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Electroencephalographic responses of anaesthetised pigs to intraperitoneal injection of sodium pentobarbital

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Abstract

Small laboratory animals are commonly euthanased via intraperitoneal (IP) injection of sodium pentobarbital. However, there is concern that animals may experience pain prior to loss of consciousness with this delivery route. The present study investigated electroencephalographic (EEG) nociceptive responses of anaesthetised pigs to IP sodium pentobarbital injection using an established minimal anaesthesia model. Thirty commercial white line entire male pigs aged 10–15 days were minimally anaesthetised with halothane in oxygen. Following 10 min of baseline EEG data collection, pigs had their tails docked using side-cutters and, after a further 5-min interval, were euthanased via IP injection of sodium pentobarbital (250 mg kg^{-1}). The summary variables median frequency (F50), 95% spectral edge frequency (F95) and total power (P_{TOT}) were derived from the EEG data. For each variable in each pig, means were calculated for the following 60-s periods: immediately prior to tail-docking (baseline 1); immediately prior to pentobarbital injection (at least 4 min after docking; baseline 2); and for two consecutive 60-s periods immediately following pentobarbital injection (P1 and P2). Statistical analyses revealed no differences between the two baseline periods, indicating that transient EEG changes induced by tail-docking had resolved prior to pentobarbital injection. IP pentobarbital injection induced a significant increase in F50 and decrease in P_{TOT} of the EEG during P1. This response is characteristic of acute nociception, indicating that conscious pigs likely perceive IP sodium pentobarbital as painful in the period prior to loss of consciousness.

Keywords: *animal welfare, EEG, euthanasia, nociception, pentobarbital, pig*

Introduction

Intraperitoneal (IP) injection of sodium pentobarbital is frequently employed for anaesthesia and euthanasia of laboratory rodents (Svendsen *et al* 2007). Whilst intravenous delivery is preferred as it achieves more rapid distribution of the agent and has lower potential to cause tissue irritation (Wolfensohn & Lloyd 2003; Svendsen *et al* 2007), IP delivery is most common in small animals, such as rodents, due to its relative ease of administration. However, there is concern that pentobarbital preparations may cause pain or irritation to the parietal and visceral peritoneum and associated tissues when delivered IP, particularly at concentrations required for euthanasia (Ambrose 1998; Wolfensohn & Lloyd 2003). Such irritation is thought to arise due to the high pH of euthanasia solutions, which have been reported as ranging from pH 10–12.5 (Wadham 1997; Ambrose 1998).

Previous studies have investigated pain and/or distress associated with IP pentobarbital injection in rodents through measurements of behaviour, plasma stress hormone concentration and expression of c-fos (a marker of neuronal activity) in spinal nociceptive neurons following peripheral stimulation (Svendsen *et al* 2007). Ambrose (1998) reported writhing in rats (*Rattus norvegicus*) in response to IP sodium pentobarbital injection. Abdominal writhing is a recognised sign of pain in rats that can be experimentally induced through injecting a known irritant, such as acetic acid, into the peritoneal cavity (Siegmund *et al* 1957). Further, plasma cortisol was elevated in rats that were decapitated following IP sodium pentobarbital anaesthesia, relative to control rats decapitated without prior anaesthesia (Vahl *et al* 2005; Wu *et al* 2015).

Elevations in plasma stress hormone concentrations are frequently used as indices of distress induced by painful or noxious procedures. However, such measures are not specific to pain, instead providing a measure of the overall noxiousness of an experience, including both physical and emotional components (Mellor *et al* 2000). Elevations in plasma stress hormone concentration have been shown to occur in rats administered IP physiological saline solution prior to decapitation, relative to those that did not receive IP saline (Baek *et al* 2015; Wu *et al* 2015), suggesting that the process of IP injection itself is stressful, independent of the compound being delivered.

Whilst changes in behaviour and stress hormone concentration can occur in response to non-painful stressors, the expression of biomarkers, such as c-fos, produced by spinal dorsal horn neurones, may provide more specific and quantitative measures of nociception. Thermal, mechanical, chemical and electrical noxious stimuli elicit c-fos expression in the spinal cord (Hunt *et al* 1987; Willcockson *et al* 1995; Yi & Barr 1995), with expression shown to correlate with stimulus intensity (Yi & Barr 1995; Jinks *et al* 2002). IP pentobarbital injection reportedly caused an increase in spinal nociceptive neurone c-fos expression in rats, which was attenuated by concurrent lidocaine administration (Svendsen *et al* 2007). Together, these data provide evidence for pain and distress in rodents following IP pentobarbital injection.

The use of electroencephalographic (EEG) parameters is gaining popularity for the quantitative assessment of nociception in mammals. Using an established minimal anaesthesia model (MAM) (Murrell & Johnson 2006), changes in the EEG frequency spectrum associated with nociception may be studied in anaesthetised animals. The MAM involves maintaining the animal on a stable plane of light halothane anaesthesia, such that the cerebrocortex remains responsive to noxious stimuli whilst conscious perception is prevented. This provides the advantages of reducing variability in background cerebrocortical activity due to extraneous stimuli, such as novelty or handling, whilst allowing painful stimuli to be investigated without compromise to the animal's welfare.

The EEG provides a summation of electrical activity arising from neurons in the cerebral cortex. In man, changes in the frequency spectrum of the EEG have been found to mirror changes in cortical activity relating to the cognitive perception of pain (Bromm 1984). Using the MAM, nociception in response to the application of a known noxious stimulus has been demonstrated in a range of mammals, including horses (*Equus caballus*) (Murrell *et al* 2003), sheep (*Ovis aries*) (Johnson *et al* 2005a), cattle (*Bos taurus*) (Gibson *et al* 2007), deer (*Cervus elaphus*) (Johnson *et al* 2005b), pigs (*Sus scrofa*) (Haga & Ranheim 2005) and rats (Murrell *et al* 2007). In sheep, the magnitude of changes in the EEG frequency spectrum correlated well with behavioural responses to noxious stimuli in conscious animals (Ong *et al* 1997). Likewise, in man, the magnitude of changes correlated well with reports of pain intensity in response to graded noxious stimuli (Chen *et al* 1989), highlighting the quantitative value of EEG as an index of nociception. Furthermore, prior administration of effective analgesia has been shown to obtund spectral EEG responses to noxious stimuli (Haga & Ranheim 2005; Johnson *et al* 2005b; Murrell *et al* 2005; Gibson *et al* 2007; Kongara *et al* 2010, 2014), further demonstrating the specificity of this model.

In commercial situations, euthanasia of neonatal pigs is usually performed via manually applied blunt force trauma. Other methods considered acceptable for euthanasia of neonatal pigs include exposure to carbon dioxide, argon/carbon dioxide/nitrogen mixtures or carbon monoxide, non-penetrating captive bolt, inhalant anaesthetic overdose or intravenous (IV) anaesthetic overdose (Leary *et al* 2013). Although it is generally recommended that euthanasia via injected overdose of barbiturates should be administered IV, IP administration is deemed acceptable in situations where IV access may be distressful, dangerous or impractical, such as due to small animal size (Leary *et al* 2013). In the research setting, specific institutional guidelines govern euthanasia practise. For example, University of Minnesota guidelines specify that whilst adult or nursery pigs must be euthanased via IV overdose of pentobarbital, it is acceptable for neonatal swine to be euthanased via the IP route (http://www.ahc.umn.edu/rar/euthanasia.html). Thus, whilst mature research pigs would typically be euthanased via IV-administered pentobarbital, neonatal pigs may be euthanased via the IP route. No previous studies investigating the noxiousness of IP pentobarbital euthanasia in pigs were identified.

The aim of the present study was to evaluate the nociceptive responses of piglets to IP pentobarbital injection, by assessing changes in the median frequency (F50), 95% spectral edge frequency (F95) and total power (P_{TOT}) of the EEG. We hypothesised that if IP pentobarbital injection were noxious to pigs, there would be a transient increase in F50 and decrease in P_{TOT} of the EEG in response to injection.

Study animals

This study used 30 commercial white line (Large white × Landrace) entire male pigs, aged 10, 12 or 15 days (n = 10 per age). Bodyweight ranged from 3.17–6.96 kg, with a mean (± SEM) of 3.6 (± 0.07) kg in 10-day old pigs, 4.1 (± 0.09) kg in 12-day old pigs and 5.6 (± 0.09) kg in 15-day old pigs. Males only were tested, to reduce potential variability in the data set. The allocation of ten pigs per age group was based on previous nociceptive studies utilising the same model, where statistically significant effects of a noxious stimulus were identified using nine or ten animals per group (Johnson *et al* 2005b; Murrell *et al* 2005; Gibson *et al* 2007, 2009). All animals were purchased from one, single commercial premises and formed part of a larger cohort of 60 pigs used to study the post-natal development of nociceptive responses to tail-docking between birth and 15 days of age. As the pigs involved in the wider study were not yet weaned and could not be returned to the farm of origin due to biosecurity restrictions, the experimental protocol dictated that they be euthanased via IP pentobarbital injection following data collection, whilst still under anaesthesia. This presented the opportunity to investigate piglet nociceptive responses to IP pentobarbital injection, thus maximising the value of the animals by using them to test two distinct hypotheses. Based on evidence from previous studies indicating that mammalian cortical responses to nociceptive stimuli may be less developed in the very early post-natal period (eg Johnson *et al* 2009; Devonshire *et al* 2015; Kells *et al* 2017a), only data from piglets aged ten days or more were analysed in the present study.

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Materials and methods

This study was conducted with approval from the Massey University Animal Ethics Committee, New Zealand (MUAEC approval number 14/26). All procedures were undertaken in accordance with the MUAEC code of ethical conduct for the use of live animals for research, testing and teaching.

Pigs were purchased from a commercial pig farm on the day of testing and housed in a temperature-controlled (30°C), ventilated indoor facility on deep straw litter with *ad libitum* access to water until the time of testing. All experiments were conducted within 6 h (min 1.0, max 5.3 h) from the time of collection, in order to minimise distress associated with separation from the sow. The pigs had not previously undergone any painful husbandry procedures (eg castration, tooth-trimming, ear-tagging, iron injection) and had intact tails on arrival. Pigs within each age group were sourced from three separate litters, with each litter being tested at a single age.

Experiments were conducted over ten separate test days, with 2–4 pigs tested per day.

Anaesthesia

Pigs were anaesthetised with halothane (Halothane-Vet, Merial NZ Limited, Manukau City, New Zealand) vaporised in oxygen (4 L min^{-1}) delivered via facemask. Halothane delivery was maintained at 3–4% during induction and instrumentation and between 0.95 and 1.05% end-tidal concentration during the data acquisition period. End-tidal gas measurements were obtained from a side-stream sample port located at the junction of the mask and the scavenger tube, near the pig's nostrils. End-tidal halothane and CO_2 tension, SpO_2, respiration rate and heart rate were monitored throughout using an anaesthetic agent monitor (Hewlett Packard M1025B, Hewlett Packard, Hamburg, Germany). In addition, the presence of palpebral and pedal reflexes was tested periodically to ensure adequate depth of anaesthesia was maintained throughout. Rectal temperature was monitored using a digital thermometer (Q 1437, Dick Smith Electronics, New Zealand) and maintained at 38–40°C with the aid of a circulating warm-water heating blanket (T pump, Gaymar Industries Inc, NY, USA).

Electrophysiology

Subcutaneous 27-gauge stainless steel needle electrodes (Viasys Healthcare, Surrey, UK) were positioned to record EEG from the left and right cerebral cortices, with inverting electrodes placed parallel to the midline 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.

EEG signals were fed via breakout 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 Ltd, Colorado Springs, CO, USA). The digitised signals were recorded on an Apple Macintosh personal computer for off-line analysis at the conclusion of the experiment.

Experimental procedure

Following induction of anaesthesia, pigs were positioned in lateral recumbency on their left side and recording electrodes placed as described previously. Once end-tidal halothane tension was stable at 1.0 (± 0.05)%, 10 min of baseline EEG data were recorded. Pigs were then tail-docked using a pair of clean, disinfected side-cutter pliers. After a minimum interval of 5 min (range 5 min 0 s–9 min 59 s), to allow for resolution of acute EEG responses to tail-docking, pigs were euthanased via IP injection of sodium pentobarbital (Pentobarb 500, Provet NZ Pty Ltd, Auckland, New Zealand) at a dose rate of 250 mg kg^{-1} (0.5 ml kg^{-1}). The dose was delivered to the lower right abdominal quadrant, approximately 2 cm from the midline, using an 18-gauge needle. The needle was inserted fully and aspirated prior to delivery to ensure it had not punctured the intestines or abdominal blood vessels. The injection rate was not recorded; however, the same researcher performed all injections to ensure consistency in the delivery rate. Anaesthesia was maintained and EEG recording continued until death (indicated by isoelectric EEG in conjunction with respiratory and cardiac arrest). Isoelectric EEG was defined as a stable trace consisting of background noise only, with an amplitude < 1/8 of baseline (pre-stimulus) EEG (Gibson *et al* 2009).

Data analysis

EEG data from the right cerebral cortex only were analysed. Although EEG was recorded bilaterally, previous studies using the MAM have demonstrated equivalency in spectral EEG between hemispheres (Murrell *et al* 2007, 2010), suggesting data from either hemisphere alone are suitable for analysis. Data from the left cortex were collected for use in the event that right cortex data were unsuitable for analysis, for example, due to electrode displacement or the presence of extensive artefact confined to a single channel. Raw EEG recordings were inspected manually and any artefacts, such as over-scale, under-scale, nystagmus or other muscle activity, were excluded from subsequent analysis. The 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, Palmerston North, New Zealand). Fast Fourier transformation was applied to each epoch, generating sequential power spectra with 1 Hz frequency bins. To account for individual variation in baseline EEG, data from each individual were standardised to a percentage of pre-docking baseline, calculated over the 5-min period preceding tail-docking. For the purposes of statistical analyses, mean F50, F95 and P_{TOT} were calculated for four non-overlapping 60-s periods for each individual pig: immediately preceding tail-docking (Baseline 1; B1); immediately preceding pentobarbital injection (Baseline 2; B2); from 1 to 60 s after pentobarbital injection (P1); and from 61 to 120 s after pentobarbital injection (P2). A single mean value for each EEG variable was calculated for each time-period in each pig, generating a total of four data-points per pig per variable.

Table 1 Effect of piglet age (10, 12 or 15 days) and recording period (pre-tail-dock baseline, pre-pentobarbital injection and post-pentobarbital injection), and their interaction on the summary variables F50, F95 and P_{TOT} of the pig EEG.

	F50		F95		P_{TOT}	
	F-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Age	0.52	0.598	0.07	0.934	0.66	0.524
Period	48.70	< 0.001	24.33	< 0.001	48.70	< 0.001
Age × period	1.40	0.225	2.82	0.016	1.40	0.225

F50 = median frequency; F95 = 95% spectral edge frequency; P_{TOT} = total power.

In addition, the interval (s) from delivery of pentobarbital to the onset of isoelectric EEG was determined in each individual, following visual inspection of the raw EEG traces.

Statistical analysis

All statistical analyses were performed using SAS version 9.3.1 (SAS Institute Inc, Cary, NC, USA). Between-period comparisons of EEG data were performed using proc MIXED. The model incorporated age as a fixed effect, pig as a random effect and period as a repeated measure. Statistical significance was set at $\alpha = 0.05$. Where a significant period or interaction effect was identified, *post hoc* pair-wise comparisons of least square means were carried out with Bonferroni correction for multiple comparisons. Corrected values for alpha were 0.0083 (period) and 0.0017 (age × period interaction).

Analysis of variance was undertaken to compare the time to onset of isoelectric EEG between age groups. Statistical significance was set at $\alpha = 0.05$, with Tukey's HSD test performed to identify group differences. To determine whether time to onset of isoelectric EEG influenced spectral EEG variables, this variable was included as a covariate in the mixed model. No significant effect was identified; therefore, this was omitted from the final model.

Results presented as mean (± SEM) unless otherwise specified.

Results

End-tidal CO_2 varied between 26 and 58 mm Hg across all pigs, with ranges of 30–50, 32–58 and 26–58 mm Hg recorded in 10-, 12- and 15-day old pigs, respectively. It should be noted that O_2 flow was maintained at 4 L min^{-1} throughout, which may have resulted in some dilution of expired CO_2.

EEG data were successfully collected from 29/30 pigs, with data from one 15-day old pig excluded due to equipment failure during data collection. Body temperatures recorded during anaesthesia ranged from 37.0 to 39.4°C. $PECO_2$ (mixed expired carbon dioxide tension) did not exceed 57.5 mm Hg in any animal at any time during anaesthesia.

Age did not significantly influence any EEG summary variable. There was a significant effect of period on F50 and F95, and a significant age × period interaction on P_{TOT} (Table 1).

Mean F50 was significantly greater in P1 compared with B1 ($P < 0.0001$) or B2 ($P < 0.0001$; Figure 1[a]). In P2, mean F50 did not differ from P1 ($P = 0.58$) and remained higher than B1 ($P = 0.009$) or B2 ($P = 0.002$; Figure 1[a]).

Mean F95 differed between periods in an age-dependent manner (Figure 1). Whilst F95 did not differ between age groups within periods (adjusted $P \geq 0.76$ for all comparisons), within-age differences between periods were observed in 10- and 12-day old pigs. In 10-day old pigs, F95 was higher in P1 than B2 ($P = 0.03$) or P2 ($P < 0.0001$; Figure 1[b]) but did not differ to B1 ($P = 0.17$). Similarly, in 12-day olds, F95 in P1 was higher than that in P2 ($P < 0.0001$) and tended to be higher than that in B2 ($P = 0.06$) but did not differ from B1 ($P = 0.68$; Figure 1[b]).

Mean P_{TOT} was lower in P1 than B1 ($P < 0.001$) or B2 ($P < 0.001$; Figure 1[c]). In P2, mean P_{TOT} was significantly greater than P1, B1, or B2 (all $P < 0.001$; Figure 1[c]).

A summary of the changes in spectral EEG variables over time (data from all pigs combined) is provided in Figure 2. The increase in F50 and decrease in P_{TOT} following pentobarbital injection (vertical dashed line) can be clearly seen, along with the subsequent resolution of these responses.

The mean interval between administration of pentobarbital and the appearance of an isoelectric EEG trace for all pigs was 360 (± 32.4) (min 144, max 640) s. Age significantly influenced the time to onset of isoelectric EEG ($F = 8.15$; $P = 0.002$). The mean interval to onset of isoelectric EEG was greater in 10-day old pigs (490.9 [± 54.0] s) than 12-day old pigs (234.5 [± 34.1] s). However, the time to onset of isoelectric EEG in 15-day old pigs (354.9 [± 46.8] s) did not differ to that of either 10- or 12-day old pigs.

Discussion

The aim of this study was to use the MAM to determine whether administration of IP pentobarbital euthanasia induces a nociceptive response in pigs prior to loss of awareness. In mammals, cortical processing of nociceptive signals is characterised by a transient increase in F50 and concurrent decrease in P_{TOT} of the EEG (Murrell & Johnson 2006). In the present study, across all ages combined, such a characteristic nociceptive response was observed in the period immediately following IP pentobarbital injection (Figure 2).

The data analysed in this study were collected from pigs that had been subjected to the noxious stimulus of tail-docking shortly prior to being euthanased via IP pentobarbital injection. Previous studies conducted by our group have demonstrated that tail-docking of halothane-anaesthetised 20-day old pigs elicits an increase in F50 and decrease in P_{TOT} of the EEG, characteristic of nociception

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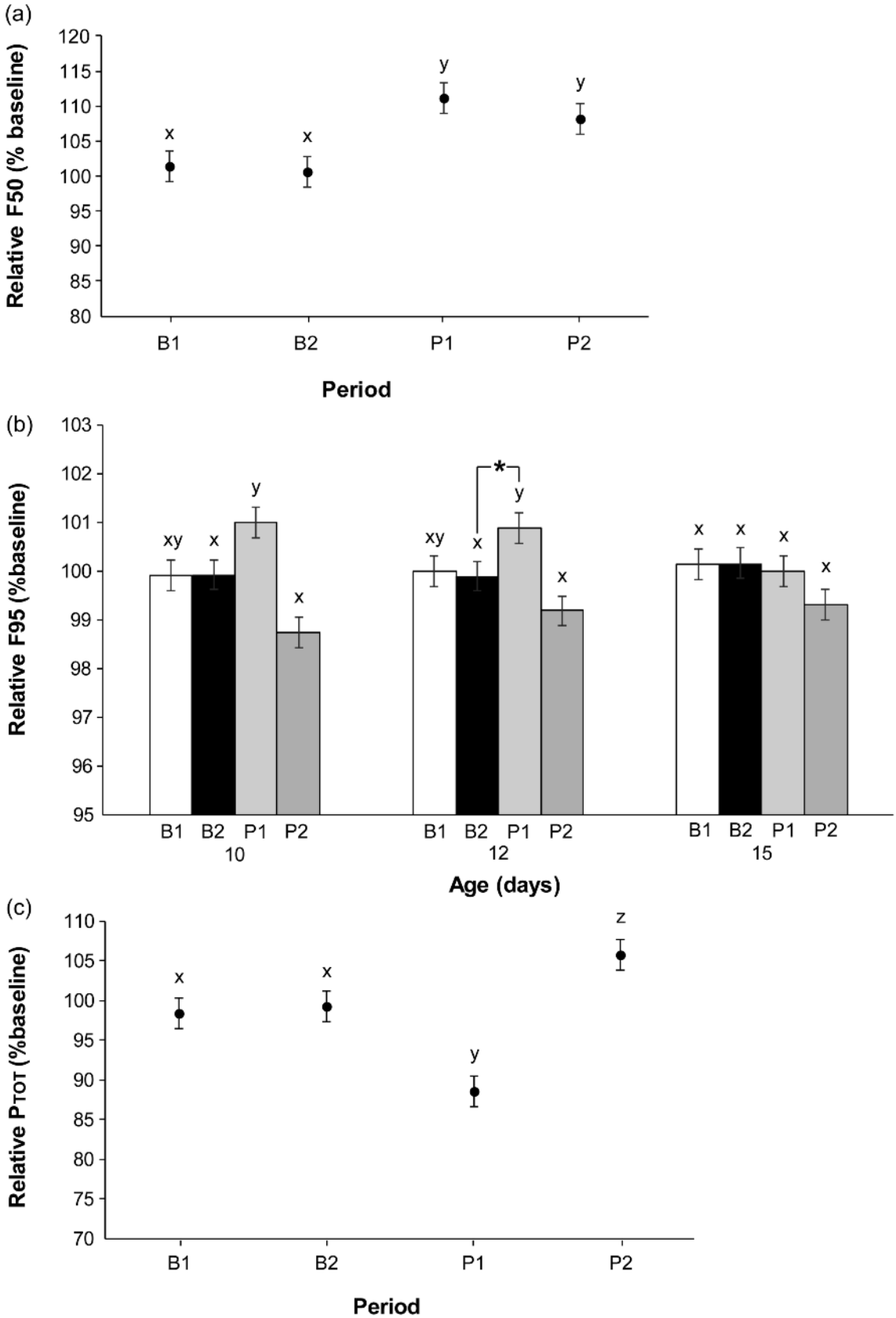
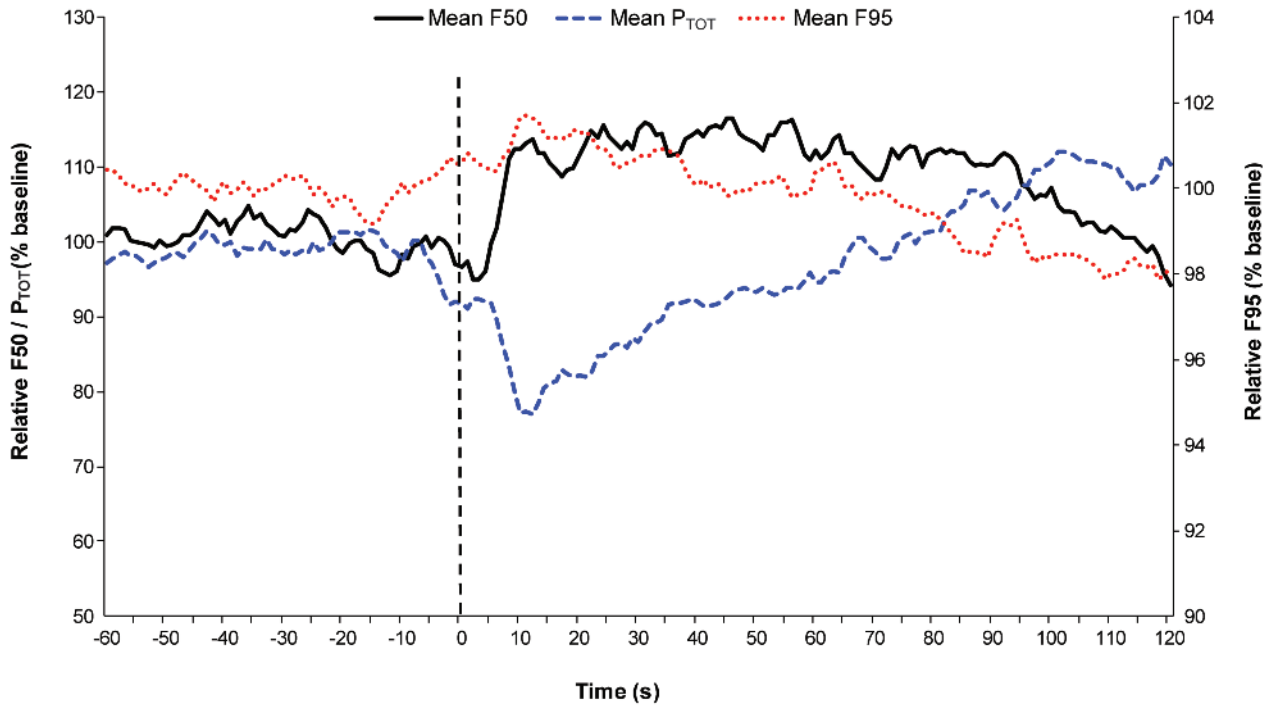
Figure 1



Comparison of mean (± SEM) for (a) F50, (b) F95 and (c) P_{TOT} of the pig EEG (n = 29) over the 60-s period prior to tail-docking (B1), the 60-s period prior to pentobarbital injection (B2), the 60-s period immediately after pentobarbital injection (P1) and the 60-s period from 61–120 s after pentobarbital injection (P2). ^{xyz} Means with different superscripts were significantly different ($P < 0.05$). * Indicates tendency toward a significant difference ($P = 0.063$).

Figure 2



The changes in mean F50 F95 and P_{TOT} of the pig EEG (all ages combined), relative to pre-treatment baseline mean, following intraperitoneal injection of sodium pentobarbital (250 mg kg^{-1}; vertical dashed line).

(Kells *et al* 2017a). In addition, such EEG changes are abolished by prior administration of topical anaesthetic to the tail (Kells *et al* 2017b), further validating the MAM as a nociceptive assessment tool in pigs. In the present study, the magnitude of changes observed in EEG summary variables following IP pentobarbital injection were comparable to those previously observed in 20-day old pigs following tail-docking using clippers or cautery iron (Kells *et al* 2017a).

Changes in the EEG frequency spectrum following noxious stimulation are typically short-lived. Previous mammalian nociceptive studies have identified EEG changes when comparing pre- and post-stimulus observation periods ranging from 10 s to 5 min. For example, Kongara *et al* (2010) compared 10-s periods in dogs (*Canis familiaris*) subjected to noxious electrical stimulation, Murrell *et al* (2007) compared 30-s periods in rats subjected to noxious mechanical, thermal or electrical stimulation, while Johnson *et al* compared 2-min periods in deer undergoing antler removal (Johnson *et al* 2005b) and 5-min periods in lambs undergoing castration (Johnson *et al* 2005a). In the present study, a 60-s period was selected for analysis as it was believed this would adequately capture transient EEG changes related to nociception, whilst preceding central suppression related to the circulatory uptake of pentobarbital. To avoid potential confounds, baseline data collected over the 60 s prior to pentobarbital injection were compared with baseline data collected over the 60 s preceding tail-docking, to ensure that EEG responses to tail-docking had resolved prior to the administration of pentobarbital. The two baseline periods were found to be equivalent for all variables, indicating complete resolution of nociceptive responses to docking prior to pentobarbital injection. However, it is possible that tail-docking may have sensitised pigs to subsequent noxious stimulation, thereby affecting the magnitude of responses to IP pentobarbital injection.

Although there were some differences in maximum $PECO_2$ recorded among pigs, this was unlikely to have had a significant effect on cerebral bloodflow over the range recorded (Olsen *et al* 2006), and therefore was unlikely to have influenced electroencephalographic values.

Pentobarbital is a short-acting oxybarbiturate, previously used for veterinary clinical anaesthesia, but nowadays primarily used as a euthanasia solution (Riviere & Papich 2009). It induces progressive central nervous system (CNS) depression, beginning with the cerebral cortex. Deep anaesthesia progresses to apnoea as the brainstem respiratory centres are depressed, with eventual death due to respiratory and/or cardiac arrest at doses sufficient for euthanasia (Leary *et al* 2013). Cortical depression is reflected by a progressive reduction in total EEG power until complete cessation of cortical electrical activity occurs, indicated by an isoelectric EEG trace.

Whilst there appears to be no information regarding the effects of pentobarbital alone on the mammalian EEG, dose-dependent changes in EEG activity with increasing depth of anaesthesia have been demonstrated for a range of other anaesthetic agents. In general, increasing anaesthesia is associated with an increase in amplitude and decrease in

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frequency of the EEG (Johnson & Taylor 1998; Otto 2007), which is reflected by an increase in P_{TOT} and reduction in F50 and/or F95 (Hudson *et al* 1983; Otto & Short 1991; Schwender *et al* 1998; Bergamasco *et al* 2003; Martin-Cancho *et al* 2003). For example, increasing depth of halothane anaesthesia is associated with reductions in F50 and F95 in humans, horses and rats (Thomsen & Prior 1996; Johnson & Taylor 1997; Antunes *et al* 2003).

In the present study, the prolonged time to onset of isoelectric EEG (mean 6 min) suggests that pentobarbital entry into general circulation, and subsequent central depression, was delayed. A transient increase in F50 and F95 and decrease in P_{TOT} of the EEG was observed in the initial 60-s period after pentobarbital injection, the opposite of that typically observed with increasing depth of anaesthesia. These data support this being a nociceptive response to injection rather than CNS depression caused by the pharmacological effects of pentobarbital.

Whilst pooling of data from all pigs revealed a significant increase in F95 immediately following pentobarbital injection, the response was found to be age-dependent. In 10- and 12-day old pigs, F95 was higher immediately following injection (P1) than immediately prior to injection (B2), whereas F95 did not differ between periods in 15-day old pigs. Given that other studies using the MAM have demonstrated patterns of increasing cortical responsiveness to noxious stimuli with increasing post-natal age (Johnson *et al* 2005a; Kells *et al* 2017a), the lack of a period effect in 15-day old pigs was unexpected. F95 did not differ to either baseline in the period 60–120 s after injection (P2), indicating resolution of responses associated with IP injection. Given that F95 is reported to respond preferentially to changes in anaesthetic agent dose (Johnson *et al* 1994; Johnson & Taylor 1998; Antunes *et al* 2003), reflecting general CNS suppression, the absence of a significant alteration in F95 during P2 suggests there was no measureable effect of pentobarbital itself on the EEG during this period.

However, F50 remained elevated relative to baseline in P2 indicating that nociceptive responses to injection were still present. This is consistent with previous studies showing that changes in F50 of the pig EEG in response to tail-docking are detectable for up to 90 s after docking (Kells *et al* 2017a). In contrast, P_{TOT} was found to increase in P2, relative to P1 or either baseline period. Whilst the initial decrease in P_{TOT} is consistent with nociception, the subsequent increase is not. A transient rebound increase in P_{TOT} following resolution of nociceptive responses may occur. However, a similar increase may occur because of cortical depression. The lack of an accompanying decrease in F95 or F50 in this period suggests this was not due to pentobarbital-mediated increase in depth of anaesthesia.

There was considerable variation among pigs in the time to onset of isoelectric EEG. Such variability suggests that the site of drug placement may have been inconsistent among animals. Whilst an effort was made to standardise the placement site based on anatomical landmarks, differences in the size and weight of pigs used in the study may have led to variation in the delivery site. There is substantial evidence that the accuracy of placement of IP injections is often poor, with reported misplacement rates ranging from 10–24% in rats and mice (*Mus musculus*) (Steward *et al* 1968; Miner *et al* 1969; Arioli & Rossi 1970; Claasen 1994) to greater than 50% in cats (*Felis catus*) (Grier & Schaffer 1990). Dose misplacement may affect subsequent absorption and metabolism, thus speed and efficacy, as well as the extent of associated irritation or pain (Svendsen *et al* 2007). In the current study, variations in dose placement may have affected subsequent drug absorption and metabolism, thus contributing to the variation in time to onset of isoelectric EEG. However, this could not be confirmed, as neither post mortem examination nor pharmacokinetic analyses were undertaken as part of this study. In addition, variations in dose placement may have influenced nociceptive responses among individuals should some sites/tissues have a higher density of nociceptors. The absence of a statistical relationship between time to isoelectric EEG and spectral EEG variables suggests this was not the case; however, this could not be confirmed without necropsy examination.

Although pig age was found to affect the time to onset of isoelectric EEG, there was no linear pattern of increasing or decreasing duration with increasing age. The finding that isoelectric EEG occurred sooner in 12- than in 10-day old pigs, but did not differ between 12- and 15-, or 10- and 15-day old, suggests this may have been a random effect (eg due to variation in dose placement) rather than a true age effect. The pentobarbital dose given was calculated on pig weight, thus accounting for differences in bodyweight that may have influenced drug absorption. Again, the lack of post mortem examination limits data interpretation.

The interval between administration of pentobarbital and the appearance of an isoelectric EEG trace is indicative of the period of potential awareness following drug administration in non-anaesthetised animals. Given that isoelectric EEG is considered incompatible with consciousness (Newhook & Blackmore 1982), awareness of sensory inputs, including noxious inputs associated with IP pentobarbital, cannot occur following the onset of irreversible cortical isoelectricity. In the present study, the mean interval between pentobarbital administration and the onset of isoelectric EEG was 6 min, suggesting conscious pigs might perceive noxious stimuli for up to several minutes following IP pentobarbital overdose. Previous studies in awake rodents have reported shorter times to loss of awareness; rats administered 150 mg kg^{-1} IP lost consciousness after 3.5 min, whilst mice administered 330 mg kg^{-1} IP pentobarbital lost consciousness after 2.2–2.6 min (Ambrose 1998). In those studies, time to loss of posture was used as an indicator of loss of consciousness. However, this measure is considered to represent the earliest onset of loss of consciousness and, as such, may not represent unequivocal loss of consciousness (Raj 1999). Whilst it is possible that loss of consciousness may occur sooner than the 6 min determined by the onset of isoelectric EEG in the present study, loss of consciousness following IP pentobarbital overdose is far from immediate and there is potential for pigs to experience pain in the interim period.

The pH of the pentobarbital solution used in the present study was 10.6. This is consistent with the pH range of 10–11.5 reported by Ambrose (1998), but lower than the 12.5 reported by Wadham (1997). It is likely that the high solution pH is largely responsible for the observed nociceptive response (Wadham 1997; Ambrose 1998), although stress hormone responses to IP injection of non-irritant substances indicate that IP injection itself may contribute to this (Baek *et al* 2015; Wu *et al* 2015).

The finding that IP injection of sodium pentobarbital at doses commonly used for euthanasia induces nociception in anaesthetised pigs is consistent with previous studies in rats that demonstrated IP pentobarbital administered at concentrations sufficient for either anaesthesia or euthanasia was noxious. Svendson and co-workers showed that 40 mg kg^{-1} pentobarbital (from a 100 mg ml^{-1} stock solution) used for short-term anaesthesia induced a significant rise in the activity of spinal nociceptive neurones, compared with the same volume of physiological saline (Svendsen *et al* 2007). Similarly, Ambrose demonstrated increased activity and writhing following administration of 150 mg kg^{-1} sodium pentobarbital (from a 100 mg ml^{-1} solution) used for euthanasia (Ambrose 1998). In both studies, adverse responses were attenuated, but not abolished, by the addition of 10 mg ml^{-1} lidocaine, a fast-acting local anaesthetic, to the pentobarbital formulation.

In the present study, a dose of 250 mg kg^{-1}, from a 500 mg ml^{-1} stock solution, was administered to pigs. A stronger stock solution was used to reduce the total volume required to achieve a lethal dose. More concentrated solutions are likely more irritant due to their higher pH; however, the reduced dose volume may be more practical to administer, particularly in larger, conscious animals. In the interest of improving animal welfare, the benefits of using a more dilute solution, or the addition of local anaesthetic, should be investigated in future studies.

It should be noted that males only were used in the present study. Although there appears to be no consistent gender bias in pain sensitivity among humans (Racine *et al* 2012), extrapolation of these results to female pigs should be undertaken with caution.

Animal welfare implications

Euthanasia of neonatal pigs via IP-administered sodium pentobarbital is likely to induce pain prior to loss of awareness. Given that IV administration in young, awake animals may be difficult due to their small size, future investigation into ways of modifying IV administration to enhance pig welfare is therefore warranted.

Conclusion

The transient increase in F50 and decrease in P_{TOT} observed following IP pentobarbital injection in pigs is consistent with an acute nociceptive response. It is therefore likely that unanaesthetised pigs would perceive IP-administered pentobarbital injection as painful in the period prior to loss of awareness.

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