Exploring the Role of Cannabidiol in a *Caenorhabditis elegans* Epilepsy Model

Abstract

The role of cannabidiol in a *C. elegans* model was analyzed using adenosine and tumor necrosis factor alpha antagonists to further elucidate the protective effects of cannabidiol in mitigating seizure activity. Convulsive-sensitive mutant *unc-49 C. elegans* were cultured in NGM agar with OP50 *E. coli* with tumor necrosis factor alpha antagonist etanercept and the adenosine antagonist ZM241385 to diminish the antiepileptic effects of cannabidiol and increase epileptiform activity in *unc-49 C. elegans*. *C. elegans* convulsions were induced with pentylenetetrazole and recorded utilizing a dissecting microscope. Furthermore, the addition of adenosine agonist methotrexate restores the protective effects of cannabidiol.

Introduction

Epilepsy is a progressive neurological disease characterized by recurrent seizures and behavioral comorbidities. A person is considered to have epilepsy if they had at least two unprovoked seizures, an abnormal electrical perturbation resulting from a network of neurons, occurring greater than 24 hours apart. Epilepsy affects people of all ages, involves 2.2 million people in the United States and greater than 65 million people worldwide, of whom 30% are pharmacoresistant to the current available antiseizure drugs. Individuals with epilepsy are divided into two categories: those that respond to medication, whose seizures are well controlled by the 24 anti-epileptic drugs approved by the FDA for use in the United States, or those that fail to respond to medication and are deemed drug resistant (Sirven, 2015).

C. elegans are transparent microscopic nematodes with a defined nervous system in which all major mammalian neurological functions are conserved including ion channels, axon guidance cues, receptors, transporters, synaptic components, and neurotransmitters (Williams, *et. al.*, 2004). *C. elegans* share many fundamental biological processes with humans and have a completely sequenced and annotated genome. *C. elegans* have a nervous system consisting of 302 neurons which exhibits complete neuronal activity (Williams, *et. al.*, 2004). Studies have revealed that *C. elegans* contain many genes homologous to mammals allowing for the nematode to be used as a biomedical tool for disease modelling, drug discovery and toxicity assessments (Xiong, Pears, & Woollard, 2017). *C. elegans* are utilized as an *in vivo* model for research on neurological conditions including neurodegenerative diseases and neurodevelopmental disorders as they are small, inexpensive to obtain and maintain, and are conducive for large scale screening (Risley, *et. al.*, 2016). Wild type *C. elegans* originate from a culture of Bristol strain *C. elegans* (N2) and all mutants have been isolated in this strain (Goldstein, 2016). The *unc-49 C. elegans* strain encodes a null allele of a $GABA_A$ receptor. GABA is a major inhibitory neurotransmitter in the central nervous system. Defects in GABA are associated with epilepsy, and have been linked to Dravet Syndrome (Ruffolo, *et. al.*, 2018). Pentylenetetrazol (PTZ), a chemical which induces convulsions, does not alter the behavior of wild type (N2) *C. elegans*, however the *unc-49* model demonstrates seizure like movements with exposure to PTZ permitting for screening of convulsive activity (Williams, *et. al.*, 2004).

Epilepsy syndromes may have associated gene mutations coding for channels or their accessory subunits (Sirven, 2015). Other evidence supports the potential role of inflammation in

epileptogenesis. Inflammation of the brain can perpetuate the onset of seizures (Verrotti, *et. al.*, 2007). Clinical studies have shown anti-inflammatory treatments to act as anticonvulsants. A rise in inflammatory mediators have been seen in some epilepsy syndromes. Glia, neurons, endothelial cells of the blood brain barrier, and peripheral immune cells produce inflammatory mediators (Verrotti, *et. al.*, 2007). Disruptions in the blood brain barrier has been shown to allow for progression of epilepsy and are associated with deposits of immunoglobulins (Alyu & Dikmen, 2017). Immunoglobulin involvement supports an autoimmune and inflammatory pathogenesis to epilepsy. In temporal lobe epilepsy, activation of the interleukin-1 receptors occurs prior to seizure onset. Other inflammatory proteins, cytokines and chemokines, which are secreted by astrocytes and microglia cells, facilitate cellular excitability (Verrotti, *et. al.*, 2007).

Adenosine, another inflammatory mediator, has been shown to have anticonvulsant effects mediated by G protein coupled receptors (Boison, 2013). DNA methylation patterns determine the progression of epilepsy. An increase in adenosine may reverse the DNA hypermethylation, decreasing seizure severity and activity as shown in Figure 1 (Lusardi, *et. al.*, 2015). A ketogenic diet has been shown to reduce seizure activity in transgenic mice by restoring adenosine homeostasis (Lusardi, 2015).

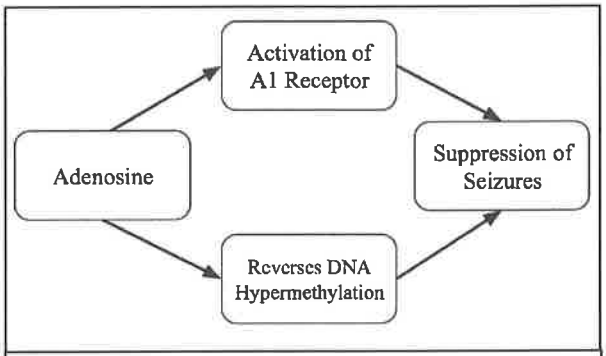

Figure 1 Adenosine activates the A1 receptor and reverses DNA hypermethylation providing suppression of seizures (Created by Student Researcher).

Tumor necrosis factor (TNF) is a pleiotropic cytokine associated with inflammation and regulation of immune cells. Anti-tumor necrosis factor are biological pharmacological agents utilized to treat several inflammatory diseases (Steeland, Libert, & Vandenbroucke, 2018). TNFa is a proinflammatory cytokine and is able to induce seizure by modulation of astrocyte glutamatergic transmission as shown in Figure 2. It has been reported that TNFa increases in the hippocampus after tonic-clonic seizures (Alyu & Dikmen, 2017). Targeting TNFa pathway by inhibiting P2Y1 receptors restores normal excitatory synapses. Both adenosine and TNFa are critical chemokines in inflammation which are implicated in epileptogenic pathways (Nikolic, 2018).

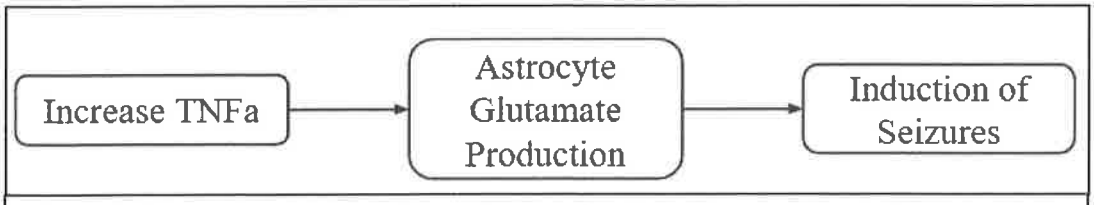

Figure 2 Seizure activity is activated by modulating glutamate production. Modulation of glutamate production is activated by increases in TNFa (Created by Student Researcher).

Tetrahydrocannabinol (THC) and cannabidiol (CBD) components of cannabis have been shown to have anticonvulsant effects. As early as 1843, these effects were documented by Dr. O'Shaughnessy who reported anti-seizure effects of cannabis in an infant with recurrent convulsive seizures (Perucca, 2017). Cannabis is a flowering plant with three species: *Cannabis sativa*, *Cannabis indica*, and *Cannabis ruderalis*. The plants have over 100 biologically active chemicals which include cannabidiol (Perucca, 2017). Until recently, the use of crude cannabis was limited due to the psychoactive component THC as shown in Figure 3 (Perucca, 2017). Epidiolex (CBD) was approved by the FDA in 2018 for the treatment of seizures in the catastrophic pediatric encephalopathies Dravet syndrome and Lennox-Gastaut syndrome, however the pathway in which cannabidiol inhibits seizure activity is unknown (Steeland, Libert, & Vandenbroucke, 2018).

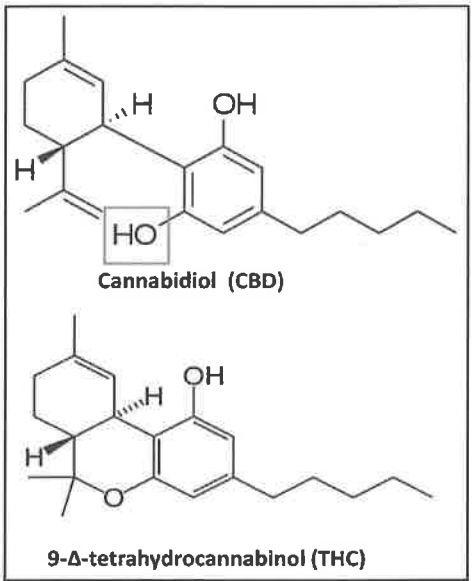

Figure 3 The non-psychoactive component cannabidiol has the addition of a hydroxyl group compared to the psychoactive component, tetrahydrocannabinol, which has a cyclic ring structure (Perucca, 2017).

Cannabidiol has been shown to affect various pathways of inflammation and possesses anticonvulsant and anti-inflammatory properties. Cannbidiol anti-seizure activity has been suggested to be mediated by adenosine reuptake and antagonism of G protein coupled receptor 55 (GPR55) (Perucca, 2017). Cannabidiol modulates the actions of adenosine receptors 1 and 2, inhibits adenosine reuptake, and modulates TNFa activity (Perucca, 2017). Cannabidiol anti-seizure effects should be mitigated by blocking adenosine pathways, validating adenosine

increase as the protective pathway. As cannabidiol blocks the GPR55 receptor, adenosine reuptake should decrease as shown in Figure 4. Therefore, the pathway in which cannabidiol inhibits seizure activity was assessed by using inflammatory mediators, adenosine and TNFa antagonists and agonists, measuring the protective effects of cannabidiol in *C. elegans unc-49* and wild type strains.

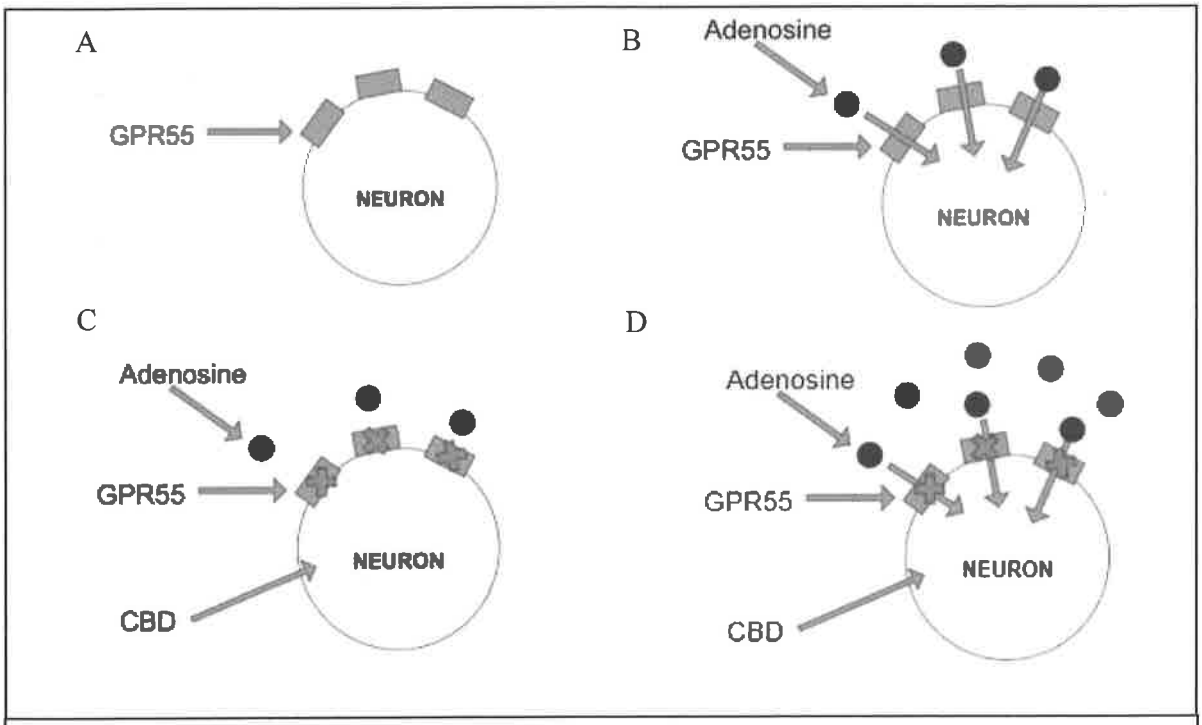

Figure 4 GPR55 receptors (A) allows for adenosine to move into the neuron (B). Addition of cannabidiol (CBD) blocks GPR55 receptors inhibiting upregulation of adenosine (C). Cannabidiol exposure prevents GRP55 adenosine reuptake, increasing extracellular adenosine concentrations (D) (Created by Student Researcher).

Materials and Methods

Nematode strain and maintenance

C. elegans unc-49(e407) were obtained from the *Caenorhabditis* genetics center (CGC; University of Minnesota, USA). Nematodes were transferred by subculturing; a sterilized scalpel was used to move a chunk of agar from the cultured plate to the fresh plate. Worms were grown and maintained on 60mm plastic Petri dishes containing OP50 *E. coli*-seeded nematode growth medium (NGM; 1mM each of $CaCl_2$ and $MgSO_4$, 25mM KH_2PO_4, 5µg/mL cholesterol, and in w/v 2% agar, 0.25% peptone, and 0.3% NaCl) at 20 °C.

Age Synchronization

Cultured adult nematodes were age-synchronized by bleaching. Adult nematodes plates were washed with M9 buffer (22mM KH_2PO_4, 42 mM Na_2HPO_4, 86 mM NaCl, and 1 mM $MgSO_4$). Nematodes were placed in 15 ml tubes and centrifuged for 4 minutes at 1500 rpm on a standard table centrifuge. The nematodes were washed until M9 buffer appeared clear of bacteria. Bleaching solution (2 parts 8% commercial alkaline hypochlorite bleach and 1 part 5 M NaOH) was added to the 15 mL tubes. M9 buffer was added to fill the tubes to stop the reaction. The tubes were centrifuged for 1 minute at 1500 rpm and the supernatant was discarded. The pellets were washed an additional three times with M9 buffer. *C. elegans* egg pellets were deposited onto seeded NGM plates using a micropipette.

Liquid-based administration of PTZ

PTZ administration was performed by incubating 1-3 days old individual young adult nematodes in 50µl liquid droplets of PTZ (7 mg/ml) dissolved in Dent's Ringer solution (Wong, *et. al.*, 2018).

Control Treatment

C. elegans unc-49 (e407) were cultured and maintained on standard NGM agar plates seeded with OP50 *E. coli.* 1- 3 days old *C. elegans unc-49* (e407) were transferred to the liquid based administration of PTZ and incubated for 15 minutes. Seizure duration, frequency, and intensity of convulsions was observed under light microscopy.

Cannabidiol Dose Response

C. elegans unc-49 (e407) were cultured and maintained on standard NGM agar plates seeded with a mixture of OP50 *E. coli* cultured with cannabidiol (1 drop/2.4ml of nutrient broth). Cannabidiol (50 mg/ml) at varying concentrations (10%, 25%, 50%,100%) was spread to cover the entire surface of the plate to determine the optimal concentration and exposure times without inducing death or other motility impairments. *C. elegans* were observed by microscopy at 24, 48, and 72 hour intervals to determine the optimal exposure duration and worm maturation.

Pharmacological Treatments

C. elegans unc-49(e407) were cultured and maintained on standard NGM agar plates seeded with OP50 *E.* coli cultured with cannabidiol (1 drop/2.4ml of nutrient broth). 100µl of cannabidiol (1 drop/2.4ml of nutrient broth) was spread to cover the entire surface of the plate at 20°C and placed under a laminar flow hood for 30 minutes. Age synchronized 1- 3 days old *C. elegans unc-49*(e407) were transferred to the liquid based administration of PTZ and incubated for 15 minutes. Seizure duration, frequency, and intensity of convulsions was observed and recorded under light microscopy. This process was repeated with the addition of adenosine antagonist ZM241385 (0.33734ug/ml), TNF alpha antagonist (etanercept) (50mg/ml), and of adenosine agonist (methotrexate) (10mg/ml). 100µl of each chemical were spread on individual plates already treated with 100µl of cannabidiol spread on the surface. Adenosine agonist (methotrexate) was only added to the plates in conjunction with adenosine antagonist (ZM241385) and cannabidiol.

Scoring of seizure activity

Seizure- like head bobbing movements were recorded by microscopic visualization with videography, and defined as an extension and retraction of the anterior pharyngeal region on the worm along the posterior axis or thrashing, movement from head to tail. Frequency of seizure activity in *C. elegans* was scored by occurrence (1) versus non-occurrence (0). Intensity was measured on a scale of 0-2, being nonconvulsive, mild convulsions, major convulsions. Duration of seizure was recorded with a timer by direct observation and videography. Convulsions were recorded over 30 second durations.

Results

Control Treatment

Unc-49 C. elegans convulsions were analyzed by videography with a dissecting microscope. Convulsions were identified as "head bobbing" where the posterior half of the animal is immobile and the anterior muscle contraction occur repeatedly, and continuous shaking. *Unc-49 C. elegans* cultured and maintained on standard NGM agar plates seeded with OP50 *E. coli* and transferred to the liquid based administration of pentylenetetrazol (PTZ) exhibited seizure activity in 100% of the control trials. The frequency and intensities for the

C. elegans in the control trials was 1 and 2 respectively, having a statistical significance of 1.000 as shown in Tables 1 and 2.

Table 1 The mean frequency of convulsions in control and cannabidiol trials recorded as non-occurrence (0) versus occurrence (1) (Created by Student Researcher).

	V1	N	Mean	Std. Deviation	Std. Error Mean
Frequency	Control	42	1.00	.000	.000
	CBD	33	.15	.364	.063

Table 2 The intensity of convulsions in control and cannabidiol trials recorded on a scale of 0-2, being nonconvulsive, mild convulsions, major convulsions, respectively (Created by Student Researcher).

	V1	N	Mean	Std. Deviation	Std. Error Mean
Intensity	Control	42	2.00	.000	.000
	CBD	33	.15	.364	.063

The control trials had a mean frequency of 1.00 and a mean intensity of 2.00 compared to frequency and intensity of trials in which *unc-49 C. elegans* were cultured and maintained with cannabidiol, which had a mean frequency and mean intensity of 0.15 as shown in Table 1 and 2.

Cannabidiol Dose Response

The cannabidiol dose response trial was completed to determine the optimal concentration and exposure times without inducing death or other motility impairments. Observation of *C. elegans* were made by microscopy at 24, 48, and 72 hour intervals. A total of two trials were performed consisting of 16 plates in each trial. Four plates were designated to each concentration of 10%, 25%, 50%, 100% CBD spread on the plate surface. *C. elegans* death was not observed consistently when exposed to CBD nor did the *C. elegans* have motility impairments. In the second trial *E. coli* was cultured with CBD and additional CBD was spread on the plate surface and the 1-3 days old nematodes were picked and placed separately on the plates so additional eggs on the plates were minimized. At the maximum concentration of 100% CBD exposure over 72 hours, *C. elegans* mortality was not compromised compared with lower

concentrations of CBD demonstrating the optimal concentration as shown in Table 3. This concentration was utilized in pharmacological treatment trials.

Table 3 The percent of *C. elegans* alive at 72 hours after exposure to cannabidiol at varying concentrations (Created by Student Researcher).

Concentration of Cannabidiol	10%	25%	50%	100%
% of *C. elegans* alive after 72 hours	69	87.5	75	75

Pharmacological Treatments

Seizure activity was further exhibited in 100% of the trials with *unc-49 C. elegans* individually cultured with ZM241385 and TNFa (etanercept). The mean frequency of the control, ZM241385, and TNFa (etanercept) trials was 1.00 with a statistical significance of 1.000 as shown in Table 4 and Table 5. The mean frequency of the methotrexate and CBD trials compared to the mean frequency of control trials had a statistical significance of 0.000 as shown in Table 5.

Table 4 The mean frequency of the control, ZM241385, and TNFa (etanercept) trials recorded as non-occurrence (0) versus occurrence (1) (Created by Student Researcher).

	N	Mean	Std. Deviation	Std. Error
Control	42	1.00	.000	.000
ZM	91	1.00	.000	.000
TNF	911	1.00	.000	.000

Table 5 Multiple comparison of the frequency of convulsions in control, ZM241385, and TNFa (etanercept) trials. Control, ZM241385, and TNFa trials all exhibited the same frequency of 1.00 having a statistical significance of 1.000 (Created by Student Researcher).

Dependent Variable: Frequency		
Tukey HSD		
(I) V1	(J) V1	Sig.
Control	ZM	1.000
	Methotrexate	.000
	TNF	1.000
	CBD	.000

The intensity of the control, ZM241385, and TNFa (etanercept) trials was 2.00 as these respective trials had a mean difference in intensity of 0.000 and a statistical significance of 1.000 as shown in Table 6 and Table 7. The intensity of the methotrexate and CBD trials compared to the intensity of control trials had a statistical significance of 0.000 as shown in Table 7.

Table 6 Multiple comparisons of the intensity of convulsions in control, ZM241385, and TNFa (etanercept) trials. Control, ZM241385, and TNFa trials all exhibited the same intensity of 2.00 having a statistical significant of 1.000 (Created by Student Researcher).

Dependent Variable: Intensity						
Tukey HSD						
(I) V1	(J) V1	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	ZM	.000	.015	1.000	-.04	.04
	TNF	.000	.013	1.000	-.03	.03

Table 7 Intensity of the control compared by multiple analysis to ZM241385, methotrexate, TNFa (etanercept), and cannabidiol. A difference in the intensities of methotrexate and cannabidiol compared to the control is depicted by a significance of 0.000 (Created by Student Researcher).

Dependent Variable: Intensity		
Tukey HSD		
(I) V1	(J) V1	Sig.
Control	ZM	1.000
	Methotrexate	.000
	TNF	1.000
	CBD	.000

Mean duration of *unc-49 C. elegans* convulsions from the control trial in which *unc-49 C. elegans* were cultured and maintained on standard NGM agar plates seeded with OP50 *E. coli* and transferred to the liquid based administration of pentylenetetrazol (PTZ) was 15.35 minutes. Mean duration of *unc- 49 C. elegans* convulsions cultured and maintained with cannabidiol was 0.17 minutes as shown in Table 8.

Table 8 Mean duration of convulsions in *C. elegans* control compared to CBD trials (Created by Student Researcher).

	V1	N	Mean	Std. Deviation	Std. Error Mean
Duration (min)	Control	42	15.3505	7.50826	1.15855
	CBD	33	.1788	.42965	.07479

The duration of the *unc-49 C. elegans* convulsions in the control, ZM241385, TNFa (etanercept), and methotrexate trials are shown in Figure 5. As expected, duration of convulsions was maximized in control trials and minimized in CBD trials. The protective effects of CBD are significantly ($p < 0.000$) inhibited by adenosine antagonist ZM241385 with a mean seizure duration of 6.79 minutes. The protective effects of CBD are partially reversed with

adenosine agonist methotrexate having a mean seizure duration of 4.16 minutes. TNFa inhibition partially reversed the effects of CBD, increases seizure duration by 3.49 minutes, suggesting TNFa pathway as contributing mechanism.

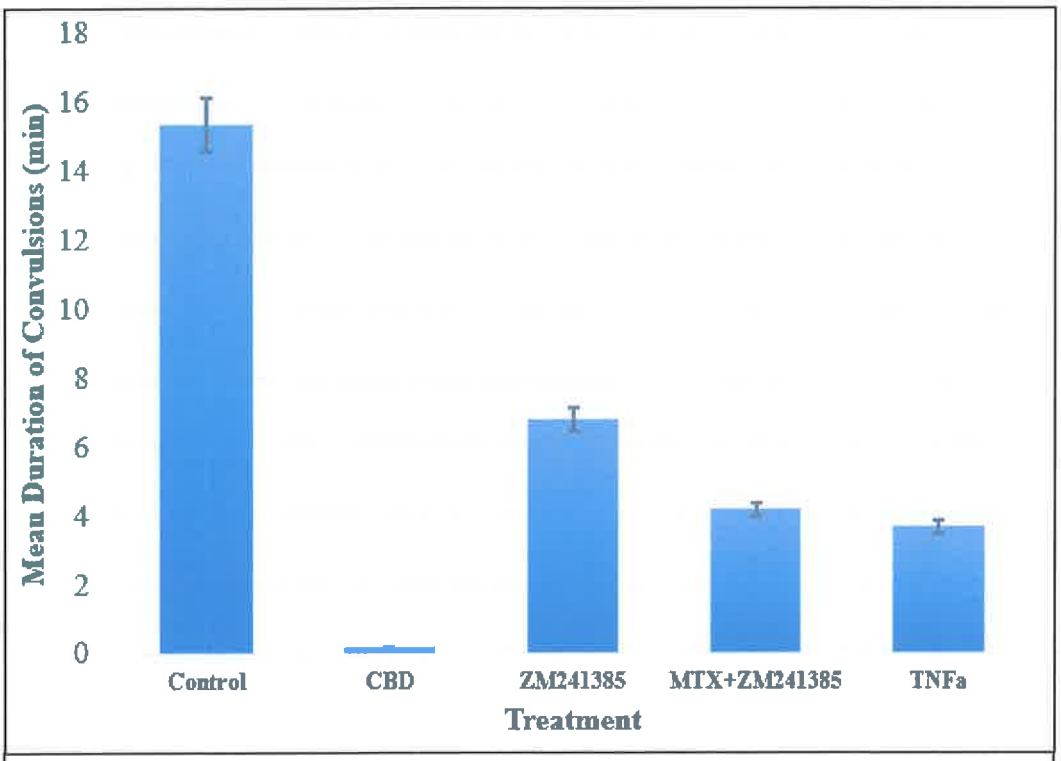

Figure 5 Comparison of the mean duration of convulsions in control, cannabidiol, ZM241385, ZM241385, methotrexate (MTX), and TNFa antagonist (etanercept) (Created by Student Researcher).

Discussion

High mortality rates are associated with drug resistant seizures (Devinsky, *et. al.*, 2017). Cannabidiol has been shown to reduce seizure activity in Dravet and Lennox Gastaut Syndrome but the mechanism of action of cannabidiol as an anti-epileptic drug is unknown (McCoy, *et. al.*, 2018). This study demonstrates that the anti-seizure effects of cannabidiol are mitigated in a *C. elegans* model utilizing *unc-49 C. elegans* by blocking adenosine pathways, validating that increases in the inflammatory mediator adenosine serves as a protective pathway. In addition, inhibition of TNFa with etanercept partially reverses the effects of cannabidiol implicating the inflammatory cytokine TNFa as a potential mechanistic pathway.

The *unc-49 C. elegans* mutant demonstrated convulsive activity with exposure to PTZ, as previously demonstrated (Wong, *et. al.*, 2018). The data supports that cannabidiol exposure at 100% concentrations does not impair motility or increase mortality. The cannabidiol dose response trial was repeated as the amount of *C. elegans* on each plate increased over the time intervals recorded. This was due to the presence of eggs which remained on the plates. During the second trial, shown in Table 3, *C. elegans* populations remained stable as *C. elegans* were picked decreasing the ability of eggs to hatch on each plate. The concentration of 100% cannabidiol exposure exhibited consistent survival rates and was determined to be the optimal dose. *Unc- 49 C. elegans* cultured and maintained on standard NGM agar plates seeded with OP50 *E. coli* cultured with cannabidiol (1 drop/2.4ml) and additionally treated with cannabidiol (1 drop/2.4ml) spread to cover the entire surface of the plate was determined to be the most effective preparation in preventing seizure activity in nematodes treated with PTZ.

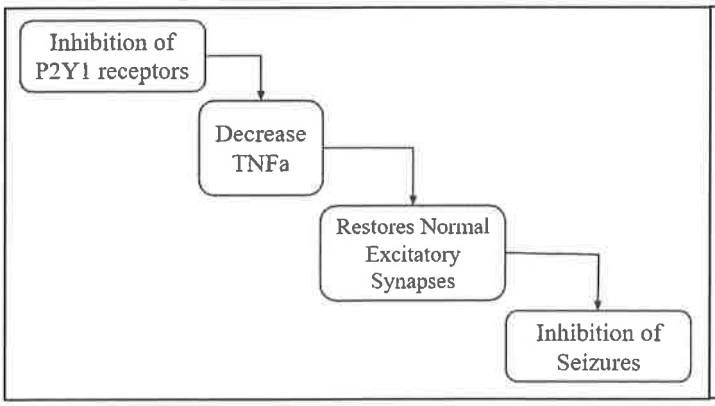

Figure 6
Mechanism of seizure inhibition by decreasing the inflammatory cytokine TNFa (Created by Student Researcher).

Various inflammatory pathways are affected by cannabidiol. Adenosine has been identified as an endogenous agent of the brain with potent and long lasting antiepileptic properties (Lusardi, *et. al.*, 2015). It has been suggested that cannabidiol anti-seizure activity is mediated by adenosine reuptake and antagonism GPR55. An increased level of tumor necrosis factor alpha (TNFa), a cytokine involved in the regulation of astrocyte glutamate release, is evident during epileptic seizures. This direct relationship is shown in Figure 6 (Nikolic, *et. al.*, 2018).

This current study examined adenosine antagonist ZM241385 and tumor necrosis factor alpha antagonist (etanercept) to determine their role in mitigating the protective effects of cannabidiol and increase epileptiform activity in *unc-49(e407) C. elegans.* Cannabidiol reduces

seizure duration, frequency and intensity in *C. elegans unc-49* model induced by PTZ. The *unc-49 C. elegans* cultured with methotrexate, ZM241385, and CBD displayed seizure activity with lessened intensity and shorter duration times compared with both control and ZM241385 trials implicating adenosine as key pathway involved in cannabidiol mechanism of anti-seizure activity. The mean seizure duration of adenosine antagonist ZM241385 trials was 6.79 minutes for the *C. elegans* observed. The anti-epileptic effects of cannabidiol were reversed by both the adenosine antagonist ZM241385, and by TNFa antagonist (etanercept). The adenosine antagonist restored seizure duration by 6.62 minutes, and the TNFa antagonist restored seizure duration by 3.5 minutes. This demonstrates that cannabidiol anti-epileptic effects are mitigated by both adenosine antagonist and TNFa antagonists. Furthermore, the addition of the adenosine agonist methotrexate to the adenosine antagonist ZM 241385, was shown to reduce seizure duration by 2 minutes, restoring the anti-seizure effects of cannabidiol. The inflammatory mediator adenosine serves a key role in cannabidiol anti-epileptic properties. The protective effects of cannabidiol are notably inhibited by adenosine antagonist ZM 241385 and only partially reversed with adenosine agonist methotrexate. The partial restoration is likely due to the potent inhibition of adenosine receptors by ZM 241385, causing incomplete ability to fully restore intracellular adenosine levels by methotrexate. Further research with a less potent adenosine antagonist could establish if complete reversal can be achieved with methotrexate. The TNFa inhibitor was less effective in reversing cannabidiol anticonvulsant activity compared with adenosine antagonist. However, there was a demonstrated increase in seizure duration. Inhibition of TNFa reduced seizure duration compared with the control PTZ treated group as predicted. The anti-seizure effect of cannabidiol was partially reversed by TNFa antagonist. This suggests that there is likely greater complexity of TNFa in epileptogenesis as reductions in TNFa mitigated seizure activity, however the protective effects of cannabidiol are partially reversed with inhibition of TNFa.

Future Work

This study has established a *C. elegans* model exploring the role of cannabidiol and inflammatory mediators to elicit the pathway in which cannabidiol is an effective anti-epileptic drugs. Cannabidiol has been proposed to involve other molecular targets in animal epilepsy models including serotonin pathways, specifically 5HT1A receptors. Further investigations can

explore serotonin agonist and antagonists in a *C. elegans* model to further elicit the mechanisms of cannabidiol anti-epileptic activity. Multiple pathways are likely involved in cannabidiol's mechanism of action and should be investigated further to understand treatments for drug resistant epilepsy.

Conclusion

The implications of the current research highlight a potential role of cannabidiol for refractory forms of epilepsy. Understanding the mechanisms of cannabidiol in mitigating seizures will expand the therapeutic possibilities. Clinical evidence has shown the potential role of inflammation in epilepsy. Steroids and other anti-inflammatory therapies have served as anticonvulsants. Inflammatory processes in epilepsy are seen by increased levels of inflammatory mediators during seizure activity (Verrotti, *et. al.*, 2007). The role of cannabidiol in inflammatory pathways implicated in this study suggest potential benefits for inflammatory seizure disorders such as Rasmussen encephalitis, aseptic encephalitis, and febrile seizures.

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