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Influence of Running on the Seizure Threshold

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Abstract

Cyclooxygenase-2 (COX-2) is one of two COX enzymes involved in the conversion of free arachidonic acid (AA) to prostaglandin H_2, a precursor for prostaglandin synthesis. COX-2 expression has been detected in the cerebral cortex, hippocampal formation, and amygdala, with its expression localized to the dendritic spines of excitatory neurons. In addition to its localization in excitatory neurons, COX-2 expression is regulated by neuronal activity, suggesting a modulatory role of COX-2 in synaptic transmission. COX-2 contributes to numerous functions in the normal brain, including learning and memory. Findings from our lab suggest that COX-2 also contributes to the maintenance of the seizure threshold, which is an innate property of the brain that depends on the homeostatic balance between excitation and inhibition. For example, pharmacological inhibition of COX-2 increased and neuronal overexpression of COX-2 in the CNS decreased the severity and incidence of convulsive seizures. Voluntary exercise has also been shown to play a role in reducing seizure frequency and severity. However, the mechanism behind how it does so remains poorly understood. The elevated seizure threshold that results from both elevated COX-2 levels and exercise potentially suggests that changes in COX-2 expression mediate the effects of running on the seizure threshold. Here I hypothesize that running will increase the expression of COX-2 in the hippocampus, resulting in increased prostaglandin production that will in turn raise the seizure threshold. To test this, CD-1 mice were housed in running wheel cages for 4 weeks before testing the seizure threshold. Seizures were induced through the use of pentylenetetrazole (PTZ) and scored based on their severity as a measure of the seizure threshold. To measure COX-2 expression and activity, brain tissue samples were collected for COX-2 immunostaining and assayed for Prostaglandin E_2, a downstream product of COX-2 activity. While COX-2 expression and PGE_2 levels were elevated after running, we did not see an elevation of the seizure threshold in mice belonging to the running wheel experimental group. However, unlike other studies examining the effects of running on the seizure threshold, tests of the seizure threshold in the current study were performed six days post running rather than immediately after running had concluded. The difference in the results presented here from those reported previously could suggest that the effects of exercise on the seizure threshold are transient and require ongoing participation.

Executive Summary

Seizures are episodes of abnormal or excessive excitation in the brain that results in behavioral responses such as convulsions. Normally, there is a homeostatic balance between excitation and inhibition in the brain. However, in the case of a seizure this balance is disrupted in favor of excess excitation. This homeostatic balance between excitation and inhibition is referred to as the seizure threshold, with a higher seizure threshold making an individual less susceptible to a seizure than a lower seizure threshold. If the seizure threshold becomes lowered to the point where an individual has multiple unprovoked seizures, then they are said to have epilepsy.

Within the brain there are neuromodulatory molecules that regulate neuronal activity and help maintain a high seizure threshold, thus preventing seizures. One molecule believed to be involved in seizure threshold maintenance is the protein cyclooxygenase-2 (COX-2). COX-2 is an enzyme in the arachidonic acid pathway, where it is responsible for converting free arachidonic acid into Prostaglandin H_2, which is a precursor for prostaglandin synthesis. COX-2 is expressed in excitatory neurons in a number of brain regions, including the hippocampus, which is region of the brain associated with the excitatory neuronal activity that occurs during seizures. COX-2's role in modulating seizure activity has been well documented by members of our lab. When COX-2 expression was inhibited prior to the induction of a convulsive seizure, the severity of the resulting seizures was significantly increased. Conversely, when COX-2 was genetically overexpressed in neurons, the incidence of convulsive seizures was significantly reduced. Taken together, these findings suggest that COX-2 maintains the seizure threshold.

A number of factors are also known to alter the seizure threshold. One of these factors is exercise, specifically running, which has been demonstrated to elevate the seizure threshold.

Over the last 20 years, as little as 30 days of running has been shown to decrease the incidence of convulsive seizures and reduce the likelihood of developing epilepsy. While the effects of exercise on the seizure threshold have been demonstrated numerous times, exactly how it does so remains poorly understood. One possibility is that changes in COX-2 expression mediate the effects of running on the seizure threshold. Here I hypothesize that running will increase the expression of COX-2 in the hippocampus, resulting in increased prostaglandin production that will in turn raise the seizure threshold. To test this hypothesis, two specific goals were outlined for this project. The first one being to determine if COX-2 expression and activity was increased after running and then to confirm the previously demonstrated effects of running on the seizure threshold.

To address these goals, CD-1 mice were housed in running wheel cages for four weeks before testing the seizure threshold. Seizures were induced through the use of pentylenetetrazole (PTZ), a chemical convulsant, and scored based on their severity as a measure of the seizure threshold. To measure COX-2 expression and activity, brain tissue samples were collected for COX-2 immunostaining and assayed for Prostaglandin E_2, a downstream product of COX-2 activity.

The results showed that basal levels of COX-2 expression were elevated in the hippocampus after running. PGE_2 levels were increased as well, indicating that COX-2 activity and expression are both increased by running. Despite these increases, the results indicated that participation in voluntary running did not significantly affect the seizure threshold. In the current study, however, the seizure threshold was tested six days post running, which differs from previous studies performed in our lab and by other groups, who assessed changes in the seizure threshold immediately after running had concluded. It is possible then that by six days post

running the beneficial effects of exercise have been lost, which suggests the running induced changes in the seizure threshold are not persistent. Additionally, COX-2 expression was only measured immediately after running had ended and was not analyzed at six days post running when the seizure threshold was tested. Therefore, it is crucial that in future studies COX-2 expression in measured at later time points to determine if changes in COX-2 expression over time are responsible for the transient effects of running on the seizure threshold.

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Acknowledgements

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Chapter 1

Introduction

Epilepsy

Epilepsy is a neurological disease defined by multiple unprovoked seizures[1,2]. It is the fourth most common neurological disease, with 2.2 million people currently living with epilepsy in the US[3]. While the cause of many types of epilepsy remain unknown, traumatic brain injury and stroke are known to increase the likelihood of an individual developing epilepsy. Risk factors like these and others contribute to the estimation that 1 in 26 people will develop epilepsy during their lifetime[4].

The development of epilepsy is thought to result from the progressive reduction of a person's seizure threshold. The seizure threshold is the balance between excitation and inhibition in the brain[5]. A disruption of this homeostatic balance in favor of elevated excitation can result in excessive electrical activity, otherwise known as a seizure[1]. When the seizure threshold is high, as is the case in the normal brain, increases in the relative levels of excitation are less likely to result in a seizure. On the other hand, lowering the seizure threshold results in an increased likelihood of an individual experiencing a seizure[6]. In the case of epilepsy development, the seizure threshold is reduced over time, resulting in a hyper excitable state where there is an increased incidence of spontaneous seizures **(Figure 1).** There are numerous ways in which this could occur, some of which are: alterations in the amount of excitatory and inhibitory

neurotransmitters, a change in the number of select neurotransmitter receptors, changes in synaptic connectivity, and disruptions in neuronal ion channels.

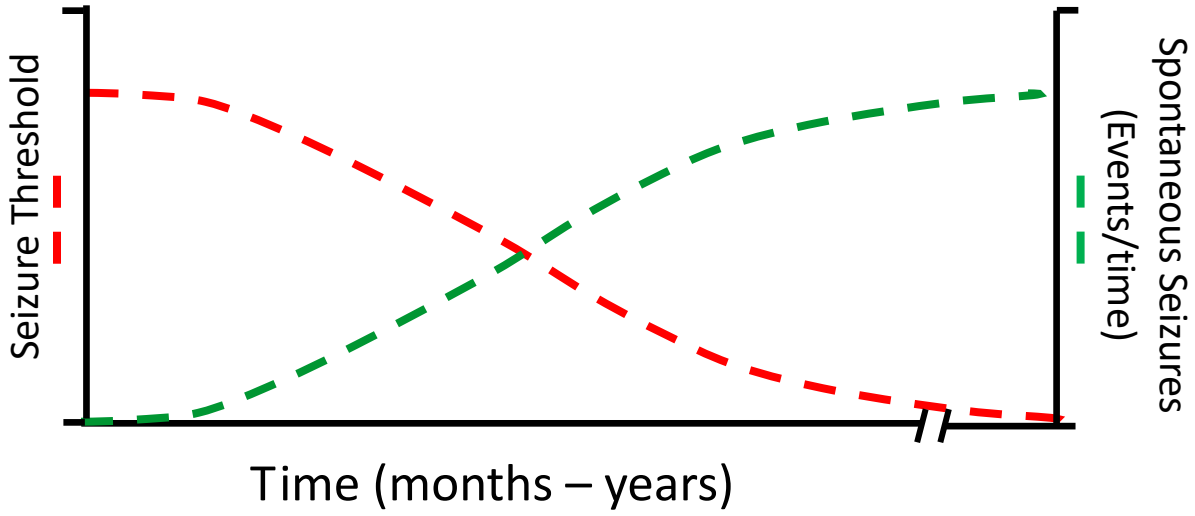


Figure 1. Schema depicting the relationship between seizure threshold (red) and incidence of spontaneous seizures (green). A reduction of the seizure threshold (red dashed line) and resulting increase in spontaneous seizures (green dashed line) over time represent the process of epileptogenesis, the development of epilepsy.

Temporal lobe epilepsy (TLE) is the most common focal epilepsy and accounts for roughly 80% of individuals living with epilepsy[7]. The hippocampal formation is a brain region in the temporal lobe that is considered to be centrally involved in the circuitry of TLE[8]. This brain region, which is normally involved in learning and memory formation[9], is classically divided into three sub regions: the dentate gyrus (DG), CA3, and CA1. Each sub region is composed of separate populations of neurons that are connected via the trisynaptic circuit. The trisynaptic circuit is a sequential series of synaptic connections from the entorhinal cortex to the DG, the DG to the CA3 and the CA3 to CA1, with the CA1 sending projections back to the entorhinal cortex[10]. The DG is referred to as the "gatekeeper" of the hippocampus, as it is the major point of entry for information coming from the entorhinal cortex. Axons from the entorhinal cortex travel

via the performant pathway and synapse on neurons of the DG. Axons from these neurons, referred to as mossy fiber projections, synapse on the pyramidal neurons of the CA3, which then send axons via the Schaffer collateral pathway to synapse on neurons of the CA1. In models of temporal lobe epilepsy, the hippocampus undergoes complex changes that result in the rewiring of these synaptic connections, including loss of normal connections and gain of new aberrant connections[11]. This, together with functional changes in neurons, is thought to increase the excitatory activity of the trisynaptic circuit, making the brain prone to hyper-excitability[1].

The goal of antiepileptic therapy is to raise the seizure threshold. Currently the most common treatments for epilepsy are pharmacological. However, antiepileptic drugs (AEDs) are ineffective in controlling seizures in roughly 20-40% of cases or are accompanied by unwanted side effects, making them difficult to tolerate[12]. Resective surgery and vagus nerve stimulation may also be used to control the occurrence of seizures in cases where medication is ineffective[13,14]. Resective surgery can be performed in cases where the origin of seizure activity has been identified and is contained to a localized region of the brain[13]. In these instances, physicians are able to remove or sever synaptic connections in the areas responsible for seizure activity. Vagus nerve stimulation is performed to partially control seizures in some, by sending electrical impulses to the brain via the vagus nerve, which has been shown to alter blood flow and levels of inhibitory and excitatory neurotransmitters [14,15]. In many cases, however, seizures persist despite these interventions[3]. A greater understanding of the mechanisms that underlie the seizure threshold in the normal brain has the potential to help identify new therapeutic targets for the prevention and reduction of epileptic seizures. As a result, increased attention has been given to the study of endogenous neuromodulators and their potential role in the maintenance of the seizure threshold.

Cyclooxygenase-2 (COX-2)

Cyclooxygenases (COX) are enzymes involved in the synthesis of prostaglandins (**Figure 2**), which are potent autocrine and paracrine signaling molecules that affect many cellular functions under normal and pathological conditions. Prostaglandins are derived from arachidonic acid (AA), a polyunsaturated fatty acid that is stored in membrane phospholipids. AA is cleaved from the membrane by phospholipase A (PLA_2) which can be activated by various stimuli[16,17]. In the brain, AA release is coupled to excitatory neuronal activity[18]. Upon its release, AA is converted to prostaglandin H_2 (PGH_2) by COX enzymes[17]. There are two COX isozymes, COX-1 and COX-2. These isozymes have very similar structures and enzyme kinetics but differ in their expression pattern. COX-1 is expressed basally throughout the entire body, while COX-2 expression is low or undetectable normally in most tissues but can be rapidly induced in response to growth factors or pro-inflammatory signals. An exception to this is found in the CNS where COX-2 is expressed basally by excitatory neurons[19].

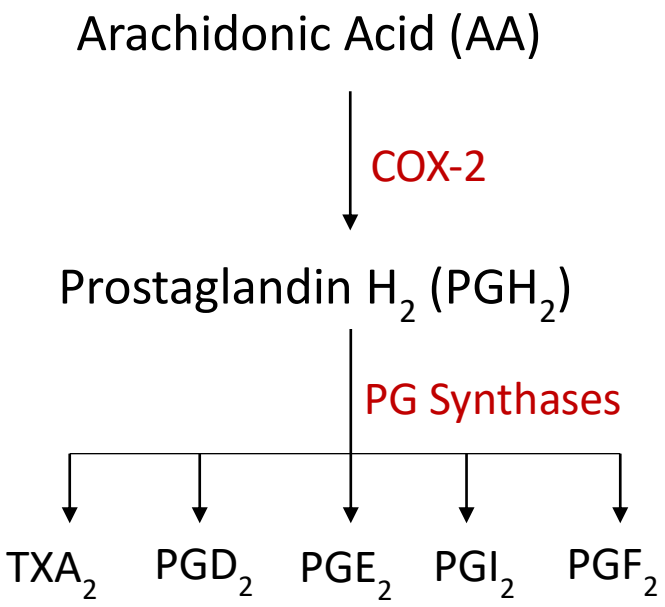


Figure 2. Cyclooxygenase pathway of Arachidonic Acid metabolism

Expression of COX-2 is particularly prominent in the cerebral cortex and the CA3 region of the hippocampus, where it is localized to the dendritic spines of excitatory neurons[19]. The localization of COX-2 to dendritic spines suggests it may have a modulatory role in synaptic transmission. In the hippocampus, COX-2 expression is regulated by excitatory neuronal activity[20]. Thus, an increase in activity is accompanied by a subsequent increase in COX-2 expression. This relationship suggests that COX-2 is involved in modulating neuronal activity in processes that involve the hippocampus, such as memory and long-term potentiation (LTP). Consistent with this theory, pharmacological inhibition of COX-2 produced amnesia like effects in chicks subjected to passive avoidance tasks[21]. Furthermore, mouse hippocampal slices treated with COX-2 inhibitors prior to high frequency electrical stimulation saw a reduction in the amplitude of the post synaptic potential, indicating a reduction in LTP[22].

Additionally, evidence suggests that COX-2 modulates neurogenesis and contributes to the enhanced proliferation and differentiation of newly generated neurons. Adult neurogenesis occurs in select regions of the brain, including in the dentate gyrus of the hippocampal formation, where is it thought to be involved in learning and memory[23]. In a study done by Ma and colleagues, the upregulation of COX-2 protected newly formed hippocampal neurons from acute stress[24]. Further support was provided by additional studies that demonstrated COX-2 inhibition, either through pharmacological block or genetic deletion, reduced the amount of cell proliferation and neural differentiation in the dentate gyrus[25].

Numerous studies have also assessed the role of COX-2 in epilepsy and seizure generation. In particular, there is evidence that COX-2 activity contributes to the maintenance of a high seizure threshold, thereby preventing the generation of spontaneous seizures. For instance,

mice that were treated with rofecoxib, a selective inhibitor of COX-2, prior to the induction of seizures with a chemoconvulsant, demonstrated an increase in seizure severity in comparison to control mice receiving vehicle[26]. Alternatively, transgenic mice overexpressing COX-2 in the cortex and CA3 of the hippocampus had a significantly lower incidence of convulsions in response to a chemoconvulsant stimulus as compared to wild type mice[27]. Together these studies provide support for the idea that COX-2 is an endogenous modulator of the innate seizure threshold.

The action of COX-2 in seizure threshold maintenance could be mediated by the enzymatic products of COX-2 activity, known as prostaglandins (**Figure 2**). While prostaglandin levels in the brain are usually low, their synthesis is elevated following a chemically or electrically induced seizure[28]. Inhibition of these prostaglandin levels prior to injection with a chemoconvulsant correlated with an increased severity and duration of convulsions, suggesting the elevated expression of prostaglandins produced anticonvulsive effects[28]. Studies examining the effects of specific prostaglandins on seizure generation have found that in rats, injections with prostaglandin D_2 (PGD_2) five minutes prior to injection with a chemoconvulsant significantly increased the latency to convulsion as compared to controls receiving the vehicle[29]. Additionally, when rats were injected with a pharmacological agonist of the prostaglandin E_2 (PGE_2) EP_2 receptor before seizures were induced with a chemoconvulsant, a significant increase in the latency to convulsion was similarly observed[30].

Exercise

In addition to endogenous modulators, there has been increasing interest in identifying lifestyle changes, such as regular participation in exercise, that can alter the seizure threshold.

Exercise has a number of beneficial effects on the brain. It has been demonstrated to improve learning and memory, in addition to reducing the likelihood of suffering from certain neurological conditions[31,32]. For example, adults who exercise regularly were diagnosed with dementia less often and saw a reduction in the cognitive decline associated with aging[33,34]. Additionally, exercise has been shown to induce neurogenesis in the dentate gyrus of the hippocampus. In a study done by Van Praag and colleagues, mice were housed with running wheels for 29 days and injected with BrdU, a synthetic nucleoside used to label newly generated neurons, once daily for the first 12 days. They found that mice in the running group had a significant increase in the number of BrdU-postitive cells in the DG of the hippocampus both immediately after running had ended and four weeks later. Four weeks later the runners also showed a significantly higher number of BrdU-postive cells that had differentiated into neurons as compared to the sedentary controls. The results of this study indicate that running increases both cell proliferation and survival in the dentate gyrus[35].

In a follow up study, Van Praag and colleagues examined the effects of running induced neurogenesis on learning. They found that mice that had been subjected to at least 30 days of voluntary running had a significantly higher rate of acquisition of complex Morris Water Maze tasks, a measure of hippocampal-dependent learning. Additionally, differences in long term potentiation between groups was assessed through the administration of high frequency stimulation to the DG and CA1 of hippocampal sections. The exercised mice had significantly higher levels of LTP in the DG, while no difference in CA1 LTP was seen between the running and control groups[36]. In this follow up study they also confirmed the increase in BrdU-positive neurons in the DG of the running group. Together the results of these two studies suggest that

increased proliferation of neurons in the DG contributed to enhanced water maze performance and elevated levels of LTP.

Seizure severity and incidence is also affected by exercise. For instance, in a temporal lobe model of epilepsy, the frequency of spontaneous seizures in epileptic rats was reduced during the 45 day running protocol as compared to the control and sham groups[37]. Exercise has also been shown to reduce the susceptibility to epileptogenesis, or epilepsy development. In rats containing brain lesions, 30 days of running reduced the severity of pilocarpine-induced seizures as compared to injured rats that were sedentary[38]. It was concluded that exercise reduced the process of epileptogenesis initiated by the brain lesion. Additionally, in the kindling model of epileptogenesis, daily administrations of electrical or chemical stimuli cause a progressive reduction in the seizure threshold. Consistent with other models of epileptogensis, rats subjected to 45 days of running prior to the kindling paradigm required a significantly greater number of electrical stimulations to develop the epilepsy phenotype than the control or sham groups[39]. These results are relevant as epilepsy is a neurological disease that can be acquired following a neurological insult, which is what the brain lesion and kindling stimulations mimic. Currently, no known cure to prevent the development of epilepsy exists. However, findings from both these studies suggest that running could decrease an individual's susceptibility to epilepsy development following neurological injury. Running has shown similar effects in uninjured rodents as well. Previous unpublished data from our lab showed that 28 days of voluntary running prior to seizure induction reduced the incidence of convulsions in an acute seizure model in mice.

While the benefits of exercise, in particular running, have been demonstrated by multiple groups over the last 20 years, little is known about the mechanism behind these effects. This

study aimed to investigate the role of COX-2 in mediating these effects, with our expectation that running will increase the expression of COX-2 in the hippocampus, resulting in increased prostaglandin production that will in turn raise the seizure threshold.

Chapter 2

Methods

Husbandry

Male 6-week-old CD-1 mice were purchased from Charles River Laboratories. The mice were housed in a temperature and humidity-controlled vivarium at Syracuse University that maintains a 12-hour light-dark schedule. Mice were housed in pairs and provided food and water *ad libitum*. All animal protocols were approved by the Syracuse University IACUC and adhered to the guidelines of the NIH handbook and AVMA.

Running Paradigm

Following a week of acclimation to the facilities at Syracuse University, mouse pairs were transferred to new cages that either contained a running wheel (Lafayette Instrument Mouse Activity Wheel Chambers; Model 80820F) or did not. In each study, four experimental cages contained running wheels while two cages did not (control). For the former, the number of rotations were recorded and reset every 24 hours for 28 consecutive days. On days 29-34 each animal was handled in order to reduce stress prior to testing changes in seizure threshold and COX-2 activity.

Acute Seizure Model

Pentylenetetrazole (PTZ)-induced seizures were used to test the innate seizure threshold. PTZ is a convulsant agent, which provokes seizure responses by indirectly enhancing excitation through

the its ability to reduce inhibition. Injection solutions were prepared immediately prior to use in 0.9% filter sterilized saline. All PTZ solutions were administered in an injection volume of 0.1 mL solution per 10 g body weight of the mouse. Injections were made into the peritoneal space and behavior was observed for 20 minutes following PTZ injection. The observed behavior was quantified based on the seizure scoring system shown in **Table 1**[26]. Normal behavior (0) includes activities such as grooming, socializing, exploration, digging, and climbing. Hypomobility constitutes a score of one (1). Animals exhibiting this behavior pay little attention to other animals in the cage and sit motionless for extended periods of time. If movement occurs during this stage, it is slow, with the animal's body elongated and close to the floor of the cage. A score of two (2) is defined as the presence of at least two separate myoclonic seizures. Common characteristics of myoclonus are brief episodes of flexing of the neck, distinct twitches involving the whole body, and in more severe instances, the presence of a Straub reaction (rigid upright tail extension). Convulsive seizures are defined as either a score of three (3) or four (4). A score of three (3) does not impair the animal's ability to remain in an upright position. A score of four (4) involves the loss of ability to maintain an upright posture during a convulsion. Both a score of three and four are followed by excessive salivation and a period of hypomobility. The highest score attained during the observation period was recorded. In the case of a convulsive seizure, the time in seconds until the convulsion onset (latency) was recorded.

Table 1. Seizure severity scoring system.

Score	Description	Category
4	Generalized convulsion with loss of righting	Convulsive seizures
3	Generalized convulsion with righting reflex	
2	Myoclonus	Non-convulsive seizures
1	Hypomobility	
0	Normal Behavior	

Tissue Harvest

Three hours after PTZ injection, mice were administered an anesthetic mixture of ketamine and xylazine in an injection volume of 0.1 mL solution per 10 g body weight for a final dose of 100 and 10 mg/kg, respectively. Reflexes were tested 7 – 10 minutes after injection by pinching the base of the tail and the center of the foot pad with forceps. The mouse was deemed fully anesthetized when no response was elicited. Once in deep anesthesia the thoracic cavity was opened to expose the heart in order to exsanguinate the mouse via cardiac perfusion. To begin the perfusion, the right atrium of the heart was punctured to allow blood to escape the circulatory system. The blood was then flushed out with 15 mL of room temperature phosphate-buffered saline (PBS) using a syringe inserted into the left ventricle. The structural integrity of the tissue was maintained by subsequent perfusion with 15 mL of room temperature 4% paraformaldehyde (PFA) in PBS. After fixation, the whole brain was removed from the cranium and submerged in 15 mL PFA overnight in a refrigerator. Approximately 12 hours later, tissue samples were transferred to a PBS solution containing 20% sucrose and returned to the refrigerator. Once the tissue had sunk to the bottom of the tube (~24 hours later), brains were

washed by dipping in a petri dish of PBS and placed in Optimum Cutting Temperature solution (OCT, Tissue-Tek), then flash frozen with dry ice-cooled ethanol and stored in an ultra-low temperature freezer at -80°C.

Sectioning and Immunohistochemistry

Embedded blocks of brain tissue were removed from freezer storage and allowed to equilibrate to -20°C in a cryostat for ~30 minutes prior to sectioning. Tissue blocks were mounted on the microtome using OCT and 60 μm thick sections were cut and discarded until the hippocampal region was detected. The section thickness was then reduced to 14 μm and every third section was collected and mounted on a microscope slide specifically designed to optimize adherence of the section (Superfrost Plus, Fisher). Nine microscope slides containing 4-5 sections each were collected from each tissue sample. Slides were stored at -20 °C until they could be processed for analysis of COX-2 protein expression.

COX-2 protein expression in tissue sections was assessed by immunohistochemistry (IHC). The IHC protocol utilized by the J. Hewett lab is a three-day process that requires both a primary and secondary antibody. Slides were removed from the freezer and allowed to dry for 5 minutes at room temperature. Once dry, an Aqua-Hold Pap Pen (Electron Microscope Sciences) was used to draw a continuous border around the brain sections, preventing the staining solutions added in subsequent steps from running off the surface. Next, the tissue was rehydrated by incubating with PBS for 5 minutes and made permeable by adding PBS with 0.25% Triton-X 100 detergent (PBT). After incubating the sections at room temperature for 15 minutes, excess liquid was removed from the slides before the addition of 100 μL of blocking solution (5% donkey serum, 0.25% PBT, 1% BSA), which blocks non-specific binding of antibody to unintended proteins in the brain sections. The slides were then incubated in the blocking solution

overnight at 4°C. On the second day, the blocking solution was removed and 100 μL of the primary antibody solution was added. A rabbit polyclonal anti-COX2 antibody **(**1:250 Cayman Chemical Cat# 160106 RRID: AB_10077935) was used herein to observe COX-2 protein expression in tissue sections. The sections were incubated overnight at 4 °C with this antibody, which was diluted in modified blocking solution (1% donkey serum, 0.25% PBT, 1% BSA) overnight at 4 °C. Following this incubation, the primary antibody was collected, 0.01% sodium azide preservative was added, and solutions stored for reuse. The sections were then washed (3x 15 minutes each) with PBS and incubated for 2.5 hours at room temperature in 100 μL of secondary antibody solution containing a donkey anti-rabbit antibody (7.5 μg/mL Jackson ImmunoResearch Labs Cat# 711-545-152 RRID: AB_2313584) that was conjugated with a fluorescent tag (Alexaflour 488). The secondary antibody, which was diluted in modified blocking solution (1% donkey serum, 1% BSA, 0.25% PBT), permitted the detection of the primary antibody and hence COX-2 by fluorescence microscopy. This and all subsequent steps were performed in the dark to shield the fluorescent tags from room light, which can destroy (bleach) the fluorophores. After incubation with secondary antibody, the solution was removed and the slides were washed with PBS (5 x 10 minutes each). The slides were then submerged for five minutes in a solution containing DAPI (diamidino-2-phenylindole), which is a fluorescent dye that stains the nucleus of all cells and is used as a cell marker. The slides were washed a final time with PBS for 5 minutes, then covered with antifadent medium (Citifluor AF1, Electron Microscopy Sciences) and cover slip. Slides were stored at 4 °C in a light proof container until they were imaged. Brain sections from each treatment group were processed together using the same reagents.

Brain sections were visualized using a Zeiss Axio Imager A2 microscope fitted with EC Plan-NeoFluar objectives and a set of fluorescent filters designed to detect fluorophores with different excitation and emission spectra. This allowed distinction between the 2nd antibody and DAPI fluorescent signals. Fluorescence was stimulated using an X-cite 120XL Iris FL illuminator and images were acquired using an AxioCam MRc digital camera and ZEN 2 software (Zeiss, Version 2.0.0.0). Separate images of COX-2 and DAPI stains in the dentate gyrus (DG), CA1, and CA3 areas of the hippocampal formation were taken from each sample.

Quantification of COX-2 Expression

Adobe Photoshop CC 2018 was used to increase the contrast between the background and specific staining, as well as to overlay COX-2 and DAPI images. The thresholds used to increase the contrast remained constant for all images (24-130 for COX-2 and 24-255 for DAP1). NIH Image J (version 1.50i) was used to quantify the fluorescent intensity of COX-2 staining. With this program, jpg images of COX-2 staining were converted to an 8-bit grey scale before setting the maximum and minimum fluorescent threshold. The threshold was set using an image of the CA3 from the control group. This threshold (23-255) was used for analysis of the rest of the images. Rectangular boxes were used to designate areas for fluorescent intensity measurement and the average of three to seven measurements per region were averaged to determine the mean intensity density of COX-2 staining in each region of the hippocampus. For each brain region, two biological replicates were analyzed, and the results averaged to determine the final mean intensity density.

Prostaglandin E_2 Assay

A separate study was performed to assess changes in prostaglandin E_2 (PGE_2) levels in brain tissue in the PTZ-induced acute seizure model. PTZ solutions were prepared and administered as described in the acute seizure model section. Seizure behavior was monitored and scored for five minutes following PTZ injection, after which mice were placed in an isoflurane chamber until sedated (~one minute). Next, mice were rapidly decapitated using a guillotine specifically designed for this purpose. The head was immediately placed in liquid nitrogen for three minutes to allow the sample to freeze thoroughly. This approach to harvest brain tissue samples was chosen to minimize further PGE_2 production[28]. Frozen samples were stored at -80 °C until brain tissue could be removed and prepared for measurement of PGE_2 via ELISA.

The ELISA was performed using a commercially available kit following the manufacturers protocol (Cayman Chemicals Prostaglandin E_2 Express ELISA Kit, Item No. 500141). The frozen head was allowed to thaw on ice for 30 minutes before the skull was opened and the brain tissue was removed. Each hemisphere of the brain was placed into its own pre-weighed micro centrifuge tube and wet weight of the tissue sample was determined. Homogenization buffer (0.1 M phosphate, 1mM EDTA, 10 μM indomethacin) was added to each tube in a ratio of 2 mL per gram of tissue. The samples were then homogenized manually with a pestle until no tissue fragments were visible and spun for 20 minutes at 12,000 x g in a 4 °C centrifuge. Cleared supernatants were transferred to a clean tube and either used immediately or frozen at -80 °C.

Statistical Analysis

All statistical tests were performed using GraphPad Prism 6.03. To calculate the significance of the percent incidence of convulsions between treatment groups, a Chi-squared contingency test was used. Latency to convulsive seizure was tested for significance using a one-tailed t-test due to our hypothesis that latency would increase after running. Seizure severity was analyzed using Mann-Whitney U tests. PGE_2 values were transformed via a log function and equal variance was confirmed using an F-test. A one tailed t-test was used to determine significance between mean PGE_2 levels as our hypothesis predicted an increase after running.

Chapter 3

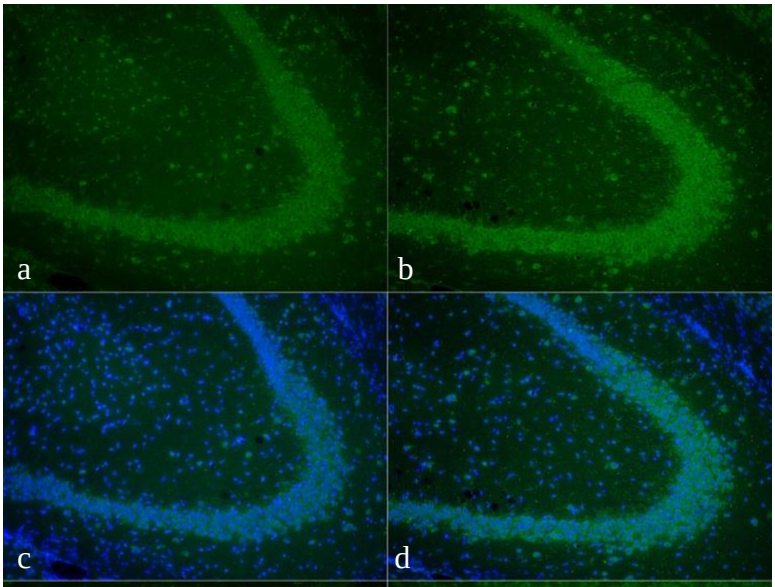
Results

Basal COX-2 protein expression in the hippocampus

To assess changes in basal COX-2 protein expression in the experimental running group, brain tissue samples were collected immediately after the 28 days of running had concluded. Tissue samples were sectioned and stained for COX-2 protein expression and Image J was then used to quantify COX-2 expression in each region of the hippocampus as described in the methods. Although basal expression of COX-2 protein was detected at varying levels in the CA3, CA1, and DG of control animals (**Figure 3Aa, Ba, Ca**), it was highest in the CA3 (**Figure 3D-F**). This protein expression was elevated in the CA3 and CA1, but not the DG, after completion of the running protocol (**Figure 3Ab, Bb, Cb, D-F**). The mean CA3 intensity increased fourfold in the running wheel experimental group (**Figure 3d**) and the mean intensity increased threefold in the CA1 of the running wheel group (**Figure 3e**), whereas intensity was approximately equal between groups in the DG (**Figure 3F**). The increases in intensity seen in the CA3 and CA1 could not be analyzed for statistical significance due to the small sample size (n=2) in both the control and running wheel groups. Despite this, the results indicate a trend towards increased basal COX-2 protein expression in select regions of the hippocampus immediately after cessation of running.

CONTROL RUN

A



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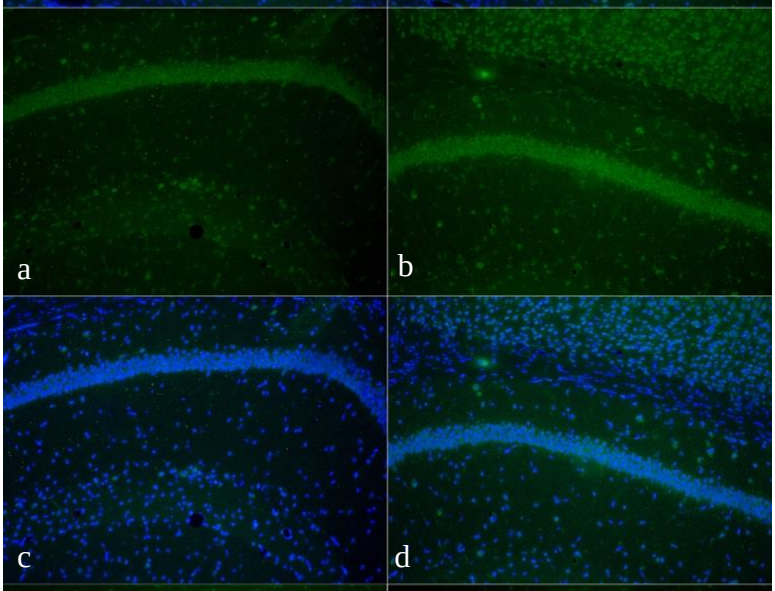

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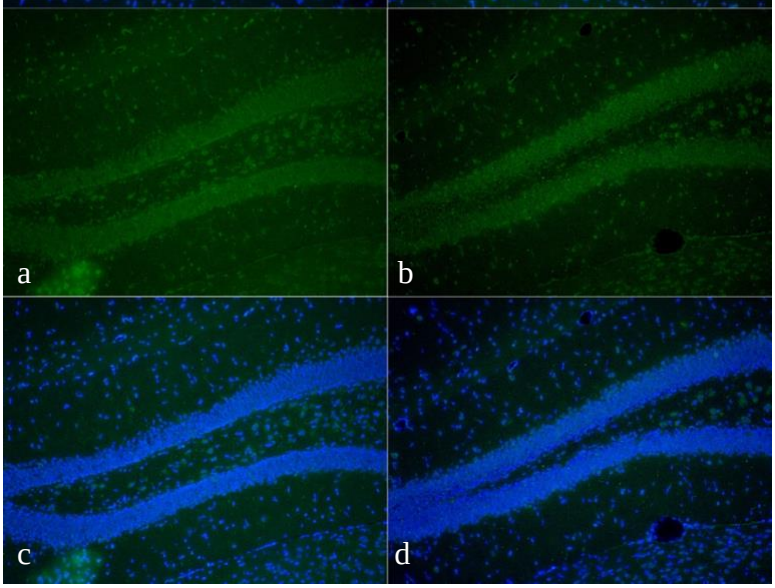

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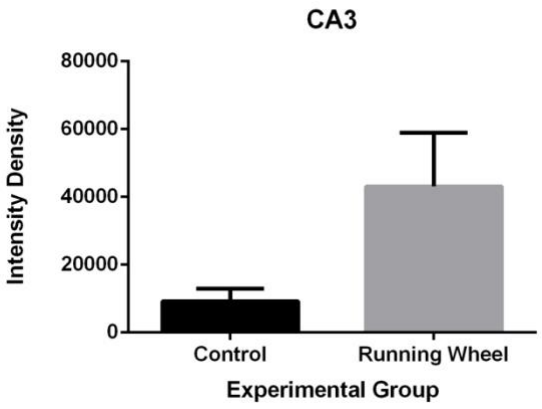

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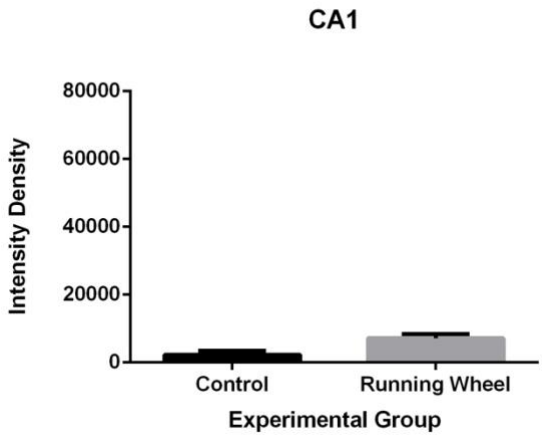

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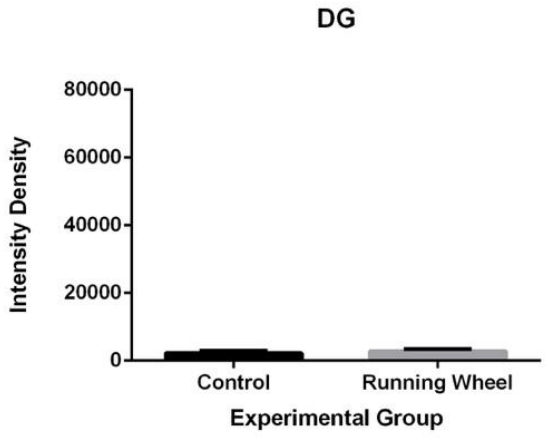

Figure 3. Effects of running on COX-2 expression in the hippocampal formation
COX-2 expression in CA3 **(A)**, CA1 **(B)**, and DG **(C)**. Immunostaining for COX-2 alone (Aa/b, Ba/b, Ca/b). Overlay of COX-2 immunostaining and DAPI (Ac/d, Bc/d, Cc/d). Green, COX-2 protein; Blue, DAPI nuclear stain. Quantification of COX-2 immunostaining was performed using ImageJ (threshold 23,255). Intensity density reported for each experimental group (n=2) in the **(D)** CA3 **(E)** CA1 and **(F)** DG of the hippocampal formation. Intensity density expressed as mean ± SEM. Higher mean intensity density indicative of greater COX-2 immunostaining.

PGE_2 levels six days after running

To test alterations in seizure threshold and COX-2 activity, mice were treated with the convulsant agent pentylenetetrazole (PTZ). After 28 days of running and five subsequent days of handling, mice were treated with 44 mg/kg PTZ and seizure responses and COX-2 activity were assessed. Mice provided free access to a running wheel had a median seizure score of 1.5, which was identical to the median seizure score seen in the sedentary control group (**Figure 4a**).

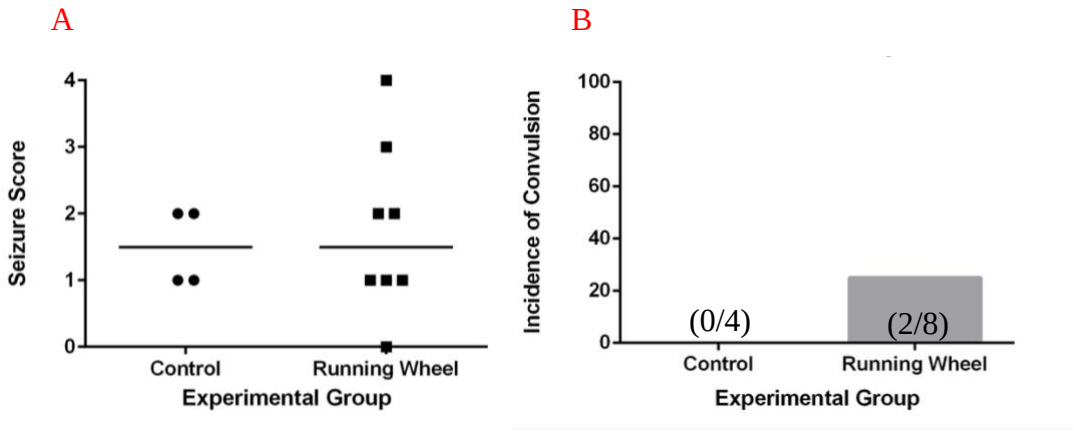

Figure 4. Seizure score and incidence of convulsion at 44 mg/kg PTZ
Control (n=4) and running wheel (n=8) mice were administered 44 mg/kg PTZ through i.p. injection. Behavior was observed for 5 minutes and scored on a five-point scale as described in Methods. **(A)** Effects of running on seizure severity. Each symbol indicates the maximum seizure score reached by individual mice, whereas the horizontal line illustrates the median seizure score for all mice. Mann Whitney U tests determined seizure severity was not significantly different between experimental groups ($p>0.9515$). **(B)** Effects of running on incidence of convulsions. The number of mice in each experimental group that experienced a convulsive seizure (score of 3 or 4) is graphed as a percentage of the total number of individuals in each group, which can be seen in the parentheses within bars. Differences in the incidence of convulsions between groups were compared using a chi-squared contingency test and a statistically significant difference was not seen ($p=0.5152$).

PTZ injections only produced convulsions in two out of 12 total individuals tested, both of which belonged to the running wheel group (**Figure 4b**). For both seizure score and incidence of convulsion, there was no statistically significant difference between the running and control groups ($p>0.9999$, $p=0.5152$ respectively) (**Figure 4**).

Tissue PGE_2 levels were measured to determine if COX-2 activity was modulated by physical activity (**Figure 5**). PGE_2 was assessed five minutes after 44mg/kg PTZ injection as described in Methods. PGE_2 levels in samples collected from this study were roughly equivalent between individuals in the control group, whereas the running wheel group exhibited more variation, and this was significantly greater than the levels seen in the sedentary controls ($p=0.0202$) (**Figure 5a**). PGE_2 levels were much higher in two individuals in the running group, relative to the rest of the individuals in this experimental group. These two individuals, indicated by red circles, correspond to the two mice that had convulsive seizures (**Figure 4**). Interestingly, a comparison between mice that did not exhibit convulsions still showed a statistically significant elevation in mean tissue PGE_2 levels in the running group relative to control mice. ($p=0.0415$) (**Figure 5b**).

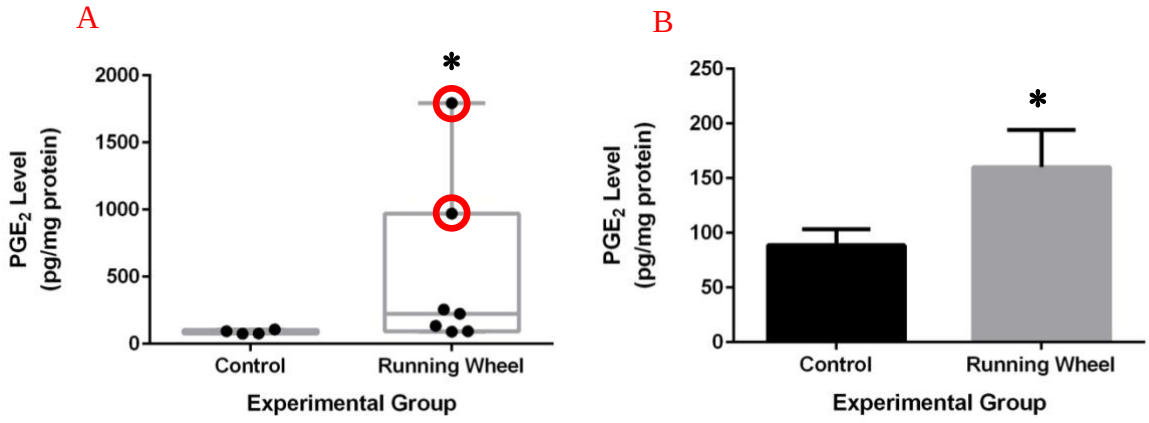

Figure 5. Effects of Running on PGE_2 levels in whole brain samples
Total PGE_2 levels were measured in whole brain samples collected 5 minutes after 44 mg/kg PTZ injection. Supernatant from tissues sample homogenates was used in the PGE_2 assay as described in Methods. **(A)** All mice. Each symbol represents the PGE_2 level in individual mouse brain samples from the control (n=4) and running wheel (n=7) experimental groups. Data is expressed as mean pg PGE_2/mg protein. Red circles indicate mice that experienced a convulsive seizure (score of 3 or 4). **(B)** Non-convulsing mice only. Data points corresponding to mice that experienced a convulsive seizure were removed to compare mean PGE_2 levels in non-convulsive animals in the control (n=4) and running wheel (n=5) experimental groups. Data is expressed as mean pg PGE_2/mg protein ± SEM. An asterisk (*) represents significant difference in PGE_2 levels between experimental groups as determined by a one-tail t-test (p=0.0202, p=0.0415 respectively).

Voluntary running and the seizure threshold

Since the dose of 44 mg/kg PTZ did not elicit a convulsive response in control mice, it was not possible to assess the effects of running on the seizure threshold. Thus, another study was performed using 52.5 mg/kg PTZ (**Figure 6**). Seizure severity, convulsive seizure incidence, and latency to convulsive seizure were subsequently recorded. Mice subjected to voluntary running had a median seizure score of 3, which was not significantly different than the median seizure score of 2.5 seen in the sedentary controls (p=0.7556) (**Figure 6A**). The percentage of mice in each group that experienced convulsive seizures in response to PTZ injections also did not differ significantly (p=0.5475), with 50% experiencing convulsions in the control group and 75% in the running wheel group (**Figure 6B**). Additionally, the median

latency to convulsion was not statistically different between the running and control groups (200 and 400 seconds respectively) (p=0.2830) (**Figure 6C**).

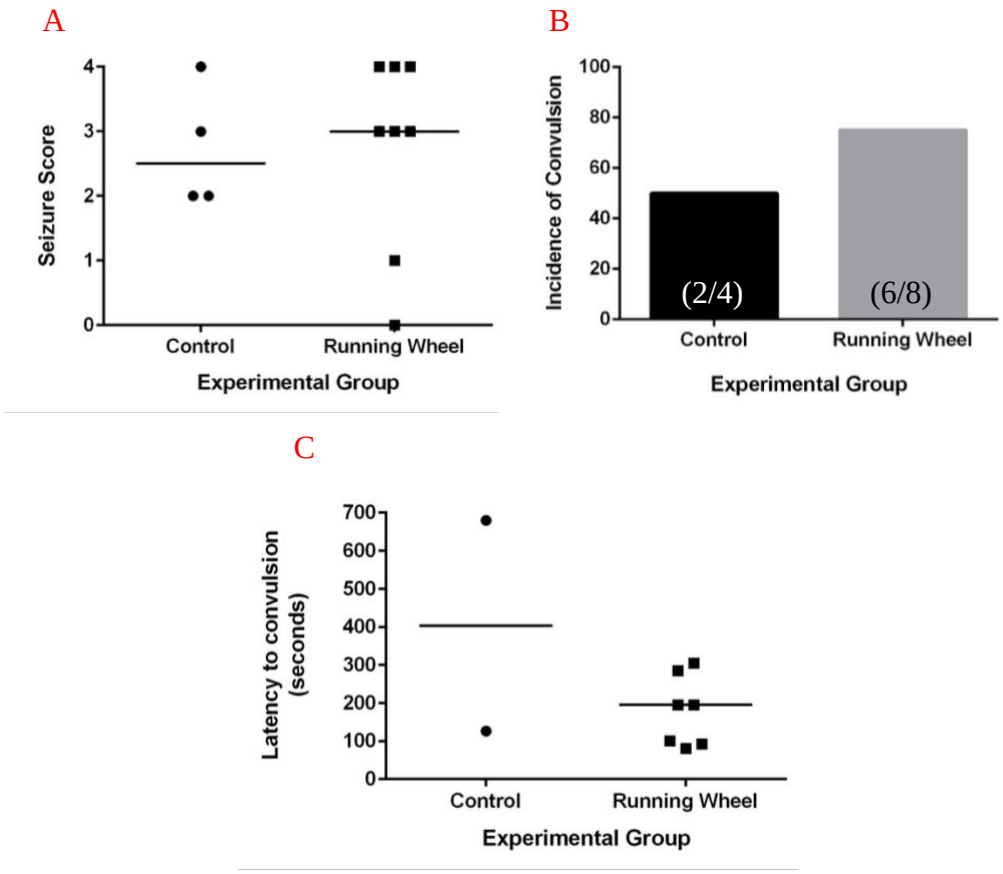

Figure 6. Seizure score and incidence of convulsion at 52.5 mg/kg PTZ
Control (n=4) and running wheel (n=8) mice were administered 52.5 mg/kg PTZ i.p. Behavior was observed for 20 minutes and scored on a five-point scale as described in Methods. **(A)** Effects of running on seizure severity. Each symbol indicates the maximum seizure score reached by individual mice, whereas the horizontal line illustrates the median seizure score for all mice. Mann Whitney U tests determined seizure severity was not significantly different between experimental groups (p=0.7556). **(B)** Effects of running on incidence of convulsions. The number of mice in each experimental group that experienced a convulsive seizure (score of 3 or 4) is graphed as a percentage of the total number of individuals in each group, which can be seen in the parentheses within bars. Differences in the incidence of convulsions between groups were compared using a chi-squared contingency test and a statistically significant difference was not seen (p=0.5475). **(C)** Effects of running on latency to convulsive seizure. Each symbol represents the time in seconds until onset of the convulsion (score or 3 or 4) for mice in A. Horizontal bars indicate the median latency in seconds for all mice in each experimental group. A one tailed t-test found no significant difference in latency between groups (p= 0.2830).

Effects of neuromodulators on the seizure threshold can be subtle and easily overwhelmed by too strong a seizure stimulus[26]. This raised the possibility that the dose of PTZ used for this study may have been too high. The PTZ dose used for this study was selected based on an initial dose-response study in which the sample size was small. Thus, a second, more extensive (n=24), dose response study was completed (**Figure 7**). This analysis demonstrates that PTZ lowers the seizure threshold in a dose-dependent manner. The percent convulsions for 38, 42, 46, and 50 mg/kg PTZ was 16.7, 0, 50 and 50, respectively. These results show that doses above 42 mg/kg most consistently elicited convulsive responses. Based on these results, 46 mg/kg was selected for a follow up study due to the greater range of seizure scores it provoked.

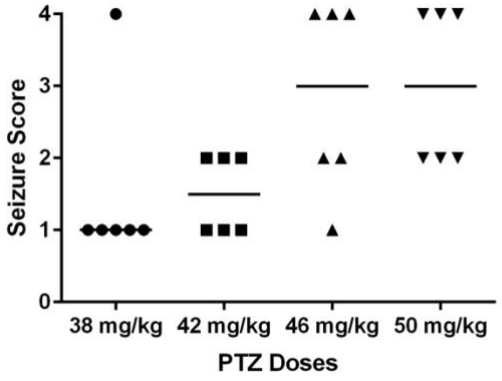

Figure 7. PTZ dose response curve
CD-1 mice were dosed with 38, 42,46, or 50 mg/kg PTZ through i.p. injection. Behavior was monitored for 20 minutes and scored on a five-point scale as described in Methods. Individual points represent maximum seizure score for each individual in response to the specified PTZ dose.

However, this dose generated median seizure scores of 3.5 and 3 for the sedentary and running groups, respectively, and convulsions occurred in all members of both groups except one (**Figure 8A and B**). Latency to convulsive seizure was also analyzed for differences between groups. The median latency in seconds for each group was 150 and 225, for the control and running treatment groups, respectively, which was not statistically different (p=0.2980) (**Figure 8C**). The high incidence of convulsions in both groups indicated that the PTZ dose used was too high. In hindsight, it is important to note that the mouse housing conditions between this and the

dose-response study differed slightly in that the latter was performed with mice that were house in conventional cages, which could explain the discrepancy.

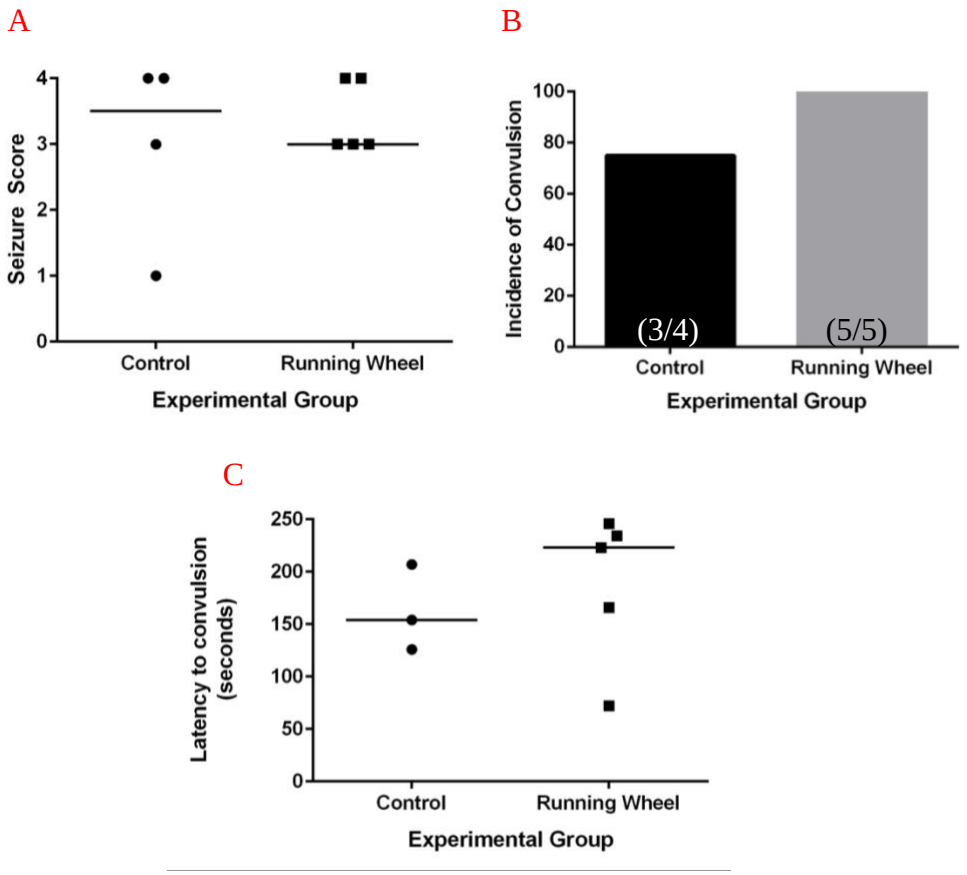

Figure 8. Seizure score and incidence of convulsion at 46 mg/kg PTZ
Control (n=4) and running wheel (n=5) mice were administered 46 mg/kg PTZ through i.p. injection. Behavior was observed for 20 minutes and scored on a five-point scale as described in Methods. **(A)** Effects of running on seizure severity. Each symbol indicates the maximum seizure score reached by individual mice, whereas the horizontal line illustrates the median seizure score for all mice. Mann Whitney U tests determined seizure severity was not significantly different between experimental groups ($p>0.9999$). **(B)** Effects of running on incidence of convulsions. The number of mice in each experimental group that experienced a convulsive seizure (score of 3 or 4) is graphed as a percentage of the total number of individuals in each group, which can be seen in the parentheses within bars. Differences in the incidence of convulsions between groups were compared using a chi-squared contingency test and a statistically significant difference was not seen ($p=0.4444$). **(C)** Effects of running on latency to convulsive seizure. Each symbol represents the time in seconds until onset of the convulsion (score of 3 or 4) for mice in A. Horizontal bars indicate the median latency in seconds for all mice in each experimental group. A one tailed t-test found no significant difference in latency between groups ($p= 0.2980$).

To complete the analysis of running on the seizure threshold, a final study was performed using 42 mg/kg PTZ (**Figure 9**). As in the previous studies, participation in the running experimental treatment did not have a significant effect on the seizure threshold compared to sedentary control mice. However, both seizure severity and incidence of convulsions were very low at this dose. The running wheel and control mice exhibited a median score of 1 and 1.5, respectively (p>0.9999) (**Figure 9A**). None of the mice in the control group and only one out of six in the running group experienced a convulsion (p=1.0000) (**Figure 9B**). Latency to convulsion could not be analyzed in this study due to the absence of convulsions in the control group. Taken all together, these three running wheel studies suggest that running did not have a significant effect on the seizure threshold.

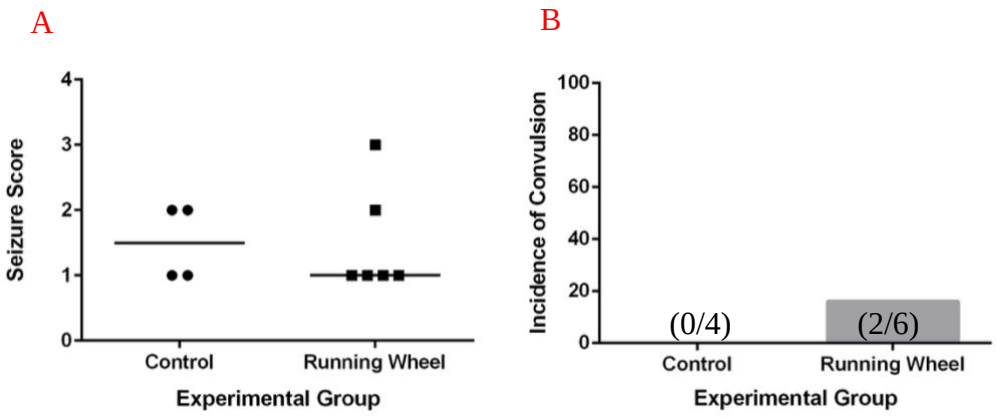

Figure 9. Seizure score and incidence of convulsion at 42 mg/kg PTZ
Control (n=4) and running wheel (n=6) mice were administered 42 mg/kg PTZ through i.p. injection. Behavior was observed for 20 minutes and scored on a five-point scale as described in Methods. **(A)** Effects of running on seizure severity. Each symbol indicates the maximum seizure score reached by individual mice, whereas the horizontal line illustrates median seizure scores for all mice. Mann Whitney U tests determined seizure severity was not significantly different between experimental groups (p>0.9999). **(B)** Effects of running on incidence of convulsions. The number of mice in each experimental group that experienced a convulsive seizure (score of 3 or 4) is graphed as a percentage of the total number of individuals in each group, which can be seen in parentheses within bars. Differences in the incidence of convulsions were compared using a chi-squared contingency test and a statistically significant difference was not seen (p=1.000).

Chapter 4

Discussion

Running increased basal COX-2 expression in the CA3 and CA1 of the hippocampus

The role of COX-2 expression in the brain during seizure activity is widely debated, with reports of both anticonvulsive and deleterious effects[11,27]. The time of COX-2 expression appears to contribute to the different effects of COX-2, with elevated expression prior to the induction of a convulsion responsible for its anticonvulsive properties. For example, overexpression of COX-2 in neurons prior to seizure induction raised the seizure threshold in a PTZ model of acute seizures[27]. In contrast, elevated COX-2 expression in the hippocampus that persisted 6-24 hours after pilocarpine seizures resulted in excitotoxicity[40]. The different effects of COX-2 in regards to seizure activity may also depend on the seizure model used. In this regard, Jung and colleagues showed that COX-2 inhibition attenuated spontaneous seizure activity in a pilocarpine-induced model of epilepsy[11]. For the purpose of these studies, we hypothesized that elevated COX-2 protein expression in the hippocampus prior to seizure induction would exhibit anticonvulsive effects as that is what has been previously demonstrated in the acute PTZ seizure model used[27].

The results demonstrated a trend toward increased basal COX-2 expression in the CA3 and CA1 of the hippocampus after running. Mice belonging to the running wheel group also displayed a greater degree of variation in COX-2 expression, particularly in the CA3 region of the hippocampus. While these results provide preliminary evidence for a relationship between

running and COX-2 expression, they are limited by their small sample size (n=2 for both treatment groups). The small sample size in this case is due to problems encountered in the immunohistochemistry (IHC) procedure. For most of the samples, high levels of background and nonspecific staining occurred. Multiple experiments were designed to determine the source of error including: tissue type, expired or contaminated reagents, primary antibody, and secondary antibody. Some nonspecific staining was reduced after the secondary antibody was spun in the centrifuge for two minutes prior to use. However, it did not eliminate the problem completely. Further tests of the IHC protocol are required to solve the problem. While there was some background present in the samples used in the current analysis, the results in the control brain were consistent with COX-2 expression in the hippocampus reported by others. Thus, neurons of the CA3 region exhibited the highest constitutive levels of COX-2, whereas expression was much lower in CA1 and DG neurons[19,20,41]. Measures were taken to control for the background present in these samples as well, by only analyzing tissue samples stained in the same batch to ensure that any background staining was consistent across brain tissue from both the running wheel and control group. Additional replications of this study with larger sample sizes need to be performed to determine if running induces a meaningful increase in the COX-2 protein expression seen in the CA3 and CA1.

Further replications of the COX-2 expression analysis presented here are also necessary due to a potential source of bias introduced in the quantification step. Image J analysis was performed knowing which experimental group the individual belonged to. Since Image J requires the selection of areas of expression to be counted, this knowledge could impact the number of boxes drawn and the areas of expression selected. However, care was taken to reduce bias by analyzing comparable sections from the same brain regions, as well as comparable positions in

that area of interest within the same sub region of each population of neurons (i.e. CA3, CA1, DG). Multiple replicates were also counted in each brain region from each mouse. In addition to these precautions, the individual performing the measurements in future studies should be blind to the experimental group of the sample being analyzed.

Contingent on these results being verified in future studies, the ability of running to increase basal levels of COX-2 expression in the hippocampus could, in part, explain the reduction in convulsive seizures after exercise as elevated COX-2 levels are associated with an increased seizure threshold.

Running increased PGE_2 levels in whole brain samples

PGE_2 is one of many downstream products of COX-2 activity. Of the prostaglandins produced, PGE_2 production is tightly coupled to COX-2 activity and is present at high levels in the hippocampus where it has been demonstrated to regulate membrane excitability[42]. While high levels of PGE_2 and other prostaglandins after a convulsion are associated with both anticonvulsive and pathological outcomes, inhibition of prostaglandins prior to seizure induction increases seizure activity[28]. Evidence from numerous studies supports the assertion that PGE_2, as well as other prostaglandins, could possess anti-convulsive properties when expressed prior to seizure induction[29,30].

The current analysis of PGE_2 levels in mice that received a chemoconvulsant but did not experience a convulsive seizure, found that PGE_2 was significantly increased in the running wheel group. PGE_2 levels were also measured for the two mice that had experienced a convulsive seizure. These two mice had elevated PGE_2 levels exceeding those detected in mice that did not have convulsions, which is consistent with reports that prostaglandin levels are

increased following a convulsive seizure (**Figure 9**)[28]. Of note, however, is that tissues analyzed for PGE_2 levels were obtained from animals injected with PTZ six days after running had ended. Thus, prostaglandin levels were measured six days after COX-2 protein expression was analyzed previously. Due to the difference in time points at which these measurements were taken, current estimations of PGE_2 levels, and thus COX-2 activity, after running could be conservative. For example, it is possible that COX-2 expression may be upregulated transiently after running and could have decreased six days later when PGE_2 was measured. A priority in follow up studies by future investigators should be to analyze tissue prostaglandin levels immediately after cessation of running. PGE_2 is not the only prostaglandin produced from COX-2 activity, as illustrated in **Figure 2**. Thus, follow up studies examining the effects of running on different prostaglandins could provide important information regarding the anticonvulsive properties of prostaglandins and their role in the effects of exercise on the seizure threshold.

Running did not have a significant effect on the seizure threshold six days post running

Despite evidence that COX-2 expression and PGE_2 levels were elevated after running, the seizure threshold was not significantly affected by participation in wheel running. These results differ from the widely reported benefits of running on seizure activity discussed previously and from previously obtained data within our own lab. This striking difference suggests a potential confounding factor in the studies presented currently.

There are a number of factors that could have contributed to the difference in results seen herein, one being that due to technical difficulties obtaining the proper dose of PTZ, the sample size for each dose was very small. Thus, additional replicates at each dose may be necessary. Another possible explanation is that the current protocol used herein was modified relative to

that used by the previous undergraduate student in our lab. As reported in this paper, animals were removed from their running wheel cages and handled for five consecutive days before seizures were induced. During these five days, the mice did not have access to a running wheel. Running wheel studies done in the lab previously did not include the five days of handling and mice were given access to a running wheel until seizures were induced (**Figure 10**).

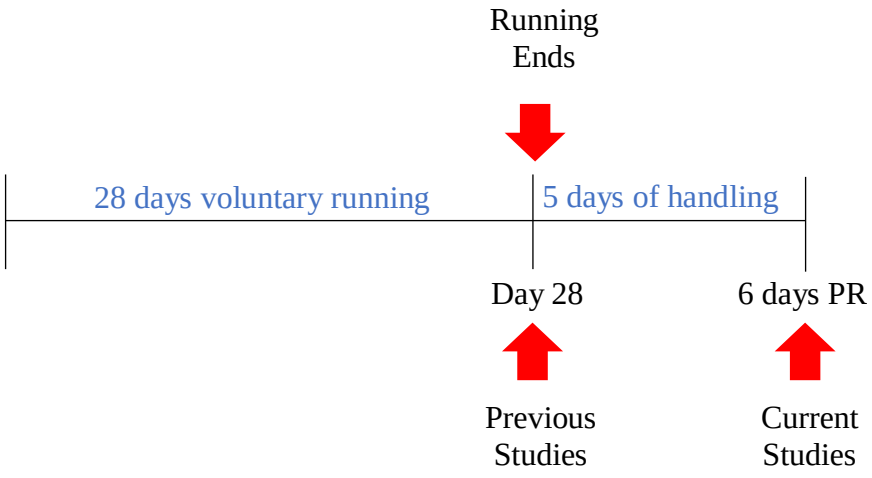

Figure 10. Schema representing differences in running wheel protocols

Other studies assessing the effects of running on the seizure threshold also performed tests immediately after or while currently engaged in running[38,39,43]. It is possible that by six days post running (five days of handling + day of PTZ injection), the beneficial effects of exercise have been lost. This could potentially suggest that the effects of running on the seizure threshold are transient and require regular participation in the activity to see the beneficial effects. This would have important clinical ramifications regarding the recommendation of running as a potential therapy. Future studies exploring differences in seizure susceptibility at different time points after running has ended (ex: 0 days, 2 days, 1 week) are required to determine if this hypothesis is true.

Additionally, the elevation in COX-2 protein expression that is reported here was seen immediately after running had concluded and was not assessed six days post running. Therefore, it is crucial that COX-2 expression is analyzed at different time points after running as well to determine if changes in COX-2 expression over time are responsible for the possible transient effects of running on the seizure threshold.

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