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

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Running title: Amygdala mediates respiratory responses

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ABSTRACT

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 and in tidal volume 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 (70-90dB). 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.

Key words: 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 (4) and (14) for reviews). Anxiety is one such emotional state; it is physiologically associated with defense mechanisms or fight-or-flight response. Using various laboratory stressors, human studies 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 (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 ponto-medullary mechanisms (9, 30).

Preclinical research has recently started investigating respiratory responses in animal models of anxiety. Early studies lacked non-intrusive and precise techniques for assessing respiratory indices in non-anesthetized animals. Among modern methods, whole-body plethysmography represents a promising approach, as it is entirely non-invasive and thus does not introduce any confounding factors. Using this method, Kinkead and colleagues 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 brainstem (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 to 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-induces 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 the amygdala in cardiovascular responses to various stressors, only 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 anaesthetized 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 the increase in respiration (21). All these findings suggest that amygdala may be involved in processing some aspect of anxiety-induced respiratory effects, but do not directly prove its involvement. Obtaining such direct evidence in animal experiment 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 and visual stimuli and by a prolonged stressor (restraint).

METHODS

Animals

Eight outbred male Wistar rats, weighing 350-400 g, were received 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 19.00). 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 hours 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 counterbalanced within-subjects design. Each animal underwent the protocol with all three types of microinjection. Muscimol was from purchased Sigma Aldrich (USA).

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 l, wall thickness 3 mm) with animal bedding 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 liters per minute. 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, USA), 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 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-minute “acclimatization” period, followed by presentation of six brief acoustic stimuli of progressively increasing intensity (500 msec 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 inter-stimulus intervals as shorter intervals resulted in habituation. All stimuli were presented when animals were awake, quiet, and their breathing was slow (<150 cpm) and regular (without obvious accelerations or decelerations) for at least 10 seconds. Subsequently, 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, Sydney, Australia). Respiratory rate was computed online with subsequent off-line verification using LabChart software (Version 7.1, ADInstruments, Sydney, Australia). 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 variation compared with baseline.

Acclimatization and restraint
For characterizing respiratory pattern during acclimatization and restraint we used four parameters: mean respiratory rate ($\text{RespR}_{\text{mean}}$) was computed by LabChart software from peaks in the respiratory signal, coefficient of variation, $K_{\text{var}} = \text{S.D.}/\text{RespR}_{\text{mean}} * 100\%$, dominant respiratory rate $\text{RespR}_{\text{dom}}$ – respiratory frequency at which animal spent most of time during recordings and the percentage of time spent at high respiratory frequency, $\%HF$. For the latter two measures, using IgorPro software (Wavemetrics, USA), we first constructed time histograms for each recording with bin width equal 10 cycles/min; an example of such histogram is shown in Fig. 1B. This graphic representation indicates how much time (in ms) animals spent at a given respiratory frequency. The mode of such histograms represent dominant respiratory rate ($\text{RespR}_{\text{dom}}$). Respiratory rate is relatively stable during periods of no locomotor activity, but 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 $AUC_{250-650}/AUC_{0-650}$ ($AUC = \text{area under the curve}$). We computed these for values for the following 5-min intervals: eight epochs of acclimatization, baseline before restraint and three epochs during restraint.

Acclimatization data were first analyzed by 8x3 within-subjects ANOVAs (interval x pre-treatment) for each of the four respiratory parameters measured ($\text{RespR}_{\text{mean}}$, $\text{RespR}_{\text{dom}}$, $K_{\text{var}}$ and $\%HF$). If the ANOVA indicated a significant main effect of drug pre-treatment, 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 8x3 ANOVA indicated a significant interaction between the effects of time interval and drug, three 8x2 ANOVAs (interval x muscimol vs. saline, interval x...
muscimol vs. control muscimol, interval x 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 pre-treatment, post LSD tests were performed between the drug pre-treatments. For the restraint data, we calculated changes from baseline for each index (Δ) and performed a 3x3 within-subjects ANOVA (drug pre-treatment x interval) on these computed Δ indices. A similar approach to acclimatization analysis was taken for the restraint analysis. If a 3x3 ANOVA indicated a main effect of drug pre-treatment, we performed a pairwise comparison of main effects of drugs. If one of these comparisons was significant, we performed post-hoc LSD test. If, however, a 3x3 ANOVA indicated a significant interaction between the effects of drug and time interval, we performed three 3x2 ANOVAs testing different pairs of drug pre-treatments. Subsequently, we performed 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 latency of responses. Both respiratory rate response 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, TV amplitude and latency) were then analyzed by within-subjects ANOVAs (stimulus intensity x pre-treatment). 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 pre-treatments that were shown to have significantly different main effects. If the within-subjects 6x3 (3x3 for Latency) ANOVAs indicated significant interactions between the effects of drug and stimulus intensity, three 6x2 (3x2 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 pre-treatment, post LSD tests were performed between the drug pre-treatments.

**Light stimulus**

Mean values of respiratory rate ($\text{RespR}_{\text{mean}}$) and tidal volume ($\text{Vt}$) were determined for two 30-s intervals – one immediately before presentation of light and one during the light stimulus. We firstly performed paired t-tests for these two values for saline pre-treatment to describe a general pattern of response to this stimulus. Secondly, we computed delta respiratory rate ($\Delta \text{RespR}_{\text{mean}}$) and delta tidal volume ($\Delta \text{Vt}$) 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 $\Delta \text{RespR}_{\text{mean}}$ and $\Delta \text{Vt}$ with post-hoc LSD test.

**RESULTS**

**Respiratory pattern during acclimatization period**

An 8x3 within subjects ANOVA indicated a significant main effect of time on the mean respiratory rate during the acclimatization period, $F (7, 49) = 11.904, p < .001$. After being placed into the plethysmograph, rats displayed an elevated respiratory rate of 214±21 cpm on the saline trials during the first 5 minutes, which gradually declined to 122±18 cpm during the last 5-minute interval of the 40-minute acclimatization; this
is illustrated in Fig. 1A. 8x3 within subjects ANOVA indicated a significant interaction between drug pre-treatment and interval number in the percentage of high frequency respiratory rate (%HF) index, $F(14, 98) = 2.293$, $p = .009$. Three 8x2 (time interval x 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 pre-treatment trials in %HF, $F(7, 49) = 3.990$, $p = .002$. Post-hoc LSD analysis revealed that muscimol microinjection into the amygdala significantly decreased %HF during the first 5-minute interval from 43.2±6.8% to 22.1±7.8% ($p = .008$). Injections of muscimol dorsal to the amygdala did not produce responses that were different from those who 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 $+13 \pm 9$ cpm in response to the lowest intensity stimulus to $+276 \pm 67$ 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 80dB stimulus averaged from all saline trials. A 6x3 within-subjects ANOVA revealed a significant interaction between the drug pre-treatment (saline vs. muscimol to the target area vs. control muscimol injection) and the stimulus intensity in the amplitude of respiratory rate response, $F(10, 70 =2.748$, $p = .006$. 


Respiratory rate responses to the acoustic stimuli were linearly dependent upon stimulus intensity, $F(1, 7) = 24.435, p = .002$. A muscimol vs. saline 6x2 ANOVAs (intensity x drug pretreatment) indicated a significant interaction between the effects of intensity and drug pretreatment, $F(5, 35) = 3.862, p = .007$. 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 ($p = .009$, $p = .021$ and $p = .043$, respectively; Figure 4A). A muscimol vs. control muscimol 6x2 ANOVA (intensity x drug pretreatment) indicated a significant main effect of drug, $F(1, 7) = 5.859, p = .046$. Post-hoc LSD test revealed that muscimol to the amygdala microinjection significantly inhibited respiratory rate responses to the 70 and 90 dB intensity stimuli ($p = .036$ and $p = .043$, respectively) compared with muscimol dorsal to the amygdala microinjection. A control muscimol vs. saline 6x2 ANOVA (intensity x drug pretreatment) indicated no significant main effects of drug ($F(1, 7) = 2.047, p = .196$) or an interaction between the effects of drug and of intensity ($F(5, 35) = 1.356, p = .265$).

We also found a significant main effect of stimulus intensity on the tidal volume, $F(10, 70) = 11.590, p < .001$. Tidal volume responses to the acoustic stimuli were linearly dependent upon stimulus intensity ($F(1, 7) = 19.882, p = .003$) and were ranging from $10 \pm 23\%$ increase over baseline after a 40 dB stimulus to $186 \pm 57\%$ increase over baseline after a 90 dB stimulus on saline trials (Figure 4B). There was no significant main effect of the drug pre-treatment on tidal volume responses ($F(2, 14) = 1.982, p = .175$) or an interaction between the effects of drug and of intensity ($F(10, 70) = 1.227, p = .289$).
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 msec latency of a response to the 70 dB stimulus to 65 ± 15 msec latency of a response to the 90 dB stimulus. There was a significant main effect of intensity of stimuli on latency of responses, $F(2, 14) = 20.046, p < .001$. Muscimol microinjection failed to significantly affect latencies of any responses (Figure 4C).

Presentation of a light stimulus elevated the mean of respiratory rate from 83 to 242 cpm and also increased tidal volume by 21% compared with baseline. Muscimol microinjection significantly decreased the response in respiratory rate mean, but not in tidal volume, during presentation of a 30-second light stimulus compared with the saline microinjection trial, $t(7) = 4.74, p = .002$ (Figure 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, $t(7) = 4.71, p = .002$. A difference between the saline and control muscimol pretreatment trials was only marginally significant, $t(7) = 1.833, p = .055$.

Respiratory responses to restraint stress.

In the trials with saline pre-treatment, restraint stress significantly elevated the mean respiratory rate ($\text{RespR}_{\text{mean}}$; from 85 ± 6 to 157 ± 7 cpm, $t(7) = 4.678, p = .001$), the dominant respiratory rate ($\text{RespR}_{\text{dom}}$; from 78 ± 3 to 129 ± 10 cpm, $t(7) = 3.802, p = .004$) and the fraction of high-frequency respiratory rate (%HF; from 3.1 ± 2.5% to 13.8 ± 2.4%, $t(7) = 3.700, p = .004$) during the first 5 minutes of restraint. An example of a raw trace of respiratory rate recording is presented in Figure 6A. A similar pattern
was observed on the control muscimol dorsal to the amygdala trials (Fig. 6A).

Muscimol microinjection into the amygdala abolished the increase in $\text{RespR}_{\text{mean}}$, $t(7) = .767, p = .234$ (Fig. 6B), and significantly attenuated the increase in $\text{RespR}_{\text{dom}}$, $t(7) = 3.094, p = .009$ (Fig. 6C), and $\%\text{HF}$, $t(7) = 2.020, p = .042$ (Fig. 6E). Respiratory rate during the first 5-minute interval of restraint stress after the control muscimol microinjection dorsal to the amygdala was significantly lower than that after saline pre-treatment ($t(7) = 2.176, p = .033$), but higher than that after muscimol to the amygdala microinjection ($t(7) = 3.157, p = .008$).

We found significant main effects of drug ($F(2, 14) = 4.591, p = .029$) and time interval ($F(2, 14) = 10.54, p = .002$) on $\Delta\text{RespR}_{\text{mean}}$. The main effect of muscimol pretreatment was significantly different from the main effect of saline pretreatment ($p = .009$) and marginally different from the control muscimol microinjection ($p = .098$). Post-hoc LSD test indicated that muscimol significantly inhibited $\Delta\text{RespR}_{\text{mean}}$ during the first ($p = .004$) and second ($p = .042$) 5-minute intervals of restraint. We also found significant interactions between the drug pre-treatment and the interval of the restraint in $\Delta\text{RespR}_{\text{dom}}$ ($F(4,28) = 3.961, p = .011$; and $F(8,56) = 4.345, p < .001$). Three 3x2 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, $F(2, 14) = 5.057, p = .022$. Post-hoc analysis indicated that microinjection of muscimol to the amygdala significantly decreased responses in the dominant respiratory rate during the first 5-minute interval of restraint ($p = .024$). Lastly, there was a significant main effect of drug on $\Delta\%\text{HF}$, $F(2, 14) = 5.169, p = .021$. The main effect of muscimol microinjection was significantly different from the saline microinjection ($p = .003$) and marginally
different from the control muscimol microinjection ($p = .055$). Post-hoc LSD test indicated that muscimol significantly inhibited $\Delta%HF$ during all three 5-minute intervals ($p = .009$, $p = .008$ and $p = .017$, 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-minute interval of the restraint. Control microinjection of muscimol dorsal to the amygdala did not significantly inhibit respiratory response to restraint. All data values and results of statistical analysis are presented in Figure 7.

An example of histologically verified microinjection and a summary diagram of injection sites in 8 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 (8) for review). Despite firmly established
link between negative emotional state and respiration (4, 14), only few previous animal and human studies provide evidence that the amygdala may be involved in respiratory control (see Introduction). One early work where this has been directly confirmed reported that surgical lesion of the amygdala in two epileptic patients resulted in a decrease 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. Our work is thus 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 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
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 (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 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 intensity of the acoustic stimuli, whereas their latency is inversely proportional to the intensity. Of major relevance is the fact
that the reported here inhibition 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 seconds 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 in conducting pathways as well as basal differences in respiratory rhythms, as humans’ resting respiratory rate is approximately 12-15 cycles per minute, while rats’ resting respiratory rate is varies from 80 to 100 cycles per minute.

Another novel finding of our study is the sensitivity of tidal volume responses to the amygdala blockade. 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, and inhibition of the amygdala significantly attenuated these volume responses. This finding indicated that amygdala is involved in modification of both frequency and magnitude of rhythmic respiratory neural discharges. 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 grasp 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 minutes 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 extends 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 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 is against 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. It is thus evident that assessment of the mean respiratory rate and the %HF has 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 regards to changes in 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). Similarly 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 amygdaloid 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, 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, in order to produce a coordinated pattern of autonomic response (7, 35).

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 nuclei 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 various stresses. Firstly, compared to commonly used cardiovascular indices, respiratory changes are more sensitive as they have lower thresholds (23). Secondly, 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 is much greater compared to other autonomic indices or of evoked potentials, making this index readily assessable and quantifiable on single-trial experiments. This methodology is also simple and non-invasive. Given demonstrated links between respiration and anxiety state, as discussed earlier, the current methodology can potentially be used as a non-invasive measure of anxiety in animals that is directly translatable to humans.

GRANTS

This work was supported by a Postgraduate Scholarship (PB 10S 5462) from the National Heart Foundation of Australia.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
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Figure 1. Respiratory rate during acclimatization. (A) Raw trace of respiratory rate during 40 minutes of acclimatization of one of the rats. (B) A 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).

Figure 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) pre-treatment. The amygdala inhibition with muscimol decreased percentage of high frequency respiratory rate (D) during the first 5-minute interval, but did not affect it for the remainder of the acclimatization period: ** - significant difference between muscimol and saline pre-treatment trials, $p < .01$.

Figure 3. Raw trace of respiratory recording illustrating the response to a 80-dB white noise (500msec) acoustic stimulus (A). Respiratory rate response to a 80-dB white noise (500msec) stimulus averaged from 8 rats after saline microinjection (B).
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 pre-treatment trials, $p < .05$; ** - significant difference between muscimol and saline pre-treatment trials, $p < .01$.

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 seconds 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).

Respiration during restraint. (A) An example of a raw respiratory rate recording after saline microinjection during baseline and a 15-minute restraint. Microinjection of muscimol to the amygdala significantly decreased the mean (B) of respiratory rate during all three 5-minute 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-minute interval, but not during the first. Each data point represents a mean of 30 seconds of 8 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 $p < .05$; ** - significant difference with $p < .01$; *** - significant difference with $p < .001$.

Figure 7. Microinjection of muscimol to the amygdala significantly decreased the deltas of the mean of respiratory rate (A) during the first and second 5-minute interval of restraint and of the dominant respiratory rate (B) during the first 5-minute 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 seconds of 8 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 $p < .05$; ** - significant difference with $p < .01$.

Figure 8. Histological verification of microinjection sites into the amygdala. 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 centers of successful microinjection sites (gray 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; mb, mamillo-thalamic tract; OT, optic tract; III, third ventricle.
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