Spring 5-2016

The Influence of the Estrous Cycle on Acute Seizure Activity

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Abstract

Epilepsy is a neurological disease that is characterized by spontaneous seizure activity. Seizures are excitatory events that result from the abnormal excessive and hypersynchronous firing of populations of neurons in the brain. Catamenial epilepsy is a condition in which seizure threshold fluctuates during the female menstrual cycle. It is commonly hypothesized that this fluctuation is due to the changes in estradiol:progesterone ratio that occur over the course of the menstrual cycle (Herzog, 2008). It has been shown in many studies that estradiol has proconvulsant properties whereas progesterone possesses anticonvulsant properties. Considering the pattern of hormonal variation and the effects of sex hormones on seizure threshold, a female would be most susceptible to seizure activity when estradiol levels are high and/or immediately following progesterone withdrawal. Cyclooxygenase-2 (COX-2) is an enzyme that facilitates the first committed step in the metabolism of arachidonic acid to biologically active prostaglandins. It has been shown to serve as an endogenous suppressor of seizures. Its expression is constitutively expressed in subsets of excitatory glutamateric neuronal populations and it is intensely induced under conditions of strong excitation, such as occurs during convulsive seizures (Hewett, 2006). Inhibitors of COX-2 enhance the sensitivity to and severity of acute seizures. However, its role in the fluctuation of seizure threshold in catamenial epilepsy remained to be explored. Given that both COX-2 and progesterone possess anticonvulsive properties, the goal of this study was to examine the possible correlative relationship between these two neuromodulators. Considering the influence of female sex hormones on seizures, it was posited that both seizure sensitivity and brain COX-2 expression levels will vary over the course of the estrous cycle and that the latter will be inversely related to the former. Studies were performed with female mice, which were treated with pentylenetetrazol (PTZ), a GABA-A receptor inhibitor, to model acute seizures. Results showed that COX-2 expression does indeed fluctuate consistently over the timeframe of the estrous cycle and that this may correlate with hormonal changes in the brain. An additional related study was performed to examine the possibility that sensitivity to PTZ differed between female and male mice. It was observed that females were more resistant to PTZ-induced convulsion than males. This project was a new direct of the research in the Hewett laboratory. The results established an animal model of catamenial epilepsy in mice that will provide the basis for future studies to examine the role of neuromodulators in seizure sensitivity.

Executive Summary

Fluctuations in excitatory and inhibitory activities within the neurocircuitry of the brain underlie proper physiologic function and homeostasis. Deviations outside the normal range can result in transient or permanent dysfunction. In the epileptic brain, surges in excitatory activity in circuits can lead to seizure behavior, which can have devastating consequences on normal daily function. Catamenial epilepsy is a condition in which seizure threshold fluctuates during the female menstrual cycle. It is commonly hypothesized that this fluctuation is due to the changes in estradiol:progesterone ratio that occur over the course of the menstrual cycle (Herzog, 2008). Estradiol, the most common form of estrogen found in the body, has been shown to possess proconvulsant properties. In contrast, progesterone and its main metabolite allopregnalone have been shown to possess anticonvulsant properties.

The estrous cycle of female mice is analogous to the human menstrual cycle. It is divided into stages as follows: 1) Proestrus lasts ~1 day starting with a rapid and transient increase in circulating estradiol levels. The corresponding period of the menstral cycle correlates with a higher seizure probability in catamenial epilepsy. This is followed by a surge of progesterone and lower seizure probability; 2) Estrus occurs the next day and is characterized by a rapid decrease in progesterone levels, resulting in “progesterone withdrawal”. This is a second period of elevated seizure risk in catamenial epilepsy; 3) Metestrus and 4) Diestrus last for ~2 days and corresponds to very low sex hormone levels. From here, the cycle can repeat or enter anestrus if proper stimulation isn’t present. Considering this pattern of hormonal variation and the effects of sex hormones on seizure threshold, a female mouse could be most susceptible to seizure activity during proestrus when estradiol levels are high and/or in early estrus immediately following

progesterone withdrawal. Susceptibility to seizure activity would therefore be lowest at midnight between metestrus and diestrus 2 due to low levels of estradiol coupled with raised progesterone levels (Scharfman, 2014).

A primary focus of the Hewett laboratory is to elucidate the role of neuromodulators in seizures and epilepsy. Neuromodulators are defined as non-neurotransmitter substances that control the the function of neurotransmitters, and thus, the process of neurotransmission. Cyclooxygenase-2 (COX-2) is an enzyme that is involved in the metabolism of arachidonic acid to bioactive prostaglandins. It is constitutively expressed by subpopulations of glutamatergic neurons of the hippocampus, cortex, amygdala and spinal cord. Its expression is particularly high in pyramidal neurons of the CA3 layer of the hippocampus. In contrast, basal expression of COX-2 is very low or non-detectable in the glutamatergic neurons of the dentate gyrus, which synapse on the CA3 neurons. However, its expression is markedly and transiently induced in this neuronal population under conditions of strong excitation, such as occurs during convulsive seizures. The level of neuronal COX-2 activity and expression is positively correlated with excitatory NMDA receptor-dependent synaptic transmission. In the normal brain, COX-2 functions as a neuromodulator of brain physiology, exemplified by its role in long-term potentiation. It also contributes to certain behavior activities, such as learning and memory. Under pathophysiological conditions, such as occurs in the epileptic brain, COX-2 may serve as an endogenous suppressor of seizures (Hewett, 2006). However, its role in the fluctuation of seizure threshold in catamenial epilepsy remains to be explored. It is posited that seizure threshold will vary over the course of the estrous cycle and that brain COX-2 expression levels will increase or decrease with a reduction or enhancement of seizure sensitivity, respectively.

This necessitated establishing and characterizing a new animal model in the laboratory to study the role of neuromodulators in catamenial epilepsy.

This project consisted of three specific aims utilizing outbred female and male CD-1 mice, the genetic background of which more accurately reflects the human condition than inbred strains. Acute seizures were modeled using pentylenetetrazol (PTZ), a GABA-A receptor inhibitor, which increases excitatory electrical activity in the brain. Since the Hewett laboratory hadn't previously used female mice in this model, the goal of the first study was to examine the seizure response in female mice and to determine whether this differed from male mice. It was observed that males had a higher sensitivity to PTZ-induced seizure while the females were more resistant to convulsion. The goal of the second study was to subject female mice to a protocol to synchronize estrous and examine the changes in brain COX-2 expression over five consecutive days. The results suggest that COX-2 expression may indeed fluctuate during the estrous cycle. The goal of the third study was to examine the acute seizure susceptibility over the course of the 5 day estrous cycle and to correlate this with changes in COX-2 expression in study 2. While there was a trend for fluctuation in seizure sensitivity, the number of observations must be increased in follow-up studies in order to confirm this. Nevertheless, this project has established and characterized a model of catamenial epilepsy in the Hewett laboratory that will permit further examination of the hypothesis of this project.

Acknowlegments

I would first and foremost like to thank my advisor and principal investigator, Professor James Hewett for giving me the opportunity to develop this study and for his continued guidance. I would also like to thank our lab manager Miriam Gladstone-Helak for the great deal of time she spent training me and for being a source of constant support. Many thanks to the graduate students in the lab, Spandita Dutta and Yifan Gong, for their help and comraderie. To my undergraduate peers in lab, Kelsey Schuch, Alicia Warnecke and Ed Datig, I thank you for your encouragement and friendship. Thanks also to my Capstone reader, Melissa Pepling and distinction thesis reader Mark Braiman. Lastly, thank you to the Coronat Scholarship and Syracuse University Biology department without which, this project would not have gotten off the ground.

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Chapter 1

Introduction

Epilepsy and Seizures

Epilepsy is one of the most common neurological disorders that can strike at any age, with an estimated lifetime risk of 1 in 26 (Hesdorffer et al., 2011; Hirtz et al., 2007). It is a disorder that affects the function of the brain specifically and, while genetic mutations and developmental abnormalities have been linked to epilepsy, the etiology in most cases is unknown. However, a number of risk factors are known to predispose individuals to acquiring epilepsy, including traumatic brain injury, hemorrhage, stroke, infection and tumors (Herman, 2002; Lowenstein, 2009). The defining feature of epilepsy is spontaneous seizure activity. Diagnosis requires a history of at least two unprovoked or "reflex" seizure events separated by more than one day or a single seizure with a high probability of having a second over the next decade (Fisher et al., 2014).

Seizures are excitatory events that result from the abnormal excessive and hypersynchronous firing of populations of neurons. Disruption in the balance between the two main neurotransmitters in the brain, γ-aminobutyric acid (GABA) and glutamate, are thought to be primarily responsible for the atypical neuronal activity associated with epilepsy (McNamara et al., 2006). GABA, the primary inhibitory neurotransmitter (Nicoll et al., 1990), is released from GABAergic neurons and antagonizes excitatory electrical activity via binding to specific receptors on pre and post-synaptic cell membranes. The GABA-A receptor is a ligand-gated ion channel that permits the flow of chloride ions into the cytoplasm (Sigel and Steinmann, 2012), resulting in hyperpolarization of the plasma membrane below its resting potential of ~-60mV

(more negative) thus making it more difficult for excitation of an action potential to occur. Glutamatergic neurons produce glutamate which, although the precursor to GABA, plays an opposing role in the brain. Serving as the main excitatory neurotransmitter (Meldrum, 2000), it binds to ligand-gated ion channels (Traynelis et al., 2010), such as α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors, that allow the influx of positively charged ions into the cytoplasm. This depolarizes the membrane (making it more positive), opening voltage-gated sodium channels in the axon to stimulate an action potential. The balance of inhibitory and excitatory forces in the brain is referred to as the "seizure threshold". The seizure threshold is a set point in the brain that determines the probability of seizure activity. Seizure threshold and probability are inversely related. They are innate properties of the normal brain homeostasis, whereby seizure threshold is elevated to maintain a low probability of abnormal neuronal activity that underlies the seizure event. During seizure activity, groups of neurons are overexcited either due to excessive stimulation by glutamate and/or the inability of GABA to impede neuronal action. This imbalance of excitatory and inhibitory action lowers the seizure threshold and may result in the convulsive activity common to many epileptic episodes.

Catamenial Epilepsy

The female reproductive hormones, estradiol and progesterone, are steroid hormones derived from cholesterol. In addition to important roles in regulating the reproductive cycle, they are neuroactive hormones that can modulate various functions of neurons in the brain (Veiga et al., 2004). For example, estradiol can affect cognition via regulation of synaptic plasticity in the hippocampus (Barha and Galea, 2010; Liu et al., 2008; Woolley, 1998). Catamenial epilepsy

affects nearly 40% of women who suffer from epilepsy (Herzog, 2008). It is a condition in which seizure threshold fluctuates during the female menstrual cycle in one of three distinct patterns (Herzog et al., 1997). Female reproductive hormones can modulate brain function by affecting GABAergic and glutamatergic neurotransmission (Zheng, 2009). It is commonly hypothesized that alterations in seizure threshold over the course of the menstrual cycle are due to changes in estradiol:progesterone ratio (Reddy, 2009). Estradiol is the most common form of estrogen found in the body. Results from many studies provide compelling evidence that estradiol, specifically its 17β isoform, has proconvulsant properties (Marcus et al., 1966). Estradiol has been shown to increase neuronal excitability and lower the seizure threshold by inhibiting GABA synthesis as well as enhancing glutamatergic transmission by increasing NMDA receptor density on synapses. This is thought to predispose epileptic females to seizures (Scharfman, 2006). In contrast to estradiol, progesterone and its main metabolite allopregnalone have been shown to possess anticonvulsant properties (Kokate et al., 1994; Reddy et al., 2004; Reddy et al., 2001). Indeed, results from clinical trials suggest that progesterone may be an effective therapy for some women suffering from catamenial epilepsy (Herzog, 2015). Two main mechanisms have been proposed to explain this. The first suggests that progesterone interacts with the progestin receptors PR-A and PR-B. This activation inhibits the function of the estrogen receptors Erα and Erβ, hindering the proconvulsant effects of estradiol. Secondly, allopregnalone, a product of the metabolism of progesterone by 5α-reductase, enhances the actions of GABA through the GABA-A receptor.

Estrous Cycle

The estrous cycle of female mice is analogous to the human menstrual cycle. The estrous cycle has 4 main stages; Proestrus, Estrus, Diestrus 1(metestrus) and Diestrus 2. Proestrus lasts ~1 day starting with a rapid and transient increase in circulating estradiol levels, peaking mid-morning. This is followed by a surge of progesterone corresponding to ovulation. Estrus occurs the next day, and is characterized by a rapid decrease in progesterone levels, resulting in "progesterone withdrawal". Diestrus lasts about 2 days with very low hormone levels and minimal fluctuations. From here, the cycle can repeat or enter anestrus if proper stimulation isn't present. Considering this pattern of hormonal variation and the effects of sex hormones on seizure threshold, a female mouse could be most susceptible to seizure activity during proestrus when estradiol levels are high and/or in early estrus immediately following progesterone withdrawal. Susceptibility to seizure activity would therefore be lowest at midnight between metestrus and diestrus 2 due to low levels of estradiol coupled with raised progesterone levels (Scharfman, 2014).

COX-2, seizures, and epilepsy

Cyclooxygenase-2 (COX-2) is key enzyme in the metabolism of arachidonic acid to bioactive prostaglandins (Figure 1). Arachidonic acid is an essential fatty acid that is stored in the sn-2 position of membrane bilayer phospholipids (Brash, 2001). The cyclooxygenase pathway can be divided into 3 separate reactions. In the initial reaction (step 1, Figure1), extracellular signals induce release of arachidonic acid via activation of phospholipase A_2 (PLA_2), which is a large family of intracellular and extracellular enzymes (Burke and Dennis, 2009).

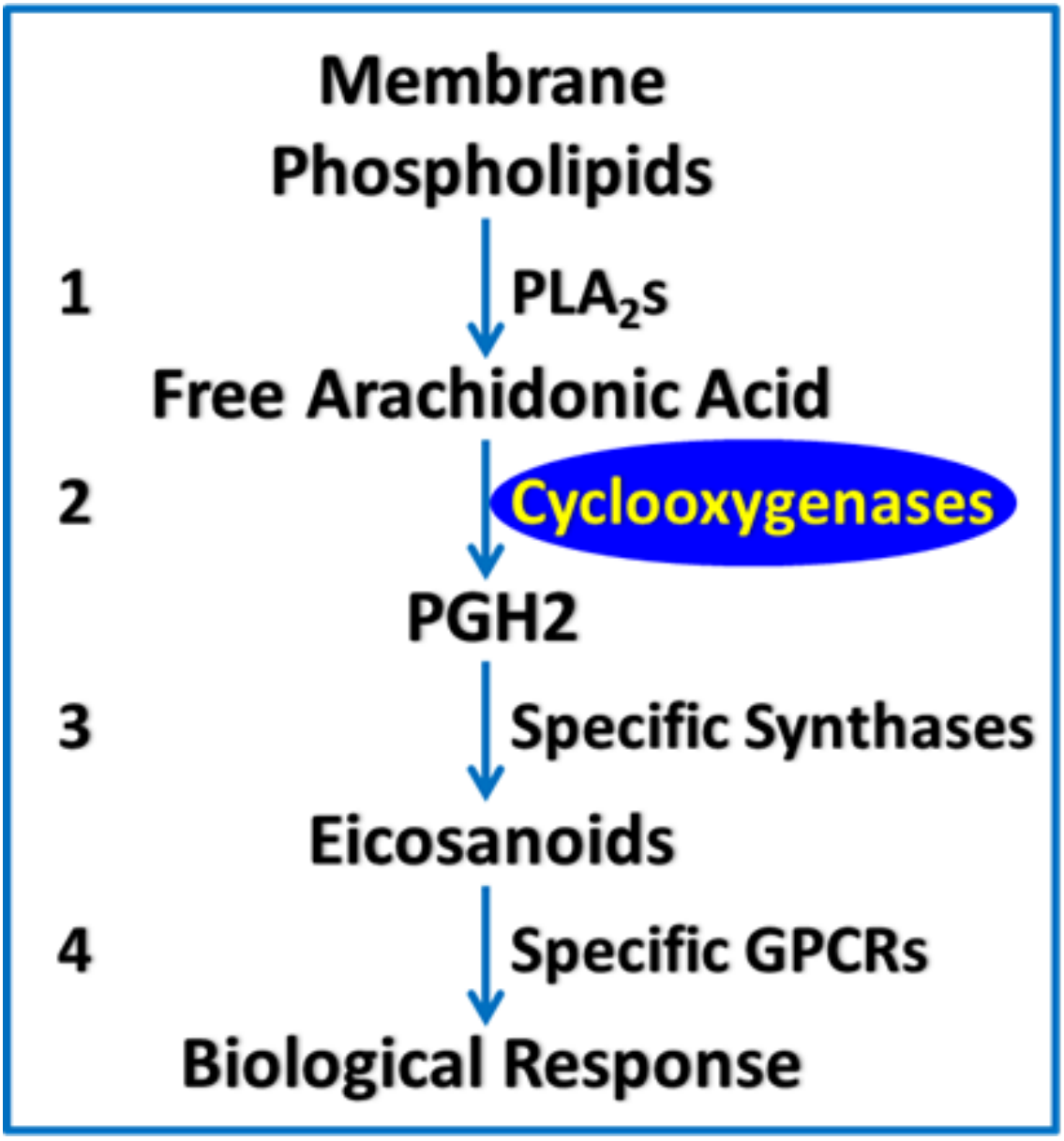

Figure 1. *Arachidonic acid metabolism*. See text for description.

In the brain, excitatory synaptic transmission induces release of glutamate from the presynaptic glutamatergic neuron, which binds to NMDA receptor on the post-synaptic neuron and induces activation of cytoplasmic calcium-dependent PLA_2 (PLA2G4A) (Taylor et al., 2008; Taylor and Hewett, 2002). Free arachidonic acid serves as a substrate for COX-2 in the second reaction of

the cascade (step 2, Figure 1). COX-2 is a *bis*-oxygenase that catalyzes a two step reaction (Smith et al., 1996): 1) the cyclooxygenease reaction catalyzes the incorporation of two molecules of molecular oxygen into arachidonic acid to form the endoperoxide, Prostaglandin G_2 and 2) a peroxidase reaction which employs heme iron to catalyze the two electron reduction of PGG_2 to form PGH_2. Hence, the enzyme has two separate catalytic sites that may function independently under certain conditions. PGH_2 serves as a substrate in the third reaction to produce the different biologically active prostaglandins (e.g., PGD_2, $PGE_{1/2}$, PGF_2, and PGI_2). Each of these prostaglandins is produced by the catalytic activity of a different synthase (step 3, Fig 1). These synthases may be expressed in a cell-type specific manner and contribute to the different biological activities of prostaglandins via binding to and activating ligand-specific G protein-coupled receptors on target cells (step 4, Figure 1).

The level of prostaglandin production in the brain is largely dependent on the level of COX-2 expression. Thus, over-expression of the enzyme in neurons leads to >10-fold increase in the basal level of prostaglandins (Andreasson et al., 2001). COX-2 is constitutively expressed by subpopulations of glutamatergic neurons of the hippocampus, cortex, amygdala and spinal cord (Breder et al., 1995; Joseph et al., 2006; Yamagata et al., 1993). Basal expression levels are particularly high in pyramidal neurons of the CA3 layer of the hippocampus (Claycomb et al., 2011). In contrast, basal expression of COX-2 is very low or non-detectable in the glutamatergic neurons of the dentate gyrus, which synapse on the CA3 neurons. However, expression in this population is markedly and rapidly induced under conditions of strong excitation, such as occurs following an acute convulsive seizure (Claycomb et al., 2011). Like arachidonic acid release (see above), the level of neuronal COX-2 expression is positively coupled with excitatory NMDA receptor-dependent synaptic transmission (Figure 2).

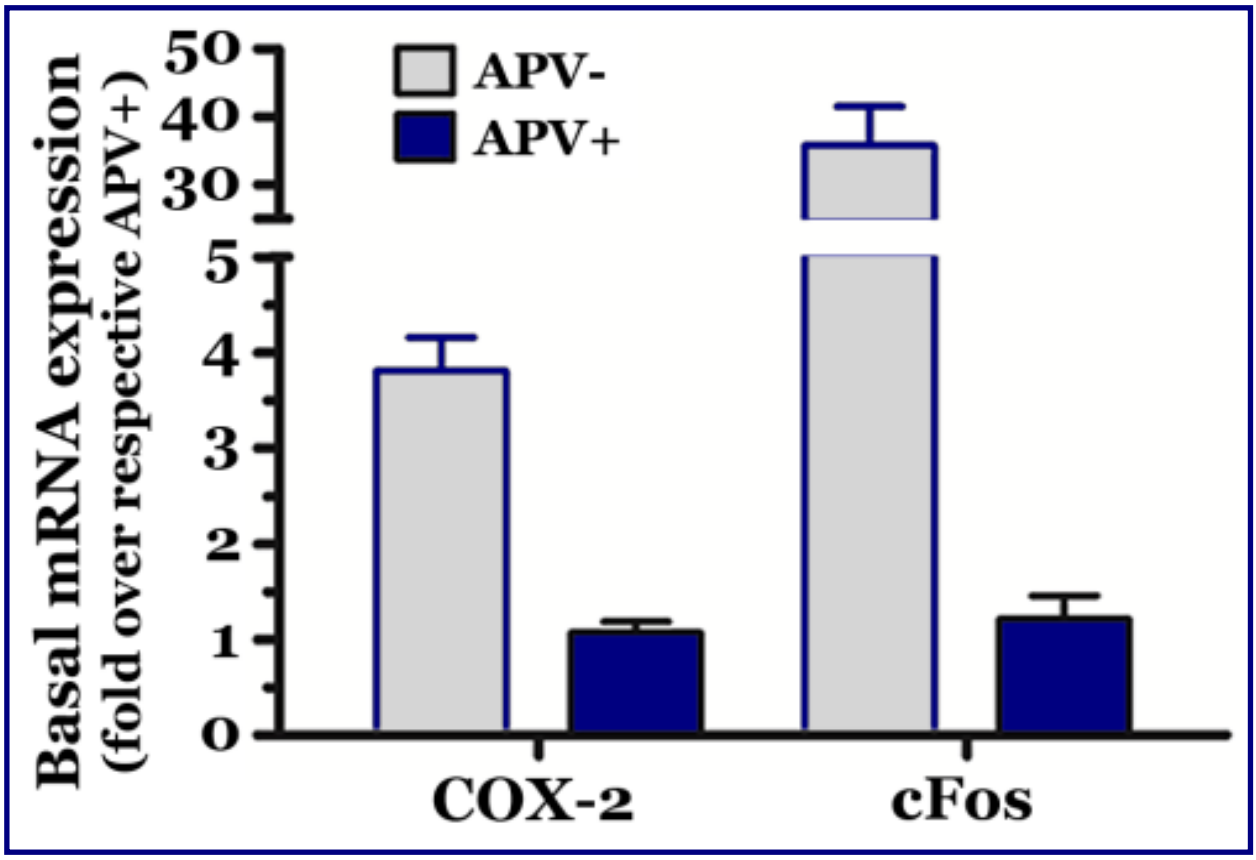

Figure 2. *Basal expression of COX-2 and cFos in cultures of glutamatergic neurons is dependent on NMDA receptor activity*. Cells were treated without or with the NMDA receptor antagonist, APV, and mRNA expression was determined by RT-qPCR analysis. β-actin was used as an internal control for each sample. Results are from duplicate samples prepared from 3 different harvests. (Data from Y. Gong in Hewett lab).

In the normal brain, COX-2 functions as a neuromodulator of brain physiology, exemplified by its role in neuroplasticity (Chen et al., 2002; Le et al., 2010; Murray and O'Connor, 2003). It also contributes to certain behavior activities, such as learning and memory (Holscher, 1995; Rall et al., 2003; Sharifzadeh et al., 2005). Under pathophysiological conditions, such as occurs in the epileptic brain, COX-2 may serve as an endogenous suppressor of seizures. In support of this notion, the selective COX-2 inhibitor, rofecoxib, increased the incidence and severity of convulsions compared to vehicle-treated control CD-1 or C57BL/6

mice (Figure 3A) (Claycomb et al., 2012). In contrast, transgenic mice overexpressing COX-2 in neurons exhibited a decrease in the incidence of acute convulsions relative to non-transgenic littermate controls (Figure 3B). However, its role in the fluctuation of seizure threshold in catamenial epilepsy remains to be explored.

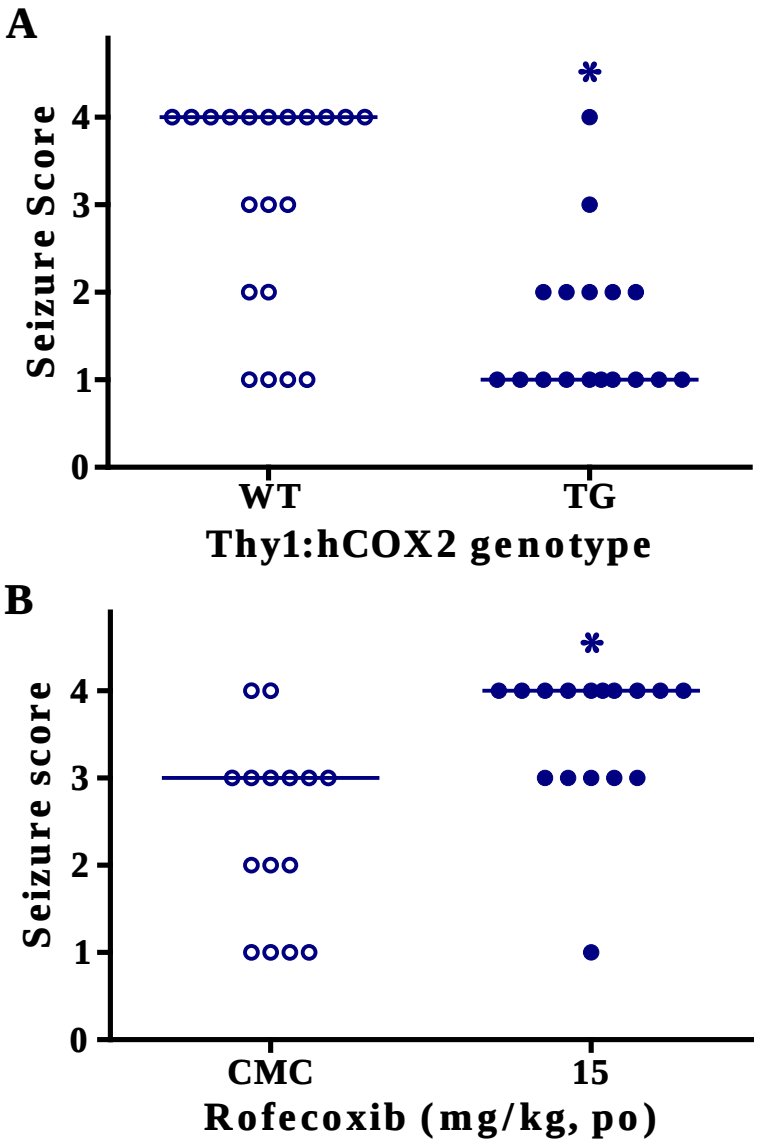

Figure 3. *COX-2 activity suppresses acute convulsions*. **A)** *COX-2 over-expression*. Seizure activity was induced by PTZ (32mg/kg, ip) in non-transgenic (WT) or COX-2 over-expresser transgenic (TG) littermates (Thy1:hCOX2). **B)** *COX-2 inhibition*. CD-1 mice were treated with vehicle (CMC) or rofecoxib (15mg/kg, po) 3 hr prior to PTZ (50mg/kg, ip). Maximum seizure scores (within 20-30 min following PTZ) are shown for individual mice. *, significantly different from control (Mann Whitney test, $p<0.05$). PTZ dose is tailored to the sensitivity of mouse strains used.

Specific Aims

Given that both COX-2 and progesterone possess anticonvulsive properties, the goal of this project was begin to explore the possible relationship between these two neuromodulators. Considering the influence of female sex hormones on seizures, it is posited that seizure threshold will vary over the course of the estrous cycle and that this changes in brain COX-2 expression levels will correlate inversely with changes in seizure threshold. This project can be divided into 3 separate studies.

Study 1. CD-1 Dosing Curve

To determine the dose of PTZ needed to elicit a convulsive response in female mice as well as to examine possible differences in the seizure responses of male mice.

Study 2. Basal COX-2 Expression

To determine if basal levels of COX-2 mRNA expression fluctuate over the course of the estrous cycle.

Study 3. Synchronized PTZ Responses

To determine if female seizure threshold fluctuates over the course of the estrous cycle and its possible correlation COX-2 expression levels.

Chapter 2

Methods

Mouse husbandry

Female and male 6-week old CD-1 mice were purchased from Charles River Laboratories and housed in the temperature- and humidity-controlled vivarium at Syracuse University for a minimum of one week to allow acclimation to the facility. Mice were maintained on a 12 hr light-dark schedule and allowed both food and water *ad libitum*. All procedures were approved by the Syracuse University Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the *National Academy of Sciences Guide for the Care and Use of Laboratory Animals* (NRC, 2011).

Synchronization of Mice

Female mice were housed 2 per cage and left to acclimatize for three weeks to reduce confounding effects of stress and environmental changes. Their estrous cycles were synchronized via exposure to soiled bedding from male cages for 96 hours prior to use in studies. This technique, so named the “Whitten Effect” suggests pheramones from the urine-soaked bedding of males can stimulate females’ estrous cycles (Whitten et al., 1968). Prior to all procedures, mice were handled for 5 consecutive days to acclimatize them to the experimenter and to reduce the influence of acute stress from handling.

Acute Seizure Model

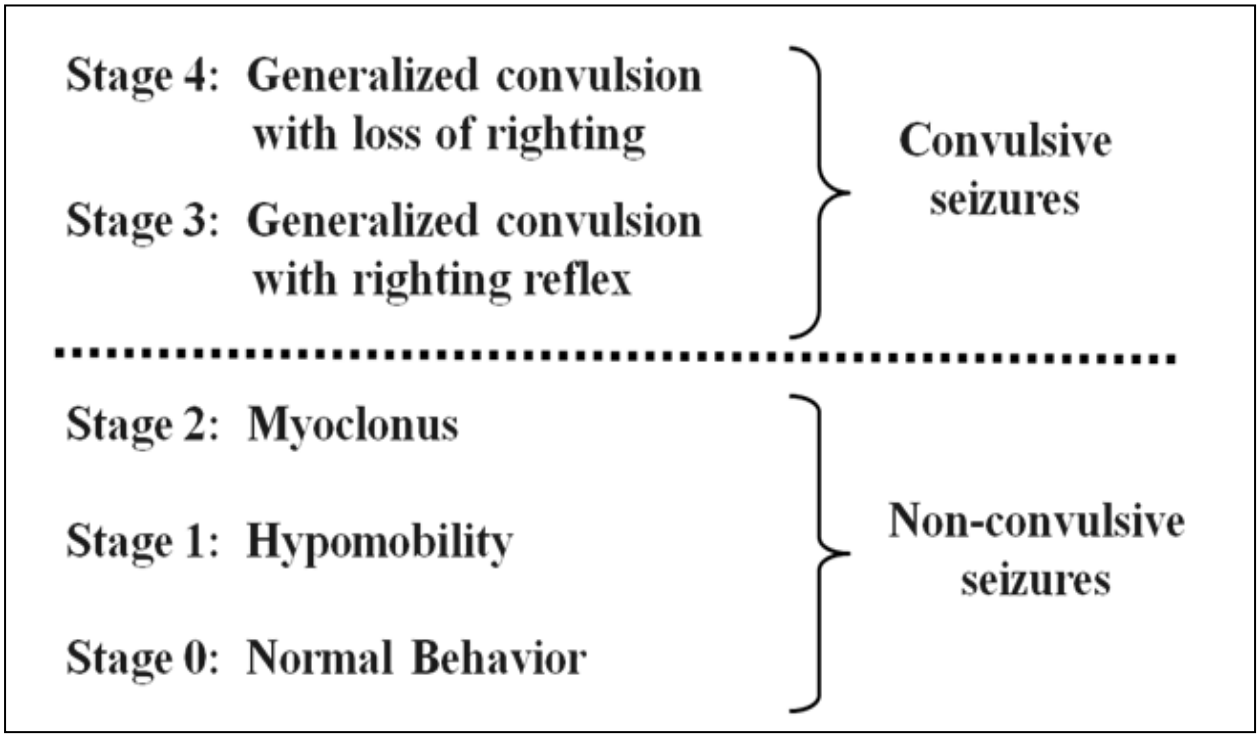

Figure 4. *Seizure scoring rubric.* (Y. Gong in Hewett Lab)

Pentylenetetrazole (PTZ) is a GABA-A inhibitor commonly used to model acute seizures (Holmes, 2007; Huang et al., 2001; White, 1997). PTZ injection solutions were prepared fresh in normal saline and administered in a volume of 10mL/kg body weight as described previously (Claycomb et al., 2011). Injections were made into the peritoneal space surrounding the abdomen organs and seizure behavior was observed for 30 minutes. Seizure severity was quantified using an established scoring system outlined in Figure 4 (Ferraro et al., 1999). Normal behavior (stage 0) includes socializing with cage mates, grooming, exploring the cage, and eating. Hypomobility (stage 1) is characterized by a lack of activity and movement. These mice no longer engage in their normal activities and instead isolate themselves in the cage without moving. Myoclonus (stage 2) is when the mouse suddenly exhibits two or more spontaneous

jerks or twitches involving the entire body. Convulsive seizures are divided into two levels of severity: generalized convulsion without (stage 3) or with (stage *4*) loss of righting, or it ability to remain on its four legs.

Tissue Sample Collection

Three hours after PTZ injection, mice were anesthetized fully with a general anesthetic solution consisting of a mixture of ketamine/xylazine (100/10 mg/kg). Full anesthesia was confirmed by lack of response to tail and foot pinch with tissue forceps. After opening the body cavity and exposing the heart, a cardiac puncture of the right atrium was performed and right atrial blood samples were taken. After the blood had sat at room temperature for ~30 minutes, blood serum was isolated by centrifuging at 3000 rpm for 10 minutes at 4ºC. The serum was then isolated from clot and stored at -80ºC. The mice were then exsanguinated by transcardial perfusion of 15 mL of PBS via the left ventricle of the heart. Brains were dissected and cortical and hippocampal tissue samples collected and flash frozen on dry ice in ethanol prior to storage at -80ºC.

Analysis

Total RNA extraction was performed using 1mL Trizol Reagent (Invitrogen) per tissue sample following the manufacturer's protocol. RNA concentration and purity were assessed using a Nanodrop spectrophotometer. Reverse transcription to generate cDNA was then performed on the RNA samples using M-MLV reverse transcriptase essentially as described previously (Hewett et al., 1999). Resulting cDNA samples were amplified by PCR for β-actin to confirm the integrity of the samples prior to proceeding with quantification. Quantitative PCR for COX-2 was then run. β-actin was simultaneously run on all samples as an internal control. Comparisons

of COX-2 mRNA concentrations of each of the five samples was used to determine whether there was an overall fluctuation of COX-2 expression over the estrous cycle.

Experimental Design

Estrous Study 1

The goal of the first study was to create a preliminary dosing curve to determine the proper amount of pentylenetetrazol (PTZ) required to elicit a convulsive response in females. Twenty female CD-1 mice were dosed with PTZ via IP injection over the course of 5 days (4 per day) and observed for 30 minutes. On day 1, one mouse was dosed with saline as a control while the other three received dosages of 42, 46 or 50 mg/kg respectively. After failing to elicit a convulsive response at any of these doses, the dosing paradigm was shifted up to 50, 55, or 60 mg/kg for the remainder of the study as this had been an effective range for a fellow labmate using male CD-1's. The seizures were scored based on a scaled numbering system used throughout the lab (Figure 4). Three hours after seizure scoring was completed, cortex and hippocampal tissue samples were harvested via cardiac perfusion to use for training purposes. The scores recorded were used to create a dosing curve on Prism software and determine a 50% convulsion dosage to be used in future studies. The second round of this study was performed following the same design as the first except that males were dosed simultaneously to examine the difference in seizure scores between the sexes. Ten CD-1 females and 10 CD-1 males were dosed with either 50, 55 or 60 mg/kg PTZ and observed for 30 minutes to assess seizure score based on the numbering system in Figure 4. Three hours after dosing, all subjects were perfused to harvest cortex and hippocampal tissue samples as well as blood serum samples. These samples were analyzed via QPCR and ELISA.

Estrous Study 2

This study involved inducing estrous cycles in females to collect brain tissue and blood samples on each of the five days of estrous to serve as a basis in which to compare COX-2, progesterone, and estradiol levels to those after an induced seizure. Twenty CD-1 females were housed 2 per cage and allowed to acclimate to the vivarium for 3 weeks prior to handling. Ninty six hours prior to perfusion, soiled male bedding was placed into the cage to induce estrous. On five consecutive days, four mice were perfused and blood serum and hippocampal and cortex tissue samples were collected for QPCR and ELISA analysis.

Estrous Study 3

The last study used cycled females dosed with PTZ to observe the fluctuations of acute seizure susceptibility over the course of the 5-day estrous cycle. Twenty CD-1 females were housed 2 per cage and allowed to acclimate to the vivarium for 3 weeks prior to handling. 96 hours prior to perfusion, soiled male bedding was placed into the cage to induce estrous. On five consecutive days, four mice were dosed with 55 mg/kg PTZ and observed for 30 minutes. Three hours after dosing, all subjects were perfused for harvesting of cortex and hippocampal tissue samples and blood serum samples. QPCR and ELISA was conducted to analyze these samples for COX-2, progesterone and estradiol levels.

Chapter 3

Results

Estrous Study 1

After adjusting the PTZ dosing paradigm to the range of 50, 55, and 60 mg/kg it was determined that these dosages were more useful in studies to follow as they elicited a more diverse array of seizure scores in the convulsive category. As shown in the intial dosing curve (Figure 5) the 55 mg/kg dose was stimulating both convulsive and non-convulsive responses in females and was therefore selected as the dose to be used in following studies. Tissue samples collected were used for practice in analytical techniques. The comparative study using both male and female CD-1's indicated that the male cohort had a higher sensitivity to PTZ-induced acute seiure as they scored higher on the convulsion scale than did females dosed in parallel (Figure 5). Specifically at the 50 mg/kg dose, zero of eleven females had a convulsize seizure (score of 3 or 4) and in five of six males a convulsive seizure was elicited. At 55 mg/kg and 60 mg/kg, the experimental group size is too small to be statistically significant so further replications of this study must be done in order to conclude sex differences in response at those doses (Figure 6).

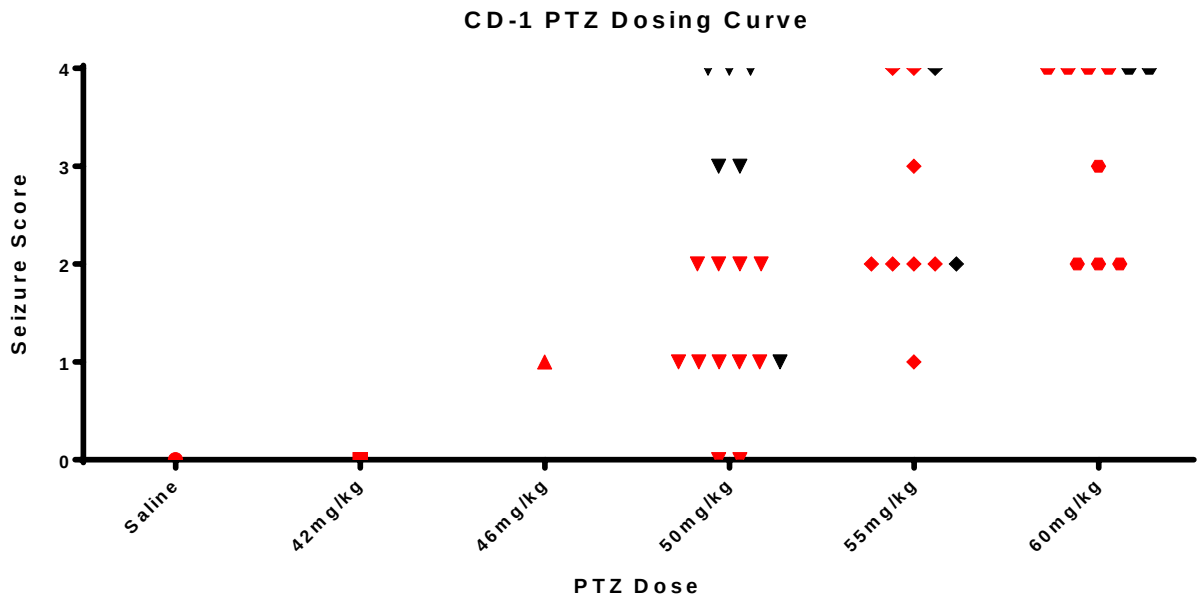

Figure 5. *Dose-response relationship for acute PTZ-induced seizure activity in female and male mice*. Each point represents the maximal seizure score for individual mice. Red symbols represent female responses; Black symbols represent male responses. Seizure severity was scored as described in Figure 4.

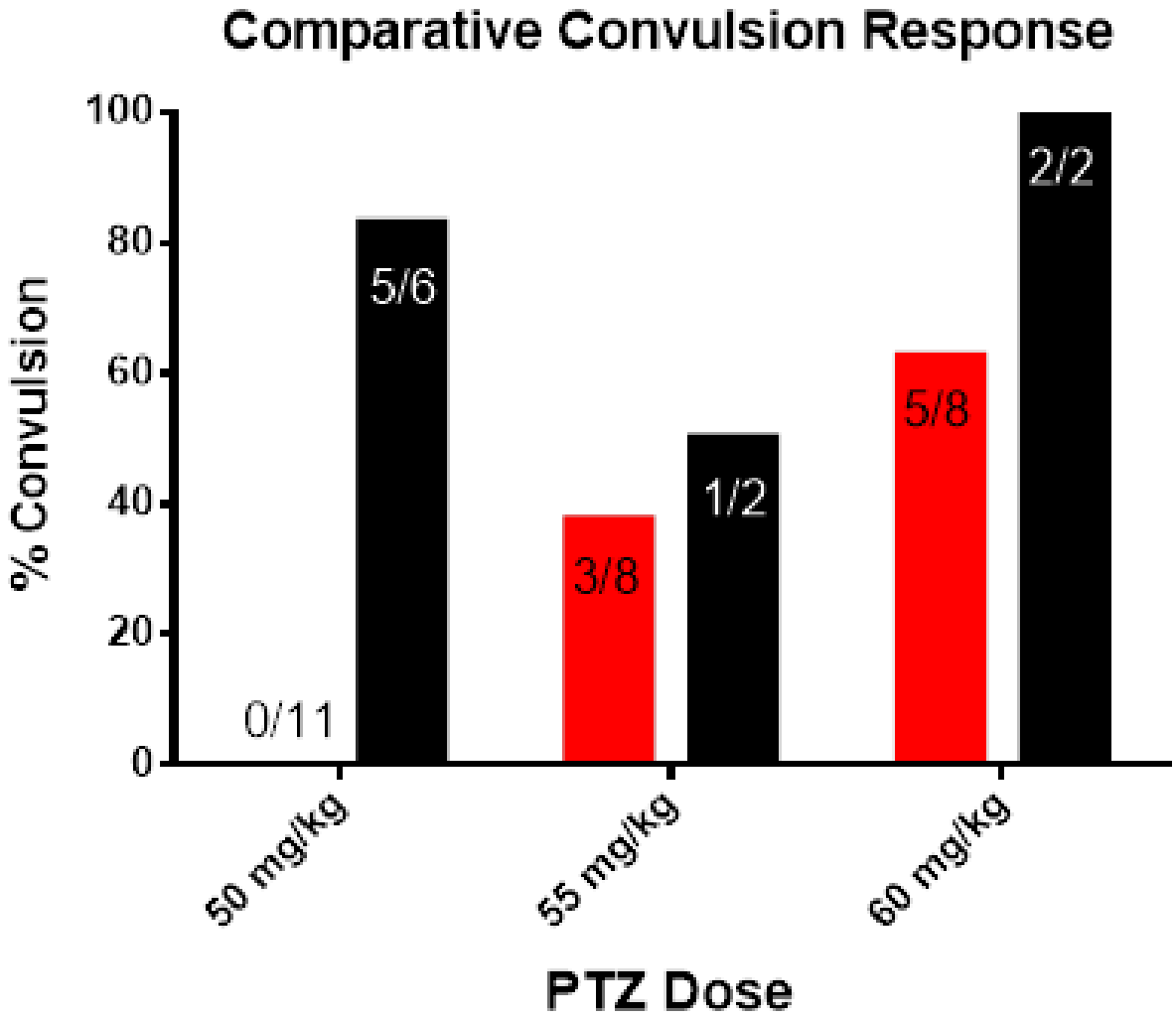

Figure 6. *Incidence of convulsive seizure responses in female and male mice*. Data shows mice with seizure severity scores of 3 or 4 compiled from Figure 5. Red bars represent female responses and black represents male responses.

Estrous Study 2

QPCR analysis of cortical tissue harvested from 20 synchronized females, four on each of the five days of estrous, shows that there is a significant decrease in basal COX-2 mRNA expression on day 2 in comparison to day 1 (Figure 7). There were no statistically significant changes in expression at day 3, 4 or 5 in comparison to day 1. This suggests that COX-2 levels may fluctuate over the course of the estrous cycle as hypothesized.

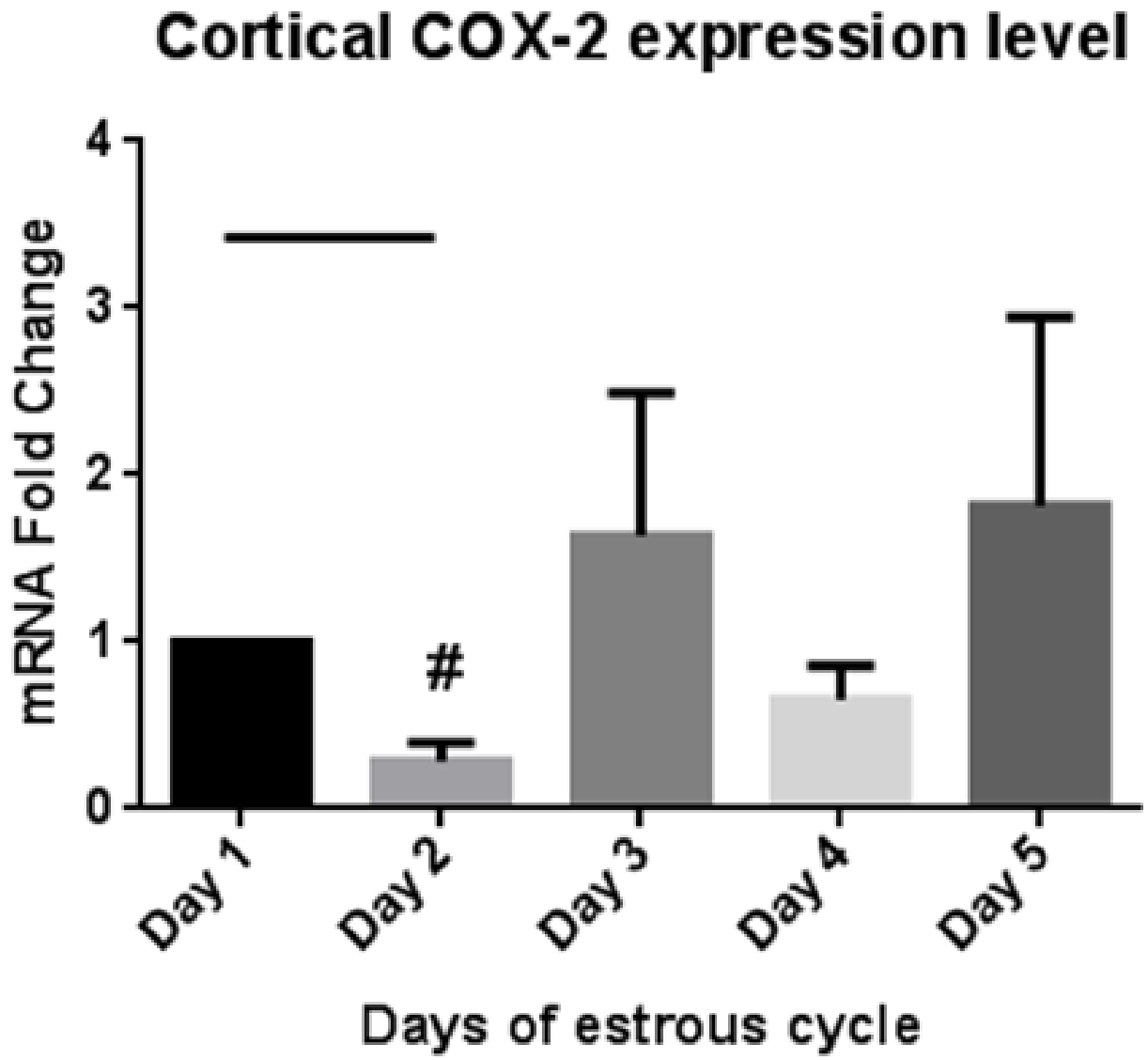

Figure 7. *Analysis of basal COX-2 mRNA expression over the course of the estrous cycle in cortex samples*. (#) indicates significant change in COX-2 mRNA expression from day 1.

Estrous Study 3

This study examined the effect of the estrous synchronization paradigm on PTZ-induce COX-2 mRNA expression in the brain and seizure severity. COX-2 expression in the cerebral cortex of synchronized mice did not differ significantly over the course of five consecutive days.

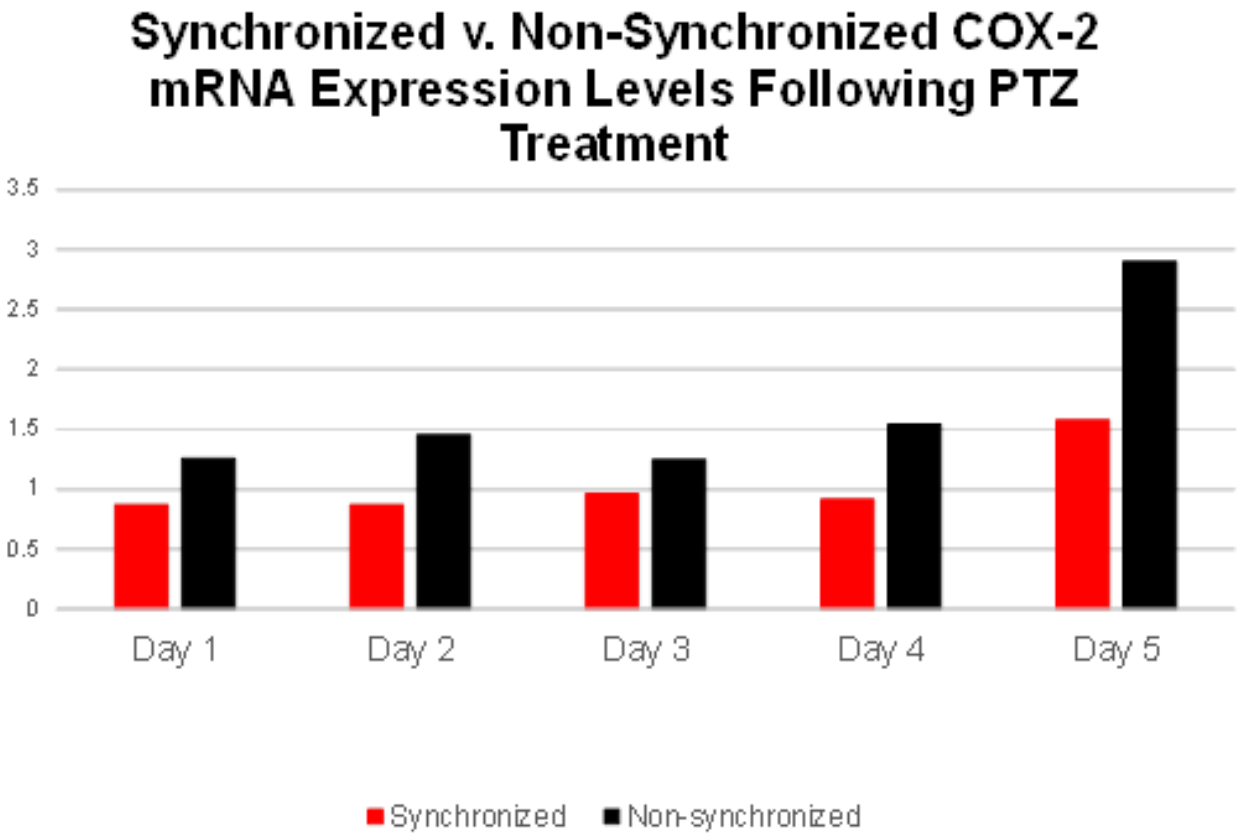

Figure 8. *Analysis of PTZ-induced changes in COX-2 mRNA expression over the course of the estrous cycle in cortex samples*. Naïve female mice (black bars) or mice subjected to the estrous synchronization paradigm (red bars) were treated with 50 mg/kg PTZ. Brain tissue was harvested 3 hours after PTZ administration and COX-2 expression assessed by RT-qPCR as described in Methods. See Figure 9 for acute PTZ-induced seizure scores for this analysis.

Moreover, exposure of female mice to male bedding in the synchronization of estrous paradigm did not significantly alter the acute PTZ-induced seizure response compared to naïve unsynchronized mice (Figure 9). There does appear to be a somewhat cyclic pattern to the scores of the synchronized group but without a larger experimental group, a statistically significant conclusion cannot be drawn. It is important to note that although the control group wasn't subjected to the synchronization paradigm, the females may have still been cycling in estrous (i.e., not anestrus) resulting in the varied seizure score observed in this group.

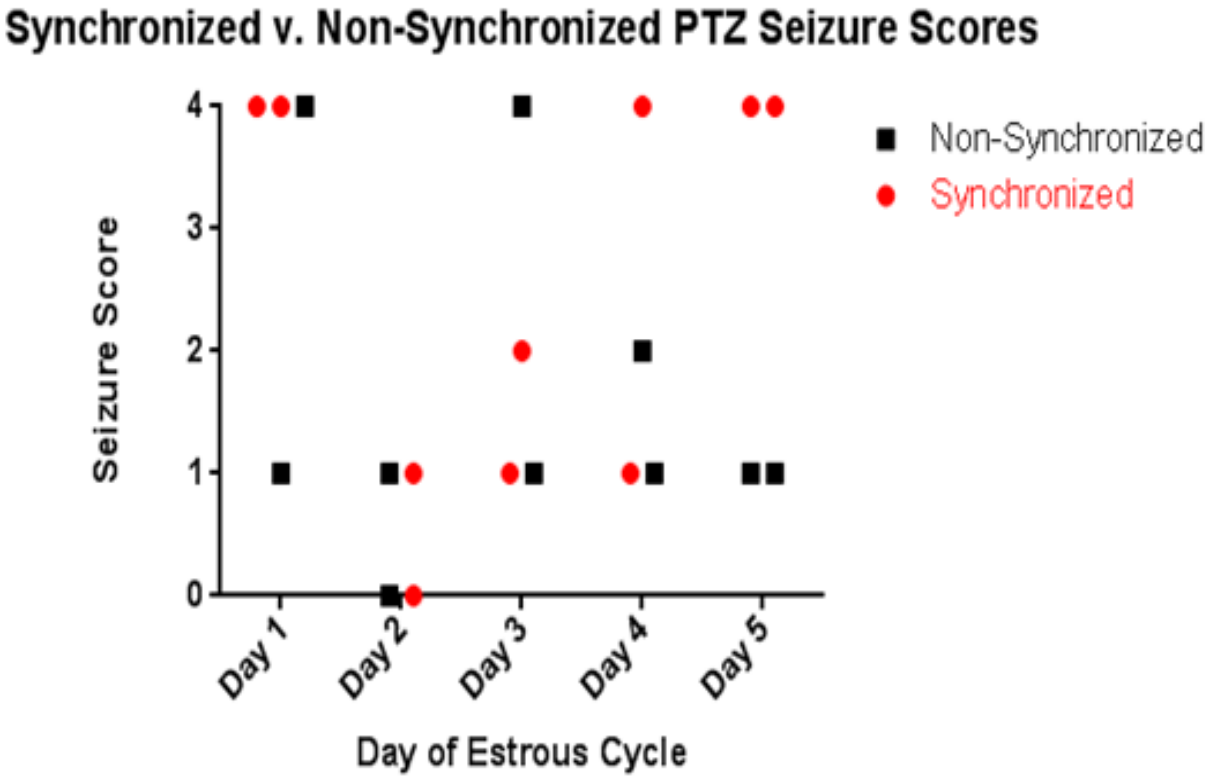

Figure 9. *Analysis of PTZ-induced acute seizure response over the course of the estrous cycle in cortex samples*. The acute PTZ-induced seizure response of mice used for COX-2 expression analysis in Figure 8. Naïve female mice (black symbols) or mice subjected to the estrous synchronization paradigm (red symbols) were treated with 50 mg/kg PTZ. Seizure severity was scored as described in Figure 4.

Chapter 4

Discussion

Epilepsy is the fourth most prevalent neurological disorder in existence, following migrane, stroke and Alzheimer's disease. It is estimated that there are 50 million cases in the world which puts a significant financial burden on the global economy. As a seizure disorder, treatments for epilepsy focus on reducing the chance of spontaneous seizure activity. This primarily depends on maintaining a balance of excitatory (glutamate) and inhibitory (GABA) signals within the brain. Disruption of this homeostasis through an overexpression of glutamate or low levels of the GABA can potentiate excessive neuronal activity, leading to convulsive behavior. The point at which seizure activity can occur due to an inbalance of these signals is thus called the seizure threshold. Estradiol and progesterone, two cholesterol based hormones found in the female brain, can also play a role in modulating the seizure threshold. Estradiol is a known pro-convulsant as it stimulates glutamate release as well as inhibiting GABA synthesis. Progesterone has an opposing effect as an anticonvulsant. This hormone is able to block the excitatory activity of estradiol and encourages GABA release. Catamenial epilepsy is a phenomenon in which seizure threshold fluctuates over the course of the menstrual cycle in females; high susceptibility to seizure when estradiol levels are elevated and low susceptibility when progesterone levels are higher. Progesterone is therefore hypothesized to be a useful component of antiseizure medications for those females who suffer from epilepsy. COX-2 is another known anticonvulsant but whose role has yet to be defined in catamenial epilepsy. Fluctuations in COX-2 expression may also be a factor in explaining the variation in seizure

susceptibility throughout the estrous cycle. This study hoped to determine the role of COX-2 in catamenial epilepsy as it may help maintain a higher seizure threshold.

As mentioned previously, this project was not without some challenges. As it was new protocol to the lab, significant research as well as some trial and error was required to attain the expected results. The first of these issues came when the dosing paradigm (42, 46, 50 mg/kg) traditionally used in CD-1 male mice did not elicit a similar response in the female cohorts. As it did not appear to be a user issue because the males were still responding as normal, it was concluded that female CD-1 mice had a higher latent seizure threshold than males and therefore needed a higher dosage of PTZ to stimulate convulsive seizure activity. A higher dosing paradigm (50, 55, 60 mg/kg) produced a wider range of seizure reponses and was therefore selected to be used in furture studies. This higher dose (55 mg/kg) proved to be problematic as well. When synchronized females were dosed, their seizures were significantly more severe than in the non-synchronized females. This made tissue collection difficult and no conclusions about fluctuations in seizure susceptibility could be made since the females were being overdosed. Again, the PTZ dosage was shifted to a slightly lower dose (50 mg/kg). This dose worked well in attaining varied scores in both synchronized and non-synchronized female mice. With these issues resolved, conclusive studies could be performed to identify effects the estrous cycle had on seizure threshold and COX-2 expression.

In examining the data collected from studies 2 and 3, it is apparent that basal COX-2 mRNA expression levels fluctuate during estrous with a significant decrease in COX-2 levels occurring on day 2. Mice treated with PTZ prior to tissue harvest, however, show little in terms fluctuation. There was no significant difference in COX-2 levels of synchronized females after PTZ to those non-synchronized and treated with PTZ. The seizure scores of the two groups at 50

mg/kg were not significantly different either but there did appear to be a cyclic pattern to the scores of the synchronized cohort. It is important to note as well, that in comparing the seizure scores of synchronized females to those non-synchronized treated with 55 mg/kg PTZ that synchronized females had higher scores across the entirety of the estrous cycle. This suggests that synchronization lowers females' seizure threshold. It is also interesting that synchronized females experienced the lowest seizure scores on day 2, similarily to the lowest levels of COX-2 mRNA expression. This could indicate that COX-2 expression correlates inversely to seizure threshold as hypothesized.

Considering the promising results obtained through these few studies, further examination of these hypotheses should be continued. First and foremost, many of these experiments should be repeated with more animals. Larger experimental groups will allow a more conclusive result with statistically analyses. Repeats of these studies may rule out any outliers that cannot be determined in small numbers. Continuing analysis of the tissue samples as well as serum collected will help solidify conclusions made in this paper as well. Due to time constraints only cortical samples were analyzed so far. QPCR on the hippocampal tissue may prove useful in corroborating fluctuations of COX-2 expression in the brain. Serum analysis via ELISA will help correlate hormonal levels to that of COX-2 as well as trends in seizure threshold. As of now days 1-5 are arbitrarily based on when the study began. Vaginal smearing prior to tissue collection or PTZ treatment would allow for the determination of the exact phase of estrous the mouse was in based on the cell types present.

The purpose of these studies was to gain better understanding of the phenomenon that is catamenial epilepsy and the epileptic condition in general. However, preliminary these results

may be, they indicate trends that may prove useful to future study and discovery. Development of these protocols will allow for more extensive studes to be conducted in the future.

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