The Developmental and Behavioral Effects of Δ9-Tetrahydrocannabinol on a Spastic Mutant

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The Developmental and Behavioral Effects of Δ⁹-Tetrahydrocannabinol on a Spastic Mutant

Kennesaw State University MSIB Thesis Summer 2019

Victoria Mendiola
Dr. Lisa Ganser
Dr. Martin Hudson
Dr. Bill Ensign
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Abstract

Hyperekplexia is a neurological disorder affecting infants and can be characterized by excessive startle reactions in response to unexpected stimuli. This disease can be modeled in zebrafish in the bandoneon (beo) mutant, where the glrrb mutation results in glycine receptor (GLYR) loss of function glycine receptors and ultimately, improper inhibitory signaling. Though hyperekplexia is a glycine-based signaling disorder, disease symptoms are often alleviated with GABA-agonistic benzodiazepines. Exogenous cannabinoids also modulate GABAergic signaling, and THC, one of the natural compounds that prompted the discovery of the endocannabinoid system mimics the effects of endogenous cannabinoids at endocannabinoid and non-cannabinoid sites in the central nervous system. These cannabinoids also have therapeutic effects in animal models of seizures and epilepsy, leading to the aims of the present study to examine the developmental and behavioral influences of THC on wild-type (WT) and bandoneon (beo) embryos and to compare these effects to those observed after exposure to Lorazepam, a common benzodiazepine used to mitigate spasticity in hyperekplexics. Since both THC and Lorazepam restore balanced signaling, we hypothesized that THC would alleviate spasticity and Lorazepam would serve as a positive control in beo embryos. We assessed embryonic growth parameters at 24, 48, 72, 96, and 120 hours post fertilization (hpf) following THC (0.2, 0.6, 2.0 and 5.0 mg/L) and Lorazepam (0.02 and 0.2 mg/L) exposure at 0-1hpf in WT and beo embryos. In WT and beo embryos, survival and hatching rates were inversely proportional to THC concentration confirming that THC exposure slows development and can impact survivorship. Morphological anomalies, however, increased with higher THC concentrations. We then assessed embryonic behavior using the innate escape response essential to survival at 24, 48, 72, 96, and 120 hpf following THC (0.2 and 2.0 mg/L) and Lorazepam (0.2 and 0.02 mg/L) exposure. Impacts on escape behavior varied, and when dosed with Lorazepam and THC, individuals displayed similar response times to controls at 24, 96, and 120 hpf. Significant differences in escape behavior times were measured at 48 and 72 hpf, specifically during the coil to relax phase of the escape response. During this time, beo control individuals were significantly different than 0.2 mg/L Lorazepam (p<0.0003 and p<0.0001 respectively) and 0.2 mg/L THC (p<0.0014 and p<0.0021 respectively). 0.2 mg/L THC was significantly different from 0.2 mg/L Lorazepam during the coil to relax phase of the escape response at 48 and 72 hpf (p<0.0014 and p<0.0002 respectively) and the total response time at 72 hpf (p<0.0201). These data suggest that THC exposure can deleteriously affect development and the establishment of neural circuits essential to survival, but can also alleviate spasticity symptoms depending on developmental stage and concentration.
Chapter One: Introduction

The Nervous System

Using the body’s ability to detect change and activate internal regulatory systems for regaining physiological setpoints, homeostatic mechanisms maintain a steady-state internal environment that optimizes cellular function and reproduction. As one of the two main homeostatic control systems, the nervous system receives and integrates stimuli to dictate responsive actions throughout the body (O’Leary and Wyllie, 2011). Composed of two main parts, the nervous system collaborates between the central and peripheral branches to maintain a functioning, homeostatic system. The central nervous system (CNS) includes the brain and spinal cord that integrate our sensory inputs. The peripheral nervous system (PNS) is composed of any neuronal tissue outside of the CNS that detects stimuli, delivers these afferent inputs to the central nervous system, and then relays the necessary, physical responses to these stimuli that return the body to homeostasis. Together, the central and peripheral nervous systems create a network of neuronal circuits extending throughout the body with all pathways leading to and from the spinal cord and brain.

Sensory stimuli such as touch, light, temperature, chemical, and pain are a few of the inputs received and interpreted by the nervous system. In a homeostatic system, stimuli are detected by somatic sensory, special sensory receptors, or visceral sensory receptors that detect changes in the environment. Once detected, an electrochemical message representing the sensory stimulus is sent through the PNS as an afferent signal, and then to the CNS for information processing and integration. Following integration, the CNS sends another electrochemical signal back through the PNS to modulate the required signal to produce an appropriate somatic or autonomic response at an efferent target.

In order to maintain a homeostatic system in a constantly changing environment, the nervous system must be able to adapt to constant stimulation and make experience-mediated alterations to avoid noxious stimuli or to seek stimuli that increase fitness. Throughout an individual's lifetime, the nervous system integrates experiences by constantly finding new paths then establishing, refining, and strengthening new neuronal connections. This neural plasticity allows for both functional and structural modifications to maintain normal brain function and optimize survival in response to novel or former stimuli. Neuronal stimulation is essential for these plasticity mechanisms in order to acquire new functions and for neurons underlying behaviors and memory to remain robust and stable (von Bernhardi et al., 2017). Homeostatic plasticity, therefore, refers to the ability of neurons to regulate their own
growth and excitability in response to stimulation and consists of an overall dynamic maintenance of balanced synaptic establishment and refinement, neuronal density, and circuit excitability (Tien and Kerschensteiner, 2018).

For example, in zebrafish the earliest embryonic stages serve as critical periods for neuronal synapse construction and priming when novel exposure and re-exposure to stimuli from the nascent neuronal connections underlying survival behaviors. During neurogenesis, undifferentiated neural progenitor cells develop into the neuronal circuits that detect stimuli, integrate these signals, and produce appropriate responses. Throughout the lifetime of an organism, these circuit connections grow, change, strengthen, or are lost, depending on experience (O’Leary and Wyllie, 2011; Kleim and Jones, 2008).

**Inhibitory and Excitatory Neurotransmitters**

Communication in the CNS relies upon neurotransmitters that act as chemical messengers between neurons to convert sensory information into patterns of action potentials that relay to the brain in a process called neural or sensory coding (Gonzalez-Espinosa and Guzman-Mejia 2014; Schaeffner et al., 2018). Circuit components perpetuate neural coding through repeated communication of neurotransmitters across synapses to establish connections that stimulate or block responses from subsequent neurons or target tissues in the circuit. Mechanistically, as an action potential is propagated down the axon of a neuron, voltage-gated Na⁺ channels on the membrane open, allowing Na⁺ to flood then depolarize the neuron. Once the action potential reaches the axon’s presynaptic terminal, voltage-gated Ca²⁺ channels open so the influx Ca²⁺ mediates the fusion of vesicles that contain neurotransmitters with the presynaptic membrane to release neurotransmitters into the synapse (Raghavan et al., 2019). These neurotransmitters bind to specific receptors on the postsynaptic membrane of the next neuron in sequence, and depending on the neurotransmitter, activate or inhibit another postsynaptic potential (Fletcher, 2016). Through sensory coding, an elaborate network of neurons translates sensory stimuli into physical actions throughout the body (Marty, 1989).

Neurotransmitters can elicit excitatory to elicit a response or inhibitory responses to modulate signal propagation along a circuit (Bloom, 2015). Balanced excitatory and inhibitory signaling among neurons is an essential component of reciprocal inhibition and coordinated movement (Hamm and Alexander 2010). The inhibitory neurotransmitter, gamma-aminobutyric acid (GABA), is the most
common inhibitory neurotransmitter in the brain, while the other inhibitory neurotransmitter, glycine (GLY) is the main inhibitory signaling molecule in the brainstem and spinal cord.

There are two main types of post-synaptic receptors that neurotransmitters can bind to: metabotropic receptors and ionotropic receptors. Once bound, ionotropic receptors open ligand-gated ion channels directly associated with the receptor. Metabotropic receptors are indirectly linked with ion channels on the plasma membrane or within the cell, and must therefore must act through signal transduction proteins, such as G-proteins (Purves et al., 2001).

GABA receptors (GABAR) are composed of four alpha (α) subunits for ligand binding and two beta (β) subunits for structure (Lynch 2004). Two types of GABA receptors are present in the body: ionotropic GABAA and GABAc receptors (GABAAr and GABAcR) and metabotropic GABAb receptors (GABAbR). When GABA binds GABAAr or GABAcR, the associated Cl− channel opens, allowing an influx of negatively charged anions into the cell and slowing or blocking the propagation of the action potential by raising the neuron’s threshold (Fagiolini et al., 2004), while GABAbR use a secondary messenger mechanism to communicate through indirect coupling with K+ channels to cause a decrease in Ca2+ influx when activated, effectively preventing the release of neurotransmitters into the synapse if located on the pre-synapse or hyperpolarizing the post synaptic neuron. GABAbR are heterodimers of GABAb1 (α) and GABAb2 (β) subunits that are able to mediate both pre and postsynaptic action potentials and always modulate inhibitory signaling (Siegel et al., 1999). Another example of ionotrophic receptors are postsynaptic glycine receptors (GlyR) in the spinal cord and brainstem that are responsible for fast inhibitory neurotransmission (Delgado et al., 2008; Bakas et al., 2017). GlyR and GABAAr overlap in their sites of action and cooperatively contribute to inhibitory signaling (Waxham et al., 2014).

Inhibitory neurotransmitters, such as GABA and GLY, mechanistically work to diffuse the action potential from continuing increasing threshold at their targets. GABA has well-known presynaptic inhibitory effects throughout the CNS, while the presynaptic role of GlyR remains unclear except that presynaptic function is restricted to neurodevelopmental critical periods of cortical synapse formation (Kunz et al., 2012). As these nascent circuits establish their first connections, GlyR govern excitatory signaling of glutamate (GLUT) and dopamine (DA) through modulation of Cl− ion gradients across the membrane. During development, intracellular Cl− is high, because of the increased presence and action of the Na+, K+, Cl− co-transporter, KCC1, and as the nervous system matures, KCC2 expression gradually increases, establishing the high extracellular Cl− gradient of the mature nervous system environment (Kunz et al., 2012). Because of this early, high intracellular Cl− gradient, when GLY binds and gates
presynaptic GLYR, Cl− slowly exits the cell, lowering the threshold for opening voltage-gated Ca^{2+} channels, that subsequently trigger GLUT release into the synapse (Waseem and Federovich, 2010). As the nervous system matures, extracellular Cl− gradients are high due to an increase in KCC2 and decrease in KCC1 expression, as well as the major shift to post synaptic expression of GLYR, establishing the inhibitory signaling mechanisms necessary for balanced signaling (Kunz et al., 2012). Even after the switch from presynaptic to postsynaptic expression, GLYR continues to play an indirect role in controlling GLUT and DA release in the CNS by blocking presynaptic GABA release from the presynaptic neurons that govern DA communication (Ye et al., 2004). GABA mediates the release of DA in tegmental DA circuits by binding GABAR on presynaptic DA neurons while GLY acts as a DA agonist by activating GlyR on presynaptic GABA neurons (Ye et al., 2004).

Without inhibitory neurotransmitters diffusing action potentials, constant excitatory signaling would impede homeostasis, thus tipping the E/I balance towards excitatory. For example, excitation of both target and opposing muscle groups would result in the loss of coordinated movement, or spasticity. Many diseases of spasticity or uncoordinated locomotor phenotypes result from the lack of inhibitory signaling leading to hyper-excitatory messages simultaneously stimulating opposing muscle groups.

**Reciprocal Inhibition in Movement Disorders**

Patterned motor behaviors require E/I balance among locomotor circuits to signal reciprocal inhibition of the muscle groups opposing contracting muscle groups. If the signaling balance favors only excitation, both opposing muscle groups contract, resulting in active paralysis, but when only inhibitory neurotransmitters are communicated within the circuit, the opposing muscle groups experience flaccid paralysis. Even mild disruption of E/I balance influences the signaling necessary for the reciprocal inhibition that governs smooth, patterned movements, resulting in spastic, uncoordinated motor patterns.

Muscle spasticity is a phenotypic consequence of E/I imbalance. Spasticity is a primary symptom in many motor function disorders, such as Parkinson’s disease, seizure disorders, epilepsy, efferent signaling diseases, paralysis, and hyperekplexia (Andermann et al., 1980; Dietz and Sinkjaer 2007; Harvey et al., 2008; Lynch et al., 2004). Synthetic drugs can often alleviate the lack of voluntary control and pain associated with spastic movement disorders.
Muscle Spasticity and Treatments Associated with Hyperekplexia

One specific movement disorder, hyperekplexia, or human startle disease, is a rare, hereditary, neurological disorder that affects approximately 1 in every 400,000 people (Hyperekplexia, NORD 2013) and causes hypertonia that impedes voluntary movement. The disease is usually diagnosed shortly after birth, when startle stimuli induce body-wide spinal seizures resulting in sustained contractions of the diaphragm that inhibit breathing. Hyperekplexia can affect both genders with disease phenotypes present, but attenuating in severity through adulthood.

Hyperekplexia is often confused with seizure disorders, but unlike seizure disorders, patients who suffer from hyperekplexia retain consciousness and show electroencephalograms (EEGs) that do not correlate with those typical of most seizure disorders (Davies et al., 2010; Zhou et al., 2002). Hyperekplexia can be inherited via dominant or recessive genes through deletions, frameshift, missense, or nonsense mutations of the genes GLRA1 (OMIM# 138491), GLRB (OMIM#138492), SLC6A5 (OMIM# 604159), GPHN (OMIM # 603930) and ARHGEF9 (OMIM # 300429) (Davies et al., 2010; Harvey et al., 2008; Eulenburg et al., 2006; Rees et al., 2001, 2002, 2003 and 2006; Carta et al., 2012; Alber et al., 2017; Zhang et al., 2011; Bode et al., 2014; Shiang et al., 1993; Villman et al., 2009). Of the five genes associated with hyperekplexia, three are related to inhibitory neurotransmitter transmission. GLRA1 and GLRB both encode for glycine receptor proteins, with GLRA1 representing the alpha subunit for which mutations causing hyperekplexia are most common. GLRA1 encodes both ligand binding and pore lining proteins of the GlyR. GLRB mutations resulting in hyperekplexia target βA or βB subunits of GlyR and are much less common than those affecting GLRA1. SLC6A5 encodes for the glial glycine transporter type 2 (GlyT2), for which mutations that cause hyperekplexia are even more rare. Overall, mutations to the GLRA1, GLRB and SLC6A5 genes result in the malfunctioning neurotransmission of glycine that underlie the startle-induced bouts of spasticity that are characteristic of hyperekplexia.

Treatments such as Botulinum toxin A are relatively ineffective, and benzodiazepines are highly addictive, further compromising a homeostatic system (Grazko et al., 1995; Chang et al., 2013). Historically, diazepam, a GABA agonist has been used for the treatment of muscle spasticity (Andermann et al., 1990), and recently treatments such as Clonazepam, a benzodiazepine that acts as a GABA agonist, have been used with some success. Unfortunately, Clonazepam is only able to mitigate the hypertonic effects of hyperekplexia instead of acting at the glycnergic root of the problem (Ryan et al., 1992). Current studies regarding the use of DH-CBD, a synthetic cannabinoid modified slightly from cannabidiol (CBD), has been used to alleviate the muscle spasticity associated with hyperekplexia.
through the suppression of pain sensitivity. This mechanism uses DH-CBD to reduce the diminished spinal glycine release through a presynaptic mechanism (Xiong et al., 2012).

**The Zebrafish as an Animal Model**

Zebrafish (*Danio rerio*) have been used as an embryological tool for research due to their developmental advantages, such as their quickly dividing cells, transparent chorion, rapid and extensively classified development, thoroughly sequenced genome, and highly characterized escape response behaviors (Jones and Huffman 1957; Burgess and Granato 2007). The zebrafish has many orthologous genes with humans, such as those for GABAR proteins (Wilkinson et al., 1983; Delgado et al., 2008) GlyR proteins (Imboden et al. 2001; Ganser et al., 2013), the components of the endocannabinoid system (Krug and Clark 2015), and CB1 receptors (Lam et al., 2006; Elphick et al., 2012; Oltrabella et al., 2017) making them ideal animal models for translational studies.

Zebrafish have a highly characterized escape response (also called the startle response, c-start response or escape circuit) that is often used as a behavioral assay to indicate neural circuit anomalies. The escape response is an innate reaction to predatory stimuli that consists of simple pathways from sensory integration to motor output that is quantifiable from 22 hours post fertilization (hpf) as a series of movements away from noxious stimuli (Hale et al., 2016). Depending on the developmental stage and the location of the stimuli on the body, various cells detect and integrate the signal. Once the zebrafish embryo is stimulated, it contracts the entire side of its body to produce a c-shaped bend, and then uncoils to a fully relaxed state where muscles on both sides are relaxed (Fig 1. A1-3, Fig 3).

Embryonic zebrafish have often been used for behavioral assays due to their detailed escape behaviors that parallel detailed neuronal and developmental stages. To elicit the response, most researchers use tactile stimulation invoked by a brief, gentle touch to the side of the tail or yolk sac with a Tungsten wire or fine dental pick. During development, key motor neurons are born during gastrulation, which occurs approximately 5 hpf in the zebrafish (Webb and Miller 2007; Ahmed et al., 2018). As early as 15 hpf, the nervous system begins innervating at neuromuscular junctions with growing somite muscles to establish movement control. From 17 to 21 hpf, spontaneous coiling is modulated by local spinal interneurons to these developing somite muscles and is independent of sensory stimulation (Hirata et al., 2010). Later, around 21 to 27 hpf, touch induced coils are apparent where head and yolk stimulation activates trigeminal sensory neurons, while trunk and tail sensory stimulation triggers Rohan Beard neurons in the spinal cord and hindbrain (Hirata et al., 2010; Drapeau,
2002) to activate commissural Mauthner neurons that stimulate motor neurons and axial muscles on the side opposite to the stimuli (Eaton et al., 2001). Commissural local glycinergic interneurons inhibit the opposing Mauthner neurons from stimulating muscles ipsilateral to the stimulus. During this time, specifically around 24 hpf, diencephalospinal dopaminergic (DDN) neurons begin extending from the diencephalon to the spinal cord to encourage peripheral motor neuron growth (Lambert, 2016).

As an individual grows from 48 to 72 hpf, touch induced coils translate into coordinated swimming when dopaminergic inputs from the diencephalon and midbrain extend to innervate the hindbrain reticular formation. During this time, increasing innervation of the hindbrain reticular system to the Mauthner neurons induces fictive swimming circuits that are slow acting compared to fast acting escape behaviors. After 96 hpf, afferent inputs are primarily received via vestibulocochlear cranial nerves (CN8), including the octavolateralis (lateral line) systems and integrated through growing inputs onto Mauthner neurons and reticular based swimming circuits. Overall, the startle response is a cooperative contribution between the nervous system and muscular-skeletal system for integration and appropriate physical responses.

Figure 1. Stimulation of the escape response in 24 hpf zebrafish embryos (A1-3), 48 hpf zebrafish embryos (B1-3), and 72 hpf zebrafish embryos (C1-3). A1-3. 21 hpf to 27 hpf embryos receive stimulation through CN5 or Rohan bear neurons that relay the signal to Mauthner neurons to instigate spontaneous coiling. B1-3. In 48 hpf embryos, increasing DAergic innervation...
into the hindbrain and further onto Mauthner neurons translates spontaneous coiling to touch induced locomotor responses. C1-3. Embryos at 72 hpf receive stimulation through CN8, and are able to perform coordinated movements away from stimuli.

Figure 2 (left). Simple diagram of CN8 stimulation of Mauthner neurons to primary motor neurons in a zebrafish embryos approximately 96 hpf. Hair cells receive mechanostimulation and relay the stimuli to octavolateralis afferent neurons. This stimuli is then either relayed to commissural spinal fiber neurons or Mauthner neurons. The Mauthner neurons decussate and extend down the spinal cord to stimulate primary motor neurons to contract on the side opposite to the stimulation. On the same side that the stimulation was received, commissural local glycinergic inhibitory interneurons inhibit opposing Mauthner neurons from stimulating muscles ipsilateral to the stimuli.

The Bandoneon Mutant

Many animal models have been created using mutations associated with hyperekplexia (Hirata et al., 2005, 2010; Ganser et al., 2013; Harvey et al., 2008; Becker et al., 2002), but these animal models have more severe disease phenotypes in humans (Chung et al., 2010; Harvey et al., 2008). The bandoneon (beo) mutant is from a WT Tübingen line (TL) and is classified as an accordion mutant due to its abnormal startle response (Haffter et al., 1996; Hirata et al. 2008). Beo mutants are used as an animal model for diseases associated with spasticity, specifically hyperekplexia, because of their bouts of startle-induced spasticity resulting in bilateral contractions in the trunk (Hirata et al., 2008). These simultaneous, bilateral trunk contractions result a phenotypic shortening of the overall trunk in response to touch that give the appearance of the folds in an accordion (Hirata et al., 2008). This phenotype is a result of a mutation in the glrbβ, the gene that encodes for the beta subunit of the glycine receptor (Granato et al., 1996; Hirata et al., 2005).

Due to this mutation, the loss of glycinergic signaling via the glycine receptor results in imbalanced signaling to muscles causing spastic motor behaviors and active paralysis that can be seen in zebrafish as early as one day post fertilization (dpf). Although the defective glycine receptor does not disrupt spontaneous coiling at 22 hpf, homozygous beo mutants begin displaying the hyperekplexia
phenotype at 24 hpf, with increasingly severe morphological defects caused by continued muscle strain beginning at 48 hpf that will continue to increase with severity. Interestingly, mutations to GLRB produce a more severe phenotype than the more common GLRA1 mutations, even though the two subunits form the same pentameric GlyR complex (Ganser et al., 2013).

While glrbb is expressed from day one during development, the GLRA subunit is not expressed until day three suggesting that the implications of improper GLRB function during development will be more severe. The loss of GLRB results in an upward curved tail phenotype at approximately 48 hpf due to the constant bilateral contractions (Low et al., 2018). Glrbb expression early in development in the reticulospinal and spinal neurons along with the malfunction of the beta subunit correlates with the expression of hyperekplexia phenotypes in beo mutants. Like the GLRB beta subunit, the expression of the cnr1 gene, encoding for CB1 receptors, begins at 24 hpf in the developing central nervous system suggesting our hypothesis that interactions between the CB1 receptor and inhibitory receptors, when facilitated by THC, may alleviate spasticity in hyperekplexia patients by restoring reciprocal inhibition and E/I balance.

**Restoring Reciprocal Inhibition and Treatment**

Restoring reciprocal inhibition would require restoring inhibitory signaling. Though spasticity disorders such as hyperekplexia are glycineric based signaling diseases, patients are regularly treated with GABA agonist drugs. On the GABA receptor, binding sites are present where drugs can bind and manipulate the function of the receptor. Benzodiazepines such as Lorazepam, Clonazepam, and Diazepam can bind to the benzodiazepine site on the GABAR and cause an influx of chloride ions, ultimately raising the threshold of the cell to slow or stop further propagation of action potential down the neuron. Benzodiazepines are used to treat a wide variety of diseases from anxiety to spasms (Tallman et al., 1980; Crawley and Goodwin 1980; Wu et al. 2016). Unfortunately, because benzodiazepines do not target the defective glycineric signaling components, and instead rely upon GABAergic signaling to restore reciprocal inhibition (Walters et al., 2000; Haefely et al., 1975), they are not treating the problem directly.
The Endocannabinoid System

Of the regulatory systems that contribute to the chemical messenger network associated with the nervous system, the endocannabinoid system is a recent discovery and now one of the most studied retrograde signaling systems in the brain (Zou et al., 2018). The endocannabinoid system is a biological control mechanism composed of endogenous cannabinoids that act as lipid based retrograde neurotransmitters. Cannabinoid receptors (CBR) bind ligand on the cell surface or on intracellular compartments. Endogenous cannabinoids, such as arachidonoyl glycerol (2-AG) and anandamide (AEA), are lipid mediators synthesized locally in response to depolarization and Ca\(^{2+}\) influx, and on demand to act as retrograde messengers that mediate feedback inhibition and moderate synaptic plasticity. Although the intracellular signaling mechanisms for the exocytosis of newly synthesized endocannabinoids is unknown (Wilson and Nicoll, 2002), extracellular endogenous cannabinoids are rapidly deactivated by reuptake and degradation enzymes such as fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (Blankman et al., 2007).

AEA and 2-AG act as agonists to endocannabinoid receptors (Busquets-Garcia, et al., 2018). These endocannabinoids are derived from arachidonic acid and are involved in the suppression of synaptic transmission (Zou et al., 2018). AEA is synthesized through the hydrolysis of N-arachidonoyl phosphatidylethanolamine, catalyzed by phospholipase, and has the ability to bind the VR1 receptor present in primary afferent neurons that are known to play a role in nociceptive pain. The other endogenous cannabinoid, 2-AG, is synthesized from the cleavage of inositol-1,2-diacylglycerol and catalyzed by phospholipase C. Unlike AEA, 2-AG is found at 50-100 times higher levels in the brain (Reggio et al. 2010). Once described as circuit breakers (Katona and Freund, 2012), endogenous cannabinoids interact with cannabinoid receptors and other types of receptors such as the G-protein coupled receptor, GPR5, or the transient receptor potential vanilloid receptor once (TRPV1) that is involved in the regulation of body temperature (Di Marzo et al., 2001). These connections to other receptors and regulatory systems in the body contribute to the impact of the endogenous cannabinoid system on homeostatic regulation. Unlike neurotransmitters, however, endocannabinoids are unable to diffuse freely due to their hydrophobic nature (Zou et al., 2018).

Endocannabinoid receptors play an important role in the function of the endocannabinoid system by binding endogenous, synthetic, and phytocannabinoids to alter neurotransmitter signaling (Kano et al., 2003). So far, two endocannabinoid receptors have been identified. The lesser studied of the two, the CB2 receptor, was discovered in 1993 by Munro and colleagues, and is found on immune
tissues as a G-protein coupled receptor whose expression pattern follows inflammation and is often localized to microglia. Although the CB2 receptor has significant effects on regulating a homeostatic system, the other receptor involved in cannabinoid signaling has a wide expression pattern in the central nervous system and plays an essential role in homeostatic regulation as a secondary messenger.

Discovered in 1990 by Matsuda and colleagues, the CB1 receptor was identified as a G-protein coupled receptor that mediates the inhibition of both inhibitory and excitatory neurotransmitters at axon terminals (Matsuda et al., 1990; van der Stelt and Di Marzo, 2003). As a result of alternative splicing, three isoforms of CB1R have been identified, two of which are the long and short form, where the typical long-form of the CB1R is found in the brain and skeletal muscle (Zou et al., 2018). The activation of CB1 leads to the suppression of the opioid agonist cholecystokinin (CCK) (Katona et al., 1999, 2001), to lessen the neural and behavioral effects of opioids. For example, the CB1 receptor has been found to mediate nearly all supraspinal effects of marijuana, a popular psychoactive drug used for recreational and medicinal purposes (Wilson and Nicoll, 2002). Expression of the CB1 receptor has been found in high levels in central nervous system components that regulate reciprocal inhibition and coordinated movement, including the neocortex, hippocampus, basal ganglia, cerebellum, and brainstem (Wilson and Nicoll 2002; Herkenham et al., 1990; Zou et al., 2018). Lower levels of CB1R expression have been found in the cerebral cortex, septum, amygdala, hypothalamus, and parts of the brainstem and dorsal horn of the spinal cord. Alternatively, CB1R expression has been seen in the sympathetic nervous system (Zou et al., 2018). The CB1 receptor has been found on pre and postsynaptic neurons (Katona et al., 1999, 2001), specifically localized on axon terminal ends, but can also be found on the cell surface or intracellular compartments (Freund et al., 2003; Zou et al., 2018).

Genetically, the coding region for the CB1 receptor, cnr1 (OMIM # 114610), lacks introns, and thus skips a processing event during translation (Busquets-Garcia et al., 2018). In zebrafish, the CB1 receptor is present as early as 2 hours post fertilization (hpf) at the three-somite stage (Migliarini and Carnevali, 2009) and continues to peak in the telencephalon at 96 hpf (Lam et al., 2006). Oddly, regions such as the hypothalamus (Wittman et al., 2007) that contain low levels of CB1 receptors are more cannabinoid sensitive and dependent than regions with higher expression of CB1 receptors (Breivogel et al., 1997).

Endocannabinoids mechanistically act as retrograde signaling molecules, a theory confirmed by mechanisms of depolarization-induced suppression of inhibition and excitation (Zou et al., 2018). Depolarization induced suppression of inhibition (DSI) is the mechanism by which CB1 decreases the
probability for vesicles containing neurotransmitters to be released from local synapses to suppress neurotransmitter communication across the synapse (Wilson and Nicoll, 2002; Iversen, 2003; Kendall et al., 2017). While the CB1R can act through a G-protein dependent or independent mechanisms, it typically acts through a G-protein coupled receptor mechanism (Zou et al., 2018) to open a K⁺ channel, allowing the efflux of K⁺ from the presynaptic terminal and increasing the threshold for opening voltage-gated Ca²⁺ that drive the pre-synaptic docking and release of neurotransmitter-containing vesicles into the synapse (Wilson and Nicoll, 2002). Endocannabinoid signaling participates in long term and short-term depression at both excitatory and inhibitory synapses, adding to the implications and complexity of the involvement of the endocannabinoid system in neuronal signaling (Zou et al., 2018).

**Phytocannabinoid Stimulation of the Endocannabinoid System**

The discovery of the endocannabinoid system was prompted by the recreational cannabinoid drug, marijuana, a product of the angiosperm *Cannabis sativa* (Mack and Joy, 2000). Marijuana contains phytocannabinoid compounds that can trigger the endocannabinoid system to mimic the effects of endogenous cannabinoids. Now being examined for its medical purposes, *C. sativa* has become a popular topic for political and social debate due to its ability to trigger the endocannabinoid system, resulting in medicinally relevant effects like hyperalgesia, anti-emesis, anti-anxiety, and anti-spasticity, but also remaining controversial because of its accompanying hallucinogenic effects.

Of the hundreds of phytocannabinoids produced by *C. sativa*, Δ⁹-tetrahydrocannabinol (THC) was the first to be synthesized and identified as the main psychoactive constituent of marijuana by Mechoulam and colleagues (1964). THC is a sticky resin that is insoluble in water and acts as a partial agonist of CB1 and CB2 (Huestis et al., 2001), with the ability to bind important non-cannabinoid receptor sites, specifically at GABAergic synapses (Wilson and Nicoll, 2002; Xiong et al., 2012). Because of its affinity for GABAergic synapses, inhibitory axon terminals are more sensitive and the inhibitory effects of GABA are enhanced by THC (Wilson and Nicoll, 2002; Hoffman and Lupica, 2013).

In zebrafish, THC depresses locomotor activity (Stewart et al., 2014; Krug et al., 2015; Breivogel et al., 1998) by slowing movement in adult zebrafish (Stewart et al., 2014) and larval zebrafish (Akhtar et al., 2013; Thomas 1975) specifically causing motor inhibition at the GABAbR in the basal ganglia (Romero et al., 1996). The disruption of endocannabinoid signaling caused by THC makes this molecule an important synthetic modulator of the physiological maintenance of the endocannabinoid and other dependent systems. Because THC binds to CB1 and also to bind to non-endocannabinoid sites (Hoffman
and Lupica, 2013), brief exposure to THC during gastrulation in zebrafish is able to alter motor neuron development (Ahmed et al., 2018).

**Chapter Two: Aims**

By analyzing the escape circuit behaviors in zebrafish, we are able to use the hyperekplexia *beo* mutant of *GLRBb* to quantify the effects of THC on efferent/motor malfunction by measuring the time between afferent stimulus application, efferent muscular contraction, and relaxation points of the escape behavior. The aims of the study are to:

**Aim 1**: Determine the possible deleterious effects of THC on the zebrafish developing nervous system.

A) Behavioral abnormalities, including spasticity within the escape circuit or non-responsiveness to stimuli will be quantified in several experimental groups, including a benzodiazepine, Lorazepam, that serves as an inhibitory neurotransmitter agonist to mimic the hypothesized effects of THC at restoring reciprocal inhibition. For behavioral evaluation of THC on a spastic mutant, the zebrafish escape circuit and its signaling components will be examined to assess the ability of THC to restore reciprocal inhibition.

B) Developmental and morphological changes associated with THC exposure, specifically maturation rates, hatching rates, differences in length, and somatic anomalies will be assessed every 24 hours after five-day after chronic THC exposure.

**Aim 2**: Identify embryonic THC induced changes in inhibitory signaling within the escape response circuit. The compensatory mechanisms by which reciprocal inhibition is restored by THC and Lorazepam will be quantified among the same treatment groups. Ineffective glycine receptors in the *beo* mutant underlie the spastic motor behaviors in the hyperekplexia model, but because hyperekplexics have some success in alleviating spasticity with GABA agonists, like the benzodiazepine lorazepam, the present study seeks to identify whether GABAergic signaling compensates for defective GLY signaling. We also analyze whether THC, an indirect GABA agonist, enhances the release of GABA triggering the receptor itself, likewise induces the compensation in the absence of GLY signaling to restore reciprocal inhibition. To understand how Lorazepam and THC can attenuate spastic behaviors can attenuate in the *beo* mutant, the expression GABAR2 will be quantified using immunofluorescence. Utilizing the same treatment groups as in Aim 1, midbrain and caudal hindbrain sections of the embryos will be quick
frozen, labeled with antibodies against GABAb receptor proteins and analyzed under a confocal microscope. Differences in puncta expression will be measured in Fluorescence Arbitrary Units (FAU) and compared among treatments to determine the differences in GABAR2 expression and signaling as a result of THC and Lorazepam treatment.

Chapter Three: Methods

Animal Care and Use

*Danio rerio* embryos were bred from at WT strain from a local fish store, Optimum Aquarium (Kennesaw, GA 30144) and TAB WT, a cross between TL and AB WT strains, (University of Miami Zebrafish Facility, Coral Gables, FL). *Beo* mutant embryos (ta86d) were obtained from ZIRC (University of Oregon, 97403-5274) in February 2018 and raised to sexual maturity. All fish were kept on a PENTAIR fish systems (Pentair Aquatic Habitats, Apopka, FL) at 27.8°F to 28.2°F, approximately 6.8 to 7.2 pH, and in groups of approximately 20 separated by sex and strain in 10-liter aquaria. Mounted lights maintained a 14h/10h light/dark cycle and fish were fed a combination of newly hatched live brine shrimp and Zeigler zebrafish diet (Pentair Aquatic Ecosystems, Apopka, FL). All experiments were conducted in compliance with Kennesaw State University’s Institutional Animal Care and Use Committee guidelines, protocol Ganser 16-010.

Embryo Collection and Rearing

Heterozygous F₀ breeding groups of 2:4 or 4:6 females to males were placed under a black box in false bottom breeding containers with dividers separating the sexes overnight. The following morning, dividers were removed at first light, and the fish were allowed to breed for approximately one hour. Embryos were collected within 1-hour post fertilization and dosed with varying concentrations of THC, Lorazepam, DMSO, or plain egg water before they reached the age of 2 hpf. Embryos were kept in a Benchmark my temp mini incubator at 28.1°F when data were not being collected. Water and drug solutions were changed every 24 hours and any debris or dead embryos were removed.
**Electrical Stimulation**

We affixed two nails to the bottom of a petri dish using aquarium-safe silicone to serve as electrodes for the electrical stimulation of embryos in the petri dish. The electrodes were then connected to an iWorx LabScribe (Dover, NH) stimulator controlled by the program iWorx Neuromuscular Physiology activity. WT individuals were placed in between the electrodes and stimulated with a single electrical shock (amplitude= 0.5, frequency=1 Hz, and pulse width= 5 ms). Startle response was quantified every 24 hours for three days by identifying the start of the response, the top of the coil, and the relax (Figure 3).

**Drugs and Exposure**

THC in ethanol (25 mg/mL THC in EtOH) and lorazepam were obtained from Sigma Aldrich (St. Louis). Biologically relevant dosage concentrations were determined via previous literature (THC: Thomas 1975, Akhtar et al., 2013 / Lorazepam: Biolotta et al., 2004, Sylvan et al., 2010 or Ethanol: Reimers et al., 2004, Carvan et al., 2004; Basnet et al., 2019) and were dosed using a logarithmic scale. Lorazepam required a Dimethylsulfoxide (DMSO) vehicle to incorporate into embryo water, thus a DMSO vehicle control group was analyzed for its interactions with development and behavior. Lorazepam was dissolved in DMSO at a 1:1 dilution (10mg Lorazepam/ 10 mL DMSO). Because the ethanol vehicle for THC did not have a significant effect on development or behavior in previous experiments on wildtypes, ethanol was not included in this analysis. Drugs were put into solution with egg water (ZFIN.com) and kept in light sensitive containers at room temperature. Embryos were dosed at 2 hpf after collection.

**Developmental Assays**
Daily mortality and hatching rate counts with groups of ten or twenty individuals per dosage were quantified every 24 hours for five days. Debris and dead individuals were removed after counting and water was replenished daily. Pictures were taken with a Zeiss V12 microscope using ZenBlue to record morphological defects.

**Somite Counting**

At 24 hpf, embryos were dechorionated and pictures were taken using a Zeiss Discovery v12 microscope. Somites were quantified and recorded by hand and recorded by multiple observers blinded to the treatment groups.

**Behavioral Assays**

Individuals were dosed by 2 hpf, dechorionated at 24 hpf, and separated into individual wells of a 48-well plate for individual identification. Every 24 hours, the startle response was evoked via tactile stimulation for both WT and beo embryos and quantified using three identifying stages of the response: the start, the coil, and relax (Figure 3).

**Molecular Assays**

Immunohistochemistry was performed on individuals at 72 hpf that had received dosages of 2 mg/L THC, 0.2 mg/L Lorazepam, or a DMSO vehicle control to identify puncta representing GABBR2 expression and quantify expression in fluorescence arbitrary units (FAU). GABBR2 rabbit polyclonal primary antibody from ThermoFisher (Waltham, MA) was diluted 1:1000 and counter labeled with Alexa568goat^rabbit secondary antibody at a 1:1000 concentration. Imaging was performed on a Zeiss 700 Confocal Microscope. A positive control tissue absent of primary antibody was used to set pinhole and gain parameters that were standard on all other analyzed tissues. Images were then analyzed for mean fluorescence using ImageJ in 40x40mm squares, and a one-way ANOVA to detect differences in mean GABBR2 expression among treatments.

**Chapter Three: Results**

Tactile stimulation reliably elicits the escape response (Kimmel et al., 1974; Hale et al., 2016) but can be skewed by human errors in touch placement, touch force, and the type of instrument used. In an effort to eliminate experimental error, we examined the variability in WT individual response times
using tactile and the alternative electrical stimulation. We found that electrical stimulation had less
variability in the total escape response time at 48hpf and 72hpf (Figure 4). Interestingly, at 48hpf, touch-
stimulated embryos took significantly longer to complete the coil to relax phase (indicating muscle
relaxation) between electrical (Mean= 18.01 ± 0.73) and tactile (Mean = 24.02 ± 1.78) stimulation
(p<0.0048), compared to 72hpf when the muscle contraction phase revealed significant differences in
the response time between electrical and tactile stimuli (p<0.0001, Tactile Mean 14.21 ± 0.6, Electrical
Mean 18.3 ± 0.62). Both discrepancies during the coil to relax phase at 48 hpf and the start to coil
phase at 72 hpf resulted in an overall significant difference in the total response time (48 hpf p<0.0048;
Tactile Mean= 56.32 ± 3, Electrical Mean= 47.26 ± 1.33 and 72 hpf p<0.0013; Tactile Mean = 24.85 ±
0.87, Electrical Mean= 29.15 ± 0.89).

Figure 4. Electrical vs Tactile response times. (Start to coil: tactile 24 hpf n = 47, electrical 24hpf n=80, tactile 48 hpf n= 82,
 electrical 48hpf n=91, tactile 72 hp f n=52, electrical n =76) (coil to relax: tactile 24 hpf n= 47, electrical 24hpf n =90, tactile 48
hpf n =91, electrical 48hpf n=82, tactile 72 hp f n=52, electrical 72hp f n = 76) (total: tactile 24 hpf n = 47, electrical 24hpf n=80,
tactile 48 hpf n=82, electrical 48hpf n=91, tactile 72 hpf n =52, electrical 72hp f n =76). P value *n>0.0332, ** n> 0.0021,
***n>0.0002, ****n>0.0001.
Figure 5. Morphological defects in WT individuals treated with 2 mg/L THC and 5 mg/L THC. A and B. WT embryos at 72 hpf and 96 hpf respectively treated with 5 mg/L THC presenting with bent axis (BA), blunt-ended tail (BT), yolk sac edema (YSE), and cardiac edema (CE). C. WT embryo at 120 hpf treated with 2 mg/L THC presenting with an enlarged swim bladder (ESB). D. WT embryo at 96 hpf treated with 2 mg/L THC remaining in the chorion.

In order to examine the effects of THC on survival and hatching rate and to identify a relevant concentration for treatment, embryos were dosed with 0.2 mg/L THC, 0.6 mg/L THC, and 2 mg/L THC. We used an ethanol vehicle to introduce THC directly into the embryo water, and replaced the water and treatment every 24 hours. With each 24-hour treatment change, survival and hatching rates were measured in all experimental groups.

Overall survival and hatching rates were negatively impacted by increasing doses of THC. Approximately half of control and 0.2 mg/L THC survived until 120 hpf, while less than half of 0.6 mg/L THC and 2 mg/L THC survived until 120 hpf (Figure 6). From 48 hpf to 72 hpf, at 2 mg/L THC, three entire clutches died off and from 72 hpf to 96 hpf, another two clutches died off so there were a total of five clutches at the end of five days. From 48 hpf to 72 hpf, three entire 2 mg/L THC treatment replicates died, and from 72 to 96 hpf, another two treatment replicates died, leaving a total of five replicates at the end of five days. From 48 to 72 hpf, three 0.6 mg/L THC treatments replicates also died.

Similar to survival rates, control and WT groups had hatching rates of approximately half after five days. 42% of 0.6 mg/L THC treated individuals hatched out of their chorion by day five, while only 35% of 2 mg/L THC treated individuals emerged from their chorion 5 days post fertilization, suggesting that THC has an effect on chorion emergence rate (Figure 5).
Figure 6. WT survival and hatching rates.

A. WT survival rates for all experiments after THC treatment for 120 hpf.

B. WT hatching rates for 120 hpf after THC exposure.

Table 1: Survival rates (%) for petri dishes with 10 individuals in each. Control n=9, 0.2 mg/L THC n=4, 0.6 mg/L THC n=4, 2 mg/L THC n=5.

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<td>120 hpf</td>
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Table 2: Survival rates (%) for petri dishes with 20 individuals in each. Control n=8, 0.2 mg/L THC n=10, 0.6 mg/L THC n=9, 2 mg/L THC n=10.

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The number of individuals per petri dish influenced survival and hatching rates for WT individuals. Tables 1-4 reveal the effect of crowding on survival (Tables 1 and 2) and hatching (Tables 3 and 4) percentages where overall, the individuals in groups of 20 had an overall lower survival and hatching rate than individuals in groups of 10.

Next, we examined the effects of THC on beo embryos using only the 2 mg/L THC, 0.2 mg/L THC and adding the hypothesized positive control, Lorazepam at 0.2mg/L and 0.02 mg/L dosages. Lorazepam is a GABA agonist and is often used to treat diseases of spasticity (Wu et al., 2016). Overall, beo survival and hatching rates do not mirror WT survival and hatching rates (Figure 7A and 7B). Beos have a high mortality rate at 24 hpf and survivorship seems to plateau for all treatment groups except 0.2 mg/L Lorazepam (Figure 7A). Compared to WT embryos, beo embryos dosed with 0.2 mg/L THC had similar survival rates to controls (Figure 6A and 7A). Beo embryos dosed with 0.2 mg/L Lorazepam had the lowest survival rate (>50%) after 120 hpf of chronic exposure (Figure 7A). When we examine the effects of THC and Lorazepam on hatching rate, we see that 100% of beo embryos hatch after 120hpf and again, that 0.2 mg/L Lorazepam has the slowest hatching rate compared to THC, 0.02 mg/L Lorazepam, DMSO vehicle control, and control embryos (Figure 7B).

### Table 3
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<td>72 hpf</td>
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Hatching rates (%) for petri dishes with 10 individuals in each. Control n=9, 0.2 mg/L THC n=6, 0.6 mg/L THC n=6, 2 mg/L THC n=5.

### Table 4
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Survival rates (%) for petri dishes with 20 individuals in each. Control n=8, 0.2 mg/L THC n=10, 0.6 mg/L THC n=9, 2 mg/L THC n=10.
While the bilateral dorsiflexion, or accordion phenotype is apparent as early as 24 hpf in beo mutants, morphological defects do not appear until 48 hpf. The absence of reciprocal inhibitory glycnergic signaling underlies the accordion phenotype, thus placing prolonged strain on forming muscles in early stages of development and likely resulting in beo-typical morphological defects such as upward curved tail or ‘kinks’ along the spinal cord (Figure 9).

![Figure 7. Beo survival and hatching rates.](image_url)

A. Beo survival rates for embryos after THC, Lorazepam, and DMSO treatment for 120 hpf.

B. Beo hatching rates for embryos after THC, Lorazepam and DMSO treatment for 120 hpf.
At 24 hpf, embryos should have approximately 30 somites (Stickney et al. 2000). Thus in order to determine if the beo mutation has an effect on developmental progression, we compared the number of somites present in beo embryos and WT embryos at 24 hpf. It is important to eliminate developmental patterning delays as a cause for morphological defects present in beo mutants because the beo phenotype does not appear until 24 hpf (Hirata et al., 2005). Ultimately, WT and beo control embryos did not differ significantly in somite number at 24 hpf (Figure 10).

![Figure 9. Morphological defects displayed in beo embryos at 48 hpf to 120 hpf. Arrow heads indicate morphological defects consistent with beo phenotype.](image)

![Figure 10. Number of somites in beo and WT embryos at 24 hpf.](image)

No significant difference between the numbers of somites in beo mutants compared to WT embryos at 24 hpf. WT control embryos had an average of 28.7 somites and beo embryos had an average of 28.4 somites. Beo control n=11; 28.36±0.5439 (SEM) and WT control n =13; 28.69±0.835 analyzed using a t-test.
The number of individuals displaying the beo phenotype was quantified after five days through the confirmation of a spastic startle response and morphological defects present. Out of four clutches collected and dosed with THC and Lorazepam, approximately one quarter of every dosage displayed the beo phenotype (Figure 11).

Aside from common morphological differences in beo embryos, behavioral anomalies were more frequent in beo embryos compared to WT embryos. In Figure 12, beo embryos varied in their ability to elicit a response from an electrical stimulus. The ability of an individual to respond was quantified by any sign of response, whether it is c-start, fictive swimming, or a twitch. At 24, 72, and 96 hpf, beo embryos in the DMSO vehicle control group showed a significant number of individuals that did not respond to the stimuli (1 at 24 hpf, 2 at 72 hpf, and 1 at 96 hpf). Embryos dosed with 2 mg/L THC did not respond to stimuli when they were 48, 72 or 120 hpf. At 96 hpf, both 0.02 mg/L Lorazepam and 0.2 mg/L Lorazepam had instances where one individual did not respond.
We analyzed escape behavior in beo mutants to identify differences in the response compared to WT embryos, but also to measure whether THC and Lorazepam improve the beo escape response. Mirroring the THC experiments on WT, escape behavior was recorded and measured in beo embryos from different treatment groups. We quantified the total response time and also analyzed the time from stimulus to coil, indicating the contractile reaction, as well as the relaxation phase; by measuring the time it took embryos to move from the top of the coil to a relaxed position. In Figure 13, beo mutants took significantly longer compared to WT to uncoil from the touch-induced C-bend at 48, 72, and 96 hpf. Total beo escape response times were significantly longer and varied more than in WT at 24, 96, and 120 hpf.

When comparing beo and WT response times, the start to coil response time at 48 hpf was not significant (p =0.0544) and at 120 hpf (p =0.0985). The coil to relax response time was also not significantly different at 120hpf (p=0.0858), nor did the total escape response time (p = 0.0833; Figure 13). Because some of these times seem to be nearing significance, perhaps a larger sample size would better explain the differences in beo and WT response times.
Because aberrant inhibitory glycineric signaling underlies the spastic behaviors common among beo mutants, we hypothesized that inhibitory agonists, THC and Lorazepam, would help to alleviate spasticity by balancing the overwhelming excitatory signaling with inhibition through means other than glycineric signaling. However, THC and Lorazepam treatments slowed the beo escape response even more. When dosed with Lorazepam and THC, individuals displayed similar response times to control at 24, 96, and 120 hpf. Significant differences in escape behavior times were measured at 48 and 72 hpf, specifically during the coil to relax phase (Figure 14). Embryos dosed with 2 mg/L THC took significantly longer to coil compared to controls (48 hpf =p<0.0003, 72hpf =0.0024). At 72hpf the total response time of the 2 mg/L THC group was also significantly greater than controls (p<0.0001) and from 0.2 mg/L THC (p<0.0011). Overall, at 48 hpf and 72 hpf during the coil to relax phase, beo control individuals took significantly less time to complete the coil to relax phase than 0.2 mg/L Loraz (48 hpf: p<0.0003 and 72 hpf: p<0.0001) and 0.2 mg/L THC (48 hpf: p<0.0014 and 72 hpf: p<0.0021). Embryos treated with 0.2 mg/L THC took significantly less time to complete the coil to relax phase at 48 and 72 hpf compared to 0.2 mg/L Lorazepam- treated embryos (48 hpf: p<0.0014 and 72 hpf: p<0.0002) and in total response time at 72 hpf (p<0.0201). Tables 5 and 6 display mean response times for beo and WT embryos with SEM.

Delays in escape response increased by 72 hpf, however, with 0.2 mg/L Lorazepam embryos averaged significantly slower stimulus to coil responses compared to embryos in other treatment groups. As with the 48 hpf embryos, at 72 hpf, the majority of anomalous behavior was measured at the coil to relaxation phase of the escape response. Control beo embryos treated with 0.2 mg/L THC did not differ in the average time from coil to relax, but embryos exposed to lorazepam and 2 mg/L THC were significantly slower in these groups. Total escape response time at 72 hpf differed among treatments similarly to the relaxation phase measurements.
To examine the ability of THC to compensate for lack of glycinergic signaling through its interaction with GABA receptors, we measured immunofluorescence in fluorescence arbitrary units (FAU) of GABBR2 expression in the midbrain versus the caudal hindbrain sections of embryos treated with 0.2 mg/L Lorazepam and 2 mg/L THC through the use of fluorescence arbitrary units (FAU) in Image J. Figure 16 shows a midbrain (A) and hindbrain (B) section of a 72 hpf beo embryo staining for the GABA receptor. Individuals dosed with Lorazepam and THC showed no significant difference compared to control and vehicle control individuals (Figure 15).

Figure 13. Beo and WT response times broken down into day and escape response part compared using a non-parametric Mann-Whitney ANOVA.
Figure 14. Beo response times at 48 hpf and 72 hpf in embryos dosed with Lorazepam and THC. Results were analyzed using a one-way ANOVA where * = p<0.01, ** = p<0.001, and *** = p<0.0001.
Figure 15. Fluorescence Arbitrary units (FAU) for 72hpf beo embryos dosed with DMSO, 0.2 mg/L Lorazepam and 2 mg/L THC. A one-way ANOVA indicates no significant difference in GABBR2 expression among treatments (p=0.0838).
Chapter Four: Discussion

Electrical stimulation as an alternative to tactile stimulation of the escape response in larval zebrafish

Drug exposure during development causes a number of issues ranging from improper neuronal signaling to morphological defects (Ahmed et al., 2018; Carty et al., 2017). Using the zebrafish as an animal model for toxicity, developmental, and behavioral assays allows us to comparatively examine the consequences of drug exposure on the neural circuitry that governs the escape behaviors that are essential for survival. Because neuronal development is so complex in the early embryonic stages, an umbrella method throughout development to elicit escape response behaviors such as tactile stimulation leaves room for discrepancies that can affect experimental results. Therefore, this research examined electrical stimulation as an alternative to tactile stimulation during early embryonic development. One predetermined, short electrical pulse was sent through the water in order to mimic a whole-body response instead of single point stimulation. By evoking a response through whole-body stimulation, there was little room for discrepancies in the neuronal pathway that received the stimuli. Researcher-dependent variables such as the force of the contact, the location on the embryos spinal cord of the contact, the duration of contact time, and the utensil used for contact affects the consistency of responses elicited by the embryo. In addition, researchers must control for external variables such as environmental conditions (light or dark, hot or cold, depth of the water, etc.) and organismal-dependent variables such as body positioning or developmental stage. Electrical stimulation removes inconsistencies associated with researcher-dependent variables and as seen in Figure 4, has significantly different average response times at 48 and 72 hpf. The 48 and 72 hpf developmental stages mark critical periods of neurodevelopment when escape circuits are transition in signal detection, connections to the CNS, and efferent response circuits and altered placement of tactile stimuli can produce variable responses. At 48 hpf, tactile stimulation yielded a longer response times during the coil to relax phase compared to electrical stimulation. The difference in times was enough to affect the overall response time. At 72 hpf, tactile stimulation produced shorter response times than electrical stimulation, which affected the total response time. It is possible that because innervation of the hindbrain reticular system onto Mauthner neurons increases during the 48 to 72 hpf developmental time periods, and the consistency of electrical stimulation becomes more apparent at different stages of the escape response (i.e. start to coil vs. coil to relax).
Although electrical stimulation provided more responses that took less time overall than tactile stimulation in WT embryos, beo mutants did not respond as well to the whole body stimulation and would often fail to respond to the stimuli altogether.

**THC affects development**

As one of the most commonly used, easily accessible, and often exploited drugs in the world, marijuana has persisted throughout history as a recreational and medicinal compound (Substance Abuse Center for Behavioral Health Statistics Quality 2015). The widespread and diversifying uses for marijuana provided probable cause to researchers to further explore the effects of THC on the developing central nervous system. The psychoactive constituent of marijuana, THC, has a significant amount of controversy associated with its usage due to its ability to bind to receptors throughout the body and alter physiological set points in essential neuronal circuitry (Iversen, 2003; Hoffman and Lupica, 2013).

In adults, THC influences memory, movement, and judgement but little is known about the effects of THC on a developing embryo (Thomas 1975; Akhtar et al., 2013; Carty et al. 2017; Ahmed et al. 2018). In this study, we examined the effects of THC on development and behavior. Our developmental assays show that when treated with high dosages of THC, zebrafish embryos begin showing obvious morphological defects such as a yolk sac edema, cardiac edema, and bent axis. These morphological defects were especially apparent in individuals dosed with 5 mg/L THC, while individuals dosed with a lower concentration of 2 mg/L THC showed less severe symptoms and typically appeared at 72-96 hpf (Figure 5).

Due to the severity of the morphological defects seen in 5 mg/L dosages of THC, the embryos were unable to swim and seek food properly, and ultimately, did not survive past 10 dpf. The severity of morphological defects increased parallel to the increased dosage of THC, thus suggesting that at higher dosages, THC is toxic to the developing embryo. Therefore, in order to examine THC as a potential therapeutic for spasticity diseases, treatment concentrations lower than 2 mg/L are examined throughout this study.

If higher doses of THC resulted in more severe defects, what level of THC can be used that will still be effective without adverse consequences? To answer this question, three concentrations of THC were identified (2.0, 0.6, and 0.2 mg/L) and used to investigate the effect of THC on survival and hatching over the span of five days in both WT and beo embryos. Overall, embryos treated with 0.2 mg/L THC had the most similar survival and hatching rates to control embryos, while embryos treated
0.6 mg/L THC had similar survival and hatching rates with the 2.0 mg/L THC dosage group, having lower rates in both categories compared to the other treatments (Figure 6). Again, these results are consistent with previous research suggesting that embryos exposed to lower doses of THC result in similar survival and hatching rates more similar to control embryos (Ahmed et al., 2018, Thomas 1975).

**Size of beo rearing group effects survival and hatching**

During the survival and emergence assays, groups of 10 or 20 individuals were used in each petri dish based on the number of eggs that were collected that day. Interestingly, group size had an effect on survival and emergence rates. Groups of 20 individuals had an overall lower survival and hatching rates for each dosage than groups with 10 individuals (Tables 1-4). Groups of both 10 and 20 individuals treated with 0.2 mg/L THC had higher survival and hatching rates than control groups. Because THC has antimicrobial properties, the lower dosage of THC could have kept the microbial burden lower for embryos, improving health, survival, and hatching rates (van Kilneren et al. 1976; Appendino et al., 2008) (ZFIN.com). In the present study, embryos in groups of 20 treated with 0.2 mg/L THC and 0.6 mg/L THC had higher hatching rates than control and 2 mg/L THC treated groups (Table 4). It is also possible that the groups with 20 embryos had more collisions within the petri dish, causing the chorions to weaken and break open sooner than embryos in groups of 10.

**THC differentially affects WT and beo embryos**

While WT embryos displayed morphological results consistent with previous studies, beo embryos had different patterns of survival and hatching than WT (Figure 7). Survival and hatching rates of beo embryos were examined for five days after being dosed with varying concentrations of THC and the GABA agonist Lorazepam. Beo embryos displayed steep decreases in survival rates at 24 hpf and increases in hatching rate compared to the steady increase and decrease of WT embryos (Figures 6 and 7). The drastic increase in hatching rate from 48 hpf to 72 hpf could correlate to the onset of the spastic phenotype of beo mutants. WT and spastic embryos were kept in the same petri dish for these experiments increasing the possibility that the spastic individuals caused a greater disturbance in the petri dish than in dishes with all WT individuals, causing some WT embryos to break through their chorion prematurely.

In addition to being a lethal mutation, defective glycinergic signaling yields morphological defects likely resulting from strain caused by unsolicited muscle contractions. As seen in Figure 9, morphological defects appear as early as 48 hpf and increase in severity as the individual grows. These
date correlate with previous research suggesting that due to the early onset of spastic behavior, the muscle strain caused by spastic behavior can lead to morphological defects (Ganser et al., 2013).

Though beo embryos eventually develop morphological anomalies, defective glycineric signaling does not perturb developmental progression before spastic behaviors become apparent. At 24 hpf, embryos should have approximately 30 somites (Stickney et al., 2000), and in the present study, beo embryos and WT embryos had approximately the same number of somites by 24 hpf (Figure 10). These data suggest that glycineric signaling is essential for development, but the beo mutation does not perturb developmental progression in the elongation phase (Chalphin and Saha, 2010). These findings support previous research showing that the glrb mutation does not perturb body patterning and spontaneous coiling prior to 24 hpf in these embryos (Hirata et al., 2005).

**THC effects beo behavior**

Embryo response time was quantified by using identified time points during the startle response (Figure 3). Beo embryos were able to perform the initial start to coil movements of the escape response similar to WT but were significantly slower at uncoiling than WT at 48, 72, and 96 hpf (Figure 13). Interestingly, the overall time needed to perform an entire set of movements was significantly different in beo embryos compared to WT embryos at 24, 96 and 120 hpf, while neither the coil nor the relax phase of the startle response was significantly different between WT and beo embryos at 24 hpf (Figure 13). The average response time for WT individuals was consistently lower than beo embryos (Figure 13).

The significant increase in average time taken during the coil to relax phase at 48 and 72 hpf in beo embryos treated with THC compared to control embryos shows that at these time points, THC is able to slow the second part of the startle response enough to cause a difference in overall escape response time (Figure 14). Interestingly, the lower dose of THC (0.2 mg/L THC) has response times similar to beo control response times, suggesting that 0.2 mg/L THC is not a strong enough dosage to facilitate THC binding and compensation for the loss of glycineric signaling. Comparatively, 0.2 mg/L Lorazepam and 2 mg/L THC have similar response times suggesting that the higher dosage of THC is able to mimic the GABA agonist Lorazepam.

Because glycineric signaling is dominant in spinal locomotor responses such as the escape response, THC and Lorazepam were only effective in the brain, therefore unable to alter spinal cord signaling (Chalphin and Saha, 2010). Specifically at 48 and 72 hpf, THC and Lorazepam appear to affect behavior the most (Figure 14). The hindbrain reticular formation connections are the most influential at
these developmental stages, especially with inputs from the trigeminal afferents leading the primary response at this time to be governed by GLUT/GLY at the spinal cord. Even after spiral fiber innervation of the hindbrain coordinates the fast c-bend response, GABA tempers inhibition in a feed-forward manner, but the response itself is still dominated by GLY.

When examined with fluorescence microscopy to measure fluorescence intensity for GABAR2 expression, midbrain and hindbrain tissues from THC and Lorazepam- treated individuals showed the presence of GABBR2 expression (Figure 16), though there were no significant differences in GABBR2 expression among all treated individuals (Figure 15).

**Chapter Five: Conclusions**

This study contributes to the growing database of the effects of THC on a developing organism and the neural circuitry that is essential for innate escape behaviors. Through behavioral and developmental studies, this research identified 2 mg/L THC to be the approximate amount of THC necessary to affect behavioral response time without causing lethal morphological defects. Through immunohistochemistry and measuring GABBR2 expression, the present study shows that the neuroplastic changes associated with THC exposure during development remain unknown. In conclusion, the therapeutic benefits and severity of potential defects associated with THC depend on the exposure concentration and duration, but may not necessarily have a lasting effect on the neural circuitry to alleviate a disorder such as hyperekplexia. Further research must be done to ultimately understand the role of the endocannabinoid system in seizure models and examine possible alternatives to harmful drugs currently being used to treat spasticity.

**Chapter Six: References**


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