GABAergic Control of the HPA axis as a Treatment Target for Epilepsy

A thesis

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Abstract

In response to stress, the hypothalamic pituitary adrenal (HPA) axis is activated and the stress hormone corticosterone is released from the adrenal gland in mice. Stress is a common trigger for seizures, and epileptic patients have increased levels of stress hormones. We hypothesize that HPA activation following a single seizure episode may result in future seizure susceptibility due to the pro-convulsant effects of corticosterone. Our data demonstrate that a single seizure episode is sufficient to activate the HPA axis and increase circulating levels of corticosterone. Consistent with the pro-convulsant effects of corticosterone, attenuating the output of the HPA axis reduced seizure susceptibility. Our data suggest that increased levels of corticosterone during and following seizures could be mediating the creation of a pro-epileptic environment. Thus, insight into the regulation of the HPA axis associated with seizure activity might have significant implications for seizure control. Neurons controlling the HPA axis are largely regulated by GABAergic inhibition. The chloride co-transporters KCC2 and NKCC1 maintain the chloride gradient which is essential for $GABA_A$ mediated hyperpolarization. Here we demonstrate that KCC2 is down-regulated and functionally impaired, and NKCC1 up-regulated, in the paraventricular nucleus (PVN) of the hypothalamus, compromising GABAergic inhibition and contributing to HPA hyper-excitability following seizures. Further our data suggest that treatment with the NKCC1 inhibitor bumetanide decreases seizure susceptibility in adult animals. These data suggest that impaired GABAergic inhibition in neurons controlling the HPA axis create a pro-epileptic environment and bumetanide may be a novel target for seizure control.

Introduction

Stress induces a physiological response leading to the release of corticosterone in mice (cortisol in humans) which is mediated by the hypothalamic-pituitary-adrenal axis. In response to stress, parvocellular neurons residing in the paraventricular nucleus (PVN) of the hypothalamus release corticotropin releasing hormone (CRH) leading ultimately to corticosterone release. The CRH neurons controlling the HPA axis are regulated by substantial GABAergic inhibitory control, for review see (Herman et al., 2004).

The inhibitory action of GABA relies on a chloride gradient set by two chloride transporters, KCC2 and NKCC1. NKCC1 is driven by the inward gradient for sodium to load chloride into the cell (Kahle et al., 2008). This chloride transporter is predominantly expressed early in development in both humans and rodents, and is responsible for maintaining the high levels of intracellular chloride necessary for the depolarizing actions of GABA at this stage in development (Kahle et al., 2008; Blaesse et al., 2009). In adults, NKCC1 levels drop considerably compared to neonates, and KCC2 expression increases (Dzhala et al., 2005). KCC2 extrudes chloride, coupling this transport to the outward gradient for potassium, resulting in low levels of intracellular chloride in adults; 7 mM inside vs. 140 mM outside (Ben-Ari, 2002). Thus KCC2 is responsible for the inhibitory actions of GABA, by creating a hyperpolarizing equilibrium potential for chloride.

Both NKCC1 and KCC2 have large N- and C- terminal cytoplasmic domains that have been shown to have sites for posttranslational modification. The activity of NKCC1 appears to be most readily controlled by the phosphorylation state of a number of threonine residues in the N-terminal domain (Darman and Forbush, 2002), whereas the regulation of KCC2 seems to be dependent on phosphorylation at tyrosine and serine residues in the C-terminal domain. Phosporylation can inhibit KCC2 driven chloride transport. WNK3 and WNK1 kinases act to reduce KCC2 activity and Src kinase acting at Y903/1087 decreases surface stability of KCC2 (Kahle et al., 2005; Rinehart et al., 2009; Lee et al., 2010). Phosporylation of KCC2 can also enhance activity. PKC dependent phosphorylation at S940 promotes surface stability and activity (Lee et al., 2007).

As previously mentioned, the stress response is governed by GABAergic inhibition, and the inhibitory actions of GABA rely on chloride co-transporter function. It is known that stress is a common trigger for seizures, and epileptics have increased levels of stress hormones (Sperling et al., 2008), which is recapitulated in animal models (Talmi et al., 1995; Taher et al., 2005). Additionally, status epilepticus

(SE) is a potent activator of the HPA axis (Daniels et al., 1990). We recently demonstrated that KCC2 plays a role in the activation of the HPA axis in response to stress (Sarkar et al., 2011). Here we demonstrate that the HPA axis is activated following seizures in mice and exogenous corticosterone is sufficient to increase seizure susceptibility. We sought to investigate if changes in KCC2 underlie the activation of the HPA axis following kainic acid (KA) induced seizures in mice and whether preventing the activation of the HPA axis in this model with antalarmin or bumetanide could be of therapeutic value.

Results

Using the kainic acid (KA) model of Temporal Lobe Epilepsy (TLE) (Ben-Ari, 1985; Sperk, 1994), we observed that KA treatment activated the HPA axis. Mice receiving a 10 mg/kg i.p. KA treatment had much greater corticosterone (cort) levels than mice receiving vehicle treatment, as has been reported (Daniels et al., 1990); we also noted slightly increased HPA activation with a larger (20 mg/kg i.p.) KA dose (Veh: 49.9 ± 13.8 ng/ml, 10 mg/kg KA: 168.0 ± 51.7 ng/ml, 20 mg/kg KA: 249.6 ± 60.4 ng/ml; **Figure 1**; n=3-8, * indicates $p<.05$ compared to vehicle). To determine if the increased corticosterone levels following seizures could create a pro-epileptic environment, we exposed mice to corticosterone continuously for 7 days and compared their seizure susceptibility in response to 20 mg/kg KA to the susceptibility of mice that were not continuously exposed to corticosterone. Corticosterone treatment increased susceptibility to an acute seizure episode, increasing percent time seizing (Veh: 76.7 ± 5.8 %, Cort: 89.8 ± 1.6 %; **Figure 2A**; n=6-7, * indicates $p<.05$ compared to veh) and decreasing latency to seizure onset (Veh: 451.4 ± 85.2 s, Cort: 259 ± 48.3 s; **Figure 2B**; n=6-7, * indicates $p<.05$ compared to veh). Altering neuronal excitability could be affecting the latency to a first seizure, while inhibiting endogenous anti-excitability mechanisms could affect overall percent time seizing. To further examine the effect of HPA axis activation on seizure susceptibility, we blocked activation of the HPA axis using the CRF-1 receptor antagonist antalarmin. Treatment with antalarmin (20 mg/kg/day) for seven days in drinking water decreased seizure susceptibility. This antalarmin treatment protocol decreased percent time seizing compared to vehicle (Veh: 76.7 ± 5.8 %, Anta7: 27.3 ± 12.8 %; **Figure 2A**; n=6, * indicates $p<.05$ compared to veh) and increased latency to seizure onset (Veh: 451.4 ± 85.2 s, Anta7: 1119.2 ± 211.2 s; **Figure 2B**; n=6, * indicates $p<.05$ compared to veh). Acute 30 minutes antalarmin (20 mg/kg i.p.) pre-treatment did not decrease percent time seizing (Veh: 76.7 ± 5.8 %, Anta: 74 ± 9.5 %; **Figure 2A**; n=6, $p>.05$ compared to veh) or increase latency to seizure onset (Veh: 451.4 ± 85.2 s, Anta: 354.2 ± 73.1 s; **Figure 2B**; n=6, $p>.05$ compared to veh). These data together indicate: seizures activate the HPA axis, activation of the HPA axis enhances seizure severity, and blocking HPA axis activation before and during a single seizure episode can reduce the time spent seizing during that episode.

Using the kainic acid model of TLE we did not find that inducing a seizure episode with KA 24 hours prior to another KA challenge increased susceptibility to the second seizure episode, which is a well-accepted observation with the kainic acid model (Sperk, 1994; Marksteiner et al., 1990). The “n” numbers of this study will need to be increased in order to reproduce this result. The animals that were pretreated with 10 mg/kg i.p. KA 24 hours prior to an additional 10 mg/kg i.p. KA treatment did not seize more than

controls (Veh: 69.1 ± 6.9 %, KA: 72 ± 10.7 %; **Figure 3A**; n=3-6, p>.05 compared to veh), and had no decrease in the latency to seizure onset (Veh: 496.7 ± 161.3 s, KA: 1129.2 ± 506.4 s**; Figure 3B**; n=3-6, p>.05 compared to vehicle). This result is examined further in the discussion, but the failure of this control experiment was disconcerting. We found bumetanide (2 mg/kg i.p.) pre-treatment 30 minutes prior to the second dose of KA significantly reduced percent time seizing (KA: 72 ± 10.7 %, Bu: 20.1 ± 8.9 %; **Figure 3A**; n=6, * indicates p<.05 compared to KA) but not latency to seizure onset, compared to KA (KA: 1129.2 ± 506.4 s, Bu: 1835 ± 299.7 s; **Figure 3B**; n=6, p>.05 compared to KA). This indicated that promoting GABAergic inhibition under epileptic conditions by blocking the NKCC1 transporter in CRH neurons can be anti-epileptic. Antalarmin treatment 30 minutes prior to the second KA treatment also reduced time seizing, but not significantly (KA: 72 ± 10.7 %, Ant: 49.4 ± 18.9 %; **Figure 3A**; n=6, p>.05 compared to KA), and did not affect the latency to seizure onset (KA: 1129.2 ± 506.4 s, Ant: 529.2 ± 203.5 s; **Figure 3B**; n=6, p>.05 compared to KA). Seven day antalarmin treatment between two KA episodes spaced 1 week apart significantly attenuated the severity of the second seizure episode, reducing time seizing (Veh: 81.8 ± 10.4 %, Ant: 23.3 ± 5.7 %; **Figure 3C**; n=6, ** indicates p<.001 compared to veh) and increasing the latency to the first seizure compared to vehicle (Veh: 330.8 ± 186.3 s, Ant: 4220.8 ± 499.5 s; **Figure 3D**; n=6, ** indicates p<.001 compared to veh). Although these data need to be expanded, there is some indication that increases in seizure susceptibility due to prior seizure activity can be controlled by inhibiting HPA axis activation.

Hippocampal sclerosis, or selective cell death in the hippocampus, is a hallmark of both clinical TLE (Engel, 1996) and the kainate animal model of TLE (Ben-Ari, 1985; Sperk, 1994). We hypothesized that bumetanide and antalarmin, which were effective in reducing seizure severity, would also inhibit sclerosis in the hippocampus. Thus far the data was inconclusive. There was no reduction in CA1 cell number 72 hours after SE induced with 30 mg/kg KA, compared to controls (Veh: 459.5 ± 109.5 cells, KA: 462.5 ± 180.2 cells; **Figure 4A**; n=3 mice, 11-18 sections, p>.05 compared to vehicle). Thirty minute pretreatment with antalarmin or bumetanide before seizure induction had no effect on cell number compared to vehicle or kainate treatment alone (Ant: 390 ± 77.8, Bu: 446.2 ± 77.7 cells; **Figure 4A**; n=3 mice, 11-21 sections, p>.05 compared to vehicle or KA). There was no KA induced cell loss in CA3, a hippocampal region particularly sensitive to seizure induced cell death (Veh: 981.9 ± 94.3 cells, KA: 1034.9 ± 198.3 cells; **Figure 4B**; n=3 mice, 7-32 sections, p>.05 compared to vehicle). Thirty minute pretreatment of bumetanide or antalarmin had no effect on CA3 cell number compared to vehicle or kainate alone (Veh: 981.9 ± 94.3 cells, KA: 1034.9 ± 198.3 cells, Ant: 975.5 ± 155.7, Bu: 926.1 ± 163.0

cells; **Figure 4B**; n=3 mice, 15-27 sections, p>.05 compared to vehicle or KA). This result is examined further in the discussion.

It has been shown previously that mice who experience status epilepticus (SE) show increased susceptibility to future seizures up to a month later (Sperk, 1994). We sought to investigate this in our model using the following protocol: mice were subjected to a 10 mg/kg KA dose at time 0, followed by another 10 mg/kg dose at a second time point. Seizure activity was recorded immediately following the second KA dose. Control mice received KA at the second time point only, allowing us to compare differences in seizure activity between animals that had previously experienced seizure activity to those that had not. We chose time points of 1 week, 1 month, and 3 months following an initial induction of SE with 10 mg/kg KA. There were no differences in susceptibility from kainate treated mice compared to control for any time point. There was no difference in percent time seizing at 1 week (Veh: 55.2 ± 10.6 %, KA: 35.0 ± 2.3 %; **Figure 5A**; n=2-4 mice, p >.05 compared to vehicle), 1 month (KA: 60.6 ± 7.2 %, Veh: 67.7 ± 5.7 %; **Figure 5A**; n=4 mice per group, p>.05 compared to vehicle), or 3 months (Veh: 52.5 ± 3.5 %, KA: 38.1 ± 4.0 %; **Figure 5A**; n=5-12 mice, p>.05 compared to vehicle). Nor did we see differences in latency to seizure onset at 1 week (Veh: 1037.1 ± 663.4 s, KA: 2012.3 ± 56.8 s; **Figure 5B**; n=2-4 mice, p>.05 compared to vehicle), 1 month (Veh: 401 ± 142.7 s, KA: 633.3 ± 177.3 s; **Figure 5B**; n=4, p>.05 compared to vehicle) or 3 months (Veh: 638 ± 116.2 s, KA: 738.4 ± 95.2 s; **Figure 5B**; n=5-12, p>.05 compared to vehicle). These data show further studies are needed with increased "n" numbers to evaluate the time course of increases in seizure susceptibility.

We also wondered if the change in seizure susceptibility due to an initial seizure episode would be due to continued seizure activity or acutely induced physiological changes. One hypothesis regarding increases in seizure susceptibility due to previous seizures could be that the seizures never completely terminate. Therefore we performed electroencephalogram (EEG) recordings to determine how long seizure activity lasts in response to a single 10 mg/kg dose of KA. **Figure 5C** shows the cumulative percent time seizing in two hour epochs following administration of a single 10 mg/kg dose of kainic acid. Most animals stopped seizing by 8 hours, although 1 animal was still having significant seizures into hour 24 (**Figure 5C**; n=5 mice, each trace shows seizure activity of 1 mouse). A majority of our mice (3/5) showed minimal ≤ 2 % seizure frequency after 6 hours (**Figure 5C**), and 4/5 showed minimal seizure frequency after 8 hours (**Figure 5C**). Taken together, the data demonstrate that hypothesized long lasting changes in seizure susceptibility will be due to acute changes in brain physiology, not due to continuous seizure activity. If seizures from the initial episode did not terminate before the seizure

susceptibility was tested with another round of chemo-convulsant, than the ongoing seizure activity could confound hypothesized changes in seizure susceptibility.

To determine if seizure induced activation of the HPA axis is associated with a de-phosphorylation and down-regulation of KCC2 in CRH neurons (Sarkar et al., 2011); we examined KCC2 levels 2 hours after a 20 mg/kg KA treatment (**Figure 6A**). Kainic acid treatment significantly reduced total KCC2 levels compared to vehicle treatment (Veh: 70.7 ± 2.7, KA: 57.5 ± 2.4 OD units/50 ug protein; **Figure 6B**; n=9-12,* indicates p<.05 compared to vehicle). KCC2 is phosphorylated at residue Ser940 of the C-terminal intracellular domain and reductions in phospo S940 KCC2 lead to impaired chloride extrusion and decreased KCC2 membrane stability (Lee et al., 2007). The data indicate Ser940 phospho-KCC2 was significantly reduced in KA treated compared to vehicle treated animals (Veh: 40.7 ± 5.9, KA: 25.0 ± 2.9 OD units/50 ug protein; **Figure 6C**; n=4-5, * indicates p<.05 compared to vehicle). The NKCC1 protein is not thought to be an important contributor to chloride flux in adult animals, because of the minimal expression after development (Bragin et al., 2009). However under pathological conditions, NKCC1 can increase in expression and contribute to reductions in inhibitory GABAergic function by facilitating Cl^- influx, resulting in a more depolarized E_{Cl} (Barmashenko et al., 2011). The data demonstrate an increase in total NKCC1 levels following KA treatment compared to vehicle treated mice (Veh: 27.8 ± .8, KA: 32.8 ± 2.0 OD units/50 ug protein; **Figure 6D**; n=9, * indicates p<.05 compared to vehicle). Follow up studies should look at N-terminal phosphorylation of NKCC1, an indicator of surface stability and function.

To further investigate the role of HPA axis regulation on seizure susceptibility, we utilized a novel mouse model in which KCC2 is knocked out specifically in CRH neurons (KCC2/CRH mice: loxed KCC2 mice, generously provided by Dr. Steve Moss, Tufts University School of Medicine, Boston, MA), were crossed with CRH-Cre mice (Mutant Mouse Regional Research Center). KCC2 function has been shown to be an important modulator of the activity of these neurons (Sarkar et al., 2011). Knocking out KCC2 in CRH neurons is sufficient to increase baseline corticosterone levels compared to mice that have intact KCC2 function in CRH neurons (Cre-/- : 68.1 ± 18.3 ng/ml, Cre +/+: 154.2 ± 40.0 ng/ml; **Figure 7A**; n=6-7, * indicates p<.05 compared to cre -/-). To investigate the impact of this HPA axis hyperexcitability on seizures, we assessed seizure susceptibility in response to 20 mg/kg kainic acid. KCC2/CRH mice exhibit increased seizure severity (time seizing- Cre -/-: 39.2 ± 7.8 %, Cre+/+: 62.6 ± 4.0%; **Figure 7B**; n=6, *indicates p<.05 compared to cre -/-), and decreased latency to seizure onset compared to cre -/- mice (Cre -/-: 421.5 ± 58.4 s, Cre +/+: 212.9 ± 47.0 s; **Figure 7C**; n=6, * indicates p<.05 compared to cre -/-).

These data support our hypothesis that deficits in GABAergic control of CRH neurons lead to pathological increases in corticosterone and increased seizure susceptibility.

Methods

Animal Handling

Adult 3-6 month old mice were housed at the Tufts University School of Medicine, Division of Laboratory Animal Medicine. Mice were group housed, with 5 maximum animals per cage in clear plastic cages within a temperature and humidity controlled environment. There was a 12 hour light/dark cycle (light on at 7:00 A.M.) and free access to standard laboratory chow and tap water. Seizures in mice were induced by i.p. injection of 10, 20 or 30 mg/kg kainic acid (as described in each experiment). KCC2/CRH mice were generated by crossing floxed KCC2 mice generously provided by Dr. Steve Moss (Tufts University School of Medicine, Boston, MA), with CRH-Cre mice (Mutant Mouse Regional Research Center). Cre expression in the CRH-Cre; Tg(Crh-cre)KN282Gsat; mice has previously been shown to be specific for CRH neurons by in situ data as previously described (Sarkar et al., 2011). Animals were used in accordance with protocols approved by the Tufts University Institutional Animal Care and Use Committee.

Surgery and Recording

Electroencephalography was recorded using equipment from Pinnacle Technology (8200 Series, 3 Channel EEG/EMG, Kansas). EEG recording headmounts with EEG and EMG leads were implanted surgically, with (2) .10" and (2) .12" screws contacting the cortex and acting as leads. Briefly, mice were anesthetized using 111 mg/kg ketamine and 11 mg/kg xylazine, a standard dose (Tkatchenko and Tkatchenko, 2010). Using a stereotaxic apparatus, incisions were made on top of the skull and headmounts were glued into place 3.0 mm anterior to bregma. After the glue dried, pilot holes were drilled with a 23 g needle using 4 predrilled locations on the headmount as as guides. Screws serving as leads were inserted into the predrilled holes in the headmount and screwed through the pilot holes in the skull and rested in the cortex. A conductive silver epoxy was used to strongly electrically couple the screws to the headmounts. The entire unit was secured to the skull with dental cement. The mice were allowed to recover at least 5 days after implantation before recording EEGs.

To record EEGs, mice were placed in a recording chamber and allowed to roam while being loosely tethered to amplifying and recording equipment. 10 minutes of baseline data was collected before kainate or vehicle injection, with primary recording immediately following. Data was recorded and analyzed using AD Instruments Lab Chart software. Most recording periods lasted 2 hours (**Figs. 2, 3, 5, 7**), although some mice were recorded for 24 hours (**Figure 5**).

Drug treatments

Some animals were implanted with a subdermally placed time release corticosterone tablet just caudal to the base of the skull, during the EEG implant surgery. For these corticosterone treated animals, EEG recording was performed 1 week after surgery, giving 1 week of corticosterone supplementation (**Figure 2**). In parallel to these artificial increased HPA tone studies, a set of control animals were given antalarmin 20 mg/kg/day in drinking water to study artificial decreased HPA tone (**Figure 2**). Some animals were studied for the time course of multiple KA induced seizures. These mice were subjected to a 10 mg/kg KA dose at time 0, followed by another 10 mg/kg dose at a second time point. Seizure activity was recorded immediately following the second KA dose. Control mice received KA at the second time point only, allowing us to compare differences in seizure activity between animals that had previously experienced seizure activity to those that had not. We chose time points of 1 week, 1 month, and 3 months following an initial induction of SE with 10 mg/kg KA (**Figs. 2, 3, 5**). Some of these animals that received two doses of KA were given bumetanide (2 mg/kg i.p.; **Figure 3**) or antalarmin (20 mg/kg i.p.; **Figure 3**) 30 minutes prior to the second KA dose.

Western Blot

Protein quantification was performed with western blotting as previously described (Sarkar et al., 2011). Animals were anesthetized with isofluorane, killed by decapitation, and the PVN was rapidly removed. The tissue was sonicated in lysis buffer [containing in mM: 10 $NaPO_4$, 100 NaCl, 10 sodium pyrophosphate, 25 NaF, 5 EDTA, 5 EGTA, 2% Triton X-100, 0.5% deoxycholate, 1 sodium vanadate, pH 7.4) in the presence of a protease inhibitor cocktail (Complete Mini, Roche and fresh PMSF). The lysate was incubated on ice for 30 min, centrifuged for 10 minutes at 14,000 rpm at 4^oC. The supernatant was collected and protein concentrations were determined using the DC protein assay (Bio-Rad). 50 ug total protein (for all KCC2, Ser940 and NKCC1) was loaded onto a 10% SDS polyacrylamide gel, electrophoresis was performed at 100 V with Bio-Rad apparatus, and transferred at 30 Volts 4^o overnight onto an Immobilon-P membrane (Millipore). The membrane was blocked in 10% non-fat millk and probed with a polyclonal antibody for KCC2 (Millipore 1:1000), Ser940 (Generous gift from Dr. Steve Moss, Tufts University School of Medicine, Boston, MA 1:1000) and NKCC1 (Abcam 1:1000). The blots were incubated with anti-rabbit HRP IgG (GE Healthcare 1:2000) and visualized with ECL (GE Healthcare). Optical density measurements were determined using the NIH ImageJ software.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Sarkar et al., 2011; Ghosal et al., 2009). Briefly, the mice were treated with vehicle, 2 mg/kg bumetanide i.p., or 20 mg/kg antalarmin i.p. 30 minutes before 30 mg/kg kainate i.p. Mice were monitored to verify SE. 72 hours later they were anesthetized with isofluorane, killed by decapitation and the brain was rapidly removed. Brains were fixed in 4% paraformaldehyde overnight at 4^{o}C, cryoprotected in 10-30% sucrose, frozen at -80^{o}C, and 40 µm sections were cut on a Leica cryostat. The sections were blocked in .3% Triton X-100, 10% Normal Goat Serum (Vector) in PBS for 1 hour at RT. The floating sections were incubated NeuN conjugated to Alexa 488 primary antibody (Millipore 1:100) in blocking buffer from above. Images were taken on a Nikon fluorescent microscope, using a 20X objective and then analyzed using NIH ImageJ software.

Corticosterone measurements

SE was induced in mice with 20 mg/kg Kainic Acid (Sigma), and after two hours, animals were anesthetized with isofluorane, decapitated and trunk blood was collected. Plasma was isolated via centrifugation at 14,000 rpm for 5 minutes at RT using specialized blood collection tubes (Teromo, MD). Corticosterone was measured using a competitive EIA kit following manufacturer's instructions (Enzo Life Sciences, NY). Briefly, each reaction well is coated with donkey anti-sheep antibody. The sample, alkaline phosphatase linked to corticosterone, and a polyclonal sheep antibody to corticosterone are all added to the reaction. A molecule is added that emits light when acted on by the alkaline phosphatase. The more corticosterone that is in the sample to be studied, the more it will compete off the phosphatase linked corticosterone from the corticosterone antibody. After washing, the samples that competed off more of the phosphatases, thus having higher corticosterone concentrations, will show emit less color. Duplicate 5 µl plasma samples were tested and absorbance measurements were taken at 415 nm and compared to a standard curve. Samples from different experiments were run in parallel.

Statistics

Statistics were performed using 1 way ANOVA on Figures 1, 2, 3A-B, 4A-B. Students t-test was used on Figure 3C-D, , 5A-B, 6 and 7. Data are presented as mean ± SEM. Significant differences ($p<.05$) between the different groups are indicated in the figures by *, and very significant differences $p<.001$ are indicated by **.

Discussion

This is one of the first studies to examine the anti-epileptic efficacy of bumetanide in adult animals. The data also indicate a novel mechanism of therapeutic action for bumetanide: modulating seizure induced HPA axis activation. GABA has been shown to be excitatory in adult human TLE patients (Cohen et al., 2002), and a down-regulation of KCC2 was thought to be the cause (Huberfeld et al., 2007). Decreased levels of KCC2 have been shown in animal models of TLE (Pathak et al., 2007)(Barmashenko et al., 2011). Thus, changes in the efficiency of GABA mediated hyperpolarization, due to seizure activity induced alterations in KCC2 (and presumably E_{Cl}) are readily observed in the clinic and in animal models. Our results build on previous studies that have shown drug treatments that protect or restore the chloride gradient and promote GABAergic inhibition have shown therapeutic promise (Dzhala et al., 2005; Brandt et al., 2010).

HPA axis and seizure susceptibility

We have shown that seizure episodes are a potent activator of the HPA axis (**Figure 1**), in agreement with others (Daniels et al., 1990; Mazarati et al., 2009). Clinical literature has shown high levels of cortisol can create a pro-epileptic environment; perhaps indicating that corticosterone can create a feed forward cycle of seizure activity (Sperling et al., 2008; Schmid-Schonbein, 1998). Our data agree with this clinical finding, as modulating the HPA axis significantly altered seizure susceptibility. We observed increased seizure activity by artificially increasing HPA tone with chronic (1 week) corticosterone treatment (**Figure 2**). We also observed reduced seizure activity after down-regulating HPA activity by chronic (1 week) blockade of CRH receptors with antalarmin (**Figure 2**). This latter finding was quite interesting as the animals were seizure naïve; suggesting HPA activation even *within* a seizure episode contributes to the severity of that episode. Activation of the HPA axis also plays a role in the increases in seizure susceptibility seen subsequent to an initial seizure insult. Blocking HPA activation after an initial KA insult with bumetanide 30 minutes before a second KA challenge reduced seizure severity (**Figure 3**). An acute antalarmin treatment 30 minutes prior to a second KA challenge trended to a reduction in the time spent seizing, but the “n” numbers need to be increased to determine if the effect is significant (**Figure 3**). These results were interesting as the stress response during the initiating seizure episode did not have to be blocked in order to see seizure reduction in subsequent episodes. Therefore it seems the stress response system was put into a hyper-responsive state by the initial seizure insult, and blocking this HPA axis hyper-responsiveness with a 30 minute drug treatment prior to the second seizure challenge led to a reduction in seizure severity. Seven day treatment with antalarmin

blocked the increase in seizure severity seen with multiple KA doses 1 week apart (**Figure 3**). Further studies should see if reductions in corticosterone levels are seen with drug treatments to verify modulation of the HPA axis, and confirm our hypothesized mechanism. Additionally, electrophysiological studies should be performed on CRH neurons to determine if KA treatment alters the chloride gradient and E_{GABA} to impair GABAergic inhibition in CRH neurons. We could also verify that bumetanide is acting as an anti-epileptic by restoring E_{GABA} and promoting GABAergic inhibition in these same neurons.

Data on stress and epilepsy is confounding if the time course of the stress is not taken into account. Acute stressors have been shown to be anti-epileptic (Reddy and Rogawski, 2002). That particular study indicated a 15 or 30 minute pretreatment with stress derived neurosteroids protected rats against PTZ induced seizures for at least 30 minutes. These anti-convulsant actions are most likely due to the actions of stress-derived neurosteroids on $GABA_A$ δ containing receptors in the molecular layer of the dentate gyrus. The molecular layer of the dentate gyrus, "the hippocampal gate," is rich in δ containing receptors (Maguire et al., 2009), and neurosteroids are known to enhance tonic inhibition by acting on δ containing receptors (Sarkar et al., 2011). Therefore, if an animal is subjected to an acute stress immediately preceding an epileptic insult, the increased tonic inhibition in the dentate gyrus will dampen convulsant activity. These neurosteroid derivatives of corticosterone lose their ability to dampen dentate excitability under chronic epileptic conditions, as neurosteroid sensitive $GABA_A$ δ subunits in the dentate are down-regulated under these conditions (Peng et al., 2004). Chronically elevated corticosterone seen epileptic patients, due to a hyper-activated HPA axis (Zobel et al., 2004), is proconvulsant, as we (**Figure 2**) and others have shown (Talmi et al., 1995; Kumar et al., 2007). These effects are most likely mediated by cort's actions in the hippocampus, such as cell damage, excitation and calcium influx (for review see (Sapolsky et al., 1985) or (Joels, 2009)

KCC2 alterations in disease

Interestingly, dis-inhibition of CRH neurons via KCC2 and phospho ser940 KCC2 down-regulation has been shown to be a normal physiological response to 30 minute restraint stress, so perhaps epileptic episodes are a hijacking, or hyper-activating of endogenous stress response systems (Sarkar et al., 2011). We thoroughly showed that the hyperactivation of the HPA axis in epilepsy was due to reduced inhibition on CRH neurons in the PVN, noting decreased levels of KCC2, phospho Ser940 KCC2, and increased NKCC1 levels (**Figure 6**), which combine to abolishing the inhibitory actions of GABA. Electrophysiological data to confirm this should be included in future studies. Consistent with the role

of KCC2 regulation of CRH neurons on activation of the HPA axis in response to seizures induced with KA, KCC2/CRH knockout mice exhibit increased corticosterone levels and increased seizures susceptibility (**Figure 7**).

Loss of GABAergic inhibition is not restricted to CRH neurons of the PVN, it has been shown in magnocellular vasopressin neurons in the paraventricular and supraoptic nuclei (Haam et al., 2012), in dissociated hippocampal neurons after glutamate exposure (Lee et al., 2011), and dentate gyrus granule cells after SE (Pathak et al., 2007). One idea is that GABA shifts to depolarizing as a recovery mechanism in response to injury, a process shown to happen in after peripheral nerve injury, mediated by microglial release of BDNF (Coull et al., 2005). These neurons had general impairments in neuronal maturation and synaptogenesis (Wang and Kriegstein, 2011; Cherubini et al., 2011). It has also been reported that blocking GABA in developing neurons (where GABA is excitatory) lead to deficits in neuronal migration (Manent et al., 2005).

Modulators of chloride co-transporters have become an attractive potential therapy in neonatal seizures because of the physiological excitatory GABA at this developmental stage. bumetanide attenuated EEG seizures in neonatal rats, and seems to be a promising adjunct to conventional benzodiazepine (BZD) therapy in neonatal electroclinically uncoupled seizures (Dzhala et al., 2005; Glykys et al., 2009). This fascinating work is based on the progressive caudal to rostral expression pattern of KCC2 in development, whereby GABA remains excitatory for an extended period of time in the cortex compared to sub-cortical structures. Conventional BZDs suppressed the motor effects of seizures because GABA was inhibitory in these subcortical neurons; only by adding bumetanide were cortical seizures effectively controlled, indicating shifts in E_{Cl} were necessary before GABA became inhibitory. Kahle et al. (2008) readily admit potential side effects of bumetanide treatment warrant further study, citing that infants treated with diuretics like bumetanide commonly show developmental defects. Our studies are most encouraging because we show bumetanide treatment has promise in adults; a clinical population in need of improved therapies and not subject to these developmental side effects. Taken together, our data add to these promising results, revealing impaired GABAergic function is characteristic of adult models of TLE as well.

Future Directions

Hippocampal sclerosis is a hallmark of clinical TLE and animal models of TLE (Ben-Ari, 1985). The data did not show kainate induced sclerosis upon multiple attempts. Therefore the data did not allow a

conclusive determination on whether bumetanide or antalarmin treatment reduced hippocampal damage induced by seizures (**Figure 4**). This could have been caused by errors in IHC methodology, incomplete SE induction, or low "n" numbers. We could have also examined cell damage with NPY staining for mossy fiber sprouting, or TUNEL for DNA damage. The data did not show seizure induced changes in seizure susceptibility at 24 hours, 1 week, 1 month or 3 months (**Figure 5**). Kainate induced increased seizure susceptibility has previously been shown to last at least a month (Sperk, 1994), so this results was surprising. We had very low "n" numbers in our 1 week data as repeat experiments provided unusual and inconsistent results. Examination of the EEG lead placement in some animals revealed the placement was too far posterior, which resulted in just half of the leads recording data. This could explain inconsistent results for the time course studies. Additionally, more "n" numbers could have been added to the study to determine if there were significant changes in seizure susceptibility. The time course and the cell death data should be subject to further experimentation. These data therefore represent a starting point to further explore the relationship between corticosterone and seizure severity in animal models of TLE.

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Figure legends

Figure 1. The HPA axis is activated upon induction of seizure activity with kainic acid in a dose dependent manner. Average corticosterone levels reveal robust increases in HPA activation following seizures induced by 10 mg/kg KA i.p. or 20 mg/kg KA i.p. n=3-8 mice per group, * indicates p<.05 compared to vehicle.

Figure 2. Corticosterone increases acute seizure severity and blocking HPA axis activation decreases seizure susceptibility. A. Average percent time seizing in response to 10 mg/kg kainic acid in vehicle, cort, seven day chronic antalarmin (20 mg/kg/day in drinking water) treated or acute antalarmin (20 mg/kg i.p. 30 minutes prior to epileptic induction) treated animals. B. The average latency to seizure onset in response to 10 mg/kg kainic acid in vehicle, cort, seven day chronic antalarmin (20 mg/kg/day in drinking water) or acute antalarmin (20 mg/kg i.p. 30 minutes prior to epileptic induction) treated animals. n=6-7 mice per group, * indicates p<.05 compared to vehicle.

Figure 3. Blocking the seizure induced HPA axis response with bumetanide or antalarmin reduces seizure susceptibility. A. The average percent time seizing 24 hours after vehicle or 10 mg/kg kainate treatment. bumetanide (2 mg/kg i.p.) and antalarmin (20 mg/kg i.p.) treatments were given 30 minutes prior to second KA dose. B. The average latency to seizure onset 24 hours after vehicle or 10 mg/kg kainate treatment. bumetanide (2 mg/kg i.p.) and antalarmin (20 mg/kg i.p.) treatments were given 30 minutes prior to second KA dose. C. The average percent time seizing in response to a second 10 mg/kg KA treatment given 1 week after a first 10 mg/kg KA treatment. antalarmin (20 mg/kg/day in drinking water) or vehicle was given between the two KA doses. D. The average latency to seizure onset in response to a second 10 mg/kg KA treatment given 1 week after a first 10 mg/kg KA treatment. antalarmin (20 mg/kg/day in drinking water) or vehicle was given between the two KA doses. N=6 mice per group, * indicates p<.05 compared to vehicle or KA.

Figure 4. Antalarmin and bumetanide protection from seizure induced hippocampal sclerosis data is inconclusive. A. Average cell loss determined using NeuN staining in the CA1 (A) and CA3 (B) regions of the hippocampus 72 hours following treatment with 30 mg/kg KA. n=3 animals. p>.05 compared to vehicleor KA.

Figure 5. Time course of seizure susceptibility after an initial seizure insult. A. The average percent time seizing 1 week, 1 month and 3 months after vehicle or 10 mg/kg kainate treatment. B. Average latency to seizure 1 week, 1 month and 3 months after vehicle or 10 mg/kg kainate treatment. C. Percent time seizing for 5 individual representative mice in a 24 hour period, following one dose of 10 mg/kg i.p. KA. n=2-12 animals per group.

Figure 6. De-phosphorylation and down-regulation of KCC2 and up-regulation of NKCC1 in the PVN following seizure activity. A. Representative western blots of KCC2, Ser940 phospho KCC2 and NKCC1, in total protein from the PVN isolated from vehicle and 20 mg/kg i.p. kainate treated mice 2 hours after treatment. B. Average optical density measurements of KCC2, phospo S940 KCC2 and NKCC1 from control and kainate treated mice in the PVN. n=4-12 per group, * indicates p<.05 compared to control.

Figure 7. Mice lacking KCC2 in CRH neurons (KCC2/CRH mice) have increased corticosterone levels and increased seizure susceptibility. A. Baseline corticosterone levels were increased in KCC2/CRH mice compared to control (Cre -/-). B. KCC2/CRH mice show increased percent time seizing compared to controls (Cre -/-). C. KCC2/CRH mice show shorter latency to first seizure compared to controls (Cre -/-). n=6-7 mice per group, * indicates $p<.05$ compared to cre -/- mice.

Figures

Figure 1.

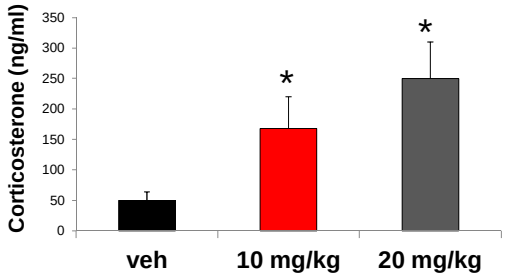
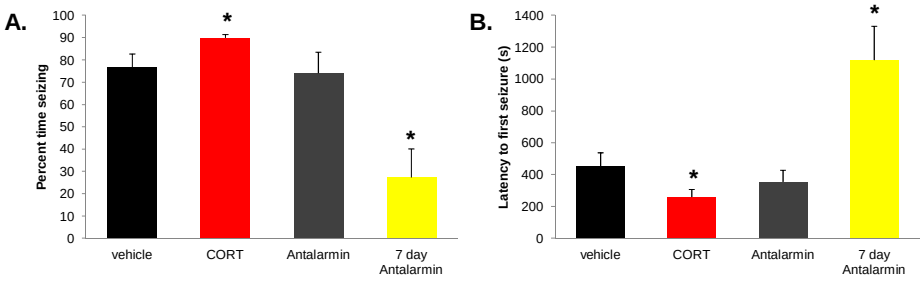


Figure 2.



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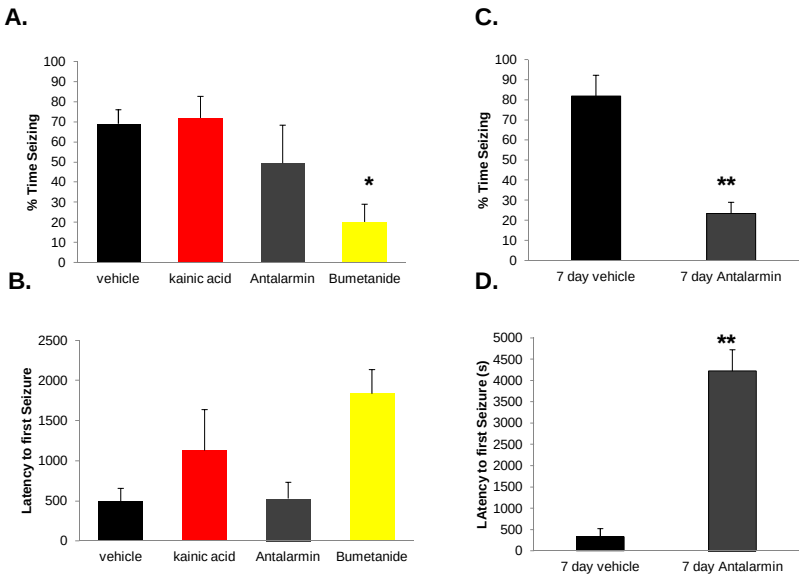


Figure 4.

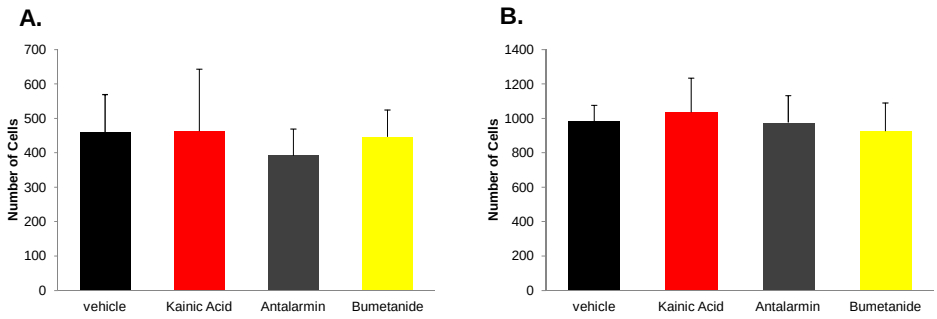


Figure 5.

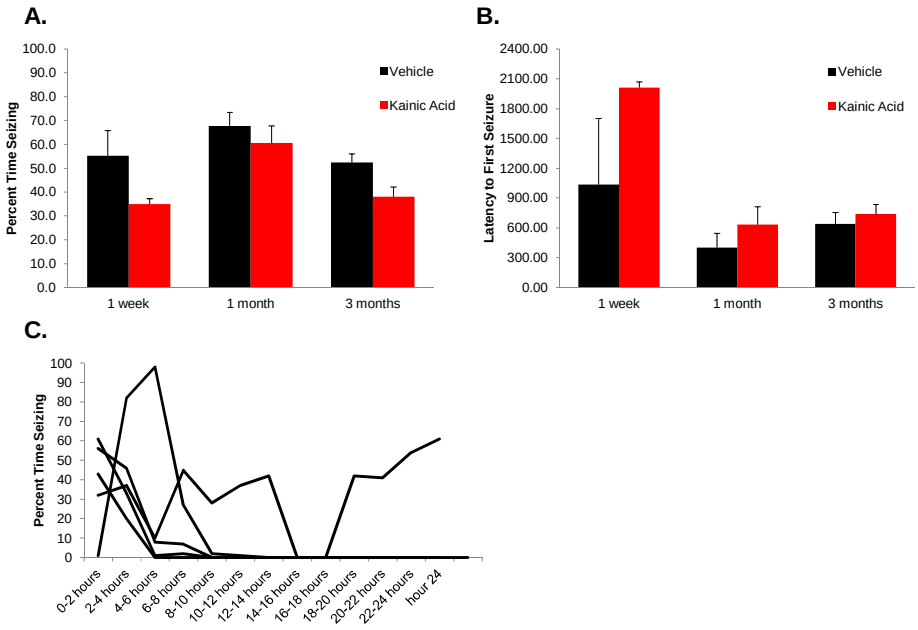


Figure 6.

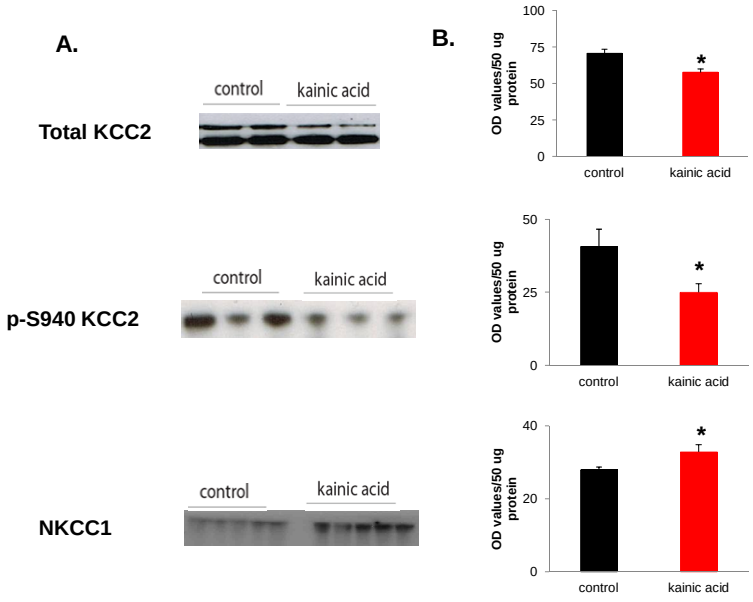
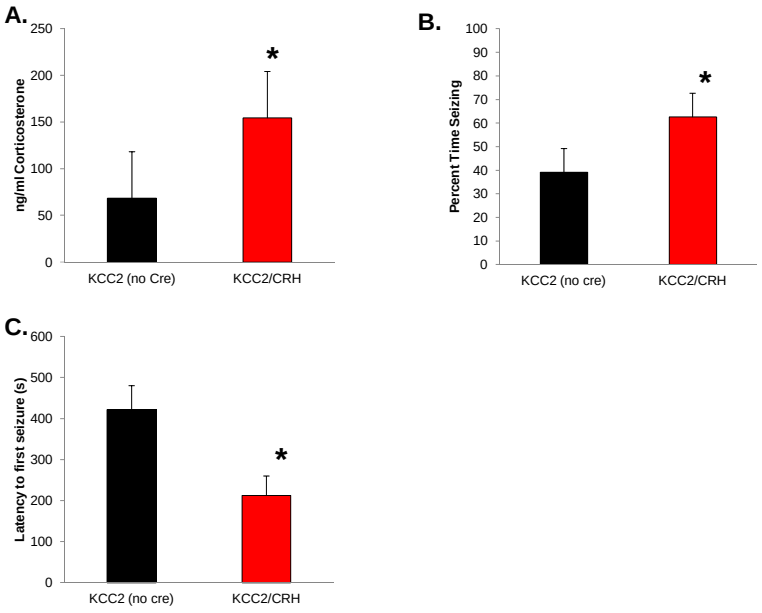


Figure 7.


A.
ng/ml Corticosterone
250
200
150
100
50
0
*
KCC2 (no Cre)
KCC2/CRH
B.
Percent Time Seizing
100
90
80
70
60
50
40
30
20
10
0
*
KCC2 (no cre)
KCC2/CRH
C.
Latency to first seizure (s)
600
500
400
300
200
100
0
*
KCC2 (no cre)
KCC2/CRH

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