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Seizure-induced disinhibition of the HPA axis increases seizure susceptibility

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Abstract

Stress is the most commonly reported precipitating factor for seizures. The proconvulsant actions of stress hormones are thought to mediate the effects of stress on seizure susceptibility. Interestingly, epileptic patients have increased basal levels of stress hormones, including corticotropin-releasing hormone (CRH) and corticosterone, which are further increased following seizures. Given the proconvulsant actions of stress hormones, we proposed that seizure-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis may contribute to future seizure susceptibility. Consistent with this hypothesis, our data demonstrate that pharmacological induction of seizures in mice with kainic acid or pilocarpine increases circulating levels of the stress hormone, corticosterone, and exogenous corticosterone administration is sufficient to increase seizure susceptibility. However, the mechanism(s) whereby seizures activate the HPA axis remain unknown. Here we demonstrate that seizure-induced activation of the HPA axis involves compromised GABAergic control of CRH neurons, which govern HPA axis function. Following seizure activity, there is a collapse of the chloride gradient due to changes in NKCC1 and KCC2 expression, resulting in reduced amplitude of sIPSPs and even depolarizing effects of GABA on CRH neurons. Seizure-induced activation of the HPA axis results in future seizure susceptibility which can be blocked by treatment with an NKCC1 inhibitor, bumetanide, or blocking the CRH signaling with Antalarmin. These data suggest that compromised GABAergic control of CRH neurons following an initial seizure event may cause hyperexcitability of the HPA axis and increase future seizure susceptibility.

Keywords

stress; epilepsy; GABA; KCC2; HPA axis; seizures

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1.1 Introduction

Robust anecdotal evidence from the clinic suggests that there is a link between stress and epilepsy (for review see (Maguire and Salpekar, 2013)). The majority of patients with epilepsy self-report that stress exacerbates and/or triggers their seizures (Nakken et al., 2005; Sperling et al., 2008; Neugebauer et al., 1994; Frucht et al., 2000; Haut et al., 2003; Haut et al., 2007) (for review see (Lai and Trimble, 1997; Maguire and Salpekar, 2013)). Interestingly, some studies have demonstrated anticonvulsant effects of stress hormones, such as deoxycorticosterone (Reddy and Rogawski, 2002), which has been attributed to the production of stress-derived neurosteroids (Reddy and Rogawski, 2002). In addition, adrenocorticotrophic hormone (ACTH) is an effective treatment for infantile spasms (Snead et al., 1983; Arya et al., 2012; Jaseja and Jaseja, 2013). These studies demonstrate the complex relationship between steroid hormones and epilepsy (for review see (Sawyer and Escayg, 2010)), which likely depends upon the age of the subject, the type of steroid hormone, and duration of exposure. However, there is substantial evidence demonstrating the pro-convulsant actions of stress and stress hormones, including corticotropin-releasing hormone (CRH) and corticosterone (for review see (Joels, 2009)). Consistent with the role of stress in epilepsy, cortisol is elevated in patients with epilepsy and is further increased following seizures (Culebras et al., 1987; Tunca et al., 2000; Abbott et al., 1980; Pritchard et al., 1985). Furthermore, cortisol levels are positively correlated with seizure frequency in patients with epilepsy (Culebras et al., 1987; Galimberti et al., 2005). Given the pro-convulsant actions of stress hormones, we hypothesized those seizure-induced elevations in stress hormone levels may foster a proconvulsant environment and contribute to further seizure susceptibility.

The production of stress hormones is mediated by the hypothalamic-pituitary-adrenal (HPA) axis, involving the release of CRH from the hypothalamus, which acts in the anterior pituitary to signal the release of ACTH, which then triggers the release of cortisol from the adrenal cortex in humans (corticosterone in rodents). CRH neurons are at the apex of HPA axis control and govern the production and release of stress hormones. These neurons receive input from numerous different brain regions and are regulated by multiple neurotransmitter systems (for review see (Herman et al., 2003; Larsen et al., 2003; Ulrich-Lai and Herman, 2009)). Ultimately, the activity of these neurons is tightly regulated by GABAergic inhibition (for review see (Herman et al., 2004; Decavel and van den Pol, 1990)). Our laboratory has recently uncovered the mechanisms through which CRH neurons overcome this robust GABAergic constraint to elicit the body's physiological response to stress (Sarkar et al., 2011). However, the mechanisms mediating elevations in stress hormone levels following seizures are unknown.

Alterations in GABA_A receptor (GABA_AR) subunit expression have been shown to occur in many brain regions in both animal models of epilepsy and in patients with epilepsy (Zhang et al., 2007) (for review see (Sperk et al., 2009)). The majority of these studies have focused on changes in GABA_AR subunit expression and GABAergic inhibition in the hippocampus following seizures. It is unknown whether similar changes occur in the paraventricular nucleus (PVN) of the hypothalamus following seizures which may alter GABAergic control of CRH neurons and contribute to elevations in the levels of stress hormones. We hypothesize that seizures induce deficits in the GABAergic control of CRH neurons, resulting in hyperexcitability of the HPA axis which may contribute to further seizure susceptibility.

Here we demonstrate that seizures impair the GABAergic regulation of CRH neurons by inducing a collapse in the chloride gradient. The chloride gradient in neurons is maintained by the K⁺/Cl⁻ co-transporter (KCC2) in the adult brain (Rivera et al., 1999; Payne et al.,

2003;Rivera et al., 2005) which is necessary for the inhibitory actions of GABA. The extrusion of chloride by KCC2 is opposed by the active transport of chloride into the cell by the Na-K-Cl co-transporter 1 (NKCC1). Our data demonstrate that there is a dephosphorylation of KCC2 residue Ser940 and a downregulation of KCC2 and an increase in NKCC1 in the PVN following seizures induced with kainic acid. Our lab previously demonstrated a role for KCC2 in mediating stress-induced activation of CRH neurons and elevations in corticosterone. Our data suggest that seizures activate the HPA axis using mechanisms similar to stress. Alterations in KCC2 expression in the PVN following seizures is associated with a reduction in the amplitude of sIPSPs and even depolarizing actions of GABA on CRH neurons, an increased firing rate of CRH neurons, elevations in circulating corticosterone, and increased seizure susceptibility. Blocking seizure-induced activation of the HPA axis with a CRH receptor antagonist, Antalarmin, or preventing the collapse in the chloride gradient with an NKCC1 antagonist, bumetanide, prevented future seizure susceptibility. These data suggest that changes in the regulation of the HPA axis following an initial seizure episode may result in HPA axis hyperexcitability and increased seizure susceptibility. Thus, insight into the mechanisms contributing to the dysregulation of the HPA axis associated with epilepsy may have significant therapeutic potential for seizure control.

1.2 Methods

1.2.1 Animal Handling

Mice expressing green fluorescent protein (GFP) specifically in CRH neurons have previously been characterized in our laboratory (Sarkar et al., 2011). Adult male CRH-GFP mice (C57Bl/6 background) were bred and housed at the Tufts University School of Medicine, Division of Laboratory Animal Medicine. The animals (5/cage) were housed in clear plastic cages in a temperature-, and humidity-controlled environment with a 12 h light/dark cycle (light on at 7 a.m.), and were maintained on an *ad libitum* diet of lab chow and water. Animals were handled according to protocols approved by the Institutional Animal Care and Use Committee of the Tufts University School of Medicine.

1.2.2 Treatments

Kainic acid—Kainic acid (Sigma) was dissolved in sterile injection saline (0.9% sodium chloride) and either 10mg/kg or 20mg/kg was delivered by intraperitoneal (*i.p.*) injection. For acute corticosterone measurements, mice were injected with either 10mg/kg or 20mg/kg kainic acid and blood samples were collected 2 hours following kainic acid administration. Corticosterone levels were also measured 7 days following seizures induced with 10mg/kg kainic acid in vehicle, Antalarmin, or bumetanide treated mice. Fresh kainic acid was prepared immediately prior to use.

Pilocarpine—Pilocarpine (Sigma) was dissolved in sterile injection saline (0.9% sodium chloride) and a single dose of 340 mg/kg was delivered by intraperitoneal (*i.p.*) injection 30 mins following treatment with scopolamine (1 mg/kg) (Peng et al., 2004). Mice were sacrificed 2 hours following treatment with either vehicle or 340 mg/kg pilocarpine and the brain and trunk blood was collected.

THIP—A blood sample was collected by submandibular bleed twenty-four hours before mice received treatment with THIP (10 mg/kg, *i.p.*) 30 min prior to administration of 20mg/kg kainic acid. Vehicle-treated mice, administered sterile injection saline (0.9% sodium chloride), were used as controls. Two hours following kainic acid treatment, trunk blood was collected for corticosterone measurements.

Bumetanide—Bumetanide was dissolved in sterile injection saline (0.9% sodium chloride) and 0.2mg/kg (Dzhala et al., 2008) was delivered by *i.p.* injection daily for 1 week between the initial dose of kainic acid and 2nd dose 7 days later. For corticosterone measurements, trunk blood was collected 30 mins after the final dose of bumetanide. For EEG recordings, the final dose of bumetanide was administered 30 mins prior to kainic acid injection (10mg/kg, *i.p.*).

Antalarmin—Antalarmin was administered *ad libitum* in drinking water for 7 days post the initial dose of kainic acid. Antalarmin (10mg) was dissolved in up to 100 μ l EtOH and then added to 100 ml of drinking water. Mice were given *ad libitum* access to drinking water containing 10mg of Antalarmin/100ml drinking water for 7 days between the 1st and 2nd dose of kainic acid (10mg/kg). For corticosterone measurements, trunk blood was collected on the 7th day of Antalarmin treatment. For EEG recordings, on the 7th day of Antalarmin treatment, seizures were induced with 10mg/kg kainic acid and EEG was recorded for 2 hours.

Corticosterone—Mice were anesthetized with 100mg/kg ketamine and 10mg/kg xylazine until unresponsive to a foot pinch and were either sham implanted or implanted with a 21-day release 10mg corticosterone pellet (Innovative Research of America, Sarasota, FL). The hair from the incision site on the back of the neck was clipped, swabbed with ethanol and iodine prior to making the incision. A small 1 cm incision was made on the back of the neck and a small slow-release pellet (or nothing for sham) was placed underneath the skin. The mice were allowed to recover and were exposed to corticosterone for 7 days prior to kainic acid treatment (20mg/kg).

1.2.3 Electrophysiological Recordings

Animals were anesthetized with isoflurane, decapitated, and the brain rapidly removed. 350 μ m thick coronal sections, including the PVN, were prepared in ice cold (4–8°C) artificial cerebral spinal fluid (ACSF) using a Leica vibratome. The intact coronal brain slices were stored oxygenated at 34°C for 1 hr prior to recording. Slices containing CRH-GFP neurons in the PVN were placed into a recording chamber maintained at 34°C and perfused with normal ACSF (nACSF) containing 126 mM NaCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂ and 10 mM dextrose (300–310 mOsm). Adequate O₂ tension and physiological pH (~7.4) were maintained by continually bubbling the media with a gas mixture: 95% O₂ / 5% CO₂.

Cell attached recordings were used to determine the spontaneous firing rate of CRH neurons. Whole cell recordings were performed on visually identified CRH neurons as previously described (Sarkar et al., 2011). Intracellular recording solution containing 140 mM cesium-methylsulfonate, 10 mM Hepes, 0.2 mM EGTA, 5 mM NaCl, 2 mM MgATP, 0.2 mM NaGTP (~280 mOsm, pH ~ 7.25) and electrodes with DC resistance of 5–8M Ω were used for recording spontaneous excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs). EPSCs were recorded at V_H = –70 mV and IPSCs were recorded at V_H = 0 mV. Once the series resistance, whole cell capacitance, and holding current stabilized, the frequency, peak amplitude, and weighted decay (τ_w) of EPSCs and IPSCs were measured during a 5 min period at each holding potential. Tonic GABAergic currents were measured using an intracellular solution containing 140mM CsCl, 1mM MgCl₂, 10mM HEPES, and 4mM Na-ATP (~280 mOsm, pH ~ 7.25) as previously described (Maguire and Mody, 2007; Maguire and Mody, 2008; Maguire et al., 2005; Sarkar et al., 2011). Briefly, the mean current was measured during 5 ms epochs collected every 100 ms throughout the experiment. A Gaussian function was fit to these points to determine the mean holding current during a period of 1 min prior to the addition of SR95531.

Similarly, the mean current was measured for a 1 min period after all GABA_AR activity was blocked with SR95531 (>200 μ M, Sigma, St. Louis, MO). The difference in the holding current in nACSF and SR95531 was calculated as the magnitude of the tonic GABAergic current. Series resistance and whole-cell capacitance were continually monitored and compensated throughout the course of the experiment. Recordings were eliminated from data analysis if series resistance increased by > 20%.

Inhibitory postsynaptic potentials (IPSPs) were recorded in nACSF containing 3 mM kynurenic acid with an internal solution containing 130 mM K-gluconate, 10 mM KCl, 4 mM NaCl, 10 mM HEPES, 0.1 mM EGTA, 2 mM Mg-ATP, 0.3 mM Na-GTP (pH=7.25, 280-290 mOsm) and 50 μ g/ml gramicidin. Perforated patch recordings with gramicidin were employed to maintain the native ionic gradients. IPSPs were recorded in the I=0 configuration to maintain the native resting membrane potential. Access resistance of < 20 M Ω was achieved within 20-30 min of establishing the G Ω seal under perforated patch clamp conditions, similar to previous studies (Sarkar et al., 2011). Once series resistance dropped below 20M Ω and stabilized, the direction of GABA-mediated postsynaptic potentials (hyperpolarizing versus depolarizing) was determined and the frequency and peak amplitude of postsynaptic potentials were measured over a 5 min period. Series resistance and capacitive transients were carefully monitored throughout the experiments to confirm the stability of the perforated-patch.

Data acquisition was carried out using an Axon Instruments Axopatch 200B and Powerlab software (ADInstruments). Data analysis was performed as previously described using minianalysis software (Synaptosoft) (Sarkar et al., 2011).

1.2.4 Western blot analysis

Western blot analysis was carried out as previously described (Maguire and Mody, 2007; Maguire and Mody, 2008; Maguire et al., 2005; Sarkar et al., 2011). Animals were anesthetized with isoflurane, rapidly euthanized by decapitation, and the PVN was rapidly microdissected out and briefly sonicated in homogenization buffer (containing 10mM NaPO₄, 100mM NaCl, 10mM Na pyrophosphate, 25mM NaF, 5mM EDTA, 5mM EGTA, 2% Triton X-100, 0.5% Deoxycholate, 1mM Na vanadate, pH 7.4), in the presence of protease inhibitors (complete mini, Roche, and fresh PMSF). The lysate was incubated on ice for 30 min then the supernatant was collected following centrifugation at 14,000rpm for 10 min at 4°C. Protein concentrations were determined using the DC Protein Assay (BioRad). Protein (25 μ g) was loaded onto a 12% SDS-polyacrylamide gel, subjected to electrophoresis and transferred to a PDVF membrane (Immobilon P, Millipore), blocked in 10% non-fat milk, and probed with a polyclonal antibody specific for KCC2 (1:1,000, Millipore), a polyclonal antibody specific for the phosphorylated Ser940 residue on KCC2 (1:1000, a generous gift from Dr. Stephen J. Moss), a polyclonal NKCC1 antibody (1:1000, Millipore), a polyclonal antibody against the GABA_AR γ 2 subunit (AbCam, 1:1000), or a monoclonal β -tubulin antibody (1:10,000, Sigma). The blots were incubated with either peroxidase labeled anti-rabbit IgG (1:2,500, GE Healthcare) or peroxidase labeled anti-mouse IgG (1:2,500, GE Healthcare) and immunoreactive proteins were visualized using enhanced chemiluminescence (Amersham). Optical density measurements were performed using NIH Image J software.

1.2.5 Immunofluorescence

Immunofluorescence for c-fos was carried out similar to previously described (Rostkowski et al., 2013). Animals were anesthetized with isoflurane, rapidly euthanized by decapitation, and the brain was rapidly dissected out and fixed by immersion fixation in ice cold 4% paraformaldehyde. The brain was cryoprotected using a sucrose gradient (10-30% sucrose in

1 × phosphate buffered saline (PBS)), snap frozen in an isopentane bath on dry ice, and placed in the −80°C freezer until use. Free-floating sections (40 μm) were prepared using a cryostat. Sections were incubated with a polyclonal antibody specific for c-fos (1:5,000, Millipore) for 72 hours at 4°C. The sections were then incubated with Alexa Fluor® 488 Goat Anti-Rabbit IgG (1:200, Invitrogen) for 2 hours at room temperature. The sections were mounted and coverslipped with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories). Cell counts were performed using Image J software and images were acquired using a Nikon A1R confocal microscope.

1.2.6 Steroid hormone concentration determination

Plasma was isolated from trunk blood by high speed centrifugation (14,000 rpm for 5 mins). ACTH was measured by enzyme immunoassay (Phoenix Pharmaceuticals) according to manufacturer's instructions. ACTH levels were measured in duplicate samples (25 μl) and compared to a standard curve of known ACTH concentrations. Similarly, corticosterone levels were measured by enzyme immunoassay according to manufacturer's specifications (Assay Designs). Corticosterone was measured using a kit from Assay Designs which has been shown to be successful in accurately determining plasma corticosterone levels in the mouse. Briefly, duplicate 5 μl plasma samples were assayed and compared to a standard curve using a spectrophotometer (at 450 nm).

1.2.7 Electroencephalogram (EEG) recording

Mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine until unresponsive to a foot pinch. A lengthwise incision was made along the scalp and a pre-fabricated headmount (Pinnacle Technology, part #8201) was fixed to the skull with four screws, which serve as differential recording leads. The headmount was fixed to the skull using dental cement and the animal was allowed to recover for a minimum of 5 days prior to experimentation. Electroencephalogram recordings were collected using a 100x gain preamplifier high pass filtered at 1.0 Hz (Pinnacle Technology, part #8202-SE) and tethered turnkey system (Pinnacle Technology, part #8200) for 2 hrs following *i.p.* injection with either 10mg/kg or 20 mg/kg kainic acid. Epileptiform activity was measured as previously described (Klaassen et al., 2006; Maguire and Mody, 2007; Maguire et al., 2005). Seizure events were identified by the sudden onset of high amplitude activity, at least 2.5 times the standard deviation of the baseline, lasting longer than 5 s in duration. Seizure activity was also identified by consistent changes in the Power of the fast Fourier transform of the EEG, including a change in the Power and the frequency of activity over the course of the event. Abnormal periods of EEG activity which cannot be defined as a seizure, including periods of rhythmic spiking lasting longer than 30 s, along with ictal events were defined as “epileptiform activity”. These criteria have been used previously by our group (Klaassen et al., 2006; Maguire and Mody, 2007; Maguire et al., 2005) as well as by other experts in the field (Castro et al., 2012). Seizure latency was defined as the time elapsed from the injection of kainic acid to the start of the first electrographic seizure. The fraction of total time exhibiting epileptiform activity (% epileptiform activity) was calculated as the cumulative time of all epileptiform activity during a 120-min recording period divided by 120 min. LabChart Pro software (ADInstruments) was used for data acquisition and analysis.

1.2.8 Statistical Tests

Experiments involving comparison between two experimental groups (vehicle vs. kainic acid), statistical significance was determined using a Student's t-test. For experiments involving more than two experimental groups (vehicle, kainic acid, and pilocarpine or vehicle, Antalarmin, and bumetanide), statistical significance was determined using a one-way ANOVA with a Tukey's test for multiple comparisons. A Pearson's correlation

calculation was used to determine a positive correlation between epileptiform activity and corticosterone levels. All statistical tests were performed using Prism software (GraphPad).

* denotes statistical significance of $p < 0.05$.

1.3 Results

1.3.1 HPA axis activation following seizures

Circulating levels of ACTH and corticosterone levels were measured in mice 2 hours following treatment with vehicle, 20mg/kg kainic acid, or 340 mg/kg pilocarpine (**Figure 1**). ACTH levels were elevated in mice with seizures induced with kainic acid (265.6 ± 40.3 pg/ml) or pilocarpine (223.0 ± 16.7 pg/ml) compared to vehicle-treated controls (138.9 ± 7.6 pg/ml) (**Figure 1a**; $n = 7-13$ mice per experimental group; $p < 0.05$). Similarly, corticosterone levels were significantly elevated in mice following seizures induced with either kainic acid (192.2 ± 17.1 ng/ml) or pilocarpine (153.2 ± 13.7 ng/ml) compared to vehicle-treated controls (48.0 ± 6.2 ng/ml) (**Figure 1b**; $n = 7-28$ mice per experimental group; $p < 0.05$). We observe a dose-response relationship between kainic acid and the extent of elevations in circulating corticosterone. A low dose of kainic acid (10mg/kg) significantly increased circulating corticosterone levels (111.3 ± 17.7 ng/ml) compared to vehicle treated mice (48.0 ± 6.2 ng/ml). A higher dose of kainic acid (20mg/kg) increased circulating corticosterone levels even further (192.2 ± 17.1 ng/ml) (**Figure 1c**; $n = 22-28$ mice per experimental group; $p < 0.05$). Further supporting a relationship between seizures and activation of the HPA axis, a correlation analysis revealed a positive correlation between the extent of epileptiform activity and corticosterone levels ($r = 0.819$) ($n = 10$ mice; < 0.05 ; **Figure 1d**).

Seizure-induced activation of the HPA axis is also supported by evidence demonstrating c-fos activation of neurons in the PVN following seizures induced with either kainic acid or pilocarpine. There is an increase in the number of c-fos-positive neurons in the PVN two hours following seizures induced with kainic acid (20mg/kg) (193.2 ± 31.7) or pilocarpine (340mg/kg) (242.4 ± 33.0) compared to vehicle-treated mice (14.7 ± 2.6) ($n = 18$ sections, 3 mice per experimental group; < 0.05 ; **Figure 2**). These data demonstrate activation of the HPA axis in two acute epilepsy models.

To investigate whether the increased circulating levels of corticosterone are associated with activation of the HPA axis, we measured the spontaneous firing rate of CRH neurons, which are at the apex of HPA axis control, 2 hours following either vehicle, kainic acid, or pilocarpine treatment. The spontaneous firing rate of CRH neurons (Sarkar et al., 2011) in the PVN was significantly increased following seizures in mice induced with either kainic acid (7.5 ± 0.9 Hz) or pilocarpine (7.0 ± 0.9 Hz) compared to vehicle-treated mice (0.09% injection saline) (3.8 ± 0.5 Hz) (**Figure 3**; $n = 15-16$ cells, 3-4 mice per experimental group; $p < 0.05$). These data suggest that seizures induced with kainic acid activate CRH neurons and increase corticosterone levels.

1.3.2 Corticosterone exacerbates seizure susceptibility

To determine whether elevations in corticosterone levels of this magnitude are sufficient to increase seizure susceptibility, we assessed seizure susceptibility in mice treated with exogenous corticosterone. Mice were treated for 7 days with a subcutaneous, 10mg slow release corticosterone pellet and seizure susceptibility was analyzed following administration of 20mg/kg kainic acid (**Figure 4a**). Exogenous corticosterone treatment increased corticosterone (192.2 ± 17.1 ng/ml) compared to sham (57.9 ± 13.7 ng/ml) or vehicle (47.2 ± 6.9 ng/ml) treated mice, increasing circulating corticosterone to levels comparable to those following seizures induced with kainic acid (169.8 ± 19.2 ng/ml) or

pilocarpine (153.2 ± 13.7 ng/ml) (data not shown; $n = 6-9$ mice per experimental group; $p=0.93$). Representative EEG recordings of activity approximately 10 min following 20mg/kg kainic acid treatment in sham and corticosterone-implanted mice are shown in **Figure 4b**. Mice treated with corticosterone exhibited a decreased latency to the first seizure episode (239.0 ± 35.0 s) and an increased percent time exhibiting epileptiform activity for the 2 hours post-kainic acid (20mg/kg) treatment (89.8 ± 1.6 %) compared to sham-implanted mice (latency: 451.4 ± 85.2 s; percent epileptiform activity: 74.5 ± 6.5 %) (**Figure 4c**; $n = 6$ mice per experimental group; $p<0.05$). These data suggest that elevations in corticosterone, to levels similar to those observed following kainic acid-induced seizures, are sufficient to increase seizure susceptibility. Insight into the mechanisms underlying seizure-induced elevations in corticosterone levels may allow for intervention to prevent seizure susceptibility.

1.3.3 Altered inhibition of CRH neurons following seizure activity

To determine the mechanisms underlying the observed increase in the excitability of CRH neurons following kainic acid-induced seizures (**Figure 3**), we performed whole cell patch clamp recording, using a cesium methane sulfonate internal solution, to measure both spontaneous EPSCs and IPSCs by changing the command voltage ($V_H = -70$ mV and 0 mV, respectively). Two hours following kainic acid administration (20 mg/kg), there was no significant difference in the frequency (1.1 ± 0.3 Hz) or peak amplitude (21.7 ± 2.3 pA) of sEPSCs compared to vehicle-treated mice (frequency: 1.0 ± 0.1 Hz; amplitude: 18.4 ± 1.9 pA) (**Figure 5b**; $n = 14$ cells, 4 - 5 mice per experimental group; $p = 0.85$ and 0.27 , respectively). Thus, the increased excitability of CRH neurons following kainic acid treatment does not appear to be the result of increased glutamatergic excitation. Therefore, we examined potential changes in the GABAergic control of CRH neurons. Counter-intuitively, we observed a significant increase in the frequency (5.1 ± 0.7 Hz) and peak amplitude (45.8 ± 3.8 pA) of sIPSCs 2 hours following kainic acid treatment compared to vehicle-treated mice (frequency: 2.7 ± 0.5 Hz; amplitude: 26.6 ± 3.3 pA) (**Figure 5a,c**; $n = 14$ cells, 4 - 5 mice per experimental group; $p < 0.05$). The lack of change in EPSCs and the increase in IPSCs (**Figure 5**) cannot account for the increased firing rate and increased corticosterone levels (**Figure 3**) that we observe following kainic acid-induced seizures. Therefore, we assessed potential changes in tonic GABAergic inhibition, which has been shown to regulate CRH neurons (Sarkar et al., 2011). Two hours following kainic acid administration, we observe a significant decrease in the tonic GABAergic inhibition (4.6 ± 1.2 pA) in CRH neurons in the PVN compared to vehicle-treated mice (10.5 ± 2.6 pA) (**Figure 6a,b**; $n = 15 - 17$ cells, 5 mice per experimental group; $p < 0.05$). In light of these findings, we hypothesized that the loss of tonic inhibition may underlie the increased activity of CRH neurons and increase in corticosterone levels following kainic acid treatment.

1.3.4 Potentiating tonic GABAergic inhibition exacerbates seizure-induced activation of the HPA axis

Previous studies have demonstrated a role for the GABA_AR δ subunit in the tonic GABAergic regulation of CRH neurons (Sarkar et al., 2011). If loss of tonic GABAergic inhibition in CRH neurons underlies activation of the HPA axis following kainic acid-induced seizures, then we anticipated that potentiating the δ subunit-mediated GABAergic current with the GABA agonist THIP (Maguire et al., 2005), may decrease HPA axis activation following kainic acid treatment. Adult male mice were treated with a non-sedative dose (10 mg/kg) of THIP (Maguire et al., 2005) 30 min prior to treatment with kainic acid. Serum was isolated from blood samples collected 24 hours prior to THIP treatment and compared to levels obtained 2 hours following kainic acid treatment by enzyme immunoassay. We observed an unexpected seizure-induced increase in corticosterone levels

following THIP treatment (1093.6 ± 259.8 ng/ml) compared to kainic acid treatment alone (169.8 ± 19.2 ng/ml) (**Figure 6c**; $n = 6$ mice per experimental group; $p < 0.05$), suggesting that potentiating the effects of tonic GABAergic currents exacerbates the HPA axis activation induced by kainic acid treatment. These data are consistent with compromised GABAergic control of the HPA axis following seizures induced with kainic acid, similar to activation of the HPA axis following stress (Hewitt et al., 2009; Sarkar et al., 2011).

1.3.5 Seizure-induced alterations in proteins required for effective GABAergic inhibition

Seizures have been shown to alter GABA_AR subunit expression in other brain regions, and particularly well studied are changes in the hippocampus (for review see (Sperk et al., 2009)). To determine whether similar changes occur in the PVN, we performed Western blot analysis. We observe an increase in GABA_AR $\gamma 2$ subunit expression in the total protein isolated from the microdissected PVN from kainic acid-treated mice (60.2 ± 3.0 O.D. units/25 μ g total protein) compared to vehicle (41.6 ± 6.1 O.D. units/25 μ g total protein) (**Figure 7a,b**; $n = 8-10$ mice per experimental group; $p < 0.05$), consistent with the observed increase in sIPSCs following kainic acid-induced seizures (**Figure 5**). However, as stated above, these changes cannot account for the increased activity of CRH neurons observed following seizures.

Alterations in KCC2 expression, including a dephosphorylation of KCC2 Ser940 and downregulation of KCC2, have been demonstrated in the PVN following acute stress and have been implicated in activation of the HPA axis (Sarkar et al., 2011). To determine whether similar mechanisms play a role in seizure-induced activation of the HPA axis, which might help explain the elevated corticosterone levels in mice treated with THIP prior to kainic acid-induced seizures, we analyzed KCC2 expression in total protein isolated from the PVN. We observed a decrease in the phosphorylation of KCC2 residue Ser940 following kainic acid-induced seizures (27.6 ± 2.6 O.D. units/25 μ g total protein) compared to vehicle (42.8 ± 3.0 O.D. units/25 μ g total protein) (**Figure 7a,b**; $n = 10-12$ mice per experimental group; $p < 0.05$). In addition, there is a significant decrease in the total expression of KCC2 in the PVN following kainic acid treatment (57.0 ± 1.8 O.D. units/25 μ g total protein) compared to vehicle (72.9 ± 2.5 O.D. units/25 μ g total protein) (**Figure 7a,b**; $n = 13-15$ mice per experimental group; $p < 0.05$). In contrast, there is a slight, yet statistically significant increase in NKCC1 expression following kainic acid treatment in the PVN (32.8 ± 2.0 O.D. units/25 μ g total protein) compared to vehicle (27.9 ± 0.7 O.D. units/25 μ g total protein) (**Figure 7a,b**; $n = 9$ mice per experimental group; $p < 0.05$). These data suggest that compromised GABAergic control of CRH neurons following kainic acid-induced seizures may employ similar mechanisms to those involved in the activation of the HPA axis following acute stress.

1.3.6 Depolarizing effects of GABA on CRH neurons results in dysregulation of the HPA axis

To determine whether alterations in the expression of NKCC1 and KCC2 alter GABAergic control of CRH neurons, we performed perforated patch clamp recordings on CRH neurons. In order to measure the magnitude and direction of spontaneous IPSPs without altering the native ionic gradients or resting membrane potential, we used gramicidin perforated patch and $I=0$, respectively. We did not observe any difference in the resting membrane potential (V_m) of cells from vehicle (60.6 ± 4.2 mV) versus kainic acid-treated mice (62.0 ± 7.5 mV) (data not shown; $p = 0.82$). As expected, in vehicle-treated mice, the actions of GABA on CRH neurons were largely hyperpolarizing. We observed hyperpolarizing sIPSPs in 92.9 ± 5.0 % of cells from vehicle-treated mice; whereas, only 59.3 ± 9.6 % of cells from kainic acid-treated mice have hyperpolarizing sIPSPs (**Figure 8a,b**). Conversely, we observed depolarizing sIPSPs in a small percentage of cells (7.1 ± 5.0 %) from vehicle-treated mice;

whereas, a higher percentage of cells from kainic acid-treated mice ($40.7 \pm 9.6\%$) exhibited depolarizing sIPSPs (data not shown; $n = 27-28$ cells, 6-8 mice per experimental group; $p < 0.05$). Furthermore, cells from kainic acid-treated mice exhibiting hyperpolarizing GABAergic responses, showed a decrease in the peak amplitude of sIPSPs (2.6 ± 0.2 mV) compared to vehicle-treated mice (3.9 ± 0.4 mV) (**Figure 8c**) $n = 17-18$ cells, 6-8 mice per experimental group; $p < 0.05$), suggesting a change in the driving force of chloride, consistent with the loss of KCC2 expression. These data suggest that the GABAergic inhibition of CRH neurons is compromised following kainic acid-seizures, and may illustrate the mechanism by which seizures induce elevations in corticosterone.

1.3.7 Blocking activation of the HPA axis suppresses future seizure susceptibility

To determine what effect seizure-induced activation of the HPA axis has on further seizure susceptibility, mice were treated with an initial dose of kainic acid (10mg/kg) and epileptiform activity was measured for 2 hours, and 7 days later seizure susceptibility to 10mg/kg kainic acid was measured again and compared with the response to the initial dose (**Figure 11a**). Seizures induced with kainic acid (10mg/kg) is sufficient to increase corticosterone levels (100.5 ± 20.1 ng/ml) compared to vehicle-treated mice (47.2 ± 6.9 ng/ml) (**Figure 10**). Not surprisingly, the initial bout of seizures induced with kainic acid was sufficient to increase future seizure susceptibility 7 days later. The latency to the first seizure was decreased in response to the 2nd dose of kainic acid after 7 days (330.8 ± 186.3 s) compared to the initial seizure latency (1117.7 ± 520.1 s) and the percent time exhibiting epileptiform activity was increased ($81.8 \pm 10.4\%$) compared to the first dose ($44.4 \pm 13.6\%$) (**Figure 11b,c**; $n = 6$ mice per experimental group; $p < 0.05$). These data suggest that seizures induced with kainic acid increase future seizure susceptibility.

Although it is not surprising that pretreatment with a chemical convulsant increases future seizure susceptibility, this validates the model which will enable us to analyze the impact of seizure-induced elevations in corticosterone on future seizure susceptibility. However, it is first necessary to determine whether there are residual seizures following the initial dose of kainic acid. We analyzed the percent time exhibiting epileptiform activity in two hour blocks following kainic acid treatment over a 24 hour period. Our data demonstrate cessation of epileptiform activity by 10 hours post kainic acid treatment (0-2 hrs: $54.9 \pm 8.4\%$; 2-4 hrs: $46.9 \pm 13.4\%$; 4-6 hrs: $9.2 \pm 4.8\%$; 6-8 hrs: $11.3 \pm 8.6\%$; 8-10 hrs: $5.9 \pm 5.6\%$; 10-12 hrs: $0.0 \pm 0.0\%$; 12-14 hrs: $0.0 \pm 0.0\%$; 14-16 hrs: $0.0 \pm 0.0\%$; 16-18 hrs: $0.0 \pm 0.0\%$; 18-20 hrs: $0.0 \pm 0.0\%$; 20-22 hrs: $0.0 \pm 0.0\%$; 22-24 hrs: $0.0 \pm 0.0\%$) (**Figure 9**; $n = 5$ mice). These data suggest that residual seizures cannot account for the increased seizure susceptibility in response to a 2nd challenge with kainic acid.

To determine whether activation of the HPA axis and increased levels of corticosterone following an initial seizure episode can contribute to future seizure susceptibility, we analyzed the anticonvulsant effects of the CRH antagonist Antalarmin. Seven days after subjection to kainic acid-induced seizures, corticosterone levels are significantly increased (**Figure 10**). Antalarmin treatment during this seven day period (10mg/kg/day in the drinking water) was sufficient to block the seizure-induced elevations in corticosterone levels (48.4 ± 7.7 ng/ml) compared to vehicle-treated mice (100.5 ± 20.1 ng/ml), and reduce corticosterone to control levels (47.2 ± 6.9 ng/ml) (**Figure 10**; $n = 17-20$ mice per experimental group). Antalarmin was sufficient to increase the latency to the first seizure (3082.5 ± 552.4 s) and decreased the cumulative time exhibiting epileptiform activity in the two hour recording session ($25.3 \pm 6.6\%$) compared to vehicle-treated mice 7 days later (latency: 330.8 ± 186.3 s; epileptiform activity: $81.8 \pm 10.4\%$) (**Figure 11b,c**; $n = 6-10$ mice per experimental group; $p < 0.05$). It is important to note that Antalarmin treatment did not have any effect on acute seizure susceptibility to kainic acid (20mg/kg), demonstrating an equivalent percent

time spent exhibiting epileptiform activity (vehicle: 76.7 ± 5.9 %; Antalarmin: 74.0 ± 9.5 %) ($n = 6-7$ mice per experimental group). These data suggest that blocking CRH signaling following an initial seizure episode can decrease future seizure susceptibility.

To determine whether changes in the chloride gradient and compromised GABAergic inhibition may play a role in activation of the HPA axis contributing to future seizure susceptibility, we assessed the anticonvulsant effects of an NKCC1 antagonist, bumetanide, on future seizure susceptibility. Bumetanide treatment is sufficient to decrease seizure-induced elevations in corticosterone levels (56.2 ± 10.0 ng/ml) compared to vehicle-treated mice (100.5 ± 20.1 ng/ml) to levels similar to controls (47.2 ± 6.9 ng/ml) (**Figure 10**; $n = 17-20$ mice per experimental group; $p < 0.05$). Mice were treated with 10mg/kg kainic acid and 7 days later challenged with another dose of 10mg/kg kainic acid. Daily bumetanide treatment (a single dose of 0.2mg/kg bumetanide daily) for the 7 days between kainic acid treatments increased the latency to the first evidence of epileptiform activity (3458.0 ± 1585.8 s) and decreased the percent time exhibiting epileptiform activity (5.3 ± 4.9 %) compared to vehicle (latency: 330.8 ± 186.3 s; time seizing: 81.8 ± 10.4 %; **Figure 11b,c**; $n = 6$ mice per experimental group; $p < 0.05$). These data suggest that breakdown in the chloride gradient due to loss of KCC2 results in HPA axis hyperexcitability and increased seizure susceptibility. Further, restoration of the chloride gradient with bumetanide and blocking the seizure-induced activation of the HPA axis decreases future seizure susceptibility.

1.4 Discussion

Patients with epilepsy have increased cortisol levels, which has been implicated in numerous pathological consequences, including increased seizure susceptibility (for review see (Joels, 2009; Sawyer and Escayg, 2010; Roberts and Keith, 1995)) as well as the comorbidity of depression in epilepsy (Pineda et al., 2010; Sankar R and Mazarati A, 2013). However, the underlying mechanisms leading to elevated levels of cortisol in patients with epilepsy are unknown. This study demonstrates, for the first time, that an initial seizure insult can result in HPA axis activation which consequently promote further seizure susceptibility. Seizures induced with kainic acid create a pro-epileptic environment which increases seizure susceptibility. Our data demonstrate that the increased seizure susceptibility lasts for at least a week following the initial insult. The establishment of a pro-epileptic environment may last even longer than the one-week time point investigated here, and future studies will focus on the time course of HPA axis activation and how long the initial insult renders the brain more susceptible to seizures. The increased seizure susceptibility following an initial seizure insult induced with kainic acid is likely multifactorial. Previous studies report neurodegeneration, gliosis, mossy fiber sprouting, and changes in neurotransmitter systems, such as changes in GABA_AR subunit expression (Sperk et al., 1983; Sperk et al., 2009; Cronin and Dudek, 1988) following kainic acid-induced seizures, which likely play a role in further seizure susceptibility. In addition to these proposed mechanisms, here we demonstrate a role for HPA axis activation in promoting seizure susceptibility following an initial seizure insult. These studies are consistent with the idea that seizures beget seizures and validate a model for investigating potential interventions.

Previous studies have demonstrated the development of spontaneous recurrent seizures following kainic acid treatment (Williams et al., 2009). Our data suggest that there is a cessation of seizures induced with 10mg/kg kainic acid in C57Bl/6 mice after 10 hours. These conflicting data are likely due to differences in dosing regimens. Recurrent seizures following kainic acid treatment were observed using 10-fold higher doses of kainate. The mean total dose of kainate used to elicit spontaneous recurrent seizures was 2.98 ± 1.34 mg (Williams et al., 2009). In our studies, the mean dose of kainate administered was $0.25 \pm$

0.01 mg. Furthermore, the latent period observed prior to the detection of spontaneous recurrent seizures following kainic acid was 11.0 ± 9.8 days, suggesting even at this high dose of kainate, the animals do not exhibit recurrent seizures at 7 days post kainate administration. Either way, our data suggest that seizure-induced elevations in corticosterone levels may contribute to the pathophysiological mechanisms leading to future seizure susceptibility. Future studies will determine whether activation of the HPA axis plays a role in epileptogenesis.

Altered GABA_AR subunit expression has been observed in numerous animal models of epilepsy as well as in humans with temporal lobe epilepsy (Sperk et al., 2009). The vast majority of these studies focus on changes in the hippocampus. Here we tested the hypothesis that changes in GABA_AR subunit expression and function in CRH neurons of the PVN may underlie dysregulation of the HPA axis following seizures. We observed an increase in the expression of the GABA_AR $\gamma 2$ subunit (**Figure 7**) and increased phasic GABAergic currents on CRH neurons (**Figure 5**) following seizures induced with kainic acid. In addition, we measured a decrease in tonic GABAergic currents in CRH neurons following kainic acid-induced seizures. These data demonstrate that there are alterations in GABAergic inhibition of CRH neurons following seizures, consistent with our hypothesis. Interestingly, we unexpectedly observed that GABAergic inhibition onto CRH neurons becomes compromised following seizure activity, resulting in a reduction in the amplitude of sIPSPs recorded under perforated patch clamp conditions and even depolarizing sIPSPs.

In this study, we uncover the mechanism underlying seizure-induced activation of the HPA axis involving a dephosphorylation and downregulation of KCC2, resulting in depolarizing actions of GABA and increased excitability of CRH neurons. The ability of THIP to exacerbate the seizure-induced elevations in circulating corticosterone are consistent with this proposed mechanism. However, we cannot rule out off-target effects of THIP or the effects of THIP on seizure activity which may indirectly alter HPA axis responsiveness. Although we would anticipate that the potential anticonvulsant effects of THIP to decrease rather than increase seizure-induced corticosterone levels. These findings support a mechanism of seizure-induced activation of the HPA axis is similar to the mechanism underlying acute stress-induced activation of the HPA axis (Sarkar et al., 2011), which has been proposed to result from increased excitation (Lee et al., 2011; Sarkar et al., 2011). These data suggest that seizures are stressors and activate the HPA axis via a similar mechanism to stress. Following stress, changes in KCC2 and alterations in GABAergic inhibition are restricted to CRH neurons in the PVN and do not occur in the other brain regions, such as the hippocampus (Sarkar et al., 2011). In contrast, previous studies have demonstrated changes in KCC2 expression in the hippocampus following seizures (Pathak et al., 2007), which may also contribute to future seizure susceptibility. However, along with changes in KCC2, the ability of Antalarmin to decrease future seizure susceptibility convincingly implicates the activation of CRH neurons in future seizure susceptibility.

Here we demonstrate the therapeutic potential of Antalarmin in seizure control. Antalarmin is a novel target for seizure control, although others have suggested that antiglucocorticoids may be a beneficial treatment for epilepsy (Joels, 2009). However, the enthusiasm for targeting glucocorticoids has waned after failed clinical trials observed compensatory upregulation of other steroid hormones, including ACTH and cortisol (Laue et al., 1990), suppression of immune function (Laue et al., 1990), and compromising homeostatic functions by blocking the diurnal corticosterone release (for review see (Nihalani and Schwartz, 2007)). The data presented here suggests that CRH may be a useful therapeutic target for seizure control. It is unlikely that Antalarmin alone will be effective as an anti-seizure medication; however, in combination with currently used antiepileptic drugs (AEDs), Antalarmin may confer benefits for seizure control and potentially the

comorbidities associated with epilepsy, such as depression. Ongoing studies in our laboratory are investigating this hypothesis.

Here we also demonstrate potential anti-seizure actions of bumetanide in the adult. Bumetanide has previously been shown to be beneficial for the treatment of pediatric epilepsies (Dzhala et al., 2008). However, it was disregarded as a potential treatment for seizure control in adults due to the developmental profile of NKCC1, the inability of bumetanide to cross the blood brain barrier (BBB), and the rapid clearance of bumetanide (Brandt et al., 2010). In contrast, our data suggest that bumetanide does have potential for the treatment of seizures in adults. Furthermore, the data presented here suggest that targeting NKCC1 or KCC2 may have therapeutic potential for the treatment of epilepsy. The ability of bumetanide to act as an anticonvulsant in adults may be due to the breakdown of the BBB following seizures, allowing bumetanide to gain access to the brain (Nitsch and Klatzo, 1983). In addition, the downregulation of KCC2 following seizures may result in an increased activity of NKCC1 facilitating the therapeutic effect of bumetanide. In fact, we do observe an increase in NKCC1 expression in the PVN following seizures induced with kainic acid (**Figure 7**), which has also been observed in the hippocampus in the pilocarpine model of epilepsy (Brandt et al., 2010). Interestingly, it has recently been proposed that the anticonvulsant actions of bumetanide may involve actions on the HPA axis (Kahle and Kaila, 2013). Our data support this hypothesis. However, further studies are required to investigate this hypothesis which is outside the scope of the current study.

1.5 Conclusions

This study demonstrates alterations in NKCC1 and KCC2 expression in the PVN compromise GABAergic inhibition of CRH neurons, increase corticosterone levels, create a pro-convulsant environment, and ultimately contribute to increased future seizure susceptibility. Furthermore, reducing seizure-induced elevations in corticosterone levels with either Antalarmin or bumetanide treatment decreased further seizure susceptibility and may represent an important therapeutic target for seizure control.

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Abbreviations

CRH	corticotropin-releasing hormone
CORT	corticosterone
HPA	hypothalamic-pituitary-adrenal
GABA_AR	GABA _A receptor
KCC2	K ⁺ /Cl ⁻ co-transporter 2
KA	kainic acid
NKCC1	Na-K-Cl co-transporter 1

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Highlights

- Seizure-induced activation of the HPA axis
- Seizure-induced dysregulation in the GABAergic control of the HPA axis
- Compromised GABAergic inhibition of CRH neurons following seizures induced with kainic acid due to downregulation of KCC2
- Preventing seizure-induced activation of the HPA axis with Antalarmin decreased seizure susceptibility
- Preventing seizure-induced activation of the HPA axis with bumetanide decreased seizure susceptibility

Highlights

- Seizure-induced activation of the HPA axis
- Seizure-induced downregulation of KCC2 compromises GABAergic control of CRH neurons
- Antalarmin blocked activation of the HPA axis and decreased seizure susceptibility
- Bumetanide blocked activation of the HPA axis and decreased seizure susceptibility

Figure 1. Seizure-induced elevations in ACTH and corticosterone

a, Average circulating ACTH levels 2 hours following treatment with vehicle, kainic acid (20mg/kg), or pilocarpine (340mg/kg). $n = 7-13$ mice per experimental group. b, Average corticosterone levels measured 2 hours following treatment with vehicle, 20mg/kg kainic acid, or pilocarpine (340mg/kg). $n = 7-28$ mice per experimental group. c, Average circulating corticosterone levels measured 2 hours following vehicle, 10mg/kg kainic acid, or 20mg/kg kainic acid treatment. $n = 22-28$ mice per experimental group. d, The correlation calculation between the percent electrographic epileptiform activity and circulating corticosterone levels ($r = 0.819$). $n = 10$ mice per experimental group. * denotes significance ($p < 0.05$) compared to control using a one-way ANOVA with Tukey's test for multiple comparisons.

Figure 2. Seizure-induced activation of c-fos in PVN neurons

a, Representative images of c-fos immunoreactivity in the PVN of vehicle, kainic acid (20mg/kg), and pilocarpine (340 mg/kg) treated mice. b, The average number of c-fos-positive neurons in the PVN of vehicle, kainic acid, and pilocarpine-treated mice. $n = 18$ sections, 3 mice per experimental group. * denotes significance ($p < 0.05$) compared to control using a one-way ANOVA with Tukey's test for multiple comparisons.

Figure 3. Seizure-induced increase in the activity of CRH neurons

a, Representative traces of the spontaneous firing rate of CRH neurons in vehicle, kainic acid (20mg/kg), and pilocarpine (340mg/kg) treated mice. b, The average firing rate of CRH neurons in the PVN in vehicle, kainic acid, and pilocarpine treated mice. $n = 15-16$ cells, 3-4 mice per experimental group. * denotes significance ($p < 0.05$) compared to control using a one-way ANOVA with Tukey's test for multiple comparisons.

Figure 4. Increased seizure susceptibility in corticosterone-treated mice

a, Timeline of corticosterone and kainic acid treatment prior to *in vivo* EEG recording. b, Representative traces of epileptiform activity in sham and corticosterone-implanted mice at the same time post kainic acid administration (approximately 10 min post-KA administration). Each line is 1min of EEG activity for a total of 6 min in each experimental group. c, The average latency to the first seizure episode in sham and corticosterone-implanted mice following 20mg/kg kainic acid administration. d, The percent time exhibiting epileptiform activity for the 2 hours post kainic acid administration in sham and corticosterone-implanted mice. $n = 6-9$ mice per experimental group. * denotes significance ($p < 0.05$) compared to sham using a Student's t-test.

Figure 5. Increased phasic GABAergic inhibition following seizures

a, Representative traces of sIPSCs ($V_H = 0$ mV) recorded in CRH neurons 2 hours following administration of either vehicle (black) or 20mg/kg kainic acid (grey). b, The average frequency and peak amplitude of sEPSCs in CRH neurons from vehicle and kainic acid-treated mice. c, The average frequency and peak amplitude of sIPSCs in CRH neurons from vehicle and kainic acid-treated mice. $n = 14$ cells, 4 - 5 mice per experimental group. * denotes significance ($p < 0.05$) compared to vehicle using a Student's t-test.

Figure 6. Decreased tonic GABAergic inhibition in CRH neurons following kainic acid-induced seizures

a, Representative graphs of the tonic GABAergic current on CRH neurons from vehicle and kainic acid-treated mice. Each dot represents the average holding current every 100 ms before and after the addition of SR95531 (black line). b, The average tonic GABAergic inhibition in CRH neurons recorded in CRH neurons in vehicle and kainic acid-treated mice. $n = 15 - 17$ cells, 5 mice per experimental group. * denotes significance ($p < 0.05$) compared to vehicle using a Student's t-test. c, The average corticosterone levels before and after kainic acid-induced seizures in vehicle and THIP-treated mice. $n = 6$ mice per experimental group. * denotes significance ($p < 0.05$) compared to vehicle using a paired t-test.

Figure 7. Alterations in GABA-relevant proteins following seizures

a, Representative Western blots of the total protein isolated from the PVN of vehicle and kainic acid-treated mice in two independent samples per experimental group probed with antibodies against KCC2, a phosphospecific antibody for KCC2 residue Ser 940 (P-KCC2), NKCC1, the GABA_AR γ2 subunit, and β-tubulin. b, The average optical density measurements for KCC2, P-KCC2, NKCC1, and the GABA_AR γ2 subunit in the PVN of vehicle and kainic acid-treated mice. n = 8-15 mice per experimental group. * denotes significance (p<0.05) compared to vehicle using a Student's t-test.

Figure 8. Compromised GABAergic inhibition of CRH neurons following seizures

a, Representative perforated patch clamp traces of sIPSPs recorded in CRH neurons from vehicle and kainic acid-treated mice. The majority of sIPSPs in CRH neurons from vehicle-treated mice are hyperpolarizing (control). Spontaneous IPSPs recorded in CRH neurons from kainic acid-treated mice are either hyperpolarizing with a decreased amplitude (kainic acid – hyperpolarizing IPSPs) or depolarizing (kainic acid – depolarizing IPSPs) compared to those recorded in vehicle-treated mice. All sIPSPs were blocked with SR95531. **b**, The percentage of hyperpolarizing sIPSPs recorded in CRH neurons from vehicle- and kainic acid-treated mice. **c**, The average peak amplitude of hyperpolarizing sIPSPs recorded in CRH neurons from vehicle- and kainic acid-treated mice. $n = 17$ -28 cells, 6-8 mice per

experimental group. * denotes significance ($p < 0.05$) compared to vehicle using a Student's t-test.

Figure 9. Timecourse of kainic acid-induced seizures

The average percent time exhibiting epileptiform activity in 2 hour blocks following treatment with 10mg/kg kainic acid. n = 5 mice.

Figure 10. Prevention of seizure-induced elevations in corticosterone

The average corticosterone levels 7 days following kainic acid treatment in vehicle-, Antalarmin-, or bumetanide-treated mice compared to controls (no kainic acid). $n = 17-20$ mice per experimental group. * denotes significance ($p < 0.05$) compared to vehicle using a one-way ANOVA with Tukey's test for multiple comparisons.

Figure 11. Blocking seizure-induced corticosterone elevations prevents future seizure susceptibility

a, Timeline of drug treatments and EEG recording. The average latency to the first seizure detected (b) and percent time exhibiting epileptiform activity (c) in response to a second 10mg/kg dose of kainic acid 7 days following initial kainic acid treatment. $n = 6-10$ mice per experimental group. * denotes significance ($p < 0.05$) compared to vehicle using a one-way ANOVA with Tukey's test for multiple comparisons.