ABSTRACT

EFFECTS OF PSYCHOLOGICAL STRESSORS AND Δ^9-THC ACUTELY AND CHRONICALLY ON ZEBRA FINCH SONG BEHAVIOR AND DENDRITIC SPINE DENSITY. TESSA L. HOLLAND. Under the direction of Dr. Ken Soderstrom) Department of Pharmacology and Toxicology, May 2020.

This dissertation investigated song performance and dendritic spine density, following acute restraint stress in adult zebra finches and, following chronic mild stress and CB_1 receptor partial agonist Δ^9-tetrahydrocannabinol (Δ^9-THC) treatments (3 mg/kg) during sensorimotor development or adulthood. CB_1 receptor agonists and stressors have mechanistic overlap: a stressor activates glucocorticoid corticosterone release in the hypothalamic-pituitary-adrenal axis, and endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are CB_1 receptor agonists which operate as an endogenous stress buffer system that turns off the response. The endocannabinoid system is prominent during late postnatal development and may modulate important synaptic fine-tuning. Chronic CB_1 receptor agonist treatment or stressors during this developmental stage may disrupt appropriate endocannabinoid signaling mediating brain development. Male zebra finches possess a developmental, sensorimotor critical period for learning a song in a mechanism similar to language acquisition in humans. Initially in sensorimotor development, zebra finches possess a surplus of dendritic spines, which are the anatomical basis of the post-synaptic site with excitatory input and may represent morphological building blocks of learning and memory. Over time, a net elimination occurs as part of the developmental learning process. In this dissertation, acute restraint stress (30 minutes) in adults rapidly increased plasma corticosterone levels, altered performance of spectral and temporal acoustic features, and stimulated dendritic spine and c-Fos immunolabeled nuclei density in higher-order acoustic region

NCM. Δ^9-THC, the principal psychoactive component of marijuana, inhibits perceptual sensory processing, and Δ^9-THC pretreatment antagonized the effects of stress on c-Fos density in NCM in a CB_1 receptor inverse agonist/antagonist SR141716 (6 mg/kg)-reversible manner. The acute effects differed from chronic effects. In adult groups, chronic mild stress or Δ^9-THC treatments alone did not alter corticosterone levels, song acoustic features, or dendritic spine density in NCM or basal ganglia/striatal region Area X. Both chronic stress and Δ^9-THC treatments during sensorimotor song development resulted in effects persistent into adulthood, with reduced syllable entropy and dendritic spine density in Area X. These effects suggest an interference with typical developmental song learning and brain development. Adolescent brain development may be vulnerable to long-term consequences following chronic exposure to CB_1 receptor agonists or stressors, and their effects likely differ than exposure during adulthood. This distinction is important to the elucidation of mechanisms and outcomes of marijuana and psychological disorders, such as depression.

EFFECTS OF PSYCHOLOGICAL STRESSORS AND Δ^9-THC ACUTELY AND CHRONICALLY ON ZEBRA FINCH SONG BEHAVIOR AND DENDRITIC SPINE DENSITY

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By

Tessa L. Holland

APPROVED BY:

DIRECTOR OF DISSERTATION _____

Ken Soderstrom, PhD

COMMITTEE MEMBER _____

Jamie DeWitt, PhD

COMMITTEE MEMBER _____

Brian McMillen, PhD

COMMITTEE MEMBER _____

David Taylor, PhD

COMMITTEE MEMBER _____

Tuan Tran, PhD

COMMITTEE MEMBER _____

Rukiyah Van Dross, PhD

CHAIR OF THE DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY _____

David Taylor, PhD

DEAN OF THE GRADUATE SCHOOL _____

Paul Gemperline, PhD

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2-AG	2-arachidonoylglycerol
AEA	anandamide
CBD	cannabidiol
CBN	cannabinol
CBG	cannabigerol
CN	cochlear nucleus
CMM	caudal medial mesopallium
dH_2O	distilled dihydrogen monoxide
DLM	medial subdivision of the dorsolateral nucleus of the anterior thalamus
DM	dorsomedial subdivision of nucleus intercollicularis of the mesencephalon
DMSO	dimethyl sulfoxide
FAAH	fatty acid amide hydrolase
HP	hippocampus
HPA	hypothalamic-pituitary-adrenal
HVC	proper name
LLD	lateral lemniscus, dorsal nucleus
LLI	lateral lemniscus, intermediate nucleus

LLV	lateral lemniscus, ventral nucleus
lMAN	lateral magnocellular nucleus of the anterior nidopallium
MLd	dorsal part of the lateral nucleus of the mesencephalon
NCM	caudal medial nidopallium
NIf	nucleus interfacialis nidopalii
nXIIts	tracheosyringeal portion of the nucleus hypoglossus (nucleus XII)
OV	nucleus ovoidalis
PBS	phosphate-buffered saline
RA	robust nucleus of the arcopallium
Ram/Pam	nucleus retroambiguus medullaris
SEM	standard error of the mean
SO	superior olive
THC	tetrahydrocannabinol
THCA	tetrahydrocannabinolic acid
THCV	tetrahydrocannabivarin
TnA	nucleus taeniae of the amygdala

CHAPTER ONE: INTRODUCTION

General Introduction and Purpose of Project

Endocannabinoids operate as a neuromodulatory signaling system that may be more prominent during late postnatal development than in adulthood. Endocannabinoids are mediators in long-term plasticity and the refinement of neuronal networks during brain development. Since adolescence is a vulnerable developmental window, alterations of appropriate endocannabinoid signaling, such as by cannabinoid CB_1 receptor agonists or psychological stress, may produce effects that persist into adulthood. This dissertation compares the effects of acute and chronic treatment with CB_1 receptor agonists or psychological stressors on song behavior and neuronal morphology in a developmental zebra finch songbird model. Zebra finches learn a complex song during a developmental critical period in a mechanism similar to human language acquisition, and developmental treatment with a full cannabinoid agonist WIN 55,212-2 persistently reduced adult song quality (Soderstrom and Johnson, 2003), suggesting a disruption of typical developmental learning. Since zebra finches cannot learn a new song in adulthood, this model is useful for the study of the effects of pharmacological or psychological treatments on developmental critical period-sensitive effects on brain development.

Cannabis sativa is a recreational drug that is often used by adolescents and young adults (Johnston et al., 2019), and age of exposure to this drug may have mechanistic significance to its effects. The phytocannabinoid Δ^9-tetrahydrocannabinol

(Δ^9-THC) is a partial CB_1 receptor agonist and the principal psychoactive constituent of *Cannabis sativa*. Treatment with CB_1 receptor agonists inhibit learning and memory processes (reviewed by Ranganathan and D'Souza, 2006). Endocannabinoids mediate long-term depression of inhibitory synaptic transmission in the hippocampus and long-term depression of excitatory transmission in the nucleus accumbens. An acute Δ^9-THC injection (3 mg/kg) prevented these occurrences in a CB_1 receptor-dependent manner (Mato et al., 2004). In a working memory assay involving the eight-arm radial maze, acute (1.25 mg/kg) or chronic (5 mg/kg) Δ^9-THC treatments in adult rats increased the number of entry errors, and in the chronic experiments, impairment was reversible following a no-treatment period (Nakamura et al., 1991). In light of learning and memory processes that are vulnerable to disruption during the late postnatal period and the significance of endocannabinoids in these cognitive functions, this dissertation compares the effects of Δ^9-THC when given during late postnatal development or adulthood.

Endocannabinoid signaling underlies psychological stress responses. Endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are CB_1 receptor agonists and function as an endogenous stress buffer system in the hypothalamic-pituitary-adrenal (HPA) stress axis (Evanson NK et al., 2010; Hill MN et al., 2009). A stressor activates corticotropin-releasing hormone cells in the paraventricular nucleus of the hypothalamus and prompts adrenocorticotropic hormone release from the pituitary gland. This stimulates the synthesis and release of corticosterone, a glucocorticoid hormone, from the adrenal gland. Corticosterone increases endocannabinoid signaling in the paraventricular nucleus and prefrontal

cortex, which is a negative feedback mechanism, turns off the HPA axis stress response (Frodl and O'Keane, 2013). AEA and 2-AG inhibit the HPA axis under basal or stress conditions, respectively. Amygdalar AEA inhibits the HPA axis under basal conditions. Following a stressor, AEA is rapidly hydrolyzed by fatty acid amide hydrolase (FAAH), allowing a surge in corticosterone release (reviewed by Hill and Tasker, 2012). 2-AG levels begin to increase and inhibit the HPA axis, and meanwhile AEA levels begin to return to normal levels to terminate the neuroendocrine acute stress response and reinstate basal inhibition of the HPA axis (reviewed by Hill and Tasker, 2012). This dissertation compares the effects of stressors and Δ^9-THC treatments on corticosterone, song behavior, and dendritic spine density in light of the mechanistic similarities involving CB_1 receptor activation in stressors and Δ^9-THC.

Dendritic spines are small protrusions on dendrites that operate as the anatomical basis of postsynaptic sites, with typically excitatory input. An important significance of these features is that they may act as morphological building blocks of learning and memory (Bhatt et al., 2009). Processes important to brain development during late postnatal development include activity- and experience-dependent establishment of synaptic networks during brain maturation (Casey et al., 2008). In cortical regions of rodents (Blue and Parnavelas, 1983; De Felipe et al., 1997) and primates (Bourgeois and Rakic, 1993; Huttenlocher, 1990) development is associated with a general profusion of synaptic contacts followed by a reduction of spine densities to adulthood. This dissertation used Golgi-Cox staining to evaluate the developmental-dependence of changes in dendritic spine density following chronic stressors and Δ^9-THC treatments.

The global hypothesis of this dissertation is that chronic Δ^9-THC or stress treatments during zebra finch late postnatal development would alter song acoustic features and these effects would persist into adulthood. Likewise, developmental chronic Δ^9-THC or stress treatments would alter dendritic spine densities in brain regions relevant to song learning, and these effects would persist into adulthood. In contrast, chronic Δ^9-THC or stress treatments in adulthood would have no persistent effects on song features or dendritic spine density. These results would support the significance of the endocannabinoid system in developmental-dependent learning.

Cannabinoids

Endocannabinoids

Endocannabinoids are a neuromodulatory signaling system important to many brain processes, including plasticity and adolescent brain development. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are lipophilic, endogenous ligands for cannabinoid CB_1 and CB_2 receptors. The most common G-coupled receptor in the brain is the CB_1 receptor, which is widely expressed in many brain regions, including the cerebellum, hippocampus, striatum, and cortex (Tsou et al., 1998) and is highly conserved among different vertebrate species (Console-Bram et al., 2012). Endocannabinoids are produced on-demand postsynaptically from membrane phospholipids following postsynaptic depolarization and Ca^{2+} influx or activation of postsynaptic metabotropic glutamate receptors or muscarinic receptors. Synthesis of 2-AG is Ca^{2+}-dependent and typically formed from arachidonic acid with phospholipase C and diacylglycerol lipase (Murataeva et al., 2014). AEA is present in the brain at relatively lower levels than 2-AG and is synthesized from N-arachidonoyl phosphatidylethanolamine (NAPE) in multiple pathways, including NAPE-hydrolyzing phospholipase D (NAPE-PLD), phospholipase C, and phospholipase A2 (Liu et al., 2008). Endocannabinoids operate as retrograde messengers and travel backwards across the synapse to activate presynaptic CB_1 receptors, which are coupled to G_i signal transduction and inhibit cAMP production from ATP. CB_1 receptor activation inhibits presynaptic neurotransmitter release through inhibition of Ca^{2+} influx and enhancement of K+ outflow, and endocannabinoids can modulate excitation or inhibition

by inhibiting respective glutamate or GABA release. AEA has relatively higher binding affinity than 2-AG (Steffens et al., 2005) and is inactivated intracellularly by fatty acid amide hydrolase (FAAH), and monoacylglycerol lipase (MAGL) degrades 2-AG (Basavarajappa 2007).

In synaptic plasticity, endocannabinoids mediate forms of long-term depression and is a process important in the establishment of neuronal networks in learning and memory. Induction of long-term depression in the striatum is dependent on CB_1 receptor activation (Gerdeman et al., 2002) and regulated by dopamine via metabotropic glutamate receptor-dependent endocannabinoid release (Kreitzer and Malenka, 2005). Striatal synaptic plasticity underlies goal-oriented motor learning (Dang et al., 2006) and the different stages of drug addiction: the progression from early, hedonistic drug-seeking behavior to subsequent compulsive habit and dependence (Gerdeman et al., 2003). Drugs of abuse may alter endocannabinoid-mediated synaptic plasticity. Endocannabinoids mediate long-term depression of inhibitory synaptic transmission in the hippocampus and long-term depression of excitatory transmission in the nucleus accumbens. Acute, moderate Δ^9-THC administration (3 mg/kg) prevented these occurrences in a CB_1 receptor-dependent manner (Mato et al., 2004). Exogenous cannabinoid exposure may inhibit endocannabinoid-mediated synaptic plasticity in striatum, nucleus accumbens, and hippocampus and disrupt learning and memory formation, particularly during critical periods of brain development. The endocannabinoid system is more prominent during late postnatal development than at maturity. In human prefrontal cortex, mRNA expression of CB_1 receptor and endocannabinoid-synthesizing enzyme diacylglycerol lipase is greater during juvenile

and adolescent development than during adulthood (Long et al., 2012). The same developmental pattern of CB_1 receptor density occurred in rodent limbic/associative and sensorimotor cortical regions (Heng et al., 2011), and adolescence may be a vulnerable window to cannabinoid drugs.

Δ^9-Tetrahydrocannabinol

The phytocannabinoid Δ^9-tetrahydrocannabinol (Δ^9-THC) is the principal psychoactive constituent of *Cannabis sativa*. The chemical composition of *Cannabis sativa* is a constellation of chemicals and, in addition to Δ^9-THC, includes other cannabinoid constituents, including cannabidiol (CBD), cannabinol (CBN), cannabigerol (CBG), tetrahydrocannabinolic acid (THCA), and tetrahydrocannabivarin (THCV), which likely all have mechanistic relevance (Aizpurua-Olaizola et al., 2014). Δ^9-THC is the most studied compound and is a partial CB_1 receptor agonist with both peripheral and central effects. In the classical tetrad assays for cannabinoids, Δ^9-THC dose-dependently decreased spontaneous activity in the open field test, increased catalepsy, reduced body temperature, and induced analgesia in the tail-flick test. CB_1 receptor antagonist/inverse agonist SR141716 treatment reversed these effects. This indicates CB_1 receptor-dependence (Wiley and Martin, 2003).

In the mesolimbic reward pathway, Δ^9-THC increases release of dopamine to the nucleus accumbens in a manner, similar to other drugs of abuse that promotes reinforcement. Δ^9-THC increases both tonic dopamine activity, which is a tone of low-frequency firing at high-affinity dopamine D2 receptors, and phasic dopamine activity,

which occurs transiently at high-frequency bursts to low-affinity dopamine D1 receptors in response to a rewarding stimulus or cue (Oleson and Cheer, 2012). Moderate doses of Δ^9-THC did not reliably induce conditioned place preference, but induced conditioned saccharin taste aversion (Hempel et al., 2017; Hempel et al., 2016; Wakeford et al., 2016), a result suggesting the rewarding properties of Δ^9-THC may be weaker than other drugs of abuse.

Δ^9-THC impairs learning and memory processes, but the extent and duration of the type of impairments may be differentiated across development. In working memory assay eight-arm radial maze, acute (1.25 mg/kg) or chronic (5 mg/kg) Δ^9-THC treatments in adult rats increased the number of entry errors, and in the chronic experiments, impairment was reversible following a no treatment period (Nakamura et al., 1991). However, evidence suggests that Δ^9-THC may have more profound cognitive effects when given during late postnatal development. Rats given chronic Δ^9-THC treatment during adolescence showed increased spatial memory errors in a radial maze and reduced dentate gyrus dendritic spine density, dendritic length, and dendritic number when examined as adults (Rubino et al., 2009). Chronic Δ^9-THC treatment during adolescence, not adulthood, persistently decreased exploration time for a novel object, and adolescent-treated rats had more widespread changes in hippocampal proteins (Quinn et al., 2008). In humans, long-term *Cannabis* users display cognitive decline from childhood to midlife in neuropsychological tests of mental function, and adolescent-onset dependence resulted in greater IQ decline than adult-onset (Meier et al., 2012). The robust synaptic pruning occurring during adolescent brain maturation

may represent a vulnerability to *Cannabis* exposure, and more research is needed to understand the magnitude of the impact and underlying processes.

Zebra Finches as a Psychopharmacological Model

Zebra finches, which are gregarious songbirds that originated in an unpredictable, drought-prone climate in Australia (Morton and Davies, 1983), learn a complex song during a developmental sensitive period in a process that parallels human language acquisition (Bolhuis et al., 2010). During the auditory phase (25-60 days old), zebra finches memorize a template after listening to a conspecific tutor's song. During an overlapping sensorimotor phase (35-90 days old), male zebra finches begin performing a highly variable and poorly structured version of their song (Olson et al., 2016). Through practice and auditory feedback, they gradually improve their attempts to match their template. At the onset of adulthood and the end of the developmental learning window, zebra finch song is stable and stereotyped (Boogert et al., 2008).

Song is an instrument in the formation of socially monogamous pair-bonds in zebra finches. In courtship, females prefer a song with complexity and with similarity to their early life exposure to conspecific song, such as their father's (Chen et al., 2017). Song complexity may suggest the quality of the male's early life. Nestling zebra finches that experienced nutritional stress at 5-30 days old possessed song motifs with fewer syllables as adults (Spencer et al., 2003), and females showed less preference towards stressed songs (Spencer et al., 2005). Song may indicate to the female the attractiveness of a prospective partner.

Unlike the mammalian brain which is organized in layers, the zebra finch brain contains discrete song nuclei regions in descending motor and anterior forebrain pathways and an ascending auditory pathway (Brenowitz et al., 1998). A dopamine basal ganglia thalamocortical loop with Area X, medial subdivision of the dorsolateral nucleus of the anterior thalamus (DLM), and lateral magnocellular nucleus of the anterior nidopallium (lMAN) mediates developmental sensorimotor learning. During this phase, lMAN, a mistake generator (Olveczky et al., 2005), is the primary input to motor output region robust nucleus of the arcopallium (RA). In adulthood, premotor HVC becomes a more dominant input to RA (**Fig. 1.1**). Motor regions in song production, HVC (proper name) and RA, are interconnected with the ascending auditory pathway (Vates et al., 1996). Flow of information of auditory stimuli entails levels of processing to accomplish awareness, discrimination, identification, and comprehension of sound (Woolley and Casseday, 2004). Hair cells transduce the mechanical sound wave to electrical stimuli that cochlear ganglion transmit to brain. L2 (subunit of the L field) is the direct thalamic recipient and primary auditory cortex-like (**Fig. 1.2**).The other subunits of the L field, L1 and L3, and higher-order regions, caudal medial nidopallium (NCM) and caudal medial mesopallium (CMM), show higher activation when a bird hears a song (Mello and Clayton, 1994). Evidence suggests that CMM and NCM are the neural substrate of song memory (Yanagihara and Yazaki-Sugiyama, 2016).

During sensorimotor development, the endocannabinoid system is more prominent and likely has mechanistic importance, and this developmental age is vulnerable to CB_1 receptor agonists. The immunohistochemical density of CB_1 receptors was higher during development and was decreased at adulthood in motor regions HVC

and RA and anterior forebrain regions lMAN and Area X, and these regions mediate song learning (Soderstrom and Tian, 2006). Expression of diacylglycerol lipase-α (DAGLα), a key enzyme in synthesis of endocannabinoid 2-arachidonoylglycerol (2-AG), similarly peaked during sensorimotor development (Soderstrom and Wilson, 2013). The role of endocannabinoids during sensorimotor development may represent a vulnerability to exogenous CB_1 receptor agonists. Chronic WIN 55,212-2 treatments during sensorimotor development, but not during adulthood, persistently increased 2-AG levels

FIGURE 1.1: Zebra finch descending motor and anterior forebrain pathway. Parasagittal section is ~2 mm from midline, and bar = 1.5 mm.

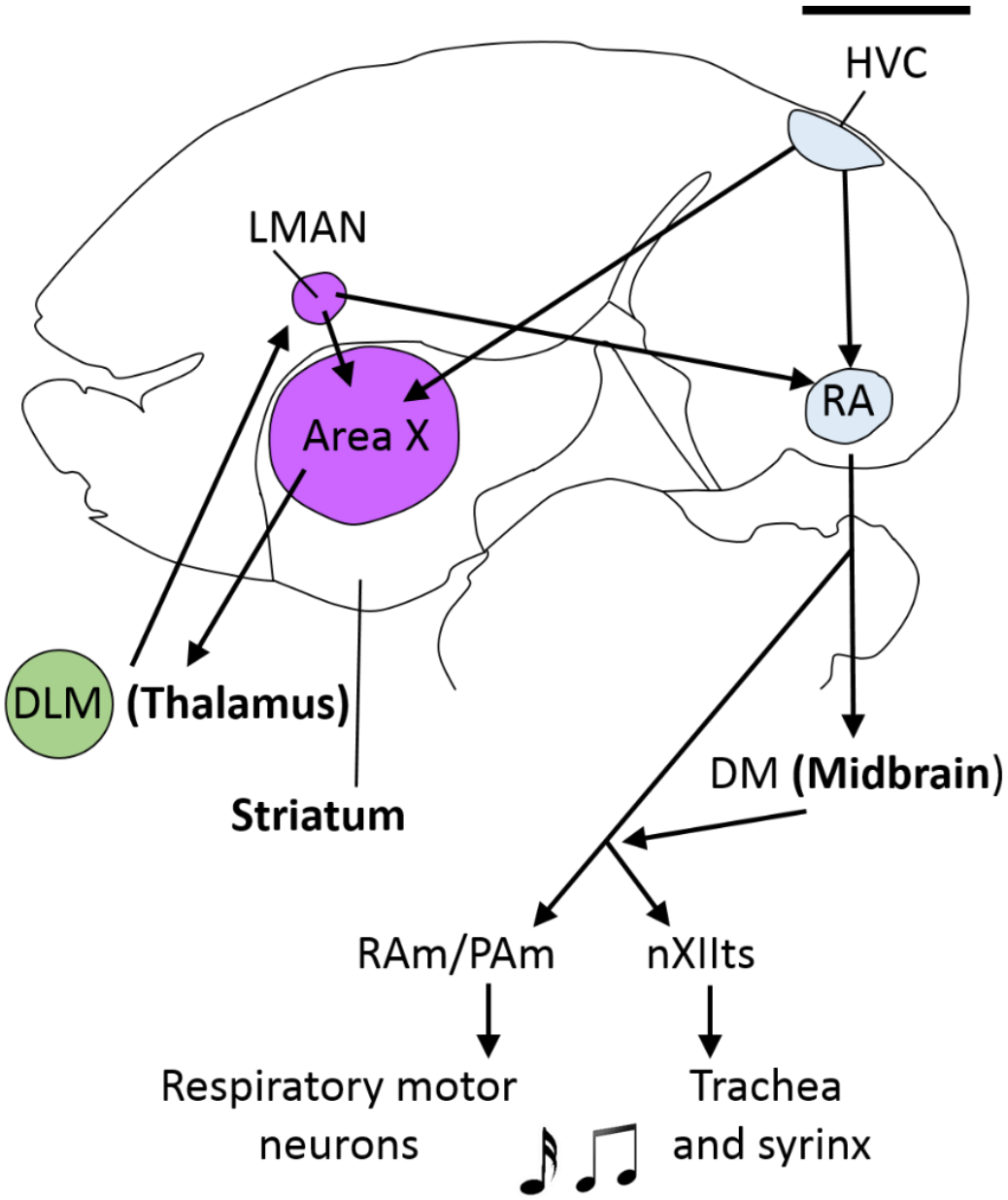
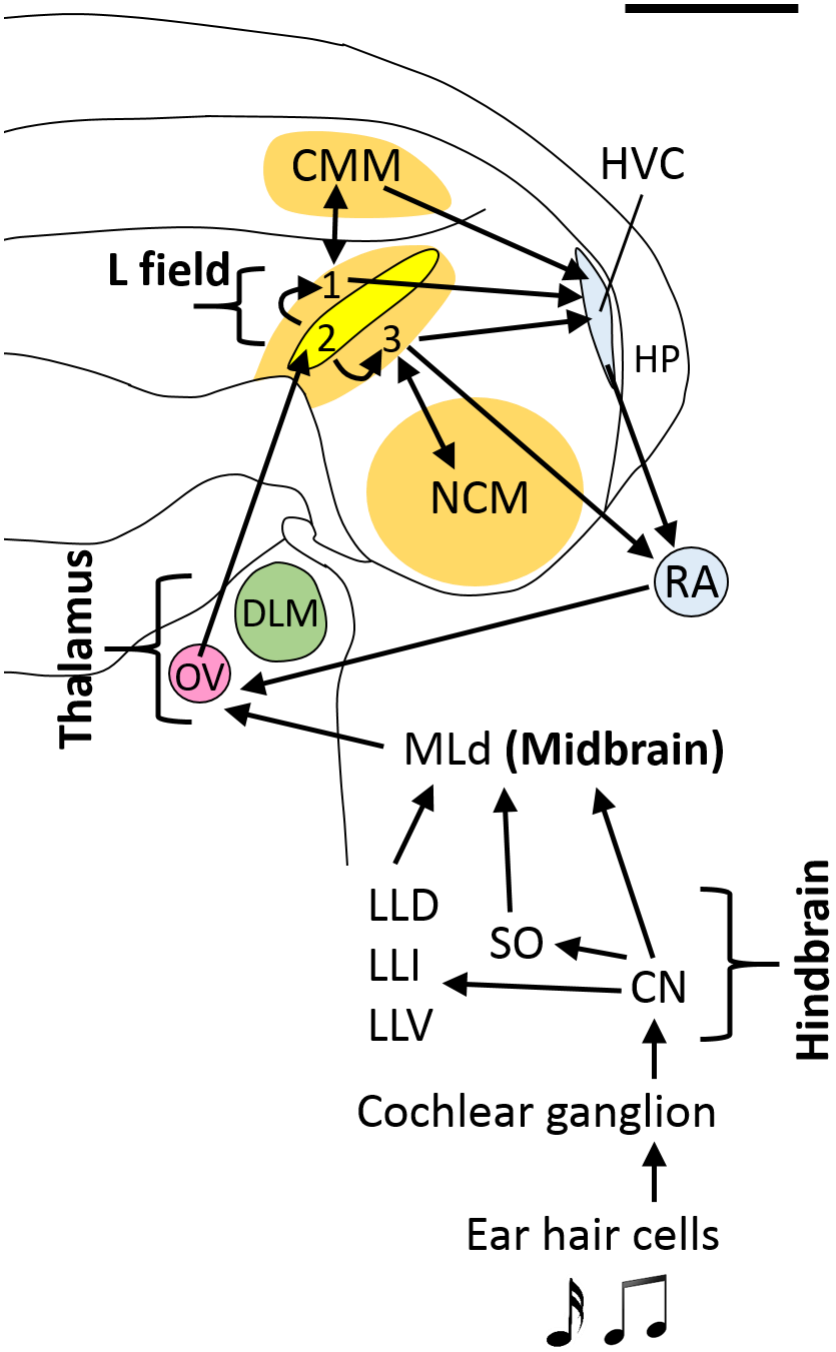

HVC
LMAN
Area X
RA
DLM
(Thalamus)
Striatum
DM (Midbrain)
RAm/PAm
nXIIts
Respiratory motor neurons
Trachea and syrinx

FIGURE 1.2: Zebra finch ascending auditory pathway. Parasagittal section is ~1 mm from midline, and bar = 1.5 mm.


CMM
HVC
L field
1
2
3
HP
NCM
RA
Thalamus
DLM
OV
MLd (Midbrain)
LLD
LLI
LLV
SO
CN
Hindbrain
Cochlear ganglion
Ear hair cells

in rostral telencephalon, which contains lMAN and Area X (Soderstrom et al., 2011), providing evidence that exogenous CB_1 receptor agonism persistently alters the endocannabinoid system in a developmentally-dependent manner. Developmental WIN 55,212-2 treatments resulted in adult songs with fewer syllables learned and less stereotypy compared to vehicle group, but adult treatment had no effect (Soderstrom and Johnson, 2003), which suggests that only the critical learning window is sensitive. WIN 55,212-2 also selectively alters neuronal morphology when administered during development. Following developmental treatment, dendritic spine density was decreased in adulthood in basal ganglia region Area X and premotor region HVC, which suggests that a disruption of the normal pruning of dendritic spines that occurs during sensorimotor development (Gilbert and Soderstrom, 2011). Following developmental WIN 55,212-2 treatments, axonal marker Nf-200 density was increased in Area X and motor regions HVC and RA, and dendritic marker MAP2 density was increased in HVC, RA, lMAN, and Area X (Gilbert and Soderstrom, 2014). Persistent alterations of the endocannabinoid system and morphology of neuronal networks in song regions may be an underlying mechanism of decreased song quality following WIN 55,212-2 treatments during sensorimotor development.

Cannabinoid CB_1 receptor agonists acutely disrupt perceptual sensory processing in zebra finches. Playback of a novel conspecific song rapidly induced expression of immediate-early gene zenk in NCM, a higher-order perceptual auditory region that resembles mammalian auditory association cortex and may be a neural substrate of song memory (Bolhuis and Gahr, 2006), and this response diminished following repeated exposure to novel song (Mello et al., 1995). Pretreatment with

cannabinoid agonist WIN 55,212-2 inhibited novel song-induced zenk expression and prevented habituation to repeated playback of song (Whitney et al., 2003). Similarly, novel song increased dendritic spine density in NCM, and WIN 55,212-2 pre-treatment inhibited this stimulation (Gilbert and Soderstrom, 2013), which suggests a disruption of auditory responsiveness.

Stress

Hypothalamic-pituitary-adrenal Axis

Endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are CB_1 receptor agonists and operate as an endogenous stress buffer system in the hypothalamic-pituitary-adrenal (HPA) stress axis (Evanson et al., 2010; Hill et al., 2009). A stressor activates corticotropin-releasing hormone cells in the paraventricular nucleus of the hypothalamus and prompts adrenocorticotropic hormone release from the pituitary gland. This stimulates the synthesis and release of corticosterone, a glucocorticoid hormone, from the adrenal gland. Corticosterone increases endocannabinoid signaling in the paraventricular nucleus and prefrontal cortex, which in a negative feedback mechanism, turns off the HPA axis stress response. AEA and 2-AG inhibit the HPA axis under basal or stress conditions, respectively. Amygdalar AEA inhibits the HPA axis under basal conditions. Following a stressor, AEA is rapidly hydrolyzed by fatty acid amide hydrolase (FAAH), allowing a surge in corticosterone release. 2-AG levels begin to increase and inhibit the HPA axis, and meanwhile AEA levels also increase to return to normal levels to terminate the neuroendocrine acute stress response and reinstate basal inhibition of the HPA axis (reviewed by Hill and Tasker, 2012).

Corticosterone binds to glucocorticoid receptors, which are present in virtually every animal cell, and the complex translocates into the cytosol and binds to glucocorticoid response elements in a transactivation mechanism. The release of corticosterone in the HPA axis following a stressor is an important component of the fight-or-flight response. While a stressor rapidly increases epinephrine and activates the

sympathetic nervous system, corticosterone release is delayed (reviewed by Romero and Butler, 2007). Corticosterone alters glucose metabolism for enhanced energy mobilization, and an acute stressor increased serum glucose levels (Armario et al., 1990). In addition to peripheral effects, corticosterone affects neuronal activity in the hippocampus and amygdala, which have a high density of glucocorticoid receptors (Morimoto et al., 1996), that alters brain function (Joels, 2011). In the hippocampus, acute corticosterone treatment decreased brain-derived neurotrophic factor (BDNF) and inhibited long-term potentiation formation (Zhou et al., 2000), that suggests impairment relevant to learning and memory. In amygdala, a corticosterone injection increased dendritic arborization (Mitra and Sapolsky, 2008), and amygdalar structural changes may facilitate the fight-or-flight response. Although acute stress effects are likely transient and adaptive for survival, evidence suggests chronic activation of the HPA axis is maladaptive. Corticosterone treatments for 21 days inhibited body weight gain, increased depression-like behavior in forced swim test, and dampened acute stressor-induced corticosterone increase in a challenge experiment, suggesting HPA axis dysregulation. A single corticosterone treatment did not produce these effects (Johnson et al., 2006). HPA axis dysregulation is associated with the neurobiology of psychological disorders, including depression (Aihara et al., 2007).

Acute stress

In songbirds, restraint stress results in endocrine, autonomic, and neurochemical effects. Restraint stress is a common acute paradigm in both rodents and songbirds that exploits the naturalistic fear of immobilization (reviewed by Pare and Glavin, 1986), but

that are relatively fewer studies in songbirds. A single restraint stressor of 5-60 minutes duration significantly increased plasma corticosterone levels rapidly in zebra finches (Ernst et al., 2016; Spencer et al., 2009; Soma et al., 2004), European starlings (Jones et al., 2016; Dickens et al., 2015), rufous-winged sparrows (Deviche et al., 2012; Deviche et al., 2010), house sparrows (Lattin et al., 2014; Fokidis et al., 2009), and snow buntings (Walker et al., 2015). In European starlings, CB_1 receptor antagonist/inverse agonist AM251 pretreatment dose-dependently potentiated this effect (Dickens et al., 2015), and implicates avian endocannabinoids in buffering the stress response. In free-ranging male rufous-winged sparrows in breeding condition, when testosterone in this species is seasonally elevated, restraint stress decreased plasma testosterone (Deviche et al., 2012; Deviche et al., 2010), and restraint stress may inhibit reproduction. Restraint stress increased heart rate in European starlings (Cyr et al., 2009), that indicates activation of the sympathetic nervous system. In zebra finches, playback of a novel conspecific song selectively induced immediate early gene ZENK expression, unlike other auditory stimuli, in higher-order perceptual auditory region NCM (Mello et al., 1992). Following restraint, conspecific song induced a ZENK response similar to other auditory stimuli (Park and Clayton, 2002), and stress may inhibit auditory discrimination.

In neural endocannabinoid content, restraint stress decreased amygdalar AEA and 2-AG, increased hypothalamic AEA, and increased hippocampal AEA in European starlings (Dickens et al., 2015). These results are dissimilar with effects observed in rodents. In the rodent amygdala, acute restraint stress decreased AEA but had no effect on 2-AG content (Hill et al., 2009; Rademacher et al., 2008). In the hypothalamus,

restraint stress increased 2-AG and had no effect on AEA (Evanson et al., 2010). In the hippocampus, restraint stress decreased AEA and increased 2-AG (Wang et al., 2012). Unlike rodents, European starlings are a species of songbird that possess a breeding season with increased HPA axis responsiveness and elevated corticosterone levels than during their molting season (Romero et al., 2000). The seasonal modulation of corticosterone by the HPA axis is important in this type of songbird, because low corticosterone levels are necessary for optimal regrowth of feathers during the molting season (Romero et al., 2005). To better understand the role of endocannabinoids in the avian stress response, more research is needed in a non-seasonal songbird, such as the zebra finch.

Chronic stress

Stressful life events can precede the onset of depression (Muscatell et al., 2009), and chronic, mild unpredictable stress is a paradigm that results in a depression-like phenotype in animals. It includes chronic administration of a variety of mild stressors, such as cage tilt, white noise, or food and water deprivation in an unpredictable schedule. A reduction of sucrose preference (anhedonia) following this paradigm is reversible by tricyclic antidepressant treatment (Willner et al., 1987). Following repeated administration of a single stressor, habituation of the acute response in serum corticosterone and cortex c-Fos mRNA occurred (Melia et al., 1994), but an unpredictable, random administration of multiple stressors prevents habituation and produces long-term effects.

Fewer studies in songbirds exist. The chronic, mild unpredictable stress paradigm alters endocrine, autonomic, reproductive success, and song behavior endpoints. In adult European starlings, the paradigms included 30 minute stressors, such as loud radio, predator calls, novel object, predator decoy, restraint stress, rocking bird's cage, or human voice. Four to five were randomly administered per day for eight to 20 days. The stress paradigms decreased baseline plasma corticosterone levels (Cyr et al., 2007; Rich et al., 2005) and glucocorticoid receptor mRNA expression in the hypothalamic paraventricular nucleus (Dickens et al., 2009). Chronic stress may persistently dysregulate the HPA axis and result in hypoactive responsivity. Chronic stress increased daytime baseline heart rate but the effects did not persist following cessation of the paradigm (Cyr et al., 2009). Following chronic stress, female European starlings had poorer reproductive success, and their nestlings had enhanced corticosterone response to acute stress (Cyr and Romero, 2007), that indicates that effects of chronic stress may extend to the next generation. In fledgling European starlings (30-50 days old), an unpredictable food restriction (4 hours daily) paradigm for three months during development increased weight in stress group during treatment period. In adulthood, the stress group, despite the absence of evidence of malnutrition following treatment, spent less time singing, sang fewer and shorter bouts, and took longer to start singing (Buchanan et al., 2003), which suggests that adult birdsong is an indication of quality of developmental environment. European starlings are open-ended song learners and can learn songs in adulthood. The current project evaluated the effects of chronic stress during sensorimotor development (50-75 days old) in zebra finches, which possess a closed, developmental critical period of song learning, and this

songbird model allows elucidation of effects on vocal learning that originate from treatments during that critical period.

Adolescence is a coming-of-age transitional stage of consequential cognitive, social, and neurobiological development. The ongoing maturation processes of important brain regions during this stage may be uniquely vulnerable to chronic stress in a way that shapes adulthood behavior and neural networks.

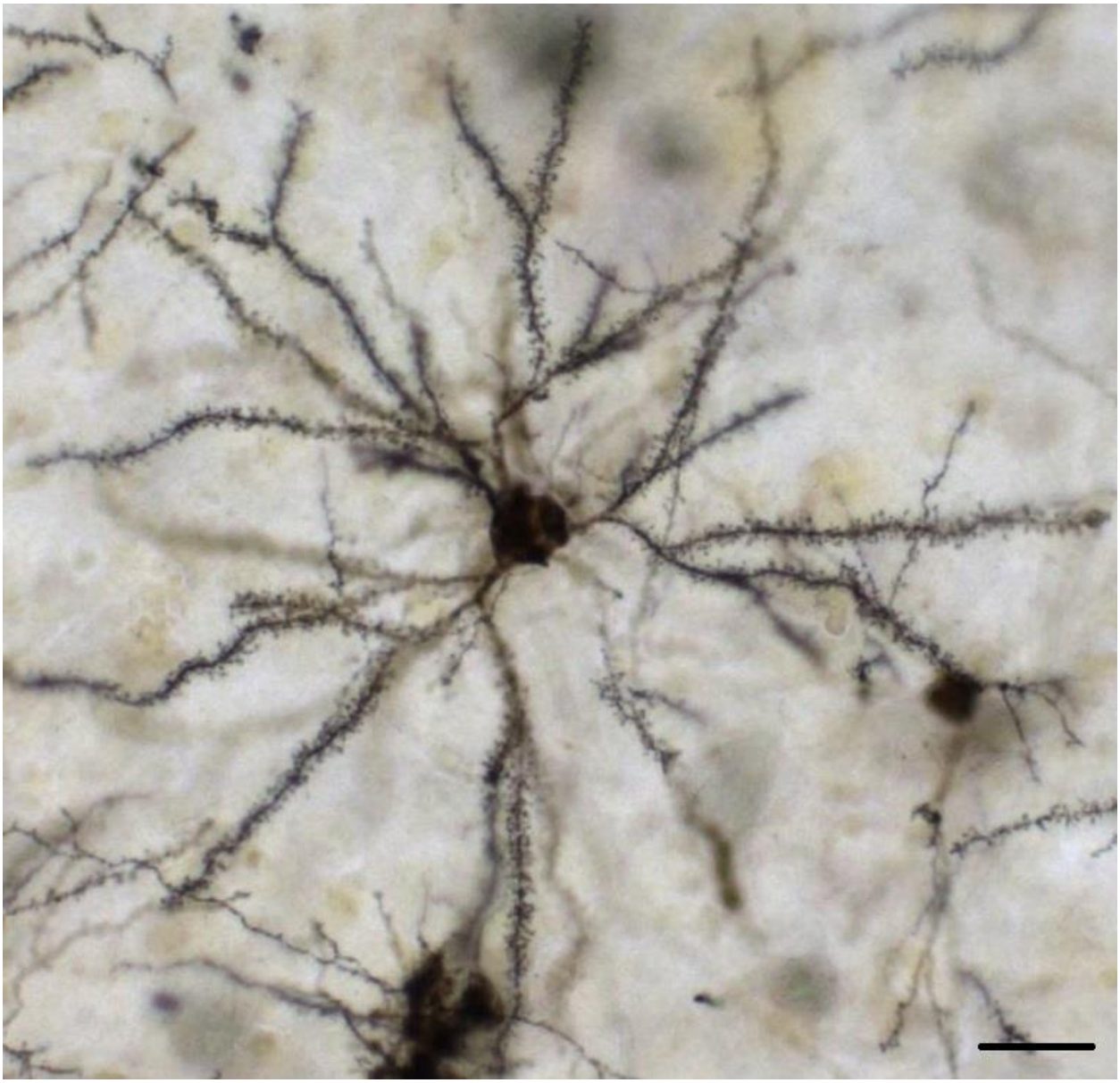
Dendritic spines

Significance and Function

Dendritic spines are small protrusions on dendrites that operate as the anatomical basis of postsynaptic sites, with typically excitatory input. An important significance of these features is that they may act as morphological building blocks of learning and memory (reviewed by Bhatt et al., 2009). Hebbian learning in synaptic plasticity states “cells that fire together, wire together,” and memories can be rapidly formed and lead to persistent structural changes to dendritic spines (Munakata and Pfaffly, 2004). In hippocampal CA1 pyramidal neurons following a spatial learning task, rats had an increased density of dendritic spines, but no change in dendritic length (Moser et al., 1994), and evidence suggests spines have dynamic populations while neurite lengths remain relatively unaltered following formation. A structure-function relationship underlies enlargement of dendritic spines, which are actin-rich. Experience and dynamics of synaptic strength drive changes to enlargement of spines in actin cytoskeleton remodeling (Hotulainen and Hoogenradd, 2010) in a mechanism that is protein synthesis-dependent (Tanaka et al., 2008).

Several methods are used to study dendritic spines. The most classical, Golgi-Cox staining (**Fig. 1.3**), uses dissected brains and heavy metal solutions of potassium dichromate, mercury chloride, and potassium chromate to impregnate approximately <5% of neurons in an apparent random manner to allow visualization with light microscopy of anatomical features, including cell bodies, axons, dendritic arborization, and spines with low background (Zaqout and Kaindl, 2016). A limitation of the Golgi-Cox technique is only net changes in spine density are observed, which makes it unclear if

FIGURE 1.3: Golgi-Cox stained spiny neuron in zebra finch NCM, 200X magnification, bar = 31 µm.



either spine formation or elimination were primarily effected, so it is a “flashbulb” approach that evaluates the overall effects on spine density at time of euthanasia. Time-lapse imaging with two-photon microscopy evaluates neurons over the course of multiple imaging sessions in live animals to evaluate the dynamics of spines, including transience or persistence of newly formed spines and the changes in individual spines over time (Holtmaat et al., 2005). Spine dynamics is important to circuit remodeling over the longitudinal course of development or during a learning task. In two-photon optogenetics, enlarged spines are labeled with a light-sensitive synaptic probe, and light induces the erasure of labeled spines. Following a rotarod training, newly potentiated spines in motor cortex were labeled with synaptic optoprobe AS-PARac1 (activated synapse targeting photoactivatable Rac1). A blue laser activated Rac1, and chronic activation of Rac1 resulted in selective shrinkage of newly potentiated spines. When animals were retested in the rotarod task, they had diminished performance despite previous evidence of learning, implicating causation in the relationship between spine formation and learning, unlike correlational evidence in previous studies. In the same study, in comparison of spines recruited in different behavioral assays, rotarod and balance beam tests potentiated different spine populations, with some overlap, in motor cortex, indicating task specificity of the function of dendritic spines (Hayashi-Takagi et al., 2015). Advanced techniques have illuminated mechanisms of the structure and function of dendritic spines with important implications in understanding neural circuitry. This dissertation used Golgi-Cox histology to evaluate net effects on spine density as a function of acute or chronic stress paradigms, Δ^9-THC treatments, and adult or late postnatal developmental age. The results are promising for future experiments

examining underlying mechanisms of these results in time-lapse evaluations of spine dynamics in newer techniques, such as two-photon microscopy.

Late Postnatal Brain Development

Processes important to CNS development during late-postnatal development include activity- and experience-dependent establishment of synaptic networks during brain maturation. In cortical regions of rodents (Blue and Parnavelas, 1983; De Felipe et al., 1997) and primates (Bourgeois and Rakic, 1993; Huttenlocher, 1990) development is associated with a general profusion of synaptic contacts followed by a reduction of spine densities to adulthood. In songbirds, similar processes occur in at least one cortical-like region necessary for zebra finch vocal learning (lMAN, Nixdorf-Bergweiler et al., 1995). Importantly, these developmental spine density reductions are inhibited by manipulations that alter normal vocal learning, including rearing in social isolation (Wallhäusser-Franke et al., 1995) and exposure to cannabinoid agonists (Gilbert and Soderstrom, 2011). Adult dendritic spine populations are relatively stable in total number compared to during development (Grutzendler et al., 2002).

Evidence suggests dendritic spine populations are sensitive to stress (Leuner and Shors, 2013), and the net dendritic spine elimination during sensorimotor development may be uniquely vulnerable to psychological stress. Glucocorticoids regulate dendritic spine dynamics in both adolescents and adults. In transcranial two-photon microscopy, cortical corticosterone injections dose-dependently increased spine formation and spine elimination in adolescents and adults, and the magnitude of

enhanced three-day spine turnover was greater in adolescents. Additional evidence in the same study is that injection of dexamethasone, which inhibits adrenocorticotropic hormone production from the anterior pituitary and suppresses corticosterone release from the adrenal gland, decreased spine turnover and inhibited the effects of corticosterone on spine turnover. Similarly, treatments with corticosterone receptor antagonists spironolactone, a mineralocorticoid receptor antagonist, and mifepristone, a glucocorticoid receptor antagonist, reduced spine turnover and inhibited corticosterone's stimulation of spine turnover (Liston and Gan, 2011), results that further implicate glucocorticoids in regulating dendritic spine remodeling. An acute multimodal stress paradigm impaired memory in novel object recognition test, which corresponded to decreased hippocampal long-term potentiation magnitude and dendritic spine density (Chen et al., 2010), that demonstrates that the effects of stress on dendritic spine populations has behavioral correlates. Chronic stress paradigms modeling depression alter hippocampal or prefrontal cortex dendritic spine density, typically causing atrophy of spine populations (reviewed by Qiao et al., 2016). Dendritic spine dynamics may be relevant to the mechanism of depression and subsequent alleviation of symptoms by antidepressants by guiding neuronal network reorganization (Castren, 2013). Olfactory bulbectomy, an animal model for depression, decreased hippocampal spine density, which was reversible by treatment with tricyclic antidepressant amitriptyline (Norrholm and Ouimet, 2001). The differing spine dynamics during adolescence and adulthood may result in different long-term effects of stress on dendritic spines during development.

CHAPTER TWO: EXPERIMENTAL METHODS

General Approach

For acute stress experiments, adult zebra finches were used, and the groups were No stress or Stress. Experiments evaluated plasma corticosterone concentration, song analysis, dendritic spine density, and c-Fos immunohistochemistry (**Fig. 2.1**).

For chronic stress experiments, developing and adult zebra finches were treated with Δ^9-THC (3 mg/kg) and chronic mild stressors. In developing and adult zebra finches, the groups were Vehicle + No Stress, Δ^9-THC + No Stress, Vehicle + Stress, Δ^9-THC + Stress, for eight groups total. Zebra finches were treated daily for 25 days followed by 25 days of no treatment, in order to allow developmental groups to mature. Experiments evaluated plasma corticosterone concentration, song analysis, and dendritic spine density (**Fig. 2.2**).

Animals

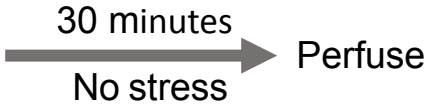
Developing (50 ± 3 days old) or adult (>100 days old) male zebra finches were obtained from a breeding colony at Brody School of Medicine, East Carolina University. All husbandry and experimental procedures were approved and supervised by their Institutional Animal Care and Use Committee. Animals were housed with an adult male tutor at ages 33-40 days old in a mixed-sex cage and then housed in a male-only cage until adulthood. Animals were single-housed during experiments. *Ad libitum* bird seed

FIGURE 2.1: Acute stress paradigms for vocal recording experiment (**A**), histology experiments (**B**), drug pretreatment experiments (**C**).

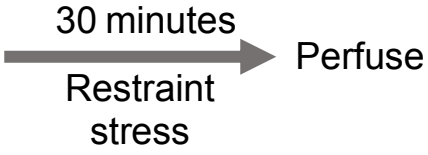
A. No stress control group

Stress group

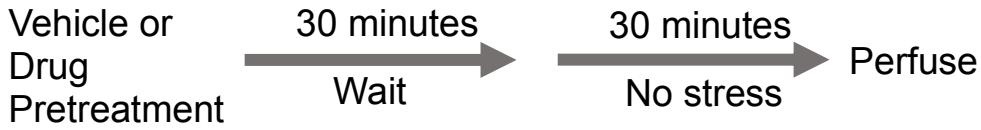
B. No stress control group



Stress group



C. No stress control group



Stress group

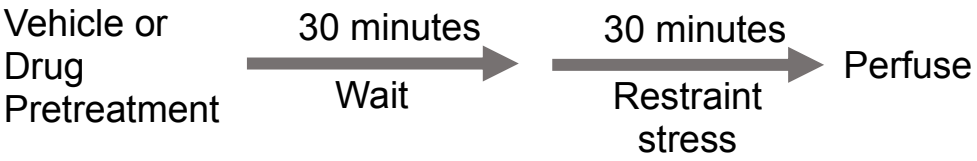
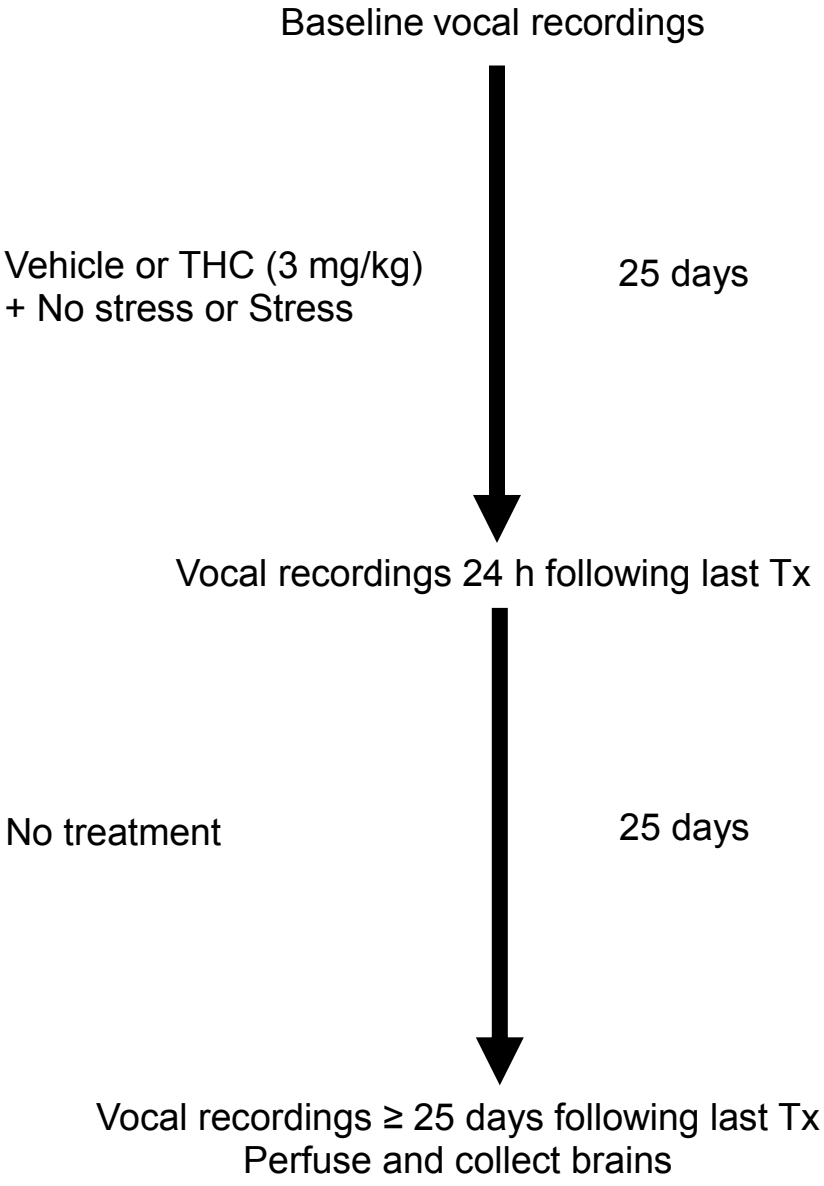


FIGURE 2.2: Chronic mild stress paradigm.


Baseline vocal recordings
Vehicle or THC (3 mg/kg)
+ No stress or Stress
25 days
Vocal recordings 24 h following last Tx
No treatment
25 days
Vocal recordings ≥ 25 days following last Tx
Perfuse and collect brains

and water were available at all times, except during restraint stress procedure.

Drugs

CB_1 receptor partial agonist Δ^9-tetrahydrocannabinol (Δ^9-THC) was obtained in ethanol (NIDA drug supply program), and ethanol was blown off with nitrogen gas. Δ^9-THC was dissolved in dimethyl sulfoxide (DMSO), and 3 mg/kg injections were prepared with Δ^9-THC + DMSO stock solution, Alkamuls EL-620, and phosphate-buffered saline (PBS) in a 1:1:18 ratio. CB_1 receptor antagonist/inverse agonist SR141716 was obtained (NIDA drug supply program), dissolved in DMSO, and 6 mg/kg injections were prepared with a 1:1:18 ratio of DMSO, Alkamuls, and PBS. Vehicle injections were DMSO, Alkamuls, and PBS, 1:1:18 ratio. Each dose had a 50 µL volume, and intramuscular injections in pectoral muscle were given with a 30 gauge needle.

Acute Stress Experiments

Paradigm

In the restraint stress + histology experiment, 24 h prior to experiment, one animal for each group (No stress and Stress) was moved from group housing to single cages. For the restraint stress procedure, first the Stress group animal's cage was removed from the room, and the animal was placed in paper bag for 30 minutes. Then the No stress animal was rapidly caught, and blood was collected from brachial vein

with a 25 gauge needle in heparin-treated capillary tubes within three minutes of capture. Equithesin was injected in pectoral muscle and transcardially perfused with ice-cold phosphate-buffered saline (PBS). Immediately following the cessation of the stressor, the Stress animal had blood collected and was similarly injected with Equithesin and perfused. When the brains were collected, one hemisphere was placed in Golgi-Cox solution for 5 days for neuronal morphology experiments, and the other hemisphere was placed in 4% para-formaldehyde for 24 h for immunohistochemistry experiments.

In the drug treatment + restraint stress + histology experiment, 24 h prior to experiment, 4 animals were moved from male-only, group housing to single housing. At 9 AM, the 4 animals were transported to laboratory, a novel environment. At 12 PM, first animal was injected with drug (vehicle, THC, SR, or SR + THC). For SR + THC groups, SR was injected first, followed by THC five minutes later. 15 minutes after the first animal, the second animal received an injection in order to stagger each animal in 15 minute increments. Stress groups and no stress groups were conducted on separate days. For restraint stress paradigm, 30 minutes following drug injection (vehicle, THC, or SR), the animal was placed in paper bag for 30 minutes. Following cessation of stressor, the bird was immediately removed and Equithesin was injected in pectoral muscle. Blood was collected from jugular vein in heparin-treated capillary tubes. A transcardial perfusion was performed with ice-cold phosphate-buffered saline (PBS), and the brain was placed in 4% para-formaldehyde for 24 h for immunohistochemistry experiments. For no stress groups, one hour following drug injection, animal had Equithesin injection, blood collection, and perfusion.

In the restraint stress + vocal recordings experiment, the day prior to initiation of recording sessions, two male birds were transported to recording room for 3 hours as an introduction to the environment. For baseline recordings, they were recorded for 3 hours, and for the post-treatment recordings on the following day, birds were recorded for 1 hour. For restraint paradigm, Stress group birds were placed in paper bag for 30 minutes immediately following transport to recording room. No Stress group birds were instead placed in recording chamber for 30 minutes prior to initiation of recording session. During recording, each male bird had a female audience bird as a social stimulus. Stress and No Stress groups were recorded separately. Two male birds were recorded at a time in visual, but not acoustic, isolation.

Song Analysis

Avisoft Bioacoustics Recorder software (4.2.22) was used for vocal recordings. Not all birds sang in the recording paradigm, and the inclusionary criteria was that at least 10 songs were performed during initial 3 hour baseline recording session. Birds were randomly assigned to groups, and 3 out of 7 No stress birds and 3 out of 8 Stress birds did not perform adequately in baseline recordings (i.e., too few songs) to be included in the experiment. Using unidirectional microphones, vocal recordings were obtained 12-3 PM at 22050 sampling rate and 16 bit format. Birds were continuously recorded, and time-stamped audio files (.wav) were created by sound-activated, internal logic trigger criteria. A threshold for trigger events was energy level 5% and range 1.8-250 kHz to omit ambient noise from being recorded. To trigger the recording, sounds must be above threshold and also >1 second in duration. Pre- and post-trigger buffer period was set to include 0.5 seconds before and 0.5 seconds after the recording was triggered.

To compare post-treatment song recordings to baseline recordings, a previous acoustic features analysis in zebra finches was adapted that compared adult songs following lMAN lesions to pre-surgery, baseline recordings (Thompson et al., 2011). To sort and to isolate the audio files that contained song syllables, the files were batch-processed with Spectrogram 13.0 (Visualization Software) to convert the audio files (*.wav) to spectrogram images (*.jpg), and images were visually inspected to delete files that did not contain song syllables.

Using Sound Analysis Pro software (version 2011.104), each bird's song files were segmented into syllables according to an amplitude threshold that was set optimally for each individual bird. These parameters were used to batch-process each bird's files to obtain acoustic feature values for each syllable. In MATLAB 7, scatter plots of acoustic features were created to assess changes in median and interquartile range (IQR) for each acoustic feature following acute restraint stress. Median and IQR were selected as measures of central tendency and variability, respectively, because they are less sensitive to outliers in scatterplots than mean and standard deviation. Syllable duration, entropy, FM, pitch, and pitch goodness were the temporal and spectral features selected to study changes in adult zebra finch song performance following acute restraint stress. Previously, the IQRs, but not the medians, of these features were altered compared to baselines following lesions of lMAN, a component of the basal ganglia-thalamocortical song circuit, in adult zebra finches (Thompson JA et al., 2011), and these features may have biological significance relevant to songbird communication.

For each bird's baseline and post-treatment recordings, four scatterplots were created in MATLAB 7 with Sound Analysis Pro output with syllable duration on the x-axis and entropy, FM, pitch, or pitch goodness on the y-axes, respectively. When acoustic features are plotted on a scatter plot, syllable clusters are apparent. Identical parameters labeling each syllable were applied to each bird's baseline and post-treatment scatterplots. For the scatterplot of each acoustic feature, x- and y-values were exported for each syllable in each syllable cluster. In Excel, the median and IQR for each syllable for each acoustic feature were calculated.

Chronic Stress Experiments

Paradigm

A previously described protocol for chronic mild stress in rodents, that induced a depression-like behavioral phenotype and altered the endocannabinoid system in rats, was adapted to the zebra finch (Hill et al., 2008). Concurrent treatment (vehicle or THC, 3 mg/kg) and daily, randomly-selected, unpredictable mild stressors were administered for 25 days followed by 25 days of no treatment to evaluate persistence of effects and to allow developing animals to reach maturation. Developing animals were treated during sensorimotor development (50-75 ± 3 days old). Animals were divided into four groups: vehicle + no stress, THC + no stress, vehicle + stress, and THC + stress. Injections were administered daily at 11 AM. Five stressors were used: food and water deprivation (1 hour), bright light (1 hour), loud white noise (1 hour), restraint stress in paper bag (30

minutes), and rubber “snake” in cage (1 hour). Per day, 2-3 stressors were randomly selected and administered at least 1 hour apart 2-7 PM.

Song Analysis

Similar recording parameters were used as in acute experiments. Three recording sessions were performed for 24 hours each to evaluate the short-term and long-term effects of treatment, and included baseline recordings, initial post-treatment recordings (24 hours following last treatment), and delayed post-treatment recordings (25 days following last treatment). An inclusion criterion was that birds had to produce at least 30 song files per recording session to have adequate numbers to conduct the song analysis. In the developing groups, one out of ten vehicle + no stress birds and two out of 11 THC + stress birds did not perform enough songs. In adult groups, one out of ten vehicle + no stress birds, three out of seven THC + no stress birds, two out of eight THC + no stress birds, and two out of eight vehicle + no stress birds did not produce enough songs.

Since the chronic experiments included developmental treatment groups and zebra finches develop greater stereotypy of syllable sequence as a function of development (Pytte et al., 2007), analysis of atypical syllable transitions was performed in addition to acoustic features. A similar strategy was used as the acute experiment analysis, but SongSeq software (v1.1) was used for sequence and acoustic feature analysis instead of MATLAB scatter plots in a previously described protocol (Daou et al., 2012).

Histology

Golgi-Cox Staining

Following transcardial perfusion with PBS, one brain hemisphere was placed in Golgi-Cox solution (5% potassium dichromate, 5% mercury chloride, 5% potassium chromate) for five days at room temperature in dark. The brains were placed in 30% sucrose solution at 4° C for > 7 days. With a vibratome, 125 µm thick sagittal sections were collected in 20% sucrose solution. Free-floating sections were placed in ice-cold serial glycerol solutions (80, 60, 40, and 20%) for 2 minutes each. Sections were placed in ice-cold dH_2O for 2 minutes and then washed in ice-cold dH_2O 3 times. Sections were placed in ice-cold gold chloride solution (50 mg/dL) for 80 minutes on ice. Sections were washed in ice-cold dH_2O 3 times and placed in ice-cold 0.2% oxalic acid solution for 1 minute 15 seconds.

Sections were removed from ice, washed in ice-cold dH_2O 3 times, and placed in 0.1% sodium thiosulfate solution for 30 minutes at room temperature and then a fresh portion of sodium thiosulfate solution for an additional 30 minutes. The sections were washed in ice-cold dH_2O 3 times and placed in 7% ammonium hydroxide for 30 minutes in the dark. The sections were washed in ice-cold dH_2O 3 times and placed in Kodak Fixer solution for 30 minutes in the dark. The sections were washed in ice-cold dH_2O 3 times and mounted to slides with 0.3% gelatin solution. When the slides were dry, they were hydrated with distilled water (dH_20) for 1 minute, serially dehydrated with ethanol solutions (50, 70, and 95%) for 1 minute each and 100% ethanol for 5 minutes, cleared with xylene for 10 minutes, and coverslipped with permount.

Neurolucida software (9.14.15) was used to create 3D neuronal reconstructions. At 100X magnification, 20 spiny neurons were identified and labeled. At 1000X magnification, five randomly-selected neurons were reconstructed, outlining cell body and dendritic trees and placing markers on each dendritic spine. Dendritic spine, dendritic length, and dendritic branching data were exported with Neurolucida Explorer software.

C-Fos Immunohistochemistry

Following transcardial perfusion with PBS, one brain hemisphere was fixed via immersion in 4% para-formaldehyde solution for 24 h. Then brain was placed in 20% sucrose solution at 4° C for > 2 days. With vibratome, 30 µm thick sagittal sections were obtained in PBS. The free-floating sections were washed 3 times in PBS for 3 minutes and pretreated with 1% hydrogen peroxide solution for thirty minutes. The sections were washed again 3 times in PBS for 3 minutes and pretreated with 5% normal goat serum solution for 30 minutes. The sections were incubated in the primary rabbit polyclonal c-Fos antibody (K-25, sc-253, lot #K0104, Santa Cruz Biotechnology), 1:3000 dilution, for 12-24 hours.

Following primary antibody incubation, the sections were washed 3 times in PBS for 3 minutes and incubated in biotinylated anti-rabbit secondary antibody (BA-1000, lot #S0320, Vector Laboratories), 1:3000 dilution, for 1 hour. Sections were washed 3 times in PBS for 3 minutes and incubated in avidin-biotin complex (ABC) solution (PK-6100, Vector Laboratories), 1:50 dilution, for 1 hour. ABC solution was prepared 1 hour

in advance. Sections were washed 3 times in PBS for 3 minutes and stained with DAB substrate in 0.1% hydrogen peroxide solution (5 mg DAB tablets, Amresco). Sections were washed 3 times for 3 minutes and mounted to slides with 0.3% gelatin solution. After drying overnight, slides were hydrated with dH_20 for 3 minutes, serially dehydrated with ethanol solutions (70, 95, and 100%) for 30 seconds each, cleared with xylene for 1.5 minutes, and coverslipped with permount.

To count the density of c-Fos-labeled nuclei, Image-Pro Plus software (6.3.0.512) was used. A macro was created to apply the same procedure to each image at 100X magnification. For each region of interest, the image was converted to grayscale 8, and the contrast between the labeled nuclei and background tissue was enhanced. The automatic count feature included objects with an area 20-500 and characterized clusters. The area measurement was based on a spatial calibration. Every collected section was included in analysis, and a mean was calculated for each animal.

Corticosterone Enzyme Immunoassay

The blood was centrifuged for 10 minutes at 10,000 rpm to separate the plasma, which was stored at -80° C for enzyme immunoassay experiments. Cayman Chemical corticosterone ELISA kit (item no. 501320) was used. The assay contains a corticosterone-acetylcholinesterase conjugate tracer that is competitive with corticosterone in the sample to bind with a limited amount of corticosterone antiserum. Ellman's reagent causes a colorimetric, enzymatic reaction. The color intensity is read with a plate reader at wavelength 412 nm and is inversely proportional to the

concentration of corticosterone in the sample. The sensitivity of the kit is 30 pg/mL. For each plate, blank, non-specific binding, total activity, and maximum binding wells were produced, and a standard curve was determined. Each sample was assessed in duplicate.

Statistics

In acute restraint stress experiments for corticosterone, song activity, dendritic spine density, and c-Fos immunohistochemistry, two-tailed Student's t-tests were used, No stress vs. Stress, alpha = 0.05. In song analysis of number of syllables and acoustic features, baseline recordings were compared to post No stress or Stress recordings for each zebra finch in a paired design. Two-tailed paired t-test or Wilcoxon signed-rank tests were used, alpha = 0.05, depending on the normality of the data. For drug pretreatment + acute stress experiments, one-way ANOVAs were used with Tukey HSD post-hoc test, and groups were considered significantly different from vehicle control if $p < 0.05$. In chronic experiments, one-way ANOVAs with Tukey HSD post-hoc tests were performed with alpha = 0.05. Syllable entropy was log transformed to normalize data.

CHAPTER THREE: RESULTS

Acute Stress

Plasma Corticosterone

An acute stressor stimulates a neuroendocrine response in the hypothalamic-pituitary-adrenal (HPA) axis that prompts the release of glucocorticoid stress hormone corticosterone from the adrenal gland. Corticosterone enters the circulation and acts on the body in ways that may be adaptive in a fight-or-flight scenario, such as energy mobilization, muscle tension, and hypervigilance to threats. Corticosterone additionally acts on HPA axis in negative feedback mechanism to turn off the acute stress response. Thirty minutes of restraint stress increased plasma corticosterone levels compared to No stress controls, 0.83 ± 0.11 vs. 1.71 ± 0.29 ng/mL ($t_6 = 8.856$, $p < 0.05$), Student's t-test, two-tailed (**Fig. 3.1**).

Song Activity

The effect of acute restraint stress on song production was measured in two ways: by evaluating the number of song files recorded with the software trigger criteria and by comparing the number of occurrences of each unique syllable in baseline and experimental recordings (**Fig. 3.2**). Mean number of song files per hour did not significantly differ (58.85 ± 18.65 vs. 47.45 ± 18.22, $t_7 = 0.432$, $p = 0.678$, Student's t-test, two-tailed). Following restraint stress, the number of syllables increased compared to baseline recordings ($p < 0.05$), Wilcoxon signed-rank test.

FIGURE 3.1: Acute restraint stress increased plasma corticosterone levels (*$p < 0.05$, Student's t-test, two-tailed) measured by enzyme immunoassay (n = 4 animals). Error bars = SEM.

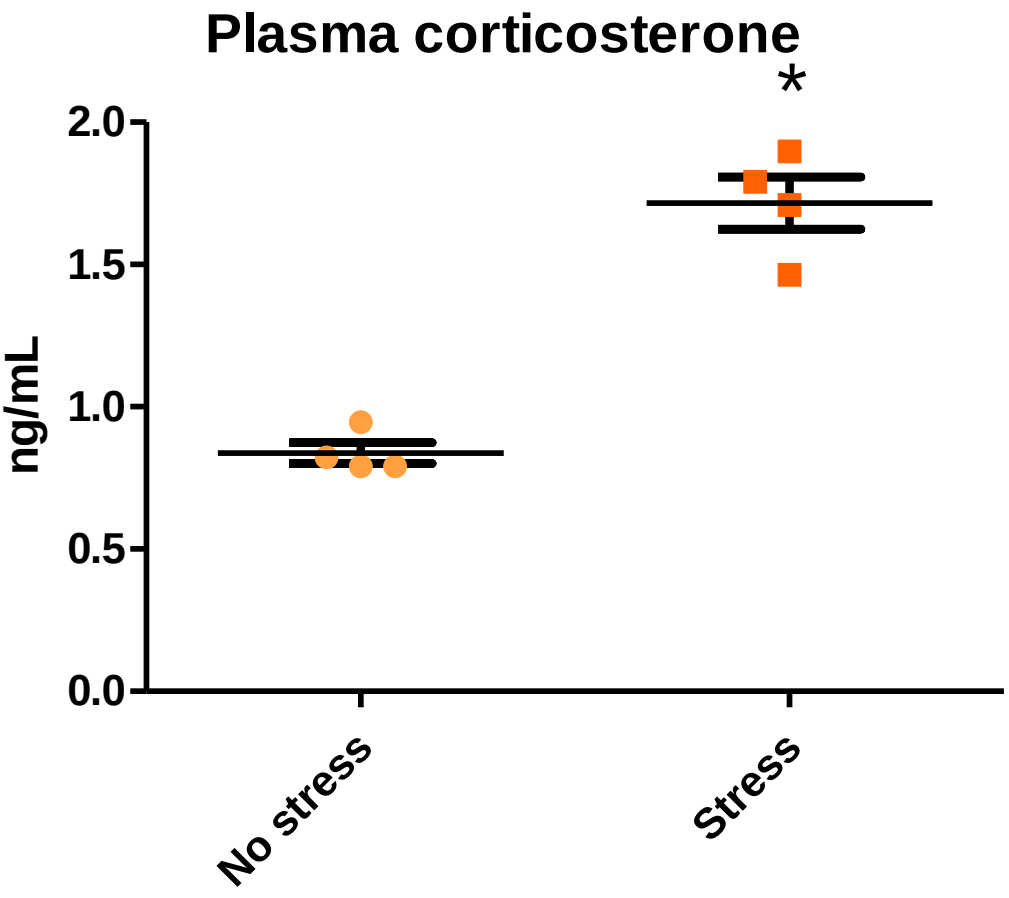
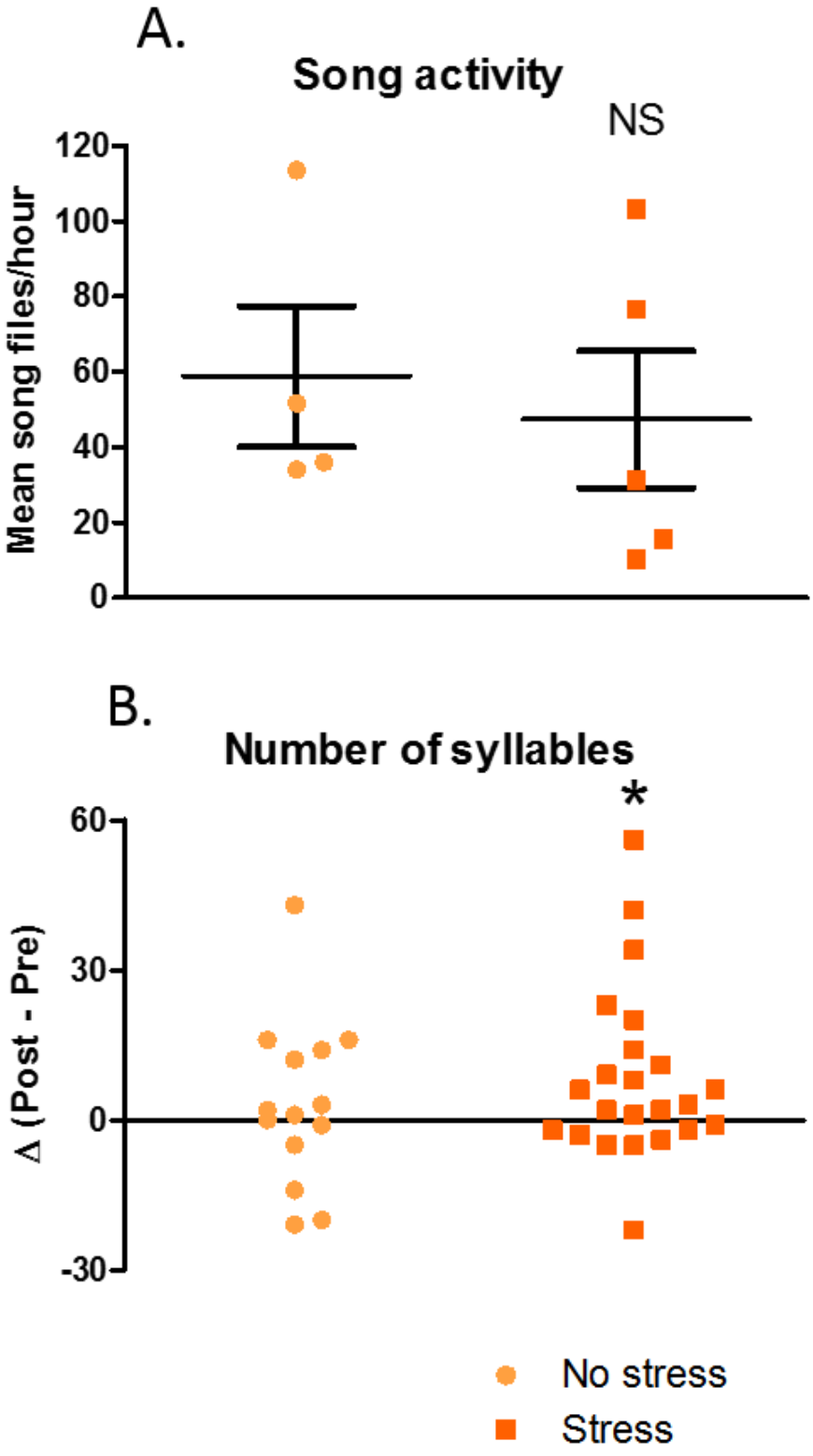

Plasma corticosterone
*
2.0
1.5
1.0
0.5
0.0
ng/mL
No stress
Stress

FIGURE 3.2: Number of song files and song syllables produced one hour following cessation of acute restraint stressor (n = 4-5 animals). Restraint stress had no significant effect on mean songs/hour **(A)**, $p = 0.16$, two-tailed paired t-test. Restraint stress significantly increased the number of syllables performed compared to baseline recordings **(B)**, $p < 0.05$, Wilcoxon signed-rank test. In **A**, each data point represents the mean songs per hour for one animal, and in **B**, each data point represents the difference in number between baseline and post-treatment recordings for one syllable. Error bars = SEM.


A.
Song activity
NS
Mean song files/hour
120
100
80
60
40
20
0
B.
Number of syllables
*
Δ (Post - Pre)
60
30
0
-30
No stress
Stress

Song Acoustic Features

Each male zebra finch sings a unique song that is learned during a developmental critical period and is composed of a sequence of syllables in a motif. The effect of acute restraint stress on adult performance of temporal and spectral features was measured by evaluating the central tendency, (median), and the variability, (interquartile range, IQR), of syllable duration, pitch, entropy, FM, and pitch goodness for each unique syllable compared to baseline recordings (**Fig. 3.3**). These five were selected, because previously, lMAN lesions in adult zebra finches increased the IQR of these acoustic features (Thompson JA et al., 2011), and these features may have biological significance in songbird communication. Following restraint stress, syllable medians of syllable duration, pitch, and entropy and IQRs of pitch goodness and FM were significantly increased compared to baseline recordings. A representative scatter plot of restraint stress-induced increases of the central tendency of syllable entropy and duration show a shift of post-stress clusters from baseline clusters, and no shift is apparent in recordings from a bird in the No Stress group (**Fig. 3.4**). Paired t-tests or Wilcoxon signed-rank tests were used, depending on the normality of data, and alpha = 0.05.

Dendritic Spine Density

Golgi-Cox stained neurons in brain regions involved in song perception, production, and learning pathways (**Fig. 3.5**) were evaluated to determine if the effects of acute restraint stress on dendritic spine density occurred globally or

FIGURE 3.3: Syllable median and interquartile range (IQR) of temporal and spectral features performed one hour following cessation of acute restraint stress (n = 4-5 animals). Compared to baseline recordings, restraint stress increased syllable medians of syllable duration, pitch, and entropy **(A, C, E)** but not pitch goodness or FM **(G, I)**. Conversely, restraint stress increased syllable IQRs of pitch goodness and FM (**H,J**) but not syllable duration, pitch, or entropy **(B, D, F)**. *$p < 0.05$, two-tailed paired t-test or Wilcoxon signed-rank test. Each data point represents the difference between baseline and post-treatment recordings for one syllable.

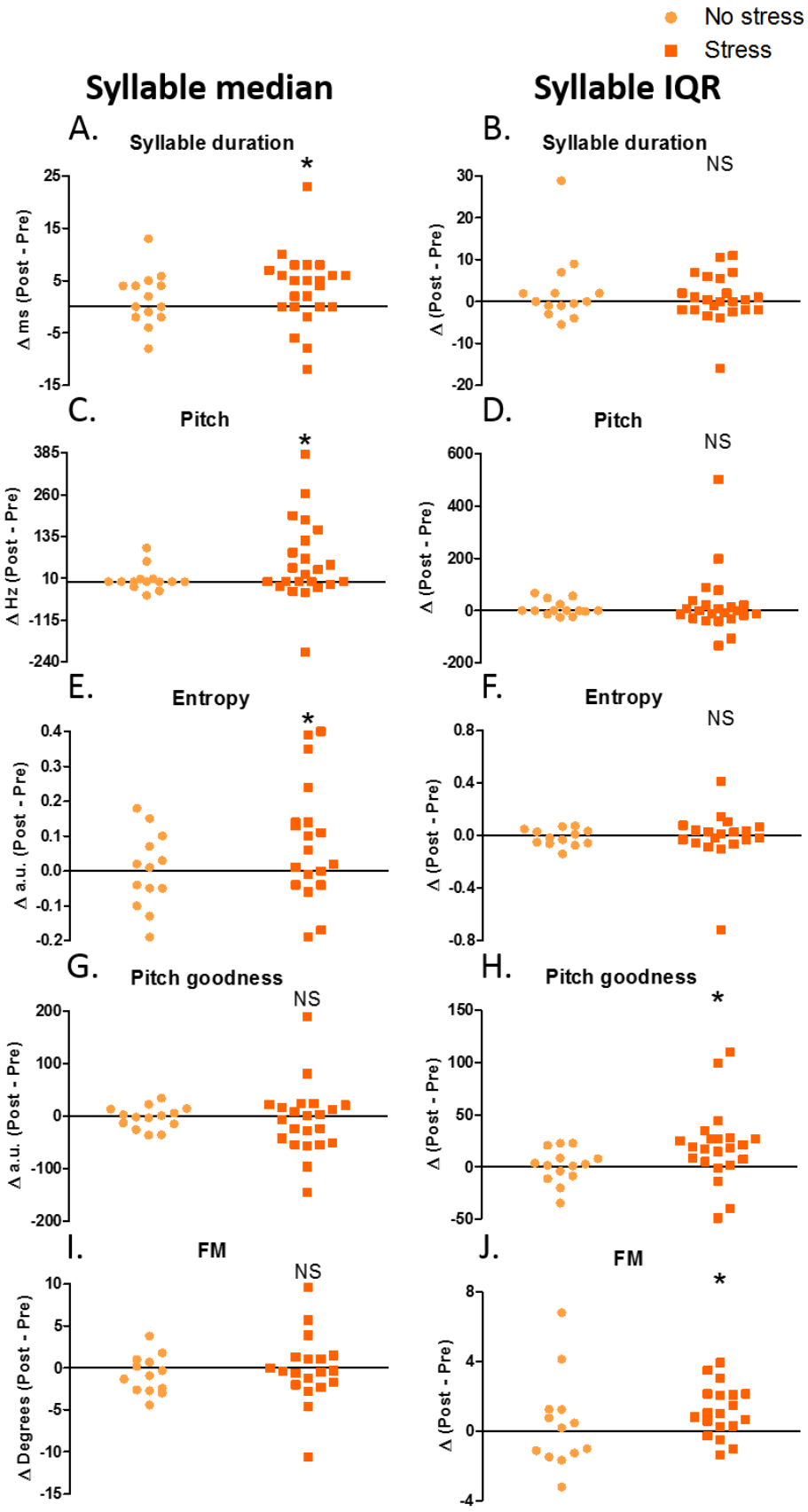
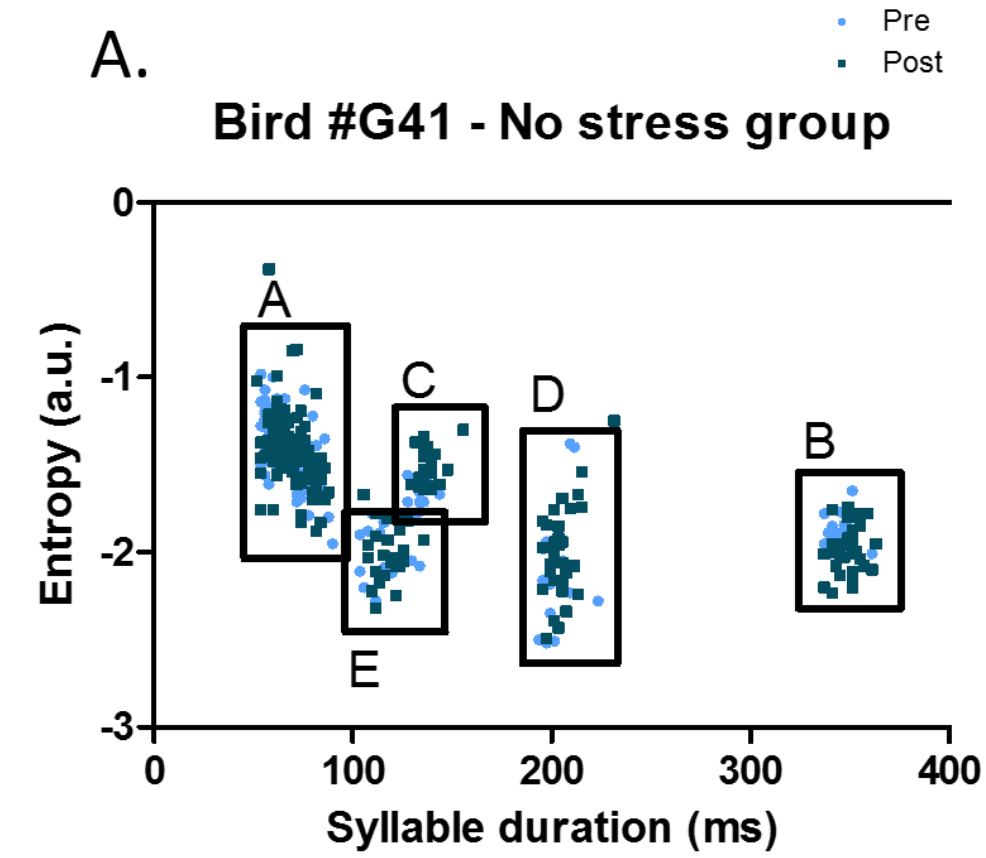

No stress
Stress
Syllable median
Syllable IQR
A.
Syllable duration
Δ ms (Post - Pre)
B.
Syllable duration
Δ (Post - Pre)
NS
C.
Pitch
Δ Hz (Post - Pre)
D.
Pitch
Δ (Post - Pre)
NS
E.
Entropy
Δ a.u. (Post - Pre)
F.
Entropy
Δ (Post - Pre)
NS
G.
Pitch goodness
Δ a.u. (Post - Pre)
NS
H.
Pitch goodness
Δ (Post - Pre)
I.
FM
Δ Degrees (Post - Pre)
NS
J.
FM
Δ (Post - Pre)

FIGURE 3.4: Scatter plots of entropy and syllable duration values for representative effects on syllable median in No stress and Stress groups. Each data point represents one syllable value, and each syllable cluster contains every syllable value for the songs analyzed. Representative scatter plot of bird #Y71 from the Stress group shows a shift in syllable clusters compared to baseline clusters.


A.
Pre
Post
Bird #G41 - No stress group
Entropy (a.u.)
0
-1
-2
-3
A
C
D
B
E
0
100
200
300
400
Syllable duration (ms)

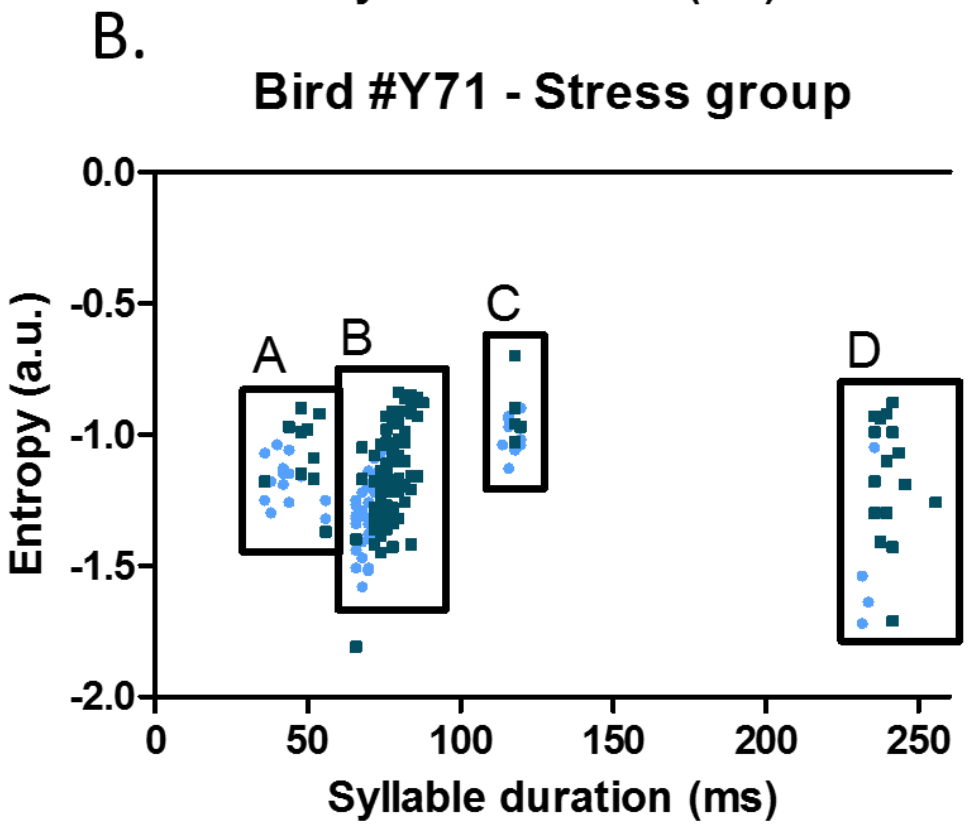
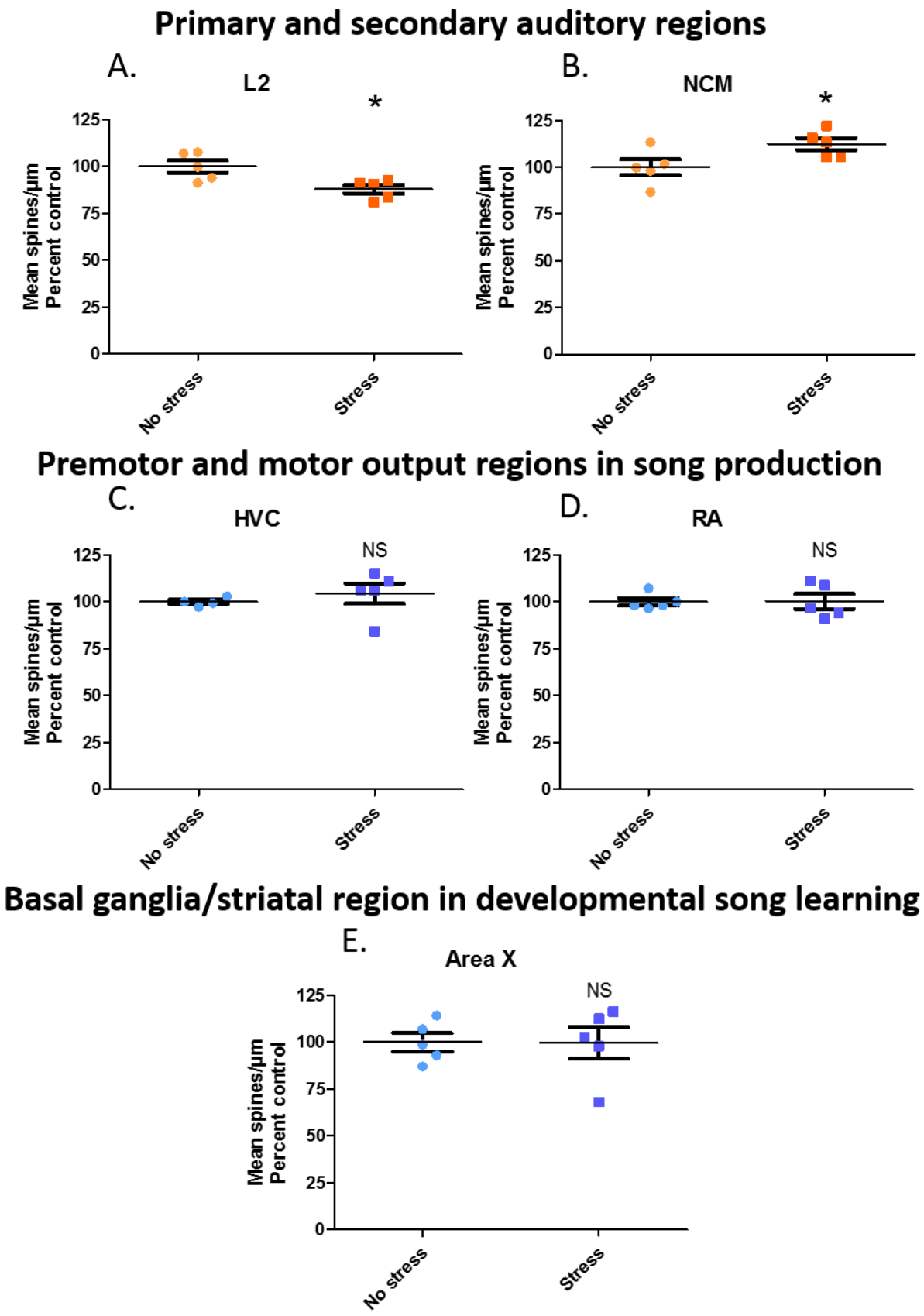

B.
Bird #Y71 - Stress group
Entropy (a.u.)
0.0
-0.5
-1.0
-1.5
-2.0
A
B
C
D
0
50
100
150
200
250
Syllable duration (ms)

FIGURE 3.5: Dendritic spine density of Golgi-Cox stained spiny neurons immediately following 30 minutes of restraint stress (n = 5 animals). The Stress group showed decreased spine density in primary auditory region L2 (*p < 0.05) and increased spine density in higher-order auditory region NCM (*p < 0.05), compared to the No Stress control group **(A, B)**, two-tailed Student's t-test. No significant effects occurred in HVC and RA, respective premotor and motor output regions in song production **(C, D)** or in Area X, a basal ganglia/striatal region in developmental song learning **(E)**. Each data point represents a mean spine density per animal. Error bars = SEM.


Primary and secondary auditory regions
A.
L2
*
B.
NCM
*
Mean spines/µm
Percent control
125
100
75
50
25
0
No stress
Stress
Premotor and motor output regions in song production
C.
HVC
NS
D.
RA
NS
Basal ganglia/striatal region in developmental song learning
E.
Area X
NS

selectively in some regions (n = 5 animals). In stress groups, overall mean dendritic spine density was increased in NCM ($t_8 = 2.32$, $p < 0.05$) and decreased in L2 ($t_8 = 2.99$, $p < 0.05$), Student's t-test, two-tailed. No significant effects occurred in HVC, RA, or Area X. In spine subtypes (**Fig. 3.6**), in L2 thin and stubby subtypes were significantly different in stress groups ($t_8 = 3.03$, $p < 0.05$; $t_8 = 3.42$, $p < 0.05$) and in NCM, only the stubby subtype was significantly different ($t_8 = 2.86$, $p < 0.05$), Student's t-test, two-tailed.

C-Fos Immunohistochemistry

Neuronal activity drives changes in dendritic spine populations, and the immediate early gene c-Fos is expressed following action potentials and acts as an indirect marker. Acute restraint stress's effects on c-Fos-labeled nuclei density followed similar trends as the effects on dendritic spine density, with effects occurring selectively in auditory regions (**Fig. 3.7 and 3.8**) and additionally avian amygdala nucleus taeniae (TnA; **Fig. 3.9**). Increases in c-Fos expression occurred in higher-order auditory regions CMM and NCM, L field subunits L1 and L3, and TnA, and acute restraint stress inhibited expression in primary cortex-like L field subunit L2 (dof = 14, $p < 0.05$), Student's t-test, two-tailed. Changes in c-Fos expression did not appear globally in other regions in the brain (**Fig. 3.10**). No significant changes were detected in premotor HVC and motor output region RA in song production, anterior forebrain regions Area X and lMAN, hippocampus, and NIf, a sensorimotor region that acts as an developmental interface between auditory and vocal motor pathways.

Modulation of Acute Stress by THC

FIGURE 3.6: Density of dendritic spine morphological subtypes following restraint stress (n = 5 animals). In L2, stress groups had significantly reduced density of thin and stubby subtypes **(A)**, and in NCM, stress groups had a greater density of the stubby subtype **(C)**, *p < 0.05, two-tailed Student's t-test. Each data point represents a mean spine density per animal. Error bars = SEM.

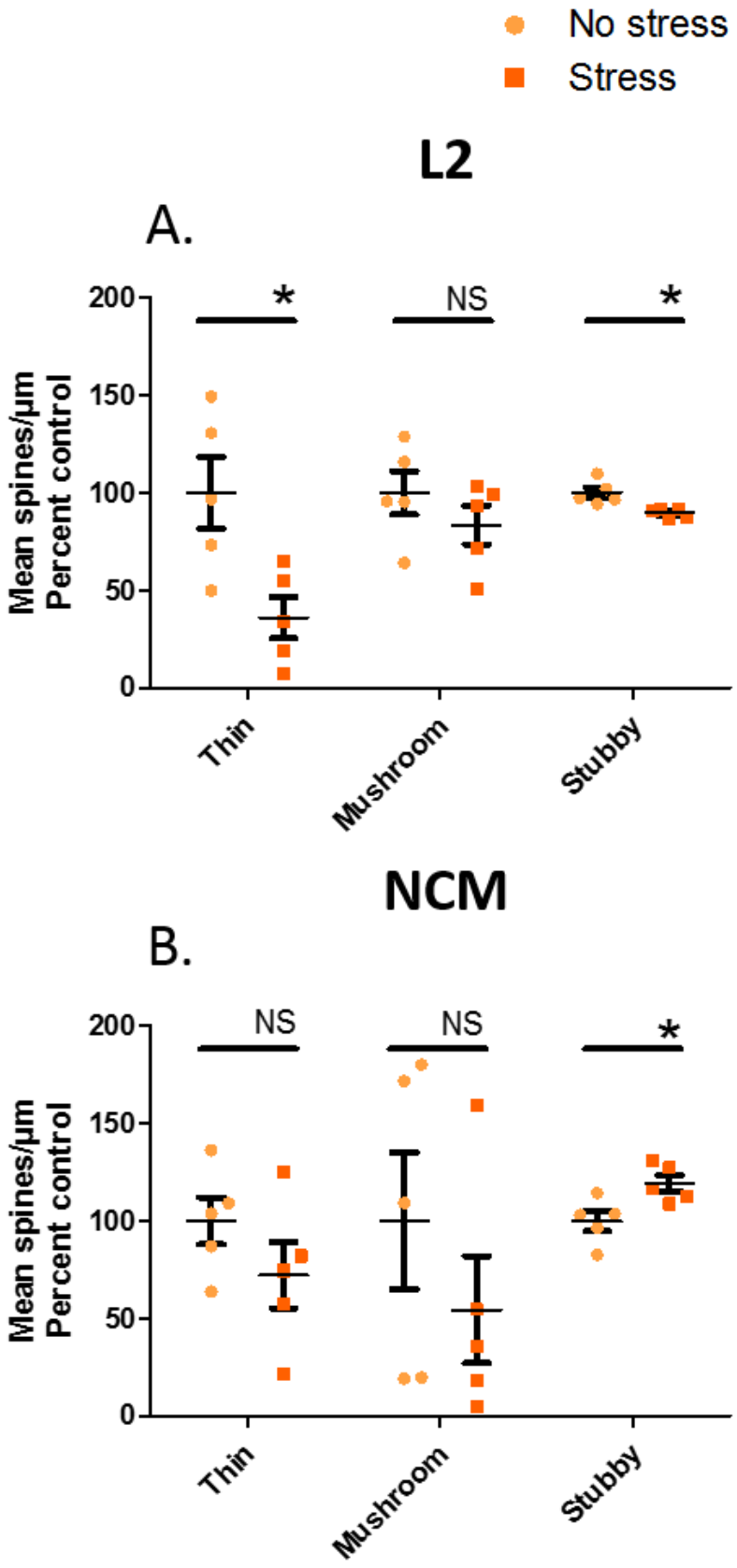

No stress
Stress
L2
A.
Mean spines/µm
Percent control
200
150
100
50
0
*
NS
*
Thin
Mushroom
Stubby
NCM
B.
Mean spines/µm
Percent control
200
150
100
50
0
NS
NS
*
Thin
Mushroom
Stubby

FIGURE 3.7: Immunohistochemical c-Fos expression following acute restraint stress in brain regions in ascending auditory pathway (n = 8 animals). In L field subunits L1 and L3 and higher order regions CMM and NCM, c-Fos density was increased **(A-D)**. In primary auditory region L2, c-Fos density was decreased following restraint stress **(E)**, and no significant changes occurred in OV, an auditory thalamus region **(F)**. *$p < 0.05$, two-tailed, Student's t-test. Each data point represents a mean c-Fos density per animal. Error bars = SEM.

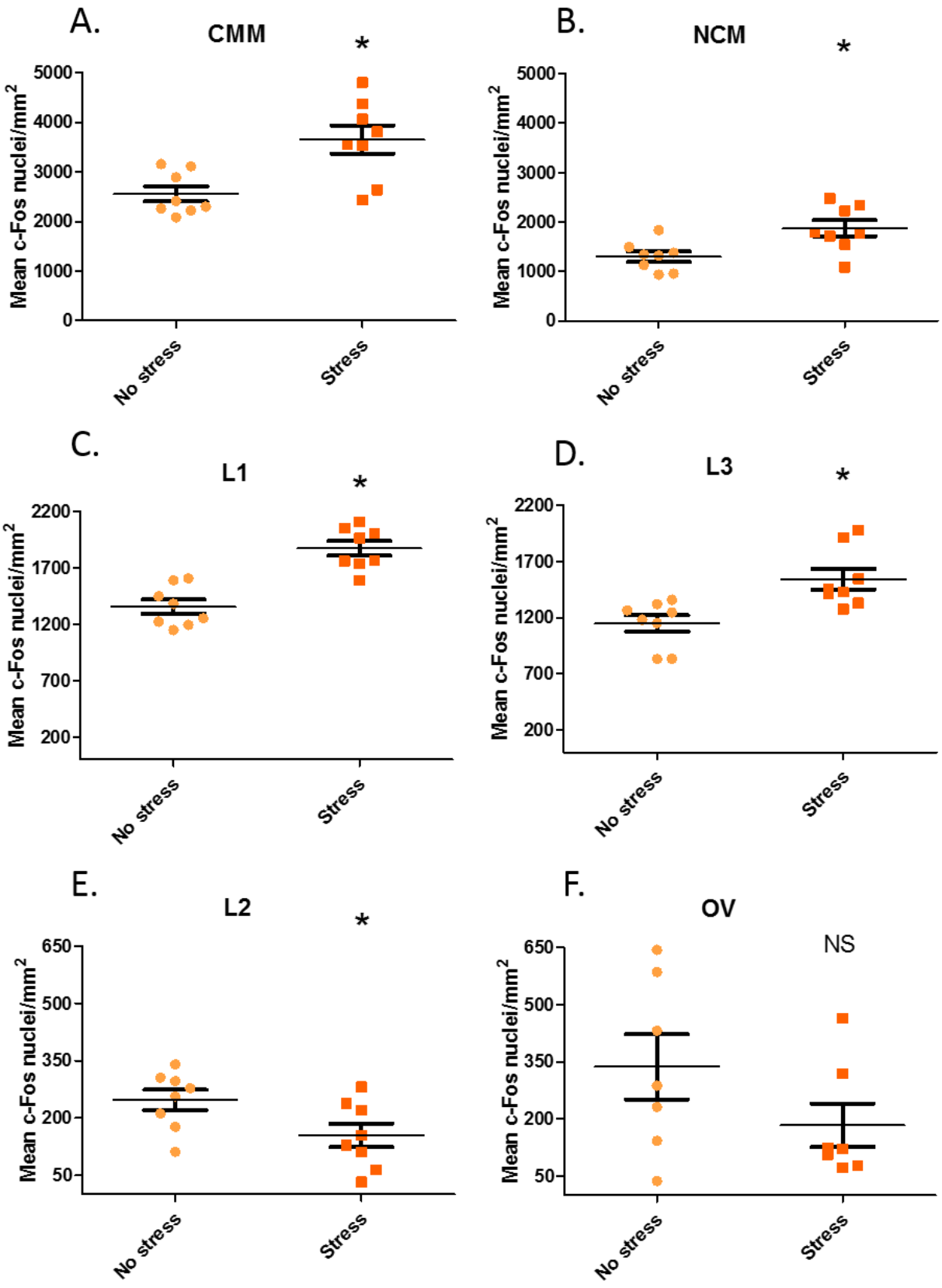

A.
CMM
*
Mean c-Fos nuclei/mm2
5000
4000
3000
2000
1000
0
No stress
Stress
B.
NCM
*
Mean c-Fos nuclei/mm2
5000
4000
3000
2000
1000
0
No stress
Stress
C.
L1
*
Mean c-Fos nuclei/mm2
2200
1700
1200
700
200
No stress
Stress
D.
L3
*
Mean c-Fos nuclei/mm2
2200
1700
1200
700
200
No stress
Stress
E.
L2
*
Mean c-Fos nuclei/mm2
650
500
350
200
50
No stress
Stress
F.
OV
NS
Mean c-Fos nuclei/mm2
650
500
350
200
50
No stress
Stress

FIGURE 3.8: Expression of c-Fos in immunohistochemistry staining following acute restraint stress, in brain regions of the auditory pathway, 25X magnification **(A-D)** and 100X magnification **(a, aa, b, bb, c, d)**. Bars = 750 µm for 25X magnification and 250 µm for 100X magnification.

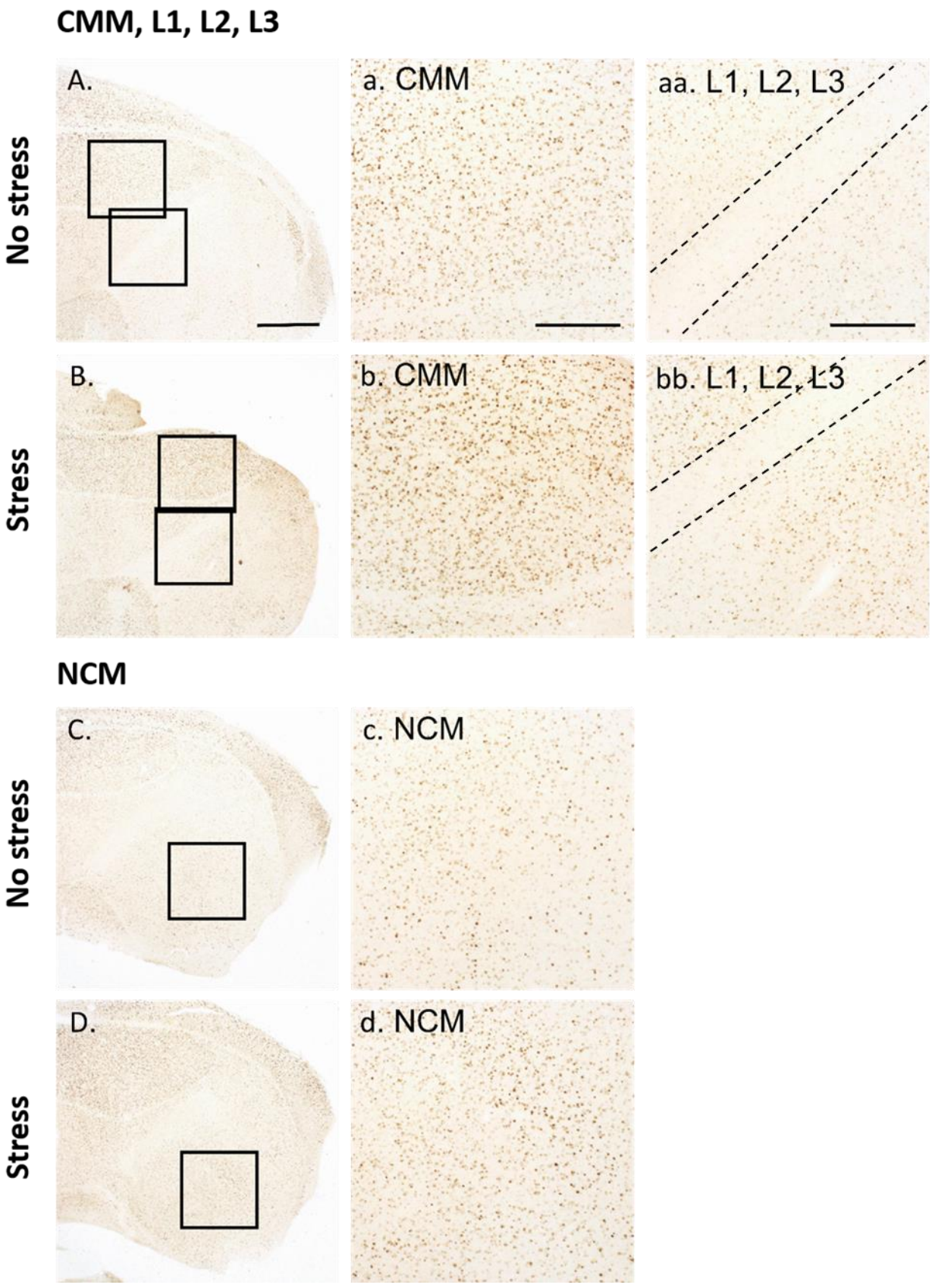

CMM, L1, L2, L3
No stress
A.
a. CMM
aa. L1, L2, L3
Stress
B.
b. CMM
bb. L1, L2, L3
NCM
No stress
C.
c. NCM
Stress
D.
d. NCM

FIGURE 3.9: Immunohistochemical c-Fos expression following acute restraint stress in avian analogue of the amygdala, nucleus taeniae (TnA; n = 8 animals). Restraint stress significantly increased c-Fos density (25X magnification, **A-B**; 100X magnification, **a-b**). Bars = 750 µm for 25X magnification and 250 µm for 100X magnification. *$p < 0.05$, two-tailed, Student's t-test. Error bars = SEM.

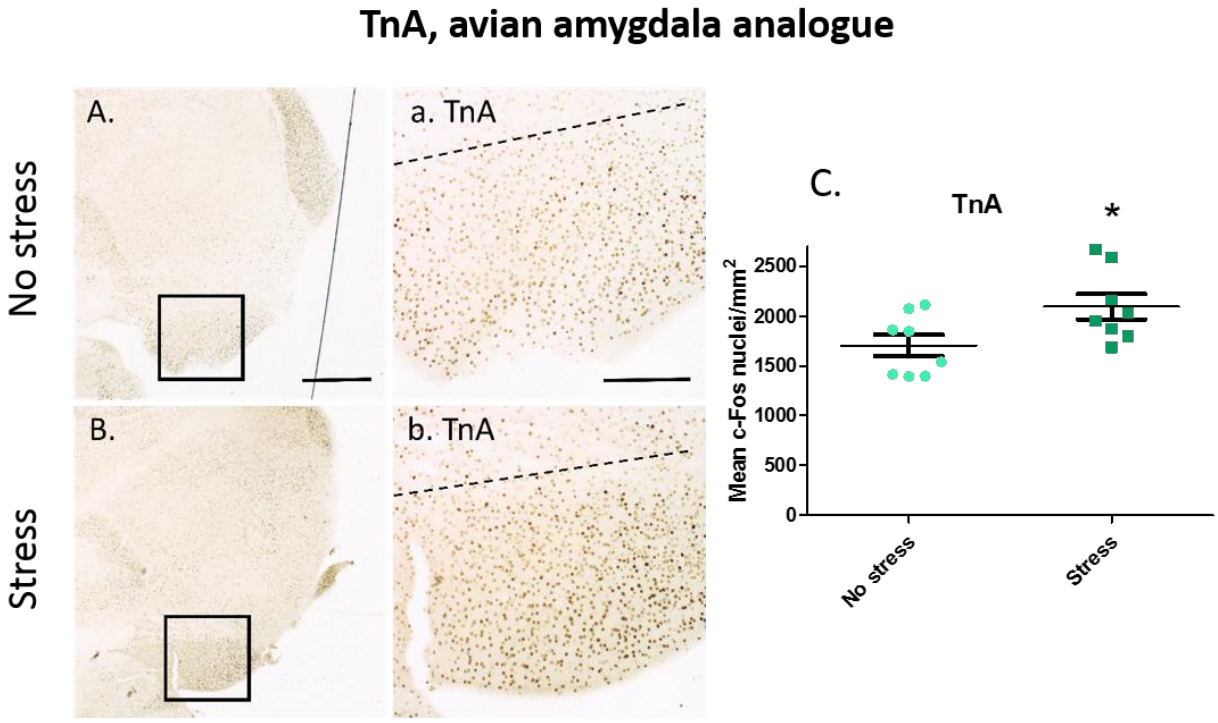
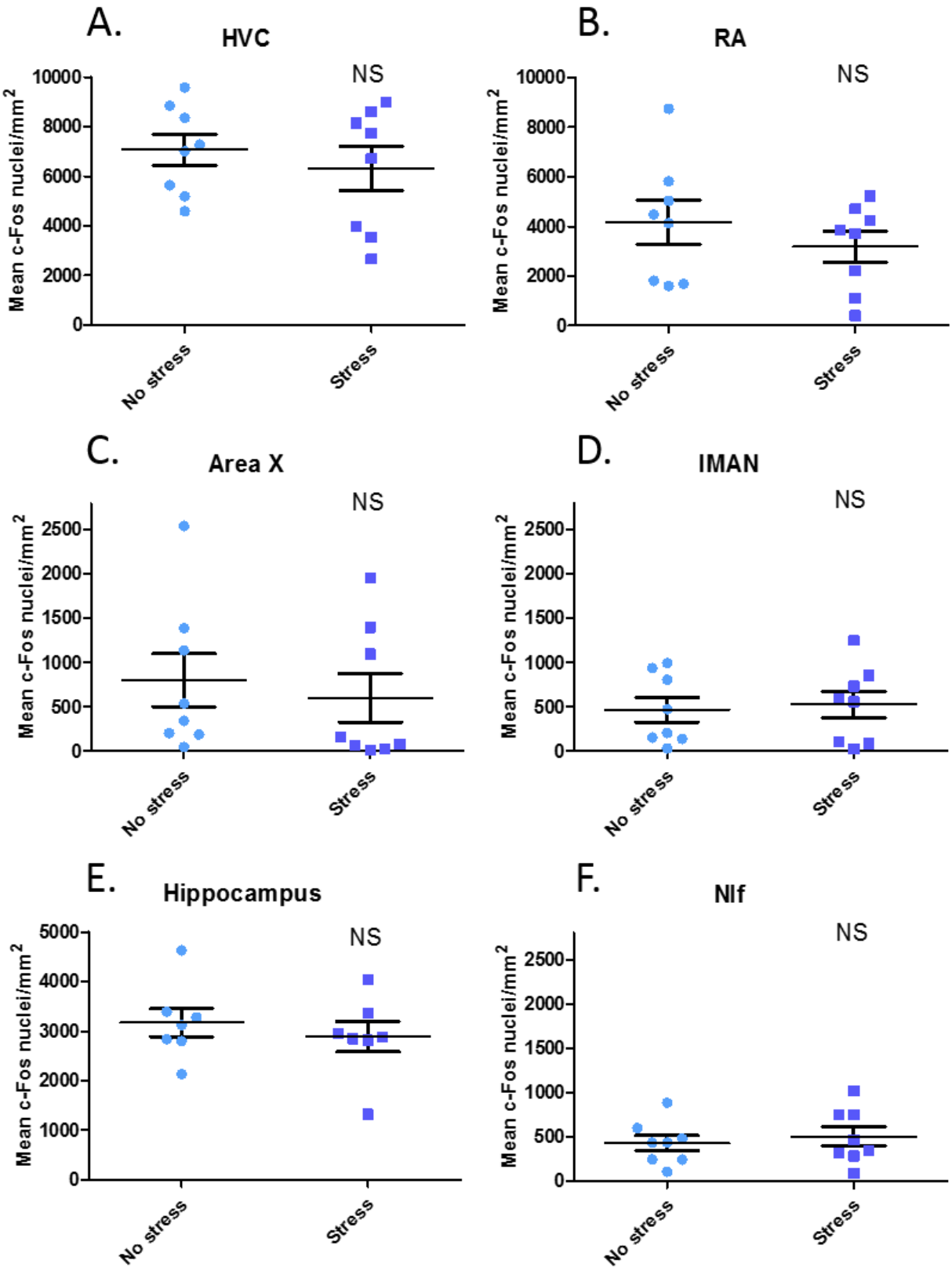

TnA, avian amygdala analogue
No stress
Stress
A.
a. TnA
B.
b. TnA
C.
TnA
*
Mean c-Fos nuclei/mm2
2500
2000
1500
1000
500
0
No stress
Stress

FIGURE 3.10: C-Fos expression in song nuclei (A-D), hippocampus (E), and NIf (F) following acute restraint stress (n = 8 animals). No significant effects were observed. Alpha = 0.05, two-tailed, Student's t-test. Error bars = SEM.


A.
HVC
NS
Mean c-Fos nuclei/mm2
10000
8000
6000
4000
2000
0
No stress
Stress
B.
RA
NS
Mean c-Fos nuclei/mm2
10000
8000
6000
4000
2000
0
No stress
Stress
C.
Area X
NS
Mean c-Fos nuclei/mm2
2500
2000
1500
1000
500
0
No stress
Stress
D.
IMAN
NS
Mean c-Fos nuclei/mm2
2500
2000
1500
1000
500
0
No stress
Stress
E.
Hippocampus
NS
Mean c-Fos nuclei/mm2
5000
4000
3000
2000
1000
0
No stress
Stress
F.
NIf
NS
Mean c-Fos nuclei/mm2
2500
2000
1500
1000
500
0
No stress
Stress

Following vehicle, THC (3 mg/kg), or SR (6 mg/kg) pretreatments and acute restraint stress, plasma corticosterone levels were measured by enzyme immunoassay (alpha = 0.05, dof = 7, one-way ANOVA, Tukey HSD post-hoc test). Both the SR + stress (8.14 ng/mL ± 1.97) and the THC (15.34 ng/mL ± 2.21) groups had significantly increased corticosterone levels compared to the vehicle group (2.53 ng/mL ± 0.32). The SR pretreatment + THC group (7.98 ng/mL ± 1.54) had significantly decreased corticosterone levels compared to the THC group. The vehicle + stress group (5.14 ng/mL ± 0.79) had greater corticosterone levels than the vehicle group but was not significantly different (**Fig. 3.11**).

In this experiment, density of c-Fos immunolabeled nuclei was also measured in the regions CMM, NCM, L1, L3, L2, and TnA in order to evaluate neuronal activity (alpha = 0.05, dof = 7, one-way ANOVA, Tukey HSD post-hoc test). The results in CMM and NCM were similar and showed inhibition of the stress response by THC (**Fig. 3.12**). In CMM, the groups vehicle + stress (3065 ± 127.3) and SR + stress (3390 ± 243.5) were significantly increased compared to the vehicle group (2314 ± 104.3). The THC + stress group (2693 ± 91.36) was decreased compared to the vehicle + stress group, suggesting that THC treatment inhibits the stress response. The SR + THC + stress group (3243 ± 168.1) was significantly increased compared to the vehicle group, indicating SR pretreatment antagonized the effect of THC on the stress response in a CB_1 receptor mechanism. The c-Fos expression followed a similar pattern in NCM as CMM. In NCM, the vehicle + stress group (1922 ± 129.3) was significantly increased compared to the vehicle group (1231 ± 94.85). The SR + stress group (1757 ± 98.34)

FIGURE 3.11: Plasma corticosterone levels following vehicle, THC (3 mg/kg), or SR (6 mg/kg) pretreatments and acute restraint stress (n = 4-15 animals), measured by enzyme immunoassay. Plasma corticosterone levels were significantly elevated in THC and SR + stress groups compared to vehicle control (*$p < 0.05$, one-way ANOVA, Tukey HSD post-hoc test). Error bars = SEM.

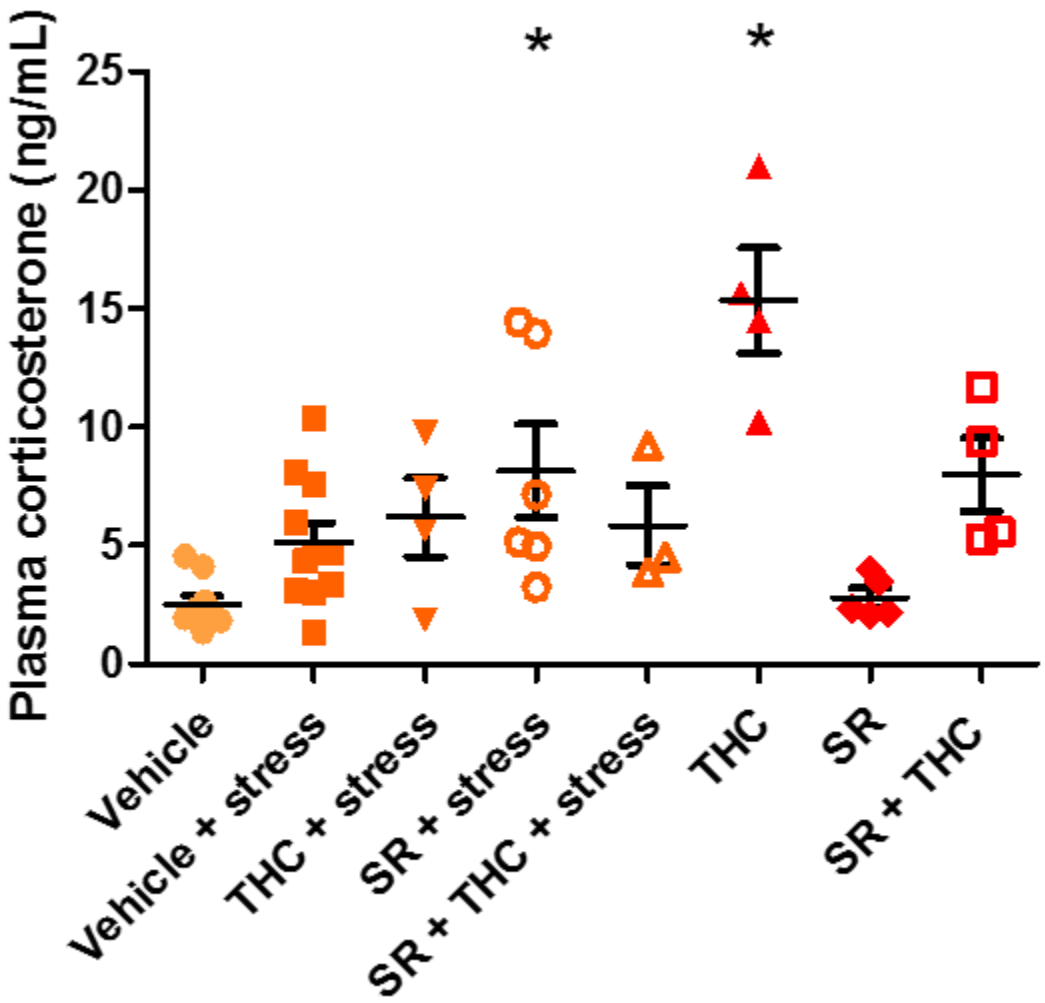
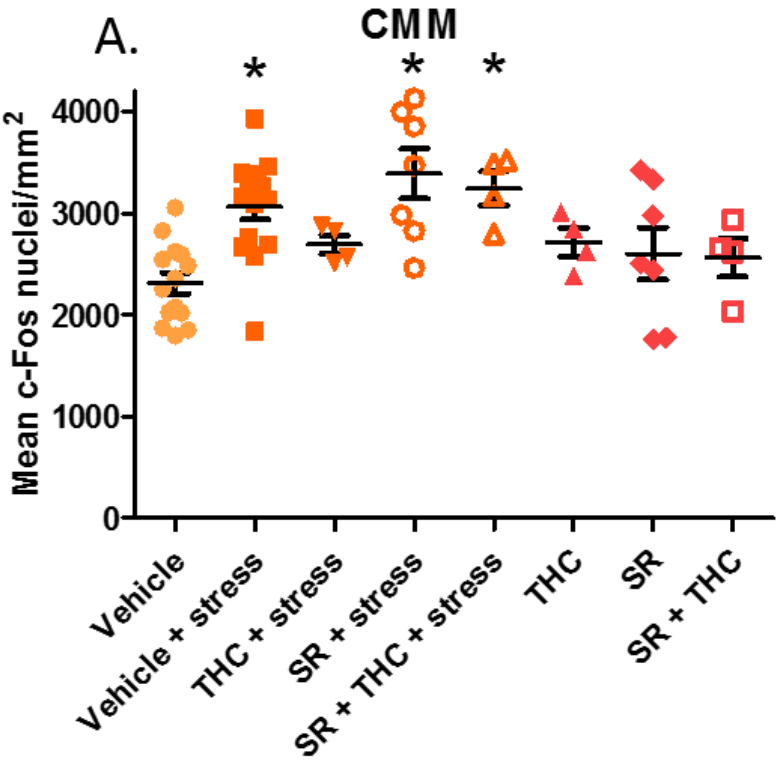
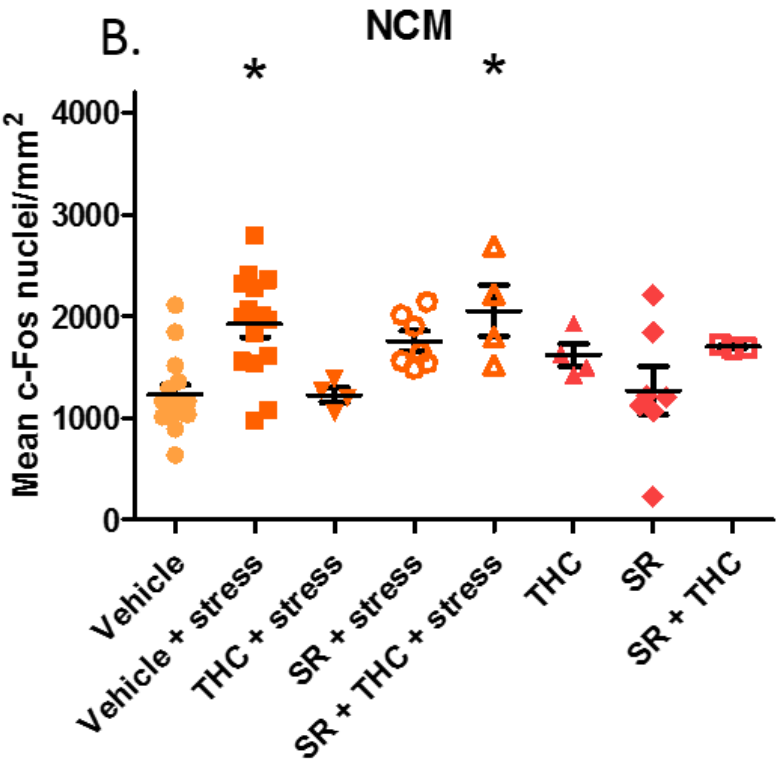

Plasma corticosterone (ng/mL)
25
20
15
10
5
0
*
*
Vehicle
Vehicle + stress
THC + stress
SR + stress
SR + THC + stress
THC
SR
SR + THC

FIGURE 3.12: C-Fos expression in CMM and NCM following vehicle, THC (3 mg/kg), or SR (6 mg/kg) pretreatments and acute restraint stress (n = 4-15 animals). In higher-order auditory regions CMM and NCM (**A-B**), THC pretreatment antagonized stress in a SR-reversible manner (*$p < 0.05$, compared to vehicle control, one-way ANOVA, Tukey HSD post-hoc test). Error bars = SEM.


A.
CMM
Mean c-Fos nuclei/mm2
4000
3000
2000
1000
0
*
*
*
Vehicle
Vehicle + stress
THC + stress
SR + stress
SR + THC + stress
THC
SR
SR + THC


B.
NCM
Mean c-Fos nuclei/mm2
4000
3000
2000
1000
0
*
*
Vehicle
Vehicle + stress
THC + stress
SR + stress
SR + THC + stress
THC
SR
SR + THC

was increased compared to the vehicle group but not significantly different (p = 0.14). The THC + stress group (1227 ± 69.13) was decreased compared to the vehicle + stress group, suggesting that THC treatment inhibits the stress response. The SR + THC + stress group (2055 ± 255.4) was significantly increased compared to the vehicle group, indicating SR pretreatment antagonized the effect of THC on the stress response in a CB_1 receptor mechanism.

Compared to CMM and NCM, the expression pattern of c-Fos in L1, L3, L2, and TnA were dissimilar. In L1 and L3 (**Fig. 3.13**), THC did not appear to inhibit the stress response as in CMM and NCM. In L1, the groups vehicle + stress (1428 ± 106.7) and SR + THC + stress (1604 ± 354.3) were significantly increased compared to the vehicle group (720.3 ± 59.14). The following groups were increased compared to the vehicle group but not significantly: THC + stress (1174 ± 14.09, p = 0.73), SR + stress (1287 ± 302.0, p = 0.12), THC (1426 ± 73.39, p = 0.10), and SR + THC (1296 ± 255.1, p = 0.30). The increased expression in the THC group compared to the vehicle group, although not significant, suggests that THC did not inhibit the stress response. The results in L3 were similar as in L1. The density in groups vehicle + stress (1308 ± 112.3), SR + THC + stress (1400 ± 292.2), and THC (1401 ± 80.91) were significantly elevated compared to the vehicle group (635.9 ± 68.48). These groups were increased compared to the vehicle group but not significantly: THC + stress (1202 ± 103.2, p = 0.15) and SR + stress (1097 ± 205.8, p = 0.15). In L2, there were no significant differences. In TnA, the SR + stress group (2400 ± 148.7) was elevated compared to the vehicle group (1810 ± 79.68) significantly (**Fig. 3.14**).

FIGURE 3.13: C-Fos expression in L1 and L3 following vehicle, THC (3 mg/kg), or SR (6 mg/kg) pretreatments and acute restraint stress (n = 4-15 animals). In L1 and L3 (**A-B**), results imply that vehicle + stress and THC groups had similarly increased c-Fos expression, although in L1, the THC group was not significantly different from vehicle control group (p = 0.10). One-way ANOVA, Tukey HSD post-hoc test, alpha = 0.05. Error bars = SEM.

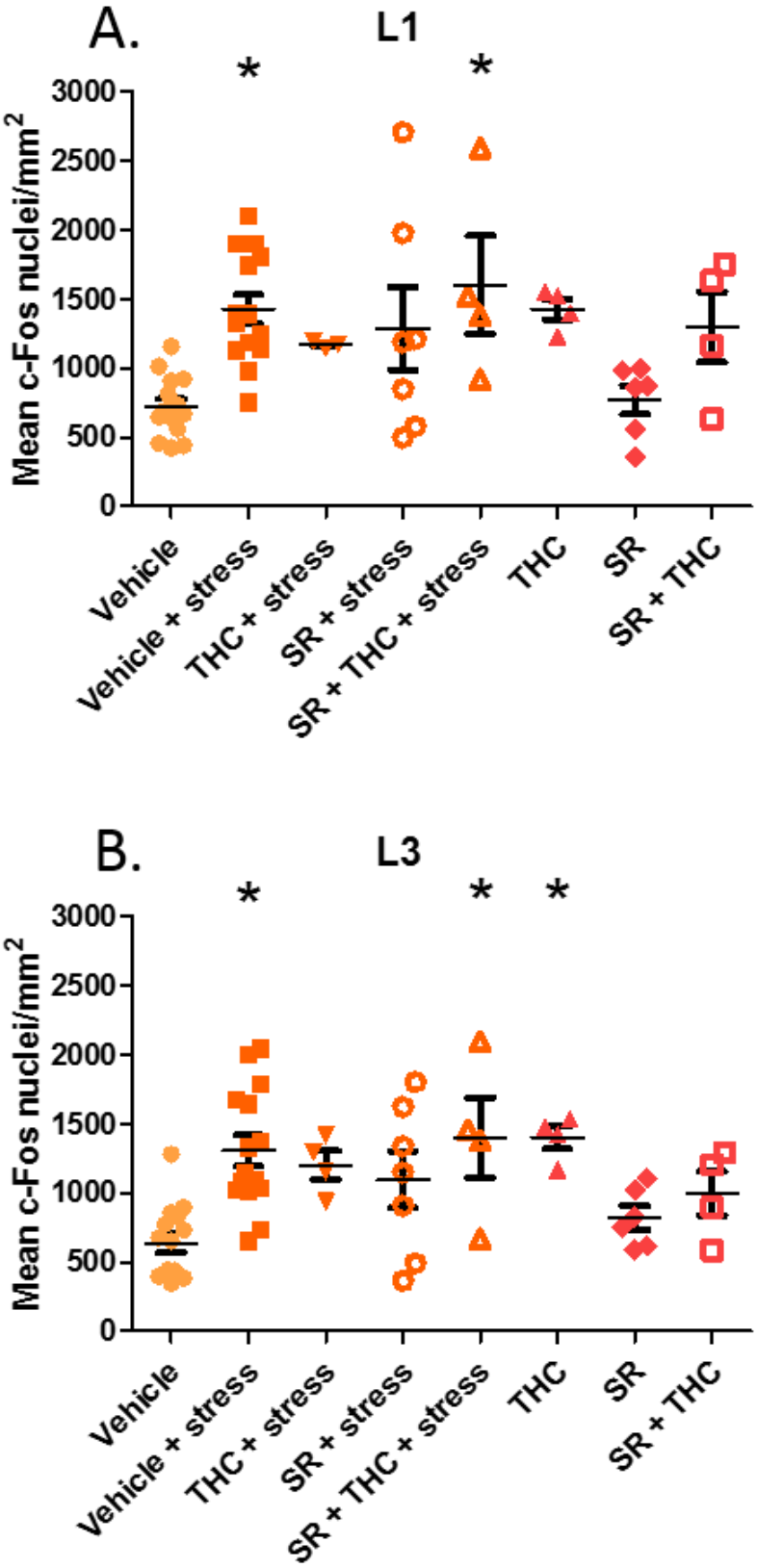
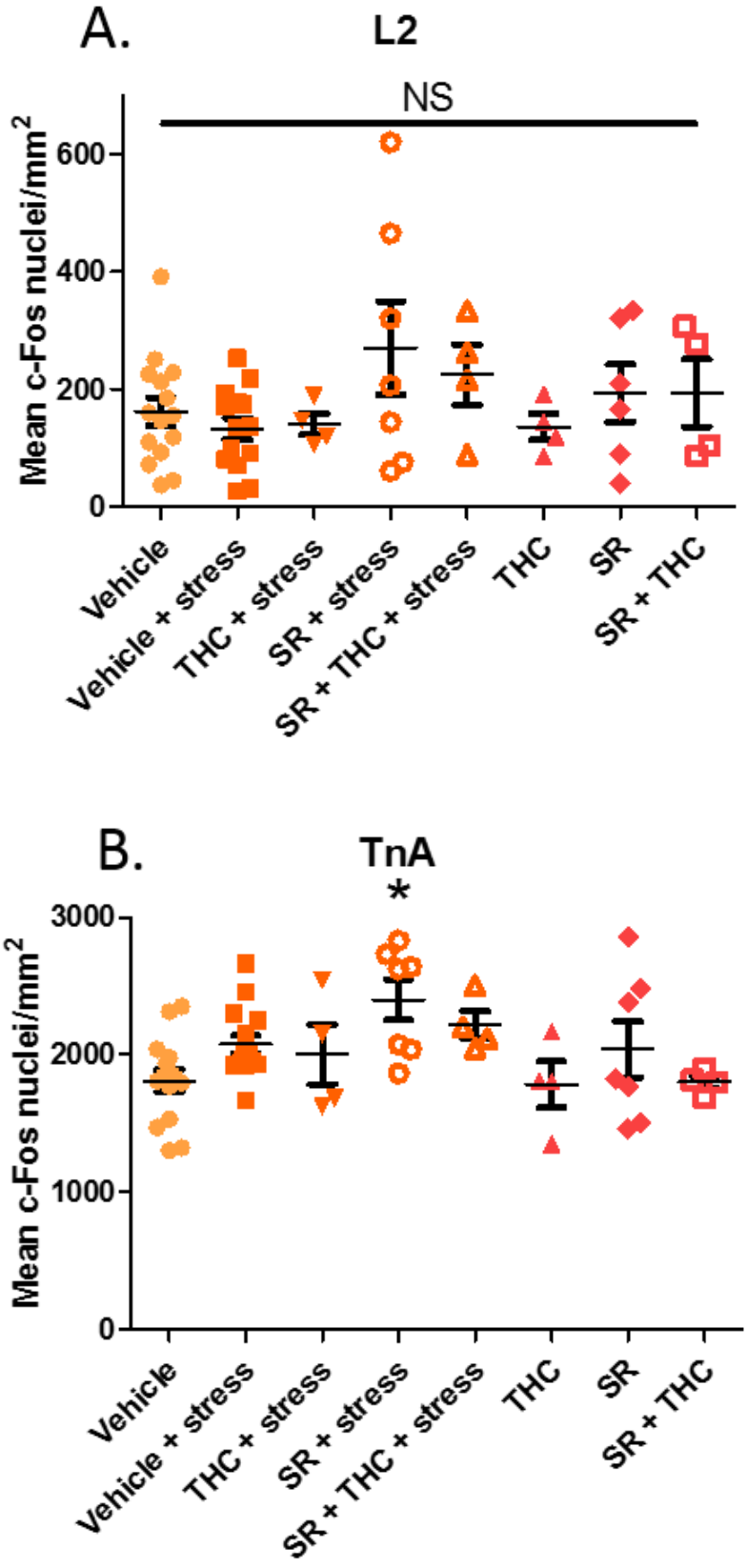

A.
L1
Mean c-Fos nuclei/mm2
3000
2500
2000
1500
1000
500
0
*
*
Vehicle
Vehicle + stress
THC + stress
SR + stress
SR + THC + stress
THC
SR
SR + THC
B.
L3
Mean c-Fos nuclei/mm2
3000
2500
2000
1500
1000
500
0
*
*
*
Vehicle
Vehicle + stress
THC + stress
SR + stress
SR + THC + stress
THC
SR
SR + THC

FIGURE 3.14: C-Fos expression following vehicle, THC (3 mg/kg), or SR (6 mg/kg) pretreatments and acute restraint stress (n = 4-15 animals). In L2 (**A**), no groups were significantly different from vehicle control, and in TnA (**B**), expression in the SR + stress group was elevated (*p <0.05, one-way ANOVA, Tukey HSD post-hoc test). Error bars = SEM.


A.
L2
NS
Mean c-Fos nuclei/mm2
600
400
200
0
Vehicle
Vehicle + stress
THC + stress
SR + stress
SR + THC + stress
THC
SR
SR + THC
B.
TnA
*
Mean c-Fos nuclei/mm2
3000
2000
1000
0
Vehicle
Vehicle + stress
THC + stress
SR + stress
SR + THC + stress
THC
SR
SR + THC

Chronic Stress

Plasma Corticosterone

In chronic experiments, plasma was collected at time of euthanasia, and results are persistent corticosterone levels following 25 days of vehicle or drug treatments and ≥ 25 days of no treatment, measured by enzyme immunoassay (**Fig. 3.15**). Developmental treatment groups were not significantly different from the vehicle group ($p = 0.86$). The adult THC + stress treatment group (7.09 ng/mL ± 2.73) had significantly increased plasma corticosterone compared to the vehicle group (1.53 ng/mL ± 0.34; $p < 0.05$), one-way ANOVA, dof = 3, Tukey HSD post-hoc test.

Song Activity

Following developmental chronic treatments, the vehicle + stress group (28.6 mean songs/hour ± 5.65) produced significantly more songs than the vehicle control group (14.4 mean songs/hour ± 4.50) 24 hours after the last treatment ($p < 0.05$, vs. vehicle + no stress, one-way ANOVA, dof = 3, Tukey HSD post-hoc test), and THC treatment prevented this effect ($p < 0.05$, vs. vehicle + stress). Following a no-treatment duration of ≥25 days, these effects were not persistent (**Fig. 3.16**).

Following adult chronic treatments, THC inhibited song activity (6.65 mean songs/hour ± 0.95), and vehicle + stress group (23.7 mean songs/hour ± 1.31) produced significantly more songs, compared to the vehicle control group (14.3 mean songs/hour ± 3.20) 24 hours after the last treatment ($p < 0.05$, one-way ANOVA, dof = 3, Tukey HSD post-hoc test). THC treatment prevented the stimulatory effect of stress

FIGURE 3.15: Persistent plasma corticosterone levels ≥25 days following chronic treatments, measured by enzyme immunoassay. No significant differences occurred following developmental treatments (**A**). THC + stress treatments during adulthood (**B**) significantly increased corticosterone levels compared to vehicle control group (*$p < 0.05$, one-way ANOVA, Tukey HSD post-hoc test). Error bars = SEM.

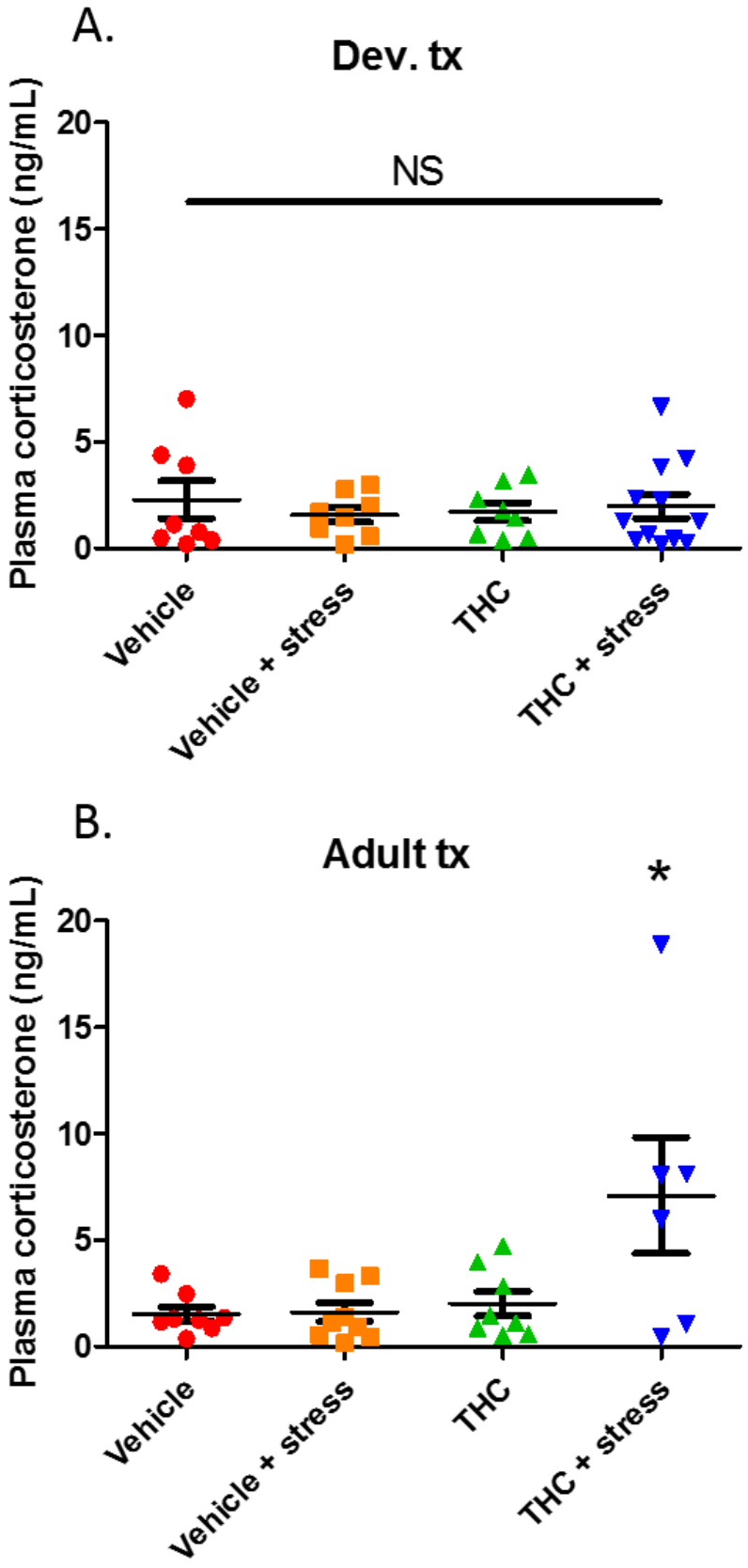
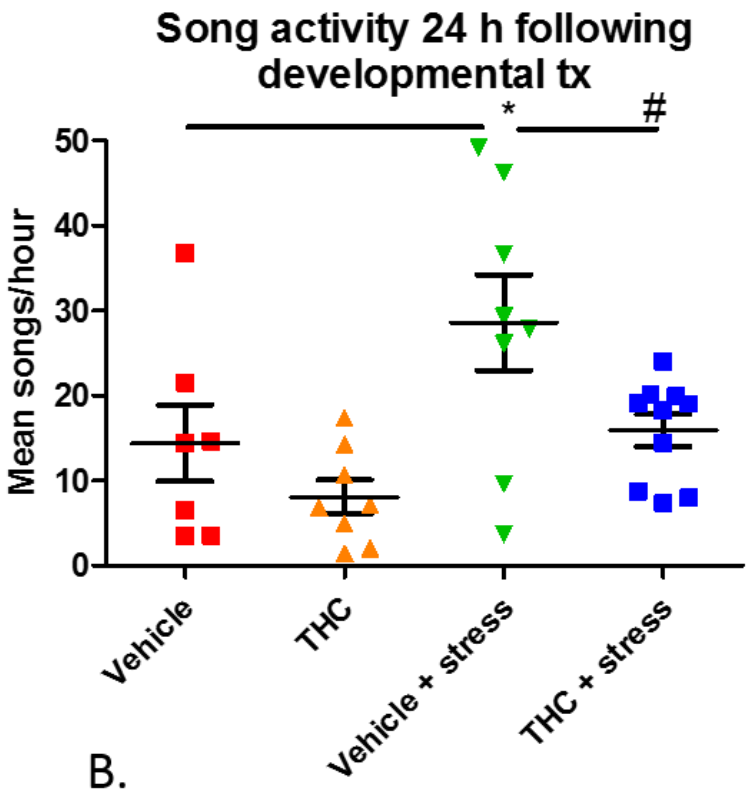
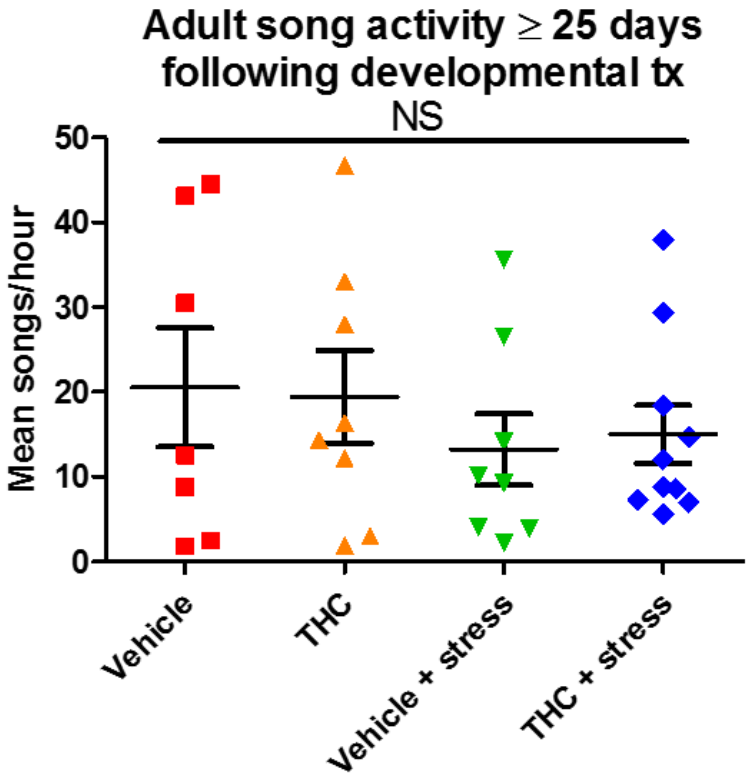

A.
Dev. tx
NS
Plasma corticosterone (ng/mL)
20
15
10
5
0
Vehicle
Vehicle + stress
THC
THC + stress
B.
Adult tx
*
Plasma corticosterone (ng/mL)
20
15
10
5
0
Vehicle
Vehicle + stress
THC
THC + stress

FIGURE 3.16: Song activity following developmental chronic treatments. 24 hours after the last treatment (A), the vehicle + stress group produced significantly more songs than the vehicle control group (*$p < 0.05$, vs. vehicle + no stress, one-way ANOVA, Tukey HSD post-hoc test), and THC treatment prevented this effect (#$p < 0.05$, vs. vehicle + stress). Following no treatment duration of ≥25 days, these effects were not persistent. Error bars = SEM.

A.


Song activity 24 h following developmental tx
*
#
Mean songs/hour
50
40
30
20
10
0
Vehicle
THC
Vehicle + stress
THC + stress

B.


Adult song activity ≥ 25 days following developmental tx
NS
Mean songs/hour
50
40
30
20
10
0
Vehicle
THC
Vehicle + stress
THC + stress

on song activity ($p < 0.05$, vs. vehicle + stress). Following a no treatment duration of ≥25 days, these effects were persistent (**Fig. 3.17**).

Song Acoustic Features

Initially, the syllable entropy medians of the THC and Vehicle + stress groups were significantly different than the vehicle control following chronic developmental treatment. After a ≥ 25 days no treatment period, the THC + stress group was also different (alpha = 0.05, one-way ANOVA, dof = 3, Tukey HSD post-hoc test). Adult treatment had no significant effect on entropy medians (**Fig. 3.18**).

The syllable entropy IQR following developmental THC or vehicle + stress treatments initially increased entropy IQRs significantly, but only the THC effects were persistent. Adult THC + stress significantly increased entropy IQRs (alpha = 0.05, one-way ANOVA, dof = 3, Tukey HSD post-hoc test). This effect was persistent following the no treatment period (**Fig. 3.19**).

The syllable medians and IQRs of other acoustic features were also evaluated, including syllable duration, pitch, FM, and pitch goodness. Entropy was the only acoustic feature that displayed significant differences as a function of treatment.

Dendritic Spine Density

In Area X following developmental treatments, the THC (89.94 ± 3.065), vehicle + stress (89.26 ± 3.519), and THC + stress (86.42 ± 2.426) groups persistently had significantly decreased spine density compared to vehicle control (100.0 ± 3.296). After adult treatments with THC + stress (111.2 ± 2.328), spine density was increased in the

FIGURE 3.17: Song activity following adult chronic treatments. 24 hours after the last treatment (**A**), THC inhibited song activity, and vehicle + stress group produced significantly more songs than the vehicle control group (*$p < 0.05$, vs. vehicle + no stress, one-way ANOVA, Tukey HSD post-hoc test). THC treatment prevented the stimulatory effect of stress on song activity (#$p < 0.05$, vs. vehicle + stress). Following a no treatment duration of ≥25 days (**B**), these effects were persistent. Error bars = SEM.

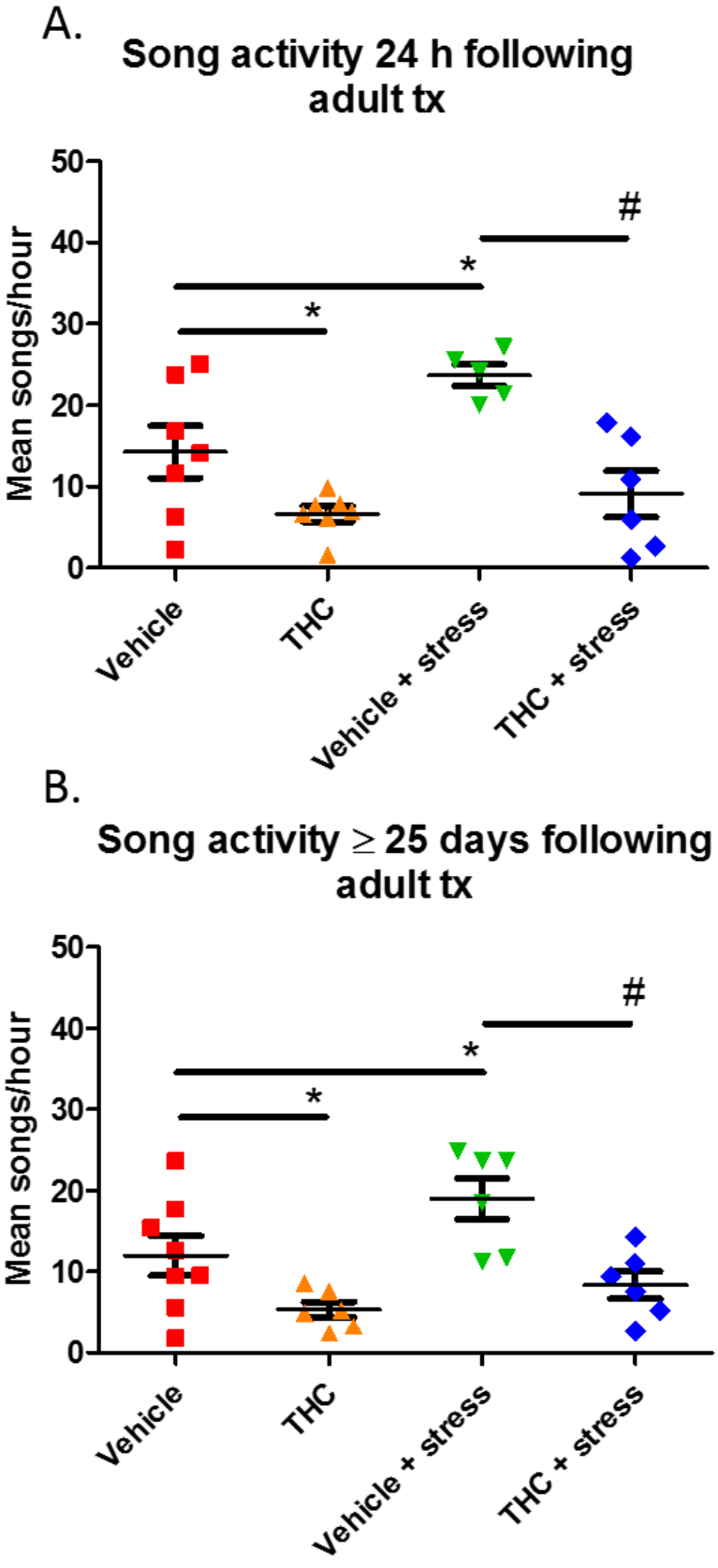

A.
Song activity 24 h following adult tx
Mean songs/hour
50
40
30
20
10
0
*
*
#
Vehicle
THC
Vehicle + stress
THC + stress
B.
Song activity ≥ 25 days following adult tx
Mean songs/hour
50
40
30
20
10
0
*
*
#
Vehicle
THC
Vehicle + stress
THC + stress

FIGURE 3.18: Syllable entropy medians following chronic treatments. Initially, THC and Vehicle + stress groups were significantly different than the vehicle control following developmental treatment (**A**), and after a ≥ 25 days no treatment period (**C**), the THC + stress group was also different (*$p < 0.05$, one-way ANOVA, Tukey HSD post-hoc test). Adult treatment had no effect on entropy medians (**B-D**). Error bars = SEM.

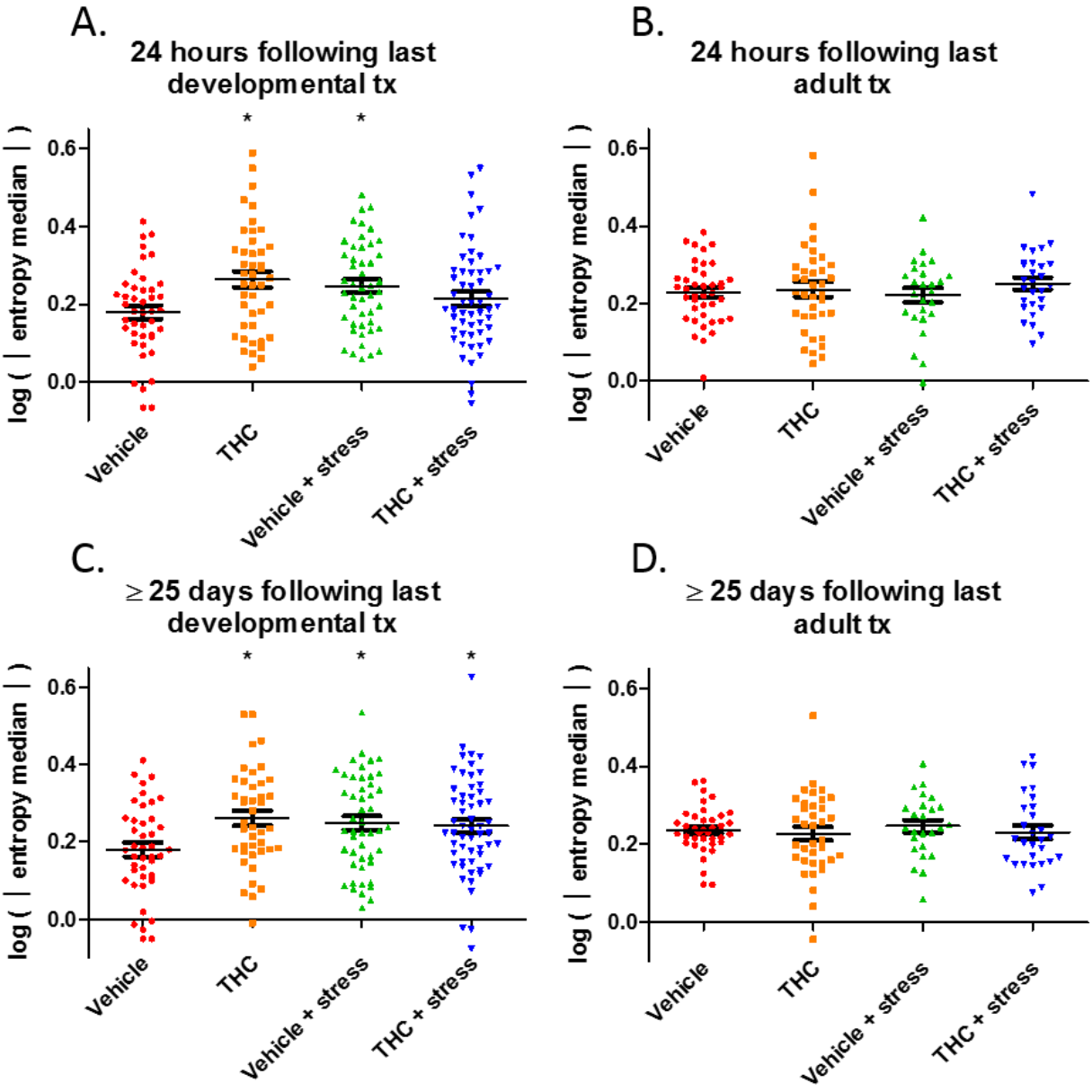
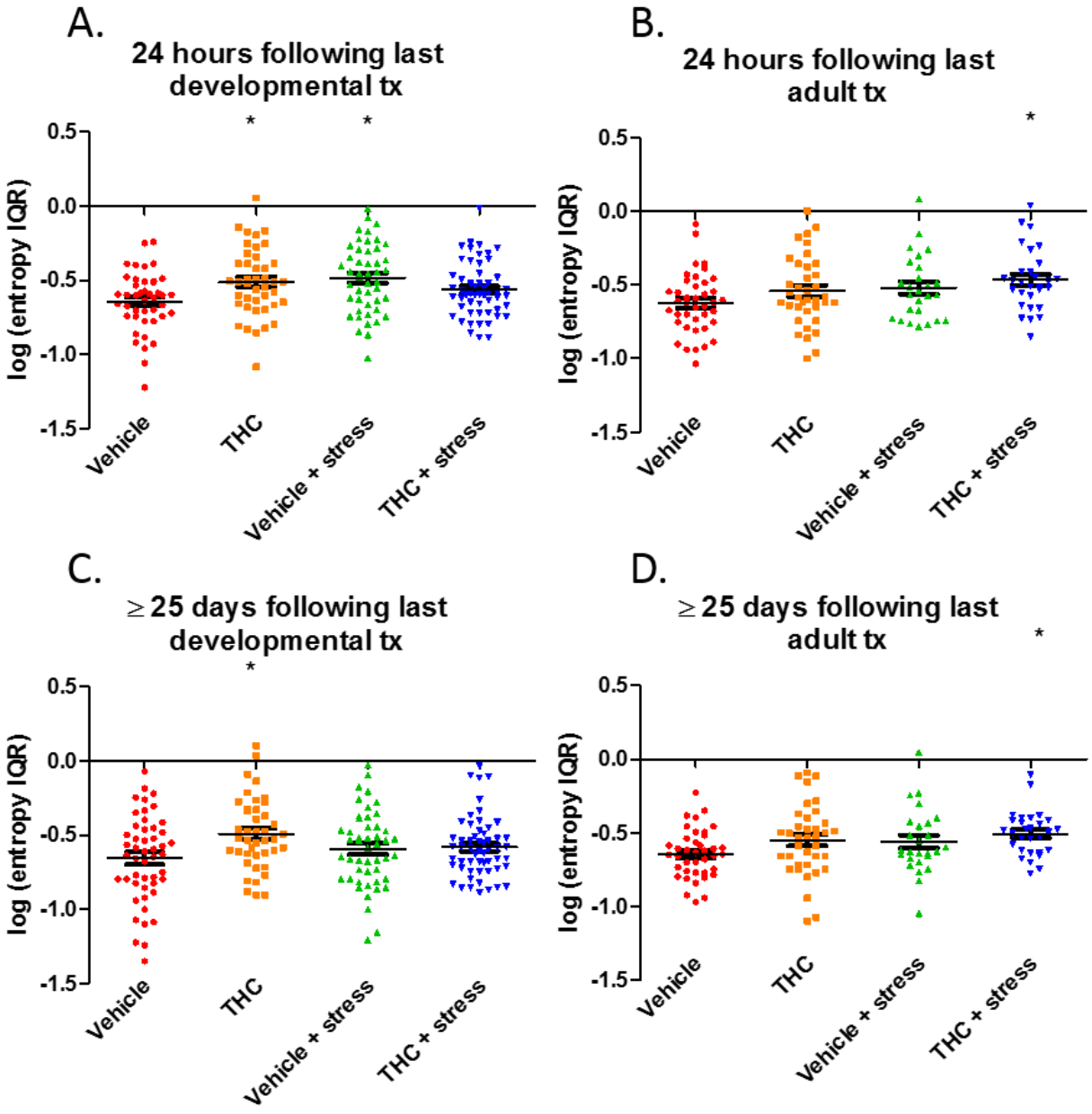

A.
24 hours following last developmental tx
B.
24 hours following last adult tx
C.
≥ 25 days following last developmental tx
D.
≥ 25 days following last adult tx
log (| entropy median |)
0.0
0.2
0.4
0.6
*
Vehicle
THC
Vehicle + stress
THC + stress

FIGURE 3.19: Syllable entropy IQR following chronic treatments. Developmental THC or vehicle + stress treatments initially increased entropy IQRs (**A**), but only the THC effects were persistent (**C**). Adult THC + stress increased entropy IQRs (**B**), and this effect was persistent (**D**) following the no treatment period (*$p < 0.05$, one-way ANOVA, Tukey HSD post-hoc test). Error bars = SEM.


A.
24 hours following last developmental tx
B.
24 hours following last adult tx
C.
≥ 25 days following last developmental tx
D.
≥ 25 days following last adult tx
log (entropy IQR)
0.5
0.0
-0.5
-1.0
-1.5
*
Vehicle
THC
Vehicle + stress
THC + stress

vehicle group (100.0 ± 2.01) in Area X (alpha = 0.05, one-way ANOVA, dof = 3, Tukey HSD post-hoc test; **Fig. 3.20**).

In HVC following developmental treatments, THC + stress (86.42 ± 2.42) significantly increased spine density compared to vehicle control (100.0 ± 3.29). After adult treatments with vehicle + stress (85.73 ± 2.12), spine density was significantly decreased compared to adults treated with vehicle (100.0 ± 2.61) in HVC (alpha = 0.05, one-way ANOVA, dof = 3, Tukey HSD post-hoc test; **Fig. 3.21**).

In NCM following developmental treatments, THC + stress (86.42 ± 2.426) significantly decreased spine density compared to vehicle control (100.0 ± 3.296). After adult treatments with THC + stress (90.66 ± 3.41), spine density was decreased compared to adults treated with vehicle (100.0 ± 2.61) in NCM (alpha = 0.05, one-way ANOVA, dof = 3, Tukey HSD post-hoc test; **Fig. 3.22**).

FIGURE 3.20: Area X dendritic spine density following chronic treatments. Following developmental treatments, the THC, vehicle + stress, and THC + stress groups persistently had decreased spine density compared to vehicle control (**A**). After adult treatments with THC + stress (**B**), spine density was increased compared to adults treated with vehicle (*$p < 0.05$, one-way ANOVA, Tukey HSD post-hoc test). Error bars = SEM.

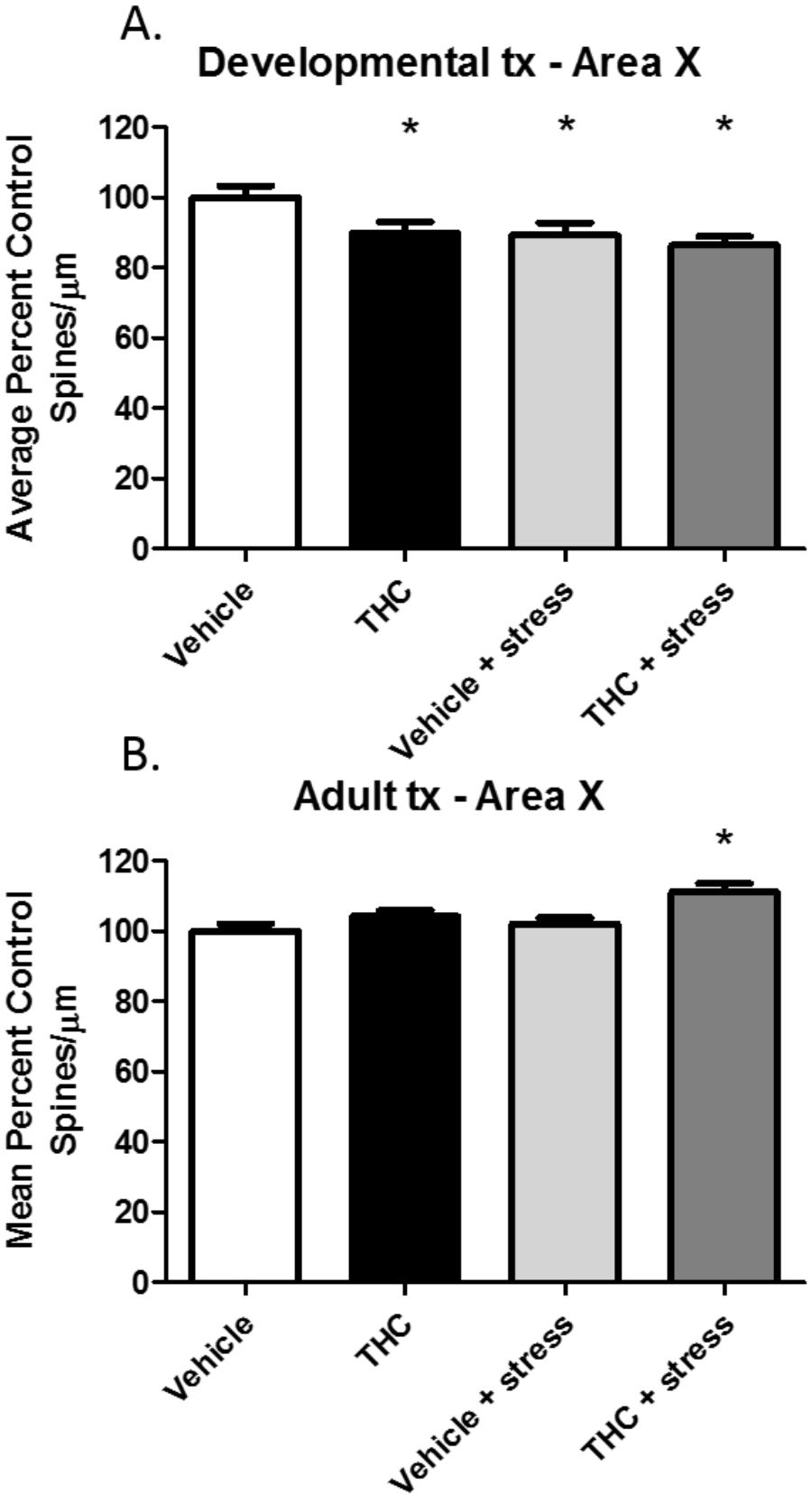

A.
Developmental tx - Area X
Average Percent Control Spines/µm
120
100
80
60
40
20
0
*
*
*
Vehicle
THC
Vehicle + stress
THC + stress
B.
Adult tx - Area X
Mean Percent Control Spines/µm
120
100
80
60
40
20
0
*
Vehicle
THC
Vehicle + stress
THC + stress

FIGURE 3.21: HVC dendritic spine density following chronic treatments. Following developmental treatments, THC + stress persistently increased spine density compared to vehicle control (**A**). After adult treatments with vehicle + stress (**B**), spine density was decreased compared to adults treated with vehicle (*$p < 0.05$, one-way ANOVA, Tukey HSD post-hoc test). Error bars = SEM.

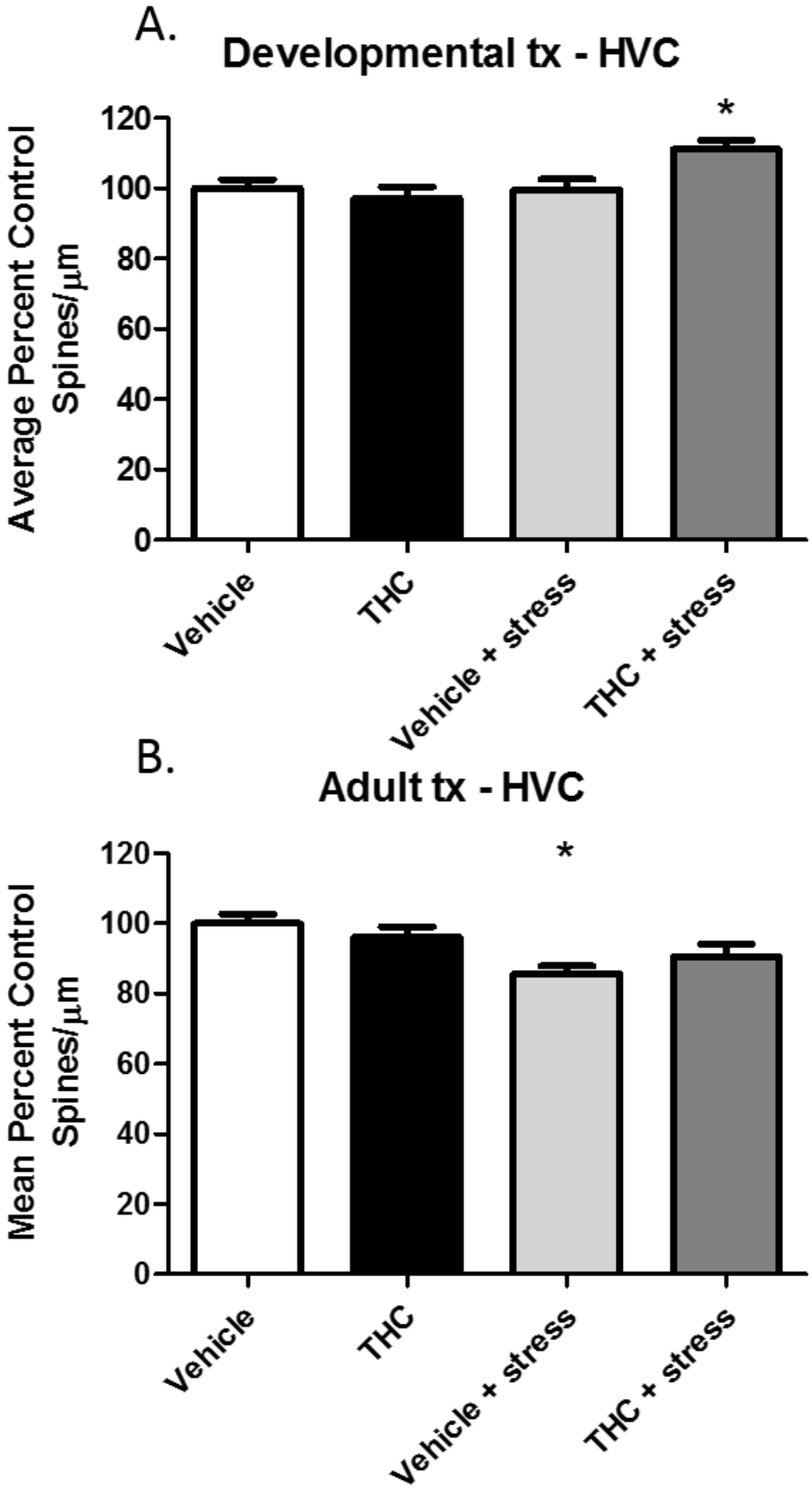
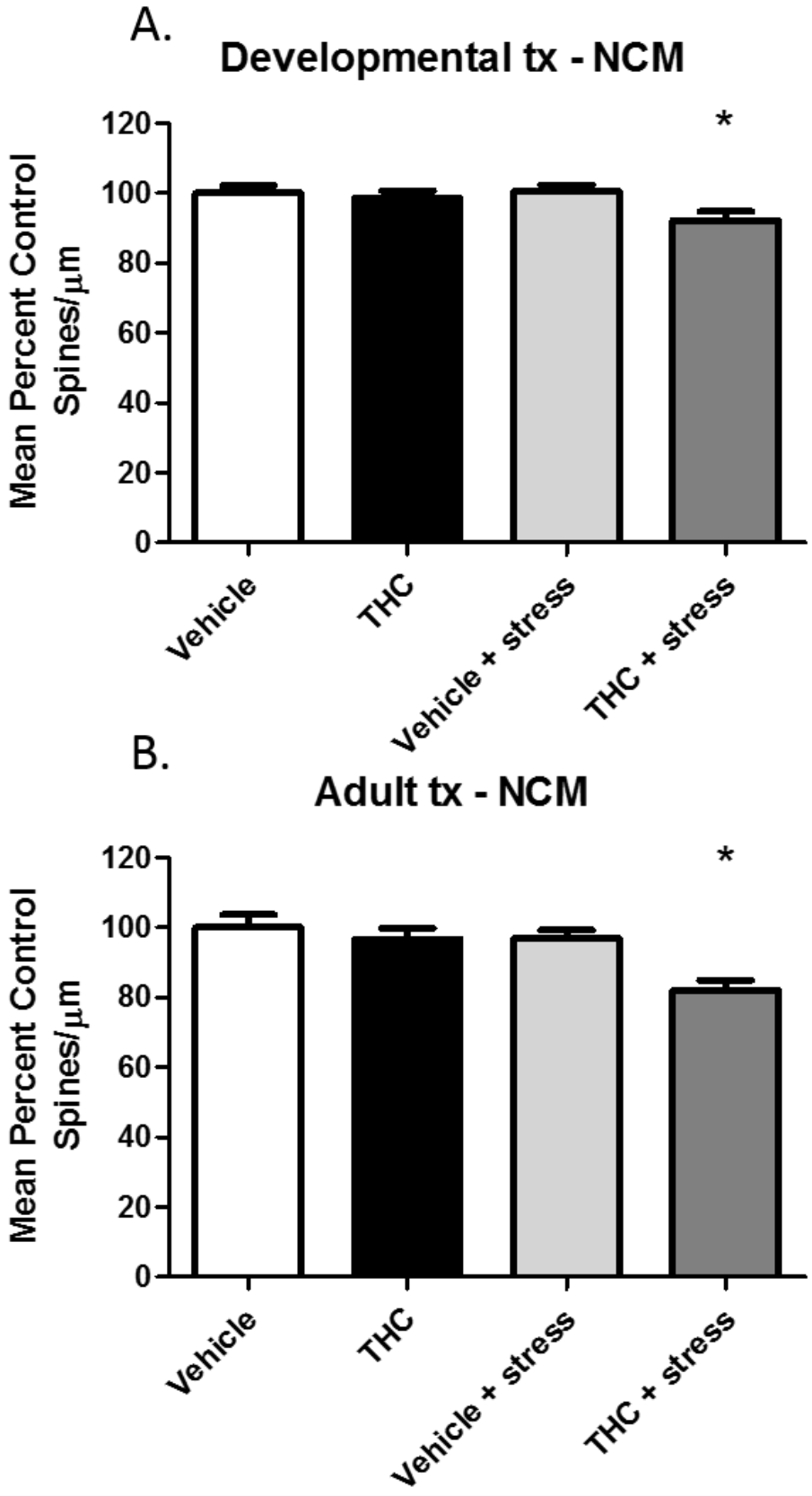

A.
Developmental tx - HVC
Average Percent Control Spines/µm
120
100
80
60
40
20
0
*
Vehicle
THC
Vehicle + stress
THC + stress
B.
Adult tx - HVC
Mean Percent Control Spines/µm
120
100
80
60
40
20
0
*
Vehicle
THC
Vehicle + stress
THC + stress

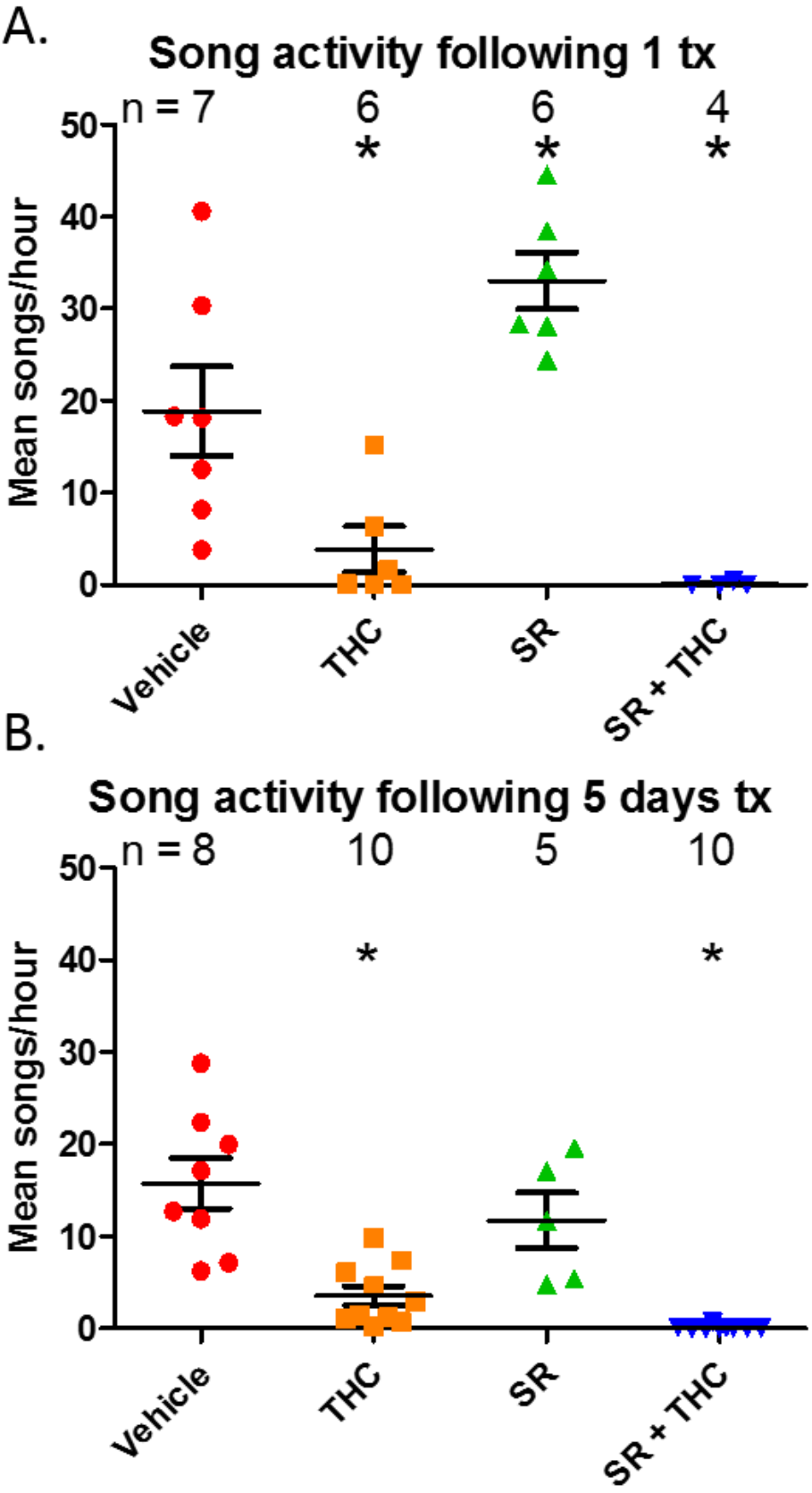
FIGURE 3.22: NCM dendritic spine density following chronic treatments. Following developmental treatments, THC + stress persistently decreased spine density compared to vehicle control (**A**). After adult treatments with THC + stress (**B**), spine density was decreased compared to adults treated with vehicle (*$p < 0.05$, one-way ANOVA, Tukey HSD post-hoc test). Error bars = SEM.


A.
Developmental tx - NCM
Mean Percent Control Spines/µm
120
100
80
60
40
20
0
*
Vehicle
THC
Vehicle + stress
THC + stress
B.
Adult tx - NCM
Mean Percent Control Spines/µm
120
100
80
60
40
20
0
*
Vehicle
THC
Vehicle + stress
THC + stress

Modulation of Song Activity by THC or SR Following One or Five Treatments

Adult zebra finches (n = 5-10) were administered five daily treatments with vehicle, THC (3 mg/kg), SR (6 mg/kg), or SR + THC. Zebra finches were recorded 24 hours following the last treatment in order to determine if SR pretreatment would antagonize the inhibitory effect of THC on song activity, alpha = 0.05, dof = 3, one-way ANOVA, Tukey HSD post-hoc test. Repeated THC treatments significantly inhibited song production (3.54 mean songs/hour ± 1.04) compared to vehicle treatment (15.79 mean songs/hour ± 2.75). SR treatment (11.73 mean songs/hour ± 2.99) had no effect. The song production of the SR + THC group did not resemble the vehicle group's, as hypothesized, but was virtually nonexistent (0.112 mean songs/hour ± 0.07). The experiment was repeated with a single vehicle, THC (3 mg/kg), SR (6 mg/kg), or SR + THC treatment in adult zebra finches (n = 4-7) followed by a vocal recording session to determine if chronic treatment was necessary to observe the results from the five treatment experiment. Similar results were found in the one treatment experiment, but an additional effect was that SR (33.02 mean songs/hour ± 3.082) significantly stimulated song activity compared to vehicle treatment (18.86 mean songs/hour ± 4.847; $p < 0.05$, dof = 3, one-way ANOVA, Tukey HSD post-hoc test; **Fig. 3.23**).

FIGURE 3.23: Song activity following one treatment (**A**) or five daily treatments (**B**) with vehicle, THC (3 mg/kg), SR (6 mg/kg), or SR + THC in adult zebra finches (n = 4-10). THC treatments inhibited song production (*p < 0.05). One SR treatment stimulated song activity (*p < 0.05), but this effect was not present following five treatments. SR pretreatment did not antagonize the effects of THC, and the SR + THC groups had inhibition of greater magnitude than THC alone (*p < 0.05, one-way ANOVA, Tukey HSD post-hoc test). Error bars = SEM.


A.
Song activity following 1 tx
n = 7
6
6
4
*
*
*
Mean songs/hour
50
40
30
20
10
0
Vehicle
THC
SR
SR + THC
B.
Song activity following 5 days tx
n = 8
10
5
10
*
*
Mean songs/hour
50
40
30
20
10
0
Vehicle
THC
SR
SR + THC

CHAPTER FOUR: DISCUSSION

Acute Stress

Song performance and the auditory system may represent defensive tools of zebra finches in a stressful scenario. Unlike some songbirds, such as European starlings and canaries, that can learn new songs in adulthood, zebra finches are age-dependent learners that can only learn their song during a critical developmental period. In adulthood, the song is crystallized and has low variability (Boogert et al., 2008). A main finding of the current study is that the medians of syllable duration, pitch, and entropy were rapidly increased following acute restraint stress compared to baseline recordings (**Fig. 3.3**). The changes to the central tendency of syllable acoustic features suggest they are singing an alternate version of their song, which was surprising since the central tendency is considered fixed and stable in adulthood. A stressful event or stimulus may trigger an alarm neurally that prompts the performance of an emergency version of their song, which may have significance to other zebra finches, such as their partner, stimulating increased vigilance and inhibition of less urgent behaviors, such as foraging for food. Acute restraint stress also increased the number of syllables and increased the IQR (variability) of pitch goodness and FM (**Fig. 3.3**), which may additionally represent a more dramatic song aurally and be a type of alarm to conspecifics who are familiar with the bird's typical song.

A short (one hour) recording duration following the cessation of the stressor was selected to obtain the acute response. An initial concern was that birds would not

perform adequate songs in this brief duration or that the stressor would suppress singing. Birds were randomly assigned to groups, and three out of seven No stress birds and three out of eight Stress birds did not perform adequately (≥ 10 songs needed) in baseline recordings, were designated nonperformers, and were not included in the study. However, all birds in both groups who performed in baseline session also performed in their subsequent session, and there was no statistical difference in song activity between No stress and Stress groups (**Fig. 3.2**). This finding indicates that future experiments evaluating acute song behavior are possible, such as a function of drug pretreatment, age, or behavioral paradigms.

Dendritic spines are the anatomical basis of the post-synaptic site that receives primarily excitatory input. Their dynamic, activity-dependent populations may represent morphological correlates of behavior. For example, an acute multimodal stressor in mice reduced hippocampal spine density and inhibited novel object discrimination, suggesting decreased recognition memory (Chen et al., 2010). Although zebra finch hippocampus has significance to spatial memory (Mayer et al., 2013), for the songbird model we analyzed brain regions relevant to the auditory pathway, song production, and song learning. The rapid effects of acute restraint stress on dendritic spine density were region-selective (**Fig. 3.5**). In primary auditory cortex-like L2, the stress group had decreased overall spine density, including reductions of thin and stubby subtypes. In higher order, perceptual auditory region NCM, spine density was increased, including an increase in the stubby subtype (**Fig. 3.6**). The spine subtypes altered by acute stress may represent less stable, more dynamic populations. L2 is the thalamo-recipient region in the brain that projects to L1 and L3 and then to CMM and NCM to transmit

information in auditory processing. L2 neurons are narrowly tuned and respond to specific spectral or temporal qualities (Nagel and Doupe, 2008). NCM is an integrative region that may contain a bird's memory of the template of song (Bolhuis and Gahr, 2006). Decreased spine density in L2 following acute stress may represent an inhibition of the responses of its narrowly-tuned neuronal populations and a "tunnel effect" on primary auditory sensitivity that filters some acoustic stimuli. Increased spine density in NCM may correspond to enhanced perception of fine auditory interpretation that may have conspecific significance. A limitation is the current research did not directly measure auditory sensitivities, and in the future, electrophysiology experiments may compare responses of different auditory regions to different auditory stimuli, such as bird's own song, conspecific song, predator vocalizations, and noise.

In rats, 30 minutes of restraint stress induced widespread c-Fos mRNA in nearly every region evaluated, including in hippocampus, amygdala, dorsal striatum, temporal cortex, parietal cortex, and frontal cortex (Cullinan et al., 1995), and for this reason, we analyzed the density of c-Fos immunolabeled nuclei in numerous zebra finch brain regions (**Fig. 3.7-3.10**). However, we did not find widespread induction, and the pattern of c-Fos distributions induced by this paradigm in mRNA with in situ hybridization may be different than in the protein with immunohistochemistry. Following restraint stress in rats with both these methods, c-Fos in immunohistochemistry was less widely induced than c-Fos mRNA (Del Bel et al., 1998). In zebra finches, thirty minutes of restraint stress increased density of c-Fos immunolabeled nuclei in selected regions in a pattern consistent with spine density results. Since non-constitutive changes in spine density are activity-dependent and influenced by neuronal activity (Yasumatsu et al., 2008), the

corresponding c-Fos results suggest the observed spine density results are driven by changes in neuronal activity following acute stress. The selectivity of effects on dendritic spine density and c-Fos density in auditory regions, not in regions mediating song production and song learning, may suggest that zebra finches' auditory system is an important defensive tool in presence of predators or other threats.

In addition to changes in auditory regions L2 and higher-order regions L1, L3, CMM, and NCM, acute stress induced c-Fos density in an avian amygdala analogue the nucleus taeniae (TnA). Following sensory input, the mammalian amygdala sends a distress signal to the hypothalamus as part of a neuroendocrine stress-response mechanism. Increased c-Fos density in TnA may represent an important interpretation of threats and relaying a message to activate autonomic and hormonal stress effects.

Following vehicle, Δ^9-THC (3 mg/kg), or SR141716 (6 mg/kg) pretreatments and acute restraint stress, plasma corticosterone was measured by enzyme immunoassay (**Fig. 3.11**). In comparison to the earlier no stress vs. stress experiment (**Fig. 3.1**), the results of the drug pretreatment + stress experiment displayed more variance, and the results yielded fewer statistically significant effects. In comparison of the methods of the two experiments, the animals in the drug pretreatment experiment were transported from the animal facility to a laboratory room in the morning before the execution of the experiment in the afternoon. This may have introduced confounding environmental factors, unlike in the earlier no stress vs. stress experiment, which was performed entirely in the animal facility. The corticosterone level of the vehicle + stress group was not significantly different from the vehicle group, which suggests the effects of restraint stress on corticosterone were not robust in this drug pretreatment experimental

paradigm. Additionally, the corticosterone level of the vehicle + no stress group (2.535 ng/mL ± 0.321) in the drug pretreatment experiment was higher than the no stress group (0.837 ng/mL ± 0.117) and, notably, the stress group (1.716 ng/mL ± 0.293) in the earlier experiment. The experimental protocol of the drug pretreatment + stress experiment may have stimulated corticosterone levels to a degree that changes as a function of treatment were more difficult to detect.

SR141716 pretreatment + stress resulted in significantly increased corticosterone levels compared to the vehicle group, and SR141716 may enhance stress responses by blocking endocannabinoid activity in a negative feedback, stress buffer system. The Δ^9-THC group exhibited increased corticosterone levels, which is in harmony with the effects observed in rodents with 1, 2, or 4 mg/kg doses (Zuardi et al., 1984). Δ^9-THC has relatively lower efficacy than endocannabinoid anandamine (AEA) in [^{35}S]GTPγS binding assays (Kearn et al., 1999). AEA inhibits the HPA axis under basal conditions (Hill and Tasker, 2012), and Δ^9-THC may displace AEA and antagonize that basal inhibition, triggering an increase in corticosterone.

Evidence supports that CB_1 receptor agonists inhibit zebra finch perceptual auditory processing (Whitney et al., 2003; Gilbert and Soderstrom, 2013). Acute restraint stress altered c-Fos density in auditory regions (CMM, NCM, L1, L3, and L2) and TnA. Δ^9-THC pretreatment was hypothesized to inhibit acute stress-induced changes in c-Fos density. In CMM and NCM, the mean expressions of the vehicle + stress groups were significantly increased compared to the vehicle groups. Δ^9-THC treatment inhibited this stress response, and SR141716 pretreatment antagonized the inhibition by Δ^9-THC, which implicates a CB_1-receptor-mediated mechanism. Δ^9-THC is

the principal psychoactive constituent of marijuana, and anxiolytic effects are commonly cited by marijuana users (Lee et al., 2007). Δ^9-THC may blunt acute stress effects, not through a neuroendocrine mechanism by decreasing corticosterone, but a psychoactive mechanism of altering the perception or interpretation of threats. Inhibition of acute stress responses following Δ^9-THC pretreatment may make zebra finches less prepared to avoid a threat, because stressor-induced activation of CMM and NCM may be adaptive for their survival. In contrast to zebra finches, humans do not risk life-or-death scenarios as frequently, and inhibition of stress responses by the psychoactive effects of Δ^9-THC may be desirable. Unlike CMM and NCM, Δ^9-THC did not inhibit c-Fos stress responses in L1, L3, L2, or TnA. CMM and NCM are higher-order auditory regions that are integrative and may contain a zebra finch's memory of song (Yanagihara and Yazaki-Sugiyama 2016), and these regions may be more vulnerable to the psychoactive effects of Δ^9-THC.

Comparison of Acute and Chronic Stress Effects

The acute and chronic stress experiments evaluated similar endpoints: plasma corticosterone levels, song activity, song acoustic features, and dendritic spine density. However, the hypotheses for the effects of acute or chronic stress on these endpoints differed. An acute stressor may induce adaptive, transient effects to aid an animal in surviving a flight-or-fight scenario. Chronic stress may induce maladaptive, long-term effects and is associated with depression- or anxiety-like behavior.

A neuroendocrine marker for acute stress is plasma corticosterone. In adult zebra finches, restraint in a paper bag for thirty minutes rapidly stimulated plasma corticosterone concentration (**Fig. 3.1**), but chronic mild stressors for 25 days did not demonstrate any significant effect on corticosterone levels (**Fig. 3.15**). These results support that the acute restraint-induced increase in corticosterone may be temporary and part of a fight-or-flight response. In the chronic experiments, only one blood sample was collected at time of euthanasia, which was following a 25 day no-treatment period. Increased corticosterone concentration may have been detected if an additional blood sample was collected immediately following the cessation of the chronic stressors.

Song activity results also differed between acute and chronic stress experiments. While acute restraint stress had no effect on song activity, measured by mean song files/hour (**Fig. 3.2**), chronic stress stimulated song activity in adults, both immediately and persistently following cessation of stressors (**Fig. 3.17**). Elevated song activity may represent a unique, songbird-specific behavioral phenotype of chronic stress in zebra finches. Acute restraint stress rapidly altered the medians and IQRs of several acoustic features in adults (**Fig. 3.3**), but the chronic stress paradigm in adults had no effect on acoustic features. Bird song is an important tool of communication. The altered acoustic features following a single stressor may signify to other birds that a threat may be temporary, and the increased song output following chronic stressors may signal that threats have occurred more frequently.

While acute restraint stress rapidly increased dendritic spine density in NCM in adults (**Fig. 3.5**), chronic stress had no effect (**Fig. 3.22**). This difference may signify that activation of NCM is beneficial in fight-or-flight scenarios, but this effect habituates

over repeated exposure to stressors. Chronic stress in adults decreased dendritic spine density in HVC (**Fig. 3.21**), but acute stress had no effect in this region (**Fig. 3.5**). Since HVC is the premotor region for song production, reduction of spine density may have mechanistic significance to the increased song activity following chronic stress.

Comparison of Developmental and Adult Treatment Effects

The effects of chronic THC or stress treatments were hypothesized to be developmentally-dependent, because sensorimotor song learning and dendritic spine pruning occurs during late postnatal developmental. Both chronic THC or stress treatments during development, but not during adulthood, resulted in similar, persistent effects on syllable entropy medians and Area X dendritic spine density (**Fig. 3.18, 3.20**). Additionally, developmental THC or stress treatments, not adult treatments, increased entropy IQRs, but the effects in the developmental stress group were not persistent into adulthood (**Fig. 3.19**). The development-dependence of these effects supports the hypothesis that sensorimotor development, a critical period for song learning, is vulnerable to exogenous CB_1 receptor agonism and to the chronic mild stress paradigm. The similarity of the results in the developmental Δ^9-THC and vehicle + stress groups suggests that the effects may have mechanistic overlap. Since endocannabinoids mediate stress responses in the HPA axis, chronic stress may increase endogenous CB_1 receptor activation and have similar developmental consequences as Δ^9-THC treatments. Area X is a striatal brain region important in developmental song learning as a part a cortical-basal ganglia-thalamic loop. Lesions in Area X in juvenile zebra finches disrupt normal song development, while lesions in adults had no effect (Sohrabji et al.,

1990). Dopaminergic neurons from ventral tegmental area (VTA) innervate Area X, and phasic dopamine firing acts as a signal for reward prediction errors (Schultz, 2016), which judges and compares the obtained reward versus the predicted reward. Dopamine prediction error encoding likely underlies developmental song learning and may signal the quality of syllable performance, compared to the predicted syllable, which is encoded within the songbird's template (Woolley, 2019). Chronic Δ^9-THC or stress treatments during development decreased dendritic spine density in Area X, and these treatments may have impaired the stabilization and strengthening of synapses underlying normal song development, that results in a net spine reduction persistent into adulthood.

It is unclear why entropy medians were decreased (i.e., less "noisy") by chronic developmental Δ^9-THC or stress treatments, and no effects were observed on other acoustic features. In contrast, acute restraint stress in adults increased entropy (**Fig. 3.3**), which is consistent with increased disorder or nonlinearities observed in "distress calls" in other organisms, including human infant cries (Koutseff et al., 2018). Zebra finch song contains different syllable types, including harmonic stacks, which typically have moderate entropy, and noisy syllables, which have relatively high entropy. Decreased entropy medians following chronic developmental Δ^9-THC or stress treatments may be the result of a shift in distribution of syllable types to increased harmonic stack types. Increased entropy IQRs following chronic developmental Δ^9-THC or stress treatments signifies increased variability in the performance of this acoustic feature, and this result suggests diminished song quality.

The effects of chronic Δ^9-THC treatments (3 mg/kg) during development on zebra finch were less profound than a previous experiment with WIN 55,212-2 (1 mg/kg), a full cannabinoid agonist. Developmental WIN 55,212-2 treatments resulted in adult songs with fewer syllables learned and less stereotypy compared to vehicle group, but adult treatment had no effect (Soderstrom and Johnson, 2003). The reduced song quality of WIN 55,212-2-treated zebra finches was apparent in visual inspection of spectrograms, which contained repeated syllables in a non-motif sequence, and this vividly supported the song analysis results. In contrast, the effects from Δ^9-THC were more subtle. Song syntax analysis was performed with SongSeq software to identify the frequency of typical and atypical syllable transition as an assessment of song quality (Daou et al., 2012), and the hypothesis was that Δ^9-THC -treated songs would have increased number of atypical transitions, which represent “mistakes” in the performance of the typical syllable motif sequence. Treatments with Δ^9-THC, or stressors, did not significantly alter the number of atypical transitions, that suggests the treatments had no effect on song syntax. These results were supported by visual inspection of the spectrograms, which all looked like typical zebra finch songs, with repeated introductory syllables followed by a consistent sequence of syllables organized in a motif. The differing effects of Δ^9-THC and WIN 55,212-2 on song syntax may be a reflection of their different efficacies, because while WIN 55,212-2 is a full cannabinoid agonist, Δ^9-THC is a partial agonist.

Chronic stress in both developing and adult zebra finches initially stimulated song activity in a Δ^9-THC -reversible manner, but the effects in the developmental groups were not persistent in adulthood (**Fig. 3.16, 3.17**). This developmental-dependence of

effects is the opposite trend as the acoustic features and Area X dendritic spine density experiments. Song production to a female is associated with the motor pathway, not the anterior forebrain pathway (**Fig. 1.1**). Late postnatal development may only be vulnerable to long-term effects relevant to sensorimotor song learning and the anterior forebrain pathway. Notably, developmentally-treated zebra finches in all groups demonstrated a relatively high level of variance in song activity, compared to adult treatment groups, following the no treatment period. The chronic drug + stressors protocol during development may persistently alter song activity, but not as a function of treatment group.

Modulation of Song Activity by THC or SR Following One or Five Treatments

The hypothesis was that the CB_1 receptor antagonist/inverse agonist SR141716 pretreatment would prevent effects of Δ^9-THC on song activity and demonstrate a CB_1 receptor mediated mechanism. These results would have significance to understanding the underlying mechanism in the chronic Δ^9-THC experiments (**Fig. 3.23**). Five daily treatments with Δ^9-THC (3 mg/kg) in adults produced the expected results of inhibition of song activity, which was similar to the results in the earlier 25 daily treatments experiment (**Fig. 3.17**). Five daily treatments with SR141716 (6 mg/kg) alone had no significant effect on song activity, but SR141716 pretreatment + THC virtually eliminated all song activity. One reason this was surprising is that SR141716 (6 mg/kg) pretreatment acted as an antagonist in the acute drug corticosterone experiment (**Fig. 3.11**) and appeared to inhibit the effects of Δ^9-THC (3 mg/kg). The SR141716 + Δ^9-THC song activity experiment was repeated with only one treatment instead of five

treatments to determine if SR141716 pretreatment would antagonize the Δ^9-THC effects on song activity in an acute paradigm. The results following one treatment were similar to those following five treatments, except SR141716 additionally stimulated song activity. This suggests that tolerance to the effects of SR141716 on song activity occurred following chronic exposure. Another reason the dramatic inhibition of song activity following SR141716 + Δ^9-THC treatments was surprising is that all the zebra finches in the SR141716 + Δ^9-THC group sang adequately in initial baseline recordings. These were executed to identify "nonperformers," and zebra finches that did not perform enough songs, according to inclusion criteria, in baseline recordings were not included in song analysis experiments. The effect of SR141716 + Δ^9-THC on song performance may involve a mechanism specific to the song system. Since ventral tegmental area (VTA) dopaminergic neurons with input to striatal Area X are active during singing, especially when directed to a female (Huang and Hessler, 2008). SR141716 may alter dopamine transmission in a way that exaggerates the inhibitory effects of Δ^9-THC on motivation to sing and rewarding effects of singing. While Δ^9-THC increases dopamine release in the striatum (Bloomfield et al., 2016), it reduced motor activity. SR141716 decreases dopamine release from VTA to nucleus accumbens (Cheer et al., 2007). Δ^9-THC may reduce motor output in striatum by inhibiting GABAergic input to the indirect dopamine pathway, and SR141716 may inhibit by reducing direct pathway dopamine.

Limitations

Zebra finches, the animal model for the experiments in this dissertation, were chosen for several reasons. Most notably, they learn a complex song during a

developmental critical period in a sensorimotor mechanism that resembles human language acquisition (Bolhuis et al., 2010). Rodents possess innate vocalizations (Kikusui et al., 2011), but not developmentally-learned, complex vocalizations, and human participants are not pragmatic for many types of molecular and behavioral experiments. A songbird model offers an unique approach to assessing the effects of cannabinoid drugs and stressors on adolescent brain development. Zebra finches additionally have other qualities that make them preferable in the laboratory setting, compared to other songbirds (Griffith and Buchanan, 2010). They are closed-learners that cannot learn a new song in adulthood (George et al., 1995), are colonial (i.e., live in groups and are characterized as relatively non-territorial; Dunn and Zann, 1996), are opportunistic breeders (Williamson et al., 2008), and do not possess seasonal hormone fluctuations (Tramontin and Brenowitz, 2000).

However, the zebra finch model also presents major limitations. Only male zebra finches were used in this dissertation's experiments, because the major focus was analysis of song learning and performance. While both male and female zebra finches memorize a song template early in development, only male zebra finches perform a song and possess song circuitry that is functional to motor production of song. Female song circuitry is less well-developed, and it is unclear if it is vestigial or possesses a function that is unique to females and unrelated to song production (Shaughnessy et al., 2019). Inclusion of female animals is significant for all biomedical research and a priority for National Institutes of Health (NIH) funding (McCullough et al., 2014). For this dissertation's experiments, that female animals were not studied may especially represent a limitation. The chronic mild unpredictable stress paradigm was used as an

animal model for depression, and women have approximately twice the prevalence of depression than men (Kessler et al., 1993). One reason may be that women have increased stress responses (Heck and Handa, 2019). Although the role of the song system is not well understood in female zebra finches, much more research is available on their auditory pathway. Since acute restraint stress increased plasma corticosterone and altered c-Fos and dendritic spine densities in auditory regions in male zebra finches, future experiments may evaluate if female zebra finches have enhanced effects, which would demonstrate sex differences in stress responses.

Another limitation is the ability to generalize the effects of developmental Δ^9-THC exposure in this dissertation to the effects of marijuana on adolescent brain development in humans. Isolated Δ^9-THC was used, and this approach enables reproducibility and facilitates pharmacological experiments with other cannabinoid drugs (e.g., SR141716, WIN 55,212-2) to elucidate mechanisms with clarity. However, *Cannabis sativa* is a plant with more than 400 constituents, including at least 60 cannabinoids (Dewey, 1986). Some of these constituents may interact with the effects of Δ^9-THC, possibly resulting in different effects of *C. sativa* compared to isolated Δ^9-THC. Cannabidiol is a non-psychoactive phytocannabinoid that is a major constituent of *C. sativa* with many pharmacodynamic effects (Demirakca et al., 2011), including low affinity for CB_1 and CB_2 receptors, 5-HT_{1A} receptor partial agonism, inhibition of anandamide degradation, and possible neuroprotective effects. Cannabidiol reduced the effects of Δ^9-THC, including anxiety (Zuardi et al., 1982; Karniol et al., 1974), euphoria (Dalton et al., 1976), and paranoia (Bhattacharyya et al., 2010), in healthy humans. In adolescents, less is known about the role cannabidiol on the effects of THC.

In adolescent rats treated for 21 days with vehicle, Δ^9-THC, or CBD + Δ^9-THC, in ascending daily doses of 1 to 3 to 10 mg/kg for each drug, CBD enhanced Δ^9-THC-induced weight loss and anxiogenic behavior in the elevated plus maze (Klein et al., 2011). In this experiment, this suggests that CBD exaggerated the effects of Δ^9-THC. Future experiments may evaluate the effects of the addition of CBD to Δ^9-THC treatments on zebra finch song development, and these results may be more translational to research on marijuana in humans.

Another limitation is the methodology used to evaluate dendritic spines. Golgi-Cox histology is a classical technique that impregnates approximately <5% of neurons in an apparent random manner, to allow visualization of anatomical features, including dendritic spines, with light microscopy. The experiments in this dissertation determined net changes at time of euthanasia following acute restraint stress and chronic THC or stress treatments. While net changes provide information about overall spine density at a population level, a shortcoming is that this technique does not fully explain changes in spine dynamics. For example if a net increase occurs, it is unclear if this is a decrease in pruning of existing spines, an increase in new spines, or a combination. Time-lapse imaging with two-photon microscopy evaluates neurons over the course of multiple imaging sessions in live animals to evaluate the dynamics of spines, including transience or persistence of newly formed spines and the changes in individual spines over time (Holtmaat et al., 2005). Spine dynamics are important to circuit remodeling over the longitudinal course of development or during a learning task.

Future Directions

A major finding included that chronic Δ^9-THC or stress treatments during development persistently altered acoustic features into adulthood. To evaluate the biological significance of these altered songs, future experiments may evaluate female zebra finch preference for these songs. The hypothesis is that females would demonstrate less preference for playback of adult songs of zebra finches treated during development with Δ^9-THC or stressors, compared to vehicle-treated songs. These results would support the conclusion that the altered songs possess diminished quality. Previous research assessed female preference for adult songs of zebra finches that had experienced nutritional stress as nestlings, and stressed songs were less complex than control songs (Spencer et al., 2003). Using an L-shaped song discrimination apparatus with a speaker in each arm, females preferred playback of control songs over stressed songs (Spencer et al., 2005). This preference is evidence that adult song complexity may reflect early developmental experiences and a male's possible fitness as a potential mate.

Chronic stressors during development or adulthood had no effect on plasma corticosterone. A shortcoming is that blood was only collected at time of euthanasia, and chronic stress may have altered corticosterone initially, following cessation of stressors, but not persistently, following the no treatment period. Or, it is possible that while baseline corticosterone did not change, chronic stressors altered HPA axis responsiveness. Future experiments may evaluate the effect of an acute stressor on corticosterone after the 25 day treatment + 25 day no treatment period. The hypothesis is that HPA axis responsiveness, evaluated by this type of challenge experiment, would be altered as a function of earlier chronic stress. In adult European starlings, the chronic

mild stress group had inhibited acute restraint-induced corticosterone increase (Rich and Romero, 2005), and chronic stress may result in decreased HPA axis responsiveness or desensitization to stressors.

Conclusion

Stressors or Δ^9-THC altered plasma corticosterone, song performance, dendritic spine density, and c-Fos expression in zebra finches, and these effects were dependent on number of treatments and age during treatments. An acute restraint stressor in adults rapidly increased corticosterone, altered performance of song acoustic features (e.g., increased syllable entropy medians), and activated higher-order auditory region NCM, including increased dendritic spine and c-Fos densities. Δ^9-THC pretreatment decreased the stress response on NCM c-Fos expression in a SR141716-reversible manner, and CB_1 agonists may be anxiolytic by inhibiting stress effects on perceptual sensory processing. Acute stress effects are perhaps a fight-or-flight response that are adaptive for a zebra finch's survival and are transient.

Chronic stress during adulthood had no long-term effect on corticosterone, NCM dendritic spine density, or song acoustic features, including syllable entropy medians. Stress stimulated song activity (mean song files/hour), but this behavioral phenotype only emerged following chronic treatments. Song activity may be songbird-specific marker of chronic stress. Sensorimotor development may be more vulnerable than adulthood to chronic Δ^9-THC or stress treatments as a consequence of the prominence of the endocannabinoid neuromodulatory system during late postnatal development.

Chronic treatments with Δ^9-THC or stress during sensorimotor development persistently decreased syllable entropy medians and dendritic spine density in Area X, a striatal region important in developmental song learning as a part a cortical-basal ganglia-thalamic loop. Adult treatments did not alter song acoustic features or Area X dendritic spine density.

These results contribute to the elucidation of mechanisms and long-term effects of marijuana abuse and psychological disorders, such as depression, during adolescence (**Fig. 3.24**). In consideration of analysis of acute vs. chronic stress effects and analysis of developmental vs. adult treatment effects in order to form a holistic conclusion, this dissertation may present evidence that zebra finches are a compelling developmental psychopharmacological model for biomedical research concerning the effects of cannabinoids and chronic stress during adolescence.

FIGURE 3.24: Proposed mechanism of acute and chronic stress

Acute stressor

↑ Corticosterone

↓

Altered cFos and dendritic spine density in auditory regions

↓

Altered auditory sensitivity

↓

Altered acoustic features, including ↑ entropy median

↓

Enhanced communication regarding potential threat

↓

Transient, adaptive fight-or-flight response

Chronic stress

Adaptive effects of first stressor are transient

↓

Chronic exposure induces dysregulation of stress response

↓

Long-term, maladaptive changes in developmental-sensitive manner

↓

A. Adult treatment inappropriately, persistently increased song activity

Depression-like behavioral phenotype

B. Developmental treatment persistently ↓ entropy median and Area X dendritic spine density

Altered sensorimotor song development

Chronic developmental Δ^9-THC treatments

More prominent endocannabinoid system during sensorimotor development

↓

Chronic developmental Δ^9-THC treatments alter acoustic features similarly as developmental stress

↓

Chronic developmental stress induces endocannabinoid system similarly as Δ^{9}-THC treatments

↓

Adolescent brain development is similarly sensitive to chronic stress or Δ^{9}-THC exposure

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APPENDIX A: APPROVAL LETTER – ANIMAL USE

East Carolina University

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

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

October 16, 2014

Ken Soderstrom, Ph.D.
Department of Pharmacology
Brody 6S-10
ECU Brody School of Medicine

Dear Dr. Soderstrom:

The Amendment to your Animal Use Protocol entitled, "Cannabinoid-Altered Vocal Development", (AUP #W207b) was reviewed by this institution's Animal Care and Use Committee on 10/16/14. The following action was taken by the Committee:

"Approved as amended"

****Please contact Dale Aycock prior to any hazard use**

A copy of the Amendment 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/jd

enclosure