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Electroencephalographic responses of anaesthetised rats to carbon dioxide inhalation

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Abstract

*Exposure to high concentrations of CO_2 is a common means of stunning and killing laboratory rodents. However, there is concern regarding the potential for animals to have aversive experiences, such as pain or breathlessness, prior to loss of awareness. This preliminary study evaluated the electroencephalographic (EEG) responses of rats (*Rattus norvegicus*) to CO_2 inhalation, using a method based on a minimal anaesthesia model previously used to assess nociception in mammals. Fifteen adult female Sprague-Dawley rats were anaesthetised with halothane in oxygen and maintained at a minimal plane of anaesthesia. EEG was continuously recorded throughout a 10-min baseline period followed by sequential exposure to 5, 15, 30 and 50% CO_2. The EEG summary variables median frequency (F50), 95% spectral edge frequency (F95) and total power (P_{TOT}) were derived from the raw EEG. The F50 of the EEG, a sensitive indicator of nociception, increased significantly above baseline during exposure to 15% CO_2, suggesting this concentration was noxious to rats. This is consistent with behavioural aversion in rats at around the same CO_2 concentration. Stimulation of the rat mucosal nociceptors only occurs at CO_2 concentrations of 37% or greater; therefore, it is hypothesised that the observed response was a result of what would have been CO_2-induced aversive respiratory sensation in conscious animals, rather than pain. This study provides some evidence that an anaesthesia model may be utilised to study the nocuous effects of low-moderate CO_2 exposure in rodents.*

Keywords: *animal welfare, carbon dioxide, electroencephalogram (EEG), euthanasia, negative affective experience, rat*

Introduction

Laboratory rats (*Rattus norvegicus*) and mice (*Mus musculus*) are used extensively in biological research. The vast majority of these are killed, whether it be at the conclusion of the research project, for the purposes of tissue harvest, or to manage the size and composition of breeding stock (Makowska *et al* 2009). Exposure to carbon dioxide (CO_2) is a common means of killing laboratory rodents. This induces central nervous system depression and subsequent loss of awareness, followed by respiratory and/or cardiac arrest and death. However, there are concerns that rodents may experience pain, breathlessness or other aversive sensations prior to loss of awareness (Hawkins *et al* 2006).

Pre-fill methods using high concentrations of CO_2 are likely to cause pain due to mucosal acidification and are generally not recommended. For example, humans report pain in the eyes, nose and throat following exposure to 40–55% CO_2 (Thurauf *et al* 1993; Chen *et al* 1995; Danneman *et al* 1997), and concentrations of 37% and greater have been shown to activate nociceptors in the rat nasal mucosa (Thurauf *et al* 1991; Peppel & Anton 1993). During forced exposure to high concentrations of CO_2, using either pre-fill or gradual induction, rats and mice displayed behavioural signs, such as increased locomotion, rearing, agitation, gasping, shaking and hyperventilation, suggesting aversive experience prior to loss of awareness (Britt 1987; Coenen *et al* 1995; Smith & Harrap 1997; Ambrose *et al* 2000).

Gradual-fill methods are proposed to be better in terms of animal welfare and cited as being conditionally acceptable by organisations such as the American Veterinary Medical Association (Leary *et al* 2013). However, behavioural studies indicate that rodents are still averse to, and avoid, CO_2 concentrations well below the threshold for mucosal nociceptor activation. For example, both rats and mice withdrew almost immediately from a chamber pre-filled with 25% CO_2 (Leach *et al* 2002). Additionally, during exposure to a gradually increasing CO_2 concentration in the presence of a food reward, rats withdrew from the chamber when the concentration reached 13.0 to 15.9% (Niel *et al* 2008). Aversion to low-moderate CO_2 concentrations is a problem, given that concentrations of 30% or

more are required to induce loss of consciousness in rats (Smith & Harrap 1997; Niel & Weary 2006), and > 50% is required for complete anaesthesia (Brosnan *et al* 2007). These findings suggest that rats are likely to experience negative sensations other than pain before loss of awareness during gradual-fill killing.

Aversion to CO_2 concentrations below those which stimulate mucosal nociceptors may be due to other unpleasant sensations, such as breathlessness or anxiety. Human subjects report breathlessness upon exposure to CO_2 concentrations as low as 8%, and rate this as severe at around 15% CO_2 (Dripps & Comroe 1947; Liotti *et al* 2001). Inhalation of CO_2 is also known to produce intense fear, autonomic and respiratory responses that can induce panic attacks in humans (Vollmer *et al* 2016). In mice, exposure to 5 or 10% CO_2 has been shown to evoke innate fear responses (Ziemann *et al* 2009; Vollmer *et al* 2016).

Measurement of cerebrocortical activity by recording the electroencephalogram (EEG) can be used to complement existing behavioural studies to investigate the unpleasantness of CO_2 inhalation at concentrations rodents are typically exposed to during gradual-fill killing. EEG analysis has been used as an adjunct to behaviour for the study of pain in animals for some time. Using a minimal anaesthesia model, quantitative changes in the power spectrum of the EEG have been used to assess responses to nociceptive stimuli and to evaluate anti-nociceptive strategies (Murrell & Johnson 2006). Whilst behavioural analysis is a valuable tool, it necessitates conscious experience of the applied noxious stimulus, and may be influenced by variables other than the applied stimulus. Under light anaesthesia, conscious perception is prevented without interrupting the relay of signals to the cerebral cortex, thus mitigating potential welfare compromise in study animals. Furthermore, responses to extraneous stimuli are reduced, thus increasing specificity and reducing variability in the data, meaning fewer experimental animals are required to detect differences among treatments.

The objective of this study was to investigate whether exposure to inspired CO_2 concentrations of 5, 15, 30 or 50% produced quantifiable changes in the EEG power spectra of the anaesthetised rat, and whether the character of any such changes was consistent with those previously reported following nociceptive stimulation.

Materials and methods

This study was conducted with approval from the Massey University Animal Ethics Committee, New Zealand (MUAEC, protocol # 09/106). All procedures were undertaken in accordance with the MUAEC code of ethical conduct for the use of live animals for research, testing and teaching.

Study animals and housing

Fifteen adult female Sprague-Dawley rats aged 4–5 months, weighing 280–440 g, obtained from the small animal production unit at Massey University, were used in the study. The rats had previously been used for teaching purposes and were destined for euthanasia. Beforehand, rats were housed in groups of five in standard laboratory cages and fed commercial rat chow (rat diet 86, Feed Processing Unit, Massey University, New Zealand) with *ad libitum* access to water. The rats were housed in a temperature-controlled (22 [± 1°C]), ventilated room on a 12-h light/dark cycle, with photoperiod 0700 to 1900h. Rats were moved to the testing laboratory in their home cages on the day of testing. Free access to food and water was available until the time of anaesthetic induction.

Experimental protocol

Each rat was placed in a custom-built 1.5-L perspex induction chamber and anaesthesia induced with 4% halothane vaporised in oxygen (2 L min^{-1}; Penlon PPV Vaporizer, Penlon Ltd, Abingdon, UK). Adequate depth of anaesthesia was assessed by lateral recumbency and the absence of withdrawal reflex to a toe pinch. Endotracheal intubation was carried out with an 18-gauge cannula, using a trans-tracheal illumination technique (Murrell *et al* 2008). After confirmation of successful intubation by capnometry (MedAir RespSense Capnograph, Nonin Medical Inc, Plymouth, MN, USA), the tracheal cannula was connected to a T-piece breathing circuit and anaesthesia maintained with 4% halothane in oxygen throughout instrumentation. Ventilation was controlled using an intermittent positive pressure ventilator (V valve ventilator, Ventronics, Bioanalytical systems Inc, IN, USA).

The head of the rat was secured in a stereotaxic apparatus and 27-G subcutaneous, stainless steel, needle electrodes (Viasys Healthcare, Surrey, UK) were positioned to record EEG and electrocardiogram (ECG) activity. A five-electrode montage was used to record EEG from both the left and right cerebral hemispheres, with inverting electrodes placed over the left and right frontal bone zygomatic processes, non-inverting electrodes over the left and right mastoid processes and a ground electrode placed caudal to the occipital process (Murrell & Johnson 2006). ECG was recorded using a base-apex configuration.

Following instrumentation, halothane delivery was reduced to achieve an end-tidal concentration of 1.5 (± 0.05)%. Once end-tidal halothane was stable at 1.5%, baseline EEG was recorded for 10 min. A concentration of 1.5% halothane was chosen to ensure a depth of anaesthesia sufficient to prevent conscious perception or arousal in response to CO_2 exposure, without significant depression of cortical activity (Murrell *et al* 2008). At the conclusion of the baseline recording period, CO_2 from a compressed cylinder (Air Liquide NZ, Penrose, AKL, New Zealand) was introduced into the anaesthetic gas mixture at a concentration of 5% (by volume) and maintained for a period of five minutes. This was followed by CO_2 concentrations of 15, 30 and 50% by volume, respectively, for five minutes each. Sampling at each concentration commenced immediately following gas adjustment (ie no stabilisation period). Following exposure to 50% CO_2, anaesthesia was maintained in oxygen for a further 15 min, after which the rat was euthanased via an intraperitoneal (IP) overdose of sodium pentobarbitone (Pentobarb 500, Provet NZ Pty Ltd, Auckland, New

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Zealand). Halothane anaesthesia was maintained at 1.5 (± 0.05)% until death was confirmed by the appearance of isoelectric EEG and ECG.

End-tidal halothane, O_2 and CO_2 were continuously monitored using a side-stream gas analyser (Hewlett Packard M1025B gas monitor, Palo Alto, CA, USA). Rectal temperature was monitored throughout using a digital thermometer (Q1437, Dick Smith Electronics, New Zealand) and maintained between 37.0 and 38.5°C with the aid of a circulating warm water heating blanket (Gaymar T/P 500, Gaymar Industries Inc, New York, USA).

EEG recording

Both EEG and ECG signals were fed via break-out boxes to separate amplifiers (Iso-Dam isolated biological amplifier, World Precision Instruments Inc, Sarasota, FL, USA). The signals were amplified with a gain of 1,000 and a band-pass of 1.0–500 Hz and digitised at a rate of 1 kHz (Powerlab 4/20, ADInstruments Pty Ltd, NSW, Australia). The digitised signals were recorded and analysed off-line at the end of the experiment.

EEG analysis

Raw EEG data were inspected manually and any artefacts, such as over-scale, under-scale, nystagmus or other muscular activity, were excluded from further analysis. Additionally, raw traces were classified into one of three categories, based on the amplitude (height) of the observed waveform: active (representing normal baseline cerebrocortical activity), transitional (having an amplitude less than 50% of baseline), or isoelectric (a stable trace consisting of background electrical noise, with an amplitude < 1/8 that of baseline) (Gibson *et al* 2009). In addition, periods of EEG containing burst suppression, characterised by periods of isoelectric EEG interspersed with high-voltage active or transitional EEG (Steriade *et al* 1994), were identified and excluded from subsequent spectral analysis. The EEG spectral variables total power (P_{TOT}), median frequency (F50) and 95% spectral edge frequency (F95) were calculated for consecutive 1-s epochs using purpose-written software (Spectral Analyser, CB Johnson, Massey University, New Zealand); data for these three variables were subjected to statistical analysis.

Statistical analysis

All statistical analyses were undertaken using SAS version 9.1 (SAS Institute Inc, Cary, NC, USA). Statistical significance was assumed when $\alpha < 0.05$. The distribution of residuals of the spectral analysis data was tested for normality using the UNIVARIATE procedure. Residuals for all three variables were found to approximate a normal distribution and were analysed using repeated measures analysis of variance.

For each individual rat, mean F50, F95 and P_{TOT} were calculated for the final 5 min of the baseline recording period. For each variable, data for the entire recording period were then standardised and displayed as percentage change from that baseline mean. To facilitate statistical analyses, mean F50, F95 and P_{TOT} were calculated for consecutive non-overlapping 30-s intervals during the 5-min baseline period and during the 5 min at each CO_2 concentration; these means were compared using the MIXED procedure. The model used an autoregressive covariance structure and included CO_2 concentration ($[CO_2]$) as the fixed effect, rat as a random effect and time as the repeated measure. Where significant main or interaction effects were identified ($P < 0.05$) Bonferroni *post hoc* tests were conducted to identify specific differences.

Terminology

Pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP Task Force on Pain Taxonomy 1994). The term 'noxious' is used to describe stimuli that cause, or have the potential to cause, tissue damage (Cervero & Merskey 1996) and is therefore applied to stimuli that are capable of evoking pain in conscious animals. However, unpleasant sensory and/or emotional experience may also arise from stimuli that do not cause, or have the potential to cause, tissue damage and therefore pain. For example, experiences, such as breathlessness, anxiety, or nausea, may arise in the absence of tissue damage, therefore it would be misleading to describe these as noxious. To avoid such confusion, stimuli capable of inducing negative affective experience in the absence of actual or potential tissue damage will hereafter be referred to as 'nocuous'.

Results

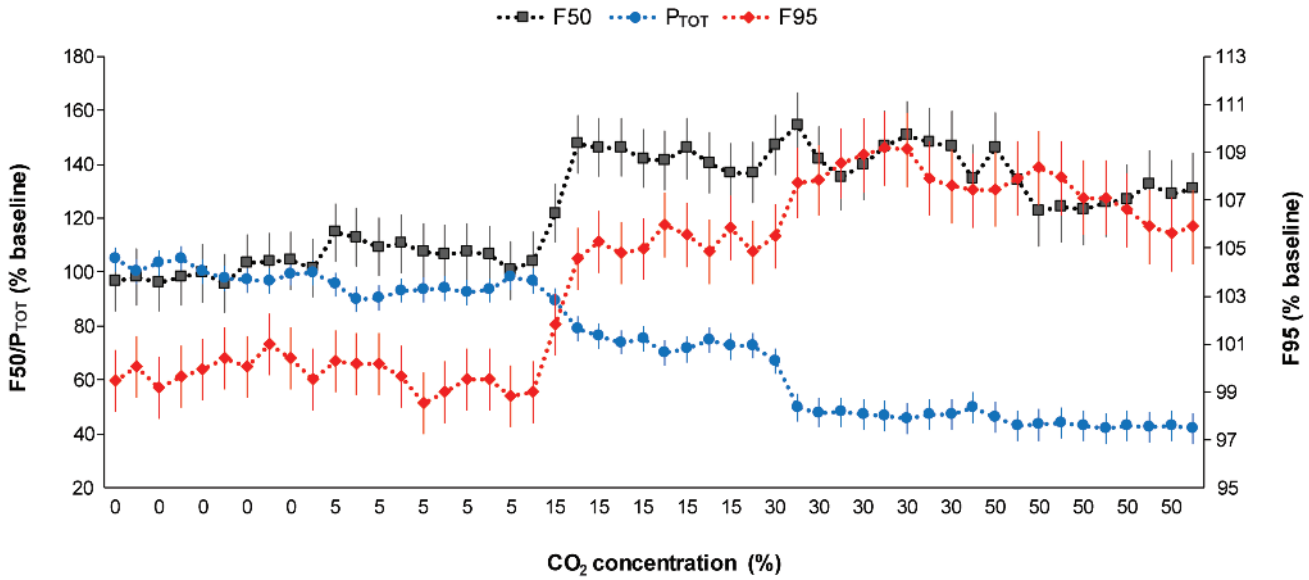
Of the 15 rats included in the study, data from only nine were suitable for analysis. One rat died during induction of anaesthesia and data from a further five rats were excluded due to the appearance of frequent burst suppression in the EEG prior to CO_2 exposure, making the data unsuitable for spectral analysis. Four of these five excluded rats subsequently died during CO_2 exposure, two at 30% CO_2 and two at 50% CO_2. Of the nine rats from which data were analysed, one exhibited signs of reduced adequacy of anaesthesia, despite end-tidal halothane remaining stable, and was euthanased with sodium pentobarbital during exposure to 30% CO_2, resulting in a partial data set. Burst suppression developed during CO_2 exposure in the EEG of 2/9 rats and persisted throughout. This was periodic and generally occurred in short bursts. Therefore, following exclusion of these periods, there was still sufficient data for spectral analysis.

The EEG typically exhibited a reduction in amplitude and increase in frequency during exposure to 15% CO_2. This continued during exposure to 30 and 50% CO_2, with EEG becoming transitional in all eight rats during exposure to 30% CO_2. Three rats developed isoelectric EEG traces, during exposure to either 30% (n = 2) or 50% (n = 1) CO_2. Upon withdrawal of CO_2, the EEG appeared to return to pre-stimulus amplitude and frequency (active EEG) within 10–15 min in 7/8 rats, with one remaining in a transitional state.

Table 1 The effects of CO_2 concentration ($[CO_2]$) and time, and their interaction, on the median frequency (F50), 95% spectral edge frequency (F95) and total power (P_{TOT}) of the rat electroencephalogram during light halothane anaesthesia.

	F50		F95		P_{TOT}	
	F-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
$[CO_2]$	4.43	0.007	10.72	< 0.0001	32.92	< 0.0001
Time	1.27	0.266	2.46	0.017	11.08	< 0.0001
$[CO_2]$ × time	1.13	0.292	1.24	0.0175	1.75	0.008

Figure 1



Change in mean median frequency (F50), 95% spectral edge frequency (F95) and total power (P_{TOT}) of the rat EEG (n = 9) during baseline (100% O_2) and sequential 5-min exposure to 5, 15, 30 and 50% CO_2. Data points represent the mean (± SEM) of consecutive non-overlapping 30-s periods.

Statistical analysis revealed a significant effect of $[CO_2]$ on the EEG (Table 1). An overview of the changes in spectral EEG variables over time during baseline and exposure to each CO_2 concentration are provided in Figure 1.

Mean F50 was significantly greater than baseline during exposure to 15 and 30% CO_2 ($P = 0.014$ and 0.018, respectively; Figure 2[a]). During exposure to 50% CO_2, F50 declined and did not differ from that during baseline or exposure to any other CO_2 concentration.

There were significant effects of both $[CO_2]$ and time on F95 (Table 1). Mean F95 not differ from baseline during exposure to 5% CO_2 ($P = 1.0$), but was significantly elevated during exposure to 15, 30 and 50% CO_2 ($P = 0.01$, 0.001 and 0.002, respectively; Figure 2[b]). Across all CO_2 concentrations, F95 was lower in the first 30-s period than the second ($P = 0.003$) or third ($P = 0.045$) 30-s periods.

Mean P_{TOT} (across all periods) decreased with increasing CO_2 concentration and was significantly lower than baseline during exposure to 15, 30 and 50% CO_2 (all $P < 0.0001$; Figure 2[c]). Additionally, mean P_{TOT} was lower during exposure to 15, 30 and 50% CO_2 than 5% CO_2, and lower during exposure to 30 and 50% CO_2 than 15% CO_2 ($P \leq 0.0001$; Figure 2[c]). There was, however, a concentration by time interaction effect, such that the differences in P_{TOT} between $[CO_2]$ were period-dependent. During exposure to 15% CO_2, P_{TOT} was lower than baseline during periods 2–10 only, lower than 5% CO_2 during periods 3–10 only, and greater than 30% during periods 1–8 only (all $P < 0.05$). In addition, there were differences in P_{TOT} between periods within $[CO_2]$. During exposure to 15% CO_2, P_{TOT} was higher in the first 30-s period than in subsequent periods, whilst during exposure to 30% CO_2, P_{TOT} was higher in the first period than periods 2–7 (all $P < 0.05$). During baseline and exposure to 5 and 50% CO_2, P_{TOT} did not differ between periods.

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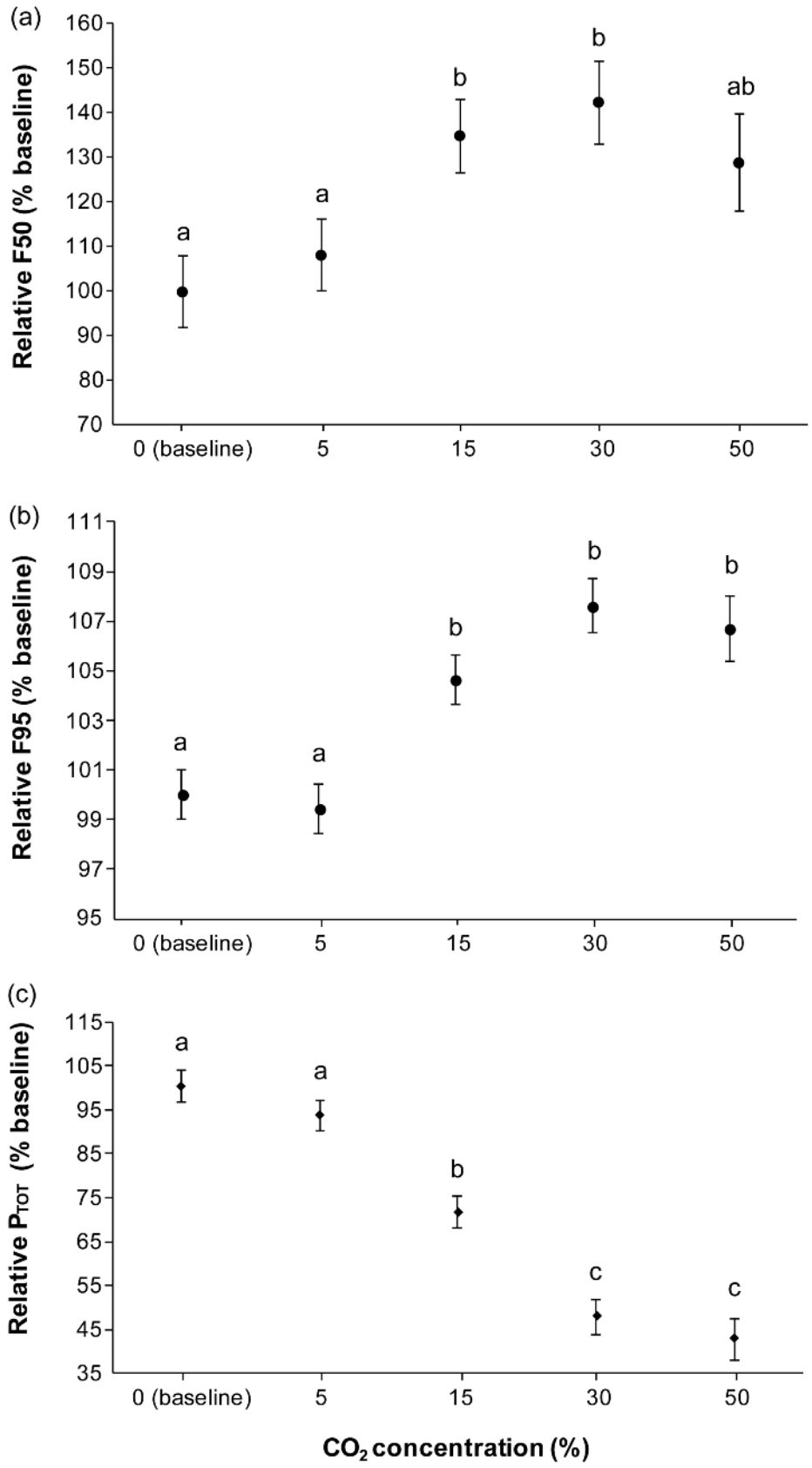
Figure 2



Comparison of the mean (± SEM) for (a) median frequency (F50), (b) 95% spectral edge frequency (F95) and (c) total power (P_{TOT}) of the EEG, relative to baseline, at each CO_2 concentration. Values with different superscript letters differed significantly ($P \leq 0.05$).

Discussion

The aim of this study was to assess the effects of exposure to increasing CO_2 concentration on the EEG of the rat, and to compare these with previously identified responses to painful stimuli in the same experimental model.

Whilst no significant changes were seen at 5% CO_2, exposure to 15% CO_2 caused EEG arousal, evidenced by significant increases in F50 and F95 and a significant decrease in P_{TOT}. This is not the first report of cortical arousal in response to exposure to moderate CO_2 concentration; in a study examining the influence of moderate hypercapnia on baseline neural and haemodynamic responses, Jones *et al* (2005) reported a marked shift toward low-amplitude, high-frequency activity in the raw EEG of urethane-anaesthetised rats exposed to 10% CO_2, with arousal persisting for the duration of exposure. In the same study, exposure to 5% CO_2 had no observable effect on the EEG.

The pattern of EEG arousal observed at 15% CO_2 in the present study is consistent with previously reported responses to nociceptive stimuli in a range of mammals (Murrell & Johnson 2006), including rats subjected to supramaximal mechanical, thermal or electrical noxious stimuli (Dominguez *et al* 2005; Orth *et al* 2005; Murrell *et al* 2007). This similarity in the character of EEG responses suggests that exposure to 15% CO_2 was noxious to rats and would have been perceived as unpleasant in conscious rats exposed to this concentration.

This is consistent with behavioural evidence of aversion in rats exposed to CO_2 concentrations in the 13.0–15.9% range (Niel *et al* 2008). However, 15% CO_2 is well below the established threshold for nociceptor activation of 37–50% CO_2 (Peppel & Anton 1993), effectively ruling this out as being a pain-related response. It is hypothesised, therefore, that the observed EEG changes were due to cortical processing of afferent impulses associated with CO_2-induced aversive respiratory sensations or direct CO_2-mediated anxiety.

Changes in the EEG frequency spectrum have not previously been used to investigate cortical responses to nocuous stimuli. However, similarities in the neurophysiology of pain and other unpleasant sensory-affective stimuli suggest that these might also induce quantifiable changes in the EEG. For example, both pain and breathlessness are recognised as being unpleasant sensations consisting of both sensory and affective (relating to subjective feelings or emotions) dimensions (von Leupoldt & Dahme 2005; Schön *et al* 2008; Lansing *et al* 2009; Beausoleil & Mellor 2015). Further, brain imaging studies have demonstrated that similar cortical structures and pathways are activated during the experience of both sensations (Banzett & Moosavi 2001; Gracely *et al* 2007; von Leupoldt *et al* 2009). In addition, several of the cortical structures activated during both pain and breathlessness also play an important role in the processing of emotions, such as fear and anxiety (von Leupoldt *et al* 2009). Whilst noxious stimuli typically induce EEG arousal in conscious and anaesthetised mammals (Murrell & Johnson 2006), reflected by an increase in F50 and corresponding decrease in P_{TOT}, the relative contributions of the sensory and affective aspects of pain processing to these EEG changes are not currently known. Should the cortical processing of affective components contribute significantly to the observed arousal, it is feasible that nocuous stimulation might also lead to EEG arousal.

The magnitude of changes in the EEG frequency spectrum correlate well with the magnitude of perceived pain in conscious people (Chen *et al* 1989), and the magnitude of behavioural responses to painful stimuli in conscious sheep (Ong *et al* 1997). Likewise, aversion studies in rats have demonstrated that higher concentrations of CO_2 are more aversive (Leach *et al* 2004; Niel & Weary 2007). Thus, if the anaesthesia model employed is sensitive enough to detect variations in the magnitude of nocuous sensory input associated with CO_2 exposure, the magnitude would be expected to increase with increasing CO_2 concentration. This was not the case. When the CO_2 concentration was increased from 5 to 15% there were increases in F50 and F95 and a decrease in P_{TOT}. A further increase to 30% CO_2 resulted in a greater reduction in P_{TOT} but no significant changes in F50 or F95, whilst there was no change in any variable when CO_2 concentration was increased to 50%. However, there are several reasons why direct comparison of responses at different CO_2 concentrations was not appropriate in this instance.

Firstly, the effects of cumulative CO_2 exposure need to be taken into account. Whilst sequential exposure to increasing CO_2 concentrations allowed us to mimic gradual-induction CO_2 killing, prolonged exposure to each concentration, without an inter-stimulus interval to allow wash-out, meant that EEG data recorded at each concentration were influenced by previous CO_2 exposure. This makes interpretation of responses to the different concentrations problematic.

Secondly, the anaesthetic properties of CO_2, itself, likely influenced EEG responses over time. The effects of increasing anaesthesia on the EEG depend on both the type and concentration of anaesthetic agent used; however, the typical response is a shift to slow-wave activity, with an increase in amplitude and decrease in frequency of the EEG (Thurmon *et al* 1996), corresponding to an increase in P_{TOT} and decrease in F50 and F95. This may be followed by the appearance of burst suppression, characterised by intermittent periods of electrical silence, before suppression in the amplitude of the EEG, leading to persistent electrical silence with deep anaesthesia (Martoft *et al* 2002; Gerritzen *et al* 2004; Murrell *et al* 2008). Although end-tidal halothane concentration was held constant in the present study, CO_2 itself has anaesthetic properties and has been shown to reduce the amount of halothane required to maintain a stable plane of anaesthesia in a dose-dependent manner (Brosnan *et al* 2007). The additive effect of CO_2 and halothane likely resulted in the rats in the study being effectively exposed to a deeper plane of anaesthesia as CO_2 concentration increased. As such, the EEG responses observed during exposure to higher CO_2 concentration should be interpreted with caution.

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Soon after the start of exposure to 30% CO_2, the EEG became transitional, as indicated by a rapid decrease in P_{TOT} which persisted during exposure to 50% CO_2. As P_{TOT} decreases, F50 and F95 increasingly represent background electrical noise as opposed to cortical activity (Gibson *et al* 2009). Because F50 and F95 provide no information on EEG power or amplitude, any major changes in P_{TOT} must therefore be taken into account when interpreting these variables (Gibson *et al* 2009). As such, values for F50 and F95 during 30 and 50% CO_2 cannot be considered to accurately represent cortical activity and should not be compared to values obtained during baseline or low CO_2 concentrations, where EEG was active.

The appearance of transitional EEG following exposure to 30% CO_2 provides further support for deepening anaesthesia at this point, as does the appearance of isoelectric EEG in three rats during exposure to 30 or 50% CO_2. This is not surprising, given that CO_2 alone induces loss of consciousness in rats at a concentration of approximately 30% (Smith & Harrap 1997; Niel & Weary 2006).

In contrast, EEG during exposure to 5 and 15% CO_2 remained active (ie amplitude > 50% of baseline) and therefore can be considered representative of cortical activity. As such, the EEG arousal observed in response to inhalation of 15% CO_2 may be attributed to cortical processing of sensations associated with CO_2 exposure during this period. Additionally, whilst very deep anaesthesia leads to EEG suppression and attendant decrease in P_{TOT}, moderate increases in anaesthetic depth have been shown to produce EEG synchronisation, characterised by an increase in amplitude and decrease in frequency with corresponding reductions in F50 and F95 (Thomsen & Prior 1996; Johnson & Taylor 1998). This is opposite to the arousal response observed at 15% CO_2, suggesting there was no significant increase in anaesthesia depth during this period.

It is possible that continual exposure to the CO_2 stimulus may have resulted in neuronal fatigue, or response saturation. It is well accepted that neuronal responses to noxious thermal stimuli decline over time with repeated stimulation (Adriaensen *et al* 1984). It is therefore possible that responses to CO_2 exposure may have been affected by prolonged stimulation.

The appearance of burst suppression has also been associated with increasing depth of anaesthesia (Yoshitani *et al* 2003). In the present study, intermittent burst suppression was evident during exposure to 5% CO_2 in two rats and persisted throughout. However, spectral EEG analysis indicated no increase in depth of anaesthesia at the time of appearance of burst suppression. In addition, burst suppression was apparent prior to CO_2 administration in several excluded data sets. Previous studies have reported no burst suppression during halothane anaesthesia, even at more than 1.5× the minimum alveolar concentration (Murrell *et al* 2008; Williams *et al* 2016). Together, this suggests that factors other than halothane or CO_2 concentration contributed to the appearance of early burst suppression in the present study.

Here, mean F95 and P_{TOT} during the first 30-s period differed to some or all of those in subsequent periods. This likely reflected a delay between adjustment of inhaled CO_2 concentration and subsequent cortical responses, such that values in the first period represented an interim point prior to equilibration at the new concentration.

Limitations/future research

To overcome the limitations associated with prolonged CO_2 exposure and distinguish between EEG responses at different concentrations, future studies could look at the effects of different CO_2 concentrations applied for a brief period only, with an inter-stimulus interval sufficient to allow a return to baseline EEG levels in-between. In addition, the order in which different concentrations are applied should be randomised among individuals. This would allow a direct comparison of the EEG effects of different CO_2 concentrations. In the present study, EEG changes generally peaked within the first minute of exposure to each CO_2 concentration. Therefore, delivery of each CO_2 concentration for 60 s should be sufficient to observe the attendant EEG responses.

In the present study, rats were endotracheally intubated, resulting in gas delivery directly to the lower respiratory tract, effectively bypassing nociceptors located in the nasal and upper respiratory mucosa. Although there are nociceptors located in the lower airway and alveolar walls (Mellema 2008), the sensitivity of these to CO_2 has not been reported. To more accurately assess the potential nociceptive effects of moderate to high CO_2 concentrations, it is recommended that CO_2 be delivered via a facemask, allowing exposure of the nasal mucosa and upper respiratory tract. In addition, delivery of CO_2 to non-ventilated rats would allow the normal ventilatory responses to hypercapnia and hypoxia to occur, likely increasing the rate of CO_2 uptake and more accurately reflecting the responses which occur during routine killing using this method.

Animal welfare implications and conclusion

Changes in the EEG frequency spectrum observed during sequential exposure to 5 and 15% CO_2 suggest that 15% CO_2 was nocuous to rats whereas 5% was not. Such evidence of a nocuous effect of CO_2 at a concentration well below that known to elicit nociceptor stimulation suggests that this arises through cortical processing of unpleasant affective sensations other than pain, such as breathlessness and/or anxiety. However, given the limitations in study design and the small sample size, further research is required to support this hypothesis.

In conclusion, the present study provides some evidence that an anaesthesia model may be utilised to study the nocuous effects of low to moderate CO_2 exposure in rats, providing an ethical model for the study of CO_2-induced negative affective experience and strategies to mitigate this.

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