Title: Early life maternal separation stress augmentation of limbic epileptogenesis: the role of corticosterone and HPA axis programming

Running title: Early life stress, HPA axis programming and epilepsy

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Abstract

Early life stress causes long-lasting effects on the limbic system that may be relevant to the development of mesial temporal lobe epilepsy (MTLE) and its associated psychopathology. Recent studies in rats suggest that maternal separation (MS), a model of early life stress, confers enduring vulnerability to amygdala kindling limbic epileptogenesis. However, the mechanisms underlying this remain unknown. Here, we tested whether hypothalamic-pituitary-adrenal (HPA) axis hyper-reactivity induced by MS - specifically the excessive secretion of corticosterone following a seizure - was involved in this vulnerability. In adult female rats subjected to MS from postnatal days 2-14, seizure-induced corticosterone responses were significantly augmented and prolonged for at least two hours post-seizure, compared to control early-handled (EH) rats. This was accompanied by reduced seizure threshold ($p<0.05$) and increased vulnerability to the kindling-induced progression of seizure duration ($p<0.05$) in MS rats. Pre-seizure treatment with the corticosterone synthesis inhibitor, metyrapone (MET) (50mg/kg sc) effectively blocked seizure-induced corticosterone responses. When delivered throughout kindling, MET treatment also reversed the MS-induced reduction in seizure threshold and the lengthened seizure duration back to levels of EH rats. These observations suggest that adverse early life environments induce a vulnerability to kindling epileptogenesis mediated by HPA axis hyper-reactivity, which could have relevance for the pathogenesis of MTLE.

Keywords: early life stress; epileptogenesis; kindling; corticosterone; metyrapone; epilepsy; HPA axis.

Introduction

The pathogenesis of mesial temporal lobe epilepsy (MTLE), the most prevalent form of drug-resistant focal epilepsy in adults (Engel, 2001), is currently viewed as a multistage process which could initiate early in life, even though seizures often do not commence until adolescence or adulthood (Scharfman and Pedley, 2006; Scharfman, 2007). Amongst the range of early life factors implicated in MTLE causation, which include birth trauma, febrile seizures or infection (Scharfman, 2007), environmental stressors may be important contributors. Early life stress, a strong risk factor implicated in several psychiatric disorders (Gunnar and Quevedo, 2007), may serve as a common causal or contributory factor for MTLE and the psychopathologies that are often comorbid with the epilepsy (Hermann, et al., 2008; Kanner, 2009; Kanner, 2012). In the last decade, a consistent body of experimental research has provided evidence to support this theory, documenting an enduring increase in vulnerability to epileptogenesis following early postnatal stress (Huang, et al., 2002; Lai, et al., 2006; Salzberg, et al., 2007; Gilby, et al., 2009; Jones, et al., 2009; Lai, et al., 2009; Cabral, et al., 2011; Kumar, et al., 2011). Research into the biological mechanisms underlying this relationship, however, remains sparse (Koe, et al., 2009; Kumar, et al., 2011).

Amongst several candidate mechanisms, a strong possibility is the propensity for early life adversity to alter hypothalamic-pituitary-adrenal (HPA) axis function in adulthood. From the abundant literature generated from a range of species showing that early life exposures influence the programming of the HPA axis, the majority of studies conclude that stressors in early life result in exaggerated HPA axis responses to stress (see Ladd, et al., 2000; Sanchez, et al., 2001; Levine, 2005; Heim, et al., 2008; Rao, et al., 2008; Lupien, et al., 2009). However, this is not a uniform finding, and different influences, including later

experiences (e.g., Ladd, et al., 2005; Goldman-Mellor, et al., 2012), the type of stressor (e.g., Richardson, et al., 2006) and genetic make-up (e.g., Tyrka, et al., 2009) can differentially impact the resultant function of the HPA axis. In our hands (Kumar, et al., 2011), early life maternal separation (MS) stress in Wistar rats leads to hyperresponsivity of the HPA axis in adulthood when compared to early handled (EH) controls. Adult stressors, such as restraint or seizures, therefore result in greater corticosterone release, and this may represent a mechanism underlying the augmented limbic epileptogenesis evoked by early postnatal stress. Evidence for pro-seizure and pro-epileptogenic effects of glucocorticoids is extensive in animal models (Joels, 2009), supporting the idea that excessive glucocorticoids can exacerbate MTLE development and progression. For example, administration of exogenous corticosterone aggravates kindling epileptogenesis in rats (Karst, et al., 1999; Taher, et al., 2005; Kumar, et al., 2007), an effect that was ameliorated with corticosteroid receptor antagonists (Kumar, et al., 2007). Conversely, removal of endogenous corticosterone by adrenalectomy reduced seizure susceptibility and severity, which were restored with glucocorticoid replacement (Cottrell, et al., 1984; Lee, et al., 1989). Also, restraint stress which elicits a corticosterone response accelerates kindling epileptogenesis (Jones, et al., 2013).

Recently, we reported that the acceleration of amygdala kindling in rats previously exposed to MS was associated with larger corticosterone responses immediately following a kindled seizure (Kumar, et al., 2011), promoting HPA axis hyperactivity as a potential key mechanism for the enhanced vulnerability to MTLE. Here, we aimed to characterise the time course of corticosterone elevation post-seizure, and to determine whether blocking seizure-induced corticosterone release was able to attenuate the MS-induced effects on kindling epileptogenesis. Specifically, we hypothesised that female rats previously exposed to early life MS would exhibit HPA hyper-reactivity manifested by an augmented and prolonged

corticosterone elevation following a seizure, and that inhibition of the corticosterone response using the corticosterone synthesis inhibitor metyrapone (MET) (Temple and Liddle, 1970) would reverse the pro-epileptogenic effects of MS. We focussed on females in this study since our previous work showed that MS increased vulnerability to kindling selectively in females (Salzberg, et al., 2007). In addition, female, but not male, MS rats displayed hyperactive HPA axis function during kindling (Kumar, et al., 2011), and HPA axis response to stress has been shown to be more prominent in females (McCormick, et al., 2002; Bale, 2006; Slotten, et al., 2006). Furthermore, there is evidence that the rate of cryptogenic temporal lobe epilepsy (for which the most common cause is MTLE) is more common in women than in men (Christensen, et al., 2005).

Materials and methods

Experimental animals

Inbred Wistar rats were mated in the Department of Zoology, University of Melbourne which was maintained at 20°C on a 12h light/dark cycle (lights on at 0600h). Pregnant rats were checked for litters daily and the day of birth was assigned postnatal day 0 (P0). Each mother was used for breeding only once. Litters were weaned on P21, and female pups were group-housed (2-3 per cage) until electrode implantation surgery. Male pups were used for other experiments not described here. All procedures were approved by the University of Melbourne Animal Ethics Committee and performed in accordance with the guidelines published by the Australian National Health and Medical Research Council (NHMRC).

Maternal separation and early handling

For the entire pre-weaning period (i.e.: P0-P21), litters were group-housed with the dams and sires, with the exception of the times of maternal separation. On P2, litters consisting of 8-12 pups were assigned to one of two separation protocols, which were carried out from P2-P14, inclusive. MS consisted of daily separation of litters from their dams and sires for 3 hours (0800 to 1100h), while early handling (EH) involved daily brief separations of 15 minutes (0800 to 0815h). First, dams and sires were removed from the home cage and placed in a quiet, separate room. Pups were then removed from the nest one at a time and placed together in a separate plastic box on a heating pad (30°C) to maintain normal body temperature. At the end of the separation period, pups were returned to the home cage, followed by the dams and sires. From P15-P21, litters were group-housed together with the dams and sires, and underwent normal rearing conditions (i.e. cleaning of cages once a week). Following weaning, rats were housed with their female littermates 2-3 per cage until electrode implantation, after which they were housed singly to avoid experimental loss. A total of 12 litters were used (5 MS, 7 EH), generating a total of 83 female pups. There were no differences between the average litter size between MS and EH litters (mean pups per litter: MS: 10.6 ± 0.9; EH: 10.6 ± 0.5; $t_{(11)}$=1.01, p=0.34), nor between the number of female pups in these litters (mean females per litter: MS: 8.2 ± 0.9; EH: 6 ± 0.7; $t_{(11)}$=0.19, p=0.85). At weaning, there was no difference in the average weight of females from the MS and EH groups (average weight of females per litter at weaning: MS: 39.1 ± 0.7g; EH: 36.5 ± 1.1g; $t_{(11)}$=1.74, p=0.11).

Electrode implantation

At seven weeks of age, rats were implanted with stimulating and recording electrodes under isofluorane anaesthesia (Jones, et al., 2009). Briefly, a midline scalp incision was made and holes were drilled in the skull to implant three extradural EEG recording electrodes, two anterior to bregma and one posterior to bregma. An additional hole was drilled to allow the

stereotactic insertion of a bipolar stimulating electrode (MS303/1, Plastics One, Roanoke, VA, USA) into the left basolateral amygdala: -3.00mm AP, -5.00mm ML from bregma; -6.50mm DV from dura. All attachments were held in place with dental acrylic. All animals were administered 4mg/kg (SC) carprofen (Rimadyl, Pfizer, Sydney, Australia) at the end of the surgery for post-operative analgesia.

Seizure threshold testing and amygdala kindling

One week after implantation, seizure threshold was determined by applying an electrical stimulation consisting of a 1s train of 1ms, 60Hz biphasic square wave pulses beginning at a current of 20μA and incrementing by 20μA every 60s. Seizure threshold was defined as the minimum current intensity required to evoke a synchronous after-discharge pattern of at least 6s on the EEG recording (Compumedics, Melbourne, Australia). Stimulations were delivered using an Accupulser Pulse Generator/Stimulator (A310, World Precision Instruments, Sarasota, FL, USA) coupled with an optically isolated, constant stimulus isolator (A360, WPI, Sarasota, FL, USA). Where the stimulus exceeded 400μA without the presence of an after-discharge, it was assumed (and later verified in each case) that the electrode was incorrectly placed, and the animal was excluded from the study. Following post-mortem verification of electrode placement, 64 animals were included in the final analyses.

Of 64 rats from which seizure thresholds were successfully obtained, 38 were randomly selected to undergo kindling, which commenced 24 hours after seizure threshold determination. The other animals (n=26) underwent sham-kindling, and were not used for further analyses in the current study. The kindling procedure involved stimulation at the threshold current twice a day (5 days/week), with kindling sessions at least 5 hours apart. This kindling protocol is widely adopted, and keeps consistency with our previous work (Taher, et al., 2005; Ali, et al., 2011; Tan, et al., 2012). Seizures were graded based on the

Racine classification scale - Class I: facial clonus; Class II: head nodding; Class III: forelimb clonus; Class IV: rearing; Class V: rearing and falling (Racine, 1972). Seizure duration was defined as the time between the end of the electrical stimulus and the cessation of the electrographic seizure as measured on the EEG recording. Stimulations were continued until 5 Class V seizures had been elicited.

Drug treatment and validation in naïve rats

We used MET (2-methyl-1,2-di-3-pyridyl-1-propanone, Sigma-Aldrich) to block the activity of 11β-hydroxylase, the enzyme responsible for the conversion of deoxycorticosterone to corticosterone, thereby preventing corticosterone synthesis (Temple and Liddle, 1970). This drug has been previously shown to efficiently block stress-induced rises in corticosterone (Calvo, et al., 1998). One hour prior to each kindling stimulation, rats received a 2mL/kg injection of MET (50mg/kg SC, dissolved in 2.5% ethanol and 97.5% saline), or vehicle (n=4/group). Efficiency of the dose and timing of MET treatment was validated in naïve rats using a restraint stress protocol. For this, naïve rats were administered MET one hour prior to 30-minute restraint stress using clear Perspex Broome rodent restraint tubes (SDR, Clinical Technology, Australia) (Jones, et al., 2013). Blood samples were obtained from the tail tip at the start and end of the 30-minute restraint period, and processed as described below to determine corticosterone levels.

Post-seizure blood sampling and plasma corticosterone measurement

Following kindling, all rats underwent surgery to implant a cannula (PVC tubing (OD: 1.2mm; ID: 0.8mm); Microtube Extrusions, NSW, Australia) into the right external jugular vein as previously described (Thrivikraman, et al., 2002). Following surgery, rats were placed in an infusion harness and the cannula connected to an infusion pump (Aladdin 1000 infusion pump, World Precision Instruments, Sarasota, FL, USA; infusion fluid: 4 units/mL

heparinised saline, flow rate 0.5mL/h) to maintain patency of the cannula. Following 48 hours of recovery, rats underwent serial blood sampling to assess the stress response to an induced seizure, and the effect of MET. At 0900h (three hours after lights-on), rats were administered their respective MET/vehicle injections. One hour following injection, blood samples (0.05mL) were taken using heparinised syringes connected to the catheter which extended out from the homecage at the following time points relative to a kindled seizure: 15 and 1 minute(s) prior to stimulation and 1, 2, 5, 10, 15, 20, 25, 30, 45, 60, 90 and 120 minutes post-stimulation. After each sample, cannulae were flushed with 4 units/mL heparinised saline to replace removed volume and maintain patency of the line. Blood samples were successfully obtained from 32 out of 38 animals.

Blood samples were centrifuged at 4°C at 10,000rpm for 10 minutes to separate plasma, which was collected and stored at -20°C. Plasma corticosterone levels were detected in 5µl plasma samples using a Corticosterone Double Antibody ^{125}I RIA Kit (MP Biomedicals, OH, USA), with all samples tested in duplicate.

Brain collection and verification of electrode placement

At the time of sacrifice, a 1s continuous electrical stimulus of 2000µA was applied to create anodal marking lesions between the tips of the bipolar electrode immediately prior to administration of a lethal dose of lethabarb (5mL/kg IP). Rats were then transcardially perfused with 300mL of 0.1M phosphate-buffered saline (PBS, pH 7.4), followed by 450mL of 4% paraformaldehyde (PFA, in 0.1M PBS, 4°C, pH 7.4) containing 0.05% potassium ferrocyanide and 0.05% potassium ferricyanide. Brains were removed and post-fixed in 4% PFA overnight at 4°C and later transferred to a 30% sucrose solution for 48 hours at 4°C before being stored at -80°C. Brains were sectioned (20µm), slide-mounted and stained with thionin (0.03%) in acetate buffer for 30 minutes, then dehydrated and coverslipped. The

position of the electrode tip presented as a blue dot where ferrous ions from the lesion react with the perfusion solution. Based on a rat atlas (Paxinos and Watson, 1998), animals with electrode placements outside the target basolateral amygdala were excluded from the study.

Statistical analyses

Our primary analyses determined whether there was a statistical interaction between early life stress and drug treatment for the variables of interest, namely corticosterone response to seizure, seizure threshold, and kindling rate and seizure duration. Statistical analyses of these variables were performed using analysis of variance (ANOVA) with repeated measures, followed by planned comparison post-hoc analyses where appropriate. For post-seizure corticosterone responses, there were no differences in corticosterone levels at 15 and 1 minute(s) pre-stimulation ($p>0.05$ for all groups, Student's t-test), so the average of the two time points was used as the baseline value. Area under the curve (AUC) for corticosterone responses was measured relative to baseline levels. For seizure duration analyses, blocks of five seizures were averaged. We used unpaired Student's t-tests to compare litter sizes and pup weights. All data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed on STATISTICA software (Tulsa, OK, USA) and GraphPad Prism (La Jolla, CA, USA).

Results

MET lowers basal and restraint stress-induced corticosterone levels

To validate the ability of MET to reduce corticosterone levels, naïve rats were subjected to 30 minutes of restraint stress, and plasma corticosterone levels were measured from plasma samples obtained from tail vein blood at the initiation (pre-restraint) and termination (post-

restraint) of the restraint. Restraint stress induced a significant rise in corticosterone levels, evident in vehicle-treated rats (p=0.001). This was significantly blocked by MET pretreatment ($F_{(1,6)}$=54.09, p=0.003; Figure 1), such that MET-treated rats had significantly lower corticosterone levels, compared to vehicle-treated rats, both pre- (p=0.009) and post-restraint stress (p=0.001). These findings indicate that MET, when delivered one hour prior to a stressor, effectively suppressed the stress-induced corticosterone response.

INSERT FIG 1 around here

MS potentiates seizure-induced corticosterone response

We then investigated the effects of early life intervention on the HPA axis response to kindled seizures, and whether MET treatment was able to block the corticosterone response to kindled seizures. In response to stimulation, all animals exhibited Class V (convulsive) seizures. Overall, rats exposed to MS displayed larger and prolonged corticosterone responses to seizures compared to EH rats, and these responses were attenuated by MET pretreatment.

Following a Class V seizure, a significant effect of early life intervention was observed ($F_{(1,14)}$=5.022, p=0.042), where MS rats had significantly higher plasma corticosterone levels at 60 (p=0.048), 90 (p=0.03) and 120 (p=0.002) minutes post-stimulation, compared to EH rats (Figure 2A). Pretreatment with MET successfully blocked the seizure-induced corticosterone surge following the seizures (Figure 2B).

When comparing the area under the curve (AUC) of corticosterone responses (Figure 2C), we observed significant effects of early life stress ($F_{(3,28)}$=9.0, p=0.005), drug ($F_{(3,28)}$=29.1,

p<0.0001), and a signficant interaction between early life stress × drug ($F_{(3,28)}$=7.220, p=0.012). Planned comparisons revealed that the AUC in vehicle-treated MS rats was significantly larger compared to vehicle-treated EH rats (p=0.0004), and that this was reduced in MET-treated MS rats (p<0.0001). When comparing MET vs vehicle treatment in EH rats, the difference in the AUC measure approached statistical significance (p=0.06).

INSERT FIG 2 around here

MS lowers seizure threshold, and this is reversed by MET

Next, we tested the influence of MS on electrically-evoked seizure threshold, and the effect of MET pretreatment. We observed a signficant early life intervention × drug interaction ($F_{(3,60)}$=29.672, p<0.0001; Figure 3). Planned comparisons determined that vehicle-treated MS rats had signficantly lower seizure thresholds than vehicle-treated EH rats (p<0.001), indicative of a hyperexcitable limbic circuitry. Further, MET treatment reversed the effect of MS by increasing seizure threshold in MS rats to the levels of EH rats (p<0.001), while having no effect on EH rats.

INSERT FIG 3 around here

MS prolongs seizure duration during epileptogenesis, and this is reversed by MET

When analysing seizure length, we again found a significant early life intervention × drug interaction ($F_{(3,34)}$=5.481, p=0.025; Figure 4A). Planned comparisons revealed that vehicle-

treated MS rats had significantly longer seizure durations compared to vehicle-treated EH rats ($p=0.026$), reaching post-hoc significance from the 16th stimulation onwards ($p<0.05$ for all points). However, MET treatment in MS rats significantly reduced seizure duration compared to vehicle-treated MS rats ($p=0.013$), lowering durations to levels of EH rats. Similar to seizure thresholds, MET had no effect on seizure duration in EH rats, intimating that MET influences only MS-exposed rats. These findings indicate that blocking the seizure-induced corticosterone surge with MET reversed the MS-induced increase in seizure duration during kindling.

INSERT FIG 4 around here

MET retards kindling rate in MS rats

When comparing kindling rates, we did not observe any interaction between drug × stress ($F_{(3,34)}=0.635$, $p=0.431$; Figure 4B). We did however, observe a significant overall effect of drug treatment on kindling rate ($F_{(3,34)}=4.375$, $p=0.044$), such that MET-treated rats progressed through the kindling stages slower than vehicle-treated rats. This effect of MET on kindling rate appeared to be primarily driven by its effect in MS rats – compared to vehicle treatment, the effect of MET approached significance ($p=0.06$) following MS, but was not apparent in EH rats ($p=0.340$).

Discussion

In agreement with previous literature (Huang, et al., 2002; Lai, et al., 2006; Salzberg, et al., 2007; Jones, et al., 2009; Lai, et al., 2009; Kumar, et al., 2011), we show here that early life stress induced by MS in rats creates a vulnerability to limbic epilepsy in adulthood, as evidenced by reduced electrical seizure thresholds and prolonged seizure duration during kindling epileptogenesis. The MS-induced effect was associated with augmented seizure-induced corticosterone responses which were sustained for at least two hours post-stimulation, compared to control EH rats. Pretreatment with MET, which effectively suppressed seizure-induced corticosterone responses, was able to reverse the vulnerability on the epilepsy-related variables induced by MS, increasing seizure thresholds and reducing seizure duration to levels observed in EH rats. This study provides strong evidence that HPA axis hyper-reactivity plays a causative role in mediating the susceptibility to limbic epilepsy following MS, and provides a mechanism of how early life stress may create a vulnerability to MTLE.

A surge in cortisol is known to occur following a seizure in epilepsy patients (Aminoff, et al., 1984; Pritchard, 1991), similar to what was observed here and by others using the rat kindling model of epilepsy (Szafarczyk, et al., 1986). Extensive literature indicating pro-seizure and pro-epileptogenic effects of corticosterone (Cottrell, et al., 1984; Weiss, et al., 1993; Karst, et al., 1999; Taher, et al., 2005; Kumar, et al., 2007) suggests that the magnitude and time course of corticosterone elevation could be important in impacting disease progression and subsequent seizures. Hyperactive HPA axis responses to stressors following early life MS is widely reported (Faturi, et al., 2010), and here was shown to occur following kindled seizures, manifesting as a prolonged elevation of corticosterone levels up to two hours post-seizure. It should be noted that this rise in corticosterone was referenced to 2 samples taken at 15 and 1 minutes prior to the seizure induction, which was done to minimise baseline fluctuations and thereby obtain an accurate and stable measure of baseline

corticosterone. In addition to diurnal rhythms, corticosterone levels undergo ultradian pulses (Lightman and Conway-Campbell, 2010), and the sampling at two timepoints minimises the consequences to our analysis of a corticosterone pulse occurring at one of these points. We observed no differences in corticosterone levels between the two sampling times, suggesting that the first sample did not itself induce a stress response, likely due to our sampling technique using an indwelling jugular catheter. An extensive body of evidence implicates the contributory role of corticosterone in epileptogenesis in animal models of MTLE, and there are several actions of corticosterone on the brain that could mediate such outcomes. This could occur indirectly via effects of glucocorticoids on limbic structure and function resulting in network alterations to promote seizures, or by direct effects of glucocorticoids on limbic excitability (Joels, 2009).

Corticosterone exerts its effects via glucocorticoid receptors (GR) and mineralocorticoid receptors (MR), which are highly expressed in the hippocampus and amygdala and are therefore well placed to influence limbic system electrophysiology. GR and MR activation influence neuronal excitability and regulate the expression of genes that are involved in maintaining membrane properties (de Kloet, et al., 2005), cell metabolism (Joels and Baram, 2009), neuronal plasticity (Mirescu and Gould, 2006; Alfarez, et al., 2009) and synaptic transmission (Venero and Borrell, 1999; Lee, et al., 2003), all of which can impact limbic excitability and create an environment that promotes seizures and facilitates seizure-induced damage. Important future work which would corroborate our findings and further establish the downstream pharmacological mechanisms of corticosterone in this context would be to use antagonists at either MR or GR. We have previously shown in rats that receive chronic corticosterone supplementation in adulthood that antagonism of both MR and GR can inhibit the pro-kindling effects of corticosterone (Kumar, et al., 2007), so it is likely that a similar outcome would be observed following early life stress. Also, corticosterone has

been shown to induce excitotoxicity in hippocampal neurons and increase their vulnerability to additional seizure insults (Kim and Yoon, 1998; Joels, et al., 2009), as well as having excitatory effects on basolateral amygdalar neurons (Duvarci and Pare, 2007; Karst, et al., 2010). The augmented corticosterone response to seizures observed in MS rats in this study would therefore be expected to synergise with the epileptogenic process and increase the vulnerability of the limbic system to subsequent seizures. This was supported by our finding of increased seizure length in MS rats during kindling, which was reversed by MET pretreatment.

The observation that MET was able to reverse the MS-induced effects on seizure threshold and seizure duration raises intriguing questions: for example, how is this achieved; is this mechanism the same for the reversal of both seizure threshold and duration; and why did MET only influence MS rats? MET effectively blocked the corticosterone rise following seizures and this rise is more pronounced in MS rats, in line with the finding that seizure length was reduced by MET only in MS rats. In this case, it may be that the excessive corticosterone produced by seizures in the MS group facilitates and enhances kindling-associated circuit remodelling, which leads to longer seizures upon stimulation. Inhibition of seizure-induced corticosterone, however, does not explain why MET elevated seizure threshold in MS rats, since this is tested at the beginning of the kindling process. It may be that circulating corticosterone levels in pre-kindling conditions were reduced by MET, thereby creating an inhibitory environment. Our data in naïve rats (see Figure 1) supports this, since we observed a reduction in basal pre-restraint corticosterone levels one hour following MET treatment. However, MET elevated seizure threshold only in MS rats, and perhaps threshold values reached a ceiling level in EH rats and could not be elevated further by MET. Another potential explanation is that MET is reversing relevant alterations to the limbic network that MS had initiated, and is therefore only effective in MS rats.

Unlike our previous findings, here we did not observe any independent effect of MS on the number of stimulations required to reach the fully-kindled state. The reasons for this are not clear, but could be attributed to differences in kindling protocols – previously we employed a rapid kindling paradigm (Salzberg, et al., 2007; Jones, et al., 2009; Kumar, et al., 2011), or perhaps differences in ambient stressors affecting both groups during the periods of separation. Nonetheless, the reduction in seizure threshold induced by MS was similar in magnitude to that observed previously. In addition, inhibition of corticosterone synthesis by MET slowed the behavioral progression of kindling, an effect which appeared most prominent in MS rats.

A limitation of the use of the kindling model to assess effects of an intervention on epileptogenesis is that if the intervention being tested has an anti-seizure effect (i.e. suppresses seizures as do current anti-epileptic medications), then the shorter, less intense seizures may also result in a decreased progression of kindling with repeated stimulations. Some evidence indicates that MET may possess acute anti-seizure effects of its own (Kaminski and Rogawski, 2011; Dhir and Rogawski, 2012), and so we therefore cannot exclude the possibility that such an effect specifically in the MS rats may have contributed to the effect on kindling observed in these animals. In addition, while MET primarily acts to block corticosterone synthesis by inhibition of 11β-hydroxysteroid-dehydrogenase type 1, alternate biochemical pathways might be relevant to the effects observed here. By restricting the synthesis of corticosterone, accumulation of its precursor 11-deoxycorticosterone and its metabolite tetrahydrodeoxycorticosterone (THDOC) would be favoured (Mellon and Griffin, 2002). THDOC is a positive modulator of $GABA_A$ receptors, and has been shown to have anti-convulsive effects (Reddy and Rogawski, 2002). Also, MET can inhibit monoamine oxidase and therefore enhance serotonergic and noradrenergic function (Drouet, et al., 2010). Given the modulatory role of these neurotransmitters on seizures (Jobe and Browning, 2005),

this may also be a potential contributor to MET's anti-epileptogenic effects. We did not however, observe any effect of MET treatment in the EH rats in this study, which argues against these being contributing mechanisms.

A related element relates to the potential interaction between early life stress and the subsequent isolation of the animals after electrode implantation surgery. We cannot rule out the possibility that the difference in corticosterone responses to seizures were the result of a combination of early and adolescent stressors, as opposed to the early life intervention alone. This is also pertinent for the effect of metyrapone, which was only evident in the early life stress group. However, our previous study (Salzberg, et al., 2007) demonstrates that, in our hands, maternal separation results in elevated anxiety-like behaviour, a phenotype linked to HPA axis function.

Over the years, there has been extensive debate about the optimal control group for studies investigating the effects of MS, with suggestions including nonhandling (NH), normal animal facility rearing (AFR), and brief handling and separation (EH) (Lehmann and Feldon, 2000; Pryce and Feldon, 2003; Pryce, et al., 2005; Macri and Wurbel, 2006). In our previous original research exploring the influence of early life environment on later epilepsy development (e.g., Salzberg, et al., 2007; Kumar, et al., 2011; Ali, et al., 2013), we elected to compare MS to EH, interventions that in most studies have been shown to have opposite neurobiological effects, including opposite effects on neuroendocrine function. The current work was designed to build on our previous reported findings by investigating the mechanism underlying the vulnerability to kindling epileptogenesis conferred by early life stress, and so we chose to keep consistency with these studies and compare MS with EH.

An additional potential limitation stems from our use of females, and the influence of the ovarian cycle. Estrous hormones have been shown to affect excitability and to modify kindling rates when rats are consistently exposed to them (Edwards, et al., 1999). However, it

is unlikely that natural variations in hormone levels caused by the ovarian cycle account for our findings as all critical procedures (e.g., after-discharge testing, kindling, and corticosterone measurements) occurred in random relation to it. Stage of the ovarian cycle would therefore have served as a source of variation tending to obscure true effects, rather than a systematic bias. Whether early life stress itself alters the ovarian cycle has not been researched. In addition, while our results are limited to females, future research should investigate the effect of MET on kindling in male rats, and the influence of this on prior exposure to early life stress.

In the current work, we have used an inbred strain of rat and a standardized environmental manipulation, so we are covering a narrow band of gene x environment interaction. Expanding this using outbred rodent populations, and ultimately translating to humans, would require large sample sizes to detect significant effects because of the associated genetic heterogeneity and environmental diversity, but should ultimately be achievable.

To summarise, the findings of our study are supportive of a mechanism which explains how maternal separation exacerbates kindling epileptogenesis, although many other potential interactive mechanisms may also be relevant (Koe, et al., 2009; Ali, et al., 2011). Our study demonstrates that early life stress programs the HPA axis resulting in excessive corticosterone release following seizures. Inhibiting corticosterone synthesis reversed the effects of MS on both seizure threshold and duration, providing further support for an aggravating role of this stress hormone in kindling epileptogenesis. These results suggest therapeutic strategies for the human condition targeting stress-mediators, particularly in high-risk groups exposed to early life psychosocial or physical stress.

Conflict of interest

The authors declare no competing financial interests.

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Figure legends

Figure 1. In naïve rats, MET reduced resting (pre-restraint) plasma corticosterone levels one hour after injection, compared to vehicle-treated rats. MET also effectively blocked the restraint stress-induced corticosterone response, such that corticosterone levels at the end of a 30-minute restraint were threefold higher in vehicle-treated rats than in MET-treated rats. **p<0.01, ***p<0.001.

Figure 2. Plasma corticosterone levels following Class V seizures, and the effect of MET. **(A)** In vehicle-treated rats, Class V seizures induced a corticosterone response that was augmented and prolonged for up to two hours post-seizure in MS rats, compared to EH rats. **(B)** MET treatment one hour prior to the seizure effectively blocked the seizure-induced corticosterone response. **(C)** AUC calculation of corticosterone responses following Class V seizures was augmented in vehicle-treated MS rats, compared to vehicle-treated EH rats, and this was significantly reduced by MET pretreatment. *$p<0.05$, ***$p<0.001$.

Figure 3. Seizure threshold was reduced in vehicle-treated rats previously exposed to MS, compared to EH, and this was reversed in rats treated with MET prior to testing. There was no effect observed of MET in EH rats. ***$p<0.001$. Sample sizes shown in bars.

Figure 4. Pre-seizure treatment with MET attenuated kindling epileptogenesis. **(A)** Seizure duration during kindling was increased in MS rats, compared to EH. This was reversed in MS rats treated with MET, while no drug effect was observed on EH rats. **(B)** Kindling rate was slowed by MET treatment, such that MET-treated rats required more stimulations to progress through kindling seizure classes. This effect appeared more prominent in MS rats than in EH rats. *$p<0.05$, **$p<0.01$.

Conflict of interest

The authors declare no competing interests, financial or otherwise, associated with this work.

Author Contributors:

Amelia Koe conducted all of the research and wrote the first draft of the paper

Michael Salzberg conceived the study, interpreted the data, received funding for the study and edited the paper

Margaret J Morris conceived the study, interpreted the data, received funding for the study and edited the paper

Terence J O'Brien conceived the study, interpreted the data, received funding for the study and edited the paper

Nigel C Jones conceived the study, interpreted the data, received funding for the study, heavily edited the paper, managed the animal ethics application associated with the work, and supervised the project

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Figure 1

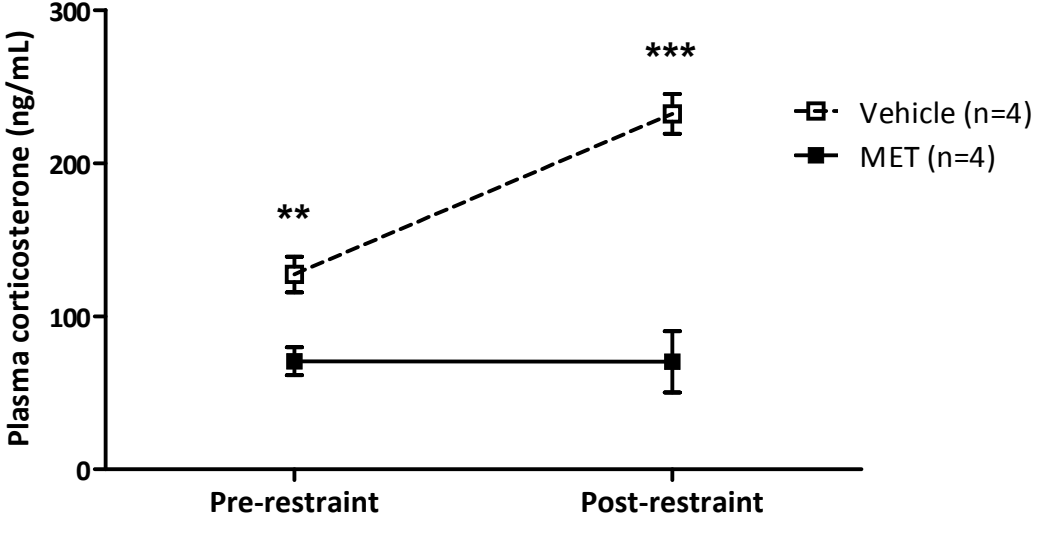
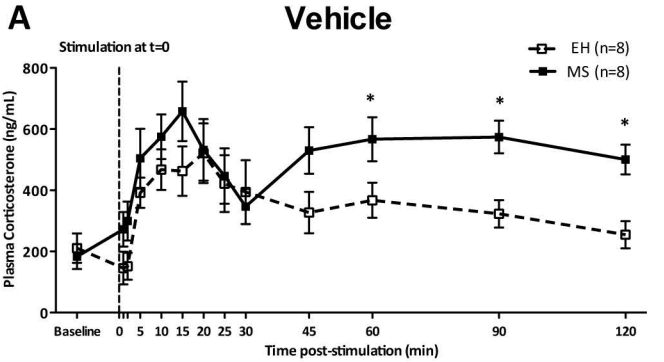
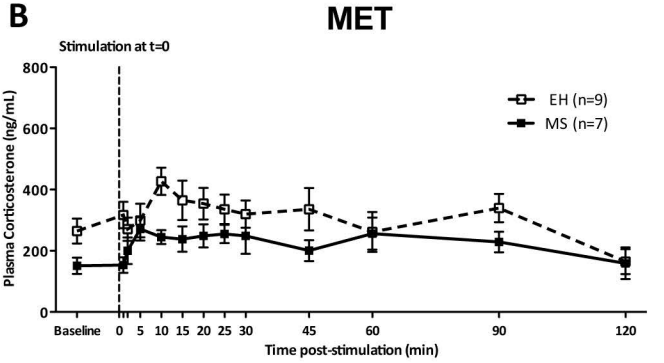

300
200
100
0
Plasma corticosterone (ng/mL)
**
***
Vehicle (n=4)
MET (n=4)
Pre-restraint
Post-restraint

Figure 2


A
Vehicle
Stimulation at t=0
EH (n=8)
MS (n=8)
Plasma Corticosterone (ng/mL)
800
600
400
200
0
Baseline
0
5
10
15
20
25
30
45
60
90
120
*
*
*
Time post-stimulation (min)


B
MET
Stimulation at t=0
EH (n=9)
MS (n=7)
Plasma Corticosterone (ng/mL)
800
600
400
200
0
Baseline
0
5
10
15
20
25
30
45
60
90
120
Time post-stimulation (min)

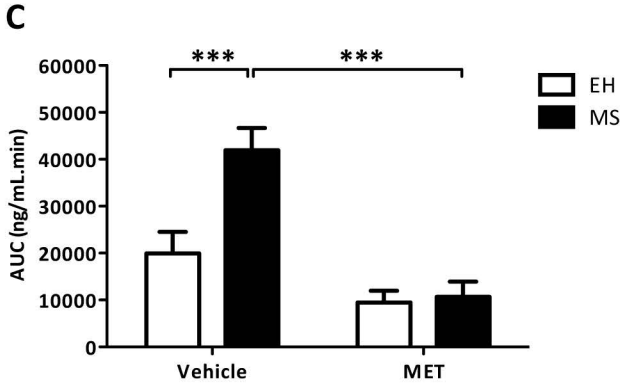
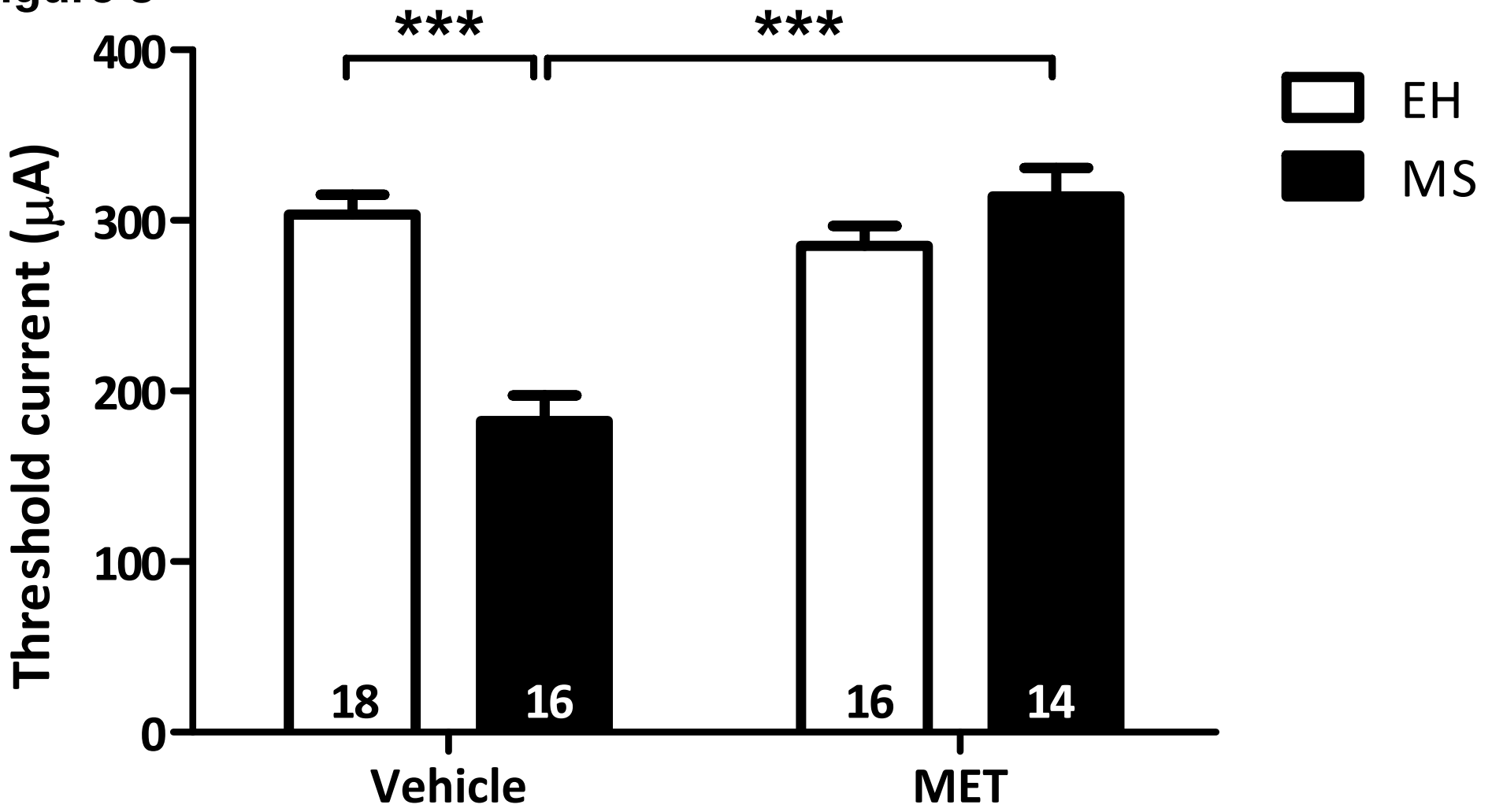

C
***
***
EH
MS
AUC (ng/mL.min)
60000
50000
40000
30000
20000
10000
0
Vehicle
MET

Figure 3


***
***
400
300
200
100
0
Threshold current (μA)
18
16
16
14
Vehicle
MET
EH
MS

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