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Ictal Bradycardia and Tachycardia Observed in the Tetanus Toxin Model of Temporal Lobe Epilepsy in the Freely Moving Rat

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Abstract

Those with epilepsy have a higher risk of sudden death than the general population; however, the mechanism of these sudden deaths is unknown. Fatal arrhythmia generation during seizure, along with changes in autonomic tone, are hypothesised to be important factors. Six Wistar rats were stereotaxically injected with tetanus toxin into the ventral hippocampus, and implanted with radiotelemeters to record electrocardiogram and electroencephalogram. The heart rate was analysed in pre-ictal, ictal and post-ictal epochs with the aim of demonstrating the changes in autonomic tone during seizure. Ictal bradycardia was observed in 89% of the seizures analysed, with post-ictal tachycardia in 93%. Further, potentially lethal arrhythmias were observed during seizure. Missed beats (59%), ventricular premature depolarisation (22%) and ventricular fibrillation (17%) are risk factors for sudden death in the general population and their presence during seizure could potentially be fatal. Whilst this study has demonstrated changes in autonomic tone and cardioarrhythmia generation; the extent of the changes and their mechanism need to be explored further. Nevertheless, this research validates the tetanus toxin model of temporal lobe epilepsy as a tool for investigating sudden death in epilepsy.

Acknowledgements

I would like to thank John Jefferys and Wei-Chih Chang for their continued guidance through this project, and for performing the implantation of the radiotelemeters. I would also like to thank all those in Neuronal Networks for their technical support and advice.

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Introduction

Around 50 million people worldwide suffer from epilepsy (WHO, 2012), with the incidence in developed countries 50 per 100,000 (Duncan et al., 2006). Epilepsy is a chronic disease of the brain that is defined by recurring and unprovoked seizures, with many seizure types identified (Duncan et al., 2006). The most destructive outcome of epilepsy is death. The risk of death in those with epilepsy is two to three times higher than the general population (Donner, 2011). Many deaths in epilepsy can be attributed to the disease, where the death is linked to a seizure, however a proportion are sudden and unexplained (Donner, 2011). In a five-year prospective study, 18% of deaths in the 4,578 patient cohort were deemed unexplained (Walczak et al., 2001). These deaths have been collectively described as sudden unexplained/unexpected death in epilepsy (SUDEP).

What is SUDEP?

SUDEP is defined as deaths in epilepsy that are sudden, unexpected, non-traumatic and non-drowning, witnessed or un-witnessed, with or without evidence of a seizure (Donner, 2011). SUDEP is given as the cause of death when all other possible causes have been eliminated; however, the poor awareness of pathologists, combined with lack of autopsy, has limited the documented number of deaths classified as SUDEP (Donner, 2011). As such, the true incidence remains unknown. In many cases, “probable SUDEP” is used to describe those deaths which satisfy the criteria above, but where no post-mortem examination was available (Donner, 2011; Annegers and Coan, 1999).

The incidence of SUDEP reported in different studies is extremely varied, as they differ on the criteria of SUDEP used, the method of study, and the population studied (Tomson et al., 2008). For example, Lhatoo et al. (2001) analysed newly diagnosed patients and showed an incidence rate

of 0.09 per 1000 patient years, whereas Dasheiff et al. (1991) studied epilepsy surgery candidates who had a SUDEP incidence rate of 9.3 per 1,000 patient years. Sillanpaa et al. (2010) found a 7% risk of SUDEP over a 40-year period of those with childhood onset epilepsy, and 12% risk in those not in five-year remission. Children have a lower incidence of SUDEP compared with adults, with the exception of those with Dravet syndrome[1] – which has a high reported rate of SUDEP (Tomson et al., 2008).

SUDEP Risk Factors

Several risk factors have been identified for SUDEP. The most important of these is experiencing frequent generalised tonic-clonic seizures, with a history of more than three in the previous 12 months (DeGiorgio et al., 2010; Donner, 2011). Active epilepsy, where the patient has failed to gain five-year remission, is also a major risk factor (Donner, 2010). Further to this, the younger the age of onset and the longer duration (over 30 years) of epilepsy have been demonstrated to increase the risk of SUDEP in adults (DeGiorgio et al., 2010; Donner, 2011). The use of anti-epileptic drugs has also been identified as a risk factor. Polytherapy, defined as the use of three or more anti-epileptic drugs, increases the risk of SUDEP by 8 times when compared to monotherapy (DeGiorgio et al., 2010; Donner, 2011). In particular, two anti-epileptic agents, lamotrigine and carbamazepine, have been implicated in SUDEP as they both have effects on the cardiovascular system (Aurlien et al., 2007; Nilsson et al., 2001). Firstly, carbamazepine was shown to reduce heart rate variability - a measure of autonomic tone - in 15 patients with epilepsy (Persson et al., 2003) implying that carbamazepine has an effect on the autonomic system as well as its anti-epileptic properties. Secondly, lamotrigine has been shown to inhibit the cardiac rapid delayed rectifier potassium ion current (I_{Kr}), the blocking of which has been associated with

[1] Dravet syndrome, or Severe Myoclonic Epilepsy of Infants (SMEI), is a drug-resistant epilepsy that occurs during the first year of life. It is characterised by frequent convulsive febrile and non-febrile seizures, frequent status epilepticus, and a mortality rate of 16% (Wolff et al., 2006).

prolonged QT interval (Donner, 2011). It is hypothesised that seizure induced metabolic acidosis could result in a fatal arrhythmia when coupled with the reduced I_{Kr} current (Danielsson et al., 2005).

Possible SUDEP Mechanisms

The mechanism, or mechanisms, which underpin SUDEP are currently unknown, with a number of hypotheses proposed. Sudden death in the general population has been associated with cardiac arrhythmias, and it has therefore been proposed that SUDEP is caused by fatal arrhythmia generation. Premature ventricular depolarisation, premature atrial depolarisations and atrioventricular block have all been observed during epileptic seizure (Opherk et al., 2002; Nei et al., 2000; Zijlmans et al., 2002). It has been proposed that the cause of such arrhythmias is through myocardial fibrosis and myofibrillar degeneration in the sino-atrial node (Reichenbach and Benditt, 1970). Other changes that influence arrhythmia generation, such prolonged QTc (Damasceno et al., 2013 and Nei et al., 2000) and elevated ST segment (Nei et al., 2000; Opherk et al., 2002; Zijlmans et al., 2002) have been associated with seizure, and therefore potentially with SUDEP.

Another hypothesis has emerged due to the similarity between SUDEP and sudden infant death syndrome (SIDS). SIDS has been associated with a defect in the brainstem-mediated response to an increase partial pressure of carbon dioxide (Donner, 2011). This respiratory arrest could also be present in SUDEP as ictal respiratory depression has been observed in numerous studies (Azar et al., 2008; Blum, 2009), with a decrease in oxygen saturation in 33% of seizures analysed by Bateman et al. (2010).

The current focus of research into the effect of seizure on the cardiovascular system centres on autonomic dysfunction, highlighting ictal tachycardia or bradycardia. Sudden death in the general population has been associated with autonomic dysfunction, with *in vivo* studies highlighting the role of the sympathetic nervous system in fatal arrhythmia generation, with

patients with high levels of circulating noradrenalin having an increased risk of sudden death (Murray and Roden, 1996).

Previous unpublished work from this group (Vivian-Griffiths, 2012) showed that electrical intrahippocampal stimulation of terminally anaesthetised rats at 20 and 40 Hz caused a decrease in heart rate. Ictal-bradycardia has also been observed in a number of other studies, with Mameli et al. (1993) producing bradyarrhythmic episodes, lasting up to 15 s, by applying penicillin G - which can trigger epileptic seizures - directly onto the thalamus and hypothalamus. Hotta et al. (2009) showed that vagal stimulation with patterns based on recording made from the vagus during seizure, induced a decrease in Wistar rats' heart rate by around 100 beats per minute (bpm). Sinus bradycardia has also been shown in humans by Nei et al. (2000), Rocamora et al. (2003) - who observed asystole and bradycardia in 5 patients - and So et al. (2000) - who observed bradycardia and apnoea in a woman with epilepsy. Bradycardia could also lead to the arrhythmias discussed above as the slowing of the pacemaker potential - induced by increased vagal activity – reduces the action potential duration and refractory period in the atria. This can in turn increase excitability of myocytes making them more susceptible to local abnormal currents.

Conversely, other studies have shown elevated heart rates associated with seizure. Damasceno et al. (2013), using Wistar audiogenic rats, showed elevated basal heart rate and prolonged QT interval and QRS complex. Leutmezer et al. (2003) analysed the heart rate changes in 145 seizures across 58 patients, and found ictal tachycardia in 86.9% of all seizures, with bradycardia only seen in 1.4%. It was also found that there was a difference in the temporal relationship between the tachycardia and seizure onset, with the change in heart rate preceding 110 seizures, occurring simultaneously in five seizure and post-ictally in 30 seizures. This finding implies that autonomic dysfunction could be caused by epileptic discharges that precede seizure.

Tachycardia was also seen by Opherk et al. (2002) who retrospectively analysed changes in

heart rate in 102 seizures from 41 patients. 99% of seizures led to tachycardia and potentially serious electrocardiograph (ECG) changes - such as ST-segment elevation and prolonged QT interval - were observed in 6% of seizures. Tigaran et al. (2003) also demonstrated ictal tachycardia in a human study of patients with drug refractory epilepsy. Finally, Zijlmans et al (2002) has shown, in a retrospective study, an increase in heart rate by at least 10 bpm in 73% of the 281 seizures analysed Zijlmans et al. (2002) also demonstrated the temporal relationship shown by Leutmezer et al. (2003), and also demonstrated bradycardia in 7% of seizures.

It is clear that the current literature is divided as to the effect of seizure on the cardiovascular system. It is apparent that arrhythmia generation is common; however, the direction of change in the heart rate is not consistent. The cause of these cardiovascular changes has been attributed to autonomic dysfunction, which needs to be explored further in order to describe the mechanisms by which this dysfunction occurs. To explore the ictal autonomic changes *in vivo*, tetanus toxin shall be injected intrahippocampally to model temporal lobe epilepsy.

Tetanus Toxin Model of Temporal Lobe Epilepsy

The tetanus toxin (TeNT) model of temporal lobe epilepsy uses the neurotoxin from *Clostridium tetani* to induce a chronic state of epilepsy, characterised by repeated seizures, via intrahippocampal injection. TeNT models different types of epilepsy dependent on the location of the injection by selectively cleaving vesicle associated membrane proteins in the γ-aminobutyric acid (GABA) interneurons. For example, a neocortical injection will model focal neocortical seizures, whereas a hippocampal injection models temporal lobe epilepsy (Jefferys and Walker, 2006). The use of intrahippocampal injections have been extensively used by this lab (Jefferys et al., 1992; Jiruska et al., 2010; Jiruska et al., 2013) and others (Mellanby et al., 1977; 1982)

TeNT is a zinc metalloprotease with two components: (1) a light chain - which is responsible

for the proteolytic activity - and (2) a heavy chain - which is responsible for the binding and internalisation of the neurotoxin (Farrar et al., 2000; Jefferys and Walker 2005). By preventing the inhibitory activity of the GABAergic neurones, a localised increase in excitatory activity occurs that can cause a partial seizure to globalise and manifest as a generalised seizure. High doses of intracranial toxin have been shown to result in cell death and a high mortality rate, whereas low doses (5 ng in 1 µl) typically induce minimal cell loss, no major morphological changes (Jefferys el al., 1992; Jiruska et al., 2010) and do not induce status epilepticus at any stage (Jefferys and Walker, 2006; Jiruska et al., 2013).

The model is reliable with seizure onset approximately three to eight days post injection, with generalised seizures observed a few days later. At their peak, seizures can happen up to 30 times per day, and are rarely beyond two minutes in duration (Jefferys and Walker, 2006; Jiruska et al., 2013). Seizures persist, with animals gaining remission six to eight weeks after onset (Jiruska et al., 2013). The clinical and behavioural features of the TeNT model of temporal lobe epilepsy have been well characterised. Seizures start with behavioural arrest and whisker twitching, continuing to forelimb movements and onto rearing and falling (Jefferys and Walker, 2006).

Ventral Hippocampus and the Cardiovascular System

Two injection sites are typically used for the induction of epileptic seizures using TeNT: (1) dorsal hippocampus (Jiruska et al., 2010; Jiruska et al., 2013) and (2) ventral hippocampus (Mellanby et al., 1977; 1982). This study injected TeNT into the ventral hippocampus, due to its association with the autonomic nervous system. The hippocampus is connected electrically to the hypothalamus by a C-shaped bundle of fibres: the fornix (Bear et al., 2007). The paraventricular hypothalamic nuclei in the hypothalamus have projections to the brainstem, namely the nucleus tractus solitarius (NTS), the dorsal vagal nucleus (DVN) and the nucleus ambiguus (NA; Mosqueda-

Garcia, 1996). These nuclei form the cardiovascular control centres, and have both parasympathetic, via the vagal nerve, and sympathetic efferents, via the glutamatergic bulbospinal pathway, to the cardiovascular system (Barrett et al., 2010).

Amongst others, the NTS is involved in cardiovascular homeostasis in normal conditions and receives inputs from the baroreceptor and chemoreceptors of the carotid sinus and aortic arch, and depending on the arterial blood pressure or the partial pressure of oxygen, will induce a change in the cardiovascular system. For example, a decrease in the afferent activity of the baroreceptors, induced by a decrease in arterial blood pressure, results in increased sympathetic outflow from the NTS to the heart and vasculature, resulting in an increased blood pressure from the increase in heart rate and induced vasoconstriction (Barrett et al., 2010; Stanfield, 2011). Therefore, injecting TeNT into the ventral hippocampus could replicate the autonomic dysfunction seen in both animal and human studies (described above).

Study Aim

This study will primarily explore whether or not epileptic seizure, induced by tetanus toxin, provokes a change to the cardiovascular system. The direction and magnitude of any cardiovascular change will be analysed, along with the duration of this change. Finally, any arrhythmias induced will be analysed. This study shall also refine the methods used to explore the effect on the cardiovascular system and validate the use of tetanus toxin as a model of exploring SUDEP.

Study Hypothesis

Tetanus toxin will induce epileptic seizures and these seizures will affect the autonomic control of the cardiovascular system, and elicit ictal tachycardia or bradycardia, along with cardioarrhythmias.

Method

This study was performed under John Jefferys' Project Licence (40/3635) and in accordance with Home Office regulations.

In total, 8 Wistar rats (260 ± 14 g; Charles River, UK) were used in this study. All animals were housed, in compliance with the Animals (Scientific Procedures) Act 1986, in the Biomedical Services Unit at the University of Birmingham, on a 12h:12h light: dark cycle with food and water available *ad libitum*. Each animal was housed with a naïve companion rat in a plastic vivarium and were monitored daily for epileptic activity (categorised using the Racine scale; see Racine, 1972 and D'Ambrosio and Miller, 2010).

Experimental Design

Two radiotelemetry systems were employed in this study. Two animals were implanted with Data Science International (DSI, US) radiotelemeters while six animals were implanted with Telemetry Research (TR) radiotelemeters (TR50BB; Millar Instruments, US).

Surgery

All surgeries were performed by Professor John Jefferys in the BMSU at the University of Birmingham. Each animal was anaesthetised using isoflurane (5% induction, 1.5-3% maintenance with oxygen at 1 Litre min^{-1}). Throughout each surgery the animal's oxygen saturation, breathing and heart rate and were monitored, with appropriate adjustments made to the level of anaesthesia to correct any deviations from normal. Furthermore, the animal's rectal temperature was maintained at 36-37°C using a heating pad.

The two radiotelemeter systems used in this study meant that the surgery required for each differed slightly. The DSI radiotelemeters used are smaller than the TR radiotelemeters, and

therefore, the location where they were implanted differed. DSI radiotelemeters can be placed in a subcutaneous cavity - created by blunt dissection - on the animal's back, whereas TR radiotelemeters must first be placed in a sterilised surgical mesh pouch and then placed in the animal's abdominal cavity. For both radiotelemeter types, the electrode wires were tunnelled subcutaneously from the radiotelemeter's location to the thorax and cervical region for the ECG and EEG respectively. Each telemeter was sterilised before implantation.

The two DSI animals were subjected to a single surgery - where TeNT was stereotaxically injected into the ventral hippocampus (Table 1) and the radiotelemeter implanted concurrently. The TR animals required two separate surgeries for the animals using TR radiotelemeters. In the first surgery, the TR radiotelemeter was placed into the abdomen, the EEG and ECG electrodes placed as described below and a steel cannula was implanted over the ventral hippocampus. In the subsequent surgery, 7-15 days later, 1 µL of 2% bovine serum albumin (Sigma-Aldrich, UK) in phosphate buffered saline (Sigma-Aldrich UK) with (n=4) or without (vehicle controls; n=2) TeNT (1-5 ng) was injected into the ventral hippocampus, via the indwelling cannula.

ECG and EEG electrode placement

The ECG electrodes were implanted into the muscle on the ventral aspect of the thorax over the rib cage by the right shoulder (negative electrode) and the lower left chest (positive electrode; as described by Kramer et al., 1993). A sterile hypodermic needle was used to tunnel into the muscle, through which the electrode was threaded (as described by Tontodonati et al., 2011 and Kramer et al., 1993) and then sutured in place using non-absorbable suture. A small plastic cap (Data Science International, US) was placed over the exposed wire tip and sutured in place to prevent it damaging neighbouring tissue.

The rat was placed into a stereotaxic frame, the skull exposed and EEG electrodes were

placed into two burr holes, drilled using a micro-drill (Circuit Medic, US), in the approximate positions shown in Figure 1. The end of each electrode wire was bent into a *U*-shape, with the base of the wire placed into the burr hole and held in place using bone cement whilst ensuring the wire makes contact with the cortex.

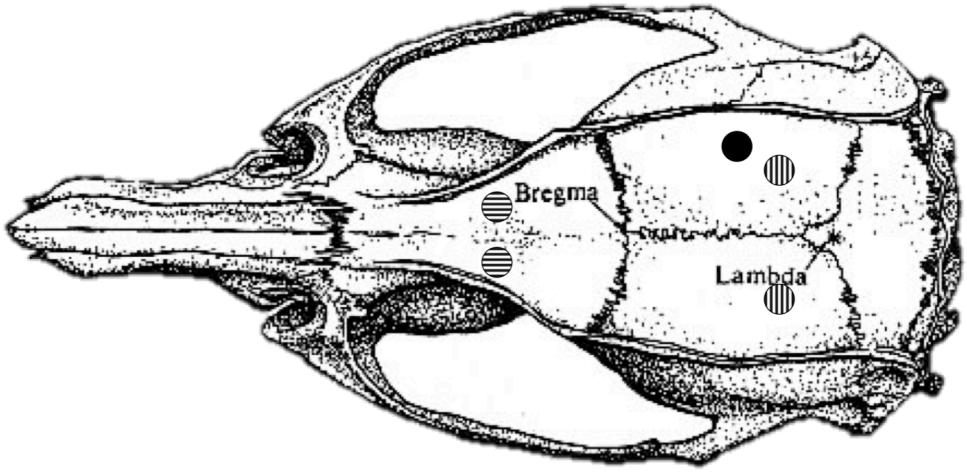

Figure 1
(Adapted from Paxinos and Watson, 1986) The approximate position of the 5 holes drilled. Horizontal lines (≡) denote the holes drilled for the anchoring screws, vertical lines show (⫼) the location of the EEG electrodes and the black circle (●) is the approximate location of the hole drilled for the indwelling cannula.

Cannula Implantation

Each cannula was made in-house to the specifications shown in Figure 2, so that is rested on the cortical surface. Each cannula was placed into a burr hole drilled at the coordinates in Table 1, using a pin chuck under stereotaxic control. By noting the final resting position below cortical surface, this allowed the subsequent stereotaxic injection to be made as accurately as possible. Each cannula was held in place using bone cement, which was extended, for stabilisation, to two anchoring screws placed on the frontal bones (Figure 1).

	Distance / mm
Anterior-Posterior *(from Bregma)*	- 4.3
Medial-Lateral *(from Bregma)*	± 4.4
Ventral Depth *(from cortical surface)*	- 7.5

Table 1

Coordinates used for stereotaxic injection into the ventral hippocampus from Paxinos and Watson (1986).

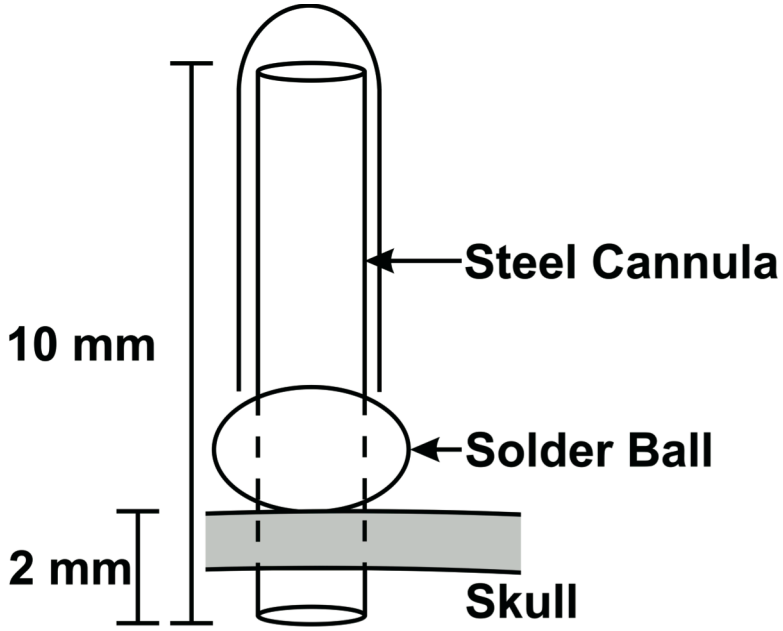

Figure 2

The specifications for the indwelling cannula. Each cannula was made from a 10 mm 21 gauge stainless steel tube with a small solder ball 2 mm from one end. This allowed the cannula to sit on the skull surface and protrude approximately 1 mm below the skull, theoretically resting on the cortical surface. Each cannula opening was protected using a plastic cap that was removed prior to the injection.

Stereotaxic Injection

In other experiments involving the dorsal hippocampus from this group, 5 ng of TeNT in 1 µL PBS/BSA is used to induce epileptic seizures. However, when this dose was injected into the ventral hippocampus it induced seizures with an exceptionally short latency period and intense seizure activity. We subsequently used 1 ng of TeNT, which proved too mild with an exceptionally long

latency period with remission gained after only two days. Therefore, a dose of 2.5 ng in 1 µL was tested and this proved optimal. This final dose had a latent period of three to five days, with seizure activity induced that did not exceed 2% of the animal's lifetime as per the project licence's limitations.

The dose that each animal received can be seen in Table 2 (see Results). 1 µL of 2% BSA in PBS with or without TeNT was stereotaxically injected into each animal's ventral hippocampus using a Hamilton syringe (25G, 7001 KH, Hamilton Company, US). For those animals with a cannula, the syringe was lowered to the base of the cannula and then lowered to the target site (Table 1) taking into account the distance the cannula protruded into the cortex. Each injection was made using a microinjection pump at 200 nL min^{-1} and left *in situ* for five minutes after the injection to prevent backflow up the needle tract. The location of the injection site was confirmed by post-mortem microscopic inspection of the hippocampus and the surrounding structures.

In Vivo Recording

EEG and ECG data were recorded continuously, along with video, using two different systems: one for each radiotelemeter type used. Two types of DSI radiotelemeter (F50-EEE and F20-EET; Data Science International, US) were implanted with multi-channel biopotential electrodes into animals with signals acquired by a PhysioTel® Receiver (RPC-1; Data Science International, US) and digitised using a Data Exchange Matrix (Data Science International, US). Signals were visualised using Dataquest A.R.T™ software (v.4.3; Data Science International, US). Data were exported offline into Spike2 (v.7.08, Cambridge Electronic Design Ltd., UK) for analysis.

Each TR implanted animal was implanted with dual biopotential telemeters (TR50BB; Millar Instruments, US), with a sampling frequency of 2 kHz and housed in a non-metallic cage on top of a SmartPad (TR180; Millar Instruments, US). Recordings were digitised using a Power1401

(Cambridge Electronic Design Ltd., UK) at a sampling frequency of 500 Hz and stored in Spike2 format.

Data Analysis

Firstly, each animal's seizure profile was analysed: the length of each seizure plotted against the time of onset post injection. In order to complete the analysis within the required period, and in order to observe the changes to the animal's HR induced by repeated seizure, and allow analysis over a longer period of time: one seizure was sampled from each of the four six-hour recordings per day. Each seizure was divided into three epochs: (1) 300s Pre-Ictal, (2) Ictal (length dependent on seizure) and (3) 300 s Post-Ictal.

Seizure Detection

Seizures were identified by rhythmic electrical activity exceeding 0.5 mV that lasted more than 20 s with a frequency exceeding 130 Hz. Figure 4 shows four example seizures. The majority of seizures analysed resembled Figure 4C.

R-Wave Detection and Heart Rate Extrapolation

An example ECG can be seen in Figure 3. This shows the components of the ECG in the rat, which differs from the human ECG in that it does not have a distinctive T-Wave. The animal's ECG had to be processed in order create the animals heart rate, whereby each R-wave was detected using an Event Detection script in Spike2 (courtesy of Steven Clifford, CED) that discriminated the R-wave from noise by a user-determined threshold and converted each R-wave into a time-point. However, this script produced many false negatives and false positives and so each epoch of ECG analysed required manual verification with any inaccuracies rectified to the best of the analyser's ability. The animal's heart rate can be calculated by the reciprocal of the intervals between

adjacent R waves. The *instantaneous frequency* function in Spike2 performed this calculation and performed fitting process so that it could be sampled at a constant frequency of 10 Hz. The resulting frequency was converted to beats per minute (bpm) by multiplying by 60.

Each epoch was analysed so that each R-R interval/heart rate was treated as a single data point and a histogram was produced that represented the proportion of the heart rates in that instantaneous frequency *bin* expressed as a percentage of the total number of data points. This allowed the modal heart rate to be established, but also identify any bradycardia or tachycardia present, as this would be represented by a shift in the distribution to lower or higher values respectively.

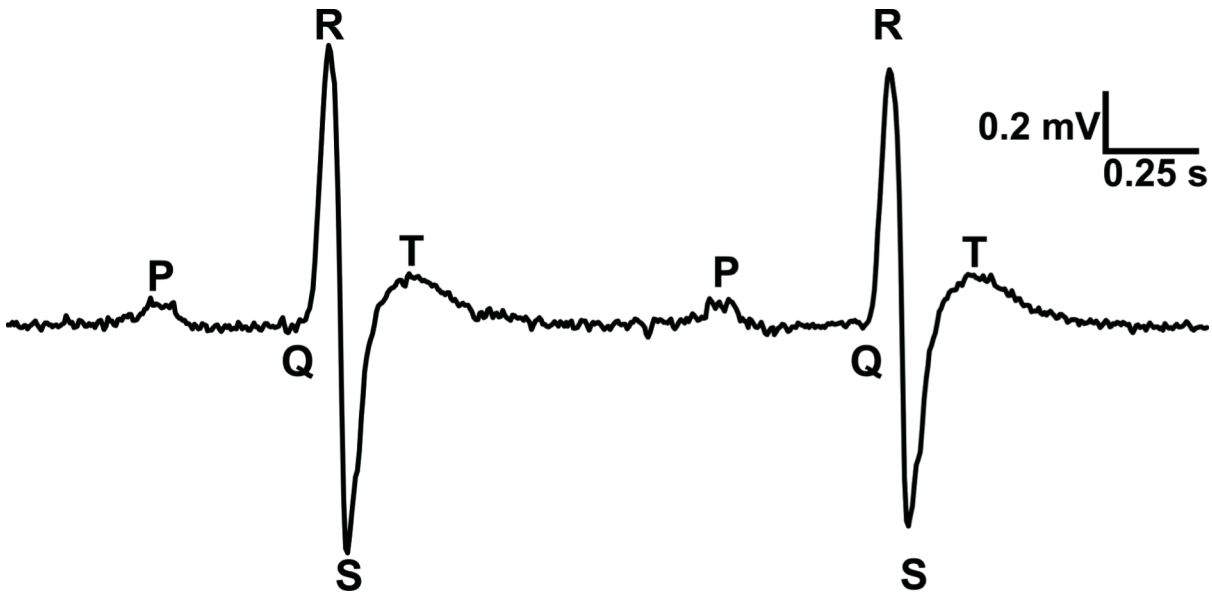

Figure 3
An example ECG of the rat. Each wave corresponds to electrical activity in the heart. The P-wave corresponds with atrial depolarisation, the QRS complex ventricular depolarisation, and the T-wave corresponds to ventricular repolarisation. The rat ECG differs to a human ECG in that it does not have a distinct T-wave.

Statistical Analysis

Statistical analysis included general linearised model and one-way ANOVA. All data was expressed as either the mode - in respect of the distribution analysis - or mean ± standard error of the mean.

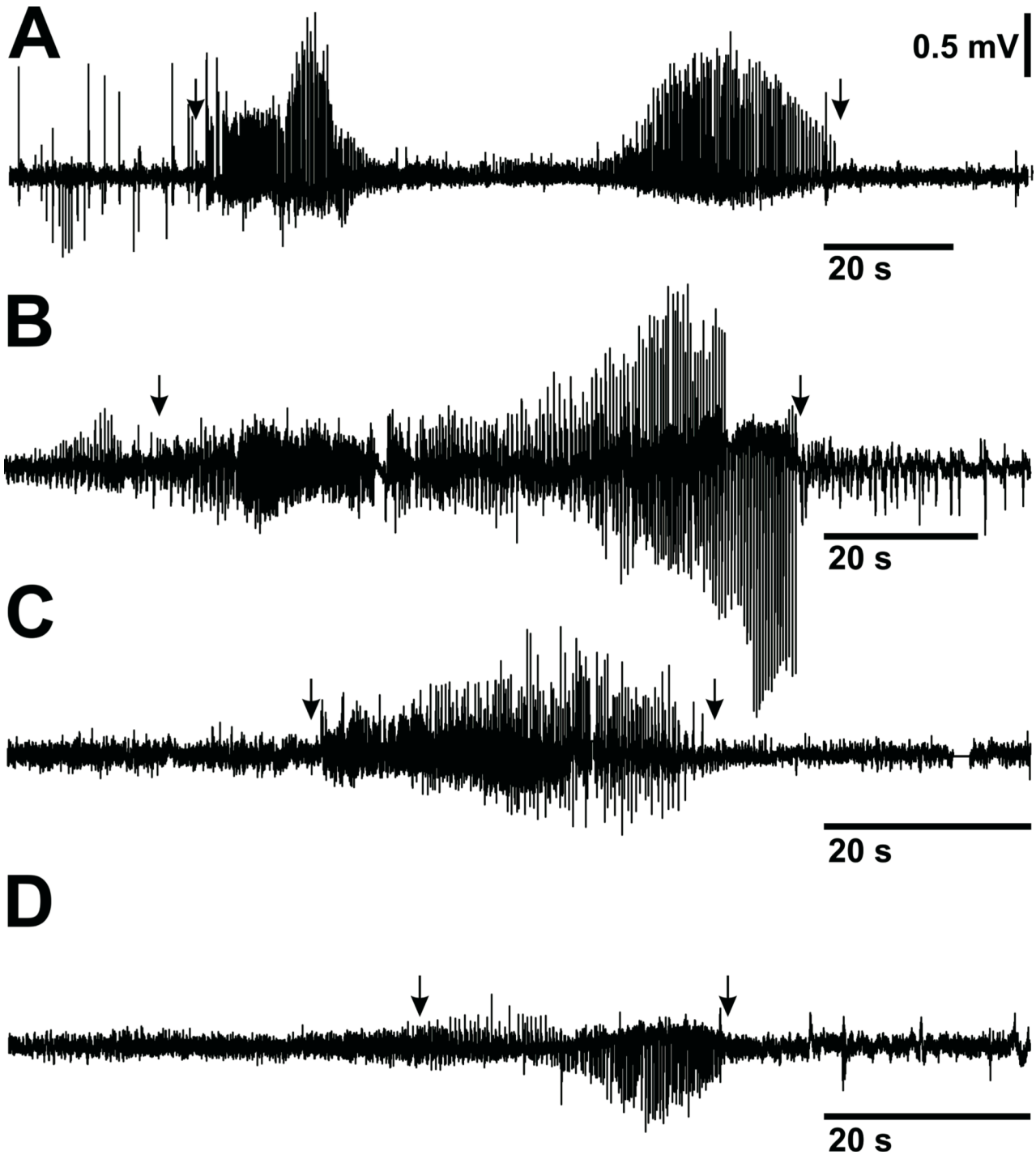

Figure 4

Examples of seizures seen in this study. A number of objective criteria were used to identify seizures and discriminate them from non-epileptic EEG, including the morphology of the seizure and the magnitude of the activity. The onset and termination of each seizure are marked by arrows, and are the same period is represented in Figure 10 and Figure 11 as a red line. The majority of seizures seen in this study resemble ***C****.*

Results

Seizures Observed

Repeated spontaneous seizures were seen in four of the six TeNT injected animals. These seizures induced behavioural changes, such as hyperreactivity, as well as EEG changes. Seizures caused EEG distortions such as those in Figure 4, with rhythmic activity exceeding 0.5 mV in amplitude, lasting an average of 65 ± 1 s for all seizures. Seizure began as behaviour arrest, with staring and sniffing automatisms, followed by unilateral or bilateral forelimb clonus, rearing, falling and in two animals (JJ130404 and JJ130703) wild running. No status epilepticus was observed at any point.

With EEG electrodes placed into the neocortex, partial seizures or epileptic activity that failed to reach the cortex may not have been observed. This may have been the case with JJ130703, as it exhibited wild running that could have been a product of a partial seizure in the ventral limbic structures or the brainstem, however the EEG recording could not corroborate this.

Animal Information

Table 2 shows a summary of the eight animals used in this study. The seizure profiles of the four animals are shown in Figure 5. JJ130404 (Figure 5A) was implanted with a DSI radiotelemeter and injected with 5 ng of TeNT in 1 µl PBS/BSA. This animal experienced seizures two days post injection but had to be killed on the third day (indicated by the dotted red line, Figure 5A) as there was a technical fault with the radiotelemeter implanted. This animal also had Racine Scale 5 seizures, average length 70 ± 8 s. It also exhibited *wild running* and had a very short latency period, which together suggested the ventral hippocampus could be more sensitive than the dorsal to TeNT.

Animal	Weight at implantation (g)	Radiotelemeter type	TeNT Dose (ng in 1 µL)	Total number of seizures	Average Seizures per day	Average Seizure Length (s)
JJ130404	268	DSI	5	13	N/A	70 ± 8
JJ130411	337	DSI	1	64	N/A	81 ± 3
JJ130513	210	TR	2.5	200	18	57 ± 2
JJ130521	294	TR	2.5	82	7	70 ± 3
JJ130701	230	TR	2.5	-	-	-
JJ130702	231	TR	Control	N/A	N/A	N/A
JJ130703	260	TR	2.5	-	-	-
JJ130704	250	TR	Control	N/A	N/A	N/A

Table 2

A summary of each animal used in this study. The seizure activity of JJ130701 and JJ130703 could not be established due to technical issues.

JJ130411 (Figure 5B) was also implanted with a DSI radiotelemeter, but was injected with 1 ng of TeNT in 1 µl PBS/BSA. This animal did not experience any seizures until nine days post injection, when it experienced 64 seizure (average length 81 ± 3 s) but entered remission 10 days post injection and did not experience another seizure, which showed the dose may be too low. JJ130513 (Figure 5C) and JJ130521 (Figure 5D) were both injected with 2.5 ng of TeNT in 1 µl PBS/BSA and experienced seizures four and three days afterwards respectively. Both animals entered a period of quiescence after an initial episode of seizures; however, a technical issue prevented data acquisition between days seven and nine post injection (indicated by shading in Figure 5C) and masked the extent of this quiescence for JJ130513. JJ130513 had 200 seizures within the first 14 days post injection, with an average length of 57 ± 2 s, and JJ130521 had 82 seizures in the same period, average length 70 ± 3 s. It was not possible to classify all seizures analysed due to an absence of evidence from the video recording.

JJ130701 and JJ130703 were also injected with 2.5 ng of TeNT in 1 µl PBS/BSA while JJ130702 and JJ130704 were the control animals. JJ130701, JJ130702 and JJ130704 opened their abdominal wounds and had to be euthanised (11, six, and six days post injection respectively), as their wounds could not be repaired more than once under the terms of our licence. JJ130701 and

JJ130702 also chewed through the electrode wires three and seven days post injection, respectively. JJ130701 was not observed seizing during the day and without EEG recording, any nocturnal seizure activity could not be confirmed. JJ130703 exhibited epileptic behaviour, namely hyperreactivity and wild running. However, its EEG recording could not corroborate the wild running as a typical seizure, as depicted in Figure 4 (see Methods). Furthermore, the animal had to be euthanised nine days post injection on welfare grounds.

Due to the above, the data from JJ130701 and JJ130703 were excluded the analysis and only six days were included in the control data.

The Normal Heart Rate

The heart rate of each control animal, derived from the ECG R-R interval, was analysed. Firstly, each epoch of extrapolated R-R intervals (Figure 6A) was analysed so that the number of times a heart rate falls into a specific 10 bpm bin (from 50-900 bpm) was counted and plotted as a frequency. Overall, the modal heart rate for both control animals was 360 bpm, with a range of 270 to 540 bpm. Figure 6A shows an example of the variation in the heart rate of JJ130704 over a six-hour period (Figure 6A), where the heart rate oscillates between 300 and 500 bpm. The modal heart rate of this epoch was 390 bpm, as shown by Figure 6B. Figure 6C shows the variation between the two animals over the four six hour epochs on each of the six days analysed. Both animals' heart rates distributed around 400 bpm with little variation day-to-day.

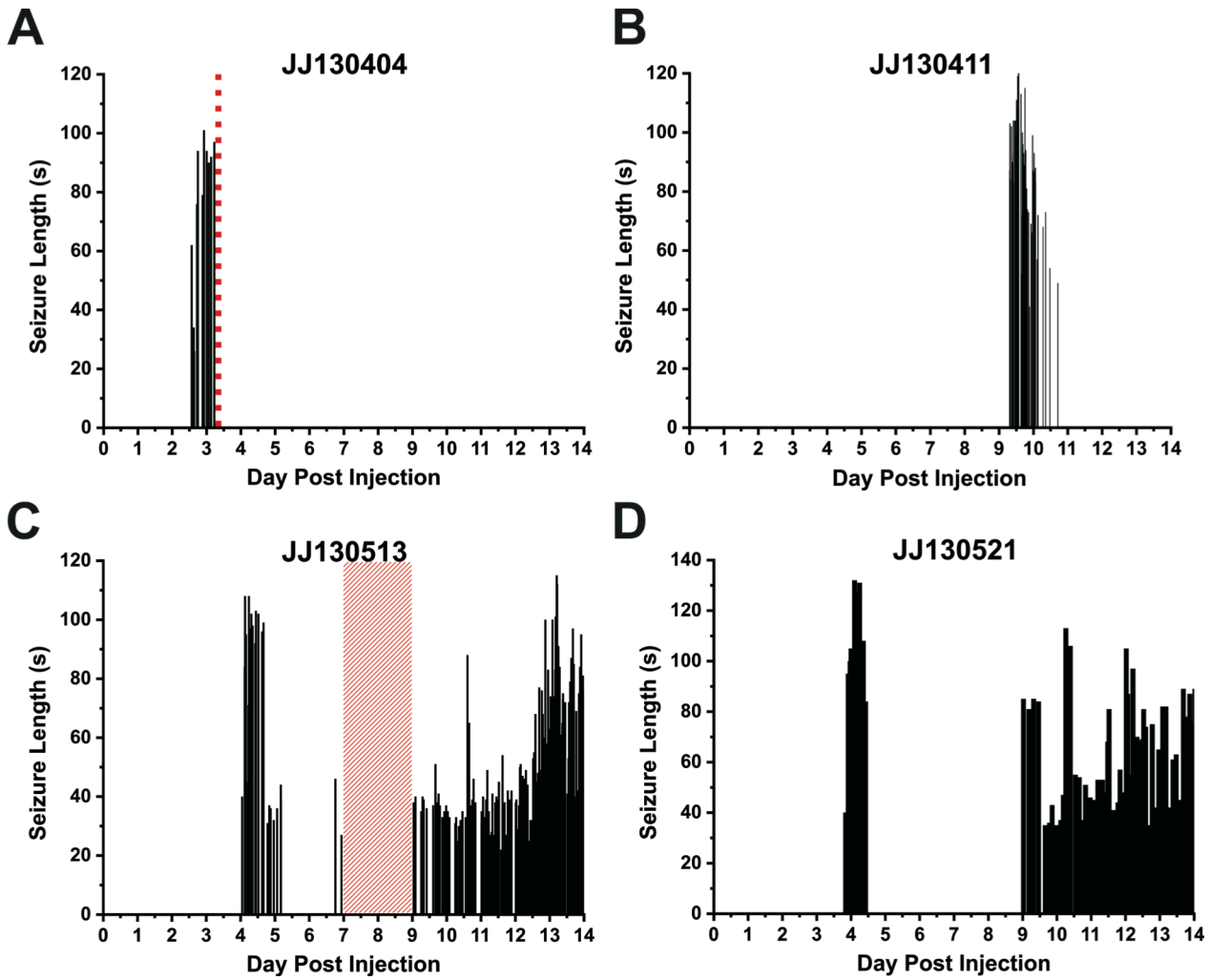

Figure 5

Seizure Profile of four of the TeNT injected animals. ***A)*** *The two days of seizures recorded from JJ130404 before the DSI radiotelemeter used failed - indicated by the red dotted line.* ***B)*** *The seizures experienced by JJ130411, which was injected with 1 ng of TeNT in 1 µl PBS. This animal experienced 64 seizures on Days 9 and 10 post injection, before experiencing remission.* ***C*** *and* ***D)*** *The seizures experienced by JJ130513 and JJ130521 respectively, with the red shaded portion of* ***C*** *depicting a period of missing data caused by technical issues. JJ130521 experienced 12 seizures on Day 3 and 4 before entering a period of quiescence until Day 9.*

The Effect of Seizure on Heart Rate

Across all four animals, bradycardia was observed during 68 (89%) of the 76 seizures analysed. An example of this ictal bradycardia is depicted in Figure 7. The average decrease across these 68 seizures was 266 ± 12 bpm, with this bradycardia lasting 38 ± 2 s. The seizure in Figure 7 elicited a decrease of 261 bpm, from 355 bpm to 94 bpm, lasting 23 s. The quantification of the bradycardia for each animal can be seen below (Table 3).

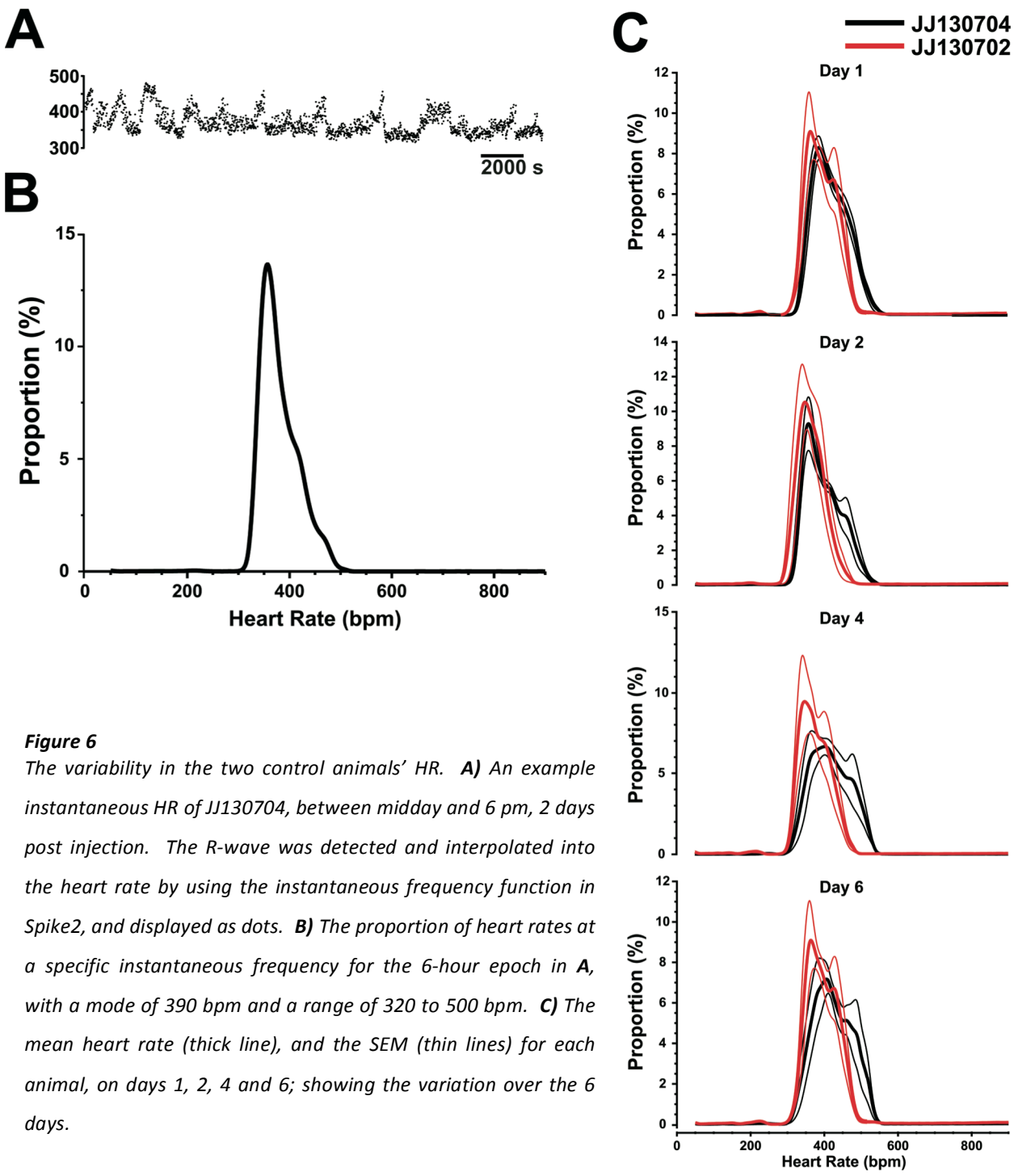

Figure 6

The variability in the two control animals' HR. ***A)*** *An example instantaneous HR of JJ130704, between midday and 6 pm, 2 days post injection. The R-wave was detected and interpolated into the heart rate by using the instantaneous frequency function in Spike2, and displayed as dots.* ***B)*** *The proportion of heart rates at a specific instantaneous frequency for the 6-hour epoch in* ***A****, with a mode of 390 bpm and a range of 320 to 500 bpm.* ***C)*** *The mean heart rate (thick line), and the SEM (thin lines) for each animal, on days 1, 2, 4 and 6; showing the variation over the 6 days.*

Post-ictal tachycardia was observed in 71 of the 76 (93%) seizures analysed, also shown in Figure 7. The heart rate increased post-ictally by a mean of 129 ± 7 bpm across all animals, with each animal's average tachycardia shown in Table 3. The duration of this post-ictal tachycardia varied, lasting a mean 863 ± 87 s.

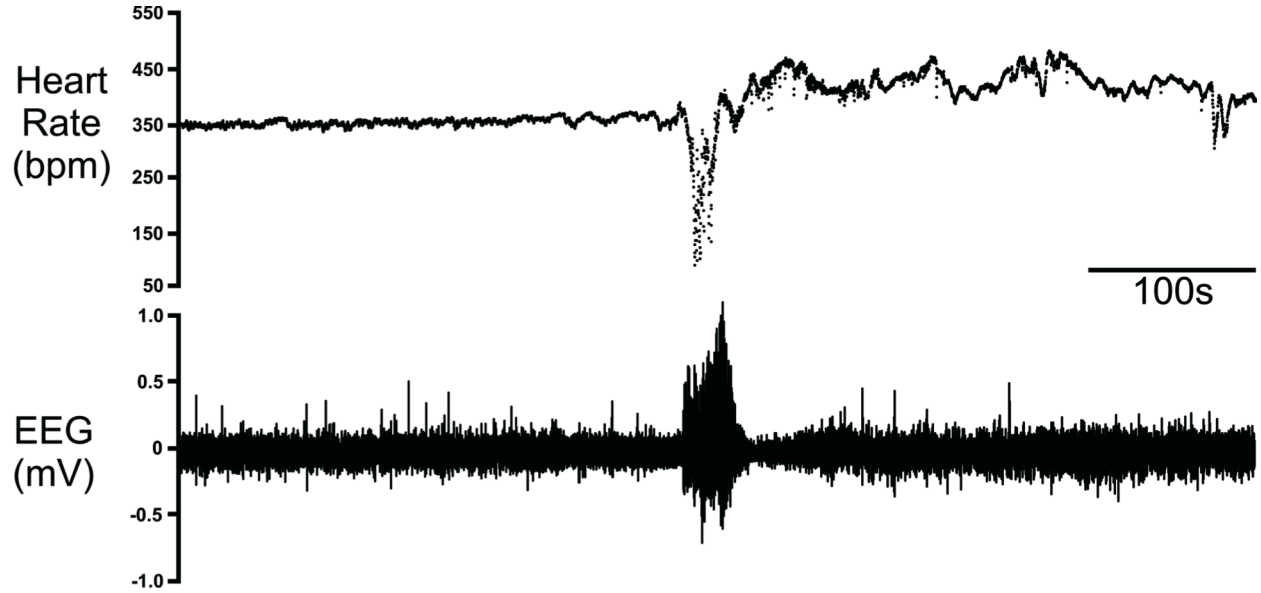

Figure 7

A representative example showing the effect of epileptic seizure on the heart rate. This seizure, taken from JJ130513, 4 days post injection, elicits a decrease in the animal's heart rate to 94 bpm; followed by a prolonged tachycardia.

	Mean Ictal Heart Rate (bpm)	Mean Magnitude of Bradycardia (bpm)	Mean Baseline: Minimum Ratio	Mean Length of Bradycardia (s)	Mean Post-Ictal Heart Rate (bpm)	Mean Magnitude of Tachycardia (bpm)	Mean Baseline: Maximum Ratio	Mean Length of Tachycardia (s)
JJ130404	332 ± 56	- 144 ± 44	0.68 ± 0.1	47 ± 6	564 ± 28	88 ± 16	1.19 ± 0.04	397 ± 182
JJ130411	182 ± 34	- 206 ± 26	0.46 ± 0.07	49 ± 6	535 ± 17	147 ± 23	1.40 ± 0.07	500 ± 241
JJ130513	111 + 10	- 326 ± 12	0.25 ± 0.02	35 ± 2	533 ± 6	96 ± 9	1.23 ± 0.02	857 ± 156
JJ130521	154 ± 21	- 222 ± 19	0.40 ± 0.05	35 ± 1	541 ± 12	169 ± 11	1.46 0.03	1078 ± 115
All Seizures	152 ± 13	- 266 ± 12	0.37 ± 0.03	38 ± 2	541 ± 6	129 ± 7	1.33 ± 0.02	863 ± 87

Table 3

A summary of the changes in heart rate induced by seizure. 68 seizures induced bradycardia, with the average magnitude for each animal displayed. All 76 seizures induced tachycardia, the data for which can also be seen.

Bradycardia and Arrhythmia

The bradycardia shown in Figure 7 was caused by two phenomena. Firstly, there was a genuine (sinus) bradycardia caused by the R-R interval increasing. Secondly, the bradycardia was caused by arrhythmias – namely missed beats. An example of missed beats induced by a seizure can be seen in Figure 8. Two missed beats, with absent p-waves, can clearly be seen in the ictal epoch, when compared to the pre- and post-ictal ECG traces (Figure 8D). Missed beats were observed in 45 (59%) of the 76 seizures analysed. Less frequently, other arrhythmias were

observed (Figure 9): including ventricular premature depolarisation indicated by arrows (during 22 seizures; 22%) and ventricular fibrillation indicated by the dotted line (Figure 9C; during 13 seizures; 17 %).

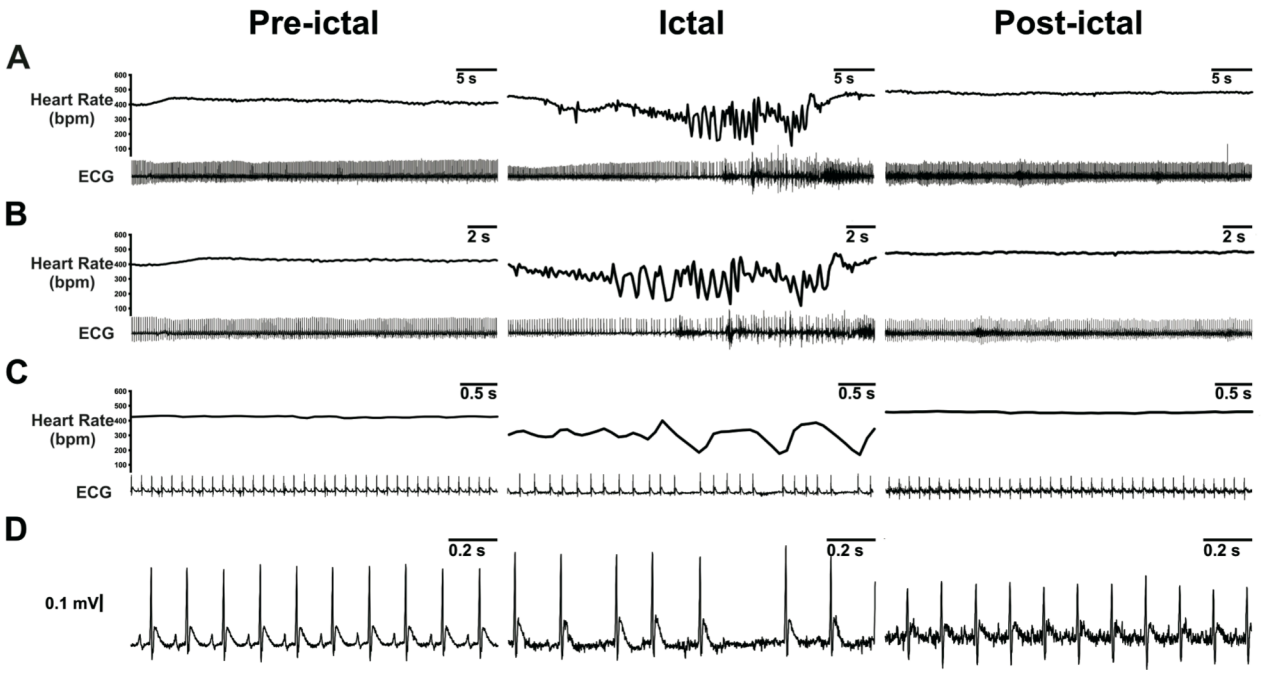

Figure 8
*An example of seizure induced missed beats and the effect on the HR, with the pre- and post-ictal periods included for comparison. Each image is a magnification of the previous, from a 45 s epoch **(A)** to a 2 s epoch **(D)**.*

The Effect of Progressive Seizure

JJ130404, JJ130411 and JJ130513 showed ictal bradycardia for all seizures. However, JJ130521 initially experienced ictal tachycardia during day three and four post injection. After the four day period of quiescence (Figure 5D), it experienced ictal bradycardia. This progression from tachycardia to bradycardia can be seen in Figure 11. Both JJ130513 (Figure 10) and JJ130521 (Figure 11) show a shift in their heart rate's distribution induced by seizure (red line). Each of the histograms were normalised to the total number of heart beats for the epoch analysed.

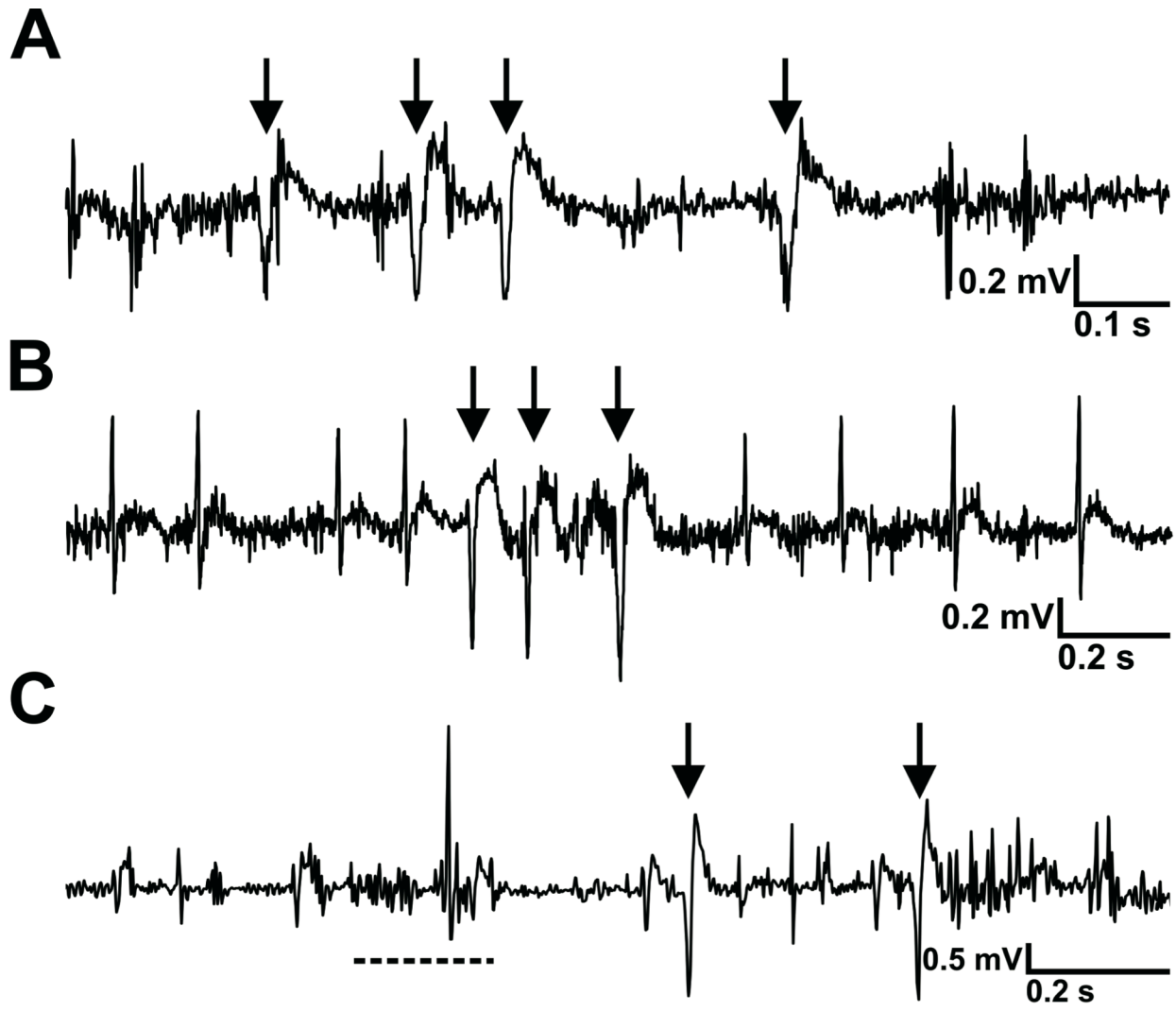

Figure 9

*Examples of the arrhythmias seen in this study. All three panels show premature ventricular depolarisations – indicated by the arrows - with **C** showing evidence of ventricular fibrillation- identified by the dotted line.*

The example seizures in Figure 10 and Figure 11 show that seizures induced a decrease in the animals' heart rate, and a shift in the ictal distribution, with the tails over the lower heart rates signifying the bradycardia. Furthermore, this bradycardia is superimposed onto a tachycardia, which continues into the post-ictal period (blue line), and is the cause of the shift to higher rates of the modal heart rate in many of the examples shown. Finally, it can be seen that the pre-ictal state (black line) varies slightly for JJ130513 (Figure 10) and is more stable in JJ130521 (Figure 11). For all seizures analysed the average pre-ictal heart rate was 412 ± 7 bpm. In JJ130521, the heart rate increases before seizure onset (Figure 11A-F). This could be a consequence of recording some

distance from the seizure onset zone and not being able to detect the beginning of the seizure.

When displaying all seizures together (Figure 12), it can be seen that the pre-ictal heart rate is very similar to the control animals' heart rate (red line, Figure 12) whereas there is a shift to the higher heart rates for both the ictal and post-ictal periods. This corroborates the individual seizure data, showing a general shift to the higher heart rates induced by seizure. During seizures, the bradycardia causes the tail over the lower heart rates, with the discontinuous appearance of this tail reflecting the missed beats.

In order to explore any heart rate difference between the three epochs and the four animals, as well as any progressive change in heart rate with successive seizures, a general linearised model was used. This model assumes factors (Animal and Epoch) and covariates (Seizure number) have a linear relationship with the dependent variable (heart rate). Each term in the model and the model as a whole (see *Sources* column, Table 4) is tested for its ability to account for the variation in the heart rate. If the significance value calculated is below α (0.05) in this study, then this variable has a significant effect on the heart rate. The result of this analysis is shown in Table 4.

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Square	F Statistic	Sig.
Corrected Model	5994804.37	12	499567.03	113.91	P < 0.001
Intercept	4859435.20	1	4859435.20	1108.05	P < 0.001
SeizureNo	18072.54	1	18072.54	4.12	P = 0.044
AnimalNo	77188.98	3	25729.66	5.87	P < 0.001
Period	3108288.61	2	1554144.31	354.38	P < 0.001
AnimalNo * Period	216117.95	6	36019.66	8.21	P < 0.001
Error	925357.19	211	4385.58		
Total	38161196.00	224			
Corrected Total	6920161.55	223			

Table 4

The result of the general linearised model used to assess the ability of each variable (Sources column) to account for the variability observed in heart rate.

Therefore, each variable assessed by this model has a significant effect on the heart rate.

There is a significant effect of the three epochs analysed ("Period": $F(2, 211) = 354.38$, $P < 0.001$), a significant effect of the four animals ("Animal No": $F(3, 211) = 5.87$, $P<0.001$) and also a significant effect of repeated seizure ("Seizure No": $F(1,211) = 4.121$, $P = 0.004$), indicating that successive seizure has an effect on the animals' heart rate.

In order to explore the change in heart rate in the three epochs, a one-way ANOVA was performed with a more stringent α-level used (0.01) to account for the fact that multiple tests have been used. A significant difference between the heart rates in all three epochs (pre-ictal, ictal and post-ictal) was observed in three of the four animals: JJ130411, JJ30513 and JJ130521 (Table 5 and Table 6). The heart rate of JJ130404 in the pre-ictal period was not significantly different from the ictal or post-ictal heart rates, with significance observed between the ictal and post-ictal heart rates.

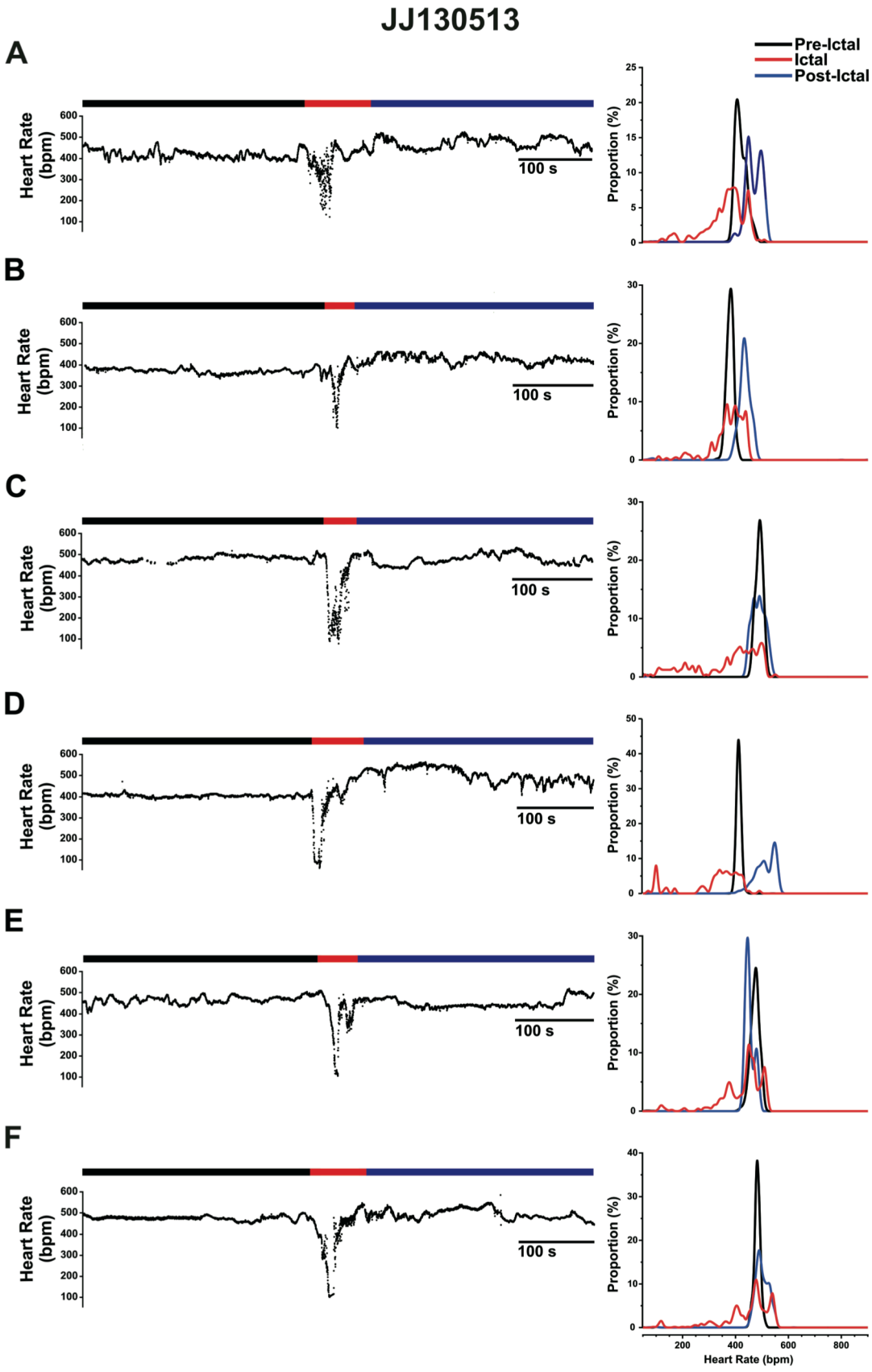

Figure 10

*The effect of seizure on JJ130513's HR four **(A)**, six **(B)**, nine **(C)**, 10 **(D)**, 12 **(E)** and 14 **(F)** days post injection. Seizure (red lines) induces a decrease from the Pre-ictal HR (black lines) with many seizures having an elevated post-ictal HR (blue lines). The bradycardia is apparent in the histograms (right panel) which show a long tail extending over the lower heart rates.*

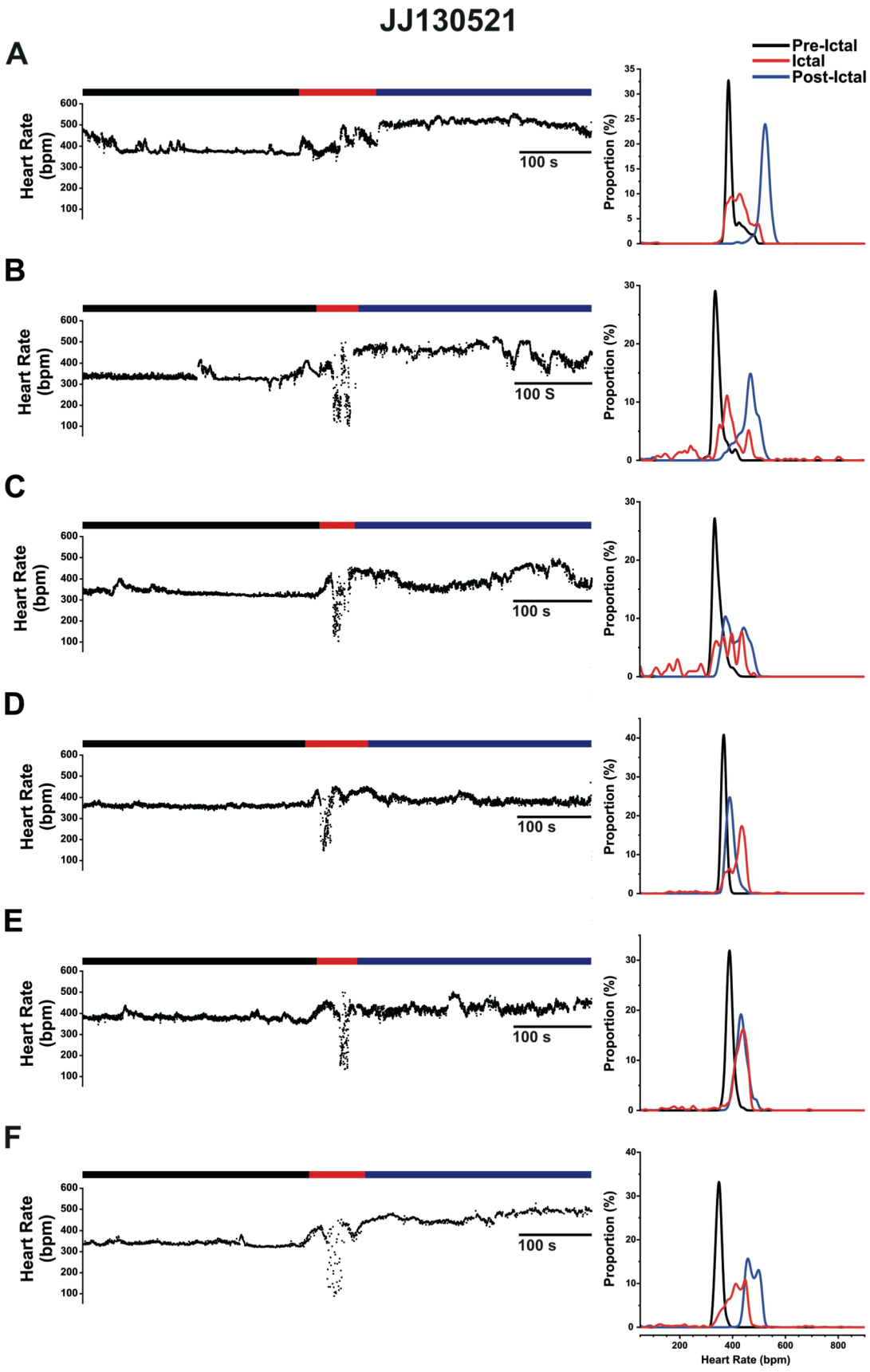

Figure 11

*The effect of seizure on JJ130521's HR four **(A)**, 10 **(B)**, 11 **(C)**, 12 **(D)**, 13 **(E)** and 14 **(F)** days post injection. Initially, the seizures (red line) induced tachycardia **(A)**, however after the 4 day period absent of seizures, ictal bradycardia was present (**B-F**).*

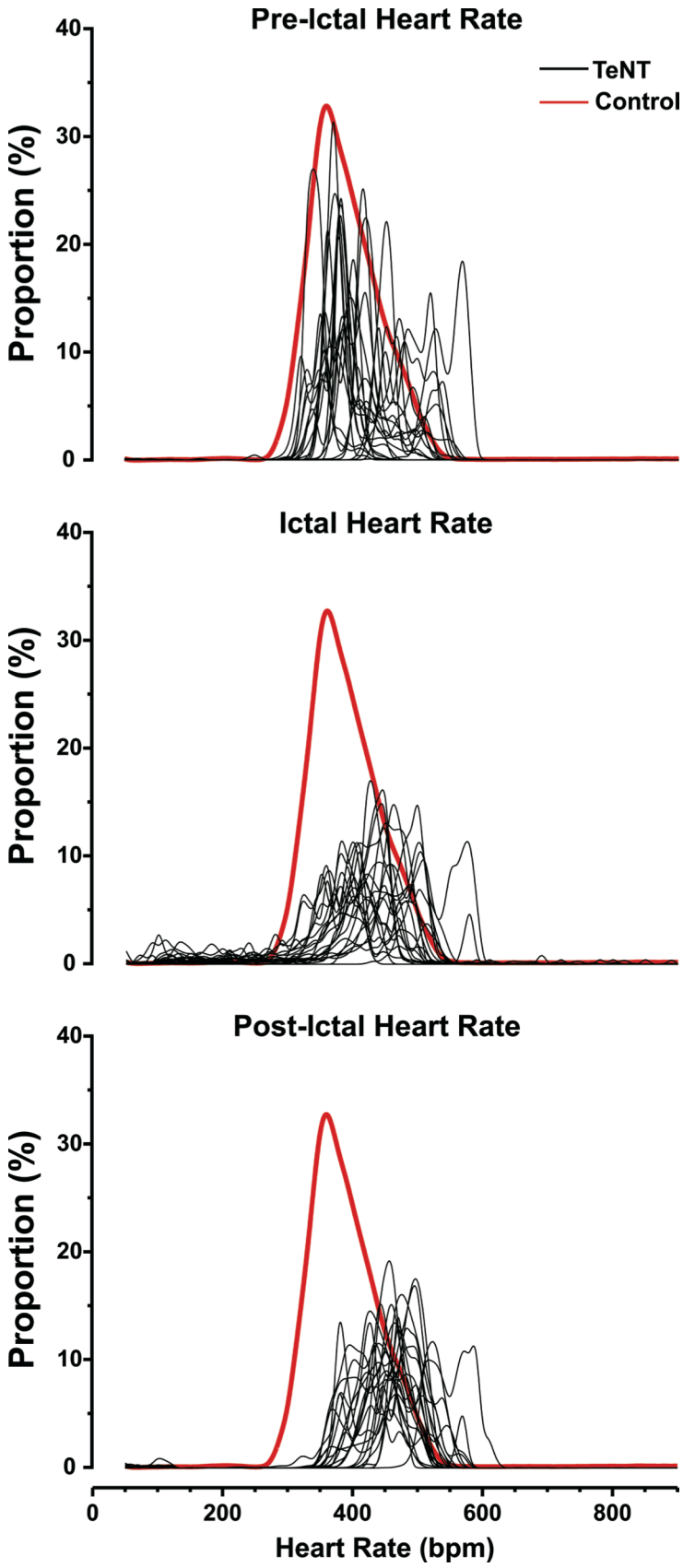

Figure 12

The distribution of the heart rates for all seizures analysed, for the pre-ictal, ictal and post-ictal periods, the red line denotes the average distribution of the heart rates of both the control animals. There is a shift in the distribution to the higher heart rates induced by seizure.

		Sum of Squares	Degrees of Freedom	Mean Square	F	Sig.
JJ130404	Between	220016.58	2	110008.29	8.940	P = 0.002
	Within	258393.38	21	12304.45		
	Total	478409.96	23			
JJ130411	Between	503038.08	2	251519.04	53.630	P < 0.001
	Within	98479.88	21	4689.52		
	Total	601517.96	23			
JJ130513	Between	3116697.90	2	1558348.95	727.190	P < 0.001
	Within	197153.32	92	2142.97		
	Total	3313851.22	94			
JJ130521	Between	2362102.17	2	1181051.08	203.500	P < 0.001
	Within	470099.39	81	5803.70		
	Total	2832201.56	83			

Table 5

The results of the one-way ANOVA assessing the variability within each animal's heart rate during the pre-ictal, ictal and post ictal periods. A significant difference was observed in each animal, and a post-hoc Bonferroni multiple comparisons test (***Table* 6)** was *performed to identify where this significance arises.*

Dependent Variable			Mean Difference	Std. Error	Sig
JJ130404	Pre-Ictal	Ictal	144.38	55.46	P = 0.05
		Post-Ictal	-87.88	55.46	P = 0.38
	Ictal	Pre-Ictal	-144.38	55.46	P = 0.05
		Post-Ictal	-232.25	55.46	P < 0.001
	Post-Ictal	Pre-Ictal	87.88	55.46	P = 0.38
		Ictal	232.25	55.46	P < 0.001
JJ130411	Pre-Ictal	Ictal	205.88	34.24	P < 0.001
		Post-Ictal	-147.13	34.24	P < 0.001
	Ictal	Pre-Ictal	205.88	34.24	P < 0.001
		Post-Ictal	-353.00	34.24	P < 0.001
	Post-Ictal	Pre-Ictal	147.13	34.24	P < 0.001
		Ictal	353.00	34.24	P < 0.001
JJ130513	Pre-Ictal	Ictal	324.51	11.67	P < 0.001
		Post-Ictal	-97.33	11.67	P < 0.001
	Ictal	Pre-Ictal	324.51	11.67	P < 0.001
		Post-Ictal	-421.84	11.57	P < 0.001
	Post-Ictal	Pre-Ictal	97.33	11.67	P < 0.001
		Ictal	421.84	11.57	P < 0.001
JJ130521	Pre-Ictal	Ictal	239.50	20.36	P < 0.001
		Post-Ictal	-169.25	20.36	P < 0.001
	Ictal	Pre-Ictal	-239.50	20.36	P < 0.001
		Post-Ictal	-408.75	20.36	P < 0.001
	Post-Ictal	Pre-Ictal	169.25	20.36	P < 0.001
		Ictal	408.75	20.36	P < 0.001

Table 6

The result of the post-hoc Bonferroni multiple comparison tests. A significant difference (α=0.01) was observed between all three epochs (pre-ictal, ictal and ictal) analysed for JJ130411, JJ130513, and JJ130521. A significant difference was only observed between the Ictal and post-ictal heart rates for JJ130404.

Discussion

This study aimed to explore the effect of seizure on the cardiovascular system, highlighting any pathological change in order to gain insight into the processes that underpin SUDEP. By implanting eight rats with radiotelemeters and continuously recording their EEG and ECG, it was possible to observe the long-term effect of seizure on the animal.

The major finding of this study was the autonomic dysfunction induced by seizure, which manifest as ictal bradycardia and ictal tachycardia. Ictal bradycardia was observed in 68 (89%) of the 76 seizures analysed across all four animal, with a mean ictal heart rate of 152 ± 13 bpm across all seizures. This finding supports previous work in this lab, which observed ictal bradycardia after electrical stimulation the dorsal and ventral hippocampus as a model of epileptic seizure (Vivian-Griffiths, 2012). Furthermore, it corroborates reports of ictal bradycardia observed in other animal models of epilepsy (Hotta et al., 2009, Mameli et al., 1993) and in human patients (Devinsky et al., 1997; Nashef et al., 1996; Nei et al., 2000; So et al., 2000; Tinuper et al., 2001; Zijlmans et al., 2002).

Post-ictal tachycardia was also observed, with a mean heart rate of 541 ± 6 bpm that persisted for 863 ± 87 s. This finding supports studies showing ictal and post ictal tachycardia in animal models (Damasceno et al., 2013) and humans (Leutmezer et al., 2003; Nashef et al., 1996; Opherk et al., 2002; Tigaran et al., 2003; Zijlmans et al., 2002). Indeed, this study supports Nashef et al. (1996) and Zijlmans et al. (2002), who had shown both seizure-induced bradycardia and tachycardia, with this period of bradycardia observed to be superimposed onto a post-ictal tachycardia in some of the seizures analysed in this study (Figure 11B, C, F).

Furthermore, the arrhythmias observed in this study (premature ventricular depolarisation and ventricular fibrillation) have also been observed in animal (Mameli et al., 1993) and human studies (Nei et al., 2000; Opherk et al., 2002; Zijlmans et al., 2002).

Origin of Bradycardia

In this study, 1-5 ng of TeNT in 1 µl of PBS/BSA was stereotaxically injected into the ventral hippocampus in order to model TLE. A prominent output of the hippocampus is the fornix, which terminates in the hypothalamus (Bear et al., 2007). The hypothalamus has been shown to influence the cardiovascular system when stimulated with penicillin G in rats (Mameli et al., 1993) and electrically in rabbits (Gellman et al., 1981). The hypothalamus itself has projections to the medulla, the location of the nucleus ambiguus (NA), the nucleus tractus solitarius (NTS) and the dorsal vagal nucleus (DVN; Barrett et al., 2010), it was therefore envisaged that seizures with foci in the hippocampus would have cardiovascular effects.

In normal conditions, three factors integrate and determine the heart rate: sympathetic neurones, parasympathetic neurons and circulating adrenaline (Stanfield 2011). Both branches of the autonomic nervous system are constantly active and the balance between the two at a given point results in the organism's heart rate.

The heart rate is slowed by increased parasympathetic outflow from the NA, via the vagus nerve. The slowing of the heart is induced by acetylcholine (ACh), released by post-ganglionic neurones, which slows the intrinsic pacemaker activity of the sinoatrial and atrioventricular node (SAN, AVN). ACh binds to M2 muscarinic receptors in the SAN. This leads to slowing of the pacemaker potential by decreasing calcium and sodium currents, via $G_i\beta\gamma$ inhibition of T-type calcium channels and Funny channels respectively, and by increasing the potassium currents by opening GIRK1 channels (Richerson, 2009; Stanfield, 2011). The average ictal heart rate observed across all 76 seizures, 152 ± 13 bpm, can be deemed bradycardic as it is pathologically lower than the normal heart rate for the Wistar rat, that ranges between 320 and 490 bpm (Pass and Freeth, 1993). This bradycardia could be a result of seizure activity driving the hypothalamus, through the fornix.

There are, however, other explanations of the bradycardia observed. For example, an increase in arterial blood pressure, as reported to occur in seizure (Damasceno et al., 2013) leads to a reflexive decrease in heart rate (Barrett et al., 2010; Richerson, 2009). Hypoxia, detected by peripheral chemoreceptors (Marshall, 1998), results in bradycardia and tachycardia. Lastly, bradycardia can also be induced by increased intracranial pressure (Barrett et al., 2010; Fodstad et al., 2006; Mangrum and DiMarco, 2000), which itself has been associated with epilepsy and seizure (Gabor et al., 1984; Minns and Brown, 1978). These could be investigated as potential confounders by recording arterial blood pressure, breathing rate, partial pressure of oxygen in the blood and by using a pressure-sensing electrode in the cranium.

Classifying Bradycardia

Sinus-node dysfunction is a common source of bradycardia and can be caused by intrinsic and extrinsic pathological influences (Mangrum and DiMarco, 2000). There are many ECG presentations of sinus-node dysfunction (Figure 13) that are caused by both intrinsic and extrinsic factors. In this study, sinus bradycardia, sinus arrest and bradycardia-tachycardia syndrome (Mangrum and DiMarco, 2000; Figure 13) have been observed, as well as bradycardia induced by missed beats. Sinus bradycardia is a result of depressed automaticity of the SAN, with increased parasympathetic stimulation resulting in a slowing of the pacemaker potential (Richerson, 2009; Magrum and DiMarco, 2000; Stanfield, 2011), with sinus arrest due to failure of impulse conduction from the SAN to the atrium.

The bradycardia observed in this study could be attributed to extrinsic influences on the SAN, such as the aforementioned increased intracranial pressure and excessive parasympathetic (vagal) tone (Barrett et al., 2010; Fodstad et al., 2006; Mangrum and DiMarco, 2000). Intrinsic causes, such as ischemia, could be confirmed with post-mortem histological evaluation of the heart

tissue. To that end, hearts were isolated post-mortem and placed into paraformaldehyde for processing by a collaborator who is currently setting up the protocols. However, as intrinsic causes are chronic, it is unlikely that they are responsible for the transient bradycardia seen in this study.

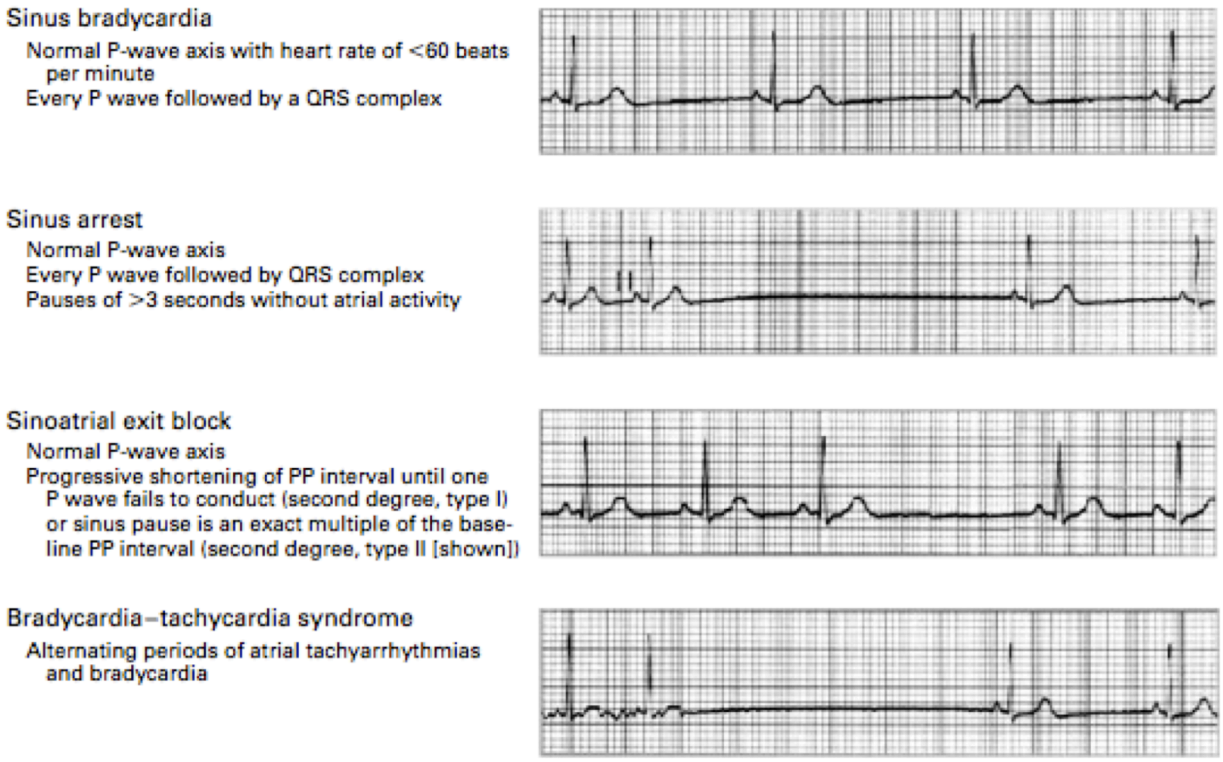

Figure 13
(Mangum and DiMarco, 2000) Types of bradycardia induced by sinus node dysfunction. Sinus bradycardia, sinus arrest and bradycardia-tachycardia syndrome have been observed in this study.

Ictal and Post-Ictal Tachycardia

This study also showed ictal and post-ictal tachycardia. Tachycardia is caused by activation of the sympathetic nervous system. Noradrenalin activates β1-adrenoceptors, leading to an increase in sodium and calcium currents in the pacemaker cells, via protein kinase C activation of the Funny channel and T-type calcium channel, respectively (Barrett et al., 2010; Richerson, 2009; Stanfield, 2011). This increase in sodium and calcium current increases the slope of the pacemaker potential, in turn increasing the frequency of action potentials in the pacemaker cell.

The hormone adrenaline also causes the heart rate to increase via the same mechanism as

noradrenaline (see above). Adrenaline is released from the adrenal medulla as a result of sympathetic stimulation via the preganglionic sympathetic neuron (Barrett et al., 2010). This generally acts to reinforce the direct sympathetic stimulation of the SAN, and could be responsible for the slow acceleration and long duration of the increase in heart rate seen in some seizures (Figure 11B, C, F).

The sympathetic and parasympathetic systems have opposite effects to each other. Therefore, the possible explanations of the bradycardia above could also be used to explain the tachycardia observed. If there was a decrease in the ABP, the baroreceptor reflex will initiate an increase in the heart rate and likewise with a low partial pressure of oxygen and increased breathing rate (Barrett et al., 2010; Richerson et al., 2009; Stanfield, 2011). These causes of tachycardia can be analysed by exploring the animal's blood pressure and breathing pattern and circulating noradrenalin levels during seizure.

Tachycardia is also induced by exercise. During the epileptic seizures induced by TeNT, the animals experienced unilateral or bilateral forelimb clonus, rearing, falling and in two animals, wild running. The enhanced muscle activity during these actions could result in tachycardia. Local vasodilation brought about by changes to the chemistry of the interstitial fluid of the muscle would induce a decrease in total peripheral resistance and fall in blood pressure, activating the baroreceptor reflex (Boulpaep, 2011). This needs to be explored further to identify whether the tachycardia observed is a result of the increased muscle activity, from autonomic dysfunction originating from the brain or from a combination of the two.

Ictal Bradycardia versus Ictal Tachycardia

Currently, no correlation between the epileptic foci and the ictal cardiovascular effects has been established; it remains unclear as to why some seizures induce tachycardia, some induce

bradycardia and others induce both (Sevcencu and Struijk, 2010). The *lateralisation hypothesis* proposes that seizures with a right-sided epileptic focus induce tachycardia, and those with left-sided epileptic focus induces bradycardia. Evidence for this hypothesis, however, is limited (Leutmezer et al., 2003; Mayer et al., 2004; Tinuper et al., 2001) with others not able to substantiate such a theory (Nei et al., 2000; Opherk et al., 2002).

Several authors have suggested that instead of a simple right-left dichotomy, seizure discharges may activate sympathetic or parasympathetic centres, inducing tachycardia or bradycardia (Leutmezer et al., 2003). Furthermore, it has been shown that parasympathetic activation predominates during partial seizure (Devinksy, 2004). To elucidate whether such selective activation occurs, or whether the cardiovascular effect is determined by the type of seizure, depth electrodes need to be placed into the hippocampus and potentially into the medulla, to explore the activity of the NA (parasympathetic) and vasomotor area (sympathetic). However, this could be difficult due to the anatomical location of these two structures within the medulla. Therefore, the autonomic balance could be assessed with the non-invasive measure of heart rate variability (see below).

Cardioarrhythmias

Cardioarrhthymas are themselves linked to sudden death in the general population, with the majority of sudden deaths caused by acute ventricular tachyarrhythmias (Huikuri et al., 2001). Missed beats were observed in 45 (59%) of the 76 seizures analysed with ventricular premature depolarisations (VPD; during 22 seizures; 22%) and ventricular fibrillation (VF; during 13 seizures; 17%) also observed. These arrhythmias are consistent with those expected by excessive autonomic activity observed in other studies (Nei et al., 2000; Opherk et al., 2000). These studies both reported that generalised seizures in humans are a risk factor for ECG abnormalities. Generalised

seizures have been identified as a risk factor for arrhythmia generation and as a risk factor for SUDEP (Donner, 2011; Nei et al., 2000). It was not possible to classify the seizures analysed in this study as generalised or partial due to the nature of the EEG, and so, correlation between the ECG abnormalities observed and the type of seizure could not be done. However, it could be assumed that those observed seizures were secondary generalised, as partial seizures would stay close to the ventral hippocampus, and perhaps circulate through the corresponding entorhinal cortex, and would not be picked up by the EEG. Increasing the number of channels used to record the EEG and also corroborating any epileptic activity with video files would aid in classifying seizures. While video recording was attempted in this study, technical issues prevented its use.

Seizures could act through two mechanisms to produce the arrhythmias seen in this study. Firstly, seizure-induced increased autonomic activity may cause arrhythmia generation (Nei et al., 2000) Secondly, repeated seizure may have a deteriorating effect on the heart; through repeated autonomic stimulation, with myocardial fibrosis and myofibrillar degeneration caused by catecholamine toxicity (Nei et al., 2000; Reichenbach and Benditt, 1970). Both myocardial fibrosis and myofibrillar degeneration have been shown in SUDEP patients (Earnest et al., 1992; Natelson et al., 1998) and could cause arrhythmia generation (Nei et al., 2000). The presence of myocardial fibrosis and myofibrillar degeneration in the animals used in this study will be explored by post-mortem histological investigation.

Previous studies have analysed the ECG waveform, analysing the QTc interval (Damasceno et al., 2013; Nei et al., 2000) and the ST-segment (Nei et al., 2000; Opherk et al., 2002; Zijlmans et al., 2002). Both long QT syndrome and ST-segment elevation have been associated with arrhythmia generation and sudden death (Barrett et al., 2010). Therefore, such analysis could provide information as to the origin of the arrhythmias observed in this study.

Method Refinement

During this study, four of the animals (JJ130701, JJ130702, JJ130703 and JJ130704) had to be killed before the desired end-point. This was due to each of them opening their abdominal wounds and exposing the electrical wires. Under the terms of the Home Office Licence, each wound could be repaired once, however each animal continued to open the wound. The other four animals did not have any issues with their wounds. The major difference between the two sets of animals was their size at the initial surgery (average weight: 277 g versus 243 g). The latter set of animals was younger, and had thinner skin. This could have meant that they were irritated by the subcutaneous wires and therefore scratched at their wounds more. Using larger rats in the future could avoid this issue.

In order to avoid this in future experiments a number of refinements to the surgery can be made in order to (1) avoid the animals opening their wounds and (2) prevent them from chewing on the electrode wires. Firstly, smaller incisions could be made; therefore limiting the number of sutures required and promoting the healing process. However, the size of the telemeter reduces the extent to which this is practical. Secondly, surgical mesh could be placed subcutaneously over the wires. This mesh is ordinarily used to repair hernias and therefore using it in this instance could promote wound healing and limit the irritation caused. Thirdly, the electrode wires could be placed away from the wound in order to reduce mechanical stress from the wire and prevent the animal from chewing through the wires if it were to open its abdominal wound. Finally, each of the younger set of animals was immediately housed with their companion rat, and this could have contributed to the wound breakdown. Housing the animals alone for a short time after the surgery may allow sufficient healing to occur before socialisation, preventing the wound breakdown observed.

Future Directions

There are a number of future investigations that need to be undertaken in order to elucidate the mechanism by which the ictal bradycardia and tachycardia were produced. Firstly, performing simultaneous recordings of the EEG, ECG, blood pressure and breathing rate will provide information of the origin of the change in heart rate. By implanting EMG electrodes to record the breathing rate, it could also be possible to identify and remove EMG artefacts from ECG recording. However, in order for all of these parameters to be recorded simultaneously, a more sophisticated radiotelemeter system would need to be employed. The DSI system has the ability to have more channels, whereas the TR system does not, however the sampling frequency is comparably low (50-100 Hz) to the TR system (1000 Hz) and does not give the resolution required to explore the subtle changes in the ECG waveform, such as QTc interval and ST segment, which are required for arrhythmia analysis.

Autonomic tone could be analysed via heart rate variability. This sophisticated and non-invasive technique calculates balance between sympathetic and parasympathetic activity by analysing the R-R interval and the variation within the intervals in the epoch analysed. A number of calculations of HRV have been developed such as the Root Mean Square of Successive RR Intervals (RMSSD, see below), which measures high-frequency vagus-mediated HRV, with a low HRV highly correlated with sudden death risk (Rauscher et al., 2011). A further technique, Fast Fourier Transformation, can be used to analyse the different frequency components of the heart rate and identify the autonomic mechanism that controls it (see Fazan Jr. et al., 2011)

$$\mathbf{RMSSD} = \sqrt{\frac{1}{\mathbf{N}-1}\sum_{j=1}^{N-1}(\mathbf{RR}_{j+1}-\mathbf{RR}_j)^2}$$

Initially, this study was going to explore heart rate variability, however due to the complexity of the changes that occur during seizure this was not possible. The number of missed

beats that were observed during seizure meant that analysing the heart rate variability of ictal periods was not possible. These missed beats were dramatic, with 45 (59%) of the 76 seizures analysed displaying them, and could be lethal. This measure could, however, be employed to analyse the interictal, pre-ictal and post-ictal periods.

Furthermore, in the current study only those seizures that were picked up by the EEG electrodes could be identified. JJ130703 exhibited wild running, a manifestation of epileptic activity in the brainstem, however the EEG did not pick up any seizure-like activity. The animal could have been experiencing partial seizures in the brainstem or hippocampus that failed to generalise and therefore was not picked up by the EEG electrodes. By placing electrodes into these structures, seizure activity could be recorded however small. This would allow a comparison between partial and generalised seizures and their effect on the cardiovascular system in the TeNT model of TLE, exploring the differences already reported (Sevcencu and Struijk, 2010).

Post-mortem analysis of the animals' heart will also clarify the origins of the arrhythmias seen in this study. SUDEP has been associated with dilated and enlarged hearts in humans (Scorza et al., 2009) therefore, investigating the TeNT injected animals' hearts and comparing them with PBS injected controls could add weight to the use of the model for investigating SUDEP. Further, analysing the SAN for myocardial fibrosis and myofibrillar degeneration or evidence of ischemia in the heart could help identify changes induced by repeated seizure.

Furthermore, the effect of pharmacological agents during seizure could also be analysed. Firstly, the effects of the anti-epileptic drugs carbamazepine and lamotrigine could be explored, as they have both been associated with a higher risk of SUDEP due to their effect on cardiac function (Danielsson et al., 2005; Donner, 2011; Persson et al., 2003). This could elucidate whether or not these anti-epileptic agents have a detrimental effect on the rat cardiac function when injected with TeNT. Secondly, antiarrhythmic agents could be explored to identify whether or not the seizure-

induced arrhythmias could be prevented. Thirdly, after further exploration into the seizure-induced changes to the autonomic system, therapeutic agents such as antihypertensives, β-blockers, calcium channel blockers and methyl atropine could be used in order to counteract the detrimental changes in heart rhythm observed in this study.

Finally, each animal injected could be time-matched with a control animal. By having a control animal and the TeNT injected animal that are exposed to the same conditions would allow for a more complete and accurate analysis, especially as the housing environment suffered from excessive noise at times - which is known to adversely affect TeNT injected animals. This comparison was planned, however due to the complications with the later four animals it was not possible.

Conclusion

In conclusion, this study observed cardiovascular changes induced by epileptic seizure. Previous studies have shown that increased autonomic tone can contribute to potential lethal arrhythmia generation (a risk factor for SUDEP). This study has shown changes in autonomic tone with ictal bradycardia and tachycardia observed, and cardioarrhythmia generation. This shows that the tetanus toxin model of epilepsy could be used to explore SUDEP, however the extent of the autonomic changes observed and the mechanism of this change needs to be explored further.

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