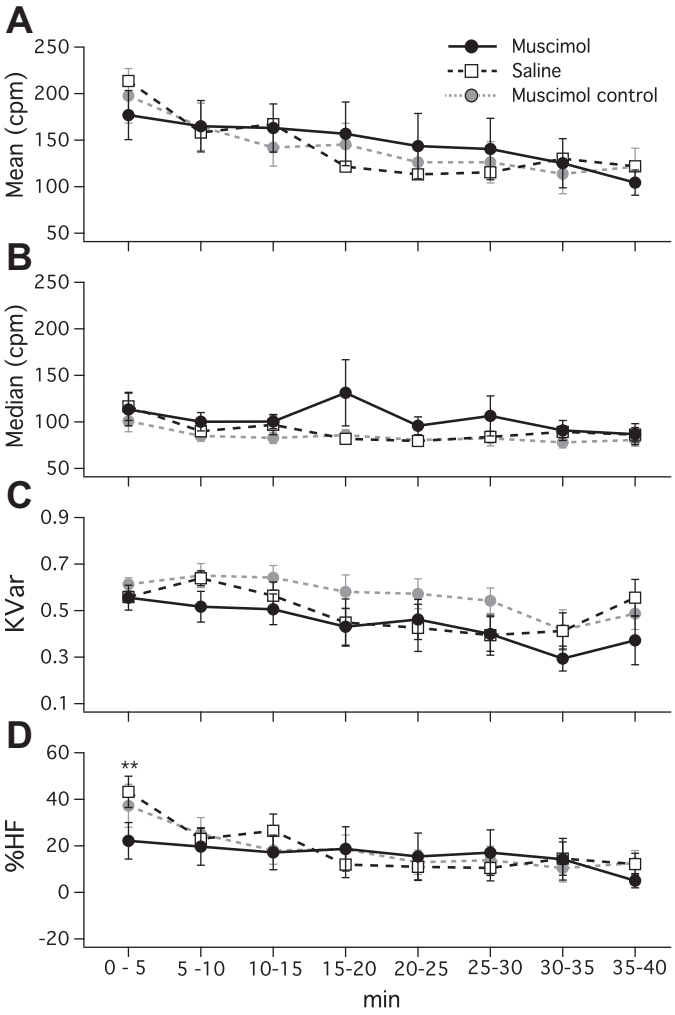

Fig. 2. Changes in respiratory indices during acclimatization period: mean respiratory rate (*A*), dominant respiratory rate (*B*), and coefficient of variation (*C*) did not differ between trials with muscimol to the amygdala (solid black line), saline (dotted black line), and muscimol dorsal to the amygdala (dotted gray line) pretreatment. The amygdala inhibition with muscimol decreased percentage of high-frequency respiratory rate (*D*) during the first 5-min interval, but it did not affect it for the remainder of the acclimatization period: **Significant difference between muscimol and saline pretreatment trials, $P < 0.01$.

90-dB stimulus on saline trials (Fig. 4*B*). There was no significant main effect of the drug pretreatment on tidal volume responses [F (2,14) = 1.982; P = 0.175] or an interaction between the effects of drug and of intensity [F (10,70) = 1.227; P = 0.289].

As described above, only latencies of respiratory responses to the 70-, 80-, and 90-dB stimuli were assessed statistically. On saline trials, latencies of respiratory responses were inversely proportional to the stimulus intensity, ranging from 240 ± 77 ms latency of a response to the 70-dB stimulus to 65 ± 15 ms latency of a response to the 90-dB stimulus. There was a significant main effect of intensity of stimuli on latency of responses, F (2,14) = 20.046; $P < 0.001$. Muscimol microinjection failed to significantly affect latencies of any responses (Fig. 4*C*).

Presentation of a light stimulus elevated the mean of respiratory rate from 83 ± 4.6 to 266 ± 18 cpm and also increased tidal volume by 21 ± 11% compared with baseline. Muscimol microinjection significantly decreased the response in respiratory rate mean, but not in tidal volume, during presentation of a 30-s light stimulus compared with the saline microinjection trial, t (7) = 4.74; P = 0.002 (Fig. 5). Also, muscimol microinjection to the amygdala resulted in a significantly greater attenuation of a respiratory response to light than the control microinjection of muscimol dorsal to the amygdala, t (7) = 4.71, P = 0.002. A difference between the saline and control muscimol pretreatment trials was only marginally significant, t (7) = 1.833, P = 0.055.

Respiratory responses to restraint stress. In the trials with saline pretreatment, restraint stress significantly elevated the mean respiratory rate [$RespR_{mean}$, from 85 ± 6 to 157 ± 7 cpm, t (7) = 4.678; P = 0.001], the dominant respiratory rate [$RespR_{dom}$, from 78 ± 3 to 129 ± 10 cpm, t (7) = 3.802; P = 0.004], and the fraction of high-frequency respiratory rate [%HF; from 3.1 ± 2.5% to 13.8 ± 2.4%, t (7) = 3.700; P = 0.004] during the first 5 min of restraint. An example of a raw trace of respiratory rate recording is presented in Fig. 6*A*. A similar pattern was observed on the control muscimol dorsal to the amygdala trials (Fig. 6*A*). Muscimol microinjection into the amygdala abolished the increase in $RespR_{mean}$, t (7) = 0.767; P = 0.234 (Fig. 6*B*) and significantly attenuated the increase in $RespR_{dom}$, t (7) = 3.094; P = 0.009 (Fig. 6*C*), and %HF, t (7) = 2.020, P = 0.042 (Fig. 6*E*). Respiratory rate during the first 5-min interval of restraint stress after the control muscimol microinjection dorsal to the amygdala was significantly lower than that after saline pretreatment [t (7) = 2.176; P = 0.033] but higher than that after muscimol to the amygdala microinjection [t (7) = 3.157; P = 0.008].

We found significant main effects of drug [F (2,14) = 4.591; P = 0.029] and time interval [F (2,14) = 10.54; P = 0.002] on Δ $RespR_{mean}$. The main effect of muscimol pretreatment was significantly different from the main effect of saline pretreatment (P = 0.009) and marginally different from the control muscimol microinjection (P = 0.098). Post hoc LSD test indicated that muscimol significantly inhibited Δ $RespR_{mean}$ during the first (P = 0.004) and second (P = 0.042) 5-min intervals of restraint. We also found significant interactions between the drug pretreatment and the interval of the restraint in $\Delta RespR_{dom}$ [F (4,28) = 3.961; P = 0.011; and F (8,56) = 4.345; $P < 0.001$]. Three 3 × 2 within-subjects ANOVAs analyzing each pair of drug pretreatments separately indicated a significant interaction between the effects of drug and time interval in the comparison between muscimol and saline trials, F (2,14) = 5.057; P = 0.022. Post hoc analysis indicated that microinjection of muscimol to the amygdala significantly decreased responses in the dominant respiratory rate during the first 5-min interval of restraint (P = 0.024). Lastly, there was a significant main effect of drug on Δ%HF, F (2,14) = 5.169; P = 0.021. The main effect of muscimol microinjection was significantly different from the saline microinjection (P = 0.003) and marginally different from the control muscimol microinjection (P = 0.055). Post hoc LSD test indicated that muscimol significantly inhibited Δ %HF during all three 5-min intervals (P = 0.009, P = 0.008, and P = 0.017, respectively). Altogether, respiratory response to the restraint stress was significantly reduced after inhibition of the amygdala compared with the saline trial, especially during the first 5-min interval of the restraint. Control microinjection of muscimol dorsal to the amygdala did not significantly inhibit respiratory

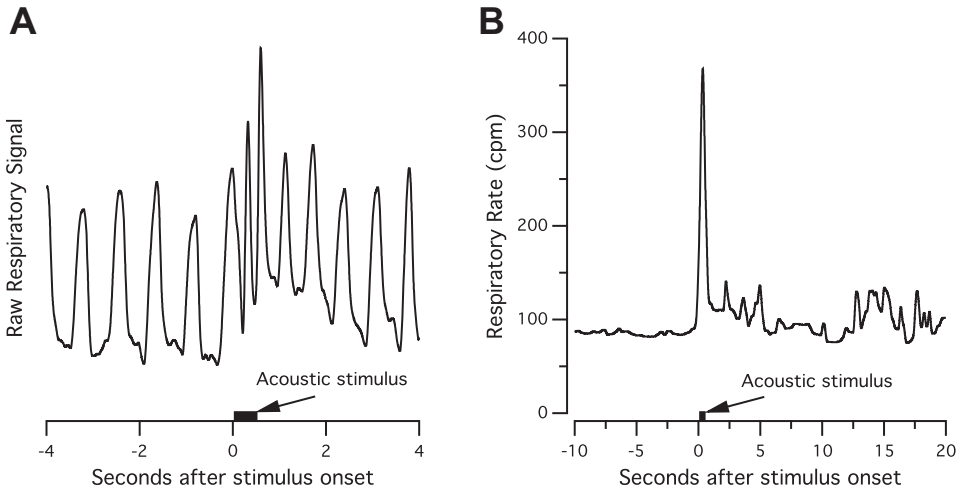

Fig. 3. Raw trace of respiratory recording, illustrating the response to a 80-dB white noise (500 ms) acoustic stimulus (*A*). Respiratory rate response to a 80-dB white noise (500 ms) stimulus averaged from eight rats after saline microinjection (*B*).

response to restraint. All data and results of statistical analysis are presented in Fig. 7.

An example of a histologically verified microinjection and a summary diagram of injection sites in eight animals are presented in Fig. 8.

DISCUSSION

This is the first study that describes the role of the amygdala in mediating respiratory responses to sudden and prolonged stressors of various intensities in conscious freely moving rats. Our principal finding is that pharmacological inhibition of neurons within the amygdala reduces tachypneic responses to high-intensity brief acoustic stimuli, to a light stimulus and to restraint stress. Of several respiratory indices employed in this study, tachypneic responses to sudden acoustic stimulation were the most sensitive to the amygdala blockade.

Involvement of the amygdala in the respiratory responses to arousal and stress. Our study was focused on the amygdala—a key brain region structure involved in processing fear and anxiety. Recent brain imaging data confirm involvement of the amygdala in panic disorder in humans (see Ref. 8 for review). Despite a firmly established link between a negative emotional state and respiration (4, 14), only a few previous animal and human studies provide evidence that the amygdala may be involved in respiratory control (see introduction). One early work in which this has been directly confirmed reported that surgical lesions of the amygdala in two epileptic patients resulted in decreases in respiratory rate response to anticipatory anxiety (20). More recently, Evans et al. (10) reported that rhythmic amygdala activation coincides with respiratory movements during mild experimental stress in humans.

Thus, our work provides the first experimental evidence in animals that the amygdala is essential for full expression of respiratory response to alerting stimuli and stress. The fact that amygdala inhibition resulted in the attenuation of amplitudes of respiratory responses to acoustic stimuli and almost completely abolished respiratory activation during restraint clearly indicates that the integrity of its neuronal circuitry is essential for respiratory activation during stress and arousal. Our results also suggest that the extent of the amygdala involvement in this activation depends on the intensity of a stressor. Indeed, inhibitory effects of the amygdala blockade were more prominent during restraint (potent stressor) than during acclimatization (milder stressor). Likewise, the intensity-dependent effect of the blockade was evident during acoustic stimulation, with no influence of the amygdala inhibition on respiratory responses to the low-intensity acoustic stimuli, and with substantial attenuation of responses to the stimuli of higher intensity.

Effects of inhibition of the amygdala on respiration during acclimatization period. During the "acclimatization" period, we observed a clear time-dependent decrease in the trends of mean and dominant respiratory rates. It must be noted that this period does not represent true baseline but rather reflects a response to a novel environment (plethysmographic chamber). Therefore, it can be argued that decreases in the trends could be due to the time-dependent dissipation of the anxiety state. We did not observe any effects of the amygdala inhibition on the mean and dominant respiratory rate, which is probably due to the relatively low potency of this stressor. However, we did find that inhibition of the amygdala reduced the %HF. Interestingly, this result contradicts our previous finding that high anxiety is associated with a reduction in the %HF (5). An increase in the %HF (that includes sniffing) could be attributed to greater exploratory behavior or due to an increase in other motor behaviors (e.g., grooming). Both of these behaviors increase mean respiratory rate, predominantly by increasing %HF (16). Exploratory behavior was generally observed at the beginning of "acclimatization", when the rat placed into the new environment has likely elevated anxiety. Grooming, however, was observed after the rat has habituated to the new environment. Therefore, a relationship between the %HF and animals' anxiety state could be biphasic, with high anxiety/fear levels being associated with a very low mean respiratory rate and zero %HF due to ultrasonic vocalizations (as observed in Refs. 3 and 37). Medium anxiety/arousal levels might be linked with a high %HF due to exploratory sniffing. Low anxiety levels, however, could be associated with either a low %HF rate during quiet rest or a high %HF during grooming, which is exhibited more often during a low anxiety state (17).

Effects of inhibition of the amygdala on respiratory responses to alerting stimuli. Acoustic stimuli evoked transient increases in respiratory rate, in accordance with our previous reports (16, 24). In the current study, we demonstrated that the magnitude of these tachypneic responses is related to the

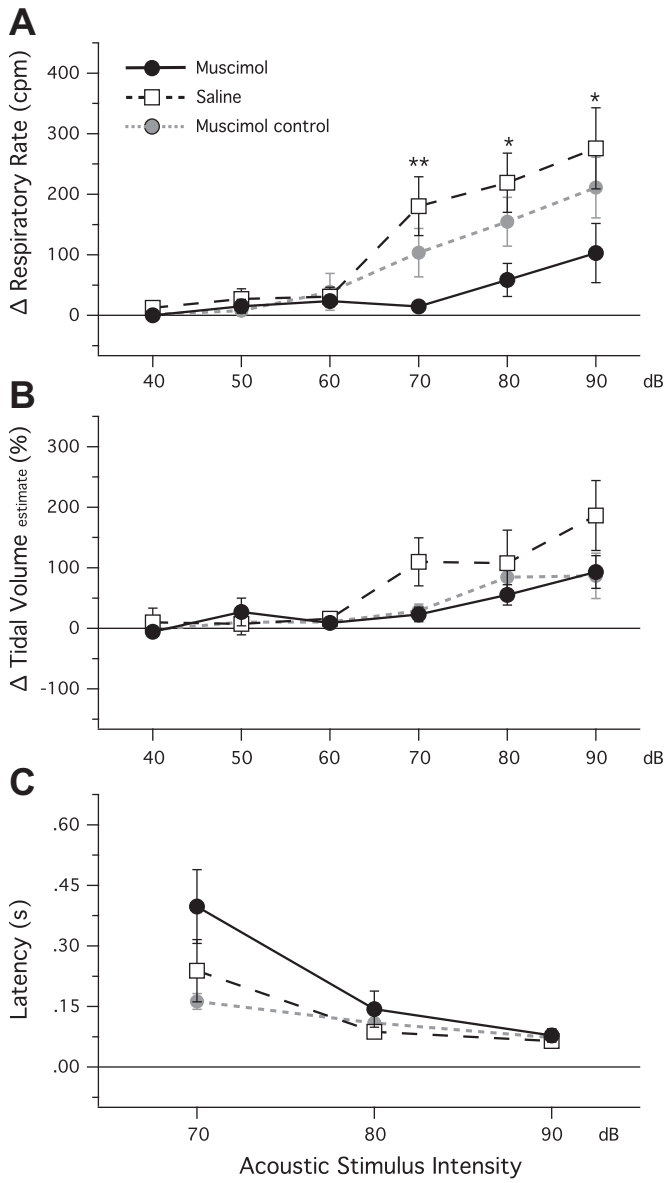

Fig. 4. Inhibition of the amygdala with muscimol significantly decreased amplitudes of respiratory responses to the 70-, 80- and 90-dB acoustic stimuli (*A*) but did not significantly affect the tidal volume responses (*B*) or latencies of these responses (*C*). *Significant difference between muscimol and saline pretreatment trials, $P < 0.05$. **Significant difference between muscimol and saline pretreatment trials, $P < 0.01$.

intensity of the acoustic stimuli, whereas their latency is inversely proportional to the intensity. Of major relevance is the fact that the reported inhibition here of respiratory responses to acoustic stimuli following amygdala blockade is similar to the effects of anxiolytic diazepam observed in our previous study (23). We can speculate that the mechanisms behind these two findings are similar in that inhibition of the amygdala mimics the "anxiolytic-like" effect of diazepam on the behavior and respiration. It is also important to note that the pattern of respiratory response to acoustic stimuli in rats is remarkably similar to that observed in humans—a very brief, sharp increase followed by a period of slightly elevated respiratory rate (27). A difference in these responses between humans and rats is the temporal dimension of it. In humans, this response was observed 1–2 s after stimulus onset, while in rats, the latency was about one order shorter. Such differences can be attributed to differences in the size and flexibility of the system, length of conducting pathways, as well as basal differences in respiratory rhythms, as humans' resting respiratory rate is ~12–15 cycles/min, while rats' resting respiratory rate varies from 80 to 100 cycles/min.

In the current study, we did not observe significant effects of the amygdala blockade on tidal volume responses. Tidal volume responses to alerting stimuli in conscious animals were previously assessed in only one study (5). Presentation of predator calls and cat odor produced significant increases in tidal volume, but there were no differences in these responses between high- and low-anxiety animals. In the current study, animals exhibited increases in tidal volume in response to high-intensity (70–90 dB) brief acoustic stimuli, while inhibition of the amygdala had no significant main effect on these responses. Previous human studies are inconsistent in the exact pattern of the tidal volume response, with some studies describing an increase in tidal volume (i.e., a gasp of air), while others describe a reduction [i.e., shallow frequent breathing (4)].

Effects of inhibition of the amygdala on respiration during restraint stress. Restraint significantly elevated the mean and the dominant respiratory rate as well as %HF. This finding confirms previously reported effects of restraint on respiration in rats (5). Pharmacological inhibition of the amygdala significantly decreased this respiratory response. Indeed, the mean and the %HF were completely unaffected by restraint after muscimol microinjection. This finding suggests that integrity of the amygdala is essential for generation of respiratory responses to prolonged high-intensity stressors. In rats, respiration is highly variable, particularly during restraint, where periods of motor activity (struggling against the restrainer) with elevated and variable respiratory rate are intertwined with periods of no activity, when respiration is fairly stable. Kabir et al. (16) analyzed respiratory rate of freely moving conscious rats over a period of 30 min and suggested that the histograms of respiratory intervals follow a bimodal distribution with a low-frequency peak, indicating a resting respiratory rate and a high-frequency peak, indicating a respiratory rate during motor activity. The current experiments further extend this finding and suggest that at least the low-frequency peak is related to animals' anxiety state, as it was significantly elevated during restraint and was significantly reduced by inhibition of the amygdala. This finding is in line with our previous study, in which rats selectively bred for high anxiety exhibited a significantly higher dominant respiratory rate during restraint than low-anxiety rats (5). However, high-anxiety rats exhibited a significantly lower %HF than low-anxiety rats in this study, which contradicts findings of the current study. This could be explained by high-anxiety rats exhibiting freezing and "helplessness" behavior during the restraint, with little active coping (i.e., struggling) and, therefore, exhibiting a lower %HF than low-anxiety rats. Thus, it is evident that assessment of the mean respiratory rate and the %HF have certain methodological problems, as both indices are highly susceptible to influences of motor activity. Assessment of the dominant respiratory rate, on the other hand, overcomes that problem and provides a more accurate index that is not contaminated by locomotion. Indeed, our findings in regard to changes in the

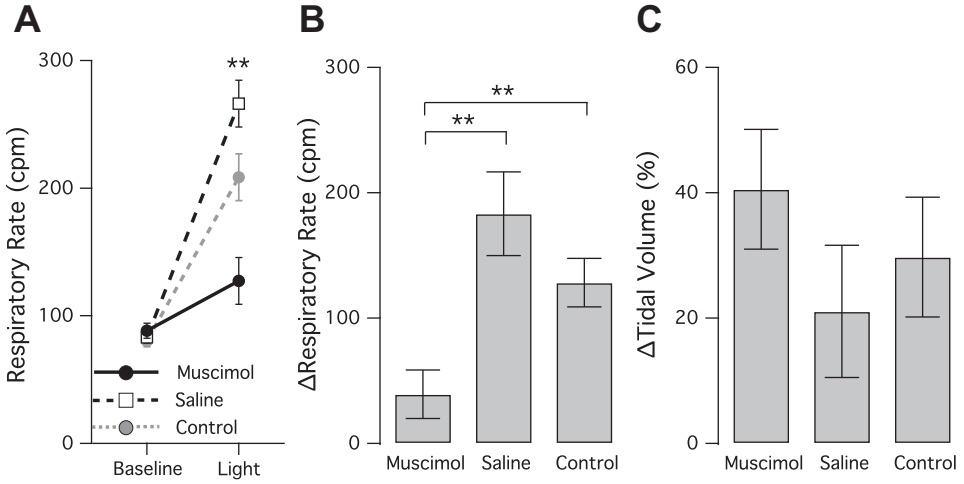

Fig. 5. Baseline respiratory rate values before and during presentation of the 30-s light stimulus (*A*). Microinjection of muscimol to the amygdala significantly decreased the mean of respiratory rate response (*B*), but not tidal volume response (*C*), to the light stimulus. Each data point represents a mean of 30 s of 8 rats after saline (dotted black line in *A*) or muscimol (solid black line in *A*) bilateral microinjection into the amygdala or after muscimol bilateral microinjection dorsal to the amygdala (dotted gray line in *A*).

dominant respiratory rate in response to various stressors are in line with human studies that assessed respiratory rate during various laboratory stressors (33, 34, 36). Similar to these human studies, we have observed elevated respiratory rate during presentation of a prolonged stressor.

Neural pathways that mediate amygdala-dependent respiratory changes. The exact neuronal pathways connecting the amygdala with the ventral respiratory group and/or the pontine respiratory group that generate respiratory neural outflow are currently unknown. Dense projections from the central amyg-

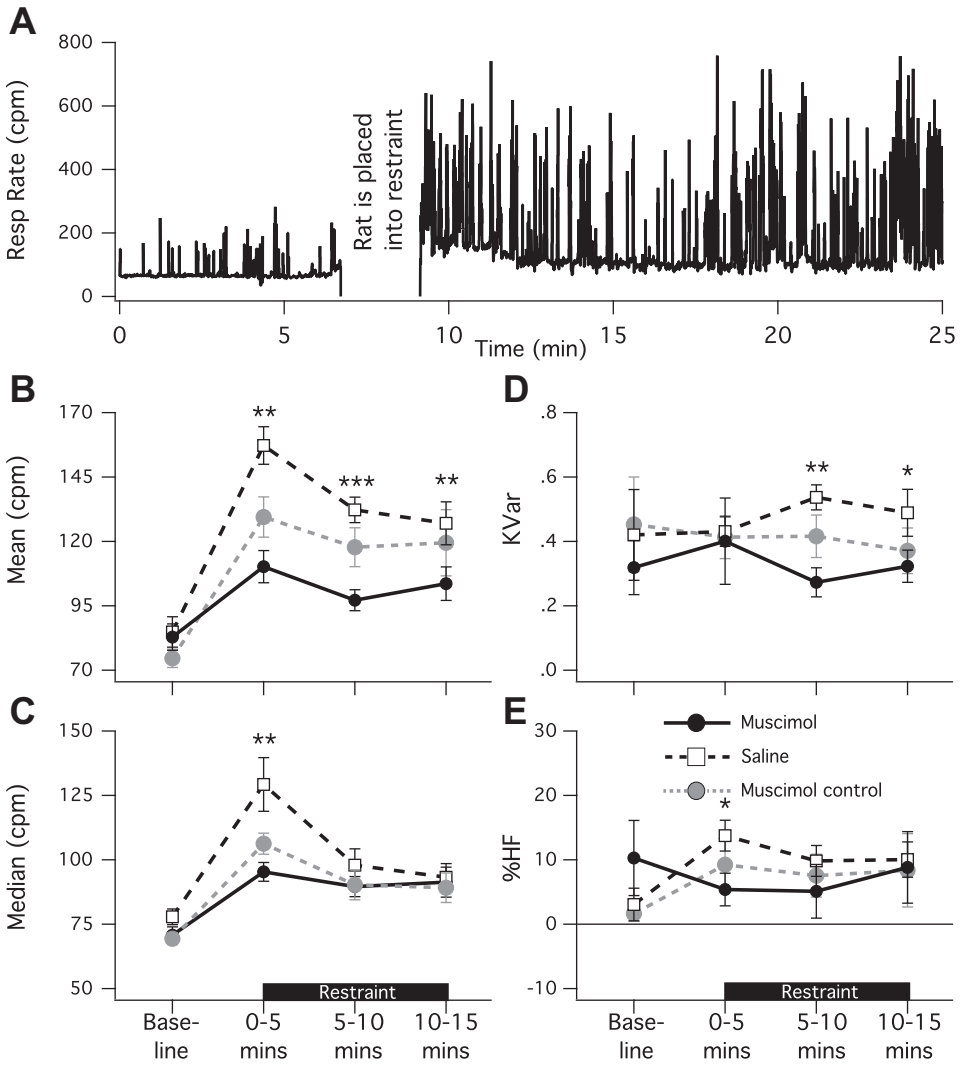

Fig. 6. Respiration during restraint. *A*: an example of a raw respiratory rate recording after saline microinjection during baseline and a 15-min restraint. Microinjection of muscimol to the amygdala significantly decreased the mean (*B*) of respiratory rate during all three 5-min intervals of restraint. Also, blockade of the amygdala significantly decreased the dominant respiratory rate (*C*), as well as the percentage of high-frequency respiratory rate (*E*) during the first 5-min epoch of the restraint. Muscimol microinjection significantly decreased the coefficient of variability of respiratory rate (*D*) during the second and third 5-min interval, but not during the first. Each data point represents a mean of 30 s of eight rats after saline (dotted black line) or muscimol (solid black line) bilateral microinjection into the amygdala or after muscimol bilateral microinjection dorsal to the amygdala (dotted gray line). *Significant difference with $P < 0.05$. **Significant difference with $P < 0.01$. ***Significant difference with $P < 0.001$.

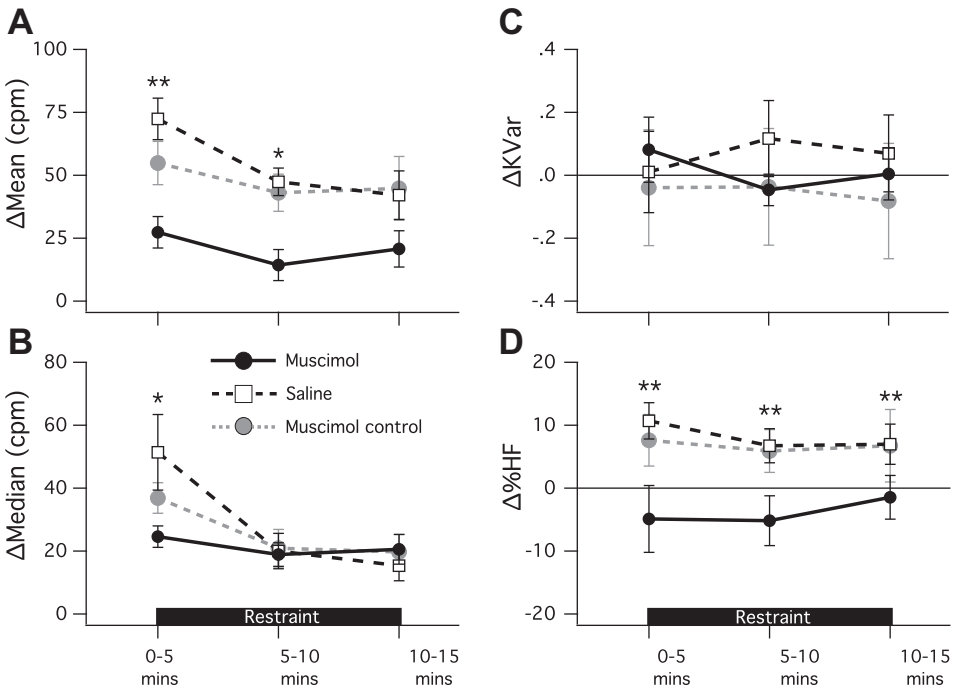

Fig. 7. Microinjection of muscimol to the amygdala significantly decreased the deltas of the mean of respiratory rate (*A*) during the first and second 5-min interval of restraint and of the dominant respiratory rate (*B*) during the first 5-min interval of restraint. Blockade of the amygdala has also significantly decreased the delta of the %HF (*D*) during all three intervals of restraint. Inhibition of the amygdala did not affect the delta of coefficient of variability of respiratory during the restraint (*C*). Each data point represents a mean of 30 s of eight rats after saline (dotted black line) or muscimol (solid black line) bilateral microinjection into the amygdala or after muscimol bilateral microinjection dorsal to the amygdala (dotted gray line). *Significant difference with $P < 0.05$. **Significant difference with $P < 0.01$.

daloid nucleus to the dorsomedial hypothalamus might be involved in mediating this response (6). This view is supported by strong "anxiolytic-like" effects of inhibition of the dorsomedial hypothalamus on both behavioral (28) and autonomic indices (29). Furthermore, the existence of distinct neuronal subpopulations in the dorsomedial hypothalamus mediating specific autonomic functions (heart rate, arterial pressure, respiration, etc.) supports the idea that dorsomedial hypothalamus integrates information from various sources, including the amygdala, to produce a coordinated pattern of autonomic response (7, 35).

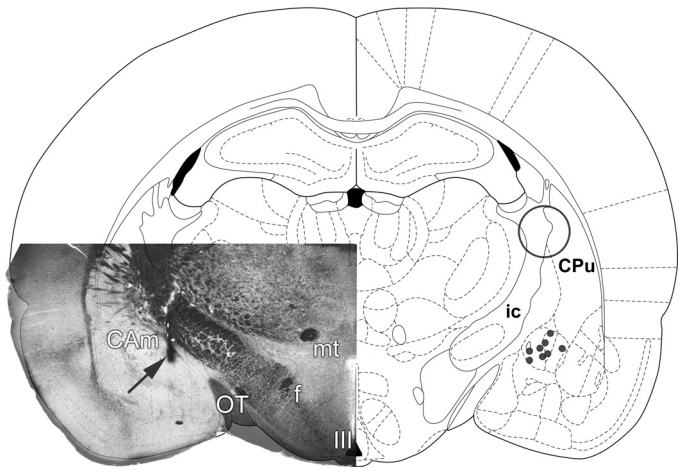

Fig. 8. Histological verification of microinjection sites into the amygdala. The left side of the picture displays a coronal section of the brain of one of the rats in the current experiment; the arrow points to a microinjection site. The right side of the picture displays centers of successful microinjection sites (solid circles) drawn on a standard coronal section diagram from the atlas of Paxinos and Watson (26). Black circle indicates an approximate location of control microinjections of muscimol. Abbreviations: CAm, central amygdaloid nucleus; CPu, caudate putamen (striatum); f, fornix; ic, internal capsule; mt, mamillo-thalamic tract; OT, optic tract; III, third ventricle.

In the current experiment, we have targeted the central nucleus of the amygdala. Electrical stimulation of this subnucleus of the amygdala has been shown to elicit an increase in respiratory rate (2). Although we cannot completely dismiss some effect of muscimol microinjection into the central amygdala on the adjacent basolateral subnucleus of the amygdala, this subnucleus does not have projections to the dorsomedial hypothalamus (6), which are believed to mediate the observed respiratory rate responses. Furthermore, neurons within the basolateral amygdala that are involved in autonomic regulation are believed to be under tonic GABAergic inhibition (31) and, therefore, they would not be sensitive to even further GABAergic inhibition. The medial subnucleus of the amygdala, on the other hand, has projections to the dorsomedial hypothalamus and can potentially contribute to the observed inhibition of respiratory rate responses. However, this subnucleus is not in close proximity of the central amygdala. If muscimol microinjection into the central amygdala did have some effect on the medial amygdala, such effect would have been minimal. Therefore, we believe that blockade of the central amygdaloid nucleus rather than other subnuclei was mainly responsible for the inhibition of the respiratory responses to the stressful stimuli and restraint stress.

Significance and Perspectives

Our results clearly demonstrate that the integrity of the amygdala is essential for full expression of respiratory responses to arousing stimuli and to stressful environment. Assessment of respiratory arousal responses has a number of advantages compared with assessment of other autonomic indices during presentation of various stresses. First, compared with commonly used cardiovascular indices, respiratory changes are more sensitive, as they have lower thresholds (23). Second, the onset of these respiratory responses is in the same range as latencies of evoked potentials, and thus, they provide

a "real-time" window in the brain activity. Lastly, the relative magnitude of respiratory rate responses or of evoked potentials is much greater compared with other autonomic indices, making this index readily assessable and quantifiable on single-trial experiments. This methodology is also simple and noninvasive. Given demonstrated links between respiration and anxiety state, as discussed earlier, the current methodology can potentially be used as a noninvasive measure of anxiety in animals that is directly translatable to humans.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

E.B. and E.N. performed experiments; E.B. and E.N. analyzed data; E.B. and E.N. interpreted results of experiments; E.B. prepared figures; E.B. drafted manuscript; D.M.H. and E.N. conception and design of research; D.M.H. and E.N. edited and revised manuscript; D.M.H. and E.N. approved final version of manuscript.

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CHAPTER 4

PRELIMBIC PREFRONTAL CORTEX MEDIATES RESPIRATORY RESPONSES TO MILD AND POTENT PROLONGED, BUT NOT BRIEF, STRESSORS

INTRODUCTION TO CHAPTER 4

Pharmacological activation of the prelimbic prefrontal cortex evokes increases in heart rate and arterial pressure in conscious unrestrained rats (Resstel and Corrêa, 2005), while activation of this area in anesthetized rats evokes a decrease in arterial pressure (Resstel et al., 2006). Activation of the prelimbic prefrontal cortex evokes an increase in respiratory rate in anesthetized rats (Hassan et al., 2013), suggesting that this structure is involved in the central respiratory network. The aim of Chapter 4 is to investigate the effects of pharmacological blockade of the prelimbic prefrontal cortex on respiratory responses to brief and prolonged stressors of various intensities in conscious unrestrained rats.

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Prelimbic prefrontal cortex mediates respiratory responses to mild and potent prolonged, but not brief, stressors☆

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ABSTRACT

The prefrontal cortex is one of the key areas of the central mechanism of cardiovascular and respiratory control. Disinhibition of the prelimbic medial prefrontal cortex elicits tachypnoeic responses in anesthetized rats (Hassan et al., J. Physiol. 591: 6069–6088, 2013). The current study examines the effects of inhibition of the prelimbic prefrontal cortex during presentation of stressors of various lengths and intensities in conscious unrestrained rats. 8 Wistar rats were implanted with bilateral guide cannulas targeting the prelimbic prefrontal cortex and received microinjections of either saline of $GABA_A$ agonist muscimol prior to recording sessions. Inhibition of the prelimbic prefrontal cortex significantly attenuated respiratory responses to a novel environment stress, 30 s light stimulus and restraint stress. It did not affect respiratory responses to 500 ms acoustic stimuli of varying intensities (40–90 dB). We conclude that the prelimbic prefrontal cortex contributes to generation of tachypnoeic responses to prolonged stressors, but does not contribute to respiratory arousal in response to brief stressors.

1. Introduction

Respiratory arousal is one of the most sensitive indices of autonomic arousal. Previous studies in humans and in animals describe tachypnoeic responses to both short and prolonged stressors (Boiten et al., 1994; Bondarenko et al., 2013; Kabir et al., 2010a; Reyes del Paso and Vila, 1993). Very little is known about the neural pathways, presumably including forebrain, involved in these non-homeostatic respiratory responses. This is in sharp contrast with the wealth of knowledge about the lower brainstem mechanisms responsible for respiratory homeostatic control (Dutschmann and Dick, 2012; Smith et al., 2013). One of the central areas that is believed to be involved in central respiratory control is the medial prefrontal cortex (mPFC). This region has direct projections to other major autonomic centres, such as the dorsomedial hypothalamus (Myers et al., 2013), the periaqueductal grey (Gabbott et al., 2005), the nucleus of the solitary tract (Terreberry and Neafsey, 1987) and the amygdala (Vertes, 2003). Electrical stimulation of the mPFC induces tachypnoea in rats (Alexandrov et al., 2007). A similar effect is observed after chemical disinhibition of this area in rats (Hassan et al., 2013).

The mPFC is comprised of two areas: prelimbic (plPFC) and infralimbic (ilPFC). Previous experiments in anaesthetized rats demonstrated that both regions contain neurons that may be involved in central respiratory control (Hassan et al., 2013). Furthermore, Powell et al. (1994) found that electrical stimulation of the plPFC in conscious restrained rabbits elicits an increase in respiratory rate. However, blockade of the plPFC neurons has never been shown to affect respiratory rate in conscious animals. Furthermore, it is unknown whether the plPFC mediates respiratory arousal in response to stressful stimuli of different lengths and potencies. The aim of the current experiment is therefore to investigate the effects of the plPFC inactivation on respiratory responses to short and prolonged stimuli and stressors of various intensities in conscious rats.

2. Methods

2.1. Animals and preliminary surgery

8 adult male outbred Wistar rats (250–350 g) were received from the University of Newcastle animal house. Animals were held on a 12-h reverse dark–light cycle (lights ON at 19.00) with unrestricted access to food and water. Experimental protocol commenced at least 7 day after animals were received. All

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experimental procedures were approved by the Animal Care and Ethics Committee of the University of Newcastle and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Animals were anaesthetized and implanted with bilateral guide cannulas targeting the prefrontal cortex prelimbic division (2.7 mm rostral, 4.0 mm ventral, 0.5 mm lateral from bregma). Carprofen (5 mg/kg) and enrofloxacin (10 mg/kg) were used as an analgesic and an antibiotic during post-operative care. After recovery (as determined by a recovery to baseline weight) and no longer than 7 days after the surgery animals were subjected to two recording sessions with at least 48 h interval between them. Twenty minutes before each recording session animals received microinjections of either $GABA_A$ agonist muscimol (20 nmol in 200 nl) or saline (equal volume) bilaterally into the plPFC in a counterbalanced within-subjects design. Muscimol was from purchased Sigma Aldrich (USA).

2.2. Experimental design

During the recording session animals were individually placed inside a whole-body plethysmograph (see Kabir et al., 2010a). This method allows online assessment of respiratory rate and an indirect assessment of the change in tidal volume by measuring the frequency and the magnitude of pressure fluctuations inside the plethysmographic chamber. Animal bedding was placed on the bottom of the plethysmograph. The apparatus was placed in a soundproof box, and constantly illuminated by a 20 lux LED light. Animals' behaviour was monitored by a video camera mounted in close proximity to the plethysmograph. For monitoring animals' gross motor activity, a piezoelectric pulse transducer (MLT1010/D, ADInstruments, Sydney, Australia) was placed under the plethysmograph.

Each recording session consisted of a 40-min acclimatization period followed by presentation of 6 acoustic stimuli (40–90 dB white noise stimuli; 500 ms duration; 50 ms rise and 50 ms fall) from a generic speaker placed underneath the recording apparatus. Following acoustic stimuli a 30-s light stimulus (2000 lux) was presented. The stimuli were presented when animals were awake, exhibited quiet and regular breathing pattern (<150 cpm) for at least 10 s and were not engaged in any motor activity (as assessed by the pressure transducer and video camera). Stimuli were presented at 5-min intervals. 10 min after presentation of the light stimulus, animals were removed from the recording chamber, placed into a tight steel mesh (restrainer) and returned into the chamber for the final 15 min of recording.

2.3. Data acquisition and analysis

Respiratory and motor signals were continuously sampled at 1 kHz and recorded using a PowerLab data acquisition system (ADInstruments, Sydney, Australia). Respiratory rate (in cycles per min, cpm) and an estimate of tidal volume (in arbitrary units) were computed online using LabChart software (Version 7.1, ADInstruments, Sydney, Australia). The apparatus used in this study did not allow reliable assessment of tidal volume during prolonged intervals as this required measurements of body temperature and air humidity. However, during short periods of time, such as during presentation of brief alerting stimuli and light, the changes in body temperature and air humidity are assumed to be negligible and therefore the change in tidal volume can be assessed more reliably. Such tidal volume responses were quantified as a % change from baseline.

For characterizing respiratory pattern during acclimatization and restraint we used four parameters: mean respiratory rate ($Resp\ Rate_{MEAN}$), dominant respiratory rate ($Resp\ Rate_{DOMINANT}$), coefficient of variation (KVar), and percentage of high frequency (>250 cpm) respiratory rate (%HF). These parameters were assessed for each 5-min interval of acclimatization (8 intervals in total), 5 min before restraint stress and for each 5-min interval of restraint (3 intervals in total). $Resp\ Rate_{MEAN}$ and KVar = SD/$Resp\ Rate_{MEAN}$ × 100% were calculated in LabChart software (SD is a standard deviation of respiratory rate data). $Resp\ Rate_{DOMINANT}$ and %HF were calculated in Igor Pro software (Wavemetrics, USA) by plotting time histograms for each of the 5-min intervals. $Resp\ Rate_{DOMINANT}$ was assessed as a mode of each of these histograms, while %HF was assessed as the ratio $AUC_{250–650}/AUC_{0–650}$ (AUC = area under the curve). See Bondarenko et al. (2014) and Carnevali et al. (2013) for more detailed descriptions of this procedure.

Acclimatization data was first analyzed by a 8 × 2 (time × drug) within-subjects ANOVA with post hoc Fisher's least significant difference (LSD) test between muscimol and saline trials for each of the 5-min intervals. Data for each of the 5-min periods of restraint was first expressed as a change (Δ) from baseline (5-min period before submission to restraint). Subsequently, a 3 × 2 (time × drug) within-subjects ANOVA was performed on these Δ indices with post hoc LSD test was performed between muscimol and saline pre-treatment trials.

To describe respiratory responses to acoustic stimuli, we assessed the amplitude of respiratory rate responses, the latency of respiratory rate responses and the changes in the estimate of tidal volume in LabChart software (Version 7.1, ADInstruments, Sydney, Australia). Amplitude of respiratory rate response (ΔResp Rate) was assessed as a maximal change in respiratory rate from baseline during the first second after the onset of the acoustic stimulus and was expressed in cycles per minute (cpm). Similarly, the change in the estimate of tidal volume (ΔTV) was assessed as a maximal change from baseline in tidal volume during the first second after the onset of the acoustic stimulus and was expressed as a % of change. Latency of respiratory rate response (latency) was assessed as a time (expressed in ms) from the onset of a stimulus presentation to the first distinctive change in the raw respiratory signal. This index could not be reliably assessed for the low intensity stimuli (40–60 dB) due to the absence of distinctive changes in respiration in response to these stimuli in some animals; therefore, Latency was only assessed for the 70–90 dB stimuli. Acoustic stimuli data was analyzed by performing a 6 × 2 (intensity × drug) within-subjects ANOVA on the ΔRR and ΔTV indices as well as a 3 × 2 (intensity × drug) within-subjects ANOVA on the Latency index. LSD test was used as a post hoc test for comparisons between muscimol and saline trials when ANOVA returned a significant interaction or a significant main effect of drug pre-treatment.

Respiratory responses to the light stimulus were characterized by assessing respiratory rate and an estimate of tidal volume. Both of these parameters were assessed as a change (ΔRR and ΔTV) from the mean of 30-s baseline immediately prior to the light stimulus presentation to the mean of 30 s during the presentation of the light stimulus. Subsequently, these indices were analyzed by paired samples *t*-tests between muscimol and saline trials.

3. Results

3.1. mPFC blockade affected respiratory pattern during acclimatization

Upon placement into the plethysmograph, the mean of respiratory rate ($Resp\ Rate_{MEAN}$) gradually declined from 232 ± 26 cpm during first 5 min to 122 ± 18 cpm during the last 5-min interval

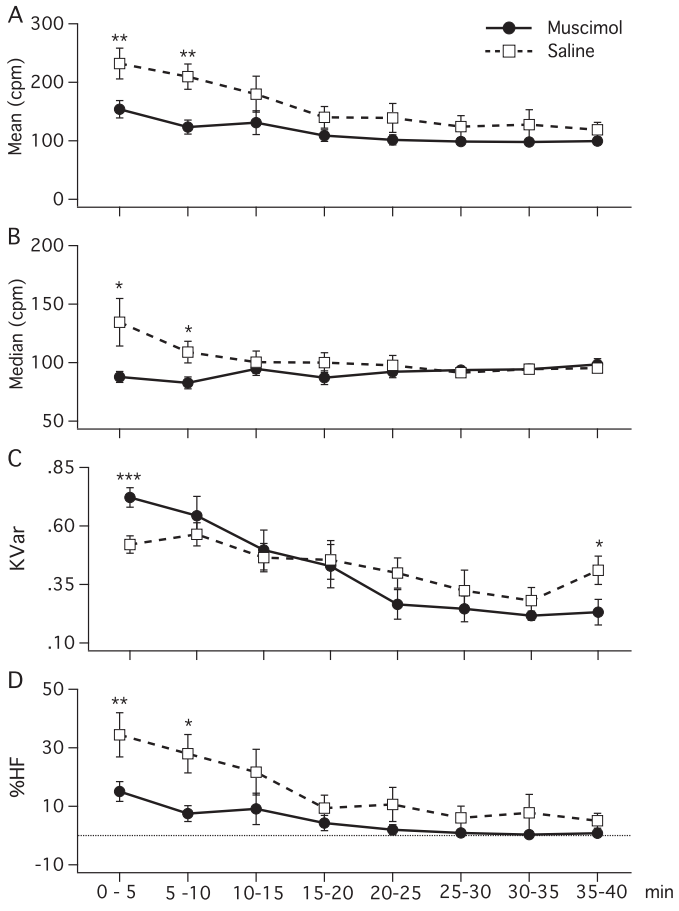

Fig. 1. Changes in respiratory indices during acclimatization period in muscimol (solid line) and saline (dotted line) pre-treatment trials. The prelimbic prefrontal cortex blockade inhibited the mean respiratory rate (A), the dominant respiratory rate (B) and the percentage of high frequency respiratory rate (D) during the first two 5-min intervals of acclimatization. It has also increased the coefficient of variability during the first 5-min interval of acclimatization, but inhibited it during the last 5-min interval. * Significant difference between muscimol and saline pre-treatment trials, $p < 0.05$; **Significant difference between muscimol and saline pre-treatment trials, $p < 0.01$; ***Significant difference between muscimol and saline pre-treatment trials, $p < 0.001$.

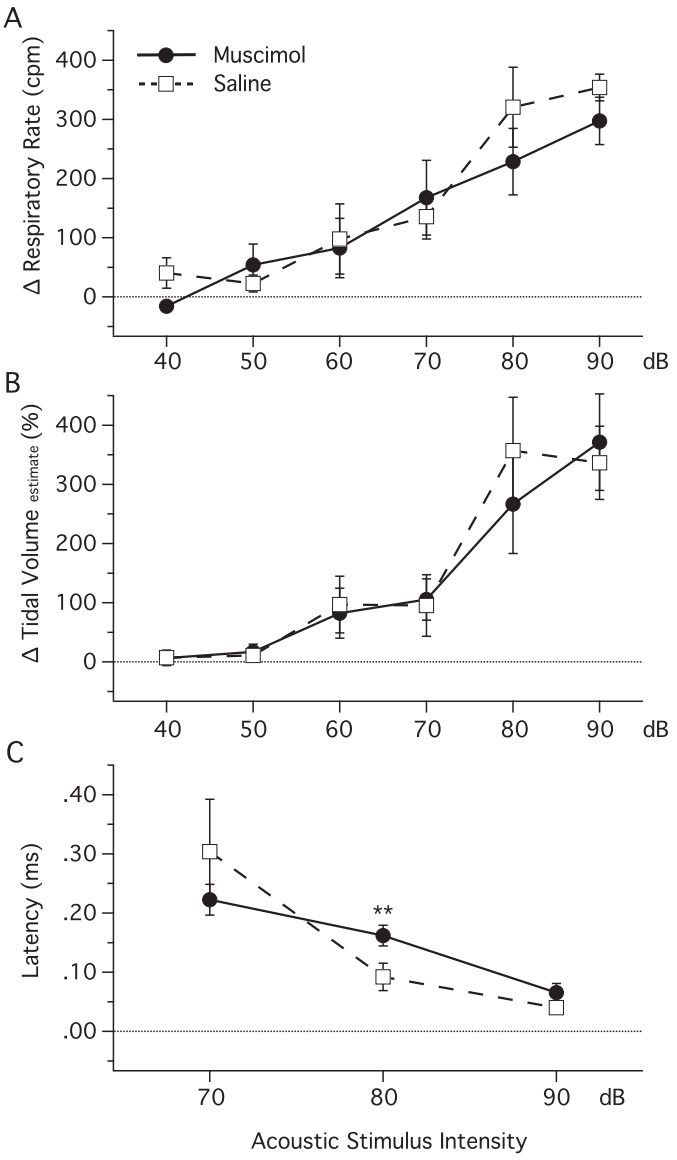

Fig. 2. Inhibition of the prelimbic prefrontal cortex had no effect on the amplitude of respiratory rate response (A) or tidal volume response (B) to acoustic stimuli. plPFC inhibition slightly, but significantly, increased latency of respiratory responses to the 80 dB stimulus (C).

(Fig. 1A). 8 × 2 (time intervals × drug) within subjects ANOVAs were performed on Resp $Rate_{MEAN}$, the dominant respiratory rate (Resp $Rate_{DOMINANT}$), the coefficient of variability (KVar) and the percentage of high frequency respiratory rate (%HF). The interaction between the effects of time and drug on the Resp $Rate_{MEAN}$ failed to reach a significance level ($F(7,49) = 1.893$, $p = 0.091$); however, main effects of both drug pre-treatment ($F(1,7) = 5.777$, $p = 0.047$) and of time ($F(7,49) = 11.012$, $p < 0.001$) were significant. Post hoc LSD test revealed that muscimol significantly inhibited Resp $Rate_{MEAN}$ during the first ($t(7) = 3.434$, $p = 0.006$) and second ($t(7) = 3.152$, $p = 0.008$) 5-min intervals. We also found significant interactions between the effects of drug and of time on Resp $Rate_{DOMINANT}$ ($F(7,49) = 5.743$, $p < 0.001$) and on KVar ($F(7,49) = 2.949$, $p = 0.012$) (Fig. 1B). Post hoc LSD test revealed that muscimol significantly inhibited Resp $Rate_{DOMINANT}$ during the first ($t(7) = 2.425$, $p = 0.023$) and second ($t(7) = 2.787$, $p = 0.014$) 5-min interval of acclimatization. Also, muscimol inhibited KVar during the first ($t(7) = 4.966$, $p = 0.001$) and eighth ($t(7) = 1.99$, $p = 0.044$) 5-min interval (Fig. 1C). Finally, the interaction between the effects of drug and of time on %HF was significant ($F(7, 49) = 2.220$, $p = 0.048$) (Fig. 1D). Post hoc LSD test indicated significant inhibition of %HF by muscimol during the first ($t(7) = 2.99$, $p = 0.010$) and second ($t(7) = 2.573$, $p = 0.019$) 5-min intervals.

3.2. mPFC blockade had no effect on respiratory responses to short acoustic stimuli

Presentation of acoustic stimuli evoked transient increases in respiratory rate that were proportional to the stimulus intensity, ranging from +40 ± 26 cpm in response to the 40 dB stimulus to +354 ± 22 cpm increase after the 90 dB stimulus (Fig. 2A). A change in tidal volume evoked by the acoustic stimuli was similarly proportional to the stimulus intensity, ranging from 7.2 ± 13% after the 40 dB stimulus to 371 ± 81% in response to the 90 dB stimulus (Fig. 2B). Latency of the respiratory responses to the acoustic stimuli was inversely proportional to the stimulus intensity, with a 0.30 ± 0.089 s latency of a respiratory response to the 70 dB stimulus and a 0.066 ± 0.016 s latency of a respiratory response to the 90 dB stimulus (Fig. 2C).

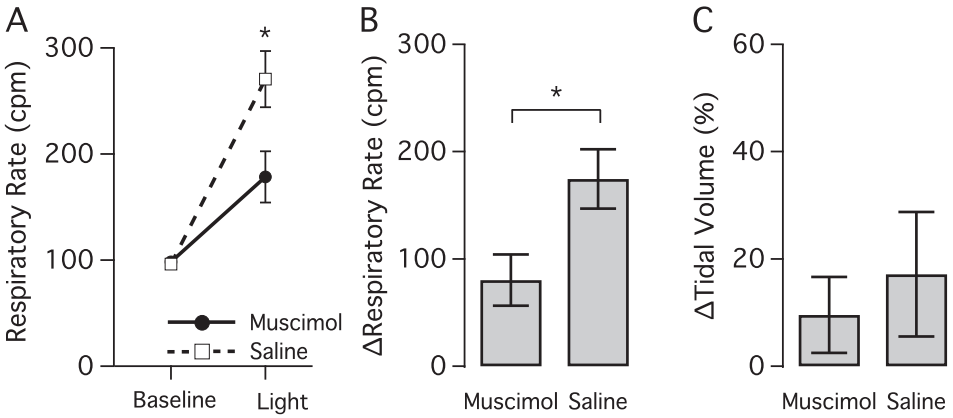

Fig. 3. Baseline respiratory rate values before and during presentation of the 30-s light stimulus (A). Microinjection of muscimol into the prelimbic prefrontal cortex significantly decreased the mean of respiratory rate response (B), but not tidal volume response (C), to the light stimulus. Each data point represents a mean of 30 s of 8 rats after saline (dotted line in A) or muscimol (solid line in A) bilateral microinjection into the prelimbic prefrontal cortex. * Significant difference between muscimol and saline pre-treatment trials, $p < 0.05$.

6×2 (intensity $\times$ drug) within-subjects ANOVAs were performed on the change in respiratory rate (ΔRR) and the change in tidal volume (ΔTV). We found a significant main effect of intensity on the ΔRR ($F(5,35) = 23.031$, $p < 0.001$), but not of the drug. Also, there was a significant linear trend between stimulus intensity and the evoked respiratory rate responses ($F(1,7) = 101.9$, $p < 0.001$). Similarly, only the main effect of intensity on the ΔTV was significant ($F(5,35) = 16.607$, $p < 0.001$). There was also a significant linear trend between stimulus intensity and the evoked tidal volume change ($F(1,7) = 41.4$, $p < 0.001$). Latencies of respiratory responses were analyzed with a 3×2 (intensity $\times$ drug) within subjects ANOVA. Similar to other indices, there was a significant main effect of intensity ($F(2,14) = 23.542$, $p < 0.001$), but not of drug, on the latency of respiratory responses to acoustic stimuli. The relationship between the stimulus intensity and the latency of respiratory responses was found to follow a linear trend ($F(1,7) = 34.2$, $p = 0.001$).

3.3. mPFC blockade attenuated tachypnoeic response to light stimulus

Fig. 3 presents analyzed respiratory data following a visual (light) stimulus presentation. Presentation of light elevated resting respiratory rate from 96 ± 4.7 cpm to 271 ± 27 cpm. Tidal volume during presentation of the light stimulus was $17 \pm 12\%$ higher than during the baseline. Paired-samples t-tests indicated that muscimol significantly inhibited the ΔResp Rate response from 174 ± 28 cpm to 80 ± 24 cpm ($t(7) = 2.179$, $p = 0.033$). However, there was no effect of muscimol on the tidal volume ($t(7) = 0.902$, $p = 0.397$).

3.4. mPFC blockade attenuated respiratory arousal during restraint stress

Submission to the restraint stress elevated the mean respiratory rate from 95 ± 3.5 cpm to 171 ± 9.7 cpm and the median of

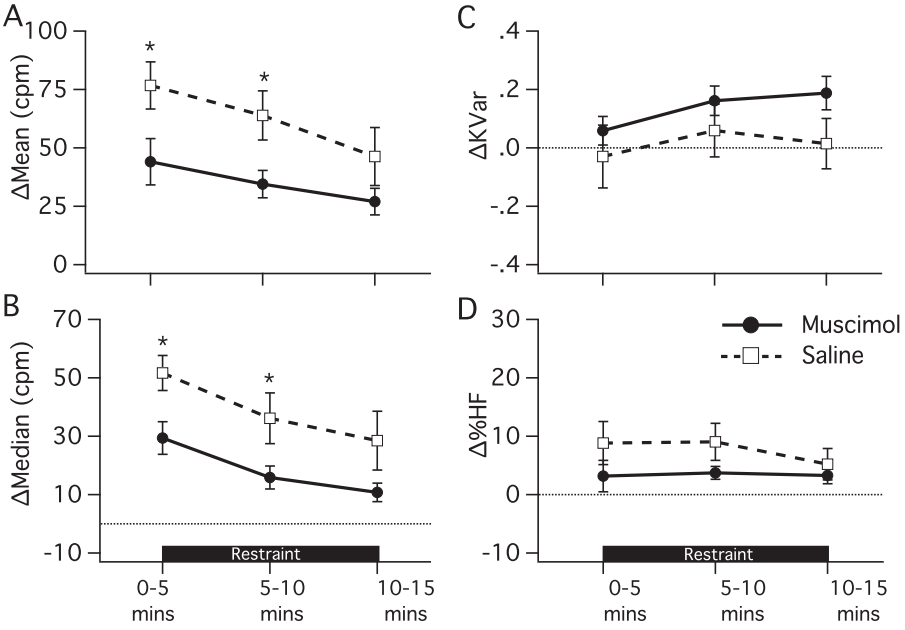

Fig. 4. Microinjection of muscimol into the prelimbic prefrontal cortex significantly decreased the deltas of the mean of respiratory rate (A) and of the dominant respiratory rate (B) during the first two 5-min intervals of restraint. Inhibition of the pre limbic prefrontal cortex did not affect the coefficient of variability of respiratory during the restraint (C) or the percentage of high frequency respiratory rate (D). Each data point represents a mean of 30 s of 8 rats after saline (dotted line) or muscimol (solid line) bilateral microinjection into the prelimbic prefrontal cortex. * Significant difference with $p < 0.05$.

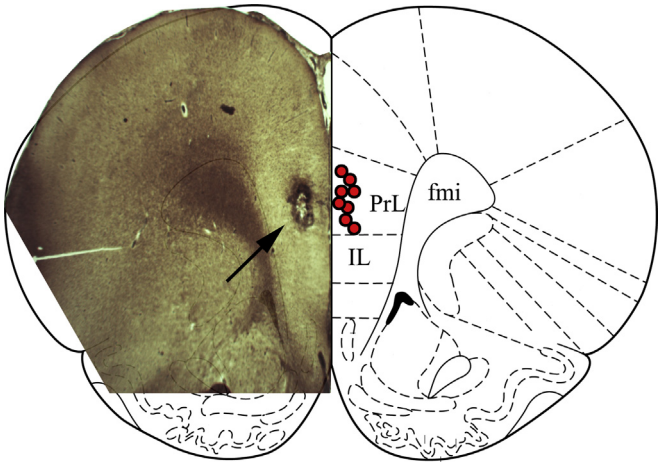

Fig. 5. Histological verification of microinjection sites into the prelimbic medial prefrontal cortex. Left side of the picture displays a coronal section of brain of one of the rats in the current experiment; the arrowhead points to a microinjection site. The right side of the picture displays centres of successful microinjection sites (red circles) drawn on a standard coronal section diagram from the atlas of Paxinos and Watson (1998). Abbreviations: fmi, forceps minor of the corpus callosum; IL, infralimbic medial prefrontal cortex; PrL, prelimbic medial prefrontal cortex.

respiratory rate from 89 ± 3.8 cpm to 141 ± 6.7 cpm. We calculated the changes (Δ) from baseline for all of the parameters for each of the 5-min intervals of restraint and analyzed this data with 3 × 2 (time × drug) within-subjects ANOVAs (Fig. 3). We found significant main effects of drug ($F(2,14) = 4.898$, $p = 0.024$) and of time ($F(1,7) = 6.301$, $p = 0.040$) on ΔResp $Rate_{MEAN}$. Post hoc LSD test indicated that muscimol significantly inhibited ΔResp $Rate_{MEAN}$ during the first ($t(7) = 2.107$, $p = 0.037$) and the second ($t(7) = 2.517$, $p = 0.020$) 5-min interval of restraint. Similarly, there were significant effects of time ($F(2, 14) = 12.191$, $p = 0.001$) and of drug ($F(1,7) = 5.777$, $p = 0.047$) on ΔResp $Rate_{DOMINANT}$. Muscimol was found to inhibit ΔResp $Rate_{DOMINANT}$ during the first ($t(7) = 2.774$, $p = 0.014$) and the second ($t(7) = 2.019$, $p = 0.042$) 5-min intervals of restraint. 3 × 2 ANOVA performed on the ΔKVar data revealed a significant main effect of time ($F(2,14) = 9.232$, $p = 0.003$), but not of drug. There were no significant effects in the Δ%HF data (Fig. 4).

An example of a histologically verified microinjection and a summary diagram of injection sites in 8 animals are presented in Fig. 5.

4. Discussion

The major finding of the current experiment is that blockade of the prelimbic medial prefrontal cortex (plPFC) inhibits respiratory responses to mild and potent prolonged stressors (novel environment, 30 s light, restraint). This blockade, however, did not affect respiratory responses to brief acoustic stimuli of mild and severe intensities, suggesting that the plPFC is involved in processing prolonged fear-related effects on respiration rather than general arousal-related respiratory changes.

We must acknowledge that the brief stimuli that were used in the current study were of the acoustic modality, while the prolonged stimuli were visual or physical. However, we believe that irrespective of the modality of the stimuli the respiratory responses are reflective of changes in the arousal level. Presentation of brief acoustic stimuli presumably evoked brief changes in arousal (a brief period of increased attention or a brief period of a defence response), which were reflected in brief tachypnoeic responses. In line with that, presentation of brief arousing stimuli of physical modality, such as a cage tap or a sudden lateral movement of the cage, produced respiratory responses that were similar in latency and duration to the respiratory responses to acoustic stimuli in the current study (Kabir et al., 2010a). Usage of acoustic stimuli, rather than physical stimuli, in the current study allowed investigation of the effects of intensity of the stimuli on the evoked respiratory responses. We observed that the magnitude of respiratory responses was proportional to the intensity of the stimuli. Presentation of prolonged stimuli, such as the novel environment or the restraint stress, presumably evoked sustained increases in arousal level (prolonged period of increased attention for the investigation of the novel environment or a prolonged period of stress), which were reflected in sustained increases in respiratory rate. A more intense stressor – restraint stress – evoked a greater increase in respiratory rate than the less intense stressor – novel environment. Taken together these observations indicate that the magnitude of the respiratory change in response to a stimulus presentation may be reflective of the change in the arousal level in response to presentation of the said stimulus.

4.1. Prelimbic prefrontal control of respiration

Hassan et al. (2013) assessed the plPFC and the infralimbic cortex (ilPFC) separately by chemical disinhibition in anaesthetized rats. Disinhibition of the ilPFC resulted in greater increases in respiratory rate than disinhibition of the plPFC; however, both regions were found to contain neurons modulating respiratory function. These results must be taken with caution, as prefrontal cortex is believed to modulate activity of other brain areas rather than have a tonic influence on them. Therefore, as activity of such brain networks was severely sedated by anaesthesia in the aforementioned study, these results must be confirmed in a conscious experiment. Our results are therefore in line with the results of Hassan et al. (2013) in that we found effects of inhibition of the plPFC on respiratory function. Our results are also in line with Powell et al. (1994), who described increases in respiratory rate in response to electrical stimulation of the plPFC in conscious restrained rabbits. Taken together, these results suggest that the plPFC modulates respiratory arousal in response to prolonged stressors, but does not contribute significantly to modulation of brief tachypnoea associated with an increase in arousal.

4.2. Dissociation between effects of the plPFC on respiration and heart rate

In general, similar structures of the central control appear to have similar effects on various autonomic indices. Inhibition of the amygdala or of the dorsomedial hypothalamus produces decreases in stress-induced tachycardic, pressor and tachypnoeic responses (Bondarenko et al., 2013, 2014; McDougall et al., 2004). Conversely, activation of these areas produces increases in heart and respiratory rates as well as an increase in arterial pressure (Applegate et al., 1983; McDowall et al., 2007). However, it does not seem to be the case with the plPFC. Chemical stimulation of the plPFC decreases plasma levels adrenocorticotropic hormone (ACTH) and corticosterone (Jones et al., 2011). Similarly, inhibition of the plPFC increases a heart rate response to restraint, but does not affect a pressor response (Tavares et al., 2009). Also, electrical stimulation of the plPFC decreases heart rate, but increases respiratory rate in conscious restrained rabbits (Powell et al., 1994). Failure to find effects of blockade of the medial PFC on cardiac parameters in some previous studies (McDougall et al., 2004) could be due to not making a distinction between the plPFC and the ilPFC in these experiments, as the plPFC and the ilPFC were shown to have opposing roles in cardiac regulation (Tavares et al., 2009). At the same time, electrical stimulation or chemical disinhibition of the plPFC and the ilPFC increases respiratory rate (Hassan et al., 2013; Powell et al., 1994). And, as was found in the current study, inhibition of

the plPFC decreases a respiratory rate response to prolonged stressors. Such dissociation between sympathetic and parasympathetic modulation by the prefrontal cortex could be related to changes in autonomic parameters during states of fear and anxiety, such as during freezing, when cardiac, arterial pressure and hormonal changes are sympathetically activated (Buijs and Van Eden, 2000), yet respiratory rate is decreased (Hegoburu, 2011).

4.3. Neural pathways

Given the opposite effects of the plPFC blockade on respiration and on heart rate (as discussed in Section 4.2) it is difficult to suggest the exact pathways from the plPFC that mediate respiratory activation without detailed investigation of the types of such projections. The plPFC was shown to have strong projections to the central nucleus of the amygdala (Vertes, 2003), which was also shown to be involved in autonomic respiratory arousal (Bondarenko et al., 2014). Yet inhibition of the amygdala by muscimol selectively inhibits respiratory arousal to potent stimuli, both brief and prolonged. On the other hand, the plPFC blockade, as shown in the current study, selectively attenuates respiratory arousal to prolonged stimuli, both mild and potent. It is therefore possible that the projections from the plPFC to the central nucleus of the amygdala are responsible for the cardiovascular rather than respiratory activation. Also, the plPFC heavily projects to the periaqueductal grey, which was suggested to mediate respiratory arousal (Dampney et al., 2013). The plPFC might mediate respiratory activation in response to stressors via projections to the dorsomedial hypothalamus (DMH). Indeed, disinhibition of this area results in potent tachypnoeic dose-dependent response (McDowall et al., 2007). Also, inhibition of the DMH almost completely abolishes respiratory activation in response to both brief and prolonged stressors of various intensities (Bondarenko et al., 2013). Therefore, projections from the plPFC to the DMH, although sparse, could mediate respiratory activation in response to stress. An alternative pathway could be via projections of the plPFC to the cuneiform nucleus, which has direct projections to the major respiratory brainstem regions, such as the Kölliker-Fuse nucleus and the nucleus tractus solitarius (Korte et al., 1992). Further research into the central respiratory pathways using methods such as optogenetic stimulation might provide better understanding of the exact mechanisms of respiratory arousal in response to stress.

4.4. Relevance for understanding panic disorders and sleep apnoea

Interestingly, dysfunction of the prefrontal cortex is linked with panic disorder (Mezzasalma et al., 2004). Panic disorder is categorized by a feeling of high heart rate and a shortness of breath. Some studies of panic disorder conducted in laboratory settings describe hyperventilation during panic attacks (reviewed in Mezzasalma et al., 2004), while such respiratory alterations were not found in patients with panic disorder during continuous ambulatory monitoring (Pfaltz et al., 2009). Also, patients with panic disorder exhibit an increased basal heart rate (Chignon et al., 1993; Cohen et al., 2000) and increased cardiac response to a recall of a panic attack (Cohen et al., 2000). As the activation of the medial PFC elicits activation of autonomic system and an inhibition of respiration, it is therefore possible that a panic attack is linked to activation of the medial PFC resulting in an increase in cardiovascular parameters with concomitant inhibition of respiration. This suggestion is in line with panic attack symptoms; yet, it requires further investigation in future studies.

Another clinical condition that is associated with the prefrontal cortex dysfunction is obstructive sleep apnoea (Beebe and Gozal, 2002). Patients with this condition experience intermittent episodes of hypoxia and hypercapnia as well as sleep disturbance, which lead to a dysfunction of the prefrontal cortex (Beebe and Gozal, 2002). Interestingly, these patients exhibit increased cardiac responses to physical stressors (Macey et al., 2013). Furthermore, children with obstructive sleep apnoea exhibit a decreased basal respiratory rate and an elevated respiratory response to arousal (Baumert et al., 2011). Lastly, cardiorespiratory coupling is decreased in patients with severe obstructive sleep apnoea (Kabir et al., 2010b). Given the involvement of the prefrontal cortex in mediating both cardiac and respiratory parameters, it is therefore possible that dysfunction of the prefrontal cortex in patients with obstructive sleep apnoea is related to such elevated respiratory and cardiac responses to stressors. Yet, at this stage, it is not known, which areas of the prefrontal cortex contribute to these autonomic disturbances. Previous findings indicate that excitation of plPFC evokes tachypnoeic and bradycardic responses (Powell et al., 1994), while excitation of the ilPFC evokes tachypnoeic and tachycardic responses (Hassan et al., 2013; Tavares et al., 2009). Such dissociation between cardiac and respiratory effects of excitation and inhibition of the plPFC and of the ilPFC may give a valuable insight into understanding of topography of the prefrontal cortex dysfunction in patients with obstructive sleep apnoea.

4.5. Significance and perspectives

Our results demonstrate involvement of the prelimbic medial prefrontal cortex in processing of respiratory activation in response to prolonged, but not short-lasting, mild and strong stressors. These results are in line with previous reports of the involvement of the prefrontal cortex in fear response processing. In the future experiments we plan to investigate blockade of the infralimbic medial prefrontal cortex – a region that is also implicated in respiratory control (Hassan et al., 2013). Pharmacological inhibition of this region was found to reduce cardiac response to stress, while inhibition of the plPFC increased it. Further investigation of the involvement of the prefrontal cortex in respiratory control may provide new valuable information for understanding of clinical conditions, such as panic disorder and obstructive sleep apnoea.

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CONCLUSIONS

The aim of this thesis was to systematically investigate respiratory responses to various stimuli and stressors, to compare them with other traditional measures of autonomic activation and anxiety, and to investigate central neuronal structures that mediate these respiratory responses. I have demonstrated that changes in respiratory rate in response to brief alerting stimuli are more sensitive than changes in a traditionally used index of autonomic activation – heart rate. Furthermore, respiratory rate during novelty stress was highly and significantly correlated with behavioural indices of anxiety as assessed by the Elevated Plus Maze. Next, I have demonstrated the involvement of the dorsomedial hypothalamic area, the amygdala and the prelimbic prefrontal cortex in mediating such respiratory activation in response to various stimuli. Inhibition of the dorsomedial hypothalamus and the adjacent perifornical area almost completely abolished respiratory responses to both short and prolonged alerting stimuli and to stress, of both low and high intensity. Blockade of the amygdala, on the other hand, significantly inhibited respiratory responses to brief and prolonged high intensity stressors, while blockade of the prelimbic prefrontal cortex inhibited respiratory responses to prolonged, but not brief, stressors.

The significance of my findings is three-fold: Firstly, they started filling the gap in the basic knowledge of the central structures that mediate respiratory activation during stress and arousal. Previous studies have implicated the dorsomedial hypothalamic area, the amygdala and the prefrontal cortex in the central control of cardiovascular parameters; the current thesis extends this knowledge and demonstrates that these structures are also involved in the control of respiratory function. Furthermore, as these experiments were performed in conscious freely moving animals, I have demonstrated the extent of the involvement of each of these structures in mediating brief and prolonged respiratory responses. Results of the current thesis show support for the suggestions that the dorsomedial hypothalamic area integrates central respiratory information (Dampney et al., 2008) as blockade of this structure abolished respiratory activation in response to restraint stress and the 40-70dB stimuli.

Furthermore, my results are in accord with previous findings by Zhang et al. (2009) that projections from the amygdala to the dorsomedial hypothalamic area mediate central respiratory activation as the effects of the DMH/PeF blockade on respiratory responses to various stimuli were stronger than or comparable with the effects of the amygdala blockade. Similarly, microinjection of muscimol into the plPFC resulted in weaker inhibition of the respiratory responses than microinjection into the DMH/PeF. As the plPFC has projections to the perifornical area and the dorsomedial hypothalamus (Vertes, 2003), it is therefore likely that the DMH/PeF region integrates respiratory information from the plPFC. Also, the plPFC has strong projections to the central amygdaloid nucleus (Vertes, 2003), suggesting that at least some aspect of the respiratory activation could be mediated by these projections. In the current thesis respiratory responses to the novelty stress was stronger after the plPFC blockade than after the amygdala blockade. Also, the plPFC blockade did not affect the magnitude of respiratory rate responses to brief acoustic stimuli. These observations suggest that it is unlikely that respiratory activation during mild stress or during brief changes in arousal level is mediated by projections from the plPFC to the amygdala. However, the initial respiratory response to the restraint stress was similar after the plPFC and the amygdala blockades; therefore, it is possible these projections mediate prolonged respiratory activation in response to high intensity stimuli. Alternatively, such projections might be non-respiratory and could be mediating cardiovascular activation during stress. These results are summarized in Figure 1.

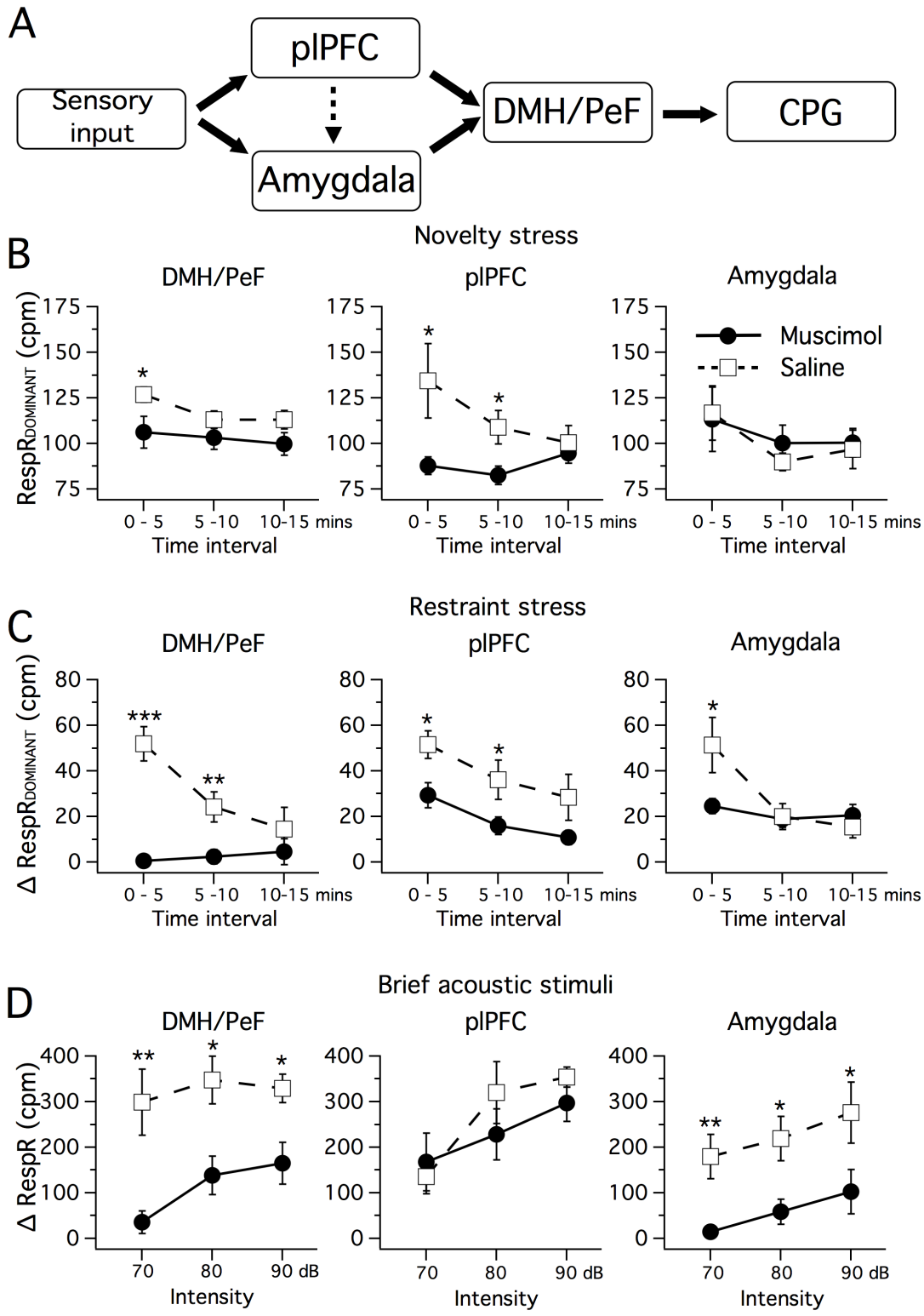

Figure 1. Summary of the thesis (A) The proposed central respiratory network that mediates stress- and arousal-induced respiratory changed based on the results of this thesis. (B) Inhibition of the dorsomedial hypothalamus and the perifornical area (DMH/PeF) and of the prelimbic prefrontal cortex (plPFC), but not of the amygdala, significantly attenuated the dominant (locomotion-independent) respiratory rate during novelty stress. (C) Blockade of

the DMH/PeF abolished respiratory response to the restraint stress (left graph); inhibition of the plPFC significantly attenuated this response preserving the time-dependent reduction of the dominant respiratory rate (center); while inhibition of the amygdala attenuated the dominant respiratory rate response during the first 5-minute interval and abolished the time-dependent reduction of the dominant respiratory rate. (D) Inhibition of the DMH/PeF and the amygdala attenuated respiratory responses to the brief acoustic stimuli (70-90dB). The magnitude of this effect was very similar after blockade of the two structures. Inhibition of the plPFC, on the other hand, did not affect respiratory rate responses to these stimuli.

Secondly, organization of the central respiratory network that I have partially described has implication for various clinical conditions, in which respiration is adversely affected. For example, dysfunction of the prelimbic prefrontal cortex has been demonstrated in patients with sleep apnea (Beebe and Gozal, 2002) and panic disorder (Mezzasalma et al., 2004). Also, hyperactivity of orexin neurons in the dorsomedial hypothalamic area has been suggested as a cause of panic disorder (Johnson and Shekhar, 2012). Dysfunction of respiratory neurons in the amygdala or the dorsomedial hypothalamus might also be involved in sudden unexpected death in epilepsy, during which patients spontaneously stop breathing after a seizure in the amygdala (Richerson, 2014). Lastly, organization of the central respiratory network might give insights into the progression of neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, as recent observations suggest that respiratory network is one of the first networks to be affected by neurodegeneration (Dutschmann, 2014).

Thirdly, the principal translational outcome of my work is developing and validation of a new locomotion-independent test of anxiety in preclinical studies. Currently there is quite limited choice of anxiolytic drugs with the most common anxiolytic drugs, benzodiazepines and selective serotonin reuptake inhibitors, acting on the neurotransmitter release and activity. The existing anxiety tests in animals are based on their ambulation, which itself can also be

affected by the anxiolytic drugs, giving a false positive result of the effects of the drug on animals' anxiety. A locomotion-independent index, such as the magnitude of respiratory activation during presentation of various stressors, could therefore be a much more accurate measure of animals' anxiety.

In the future we also aim to investigate involvement of the periaqueductal grey and the infralimbic prefrontal cortex – the two structures that were also implicated in the central respiratory control. Stimulation of the periaqueductal grey and of the infralimbic prefrontal cortex produces tachypnoeic responses in anesthetized animals (Hassan et al., 2013; Hayward et al., 2003). Also, inhibition of the infralimbic prefrontal cortex reduces cardiac and pressor responses to the restraint stress in conscious animals (Fassini et al., 2014). Therefore, investigation of effects of blockade of these two structures on respiratory responses to various stimuli and stressors in conscious animals is required to further establish and describe their contribution to mediating respiratory activation during stress and arousal.

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