EFFECTS OF CANNABIDIOL ON VOCAL LEARNING AND RECOVERY FROM CNS DAMAGE

by

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Cannabidiol (CBD), a non-euphorigenic compound derived from *Cannabis,* shows promise for improving recovery following cerebral ischemia and was recently effective for the treatment of childhood seizures caused by Dravet and Lennox-Gastaut syndromes. This condition is associated with developmental delays, including language deficits. In addition to clinical evidence of anti-seizure efficacy, quality of life assessments indicates improved cognitive function that includes speech. These reports suggest that CBD may have efficacy to improve vocal learning. Based on clinical observations, we hypothesize that CBD has distinct efficacy to mitigate CNS damage and promote vocal learning. To test this hypothesis, we have emplloyed a songbird, the male zebra finch, as a novel pre-clinical animal model. To assess the efficacy of CBD to mitigate CNS damage, we used adult birds, which received bilateral microlesions of HVC (used as a proper name) a pre-vocal motor cortical-like brain region that drives song learning. Moreover, to examine the efficacy of CBD to promote vocal learning, we used juvenile birds that received unilateral lesion of lMAN that plays a major role during the sensorimotor stage of vocal learning. Their songs were analyzed using Kullback–Leibler (KL) distance, syntax and production analysis to confirm the effects of CBD. Our results indicate that 10 and 100 mg/kg CBD

effectively reduced the time required to recover vocal phonology and syntax. In the case of phonology, the magnitude of microlesion-related disruptions were also reduced. Also, our results demonstrated ability of CBD to improve vocal learning following damage to lMAN that plays a major role in vocal learning during sensorimotor stage. Treatment with 10mg/kg CBD for 10 days improved vocal learning in terms of phonology and measures of syntax. These results suggest CBD holds promise to improve functional recovery of complex learned behaviors following brain injury and therapeutic promise for treatment of speech- and/or language-related disorders. In addition to demonstrating CBD efficacy, the work represents establishment of an important new animal model to screen drugs for efficacy to improve vocal recovery and vocal learning.

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DEDICATION

To

My Father

A strong and gentle soul who taught me to trust in Allah, believe in hard work and that so much could be done with little

My Mother

whose affection, love, encouragement and prays of days and nights make me able to get such success and honor

My Lovely Wife

whose love is the greatest gift of my life and whose commitment, support and patience are true models for all

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LIST OF SYMBOLS OR ABBREVIATIONS

Δ-9-THC: Delta-9-tetrahydrocannabinol

2-AG: 2-arachidonoyl glycerol

AC: Adenylyl cyclase

AEA: Anandamide

AIDS: Acquired immunodeficiency syndrome

Area X: Area X of striatum

BC: Before Christ

cAMP: Cyclic adenosine monophosphate

CB1: Cannabinoid receptor 1

CB2: Cannabinoid receptor 2

CBD: Cannabidiol

CBN: Cannabinol

CBL: Cannabicyclol

CBG: Cannabigerol

CNS: Central nervous system

DV: Dravet syndrome

ECs: Endocannabinoids

ENT: Equilibrative nucleoside transporter

FAAH: Fatty acid amide hydrolase

FDA: Food and Drug Administration

GPCRs: G protein-coupled receptors

GPR55: The orphan G-protein-coupled receptor

HIV: The human immunodeficiency virus

HVC: Used as a proper name

LGS: Lennox-Gastaut syndrome

lMAN: lateral magnocellular nucleus of the anterior nidopallium,

MAGL: Monoacylglycerol lipase

MAP: Mitogen-activated protein

MES: Maximal electroshock

PPARγ: Peroxisome proliferator-activated receptor gamma

PTZ: Pentylenetetrazole

PKA: Protein kinase

RA: Robust nucleus of the arcopallium

ROS: Reactive oxygen

RNS: Reactive nitrogen species

THC: Delta-9-tetrahydrocannabinol

VTA: Ventral tegmental area

CHAPTER 1: INTRODUCTION

1.1 Cannabis

1.1.1 Cannabis use over thousands of years

Over thousands of years, Cannabis has been used in many parts of the world for its effects to alter CNS activity. Before the sixth century BC, Cannabis was used by the Assyrians for its medical properties, as well as its psychoactive effects (Mechoulam and Parker 2013). In addition, in the Middle East, the use of Cannabis ever since has continued (Rosenthal 1971). In China, its dual effects were known and are documented in the Ben Ts'ao which is the Chinese pharmacopeia written in the first century AD. This source recommended Cannabis use for different diseases and noted problems with its euphoric effects when used in excess (Mechoulam and Parker 2013). The Napoleonic soldiers introduced Cannabis when to Europe they returned from Egypt (Rosenthal 1971). So, from that time virtually all civilized people have used Cannabis for its medical and euphoric effects.

The psychological effects of Cannabis have been known since 1845 when Moreau in his book, Hashish and Mental Illness, documented associated hallucinations, delusions, errors of space and time, fluctuations of emotions and irresistible impulses (Moreau 1973). The medical use of Cannabis was not reliable in Europe, because the governments were worried about their psychological effects (Mechoulam and Parker 2013). In addition, before 1960s, research on Cannabis was limited to a few groups of scientists (Mechoulam and Parker 2013). However, now with the great development in the field of research and the diversity of techniques, research has flourished on hashish, especially after the discovery of many therapeutic benefits for some of its components.

The genus Cannabis consists of three species, which have useful amounts of psychoactive cannabinoids: Cannabis indica, Cannabis ruderalis and Cannabis sativa (Ben Amar 2006). Cannabis has more than 460 known compounds; and more than 60 of these are cannabinoids structurally-related to the most psychoactive delta-9-tetrahydrocannabinol (Δ-9-THC) (Ben Amar 2006). Other notable cannabinoids include, cannabidiol (CBD), cannabinol (CBN), cannabicyclol (CBL), and cannabigerol (CBG); these have less psychoactive effects than Δ-9-THC, but still play a role in the effects of Cannabis (Gordon, Conley, and Gordon 2013). Δ-9-THC, CBD and CBN are the most studied cannabinoids (Gordon, Conley, and Gordon 2013). Δ-9-THC acts as a partial agonist for both Cannabinoid receptor type 1 (CB1) and Cannabinoid receptor type 2 (CB2) (Gaston and Friedman 2017). CBD has no euphorigenic effects (Devinsky et al. 2014). CBN is a degradation product of Delta-9-tetrahydrocannabinol and has a moderate psychotropic effect (Gordon, Conley, and Gordon 2013). In humans, the CB1 receptors are mainly found in the central nervous system (CNS) and the CB2 receptors are mainly found in peripheral tissue, including cells involved in inflammation and immunity (Gaston and Friedman 2017). Because of these receptors, Cannabis products have diverse effects in different systems.

1.1.2 Medical uses

Medical Cannabis has many potential beneficial effects such as helping in reducing chronic pain and attenuating muscle spasms (Whiting et al. 2015). In addition, it is effective in reducing nausea and vomiting during chemotherapy, improving sleep, improving tics in Tourette syndrome and improving appetite in HIV/AIDS (Whiting et al. 2015). Cannabinoids have also been recommended for anorexia, arthritis, migraine, and

glaucoma (Sachs, McGlade, and Yurgelun-Todd 2015). The legal use of marijuana is increasing. In the USA: 35 states have legalized marijuana use for medical purposes (National Conference of State Legislatures 2019)

1.2 Endocannabinoid system

The main effects of cannabinoids are produced through the endocannabinoid system. The endocannabinoid system consists of the cannabinoid receptors (mainly CB1 and CB2 receptors), their endogenous agonists: the endocannabinoids (ECs) anandamide (N-arachidonoylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG), and the enzymes responsible for their synthesis, degradation and uptake (Szabo and Schlicker 2005).

1.2.1 The Cannabinoid Receptors:

1.2.1.1 The CB1 Receptor

In humans, the CB1 receptors are mainly found in the central nervous system (CNS) (Gordon, Conley, and Gordon 2013). CB1 receptors are the most abundant GPCRs (G protein-coupled receptors) in the brain (Mackie 2006). In addition, they are expressed in the sensory and motor regions consistent with the important role of CB1 receptors in motivation and cognition (Mechoulam and Parker 2013). CB1 is activated by: endocannabinoids, such as AEA and 2-AG; plant phytocannabinoids, such as the compound Δ-9-THC which, as noted above, is the principal active constituent of Cannabis; or synthetic compounds such as Nabilone; and several compounds related to carboxamide (Mechoulam and Parker 2013).

The CB1 receptors are found primarily on the presynapse of central and peripheral neurons (Gordon, Conley, and Gordon 2013). These locations facilitate the inhibition of

neurotransmitter release, which is one of the functions of the endocannabinoid system. Most CB1 receptors are coupled through Gi/o proteins (Mechoulam and Parker 2013). Activation of CB1 receptors causes reduced accumulation of cyclic adenosine monophosphate (cAMP) and this leads to inhibition of cAMP-dependent protein kinase (PKA) (Mechoulam and Parker 2013). In addition, CB1 receptor activation leads to stimulation of mitogen-activated protein (MAP) kinase activity, which is a mechanism by which cannabinoids affect synaptic plasticity, cell migration, and possibly neuronal growth (Howlett et al. 2002). CB1 receptors are also coupled to several types of calcium and potassium channels (Mechoulam and Parker 2013). The expression of these kinds of coupled receptors has an effect on myelin sheath formation, that might help to treat some demyelinating diseases such as multiple sclerosis (Mato et al. 2009).

1.2.1.2 The CB2 Receptor

In humans, the CB2 receptors are mainly found in the immune system cells (Mechoulam and Parker 2013) such as monocytes, B-cells, macrophages, and T-cells (Basu, Ray, and Dittel 2011). Similar to the CB1 receptors, CB2 receptors produce their effect by inhibition of the activity of adenylyl cyclase (AC) through activation of Gi/Goα subunits (Demuth and Molleman 2006). CB2 receptor expression under pathological conditions is activated to mediate immunosuppressive effects and enhance neuroprotection (Pacher and Mechoulam 2011).

1.2.2 Endogenous Cannabinoid Ligands

The discovery of the CB1 and CB2 receptors suggested that endogenous cannabinoids are presumably present in the human body because the constituents of Cannabis plant binds to these receptors to produce their effects. So, Mechoulam and his

group worked to identify some endogenous molecules that act on these receptors. In 1992, Mechoulam's group isolated a compound from brain, which they named anandamide (AEA) (Devane et al. 1992). In addition, in 1995 they also isolated and identified a second compound from peripheral tissue that they named 2-arachidonoyl glycerol (2-AG) (Mechoulam et al. 1995). The structures of these two compounds are shown in Figure 1.1. Then, scientists started investigating the effects of these endogenous cannabinoids. Unlike other neurotransmitters, such as acetylcholine, serotonin, and dopamine, these two endogenous cannabinoids are not stored in vesicles, but are synthesized when they are needed (Mechoulam and Parker 2013). Moreover, their action is mostly presynaptic not postsynaptic like most neurotransmitters (Mechoulam and Parker 2013).

After synthesis of anandamide and 2-arachidonoyl glycerol in postsynaptic neurons, they cross the synapse to activate the cannabinoid presynaptic receptor that inhibits release various of neurotransmitters (Howlett et al. 2002). This is the main role of the endocannabinoids (Mechoulam and Parker 2013). Inside the cell, anandamide is hydrolyzed by fatty acid amide hydrolase (FAAH) to arachidonic acid and ethanolamine (Wilkerson et al. 2017). 2-arachidonoyl glycerol is hydrolyzed by Monoacylglycerol lipase (MAGL) to arachidonic acid (Wilkerson et al. 2017) (see Figure 1.2). Inhibition of these enzymes prolongs the activity of these endocannabinoids (Mechoulam and Parker 2013).

1.3 Cannabidiol

The two most abundant psychoactive components in Cannabis are the euphorigenic ∆-9-THC and the non-euphorigenic (CBD). The term of non-euphorigenic indicates a lack efficacy to produce a 'high' like ∆-9-THC; however CBD does have some

psychoactive effects including anti-anxiety, anti-depressant and other behavioral effects (Devinsky et al. 2014). In addition to the advantage of non-euphorigenic effects of CBD, this drug is currently of great interest because it has many effects and multiple pharmacological targets. In the last few decades, there are many studies that have investigated CBD effects finding that CBD attenuates damage of the brain following ischemic and/or neurodegenerative conditions (Devinsky et al. 2014; ElBatsh et al. 2012). Also, it has positive effects in many neuropsychiatric disorders such as anxiety and depression (ElBatsh et al. 2012; Almeida et al. 2013; Bergamaschi et al. 2011; Devinsky et al. 2014). Moreover, CBD has anti-inflammatory, neuroprotective, and antiepileptic effects (Devinsky et al. 2014a).

The mechanisms of action of CBD to produce these effects are still unclear, but many observations suggest it can act as an agonist at the 5-$HT1_A$ receptor and through which it produces anxiolytic effect (Gomes, Resstel, and Guimarães 2011; Connors et al. 2014) and reduces neuropathic pain (Palazzo et al. 2006). Also, it binds with α3 and α1 glycine receptors (Pertwee 2008) which may lead to reduced schizophrenic symptoms (Devinsky et al. 2014). CBD has also been shown as an antagonist at the orphan G-protein-coupled receptor GPR55 (Devinsky et al. 2014) and based on some studies antagonizing this receptor will play a role to reduce inflammation (Montecucco et al. 2016). In addition, CBD enhances adenosine A1 receptor activity via blocking equilibrative nucleoside transporter activity (ENT) (Devinsky et al. 2014), which will produce anti-inflammatory effects (Carrier, Auchampach, and Hillard 2006). CBD has a polyphenolic nature (see Figure 1.1) making it a potent antioxidant (Devinsky et al. 2014).

In addition to these target sites, CBD is effective against maximal electroshock (MES) and pentylenetetrazole (PTZ)-induced seizures (Devinsky et al. 2014).

1.3.1 Potential Clinical Implications

During recent years, there have been many important clinical trials done to study the potential effects of CBD in pain management. A combination of CBD and Δ-9-THC has significantly reduced acute pain scores in postoperative patients (Holdcroft et al. 2006), as well as reduced chronic pain associated with rheumatoid arthritis, multiple sclerosis, peripheral neuropathy, and central neuropathic pain (Zajicek et al. 2003; Rog et al. 2005). In Canada, Sativex®, a combination of CBD and Δ-9-THC (1:1 ratio), was approved to treat neuropathic pain in patients suffering from multiple sclerosis and in cancer patients as an adjunctive analgesic medication. In addition, Cannador®, containing CBD and Δ-9-THC in a 2:1 ratio, was registered in different countries and used in several clinical trials to reduce spasms, muscle stiffness, and pain in multiple sclerosis (Holdcroft et al. 2006). Moreover, other clinical trials have been investigating the effect of CBD-enriched Cannabis as a therapy to treat certain types of epilepsies such as Dravet syndrome (DS) and Lennox-Gastaut syndrome (LGS). Results showed a reduced seizure frequency in both DS (Devinsky et al. 2017) and LGS patients (Devinsky et al. 2018; Thiele et al. 2018). Additionally, several parents of these patients reported improved alertness without side effects such as drowsiness and fatigue, that are associated with current antiepileptic drugs (Devinsky et al. 2014; Oakley, Kalume, and Catterall 2011). Because of these positive effects of CBD, Food and Drug Administration (FDA) approved Epidiolex® (CBD) to treat patients who suffer from LGS and DS.

In addition, CBD has a positive effect on schizophrenic patients. One clinical trial compared a standard antipsychotic, amisulpride, and CBD to reduce acute schizophrenia in 33 patients on a timeline of more than four weeks. Both drugs showed similar significant improvements (Leweke et al. 2012). However, CBD produces its effect without amisulpride's side effects, such as extrapyramidal symptoms, elevated serum prolactin, and weight gain (Leweke et al. 2012). Furthermore, according to several human and animal studies, CBD has anxiolytic effects (Devinsky et al. 2014). A recent study confirmed an anxiolytic effect in patients with social anxiety disorder using CBD whereby patients have shown changes in blood flow in both paralimbic and limbic brain regions (Crippa et al. 2011). Furthermore, several studies found that CBD to attenuate brain damage following ischemic and/or neurodegenerative conditions (ElBatsh et al. 2012; Devinsky et al. 2014).

1.3.2 Pharmacokinetics

CBD is a highly lipophilic compound with a high volume of distribution, approximately 32 L/kg, and rapid distribution into the adipose tissue, brain and other organs (Devinsky et al. 2014). It has also been estimated with high protein binding and it may accumulate in adipose tissue especially in chronic administration (Devinsky et al. 2014). Similar to most cannabinoids, the metabolism of CBD occurs in the liver where it is hydroxylated by the CYP2C (8/9/19) CYP3A (2/4) to form OH-CBD (Devinsky et al. 2014). After further metabolism, the metabolites are excreted in the feces and urine and its terminal half-life is estimated between 18 and 32 hours (Devinsky et al. 2014).

1.3.2.1 Adverse events

In humans, multiple studies of CBD safety have shown that CBD is well tolerated in a wide dosage range. It does not produce significant side effects in terms of the central nervous system, does not have significant effects on vital signs or changing mood, having been studied at doses of up to 1500 mg/day (orally) or 30 mg (intravenously) in acute and chronic administration (Bergamaschi et al. 2011). A study of Thiele et al. shows that CBD produces some side effects such as vomiting, pyrexia, loss of appetite, somnolence, and diarrhea (Thiele et al. 2018). In addition CBD inhibited production of Interleukin 8 and 10 to induce apoptosis of lymphocytes in vitro (Srivastava, Srivastava, and Brouhard 1998; Wu et al. 2008).

Based on screening of Devinsky et al., most of the studies were done in adults (Devinsky et al. 2014). Therefore, more studies need to assess the CBD pharmacokinetics and toxicity in children.

1.3.3 CBD*, neuroprotection and neuropsychiatric disorders*

Millions of people in the world are suffering from neuropsychiatric disorders such as anxiety, depression, schizophrenia (World Health Orgnization 2018). These disorders are among the most complex medical conditions that cause incapacity (Department of Health Statistics and Information Systems and WHO 2013). Although these neuropsychiatric disorders are caused by several interaction factors, such as the environment, medications and genes (ROY et al. 2014; Levinstein and Samuels 2014), the specific etiology of these disorders are not well understood which causes patients to have a limited access to suitable treatments (Levinstein and Samuels 2014).

Within the last few years, many researchers have worked to find new therapeutic targets for these neuropsychiatric disorders and focused on the neuroplastic cellular processes to treat or reduce symptoms of these disorders through neuroprotective mechanisms such as oxidative stress, neurotrophic factors and immune mediators (Kalivas and O'Brien 2008; Alline C. Campos et al. 2016; Bredt et al. 2015).

CBD shows a large spectrum of potential therapeutic properties in animal models as well as in humans, including antidepressant, antianxiety (A. C. Campos et al. 2012; A. W. Zuardi et al. 1993), anti-inflammatory (Mori et al. 2016; Shimon Ben-Shabat et al. 2006), neuroprotective (A. C. Campos et al. 2012) and immunomodulatory (Kozela et al. 2010). Moreover, CBD reduces the inflammatory cytokines production and activation of microglial cells (Napimoga et al. 2009; Kozela et al. 2011).

Compared with other cannabinoids like THC, CBD has a better safety profile. For example high doses of CBD (reaching 1500 mg/day) are well tolerated in experimental animals and humans (Bergamaschi et al. 2011). It does not alter blood pressure, temperature of the body, heart rate, or produce catalepsy (Bergamaschi et al. 2011). Therefore, it has better safety profile, possibly because it does not have a direct effect at cannabinoid receptors (Scuderi et al. 2009).

The mechanism of action for CBD to produce these potential neuroprotective effects, particularly in neuropsychiatric disorders are still unclear; however, it may be due to its multiple pharmacological targets (Alline C. Campos et al. 2016).

1.3.3.1 The endocannabinoid system

Several cannabinoid agonists were shown to enhance neuroprotection through activation of CB1 and CB2 receptors (García-Arencibia et al. 2007; Kreutz et al. 2009).

Although many in vitro studies found that CBD has a low affinity for these receptors (Pertwee 2008), some effects of CBD seem to have an impact on these receptors. These effects may be through an increase of anandamide level by inhibition of its metabolism/uptake (Bisogno et al. 2001; Alline Cristina Campos et al. 2013). In addition, AM251, the inverse agonist of CB1 receptor, blocked the effects of CBD in conditioned fear on extinction and reconsolidation (Stern et al. 2012). Moreover, The CB2 receptor inverse agonist AM630, attenuated CBD effects on reducing acute and apoptotic brain damage through effecting on production of TNF-,COX-2 and IL-6 (Castillo et al. 2010).

1.3.3.2 $5HT_{1A}$ receptors

Previous studies suggested that CBD produces many effects through interaction with serotonin receptors (Resstel et al. 2009; Fogaça et al. 2014). Until now there are seven types of serotonin receptors have been discovered: six of them are G-protein coupled and one iontropic. The $5HT_1$ type is $G_{i/o}$ protein coupled and have five subtypes: $5HT_{1A}$, $5HT_{1B}$, $5HT_{1D}$, $5HT_{1E}$, and $5HT_{1F}$. These receptors are located in pre-synaptic membranes and also present post-synaptically in many brain regions (European Behavioural Pharmacology Society et al. 1997). According to the opinion of Fernández-Ruiz et al is that the main receptor that produces neuroprotective effect of CBD is $5HT_{1A}$ (Fernández-Ruiz et al. 2013). As Compos et al. and Resstel et al. have indicated CBD produces anxiolytic effects by activation of $5HT_{1A}$ receptors (Alline Cristina Campos et al. 2013; Resstel et al. 2009). In addition to anxiolytic effects, a study by Linge et al. shows that CBD induced antidepressant effects by working as an agonist at these receptors (Linge et al. 2015).The neuroprotective effects of CBD has also been linked with $5HT_{1A}$-mediated mechanisms. Pretreatment with WAY100635 which is $5HT_{1A}$ receptor

antagonist prevented CBD reduction of damage in brain tissue caused by cerebral artery occlusion (Hayakawa et al. 2007; Mishima et al. 2005).

1.3.3.3 Oxidative stress and peroxisome proliferator-activated receptor gamma (PPARγ)

Oxidative stress is described as an imbalance between the production of free radicals and the ability of the organism to detoxify their effects by producing antioxidants (Alline C. Campos et al. 2016). The production of free radicals such as reactive oxygen/nitrogen species (ROS/RNS) can be unsafe to the human body because these compounds are very reactive with other compounds inside the body and will negatively impact fatty acids, proteins, and DNA leading to cellular death (Niedzielska et al. 2016; Pisoschi and Pop 2015).

Cannabidiol has a polyphenolic nature (see Figure 1.1) making CBD a potent antioxidant. Moreover, many studies show CBD reduced tyrosine nitration, malondialdehyde (MDA) levels and decreased apoptosis and neural cell death (El-Remessy et al. 2003, 2006). In addition, CBD produces antioxidant effects through reducing ROS accumulation, caspase-3 levels, lipid peroxidation, and DNA fragmentation in Alzheimer disease and multiple sclerosis models (Iuvone et al. 2004). Moreover, CBD reduces production of ROS cell death induced by hydrogen peroxide (H_2O_2) (Mecha et al. 2012). Beside the effect of CBD on ROS production, CBD activates peroxisome proliferator-activated receptor gamma (PPARγ) which has an effect to reduce cellular proliferation, apoptosis and reduction of damage induced by free radicals (Rodrigues et al. 2014; Scuderi, Steardo, and Esposito 2014).

1.3.3.4 Immune mediators, BDNF, and other related mechanisms

The beneficial effects of CBD on neuropsychiatric disorders have also been associated with its effect on pro-inflammatory cytokines and brain-derived neurotrophic factor (BDNF) expression. Low brain levels of BDNF and excess pro-inflammatory cytokines were associated with poor cognitive performance (Alline C. Campos et al. 2016). These effects were reduced by CBD treatment (Barichello et al. 2012). In addition, CBD decreased activation of microglia in models of Alzheimer's disease and schizophrenia (Gomes et al. 2015; Mecha et al. 2013). CBD also decreased release of IL-6 and IL-17, which reduced the severity of experimental autoimmune encephalomyelitis (EAE) (Campos et al. 2016)

1.3.3.5 Inhibition of adenosine uptake

Activation of adenosine signaling, by increasing extra-cellular levels of adenosine (Mijangos-Moreno et al. 2014), was suggested to mediate part of CBD effects such as anti-inflammatory, immunosuppressive and neuroprotective (Carrier, Auchampach, and Hillard 2006). In addition, the work of Mechaet al. shows antagonists of the A2A receptor prevented CBD neuroprotective effect (Mecha et al. 2013).

These mechanisms of CBD clearly indicate that CBD can be a new opportunity to treat several brain disorders.

1.3.4 CBD *in Dravet and Lennox-Gastaut syndromes*

Dravet and Lennox-Gastaut syndromes are rare, genetic forms of childhood epilepsy and associated with developmental delays (Devinsky et al. 2016). Both start in the first year of life and are difficult to treat (Devinsky et al. 2016). CBD has recently been demonstrated as an effective for the treatment of Lennox-Gastaut syndrome (Devinsky

et al. 2018; Thiele et al. 2018a) and drug-resistant seizures in children afflicted with Dravet syndrome (Devinsky et al. 2017). This places CBD among the few treatments useful in these conditions. In addition to seizures, children with Dravet syndrome also typically suffer from cognitive impairment and developmental delays (Dravet and Oguni 2013). Although not yet supported by controlled clinical studies, survey-based quality of life assessments suggest that CBD treatment, in addition to having anti-seizure efficacy, may also mitigate associated cognitive deficits, and improve social interaction (Rosenberg et al. 2017).

1.4 Vocal defect disorders

According to Morris et al. about 17.9 million adults have difficulty using their voice in the US (Morris et al. 2016). In addition, 9% of young children have speech sound disorder (National Institute on Deafness and Other Communication Disorders (NIDCD) 2016). Because a defect in vocal production can give rise to different effects, such as impaired social development and social interactions, more research should be focused on improving vocal learning (Fusaroli et al. 2016). There are many disorders that cause or are associated with vocal defects including stroke and autism (Özbal Koç et al. 2016; Mayo clinic staff 2015).

Globally, approximately 21.7 million people have autism disorder in 2013 (Global Burden of Disease Study 2013 Collaborators 2015). In the United States, about one in 68 children (about 1.5%) were diagnosed with autism in 2014, and in 2012 was one in 88 children (Autism and Developmental Disabilities Monitoring Network Surveillance Year 2008 Principal Investigators and Centers for Disease Control and Prevention 2012; Blumberg et al. 2013). One of the characteristics that is associated with autism is a vocal

learning defect (Condro and White 2014; Panaitof 2012) and about 50% or more of these patients displayed early abnormal acoustic patterns (Fusaroli et al. 2016; Paul et al. 2005; Rogers et al. 2006; Shriberg et al. 2001). In addition to autism, vocal defects can be caused by stroke that occludes blood flow to the part of the brain that is responsible for activating the larynx (Özbal Koç et al. 2016; Mayo clinic staff 2015; NIH 2015; Ito et al. 2008). Consequently, the need to find a drug to combat this disorder is highly desired. This project investigated the effects of CBD, and how it `mitigates CNS damage and improves vocal learning.

1.5 Zebra Finch Model

The zebra finch songbird is a good model for the study of auditory and speech disorders for many reasons: in a manner similar to humans, male zebra finches learn how to produce a form of vocal communication (Scharff and Nottebohm 1991). Both species must learn their respective forms of communication from the sounds of adults of the species (Doupe and Kuhl 1999). In the case of a human, a baby will listen to the parents, and after a few months, he will start to talk. A similar process occurs with baby zebra finches; he will listen to his tutor, and after few weeks, he will start to sing. Also, both species use their auditory system as well as their vocal motor production of song to learn to vocalize (Doupe and Kuhl 1999; Brainard and Doupe 2002). In addition, both the zebra finches and humans have vocal learning and motor brain pathways (Nottebohm 2005; Simmonds 2015). The learning and production of bird song involves distinct brain areas that are divided into two pathways: a vocal learning pathway and a vocal motor pathway (Simmonds 2015). The vocal learning pathway involves these areas: lMAN, Area X , and DLM whereas the vocal motor pathway involves these areas: HVC and RA (Nottebohm

2005; Simmonds 2015). Similar to the birds, humans have vocal learning and vocal motor pathways that are involved in learning and producing vocalizations (Simmonds 2015). The vocal learning pathway involves these areas: cortex, striatum, and thalamus. The vocal motor pathway involves these areas: premotor cortex and motor cortex (Simmonds 2015) (see Figure 1.3) .In conclusion both species have similarity in using their auditory system, listening to a tutor for vocal learning, having vocal learning and motor brain pathways and have similar neuronal circuits. For these reasons, the zebra finch is a good candidate to test the hypotheses of this project.

1.5.1 Neural pathways in the song system

Neural pathways in the bird's song system starts from (vocal motor pathway) HVC, which sends a message to the (vocal learning pathway) Area X and RA in vocal motor pathway. In the vocal motor pathway, a message goes to RA from HVC, then from RA, the message will travel to the hypoglossal nerve (nXIIts), which controls muscles of the syrinx. After the completion of the neural pathways, the song of the bird will be produced (Mooney 2009; RODERICK A. SUTHERS 2004). Whereas in the vocal learning pathway, a message is sent from HVC to Area X, then to the DLM, then it will reach to lMAN, after that vocal motor pathway will receive the message from lMAN through RA (Mooney 2009; RODERICK A. SUTHERS 2004). According to previous studies, when birds are young, lMAN plays a large role in controlling activity in RA and is essential for birds to learn song (Scharff and Nottebohm 1991). As learning is completed, HVC assumes primary control over RA activity (Reiner et al. 2004).

1.6 Goal of Research and Statement of Hypothesis

About 17.9 million adults and 9% of young children have difficulty using their voice in the US (National Institute on Deafness and Other Communication Disorders (NIDCD) 2016). This defect can be caused by or associated with some diseases such as stroke, trauma and autism (Godoy et al. 2014; Mayo clinic staff 2015; Özbal Koç et al. 2016). In addition, children with Dravet syndrome typically suffer from cognitive impairment and developmental delays which are associated with vocal defects (Dravet and Oguni 2013). Moreover, a vocal production and learning defect can give rise to other problems including impaired social development and interaction (Fusaroli et al. 2016). Consequently, the need to find a drug to combat this disorder is highly desired.

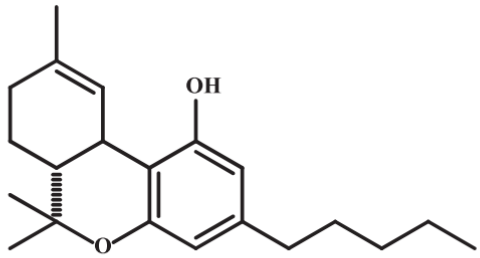
Moreover, survey-based quality of life assessments suggests that CBD treatment, in addition to having anti-seizure efficacy, may also mitigate associated cognitive deficits, and improve social interaction (Rosenberg et al. 2017). Importantly, quality of life assessments also suggests improved language, but better efficacy in children with families that relocated for treatments indicate a contributing placebo effect (Press, Knupp, and Chapman 2015). In addition to this observation, anxiolytic effects of CBD which will help to reduce stress that might be helping to improve vocal learning. Therefore, these effects as well as other effects such as anti-inflammatory and antioxidant effects will support the hypothesis of this project that CBD has distinct efficacy to mitigate CNS damage and promote vocal learning using zebra finches as a model. Stereotaxic techniques were used to make electrolytic lesions. This electrolytic lesions will target HVC to disrput vocal motor pathways and lMAN to make a defect in vocal learning. Then, the effects of CBD will examine.

Specific Aim 1: To determine whether CBD has distinct efficacy to mitigate CNS damage. The working hypothesis is that CBD will speed recovery time of song and will reduce the extent of phonetic change after a partial lesion of the pre-motor brain region, HVC. In effort to identify the effect of CBD on CNS damage, three different types of songs analyses were measured: KL distances analysis was used to assess the effect of CBD to improve acoustic features, the sequence analysis was measured to investigate the ability of CBD to improve syntax and production analysis was used to assess vocal motor activity.

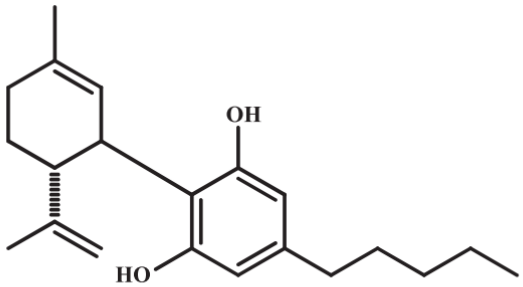
Aim 2: To evaluate whether CBD has an impact to promote vocal learning. Our working hypothesis is that CBD will enhance normal vocal learning as well as improve vocal learning following unilateral lMAN lesions. We will measure the effects of CBD using K-L distance measures, sequence analysis and production analysis in order to know if CBD has ability to improve vocal learning.

At the completion of this study, we will have further understanding of the effects of CBD to improve recovery following damage to a pre-vocal motor brain region. This may help us to develop a new therapeutic intervention for treatment of speech- and/or language-related disorders. In addition to demonstrating CBD efficacy, the study will provide a pre-clinical animal model suitable for evaluation of drugs that modulate vocal learning and behavior.

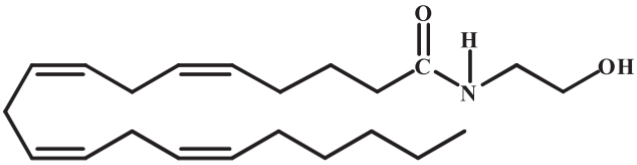
Figure 4.1: Structures of the phytocannabinoids Δ-9-tetrahydrocannabinol and cannabidiol and the endogenous cannabinoids 2 arachidonoyl glycerol and anandamide.



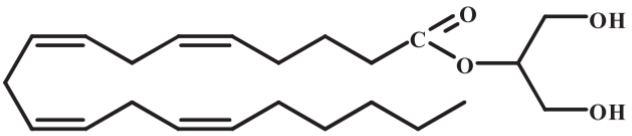
Δ^9-tetrahydrocannabinol (Δ^9-THC)



cannabidiol (CBD)



arachidonoyl ethanolamide (anandamide)



2-arachidonoyl glycerol (2-AG)

(Adapted from Mechoulam and Parker 2013)

Figure 1.2: Endocannabinoid hydrolysis.

In the nervous system, anandamide is degraded primarily by FAAH to produce arachidonic acid and ethanolamine, and 2-AG is degraded primarily by MAGL to produce arachidonic acid and glycerol.

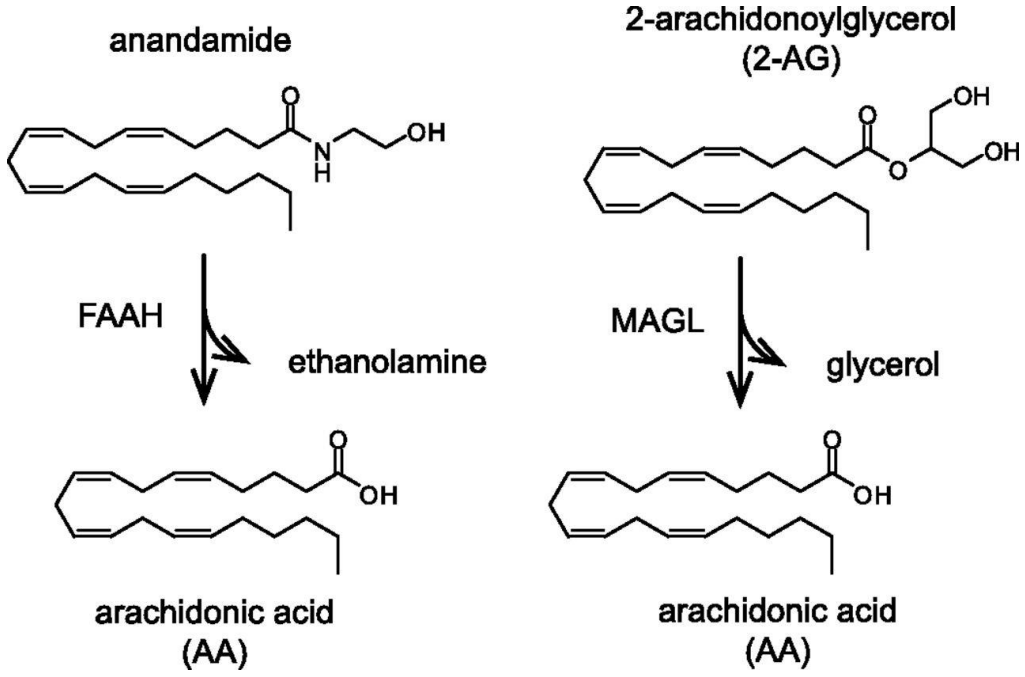
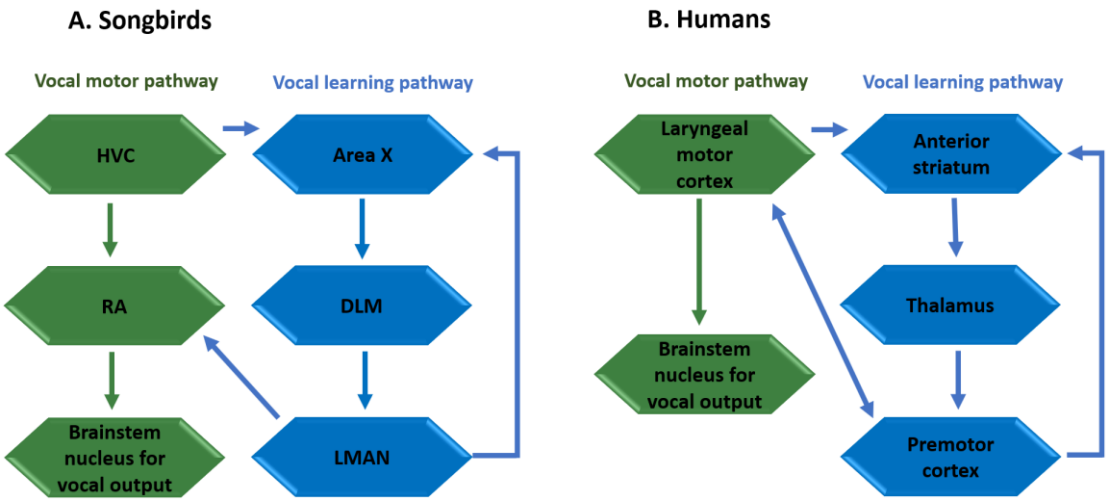

anandamide
O
N
H
OH
FAAH
ethanolamine
O
OH
arachidonic acid
(AA)
2-arachidonoylglycerol
(2-AG)
O
O
OH
OH
MAGL
glycerol
O
OH
arachidonic acid
(AA)

Figure 1.3: Vocal motor and learning pathways on human and zebra finch.

(i) The learning and production of a bird song involves distinct brain areas that are divided into two pathways: a vocal learning pathway and a vocal motor pathway. The vocal learning pathway (shown in blue) involves these areas: lMAN, Area X, and DLM whereas the vocal motor pathway (shown in green) involves these areas: HVC, RA and brainstem nucleus for vocal output. **(ii)** Similar to the bird, humans have vocal learning and vocal motor pathways that are involved in learning and producing vocalizations. The vocal learning pathway (shown in blue) involves these areas: anterior striatum, thalamus and premotor cortex. The vocal motor pathway (shown in green) involves these areas: laryngeal motor cortex and brainstem nucleus for vocal output

Vocal Pathways



CHAPTER 2: CANNABIDIOL IMPROVES VOCAL LEARNING-DEPENDENT RECOVERY FROM, AND REDUCES MAGNITUDE OF DEFICITS FOLLOWING DAMAGE TO A CORTICAL-LIKE BRAIN REGION IN A SONGBIRD PRE-CLINICAL ANIMAL MODEL

2.1 Introduction

Cannabidiol (CBD) is one of many molecules derived from *Cannabis* that is structurally-related to the principal eurphorigenic cannabinoid, Δ^9-tetrahydrocannabinol (THC). Although not associated with euphoria like THC, CBD is clearly psychoactive and is known to interact with many cellular macromolecules expressed within CNS (reviewed by Ibeas Bih et al., 2015; Soderstrom et al., 2017).

CBD was recently demonstrated as effective for the treatment of Lennox-Gastaut syndrome (Devinsky et al., 2018; Thiele et al., 2018) and drug-resistant seizures in children afflicted with Dravet syndrome (Devinsky et al., 2017). This places CBD among few treatments useful in these conditions. In addition to seizures, children with Dravet syndrome also typically suffer from cognitive impairment and developmental delays (Dravet and Oguni, 2013). Although not yet supported by controlled clinical studies, survey-based quality of life assessments suggest that CBD treatment may also mitigate associated cognitive deficits, and improve social interaction including language (Press, Knupp, and Chapman 2015; Rosenberg et al. 2017).

In addition to reports of improved cognition in children with seizure disorders, accumulating evidence indicates that CBD improves function in models of Alzheimer's and ischemic stroke (Ceprián et al. 2017; Cheng et al. 2014; Hayakawa et al. 2007; Mori

et al. 2016). Combined, this evidence suggests CBD may have distinct neuroprotective efficacy to promote recovery of complex behaviors following CNS disruption. In order to test this hypothesis, a songbird model has been applied. Songbirds, like the zebra finch, are among few vocal learning animals and are the only species well-suited to laboratory use (Petkov and Jarvis, 2012). Because song is complex and must be learned, the songbird model provides a means to evaluate drug effects on a behavior that depends upon higher brain function.

In adult zebra finches, electrolytic destruction of a small part (about 10%) of the cortical-like pre-motor region HVC (proper name, see Figure 1A) results in a temporary disruption of vocal patterns that recover over about seven days (Thompson and Johnson, 2007). Recovery from microlesions depends upon the ability of birds to hear, as deafened birds do not regain ability to produce typical song patterns (Thompson et al., 2007). Hearing-dependence indicates recovery requires auditory feedback necessary for adult sensorimotor vocal learning. Thus, the model allows assessment of learning-dependent recovery of a cognitively-complex behavior following CNS disruption – and evaluation of potential drug effects on this process. Further application of this model will allow the mechanism(s) of CBD action to be elucidated with potential relevance to positive cognitive effects reported in seizure trials.

2.2 Materials and Methods

2.2.1 Materials

Unless otherwise indicated, all materials and reagents were purchased from Sigma or Fisher. CBD was provided as a >95% pure crystalline powder by GW Research Ltd, Cambridge, UK. The 5-HT_{1A}–selective antagonist WAY-100,635 (WAY) was purchased from Tocris, USA. CBD was suspended in vehicle from 10 mM ethanol stocks. Vehicle consisted of a suspension of 2:1:17 Ethanol:Alkamuls EL-620 (Rhodia, Cranberry, NJ):phosphate-buffered saline. Daily ethanol dosages were 0.33 mg/kg - lower than that voluntarily consumed (Olson et al., 2014). WAY was diluted from a 10 mM stock in water and diluted for injection in sterile PBS.

2.2.2 Experimental design

The treatment plan is summarized in Figure 1B. Experiments spanned twenty days. During the first three recording days, no treatments were given. Pre-treatment recordings were used to generate baseline measures to which later daily recordings could be compared. After baseline recordings, once daily treatments began in the morning in a volume of 50 μl IM to pectoralis. Six daily treatments were given prior to surgical procedures for two reasons: (1) to assess potential for CBD administration without other interventions to alter vocal behavior, and (2) in an effort to allow CBD, a lipophilic drug with large volume of distribution and elimination half-life, to approximate steady-state levels (Gamble et al. 2018). Surgeries were done on day 10 and treatments and recordings continued to follow recovery to day 20.

2.2.3 Animals and audio recording environment

Adult male zebra finches (>90 days of age) were raised in our breeding aviary and maintained at 78°F on a 12/12 light/dark cycle. They were individually housed in standard finch cages (9”x11”x17”) placed within recording chambers with *ad libitum* food and water. Chambers were fitted with individual microphones and lights. Birds were visually isolated while continuously recorded. Sound Analysis Recorder software (Tchernichovski et al., 2000) was used to record vocalizations and store in Waveform Audio File (WAV) format. Birds were placed in recording chambers several days (3 to 5) prior to experiments. Animals not producing at least 500 song bouts per day during the last three habituation days were excluded. All animal procedures were approved by the East Carolina University Animal Care and Use Committee.

Intended group numbers were n=6 animals per treatment (vehicle, 1, 10, 100 mg/kg CBD and 0.1 mg/kg WAY + 10 mg/kg CBD, Figure 1C) and surgery condition (Microlesion, Sham and No-microlesion). One of the 10 mg/kg CBD animals in the microlesion group had lesion extent = 1.9%, exhibited minimal, sham-like effects on vocal behavior, and was therefore removed from the study (n=5). The 5-HT_{1A} receptor antagonist WAY was used in an attempt to reverse CBD effects. This was done because evidence suggests that CBD acts, at least in part, through agonism of the 5-HT_{1A} receptor in other systems (Resstel et al. 2009; Russo et al., n.d.). Only microlesion group animals received the combined 0.1 mg/kg WAY + 10 mg/kg CBD drug treatment (n=6). Microlesion group animals sang an overall mean of 5.9 (+/- 0.12) motif syllables (range =

5-7 syllables) and motif complexity did not differ significantly across the five treatment groups (1-way ANOVA, $F(4,24)=0.10$, $p=0.99$).

2.2.4 Microlesion surgeries

Bilateral HVC microlesions were made following the procedure previously described (Thompson et al., 2007) except that animals were administered the anti-inflammatory drug meloxicam (1 mg/kg) prior to procedures and were anesthetized with isoflurane. Birds were secured in a stereotaxic instrument and the bifurcation at the midsagittal sinus was used as stereotaxic zero. Small craniotomies were placed over HVC bilaterally. For approximately 10% destruction of HVC, four locations were targeted: 2.4 and 2.8 mm bilaterally from stereotaxic zero to a depth of 0.6 mm. Microlesions were made with 100 μA for 35s. Birds recovered in a warm incubator and were returned to recording chambers. Sham-group animals were subjected to all of the steps described above (including anesthesia, craniotomies and suturing) except no current was passed. No-microlesion group animals were transported to the operating room without other manipulation.

2.2.5 Lesion extent

Following final recordings, animals were overdosed with Equithesin. Brains were fixed with 4% paraformaldehyde, blocked down the midline and both hemispheres sectioned through HVC at 40 μm. Sections were Nissl stained and those containing HVC were imaged at 40X. Lesion damage extent was calculated by tracing borders of both HVC and infarcts, if present, using ImagePro Plus software. The total area of HVC and

the infarct were summed and percent lesion extent expressed as infarct area divided by HVC area x 100%.

2.2.6 KL distance measures of phonology

Animals were recorded continuously over the entire 20-day experimental period. Recording files were input to Sound Analysis Pro 2011 software (SAP, Tchernichovski et al., 2000) to segment song bouts into their separate syllable components (uttered sounds separated by silence). Segmentation was accomplished by thresholding based upon amplitude, entropy, syllable and syllable gap durations. Thresholding was optimized for each animal, all other procedures were done objectively. Others have reported (Wu et al., 2008) and we confirm that analyses of fewer than approximately 300 syllables is associated with underestimated phonology quality. Syllable numbers rarely reached 300 on microlesion days and so these days were excluded from analyses. Other days not meeting this criteria (typically due to recording equipment malfunction) were also excluded.

SAP characterizes individual syllables by their spectral structure through measures of acoustic features (e.g. syllable duration, amplitude, pitch, etc.) Acoustic feature measures were used to assess phonology via calculation of Kullback-Leibler (KL) distances using the methods developed by (Daou et al., 2012; Wu et al., 2008) that compare distances between 2D probability distributions of vocal acoustic features. Greater KL distance measures reflect increased phonological divergence across the vocalizations compared. We used acoustic measures from baseline recordings (days 1–3, Fig. 1B) as the “template” distribution, and recordings from following treatment days as

individual “targets”. KL distances between template and target distributions were calculated using software we developed (KLFromRecordingDays available as described in (Soderstrom and Alalawi, 2017). Because baseline days were used as the template, KL distances for this period = 0, and higher values observed on following days represent phonological divergence from baseline measures. Significant KL distance measure outliers, detected by the method described by Grubbs, 1969, were excluded from statistical analysis.

2.2.7 Typical syllable transition measures of syntax

Syntax was measured from the frequency of typical syllable transitions that were calculated using SongSeq software according to the method described by Daou et al., 2012. SongSeq uses data exported from SAP to identify distinct syllables. Pre-treatment day three was used as the template to compare target datasets generated from recordings made during each treatment day. Distinct syllable types were identified by clustering within a plot of an acoustic feature measure on the y-axis and syllable duration on the x-axis. One or two acoustic features were used as necessary to distinguish two-dimensional syllable clusters. These parameters were optimized for each subject. Once syllable clusters were identified, SongSeq calculated probabilities of each possible pair of syllable types being produced on that recording day. The highest probability transition on the baseline day for each syllable type was designated the “typical transition” for that syllable pair. The percentage of typical transitions for each treatment day was then calculated as the number of typical transitions divided by total transitions x 100%.

2.2.8 Vocal production measures

To measure vocal production, recordings in WAV format were sorted using the method described by Wu et al., 2008 to ensure each documented an incidence of singing (not only calls/extraneous sounds). Sorted song files were counted and each was considered a bout of singing. The daily average number of song bouts during baseline days 1–3 were determined and used to calculate percent baseline song bouts from counts taken for each pre- and post-microlesion day.

2.2.9 Statistical analyses

Potential lesion extent differences across drug treatment groups were assessed using 1-way ANOVA. Lesion extent data are expressed as means +/- SEM. To assess differences in phonology, syllable sequencing and vocal production over experiment day, across treatment groups and microlesion conditions, we used a mixed-effects modeling approach with SPSS software (version 22). This method controls for lack of independence of repeated measures derived from single animals (e.g. 20 daily measures from each animal, Aarts et al., 2014).

For mixed model analysis of phonology, syntax and production data, individual animals were treated as random subjects and lesion group (microlesion, sham-microlesion, no-microlesion), drug treatment (vehicle, 1, 10, 100 mg/kg CBD, 0.1 mg/kg WAY + 1 mg/kg CBD) and experiment day were used as fixed factors. For all mixed model analyses, the variance components covariance structure and the maximum likelihood method were used. Fixed explanatory variables were successively added to models.

Improvements to simpler models gained by variable additions were determined through likelihood ratio (LR) tests of differences between -2*log likelihood values from the fit of each model to vocal behavior data.

For each assessment, models included animal ID as a random factor to control for repeated measures, and experimental day was added as the first fixed explanatory variable. As subsets of animals were assigned to drug treatment groups (vehicle, 1, 10 and 100 mg/kg CBD and 0.1 mg/kg WAY + 10 mg/kg CBD) and these groups were further divided into lesion condition (microlesion, sham- and no-microlesion), microlesion group nested within CBD dosage were added as fixed factors. Differences between vehicle control and CBD-treated groups were determined from pairwise comparisons using the Bonferroni post-hoc correction. Probabilities less than 0.05 were considered significant. Mixed model statistics reported below are mean differences, with bracketed 95% confidence intervals. Figures three, four and five summarize means +/- SEM.

2.3 Results

2.3.1 Microlesion extent

The overall mean lesion extent was 8.6% +/- 0.7, (see Figure 2A). No significant differences were observed across groups following one-way ANOVA ($F[4, 24] = 0.38$, $p=0.82$). Notably, microlesion effects on syllable acoustics appear restricted to those that are part of learned vocalizations – unlearned, instinctive, call-type syllables appear normal (Figure 2C and D).

2.3.2 Mixed model fit to phonology, sequence and vocal production data

The mixed model fit of vocal behavior measures to explanatory variables was optimized as described above. Adding experimental day as a fixed factor to models with animal ID as a random factor significantly improved their fit to: (1) KL distance measures of phonology (note that these data were normalized by log transformation prior to analysis and that Figure 2.3 depicts untransformed means +/- SEM, likelihood ratio [LR] = 927 – 353 = X^2 574, 1 d.f., $p < 0.001$); (2) percent typical transition data (LR = 932 – 431 = X^2 501, 1 d.f., $p < 0.001$) and; (3) vocal production data (LR = 11919 - 11250 = X^2 670, 1 d.f., $p < 0.001$). These results demonstrated significant differences across experimental day for each measure. Next, microlesion condition group (microlesion, sham-microlesion and no-microlesion) nested within treatment group (vehicle, 1, 10, 100 mg/kg CBD, and for the 0.1 mg/kg WAY + 10 mg/kg CBD microlesion group) were added as fixed factors to models. These additions further improved model fit to: KL distance data (LR = 353 – 5.6 = X^2 347.4, 2 d.f., $p < 0.001$); percent typical transitions (LR = 431 – 201 = X^2 230, 2 d.f., $p < 0.001$) and vocal production (LR = 11250 – 11076 = X^2 174, 2 d.f., $p < 0.001$). This demonstrated significant differences across lesion condition and drug treatment groups for each vocal behavioral measure.

2.3.3 CBD improved phonology

KL distance measure differences across microlesion condition groups at each CBD dosage are summarized in Figure 2.3. Comparing microlesion group animals (Figure 3A) to sham- and no-microlesion groups (Figure 3C and D) demonstrated that, as previously

reported, HVC microlesions reversibly disrupted phonology for about seven days (Thompson et al., 2007; Thompson and Johnson, 2007).

Consistent with a protective effect of CBD, animals with microlesion treated with 10 or 100 mg/kg CBD showed significantly lower KL distance measures on multiple recovery days than vehicle controls (Figure 3A). Compared to VEH controls the 10 mg/kg CBD dosage significantly reduced KL distances on all recovery days (by: 0.74 [0.11-1.37], $p=0.011$; 1.20 [0.61-1.79], $p<0.001$; 0.95 [0.43-1.47], $p<0.001$; 0.82 [0.35-1.28], $p<0.001$; 0.71 [0.25-1.17], $p<0.001$; 0.64 [0.17-1.12], $p=0.002$; 0.78 [0.33-1.24], $p<0.001$; 0.77 [0.30-1.24], $p<0.001$; 0.58 [0.10-1.06], $p=0.007$ and; 0.70 [0.21-1.20] $p=0.001$, respectively). The 100 mg/kg CBD dosage reduced phonology disruptions on recovery days 1-5 and 7-10 (by 0.85 [0.24-1.46], $p=0.001$; 0.87 [0.30-1.43], $p<0.001$; 0.64 [0.15-1.13], $p=0.003$; 0.58 [0.14-1.02], $p=0.003$; 0.48 [0.04-0.91], $p=0.024$; 0.47 [0.034-0.90], $p=0.026$; 0.51 [0.07-0.95], $p=0.013$; 0.048 [0.02-0.93], $p=0.032$ and; 0.51 [0.04-0.99] $p=0.024$, respectively). Treatment with the 5-HT_{1A} antagonist WAY (0.1 mg/kg) prior to administration of 10 mg/kg CBD resulted in significantly lowering KL distances on recovery days 2-5 and 7 (by 0.95 [0.39-1.51], $p<0.001$; 0.70 [0.21-1.20], $p=0.001$; 0.55 [0.11-0.99] $p=0.006$; 0.49 [0.049-0.93], $p=0.019$ and; 0.51 [0.07-0.94], $p=0.011$, respectively), five fewer days than 10 mg/kg CBD alone.

2.3.4 CBD modestly improved syntax in microlesioned animals

Microlesion effects to disrupt syntax as measured by percent typical syllable transitions are evident from comparisons of Figure 4A to surgery control groups in Figure 4B and C. In contrast to phonology measures, the magnitude of syntax disruptions were

similar across treatment groups (Figure 4A). Also contrasting with phonology results was a modest treatment effect observed in sham-lesioned 10 mg/kg CBD-treated animals that had significantly lower syntax on recovery day 8 (-22.4% [-0.2 to -45.1], p=0.046, Fig 4B). This suggests an additive effect with the craniotomy procedure that is perhaps consistent with promotion of hypolocomotor efficacy (Britch et al. 2017).

Although the magnitude of syntax disruptions one day post-microlesion were similar across treatment groups, CBD-treated animals appeared to recover more rapidly than vehicle controls. This trend was significant in the case of 10 and 100 mg/kg CBD-treated groups. The 10 mg/kg CBD-treated group produced significantly higher percentages of typical syllable transitions than vehicle controls on recovery days 4 and 6 (by: 22.5% [1.5-44.9], p=0.049 and; 24.3% [3.0-45.6], p=0.014, respectively). The 100 mg/kg CBD improved syntax recovery on day 4 (by: 22.8% [1.5-44.2], p=0.028).

2.3.5 Complex CBD effects on vocal production

Consistent with earlier reports (Thompson et al. 2007) HVC microlesions effectively reduced vocal output of all treatment groups to a fraction of baseline levels (Figure 2.5A). Only 10 mg/kg CBD showed evidence of improved recovery of singing behavior relative to vehicle controls on recovery day 2 (by 68.6% [10.0-127.1], p=0.011). An interesting pre-microlesion trend toward a reduction in vocal activity was observed in the group treated with both WAY and CBD, which suggests a role for 5-HT_{1A} signaling in vocal motor processes, although this apparent difference was not statistically significant. Following microlesion procedures both 1 mg/kg CBD and WAY + CBD treatment groups exhibited significantly lower vocal output than vehicle controls. For the 1 mg/kg CBD

group this was on recovery days 5 (by 52.2 [1.7-102.8], p=0.038) and 9 (by 59.8 [1.6-117.9], p=0.039). For the WAY + CBD group this was on recovery day 9 (by 60.2 [2.04-118.3], p=0.037). Differential efficacy of 1 and 10 mg/kg CBD on vocal production suggests a complex, hormetic dose-response relationship that has been well-documented for CBD in other systems (e.g. Gallily et al. 2015; Antonio W Zuardi et al. 2017). Note that such “inverted U-shaped” responsiveness is also characteristic of lesion studies (Calabrese,2008). The sham procedure clearly reduced vocal output in controls and animals treated with 100 mg/kg CBD (Figure 5B). Both 1 and 10 mg/kg appeared to mitigate this sham effect with 1 mg/kg producing significant improvement relative to vehicle on the first recovery day (by 60.20% [5.35-115.00], p=0.023).

2.4 Discussion

2.4.1 CBD effects on microlesion recovery

Two of the three vocal behavior measures employed in this study (phonology and syntax) clearly indicate CBD is effective in mitigating effects of HVC microlesions (Figures 3A and 4A). The 1 and 10 mg/kg dosages appeared to modestly improve recovery of vocal output (Figure 5A). Improved recovery appears to involve both preventing effects following HVC damage (secondary to tissue damage itself), and to hastening the process.

Interaction with processes secondary to tissue damage is indicated by the lack of differences across treatment groups in the amount of HVC destroyed (by an average of 8.6% across all groups, Figure 2A). Thus, the effect of CBD was not to reduce the size of the infarct, and disruptive effects of HVC microlesions must involve processes distinct

from and secondary to tissue damage itself. Given similarities noted in studies employing ischemic stroke models (Ceprián et al., 2017; Mori et al., 2017) these secondary processes may include reductions of excitotoxicity, neuroinflammation, metabolic derangement, gliosis and/or protection of astrocyte function. Whatever the secondary processes are (and these will be important to address in future studies), their mitigation improves ability of adult songbirds to relearn to produce memorized vocal patterns. Elucidation of neurophysiological effects of CBD in this model may prove relevant to its clinical efficacy and potential to improve cognition in children with seizure disorders.

That relearning is required is evident from the fact recovery of vocalizations depends upon ability of birds to hear themselves sing. Deafened animals don't receive the auditory feedback necessary for sensorimotor relearning and do not recover high-quality song (Thompson et al., 2007). It may also be important that the microlesion effect is restricted to motif syllables and does not appear to impair instinctive calls (Figure 2C and D). This suggests that CBD prevents degradation of a learned behavior, consistent with improved memory in dementia models (Karl et al., 2017). Prior work has demonstrated lesions of lMAN (the output nucleus of the learning-essential anterior forebrain pathway [AFP, Bottjer et al., 1984]), prior to HVC microlesions, prevent impaired vocal behavior (Thompson and Johnson, 2007). This demonstrates that effects of HVC microlesions cannot be attributable to damage of HVC itself, but must follow from some related disruption involving lMAN. Therefore, brain regions and circuits outside of the motor-related microlesion target HVC must be involved in the efficacy of CBD to mitigate vocal impairment.

Brain regions with established relevance to the HVC microlesion model, and interconnections between them are summarized in Figure 1A. The different pathways and nuclei within these separate vocal control and production circuits have distinct effects on vocalizations. Activity within HVC is most clearly associated with syllable timing (Hahnloser et al., 2002) and those within lMAN and RA appear more important to phonology (Kao et al., 2005; Sober et al., 2008; Vu et al., 1994). As song is modulated by midbrain dopaminergic neuronal activity (Hara et al., 2007) vocal production also involves motivation systems important for incentive learning and reward. Thus, effects of CBD upon vocal production, syntax and phonology provide insight to potential brain nuclei, circuits and systems mechanistically involved.

2.4.2 CBD effects on vocal production

Relative to phonology and syntax measures, effects to improve vocal production were more modest and variable (Figure 5A). Variability may involve an “inverted U-shaped” hormetic dose-response relationship for this effect that has been described for CBD in other systems (Gallily et al., 2015; Zuardi et al., 2017). Modest efficacy in this motor-dependent measure may make sense in the context of established ability of CBD to reduce motor activity in rats (Espejo-Porras et al., 2013) although this rodent effect is thought attributable to striatal circuits. The songbird vocal motor system (Figure 1A) is likely more relevant to effects of CBD on vocal production observed here. This pathway is notably present in brains of vocal-learning mammals (e.g. humans, Petkov and Jarvis, 2012).

2.4.3 CBD effects on syntax

CBD accelerated recovery of typical syllable transitions as indicated by recovery in CBD-, but not vehicle-treated microlesioned animals, to pre-microlesion levels by the end of experiments (Figure 4A). Both 10 and 100 mg/kg CBD treatments were effective in improving this measure of syntax.

A potentially-important feature of syntax responses includes a trend in 1 and 10 mg/kg CBD-treated animals to exhibit a biphasic syntax recovery, with an initial rapid response over five to six days followed by a flat response or even regression in the 10 mg/kg group (Figure 4A). Regression in the 10 mg/kg group late in the recovery period is also suggested in sham-microlesioned animals, where typical transitions were significantly reduced relative to control on day 8 (Figure 4B). In addition to adding to evidence of complex CBD dose-response relationships, possible biphasic recovery suggests involvement of temporally-distinct physiological processes. Doses of 1 and 10 mg/kg CBD appear more effective in mitigating the earlier of these processes. The higher 100 mg/kg CBD dose demonstrated less of a biphasic restoration of typical syllable transitions, a result suggesting potentially distinct efficacy to improve syntax.

2.4.4 CBD effects on phonology

The most conspicuous CBD effect observed was reduction of the magnitude of KL distance measures immediately following microlesions. All CBD treatments significantly reduced phonological disruptions (Figure 3A) but in the case of both 10 and 100 mg/kg dosages the magnitude of this effect was particularly large. Again, this is intriguing given

that our microlesion target was HVC, a motor region most clearly associated with syntax-relevant syllable timing. Phonology appears more dependent upon the AFP and its lMAN output to RA (Figure 1A, Kao et al., 2005; Vu et al., 1994). Given that ability of microlesions to disrupt vocalizations depends upon intact lMAN (as discussed above, and see Thompson et al., 2007) a simple potential mechanism for observed ability of CBD to nearly eliminate phonology disruptions includes interference with lMAN activity. CBD inhibition of lMAN output is a hypothesis to be tested, and could follow from effects within any of the nuclei that comprise the AFP (Area X, DLM, lMAN, Figure 1A) or via action as far upstream as the HVC projection to Area X.

Finally, the fact that KL distances in 10 and 100 mg/kg CBD-treated animals never approached vehicle control levels, even immediately following microlesions (Figure 3A), suggests the six-day pretreatment period was an important factor. This has implications for potential therapeutic use of CBD in cases of CNS trauma that will depend upon after-incident administration. Thus, it will be important in future studies to establish relative contributions of pre- vs. post-microlesion CBD treatments.

2.4.5 Potential mechanism of action

Given evidence of CBD agonism of 5-HT_{1A} receptors (Resstel et al., 2009; Russo et al., 2005), we used an antagonist, WAY, as a first step toward identifying potential mechanisms. Originally described as a selective antagonist (Fletcher et al., 1995), it is now clear WAY also interacts with D_4 dopamine, $alpha_{1A}$-adrenergic and 5-HT_{2B} receptors with about 10X lower affinity than for 5-HT_{1A} (16.4, 19.9, 24 nM, respectively vs. 2.2 nM, Chemel et al., 2006; Fletcher et al., 1995). To minimize non-5-HT_{1A} effects, we employed

a very modest dosage of 0.1 mg/kg (note this dosage is >10X lower than required for rat discrimination [1.4 mg/kg, Marona-Lewicka and Nichols, 2009]). WAY pretreatment prior to 10 mg/kg CBD reduced the number of days with improved phonology and syntax by five and two, respectively (Figsure 3A and 4A). Partial reversal of CBD efficacy suggests 5-HT_{1A} involvement, but additional controls are needed for clear determination. Given the numerous macromolecular targets of CBD (reviewed by Ibeas Bih et al., 2015; Soderstrom et al., 2017) it is possible, if not likely, that multiple targets are relevant to the efficacy observed.

2.5 Conclusions

We have demonstrated ability of CBD to improve recovery following damage to a pre-vocal motor brain region. This improved recovery included both reduction of the magnitude of lesion effects, and less time required for restoration of vocal patterns. All CBD dosages showed efficacy, with 10 mg/kg appearing superior in phonology and on measures of syntax. Taken together, our results suggest CBD holds therapeutic promise for treatment of speech- and/or language-related disorders. This microlesion model will allow identification of mechanisms responsible for CBD-improved vocal behavior, and provide insight to signaling systems that control vocal learning and production. In addition to demonstrating CBD efficacy, the work has established a pre-clinical animal model suitable for evaluation of drugs that modulate vocal learning and behavior.

Figure 2.1: Experimental approach.

A, camera lucida-type drawing illustrating brain regions and circuits relevant to CBD effects on vocal behavior. Black shading corresponds to song regions within the section traced (HVC, RA, lMAN, Area X) and grey shading indicates striatum. Light grey areas with dashed borders indicate relevant regions not present in the traced section. Rose arrows indicate connections of the anterior forebrain pathway (AFP), a cortico-basal ganglia-thalamic loop critical for sensorimotor vocal learning (reviewed by Perkel, 2004). Note output from lMAN to the vocal motor output region, RA (Bottjer et al., 1989). Blue-green indicates vocal motor pathways. Note output from pre-motor HVC to the basal ganglia region, Area X. Gold arrows indicate auditory input to the motor system (Kelley and Nottebohm, 1979; Vates et al., 1996) and from the ventral portion of the intermediate arcopallium (AIV) to dopaminergic neurons within substantia nigra (SN)/ventral tegmental area (VTA, Mandelblat-Cerf et al., 2014). Note SN/VTA dopaminergic projections to spiny interneurons within Area X of striatum (Ding and Perkel, 2002). Rostral is right, dorsal is up and bar = 1 mm. Abbreviations: DLM (nucleus dorsolateralis anterior, pars medialis), HVC (proper name), lMAN (lateral magnocellular nucleus of the anterior nidopallium), CM (caudal mesopallium), RA (robust nucleus of the arcopallium). B, experimental time-line for audio recording, drug treatment, and surgery. C, summary of treatment groups used.

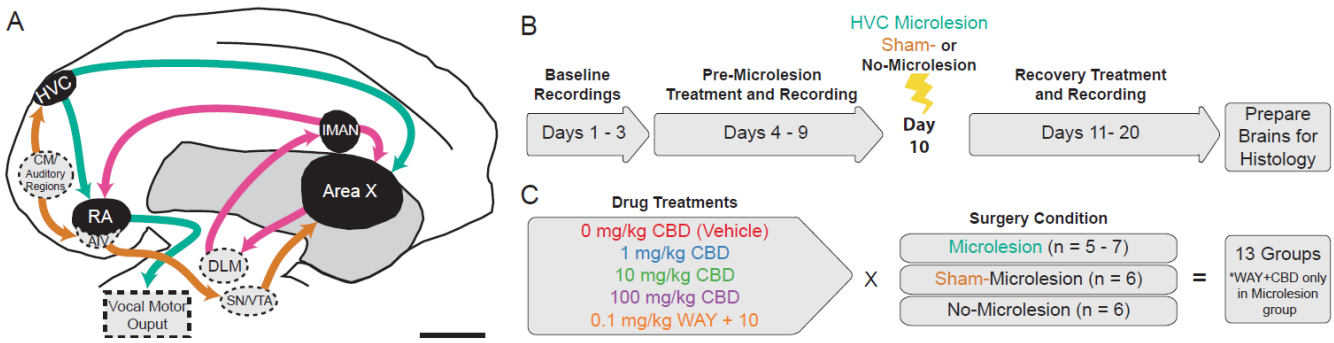

A
HVC
CM/
Auditory
Regions
RA
AIV
IMAN
Area X
DLM
SN/VTA
Vocal Motor
Ouput
B
HVC Microlesion
Sham- or
No-Microlesion
Baseline
Recordings
Days 1 - 3
Pre-Microlesion
Treatment and Recording
Days 4 - 9
Day
10
Recovery Treatment
and Recording
Days 11- 20
Prepare
Brains for
Histology
C
Drug Treatments
0 mg/kg CBD (Vehicle)
1 mg/kg CBD
10 mg/kg CBD
100 mg/kg CBD
0.1 mg/kg WAY + 10
X
Surgery Condition
Microlesion (n = 5 - 7)
Sham-Microlesion (n = 6)
No-Microlesion (n = 6)
=
13 Groups
*WAY+CBD only
in Microlesion
group

Figure 2.2: Effects of microlesions.

A, lesion extent did not differ across drug treatment groups (mean=8.6 +/- 0.7%, one-way ANOVA (F[4, 24]=0.38, *p*=0.82). B, illustration of method used to measure lesion extent of Nissl-stained parasagittal sections (dorsal up, rostral right, 12.5X inset bar = 1 mm, 40X bar = 300 μm). All HVC-containing sections were imaged. ImagePro software was used to trace both HVC and infarcts, if present. Lesion extent = infarct area/HVC area x 100%. C, audiospectrogram examples of song illustrate behavioral effects of microlesions in a vehicle control animal. On Baseline Day 1 motifs (indicated by bars) are produced with consistent syntax, and syllables (sound separated by silence) show good phonetic structure. Syntax and phonology of motif syllables are disrupted on Post-Microlesion Day 1, although phonetics of instinctive, unlearned calls appear unaffected. Phonology has improved by Post-Microlesion Day 3 while syntax remains impaired. By the final day of the experiment (Post-Microlesion Day 10) motif syntax and phonology have recovered. D, sonogram examples of microlesion effects on song in an animal treated with 10 mg/kg CBD shows good syntax and phonology on Baseline Day 3. Effects of the microlesion procedure on syntax and phonology are apparent on Post-Microlesion Day 1, although relative to the vehicle-treated control, effects are modest. Phonology and syntax structure have improved by Post-Microlesion Day 3. By Post-Microlesion Day 10, syntax and phonology have recovered.

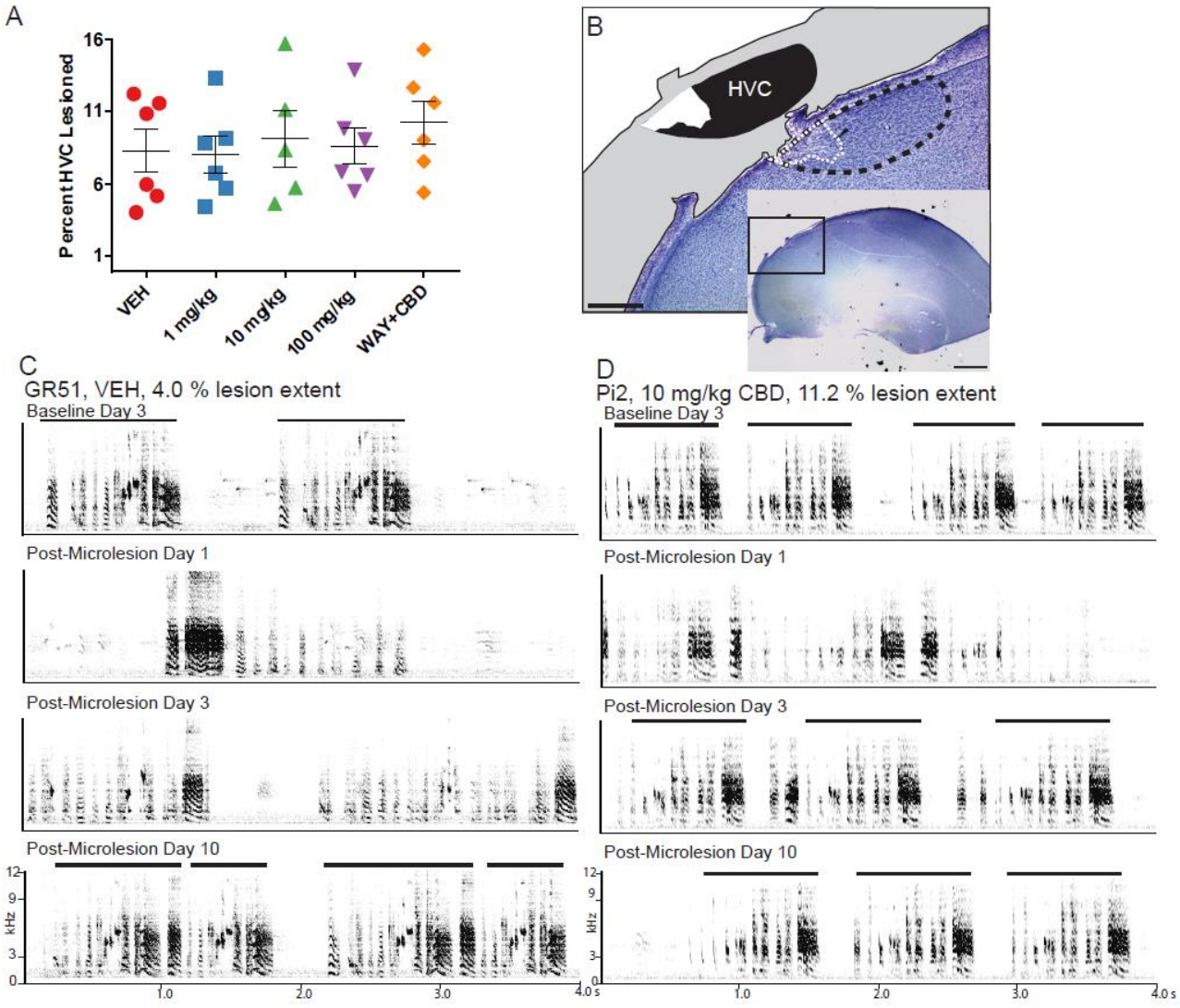

A
Percent HVC Lesioned
16
11
6
1
VEH
1 mg/kg
10 mg/kg
100 mg/kg
WAY+CBD
B
HVC
C
GR51, VEH, 4.0 % lesion extent
Baseline Day 3
Post-Microlesion Day 1
Post-Microlesion Day 3
Post-Microlesion Day 10
kHz
12
9
3
0
1.0
2.0
3.0
4.0 s
D
Pi2, 10 mg/kg CBD, 11.2 % lesion extent
Baseline Day 3
Post-Microlesion Day 1
Post-Microlesion Day 3
Post-Microlesion Day 10
kHz
12
9
3
0
1.0
2.0
3.0
4.0 s

Figure 2.3 :KL distance measures of phonology by surgery and drug treatment group.

A, in microlesioned animals, KL distances are clearly increased in VEH and 1 mg/kg CBD-treated animals indicating significant disruption of phonology relative to baseline. Asterisk color indicates significant differences from VEH (mixed-model ANOVA with Bonferroni correction for post-hoc comparisons). Both 10 and 100 mg/kg CBD treatments significantly reduced KL distance measures relative to VEH immediately post-microlesion suggesting protective effects produced by the six-day pre-treatment period. 10 mg/kg CBD was still effective following 0.1 mg/kg of the 5-HT_{1A}-selective antagonist WAY, although on fewer days than 10 mg/kg alone suggesting possible involvement of this receptor in the mechanism responsible for CBD efficacy. Panel B shows phonology trajectories from representative VEH- and 10 mg/kg CBD-treated animals (see Fig 2 C and D for sonogram examples). Panels C and D demonstrate little phonological change in sham- and no-microlesion control groups across treatment groups. Hatched line represents mean VEH-microlesion group trajectory. Shown are mean values +/- SEM.

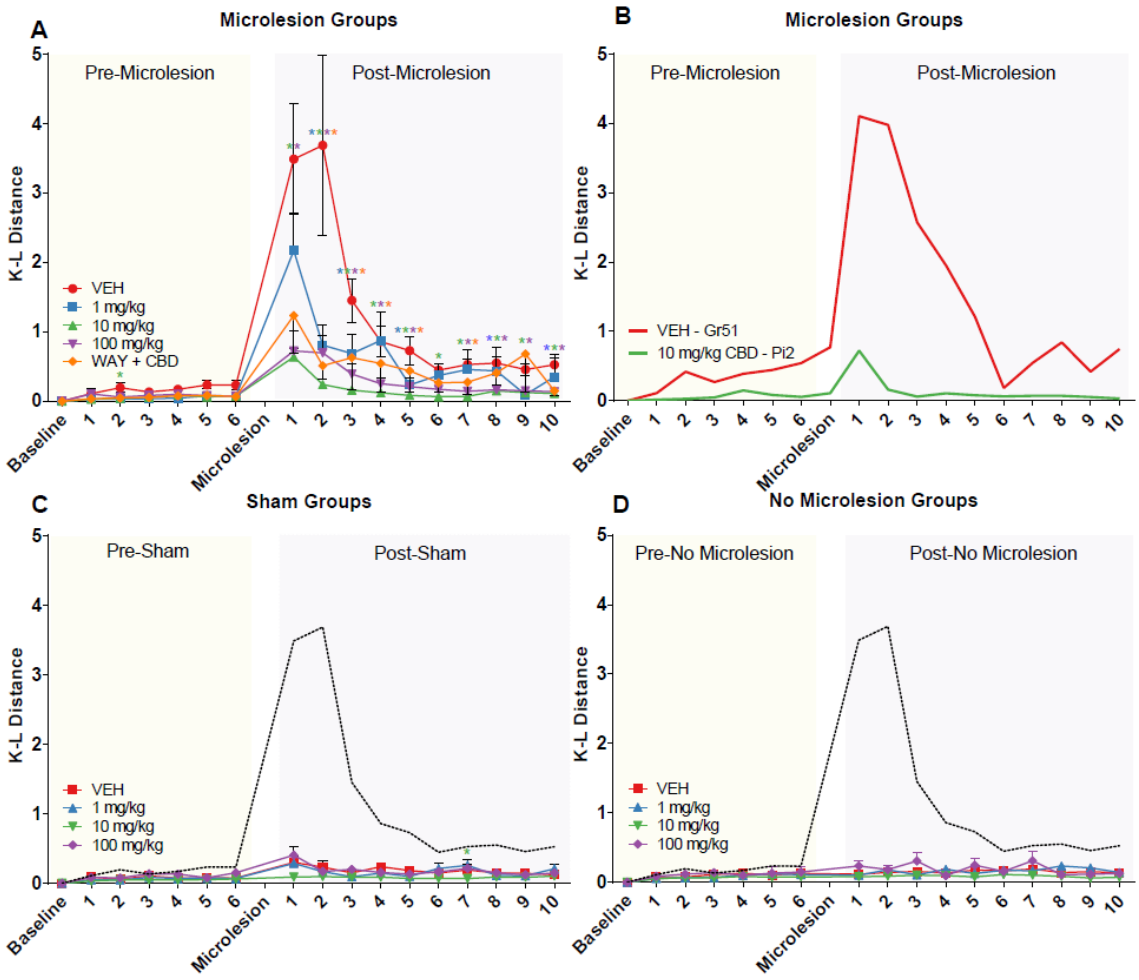

A
Microlesion Groups
Pre-Microlesion
Post-Microlesion
K-L Distance
VEH
1 mg/kg
10 mg/kg
100 mg/kg
WAY + CBD
Baseline
Microlesion
B
Microlesion Groups
Pre-Microlesion
Post-Microlesion
K-L Distance
VEH - Gr51
10 mg/kg CBD - Pi2
Baseline
Microlesion
C
Sham Groups
Pre-Sham
Post-Sham
K-L Distance
VEH
1 mg/kg
10 mg/kg
100 mg/kg
Baseline
Microlesion
D
No Microlesion Groups
Pre-No Microlesion
Post-No Microlesion
K-L Distance
VEH
1 mg/kg
10 mg/kg
100 mg/kg
Baseline
Microlesion

Figure 2.4: Syntax quality measured through percent typical syllable transitions by surgery and drug treatment group.

A, in microlesioned animals, typical transitions were significantly reduced in all treatment groups to a similar degree immediately post surgery. In 10 and 100 mg/kg CBD treatment groups syntax recovery was improved relative to VEH as indicated by significantly higher typical transitions on at least one recovery day. Asterisk color indicates significant treatment group differences determined by mixed-models post-hoc tests with the Bonferroni correction for multiple comparisons. Similar to effects on phonology, 0.1 mg/kg WAY + 10 mg/kg CBD resulted in two fewer days with significantly better syntax than CBD alone. Both 1 and 10 mg/kg CBD appeared to improve syntax recovery at a higher rate early in the recovery period, followed by a flattened response later. 100 mg/kg showed consistent efficacy over recovery days. B, the sham procedure had little effect on syntax measures. Significantly lower syntax in 10 mg/kg CBD-treated sham controls relative to VEH late in the recovery period is similar to the leveled-off response observed in microlesioned animals, and may indicate an interaction with the craniotomy procedure. C, no effect on syntax was observed in no-microlesion controls. Shown are mean values +/- SEM.

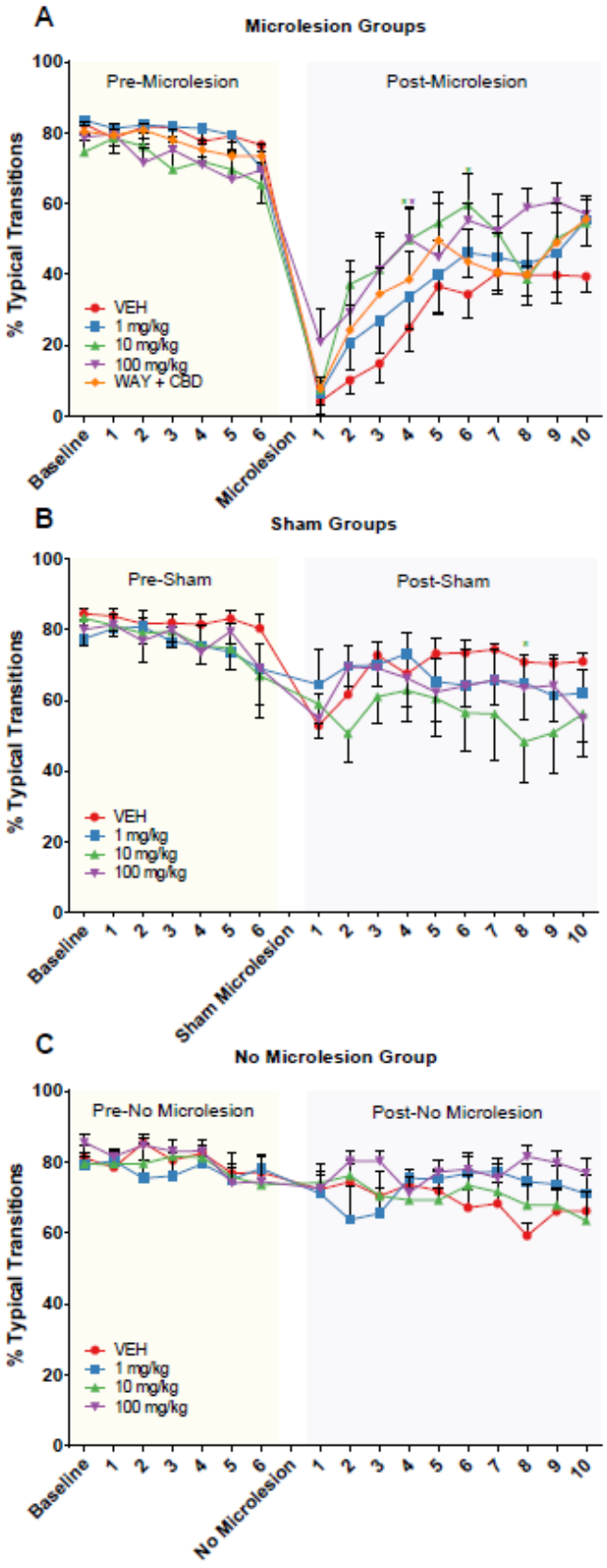
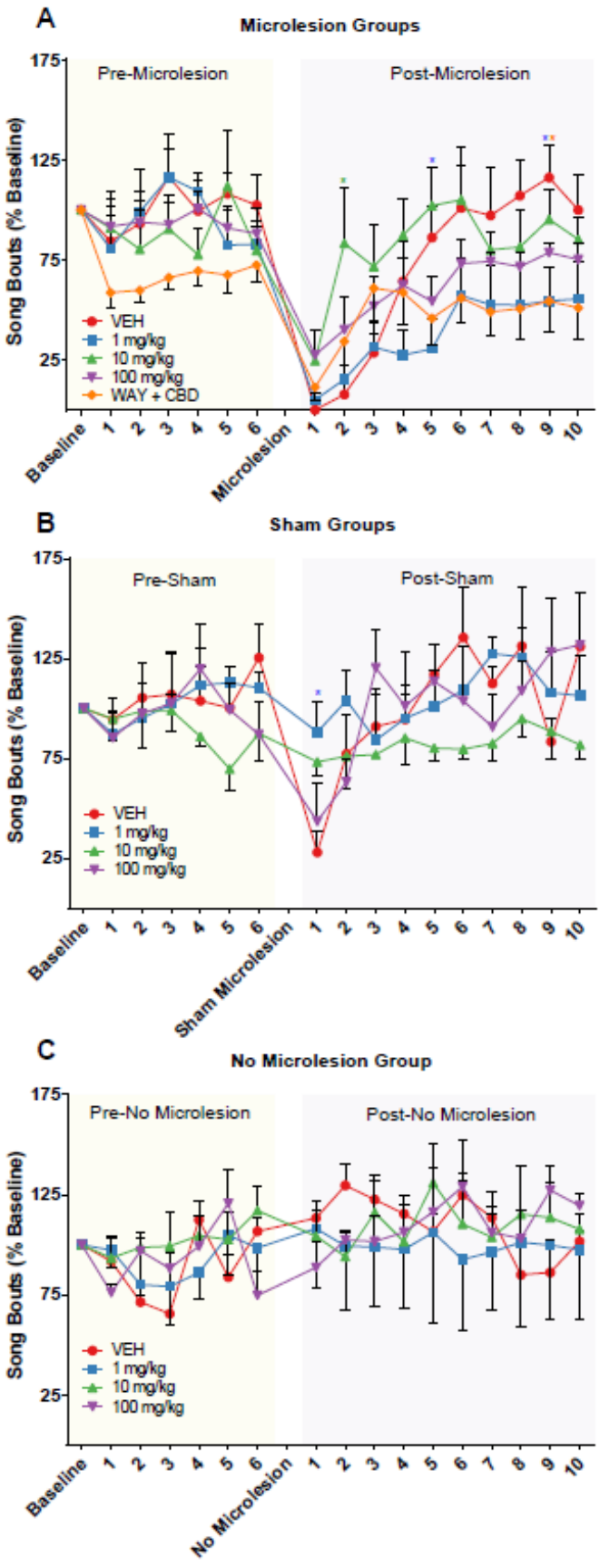

A
Microlesion Groups
Pre-Microlesion
Post-Microlesion
% Typical Transitions
VEH
1 mg/kg
10 mg/kg
100 mg/kg
WAY + CBD
Baseline
Microlesion
B
Sham Groups
Pre-Sham
Post-Sham
Sham Microlesion
C
No Microlesion Group
Pre-No Microlesion
Post-No Microlesion
No Microlesion

Figure 2.5: Vocal output measured by percent baseline song bout production.

A, microlesions almost eliminated vocal production across all treatment groups. 10 and 100 mg/kg CBD appeared to improve recovery relative to other groups on recovery days two and five, respectively (mixed-models post-hoc tests with the Bonferroni correction for multiple comparisons). Both 1 mg/kg CBD and CBD+WAY resulted in significantly lower vocal production on recovery day 10, suggesting a differential, hormetic CBD dose-response and potential 5-$HT1_a$ receptor activity to promote vocal behavior. B, the sham procedure also appeared to reduce output in VEH and 100 mg/kg-treated animals. 1 mg/kg CBD significantly improved vocal output on recovery day 1. C, the no-microlesion procedure did not produce a significant effect on vocal output in any of the treatment groups. Shown are mean values +/- SEM.


A
Microlesion Groups
Pre-Microlesion
Post-Microlesion
Song Bouts (% Baseline)
175
125
75
25
VEH
1 mg/kg
10 mg/kg
100 mg/kg
WAY + CBD
Baseline
1 2 3 4 5 6
Microlesion
1 2 3 4 5 6 7 8 9 10
B
Sham Groups
Pre-Sham
Post-Sham
Song Bouts (% Baseline)
175
125
75
25
VEH
1 mg/kg
10 mg/kg
100 mg/kg
Baseline
1 2 3 4 5 6
Sham Microlesion
1 2 3 4 5 6 7 8 9 10
C
No Microlesion Group
Pre-No Microlesion
Post-No Microlesion
Song Bouts (% Baseline)
175
125
75
25
VEH
1 mg/kg
10 mg/kg
100 mg/kg
Baseline
1 2 3 4 5 6
No Microlesion
1 2 3 4 5 6 7 8 9 10

CHAPTER 3: CANNABIDIOL IMPROVES VOCAL DEVELOPMENT FOLLOWING DAMAGE TO IMAN, A REGION IMPORTANT FOR SENSORIMOTOR VOCAL LEARNING

3.1 Introduction

Cannabidiol (CBD) is one of many molecules derived from *Cannabis* that is structurally-related to the principal eurphorigenic cannabinoid, Δ^9-tetrahydrocannabinol (THC). Although not associated with euphoria like THC, CBD is clearly psychoactive and is known to interact with many cellular macromolecules expressed within CNS (reviewed by Ibeas Bih et al., 2015; Soderstrom et al., 2017).

Because of a range of efficacies and array of cellular targets CBD holds promise for the treatment of many disorders, notably including seizures, muscle spasms, anxiety, and autism. Recently, there have been several important clinical trials done to demonstrate CBD is effective in reducing spasms, muscle stiffness, and pain associated with multiple sclerosis (Holdcroft et al. 2006). Moreover, other clinical trials have demonstrated CBD is an effective therapy for treatment children suffering from intractable seizures such as Dravet syndrome and Lennox-Gastaut syndrome. Results showed a reduced seizure frequency by approximately 43% for Dravet and Lennox-Gastaut syndrome patients (Thiele et al. 2018; Trial of CBD for Drug-Resistant Seizures in the Dravet Syndrome 2017), and it produces positive effects with promising results (Tzadok et al. 2016). Among these results is suggestion that CBD may improve vocal learning.

In addition to childhood seizure disorders, several other vocal learning and production-related pathologies remain difficult to treat. Among these disorders are stroke

(Godoy et al. 2014; Mayo clinic staff 2015; Özbal Koç et al. 2016), trauma, and autism. In the US, there are about 7.5 million people that have difficulty using their voices (National Institue on Deafness and Other Communication Disorders (NIDCD) 2016). Globally in 2013 (Global Burden of Disease Study 2013 Collaborators 2015), approximately 21.7 million people had been diagnosed with autism spectrum disorder. In the United States, about one in 68 children were diagnosed with autism in 2014 (Autism and Developmental Disabilities Monitoring Network Surveillance Year 2008 Principal Investigators and Centers for Disease Control and Prevention 2012; Blumberg et al. 2013). One of the characteristics that is associated with autism is a vocal learning defect (Condro and White 2014; Panaitof 2012) and about 50% or more of these patients displayed early abnormal acoustics patterns (Fusaroli et al. 2016; Paul et al. 2005; Rogers et al. 2006; Shriberg et al. 2001).

We have developed a unique songbird preclinical animal model to investigate drug effects to improve learning-dependent recovery following damage to a vocal cortical-like brain region. In the model, CBD was found to improve vocal recovery following electrolytic ablation of about 10% of pre-vocal motor HVC (used as a proper name). This partial damage is recovered from in about seven days in untreated animals. CBD administered once daily via IM injection of 10 mg/kg significantly reduced time to recover both vocal phonology and syntax. In the case of phonology, the magnitude of lesion-related deficits were also reduced. This experiment, done in adult animals, made us consider if similar efficacy to promote vocal learning would be observed during the songbirds' critical period for vocal development during which song patterns are memorized and practiced. Such efficacy would suggest CBD holds therapeutic promise for treatment of speech- and/or

language-related disorders, including those associated with autism.

Because of the need to find a drug effective for treating disorders like autism that is related to vocal learning, this project investigated the effect of CBD to improve vocal learning during the time that it is naturally acquired.

Zebra finch is a good model for testing this hypothesis as, in a manner similar to humans, male zebra finches learn how to produce a form of vocal communication (Scharff and Nottebohm 1991). In both humans and zebra finches, youngsters listen to and memorize vocal patterns produced by tutors, this called sensory learning stage. Then in sensorimotor learning stage, learners start to practice producing vocalizations, refining them through auditory feedback until a good copy is produced (Doupe and Kuhl 1999; Brainard and Doupe 2002). Convergent pathways control vocal learning and production in both the zebra finch and humans (Nottebohm 2005; Simmonds 2015). These pathways include: one in rostral telencephalon for vocal learning termed the “anterior forebrain pathway” (AFP) and a caudal vocal motor pathway (Simmonds 2015). The vocal learning pathway in zebra finches includes lMAN, Area X , and DLM whereas the vocal motor pathway includes HVC and RA (Nottebohm 2005; Simmonds 2015) (Figure1.3). The vocal learning pathway in humans involves these areas: cortex, striatum, and thalamus (Simmonds 2015). The vocal motor pathway involves these areas: premotor cortex and motor cortex (Simmonds 2015) (Figure 1.3) . For these reasons, we used zebra finch to test the hypotheses of this project.

3.2 Materials and Methods

3.2.1 Materials

Unless otherwise indicated all materials and reagents were purchased from Sigma or Fisher. CBD was provided by GW Pharmaceuticals, Cambridge, UK. Two CBD dosages were employed: 0 (vehicle control), 10 mg/kg. CBD suspended in vehicle from 10 mM ethanol stocks. Vehicle consisted of a suspension of 2:1:17 Ethanol:Alkamuls EL-620 (Rhodia, Cranberry, NJ):phosphate-buffered saline. Equithesin was prepared from reagents (40 % propylene glycol, 10 % ETOH, 5 % chloral hydrate, 1 % pentobarbital).

3.2.2 Animals and audio recording environment

Male zebra finches were raised in our breeding aviary. At about day 25 of age, they were transferred to tutor cage until day 45 of age. Then, they were individually housed in small cages (29 x 22 x 42 cm) with *ad libitum* food and water. There were four recording periods: at about 45, 60,75, 90 and 94 day of their age and in these times, animals were placed in recording chambers. Birds were visually isolated during the vocalization recording and they were not female-directed. Each recording chamber was fitted with a microphone and an individual light. Sound Analysis Pro 2011 software (Tchernichovski et al. 2000) was used to record vocalizations and store them in Waveform Audio File Format. Animals were maintained at 78° F on a 12/12 light/dark cycle. All animal procedures were approved by the East Carolina University Animal Care and Use Committee.

Intended group numbers were n = 6 animals per treatment and surgery condition. Thus, a total of fifty-four animals were used in these studies (Table 1). Animals sang an

overall mean of 5.93 (+/- 0.843) motif syllables (range = 4-8 syllables) and motif complexity did not differ significantly across the groups (2-way ANOVA, $F(8,45)=0.493$, $p=0.855$) (see Table 2).

3.2.3 Experimental design

To test the effect of CBD on vocal learning we used juvenile male birds in the auditory learning stage. Because these birds do not develop secondary sex characteristics before the age they were needed for these developmental experiments, PCR was used to determine their sex following the procedure previously described (Thompson et al. 2007). Adult males tutored these juvenile birds. At age 35 days, which is approximately the end of the auditory learning stage and beginning of the sensorimotor learning stage overlap; the unilateral lMAN lesions were done from late-morning to early-afternoon. Three groups of developmental birds were treated with CBD and VEH once daily treatments were delivered in the morning in a volume of 50 ul IM to pectoralis. These three groups are: First group received VEH from day 30 to day 90; Second group received 10 mg/kg CBD for 10 days from day 30 to day 40, and VEH from day 41 to day 90; Third group received 10 mg/kg CBD from day 30 to day 90. CBD injections started 5 days before surgery to reach to a presumed steady state level before surgery. Also, no-surgery and sham controls were evaluated with all three dosages. To determine if the lesion produces a confounding effect, sham groups were required. Also, no surgery groups are important to confirm the anesthetic agent (isoflurane) that is used during surgery does not produce a confounding effect. The number of birds used were 54 males, 6 birds for each group. At adulthood period at approximately day 90 of age, songs were recorded and

used for an entirely objective analysis. Finally, to confirm the location of lesions and measure their size, the birds were euthanized and the brains were fixed. Sham group birds received all these processes except for the destroying of IMAN. Whereas no surgery animals did not undergo surgeries. The treatment plan employed is summarized in Figure 3.1 B and C.

3.2.4 Microlesion surgeries

For animals in the lesion group, each was subjected to unilateral craniotomy over lMAN following the procedure previously described (Thompson et al. 2007) except that animals were administered the anti-inflammatory drug meloxicam (1 mg/kg) 30 min prior to procedures and were anesthetized by isoflurane inhalation. For these procedures, birds were deeply anesthetized by face mask (4% isoflurane in O_2 at 1 L/min) and secured in a stereotaxic instrument. Once secured, anesthesia was maintained with 2.5% isoflurane in O_2 at 1 L/min by mouth through a small diameter tube passed through a hole drilled through the bite bar. The skull was exposed by cutting the scalp down the midline and retracting the skin using forceps. To determine lMAN location, the bifurcation at the midsagittal sinus was used as stereotaxic zero. A small craniotomy was placed over the location of lMAN in right hemisphere. To produce lesion that destroy approximately 100 % of right lMAN, we placed an electrode (Teflon insulated tungsten; 200 um diameter; A-M Systems, Everett, WA) in one location. At 3.8 mm anterior from stereotaxic zero, mirrored laterally from the midline 1.8 mm, penetration was to a depth of 2.7 mm, and for 3.5 min the current was set at 100 uA. Wounds were closed with interrupted sutures and birds were placed in a warm incubator until awake and active and then returned to home

cages. Animals in the sham-lesion group were subjected to all of the steps described above for the lesion group except that no current was passed through the electrodes. Therefore, sham birds were exposed to meloxicam analgesia, anesthesia with isoflurane, craniotomies, electrode insertion and interrupted sutures. Animals in the no-lesion control group were transported to the operating room with lesion and sham-group animals, but were not subjected to any other manipulation. Thus, the no-lesion group served to control for potential effects of the craniotomy procedure itself, including analgesic and anesthetic drug treatments.

3.2.5 Lesion extent

Following recording, animals were overdosed with Equithesin. Brains were removed and post-fixed in paraformaldehyde at 4^{o} C overnight. Post-fixed brains were blocked parasagittally down the midline and right hemisphere sectioned through lMAN at 40 microns. Sections were mounted on gelatin-subbed slides and Nissl stained. Sections containing lMAN lesion were imaged at 40 X. The extent of lesion damage was calculated by tracing borders of lMAN and infarcts, if present, using ImagePro Plus software. The total area of lMAN and the infarct were summed, and percent lesion extent expressed as infarct area divided by lMAN area x 100 % (Figure 3.2).

3.2.6 Vocal production measured by measuring duration of singing per hour

To measure vocal production, recordings in wave form audio file format were sorted and duration for each syllable were calculated using SAP2011. To ensure that all syllables are motive syllables not calls or introductory notes KLFromSAPTables software

version 2 was used to assign motive and non-motive syllables. Then, MySQL Workbench 6.3 CE was used to create a new syllables table for motive syllables only. After that the syllables durations were summed and divided by hours of recording to find out duration of singing per hour. Then to measure the variability in vocal production, the percentage of duration of singing for each animal was divided by the average of duration of singing for VEH-no lesion animals.

3.2.7 KL distance measures of phonology

Animals were recorded at days 45, 60, 75, 90, and 94 of age using Sound Analysis Recorder software (Tchernichovski et al. 2000) . Recordings in Waveform Audio File Format were input to Sound Analysis Pro 2011 (Tchernichovski et al. 2000) to segment song files into their separate syllable components. Segmentation into syllables using SAP was accomplished by thresholding based upon amplitude, entropy, syllable and syllable gap durations. Values of these acoustic features used for thresholding were optimized for individual animals. After this optimization, further analysis of segmented syllables was accomplished entirely objectively. SAP characterizes individual syllables by their spectral structure through measures of acoustic features (e.g. syllable duration, mean amplitude, mean pitch, mean FM, mean AM^2, mean entropy, mean goodness of pitch, mean mean frequency, pitch variance, FM variance, entropy variance, goodness of pitch variance, mean frequency variance, AM variance). SAP saved acoustic feature measures in MySQL database tables. We used these MySQL tables to measure phonology for each animal via calculation of Kullback-Leibler (KL) distances using methods developed by Daou et al. 2012; Wu et al. 2008 that compare distances between 2D probability

distributions of vocal acoustic features. Greater KL distance measures reflect increased phonological divergence across the vocalizations compared. We used acoustic measures from VEH-no lesion animals recording as “template” distribution, and recordings from each group of treatments as individual “target”. KL distances between template and target distributions were calculated using software we developed (KLFromRecordingDays available for download as described in Soderstrom and Alalawi 2017).

3.2.8 Typical syllable transition measures of syntax

Syntax was measured from the frequency of typical syllable transitions that were calculated using SongSeq software according to the method described by Daou et al., 2012. SongSeq uses data exported from SAP to identify distinct syllables. Day 90 was used as the template to compare target datasets generated from recordings made during each time point (45, 60, 75 and 90). Distinct syllable types were identified by clustering within a plot of an acoustic feature measure on the y-axis and syllable duration on the x-axis. One or two acoustic features were used as necessary to distinguish two-dimensional syllable clusters. These parameters were optimized for each subject. Once syllable clusters were identified, SongSeq calculated probabilities of each possible pair of syllable types being produced on that recording time point. The percentage of typical transitions for each treatment day was then calculated as the number of typical transitions divided by total transitions x 100%. Then to measure the variability in syntax, the percentage of typical transition for each animal was divided by the average percent typical transitions for VEH-no lesion group animals.

3.2.9 Statistical analyses

To assess differences in phonology, syllable sequencing and vocal production over experiment day, across treatment groups and microlesion conditions, we used a mixed-effects modeling approach with SPSS software (version 24). This method controls for lack of independence of repeated measures derived from single animals (e.g. four time points measures from each animal, Aarts et al., 2014).

For mixed model analysis of phonology, syntax and production data, individual animals were treated as random subjects and lesion group (microlesion, sham-microlesion, no-microlesion), drug treatment (vehicle, CBD 30-40 and CBD 30-90) and time point were used as fixed factors. For all mixed model analyses, the variance components covariance structure and the maximum likelihood method were used. Fixed explanatory variables were successively added to models. Improvements to simpler models gained by variable additions were determined through likelihood ratio (LR) tests of differences between -2*log likelihood values from the fit of each model to vocal behavior data.

For each assessment, models included animal ID as a random factor to control for repeated measures, and time point was added as the first fixed explanatory variable. As subsets of animals were assigned to drug treatment groups (vehicle, CBD 30-40 and CBD 30-90) and these groups were further divided into lesion condition (microlesion, sham- and no-microlesion), microlesion group nested within treatment group were added as fixed factors. Differences between vehicle control and CBD-treated groups were determined from pairwise comparisons using the Bonferroni post-hoc correction.

Probabilities less than 0.05 were considered significant. Figures 3.3, 3.6 and 3.7 summarize means +/- SEM.

3.3 Results

3.3.1 CBD treatment for short period improves phonology

When songs of birds were analyzed by KL distance to see the effect of CBD on phonology in lesion groups at four different ages 45, 60, 75, and 90 days, are demonstrated in Figures 3.3. Compared to animals that received vehicle treatment from day 30 to 90 days (Figure 3.3 A), the animals that received unilateral lMAN lesion treated by 10mg/kg CBD from 30 to 40 days of age (called CBD 30-40) have improved KL distance variability in adulthood age (age 90, $F[2,210] = 5.68$, $p=0.047$). Whereas, CBD 30-90 treatment did not significantly alter KL distance comparing to VEH at all time points. Sonogram examples of CBD effects on song in an animal received unilateral lMAN lesion shows in Figure 3.4. In addition, the effect of short period treatment by CBD from day 30 to 40 was evaluated in different lesion conditions by KL distance, as shown in Figures 3.3 B. At age 60 and 90 days, CBD 30-40 treatment significantly improved KL distance in lesion animals comparing to no lesion, ($F[2,210] = 3.06$, $p = 0.049$ and $F[2,210] = 5.919$, $p=0.003$, respectively). Whereas, CBD 30-40 treatment did not significantly alter KL distance in sham animals compared to no lesion at all time points. Sonogram examples of CBD effects on song in an animal that had received unilateral lMAN is lesion shown in Figure 3.5.

3.3.2 CBD treatment for short period speeds maturation of singing

The effect of CBD treatment on syntax quality across lesion groups at four time points 45, 60, 75 and 90 days of the animals ages are summarized in Figure 3.6. This figure shows ability of CBD treatment for short period to speed maturation of syllable transitions in lesion animals. At age 45 days, CBD 30-40 treatment significantly improved typical syllable transitions comparing to VEH, $F[2,150.81] = 7.148$, $p = 0.001$. Whereas, CBD 30-90 treatment did not significantly alter typical syllable transitions comparing to VEH at all time points (Figure 3.6 A). Looking at sonograms there appears to be an effect of CBD to hasten maturation of syllable transitions. This is made evident by higher typical syllable transitions percentage in the CBD 30-40 group at Day 45 comparing to VEH and CBD 30-90 (Figure 3.4). In addition, the effect of the short period treatment with CBD from day 30 to 40 was evaluated in different lesion conditions by typical syllable transitions percentage as shown in Figures 3.6 B. At age 45 and 60 days, CBD 30-40 treatment significantly improved typical syllable transitions in lesion animals comparing to no lesion, $F[2,150.81] = 7.71$, $p = 0.001$. Whereas, CBD 30-40 treatment did not significantly alter typical syllable transitions percentage in sham animals compared to no lesion at all time points. In Figure 3.5 sonogram examples of CBD effects on song in an animal received either unilateral lMAN lesion, sham or no lesion surgery, shows high typical transitions for animals that received unilateral lMAN lesion and treated by CBD 30-40 at age 45 and 60 days compared to VEH and CBD 30-90 and this suggests that CBD has an effect to hasten maturation of syllable transitions.

3.3.3 CBD treatment does not affect vocal output in IMAN lesioned animals

Figure 3.7 shows the effect of CBD on vocal output of lesion animals. CBD does not significantly alter amount of singing per hour in both CBD 30-40 and CBD 30-90 comparing to VEH group (Figure 3.7 A) $F[2,148.74] = 0.523$, $p = 0.6$. In like manner CBD 30-40 does not significantly alter amount of singing per hour in all lesion conditions compared to no lesion group, shown in Figure 3.7 B, $F[2,148.74] = 0.27$, $p = 0.764$.

3.4 Discussion

3.4.1 CBD effects on syntax

CBD treatment for a short period (from day 30 to day 40 of animals ages) appears to have accelerated maturation of singing in animals that received unilateral lMAN lesion in terms of syntax compared to lesioned animals that received VEH (Figure 3.6 A) and to no lesioned animals that received CBD for 10 days (30 to 40 day of their ages) (Figure 3.6 B). Although all animals in adulthood (90 days) reached to approximately the same level of typical transitions (a measure of syntax), CBD 30-40 animals had significant higher typical transition at age 40 and 60 days. This effect of short period treatment of CBD likely involves processes distinct from and secondary to tissue damage itself not associated with “rewiring” circuits that control learned vocal behavior. Given similarities noted in studies employing ischemic stroke models (Ceprián et al. 2017; Mori et al. 2016), these secondary processes may include reductions of excitotoxicity, neuroinflammation, metabolic derangement, gliosis and/or protection of astrocyte function. Whatever the secondary processes are, these will be important to address in future studies. Moreover, long period treatment of CBD does not improve syntax (Figure 3.6 A) and this was similar

to our findings in first project (Figure 2.4 A), CBD has biphasic effects and this biphasic effect suggests involvement of temporally-distinct physiological processes. Moreover, this variability between long and short periods of treatment with CBD may involve an “inverted U-shaped” hormetic dose-response relationship for this effect that has been described for CBD in other systems (Gallily et al., 2015; Zuardi et al., 2017).

In addition, the findings of Scharff and Nottebohm demonstrated bilateral destruction of lMAN prevents vocal learning in zebra finch birds (Scharff and Nottebohm 1991). Furthermore, based on unpublished personal communication, unilateral lesions of lMAN prior to sensorimotor vocal development allows for learning of song, but with some deficits when compared with control animals. As mentioned before, our results suggest CBD helped to speed maturation of song in terms of syntax in unilateral lMAN lesioned animals. This result demonstrates that the effect of CBD to improve or speed maturation of syntax cannot be attributable to its effect on lMAN itself only, but must follow from some related disruption involving HVC, a motor region most clearly associated with syntax-relevant syllable timing (Hahnloser, Kozhevnikov, and Fee 2002) or increase activity of not lesioned lMAN in the second side of the brain . Therefore, other brain regions and circuits must be involved in the efficacy of CBD to speed maturation of singing in sensorimotor stage.

3.4.2 CBD effects on phonology

CBD treatment for a short period significantly improved KL distance variability in adulthood time in animals that received unilateral lMAN lesion in terms of phonology compared to lesioned animals received VEH (Figure 3.3 A), but comparing to no lesioned

animals the effect of CBD 30 to 40 days started early at age 60 days (Figure 3.3 B). According to previous studies the activity within lMAN and RA appear more important to phonology (Kao, Doupe, and Brainard 2005; Sober, Wohlgemuth, and Brainard 2008; Vu, Mazurek, and Kuo 1994). Therefore, the effect of CBD on phonology suggests that CBD plays a role through lMAN, RA or both to produce this effect.

3.4.3 CBD effects on vocal production

In addition to using phonology and syntax analysis to assess the effect of CBD to improve vocal learning, vocal output analysis was used to examine the effect of CBD on motor activity after CNS damage. However, CBD did not produce statistically significant effects on vocal learning compared to lesioned animals received VEH (Figure 3.7 A) and to No-Lesion group animals received CBD (30 – 40) (Figure 3.7 B). Because CBD does not have direct effects on CB1, the dose used in this experiment may be less than the effective dose to produce an effect in motor activity through interacting with $5HT_{1A}$ receptors. Espejo-Porras et al used 20 mg/kg to demonstrate the ability of CBD to affect motor activity (Espejo-Porras et al. 2013).

3.5 Conclusions

We have demonstrated the ability of CBD to improve vocal learning following damage to lMAN that plays a major role in vocal learning during the sensorimotor stage. Treatment with 10 mg/kg CBD for 10 days was effective at improving vocal learning in terms of phonology and on measures of syntax. Therefore, our results suggest CBD holds therapeutic promise for treatment of speech- and/or language-related disorders. In

addition to demonstrating CBD efficacy, the work has established a pre-clinical animal model suitable for evaluation of drugs that modulate vocal learning.

Table 3.1: Summary of animals used for each group.

A total of 54 animals were included in the analysis and 52 animals were excluded: 14 animals died before end of the experiment, sexing by PCR resulted in 11 erroneous sex assignments, and 27 were excluded because lesion misses and/or extent less than 100%.

Groups		No. of All Animals	Died before measuring lesion extent	Sexing error: Not male	Their lesion extent less than 100%	No. of animals which achieved the requirements
VEH	No lesion	7	0	1	0	6
	Sham	8	1	1	0	6
	lesion	19	3	3	7	6
CBD 30-40	No lesion	7	0	1	0	6
	Sham	6	0	0	0	6
	lesion	26	6	3	11	6
CBD 30-90	No lesion	7	0	1	0	6
	Sham	7	1	0	0	6
	lesion	19	3	1	9	6

Table 3.2: Similar motif syllable numbers across treatment groups.

No treatment group sang significantly more- or less complicated song patterns in adulthood age when compared to other groups (2-way ANOVA, $F(8,45)=0.493$, $p=0.855$)

Group		Mean Motif Syllables	95% CI	
			Lower	Upper
No lesion	CBD 30-40	6.0	5.3	6.7
	CBD 30-90	5.7	4.9	6.4
	VEH	6.0	5.3	6.7
Sham	CBD 30-40	5.8	5.1	6.6
	CBD 30-90	6.3	5.6	7.1
	VEH	6.2	5.5	6.9
lesion	CBD 30-40	5.5	4.8	6.2
	CBD 30-90	5.8	5.1	6.6
	VEH	6.0	5.3	6.7

Figure 3.1: Experimental approach.

A, camera lucida-type drawing illustrating brain regions and circuits relevant to CBD effects on vocal behavior. Black shading corresponds to song regions within the section traced (HVC, RA, lMAN, Area X) and grey shading indicates striatum. Light grey areas with dashed borders indicate relevant regions not present in the traced section. Rose arrows indicate connections of the anterior forebrain pathway (AFP), a cortico-basal ganglia-thalamic loop critical for sensorimotor vocal learning (reviewed by Perkel, 2004). Note output from lMAN to the vocal motor output region, RA (Bottjer et al., 1989). Blue-green indicates vocal motor pathways. Note output from pre-motor HVC to the basal ganglia region, Area X. Gold arrows indicate auditory input to the motor system (Kelley and Nottebohm, 1979; Vates et al., 1996) and from the ventral portion of the intermediate arcopallium (AIV) to dopaminergic neurons within substantia nigra (SN)/ventral tegmental area (VTA, Mandelblat-Cerf et al., 2014). Note SN/VTA dopaminergic projections to spiny interneurons within Area X of striatum (Ding and Perkel, 2002). Rostral is right, dorsal is up and bar = 1 mm. Abbreviations: DLM (nucleus dorsolateralis anterior, pars medialis), HVC (proper name), lMAN (lateral magnocellular nucleus of the anterior nidopallium), CM (caudal mesopallium), RA (robust nucleus of the arcopallium). B, summary of treatment groups used. C, experimental time-line for audio recording, drug treatment, and surgery.

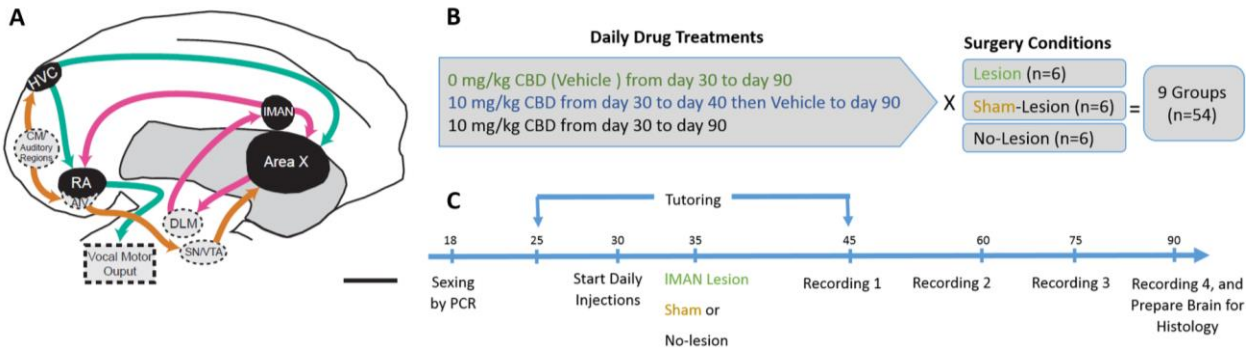

A
HVC
CM
Auditory
Regions
RA
AV
IMAN
Area X
DLM
SN/VTA
Vocal Motor
Ouput
B
Daily Drug Treatments
0 mg/kg CBD (Vehicle) from day 30 to day 90
10 mg/kg CBD from day 30 to day 40 then Vehicle to day 90
10 mg/kg CBD from day 30 to day 90
X
Surgery Conditions
Lesion (n=6)
Sham-Lesion (n=6)
No-Lesion (n=6)
=
9 Groups
(n=54)
C
Tutoring
18
25
30
35
45
60
75
90
Sexing
by PCR
Start Daily
Injections
IMAN Lesion
Sham or
No-lesion
Recording 1
Recording 2
Recording 3
Recording 4, and
Prepare Brain for
Histology

Figure 3.2: Effects of unilateral lMAN lesion.

Illustration of method used to confirm lMAN lesion of Nissl-stained parasagittal sections (dorsal up, rostral right, A, 12.5X inset bar = 1000 um, B,100X bar = 100 um).

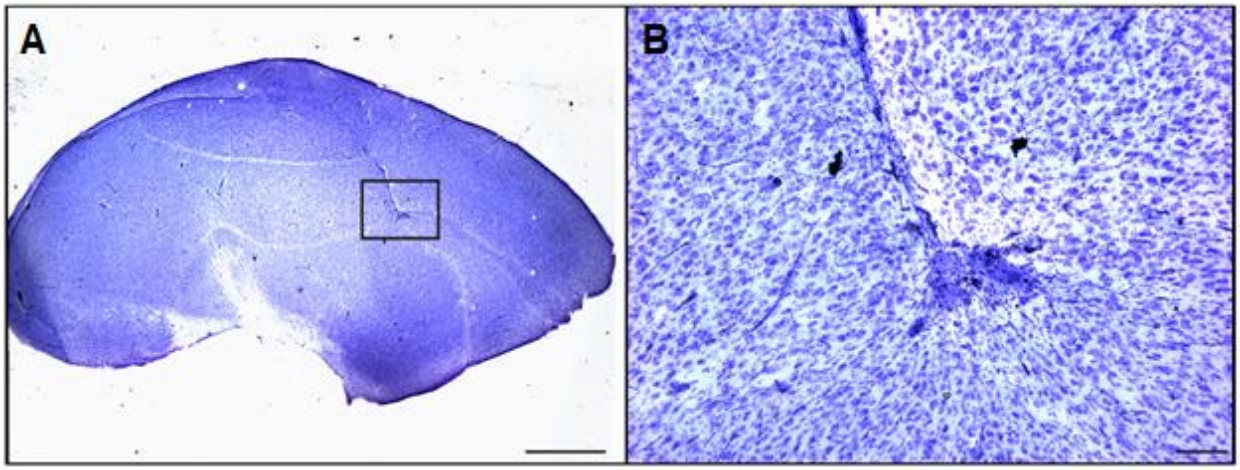

A
B

Figure 3.3: CBD improves vocal phonology in lesion animals.

KL distance measures of phonology at four time points 45, 60, 75 and 90 days of their age was calculated. Treatment groups received 60 daily IM injections of either: VEH = vehicle once daily beginning at 30 days of age; CBD 30-40 = 10 mg/kg CBD from 30 to 40 days of age and then vehicle to 90 days or; CBD 30-90 = 10 mg/kg CBD from 30 to 90 days of age. A, KL distance measures of phonology for different treatment groups in unilateral lMAN lesion animals. All animals received unilateral lMAN lesions at age of 35 days. At age 90 days, CBD 30-40 treatment significantly improved KL distance compared to VEH. Whereas, CBD 30-90 treatment did not significantly alter KL distance compared to VEH at all time points. B, KL distance measures of phonology for different lesion groups. All animals received 60 daily IM injections 10 mg/kg CBD from 30 to 40 days of age then vehicle to 90 days. At age 60 and 90 days, CBD 30-40 treatment significantly improved KL distance in lesion animals compared to no lesion. Whereas, CBD 30-40 treatment did not significantly alter typical syllable transitions in sham animals compared to no lesion at all time points. Asterisk color indicates significant lesion group differences determined by mixed-models post-hoc tests with the Sidak correction for multiple comparisons.

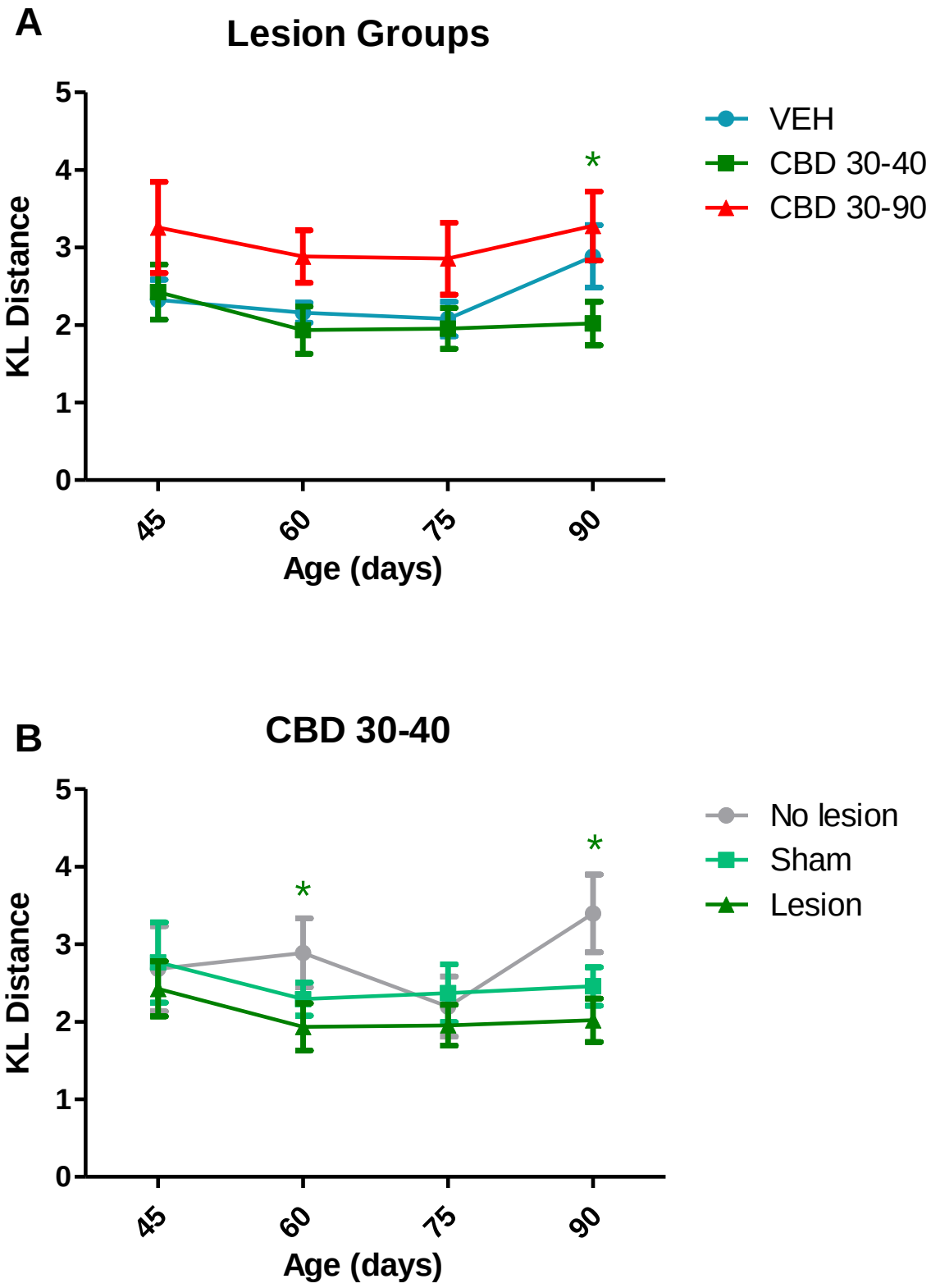

A
Lesion Groups
KL Distance
Age (days)
45
60
75
90
0
1
2
3
4
5
*
VEH
CBD 30-40
CBD 30-90
B
CBD 30-40
KL Distance
Age (days)
45
60
75
90
0
1
2
3
4
5
*
*
No lesion
Sham
Lesion

Figure 3.4: Sonogram examples of CBD effects on song in animals that received unilateral lMAN lesions

All animals received a unilateral lMAN lesion at age of 35 days. sonograms shows good syntax and phonology for the animals were treated by CBD 30-40 for all four time points compared to VEH and CBD 30-90.

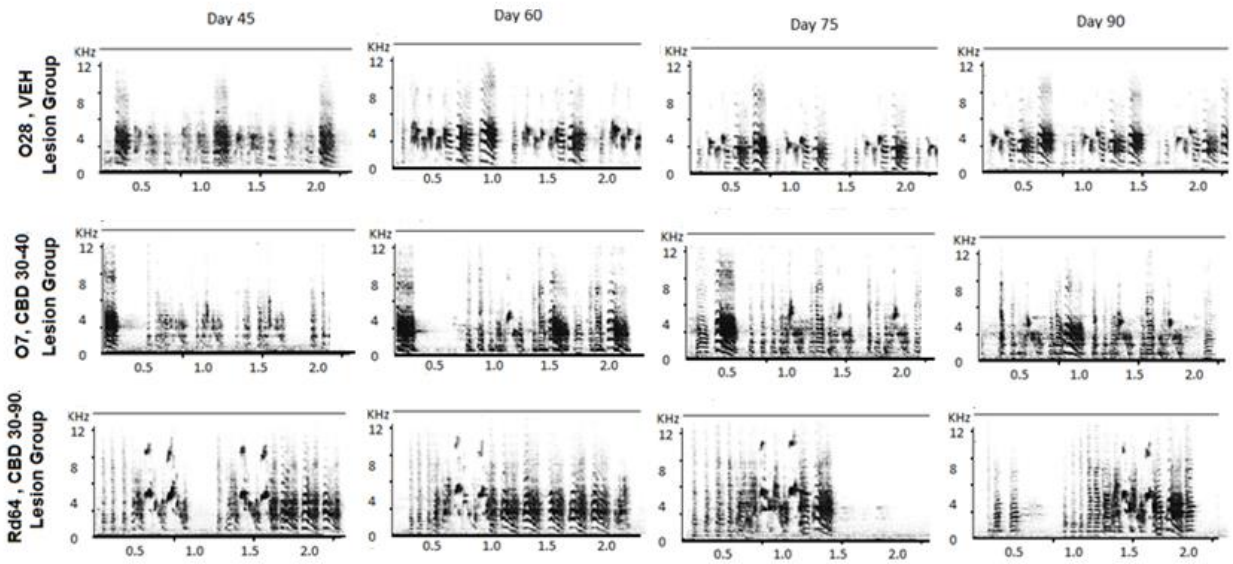

Day 45
Day 60
Day 75
Day 90
O28 , VEH Lesion Group
O7, CBD 30-40 Lesion Group
Rd64 , CBD 30-90 Lesion Group
KHz
12
8
4
0
0.5
1.0
1.5
2.0

Figure 3.5: Sonogram examples of CBD effects on song for animals received either unilateral lMAN lesion, sham or no lesion surgery.

All animals received 10 mg/kg CBD from 30 to 40 days of age and then vehicle to 90 days. Sonograms show good syntax and phonology for animals were received unilateral lMAN lesion compared to no lesion and sham animals.

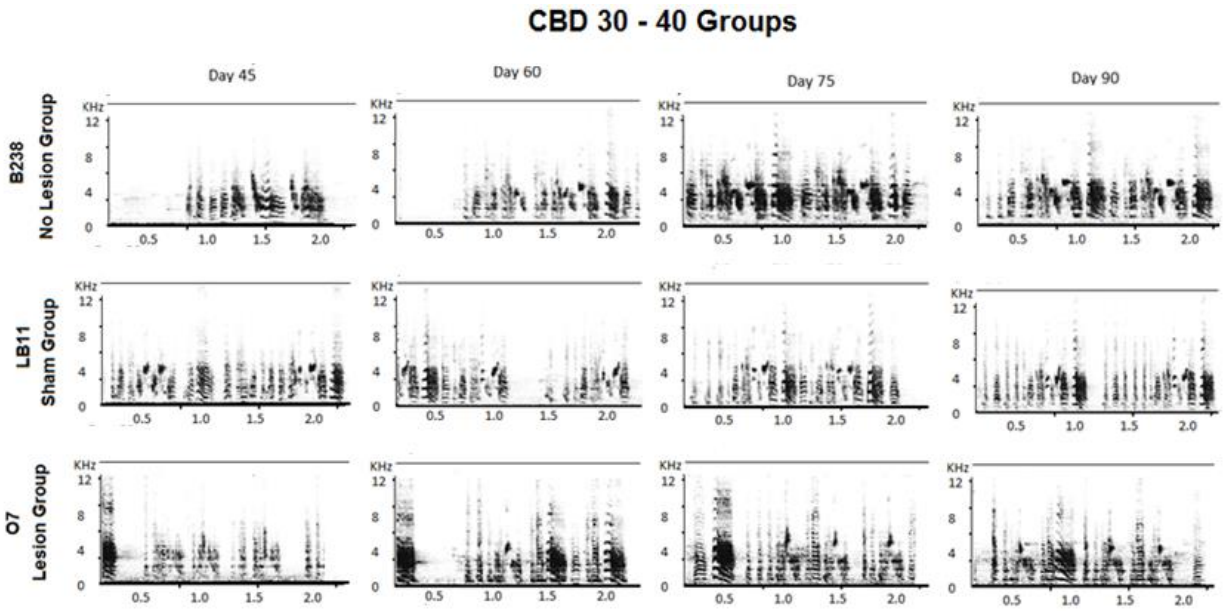
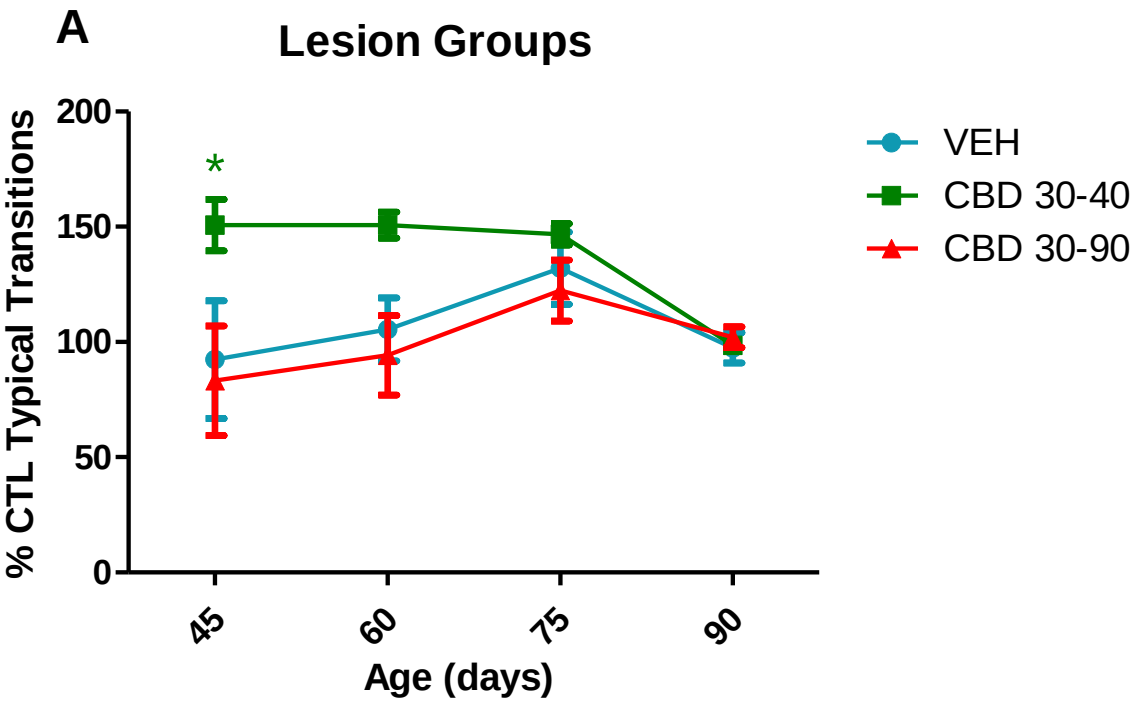

CBD 30 - 40 Groups
Day 45
Day 60
Day 75
Day 90
B238
No Lesion Group
LB11
Sham Group
O7
Lesion Group
KHz
12
8
4
0
0.5
1.0
1.5
2.0

Figure 3.6: CBD speeds maturation of singing in lesion animals.

Syntax quality measured through percent control typical syllable transitions at four time points 45, 60, 75 and 90 days of their age was calculated. The zebra finches received 60 daily IM injections of either: VEH = vehicle once daily beginning at 30 days of age; CBD 30-40 = 10 mg/kg CBD from 30 to 40 days of age and then vehicle to 90 days or; CBD 30-90 = 10 mg/kg CBD from 30 to 90 days of age. A, Syntax quality was measured for different treatment groups in unilateral lMAN lesion animals. At age 45 days, CBD 30-40 treatment significantly improved typical syllable transitions compared to VEH. Whereas, CBD 30-90 treatment did not significantly alter typical syllable transitions compared to VEH at all time points. B, Syntax quality was measured for different lesion groups. CBD 30-40 treatment significantly improved typical syllable transitions in lesion animals comparing to no lesion. Whereas, CBD 30-40 treatment did not significantly alter typical syllable transitions in sham animals compared to no lesion at all time points. Asterisk color indicates significant lesion group differences determined by mixed-models post-hoc tests with the Sidak correction for multiple comparisons.


A
Lesion Groups
% CTL Typical Transitions
200
150
100
50
0
*
45
60
75
90
Age (days)
VEH
CBD 30-40
CBD 30-90

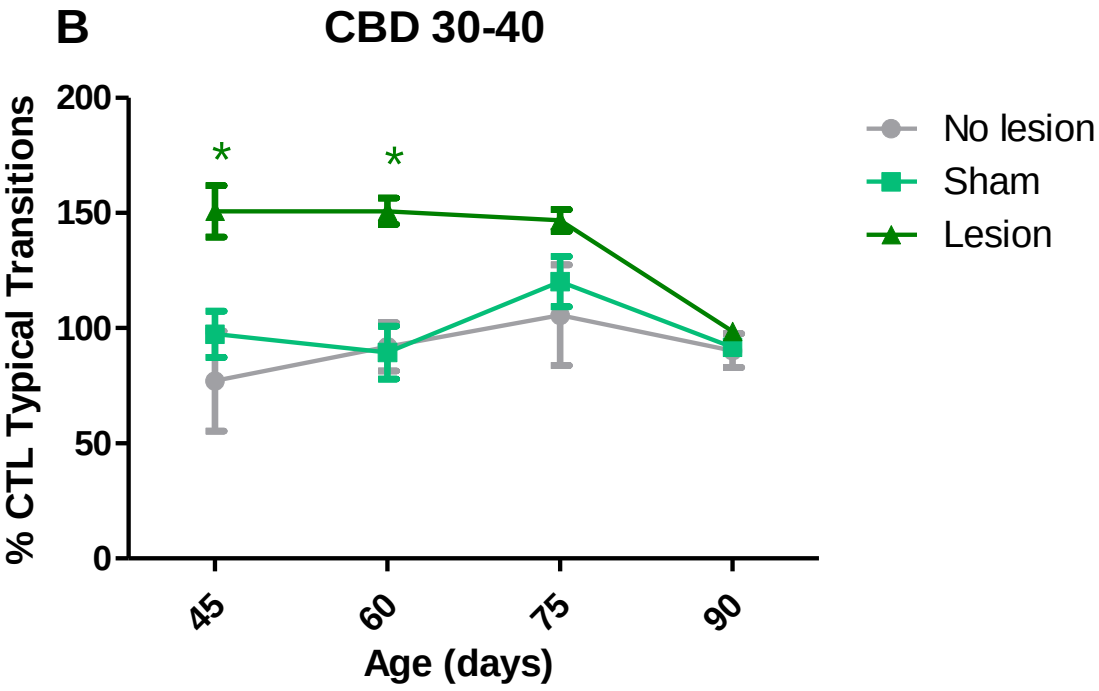
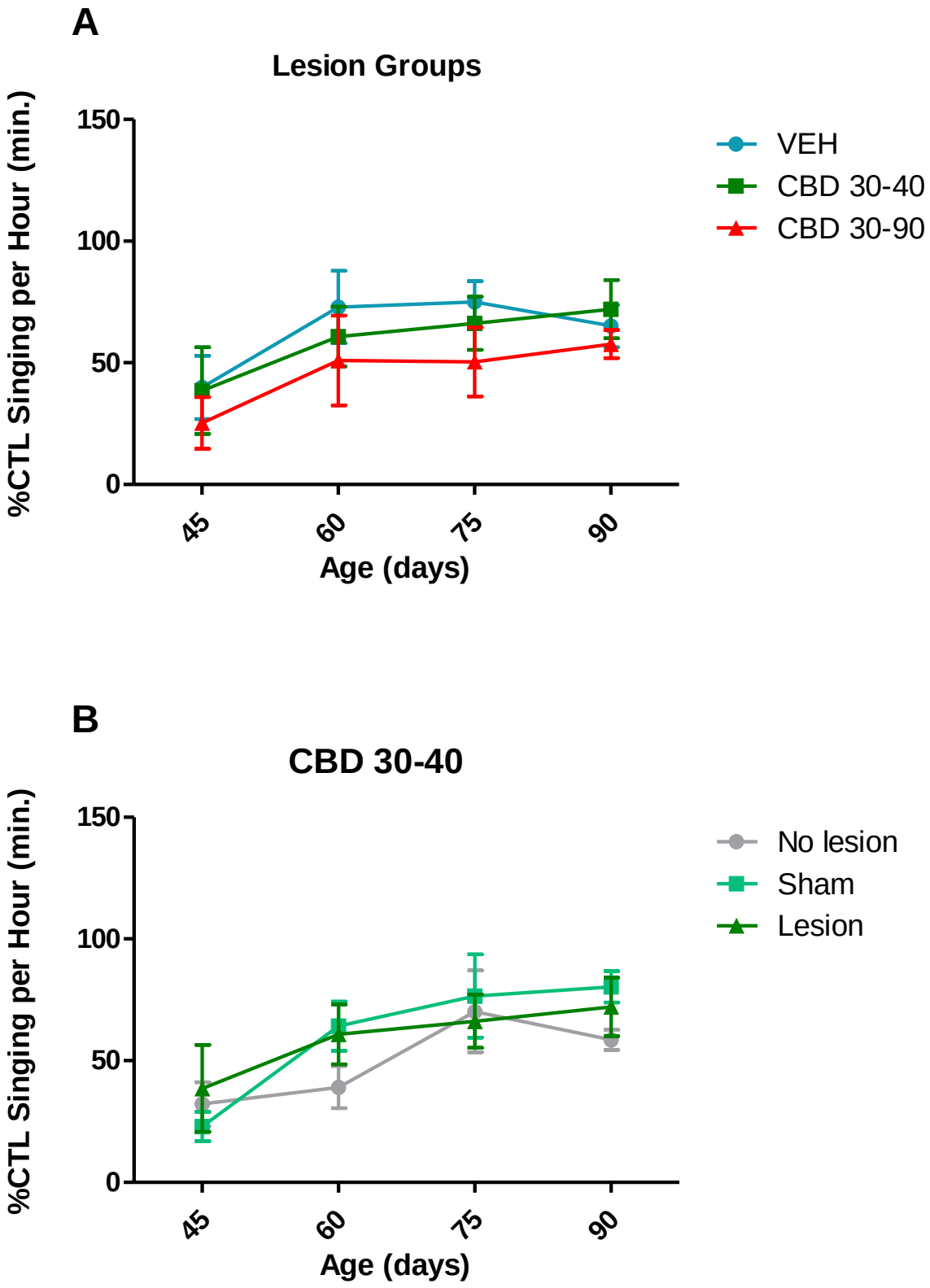

B
CBD 30-40
% CTL Typical Transitions
200
150
100
50
0
*
*
45
60
75
90
Age (days)
No lesion
Sham
Lesion

Figure 3.7: Effect of CBD on vocal output.

Vocal output was measured by percent control singing per hour at four time points 45, 60, 75 and 90 days of their age. Treatment groups received 60 daily IM injections of either: VEH = vehicle once daily beginning at 30 days of age; CBD 30-40 = 10 mg/kg CBD from 30 to 40 days of age and then vehicle to 90 days or; CBD 30-90 = 10 mg/kg CBD from 30 to 90 days of age. A, amount of singing was measured for different treatment groups in unilateral lMAN lesion animals. All animals received unilateral lMAN lesion at age of 35 days. All treatments did not significantly alter %CTL singing per hour compared to VEH at all time points. B, amount of singing was measured for different lesion groups in animals received CBD 30-40. All lesion conditions did not significantly alter %CTL singing per hour compared to VEH at all time points.


A
Lesion Groups
%CTL Singing per Hour (min.)
150
100
50
0
45
60
75
90
Age (days)
VEH
CBD 30-40
CBD 30-90
B
CBD 30-40
%CTL Singing per Hour (min.)
150
100
50
0
45
60
75
90
Age (days)
No lesion
Sham
Lesion

CHAPTER 4: GENERAL CONCLUSION AND FUTURE DIRECTIONS

The work in this dissertation was designed to accomplish two research goals: 1) to investigate the efficacy of CBD to improve learning-dependent vocal recovery following electrolytic destruction of a small part of a vocal motor brain region (HVC) which controls vocal production in adulthood, and; 2) to assess the effect of CBD to improve vocal learning after unilateral lesions of a vocal learning brain region (lMAN) which plays a major role during the sensorimotor stage of vocal learning. The central hypothesis was that CBD has distinct efficacy to mitigate CNS damage and promote vocal learning.

Aim 1, the investigation of the effect of CBD to mitigate the effects of CNS damage in terms of vocal production was experimentally addressed in three steps: Firstly, establishing a new pre-clinical animal model for screening drugs for efficacy to improve vocal behavior. The HVC microlesion model succeeded as a model to assess and show the efficacy of CBD to improve vocal behavior comparing to untreated animals.

Secondly, the ability of CBD to improve vocal learning following damage to lMAN that plays a major role in vocal learning during sensorimotor stage was examined. To test this, three different types of song analyses were measured: KL distances analysis was used to assess the effect of CBD to improve acoustic features, the sequence analysis was measured to investigate the ability of CBD to improve syntax and production analysis was used to assess vocal motor activity. Our results show that CBD treatment for a short period significantly has improved KL distance variability in adulthood time (not production).

Thirdly, was determined whether CBD produces these effects through interacting with $5HT_{1A}$ receptors. To test this, $5HT_{1A}$ antagonist (WAY) was injected to the animals

before receiving CBD to see whether the $5HT_{1A}$ antagonist would reverse the CBD effects. Our results show that the mechanism of CBD to improve recovery and mitigate CNS damage include $5HT_{1A}$ agonism, but likely other targets also involved.

Aim 2 determined whether CBD has the ability to improve vocal learning following damage to the major circuit in sensorimotor learning stage, lMAN, that was addressed in two steps: Firstly, establishing a new pre-clinical animal model for screening drugs for efficacy to improve sensorimotor vocal learning. The unilateral lMAN lesion model succeeded in demonstrating the ability of CBD to improve vocal learning compared to untreated animals.

Secondly, examining the efficacy of CBD to improve vocal learning. To test this, three types of song analyses were used: KL distances analysis was used to assess the effect of CBD to improve acoustic features, the sequence analysis was used to investigate the ability of CBD to improve syntax and production analysis was used to assess vocal motor activity. Our results show that CBD treatment sped maturation of singing in animals that received unilateral lMAN lesion in term of syntax and in term of phonology CBD improved KL distance variability in adulthood time. However, CBD does not have statically significant effect on vocal motor activity.

Taken together, the research described in chapters 2 and 3 supports the central hypothesis that CBD has distinct efficacy to mitigate CNS damage and promote vocal learning. In addition, this project provides a valuable and great addition to the research through establishment of an important new animal model to screen drugs for efficacy to improve vocal learning and production; and provides a treatment that has an ability to improve speech and language disorders related to CNS trauma.

Disruptions in speech and vocal communication are markers of several neuropsychiatric disorders, most remarkably autism spectrum disorders. Historically, animal models have been an effective approach for developing and examining disease-relevant therapeutics. The unique aspects of human language when compared to vocal behaviors for other animals make finding a suitable animal model to examine the speech disorders potentially more challenging. In addition, the FDA requires at least the use of two animal models for any new therapeutic. To our knowledge this project provides the first animal model to screen drugs for efficacy to improve vocal learning and production. Songbirds are among the few vocal learning animals and the only examples well-suited to laboratory use (Petkov and Jarvis 2012). Zebra finches have many similarities to humans in-terms of vocal communication and production (as mentioned above) that make them a good candidate to assess effect of drugs having effects on CNS damage and processes of vocal learning. The targeting of HVC of this animal by electrolytic lesion (a small part about 9% of HVC) results in a temporary disruption of vocal patterns that recovers over about seven days (Thompson and Johnson, 2007). Recovery from these microlesions depends upon the ability of birds to hear, as deafened birds do not regain ability to produce typical song patterns (Thompson et al., 2007). Auditory dependence indicates that recovery of song requires auditory feedback that is part of sensorimotor learning. Thus, this pre-clinical animal (the songbird HVC microlesion) model will speed and open the closed door to assess drug effects upon both CNS damage and processes of vocal learning and this will help to discover a treatment for many people suffering from difficulty in speech and vocal communication such as autism patients.

In addition to establishing a new and exclusively-useful pre-clinical animal model, the main finding of this project is to provide a promising drug for the treatment of speech and language disorders related to CNS trauma. Our findings show CBD has the ability to reduce the effects of HVC microlesions. This effect appears to involve both preventing deficits following HVC damage (secondary to tissue damage itself), and to a reduction in the amount of time required for recovery. These effects suggest that CBD reduces microlesion included deficits via interactions with processes secondary to tissue damage is based upon lack of significant differences across treatment groups in the amount of HVC destroyed (about 9% in all groups). Thus, the effect of CBD was not to reduce the size of the infarct. This indicates that disruptive effects of HVC microlesions must involve processes distinct from and secondary to the tissue damage itself. These secondary processes may include CBD-induced reductions in gliosis, metabolic derangement, excitotoxicity, neuroinflammation and/or protection of astrocyte function. These will be important to address in future studies. A proposed experiment may involve using proton magnetic resonance spectroscopy (H^+-MRS) to assess the effect of CBD to reduce metabolic derangement (by measuring the ratio of lactate to N-acetylaspartate in damaged tissue), excitotoxicity (by measuring the ratio of glutamate to N-acetylaspartate), and protected astrocyte function (by measuring the ratio of myoinositol to creatine) after HVC microlesions. In addition, it is important to perform immunohistochemistry studies to assess ability of CBD to reduce neuronal loss and apoptosis (using TUNEL assay), astrogliosis (using an anti-GFAP antibody) and microglial proliferation (using an anti-Iba1 antibody). The results of these experiments will help us to understand if CBD reduces microlesion effects via interaction with processes

secondary to tissue damage. Whatever the secondary processes are, their mitigation improves ability of the songbirds to relearn to produce memorized vocal patterns.

The ability of the songbirds to relearn after HVC microlesions depends upon the ability of birds to hear their songs. According to Thompson et al. deafened animals do not recover high-quality song because they don't receive the auditory feedback necessary for sensorimotor relearning (Thompson et al. 2007). It may also be important that the microlesion effect is restricted to motif syllables and does not appear to impair instinctive calls (Fig. 2C and D). Thus, CBD may prevents degradation of a learned behavior which consistent with findings of Karl et al that CBD improved memory in dementia models (Karl, Garner, and Cheng 2017)

Additionally, previous work observed that birds subjected to lesions of lMAN prior to HVC microlesions exhibit no clear effect of the procedure (Thompson and Johnson 2007). In addition, our results detected that CBD markedly reduced phonological disruptions after HVC microlesion. Again, this is intriguing given our microlesion target was HVC, a motor region most clearly associated with syntax-relevant syllable timing. Phonology looks more dependent upon the anterior forebrain circuit and its lMAN output to RA (Vu et al., 1994; Kao et al., 2005). This means that the ability of microlesions to disrupt vocalizations depends upon an intact lMAN, a simple potential mechanism for CBD to almost eliminate phonology disruptions might include interference with lMAN activity. Does CBD produce a pharmacological lMAN lesion? CBD inhibition of lMAN output is a hypothesis to be tested. To test this hypothesis quantitative EM techniques will be used to quantify the number of synapses in motor cortical region RA derived from

HVC and lMAN after receiving HVC microlesions and; CBD (or VEH). The ratio of HVC:lMAN derived synapses in RA increases during normal development because HVC is controlling RA in adult birds. We will test the hypothesis that exposure to CBD treatment after HVC microlesion will increase the ratio of HVC:lMAN by preventing or decreasing lMAN control over RA activity. If our hypothesis is true, this will indicate that exposure to CBD after a HVC microlesion that CBD produces a pharmacological lMAN lesion or inhibition of lMAN output and this will explain how CBD improves vocal learning after CNS damage.

Moreover, Additional studies are needed to assess the effect of CBD on postoperative lesions through injecting CBD only after doing lesions. This has implications for potential therapeutic use of CBD in cases of CNS trauma that will likely depend upon after-incident administration. Finally, experiments assessing the accumulation of its metabolites in the brain and other organs after chronic administration will be important to understand more about its pharmacokinetics.

Summary

In this project:

- Two pre-clinical models for screening drugs for efficacy to improve vocal behavior and vocal learning have been developed.
- CBD improves recovery and reduces magnitude of CNS damage effects.
- CBD improves sensorimotor relearning.
- Mechanism of these effects may include, in-part, 5-HT1A agonism.
- CBD improves vocal learning and appear to speed maturation of singing.

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APPENDIX

ANIMAL CARE AND USE COMMITTEE APROVAL LETTER

East Carolina University
Tomorrow starts here.

Animal Care and
Use Committee
212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834-4354

252-744-2436 office
252-744-2355 fax

August 7, 2018

Ken Soderstrom, Ph.D.
Department of Pharmacology
Brody 6S-10
East Carolina University

Dear Dr. Soderstrom:

Your Animal Use Protocol entitled, "Effects of Cannabidiol on Recovery of Vocal Behavior" (AUP #W247a) was reviewed by this institution's Animal Care and Use Committee on August 7, 2018. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure