Molecular Mechanisms Underlying Cannabidiol-Improved Vocal Recovery Following Damage to a Songbird Vocal Pre-Motor Cortical-Like Region

by

Mark Andrew Tripson

May, 2023

Director of Dissertation: Dr. Ken Soderstrom

Department of Pharmacology & Toxicology at the Brody School of Medicine, ECU

ABSTRACT

The non-euphorigenic phytocannabinoid CBD has been found to be effective in treating childhood-onset epilepsies, conditions that are often associated with developmental delays, including vocal communication. Zebra finch song is a complex behavior that is learned during a sensitive period of vocal development, making it a promising model for understanding the mechanisms responsible for potential CBD-related improvements in vocal learning. Like language, the quality of adult zebra finch song is maintained through continuous sensorimotor maintenance and refinement, involving brain regions that control vocal learning and production. One of these brain regions, HVC, is a pre-vocal motor cortical-like region that when partially lesioned temporarily disrupts vocal behavior. Recovery from HVC microlesions typically takes about seven days without treatment. However, treatment with CBD has been shown to both speed recovery and reduce the acute magnitude of disruptions. Given the anti-inflammatory properties of CBD in seizure and other models, we suspected involvement of similar mechanisms in vocal recovery. To test this, we investigated CBD modulation of post-lesion expression of inflammatory cytokines, markers of neuronal stress, microglial migration, and changes in synaptic densities

within relevant song control regions. Results indicate that CBD-improved vocal recovery is associated with reduced oxidative stress and anti-inflammatory activity. This decrease in inflammation and stress marker expression was associated with reduced density of microglia staining within song regions afferent to the lesion target, HVC (including learning-essential Area X [basal ganglia] and vocal motor RA [motor cortex]). Furthermore, we measured densities of excitatory synapses within Area X and RA, finding significant lesion-related decreases that were largely reversed by CBD. This synaptic protection was associated with BDNF/Arc/MSK1 upregulation, implicating mechanisms important to homeostatic synaptic scaling. Overall, this work indicates that CBD improves post-CNS damage recovery of learned vocal behavior by promoting multiple homeostatic mechanisms. This efficacy may generalize to sensorimotor skills learned by other vertebrates and suggests potential application to TBI and related disorders.

MOLECULAR MECHANISMS UNDERLYING CANNABIDIOL-IMPROVED VOCAL RECOVERY FOLLOWING DAMAGE TO A SONGBIRD VOCAL PRE-MOTOR CORTICAL-LIKE REGION

A Dissertation

Presented to the Faculty of the Department of Pharmacology & Toxicology

Brody School of Medicine at East Carolina University

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Pharmacology & Toxicology

By

Mark Andrew Tripson

May, 2023

Director of Thesis: Ken Soderstrom, Ph.D.

Thesis Committee Members:

Lisa Domico, Ph.D.

Karen Litwa, Ph.D.

Srinivas Sriramula, Ph.D.

David Taylor, Ph.D.

DEDICATIONS

This dissertation is dedicated to my grandmother, Mary Tripson.

<h1 style="text-align:center">Table of Contents</h1>

List of Tables

List of Figures

List of Abbreviations and Symbols

2-AG   2-arachidonoyl glycerol

5-HT   5-hydroxytryptamine

5-HT1A   5-hydroxytryptamine receptor subtype 1A

AEA   Arachidonyl ethanolamide (anandamide)

AFP   Anterior forebrain pathway

AMPA   α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ARC/ARG 3.1   Activity-regulated cytoskeleton-associated protein/gene 3.1

ARE   Antioxidant response element

ASD   Autism spectrum disorder

BDNF   Brain derived neurotrophic factor

CB1   Cannabinoid receptor type 1

CB2   Cannabinoid receptor type 2

CBD   Cannabidiol

CNS   Central Nervous System

CTCF   Corrected total cellular fluorescence

DHE   Dihydroethidium

DLM	Dorsolateral Thalamus
DSE	Depolarization-induced suppression of excitation
DSI	Depolarization-dependent suppression of inhibition
eCB	endocannabinoid
ECS	Endocannabinoid System
EEG	Electroencephalogram
FAAH	Fatty acid amide hydrolase
FABP	Fatty acid binding protein
GABA	γ-aminobutyric acid
GLUA1	Glutamate receptor ionotropic AMPA 1
GSH	Glutathione
GSHPx	Glutathione peroxidase
HD	Huntington's Disease
IDO	Indoleamine-2,3-dioxygenase
IL	Interleukin
KL	Kullback-Leibler
lMAN	lateral portion of the magnocellular nucleus of the anterior neostriatum
LMC	Laryngeal motor cortex

LTD	Long-term depression
LTP	Long-term potentiation
MAGL	Monoacylglycerol lipase
MDA	Malonaldehyde
MS	Multiple Sclerosis
MSK1	Mitogen- and Stress-Activated Protein Kinase 1
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NRF2	Nuclear factor erythroid 2-related factor 2
PD	Parkinson's Disease
PPAR	Peroxisome proliferator-activated receptor
PSD95	Post-synaptic density protein 95
PTSD	Post-Traumatic Stress Disorder
PTZ	Pentylenetetrazole
RA	Robust nucleus of the archistriatum
ROS	Reactive oxygen species
RT-PCR	Real time polymerase chain reaction
SAP	Sound Analysis Pro
SN	Substantia Nigra

SOD2	Superoxide dismutase 2
TBI	Traumatic brain injury
THC	Δ9-tetrahydrocannabinol
TNFα	Tumor necrosis factor α
TRPV1	Transient receptor potential vanilloid 1
VGLUT2	Vesicular glutamate transporter
VEH	Vehicle
VTA	Ventral Tegmental Area
XO	Xanthine oxidase

CHAPTER ONE: Introduction

1.1 Phytocannabinoids and the Endocannabinoid System

Cannabis sativa, a plant indigenous to eastern Asia, has been used for centuries to elicit both medicinal, as well as, psychotropic effects[1]. Many of the bioactive molecules it produces have been studied for decades eventually leading to the discovery of the endocannabinoid system (ECS)[2]. These efforts have resulted in identification of hundreds of constituents called phytocannabinoids, with delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) being the most well-known. Until recently, interest in CBD had been eclipsed by a focus on the more-dramatically effective and euphorigenic THC. First isolated in 1964, THC is responsible for the euphorigenic "high" that has been the leading argument for prohibition of cannabis throughout modern culture. This euphoria is well understood showing partial agonism for cannabinoid type 1 (CB1) and cannabinoid type 2 (CB2) receptors of the ECS[3] and it was THC that ultimately led to the identification of ECS and endogenous signaling components, endocannabinoids (eCB).

After its discovery in 1988, the ECS has emerged as an essential component of the development and continued dynamics of the central nervous system (CNS). Through unique retrograde signaling, endogenous eCB's, principally 2-arachidonyl glycerol (2-AG) and arachidonoyl ethanolamine (AEA), act as key modulators of synaptic plasticity at both excitatory and inhibitory synapses[4]. Major forms of this plasticity, long-term potentiation (LTP), long-term depression (LTD), depolarization-dependent suppression of inhibition (DSI), and depolarization-induced suppression of excitation (DSE) are important cellular processes involved in learning and memory[5], [6]. The association with these events has led the ECS to be viewed as a regulator of communication in the CNS, while damage and dysfunction of the system are associated with pathologies of short- and long-term memory, as well as learning and social behaviors[7], [8].

Components of the ECS, like the CB1 receptor, are found throughout the CNS and produce a range of effects, including altered perception, mood, and cognition, as well as impairment of motor coordination and memory[9]. Partial agonism by THC has also been shown to exert dopaminergic signaling in the mesolimbic pathway (ventral tegmental area (VTA) and substantia nigra (SN), which primarily projects to the striatal complex[10]) which is the basis for the reinforcing properties of cannabis[11]. On the other hand, although structurally similar to THC, CBD does not have affinity for cannabinoid receptors and does not produce euphoria, but shows potential therapeutic utility through a vast array of cellular targets[3].

Recently, CBD is receiving increased therapeutic attention as a botanically-derived formulation, Epidiolex, has been approved for marketing in the US to treat childhood seizure disorders[12]. The FDA approval of Epidiolex in 2018 has helped shift the focus towards the therapeutic potential of CBD, leading to current investigations for potential use in various psychiatric, neurodegenerative, inflammatory, and neoplastic diseases[13]. Despite this recent interest, a potential problem using CBD therapeutically appears to be the lack of selectivity. This drug interacts with and modifies activity of multiple cellular targets and appears to have unique properties that are often inconsistent in the literature[14], [15]. This inconsistency may be attributable to issues with co-isolation of other potentially bioactive molecules. For example, purified CBD extracts contain at least traces of other cannabinoids, including CNS-active, THC[16]. It is becoming clear that CBD efficacy is influenced by THC content, underscoring the importance of consistent, carefully-controlled formulations[17].

CBD holds great potential as a therapeutic agent for a wide range of conditions despite its mechanism of action remaining nonselective and largely unknown. With careful formulation, and appropriate models, the specific cellular targets of CBD can be identified, allowing for more

effective therapeutic applications. As more research is conducted on the therapeutic applications of cannabis, it is becoming evident that phytocannabinoids show immense potential in addressing various medical conditions, and CBD could provide further understanding of the therapeutic value of cannabis.

1.2 A Comprehensive Look at CBD's Therapeutic Indications

With the legalization of cannabis and cannabis products in some countries and states, as well as recent FDA approval of cannabis-derived formulations, interest in CBD has been rekindled, leading to numerous studies and clinical trials. Despite an elusive mechanism of action for CBD, it has been shown to be effective in the treatment of various conditions, including childhood seizure disorders, neurodegenerative diseases, brain injuries and multiple sclerosis[12], [18].

Currently, there are two marketed formulations of therapeutics that contain botanically derived CBD, Epidiolex and Sativex. Sativex, is a patented 1:1 ratio of THC and CBD used as a sublingual spray approved in several countries for the treatment of cancer-related pain, as well as spasticity and pain associated with multiple sclerosis (MS)[18], [19]. Sativex is not currently approved in the United States, but with growing rhetoric, cannabis products are moving towards mainstream acceptance. CBD has powerful anti-inflammatory and antioxidant activity, and it is thought to be this action which contribute to its therapeutic efficacy in MS[15]. In addition to its positive effects on MS, Sativex has also been shown to be effective in the treatment of neuropathic pain, cancer pain, and rheumatoid arthritis[20]–[22]. Despite these approved uses, the exact mechanism by which Sativex works is still unknown, although the combination of THC and CBD is believed to exhibit synergistic effects[23], [24]. Needless to say, this action is complex, and further adds to the complexity of activity associated with botanical extracts.

3

Conversely, Epidiolex, is a purified CBD extract (containing >99% CBD) that has been approved for the treatment of seizures associated with two rare forms of childhood epilepsy, Lennox-Gastaut and Dravet syndrome[12]. The efficacy of CBD in reducing seizures has been attributed to its ability to modulate gamma-aminobutyric acid (GABA) neurotransmission and voltage-gated calcium channels, but this is a proposed mechanism and a more complete understanding has yet to be determined[25]. Isolated CBD has also been shown to have neuroprotective efficacy through its anti-inflammatory, and anti-oxidative activity in animal models of Parkinson's disease (PD), Huntington's disease (HD), and Alzheimer's disease (AD)[26].

Consistent evidence in the scientific literature has shown that the treatment of these neurodegenerative disorders largely depend on the anti-inflammatory and anti-oxidative properties of CBD[2], [27], [28]. These properties hold significant promise in specifically treating unregulated or sustained inflammation, which can lead to tissue damage and worsen degenerative diseases, developmental disorders, and traumatic injuries. Notably, all of these conditions are linked to cognitive dysfunction, making the preservation of cognitive function a vital treatment goal. Recent evidence suggests that CBD may offer a protective effect on learned behaviors, including those acquired during extended periods of sensorimotor development[29], thus presenting a promising therapeutic avenue for preventing cognitive decline caused by neurodegenerative diseases.

Aside from potential applications in neurodegenerative disorders, CBD has demonstrated effectiveness in treating a broad range of other medical conditions. Chronic use of this compound has been found to possess anti-anxiety and anti-depressant properties, making it a promising therapy for conditions such as post-traumatic stress disorder (PTSD) and general anxiety disorders[26]. Additionally, the anti-inflammatory and anti-oxidative properties suggest that CBD

could be a valuable treatment option for traumatic brain injury (TBI), stroke, ischemia, and inflammatory bowel disease[30].

Although we have seen Sativex marketed for neuropathic pain containing THC, isolated CBD has shown analgesic efficacy and may be useful in the treatment of chronic pain. These effects are thought to be mediated by its ability to modulate the activity of several receptors and ion channels involved in the regulation of pain perception, as well as voltage-gated sodium and potassium channels[15]. These targets are involved in more than just inflammation, but also modulate nociceptive signaling contributing to the development and maintenance of chronic pain. Understanding this specific action could be applied to many other pathologies and replace current therapeutics. For example, clinical studies have already investigated the use of isolated CBD in the treatment of neuropathic pain, inflammatory pain, and cancer pain, and found that CBD may be effective in reducing associated pain scores and improving quality of life in some patients while maintaining a low side effect profile compared to its counterpart, THC[31].

Despite the challenges and limitations associated with using CBD therapeutically, it is becoming increasingly clear that phytocannabinoids like CBD hold great promise for the treatment of a variety of medical conditions and the utility seems to be growing exponentially. With more research into CBD's mechanism of action, it is evident that different formulations can be developed to have a greater therapeutic utility through interacting with a vast array of cellular targets. Botanically-derived formulations such as Sativex and Epidiolex have already been approved for the treatment of spasticity, pain and epilepsy, but further research is needed to fully understand CBD use in other conditions such as neurodegenerative diseases, TBI, PTSD, general anxiety, and inflammatory disorders.

CHAPTER TWO: CBD's Multifaceted Mechanisms for Neuroprotection

2.1 Anti-inflammatory activity of CBD

The therapeutic potential of CBD has been demonstrated in anxiety, depression, neurodegeneration, cancer, and epilepsy, to name a few. Although containing great potential, the mechanism of action of CBD is complex and involves interaction with various molecular targets in the CNS. Although convoluted, it is defined throughout the literature that CBD has consistent anti-inflammatory activity that may help to mitigate the damaging effects of previously mentioned neurodegenerative disorders such as AD, PD, and MS[32], [33]. Additionally, CBD may effectively help to decrease neuroinflammation that is known to accompany TBI, as well as developmental diseases such as Autism Spectrum Disorder (ASD)[34].

This anti-inflammatory activity in the CNS is achieved through complex nonselective interaction with several molecular targets, including receptors, ion channels, and enzymes[35]. Interestingly, these molecular targets do not include modulation of the ECS by direct CB1 or CB2 receptors activity. Although structurally similar to THC, a potent partial agonist of CB1, CBD does not have high affinity for these receptors and its mechanism likely does not involve this direct activity. However, it is important to note that recent evidence has shown CBD to act as a negative allosteric modulator at CB1, binding and promoting conformational changes that bridge transition towards an inactive receptor signaling state[36]. This in vitro work employed the use of CBD extracts, and trace amounts of other phytocannabinoids, including THC, may be present: All cannabis extracts have some trace amounts of other active cannabinoids. The source of CBD is not regulated among these studies and these trace constituents may have efficacy through cannabinoid agonist binding sites. This is important as we have demonstrated that different CBD:THC ratios have differential efficacy to promote recovery from CNS damage[17]. Additionally, in silico

models of CBD binding have not been reproduced and are not therapeutically relevant to the anti-inflammatory activity[37].

Without clear direct activity in the ECS at CB1 or CB2, CBD likely predominantly alters signaling events relating to indirect mechanisms that lead to much of its therapeutic efficacy[2]. To date, phytocannabinoids like CBD have been demonstrated to act on a wide range of targets including: Inhibition of eCB transporters by binding to fatty acid binding proteins (FABPs); decrease eCB hydrolysis mediated by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL); blockade of adenosine uptake; and agonism of transient receptor potential cation channel subfamily V member 1 (TRPV1), peroxisome proliferator-activated receptor gamma (PPARγ) and the serotonergic 5-HT1A receptors[38]. In addition to these interactions phytocannabinoids can directly reduce free radical and inflammatory mediator activity.

2.1.1 CBD activity on FABP

The eCB's AEA and 2-AG, are hydrophobic molecules that are shuttled across cell membranes to their intracellular targets by binding to specific transporters known as fatty acid binding proteins (FABPs)[39]. These small cytosolic protein transporters are responsible for the uptake and recycling of eCB's in the body, and CBD competes with eCBs for the binding of FABPs ultimately leading to an increase in eCB's available for binding to other prospective targets[40]. Specifically, CBD has been shown to bind to the FABP5 and FABP7 isomers, and play a key role in the transport and metabolism of eCB's in the epidermis, adipose tissue, brain and retina[41]. By inhibiting these transporters, this leads to a number of downstream effects on various physiological processes through activation of CB1 or CB2. It is important to note that the interaction between CBD and FABPs is complex and not fully understood, and there is some likely affinity differences between the isomers. While some studies have suggested that CBD can inhibit eCB transport by

7

directly binding to FABPs, other studies have suggested that CBD may interact indirectly and that other mechanisms may also be involved.

2.1.2 CBD activity on FAAH MAGL

CBD has been shown to inhibit the activity of both FAAH and MAGL, leading to further increased levels of eCB in the body. FAAH is responsible for breaking down AEA, while MAGL is responsible for the breakdown of 2-AG[4]. Inhibition of FAAH and MAGL by CBD leads to increased levels of AEA and 2-AG, effectively increasing activation of the CB1 and CB2 receptors[42], [43]. CBD is believed to involve non-competitive inhibition through binding to a site on the enzyme that is distinct from the active site, which prevents the normal breakdown and subsequent elevated levels of eCB's leading to therapeutic effects of anti-inflammatory, analgesic, and anxiolytic properties[44]. Increasing levels of AEA and 2-AG have also been shown to have neuroprotective effects, which may be beneficial for the treatment of neurological disorders as seen in epilepsy[45].

2.1.3 CBD activity on TRPV1 and 5HT-1A receptors

With the complex nonselective nature of CBD, there appears to be some effects mediated through agonism of TRPV1 and 5-HT1A receptors[42], [43]. CBD competes with the full agonist capsaicin for TRPV1 binding substantially increasing intracellular calcium levels. This increase in Ca2+ was similar to the full agonist thus suggesting CBD acts as an agonist at TRPV1[42]. This was confirmed in a pentylenetetrazole (PTZ) model of seizure activity, where CBD increased both seizure latency and reduced seizure duration of PTZ-induced EEG activity in the prefrontal cortex of mice[46]. These effects were reversed using selective antagonists of the TRPV1, CB1 and CB2 receptors (SB 366791, AM 251 and AM 630, respectfully) suggesting the involvement of multiple components for CBD's anticonvulsant activity.

CBD has also been shown to modulate the activity of the 5-HT1A serotonin receptors, which are involved in the regulation of anxiety, depression, and to an extent, allodynia[43]. In cells expressing 5-HT1A, CBD was shown to displace the selective 5-HT1A agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and induce guanosine 5'-O-(3-thiotriphosphate) (GTPγS) binding, as well as reduce the percentage of forskolin-stimulated cyclic AMP levels at similar levels of receptor occupancy as 5-hydroxytryptamine (5-HT) suggesting agonism of 5-HT1A receptor[47]. Experimental treatment with CBD also increases the time rats spend on the Elevated Plus Maze (Test that stimulates conflict between exploration of a novel adverse environment increased anxiety) and potentiate the effects of 8-OH-DPAT in motor activity both of which indicate the involvement of the 5-HT1A receptor[48]. Furthermore, much of the antidepressant effects of CBD and to attenuate mechanical allodynia in rat models of neuropathic pain is blocked by the selective 5-HT1A receptor antagonist WAY 100135, but not AM251, a CB1 antagonist[47], [48].

2.1.4 CBD activity on PPARγ

In addition to TRPV1 and 5-HT1a activity, CBD displays agonism of peroxisome proliferator-activated receptor gamma (PPARγ). This nuclear receptor is one of the most extensively studied ligand-inducible transcription factors related to glucose metabolism and insulin signaling[49]. CBD decreases reactive gliosis in primary astrocyte cultures, effectively decreasing inflammation by reducing leukocyte rolling and adhesion to the endothelium while displaying antioxidant activity by reducing hyperoxide toxicity in neurons[50]–[52].

2.1.5 CBD activity on inflammatory mediators

The modulation of inflammation is a key function of CBD therapeutic activity and evidence shows the ability to specifically modulate the production of cytokines, including tumor necrosis

factor alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β), which are key mediators of the inflammatory response[2], [53]. Treating human primary monocytes with varying concentrations of CBD for 22 hours showed a selective alteration in monocyte-derived IL-1β and IL-6 when activated through various toll-like receptors[54]. There has also been addition amelioration of TNFα and presence of cleaved caspase 3 after CBD treatment in epithelial tissue showing clear translational relevance as an anti-inflammatory compound decreasing pro-inflammatory immune proliferation[55]. This activity may be explained by CBD's ability to indirectly influence the ECS by increasing levels of endogenous ligands for CB2 although it seems the mechanism is much more complex given the anti-inflammatory activity is consistent in the presence of CB2 antagonists[28].

In conclusion, CBD has shown significant therapeutic potential in a range of conditions, including anxiety, depression, neurodegeneration, cancer, epilepsy, and more. Despite its complex mechanism of action, it is clear that CBD has consistent anti-inflammatory activity in the CNS, which may help mitigate the damaging effects of neurodegenerative disorders, traumatic brain injuries, and developmental diseases. CBD's nonselective interaction with various molecular targets, including FABPs, FAAH, MAGL, TRPV1, PPARγ, and 5-HT1A receptors, leads to downstream effects on various physiological processes, ultimately leading to its therapeutic effects. While there is still much to learn about CBD's mechanism of action, it is clear that this phytocannabinoid holds great potential as a therapeutic agent for a wide range of conditions. Further research is needed to better understand its full potential and to optimize its clinical use.

2.2 Antioxidant activity of CBD

In addition to its anti-inflammatory activity, the therapeutic efficacy of CBD has been found to include antioxidant properties that can be classified as direct or indirect. Direct

antioxidant activity of CBD includes the regulation of redox state through individual components of the redox system while indirect activity includes the interaction with other molecular targets associated with the redox system components.

Reactive oxygen species (ROS) and free radicals are generated during normal cellular metabolism, and they can cause oxidative damage to cellular components such as lipids, proteins, and nucleic acids[56]. Oxidative stress occurs when the generation of ROS and free radicals exceeds the antioxidant capacity of the cell. The dangers of ROS and free radicals include various negative effects such as DNA damage, lipid peroxidation, protein damage, inflammation, and aging. Oxidative stress is associated with several chronic diseases, including cancer, cardiovascular disease, and neurodegenerative disorders[57].

2.2.1 Direct antioxidant activity

Direct regulation of oxidative stress by CBD includes the interruption of free radical chain reactions, capturing free radicals or transforming them into less active forms. The hydroxyl groups of the phenol ring are mainly responsible for CBD's antioxidant activity, as free radicals produced in these reactions are characterized by many resonance structures in which unpaired electrons are mainly found on the phenolic structure[58]. CBD reduces oxidative conditions by preventing the formation of superoxide radicals, which are mainly generated by xanthine oxidase (XO) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase isoforms NOX1 and NOX4[28], [59]. Studies have shown that CBD reduces ROS production by chelating transition metal ions involved in the Fenton reaction that form extremely reactive hydroxyl radicals[60]. The Fenton reaction is a type of reaction that involves the production of highly reactive and damaging free radicals, which can cause damage to cells and tissues[61].

11

The ability of CBD to decrease β-amyloid formation in neurons by reducing the concentration of transition metal ions is also reported[62]. Here, cell survival and various cellular responses were evaluated prior to beta-amyloid peptide exposure. They found treatment with CBD significantly increased cell survival, while simultaneously reducing the production of ROS, lipid peroxidation, caspase 3 levels, DNA fragmentation, and intracellular calcium levels. Additional antioxidant activity is achieved by CBD-induced reduction of micro elements important in the activity of antioxidant enzymes. CBD prevents the reduction of microelements such as Zn or Sn, which are typically lowered in pathological conditions[63]. This helps to maintain the levels of antioxidant enzymes, which are essential for protecting the body against oxidative stress.

The neuroprotective antioxidant activity of CBD is also very interesting and seems to be more significant than that of vitamin C or α-tocopherol, two well-known antioxidants[64], [65]. These studies have shown that glutamate neurotoxicity can be inhibited with antioxidants, and CBD was shown to have significant efficacy. Furthermore, CBD is an inhibitor of tryptophan degradation by reducing indoleamine-2,3-dioxygenase (IDO) activity[66]. Tryptophan is an essential amino acid that plays a vital role in maintaining proper brain function and IDO is an enzyme that breaks down tryptophan, which can result in decreased levels of tryptophan and ultimately impact brain function. By inhibiting the activity of this enzyme, CBD may help to maintain healthy levels of tryptophan, which could have positive effects on brain function.

In addition to this direct activity of ROS and free radicals, CBD modifies the redox balance by changing the level and activity of antioxidants at the protein transcription level. By activating the redox-sensitive transcription factor referred to as the nuclear erythroid 2-related factor (NRF2), CBD increases the transcription of cytoprotective genes, including antioxidant genes[67]–[69]. NRF2 becomes phosphorylated in the presence of cellular stress and translocate across the nuclear

membrane to bind the active site of antioxidant response element (ARE) that is crucial for the initiation of healing[67]. Lastly, as regulation is an important aspect of this direct antioxidant activity, CBD was found to increase the mRNA level of superoxide dismutase (SOD) and the enzymatic activity of Cu-, Zn- and Mn-SOD, which are responsible for the metabolism of superoxide radicals[70].

To conclude the direct antioxidant activity of CBD, studies have shown that repeated doses of CBD can increase the activity of glutathione peroxidase and reductase which results in a decrease in malonaldehyde (MDA) levels[71]. Additionally, CBD was used to treat human skin cells that were exposed to UVB radiation and there was an increase in both glutathione peroxidase (GSHPx) activity and glutathione (GSH) levels[72]. Glutathione peroxidase and GSH are important antioxidant enzymes that protect us against oxidative stress. MDA is a biomarker of oxidative stress and is produced as a result of lipid peroxidation, higher MDA levels indicate cellular stress by highly reactive free radicals. By lowering ROS levels, CBD can protect non-enzymatic antioxidants, such as vitamins A, E, and C, from being oxidized. When the body is exposed to stress, the levels of GSH, as well as other antioxidants, can become depleted. By increasing the activity of glutathione peroxidase and reductase, CBD helps to maintain the levels of GSH and other antioxidants in the body.

2.2.2 Antioxidant activity through ECS signaling

CBD has been shown to have indirect antioxidant activity through its interaction with the ECS, which is involved in regulating redox balance in cells. As previously mentioned, CBD can modulate the activity of the ECS by increasing levels of endogenous ligands, which can affect the activity of cannabinoid receptors. The ECS, through its activation of peroxisome proliferator-activated receptor alpha (PPARα), is known to directly regulate the expression of antioxidant

13

enzymes such as superoxide dismutase (SOD) by interacting with their promoter regions[73]. Additionally, CBD can interact with ion channels and nuclear (PPAR) receptors indirectly increasing the activity of antioxidant enzymes and enhance the cell's ability to combat oxidative stress[74]. This can result in a decrease in ROS levels, protecting non-enzymatic functions.

2.3 Inflammation in the Brain: A Threat to Learning and Memory

In the absence of disease or injury, the CNS regularly adapts and change in response to various stimuli, which is broadly termed neuroplasticity. This widespread and well documented phenomenon modifies the effectiveness of communication in both excitatory and inhibitory synapses and positively refers to the strengthening of presynaptic input onto a postsynaptic neuron[75]. These changes, of synaptic plasticity are essential for learning, memory, and behavioral adaptations, but it is well understood that neuroinflammation can hinder this function[52], [76]. The ability to alter communication in the CNS occurs through the physiological modulation of synapse structure and density, which enables neurotransmission to be directed appropriately.

Mechanisms underlying synaptic plasticity include genomic changes that recruit biological machinery for the development, growth, and/or pruning of synaptic communication. One such mechanism is the alteration of neurotransmission across a synapse through retrograde activity, which can indirectly activate or repress brain-derived neurotrophic factor (BDNF). BDNF is involved in synaptogenesis, generating assembly of new synapses and the disassembly of old synapses.

It has been shown that eCB's directly suppress presynaptic neurotransmitter release in both glutamatergic and GABAergic synapses. This can increase or decrease neuronal efficiency,

respectively, thus shaping the ability to facilitate neuroplastic events like LTP or LTD. By enhancing synaptogenesis and subsequent strengthening adaptations, the neuroprotective efficacy of compounds altering the ECS may be characterized. Modifications to synaptic plasticity can be altered by exogenic events such as the recruitment of pro-inflammatory cytokines such as TNFα and IL-1β. This pro-inflammatory cytokine activity on hippocampal synaptosomes demonstrated a significant suppression of chemically induced LTP using glycine[77]. Suppression in the ability to produce consistent LTP's is correlated to a wide array of major brain disorders that display dysregulated AMPAR endocytosis[78] that prevent neurons to communicate efficiently, and CBD, an anti-inflammatory compound, may help ameliorate these negative consequences[79], [80].

2.3.1 Learning and Memory

The anti-inflammatory and antioxidative properties of CBD make it a promising therapeutic for neuroinflammatory diseases that can drastically affect learning and memory. The process of learning is complex and involves the establishment of circuits linking different brain regions. These circuits loop through regions of integrative cortex to motor striatum and coordinating thalamus. Motor circuits are refined through practice, leading to memory formation and programmed motor patterns that are vulnerable to disease-related neurodegeneration[81]. In all vertebrates, early learning initially involves circuits reinforced by midbrain dopaminergic input. As behavior matures, control shifts to medial dorsal circuits that are less reinforcement-dependent and more programmatic, which is necessary for the automaticity underlying motor skill mastery[82]. Because this process requires significant practice over time, the resulting motor skills are very valuable. Some skills are most easily acquired during sensitive periods of development, outside of which additional time and effort required for learning can make them practically unobtainable, and irreplaceable in the event of injury or disease. Despite the significance of this

issue, there are currently few therapeutic approaches available to protect learned behaviors, likely due to the lack of suitable animal models. One such animal model is the songbird, which is well-suited for laboratory study.

2.3.2 Zebra Finch: A Valuable Model for Neuroprotection

While some species are capable of learning throughout their life, for many species, complex motor skill learning occurs during a relatively brief period early in life. Among these species are domesticated and wild zebra finches that learn their song during a sensitive period for song acquisition that occurs between 25- and 90-days post-hatch[83], [84]. No song learning occurs before fledging (around 20 days post-hatch), and nestlings swapped between the nests of various males before reaching 18 days post-hatch learn their song exclusively from their foster father. Birds that are exposed to song recordings between 9-17 days post-hatch fail to recognize these songs in an operant procedure, indicating a highly specialized motor skill.

Within the sensitive period for song learning, the sensory memorization phase, during which an auditory model is formed, and the sensorimotor learning phase overlap to some degree. It has been challenging to determine the boundaries for each phase of song development. Males separated from their father at 35 days post-hatch were unable to develop normal song, while males isolated at 50 or 65 days post-hatch developed typical song. The proportion of shared elements between father and male offspring increased with the time spent together, with males that were isolated at day 65 days post-hatch having learned all of their father's song.

Although zebra finches are commonly regarded as closed-ended learners who sing a single stereotyped song in adulthood, increases in song stereotypy have been reported between 4 and 15 months of age[85]. This indicates that there is some plasticity for adult song, utilizing sensorimotor feedback throughout adulthood. Adult zebra finches have a limited ability to acquire an entirely

new song from an auditory model, but adjustment of the existing motor patterns for song and incorporation of a small number of new song elements have been shown to occur in adulthood when birds are reared under conditions that are suboptimal for song learning[86].

Like human language, song is learned during a sensitive period of development[87]. Also like humans, songbird vocal learning depends upon establishment of circuits linking cortical, striatal and thalamic brain regions[88]. Gene expression profiles demonstrate distinct similarities between songbird and human vocal learning[89], [90] and motor regions[91]. For example, evidence supports functional similarities between human laryngeal motor cortex (LMC) layers 2, 3 and 6 and songbird HVC, that each drive vocal motor output (from LMC layer 5 and songbird robust nucleus of the arcopallium[RA], respectively[92]). Vocal output is driven by motor cortical RA (Fig. 2.1). Under normal conditions RA integrates input from the learning circuit (lMAN) and motor circuit (HVC) to modulate acoustics and syntactic order of song syllables[93], [94]. Early in development, the song refinement relies on auditory feedback and utilizes glutamatergic connections of the anterior forebrain pathway (AFP; Area X, DLM, lMAN to RA see fig. 2.1)[95]. However, later in development we begin to see more robust communication through the number of synaptic connections from HVC to RA[96]. These circuits can be manipulated utilizing the discrete dense nuclei of zebra finch neuroanatomy, and this unique characteristic offers the ability to assess drug effects and targets that other models cannot.

Previously, using a bilateral HVC microlesion method to disrupt quality of vocalizations[97] it was established that daily treatments with 10 mg/kg CBD decreases the magnitude of song disruption and improves recovery time[29]. For the present study, to reduce animal impact and allow for statistically powerful within-subject controls, we are experimenting with a unilateral lesion approach. Our current goal is to understand the mechanisms by which CBD

protects vocal quality and improves learning-dependent recovery of a complex motor behavior after a unilateral lesion.

Accumulating evidence shows that CBD has anti-inflammatory and anti-oxidative stress activity involving immune cell activation and migration to areas of CNS injury[98]. Specifically, gene expression studies using microarray-based gene profiling indicate CBD attenuates several cellular events through modulating the expression of pro-inflammatory transcription factors, cytokines and cytokine receptors (that are notably released by microglia and controlled by the master anti-oxidant regulator, NRF2[68], [99]). Given this anti-inflammatory CBD activity, we suspected involvement of similar mechanisms in vocal recovery. To test this, we investigated CBD modulation of post-microlesion expression of inflammatory cytokines, markers of neuronal oxidative stress, microglia/macrophage infiltration and related changes in synaptic densities within relevant song control regions.

Figure 2.1 Zebra finch song neuroanatomy

Summary of song brain regions of interest. a, schematic representation of brain regions focused upon and their interconnections. Microlesions target HVC and produce vocal deficits with recovery dependent on sensorimotor learning (deafened birds do not recover[100]). Red arrows represent the anterior forebrain pathway (AFP), a cortico-basal ganglia-thalamic circuit critical for vocal learning and inducing vocal variability through adulthood. Green arrows represent vocal motor pathways. Dashed regions indicate approximate x,y locations of regions not visible in panel b. b, is a representative darkfield image used to identify sections with regions of interest and to define their borders for superimposing on images obtained later via confocal microscopy. This image was used to produce the camera lucida-type tracing in Panel A. Note the distinct nuclear organization of song regions contrasts with laminated mammalian cortex and allows targeting for lesioning, dissection, and other manipulation. Rostral is approximately right, dorsal up, and bars = 1 mm. Abbreviations: HVC (proper name), lMAN (lateral magnocellular nucleus of the anterior nidopallium), RA (robust nucleus of the arcopallium), Area X, Area X of striatum.

a

HVC

IMAN

Area X

RA

DLM

Vocal Motor
Output

b

HVC

IMAN

Area X

RA
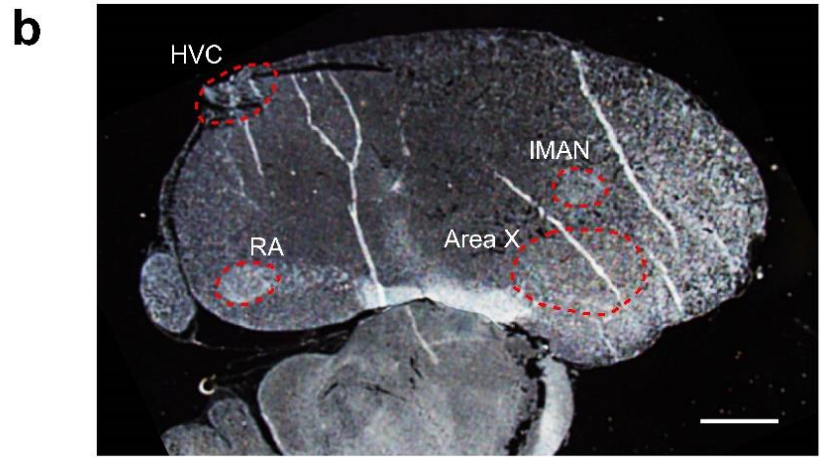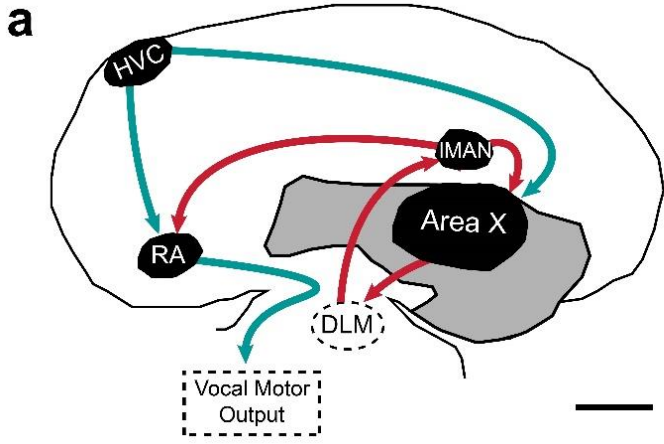
CHAPTER THREE: Inflammatory Response and Vocal Disruption Following Unilateral Microlesion

3.1 Introduction

Vocal learning and production in songbirds, like human language, depend upon the establishment of circuits linking cortical, striatal, and thalamic brain regions. The similarities between song and speech learning goes further than circuit development as it has been demonstrated this process has lateralization properties similar to humans. Lateralization refers to the phenomenon where certain functions, particularly related to speech and language, are predominantly controlled by one hemisphere of the brain. In humans, language processing and production is primarily controlled by the left hemisphere of the brain, while the right hemisphere is involved in processing nonverbal information. This lateralization of language processing is thought to have evolved as an efficient way for the brain to handle complex communication signals.

To expand, the left hemisphere is typically more involved in language processing, with specific regions such as Broca's and Wernicke's area playing important roles in speech production and comprehension, respectively[101]. The left hemisphere is also associated with processing aspects of language such as syntax, grammar, and phonology. In contrast, the right hemisphere is associated with processing nonverbal information such as spatial reasoning, visual imagery, and facial recognition. Brain damage to different regions greatly affects communication defined as either Broca's aphasia, where output of spontaneous speech is markedly diminished and there is a loss of normal grammatical structure, or Wernicke's aphasia, characterized by impaired language comprehension[102], [103].

Lateralization of speech and language has been studied in the zebra finch, but the extent of vocal disruption through a unilateral injury is currently unknown. There is prominent lateralization of song production and perception to the left hemisphere, similar to humans. Specifically, the left hemisphere of the zebra finch brain is responsible for producing and processing learned songs, while the right hemisphere is responsible for processing non-learned, innate sounds[104], [105]. The neural basis for lateralization in the zebra finch has been found to involve several brain regions, including the HVC , RA, and Area X (Figure 2.1 for neuroanatomy). Both HVC and RA, key regions in the song production pathway, has activity that appears to be lateralized to the left hemisphere[104]. Area X has been implicated in the learning and plasticity of song production and it has been shown that lesions to the left Area X can impair song learning, while lesions to the right Area X have a lower effect[106]. This suggests that lateralization of song learning may be important for the development of complex vocalizations in zebra finches and may play a role in recovery after an injury.

Acute brain injury, similar to that produced by our microlesion surgery, can cause a cascade of events that lead to neuroinflammation. Neuroinflammation is an immune response within the CNS that can be either beneficial or detrimental. This is a complex process that involves multiple cell types, including microglia, astrocytes, and peripheral immune cells[107], [108]. Following acute brain injury, quiescent microglia undergo morphological changes, become reactive and migrate to the site of injury[89]. When activated, microglia release pro-inflammatory cytokines (i.e., IL-6 and IL-1β) and chemokines (i.e., CCL2 and CXCL1), leading to the recruitment of peripheral immune cells and further amplification of the inflammatory response. While neuroinflammation can help clear damaged tissue and promote tissue repair,

prolonged or excessive inflammation can lead to secondary injury and long-term neurological deficits[110].

The value of studying CBD neuroprotection in songbirds lies in ability to identify processes modified within discrete nodes of circuits controlling vocal learning and production. Vocal control pathways in both songbirds and humans share convergent functional similarities that increase translational relevance relative to non-vocal learning species[90], [91]. A distinct difference between avian and human vocal pathways is nuclear rather than laminated organization of pallial vocal control regions. This difference imparts advantages in targeting regions for manipulation and spatial assessment; a feature we have taken advantage of with our experiments. In addition to HVC (the pre-motor cortical-like microlesion target) we have focused on its projection targets: RA (motor cortical-like) and Area X (a learning-essential striatal region). Because song recovery depends upon auditory perception-dependent sensorimotor integration (deafened birds don't improve[111]) our system uniquely models adult loss and learning-dependent recovery of a complex motor skill.

We have hypothesized that inflammation, particularly in the lesioned hemisphere, will accompany the vocal disruption resulting from HVC microlesions in our model, resembling a TBI. CBD has been shown to reduce the production of pro-inflammatory cytokines and decrease oxidative stress. In vivo models of TBI and stroke have found CBD to reduce brain swelling, improve function, reduce infarct size and improved functional outcomes as well as reduce apoptosis and promote cell survival[27], [112], [113]. We suspect that CBD-improved vocal recovery will be associated with a decrease in lesion-related inflammation.

3.2 Materials & Methods

Materials

Unless otherwise indicated, all materials and reagents were purchased from Sigma Aldrich or Thermo Fisher. Botanically derived CBD (≥98%) was provided by GW Research Ltd, Cambridge, UK. Concentrated stocks of CBD were prepared using nitrogen-sparged 100% ETOH and stored at -20 ºC. Stocks were then diluted with vehicle (2:1:17, ETOH:Alkamuls:PBS) to produce suspensions for injections of 10 mg/kg CBD. Resulting ethanol dosages were 0.33 mg/kg – lower than that voluntarily consumed by zebra finches[114].

Drug treatments

Stocks of CBD, prepared as described above, were stored in sterile 5 ml septum-capped vials at 4 ºC. Fresh stocks were prepared at least weekly. For injections drug preparations were loaded into sterile 1 cc insulin syringe with 30 ga needles. In the morning of injections while the lights were off, birds were captured by hand and the pectoralis muscle injection site was exposed by matting feathers with a small volume of 70% ETOH delivered by squirt bottle. Injections of 50 µl were made into one of four quadrants of pectoralis, rotating daily to minimize potential damage caused by repeated treatments.

Experimental design

Recording experiments spanned 20 days. Birds were acclimated to recording chamber for 3 days prior to recording. On day 4, daily injections were given for 6 days prior to the surgical procedures to allow CBD, a lipophilic drug with large volume of distribution and long elimination half-life, to approximate steady state levels[115]. On day 10, birds were given a pre-operative injection and unilateral microlesion surgeries were performed according to methods detailed below. On day 11-20, post microlesion injections were given while continuously recorded. For the

molecular experiments, they spanned 8 days. There were 6 days pre-microlesion injections, a microlesion on day 7. On day 8, birds were given a post-operative CBD injection in the morning and euthanized in the afternoon for RNA extraction, or perfusion and isolation of paraformaldehyde-fixed brain tissue.

Animals and environment

Adult male zebra finches (>90 days of age) were raised in our breeding aviary and maintained at 78 ºF on a 12/12 light/dark cycle. Males were exclusively used due to their ability to produce song. Birds were housed in standard finch cages (9"x11"x17") with ad libitum food and water. Birds were visually but not auditorily isolated, consistent with prior experiments done in recording chambers[29]. All animal procedures were approved by the East Carolina University Animal Care and Use Committee (see ethics declarations below).

HVC microlesion surgeries

To reduce animal impact and improve statistical power we modified our original bilateral microlesion model[29] to adopt a unilateral approach that allows individual subjects to serve as their own internal control. We targeted left hemispheres as evidence indicates lateralization similar to that characteristic of human language[116] further illustrating parallels between speech and birdsong (also, initial optimization experiments suggested left hemisphere HVC microlesion disrupted vocal quality to a greater degree than those targeting right hemispheres, see Supplemental Fig S1). With exception of the bilateral modification, microlesions were done as described previously[29]. Briefly, birds were anaesthetized with isoflurane and secured in a stereotaxic instrument. The bifurcation at the midsagittal sinus was used as stereotaxic zero. Small craniotomies were placed over the left HVC. For approximately 8% destruction of left HVC, two locations were targeted: 2.4 and 2.8 mm from the 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. Note these methods were adapted from those originally developed by Thompson and Johnson, 2007[97].

KL distance measures of phonology

In order to show that unilateral lesions produce vocal deficits similar to the previously used bilateral lesions, we used KL distance as a measure of vocal disruption. For KL distance measures, birds were recorded continuously for 20 days. Sound Analysis Pro (SAP) 2011 software[117] was used to segment song bouts into their separate syllable components and overall segmentation was accomplished by thresholding (optimized for each animal) based upon amplitude, entropy, syllable and syllable gap durations. SAP characterizes individual syllables by their spectral structure through measures of acoustic features. Acoustic feature measures were used to assess phonology via calculation of Kullback-Leibler (KL) distances using the previously developed methods[118], [119] that compare distances between 2D probability distributions of vocal acoustic features. The greater the amplitude of KL distance reflects a greater vocal deficit.

Quantitative RT-PCR

Gene expression experiments were performed with three biological replicates from four to five adult zebra finches per treatment group. For each animal, a sterile RNAse free 1 mm diameter biopsy punch tool was used to excise brain tissue of three regions of interest, from each hemisphere (Unlesioned right used as an internal control and lesioned left): HVC; RA; and Area X. Example dissection and brain regions shown in figure 3.1. Brain samples were homogenized in TRIzol reagent (Invitrogen, 15596026) separated using chloroform and precipitated using isopropanol. Precipitated RNA was washed and resuspended in RNase free water. RNA quality was confirmed by gel electrophoresis. Total RNA (250 ng) was used to synthesize cDNA using an iScript

synthesis kit (Bio-Rad, 1708890). Completed reactions were diluted 5-fold, in triplicate using RNAse free water. PCR was done using SYBR green supermix (Bio-Rad, 1725271). Selective amplification was confirmed using a melt curve analysis and data were obtained as cycle threshold (Ct) values using CFX Manager software (Bio-Rad). Gene expression was normalized to the endogenous control (GAPDH) and fold change was determined from the unlesioned hemisphere using the ΔΔCt method[120]. Primer sequences and information are located in table 3.1.

Fixation and cryosectioning

Adult male zebra finches (n = 3-5) were administered drug treatments for 6 days, microlesioned on day 7 and transcardial perfusions were performed 24 h later using 4% paraformaldehyde for fixation and 30% sucrose for cryoprotection. Fixed brains were blocked at the midline, placed in OCT embedding medium, frozen using a slurry of 2-methyl butane and dry ice, then sectioned at 10 µm using a cryostat kept at -20 ℃. Parasagittal sections of both right and left hemispheres from each bird were mounted on Superfrost Plus slides and stored at -20 ℃.

Immunofluorescent staining

Slides were blocked with 5% normal goat serum for 1 hour at 37 ℃. Primary antibodies targeting various proteins were diluted in 2% normal goat serum and used at optimized concentrations: anti-IL-6, 10 µg/ml (Biomatik, CAU30440); anti-IL-1β, 10 µg/ml (Mybiosource, MBS 2090494); anti-IL-10, 1:100 (BIOSS, AGO7251283); and nuclear counterstain Hoechst, 1:10000 (Thermo Fisher Scientific, H3570). Primary antibody specificity was validated via western blotting, images of which are summarized in Figure 3.2. All primary antibodies were incubated at 4 ℃ overnight. The following day, slides were washed with PBS then was incubated in corresponding secondary antibody diluted in 2% normal goat serum at 37 ℃ for 1 hour. Secondary antibodies were Alexa Fluor 488 goat anti-mouse (A32723) Alexa Fluor 647 goat anti-

mouse (A32728) Alexa Fluor 647 goat anti-rabbit (A32733). After incubation of one hour in secondary antibody, slides were washed twice with PBS for five minutes each followed by Hoechst nuclear counterstain. After fourth and final wash, cover slips were placed on slides using diamond antifade mountant (Invitrogen, P36961). Control reactions were done without primary antibodies to demonstrate lack of significant nonspecific binding of secondary antibodies (Figure 3.3).

Dihydroethidium (DHE) staining

Superoxide anions were detected via 5 µM Dihydroethidium (DHE, Invitrogen, D11347) following a previously described protocol[121]. DHE freely permeates cell membranes and reacts with cytosolic superoxide (O_2^-) producing ethidium that fluoresces red upon DNA binding. This fluorescence can then be quantified[122]. Briefly, brain tissue was rapidly dissected and flash frozen in OCT compound using a dry ice 2-methylbutane slurry. Using a freezing microtome (Epredia Microm HM525 NX Cryostat) 10 µm sections were cut and mounted on Fisher Superfrost Plus microscope slides and then 1 mL of 5 µM DHE diluted in PBS was gently pipetted onto each slide and incubated at 37 ºC for 30 minutes protected from light, rinsed two times with PBS, and imaged.

Dark-field and confocal imaging

Dark-field images for regional identification were obtained using Image-Pro Plus software (version 6.3) and an Olympus BX51 microscope equipped with a darkfield condenser at 12.5X (Fig. 3.1). Borders of regions of interest were traced from darkfield images for later superimposition over fluorescent confocal images. Regions of interest included HVC outside of infarcts, RA, and Area X. Sections that contained portions of all three regions of interest were identified to ensure equal representation across treatment conditions. Fluorescent images were obtained using a Zeiss laser scanning microscope (LSM, 700 Axio Observer) with 40X (Plan-

Apochromat/1.4 Oil DIC M27) and 10X objectives (EC Plan-Neofluar/0.30 M27). Using Zeiss ZEN Black imaging software, Z-stack images were compiled using 5 slices and analyzed after superimposing at maximum intensity using Image J.

Image analysis

Image J software was used to analyze superimposed z-stack images converted to 8-bit with a threshold applied consistently across all images within each region. All CZI-format image files were exported from ZEN Black software as greyscale tiff files and consolidated into a maximum intensity z projection. For IL-6, IL-1β, and IL-10, mean grey value of individual stain relative to Hoechst nuclear stain by song region was quantified as a percentage of the control hemisphere. Note that raw, untransformed relative fluorescence measures of cytokine expression for both lesioned and unlesioned hemispheres hemisphere are summarized next to representative images (Fig. 3.7-3.9). For DHE, mean grey values were determined and corrected for background fluorescence using the average intensity outside of each song region studied (Fig. 3.6b). Groups were compared using a percentage of the control hemisphere (Fig. 3.6c). Mean corrected total cell fluorescence (CTCF) was used to eliminate background using a consistent circular area (circular diameter of 0.5 mm): CTCF = Integrated Density – (mean song region intensity * mean background intensity

Ethics declarations

Animals were used following protocols approved by the Institutional Animal Care and Use Committee at East Carolina University (ECU-IACUC). ECU-IACUC oversees a registered research facility under the Animal Welfare Act (#55-R-0010) and has an approved Animal Welfare Assurance Statement with the Office of Laboratory Animal Welfare D16-00294. In addition, ECU

has continued full accreditation by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Statistical analysis

Statistical analyses were performed with GraphPad Prism 9.2.0 for all histological, behavioral, and gene expression data. Data are expressed as mean ± SEM. Statistical analysis was performed using a mixed-models ANOVA followed by Sidak's post hoc analysis to identify differences between groups. A p value ≤ 0.05 was considered statistically significant. Statistical results are indicated in the text and figure legends.

Figure 3.1 Illustration of micro punch technique used to isolate regions of interest

a-d, Gross dissection of fresh zebra finch brain tissue and biopsy punch locations based on fiber tracts in a sagittal section. e, Dark-field image used to identify tissue sections with all regions of interest. Dense regions are circled, and arrows indicate circuits that connect them. f, Outline of sagittal section with areas of interest highlighted. Rostral is right, dorsal up, and bar = 1 mm.
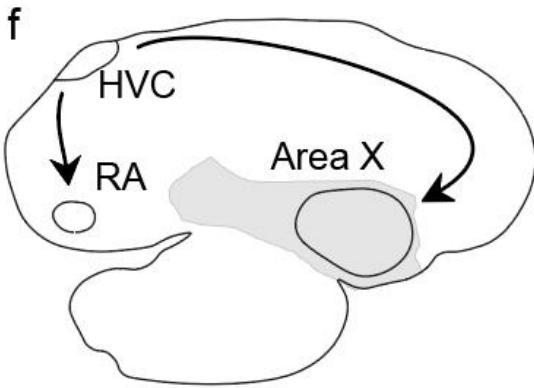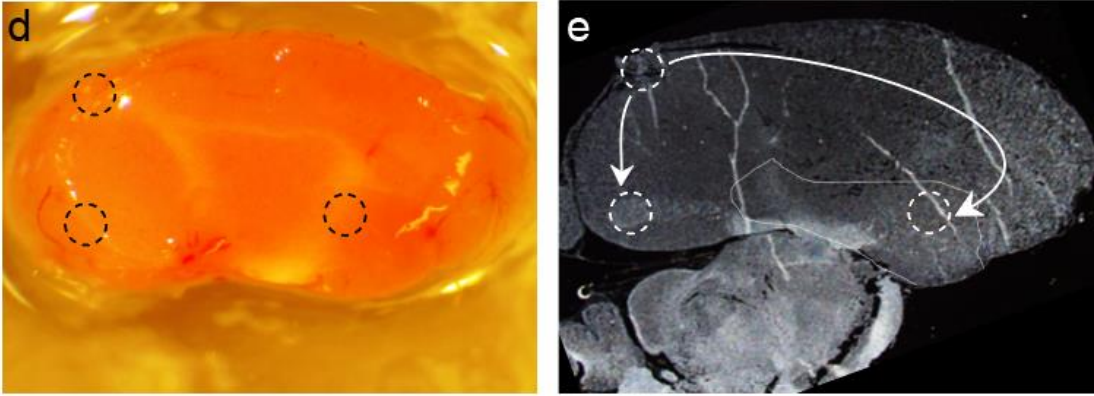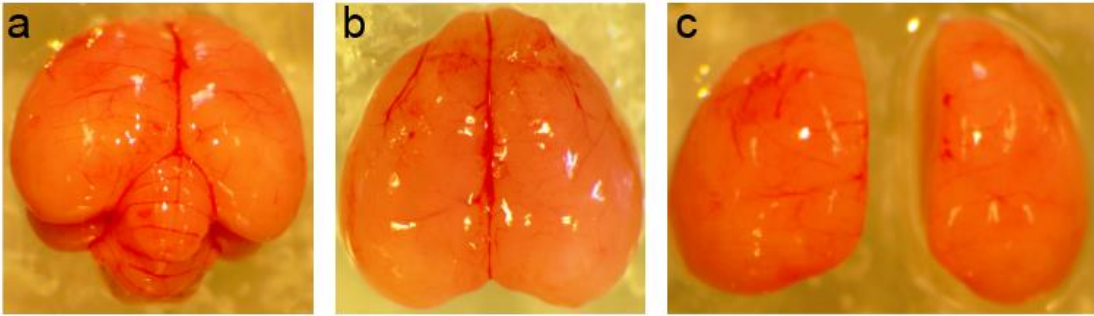
Figure 3.2 Validation of primary antibody selectivity in zebra finch brain tissue via western blot

Western blotting supports antibody selectivity with zebra finch protein.

a IL-1B
250kD —
150 —
100 —
75 —
50 —
37 —
~31kD
25 —
20 —
15 —
10 —

b IL-6
250kD —
150 —
100 —
75 —
50 —
37 —
25 —
20 — ~21kD
15 —
10 —

c IL-10
250kD —
150 —
100 —
75 —
50 —
37 —
25 —
20 — ~18kD
15 —
10 —

d vGLUT2
250kD —
150 —
100 —
75 — ~65kD
50 —
37 —
25 —
20 —
15 —
10 —

e PSD95
250kD —
150 —
100 — ~95kD
75 —
50 —
37 —
25 —
20 —
15 —
10 —

f pNRF2
250kD —
150 —
100 — ~90kD
75 —
50 —
37 —
25 —
20 —
15 —
10 —

g TMEM119
250kD —
150 —
100 —
75 —
50 — ~45kD
37 —
25 —
20 —
15 —
10 —
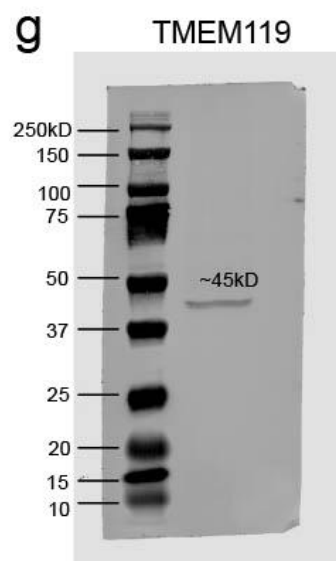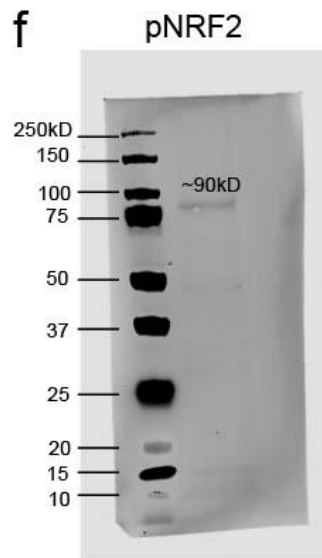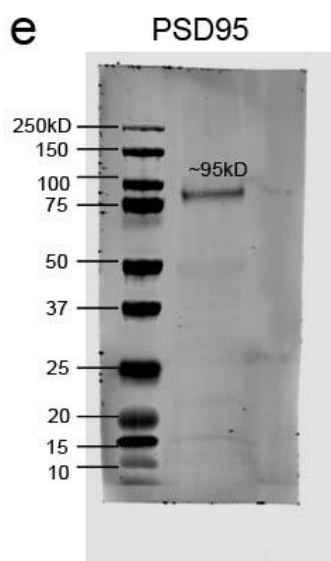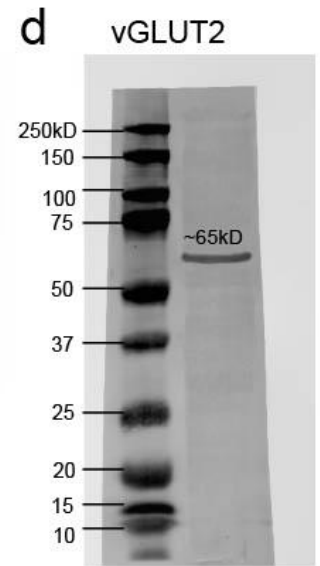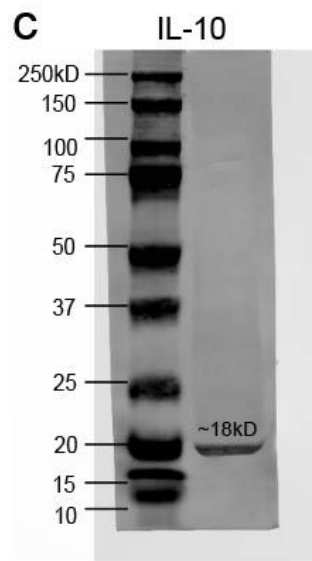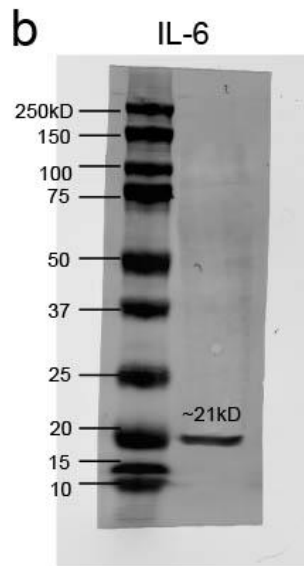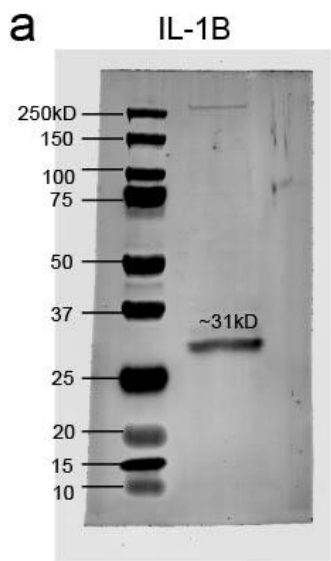
Figure 3.3 Validation of secondary antibody specificity visualized via Immunofluorescence of no-primary control staining

No primary controls for immunofluorescent staining. The samples followed the same immunofluorescence staining protocol but were incubated with only the diluent (no primary antibody) and then with the respective secondary antibody. Images were taken using the same LSM700 settings as images taken with primary antibodies.

a

	HVC		RA		Area X	
	Vehicle	10 mg/kg CBD	Vehicle	10 mg/kg CBD	Vehicle	10 mg/kg CBD

Anti-Mouse 488

Merge

b

	HVC		RA		Area X	
	Vehicle	10 mg/kg CBD	Vehicle	10 mg/kg CBD	Vehicle	10 mg/kg CBD

Anti-Mouse 647

Merge

c

	HVC		RA		Area X	
	Vehicle	10 mg/kg CBD	Vehicle	10 mg/kg CBD	Vehicle	10 mg/kg CBD

Anti-Rabbit 647

Merge

50 μm
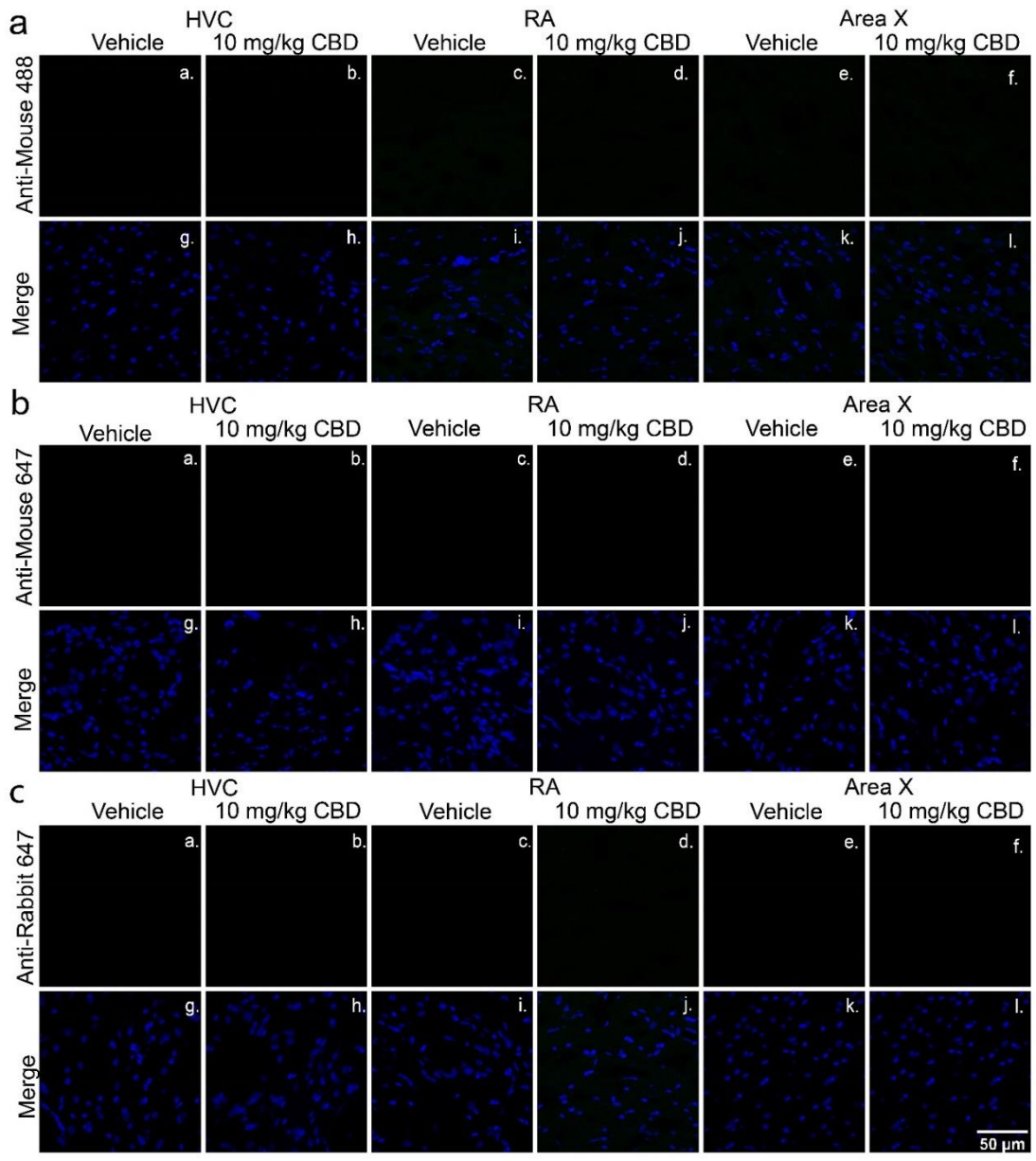
Table 3.1 qRT-PCR primer information

Table listing gene symbol, accession number, and forward and reverse primers used for quantitative RT-PCR.

Gene Symbol	Accession Number	Forward Primer (5'-3')	Reverse Primer
GAPDH	NM_OO1198610.1	TGCTGCTCAGAACATTATCCC	TTTCCCACAGCCTAGCAGCT
IL-1β	XM_002195564.2	TTCCGGTGCATCAGAGGCAGTTAT	GCACGAAGCACTTGTGGTCAATGT
IL-6	XM_002191284.2	CGTCTGCCAGAACAGCATGGAAAT	TATCCTCATTAAAGCCGGCGAGCA
IL-10	XM_002194605.4	CAAGCTCAAAGAGCTGAGGC	GCCCTTGATTTCCTCCAGCA
TNFα	XM_002197321.2	TGTCCCATCTGCACCACCTTCTTA	ATTCCCTTCCATCTGGCTTCTGT
SOD2	NM_001245469.2	ACAGGGGCGCCTACAGATAG	CATGTTCCCATACGTCAATGCC
BDNF	XM_012573739.4	TCCAGCATCTGTTGGAGAGAC	CGAAGACCTGGGTAAGCCAA
RPS6KA5 (MSK1)	XM_030274847.3	CGGAGTGCCCCTGGAAAATA	GGCCGCTTACTTTCCTCACT
Arc/Arg3.1	XM_002186825.6	TGGAAGAAGTCCATCAAGGC	TTGCGCCAGAGGAACTGGTC

3.3 Zebra Finch vocal variability demonstrates shows left hemispheric lateralization

Lateralization of speech and language has been studied in the zebra finch, but the extent of vocal disruption through a unilateral injury is currently unknown. To determine the ability of a unilateral microlesion to produce vocal deficit, we quantified the phonology of song as KL distance for both the right and the left hemisphere after their respective unilateral microlesion. A 2-way ANOVA revealed both a unilateral microlesion to the right and left hemisphere produced significant vocal disruption 24-hours after microlesions compared to baseline levels as seen in Figure 3.4 (Right by mean 1.675 [0.83 to 2.52], $p < 0.0001$; Left by mean 2.850 [2.01 to 3.70], $p < 0.0001$).

It is also important to note that left microlesions had a lasting impact on KL distance (vocal disruption) 4 days post-microlesion (Post-day 2 by mean 1.33 [0.48 to 2.2], $p = 0.0001$; Post-day 3 by mean 0.9225 [0.08 to 1.76], $p = 0.0209$; Post-day 4 by mean 0.91 [0.07 to 1.756], $p = 0.0229$) while right microlesions only had statistically significant differences 2 days after the surgery (Post-day 2 by mean 1.14 [0.30 to 1.98], $p = 0.0016$).

3.4 CBD differentially alters lesion-induced pro- and anti-inflammatory mediator protein and gene expression

In other systems CBD exhibits anti-inflammatory and antioxidative efficacy that is in part responsible for its neuroprotective activity[27], [123]. To evaluate whether CBD-improved vocal recovery involves similar anti-inflammatory efficacy, we quantified expression of several pro- and anti-inflammatory mediators (Fig. 3.5a-d). Using the micro punch dissection technique described above 24 hours after microlesions we isolated tissue for RNA extraction, cDNA synthesis and amplification from song regions within nodes of motor (HVC & RA) and learning essential circuits (Area X, note the micro punch approach used is illustrated in Fig 3.1). Mixed models ANOVA

revealed CBD treatments significantly reduced expression (relative to vehicle controls) of the pro-inflammatory mediators IL-1β (within HVC by mean = 1.33 [0.427 to 2.222], p = 0.0015; and RA by mean = 1.44 [0.542 to 2.337], p = 0.0005 and IL-6 (within HVC by mean = 0.615 [0.243 to 0.987], p = 0.0002; RA by mean = 0.563 [0.192 to 0.935], p = 0.0008; and Area X by mean = 0.435 [0.063 to 0.807], p = 0.0141, see Fig. 3.5a-c). The lesioned area, HVC and RA showed additional reduction of TNFα expression (HVC by mean 0.275 [0.087 to 0.462], p = 0.0016) (RA by mean = 0.292 [0.105 to 0.48], p = 0.0008), that was not statistically significant in Area X (by mean = 0.035 [-0.222 to 0.153], p = 0.960). In addition to decreasing expression of pro-inflammatory mediators, CBD has also been shown to upregulate the anti-inflammatory cytokine IL-10[124]. We quantified differences in IL-10 expression and found them to be significantly increased in HVC of CBD-treated birds (by mean = 0.512 [0.152 to 0.872, p = 0.0023) and RA (by mean = 0.487 [0.127 to 0.846], p = 0.004) but not in Area X (by mean = 0.008 [-0.368 to 0.352], p > 0.99, see Fig. 3.5d).

To confirm that CBD-induced anti-inflammatory patterns of gene expression result in functional protein level changes we used immunofluorescence approaches. We measured relative immunofluorescence using antibodies targeting IL-6, IL-1β and IL-10 (Representative images with individual fluorescence values in figure 3.6-3.8) and expressed results as a percentage of unlesioned hemispheres (Fig. 3.10, note that selectivity of antibodies used is supported by staining of single predominant bands of expected size: see Fig. 3.2). CBD significantly reduced lesion-related IL-6 protein expression in HVC (by mean percent = 128.9% [62.32 to 195.4], p = 0.0001), and RA (by 89.37% [22.84 to 155.9], p = 0.0063 Fig. 3.10a). For IL-1β, CBD treatment significantly decreased expression in HVC (by mean = 131.2% [53.55 to 208.8], p = 0.0007), and RA (by mean = 115.0% [37.36 to 192.6], p = 0.0026, Fig. 3.10b). Expression of IL-6, but not IL-

1β, was differentially regulated in Area X (IL-6 by 72.21% [5.679 to 138.7], p = 0.031; and IL-1β by 1.44% [-76.17 to 79.04], p > 0.9999, visualized in Fig. 3.6-3.7e). CBD significantly increased anti-inflammatory IL-10 in RA (by mean = 54.9% [9.388 to 100.4], p = 0.0147) and Area X (by mean = 66.5% [20.96 to 111.9], p = 0.0030) while there was no statistical difference in the lesioned site (HVC by mean = 21.4% [-66.85 to 24.14], p = 0.5612 Fig. 3.9c).

3.5 CBD decreases lesion-induced oxidative stress

Consistent with anti-inflammatory efficacy, CBD has been shown to affect redox balance through both direct and indirect antioxidant activity[28]. Expression of superoxide dismutase 2 (SOD2), a gene that encodes the mitochondrial protein SOD2 responsible for binding of superoxide byproducts, showed a significant CBD-related decrease within HVC (by mean fold = 0.8768 [0.269 to 1.49], p = 0.002), and RA (mean fold = 0.701 [0.093 to 1.31], p = 0.018), but not in Area X (mean fold = 0.4841 [-0.124 to 1.092], p = 0.162, see Fig. 3.5e).

To confirm that decreased SOD2 expression was accompanied by a general decrease in oxidative stress, we used the superoxide indicator dihydroethidium (DHE, see Fig. 3.10). When oxidized, DHE intercalates double-stranded genomic DNA marking nuclei with red fluorescence. In the vehicle group we found microlesions significantly increased CTCF of DHE staining within HVC (by mean = 52,215 [26,635 to 77,796], p < 0.0001), RA (by 49,460 [23,880 to 75,041], p < 0.0001), and Area X (by 29,817 [4,237 to 55,397], p = 0.0152 see Fig. 3.10a). In contrast, there were no significant differences in DHE staining between the unlesioned hemispheres of vehicle- and CBD-treated animals (Fig. 3.10b VEH Unlesioned vs CBD Unlesioned 3aa-b, e-f, i-j). (HVC, by mean = 1,777 [23,803 to 27,358], p > 0.9999; RA, by mean = 2,194 [-27,775 to 23,386], p > 0.9999; and Area X, by mean = 4,280 [-21,300 to 29,861], p = 0.9980). Comparing DHE staining within lesioned hemispheres of vehicle- and CBD-treated animals, CBD reduced intensities within

HVC (by mean = 33,153 [7,573 to 58,734], p = 0.0056), RA (by mean = 30,998 [5,417 to 56,578], p = 0.0107), and Area X (by mean = 28,089 [2,508 to 53,669], p = 0.0250 see Fig. 3.10b). Additionally, when comparing treatment groups to their respective unlesioned controls, CBD treatments significantly reduced lesion related changes in DHE expression in all regions (HVC by mean = 181.5% [53.91 to 309.1], p = 0.0045; RA by mean = 143.6% [16.04 to 271.2], p = 0.0248; and Area X by mean = 152.0% [24.40 to 279.6], p = 0.0171 see Fig. 3.10c).

3.6 Discussion

The lateralization of speech and language has been studied in the zebra finch, but the extent of vocal disruption caused by a unilateral injury is currently unknown. This gap in knowledge limits our understanding of the potential effects of drug treatments, particularly those targeting inflammation, on vocal behavior. To address this, we aimed to develop a reproducible unilateral microlesion technique in zebra finches to assess the efficacy of this approach in inducing vocal disruption and to investigate the potential utility of this model in evaluating the effects of drug treatment on vocal behavior.

To evaluate the potential utility of this model, we first assessed the efficacy of the unilateral microlesion technique by examining the extent of vocal disruption produced by unilateral HVC microlesions. Additionally, we investigated the lateralization of vocal deficit in our model to determine the hemisphere that would help identify potential molecular mechanisms for CBD-improved vocal recovery. It is important to note, previous work has employed, "no surgery" groups to control for possible effects independent of the microlesion procedure and experimental drug treatments (e.g., craniotomy manipulation per se and/or anesthesia)[29]. It was found that no significant differences were found across treatment groups within this control. Given the significant number of animals and impact, it is not a feasible group.

Our initial results indicate that left hemisphere microlesions had a greater impact on vocal deficit than right hemisphere microlesions, and the disruption was significant for four days compared to the two days seen in the right hemisphere (Fig. 3.4). This finding is consistent with previous studies suggesting that the left hemisphere is more specialized for vocal learning and production in songbirds[104]. The longer-lasting disruption seen in the left hemisphere suggests that this region may have a greater response to injury than right hemisphere.

We also investigated the potential use of cannabidiol (CBD) in protecting and promoting the recovery of lesion-disrupted vocal behavior. Results indicate that CBD neuroprotection followed powerful anti-neuroinflammatory effects, which is consistent with previous findings in mammalian systems[99] (reviewed by Kozela et al., 2017, see Figs. 2 and 4). This is important as neuroinflammation is an etiological factor in development of a spectrum of CNS disorders[32] (see chapter 1 section 1.2) such as chronic neuroinflammation triggered by post- TBI, which increases the likelihood of mood disorders and early-onset dementias[126]. Unfortunately, current therapies for chronic neuroinflammation often have significant side effects and/or diminishing efficacy, making CBD's profound anti-neuroinflammatory effects an attractive therapeutic option.

However, it has been noted that a potential problem with using CBD therapeutically is its lack of selectivity. CBD interacts with and modifies the activity of multiple cellular targets[14], [15], and purified CBD extracts may contain at least traces of other cannabinoids, including CNS-active THC[16]. We found that CBD efficacy is influenced by THC content, emphasizing the importance of consistent, carefully-controlled formulations[17]. Identifying distinct mechanisms responsible for CBD neuroprotection may lead to the development of more selective drugs, reducing potential off-target effects. Alternatively, an entourage of CBD's diverse cellular interactions may be critical to its efficacy and necessary for neuroprotection. The microlesion

model shows promise for identifying anti-neuroinflammatory mechanisms and screening potential new drugs.

CBD anti-inflammatory effects appeared greatest in the microlesioned region, HVC, and to progressively lesser extents within RA and Area X (e.g., Fig. 3.5a-c and 3.10a-c). These effects were demonstrated in the gene expression of several inflammatory mediators and confirmed using primary antibodies with high specificity to their respective protein targets (see Fig. 3.2 for confirmation of selectivity). This coincides with proximity to HVC, consistent with expected impact on shorter degenerating projections preceding longer ones. Because RA integrates output from both the Area X-associated AFP, and the posterior motor pathway (via HVC, see Fig. 2.1) we expected disrupted motor input prior to that of the AFP learning circuit[127]. The AFP has an error-generating function that introduces vocal variability critical for sensorimotor learning and modulates activity in both RA[128] and HVC (indirectly via midbrain dopaminergic nuclei[129]). Persistence of AFP error generation, under conditions of reduced HVC motor control, is consistent with microlesion-induced vocal disruption. This may explain why minimal microlesion effects are observed in AFP-deficient birds[97].

A second neuroprotection-related mechanism identified included CBD mitigation of oxidative stress, as indicated by effects on SOD2 expression within HVC and RA (Fig. 2). Oxidative stress effects were further confirmed by reduced superoxide-activated DHE staining[130] (Fig 3.11). Like cytokines, the magnitude of superoxide production varied with lesion proximity, but was significantly decreased by CBD in HVC and RA (Fig. 3.11b). Note that regional measures of DHE fluorescence are expressed relative to surrounding regions, and therefore demonstrate selective, within song circuit effects.

Given chronic administration necessary to treat chronic neuroinflammation, significant side effects and/or diminishing efficacy are common with current therapies[131]. Profound anti-neuroinflammatory effects that we and others have observed using CBD, combined with evidence of a favorable side-effect profile[65] suggests it may improve ability to manage this difficult condition.

Figure 3.4 Zebra Finch vocal variability demonstrates left hemispheric lateralization.

Unilateral HVC microlesions temporarily disrupt zebra finch song. Y-axis shows days relative to microlesions given on day 7. Using a measure of vocal variability, K-L distance[29], [132], a unilateral HVC microlesion to either the right or left hemisphere, disrupts vocalization peaking 24 hours after surgery. Left hemisphere lesions produced a greater change from baseline, although both were statistically significant. These results confirm left hemisphere lateralization similar to previous literature. n = 3.
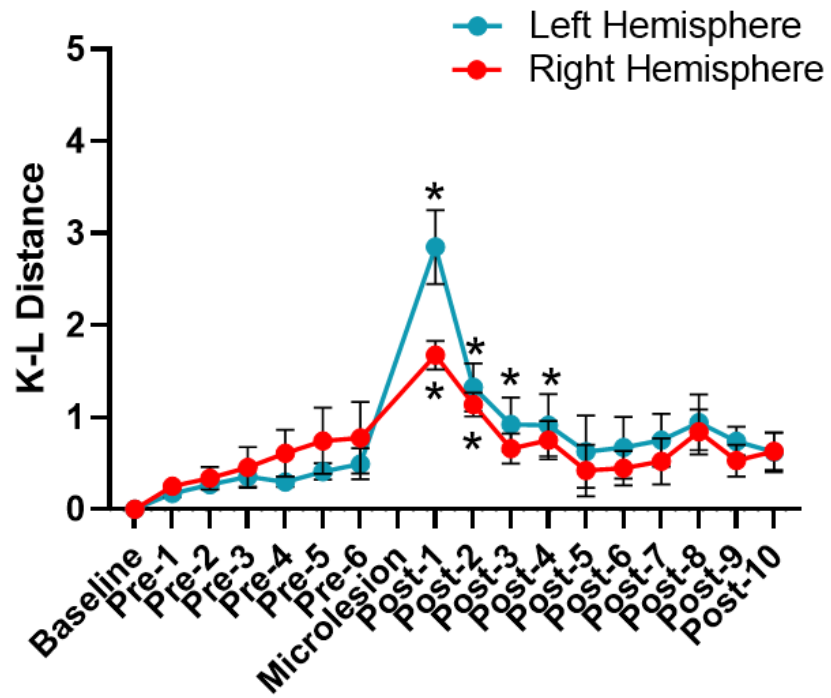
Figure 3.5 CBD promotes a pattern of anti-inflammatory and anti-oxidative stress-related gene expression via qRT-PCR

CBD promotes a pattern of anti-inflammatory and anti-oxidative stress-related gene expression. Brain regions from both vocal-motor (HVC and RA) and -learning circuits (Area X) were micropunch dissected, total RNA extracted, cDNA synthesized, and PCR amplified. Gene expression of IL-1β, IL-6, IL-10, TNFα, and SOD2 were normalized to the endogenous control (GAPDH) and fold change from the unlesioned hemisphere was expressed as 2-ΔΔCT. To quantify, cDNA from n = 5 per group was amplified in triplicate and the average cycle threshold (Ct) value was calculated. The mean Ct value was then used for further analysis. a, CBD significantly decreased mean fold expression of pro-inflammatory IL-6 in HVC, RA, and Area X. b, CBD decreased mean fold expression of IL-1β in HVC and RA but not Area X. c, TNFα was decreased in HVC and RA but not Area X. d, mean fold expression of the anti-inflammatory mediator IL-10 was increased in HVC and RA but not significantly in Area X. e, Expression of the marker of oxidative stress, superoxide dismutase 2 (SOD2) was decreased within HVC and RA. Note that tissue was obtained 24 h post-lesion and thus represents a "snap-shot" of inflammatory cytokine expression that is known to vary with time post-injury63. Group differences were assessed by mixed-models ANOVA with Sidak's multiple comparison correction.

a — IL-6

b — IL-1B

c — TNFα

d — IL-10

e — SOD2

VEH Unlesioned
VEH Lesioned
CBD Unlesioned
CBD Lesioned

Figure 3.6 CBD decreases pro-inflammatory IL-6 staining within HVC, RA and Area X

CBD alters inflammation-related IL-6 densities. Daily treatments with 10 mg/kg were associated with modulation of IL-6 protein expression 24 h after unilateral HVC lesions. Shown are representative immunofluorescent confocal images of antibody staining targeting IL-6 within motor (HVC & RA) and learning-essential (Area X) regions. aa-ah, unlesioned and lesioned hemisphere images showing representative regional distribution of IL-6 in HVC of vehicle- vs. CBD-treated birds. ba-bh, images demonstrating regional distribution of IL-6 in RA. ca-cl, confocal images demonstrating regional distribution of IL-6 in Area X. Raw values are not relative to control hemisphere but are displayed in Figure 3.9. Quantification was done as a percentage of the unlesioned hemisphere shown in Figure 3.10. Image J software was used to analyze z-stack images projected at maximum intensity and threshold was applied consistently across all images for n = 5 per group.

a

Unlesioned   Lesioned

VEH   10 mg/kg CBD   VEH   10 mg/kg CBD

IL-6

a.   b.   c.   d.

HVC

Merge

e.   f.   g.   h.

b

Unlesioned   Lesioned

VEH   10 mg/kg CBD   VEH   10 mg/kg CBD

IL-6

a.   b.   c.   d.

RA

Merge

e.   f.   g.   h.

c

Unlesioned   Lesioned

VEH   10 mg/kg CBD   VEH   10 mg/kg CBD

IL-6

a.   b.   c.   d.

Area X

Merge

e.   f.   g.   h.

50 µm
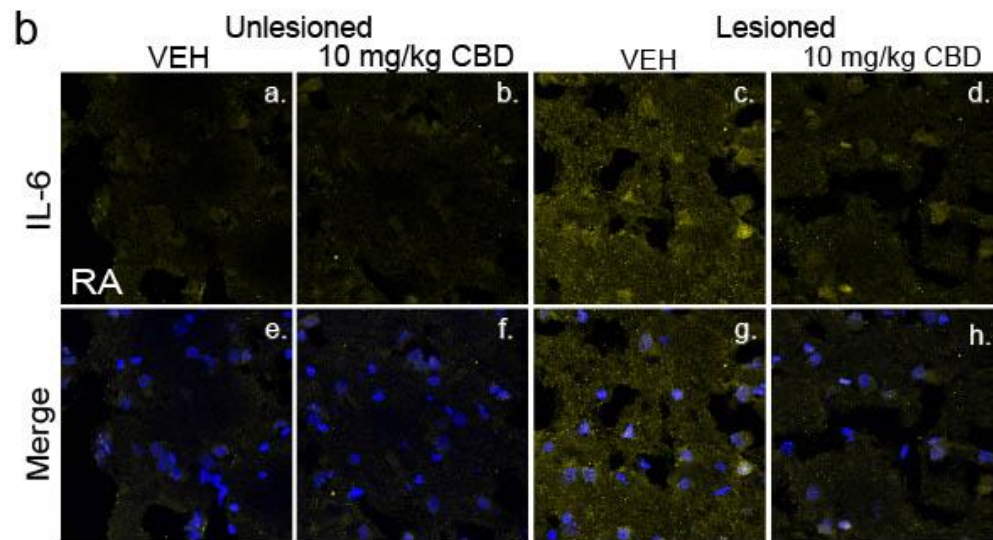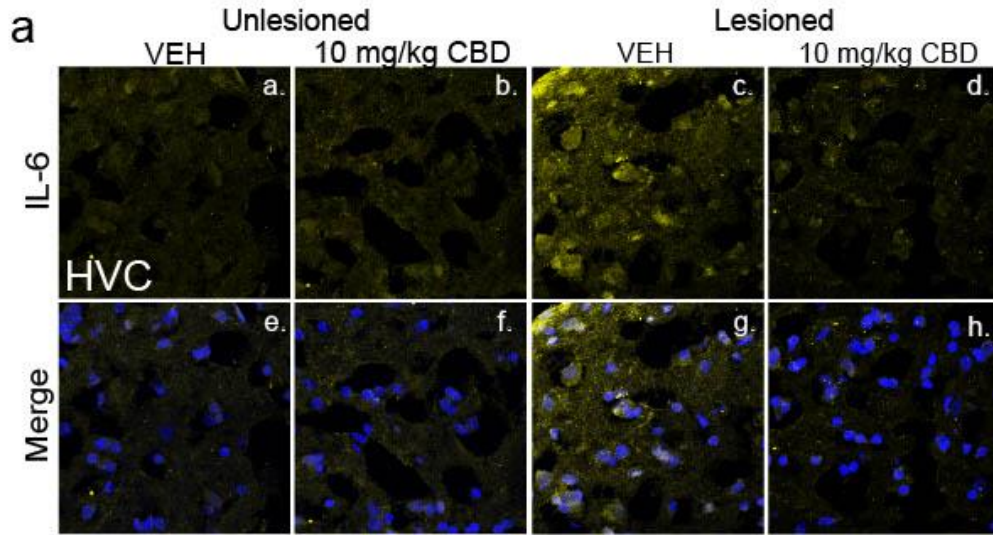
Figure 3.7 CBD decreases pro-inflammatory IL-1β staining within HVC, RA and Area X

CBD alters inflammation-related IL-1β densities. Daily treatments with 10 mg/kg were associated with modulation of IL-1β protein expression 24 h after unilateral HVC lesions. Shown are representative immunofluorescent confocal images of antibody staining targeting IL-1β within motor (HVC & RA) and learning-essential (Area X) regions. aa-ah, unlesioned and lesioned hemisphere images showing representative regional distribution of IL-1β in HVC of vehicle- vs. CBD-treated birds. ba-bh, images demonstrating regional distribution of IL-1β in RA. ca-cl, confocal images demonstrating regional distribution of IL-1β in Area X. Raw values are not relative to control hemisphere but are displayed in Figure 3.9. Quantification was done as a percentage of the unlesioned hemisphere shown in Figure 3.10. Image J software was used to analyze z-stack images projected at maximum intensity and threshold was applied consistently across all images for n = 5 per group.

a

Unlesioned | Lesioned

VEH   10 mg/kg CBD   VEH   10 mg/kg CBD

IL-1β

a.   b.   c.   d.

HVC

Merge

e.   f.   g.   h.

b

Unlesioned | Lesioned

VEH   10 mg/kg CBD   VEH   10 mg/kg CBD

IL-1β

a.   b.   c.   d.

RA

Merge

e.   f.   g.   h.

c

Unlesioned | Lesioned

VEH   10 mg/kg CBD   VEH   10 mg/kg CBD

IL-1β

a.   b.   c.   d.

Area X

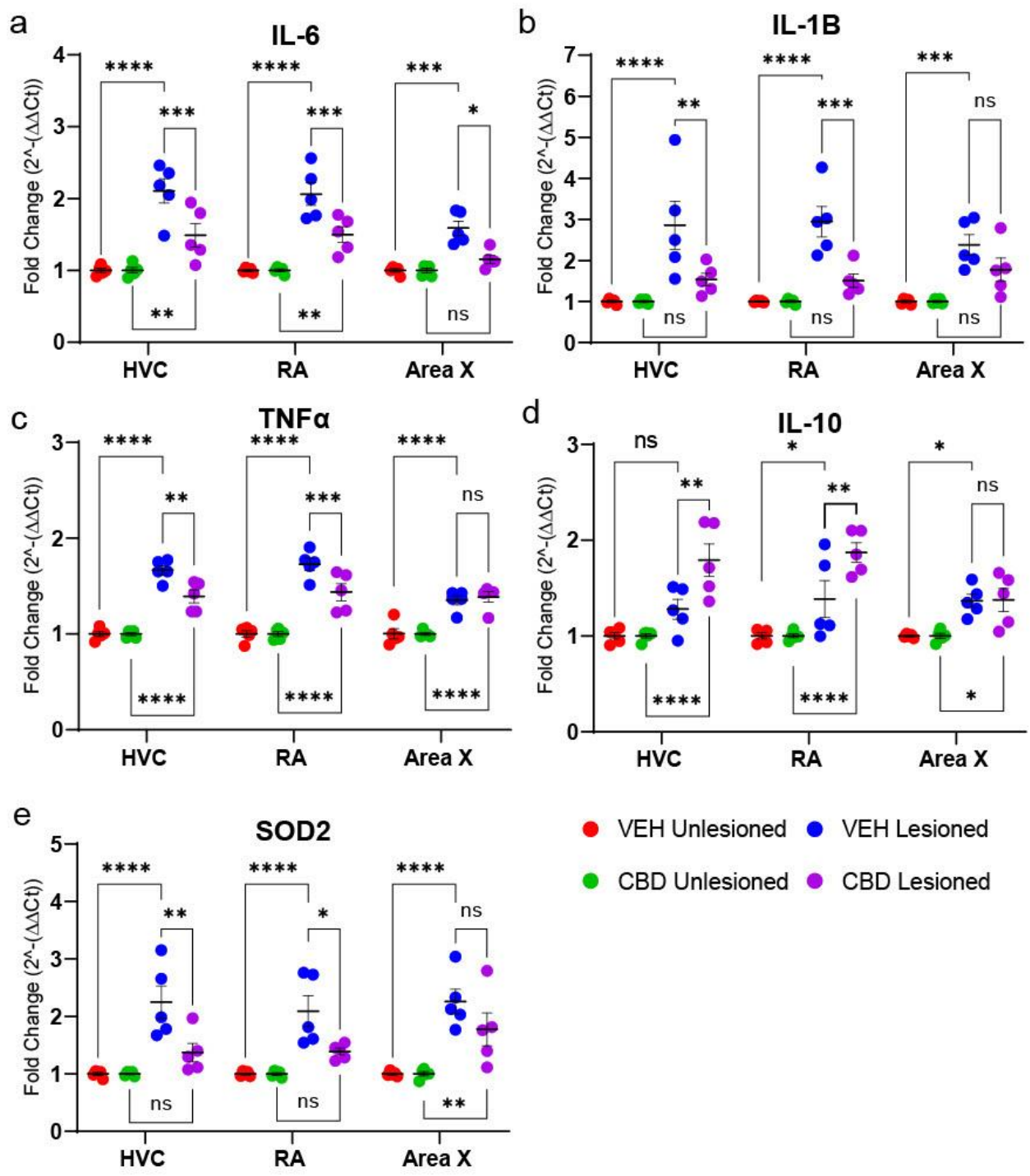Merge

e.   f.   g.   h.

50 μm

Figure 3.8 CBD increases anti-inflammatory IL-10 staining within HVC, RA and Area X

CBD alters inflammation-related IL-10 densities. Daily treatments with 10 mg/kg were associated with modulation of IL-10 protein expression 24 h after unilateral HVC lesions. Shown are representative immunofluorescent confocal images of antibody staining targeting IL-10 within motor (HVC & RA) and learning-essential (Area X) regions. aa-ah, unlesioned and lesioned hemisphere images showing representative regional distribution of IL-10 in HVC of vehicle- vs. CBD-treated birds. ba-bh, images demonstrating regional distribution of IL-1β in RA. ca-cl, confocal images demonstrating regional distribution of IL-10 in Area X. Raw values are not relative to control hemisphere but are displayed in Figure 3.9. Quantification was done as a percentage of the unlesioned hemisphere shown in Figure 3.10. Image J software was used to analyze z-stack images projected at maximum intensity and threshold was applied consistently across all images for n = 5 per group.
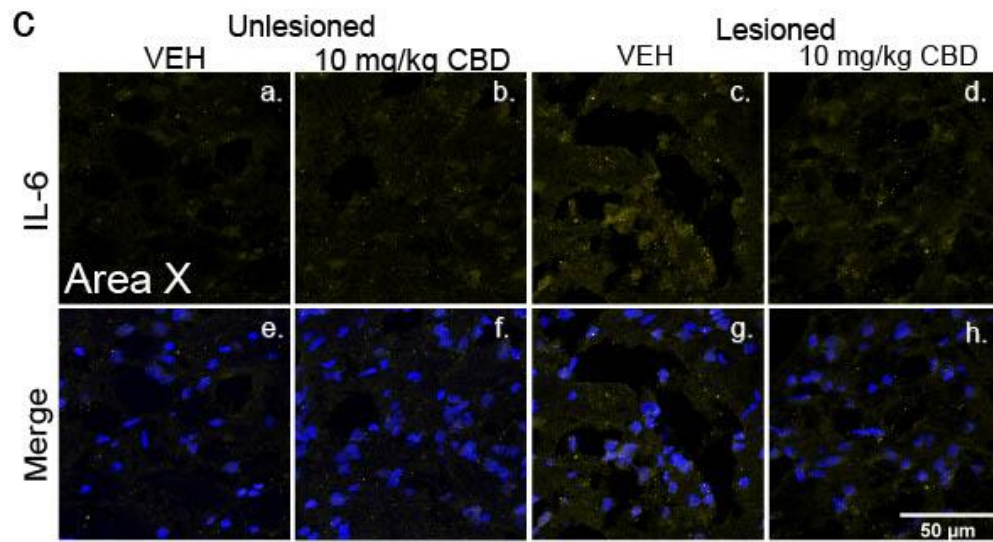
a

Unlesioned | Lesioned
VEH | 10 mg/kg CBD | VEH | 10 mg/kg CBD

IL-10

a. | b. | c. | d.

HVC

Merge

e. | f. | g. | h.

b

Unlesioned | Lesioned
VEH | 10 mg/kg CBD | VEH | 10 mg/kg CBD

IL-10

a. | b. | c. | d.

RA

Merge

e. | f. | g. | h.

c

Unlesioned | Lesioned
VEH | 10 mg/kg CBD | VEH | 10 mg/kg CBD

IL-10

a. | b. | c. | d.

Area X

Merge

e. | f. | g. | h.

50 μm
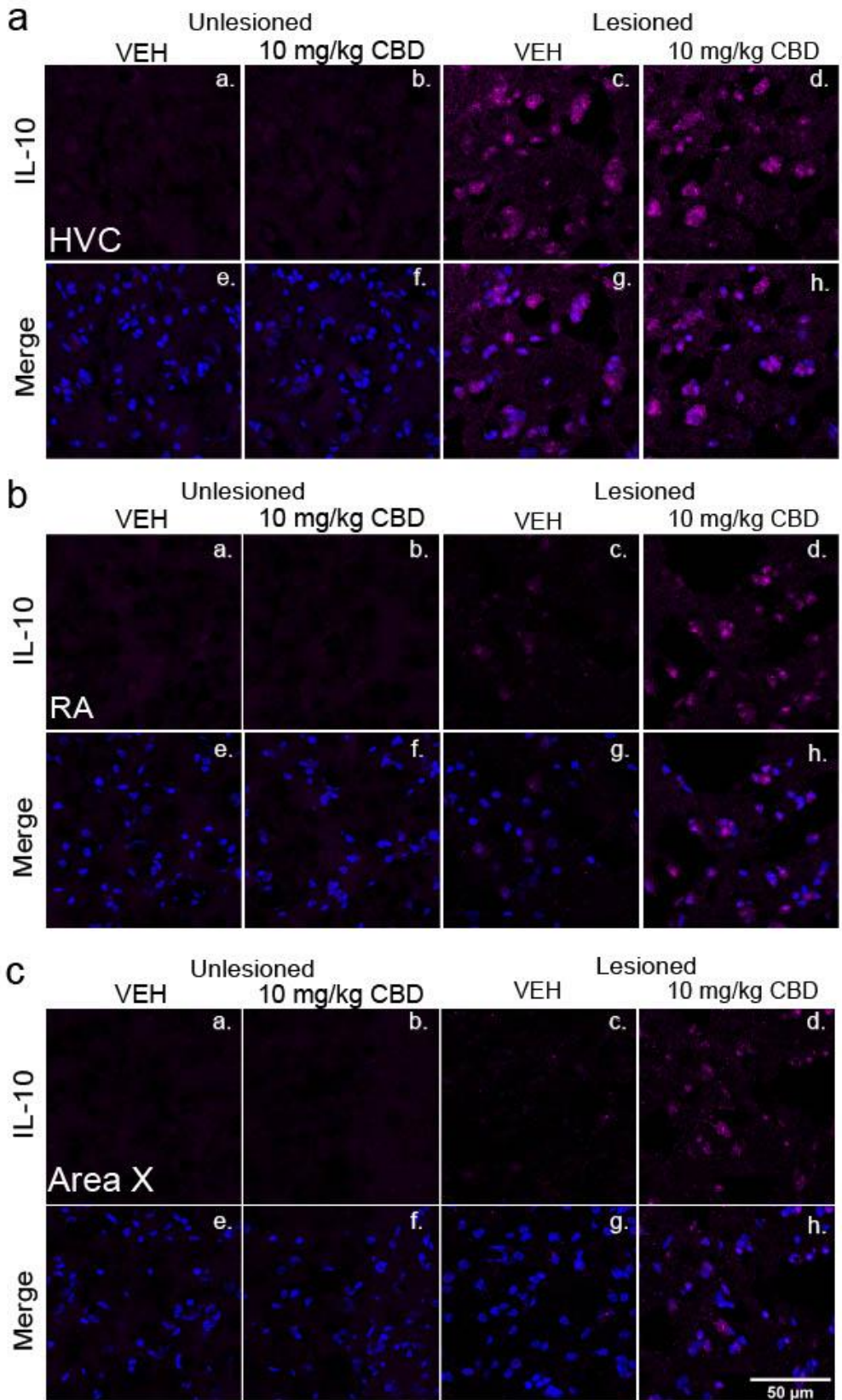
Figure 3.9 Unchanged values of relative fluorescence of individual cytokines

Quantification of relative mean grey value of pro- and anti-inflammatory cytokine protein expression. A-C, Relative fluorescence of IL-6 by song region and drug treatment. D-F, Relative fluorescence of IL-1β by song region and drug treatment. G-I, Relative fluorescence of IL-10 by song region and drug treatment.
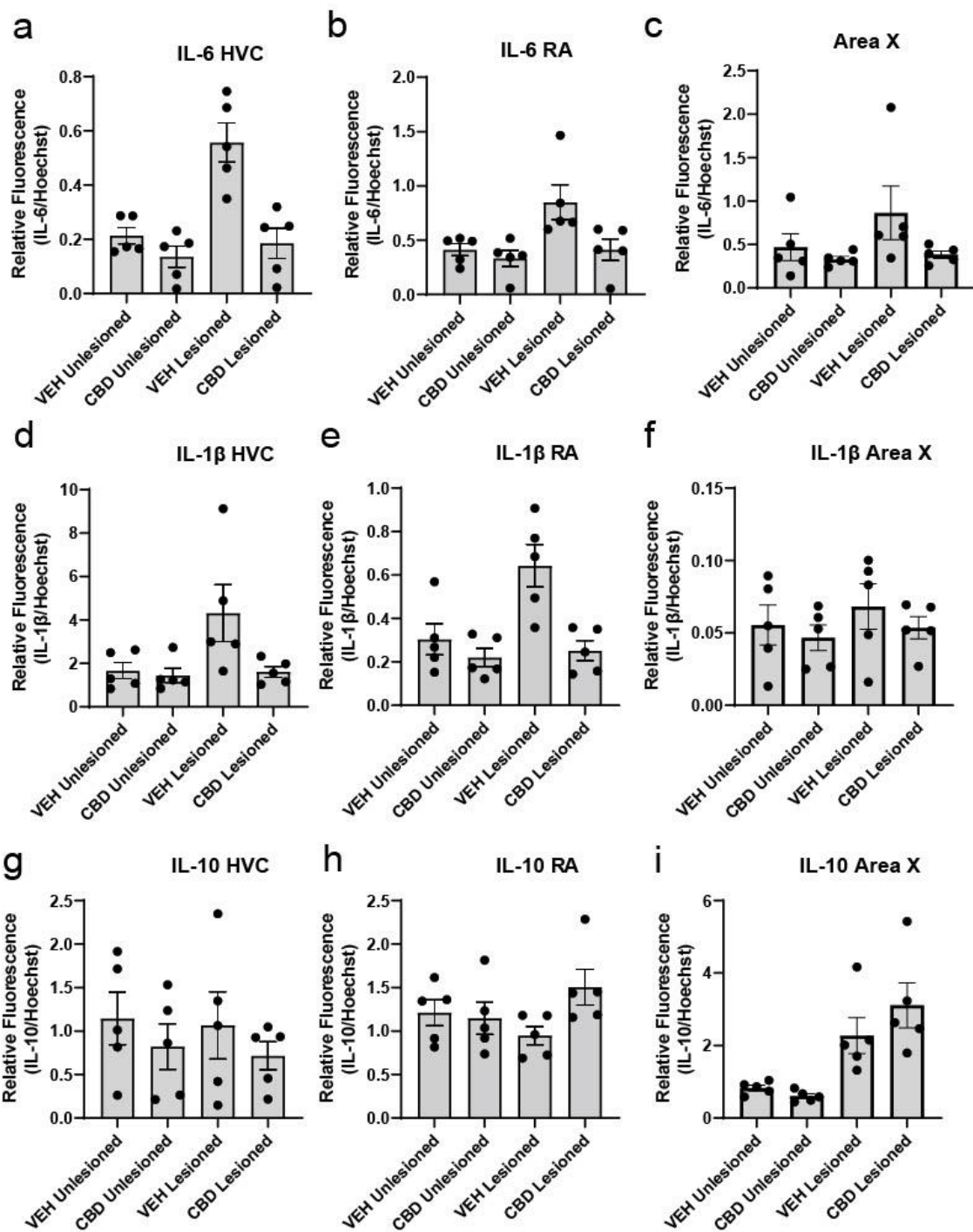
Figure 3.10 CBD alters inflammation-related cytokine densities relative to unlesioned hemisphere

Quantification of relative fluorescence as a percentage of unlesioned control. a, IL-6 change by song region and drug treatment. CBD significantly reduced IL-6 expression in HVC, RA, and Area X. b, IL-1β relative fluorescence change from unlesioned control hemisphere by song region and drug treatment. CBD significantly decreased IL-1β intensity in HVC, and RA. c, anti-inflammatory IL-10 fluorescence as percent change from unlesioned control hemisphere by song region and drug treatment. CBD significantly increased IL-10 intensity in RA and Area X. Differences were determined by mixed-models ANOVA followed by Sidak-corrected post-hoc comparisons. Image J software was used to analyze z-stack images projected at maximum intensity and threshold was applied consistently across all images for n = 5 per group.
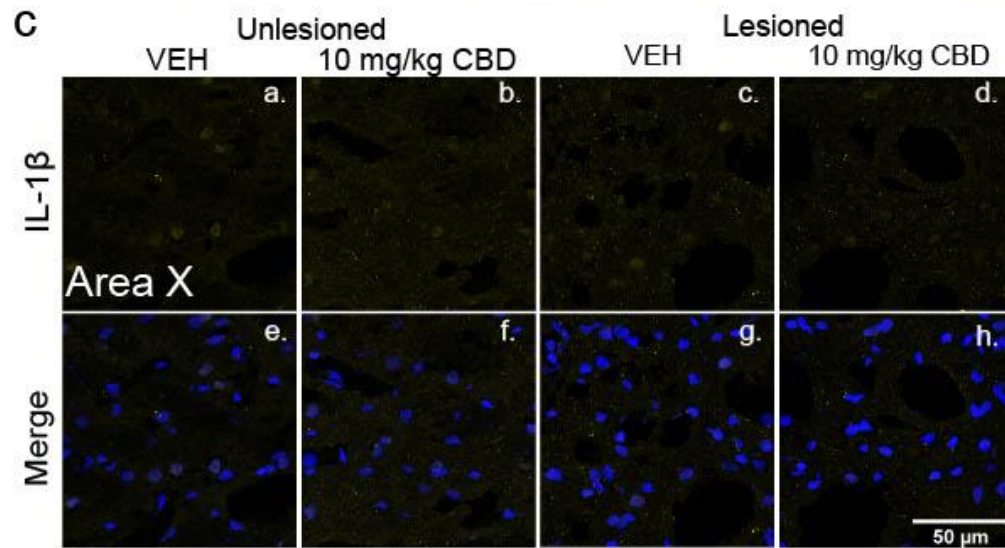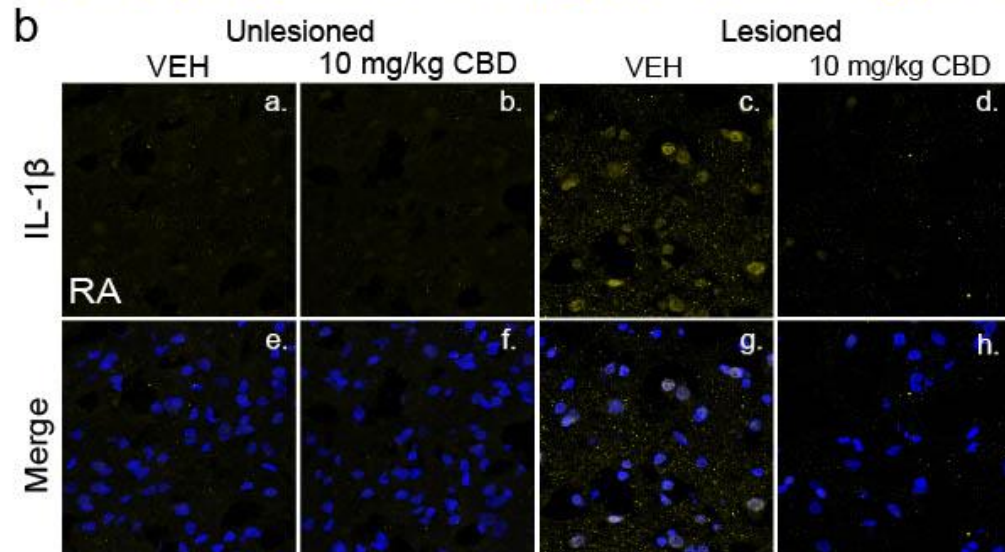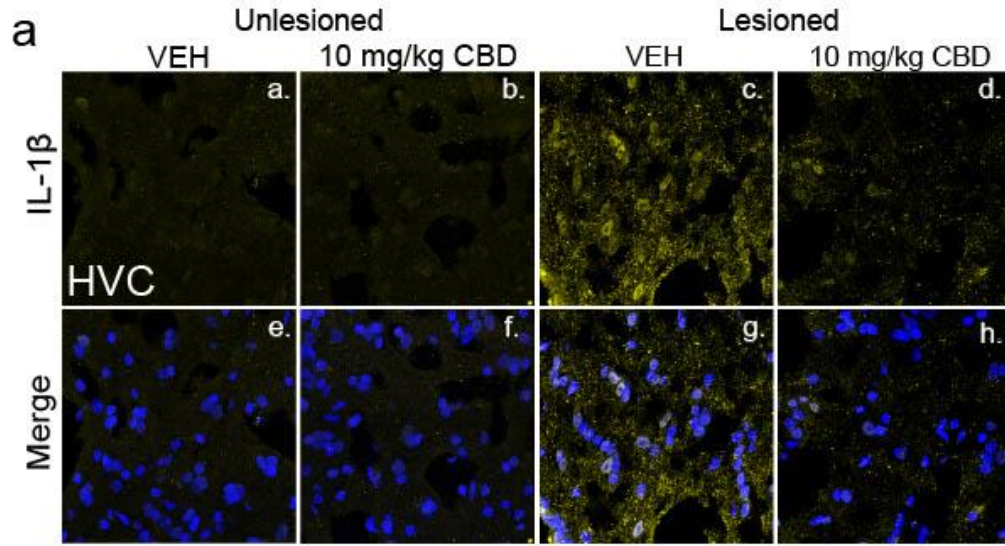
a IL-6

b IL-1B

c IL-10

● Vehicle   ● 10 mg/kg CBD
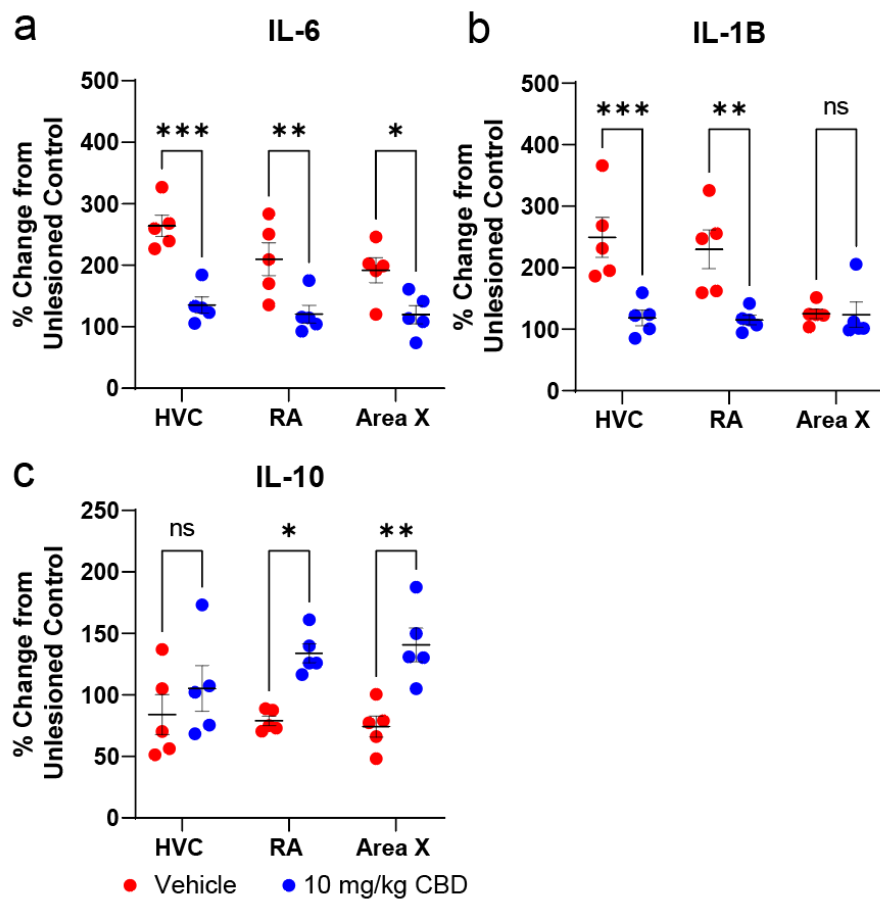
Figure 3.11 CBD decreased reactive oxygen species in lesioned hemispheres as indicated by intensity of dihydroethidium staining

CBD decreased reactive oxygen species in lesioned hemispheres as indicated by intensity of dihydroethidium staining (DHE, in red). a, representative confocal images displaying regional DHE staining indicative of reactive oxygen species (ROS) as a function of lesion condition and treatment. b, summary of mean Corrected Total Cell Fluorescence (CTCF) of DHE staining within each area using a circular area with a diameter of 0.5 mm (see methods for CTCF calculation details). In the vehicle group microlesions significantly increased total fluorescence of DHE staining within HVC, RA and Area. Increased lesion induced DHE staining was also observed in CBD treated groups although to a less significant degree. Comparing DHE staining within lesioned hemispheres of vehicle- and CBD-treated animals, CBD reduced intensities within HVC, RA, and Area X. c, CBD treatment significantly reduced lesion related changes in DHE expression in all regions as a percentage of their respective control hemisphere. The mean grey values were determined and corrected for background fluorescence using the average intensity outside of each song region studied. from n = 4 per group. Comparisons were made and significance determined using Sidak's correction for multiple comparisons following mixed-models ANOVA (n = 4 per group).

a

Unlesioned | Lesioned

VEH · 10 mg/kg CBD · VEH · 10 mg/kg CBD

Dihydroethidium

HVC — a. b. c. d.

RA — e. f. g. h.

Area X — i. j. k. l.

0.5 mm

b

Corrected Total Cell Fluorescence (a.u.)

**** **
**** *
* *
ns ns ns

HVC · RA · Area X

● Veh Unlesioned ● CBD Unlesioned
● Veh Lesioned ● CBD Lesioned

c

% Change from Unlesioned Control

** * *

HVC · RA · Area X

● Vehicle ● 10 mg/kg CBD
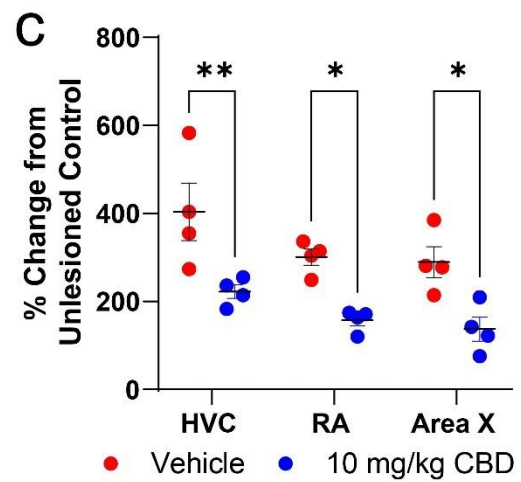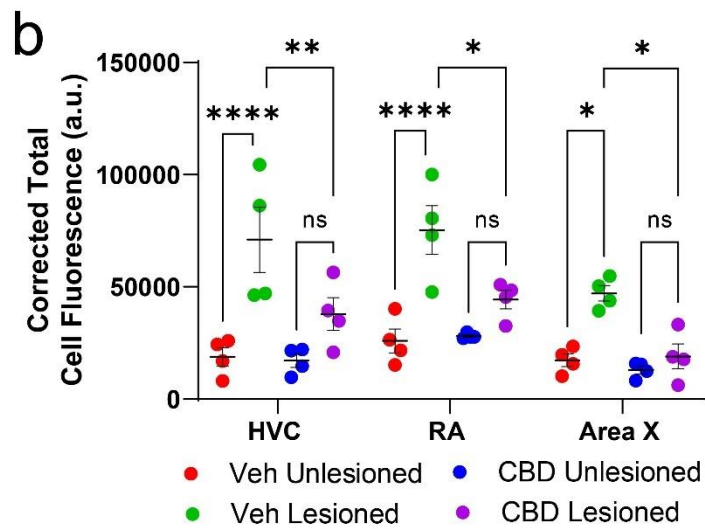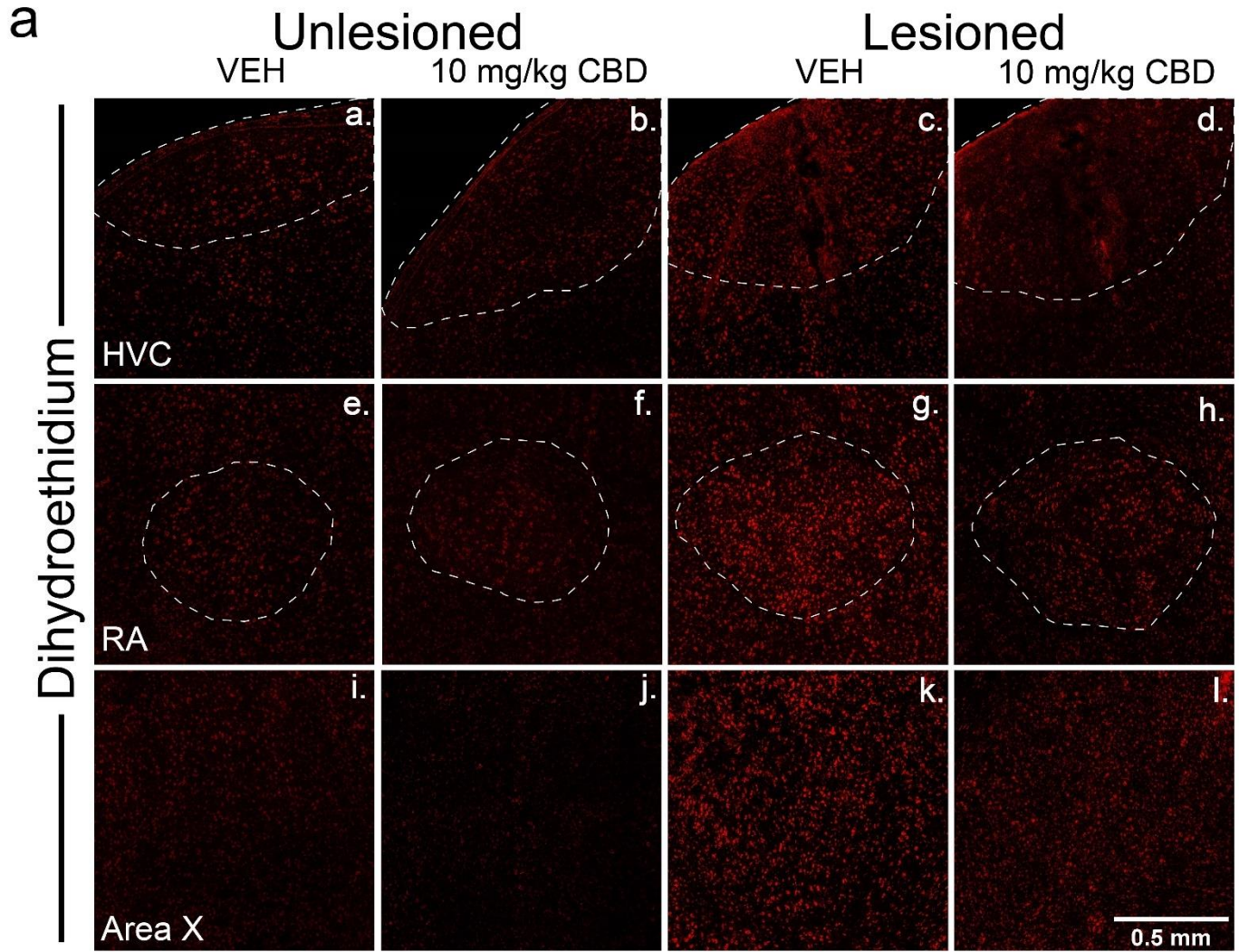
CHAPTER FOUR: Investigating the Impact of CBD on Regulators of Inflammation

4.1 Introduction

Inflammation is a complex biological response that plays a critical role in the body's defense against injury or infection. In the CNS, this response includes the activation of immune cells, including microglia and astrocytes, and a concomitant release of pro-inflammatory cytokines (i.e. IL-6 and IL-1β) that help to eliminate the source of injury or infection[133], [134]. Pro-inflammatory cytokines can recruit additional immune cells to the site of injury or infection, activate phagocytic cells to remove cellular debris and pathogens, and stimulate tissue repair mechanisms[135]. However, chronic or excessive production of pro-inflammatory cytokines can lead to pathological conditions, such as chronic inflammation and further exacerbate tissue damage[109], [136]. Therefore, proper regulation of pro-inflammatory cytokine production is essential to maintain homeostasis and promote recovery.

The activation of the NRF2 pathway has been shown to play a crucial role in the regulation of inflammation and oxidative stress. NRF2 is a transcription factor that regulates a number of genes involved in antioxidant, autophagic, and other cellular responses to inflammation and oxidative stress[67]. Under basal conditions, NRF2 is a cytoplasmic protein ubiquitylated by Kelch- like ECH-associated protein 1 (KEAP1) for proteasomal degradation[69]. In the presence of cellular stress, NRF2 becomes activated by phosphorylation leading to its translocation to the nucleus. After translocation, phosphorylated-NRF2 (pNRF2) bind promoter region of small musculoaponeurotic fibrosarcoma oncogene homologue (sMAF) proteins and induce antioxidant response element (ARE). ARE is then shown to upregulate antioxidant enzymes, detoxifying enzymes, and other anti-inflammatory mediators (IL-10)[68].

NRF2 activation is accompanied by activated microglia to regulate inflammation and tissue damage[137]. Activation and recruitment of microglia to the site of injury play a critical role in the regulation of inflammation and oxidative stress, as microglia use pro-inflammatory mediators that contribute to the inflammatory response[138]. Additionally, microglia play a key role in phagocytosis and clearance of cellular debris, which is important for promoting recovery following injury or infection[136], [139]. The relationship between microglial dynamics, inflammation, and oxidative stress is complex, and the regulation of microglial activity by NRF2 and other signaling pathways is an active area of research.

Here we investigate the role of NRF2 and microglia in regulating inflammation and oxidative stress following injury. Our goal is to better understand the mechanisms by which anti-inflammatory agents, such as CBD, regulate inflammation and oxidative stress and the role of NRF2 and microglia in these processes. Previous results show significant lesion-induced increases in pro-inflammatory mediators and oxidative markers, followed by subsequent amelioration of inflammation with CBD. Changes in pNRF2 and microglia expression may provide insight into the regulation of this response.

4.2 Materials & Methods

Drug Treatments:

Stocks of CBD, prepared according to materials (section 3.2), were stored in sterile 5 ml septum-capped vials at 4 ℃. Fresh stocks were prepared at least weekly. For injections drug preparations were loaded into sterile 1 cc insulin syringe with 30 ga needles. In the morning of injections while the lights were off, birds were captured by hand and the pectoralis muscle injection site was exposed by matting feathers with a small volume of 70% ETOH delivered by squirt bottle.

Injections of 50 µl were made into one of four quadrants of pectoralis, rotating daily to minimize potential damage caused by repeated treatments.

Experimental Design: See chapter 3 for details, summarized below.

Experiments spanned 8 days. Six daily injections were given prior to the surgical procedures to allow CBD, a lipophilic drug with large volume of distribution and long elimination half-life, to approximate steady state levels[115]. On day 7, birds were given a pre-operative injection and unilateral microlesion surgeries were performed according to methods detailed below. Note that unilateral HVC microlesions significantly, but temporarily disrupt vocalizations in a manner consistent with the bilateral approach used previously, except that the magnitude of disruptions was predictably reduced (see Fig. 3.4). On day 8, birds were given a post-operative injection in the morning and euthanized in the afternoon for perfusion and isolation of paraformaldehyde-fixed brain tissue.

Animals and Environment: Chapter 3

Microlesion Surgeries: Chapter 3

Fixation and Cryosection: Chapter 3

Immunofluorescent staining:

Slides were blocked with 5% normal goat serum for 1 hour at 37 ℃. Primary antibodies targeting various proteins were diluted in 2% normal goat serum and used at optimized concentrations: anti-TMEM119, 1:100 (Abcam, AB185337); anti-phosphorylated NRF2 (pNRF2), 1:200 (Abcam, AB76026); and nuclear counterstain Hoechst, 1:10000 (Thermo Fisher Scientific, H3570). Primary Antibody specificity were validated via western blotting images of which are summarized in Fig. 3.2. All primary antibodies were incubated at 4 ℃ overnight. The following day, slides were washed with PBS then corresponding secondary antibody diluted in 2%

normal goat serum was incubated with the tissue at 37 ºC for 1 hour. Secondary antibodies were Alexa Fluor 488 goat anti-mouse (A32723) Alexa Fluor 647 goat anti-mouse (A32728) Alexa Fluor 647 goat anti-rabbit (A32733). After incubation of one hour in secondary antibody, slides were washed twice with PBS for five minutes each followed by Hoechst nuclear counterstain. After fourth and final wash, cover slips were placed on slides using diamond antifade mountant (Invitrogen, P36961).

Confocal and dark-field imaging: Chapter 3

Image analysis:

Image J software was used to analyze superimposed z-stack images converted to 8-bit with a threshold applied consistently across all images within each region. All CZI-format image files were exported from ZEN Black software as a greyscale tiff file and consolidated into a max intensity z projection. For analysis of nuclear translocation of pNRF2, color thresholding was used to determine: (1) the area of pNRF2 colocalized with Hoechst nuclear staining and (2) the total area of Hoechst nuclear staining. pNRF2 nuclear area was then expressed as a percentage of total nuclear area and compared across groups (Fig. 4.1). For analysis of microglia within each region, mean grey value of TMEM119 staining was normalized to Hoechst-stained nuclei and calculated as percent change from unlesioned control hemisphere (Fig. 4.5).

Statistical analysis:

Statistical analyses were performed with GraphPad Prism 9.2.0 for all histological, behavioral, and gene expression data. Data are expressed as mean ± SEM. Statistical analysis was performed using a mixed-models ANOVA followed by Sidak's post hoc analysis to identify differences between groups. A p value ≤ 0.05 was considered statistically significant. Specific statistical tests performed are indicated in the text of the results and figure legends.

4.3 Regulation of inflammation pNrf2

The anti-inflammatory and anti-oxidative activity of CBD suggests involvement of NRF2-mediated regulation of redox, mitochondrial and inflammatory processes to maintain homeostasis. Results indicate CBD treatment significantly increased nuclear levels of pNRF2 within all regions of interest: in HVC (by mean percent = 19.6 [3.7 to 35.4], p = 0.0259), RA (by mean = 11.65 [2.39 to 20.9], p = 0.0262) and Area X (by mean = 7.1 [1.8 to 12.43], p = 0.0218, Fig. 4.1). Note the difference in punctate staining of (Fig 4.1aa-af) and aggregation of pNRF2 in CBD treatment that has been associated with activated microglia and subsequent decrease in pro-inflammatory signaling[140], [141]

4.4 CBD effectively reduces microglial marker TMEM119

Microglia activation, recruitment, and phagocytosis are primary inflammatory responses after injury, known to be mediated by the release of cytokines and superoxide production with downstream effects on the complement cascade[109], [135]. Previous evidence links microglial activation with transient elevation of pro-inflammatory genes (i.e. IL-1β & IL-6) that have been observed at peak neuronal death during tissue damage[135]. Given our results showing increased expression of pro-inflammatory cytokines 24 hours after the microlesion surgery, we explored the possibility of microglial involvement in CBD-improved vocal recovery. Using TMEM119 as a microglia marker, we studied lesion-induced TMEM119 staining within song regions (HVC, RA, and Area X) and CBD effects on this expression (Fig. 4.2-4.4). Results indicate within HVC, RA and Area X, TMEM119 levels were significantly lower in CBD-treated animals relative to controls (by mean 97.61% [27.59 to 167.6], p = 0.0053; 103.8% [33.77 to 173.8], p = 0.0031 and; by 87.39% [17.37 to 157.4], p = 0.0123, respectively (Fig. 4.5). Note that our present focus on

microglia represents a first step, and does not preclude likely involvement of other cell types in microlesion-induced inflammatory responses (e.g. reactive astrocytes)[142].

4.5 Discussion

The combination of anti-inflammatory and anti-oxidative CBD activity suggested involvement of a higher order, organized stress response. Consistent with this is signaling controlled by NRF2, an established central regulator of redox, mitochondrial and inflammatory mediators. Under basal conditions NRF2 is a cytoplasmic protein that upon oxidative stress is activated by phosphorylation[68]. Activated phospho-NRF2 translocates to the nucleus where it acts as a transcription factor regulating a host of genes involved in antioxidant, autophagic, misfolded protein and other cellular responses[69]. The significant CBD-related increases in nuclear phospho-NRF2 observed in our system implicates this homeostatic pathway in vocal protection. Note other activators of NRF2 signaling are clinically-relevant anti-inflammatories[143]. NRF2 is also potently activated by the botanically-derived antioxidant sulforaphane, a derivative of which is currently being evaluated in CNS hemorrhage[144]. These more selective drugs are candidates for anti-neuroinflammatory evaluation in the HVC microlesion system.

The type of pro-inflammatory cytokine expression we observe following microlesions (described above) are, in other systems, associated with microglial activation, infiltration, and phagocytosis of cellular debris. These activities may be of key importance to neuronal recovery vs. apoptosis[109]. This led us to investigate potential microglia-related activity following HVC microlesions and CBD-improved vocal recovery. Note that this investigation of microglia involvement represents an initial first step. It is highly likely that other cell types are involved in both lesion-induced inflammation and CBD's anti-inflammatory activity (e.g. reactive

astrocytes)[142]. Experiments using additional markers are currently planned and will result in a more complete characterization of cell types active in, and relevant to, our system.

Presently, using TMEM119 as a microglia marker[145] we found CBD significantly reduced staining densities consistent with reduced myeloid cell infiltration (Fig. 6A-B). This is interesting as it suggests that damage-induced myeloid cell activity, at the single early 24 hour timepoint we investigated, is associated with disruptive effects of the microlesions, and not neuroprotection. The rounded cellular appearance of TMEM119-stained cells 24 hours post-lesioning suggests, but does not prove, that microlesions increase densities of microglia in an activated phagocytic state, and that CBD treatments reduce this (as shown in Fig. 4.2aa-b). Going forward it will be important to investigate a more complete time course of lesion effects. Another caveat follows recent evidence suggesting TMEM119 may not distinguish microglia from migrating peripheral macrophages as reliably as previously thought, and that it does not distinguish activated from inactive states[146]. Because microglia can adopt a continuum of activation states: from pro-inflammatory "M1-like" to anti-inflammatory "M2-like" subtypes[147] it will be important in future studies to measure multiple markers to distinguish the types and activity of myeloid cell responses[110], [146]. Unlike other CBD-related measures, effects on lesion-increased TMEM119 densities were not reversed to unlesioned control levels. A hypothesis we are currently testing is ability of CBD to shift the relative populations of pro- to anti-inflammatory microglial species.

Figure 4.1 CBD increases nuclear localization of the antioxidant response-regulating transcription factor pNRF2

CBD increases nuclear localization of the antioxidant response-regulating transcription factor pNRF2. a, Images within song regions were taken from tissue collected 24 h after unilateral HVC lesions. b, pNRF2 nuclear staining in song regions of interest expressed as a percentage of total nuclear staining. Results indicate CBD treatment significantly increased nuclear pNRF2 within HVC, RA and Area X consistent with antioxidant responses. Image J software was used to analyze z-stack images projected at maximum intensity and threshold was applied consistently across all images. Group differences were determined using mixed-models ANOVA followed by Sidak-corrected post-hoc comparisons for n = 4 per group.

a

| | HVC | | RA | | Area X | |
| | VEH | 10 mg/kg CBD | VEH | 10 mg/kg CBD | VEH | 10 mg/kg CBD |

pNRF2
a. b. c. d. e. f.

Merge
g. h. i. j. k. l.
100 μm

b

Figure 4.2 TMEM119 staining within song regions of lesioned and unlesioned hemispheres

CBD treatments decrease density of the microglia specific marker TMEM119 within song regions of lesioned and unlesioned hemispheres. aa – h, TMEM119 immunofluorescence marks microglia, in which a high density of fluorescence is present in vehicle-treated HVC. Lower TMEM119 staining is evident in the CBD treated group. ae-h, Merge images of TMEM119 and Hoechst-stained nuclei. ba – h, TMEM119 immunofluorescence marks microglia, in which a high density of fluorescence is present in vehicle-treated RA. Lower TMEM119 staining is evident in the CBD treated group. be-h, Merge images of TMEM119 and Hoechst-stained nuclei. ca – h, TMEM119 immunofluorescence marks microglia, in which a high density of fluorescence is present in vehicle-treated Area X. Lower TMEM119 staining is evident in the CBD treated group. ce-h, Merge images of TMEM119 and Hoechst-stained nuclei.

a

	Unlesioned		Lesioned	
	VEH	10 mg/kg CBD	VEH	10 mg/kg CBD

TMEM119

HVC

a. b. c. d.

Merge

e. f. g. h.

b

	Unlesioned		Lesioned	
	VEH	10 mg/kg CBD	VEH	10 mg/kg CBD

TMEM119

RA

a. b. c. d.

Merge

e. f. g. h.

c

	Unlesioned		Lesioned	
	VEH	10 mg/kg CBD	VEH	10 mg/kg CBD

TMEM119

Area X

a. b. c. d.

Merge

e. f. g. h.

100 µm   100 µm
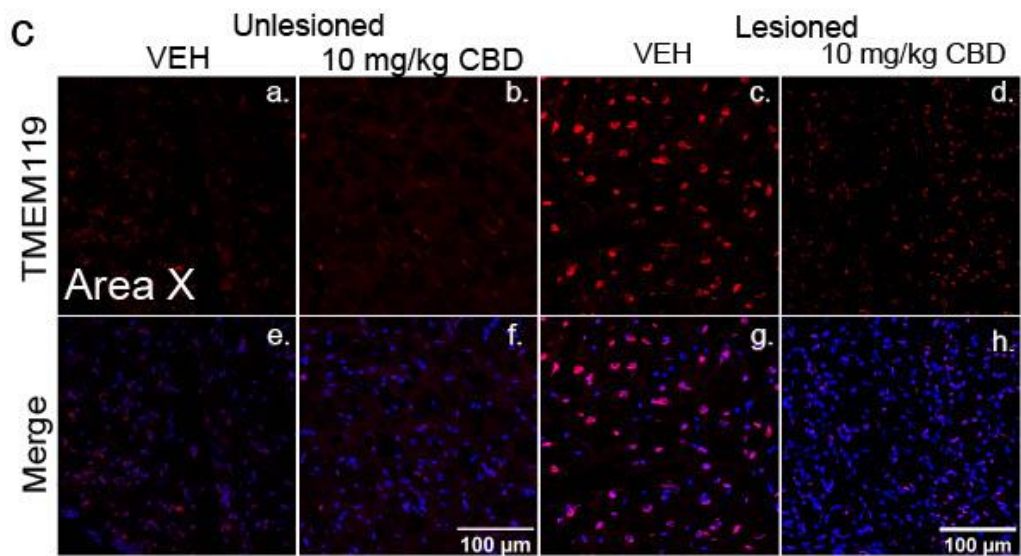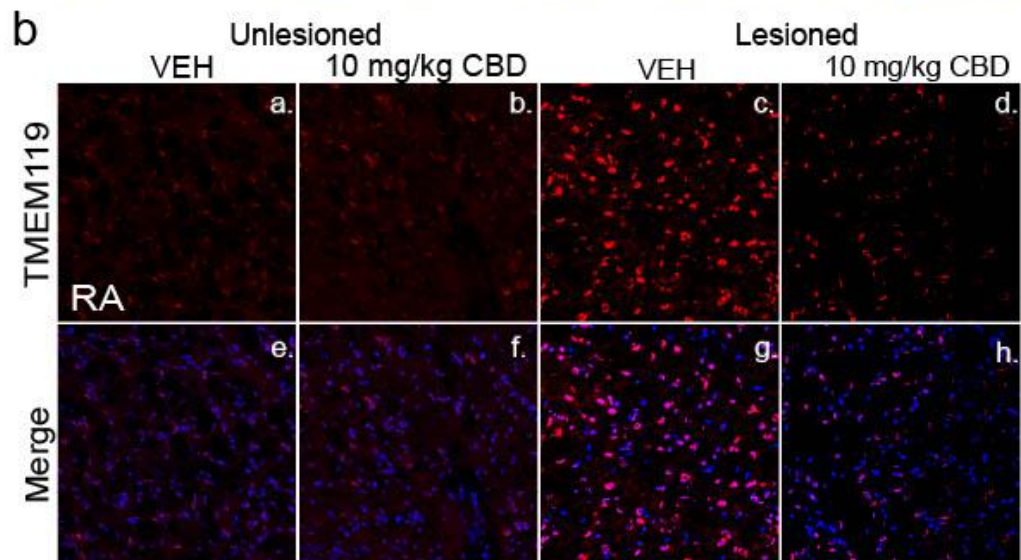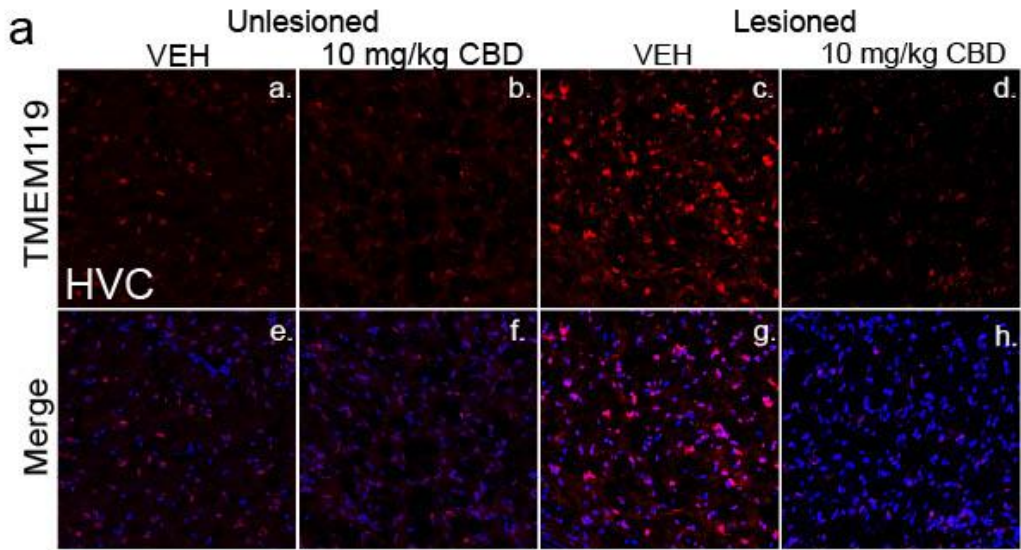
Figure 4.3 CBD treatments decrease relative staining density of TMEM119 within HVC, RA and Area X

TMEM119 density expressed as TMEM119 mean grey value relative Hoechst staining as percentage of the unlesioned hemisphere. Within HVC, RA and Area X, microglia densities were significantly lower in CBD-treated animals relative to vehicle controls. Group differences were determined using mixed-models ANOVA followed by Sidak corrected post-hoc comparisons for $n = 4$ per group.
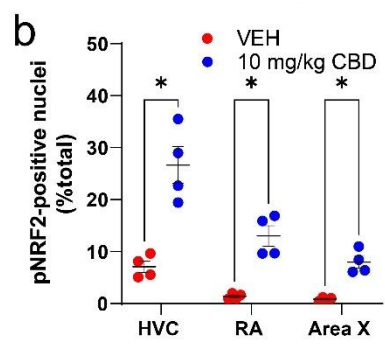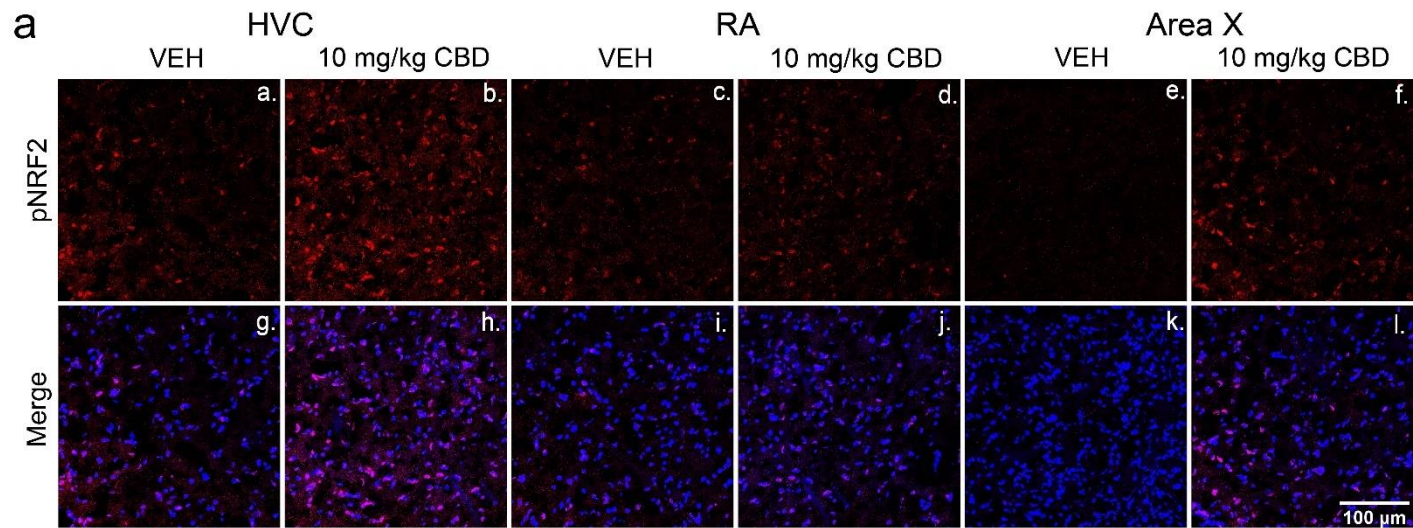
a

b
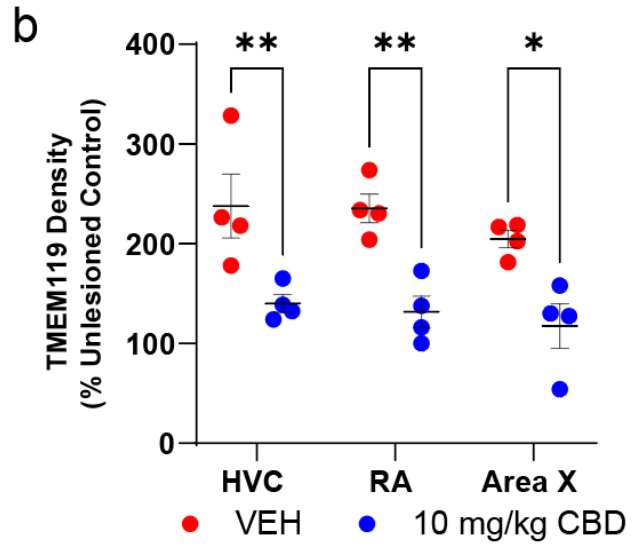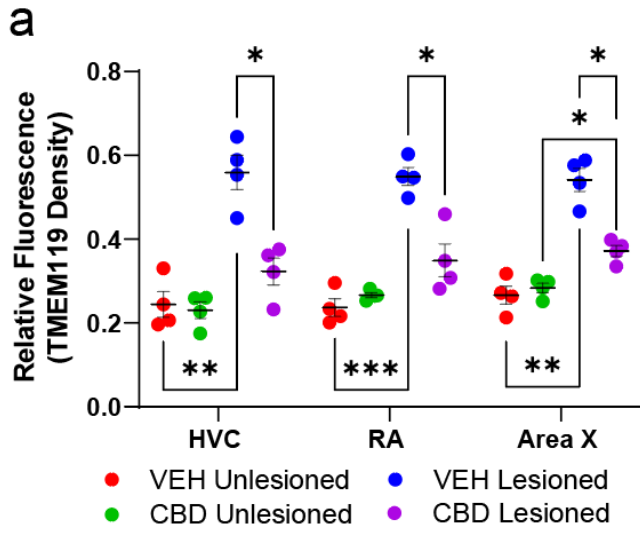
CHAPTER FIVE: Neuroplastic Changes in the Song System Contribute to CBD-Improved Vocal Recovery.

5.1 Introduction

The CNS relies on proper regulation of synaptic communication to maintain normal function and promote recovery following injury or damage. Microglia have been recognized to play a crucial role in the regulation of these connections, both during development (for example critical periods of learning, see section 2.3) and in response to injury or disease[148], [149]. One of their primary functions is to modulate these connections, especially excitatory synapses, through their ability to target degrading synapses and promote recovery. However, the same microglia can also release cytotoxic components that can damage healthy synapses and neurons, making their role in synaptic regulation dynamic and complex[139].

During development, microglia play a crucial role in the formation and elimination of synapses, especially in the context of synaptic pruning. Microglia are involved in the elimination of excess synapses, leading to a refinement of the neural circuitry. This process is critical for the proper functioning of the CNS, as it allows for the establishment of functional neural networks as seen in the zebra finch. Over the course of song crystallization, there is a shift in the circuitry used for song production that is modeled using the number of degenerating synapses[150]. Early in development, the song refinement relies on auditory feedback and utilizes glutamatergic connections of the anterior forebrain pathway (AFP; Area X, DLM, lMAN to RA see fig. 2.1)[95]. However, later in development we begin to see more robust communication through the number of synaptic connections from HVC to RA[96].

In response to injury or disease, microglia are activated and migrate towards the site of injury and release a range of factors that can modulate synaptic connections, including cytokines, chemokines, and growth factors[109]. These factors can either promote or inhibit synapse formation, depending on the type of injury or disease and the stage of recovery. Regulation of this process is critical as microglia can also damage healthy synapses and nearby neurons. With the dysregulated or chronic release of factors such as reactive oxygen species (ROS) and nitric oxide (NO), microglia can cause oxidative damage to healthy neurons and synapses leading to a loss of communication and a reduction in synaptic density[56], [139].

In this study, we aim to investigate the potential protective effects of cannabidiol (CBD) on synaptic density and plasticity following injury. We will use a combination of imaging techniques to measure the colocalization of postsynaptic density protein 95 (PSD95) and the presynaptic glutamatergic marker VGLUT2, as a measure of synaptic density. Additionally, because zebra finch vocal recovery requires sensorimotor feedback[100] we will investigate factors linking experience with neuroplasticity and homeostatic synaptic scaling[151].

5.2 Materials & Methods

Materials: Chapter 3

Drug Treatments: Chapter 3

Experimental Design: Chapter 3

Animals and Environment: Chapter 3

Microlesion Surgeries: Chapter 3

Quantitative RT-PCR: See chapter 3 for full methods

Gene expression of ARC/ARG3.1 MSK1 and BDNF were normalized to the endogenous control (GAPDH) and fold change was determined from the unlesioned hemisphere using the ΔΔCt method[120]. Primer sequences and information are located in table 3.1.

Fixation and Cryosection: Chapter 3

Immunofluorescent staining: see chapter 3 for full methods

Slides were blocked with 5% normal goat serum for 1 hour at 37 ℃. Primary antibodies targeting various proteins were diluted in 2% normal goat serum and used at optimized concentrations: anti-PSD95, 1:50 (Santa Cruz, SC-32291); anti-vesicular GLUT2 (VGLUT2) 1:500 (Cell Signaling, 715555); anti-GLUA1, 1:200 (Cell Signaling, 13185) and nuclear counterstain Hoechst, 1:10000 (Thermo Fisher Scientific, H3570).

Confocal and dark-field imaging: Chapter 3

Image analysis

Image J software was used to analyze superimposed z-stack images converted to 8-bit with a threshold applied consistently across all images within each region. All CZI-format image files were exported from ZEN Black software as a greyscale tiff file and consolidated into a max

intensity z projection. Glutamatergic density was analyzed using the colocalization of VGLUT2 and PSD95 per area of measurement (circular diameter of 100µm).

Statistical analysis

Statistical analyses were performed with GraphPad Prism 9.2.0 for all histological, behavioral, and gene expression data. Data are expressed as mean ± SEM. Statistical analysis was performed using a mixed-models ANOVA followed by Sidak's post hoc analysis to identify differences between groups. A p value ≤ 0.05 was considered statistically significant. Specific statistical tests performed are indicated in the text of the results and figure legends.

5.3 Synaptic Density

A critical microglial function includes phagocytotic clearing of axonal and synaptic debris following neuronal degeneration[139]. Evidence of CBD-related decreased microglial TMEM119 expression led us to question whether treatments also protected synaptic densities. To measure this, we compared colocalization of the pre- and post-synaptic markers, VGLUT2 and PSD-95 across treatment groups (Fig. 5.1-5.3). We saw significant lesion-related decreases in synaptic densities. In the vehicle group, unilateral microlesions decreased the synaptic density within HVC (Fig. 5.4a Veh Unlesioned vs Veh Lesioned) by mean = $0.20/\mu m^2$ [0.025 to 0.38], p = 0.272), RA by mean = $0.24/\mu m^2$ [0.11 to 0.37, p = 0.0014) and Area X by mean = $0.16/\mu m^2$ [0.007 to 0.31], p = 0.0410). Interestingly, within RA of CBD groups there was significant decrease in synaptic densities after the lesion (CBD Unlesioned vs CBD Lesioned by mean = $0.16/\mu m^2$ [0.022 to 0.30], p = 0.0234) while HVC and Area X changes were insignificant. However, CBD treatment had a profound effect on post lesion synaptic density compared to vehicle in HVC (Veh Lesioned vs CBD Lesioned, by mean = $0.19/\mu m^2$ [0.002 to 0.38], p = 0.0476) in RA (by mean = $0.18/\mu m^2$ [0.05 to 0.32], p= 0.0088, and Area X (by mean = $0.15/\mu m^2$ [0.04 to 0.26], p = 0.0122). CBD treatment

also appeared to reverse lesion related decreases within HVC (CBD Unlesioned vs CBD Lesioned, mean = 0.076/μm^2 [-0.1126 to 0.2654], p = 0.7154 and Area X (by mean = 0.094/μm^2 [-0.019 to 0.21], p = 0.1094) but decreases remained significant in RA (mean = 0.16/μm^2 [0.022 to 0.30], p = 0.0234). We then quantified the difference in synaptic density from the unlesioned to lesioned hemisphere as the number of colocalized puncta as a percentage of the unlesioned control hemisphere (Fig. 5.4b). Within HVC and RA, CBD groups had a significant increase in post-lesion synaptic density (HVC by mean = 24.4% [0.4095 to 48.41], p = 0.0.0464; RA by mean = 16.5% [3.019 to 29.90], p = 0.0186) while Area X did not differ significantly (by mean = 13.77% [12.02 to 39.56], p = 0.3336). Although not found to be significant, it may be important that CBD treatment tended to increase the synaptic densities in unlesioned hemispheres relative to vehicle, suggesting the promotion of synaptogenesis in addition to lesion protection.

5.4 Homeostatic Scaling

Zebra finch vocal recovery is dependent on auditory feedback that led us to investigate the connection between experience with neuroplasticity and regulation of synaptic strength via synaptic scaling. By quantifying expression of key plasticity-related genes via qRT-PCR we found significant treatment group differences in expression of BDNF (F[3, 36] = 28.79, $p < 0.0001$), MSK1 (F[3, 36] = 57.63, $p < 0.0001$) and ARC/ARG3.1 (F[3, 36] = 46.53, $p < 0.0001$, Fig 5.6).

Post-hoc comparisons revealed that in VEH controls lesions significantly increased BDNF expression in HVC (by 1.29 fold, 95% CI = 0.66 - 1.92, $p < 0.0001$) but not RA or Area X (Fig. 5.6a). This appears related to stimulation of BDNF expression in the lesioned region, HVC, that did not extend to its projection targets, RA and Area X. This wasn't true in CBD-treated finches where significant differences between unlesioned and lesioned-group birds were observed in each

brain region (in HVC by 1.15, 95% CI = 0.52 - 1.78, $p < 0.0001$; in RA by 1.01, 95% CI = 0.38 - 1.64, $p = 0.0007$; in Area X by 1.02, 95% CI = 0.39 - 1.36, $p = 0.0006$).

Because evidence suggests BDNF increases MSK1 expression in experience-related synaptic plasticity[152] we investigated potential CBD regulation of this kinase's expression. Although lesions tended to increase MSK1 expression in VEH controls, this was only significantly different in Area X (VEH Unlesioned vs. VEH Lesioned by 0.68, 95% CI = 0.24 - 1.11, $p = 0.0010$, Fig 11B). In contrast, CBD-treated birds had significantly increased MSK1 expression in each brain region of interest (in HVC by 1.25, 95% CI = 0.81 - 1.66, $p < 0.0001$; in RA by 1.08, 95% CI = 0.64 - 1.52, $p < 0.0001$; in Area X by 1.01, 95% CI = 0.38 - 1.64, $p < 0.0001$).

In models of homeostatic synaptic scaling, high levels of synaptic activity increase signaling of the BDNF/MSK1 pathway[153] promoting ARC/ARG3.1 expression. ARC/ARG3.1 activity promotes internalization of excitatory AMPA receptors, decreasing excitatory synaptic strength. To test whether this ARC/ARG3.1 signaling pathway is involved in our vocal recovery system we measured treatment effects on ARC/ARG3.1 expression. Interestingly, in each brain region of interest of vehicle-treated controls, lesions significantly increased ARC/ARG3.1 expression (in HVC by 0.08, 95% CI = -.40 – 0.56, $p = 0.970$; in RA by 0.56, 95% CI = 0.079 - 1.04, $p = 0.017$; in Area X by 0.62, 95% CI = 0.14 - 1.11, $p = 0.0068$, Fig 11C). Comparing vehicle- and CBD-treated lesioned birds, expression of ARC/ARG3.1 was significantly lower in RA and Area X, but not HVC, of CBD-treated finches (in RA by 0.56, 95% CI = 0.08 - 1.04, $p = 0.017$ and; Area X by 0.62, 95% CI = 0.14 – 1.11, $p = 0.007$). If, as in other systems, ARC/ARG3.1 acts to internalize AMPA receptors, we would expect higher densities following CBD treatments. We are currently investigating this possibility.

5.5 AMPA receptor regulation

AMPA receptors play a critical role in the excitatory neurotransmission in the brain. The regulation of AMPA receptors is essential for synaptic plasticity, learning, and memory formation[151]. Changes or regulation in the number of AMPA receptor subunits, such as the GLUA1 subunit, can modulate the strength of synaptic connections, which is critical for learning and memory[154]. There are several ways that AMPA receptor regulation occurs, including changes in receptor subunit composition, post-translational modifications, and changes in receptor trafficking[155], [156]. Due to changes in synaptic modifying gene expression, we looked at the post-synaptic expression of GLUA1 (Fig. 5.7-5.9) which has been shown to be internalized by ARC/ARG3.1 activity[157], [158]. Similar to an overall increase in synaptic density (Fig. 5.1-5.4), CBD treatment appeared to increase post-synaptic GLUA1 expression of the unlesioned hemisphere within RA (by mean 0.20 [0.11 to 0.29], $p < 0.0001$) but not Area X (Fig. 5.10a). Consistent with a modulation of synaptic scaling gene expression, we saw a significant decrease in post-synaptic GLUA1 expression of the vehicle group within RA (by mean 0.13 [0.04 to 0.22], $p = 0.003$) and Area X (by mean 0.099 [0.011 to 0.18], $p = 0.025$). This deficit was not significant with CBD treatment. Furthermore, the difference between lesioned groups was very large, but it is important to note these are not relative to their respective unlesioned hemisphere (RA by mean 0.33 [0.24 to 0.42] $p < 0.0001$; and Area X by mean 0.15 [0.06 to 0.24], $p = 0.001$; Fig 5.10a). When quantifying as a percentage of the unlesioned hemisphere, in both RA and Area X, CBD treatment appears to offer protection from lesion-related decreases in post-synaptic GLUA1 receptor expression (RA by mean 38.03% [57.91 to 18.14], $p = 0.0016$; and Area X by mean 40.44% [60.32 to 20.55], $p = 0.0010$; Fig 5.10b). It is important to note, expression within HVC was met with high variability likely due to microlesion surgery.

5.6 Discussion

A key function of anti-inflammatory microglial subtypes and other myeloid cells[142] in recovery from CNS damage includes preservation of synaptic densities[159]. Potential CBD protection/promotion of synaptic density was tested by measuring colocalization of PSD95 and the presynaptic glutamatergic marker VGLUT2 (note HVC projections to Area X and RA are glutamatergic[160]). As expected, HVC microlesions decreased densities within the region itself, and also within its projection targets (Fig. 5.1, see also cupric-silver evidence of degeneration[Fig. 5.5]). Less expectedly, CBD increased synaptic marker colocalization within unlesioned hemispheres, suggesting promotion of *de novo* synaptogenesis. Whether synaptogenic activity is accompanied by additional synaptoprotection remains an open question (Fig. 5.1-5.9). The decreased magnitude of vocal disruption seen following CBD treatments suggests potential protection of circuits established during song learning. Facilitating establishment of new synapses may underly CBD promotion of sensorimotor learning-dependent vocal recovery.

A mechanism by which CBD may protect excitatory synapses is through modulating synaptic scaling. This homeostatic process governs synapse sensitivity under various excitation states[161]. Synaptic scaling is regulated by a complex network of proteins and signaling pathways, including BDNF, MSK1 and Arc/Arg3.1[162], [163]. BDNF activates MSK1 that in turn acts to alter expression of Arc/Arg3.1[152]. Arc/Arg3.1 directly regulates synaptic localization of excitatory AMPA receptor subtypes in a manner critical for homeostatic protection of learning-related plasticity and memory consolidation[164]. Arc/Arg3.1 activity increases internalization of excitatory AMPA receptors, decreasing and scaling-down excitatory synaptic strength. This regulation may protect against excitotoxicity but, to the extent patterns of AMPA receptor expression are important for maintenance of song circuits established during vocal

learning, increased Arc/Arg3.1 may result in the vocal disruption observed in vehicle-treated birds. Decreased magnitudes of vocal disruption observed in CBD-treated birds may be due to reduced synaptic scaling following lesion-related excitotoxity (evident from CuAg staining, Fig 5.5).

To assess whether the gene expression results of synaptic modifying components lead to a change in GLUA1 expression, we measured the density of PSD95:GLUA1 colocalized puncta. Our findings indicate that CBD modulates the expression of post-synaptic GLUA1, which is essential for synaptic plasticity and learning[156]. Specifically, we found that CBD treatment appeared to increase post-synaptic GLUA1 expression in the unlesioned hemisphere within RA but not in Area X. This may suggest that CBD has region-specific effects on AMPA receptor regulation or that the effects of CBD may depend on the specific neural circuitry involved. We also observed a significant decrease in post-synaptic GLUA1 expression of the vehicle group within RA and Area X, suggesting lesion-related deficits in AMPA receptor regulation. This deficit was not significant with CBD treatment, indicating that CBD may protect against lesion-related decreases in post-synaptic GLUA1 receptor expression. Interestingly, when we quantified post-synaptic GLUA1 expression as a percentage of the unlesioned hemisphere, we found that CBD treatment appeared to offer protection from lesion-related decreases in both RA and Area X. These findings may have important implications for the maintenance of learning-related plasticity and memory consolidation in the brain.

This work suggests that CBD promotes post-CNS damage recovery of learned vocal behavior by promoting homeostatic mechanisms, including the preservation of synaptic density and regulation of AMPA receptor subunit expression. These findings have important implications for the potential therapeutic applications of CBD in the treatment of neurological disorders associated with synaptic dysfunction, such as TBI and related conditions. Lastly, our study

provides valuable insights into the elusive mechanisms underlying the neuroprotective effects of CBD-improved vocal recovery and highlights its potential as a therapeutic agent for the treatment of many disorders.

Figure 5.1 Identification of glutamatergic synapses within HVC of lesioned and unlesioned hemispheres

CBD treatments protect glutamatergic synaptic densities from lesion-related losses within HVC. A-T, Representative confocal images of immunofluorescence illustrating synaptic density in four groups: VEH Unleisoned (a-e), CBD Unlesioned (f-j), VEH Lesioned (k-o), and CBD lesioned (p-t). Stains are divided into columns with Hoechst, PSD95 (postsynaptic marker), and VGLUT2 (presynaptic marker) respectfully. Column four is a merge of PSD95 and VGLUT2 and column 5 shows a mask of the colocalized puncta. The synapse mask shows CBD has a significant increase in post lesion glutamatergic synapse s 24 hours after the lesions.

HVC

	Hoechst	PSD95	VGLUT2	Merge	Synapse Mask
VEH Unlesioned	a.	b.	c.	d.	e.
CBD Unlesioned	f.	g.	h.	i.	j.
VEH Lesioned	k.	l.	m.	n.	o.
CBD Lesioned	p.	q.	r.	s.	t.

50 µm

Figure 5.2 Identification of glutamatergic synapses within RA of lesioned and unlesioned hemispheres

 CBD treatments protect glutamatergic synaptic densities from lesion-related losses within RA. A-T, Representative confocal images of immunofluorescence illustrating synaptic density in four groups: VEH Unleisoned (a-e), CBD Unlesioned (f-j), VEH Lesioned (k-o), and CBD Lesioned (p-t). Stain is divided into columns with Hoechst, PSD95 (postsynaptic marker), and VGLUT2 (presynaptic marker) respectfully. Column four is a merge of the two stains and column 5 shows a mask of the colocalized puncta of PSD95 and VGLUT2. The synapse mask shows CBD has a significant increase in post lesion glutamatergic synapses 24 hours after the lesions.

RA

	Hoechst	PSD95	VGLUT2	Merge	Synapse Mask
VEH Unlesioned	a.	b.	c.	d.	e.
CBD Unlesioned	f.	g.	h.	i.	j.
VEH Lesioned	k.	l.	m.	n.	o.
CBD Lesioned	p.	q.	r.	s.	t.

50 μm

Figure 5.3 Identification of glutamatergic synapses within Area X of lesioned and unlesioned hemispheres

CBD treatments protect glutamatergic synaptic densities from lesion-related losses within Area X. A-T, Representative confocal images of immunofluorescence illustrating synaptic density in four groups: VEH Unleisoned (a-e), CBD Unlesioned (f-j), VEH Lesioned (k-o), and CBD Lesioned (p-t). Stain is divided into columns with Hoechst, PSD95 (postsynaptic marker), and VGLUT2 (presynaptic marker) respectfully. Column 4 is a merge of the two stains and column 5 shows a mask of the colocalized puncta of PSD95 and VGLUT2. The synapse mask shows CBD has a significant increase in post lesion glutamatergic synapses 24 hours after the lesions.

Area X

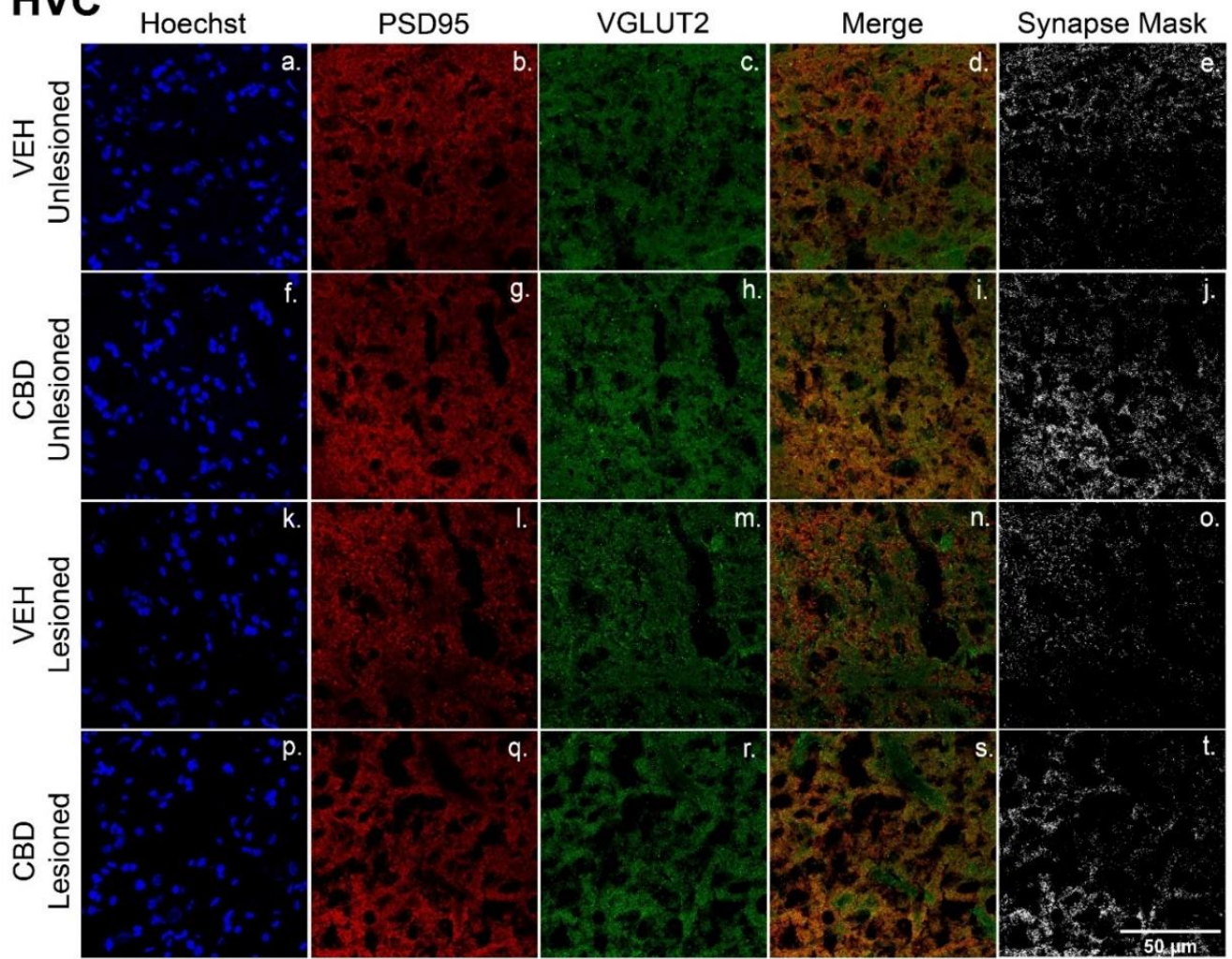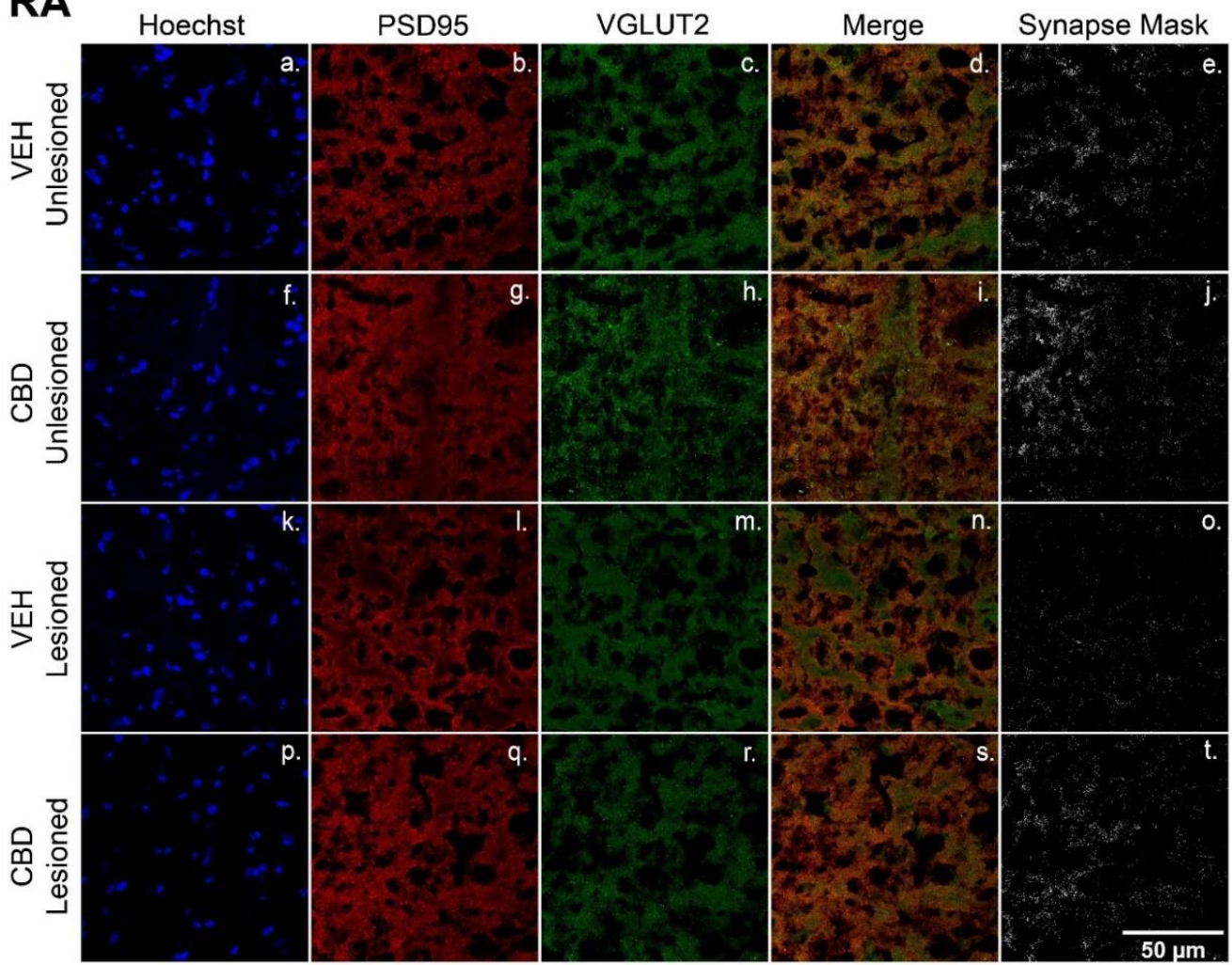|	Hoechst	PSD95	VGLUT2	Merge	Synapse Mask

VEH Unlesioned — a. b. c. d. e.

CBD Unlesioned — f. g. h. i. j.

VEH Lesioned — k. l. m. n. o.

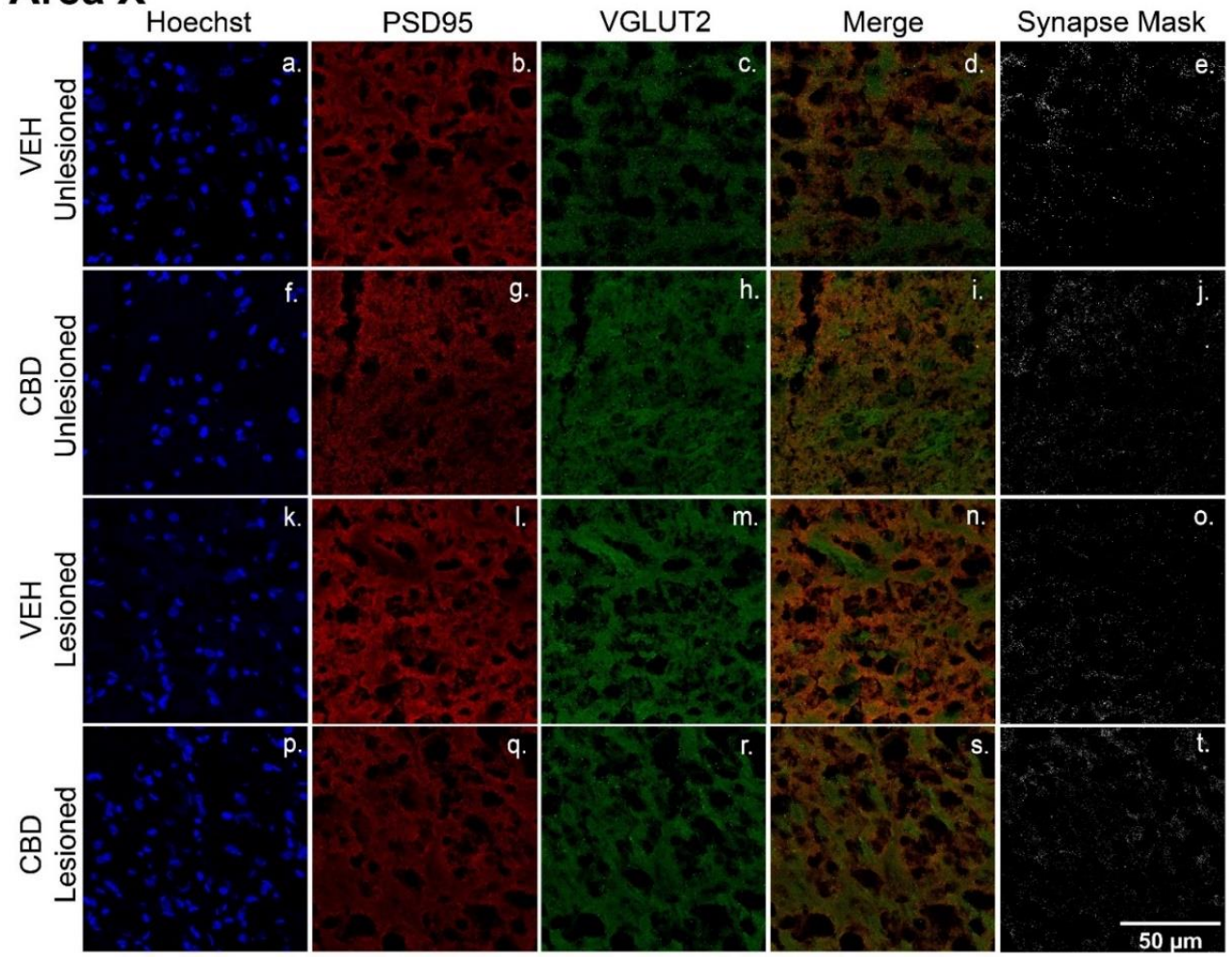**CBD Lesioned** — p. q. r. s. t.

50 µm

Figure 5.4 CBD treatments protect glutamatergic synaptic densities from lesion-related losses.

CBD treatments protect glutamatergic synaptic densities from lesion-related losses. a, Quantification of song region synaptic densities within unlesioned and lesioned hemispheres of vehicle- and CBD-treated songbirds. In the vehicle group, unilateral microlesions decreased the synaptic density in all three regions examined (HVC, RA, and Area X). Conversely, in the CBD group there was no significant lesion effects in HVC or Area X. This protection was not as robust in RA, although the deficit was decreased. CBD treatment had a profound effect on the post lesion synaptic density compared to post lesion densities of vehicle treated birds in all three regions of interest. b, Lesion related change in synaptic density expressed as colocalized puncta transformed to percentage of the unlesioned control hemisphere. Within HVC and RA, CBD groups had a significant increase in post-lesion synaptic density while Area X did not differ significantly. This indicates a significant protection of synapses in key areas of vocal production. For analysis, each z-stack image set was post-processed and projected at maximum intensity. Puncta of colocalized VGLUT-2 and PSD-95 within each region were defined for particle analysis with threshold applied from n = 5 animals per group. Glutamatergic synapse densities were then quantified as percent change from the unlesioned control hemisphere. Significance was assessed and appropriate comparisons made using mixed-models ANOVA with Sidak's correction for multiple comparisons.

a

Synaptic Density (synapses/μm²)

b

Synaptic Density (% Unlesioned Control)
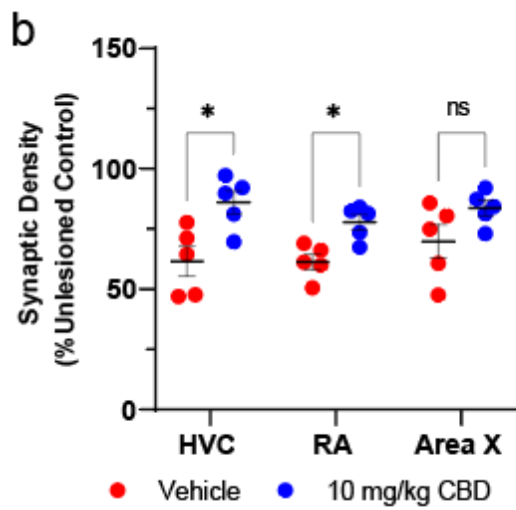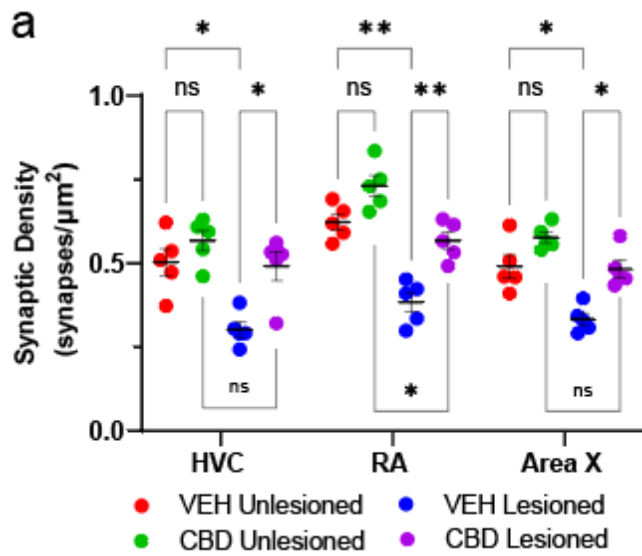
Figure 5.5 Lesion related neuronal degeneration within song regions

Amino-cupric-silver (A-Cu-Ag) staining to detect neuronal degeneration in zebra finch brain tissue 24 hours after unilateral microlesion (left is anterior, top is dorsal). a, representative image showing microlesion ablation of HVC, significant darkening of projected region RA and slight darkening of Area X. Note, there is axonal degradation from HVC to RA. b, outline of tissue with regions of interest labeled (HVC, RA, and Area X).

a

b

HVC

RA

Area X

Vocal Motor
Output

1 mm
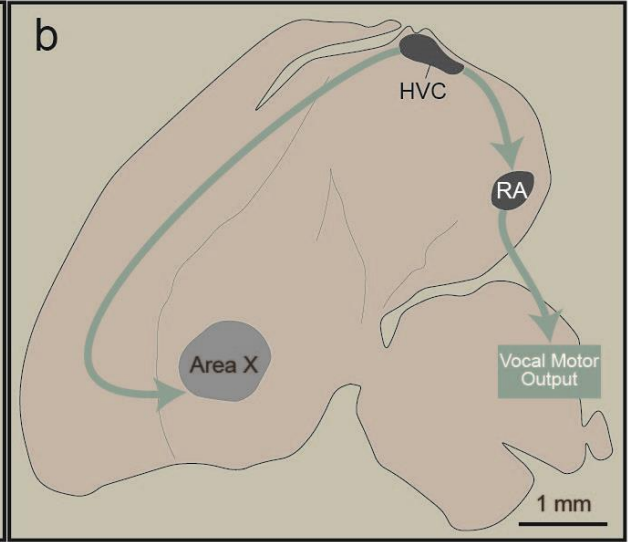
Figure 5.6 CBD influences expression of synaptic scaling regulators

CBD influences expression of synaptic scaling regulators. Y-axes = mRNA expression of BDNF, ARC/ARG3.1, and MSK1 normalized to the housekeeping control (GAPDH) and expressed as fold change from unlesioned hemispheres (2-$\Delta\Delta$CT). The cDNA samples synthesized from groups of n = 4 subjects were amplified in triplicate and means plotted. a, In RA and Area X of lesioned hemispheres, CBD significantly increased mean fold expression of BDNF relative to VEH controls. b, in HVC, RA and Area X of lesioned hemispheres CBD treatment increased mean fold expression of MSK1 over VEH. c, In RA and Area X of lesioned hemispheres, CBD suppressed ARC/ARG3.1 expression vs. VEH. Group differences were assessed by mixed-models ANOVA with Sidak's multiple comparison correction.
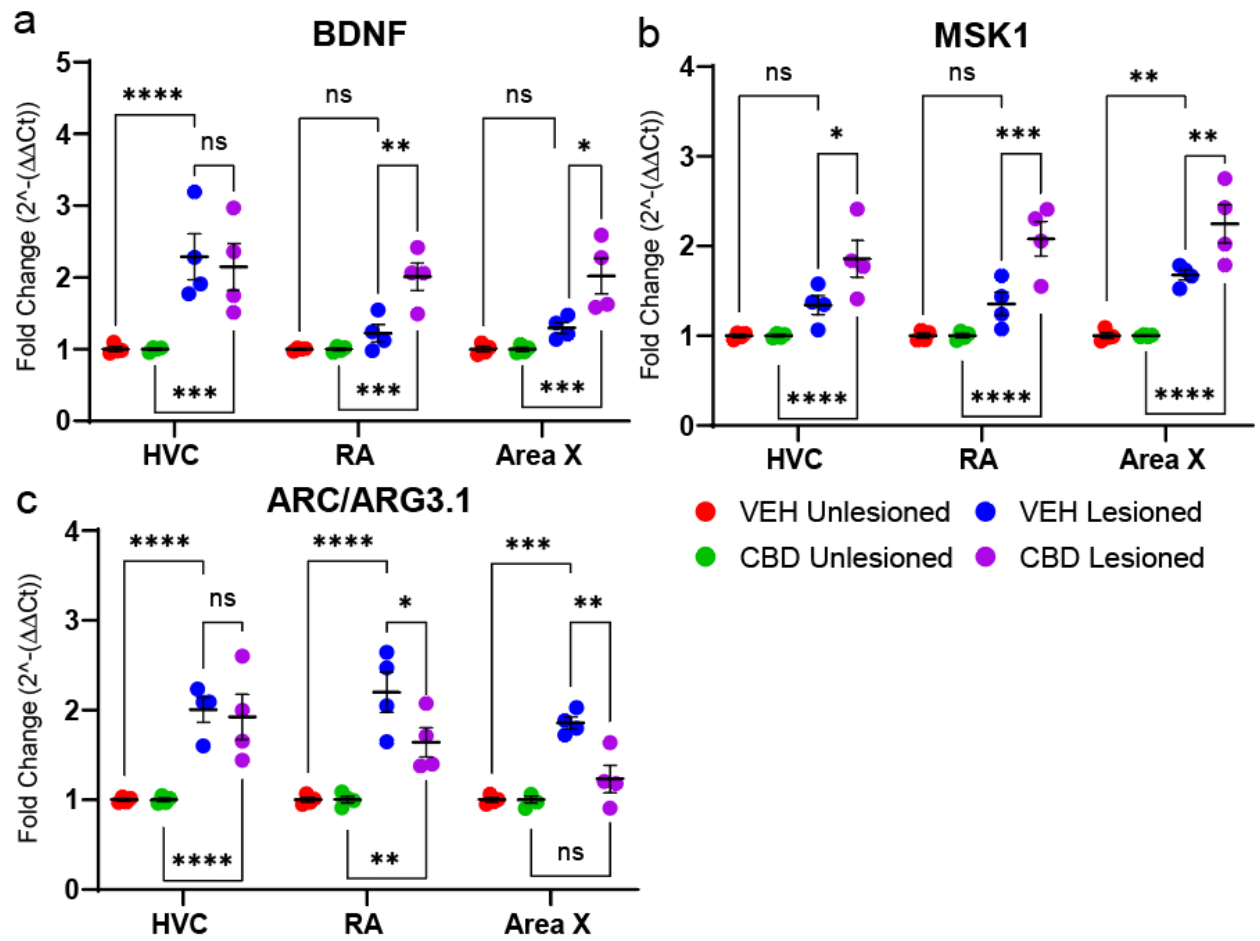
Figure 5.7 Identification of post-synaptic AMPA receptor expression within HVC of lesioned and unlesioned hemispheres

Identification of post synaptic AMPA receptor expression within HVC of lesioned and unlesioned hemispheres. A-t, Representative confocal images of immunofluorescence illustrating density of post-synaptic AMPA receptor subunit (GLUA1) in four groups: VEH Unleisoned (a-e), CBD Unlesioned (f-j), VEH Lesioned (k-o), and CBD lesioned (p-t). Stains are divided into columns with Hoechst, PSD95 (postsynaptic marker), and GLUA1 (AMPA subunit) respectfully. Column four is a merge of PSD95 and GLUA1. Column 5 shows a mask of the colocalized puncta.

HVC

Hoechst | PSD95 | GLUA1 | Merge | Post-Synaptic GLUA1 Mask

VEH Unlesioned — a. b. c. d. e.

CBD Unlesioned — f. g. h. i. j.

VEH Lesioned — k. l. m. n. o.

CBD Lesioned — p. q. r. s. t.

Figure 5.8 Identification of post-synaptic AMPA receptor expression within RA of lesioned and unlesioned hemispheres

Identification of post synaptic AMPA receptor expression within RA of lesioned and unlesioned hemispheres. A-t, Representative confocal images of immunofluorescence illustrating density of post-synaptic AMPA receptor subunit (GLUA1) in four groups: VEH Unleisoned (a-e), CBD Unlesioned (f-j), VEH Lesioned (k-o), and CBD lesioned (p-t). Stains are divided into columns with Hoechst, PSD95 (postsynaptic marker), and GLUA1 (AMPA subunit) respectfully. Column four is a merge of PSD95 and GLUA1. Column 5 shows a mask of the colocalized puncta. The synapse mask shows CBD has a significant increase in post-lesion AMPA receptor localization to the synapse 24 hours after the lesions.

RA

|	Hoechst	PSD95	GLUA1	Merge	Post-Synaptic GLUA1 Mask
VEH Unlesioned	a.	b.	c.	d.	e.
CBD Unlesioned	f.	g.	h.	i.	j.
VEH Lesioned	k.	l.	m.	n.	o.
CBD Lesioned	p.	q.	r.	s.	t.

50 μm

Figure 5.9 Identification of post-synaptic AMPA receptor expression within Area X of lesioned and unlesioned hemispheres

Identification of post synaptic AMPA receptor expression within Area X of lesioned and unlesioned hemispheres. A-t, Representative confocal images of immunofluorescence illustrating density of post-synaptic AMPA receptor subunit (GLUA1) in four groups: VEH Unleisoned (a-e), CBD Unlesioned (f-j), VEH Lesioned (k-o), and CBD lesioned (p-t). Stains are divided into columns with Hoechst, PSD95 (postsynaptic marker), and GLUA1 (AMPA subunit) respectfully. Column four is a merge of PSD95 an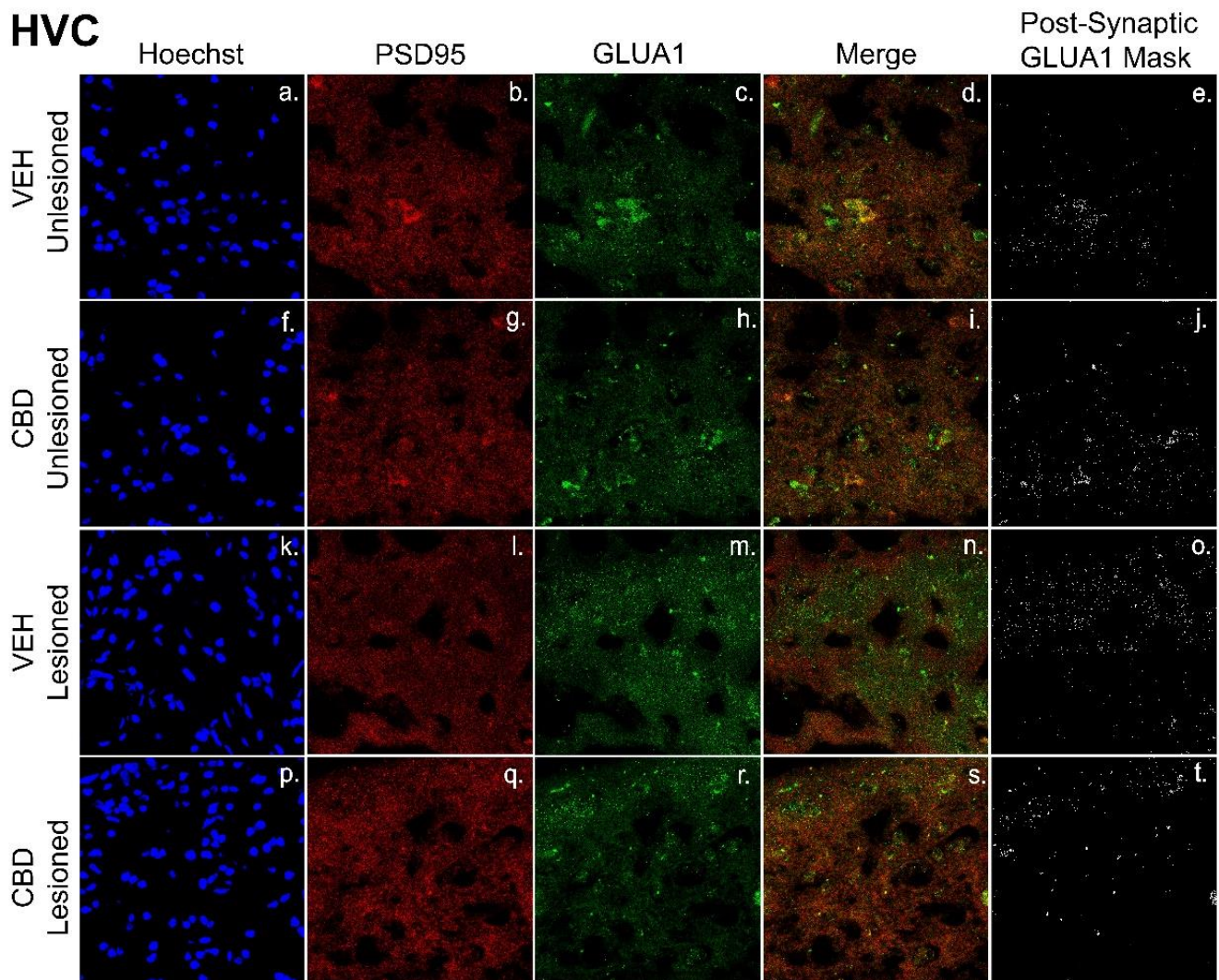d GLUA1. Column 5 shows a mask of the colocalized puncta. The synapse mask shows CBD has a significant increase in post-lesion AMPA receptor localization to the synapse 24 hours after the lesions.

Area X

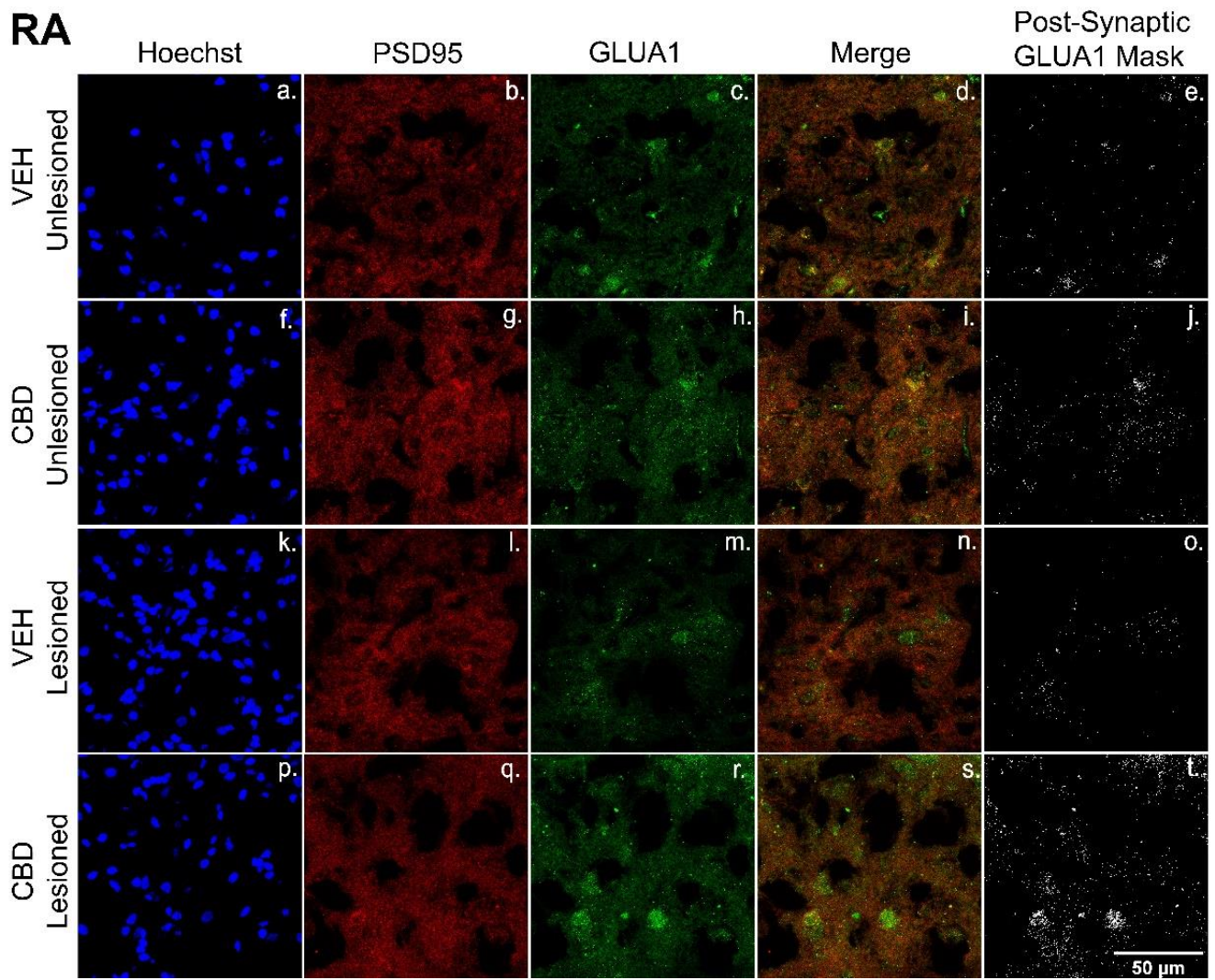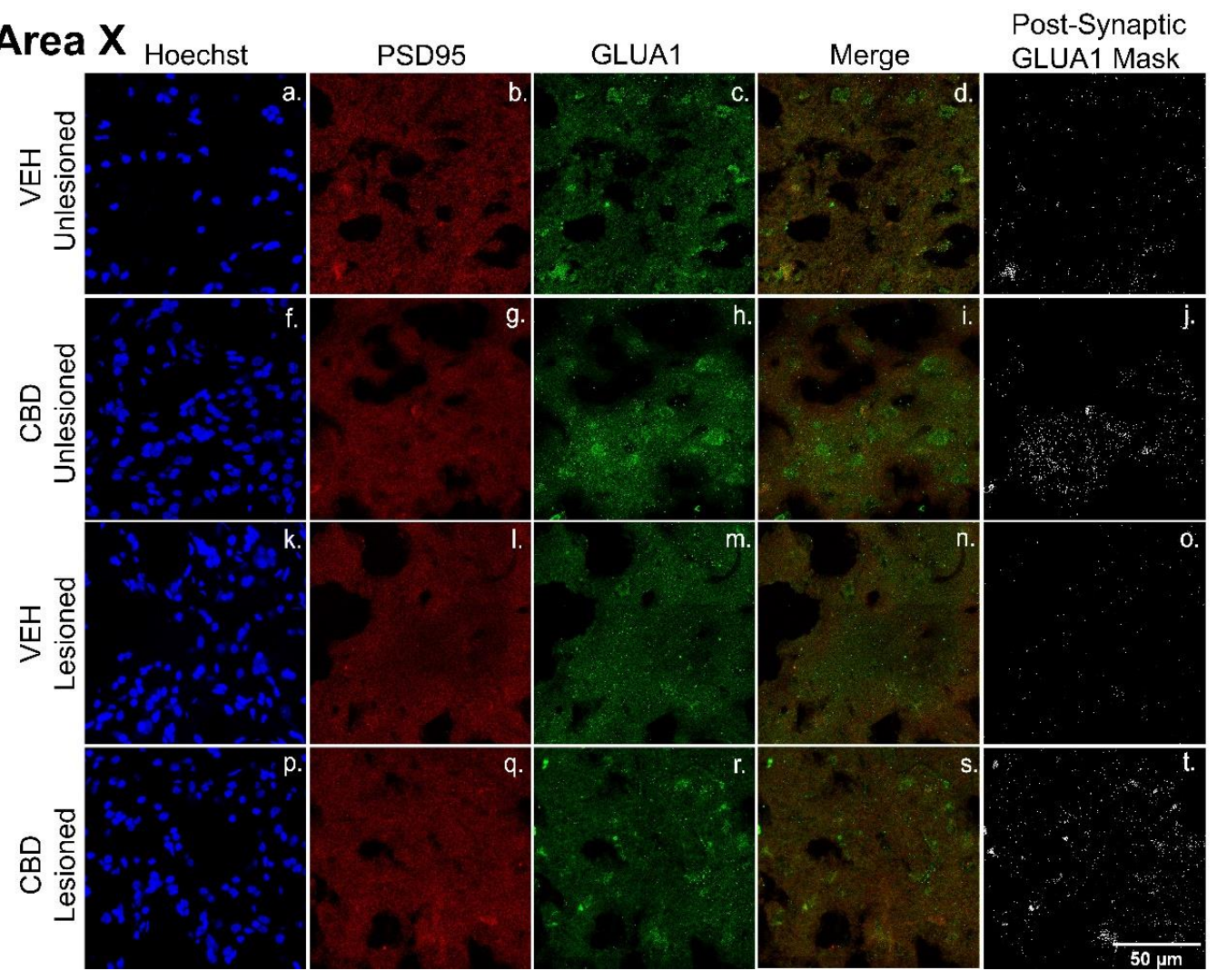| Hoechst	PSD95	GLUA1	Merge	Post-Synaptic GLUA1 Mask

VEH Unlesioned — a. b. c. d.

CBD Unlesioned — f. g. h. i. j.

VEH Lesioned — k. l. m. n. o.

CBD Lesioned — p. q. r. s. t.

50 µm

Figure 5.10 CBD treatments increase post-synaptic AMPA receptor expression

CBD treatments increase post-synaptic GLUA1 receptor expression after lesioning. a, Quantification of song region post-synaptic GLUA1 densities within unlesioned and lesioned hemispheres of vehicle- and CBD-treated songbirds. In the vehicle group, unilateral microlesions decreased the post-synaptic GLUA1 densities in two of the regions examined (RA, and Area X). Conversely, in the CBD group there were no significant lesion related changes in GLUA1 expression indicating a protection of receptor. CBD treatment had a profound effect on the post lesion receptor expression compared to post lesion densities of vehicle treated birds in RA and Area X. Note the variability in HVC, the lesioned area. b, Lesion related change in GLUA1 density expressed as colocalized puncta transformed to percentage of the unlesioned control hemisphere. Within RA and Area X, CBD groups had a significant increase in post-lesion synaptic density while HVC did not differ significantly. This indicates a significant preservation of GLUA1 in key areas of vocal learning. c-d, relative fluorescent quantification of either PSD95 or GLUA1 relative to nuclei. For analysis, each z-stack image set was post-processed and projected at maximum intensity. Puncta of colocalized GLUA1 and PSD-95 within each region were defined for particle analysis with threshold applied from $n = 3$ animals per group. Post-synaptic GLUA1 densities were then quantified as percent change from the unlesioned control hemisphere. Significance was assessed and appropriate comparisons made using mixed-models ANOVA with Sidak's correction for multiple comparisons.
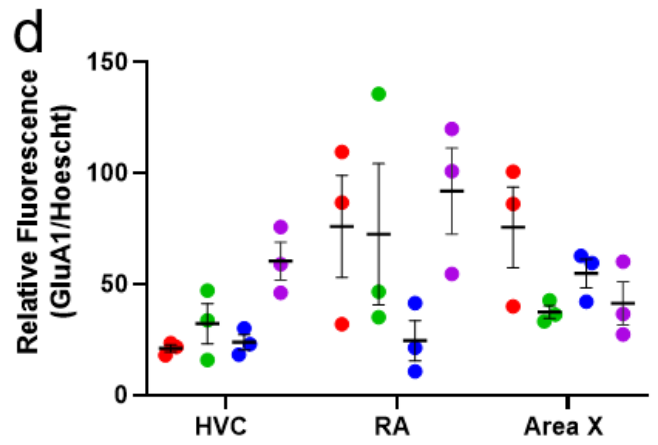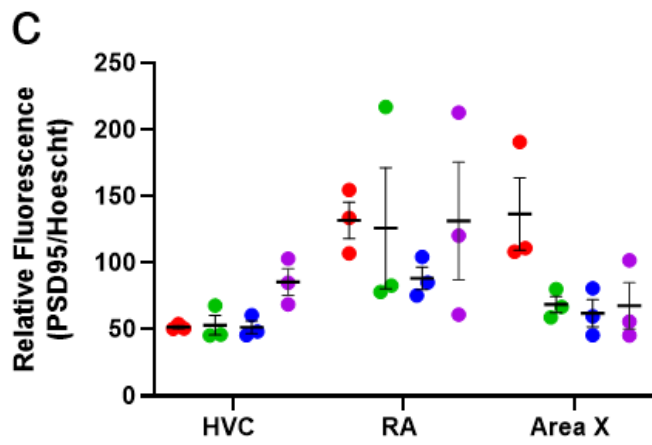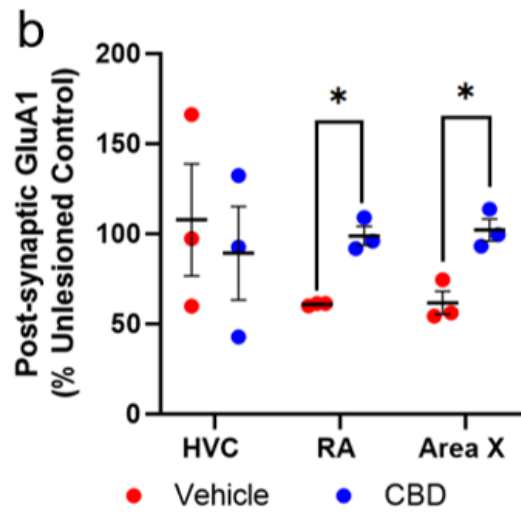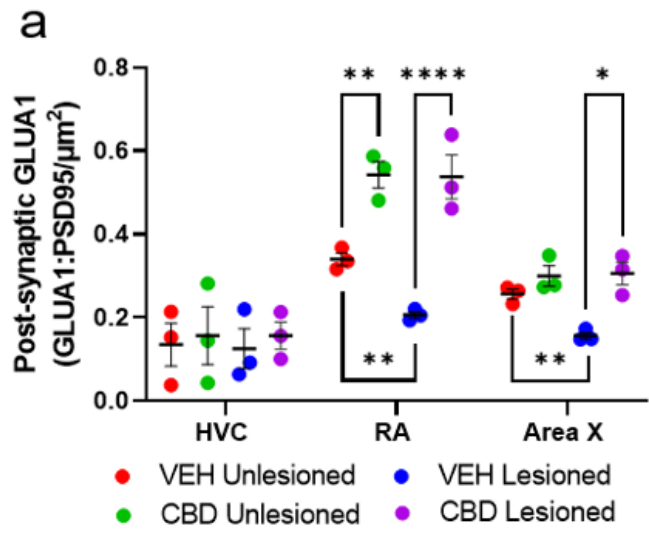
CHAPTER SIX: Summary and Concluding Statements

6.1 Summary of Findings

Vocal learning and production in songbirds have long been considered an important model system for studying the neural basis of language and speech learning. It has been demonstrated that the development of circuits linking cortical, striatal, and thalamic brain regions is critical for vocal learning and production, similar to human language. The similarities between song and speech learning also extend to lateralization properties, as has been demonstrated in humans. In this study, we investigated the lateralization properties of vocal disruption in zebra finches following a lesion to either the left or right hemisphere. Additionally, we explored the potential neuroprotective effects of cannabidiol (CBD) on lesion-disrupted vocal behavior, focusing on its anti-inflammatory and antioxidative properties. We also examined the potential involvement of microglia in CBD-induced vocal recovery and synaptic density.

6.1.1 Lateralization Properties of Vocal Disruption

Our study demonstrated that lateralization does exist in zebra finches, as a lesion to the left hemisphere showed a greater impact of vocal disruption peaking at 24hr post-lesion that lasted longer than that of a lesion to the right hemisphere. This finding is consistent with previous studies on lateralization in songbirds and adds to the growing body of evidence that the lateralization properties of song and speech learning and production are similar.

6.1.2 CBD Neuroprotection and Anti-Inflammatory Effects

Results from our study indicate that CBD neuroprotection, as seen previously using a bilateral lesion, followed powerful anti-neuroinflammatory effects, which is consistent with previous understanding of the positive efficacy of CBD in mammalian systems. The anti-

inflammatory effects appeared greatest in the microlesioned region, HVC, and to progressively lesser extents within RA and Area X. The efferent damage also seemed to coincide with proximity to the lesion, with RA having a greater modulation of inflammation, while Area X was anatomically more distal compared to RA.

6.1.3 CBD Mitigation of Oxidative Stress

We also identified a second neuroprotection-related mechanism for CBD, which included CBD mitigation of oxidative stress. This was indicated by effects on SOD2 expression within HVC and RA, as well as reduced superoxide activated DHE staining. Like cytokines, the magnitude of superoxide production varied with lesion proximity, but was significantly decreased by CBD in HVC and RA. CBD can alter oxidation through a number of mechanisms including direct and indirect pathways, so our evidence modeled a general decrease in ROS production.

6.1.4 CBD-Induced Stress Response

The combination of anti-inflammatory and anti-oxidative CBD activity suggested involvement of a higher order, organized stress response. Consistent with this is signaling controlled by NRF2, an established central regulator of redox, mitochondrial, and inflammatory mediators. The significant CBD-related increases in nuclear phospho-NRF2 observed in our system implicate this homeostatic pathway in vocal protection. The pro-inflammatory cytokine expression and increase in NRF2 translocation and activation we observe following microlesions are, in other systems, associated with microglial activation, infiltration, and phagocytosis of cellular debris. These activities may be of key importance to neuronal recovery versus apoptosis.

6.1.5 CBD and Microglia-Related Activity

Our study also investigated potential microglia-related activity following HVC microlesions and CBD-improved vocal recovery. Note that our lab did not examine astrocytes,

which are important for damage control in other models, but microglia were found to be the first step for identifying responses in this model. Our results indicate a decrease in microglia recruitment and a rounded cellular appearance of TMEM119-stained cells 24 hours post-lesioning. This structural change in appearance suggests that microlesions increase densities of microglia in an activated phagocytic state and CBD treatments reduce this. Additionally, it is important to note that microglia did not return to unlesioned or control numbers, which may indicate a persistent anti-inflammatory "M2" microglia phenotype.

6.1.6 CBD and Synaptic Density

Microglia are involved in the elimination of excess synapses leading to a refinement of the neural circuitry, which is critical for the proper functioning of the CNS. We investigated the potential protection/promotion of synaptic density by CBD, which was tested by measuring colocalization of PSD95 and VGLUT2. As expected, HVC microlesions decreased densities within the region itself and also within its projection targets. The decreased magnitude of vocal disruption seen following CBD treatments suggests potential protection of circuits established during song learning. Facilitating the establishment of new synapses may underlie CBD promotion of sensorimotor learning-dependent vocal recovery.

6.1.7 CBD and Synaptic Scaling

A mechanism by which CBD may protect excitatory synapses is through modulating synaptic scaling, which is regulated by a complex network of proteins and signaling pathways, including BDNF, MSK1, and Arc/Arg3.1. Arc/Arg3.1 activity increases internalization of excitatory AMPA receptors, decreasing and scaling-down excitatory synaptic strength. We found that 24 hours after lesioning, CBD treatment significantly regulated the expression of Arc/Arg3.1,

potentially protecting against excitotoxicity. However, patterns of AMPA receptor expression are important for maintenance of song circuits established during vocal learning, and increased Arc/Arg3.1 may result in the vocal disruption observed in vehicle-treated birds. The decreased magnitudes of vocal disruption observed in CBD-treated birds may be due to reduced synaptic scaling following lesion-related excitotoxicity.

6.1.8 CBD and GLUA1 Subunit Expression

To assess whether the gene expression results of synaptic modifying components lead to a change in GLUA1 subunit expression, we measured the density of PSD95 and GLUA1 colocalized puncta. We found that CBD treatment appeared to increase post-synaptic GLUA1 expression in the unlesioned hemisphere within RA but not in Area X. This may suggest two things: (1) CBD has region-specific effects on AMPA receptor regulation; (2) The effects of CBD may depend on the timing and proximity of neural circuitry involved. We also observed a significant decrease in post-synaptic GLUA1 expression of the vehicle group within RA and Area X, suggesting lesion-related deficits in AMPA receptor potentiation. This deficit was not significant with CBD treatment, indicating that CBD may protect against lesion-related decreases in post-synaptic GLUA1 receptor internalization. Furthermore, lesion related GLUA1 deficits were blunted with CBD treatment and in some cases expression increased. Taken together these results confirm lesion-related decrease in GLUA1 expression in vehicle treated groups that was maintained with CBD treatment, and a decrease in GLUA1 internalization consistent with our synaptic scaling gene expression.

6.1.9 Concluding statements

In conclusion, our study adds to the growing body of evidence that the lateralization properties of song and speech learning are similar. We demonstrated that a lesion to the left hemisphere had a greater impact on vocal disruption in zebra finches than a lesion to the right hemisphere. We also identified potential neuroprotective effects of CBD, including its anti-inflammatory and antioxidative properties, and its ability to modulate microglia-related activity. CBD may also promote the preservation of synaptic densities and protect against excitotoxicity through the modulation of synaptic scaling. Our findings suggest that CBD has potential as a therapeutic agent for the treatment of neural damage-related disorders, but caution is advised with the growing industry and unregulated over the counter products containing trace compounds that may differentially affect the efficacy of CBD. Further studies are needed to explore this mechanism of CBD-induced vocal recovery as this research serves as a foundation for the neuroplasticity related neuroprotection that CBD displays.

6.2 Future research

As research into the therapeutic properties of CBD continues to expand, there is growing interest in its potential for protecting against neurodegeneration following TBI, as current treatments are associated with adverse reactions. While previous studies have demonstrated CBD's ability to promote the homeostatic cellular process of autophagy[165], which is key for removing cellular debris and promoting repair, it remains unclear whether CBD can protect circuits underlying learned behavior. Therefore, future research should investigate the role of autophagy in CBD-mediated neuroprotection following TBI-induced neuroinflammation by examining the expression and distribution of LC3, a key marker of autophagy, in immunofluorescence assays.

Autophagy processes may help define the synaptic protection illustrated above, as main components of neuronal remodeling are degraded through autophagy[166].

In addition to investigating the role of autophagy in CBD-mediated neuroprotection following TBI-induced neuroinflammation, future research should also aim to determine whether CBD can drive populations of microglial subtypes towards anti-inflammatory (M2) activation. Our results indicate that treatment does not completely ameliorate TMEM119 levels which might identify an anti-inflammatory component to the glial population. We also have demonstrated that CBD treatment is associated with an upregulation of IL-10 expression, which is known to promote activation of anti-inflammatory (M2 subtype) microglia. This confirms previous work showing CBD mitigates experimental autoimmune encephalomyelitis (EAE)[167]. Although this was a combination THC:CBD drug effect, isolated CBD compounds show similar activity[168], [169]. Relative populations of M1 and M2 microglial subtypes following CBD treatment should be assessed and will identify the role of M2 anti-inflammatory microglia in neuroprotection, rather than the idea of M1 subtype inhibition, which has until recently been a therapeutic focus. However, it is important to note literature on microglia and their phenotypic changes are growing and changing exponentially. M1 and M2 are classical designations of their polarization states, but it is becoming clear that there is a spectrum of phenotypic changes. The M1 and M2 criteria are mainly indicative of structure and have been used as an oversimplification of the functional characteristics of these glia cells[170], [171]. It appears functional characteristics are a more reliable was to identify the population of microglia, and some markers may not be as reliable as previously thought. For example, Ionized calcium binding adaptor molecule 1 (IBA1), first described in 1998 and ubiquitously used throughout the scientific community, does not discern differences in

functional capabilities of microglia such as surveillance, synaptic pruning, phagocytosis, of antigen presentation[170].

Future research may also look more closely at subtypes of AMPA receptors such as GLUA2 and identify dimerization of GLUA1 and A2. Due to this work showing CBD alters expression of BDNF, MSK1, and Arc/Arg3.1, there may be regulation of AMPA receptor distribution that goes further than the subtype we have addressed. To identify differences in the subtypes and to confirm a post synaptic presence, super-resolution STORM immunofluorescence imaging techniques should be used. This will offer greater insight into the relative synaptic membrane and internalized receptor densities. It would be expected to demonstrate that CBD will have effects on reducing AMPA receptor internalization (similar to these findings) within motor cortical RA and may increase synaptic densities within Area X.

While our previous discussions have focused primarily on the role of microglia in TBI-induced neuroinflammation and the potential neuroprotective effects of CBD, it is important to note that astrocytes also play a critical role in the inflammatory response following TBI and stroke[125], [142]. Astrocytes are key regulators of the blood-brain barrier and are involved in a wide range of neuroprotective functions, including the modulation of inflammation and regulation of synaptic plasticity[172]. Therefore, in future research, the effects of CBD on astrocyte activation and function following TBI-induced neuroinflammation should be evaluated. Specifically, it is important to examine changes in astrocyte morphology, gene expression, and signaling pathways following CBD treatment, and determine the extent to which CBD-mediated neuroprotection involves modulation of astrocyte function. By elucidating the role of astrocytes in TBI-induced neuroinflammation and the potential neuroprotective effects of CBD on these cells, this could lead

to the development of novel therapeutic strategies that target multiple cell types involved in the inflammatory response following TBI.

Lastly, it is important for future research to determine the minimally effective CBD treatment regimen for neuroprotection following TBI-induced neuroinflammation (HVC microlesions). While our microlesion model was established using a six-day pre-treatment regimen intended to produce steady-state CBD (lipophilic drug with large volume of distribution and long elimination half-life) levels prior to microlesions, we have yet to determine if neuroprotection depends upon pre-lesion CBD loading. Given that treatments are typically given post-injury in translational settings, it is crucial to determine the shortest effective CBD treatment regimen, with a focus on post-lesion dosing. To this end, it is important to conduct a series of experiments varying the duration and timing of CBD treatment to determine the optimal treatment regimen for neuroprotection. This could have important implications for the clinical use of CBD in the treatment of TBI-induced neurodegeneration, and could help to guide the development of novel therapeutic strategies for this devastating condition.

References

[1]   P. Morales, D. P. Hurst, and P. H. Reggio, "Molecular Targets of the Phytocannabinoids-A Complex Picture," *Prog Chem Org Nat Prod*, vol. 103, pp. 103–131, 2017, doi: 10.1007/978-3-319-45541-9_4.

[2]   S. Burstein, "Cannabidiol (CBD) and its analogs: a review of their effects on inflammation," *Bioorg Med Chem*, vol. 23, no. 7, pp. 1377–1385, Apr. 2015, doi: 10.1016/j.bmc.2015.01.059.

[3]   R. G. Pertwee, "The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: Δ9-tetrahydrocannabinol, cannabidiol and Δ9-tetrahydrocannabivarin," *Br J Pharmacol*, vol. 153, no. 2, pp. 199–215, Jan. 2008, doi: 10.1038/sj.bjp.0707442.

[4]   J.-Y. Xu and C. Chen, "Endocannabinoids in Synaptic Plasticity and Neuroprotection," *Neuroscientist*, vol. 21, no. 2, pp. 152–168, Apr. 2015, doi: 10.1177/1073858414524632.

[5]   K. Whalley, "Balancing LTP and LTD," *Nat Rev Neurosci*, vol. 8, no. 4, Art. no. 4, Apr. 2007, doi: 10.1038/nrn2123.

[6]   G. Terral, M. Varilh, A. Cannich, F. Massa, G. Ferreira, and G. Marsicano, "Synaptic Functions of Type-1 Cannabinoid Receptors in Inhibitory Circuits of the Anterior Piriform Cortex," *Neuroscience*, vol. 433, pp. 121–131, May 2020, doi: 10.1016/j.neuroscience.2020.03.002.

[7]   D. M. Kerr, L. Downey, M. Conboy, D. P. Finn, and M. Roche, "Alterations in the endocannabinoid system in the rat valproic acid model of autism," *Behavioural Brain Research*, vol. 249, pp. 124–132, Jul. 2013, doi: 10.1016/j.bbr.2013.04.043.

[8]   B. Pan, W. Wang, P. Zhong, J. L. Blankman, B. F. Cravatt, and Q. Liu, "Alterations of Endocannabinoid Signaling, Synaptic Plasticity, Learning, and Memory in Monoacylglycerol Lipase Knock-out Mice," *J Neurosci*, vol. 31, no. 38, pp. 13420–13430, Sep. 2011, doi: 10.1523/JNEUROSCI.2075-11.2011.

[9]   L. Karila *et al.*, "Acute and long-term effects of cannabis use: a review," *Curr Pharm Des*, vol. 20, no. 25, pp. 4112–4118, 2014, doi: 10.2174/13816128113199990620.

[10]   S. Ikemoto, "Brain reward circuitry beyond the mesolimbic dopamine system: A neurobiological theory," *Neurosci Biobehav Rev*, vol. 35, no. 2, pp. 129–150, Nov. 2010, doi: 10.1016/j.neubiorev.2010.02.001.

[11] C. H. Ashton, "Pharmacology and effects of cannabis: A brief review," *The British Journal of Psychiatry*, vol. 178, no. 2, pp. 101–106, Feb. 2001, doi: 10.1192/bjp.178.2.101.

[12] O. Devinsky *et al.*, "Trial of Cannabidiol for Drug-Resistant Seizures in the Dravet Syndrome," *N Engl J Med*, vol. 376, no. 21, pp. 2011–2020, May 2017, doi: 10.1056/NEJMoa1611618.

[13] H. Meissner and M. Cascella, "Cannabidiol (CBD)," in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2022. Accessed: May 06, 2022. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK556048/

[14] K. Soderstrom, E. Soliman, and R. Van Dross, "Cannabinoids Modulate Neuronal Activity and Cancer by CB1 and CB2 Receptor-Independent Mechanisms," *Front Pharmacol*, vol. 8, p. 720, 2017, doi: 10.3389/fphar.2017.00720.

[15] C. Ibeas Bih, T. Chen, A. V. W. Nunn, M. Bazelot, M. Dallas, and B. J. Whalley, "Molecular Targets of Cannabidiol in Neurological Disorders," *Neurotherapeutics*, vol. 12, no. 4, pp. 699–730, Oct. 2015, doi: 10.1007/s13311-015-0377-3.

[16] T. Ohtsuki, J. B. Friesen, S.-N. Chen, J. B. McAlpine, and G. F. Pauli, "Selective Preparation and High Dynamic-Range Analysis of Cannabinoids in 'CBD Oil' and Other Cannabis sativa Preparations," *J Nat Prod*, vol. 85, no. 3, pp. 634–646, Mar. 2022, doi: 10.1021/acs.jnatprod.1c00976.

[17] R. M. Hodges *et al.*, "Δ9-Tetrahydrocannabinol Differentially Alters Cannabidiol Efficacy in Recovery of Phonology and Syntax Following Damage to a Songbird Cortical-Like Brain Region," *Cannabis Cannabinoid Res*, Sep. 2022, doi: 10.1089/can.2022.0073.

[18] S. Giacoppo, P. Bramanti, and E. Mazzon, "Sativex in the management of multiple sclerosis-related spasticity: An overview of the last decade of clinical evaluation," *Multiple Sclerosis and Related Disorders*, vol. 17, pp. 22–31, Oct. 2017, doi: 10.1016/j.msard.2017.06.015.

[19] E. Russo, "Cannabinoids in the management of difficult to treat pain," *TCRM*, vol. Volume 4, pp. 245–259, Feb. 2008, doi: 10.2147/TCRM.S1928.

[20] R. M. Langford *et al.*, "A double-blind, randomized, placebo-controlled, parallel-group study of THC/CBD oromucosal spray in combination with the existing treatment regimen,

in the relief of central neuropathic pain in patients with multiple sclerosis," *J Neurol*, vol. 260, no. 4, pp. 984–997, Apr. 2013, doi: 10.1007/s00415-012-6739-4.

[21]   J. R. Johnson, M. Burnell-Nugent, D. Lossignol, E. D. Ganae-Motan, R. Potts, and M. T. Fallon, "Multicenter, Double-Blind, Randomized, Placebo-Controlled, Parallel-Group Study of the Efficacy, Safety, and Tolerability of THC:CBD Extract and THC Extract in Patients with Intractable Cancer-Related Pain," *Journal of Pain and Symptom Management*, vol. 39, no. 2, pp. 167–179, Feb. 2010, doi: 10.1016/j.jpainsymman.2009.06.008.

[22]   D. R. Blake, P. Robson, M. Ho, R. W. Jubb, and C. S. McCabe, "Preliminary assessment of the efficacy, tolerability and safety of a cannabis-based medicine (Sativex) in the treatment of pain caused by rheumatoid arthritis," *Rheumatology (Oxford)*, vol. 45, no. 1, pp. 50–52, Jan. 2006, doi: 10.1093/rheumatology/kei183.

[23]   E. B. Russo, "Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects," *Br J Pharmacol*, vol. 163, no. 7, pp. 1344–1364, Aug. 2011, doi: 10.1111/j.1476-5381.2011.01238.x.

[24]   D. Ryan, A. J. Drysdale, R. G. Pertwee, and B. Platt, "Differential effects of cannabis extracts and pure plant cannabinoids on hippocampal neurones and glia," *Neuroscience Letters*, vol. 408, no. 3, pp. 236–241, Nov. 2006, doi: 10.1016/j.neulet.2006.09.008.

[25]   R. A. Gray and B. J. Whalley, "The proposed mechanisms of action of CBD in epilepsy," *Epileptic Disord*, vol. 22, no. S1, pp. 10–15, Jan. 2020, doi: 10.1684/epd.2020.1135.

[26]   A. C. Campos, M. V. Fogaça, A. B. Sonego, and F. S. Guimarães, "Cannabidiol, neuroprotection and neuropsychiatric disorders," *Pharmacol Res*, vol. 112, pp. 119–127, Oct. 2016, doi: 10.1016/j.phrs.2016.01.033.

[27]   M. Ceprián, L. Jiménez-Sánchez, C. Vargas, L. Barata, W. Hind, and J. Martínez-Orgado, "Cannabidiol reduces brain damage and improves functional recovery in a neonatal rat model of arterial ischemic stroke," *Neuropharmacology*, vol. 116, pp. 151–159, Apr. 2017, doi: 10.1016/j.neuropharm.2016.12.017.

[28]   S. Atalay, I. Jarocka-Karpowicz, and E. Skrzydlewska, "Antioxidative and Anti-Inflammatory Properties of Cannabidiol," *Antioxidants (Basel)*, vol. 9, no. 1, p. 21, Dec. 2019, doi: 10.3390/antiox9010021.

[29]   A. Alalawi, J. C. Dodu, M. Woolley-Roberts, J. Brodie, V. Di Marzo, and K. Soderstrom, "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," *Neuropharmacology*, vol. 158, p. 107716, Nov. 2019, doi: 10.1016/j.neuropharm.2019.107716.

[30] J. Fernández-Ruiz, M. A. Moro, and J. Martínez-Orgado, "Cannabinoids in Neurodegenerative Disorders and Stroke/Brain Trauma: From Preclinical Models to Clinical Applications," *Neurotherapeutics*, vol. 12, no. 4, pp. 793–806, Oct. 2015, doi: 10.1007/s13311-015-0381-7.

[31] M. E. Lynch, P. Cesar-Rittenberg, and A. G. Hohmann, "A Double-Blind, Placebo-Controlled, Crossover Pilot Trial With Extension Using an Oral Mucosal Cannabinoid Extract for Treatment of Chemotherapy-Induced Neuropathic Pain," *Journal of Pain and Symptom Management*, vol. 47, no. 1, pp. 166–173, Jan. 2014, doi: 10.1016/j.jpainsymman.2013.02.018.

[32] R. J. Boyd, D. Avramopoulos, L. L. Jantzie, and A. S. McCallion, "Neuroinflammation represents a common theme amongst genetic and environmental risk factors for Alzheimer and Parkinson diseases," *J Neuroinflammation*, vol. 19, no. 1, p. 223, Sep. 2022, doi: 10.1186/s12974-022-02584-x.

[33] B. N. Dugger and D. W. Dickson, "Pathology of Neurodegenerative Diseases," *Cold Spring Harb Perspect Biol*, vol. 9, no. 7, p. a028035, Jul. 2017, doi: 10.1101/cshperspect.a028035.

[34] C. V. A. Toscano, L. Barros, A. B. Lima, T. Nunes, H. M. Carvalho, and J. M. Gaspar, "Neuroinflammation in autism spectrum disorders: Exercise as a 'pharmacological' tool," *Neuroscience & Biobehavioral Reviews*, vol. 129, pp. 63–74, Oct. 2021, doi: 10.1016/j.neubiorev.2021.07.023.

[35] O. Devinsky *et al.*, "Cannabidiol: Pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders," *Epilepsia*, vol. 55, no. 6, pp. 791–802, Jun. 2014, doi: 10.1111/epi.12631.

[36] R. B. Laprairie, A. M. Bagher, M. E. M. Kelly, and E. M. Denovan-Wright, "Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor," *Br J Pharmacol*, vol. 172, no. 20, pp. 4790–4805, Oct. 2015, doi: 10.1111/bph.13250.

[37] H. Chung, A. Fierro, and C. D. Pessoa-Mahana, "Cannabidiol binding and negative allosteric modulation at the cannabinoid type 1 receptor in the presence of delta-9-tetrahydrocannabinol: An In Silico study," *PLOS ONE*, vol. 14, no. 7, p. e0220025, Jul. 2019, doi: 10.1371/journal.pone.0220025.

[38] S. Bonaccorso, A. Ricciardi, C. Zangani, S. Chiappini, and F. Schifano, "Cannabidiol (CBD) use in psychiatric disorders: A systematic review," *NeuroToxicology*, vol. 74, pp. 282–298, Sep. 2019, doi: 10.1016/j.neuro.2019.08.002.

[39] C. J. Fowler, "Transport of endocannabinoids across the plasma membrane and within the cell," *FEBS J*, vol. 280, no. 9, pp. 1895–1904, May 2013, doi: 10.1111/febs.12212.

[40] D. G. Deutsch, "A Personal Retrospective: Elevating Anandamide (AEA) by Targeting Fatty Acid Amide Hydrolase (FAAH) and the Fatty Acid Binding Proteins (FABPs)," *Frontiers in Pharmacology*, vol. 7, 2016, Accessed: Feb. 21, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fphar.2016.00370

[41] M. W. Elmes *et al.*, "Fatty Acid-binding Proteins (FABPs) Are Intracellular Carriers for Δ9-Tetrahydrocannabinol (THC) and Cannabidiol (CBD)," *J Biol Chem*, vol. 290, no. 14, pp. 8711–8721, Apr. 2015, doi: 10.1074/jbc.M114.618447.

[42] T. Bisogno *et al.*, "Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide," *British Journal of Pharmacology*, vol. 134, no. 4, pp. 845–852, 2001, doi: 10.1038/sj.bjp.0704327.

[43] D. L. de Almeida and L. A. Devi, "Diversity of molecular targets and signaling pathways for CBD," *Pharmacology Research & Perspectives*, vol. 8, no. 6, p. e00682, 2020, doi: 10.1002/prp2.682.

[44] M. P. Baggelaar, M. Maccarrone, and M. van der Stelt, "2-Arachidonoylglycerol: A signaling lipid with manifold actions in the brain," *Progress in Lipid Research*, vol. 71, pp. 1–17, Jul. 2018, doi: 10.1016/j.plipres.2018.05.002.

[45] F. M. Leweke *et al.*, "Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia," *Transl Psychiatry*, vol. 2, no. 3, p. e94, Mar. 2012, doi: 10.1038/tp.2012.15.

[46] L. R. Vilela *et al.*, "Anticonvulsant effect of cannabidiol in the pentylenetetrazole model: Pharmacological mechanisms, electroencephalographic profile, and brain cytokine levels," *Epilepsy Behav*, vol. 75, pp. 29–35, Oct. 2017, doi: 10.1016/j.yebeh.2017.07.014.

[47] E. B. Russo, A. Burnett, B. Hall, and K. K. Parker, "Agonistic Properties of Cannabidiol at 5-HT1a Receptors," *Neurochem Res*, vol. 30, no. 8, pp. 1037–1043, Aug. 2005, doi: 10.1007/s11064-005-6978-1.

[48] A. C. Campos and F. S. Guimarães, "Involvement of 5HT1A receptors in the anxiolytic-like effects of cannabidiol injected into the dorsolateral periaqueductal gray of rats," *Psychopharmacology*, vol. 199, no. 2, pp. 223–230, Aug. 2008, doi: 10.1007/s00213-008-1168-x.

[49] K. K. Ryan, B. Li, B. E. Grayson, E. K. Matter, S. C. Woods, and R. J. Seeley, "A role for central nervous system PPAR-γ in the regulation of energy balance," *Nat Med*, vol. 17, no. 5, pp. 623–626, May 2011, doi: 10.1038/nm.2349.

[50] H. T. Philpott, M. O'Brien, and J. J. McDougall, "Attenuation of early phase inflammation by cannabidiol prevents pain and nerve damage in rat osteoarthritis," *PAIN*, vol. 158, no. 12, p. 2442, Dec. 2017, doi: 10.1097/j.pain.0000000000001052.

[51] A. J. Hampson, M. Grimaldi, M. Lolic, D. Wink, R. Rosenthal, and J. Axelrod, "Neuroprotective Antioxidants from Marijuanaa," *Annals of the New York Academy of Sciences*, vol. 899, no. 1, pp. 274–282, 2000, doi: 10.1111/j.1749-6632.2000.tb06193.x.

[52] G. Esposito *et al.*, "Cannabidiol Reduces Aβ-Induced Neuroinflammation and Promotes Hippocampal Neurogenesis through PPARγ Involvement," *PLOS ONE*, vol. 6, no. 12, p. e28668, Dec. 2011, doi: 10.1371/journal.pone.0028668.

[53] G. W. Booz, "Cannabidiol as an emergent therapeutic strategy for lessening the impact of inflammation on oxidative stress," *Free Radic Biol Med*, vol. 51, no. 5, pp. 1054–1061, Sep. 2011, doi: 10.1016/j.freeradbiomed.2011.01.007.

[54] S. Sermet, J. Li, A. Bach, R. B. Crawford, and N. E. Kaminski, "Cannabidiol selectively modulates interleukin (IL)-1β and IL-6 production in toll-like receptor activated human peripheral blood monocytes," *Toxicology*, vol. 464, p. 153016, Dec. 2021, doi: 10.1016/j.tox.2021.153016.

[55] D. D. Filippis *et al.*, "Cannabidiol Reduces Intestinal Inflammation through the Control of Neuroimmune Axis," *PLOS ONE*, vol. 6, no. 12, p. e28159, Dec. 2011, doi: 10.1371/journal.pone.0028159.

[56] D. S. A. Simpson and P. L. Oliver, "ROS Generation in Microglia: Understanding Oxidative Stress and Inflammation in Neurodegenerative Disease," *Antioxidants (Basel)*, vol. 9, no. 8, p. 743, Aug. 2020, doi: 10.3390/antiox9080743.

[57] L. M. Achzet, C. J. Davison, M. Shea, I. Sturgeon, and D. A. Jackson, "Oxidative Stress Underlies the Ischemia/Reperfusion-Induced Internalization and Degradation of AMPA Receptors," *Int J Mol Sci*, vol. 22, no. 2, p. 717, Jan. 2021, doi: 10.3390/ijms22020717.

[58] R. S. Borges *et al.*, "Understanding the Molecular Aspects of Tetrahydrocannabinol and Cannabidiol as Antioxidants," *Molecules*, vol. 18, no. 10, pp. 12663–12674, Oct. 2013, doi: 10.3390/molecules181012663.

[59] H. Pan *et al.*, "Cannabidiol Attenuates Cisplatin-Induced Nephrotoxicity by Decreasing Oxidative/Nitrosative Stress, Inflammation, and Cell Death," *J Pharmacol Exp Ther*, vol. 328, no. 3, pp. 708–714, Mar. 2009, doi: 10.1124/jpet.108.147181.

[60] C. Hamelink, A. Hampson, D. A. Wink, L. E. Eiden, and R. L. Eskay, "Comparison of Cannabidiol, Antioxidants, and Diuretics in Reversing Binge Ethanol-Induced Neurotoxicity," *J Pharmacol Exp Ther*, vol. 314, no. 2, pp. 780–788, Aug. 2005, doi: 10.1124/jpet.105.085779.

[61] "Fenton Reaction - an overview | ScienceDirect Topics." https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/fenton-reaction (accessed Feb. 22, 2023).

[62] T. Iuvone, G. Esposito, R. Esposito, R. Santamaria, M. Di Rosa, and A. A. Izzo, "Neuroprotective effect of cannabidiol, a non-psychoactive component from Cannabis sativa, on beta-amyloid-induced toxicity in PC12 cells," *J Neurochem*, vol. 89, no. 1, pp. 134–141, Apr. 2004, doi: 10.1111/j.1471-4159.2003.02327.x.

[63] A. A. Fouad, W. H. Albuali, A. S. Al-Mulhim, and I. Jresat, "Cardioprotective effect of cannabidiol in rats exposed to doxorubicin toxicity," *Environ Toxicol Pharmacol*, vol. 36, no. 2, pp. 347–357, Sep. 2013, doi: 10.1016/j.etap.2013.04.018.

[64] A. J. Hampson, M. Grimaldi, J. Axelrod, and D. Wink, "Cannabidiol and (−)Δ9-tetrahydrocannabinol are neuroprotective antioxidants," *Proc Natl Acad Sci U S A*, vol. 95, no. 14, pp. 8268–8273, Jul. 1998.

[65] K. Iffland and F. Grotenhermen, "An Update on Safety and Side Effects of Cannabidiol: A Review of Clinical Data and Relevant Animal Studies," *Cannabis Cannabinoid Res*, vol. 2, no. 1, pp. 139–154, 2017, doi: 10.1089/can.2016.0034.

[66] L. Hornyák *et al.*, "The Role of Indoleamine-2,3-Dioxygenase in Cancer Development, Diagnostics, and Therapy," *Frontiers in Immunology*, vol. 9, 2018, Accessed: Feb. 22, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fimmu.2018.00151

[67] S. Vomund, A. Schäfer, M. J. Parnham, B. Brüne, and A. von Knethen, "Nrf2, the Master Regulator of Anti-Oxidative Responses," *Int J Mol Sci*, vol. 18, no. 12, p. 2772, Dec. 2017, doi: 10.3390/ijms18122772.

[68] M. J. Goodfellow, A. Borcar, J. L. Proctor, T. Greco, R. E. Rosenthal, and G. Fiskum, "Transcriptional activation of antioxidant gene expression by Nrf2 protects against mitochondrial dysfunction and neuronal death associated with acute and chronic neurodegeneration," *Exp Neurol*, vol. 328, p. 113247, Jun. 2020, doi: 10.1016/j.expneurol.2020.113247.

[69] C. Tonelli, I. I. C. Chio, and D. A. Tuveson, "Transcriptional Regulation by Nrf2," *Antioxid Redox Signal*, vol. 29, no. 17, pp. 1727–1745, Dec. 2018, doi: 10.1089/ars.2017.7342.

[70] M. Rajesh *et al.*, "Cannabidiol attenuates cardiac dysfunction, oxidative stress, fibrosis, inflammatory and cell death signaling pathways in diabetic cardiomyopathy," *J Am Coll Cardiol*, vol. 56, no. 25, pp. 2115–2125, Dec. 2010, doi: 10.1016/j.jacc.2010.07.033.

[71] B. Costa, A. E. Trovato, F. Comelli, G. Giagnoni, and M. Colleoni, "The non-psychoactive cannabis constituent cannabidiol is an orally effective therapeutic agent in rat chronic inflammatory and neuropathic pain," *Eur J Pharmacol*, vol. 556, no. 1–3, pp. 75–83, Feb. 2007, doi: 10.1016/j.ejphar.2006.11.006.

[72] A. Jastrząb, A. Gęgotek, and E. Skrzydlewska, "Cannabidiol Regulates the Expression of Keratinocyte Proteins Involved in the Inflammation Process through Transcriptional Regulation," *Cells*, vol. 8, no. 8, p. 827, Aug. 2019, doi: 10.3390/cells8080827.

[73] C. A. Gallelli *et al.*, "Modulation of the Oxidative Stress and Lipid Peroxidation by Endocannabinoids and Their Lipid Analogues," *Antioxidants (Basel)*, vol. 7, no. 7, p. 93, Jul. 2018, doi: 10.3390/antiox7070093.

[74] L. De Petrocellis, M. Nabissi, G. Santoni, and A. Ligresti, "Chapter Eight - Actions and Regulation of Ionotropic Cannabinoid Receptors," in *Advances in Pharmacology*, vol. 80, D. Kendall and S. P. H. Alexander, Eds. Academic Press, 2017, pp. 249–289. doi: 10.1016/bs.apha.2017.04.001.

[75] R. G. M. Morris, "D.O. Hebb: The Organization of Behavior, Wiley: New York; 1949," *Brain Research Bulletin*, vol. 50, no. 5, p. 437, Nov. 1999, doi: 10.1016/S0361-9230(99)00182-3.

[76] W.-S. Gui *et al.*, "Interleukin-1β overproduction is a common cause for neuropathic pain, memory deficit, and depression following peripheral nerve injury in rodents," *Mol Pain*, vol. 12, p. 1744806916646784, May 2016, doi: 10.1177/1744806916646784.

[77] G. A. Prieto, L. Tong, E. D. Smith, and C. W. Cotman, "TNFα and IL-1β but not IL-18 Suppresses Hippocampal Long-Term Potentiation Directly at the Synapse," *Neurochem Res*, vol. 44, no. 1, pp. 49–60, Jan. 2019, doi: 10.1007/s11064-018-2517-8.

[78] Z. Dong *et al.*, "Long-term potentiation decay and memory loss are mediated by AMPAR endocytosis," *J Clin Invest*, vol. 125, no. 1, pp. 234–247, Jan. 2015, doi: 10.1172/JCI77888.

[79] S. Casciato, J. Fareen, and M. Polinati, "The Effect of Cannabidiol (CBD) on the Short-Term Memory of young Drosophila melanogaster".

[80] A. Batalla, J. Bos, A. Postma, and M. G. Bossong, "The Impact of Cannabidiol on Human Brain Function: A Systematic Review," *Front Pharmacol*, vol. 11, p. 618184, 2020, doi: 10.3389/fphar.2020.618184.

[81] M. M. McGregor and A. B. Nelson, "Circuit Mechanisms of Parkinson's Disease," *Neuron*, vol. 101, no. 6, pp. 1042–1056, Mar. 2019, doi: 10.1016/j.neuron.2019.03.004.

[82] D. M. Lipton, B. J. Gonzales, and A. Citri, "Dorsal Striatal Circuits for Habits, Compulsions and Addictions," *Frontiers in Systems Neuroscience*, vol. 13, 2019, Accessed: Mar. 06, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fnsys.2019.00028

[83] S. Aamodt, "Singing in the brain: song learning in adult zebra finches," *Nat Neurosci*, vol. 2, no. 7, Art. no. 7, Jul. 1999, doi: 10.1038/10139.

[84] S. Yanagihara and Y. Yazaki-Sugiyama, "Auditory experience-dependent cortical circuit shaping for memory formation in bird song learning," *Nat Commun*, vol. 7, no. 1, Art. no. 1, Jun. 2016, doi: 10.1038/ncomms11946.

[85] C. L. Pytte, M. Gerson, J. Miller, and J. R. Kirn, "Increasing stereotypy in adult zebra finch song correlates with a declining rate of adult neurogenesis," *Dev Neurobiol*, vol. 67, no. 13, pp. 1699–1720, Nov. 2007, doi: 10.1002/dneu.20520.

[86] S. M. H. Gobes, R. B. Jennings, and R. K. Maeda, "The sensitive period for auditory-vocal learning in the zebra finch: consequences of limited-model availability and multiple-tutor paradigms on song imitation," *Behav Processes*, vol. 163, pp. 5–12, Jun. 2019, doi: 10.1016/j.beproc.2017.07.007.

[87]   A. J. Doupe and P. K. Kuhl, "BIRDSONG AND HUMAN SPEECH: Common Themes and Mechanisms," *Annu. Rev. Neurosci.*, vol. 22, no. 1, pp. 567–631, Mar. 1999, doi: 10.1146/annurev.neuro.22.1.567.

[88]   R. Mooney, "Neurobiology of song learning," *Curr Opin Neurobiol*, vol. 19, no. 6, pp. 654–660, Dec. 2009, doi: 10.1016/j.conb.2009.10.004.

[89]   A. R. Pfenning *et al.*, "Convergent transcriptional specializations in the brains of humans and song-learning birds," *Science*, vol. 346, no. 6215, p. 1256846, Dec. 2014, doi: 10.1126/science.1256846.

[90]   E. D. Jarvis, "Evolution of vocal learning and spoken language," *Science*, vol. 366, no. 6461, pp. 50–54, Oct. 2019, doi: 10.1126/science.aax0287.

[91]   B. M. Colquitt, "Organizational Conservation and Flexibility in the Evolution of Birdsong and Avian Motor Control," *Brain Behav Evol*, vol. 97, no. 5, pp. 255–264, 2022, doi: 10.1159/000525019.

[92]   E. Jarvis, "Evolution of vocal learning and spoken language," 2019. https://www.science.org/doi/10.1126/science.aax0287 (accessed May 10, 2022).

[93]   S. J. Sober, M. J. Wohlgemuth, and M. S. Brainard, "Central Contributions to Acoustic Variation in Birdsong," *Journal of Neuroscience*, vol. 28, no. 41, pp. 10370–10379, Oct. 2008, doi: 10.1523/JNEUROSCI.2448-08.2008.

[94]   A. C. Yu and D. Margoliash, "Temporal hierarchical control of singing in birds," *Science*, vol. 273, no. 5283, pp. 1871–1875, Sep. 1996, doi: 10.1126/science.273.5283.1871.

[95]   C. Scharff and F. Nottebohm, "A comparative study of the behavioral deficits following lesions of various parts of the zebra finch song system: implications for vocal learning," *J Neurosci*, vol. 11, no. 9, pp. 2896–2913, Sep. 1991, doi: 10.1523/JNEUROSCI.11-09-02896.1991.

[96]   S. C. Woolley, "Dopaminergic regulation of vocal-motor plasticity and performance," *Current Opinion in Neurobiology*, vol. 54, pp. 127–133, Feb. 2019, doi: 10.1016/j.conb.2018.10.008.

[97]   J. A. Thompson and F. Johnson, "HVC microlesions do not destabilize the vocal patterns of adult male zebra finches with prior ablation of LMAN," *Devel Neurobio*, vol. 67, no. 2, pp. 205–218, Feb. 2007, doi: 10.1002/dneu.20287.

[98] H. Li *et al.*, "The non-psychoactive phytocannabinoid cannabidiol (CBD) attenuates pro-inflammatory mediators, T cell infiltration, and thermal sensitivity following spinal cord injury in mice," *Cellular Immunology*, vol. 329, pp. 1–9, Jul. 2018, doi: 10.1016/j.cellimm.2018.02.016.

[99] E. Kozela, A. Juknat, and Z. Vogel, "Modulation of Astrocyte Activity by Cannabidiol, a Nonpsychoactive Cannabinoid," *IJMS*, vol. 18, no. 8, p. 1669, Jul. 2017, doi: 10.3390/ijms18081669.

[100] J. A. Thompson, W. Wu, R. Bertram, and F. Johnson, "Auditory-dependent vocal recovery in adult male zebra finches is facilitated by lesion of a forebrain pathway that includes the basal ganglia," *J. Neurosci.*, vol. 27, no. 45, pp. 12308–12320, Nov. 2007, doi: 10.1523/JNEUROSCI.2853-07.2007.

[101] Y. Zhang *et al.*, "Study on the pathogenic mechanism of Broca's and Wernicke's aphasia," *Neurol Res*, vol. 28, no. 1, pp. 59–65, Jan. 2006, doi: 10.1179/016164106X91889.

[102] A. B. Acharya and M. Wroten, "Broca Aphasia," in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2022. Accessed: Feb. 28, 2023. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK436010/

[103] A. B. Acharya and M. Wroten, "Wernicke Aphasia," in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2022. Accessed: Feb. 28, 2023. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK441951/

[104] O. R. Floody and A. P. Arnold, "Song lateralization in the zebra finch," *Horm Behav*, vol. 31, no. 1, pp. 25–34, Feb. 1997, doi: 10.1006/hbeh.1997.1368.

[105] I. George, E. Hara, and N. A. Hessler, "Behavioral and neural lateralization of vision in courtship singing of the zebra finch," *J Neurobiol*, vol. 66, no. 10, pp. 1164–1173, Sep. 2006, doi: 10.1002/neu.20273.

[106] J. R. Chen, L. Stepanek, and A. J. Doupe, "Differential contributions of basal ganglia and thalamus to song initiation, tempo, and structure," *J Neurophysiol*, vol. 111, no. 2, pp. 248–257, Jan. 2014, doi: 10.1152/jn.00584.2012.

[107] W. L. Stone, H. Basit, and B. Burns, "Pathology, Inflammation," in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2022. Accessed: Feb. 28, 2023. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK534820/

[108] D. M. Teleanu *et al.*, "An Overview of Oxidative Stress, Neuroinflammation, and Neurodegenerative Diseases," *Int J Mol Sci*, vol. 23, no. 11, p. 5938, May 2022, doi: 10.3390/ijms23115938.

[109] Y. S. Kim and T. H. Joh, "Microglia, major player in the brain inflammation: their roles in the pathogenesis of Parkinson's disease," *Exp Mol Med*, vol. 38, no. 4, pp. 333–347, Aug. 2006, doi: 10.1038/emm.2006.40.

[110] D. Mercurio *et al.*, "Protein Expression of the Microglial Marker Tmem119 Decreases in Association With Morphological Changes and Location in a Mouse Model of Traumatic Brain Injury," *Frontiers in Cellular Neuroscience*, vol. 16, 2022, Accessed: Feb. 03, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fncel.2022.820127

[111] J. A. Thompson, W. Wu, R. Bertram, and F. Johnson, "Auditory-Dependent Vocal Recovery in Adult Male Zebra Finches Is Facilitated by Lesion of a Forebrain Pathway That Includes the Basal Ganglia," *J. Neurosci.*, vol. 27, no. 45, pp. 12308–12320, Nov. 2007, doi: 10.1523/JNEUROSCI.2853-07.2007.

[112] J. Martínez-Orgado, M. Villa, and A. del Pozo, "Cannabidiol for the Treatment of Neonatal Hypoxic-Ischemic Brain Injury," *Front Pharmacol*, vol. 11, p. 584533, Jan. 2021, doi: 10.3389/fphar.2020.584533.

[113] F. R. Henshaw, L. S. Dewsbury, C. K. Lim, and G. Z. Steiner, "The Effects of Cannabinoids on Pro- and Anti-Inflammatory Cytokines: A Systematic Review of In Vivo Studies," *Cannabis Cannabinoid Res*, vol. 6, no. 3, pp. 177–195, Jun. 2021, doi: 10.1089/can.2020.0105.

[114] C. R. Olson, D. C. Owen, A. E. Ryabinin, and C. V. Mello, "Drinking Songs: Alcohol Effects on Learned Song of Zebra Finches," *PLoS ONE*, vol. 9, no. 12, p. e115427, Dec. 2014, doi: 10.1371/journal.pone.0115427.

[115] L.-J. Gamble *et al.*, "Pharmacokinetics, Safety, and Clinical Efficacy of Cannabidiol Treatment in Osteoarthritic Dogs," *Frontiers in Veterinary Science*, vol. 5, 2018, Accessed: May 06, 2022. [Online]. Available: https://www.frontiersin.org/article/10.3389/fvets.2018.00165

[116] S. Moorman, S. M. H. Gobes, M. Kuijpers, A. Kerkhofs, M. A. Zandbergen, and J. J. Bolhuis, "Human-like brain hemispheric dominance in birdsong learning," *Proceedings of the National Academy of Sciences*, vol. 109, no. 31, pp. 12782–12787, Jul. 2012, doi: 10.1073/pnas.1207207109.

[117] "A procedure for an automated measurement of song similarity | Elsevier Enhanced Reader."
https://reader.elsevier.com/reader/sd/pii/S0003347299914161?token=3364B058EF6CF97
55B4D264D52736F8E6DD0D490A9219FA57BB6FD0D5B36A5F4CD5087D1E03B290
864E699791E215079&originRegion=us-east-1&originCreation=20230113162558
(accessed Jan. 13, 2023).

[118] A. Daou, F. Johnson, W. Wu, and R. Bertram, "A computational tool for automated large-scale analysis and measurement of bird-song syntax," *Journal of Neuroscience Methods*, vol. 210, no. 2, pp. 147–160, Sep. 2012, doi: 10.1016/j.jneumeth.2012.07.020.

[119] W. Wu, J. A. Thompson, R. Bertram, and F. Johnson, "A statistical method for quantifying songbird phonology and syntax," *Journal of Neuroscience Methods*, vol. 174, no. 1, pp. 147–154, Sep. 2008, doi: 10.1016/j.jneumeth.2008.06.033.

[120] K. J. Livak and T. D. Schmittgen, "Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method," *Methods*, vol. 25, no. 4, pp. 402–408, Dec. 2001, doi: 10.1006/meth.2001.1262.

[121] Q. Wang and M.-H. Zou, "Measurement of Reactive Oxygen Species (ROS) and Mitochondrial ROS in AMPK Knockout Mice Blood Vessels," in *AMPK*, vol. 1732, D. Neumann and B. Viollet, Eds. New York, NY: Springer New York, 2018, pp. 507–517. doi: 10.1007/978-1-4939-7598-3_32.

[122] M. Sasaki *et al.*, "Neurodegenerative influence of oxidative stress in the retina of a murine model of diabetes," *Diabetologia*, vol. 53, no. 5, pp. 971–979, 2010, doi: 10.1007/s00125-009-1655-6.

[123] M. R. Pazos *et al.*, "Mechanisms of cannabidiol neuroprotection in hypoxic–ischemic newborn pigs: Role of 5HT1A and CB2 receptors," *Neuropharmacology*, vol. 71, pp. 282–291, Aug. 2013, doi: 10.1016/j.neuropharm.2013.03.027.

[124] F. Sunda and A. Arowolo, "A molecular basis for the anti-inflammatory and anti-fibrosis properties of cannabidiol," *The FASEB Journal*, vol. 34, no. 11, pp. 14083–14092, 2020, doi: 10.1096/fj.202000975R.

[125] E. Kozela, A. Juknat, and Z. Vogel, "Modulation of Astrocyte Activity by Cannabidiol, a Nonpsychoactive Cannabinoid," *Int J Mol Sci*, vol. 18, no. 8, Jul. 2017, doi: 10.3390/ijms18081669.

[126] S. U. Bhalerao, C. Geurtjens, G. R. Thomas, C. R. Kitamura, C. Zhou, and M. Marlborough, "Understanding the neuropsychiatric consequences associated with significant traumatic brain injury," *Brain Inj*, vol. 27, no. 7–8, pp. 767–774, 2013, doi: 10.3109/02699052.2013.793396.

[127] B. P. Ölveczky, T. M. Otchy, J. H. Goldberg, D. Aronov, and M. S. Fee, "Changes in the neural control of a complex motor sequence during learning," *Journal of Neurophysiology*, vol. 106, no. 1, pp. 386–397, May 2011, doi: 10.1152/jn.00018.2011.

[128] M. H. Kao and M. S. Brainard, "Lesions of an Avian Basal Ganglia Circuit Prevent Context-Dependent Changes to Song Variability," *Journal of Neurophysiology*, vol. 96, no. 3, pp. 1441–1455, Sep. 2006, doi: 10.1152/jn.01138.2005.

[129] K. Hamaguchi and R. Mooney, "Recurrent interactions between the input and output of a songbird cortico-basal ganglia pathway are implicated in vocal sequence variability," *J. Neurosci.*, vol. 32, no. 34, pp. 11671–11687, Aug. 2012, doi: 10.1523/JNEUROSCI.1666-12.2012.

[130] F. R. M. Laurindo, D. C. Fernandes, and C. X. C. Santos, "Assessment of superoxide production and NADPH oxidase activity by HPLC analysis of dihydroethidium oxidation products," *Methods Enzymol*, vol. 441, pp. 237–260, 2008, doi: 10.1016/S0076-6879(08)01213-5.

[131] B. D. Arbo, L. E. Schimith, M. Goulart Dos Santos, and M. A. Hort, "Repositioning and development of new treatments for neurodegenerative diseases: Focus on neuroinflammation," *Eur J Pharmacol*, vol. 919, p. 174800, Mar. 2022, doi: 10.1016/j.ejphar.2022.174800.

[132] K. Soderstrom and A. Alalawi, "Software for objective comparison of vocal acoustic features over weeks of audio recording: KLFromRecordingDays," *SoftwareX*, vol. 6, pp. 271–277, 2017, doi: 10.1016/j.softx.2017.10.003.

[133] S. J. Hewett, N. A. Jackman, and R. J. Claycomb, "Interleukin-1β in Central Nervous System Injury and Repair," *Eur J Neurodegener Dis*, vol. 1, no. 2, pp. 195–211, Aug. 2012.

[134] M. Erta, A. Quintana, and J. Hidalgo, "Interleukin-6, a Major Cytokine in the Central Nervous System," *Int J Biol Sci*, vol. 8, no. 9, pp. 1254–1266, Oct. 2012, doi: 10.7150/ijbs.4679.

[135] A. D. Kraft, C. A. McPherson, and G. J. Harry, "Association Between Microglia, Inflammatory Factors, and Complement with Loss of Hippocampal Mossy Fiber Synapses Induced by Trimethyltin," *Neurotox Res*, vol. 30, no. 1, pp. 53–66, Jul. 2016, doi: 10.1007/s12640-016-9606-8.

[136] M. L. Block, L. Zecca, and J.-S. Hong, "Microglia-mediated neurotoxicity: uncovering the molecular mechanisms," *Nat Rev Neurosci*, vol. 8, no. 1, Art. no. 1, Jan. 2007, doi: 10.1038/nrn2038.

[137] A. Gugliandolo, P. Bramanti, and E. Mazzon, "Activation of Nrf2 by Natural Bioactive Compounds: A Promising Approach for Stroke?," *Int J Mol Sci*, vol. 21, no. 14, p. 4875, Jul. 2020, doi: 10.3390/ijms21144875.

[138] S. Raffaele, M. Lombardi, C. Verderio, and M. Fumagalli, "TNF Production and Release from Microglia via Extracellular Vesicles: Impact on Brain Functions," *Cells*, vol. 9, no. 10, p. 2145, Sep. 2020, doi: 10.3390/cells9102145.

[139] A. Bessis, C. Béchade, D. Bernard, and A. Roumier, "Microglial control of neuronal death and synaptic properties," *Glia*, vol. 55, no. 3, pp. 233–238, 2007, doi: 10.1002/glia.20459.

[140] A. M. Barrera-Sandoval, E. Osorio, and G. P. Cardona-Gómez, "Microglial-targeting induced by intranasal linalool during neurological protection postischemia," *Eur J Pharmacol*, vol. 857, p. 172420, Aug. 2019, doi: 10.1016/j.ejphar.2019.172420.

[141] A. I. Rojo, N. G. Innamorato, A. M. Martín-Moreno, M. L. De Ceballos, M. Yamamoto, and A. Cuadrado, "Nrf2 regulates microglial dynamics and neuroinflammation in experimental Parkinson's disease," *Glia*, vol. 58, no. 5, pp. 588–598, Apr. 2010, doi: 10.1002/glia.20947.

[142] L. Li *et al.*, "The Specific Role of Reactive Astrocytes in Stroke," *Front Cell Neurosci*, vol. 16, p. 850866, 2022, doi: 10.3389/fncel.2022.850866.

[143] S. Kourakis, C. A. Timpani, J. B. de Haan, N. Gueven, D. Fischer, and E. Rybalka, "Dimethyl Fumarate and Its Esters: A Drug with Broad Clinical Utility?," *Pharmaceuticals*, vol. 13, no. 10, Art. no. 10, Oct. 2020, doi: 10.3390/ph13100306.

[144] Evgen Pharma, "SFX-01 After Subarachnoid Haemorrhage," clinicaltrials.gov, Clinical trial registration NCT02614742, Jan. 2020. Accessed: Sep. 08, 2022. [Online]. Available: https://clinicaltrials.gov/ct2/show/NCT02614742

[145] M. L. Bennett *et al.*, "New tools for studying microglia in the mouse and human CNS," *Proc Natl Acad Sci U S A*, vol. 113, no. 12, pp. E1738-1746, Mar. 2016, doi: 10.1073/pnas.1525528113.

[146] C. Ruan and W. Elyaman, "A New Understanding of TMEM119 as a Marker of Microglia," *Front Cell Neurosci*, vol. 16, p. 902372, 2022, doi: 10.3389/fncel.2022.902372.

[147] M. Y. Wendimu and S. B. Hooks, "Microglia Phenotypes in Aging and Neurodegenerative Diseases," *Cells*, vol. 11, no. 13, p. 2091, Jun. 2022, doi: 10.3390/cells11132091.

[148] M. Andoh and R. Koyama, "Microglia regulate synaptic development and plasticity," *Developmental Neurobiology*, vol. 81, no. 5, pp. 568–590, 2021, doi: 10.1002/dneu.22814.

[149] Q. Li and B. A. Barres, "Microglia and macrophages in brain homeostasis and disease," *Nat Rev Immunol*, vol. 18, no. 4, Art. no. 4, Apr. 2018, doi: 10.1038/nri.2017.125.

[150] K. Herrmann and A. Arnold, "The development of afferent projections to the robust archistriatal nucleus in male zebra finches: a quantitative electron microscopic study," *J. Neurosci.*, vol. 11, no. 7, pp. 2063–2074, Jul. 1991, doi: 10.1523/JNEUROSCI.11-07-02063.1991.

[151] G. H. Diering and R. L. Huganir, "The AMPA Receptor Code of Synaptic Plasticity," *Neuron*, vol. 100, no. 2, pp. 314–329, Oct. 2018, doi: 10.1016/j.neuron.2018.10.018.

[152] C. J. Hunter *et al.*, "MSK1 regulates transcriptional induction of Arc/Arg3.1 in response to neurotrophins," *FEBS Open Bio*, vol. 7, no. 6, pp. 821–834, 2017, doi: 10.1002/2211-5463.12232.

[153] D. D. Cooper and B. G. Frenguelli, "The influence of sensory experience on the glutamatergic synapse," *Neuropharmacology*, vol. 193, p. 108620, Aug. 2021, doi: 10.1016/j.neuropharm.2021.108620.

[154] M. B. Kennedy, "Synaptic Signaling in Learning and Memory," *Cold Spring Harb Perspect Biol*, vol. 8, no. 2, p. a016824, Feb. 2016, doi: 10.1101/cshperspect.a016824.

[155] J. Keifer, "Regulation of AMPAR trafficking in synaptic plasticity by BDNF and the impact of neurodegenerative disease," *Journal of Neuroscience Research*, vol. 100, no. 4, pp. 979–991, 2022, doi: 10.1002/jnr.25022.

[156] J. Keifer and Z. Zheng, "AMPA receptor trafficking and learning," *European Journal of Neuroscience*, vol. 32, no. 2, pp. 269–277, 2010, doi: 10.1111/j.1460-9568.2010.07339.x.

[157] H. Zhang and C. R. Bramham, "Arc/Arg3.1 function in long-term synaptic plasticity: Emerging mechanisms and unresolved issues," *European Journal of Neuroscience*, vol. 54, no. 8, pp. 6696–6712, 2021, doi: 10.1111/ejn.14958.

[158] R. E. Carmichael, "Transcriptional and post-translational regulation of Arc in synaptic plasticity," *Developmental Biology*, p. 7, 2018.

[159] I. Sandvig, I. L. Augestad, A. K. Håberg, and A. Sandvig, "Neuroplasticity in stroke recovery. The role of microglia in engaging and modifying synapses and networks," *Eur J Neurosci*, vol. 47, no. 12, pp. 1414–1428, Jun. 2018, doi: 10.1111/ejn.13959.

[160] S. D. Gale and D. J. Perkel, "A Basal Ganglia Pathway Drives Selective Auditory Responses in Songbird Dopaminergic Neurons via Disinhibition," *J. Neurosci.*, vol. 30, no. 3, pp. 1027–1037, Jan. 2010, doi: 10.1523/JNEUROSCI.3585-09.2010.

[161] G. G. Turrigiano, "The self-tuning neuron: synaptic scaling of excitatory synapses," *Cell*, vol. 135, no. 3, pp. 422–435, Oct. 2008, doi: 10.1016/j.cell.2008.10.008.

[162] S. Chowdhury *et al.*, "Arc/Arg3.1 interacts with the endocytic machinery to regulate AMPA receptor trafficking," *Neuron*, vol. 52, no. 3, pp. 445–459, Nov. 2006, doi: 10.1016/j.neuron.2006.08.033.

[163] D. D. Cooper and B. G. Frenguelli, "The influence of sensory experience on the glutamatergic synapse," *Neuropharmacology*, vol. 193, p. 108620, Aug. 2021, doi: 10.1016/j.neuropharm.2021.108620.

[164] L. L. P. DaSilva *et al.*, "Activity-Regulated Cytoskeleton-Associated Protein Controls AMPAR Endocytosis through a Direct Interaction with Clathrin-Adaptor Protein 2," *eNeuro*, vol. 3, no. 3, p. ENEURO.0144-15.2016, Jun. 2016, doi: 10.1523/ENEURO.0144-15.2016.

[165] L. C. Koay, R. J. Rigby, and K. L. Wright, "Cannabinoid-induced autophagy regulates suppressor of cytokine signaling-3 in intestinal epithelium," *Am J Physiol Gastrointest Liver Physiol*, vol. 307, no. 2, pp. G140–G148, Jul. 2014, doi: 10.1152/ajpgi.00317.2013.

[166] Y. Liang, "Emerging Concepts and Functions of Autophagy as a Regulator of Synaptic Components and Plasticity," *Cells*, vol. 8, no. 1, p. 34, Jan. 2019, doi: 10.3390/cells8010034.

[167] Z. Z. Al-Ghezi, P. B. Busbee, H. Alghetaa, P. S. Nagarkatti, and M. Nagarkatti, "Combination of cannabinoids, delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD), mitigates experimental autoimmune encephalomyelitis (EAE) by altering the gut microbiome.," *Brain Behav Immun*, vol. 82, pp. 25–35, Nov. 2019, doi: 10.1016/j.bbi.2019.07.028.

[168] F. Borrelli *et al.*, "Cannabidiol, a safe and non-psychotropic ingredient of the marijuana plant Cannabis sativa, is protective in a murine model of colitis," *J Mol Med*, vol. 87, no. 11, p. 1111, Aug. 2009, doi: 10.1007/s00109-009-0512-x.

[169] C. D. Verrico *et al.*, "A randomized, double-blind, placebo-controlled study of daily cannabidiol for the treatment of canine osteoarthritis pain," *Pain*, vol. 161, no. 9, pp. 2191–2202, Sep. 2020, doi: 10.1097/j.pain.0000000000001896.

[170] J. Lier, W. J. Streit, and I. Bechmann, "Beyond Activation: Characterizing Microglial Functional Phenotypes," *Cells*, vol. 10, no. 9, p. 2236, Aug. 2021, doi: 10.3390/cells10092236.

[171] V. Stratoulias, J. L. Venero, M. Tremblay, and B. Joseph, "Microglial subtypes: diversity within the microglial community," *EMBO J*, vol. 38, no. 17, p. e101997, Sep. 2019, doi: 10.15252/embj.2019101997.

[172] F. Giovannoni and F. J. Quintana, "The Role of Astrocytes in CNS Inflammation," *Trends Immunol*, vol. 41, no. 9, pp. 805–819, Sep. 2020, doi: 10.1016/j.it.2020.07.007.

Appendix A: AUP w247b Amendment Approval Letter

ECU

February 14, 2023

Ken Soderstrom, Ph.D.
Department of Pharmacology and Toxicology, ECU

Subject: Protocol W247b, original approval date 07/13/2021

Dear Dr. Soderstrom:

The amendment#2 to your Animal Use Protocol entitled, "Effects of cannabidiol on recovery of vocal behavior" (AUP#W247b) was reviewed by this institution's Animal Care and Use Committee on 02/10/2023. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle prior to any hazard use

A copy of the protocols 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/Amendment and are familiar with its contents.

Sincerely yours,

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

SM/GD

enclosure

Appendix B: AUP w247b Amendment Approval Letter

ECU Animal Care and Use Committee
003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27834 - 4354
252-744-2436 office | 252-744-2355 fax

February 10, 2022

Ken Soderstrom, Ph.D.
Department of Pharmacology and Toxicology, ECU

Subject: Protocol W247b, original approval date 07/13/2021

Dear Dr. Soderstrom:

The amendment#1 to your Animal Use Protocol entitled, "Effects of cannabidiol on recovery of vocal behavior" (AUP#W247b) was reviewed by this institution's Animal Care and Use Committee on 02/09/2022. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle prior to any hazard use

A copy of the protocols 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/Amendment and are familiar with its contents.

Sincerely yours,

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

SM/GD

enclosure

www.ecu.edu

Appendix C: AUP w247b Amendment Approval Letter

ECU

July 13, 2021

Ken Soderstrom, Ph.D.
Department of Pharmacology and Toxicology, ECU

Dear Dr. Soderstrom:

Your Animal Use Protocol entitled, "Effects of cannabidiol on recovery of vocal behavior" (AUP #W247b) was reviewed by this institution's Animal Care and Use Committee on 07/13/2021. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle prior to any hazard use

A copy of the protocols 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/Amendment and are familiar with its contents.**

Sincerely yours,

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

SM/GD

enclosure

www.ecu.edu

Appendix D: AUP w247a Amendment Approval Letter

ECU — Animal Care and Use Committee
003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27354 - 4354
252-744-2436 office | 252-744-2355 fax

October 22, 2020

Ken Soderstrom, Ph.D.
Department of Pharmacology and Toxicology, ECU

Subject: Protocol W247a, original approval date 8/7/2018

Dear Dr. Soderstrom:

The amendment#8 to 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 10/19/2020. The following action was taken by the Committee:

   "Approved as submitted"

Please contact Aaron Hinkle prior to any hazard use

A copy of the protocols 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/Amendment and are familiar with its contents.

Sincerely yours,

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

SM/GD

enclosure