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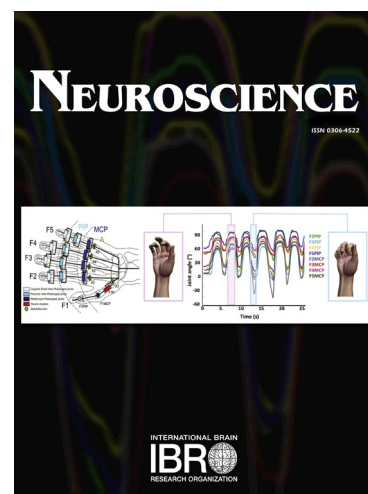
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Intrathecal application of the microglial inhibitor minocycline attenuates
sympathoexcitatory and proarrhythmogenic changes in rats with chronic temporal
lobe epilepsy

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ACCEPTED MANUSCRIPT

List of Abbreviations:

SUDEP:	sudden unexpected death in epilepsy
PACAP:	pituitary adenylate cyclase-activating polypeptide
IML:	intermediolateral cell column
RVLM:	rostral ventrolateral medulla
SNA:	sympathetic nerve activity
SE:	status epilepticus
QT interval:	a measure of the time between the start of the Q wave and the end of the T wave in the heart's electrical cycle
KA:	kainic acid
TLE:	temporal lobe epilepsy
Post-SE:	post-status epilepticus
IT:	intrathecal
ECG:	electrocardiogram
O.D.:	outer diameter
HR:	heart rate
PBS:	phosphate-buffered saline
TH:	tyrosine hydroxylase
CD:	cluster of differentiation
Iba1:	iodinised calcium binding adaptor molecule-1
AUC:	area under curve
MAP:	mean arterial pressure
PaCO ₂ :	partial pressure of carbon dioxide
QTc:	corrected QT interval
ir:	immunoreactive

Abstract:

The incidence of sudden unexpected death in epilepsy (SUDEP) is highest in people with chronic and drug resistant epilepsy. Chronic spontaneous recurrent seizures cause cardiorespiratory autonomic dysfunctions. Pituitary adenylate cyclase-activating polypeptide (PACAP) is neuroprotective, whereas microglia produce both pro- and anti- inflammatory effects in the CNS. During acute seizures in rats, PACAP and microglia produce sympathoprotective effect at the intermediolateral cell column (IML), whereas their action on the presympathetic rostral ventrolateral medulla (RVLM) neurons mediates proarrhythmogenic changes. We evaluated the effect of PACAP and microglia at the IML on sympathetic nerve activity (SNA), cardiovascular reflex responses, and electrocardiographic changes in the post-status epilepticus (SE) model of acquired epilepsy, and control rats. Chronic spontaneous seizures in rats produced tachycardia with profound proarrhythmogenic effects (prolongation of QT interval). Antagonism of microglia, but not PACAP, significantly reduced the SNA and the corrected QT interval in post-SE rats. PACAP and microglia antagonists did not change baroreflex and peripheral or central chemoreflex responses with varied effect on somatosympathetic responses in post-SE and control rats. We did not notice changes in microglial morphology or changes in a number of M2 phenotype in epileptic nor control rats in the vicinity of RVLM neurons. Our findings establish that microglial activation, and not PACAP, at the IML accounts for higher SNA and proarrhythmogenic changes during chronic epilepsy in rats. This is the first experimental evidence to support a neurotoxic effect of microglia during chronic epilepsy, in contrast to their neuroprotective action during acute seizures.

Keywords:

Microglia, Sympathoexcitation, Temporal lobe epilepsy, Rats, Proarrhythmogenic, PACAP

Introduction:

Epilepsy is a chronic brain disorder characterised by spontaneous recurrent seizures and carries a risk of sudden death that is 15-20 times higher than in normal population (Ficker et al., 1998; Nilsson et al., 1999; Eastaugh et al., 2015). Epilepsy affects about 50 million people worldwide (WHO, 2005); seizures can range from brief, barely noticeable loss of attention to major convulsions that affect the entire neuraxis. Epilepsy is associated with changes in autonomic functions, such as sympathovagal imbalance, sympathetic reflex dysfunction, tachycardia with concomitant arrhythmia or bradycardia with associated apnoea (Dütsch et al., 2006; Bateman et al., 2008; Ponnusamy et al., 2012; Massey et al., 2014; Powell et al., 2014b; Bhandare et al., 2015; Bhandare et al., 2016a). Seizure associated autonomic cardiorespiratory changes are well-documented and are thought to play an important role in a mechanism of sudden unexpected death in epilepsy (SUDEP) (Nei et al., 2004; Dlouhy et al., 2015). Interictal autonomic changes are also seen in patients with chronic epilepsy (Ansakorpi et al., 2000; Berilgen et al., 2004; Müngen et al., 2010; Lotufo et al., 2012). Nevertheless, the neuronal mechanisms causing autonomic cardiorespiratory dysfunction during chronic epilepsy are unknown.

Pituitary adenylate cyclase-activating polypeptide (PACAP), a 38 amino acid pleiotropic neuropeptide, produce neuroprotective effects (Shioda et al., 1998; Ohtaki et al., 2006; Bhandare et al., 2015) that are partly mediated through its action on microglia (Wada et al., 2013; Brifault et al., 2015). PACAP and microglia have a protective effect on sympathetic preganglionic neurons at the intermediolateral cell column (IML), where they ameliorate the sympathoexcitatory effect of acute seizures (Bhandare et al., 2015). During acute seizures, PACAP and microglia act on presympathetic rostral ventrolateral medulla (RVLM) neurons in the brainstem to promote proarrhythmogenic changes, but not sympathoexcitation (Bhandare et al., 2016a). In many cardiovascular autonomic nuclei PACAP is pressor and sympathoexcitatory (Farnham et al., 2008; Farnham et al., 2011; Inglott et al., 2011) and changes baroreflex response in trout (Lancien et al., 2011) but not in rats (Farnham et al., 2012). PACAP expression is increased in central autonomic nuclei (paraventricular nucleus) after kainic acid (KA)-induced seizures in rats (Nomura et al., 2000). Secondly, seizures produce microglial activation, and neuroinflammation

in patients and animal models (Beach et al., 1995; Shapiro et al., 2008; Eyo et al., 2014), which persist for many years during chronic epilepsy (Beach et al., 1995; Papageorgiou et al., 2015). Microglia can be pro- or anti- inflammatory in animal models of temporal lobe epilepsy (TLE) (Shapiro et al., 2008; Mirrione et al., 2010; Vinet et al., 2012; Devinsky et al., 2013). Although the pro- or anti- inflammatory state of activated microglia is a topic of debate, there is strong support for their dual role (Hanisch and Kettenmann, 2007). Short term microglial activation is considered beneficial (Mirrione et al., 2010; Vinet et al., 2012; Szalay et al., 2016), whereas chronic microglial activation is deleterious, and produces a damaging response to injury (Qin et al., 2007; Loane et al., 2014; Olmos-Alonso et al., 2016). During KA-induced acute seizures, spinal microglia have a protective effect on sympathetic preganglionic neurons (Bhandare et al., 2015), however, their role in chronic epilepsy is not known.

Thus, the aims of this study were to identify the role of PACAP and microglia in the spinal cord, during chronic epilepsy, in the regulation of central autonomic cardiovascular activity. To achieve these aims we used a model of acquired epilepsy in rats that manifest spontaneous seizures and many features of acquired epilepsy in humans— the KA-induced post-status epilepticus (post-SE) model (Morimoto et al., 2004; Powell et al., 2008; Jupp et al., 2012). The effect of intrathecal (IT) infusion of the PACAP antagonist, PACAP(6-38), and the microglial antagonist, minocycline, on sympathetic activity, cardiovascular reflex responses, and the electrocardiogram (ECG) were analysed in chronically epileptic and control rats. Microglial morphology and their phenotype in the vicinity of RVLM neurons were analysed with immunohistochemistry in epileptic and control rats.

Experimental Procedures:

Animals

The animal usage and protocols were in accordance with the Australian code of practice for the care and use of animals for scientific purposes. The protocols were approved by the Animal Care, and Ethics Committee of Macquarie University, The University of Melbourne, and the Sydney Local Health District. The epilepsy surgery and procedures were performed under isoflurane anesthesia on 17-19 weeks old

adult non-epileptic control ($n = 9$), and post-SE ($n = 15$) male Wistar rats, whereas electrophysiology experiments were performed under urethane anesthesia.

KA-induced post-SE rat model

The post-SE model of acquired epilepsy was generated by i.p. injection of the glutamate receptor agonist, KA, to induce a period of continuous seizure activity (status epilepticus) in non-epileptic rats as described previously (Hellier et al., 1998; Powell et al., 2008; Jupp et al., 2012; Powell et al., 2014b; Vivash et al., 2014). Twelve week old Wistar rats were injected with repeated low doses of KA (5mg/kg, i.p., followed by 2.5 mg/kg, i.p., injections once per hour) until SE behaviour was observed. After four hours of SE, all rats were given diazepam injection (5mg/kg i.p.) to terminate the SE. Rats were then returned to their home cages in the animal house and maintained with normal animal house care and diet.

Implantation of EEG-ECG electrodes in post-SE and control rats

Seven weeks after KA-induced SE ($n = 15$) (or saline administered controls ($n = 9$)), two ECG electrodes and four EEG electrodes were implanted in each rat under isoflurane anesthesia (5% during induction, 2.5-1.5% for maintenance) in oxygen (2.0 L/min during induction, 0.5-1.0 L/min for maintenance) as detailed in supporting data by (Powell et al., 2014b). Two small incisions were made to expose the thoracic muscle directly above the heart, and to expose the muscle overlying the xiphoid process of the sternum. The distal end of an ECG lead (13 cm; PlasticsOne, USA) was sutured to each muscle using polypropylene, 4-0 (SharpPoint, USA). The leads of both ECG electrodes were then tunneled up through the left side of the neck subcutaneously, and the skin layer was sutured (polyglycolic, 4-0; LOOK™, USA). A single midline incision was then made on the scalp to expose the skull. Two ECG leads were located and tunneled through the neck to allow protrusion of the leads out of the incision site on the scalp. Each rat was then placed into a stereotaxic frame, and four extradural electrodes comprised of gold-plated sockets attached to stainless steel screws (O.D. 1 mm; Plastics One, USA) were implanted into the skull: one on each side approximately 2 mm from the midline, 3 mm anterior to the Bregma, one at approximately the centre of the midline, and another 6 mm posterior to the Bregma, 4 mm right of the midline. The electrodes were fixed to the skull using dental cement (Vertex-Dental, The Netherlands), and the animals observed until recovery.

In vivo EEG-ECG recordings

One week after recovery from the surgery, a continuous 24 h video-EEG-ECG recording was acquired for one week using Compumedics EEG acquisition software (Profusion EEG 4 v4.3, Australia) digitised at 2048Hz as previously described (Powell et al., 2008; Powell et al., 2014b). Each recording was reviewed for seizure activity, and the start and end of a seizure was manually marked on the EEG to allow quantification of the number of seizures and seizure duration. Seizure activity on the EEG was defined as per our previous work, and that of others, as the development of high-amplitude, rhythmic discharges that clearly represented a new pattern of tracing (Kharatishvili et al., 2006; Bouilleret et al., 2011). This included repetitive spikes, spike-and-wave discharges, and slow waves. The event must have lasted at least 5 s and showed an evolution in the dominant frequency, and be accompanied by behavioural change observable on the video recording consistent with a seizure. Normally, all post-SE rats develop spontaneous seizures, and none were observed in control rats.

Non-invasive tail-cuff blood pressure recordings

A week after confirmation of spontaneous recurrent seizures in post-SE rats with video-EEG recordings and in age-matched controls, blood pressure was recorded with a non-invasive tail-cuff method (IITC Life Sciences Inc., California, USA) (Farnham et al., 2011). Rats were placed in an animal holder, pre-warmed at 32°C, and acclimatised to the chamber. All animals were kept in the chamber for 10-15 min; a cuff was attached to their tail, and blood pressure was recorded in triplicate and averaged. Heart rate (HR) and systolic, diastolic, and mean blood pressure was derived from the blood pressure waveform channel.

In vivo electrophysiology

General surgical procedure: Electrophysiology surgical procedures were carried out as described previously (Bhandare et al., 2015). Briefly, rats ($n = 24$) were anaesthetised with intraperitoneal injection of 10% urethane (ethyl carbamate; 1.3–1.5 g/kg; Sigma-Aldrich). The depth of anaesthesia was controlled by periodic tail/paw pinches and observing reflex responses (withdrawal or pressor >10 mmHg). When reflex responses were observed, an additional anaesthetic was injected (30-40 mg,

10% urethane intravenous). Bronchial secretion was prevented through administration of atropine sulphate (100 µg/kg, i.p.; Pfizer (Perth) Pty Limited, WA, Australia) with the first dose of anaesthetic.

The jugular vein and right carotid artery were cannulated for the administration of drugs and fluids, and for the recording of blood pressure, respectively. Tracheostomy enabled a mechanical ventilation. ECG was recorded through three lead electrodes, where two silver electrodes were inserted into the front paws and ground electrode into the exposed muscle on the back of rat. HR was derived from the peak of the R wave and drawn as an event channel. The head mounted EEG electrodes for *in vivo* EEG-ECG recordings enabled the recording of EEG signal. After bilateral cervical vagotomy rats were artificially ventilated (Ugo Basile, Italy) with 100% oxygen in order to silence the peripheral chemoreceptors. Rats were paralysed with pancuronium bromide (0.4 mg given as a 0.2 ml bolus i.v. injection) followed by an infusion of 10% pancuronium in 0.9% saline at a rate of 2 ml/h for the duration of the experiment. Rats were then secured in a stereotaxic frame and body temperature was maintained between 36.5 and 37.5°C until the end of experiment with the use of a rectal probe and a homeothermic blanket (TC-1000; CWE). Arterial blood gases and electrolytes were analysed with an electrolyte and blood gas analyser (IDEXX Vetstat, West Brook, USA), and pH was maintained between 7.35 - 7.45 and PaCO₂ at 40 ± 2.

IT catheter placement: The muscle layer attached to the occipital bone was carefully scraped with the cotton swabs to expose the membrane over the atlanto-occipital junction. The slit was made through the membrane and the surge of cerebrospinal fluid (CSF) provided the confirmation of correct site and was stopped by holding dry cotton swab. A polyethylene tubing (O.D. = 0.50 mm; I.D. = 0.20 mm; Microtube Extrusions Pty Ltd, NSW, Australia) with a dead space of ~6 µl was inserted through a slit in the dura into the IT space of all rats and advanced caudally to the level of T5/6.

IT drug administration protocol: In all groups, drugs were administered intrathecally 10 min after recording of baseline reflex responses and flushed in with 6µl of phosphate-buffered saline (PBS). In both post-SE (*n* = 5) and control (*n* = 3) rats, 10 µl of a control injection of 10 mmol/l PBS, 1 mmol/l PACAP(6-38) or

100µg/10µl minocycline was administered intrathecally. All infusions were made over a 10 to 15 s period, as previously described (Bhandare et al., 2015).

Isolation and preparation of nerves: The procedures were performed as described previously (Abbott and Pilowsky, 2009; Farnham et al., 2011; Inglott et al., 2011; Shahid et al., 2011). The left phrenic nerve and the left greater splanchnic sympathetic nerve at a site proximal to the coeliac ganglion were isolated and dissected. The dissected nerves were cut and the distal ends were tied with 5/0 silk thread. The isolated phrenic and splanchnic nerves were placed across a bipolar stainless steel electrodes and recorded. The nerves were electrically isolated either with paraffin oil or silgel. Signals were amplified (CWE, Incorporated, BMA-931 Bioamplifiers) (sampling rate: 6 kHz, gain: 2,000, filtering: 30-3,000Hz) and noise was removed with a 50/60-Hz line frequency filter (Humbug; Quest Scientific). The sciatic nerve was isolated at the mid-thigh, tied with 5/0 silk thread, and cut distally. The left aortic depressor nerve was isolated from the cervical vagus nerve at the level of the carotid bifurcation, tied with 5/0 silk thread and cut close to the chest.

Aortic depressor and sciatic nerve stimulation and chemoreflexes protocol:

The effect of aortic depressor and sciatic nerve stimulation on splanchnic sympathetic nerve activity (SNA) was assessed to estimate the baroreflex and somatosympathetic reflex function in post-SE and control rats in described previously (Miyawaki et al., 2001; Abbott and Pilowsky, 2009). Stimuli were generated by isolated stimulators controlled by a Spike2 script (version 8.03; Cambridge Electronic Design). Stimulus threshold was determined by increasing or decreasing the stimulus voltage until no response was observed. During each experimental protocol, the aortic depressor nerve was stimulated at 4 times threshold (1-10 V, 0.2-ms pulse width, 100 cycles at 1 Hz across 100 s), and the average SNA response was analysed before, and 60, 90 and 120 min after IT treatment (Fig. 1). The left sciatic nerve was stimulated to generate the somatosympathetic response. Stimulus threshold was determined as described above, and sciatic nerve was stimulated at 4 times threshold (1-5 V, 0.2 ms pulse width, 100 pulses at 0.4 Hz across 250 s), and the average response of SNA was analysed before, and 60, 90 and 120 min after IT treatment (Fig. 1).

Peripheral chemoreceptors were stimulated by ventilating animals with 10% O₂ in N₂ for 45 sec (Abbott and Pilowsky, 2009). Central chemoreceptors were stimulated by ventilating animals with 5% CO₂ / 95% O₂ for 3 min (Abbott and Pilowsky, 2009). The observed reflex responses are generated due to stimulation of central chemoreceptors as oxygenation of the blood was maintained throughout the challenge. Both the central and peripheral chemoreflex responses were generated prior to, and at 60 and 120 min after IT treatment (Fig. 1).

Collection of blood and plasma samples and catecholamine analysis: At the conclusion of the electrophysiology experiments, 3-4 ml blood was withdrawn from the carotid artery and collected in heparinised tubes containing metabisulphite. Blood samples were immediately centrifuged at 4°C for 10 min at 900g and plasma collected and stored at -20°C until analysis. Plasma adrenaline and noradrenaline were extracted onto activated alumina, eluted, and analysed with reverse phase HPLC and electrochemical detection.

Histology

Perfusions: One millilitre of heparin was injected in each rat through the venous line at the conclusion of the electrophysiology experiments, and then rats were perfused transcardially with 400 ml of ice-cold 0.9% saline followed by 400 ml of 4% paraformaldehyde solution. Brains were removed and post-fixed in the same fixative for 18-24 h.

Sectioning and immunohistochemistry: Immunohistochemical analysis was done, as described previously (Bhandare et al., 2016a; Kapoor et al., 2016c), in $n = 3$ rats from both post-SE and control groups treated with IT PBS. Brainstems were sectioned coronally (40 µm thick) with a vibrating microtome (Leica, VT1200S). Sections were sequentially collected into five different pots containing a cryoprotectant solution, and stored at -20°C until further processing. Histological procedures were performed on free-floating sections. Sections were rinsed, blocked, and incubated in primary antibodies for 48 h: rabbit anti-CD206 (1:2,000; Abcam, Melbourne, Victoria, Australia), goat anti-Iba1 (1:1,000; Novus Biologicals, Littleton, Colorado, USA) and mouse anti-tyrosine hydroxylase (TH) (1:100; Avanti Antibodies; (Nedoboy et al., 2016)). Sections were rinsed and CD-206 (cluster of differentiation (CD)-206 is a mannose receptor present on M2 phenotype of microglia), Iba1

(iodinised calcium binding adaptor molecule-1; a microglia/macrophage specific calcium binding protein) and TH (C1 neurons) immunoreactivity was subsequently revealed by overnight incubation with the following secondary antibodies at 1:500 dilutions (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA): Alexa Fluor 488-conjugated donkey anti-rabbit, Cy3-conjugated donkey anti-goat, and Cy5-conjugated donkey anti-mouse,. Sections were rinsed, mounted sequentially on glass slides, and coverslipped with Vectashield (Vector Laboratories, Burlingame, California, USA).

Data acquisition and analysis

Electrophysiology data: Data were acquired using a Spike2 acquisition and analysis software (version 8.06; Cambridge Electronic Design), and CED 1401 ADC system (Cambridge Electronic Design). The EEG data were recorded, amplified (CWE, Incorporated, BMA-931 Bioamplifier), band-pass filtered from 1 Hz to 10 kHz, and digitised at 20 kHz with a 100x gain. The EEG raw signal was DC removed and the “gamma” frequency range (25-45 Hz) was analysed, as shown previously (Bhandare et al., 2016a). The EEG “gamma” frequency range was analysed from 5 min blocks taken 1 min before IT treatment and 60 and 120 min post-treatment. The EEG data was also manually analysed in a blinded manner for detection of seizure activity as explained above. Phrenic nerve activity was rectified and smoothed (τ 0.5 s), and analysed from 1-min blocks taken 1 min before, and 60 and 120 min after IT treatment. The phrenic nerve activity area under curve (AUC) was analysed at 60 and 120 min after IT treatment. The percent change in the AUC at 60 and 120 min were compared with the pre-treatment area that was considered as 100%. SNA was rectified and smoothed (τ 2 s), and normalized to zero by subtracting the residual activity 5-10 min after death or after nerve pinch. The integrated SNA trace was calibrated (baseline as 100% and death level as 0%) and analysed for AUC between 60 to 120 min after IT PBS, PACAP(6-38) or minocycline infusion. Mean arterial pressure (MAP) and HR were analysed from 1-min blocks taken 1 min before, and 30, 60, 90 and 120 min after IT infusion (only 120 min results are shown in graphs). Baroreflex and somatosympathetic reflex responses were analysed before, and 60, 90 and 120 min after IT treatment (Fig. 1). The percent change in baroreflex response (AUC) was calculated considering pre-treatment response as 100%. The somatosympathetic reflex response was analysed with a sigmoid curve fit analysis. A

sigmoid curve was fitted to averaged waveform of somatosympathetic response curve (both fast conducting A-fibre and slow conducting C-fibre response). The low, high, range, and slope values were calculated (only range is shown in the graph). The pre-treatment range is considered as 100% response, and percent change is calculated at 60, 90 and 120 min post-treatment. Peripheral and central chemoreflex responses were analysed as percent change in the SNA AUC at 60 and 120 min post-treatment compared to the pre-treatment response (considered as 100%).

Core body temperature, and end-tidal CO₂ were analysed from 1-min blocks taken 1 min before and 30, 60, 90, and 120 min after IT injection. In all animals, arterial blood gas levels (PaCO₂) and pH were measured 10 min before the start of the protocol, and 120 min after IT treatment. Statistical analysis was carried out in GraphPad Prism software (version 6.05). Statistical significance was determined using one-way ANOVA followed by t-tests with the Holm-Šídák correction. Multiple comparisons were done between groups. $p \leq 0.05$ was considered significant.

Calculation of corrected QT (QTc) interval: ECG raw data was processed (DC remove), wherever baseline fluctuations were prominent. QT, PR, and RR intervals were analysed from the ECG recordings. Bazett formula was used for calculation of QTc interval, where the QT interval in seconds was divided by the square root of the R-R interval in seconds (Bazett, 1920). The QTc was obtained before, and 120 min after IT injection. The QTc and PR interval statistical analysis was carried out in GraphPad Prism software (version 6.05). Statistical significance was determined using one-way ANOVA between treatment groups followed by t-tests with the Holm-Šídák correction. Multiple comparisons were done between groups. $p \leq 0.05$ was considered significant.

Histology imaging and analysis: Acquisition and analysis of histology images is performed as described previously (Bhandare et al., 2016a; Kapoor et al., 2016b; Kapoor et al., 2016c). In brief, all images were acquired, at 20X and 40X magnifications, using a Zeiss Axio Imager Z2 (Zeiss, Germany). A 0.16 mm² box was then placed within the imaged RVLM, and this area was used for analysis. The branch length and a number of endpoint processes of Iba1-labelled microglial cells in the vicinity of TH-labelled RVLM neurons was analysed using ImageJ plugin software. Statistical analysis was carried out using GraphPad Prism (version 6.05) for

chi-square test for goodness of fit. The proportions of CD206 labelled anti-inflammatory M2 microglia in the RVLM of post-SE rats were compared with control group. Statistical significance was determined using non-parametric Mann-Whitney test (Sokal and Rohlf, 2012).

Results:

Development of spontaneous recurrent seizures in post-SE rats

A typical spontaneous recurrent seizure with transition into ictal period is shown in Fig. 2, which is characterised by high-amplitude and showed a clear new pattern of tracing. Video-EEG-ECG recordings confirmed that 9 weeks after induction of KA-induced SE, almost all rats developed spontaneous recurrent seizures with 0.44 ± 0.07 seizures per day (range 0-3) and with duration of 19.7 ± 3.3 sec per day (Table 1). The seizure frequency and scores are highly variable across the post-SE rats as shown in Table 1. Whilst some of the post-SE animals may not have had seizures during recording period (a week), and is just could be due to the low frequency. None of the control rats (saline treatment) developed spontaneous seizures.

Chronic seizure-induced tachycardia and HR variability changes

The *in vivo* EEG-ECG recordings in conscious chronic epileptic rat revealed that spontaneous seizure causes a dramatic tachycardia (~ 500 bpm (beats per minute) compared with ~ 300 bpm during pre-ictal period; Fig. 3A) and was characterised by a significant variability. The HR variability was increased from 91.8% to 98.2% in post-ictal period (Fig. 3B-E). HR was significantly unstable for more than one hour after the spontaneous seizure. These changes would contribute to the development of unstable, and possibly lethal cardiovascular abnormalities.

MAP, HR and plasma catecholamines in post-SE and control rats

Non-invasive tail-cuff blood pressure measurements confirmed that there were no significant differences in MAP and HR interictally between post-SE ($n = 15$), and control ($n = 9$) rats (Table 2). The MAP was 125.8 ± 4.5 mmHg in post-SE rats compared to 129.3 ± 3.9 mmHg in controls (Table 2). Moreover, the systolic and diastolic blood pressure values were not significantly different between these two

groups (data not shown). The HR was 310 ± 8 bpm and 326 ± 10 bpm in post-SE and control rats, respectively (Table 2). Plasma catecholamine levels (adrenaline and noradrenaline) were also not significantly different between the groups. The plasma adrenaline was 1.6 ± 0.3 nmol/L and 1.9 ± 0.5 nmol/L in post-SE and control rats, respectively (Table 2). The noradrenaline concentration was also similar in both groups of rats; 3.4 ± 0.5 nmol/L in post-SE, and 3.9 ± 0.8 nmol/L in controls (Table 2).

Inhibition of microglial activation, but not PACAP, attenuates higher SNA and proarrhythmogenic changes in rats with spontaneous recurrent seizures

Our results showed that in chronically epileptic post-SE rats, the antagonism of microglial activation with minocycline at IT level significantly reduced SNA ($\Delta -25.0 \pm 10.0\%$; $p \leq 0.05$) compared to PBS-treated group ($\Delta 10.0 \pm 7.7\%$) (Fig. 4AI). More interestingly, IT minocycline treatment in control rats ($\Delta -17.1 \pm 18.5\%$) has a similar effect to PBS-treated control rats ($\Delta -17.8 \pm 19.8\%$) (Fig. 4BI). IT PACAP(6-38) does not affect SNA activity in post-SE rats ($\Delta -15.3 \pm 10.1\%$) compared with PBS treatment (Fig. 4AI). Similar results were obtained in control rats where changes in SNA were non-significant in IT PACAP(6-38) ($\Delta -18.5 \pm 8.1\%$), and PBS treatments (Fig. 4BI). MAP and HR remained unchanged in both post-SE, and control rats treated with IT PBS, PACAP antagonist, and microglia antagonist (Figs. 4AII-III and 4BII-III). In post-SE rats, the HR changes were similar in PBS ($\Delta 0 \pm 9$ bpm), PACAP(6-38) ($\Delta -8 \pm 5$ bpm) and minocycline ($\Delta 1 \pm 8$ bpm) treated rats (Fig. 4AIII). There was a similar pattern in control rats (Fig. 4BIII) treated with PBS ($\Delta 3 \pm 14$ bpm), PACAP(6-38) ($\Delta 7 \pm 10$ bpm) and minocycline ($\Delta 1 \pm 7$ bpm). MAP was found to be unaltered in post-SE (Fig. 4AII) and control groups of rats (Fig. 4BII) treated with PBS ($\Delta 0.0 \pm 2.0$ mmHg and $\Delta 6.5 \pm 4.3$ mmHg, respectively), PACAP(6-38) ($\Delta 1.3 \pm 6.7$ mmHg and $\Delta 4.7 \pm 1.4$ mmHg, respectively), and minocycline ($\Delta -4.3 \pm 4.2$ mmHg and $\Delta 13.7 \pm 8.4$ mmHg, respectively).

The ECG findings in post-SE rats treated with IT minocycline showed a reduction in QTc interval duration (Figs. 5AI, 5CIII). In post-SE rats, the QTc interval was significantly reduced at 120 min after minocycline treatment compared to the pre-treatment period ($\Delta -3.0 \pm 0.5$ ms; $p \leq 0.05$). The representative Poincare plot (Fig. 5CIII) clearly shows that IT minocycline treatment significantly reduced the QT interval. At 120 min post-treatment, the QTc interval in IT PBS and PACAP(6-38)

treated groups did not change compared to the pre-treatment period ($\Delta 1.7 \pm 1.1$ ms and $\Delta 0.5 \pm 1.3$ ms, respectively; Fig. 5AI) (representative Poincare plot Fig. 5CI-II). In control rats, none of the treatments (IT PBS, PACAP(6-38) or minocycline) had a significant effect on QTc interval (Fig. 5BI). The IT PBS, PACAP(6-38) and minocycline do not alter the PR interval values in post-SE and control rats (Figs. 5AII and 5BII).

We did not observe any changes in EEG activity, phrenic nerve activity, expired CO_2 or body temperature in any of the groups (results not shown). EEG data during electrophysiology experiments revealed that none of the post-SE or control rats had spontaneous seizures under anaesthetised experimental conditions. Blood gas analysis confirmed that PaCO_2 and pH were within the normal physiological range (PaCO_2 was 40 ± 2 and pH between 7.35-7.45) throughout the experiments.

Neither microglia nor PACAP antagonists alter baroreflex, peripheral or central chemoreflex responses in epileptic post-SE or control rats, with varied effects on somatosympathetic responses

A typical cardiovascular reflex response is shown in Figure 1A. The protocol involved stimulation of aortic depressor nerve (baroreflex; Fig. 1B), stimulation of sciatic nerve (somatosympathetic; Fig. 1C), peripheral chemoreflex (Fig. 1D) and central chemoreflex responses (Fig. 1E), which were repeated at 60 and/or 90 and 120 min after IT treatment. Baroreflex responses were unaffected with the antagonism of PACAP or microglia in both post-SE, and control rats at 60 min, 90 min, and 120 min post-treatment (Figs. 6AI and 6BI). 120 min after IT treatments, the $\% \Delta$ AUC of baroreflex response was unchanged in post-SE rats and control rats treated with PBS ($\Delta 0.6 \pm 1.7\%$ and $\Delta 3.1 \pm 0.3\%$, respectively), PACAP(6-38) ($\Delta 2.0 \pm 7.9\%$ and $\Delta -1.0 \pm 0.5\%$, respectively), and minocycline ($\Delta 1.3 \pm 2.6\%$ and $\Delta 1.3 \pm 1.5\%$, respectively).

The IT treatments in both post-SE and control rats produced varied effects on somatosympathetic reflex responses (Figs. 6AII-III and 6BII-III). Stimulation of sciatic nerve in post-SE rats treated with PBS showed significant decrease in fast (A-fibre; Fig. 6AII), and slow (C-fibre; Fig. 6AIII) conducting nerve fibre somatosympathetic responses over the time course ($\Delta -36.0 \pm 8.3\%$ and $\Delta -53.6 \pm 14.3\%$, respectively at 120 min; $p \leq 0.05$). In post-SE rats, the A- and C-nerve fibre somatosympathetic

responses were unaffected by IT PACAP(6-38) ($\Delta -29.8 \pm 17.5\%$ and $\Delta -40.8 \pm 18.4\%$, respectively at 120 min) and minocycline ($\Delta -37.4 \pm 16.3\%$ and $\Delta -43.8 \pm 21.9\%$, respectively at 120 min) treatment (Figs. 6AII-III). At 60, 90 and 120 min post-treatment, the fast conducting A-fibre somatosympathetic response was decreased in control rats treated with minocycline ($\Delta -61.0 \pm 9.9\%$, at 120 min; $p \leq 0.001$; Fig 6BII), whereas slow conducting C-fibre response was significantly reduced in rats treated with IT PBS ($\Delta -55.2 \pm 9.6\%$, at 120 min; $p \leq 0.001$) and PACAP(6-38) ($\Delta -49.0 \pm 3.1\%$, at 120 min; $p \leq 0.01$) (Fig. 6BIII).

The peripheral and central chemoreflex responses in post-SE, and control rats treated with IT PBS, PACAP(6-38), and minocycline were not altered at 60 or 120 min post treatment (Fig. 7). In post-SE rats, peripheral (Fig. 7AI) and central (Fig. 7AII) chemoreflex responses were unchanged in IT PBS ($\Delta 2.5 \pm 10.0\%$ and $\Delta 13.3 \pm 3.7\%$, respectively), IT PACAP(6-38) ($\Delta 3.9 \pm 3.7\%$ and $\Delta -1.1 \pm 3.1\%$, respectively), and IT minocycline ($\Delta 5.8 \pm 2.9\%$ and $\Delta 1.9 \pm 3.3\%$, respectively) treated groups. The similar trend was noted in control rats where change in peripheral (Fig. 7BI) and central (Fig. 7BII) chemoreflex responses were $\Delta 3.7 \pm 1.4\%$ and $\Delta 3.2 \pm 1.1\%$ in IT PBS groups, respectively, $\Delta 5.7 \pm 4.8\%$ and $\Delta -10.8 \pm 10.3\%$ in IT PACAP(6-38) groups, respectively, and $\Delta 8.8 \pm 10.0\%$ and $\Delta 6.1 \pm 1.8\%$ in minocycline-treated groups, respectively.

During spontaneous recurrent seizures, microglia are in surveillance state in the vicinity of the RVLM neurons

The morphological analysis of microglia was carried out in post-SE and control rats ($n = 3$) in the vicinity of the RVLM neurons. The brainstem sections that contain TH-immunoreactive (ir) RVLM neurons, were labelled with CD206-ir labelled anti-inflammatory M2 phenotype of microglia, and Iba1, which is a marker for all microglia. The morphological analysis demonstrated that TH-ir neurons were surrounded with typical resting microglial cells in both groups (Fig. 8A-B). In both groups microglia appeared with a round cell body, and normal long processes with few ramifications (Fig. 8A-B). Total number of microglia in each group are shown in Table 3. Activated microglia were identified with the analysis of branch length and a number of endpoint processes. Mean branch length and a number of endpoint processes of Iba1 labelled microglia were not significantly different between vehicle

control and epileptic rats (Table 3). The proportion of anti-inflammatory M2 phenotype of microglia was $12.91 \pm 3.95\%$ in post-SE rats, which was similar in controls ($8.89 \pm 2.14\%$; Table 3). These findings revealed that microglia are in a surveillance state and there are no differences in their morphology and proportion of M2 phenotype, at least in the RVLM, between epileptic and control groups.

Discussion:

The study provides direct evidence that microglia play a role in mediating increased SNA, and arrhythmogenic cardiac electrophysiological changes in chronic epileptic rats. First, spontaneous seizures cause severe tachycardia with prolongation of QT interval that persisted for more than one hour after the onset of a seizure. Secondly, antagonism of microglial activation, but not PACAP, in the spinal cord significantly reduces the SNA and seizure-induced prolongation of QTc interval in epileptic rats. Control groups showed no significant changes, however large variations could be attributed to sample size. Thirdly, neither spinal PACAP nor microglia regulate baroreflex or peripheral and central chemoreflex responses in epileptic and control rats, whereas PACAP or microglia antagonist decreases either A- or C-fibre somatosympathetic response in control rats and has no effect in post-SE rats. Fourthly, interictally epileptic rats, and controls, were normotensive with the plasma catecholamine levels in a normal range. Finally, our findings show that morphologically, microglia were in a surveillance stage in chronically epileptic rats with no difference in the number of a M2 phenotype compared with controls.

Microglia are the resident immune cells of the CNS, contributing ~10% of the total brain cell population. They respond to all types of pathological stimulus, including seizures, through activation, and adoption of either a 'pro-inflammatory M1' or 'anti-inflammatory M2' phenotype (Li et al., 2007; Lai and Todd, 2008; Loane and Byrnes, 2010; Kapoor et al., 2016a). Epileptic seizures cause extensive microglial activation in patients (Beach et al., 1995), and in animal models (Drage et al., 2002), but there is a considerable controversy surrounding the pro- (Shapiro et al., 2008) or anti-inflammatory (Mirrione et al., 2010; Eyo et al., 2014) role of microglia during and following a seizure. Previous findings demonstrated that during acute seizures, microglia protect sympathetic preganglionic neurons from excitotoxicity (Bhandare et al., 2015). Our current findings show that the SNA and prolongation of QTc interval

following spontaneous seizures were significantly reduced in epileptic rats, when the microglial activation was inhibited at the IML. These novel findings strengthen the concept that during chronic pathological insult, such as spontaneous recurrent seizures, maladaptive responses of microglia may lead to a “deleterious” activation state (M1) that triggers the release of pro-inflammatory cytokines such as IL-1 β and TNF α (Benson et al., 2015), in contrast to their beneficial activation during acute insult (Fig. 9). This concept has been proposed by others (Hanisch and Kettenmann, 2007; Gao et al., 2011; Eyo et al., 2017), and is supported by previous studies (Bhandare et al., 2015; Olmos-Alonso et al., 2016). During acute seizure, microglia can acquire a “beneficial” activation state (M2) to protect an overexcited neurons, and restore the normal homeostatic condition to limit the further damage (Fig. 9), whereas pharmacological targeting of colony-stimulating factor 1 receptors during late (chronic) phase in transgenic mice inhibits microglial proliferation and prevents the progression of Alzheimer’s-like pathology (Olmos-Alonso et al., 2016).

Minocycline inhibits p38 mitogen-activated protein kinase and acts as a microglia antagonist (He et al., 2001; Tikka et al., 2001; Ueno et al., 2013). Minocycline does not produce significant effect on the neuronal activity (Wu et al., 2002; Ueno et al., 2013) or SNA (Bhandare et al., 2015) and any possible effect is nullified with inclusion of control group.

Cardiovascular autonomic dysfunction with profound arrhythmogenic effects, including prolongation of QT interval, QT dispersion, “T”-wave inversion, and tachycardia or bradycardia, are major risks for SUDEP in humans and animals with spontaneous recurrent seizures (Opherk et al., 2002; Sakamoto et al., 2008; Metcalf et al., 2009; Brotherstone et al., 2010; Powell et al., 2014b; Eastaugh et al., 2015; Lamberts et al., 2015). Activated microglia are present in post-SE rats (Shapiro et al., 2008), and during chronic periods of epilepsy in humans (Beach et al., 1995) and animals (Shapiro et al., 2008). Our electrophysiology findings suggest that during chronic epilepsy ramified or activated microglia (“M1” phenotype) might have produced pro-inflammatory cytokines, and contributed to a persistent neuroinflammation that lead to higher SNA and prolongation of QT interval (Fig. 9), which was seen even one hour after spontaneous seizure. However, microglial morphological analysis revealed no change in their phenotype at least in the vicinity of the RVLM neurons. In this paradigm, as shown recently, physiological stimulus

alert microglia (ramified) (Vinet et al., 2012), and change their spatial distribution and extent of end point processes contacting synapses without significant morphological changes (Kapoor et al., 2016c), which might be facilitating the pro-inflammatory effects during chronic epilepsy. Microglial activation into a deleterious phenotype in Parkinson's disease is responsible for chronic neuroinflammation, and progressive neurodegeneration of dopaminergic neurons (Gao et al., 2011). Thus, increased microglial activation or alertness during the post-seizure period in chronic epilepsy might mediate the expression and release of pro-inflammatory cytokines, and neuroinflammation in the IML. This might lead to severe cardiovascular autonomic dysfunction and a higher risk of malignant cardiac arrhythmias, and SUDEP, during chronic epilepsy (Fig. 9) (Kloster and Engelskjøn, 1999; Langan et al., 2000). Moreover, deleterious microglial response during chronic epilepsy might regulate the neurodegeneration, and neuronal loss in brainstem autonomic nuclei in SUDEP and TLE patients (Mueller et al., 2014). Collectively, chronic epilepsy-induced microglial activation contributes to sympathoexcitation and severe arrhythmogenic changes in rats.

PACAP is a pleiotropic neuropeptide that achieves its effect through cAMP-mediated mechanisms. PACAP produces neuroprotective effects (Shioda et al., 1998) as well as activates sympathetic efferent neurons (Farnham et al., 2008; Farnham et al., 2011; Inglott et al., 2011). Antagonism of PACAP in the IML during acute seizures cause greater sympathoexcitation in rats (Bhandare et al., 2015), suggesting its neuroprotective effect during acute seizures. Microinjection of a PACAP receptor antagonist into the RVLM during acute seizures does not alter SNA but does ameliorate the seizure-induced arrhythmogenic effects (Bhandare et al., 2016a). Our results demonstrate that antagonism of PACAP at the IML during chronic epilepsy did not alter SNA or QTc interval, which suggests that PACAP is not modulating overall sympathetic output or arrhythmogenic effects in chronic epileptic rats. This might be due to low levels of PACAP in post-SE rats under our experimental conditions. PACAP gene expression increases after seizures, and reaches maximum at 12 h (Fig. 9) (Nomura et al., 2000). During electrophysiology experiments, we did not observe spontaneous seizures, which suggest that there might not be increased PACAP levels in post-SE rats during electrophysiology recordings. However, expression of PACAP can be upregulated after-seizures during the chronic epilepsy,

which could produce either neuroprotective or excitatory effect on sympathetic preganglionic neurons as shown previously (Farnham et al., 2008; Farnham et al., 2011; Bhandare et al., 2015; Bhandare et al., 2016b; Bhandare et al., 2016a).

Cardiovascular reflexes (baroreflex, somatosympathetic-reflex and peripheral and central chemoreflex) are crucial for regulation of arterial blood pressure, blood pH, and its chemical constituents (glucose, PaCO₂ etc.) (Pilowsky and Goodchild, 2002; Shahid et al., 2011). The baroreflex is the first line of defence during changes in blood pressure and is impaired in epilepsy patients, and animal models (Sakamoto et al., 2005; Dütsch et al., 2006). Glutamate is a major excitatory neurotransmitter, and responsible for normal maintenance of reflex responses (Miyawaki et al., 1996; Pilowsky and Goodchild, 2002; Pilowsky et al., 2009), which also plays an important role in the development of seizure (Casillas-Espinosa et al., 2012; Powell et al., 2014a; Bhandare et al., 2016b). Heart-rate baroreflex sensitivity decreases with intracerebroventricular injection of PACAP in trout (Lancien et al., 2011), whereas in rats, PACAP agonist or antagonist microinjection into the RVLM produce no significant effect (Farnham et al., 2012). In spontaneously hypertensive rats, increased microglial activation, and pro-inflammatory cytokines in PVN causes autonomic (baroreflex) dysfunction (Masson et al., 2015). However, our findings show that neither PACAP nor microglia antagonist affect the baroreflex, peripheral or central chemoreflex response in epileptic or control rats. In control rats, the microglia antagonist decreases the fast conducting, A-fibre, somatosympathetic response, whereas the PACAP antagonist decreases the slow conducting C-fibre somatosympathetic response; none of the antagonist treatments have effects in epileptic rats. The PACAP antagonist response is consistent with our other findings where PACAP(6-38) treatment did not alter the SNA and ECG findings in chronic epileptic or control rats.

Seizure activity is accompanied with increased sympathetic output, increased plasma noradrenaline levels, tachycardia and elevated blood pressure (Read et al., 2015; Bhandare et al., 2016a). However, our non-invasive blood pressure measurements and plasma catecholamine analysis did not reveal significant differences in MAP or plasma catecholamine levels interictally between epileptic and control rats. In epileptic rats, plasma catecholamine levels are reported to peak at 48 h post-seizure and then return to normal (Read et al., 2015). Differences in species, model, seizure

type, seizure frequency, and duration might explain the non-significant differences in MAP and plasma catecholamines seen in epileptic and control rats.

Sudden cardiac death in chronic epilepsy (Kloster and Engelskjøn, 1999; Langan et al., 2000) as well as cardiorespiratory autonomic dysfunction (So et al., 2000; Opherk et al., 2002; Seyal et al., 2010) is almost always associated with seizures. Therefore, it is quite likely that activated microglia-mediated neuroinflammatory changes occurring after seizure are major contributors in central autonomic cardiorespiratory dysfunction, and potentially SUDEP (Fig. 9). Overall, the current findings suggest that spontaneous recurrent seizures in chronically epileptic rats produce tachycardia with long-term prolongation of QT interval. Microglial activation in the IML contributes to higher SNA and arrhythmogenic effects in chronic epileptic rats. These findings will help to understand the biphasic microglial response at different stages in epilepsy, and assist in tailoring treatment strategies for seizure-induced central autonomic cardiovascular dysfunction with potential implications to reduce the risk of SUDEP in patients with chronic epilepsy.

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Legends:

Figure 1: *In vivo* effects of IT minocycline treatment in post-SE rat on cardiovascular reflex responses. A typical response of stimulation of aortic depressor nerve (ADN) (B), sciatic nerve (SN) (C), peripheral chemoreceptors (D) and central chemoreceptors (E) on the SNA and on HR, end-tidal CO₂, blood pressure, EEG and SNA activity (A). The protocol was repeated at 60, and/or 90 and 120 min after IT treatment.

Figure 2: A typical spontaneous recurrent seizure (top) with a transition into ictal period (bottom) in epileptic rat.

Figure 3: The spontaneous seizure-induced tachycardia and heart rate variability. The effect of spontaneous seizure on HR (A) during *in vivo* EEG-ECG recordings in post-SE rat. The spontaneous seizure produced significant tachycardia along with increased variability in HR (B-E) during post-ictal period.

Figure 4: *In vivo* effects of IT PBS, PACAP(6-38) and minocycline treatment in post-SE and control rats. Change in SNA (AUC) between 60 and 120 min post treatment in post-SE (AI) and control (BI) rats. The changes in MAP (mmHg) at 120 min post IT treatment in post-SE (AII) and control (BII) rats, and HR (bpm) at 120 min after IT treatment in post-SE (AIII) and control (BIII) rats. Statistical significance was determined using one-way ANOVA followed by t-tests with a Holm Šídák correction. Data expressed as mean $\pm$ SEM. * $p \leq 0.05$ compared with IT PBS treated post-SE group.

Figure 5: *In vivo* effects of IT PBS, PACAP(6-38) and minocycline treatment on ECG activity. Group data showing effect of IT PBS, PACAP(6-38) and minocycline treatment on: changes in corrected QT interval (Δ QTc) (ms) (AI) and PR interval (Δ PR) (ms) (AII) at 120 min post treatment compared to the pre-treatment period in post-SE rats; and changes in QTc interval (Δ QTc) (ms) (BI) and PR interval (Δ PR) (ms) (BII) at 120 min post treatment compared to the pre-treatment period in control rats. Representative Poincare plots illustrate changes in QT interval in post-SE rats from three different groups treated with IT PBS (CI), PACAP(6-38) (CII) and minocycline (CIII). IT PBS (CI) and PACAP(6-38) (CII) treatment in post-SE rats does not alter the QT interval, whereas antagonism of microglial activity significantly

reduces the QT interval (CIII) in post-SE rats. Statistical significance was determined using one-way ANOVA followed by t-tests with a Holm Šídák correction. Data expressed as mean $\pm$ SEM. * $p \leq 0.05$ compared with IT PBS treated post-SE group.

Figure 6: *In vivo* effects of IT PBS, PACAP(6-38) and minocycline treatment on baroreflex and somatosympathetic reflex responses in post-SE and control rats. The % change in baroreflex response at 60, 90 and 120 min post IT treatment compared to pre-treatment period in post-SE (AI) and control rats (BI). Effect of IT treatment on % change in fast conducting (A-fibre) somatosympathetic response at 60, 90 and 120 min post treatment compared to pre-treatment period in post-SE (AII) and control (BII) rats. The % change in slow conducting (C-fibre) somatosympathetic response at 60, 90 and 120 min post IT treatment compared to pre-treatment period in post-SE (AIII) and control (BIII) rats. Statistical significance was determined using one-way ANOVA followed by t-tests with a Holm Šídák correction. Data expressed as mean $\pm$ SEM. * $p \leq 0.05$ compared with the pre-treatment time-point in post-SE group. #### $p \leq 0.001$, ## $p \leq 0.01$, # $p \leq 0.05$ compared with the pre-treatment time-point in control group.

Figure 7: *In vivo* effects of IT PBS, PACAP(6-38) and minocycline treatment on peripheral and central chemoreflex responses in post-SE and control rats. The % change in peripheral (AI) and central (AII) chemoreflex response at 60 and 120 min post IT treatment compared to pre-treatment period in post-SE rats. The % change in peripheral (BI) and central (BII) chemoreflex response at 60 and 120 min post IT treatment compared to pre-treatment period in control rats. Statistical significance was determined using one-way ANOVA followed by t-tests with a Holm Šídák correction. Data expressed as mean $\pm$ SEM.

Figure 8: Fluorescence images of the RVLM area containing TH⁺-ir (red), Iba-1 labelled microglia (yellow) and CD206-labelled M2 microglial cells (green) in post-SE (A) and control (B) rats. Scale bar, 20 μ m. In both groups TH⁺-ir neurons (red) were surrounded with microglia with its round cell body and normal appearing processes with few ramifications (closed arrow) and no change in number of anti-inflammatory M2 microglia (open arrow).

Figure 9: A proposed mechanism of action of microglia and PACAP on sympathetic neurons at the IML during acute and chronic seizures, and its possible outcomes.

Time scale on x-axis is variable from hours to days. Under normal conditions, highly motile “surveilling” microglia continuously survey their microenvironment through direct contact with neuronal synapses and exchange molecular signals. Microglia can immediately sense the disturbed functional and structural integrity of neurons during conditions, such as seizures. Upon detection of these trigger that are higher than the activation threshold, microglia respond through reorganisation of their processes and activity profile. During acute seizures, microglia might acquire “beneficial” activation state (M2) and produce neurotropic factors, such as IL-10 and TGF- β , to protect the overexcited neurons, and limit further damage and restore normal homeostatic condition. Seizure also triggers synthesis and release of PACAP, which peaks at 12 h post seizure, and produce either neuroprotective or excitatory effects on sympathetic neurons at IML. However, chronic seizures may trigger more drastic changes in functional phenotype of microglia. During chronic seizures, maladaptive responses of microglia may lead to “deleterious” activation state (M1) that triggers release of inflammatory molecules such as IL-1 β and TNF α . This could be the mechanism for increased risk of SUDEP during chronic phase of epilepsy, and specifically during post-seizure period (as shown in orange) that is associated with central autonomic cardiovascular dysfunctions such as increased SNA and proarrhythmogenic changes.

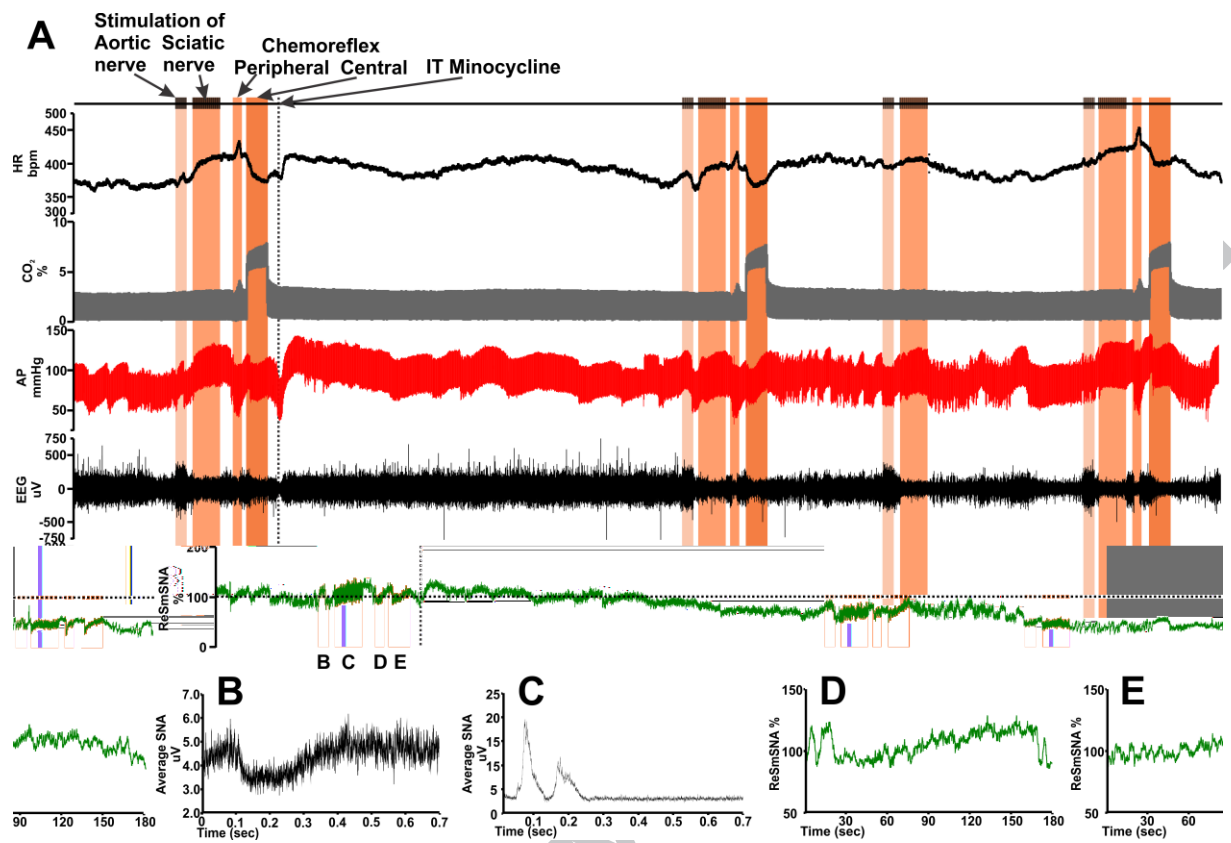
Table 1: Spontaneous seizure frequency and duration in post-SE chronic epileptic rats. Seizure frequency and duration per day in IT PBS, PACAP(6-38) and minocycline treated post-SE rats. In each group $n = 5$. Data expressed as mean $\pm$ SEM.

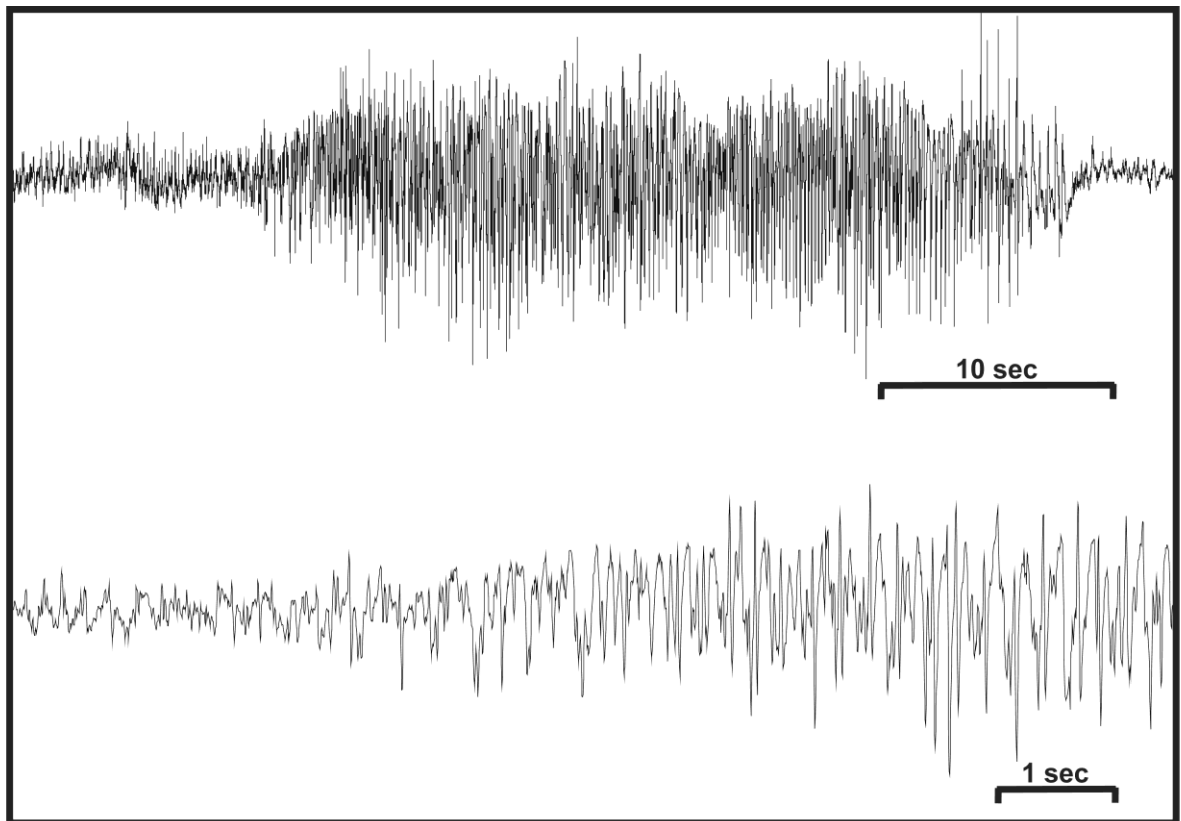
Table 2: The cardiovascular activity and catecholamine levels in post-SE chronic epileptic rats compared to control. MAP (mmHg) and HR (bpm) in post-SE, and control rats recorded under conscious conditions. Plasma adrenaline (nmol/L) and noradrenaline (nmol/L) concentration in post-SE and control group of rats. Statistical significance was determined using unpaired t-test. Data expressed as mean $\pm$ SEM.

Table 3: Morphological analysis of fluorescence images of the RVLM area containing TH⁺-ir, Iba-1 labelled microglia and CD206-labelled M2 microglial cells in post-SE and control rats. In both groups, there are no changes in number of Iba-1 labelled

microglia, number of anti-inflammatory M2 microglia or their morphology. Data expressed as mean $\pm$ SEM.

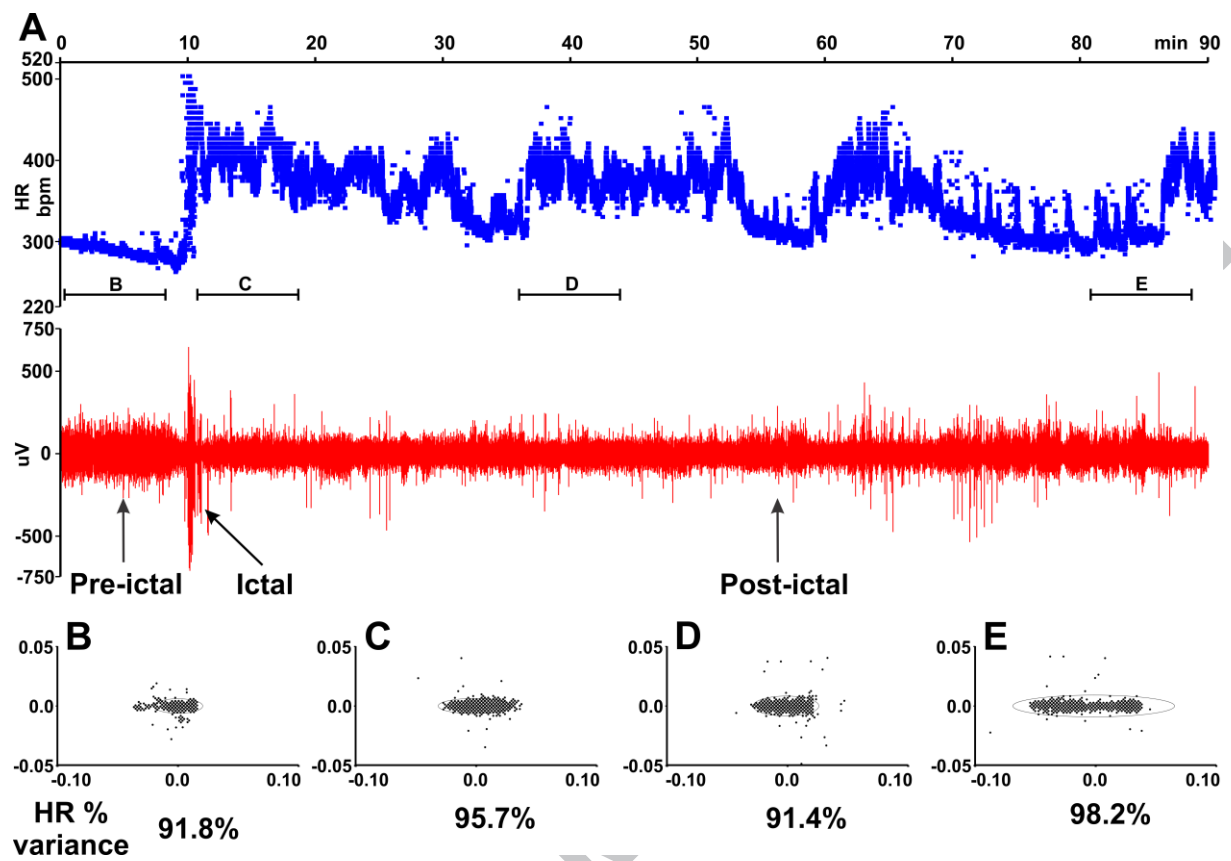
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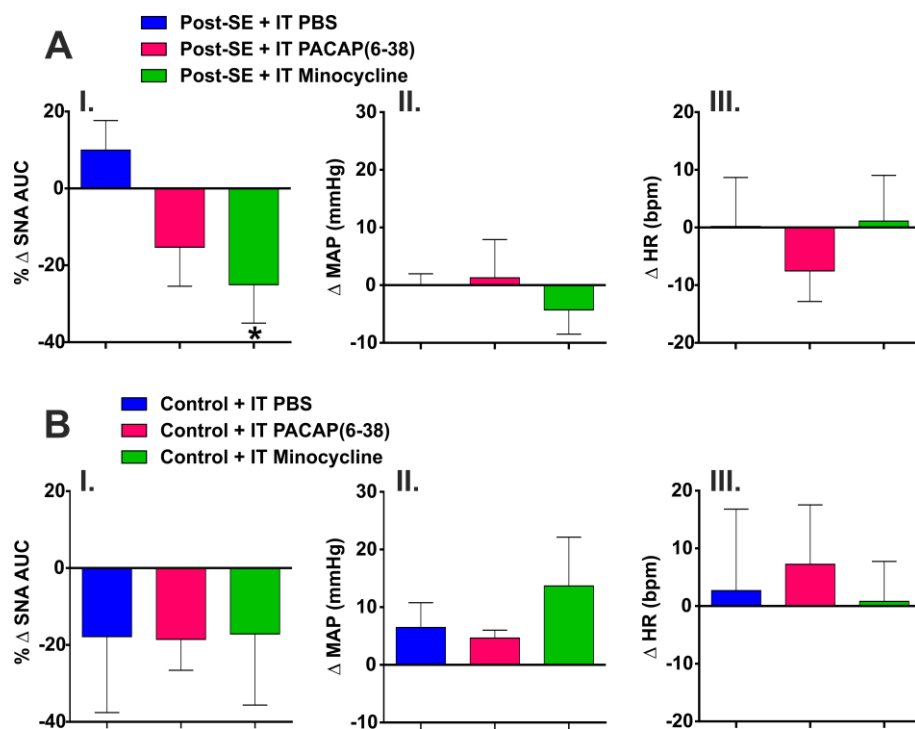


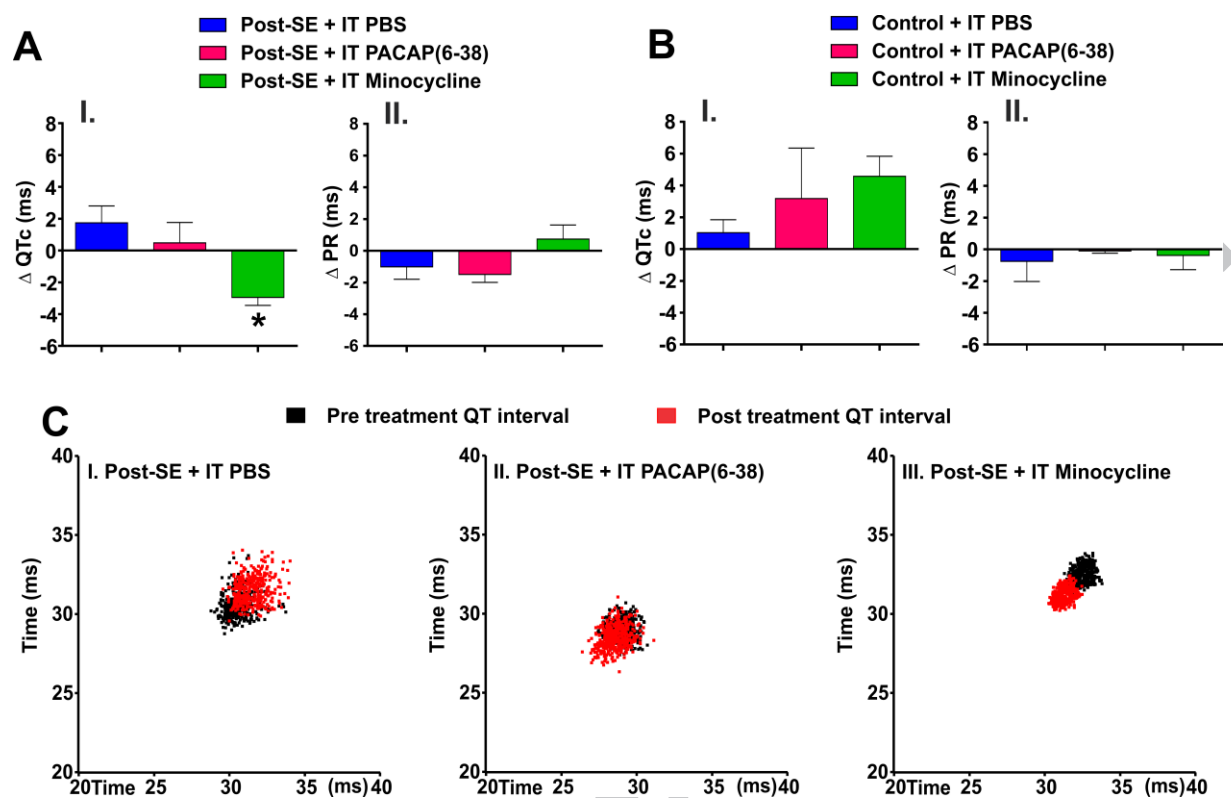


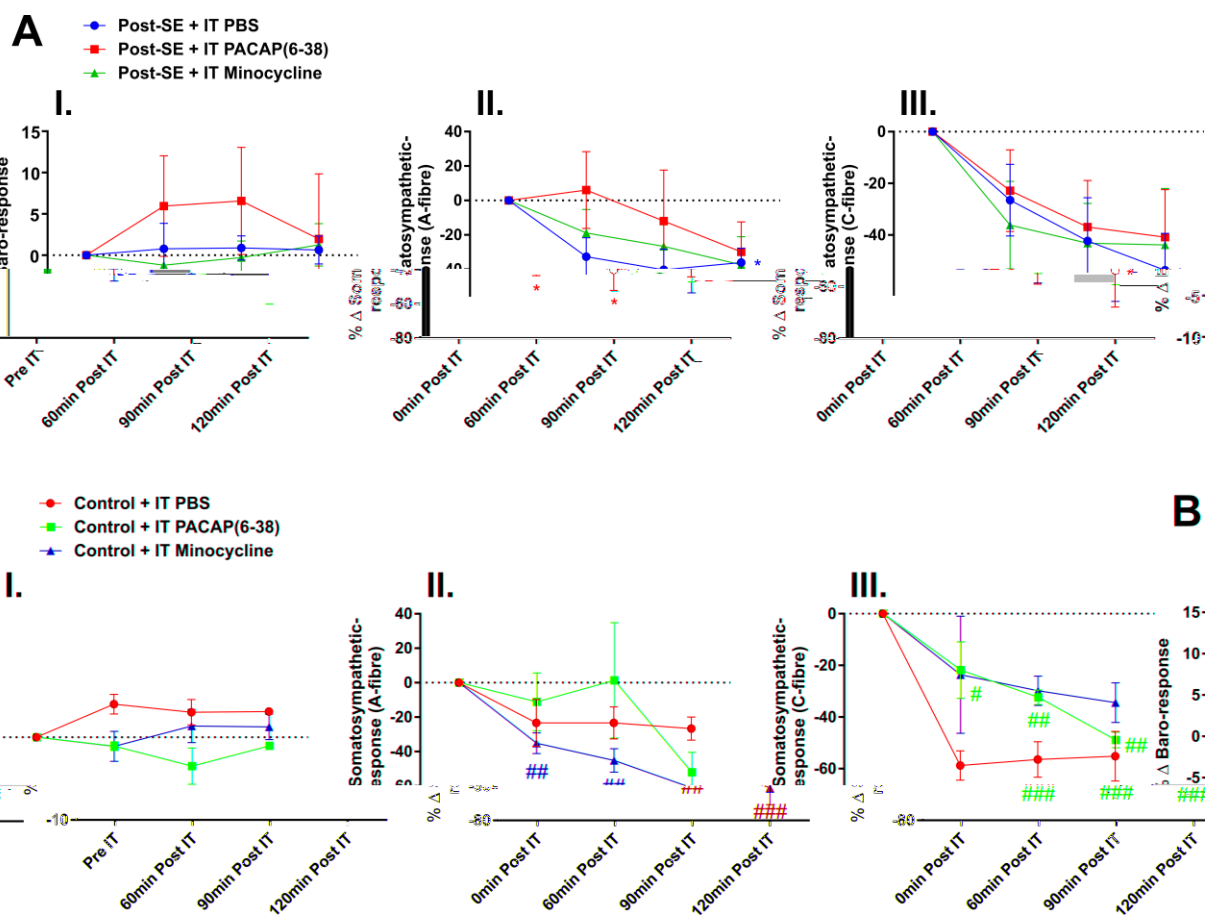
A typical spontaneous seizure in Post-SE rat

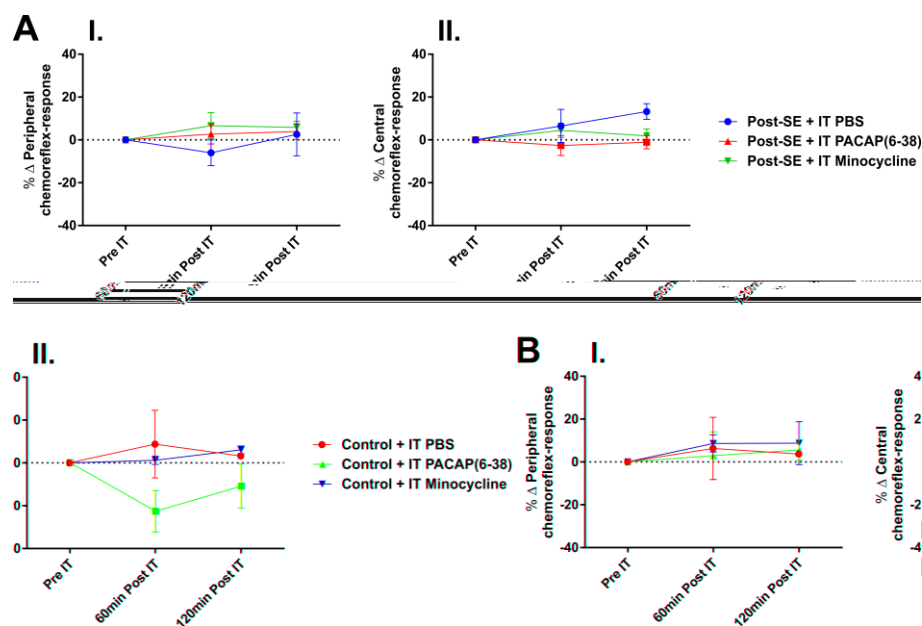
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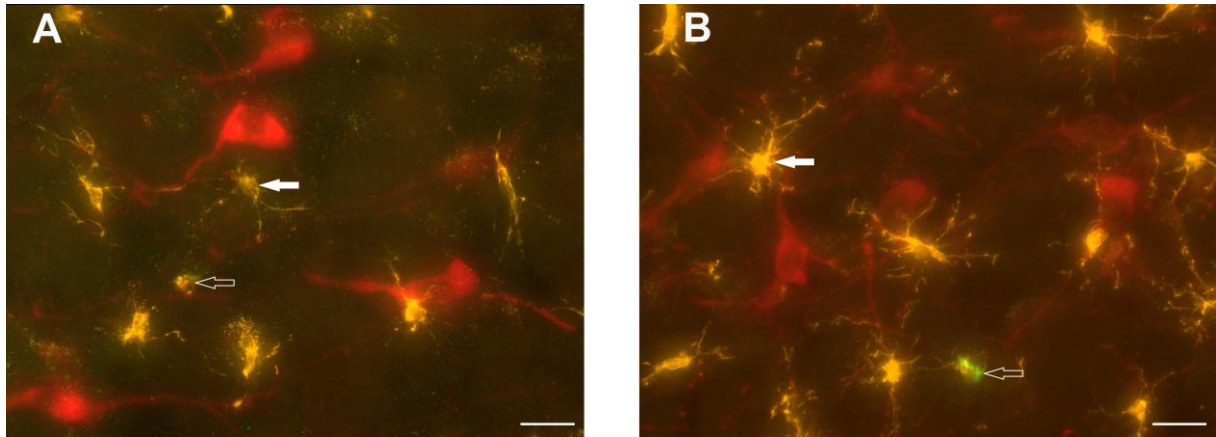












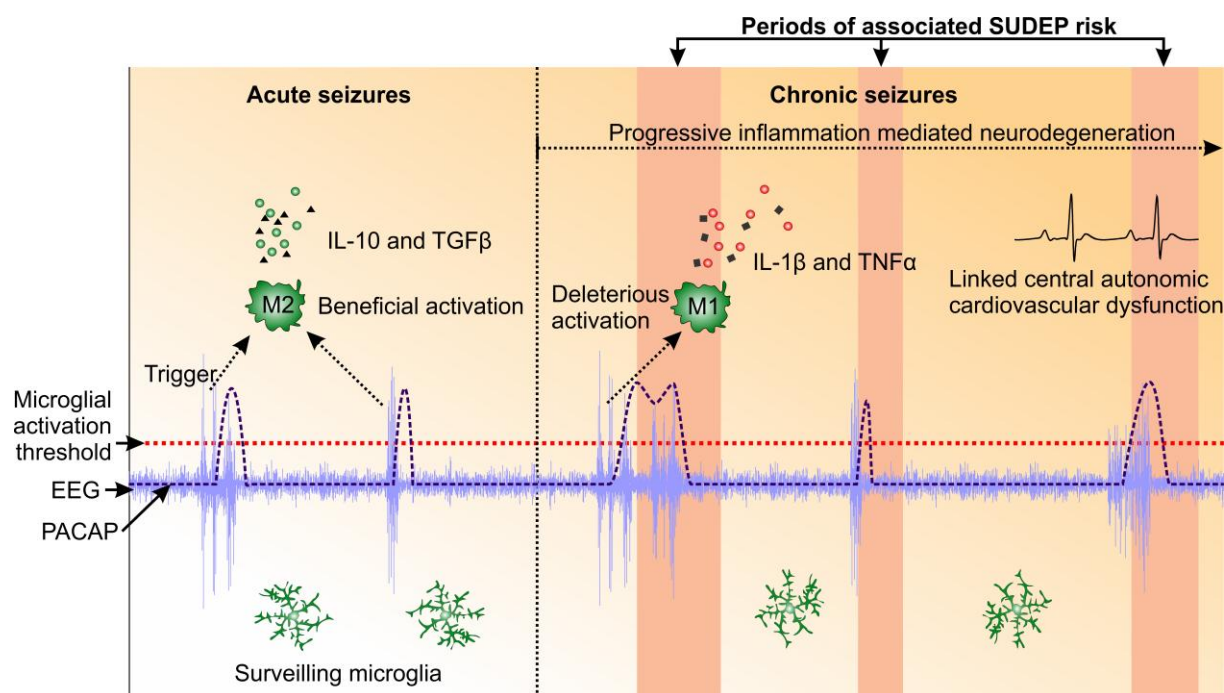


Table 1:

Post SE + IT PBS					
Animal No.	1	2	3	4	5
No. of seizures/day	0.29 ± 0.18	0.50 ± 0.50	#	0.43 ± 0.20	#
Seizure duration (sec)/ day	13.57 ± 8.87	20.50 ± 20.50	#	21.71 ± 10.36	#
Post SE + IT PACAP (6-38)					
Animal No.	1	2	3	4	5
No. of seizures/day	1.00 ± 0.53	0.71 ± 0.29	0.50 ± 0.50	0.57 ± 0.30	0.17 ± 0.17
Seizure duration (sec)/ day	44.71 ± 24.34	30.29 ± 12.74	25.0 ± 25.0	33.57 ± 16.89	5.0 ± 5.0
Post SE + IT Minocycline					
Animal No.	1	2	3	4	5
No. of seizures/day	0.86 ± 0.26	0.71 ± 0.18	0.14 ± 0.14	#	0.43 ± 0.20
Seizure duration (sec)/ day	31.14 ± 9.27	26.14 ± 7.16	4.57 ± 4.57	#	24.43 ± 12.39

Seizures were not detected in the rat during the recording period.

Table 2:

Treatment group	Post-SE	Control
MAP (mmHg)	125.80 ± 4.49	129.3 ± 3.9
HR (bpm)	310 ± 8	326 ± 10
Adrenaline (nmol/L)	1.58 ± 0.31	1.9 ± 0.49
Noradrenaline (nmol/L)	3.38 ± 0.51	3.93 ± 0.78

Table 3:

Treatment group	Post-SE	Control
No. of microglia	241	233
No. of end processes/microglia	403 ± 76	478 ± 122
Branch length (µm)/microglia	197.60 ± 5.69	224.90 ± 12.24
% CD206 labelled microglia	12.91 ± 3.95	8.89 ± 2.14

Highlights:

- Epilepsy-induced altered cardiovascular function, regulated by autonomic nervous system, is a major cause of death.
- Chronic spontaneous seizures in rats produce profound proarrhythmogenic effects.
- In epileptic rats, proarrhythmogenic and sympathoexcitatory effects are mediated by action of microglia at spinal cord.
- Neither PACAP nor microglia regulate the major cardiovascular reflex responses in epileptic rats.
- Modifying the microglial activity in epileptics might produce sympathoprotective and eventually cardioprotective effects.