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Sex Differences and Learning Related Changes in Acquisition of Associative Memory

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ABSTRACT

Understanding associative memory is fundamental for a variety of neurological and neurodegenerative diseases, however, a large proportion of this research has excluded female subjects due to unsubstantiated bias. By including intact females, ovariectomized females and males in the study of associative memory, clear sex differences in acquisition emerged. Female mice acquired the classical conditioning paradigm trace eyeblink conditioning faster than ovariectomized females and males under normal learning conditions. However, when male mice were implanted with tetrodes to record neuronal activity, acquisition was facilitated compared to intact females. These observed differences were not accounted for by the weight of the implant alone, therefore stress and neuroinflammatory factors undoubtedly play a fundamental role in these observed sex-differences. Further investigation into the mechanisms underlying sexually dimorphic behavior is necessary to develop better therapeutics for diseases and disorders that have observed sex-differences in prevalence and severity.

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LIST OF ABBREVIATIONS

AD: Alzheimer's Disease
AG1: Arginase 1
AHP: Afterhyperpolarization
AMPA: α-amino-3-hydroxy-5-methylisolazole-4-propionic acid
APOE: Apolipoprotein E
CA1: Cornu Ammonis 1
CA3: Cornu Ammonis 3
CR: Conditioned Response
CRF: Corticotropin-Releasing Factor
CRH: Corticotropin-Releasing Hormone
CS: Conditioned Stimulus
CSPGs: Chondroitin sulfate proteoglycans
DCN: Deep cerebellar nuclei
dEBC: Delay Eyeblink Conditioning
ELISA: Enzyme Linked Immunosorbent Assay
FASD: Fetal alcohol spectrum disorder
FMRI: Functional Magnetic Resonance Imaging
HPA: Hypothalamic-pituitary-adrenal
HVI: Hemispheric lobule VI
Iba-1: Ionized calcium-binding adapter molecule 1
IL-10: Interleukin ten
LH: Luteinizing hormone
LPS: Lipopolysaccharide
LTD: Long Term Depression
LTP: Long Term Potentiation
MHC II: Major Histocompatibility Complex II
mPFC: Medial Prefrontal Cortex
MRC1:Mannose Receptor
NGF: Nerve Growth Factor
NIH: National Institute of Health
NMDA: N:methyl:D:aspartate
OCT: Optimal Cutting Temperature
PBS: Phosphate-Buffered Solution
PFA: Paraformaldehyde
PICA: Posterior inferior cerebellar artery
PPAR-γ: Peroxisome Proliferation Activation Receptor gamma
SCA: Superior Cerebellar Artery
tEBC: Trave Eyeblink Conditioning

TGF-β: Transforming Growth Factor Beta
TNF-α: Tumor necrosis factor
UR: Unconditioned Response
US: Unconditioned Stimulus
Ym1: Chitinase 3-like 3

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CHAPTER ONE: INTRODUCTION

ASSOCIATIVE MEMORY

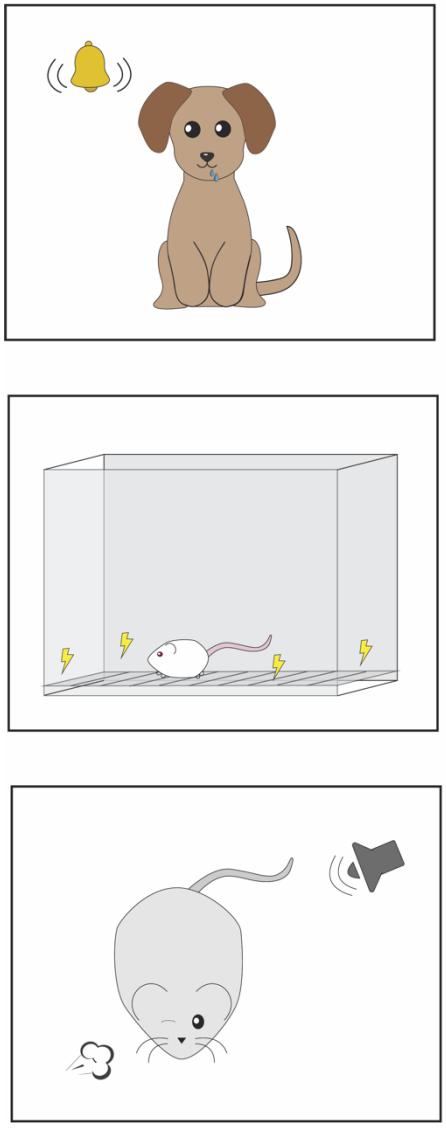

Figure 1. Associative Learning.
Top: Pavlov Conditioning
Middle: Fear conditioning
Lower: Eyeblink conditioning

Learning and memory are fundamental experiences, and understanding the mechanisms underlying learned associations is critical. Associative memory is the ability to learn a connection between two unrelated items or events. Learned connections are crucial to everyday experience and function. For example, simply learning and remembering the association between a red light and "stop" is integral to a functioning modern society. In order to investigate mechanisms of associative memory, scientists have utilized paradigms of classical conditioning. Classical conditioning has allowed neuroscientists to investigate the relationship between stimuli and learned behavior as well as the circuitry and mechanisms supporting this learned association.

Ivan Pavlov first described a classical conditioning paradigm in his work *Conditioned Reflexes* in 1927 (Gottlieb and Begej, 2014). Pavlov first used the conditioning paradigm to train dogs to salivate to a metronome after repeated pairings of the metronome with food. Repeated pairings of a neutral, conditioned stimulus (CS), with an unconditioned stimulus (US), which elicits an unconditioned response (UR), leads to a conditioned response (CR). The methodology of Pavlov's conditioning allows the experimenter strict control over the presentation of stimuli,

which are clearly defined and constrained (Gottlieb and Begej, 2014) Classical conditioning can be used to model associative learning as the subject experiences stimuli that are presented by the researcher, and this experience leads to a change in behavior that is long-lasting (Kehoe and Macrae, 2002). Two forms of classical conditioning that have been critical to the field of neuroscience are fear conditioning and eyeblink conditioning (Figure 1).

Fear Conditioning

In fear conditioning, the subject receives a neutral CS, such as a tone or light, which is paired with a strong threatening US, such as a foot shock. This pairing causes a threatened state in the subject, and in subsequent trials the subject shows stereotypical threatened behavior. An early example of fear conditioning is the "Little Albert" experiment, during which an 11-month-old boy was presented with a white rat that he curiously investigated. When the child touched the rat, an experimenter banged a steel bar behind his head, startling the child and leading him to cry and fall forward (Watson and Rayner, 1920). In subsequent presentations of the rat, the child began to cry. This fear generalized to other white, fluffy objects including a rabbit and a pair of earmuffs (Kim et al., 1996). Fear conditioning has been used across neuroscience to investigate the intersection of emotion and memory, particularly for the study of disorders including anxiety and PTSD (Maren, 2001). Rats and mice are common subjects for fear conditioning experiments and exhibit stereotypical "freezing" behavior after few CS-US pairings. Typical conditioned responses in rodents also include: decreased pain sensitivity (Lehner et al., 2010), increased blood pressure, respiration and heart rate (Iwata et al., 1986; Stiedl and Spiess, 1997), and ultrasonic distress vocalizations (Graham et al., 2009; Portfors, 2007)(Kim and Jung, 2006). The circuitry underlying fear conditioning has been thoroughly investigated, with the amygdala as a region of particular interest. In fear conditioning, subjects acquire the CS-US association in one

or a few trials, however, subjects acquire eyeblink conditioning slowly after repeated CS-US presentations. This allows experimenters to study the acquisition of the conditioned response over multiple sessions. While both fear conditioning and eyeblink conditioning are examples of classical conditioning, these paradigms employ different neural circuits and response timing (Fanselow and Poulos, 2005). Conditioned responses in fear conditioning are diffuse and can vary in behavioral response while eyeblink conditioning consists of a well-timed, specific eyeblink behavior to CS presentation (Fanselow and Poulos, 2005).

Eyeblink Conditioning

Instead of a metronome followed by food (Pavlov, 1927) or light followed by a foot shock in fear conditioning, eyeblink conditioning involves a neutral stimulus such as a tone, followed by an airpuff to the eye that causes a reflexive eyeblink response (Disterhoft and Weiss, 2017). After repeated pairings, subjects learn to close their eye prior to the US, a CR. As multiple paired CS-US trials are necessary for acquisition, conditioning occurs in bouts of trials called *training sessions*. Time between trials is defined as *intertrial interval*. *CR acquisition* can be defined as the growth in percentage of trials with CRs across training sessions. CR acquisition commonly follows a curved line, where subjects reach asymptotic performance in later training sessions. The depicted curves of CR acquisition are *learning curves* (Kehoe and Macrae, 2002). CRs are learned slowly over time, with multiple variables affecting the rate of acquisition, which will be discussed in further detail below. A distinctive feature of eyeblink conditioning is that changes in the relative timing between the onset of the CS and US affects the regions recruited for acquisition and allows researchers to compare particular types of learning and the necessary circuits (Miller, 2008).

DELAY EYEBLINK CONDITIONING

In delay eyeblink conditioning (dEBC), the CS precedes the US and the stimuli co-terminate (Figure 2). Acquisition of dEBC can occur rapidly, in one training session (Takehara-Nishiuchi, 2018). The circuitry of dEBC has been thoroughly studied, beginning with influential work from Richard Thompson (Lincoln et al., 1982; McCormick et al., 1982)(Figure 2). The cerebellum and related brainstem nuclei are widely accepted to be necessary and sufficient to support acquisition and retention of dEBC (Yang et al., 2015). Information from the CS and US is provided to the cerebellum by the pontine nuclei and dorsal accessory inferior olive. The pontine nuclei encode somatosensory, auditory and visual information from the brainstem and subcortical regions (Takehara-Nishiuchi, 2018). Mossy fibers from the pontine nuclei project this information to the deep cerebellar nuclei and granule cells in the cerebellar cortex through the cerebellar peduncle (Disterhoft and Weiss, 2017). The axons of the dorsal accessory inferior olive form climbing fibers that project to Purkinje cells and the interpositus nucleus through the inferior cerebellar peduncle (Matsushita and Ikeda, 1970; Swenson and Castro, 1983). When lesions of the anterior interpositus nucleus were made in rabbits trained on dEBC, CRs were eliminated, but URs remained. Lesions of the anterior interpositus nucleus also prevented untrained rabbits from learning dEBC (Bao et al., 1998; Disterhoft and Weiss, 2017; McCormick et al., 1982; Takehara-Nishiuchi, 2018) Furthermore, stimulation of the interpositus nucleus as the CS and stimulation of dorsal accessory inferior olive as the US was sufficient for reliable CRs and the acquisition of dEBC (Mauk et al., 1986; Steinmetz et al., 1989, 1986). Subsequent work from Yeo and Glickstein confirmed McCormick and Thompson's observations and additionally highlighted the importance of hemispheric lobule VI (HVI) of the cerebellar cortex

in dEBC (Yeo et al., 1985). Recordings obtained from HVI showed learning-related changes in large numbers of neurons (Berthier and Moore, 1986). Later studies have shown Purkinje neurons exhibited a variety of response patterns while interpositus neurons reflected CRs (Gould and Steinmetz, 1996). Hesslow and colleagues' work showed Purkinje neurons in HVI release tonic inhibitory input on anterior interpositus (Halverson et al., 2015; Jirenhed and Hesslow, 2016). Recording experiments from cerebellar cortex and interpositus nucleus show well-timed responses to drive CRs. The importance of forebrain regions to dEBC has also been thoroughly investigated. Richard Thompson's initial study of the hippocampus during dEBC showed a "neuronal model" of CRs that only occurred with conditioning (Berger and Thompson, 1978). However, lesion studies determined the hippocampus was not necessary for acquisition of dEBC (Schmaltz and Theios, 1972).

Synaptic plasticity in acquisition of dEBC has been seen in the cerebellar cortex and in the interpositus nucleus. Long-term depression (LTD) between the climbing fibers and purkinje cells have been shown in-vitro slice preparations (Feil et al., 2003; Ito and Kano, 1982). Long-term potentiation (LTP) has also been shown through slice preparation work in dEBC between mossy fibers and interpositus nucleus neurons (Pugh and Raman, 2008, 2006).

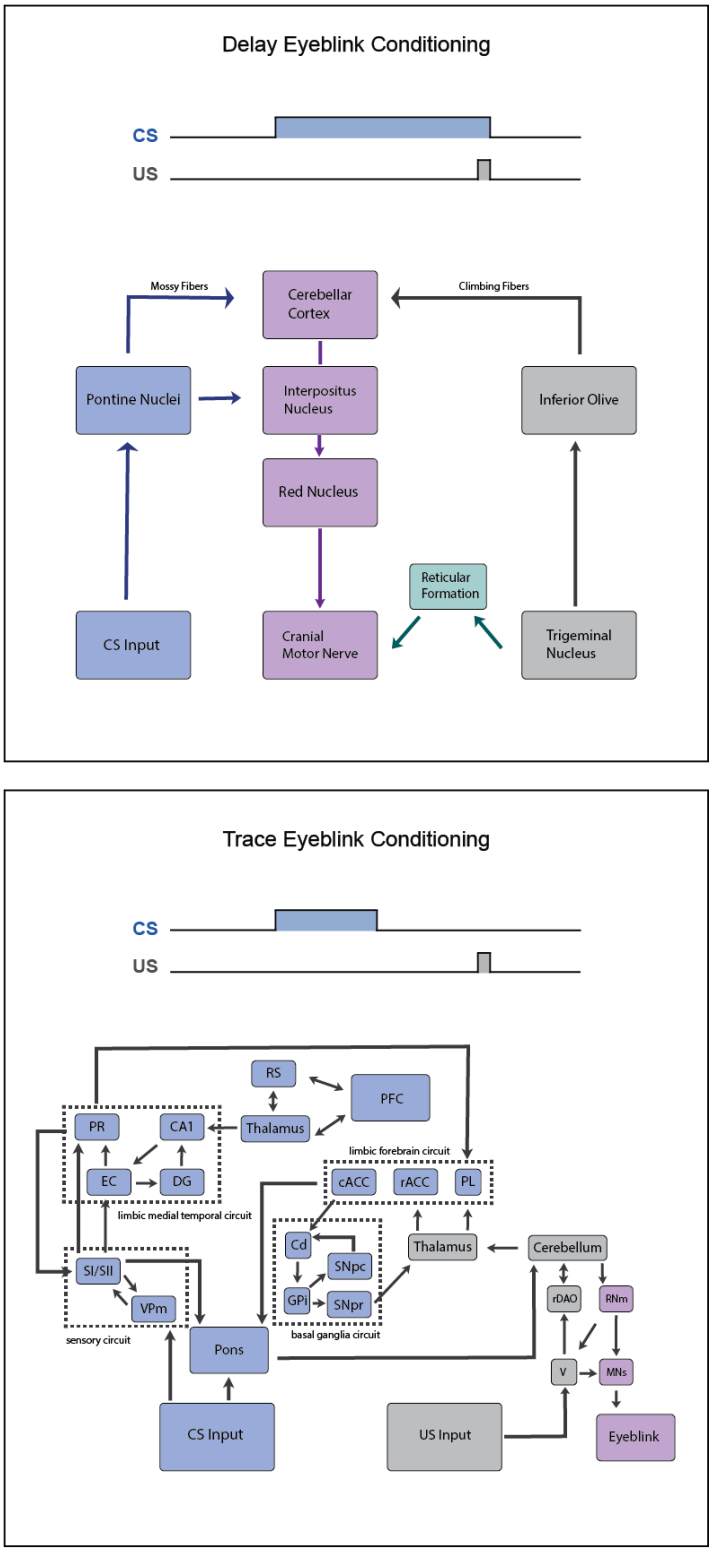

Figure 2. Eyeblink Conditioning Circuit Comparison. (Top) Delay Eyeblink Conditioning (Bottom) Trace Eyeblink Conditioning

TRACE EYEBLINK CONDITIONING

In trace eyeblink conditioning (tEBC), there is a stimulus-free interval between the presentations of the CS and US. This small modification to the paradigm greatly impacts the brain regions necessary for acquisition. In addition to the regions required for dEBC such as the cerebellum and brainstem, several forebrain regions are also necessary for tEBC including the hippocampus and medial prefrontal cortex (Kim et al., 1995; Kronforst-Collins and Disterhoft, 1998; Weible et al., 2000)(Figure 2). Unlike dEBC, tEBC requires multiple training sessions to establish reliable CRs (Takehara-Nishiuchi, 2018).

Lesions of deep-cerebellar nuclei blocked acquisition in naive rabbits, and blocked retention in trained rabbits (Woodruff-Pak and Thompson, 1985). However, in transgenic or knockout mice, mice with cerebellar disruptions were able to successfully acquire tEBC (Kishimoto et al., 2001; Woodruff-Pak et al., 2006). The complete circuitry of tEBC is under investigation, however it has been suggested that forebrain regions bridge the temporal gap to project information to the pontine nuclei. From the pontine nuclei, information is projected to the cerebellar cortex and to deep nuclei (Takehara-Nishiuchi, 2018).

Trace eyeblink conditioning is a hippocampal-dependent task as hippocampal lesions prevented the acquisition of tEBC in rabbits (James et al., 1987; Moyer et al., 1990; Port et al., 1986; Solomon et al., 1986). However, hippocampal lesions 1 month following acquisition did not prevent retention of CRs (Kim et al., 1995). Weible et al 2006 recorded from dorsal and ventral hippocampus throughout acquisition and found a variety of neuronal responses. Activity from dorsal neurons was more robust than that of ventral hippocampal neurons, suggesting a stronger role for dorsal hippocampal neurons in acquisition of tEBC (Weible et al.,

2006). Recording from the hippocampus ipsilateral to the eye receiving the airpuff illustrated changes earlier on in acquisition while the contralateral hippocampus showed significant changes later in training. Excitability of Cornu Ammonis 1 (CA1) and Cornu Ammonis 3 (CA3) hippocampal pyramidal neurons has also been shown to increase with conditioning (Moyer et al., 1996). This increase in excitability is seen through decreased afterhyperpolarization (AHP) and decreased spike frequency accommodation in pyramidal slice recordings Areas of the medial prefrontal cortex including the anterior cingulate and prelimbic cortices appear to have a role in differing phases of tEBC (Takehara-Nishiuchi, 2018). Acquisition is impaired by lesioning of the caudal anterior cingulate (Kronforst-Collins and Disterhoft, 1998; Weible et al., 2000) but remains unaffected by damage to the rostral anterior cingulate or prelimbic cortex (Kronforst-Collins and Disterhoft, 1998). However, rostral anterior cingulate and prelimbic cortex are necessary for retention as damage to these regions impairs CR expression (Oswald et al., 2010, 2008; Takehara-Nishiuchi et al., 2006). Studies of the prefrontal cortex in tEBC suggest this region serves to facilitate CS information across the trace interval, providing the final output from the forebrain to the pons (Disterhoft and Weiss, 2017).

Eyeblink Conditioning Across Species

Eyeblink conditioning is a well-studied translatable paradigm that has been utilized across species. Initially eyeblink conditioning was performed in humans, but has expanded to cover many species including rats, mice, rabbits, monkeys and ferrets (Table 1).

Human Eyeblink Studies

Human studies of eyeblink conditioning have been performed to understand various aspects of neuroscience including: basis of learning (Cason, 1922; Telford and Anderson, 1932), medial temporal lobe amnesia (Gabrieli et al., 1995), Korsakoff's (McGlinchey-Berroth et al.,

Species	Authors	Year
Mouse	Kishimoto et al	2001
	Weiss et al	2002
	Takatsuki et al	2003
	Tseng et al	2004
	Lin et al	2016
	Miller et al	2019
Rat	Hughes et al	1938
	Weiss et al	1991
	Weiss et al	1999
	Tokuda et al	2014
	Suter et al	2017
Rabbit	Gormezano et al	1962
	Woodruff-Pak et al	1985
	Moyer et al	1990
	Thompson et al	1996
	Weible et al	2006
	Miller	2008
Human	Woodruff-Pak et al	1990
	Woodruff-Pak	1993
	Soloman et al	1995
	McGlinchey-Berroth et al	1997
Other	Harrison & Buchwald	1983
	Gruart et al	1995
	Trigo et al	1999
	Chen et al	2014
	Wang et al	2019

Table 1. Eyeblink Conditioning Across Species

1995) and Alzheimer's Disease (AD). FMRI studies of humans undergoing dEBC and tEBC showed significant cerebellar activity during both tasks. As suggested by animal studies, hippocampal activity was greater in tEBC compared to dEBC (Cheng et al., 2008). Studies of patients with focal cerebellar lesions found that those with lesions of the superior cerebellar artery (SCA) that included the interpositus nucleus were impaired on tEBC acquisition (Gerwig

et al., 2006). However, patients with lesions of the posterior inferior cerebellar artery (PICA) successfully acquired the task (Gerwig et al., 2006). This data supported the hypothesis that different cerebellar structures contribute to tEBC acquisition.

Studies of bilateral medial temporal lobe amnesiacs found significant impairment in acquisition of tEBC (McGlinchey-Berroth et al., 1997; Woodruff-Pak, 1993). dEBC studies of alcoholic Korsakoff patients found these subjects were unable to learn dEBC, suggesting cerebellar damage that is typical with alcoholism (McGlinchey-Berroth et al., 1995). AD patients were impaired on dEBC compared to age-matched controls (Woodruff-Pak et al., 1990). These patients exhibited dementia compared to control subjects that were 80 years old or older. When AD patients receive training sessions across multiple days, they are able to acquire dEBC at levels similar to controls (Solomon et al., 1995). An additional element of human eyeblink conditioning is the role of awareness in acquisition. Clark and Squire found that awareness of the CS-US relationship was necessary to learn trace but not delay conditioning (Clark et al., 2002; Clark and Squire, 1999). Subjects that reported an awareness of the CS-US relationship at the conclusion of training successfully acquired tEBC. Based on these findings, Clark and Squire determined tEBC is a declarative memory task, while dEBC is nondeclarative. Distraction by an attention-exhausting task, thereby decreasing awareness, impaired acquisition of tEBC (Manns et al., 2000).

Rabbit Eyeblink Studies

Rabbits are an excellent species to study for eyeblink conditioning as they tolerate restraint, are tame animals and have a nictitating membrane that is convenient to measure (Woodruff-Pak and Thompson, 1985). Isadore Gormezano first published eyeblink conditioning

work in the albino rabbit, and a large portion of eyeblink literature has been conducted in rabbits (Gormezano et al., 1962; Woodruff-Pak and Thompson, 1985).

A significant portion of this research has been done on aged rabbits, and parallels findings of aging humans (Christian and Thompson, 2003; Kehoe et al., 1987; Woodruff-Pak and Thompson, 1985). Aged rabbits require more trials to successfully acquire tEBC compared to young adult rabbits (Thompson et al., 1996). Furthermore, the CRs of aged rabbits were not well-timed, suggesting an impaired hippocampus (Moyer et al., 2000, 1996, 1990). Recordings from CA1 neurons from naive aged rabbits had larger AHPs and increased accommodation compared to neurons from younger untrained rabbits (Moyer et al., 2000). Acquisition of tEBC increased excitability in both young and aged rabbits, reducing both the postburst AHP and spike frequency accommodation in correlation with learning (Moyer et al., 2000).

Due to their docile nature, rabbits are well-suited for imaging studies without sedation. In fact, rabbits commonly only need a single day of habituation to restraint for imaging, compared to other species like rodents that require a week or more (Schroeder et al., 2016; Weiss et al., 2018). FMRI studies of rabbits have demonstrated learning related changes in visual cortex and cerebellum with eyeblink conditioning (Miller, 2008; Miller et al., 2003), resting state networks (Schroeder et al., 2016), and learning and memory networks in resting-state FMRI of awake rabbits (Bertolino et al., 2020).

Rat Eyeblink Studies

While rabbits are docile, rats exhibit more spontaneous behaviors, which is advantageous for studying global hippocampal-dependent memory (Weiss and Disterhoft 2016). A system for freely-moving eyeblink conditioning has allowed researchers to study aging (Knuttinen et al., 2001; Weiss and Thompson, 1991), the effects of trace-interval length (Walker and Steinmetz,

2008; Weiss et al., 1999), and therapeutic effects on eyeblink conditioning (Burgdorf et al., 2011; Moskal et al., 2005). Four age-groups Male Sprague-Dawley rats were trained on dEBC: 3 months, 12 months, 18 months and 30 months. Middle aged and older rats (18 months and 30 months) were significantly impaired compared to young rats (3 months and 12 months), showing fewer CRs (Weiss and Thompson, 1991). Knuittinen and colleagues found that old and senescent male Fisher 344 x Brown Norway rats (28-35 months) showed significant impairment in acquisition of tEBC. Fifty percent of 28-29 month old rats showed significant reduction of CRs in the 250 ms trace interval task (Knuttinen et al., 2001). Long-Evans rats trained on shorter trace intervals learned tEBC at a higher rate across days and with a greater CR amplitude compared to rats trained on longer trace intervals. When rats received hippocampal lesions with ibotenic acid, they learned at a slower rate in both long and short interval groups (Walker and Steinmetz, 2008). When young Fisher 344 X Brown Norway male rats were injected with GLYX-13, a N-methyl-d-aspartate (NMDA) receptor modulator, their performance on tEBC was significantly improved compared to saline injected controls (Moskal et al., 2005). GLYX-13 improved tEBC in aged Male Fisher 344 X Brown Norway rats as well (Burgdorf et al., 2011).

Rats have also served as a model for fetal alcohol spectrum disorder (FASD), and a body of research has utilized dEBC and tEBC to study this disorder (Huebner et al., 2015; Murawski et al., 2013; Wagner et al., 2013). Eyeblink can be performed early on postnatal pups to understand hippocampal neuropathy due to neonatal alcohol exposure (Huebner et al., 2015). Impairments in both tEBC and dEBC with early alcohol exposure is behaviorally conserved across rats and humans (Murawski et al., 2013). This model may help uncover future targeted therapies to address the mechanisms behind deficits seen in FASD (Murawski et al., 2013).

Mouse Eyeblink Studies

With the development of many transgenic, knockout and Cre-recombinase technologies, mouse models have been critical for understanding molecular mechanisms underlying eyeblink conditioning. A variety of mutant mouse lines have shown reduced Purkinje neuron LTD and impaired eyeblink conditioning. Such studies have included mutant mGluR1 (Alba et al., 1994; Kishimoto et al., 2002), glutamate receptor subunit δ2 (Kishimoto et al., 2002, 2001; Takatsuki et al., 2003), CB1 receptor (Kishimoto and Kano, 2006), phospholipase Cbeta4 (Kishimoto et al., 2001; Miyata et al., 2003), glial fibrillary acidic protein (GAP) (Shibuki et al., 1996), and sodium channel Scn8A in Purkinje neurons (Woodruff-Pak et al., 2006). Mice lacking the glutamate receptor subunit δ2 showed a severe impairment in dEBC, but not tEBC (Takatsuki et al., 2003). Male Scn8A knockout mice were also impaired on dEBC, but not tEBC or Morris Water Maze, providing further support for the role of the cerebellum in dEBC (Woodruff-Pak et al., 2006).

Mutant mice have also been used to investigate Alzheimer's Disease (Ewers et al., 2006; Kishimoto et al., 2012; Kishimoto and Kirino, 2013; Weiss et al., 2002a). The presenilin 2 transgenic mice show normal CR acquisition at 3 months, but were impaired at 4, 6 and 12 months (Kishimoto and Kirino, 2013). APP+ PS1 mice were not significantly impaired on dEBC or tEBC, however cortical amyloid load was correlated with decreased tEBC performance (Ewers et al., 2006). The PDAPP mouse had decreased hippocampal volume, which was predictive of decreased learning rate (Weiss et al., 2002b).

Recent work from the Disterhoft lab has utilized genetic ablation of neural progenitor cells to investigate the role of neurogenesis in tEBC (Miller et al., 2019). The study used nestin-HSV-TK transgenic mice with valganciclovir chow to show male mice had significantly reduced acquisition compared to controls and female mice (Miller et al., 2019).

A head-fixed preparation for the mouse was developed by Chettih, McDougle, Ruffolo, and Medina (2011). This preparation allows the mouse to run atop a freely moving cylinder to reduce the stress of restraint. The head-fixed setup was utilized to investigate the importance of deep cerebellar nuclei (DCN) in the expression of CRs in dEBC and found anterior DCN was necessary for CR expression (Heiney et al., 2014). Head-fixation allows for reliable stimulus delivery and neurophysiological or imaging experiments (Heiney et al., 2014; Najafi et al., 2014).

Other Species Eyeblink Studies

Eyeblink conditioning has been performed in a variety of additional species including monkeys, cats, dogs, guinea pigs and ferrets. This versatile paradigm has allowed researchers to investigate such topics as the role of theta in tEBC (Chen et al., 2014; Wang et al., 2014), firing and timing of Purkinje cells in eyeblink conditioning (Jirenhed et al., 2017) and the underlying circuitry and kinetics of eyeblink conditioning (Gruart et al., 1995; Harrison and Buchwald, 1983; Trigo et al., 1999).

CHAPTER TWO: EFFECT OF SEX ON ACQUISITION OF TEBC

BACKGROUND

While the field of eyeblink conditioning has included a variety of species, eyeblink conditioning, and neuroscience research more generally, has largely neglected sex as a fundamental variable (Beery and Zucker, 2011a; Shansky and Woolley, 2016). This bias has led to a disparity in knowledge of fundamental differences between males and females. While many past behavioral experiments include a single sex, Shors and colleagues' work in eyeblink conditioning used both sexes, reporting that female rats outperformed male rats (Dalla and Shors, 2009). However, this finding has not been replicated in other species, including mice. It is necessary to assess sex differences in the mouse model, as mice are extensively used in preliminary clinical studies. Failure to include both sexes in the preclinical experiments that lay the foundation for future therapeutics may explain, in part, the differential effects between males and females observed in subsequent clinical trials (Soldin and Mattison, 2009). Sex-related differences have been illustrated in pharmacokinetic and pharmadynamic studies, expressing key differences in drug metabolism, efficacy and safety of frequently used drugs (Farkouh et al., 2020). While some drugs are more effective for men than women, others also have significantly greater adverse side-effects for women (Farkouh et al., 2020).

It is especially necessary to study sex-differences for therapeutics aimed at treating neurological and neurodegenerative disorders with known sex differences in severity and prevalence including schizophrenia, Alzheimer's Disease (AD) and anxiety (Zagni et al., 2016). Sex differences in schizophrenia have been well-documented (Abel et al., 2010; Goldstein et al., 2002; Leung and Chue, 2000). Onset of schizophrenia is typically 3-5 years earlier for men than women (Li et al., 2016). Response to therapeutic interventions for schizophrenia have also

shown sex-differences, with women responding better to treatment than man (Grigoriadis and Seeman, 2002; Riecher-Rössler and Häfner, 2000) and requiring 50% less hospitalizations (Desai et al., 2013; Li et al., 2016). In AD, women experience significantly greater cognitive impairment compared to men (Laws et al., 2018). Women also experience a higher risk for developing AD, which is not due to a longer life-span (Laws et al., 2018). Women have an increased susceptibility to the ApoE4 genotype (Corder et al., 1993; Liu et al., 2013; Medeiros and Silva, 2019) and show greater changes in the neural network (Damoiseaux et al., 2012) and tau pathology (Corder et al., 1993). Lifetime prevalence of an anxiety disorder is 60% greater in women compared to men (Donner and Lowry, 2013). This higher incidence is seen across many disorders including: generalized anxiety disorder, and panic disorder (Maeng and Milad, 2015). It is hypothesized that female reproductive hormones such as estrogens and progesterones have a role in the neurobiology of anxiety disorders, by modulating the central nervous system (Jalnapurkar et al., 2018).

As there are significant sex-differences observed in neurological and neurodegenerative disorders, we investigated acquisition of trace eyeblink conditioning (tEBC) in female and male C57BL/6J mice to determine the impact of sex in learning a hippocampal-dependent temporal associative memory task (Tseng et al., 2004). In this task, a neutral conditioned stimulus (CS) is paired with an aversive unconditioned stimulus (US), which causes a reflexive eyeblink response (Figure 3D). The stimuli are separated by a stimulus free “trace” interval. Repeated presentation of the paired stimuli allows the subject to learn an association over time, leading to closure of the eye before the onset of the reflexive eyeblink, a conditioned response (CR) (Figure 3E). Trace eyeblink conditioning requires many trials to successfully acquire, allowing examination of the learning process and subsequent asymptotic performance.

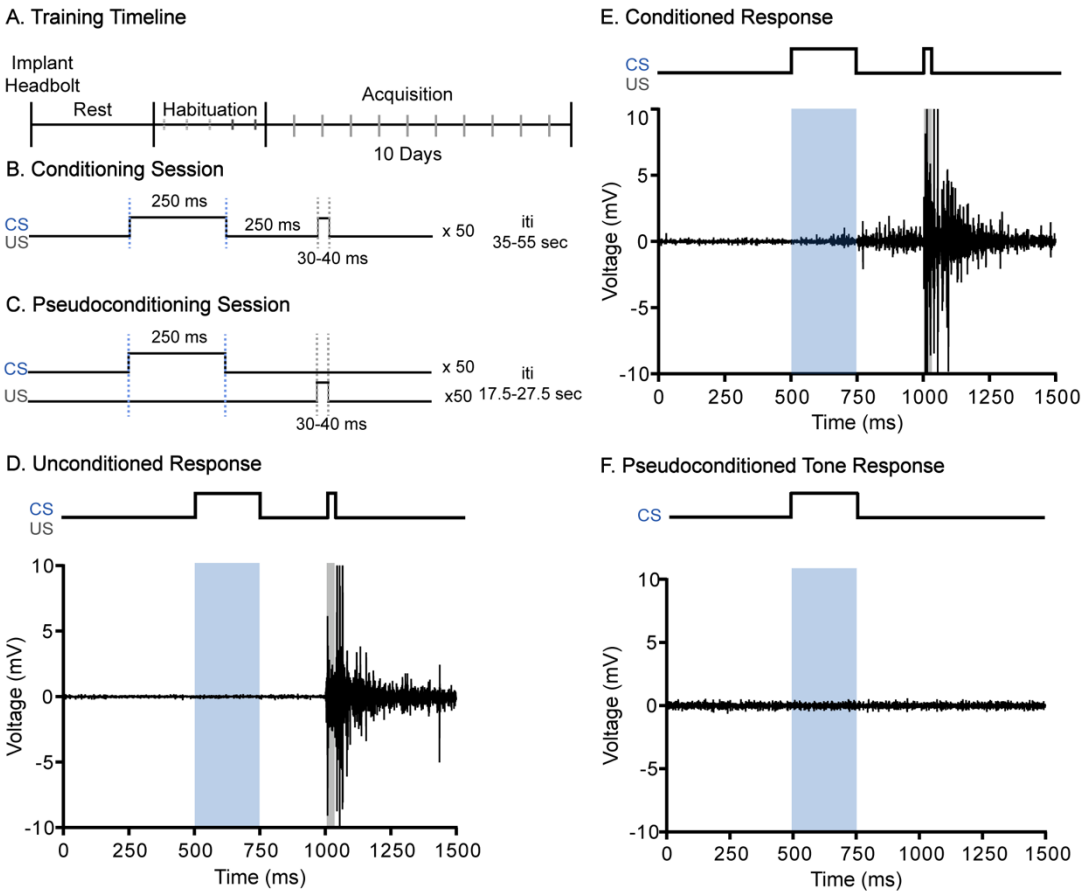

Figure 3 Experimental paradigm and example EMG traces. (A) Experimental timeline. (B) Conditioning session protocol. CS in blue, US in gray. (C) Pseudoconditioning session protocol. (D) EMG trace of unconditioned response to airpuff during early training. (E) EMG trace of conditioned response to paired tone and airpuff during subsequent training sessions. (F) EMG trace of response to tone alone pseudoconditioning trial.

When the US National Institutes of Health (NIH) announced their policy that called for the use of both male and female subjects, it was met with significant criticism (Fields, 2014; Mamlouk et al., 2020; Richardson et al., 2015). Concerns about increased behavioral variability in female rodents due to circulating hormone levels during the estrous cycle have also been widely expressed (Hughes, 2019; Meziane et al., 2007; Walf and Frye, 2007; Wong, 1979). However, recent studies suggest the estrous cycle does not need to be monitored as females without a staged estrous cycle have similar variability as males in behavioral tasks (Fritz et al., 2017; Prendergast et al., 2014). To further investigate the necessity of circulating hormones for acquisition, we included an ovariectomized female group in this study. Ovariectomy involves surgically excising the ovaries, which serves to understand the role of estrogen deficiency and cycling hormone production in animal models (Souza et al., 2019; Ström et al., 2012).

We found intact female mice acquired tEBC significantly faster than male mice, however, the presence of circulating hormones was essential for their faster learning, as ovariectomized females learned at a similar rate as males. All conditioned animals learned the associative learning task, reaching at least 60% adaptive CRs, though ovariectomized female performance was impaired on the final day of training.

MATERIALS AND METHODS

Animals

All procedures were approved by and completed in accordance with the Northwestern University Animal Care and Use Committee guidelines. Experiments were performed with young adult (3-4 months) male, intact female and ovariectomized female C57BL/6J mice. All mice (were obtained from Jackson Laboratory (Bar Harbor, Maine). Ovariectomies were performed by Jackson Laboratory at least two weeks prior to shipment. Estrous cycles of female

mice were not monitored as previous studies have demonstrated females without a staged estrous cycle had similar variability as males in behavioral tasks (Prendergast et al., 2014). All mice were housed in Northwestern University temperature-controlled facilities in a 14-hour light: 10-hour dark cycle and fed ad lib. Mice were group housed at arrival and allowed to acclimate to Northwestern University facilities for at least one week prior to experimentation. After headbolt implantation surgery, mice were housed individually.

Surgery

Male (n=29), intact female (n= 29), and ovariectomized female (n= 24) mice were implanted with a custom headbolt two weeks prior to behavioral training. Animals were briefly anesthetized with 3-4% vaporized isoflurane mixed with oxygen (flow rate: 1-2 liters/minute). Buprenorphine (0.05-2 mg/kg) was administered subcutaneously as an analgesic. The scalp was shaved, and the mouse was placed in a stereotaxic device. The scalp was sterilized with iodine and 70% ethanol, then an incision was made along the midline. The skin was retracted laterally with microclips, and the skull was cleaned with 3% hydrogen peroxide then sterile saline. Two small stainless-steel screws (00-90) were implanted to the left of midline (one in front of Bregma, and one in front of Lambda) (Figure 4). The bare stainless steel groundwire (0.005in:AM Systems: 792800) of the custom headbolt was wrapped around the screws in a figure-eight pattern to serve as a ground for EMG recordings. A thin layer of Metabond adhesive cement (Parkell) was spread over the skull, screws, and wire to secure them in place. To expose the muscle and place EMG wires, the skin surrounding the right eye was retracted. Four polyimide-coated stainless steel (0.005in: PlasticsOne: 005sw/2.0 37365 SS) wires with 2-3 mm of exposed wire were placed on the muscularis orbicularis oculi for EMG recording. The headbolt piece and base of the EMG recording wires were then secured with additional adhesive

cement. The skin was released from microclips and placed over the cement. Skin was allowed to rest naturally, and the exposed area was sealed with additional adhesive cement. Animals recovered on a warm heating pad before being returned to their home cage. Animals were allowed five to seven days to recover before habituation began.

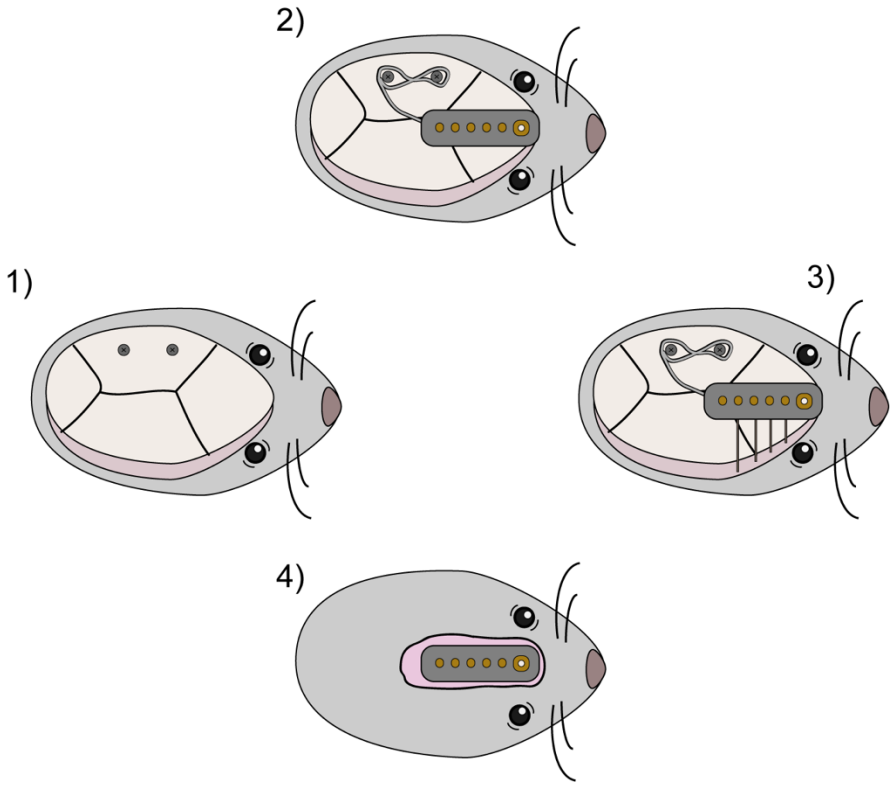

Figure 4. Headbolt Surgery Procedure. 1) Skull screws implanted 2) EMG Groundwire Wrapped Around 3) EMG wires placed in muscle surrounding eye 4)Skin glued in place around headbolt

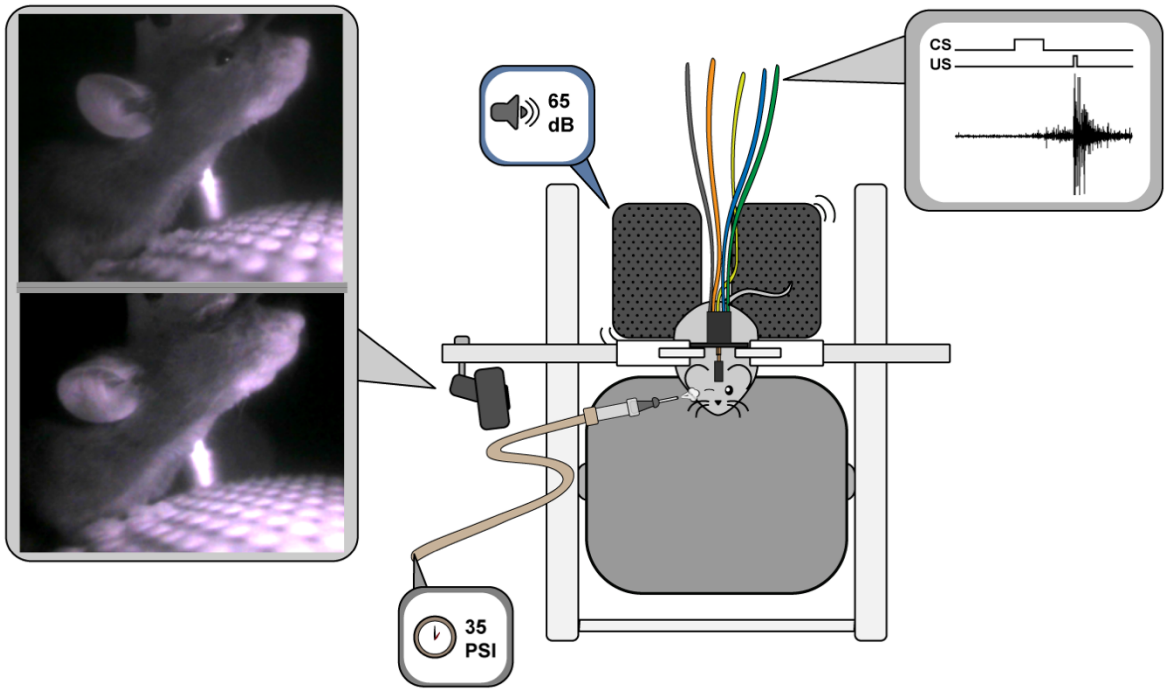

Figure 5. Schematic of eyeblink conditioning behavioral apparatus. (Upper Left) Video display of mouse with open eye during baseline. (Lower Left) Display depicting mouse with closed eyelids during a CR. (Middle) Depiction of headfixed mouse atop the freely rotating cylinder. Camera for visualizing mouse during conditioning task on left of cylinder frame. Speakers behind mouse are used to deliver tone CS. Blunted needle delivers aversive airpuff US to eye. Custom headbolt implanted on the mouse's head connects to amplifier to receive EMG signal (depicted on Right).

Trace Eyeblink Conditioning

Prior to behavioral training, mice were handled for three days for five minutes/day to habituate mice to restraint and the experimenter. After three days of handling, mice were habituated to head-fixation on a moveable cylinder apparatus for the length of a training session without the presentation of stimuli. Training began two days following habituation. Training consisted of one session per day for ten days (Figure 3A). Mice were randomly assigned to either a conditioned group or pseudoconditioned group. Conditioned animals received a 65±2 dB tone (250ms, 2kHz) conditioned stimulus (CS) paired with a 35±5 PSI corneal airpuff (30-40 ms)

unconditioned stimulus (US) (Figure 3B). Each conditioning session consisted of 50 paired CS/US trials with a random 35-55 second inter-trial interval. Pseudoconditioned animals received 50 CS-alone trials and 50 US-alone trials in pseudorandomized order with a 17.5-27.5 second inter-trial interval (Figure 3C, F). Custom routines in LabVIEW (National Instruments) were used for stimulus presentation procedures, data collection, storage, and analysis (routines available upon request). Tone intensity was calibrated with a sound meter, placed where the mouse would be, at the start of each day of training. Air pressure was calibrated with a manometer (Thermo Fisher Scientific) secured at the output of a 0.5-inch 16-gauge blunted needle before each training session. Animals were visually monitored during training through a camera (Logitech C270) attached to the frame of the cylinder apparatus (Figure 5). Trials were not presented when the animal was visibly moving.

Data Analysis

EMG signal output was amplified (x5,000) and filtered (100Hz-5kHz), then digitized at 3kHz and stored by computer. For analysis, EMG data were rectified and integrated with a 10ms time constant. A conditioned response (CR) was defined as increased EMG activity lasting at least 15ms with an amplitude at least 4 standard deviations above the mean baseline activity. Baseline activity was the average EMG activity starting 250ms before CS onset (See Figure 3E). Trials were excluded if baseline activity was 2 standard deviations above the mean baseline activity for the session. CR onset was calculated in reference to the start of the tone CS. An adaptive CR was defined as a CR that was present in the 200ms before US onset. Animals that reached at least 60% adaptive CRs were considered to have learned the task. The number of trials to 8 consecutive CRs was also used as a measurement of learning.

Data were analyzed with Bartlett's test, Two-Way Repeated Measures ANOVA or Mixed-Effects Analysis, One-Way ANOVA, and post-hoc Šídák's multiple comparisons test or Tukey's Multiple Comparison Test, when appropriate (Prism v8) (Table 2). The probability level of $p<0.05$ was used as an indicator of statistical significance. Data are expressed with standard error of the mean. Statistical tests did not include data from habituation, except for direct habituation comparison. Mice were excluded from analysis due to poor health, high startle response or failure to learn delay conditioning (intact female n=6; male n=4; ovx n=5). High startle response was defined as activity occurring during the 50ms after the start of the CS which is greater than average activity± 4SD on 2 or more sessions. Delay conditioning is non-hippocampal dependent, where the stimuli overlap (the length of the CS is extended, and the US and CS co-terminate). Failure to learn delay conditioning indicates a possible brainstem/cerebellar deficit (Cheng et al., 2008; Heiney et al., 2014; Yang et al., 2015).

RESULTS

All groups of mice displayed similarly low levels of spontaneous blinking during habituation ($F(2,64)= 0.01855$, $p= 0.9816$). Pseudoconditioned mice (male, intact female, ovx) responded comparably throughout the 10 training sessions ($F(2,14)= 0.6797$, $p= 0.5227$) and were grouped together for analysis. All conditioned mice (intact female, male, ovx) reached learning criterion (60% adaptive CRs), in contrast to the pseudoconditioned controls. As shown

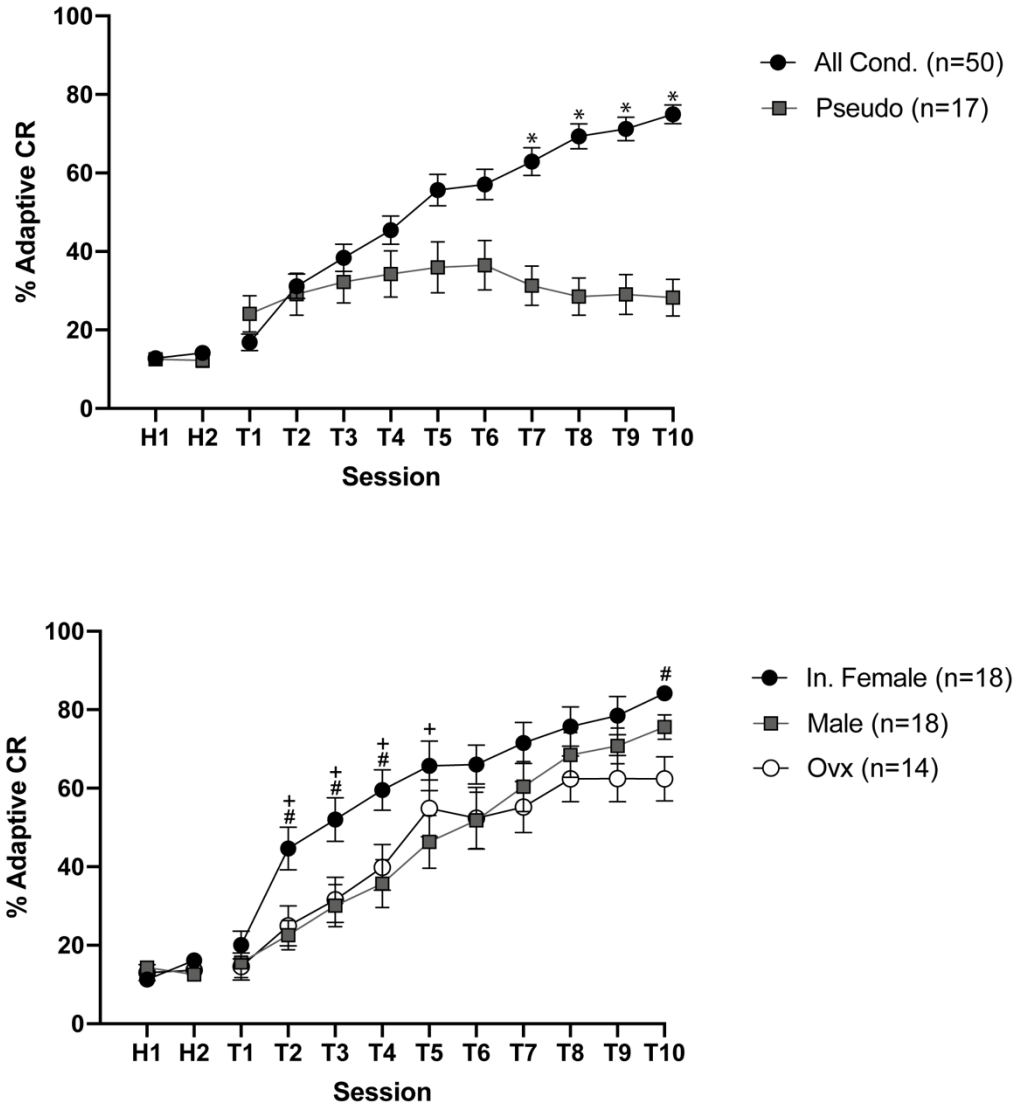

*Figure 6. Percent Adaptive CR Headbolt Animals. (Upper) Percent adaptive conditioned responses for all conditioned animals (males, intact females, ovx) and pseudoconditioned animals across two days of habituation and ten days of training. * Šídák's multiple comparisons test $p < 0.05$. (Lower) Percent adaptive conditioned responses for intact females, male and ovariectomized female animals. Intact females learn significantly faster than males and ovariectomized females. + Tukey's Multiple Comparison Test $p < 0.05$ intact female vs male. # Tukey's Multiple Comparison Test $p < 0.05$ intact female vs ovx.*

in Figure 6, a Two-Way Repeated Measures ANOVA of % adaptive CRs revealed a significant increase in adaptive CRs for the conditioned mice compared to pseudoconditioned mice ($F(1,65)= 18.55$, $p= <0.0001$).

While male, intact female, and ovx mice trained on tEBC acquired the task over the course of 10 training sessions ($F(9,423)= 76.07$, $p= <0.0001$), a significant difference between the sexes was observed ($F(2,47)= 4.447$, $p= 0.0170$). Conditioned intact female mice learned significantly faster than ovariectomized females and males [$F(2,47)= 4.447$, $p= 0.0170$: Tukey's Multiple Comparison intact female vs male, $p=0.0421$, intact female vs ovx, $p=0.0318$, male vs ovx $p=0.9581$] (Figure 6). Planned comparisons of sessions 2-5 (where initial acquisition is occurring) indicated that intact females exhibited a significantly greater percentage of adaptive CRs relative to male and ovx mice [$F(2,47)= 4.447$, $p= 0.0170$: Tukey's Multiple Comparison tests, T2, intact female vs male ($p= 0.0086$), intact female vs ovx ($p= 0.0352$), T3, intact female vs male ($p=0.0088$) intact female vs ovx ($p=0.0266$), T4, intact female vs male ($p=0.004$) intact female vs ovx ($p=0.0356$), T5, intact female vs male ($p=0.0247$)].

All intact female, male and ovariectomized female mice reached at least 60% adaptive CR by the last day of training. However, ovariectomized females were impaired during the final day of training compared to intact females (T10, Tukey's Multiple Comparison, $p=0.0167$). The variance of intact females, males and ovariectomized females was not significantly different on the first day of training (Bartlett's Test, (corrected)= 0.8188, $p=0.6641$). This further supports the findings of previous studies that variability in behavioral tasks is similar between males and females without a staged estrous cycle (Fritz et al., 2017; Prendergast et al., 2014).

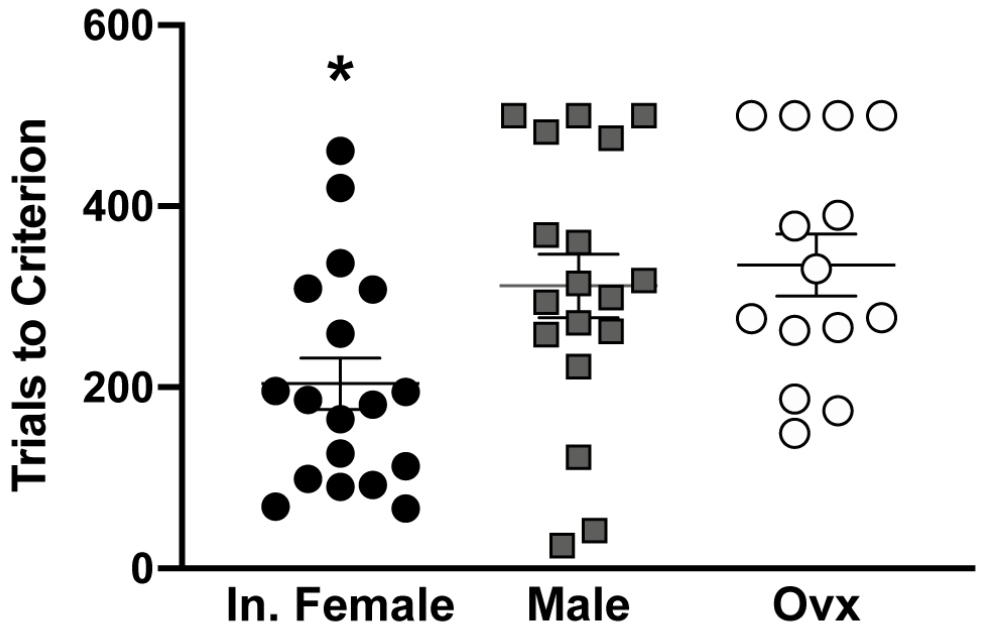

*Figure 7.Trials to 8 Consecutive CRs Headbolts. Intact females learn significantly faster than ovariectomized females and males. Intact females reach 8 consecutive CRs in 204 trials while males and ovx require 311 and 335 trials respectively. Mean ± SEM shown. * Tukey's Multiple Comparison Test $p < 0.05$ for intact female vs male and ovx.*

Number of trials to consecutive 8 CRs was also used as a measurement of learning rate. Animals that failed to reach 8 consecutive CRs by the end of 10 training sessions were scored as 500 trials, the total number of conditioning trials. Intact females reached 8 consecutive conditioned responses significantly faster than both males and ovariectomized females (Tukey's Multiple Comparison, intact female vs male, p=0.0485, intact female vs ovx, p=0.0218). On average, intact females reached 8 consecutive conditioned responses in 204 trials, while ovariectomized females required 335.1 trials and males required 311.9 trials (Figure 7). Analyses of the CR onset latency revealed a significant effect of training sessions ($F(9,422)= 2.770$, p=

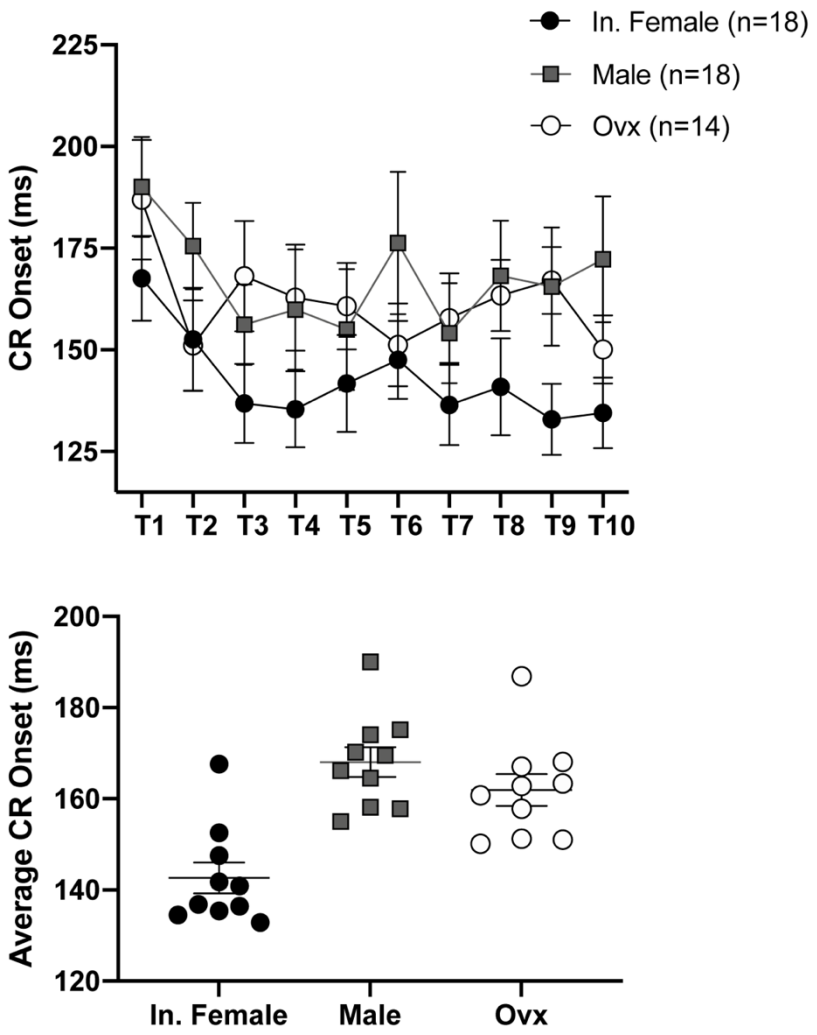

Figure 8 Average CR Onset Latency for Headbolt Animals. (Upper) Intact females respond earlier in the trial on average across all sessions. (Lower) Average CR onset latency across all training sessions. Intact Female n=18, Male n=18, Ovx n=14, per training session. Mean ± SEM.

0.0433) and a trend for a difference among the groups (Mixed-Effects Analysis, F(2,47)= 2.978, p= 0.0606) (Figure 8). Across all days of conditioning, intact females' average response onset latency was 142.6ms after the start of the CS, while males and ovariectomized females responded at 167.3ms and 161.9ms, respectively (Figure 8).

DISCUSSION:

This study set out to confirm and extend the differences in acquisition of a temporal associative memory task, trace eyeblink conditioning, due to sex and circulating ovarian hormones in mice. We found that intact female mice learned tEBC at a faster rate compared to males and ovx mice. Intact females also exhibited earlier CR onset times. These results indicate that the intact female mice learn to anticipate the aversive stimulus more quickly and respond more rapidly during the trial than males or ovx mice. These findings parallel those observed by Shors and colleagues, who found female rats learned tEBC faster than male rats (Dalla et al., 2009; Shors et al., 1998; Wood and Shors, 1998). It is important to note that these sex differences were not only confirmed across species but across distinct experimental parameters. While both studies used trace eyeblink conditioning, the experiments varied in a number of technical ways including: the length of the trace period, the modality of aversive unconditioned stimulus, and number of trials delivered each day. Additionally, our study directly compared intact female, male and ovariectomized female mice. Our results highlight the impact that sex has on learning is not dependent on specific experimental protocols.

There have been conflicting reports of the effects of ovariectomy on different forms of memory including spatial memory and object recognition. Ovariectomy has been shown to impair spatial working memory in radial arm maze and non-spatial memory in object recognition (Daniel et al., 1997; Sarkaki et al., 2008; Tao et al., 2020; Wallace et al., 2006). However, other studies have shown ovariectomy can improve or have no effect on spatial memory in Morris water maze (Bimonte-Nelson et al., 2003; Singh et al., 1994; Wilson et al., 1999). Wilson and colleagues found that ovariectomized mice did not show any impairments compared to sham-operated mice on Morris Water Maze or Y maze, indicating that ovariectomy does not impact

spatial learning (Wilson et al., 1999). One study showed that performance of ovariectomized mice depended on the time point post-surgery. In the hippocampal-dependent tasks Morris Water Maze and Novel Object Recognition, impairment was only seen 8 weeks following surgery (Tao et al., 2020). Previous tEBC studies demonstrated that the removal of ovarian hormones eliminates stress-induced sex differences and decreased performance late in training (Wood and Shors, 1998). However, this study did not directly compare intact females, males and ovariectomized females. Our present findings reveal that ovariectomy slows the learning of a temporal associative memory task in females and reduces performance on the final day of conditioning. This decline in learning may be due to decreased spine density in CA1 and medial prefrontal cortex pyramidal cells due to ovariectomy (Gould et al., 1990; Wallace et al., 2006). Studies have suggested that estradiol is neuroprotective and a decrease in estradiol levels may lead to neurodegeneration (Bohm-Levine et al., 2020a; Dubal et al., 1999). Estradiol controls levels of luteinizing hormone (LH), with ovariectomized females having elevated LH levels (Wallace et al., 2006) and low estradiol (Bohm-Levine et al., 2020a). High levels of LH have been linked to spatial memory impairment (Bohm-Levine et al., 2020a; Burnham and Thornton, 2015; Casadesus et al., 2007), potentially due to the hormone's connection with brain-derived neurotrophic factor (BDNF) (Bohm-Levine et al., 2020b). BDNF is involved in synaptic plasticity and dendritic spine maintenance (Luine and Frankfurt, 2013; Vigers et al., 2012). These mechanisms may explain why, in our study, ovariectomized females were initially impaired, although they still successfully acquired the task and reached the learning criterion of 60% adaptive CRs.

Our findings suggest that cycling ovarian hormones are necessary for the enhanced learning rate in females. Enhancement of associative learning due to ovarian hormones, such as

estrogen, is in line with evidence that estrogen plays a functional role in learning and memory formation. It is known that exposure to estrogen in young female rodents increases density of dendritic spines in CA1, neurogenesis in dentate gyrus and synaptic plasticity (Frick et al., 2018; Gould et al., 1990; Smith et al., 2009; Woolley and McEwen, 1993). Intracellular estrogen receptors have been found in both male and female rodent hippocampal pyramidal neurons and glia (Galea et al., 2017). Estrogen receptors ERα and ERβ interact with metabotropic glutamate receptors to activate extracellular signal-regulated kinase/mitogen activated protein kinase signaling, leading to increased cAMP response element-binding protein (CREB) phosphorylation and CREB-dependent gene transcription (Boulware et al., 2013, 2005). These mechanisms may mediate intact females' enhancement in acquisition of this hippocampal-dependent associative memory task.

In ovariectomized female rats, increased levels of estrogen have been shown to enhance acquisition of tEBC as compared to vehicle treated rats, albeit at supraphysiological doses (Leuner et al., 2004). Additionally, it was reported that female rats acquire tEBC at a faster rate when in proestrus (high estrogen levels), compared to rats at either estrus or diestrus (Shors et al., 1998). However, there is rapid fluctuation of ovarian hormones within each phase of the estrous cycle (Frick et al., 2015). During the proestrus phase, in particular, hormone levels rise during the day and peak in the evening. Therefore, it is difficult to assess learning and memory within a single phase of the cycle (Frick et al., 2015), especially for tEBC which requires several daily training sessions to acquire.

While we did not measure the estrous cycle, we found that intact female mice learned tEBC at a faster rate than male and ovariectomized female mice in the absence of staged estrous cycle. Moreover, the variability of the intact female mice was not significantly different than that

of male and ovariectomized mice, further supporting a previous report that intact females without staged estrous cycle had similar variability as males (Prendergast et al., 2014).

Estrogens may also play a role in male mice during acquisition of trace eyeblink conditioning. Testosterone is aromatized to estradiol in the central nervous system and has been shown to enhance cognition in humans and animals (Edinger and Frye, 2007; Luine, 2014). Estrogen receptor agonists increase CA1 spine density in vivo and in vitro in males, indicating that estrogen may also influence learning in males (Jacome et al., 2016; Koss and Frick, 2017; Murakami et al., 2015, 2006). Gonadectomy has also been shown to impair male performance on a variety of tasks (Frye et al., 2004) including object recognition (Ceccarelli et al., 2001), T-maze (Kritzer et al., 2001) and radial arm maze (Harrell et al., 1990).

Our results confirm and extend the findings that intact females learn significantly faster than both ovariectomized females and males on the trace eyeblink conditioning task. Though all conditioned animals acquired the task, ovariectomized females' performance was impaired compared to that of intact females on the final day of training. These differences in learned responses cannot be attributed to sensitization to stimuli or differences in spontaneous blink rate, since no difference was observed in spontaneous blink rate or response to the tone among the female, male and ovariectomized female pseudoconditioned controls. Overall, these results emphasize the need for inclusion of both females and males in behavioral neuroscience studies. Behavioral tasks are used as the benchmark for clinical drug studies for neurological and neurodegenerative disorders. If sex is not factored in as a biological variable, critical differences essential to successful treatments may go undetected.

Figure		Test	F-Value	P-value	Standard Omega-Square	R Squared	Mean Difference
Figure 3	**Habituation**	**Two-Way Repeated Measures ANOVA**	2,64				
		Sex	0.01855	0.9816	0.03724		
	Variance	Ordinary One-Way ANOVA Bartlett's Test	Bartlett's Statistic				
		Trained In. Female, Male, Ovx	0.8188	0.6641			
	Pseudo	**Repeated Measures ANOVA**	2,14; 9,126; 18,126				
		Sex	0.6797	0.5227	4.700		
		Session	0.9406	0.3991	2.801		
		Interaction (Session * Sex)	0.4007	0.9857	2.387		
	Conditioned Vs Pseudo	**Two-Way Repeated Measures ANOVA**	1,65; 9,585; 9,585; 65,585				
		Group	18.55	<0.0001	10.25		
		Session	21.74	<0.0001	8.267		
		Interaction (Session * Group)	18.22	<0.0001	6.926		
		Subject	13.07	<0.0001	35.89		
		Šídák's multiple comparisons test					
		T5		0.141			19.72
		T6		0.0899			20.57
		T7		0.0001			31.650
		T8		<0.0001			40.820
		T9		<0.0001			42.14
		T10		<0.0001			46.69
	Sex Difference	**Two-Way Repeated Measures ANOVA**	2,47; 9,423; 18,423; 47,423				
		Sex	4.447	0.017	5.962		
		Session	76.07	<0.0001	37.09		
		Interaction (Session * Sex)	1.471	0.0961	1.434		
		Subject	12.37	<0.0001	31.51		
		Tukey's Multiple Comparisons Test					Predicted (LS) Mean Difference
		In. Female, Male		0.0421			14.04
		In. Female, Ovx		0.0318			15.72
		Male, Ovx		0.9581			1.678
		Tukey's Multiple Comparisons Test					
		T2					Predicted (LS) Mean Difference
		In. Female, Male		0.0086			22.04
		In. Female, Ovx		0.0352			19.68
		Male, Ovx		0.9524			-2.353
		T3					
		In. Female, Male		0.0088			21.98
		In. Female, Ovx		0.0266			20.5
		Male, Ovx		0.9808			-1.484
		T4					
		In. Female, Male		0.004			23.78
		In. Female, Ovx		0.0356			19.65
		Male, Ovx		0.8605			-4.133
		T5					
		In. Female, Male		0.0247			19.38
		In. Female, Ovx		0.3594			10.81
		Male, Ovx		0.525			-8.57
		T10					
		In. Female, Male		0.4758			8.609
		In. Female, Ovx		0.0167			21.81
		Male, Ovx		0.2186			13.2

Figure 4	8 Consecutive CRs	**Ordinary One-Way ANOVA**	2,47				
		Sex	4.651	0.0144		0.1652	
		Tukey's Multiple Comparisons Test					
		In. Female, Male		0.0485			-107.9
		In. Female, Ovx		0.0218			-131.1
		Male, Ovx		0.8775			-23.13
Figure 5	CR Onset	**Mixed-Effects Analysis**	9,422; 2,47;18,422				
		Session	2.770	0.0433			
		Sex	2.978	0.0606			
		Interaction (Session * Sex)	0.5707	0.9202			
		Tukey's Multiple Comparisons Test T9					
		In. Female, Male		0.1464			-31.63
		In. Female, Ovx		0.0210			-34.21
		Male, Ovx		0.9858			-2.580
		Ordinary One-Way ANOVA	2,27				
		Sex	15.40	<0.0001		0.5035	
		Tukey's Multiple Comparisons Test					
		In. Female, Male		<0.0001			-25.42
		In. Female, Ovx		0.0011			-19.28
		Male, Ovx		0.4167			6.133

Table 2. Statistical Table for Chapter Two

CHAPTER THREE: SEX-DEPENDENT EFFECTS OF CHRONIC MICRODRIVE IMPLANTATION ON ACQUISITION OF TRACE EYEBLINK CONDITIONING

BACKGROUND

Electrophysiology has allowed neuroscientists to study a variety of phenomena including the activity of individual neurons and larger population encoding of groups of neurons (Obien et al., 2015). Fundamental neuroscience advances have come from extracellular recordings over the last half a century, including: place cells (O'Keefe and Dostrovsky, 1971) orientation-selective cells (Hubel and Wiesel, 1959) and "face cells" (Gross et al., 1972; Szostak et al., 2017)(Figure 9). While these studies consisted of single electrode recordings in a particular region of the brain,

Figure 9. Timeline of Neuronal Recording Technology. (From Szostak et al 2017)

technology has since developed to allow for the study of groups of neurons through arrays of microelectrodes (Hong and Lieber, 2019).

In addition, the development of “tetrode” recordings vastly expanded the field. By twisting together four wires and heating the insulation, these electrodes allowed researchers to distinguish between multiple cells on a single electrode (Dragoi, 2012). These advances led to many discoveries including: grid cells (Hafting et al., 2005), time cells (Manns et al., 2007) and single neuron representations of individuals “Jennifer Aniston" cells (Quiroga et al., 2005). In-vivo recording remains a critical tool for neuroscience research, expanding with new technologies (Hong and Lieber, 2019). Current advances include dual optogenetic and microelectrode recording, MRI-compatible microdrives (Jog et al., 2002), and silicon probes. Therefore, it is necessary to understand how the implementation of chronic recordings affects acquisition of learning and memory.

Our preliminary microdrive tEBC experiments showed a clear sex difference in acquisition, leading us to question the potential effect of the weight of the chronic implant on learning. Stress exposure can activate the hypothalamic-pituitary-adrenal (HPA) axis, leading to production of glucocorticoids, neurotransmitters and neuropeptides (Lindau et al., 2016). Glucocorticoids alter many regions of the brain, including the hippocampus, which contains a large number of glucocorticoid receptors (Moreira et al., 2016). The hippocampus contains two forms of adrenal steroid receptors, Type I (mineralocorticoid) and Type II (glucocorticoid), both of which affect excitability, plasticity and neurochemistry (McEwen, 2000). Glucocorticoids affect the brain through multiple pathways, including by directly stimulating the release of excitatory amino acids and indirectly regulating glutamate and GABA release through

endocannabinoids (McEwen, 2017). Chronic stress causes neurodegeneration due to a decrease in neurogenesis (McEwen, 2000; Pham et al., 2003), increased glutamate (Karst et al., 2005; Magariños et al., 1997; Wiegert et al., 2006), decreased Brain Derived Nerve Growth Factor (NGF) (Bath et al., 2013; Lakshminarasimhan and Chattarji, 2012; Smith and Cizza, 1996), and retraction of dendrites (Gould et al., 1997; Magarinos and McEwen, 1995; Sousa et al., 2000) leading to impaired hippocampal-dependent function (Moreira et al., 2016). Corticotropin-releasing factor (CRF) is also released from the hypothalamus and controls autonomic and behavioral responses to stress (Alderson and Novack, 2002). CRF has been shown in vitro to also inhibit dendritic branching in the hippocampus (Chen et al., 2004; McEwen, 2006).

Stress exposure before learning can either enhance (Domes et al., 2002; Smeets et al., 2007) or impair acquisition (Diamond et al., 2006; Elzinga and Roelofs, 2005; Kirschbaum et al., 1996)(Schwabe et al., 2012). Salehi, Cordero and Sandi demonstrated that rats performed at varying rates on radial six-arm water maze depending on the level of corticosterone (Salehi et al., 2010).This behavioral work supported the u-shape relationship between circulating glucocorticoids and LTP. At low levels of glucocorticoids, LTP is increased, but at high levels of corticoids, LTP is impaired (Bennett et al., 1991; Diamond et al., 1992). High levels of glucocorticoids can also lead to neuronal atrophy in animals and humans (Landfield et al., 1978; Lupien et al., 1998; Lupien and Lepage, 2001; Uno et al., 1989).

In experimental animals, effects of stress can be investigated by influencing the level of stress hormones directly pharmacologically or through environmental factors (Lindau et al., 2016). Implanting corticosterone pellets in the back of male rats led to impairment on radial 8-arm maze (Endo et al., 1996). These corticosterone implanted rats required significantly more

trials to acquire the task. When stressed under the chronic unpredictable stress paradigm, male Laca mice were impaired on the Elevated-Plus Maze and Morris Water Maze (Rinwa and Kumar, 2014). Rats stressed with chronic restraint had a longer location latency on water maze compared to unstressed controls (Ghadrdoost et al., 2011). Restraint stress also impaired male Sprague-Dawley rats' performance on radial arm maze and Y-maze (Bowman et al., 2001; Kleen et al., 2006).

The following experiment investigated if the microdrive implant serves as a chronic stressor to the mice, which thereby affected the rate of acquisition in a sex dependent manner. Sex differences in stress have been shown through the HPA stress response (Critchlow et al., 1963; Handa et al., 1994) behavior (Bowman et al., 2001; Luine et al., 1994), morphology (Galea et al., 1997a; Watanabe et al., 1992), and neurochemical responses to stress (Beck and Luine, 2002)(Bowman et al., 2003). Female rodents have higher baseline corticosterone levels compared to males (R. J. Carey et al., 1995; Critchlow et al., 1963; Figueiredo et al., 2002). Female rodents express less Type I receptors compared to males, and bind less corticosterone (Lesuis et al., 2018; Turner, 1992). Chronic stress changes astrocyte complexity and physiology in mPFC of males (Bender et al., 2016; Bollinger et al., 2019; Tynan et al., 2013). Astrocytic density is greater and more complex in medial amygdala in males (Pfau et al., 2016), but greater in females in the hippocampus (Bollinger et al., 2019; Garcia-Segura et al., 1988). These differences may account for the sex differences in observed behaviors.

Sex dependent effects of stress on learning have been also shown on a variety of behavioral tasks. Tracey Shors showed that when stressed with a repeated tail shock, male rats acquired tEBC faster than their female and unstressed male counterparts (Shors et al., 1998;

Wood and Shors, 1998). Similarly, when injected with high-doses of corticosterone, eyeblink conditioning acquisition was facilitated in male Long-Evans rats, but had no impact on acquisition in female rats (Wentworth-Eidsaune et al., 2016). Corticosterone treatment enhances male rats learning on an auditory fear conditioning task, but impaired female rats (Lesuis et al., 2018). When restrained, female and male rats are both impaired Y-Maze early in testing. However, in subsequent minutes, sex differences are observed (Conrad et al., 2003). Stressed females recover, while stressed males continue to show impairment (Conrad et al., 2003). When stressed with inescapable shocks, male rats showed longer-lasting effects compared to females on general ambulation, rearing and elevated-plus maze (Steenbergen et al., 1991). On a variety of tasks, sexual dimorphic behaviors have been observed when animals are stressed (Beck and Luine, 2002). These sexually dimorphic behavioral responses to stress suggest stress may underlie the behavioral differences initially observed in chronically implanted animals.

To measure stress with a noninvasive technique, corticosterone was measured from fecal samples. Unlike collecting blood samples from the tail vein or orbital sinus, fecal samples can be collected frequently without causing animals stress from handling, restraint and sample collection (Touma et al., 2004). Fecal samples also measure circulating hormone levels over a longer term compared to the short time course of blood samples. Peak levels of corticosterone metabolites were measured in feces 8-10 hours after injection of adrenocorticotropic hormone (ACTH) (Touma et al., 2004). We used the fecal sample technique, which allowed us to compare stress levels across groups without directly interfering with the animals and causing further stress.

This study investigated the behavioral sex differences observed with chronic implantation of a microdrive array by comparing the effects of a "dummy drive" to fully implanted arrays with tetrodes introduced into the brain and to smaller headbolt implants. We investigated the levels of corticosterone as previous stress studies exhibited similar behavioral tEBC results in male and female rats (Wentworth-Eidsaune et al., 2016; Wood and Shors, 1998). When male C57Bl6 mice were implanted a chronic microdrive array with tetrodes lowered into the cortex, tEBC acquisition was facilitated, but female mice were impaired with both microdrive and "dummy drive" implantation compared to headbolt controls. These experiments emphasize the need for inclusion of both sexes in research, to evaluate possible sex-specific effects that neuroscience tools and technologies may produce.

METHODS:

Animals

All procedures were approved by and completed in accordance with the Northwestern University Animal Care and Use Committee guidelines. Experiments were performed with young adult (3-4 months) male, intact female and ovariectomized female C57BL/6J mice. All mice were obtained from Jackson Laboratory (Bar Harbor, Maine). Ovariectomy was performed by Jackson Laboratory at least two weeks prior to shipment. Estrous cycles of female mice were not monitored as previous studies have demonstrated females without a staged estrous cycle had similar variability as males in behavioral tasks (Prendergast et al., 2014). All mice were housed in Northwestern University temperature-controlled facilities in a 14-hour light: 10-hour dark cycle and fed ad lib. Mice were group housed at arrival and allowed to acclimate to Northwestern University facilities for at least one week prior to experimentation. After implantation surgery, mice were housed individually.

Surgery

Headbolt Surgery

Male (n= 18), intact female (n= 18) and ovariectomized female (n=14) mice were implanted with a custom headbolt two weeks prior to behavioral training. Animals were briefly anesthetized with 3-4% vaporized isoflurane mixed with oxygen (flow rate: 1-2 liters/minute). Buprenorphine (0.05-2 mg/kg) was administered subcutaneously as an analgesic. The scalp was shaved, and the mouse was placed in a stereotaxic device. The scalp was sterilized with iodine and 70% ethanol, then an incision was made along the midline with a scalpel blade (No 15). The skin was retracted laterally with microclips, and the skull was cleaned with 3% hydrogen peroxide then sterile saline. The skull was scored with the scalpel blade to promote adhesion of the dental cement. Two small stainless-steel screws (00-90) were implanted to the left of midline (one in front of Bregma, and one in front of Lambda). The bare stainless steel groundwire (0.005in:AM Systems: 792800) of the custom headbolt was wrapped around the screws in a figure-eight pattern to serve as a ground for EMG recordings. A thin layer of Metabond adhesive cement was spread over the skull, screws, and wire to secure them in place. To expose the muscle and place EMG wires, the skin surrounding the right eye was retracted. Four polyimide-coated stainless steel (0.005in: PlasticsOne: 005sw/2.0 37365 SS) wires with 2-3 mm of exposed wire were placed on the muscularis orbicularis oculi for EMG recording. The headbolt piece and base of the EMG recording wires were then secured with additional adhesive cement. The skin was released from microclips and placed over the cement. Skin was allowed to rest naturally, and the exposed area was sealed with additional adhesive cement. Animals recovered on a warm heating pad before being returned to their home cage. Animals were allowed five to seven days to recover before habituation began.

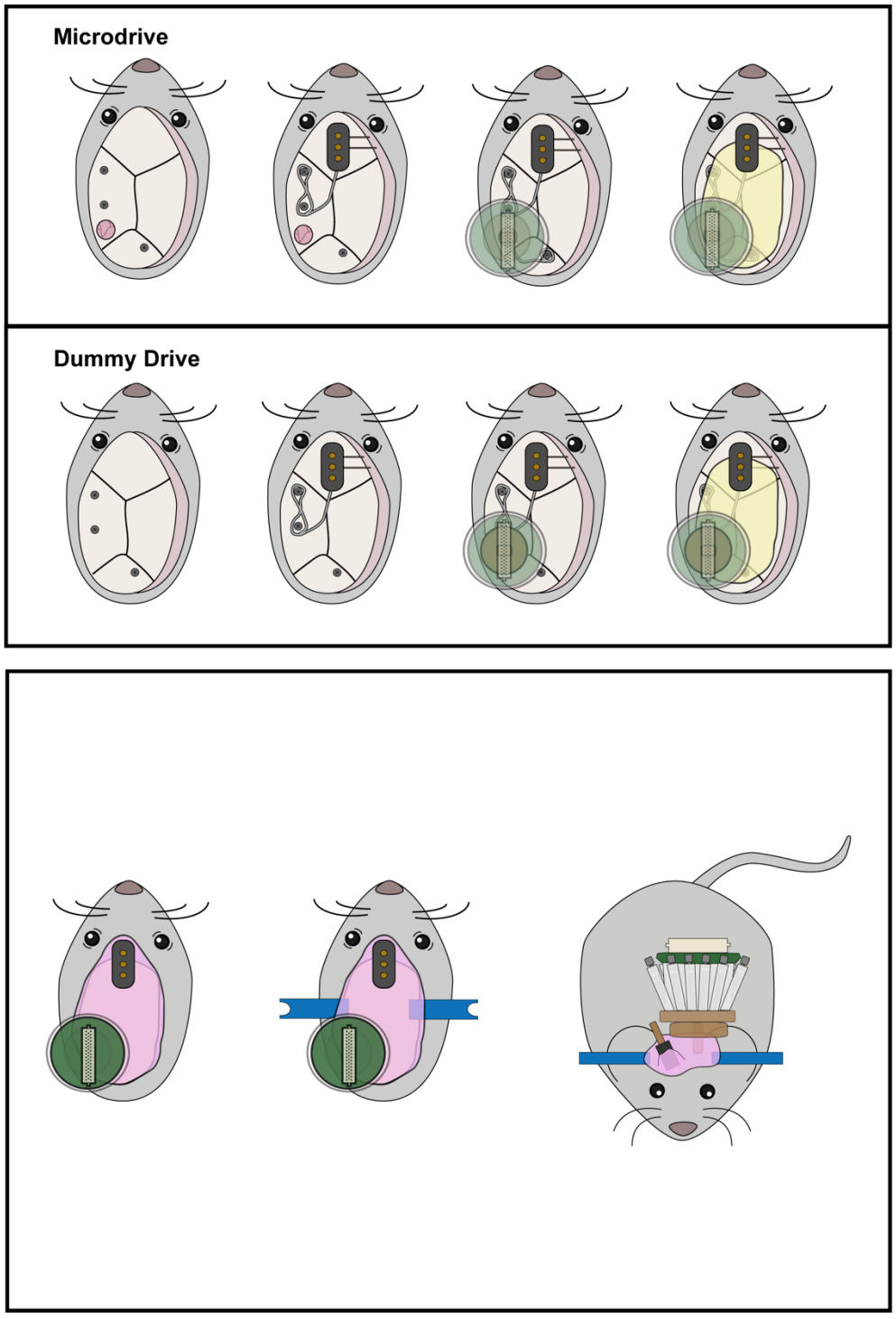

Figure 10 Surgical Procedure for Dummy Drive and Microdrive mice.

Dummy Drive Implant Surgery

Animals in the Dummy Drive group (Males n= 16 , Intact Females n= 15, Ovariectomized Females n= 4) were implanted with a modified headbolt containing one coated stainless steel groundwire (0.0110 in coated: AM Systems: 79200) and two polyimide-coated stainless steel (0.005in: PlasticsOne: 005sw/2.0 37365 SS) wires similarly to the process previously described (Figure 10). Dummy Drive animals were also implanted with a Neuralynx Halo-10-Mini Microdrive with either a Quick Clip or Omnetics electronic interface board (EIB) connector (Neuralynx, Bozeman, MT) (Figure 10). The exit tip of the Halo-10-Mini Microdrive was covered with silicone lubricant (Danco 88693P) and the microdrive was cemented to the skull with Metabond dental adhesive (Parkell Inc.) and Hygenic dental cement (Patterson Dental). After the Microdrive was secured and the skin was placed and cemented in its natural state, 3D printed head-fixation bars were cemented perpendicular to the skull, above the ears (Figure 10). Animals recovered on a warm heating pad before being returned to their home cage. Animals were allowed five to seven days to recover before habituation began. Chow was placed in a glass bowl (Amazon, B08KNTWCDD) at the bottom of the cage as the wire-top was removed to prevent damage to the implant. After surgery, mice were housed individually.

Microdrive Implant Surgery

Microdrive Implant animals (Males n= 11 , Intact Females n= 14, Ovariectomized Females n= 6) were implanted with the same modified headbolt as the Dummy Drive group and a Omnetics connector Custom 7-Degree Neuralynx Halo-10-Mini Microdrive (n= 19) or custom 3D-printed microdrive (n= 12)(Figure 10). Prior to implantation of the modified headbolt, the skull (bregma-lambda) was leveled. Two stainless-steel skull screws were implanted on either side of the coronal suture (0-80 Screw, 91772a049, McMaster Carr) for the headbolt ground. An

additional stainless steel skull screw (00-90) was implanted above the cerebellum for the Microdrive ground wire. A craniotomy was made (Drill bit: Stoeling Co: 0.45mm: 514551) at AP: +3.3 ML: -3.2 . The exit tip of the Microdrive was coated in silicone lubricant and the Microdrive was lowered into place (Figure 10). The tips of the tungsten tetrodes extended past the exit tip of the Microdrive and were lowered marginally into the cortex at implantation. The microdrive was cemented in place with multiple coats of Metabond dental adhesive and dental cement. After the skin was placed naturally around the dental cement and secured, 3D printed head-fixation bars were cemented in place as well. Animals recovered on a warm heating pad before being returned to their home cage. Chow was placed in a glass bowl (Amazon, B08KNTWCDD) at the bottom of the cage as the wire-top was removed to prevent damage to the implant. After surgery, mice were singly-housed.

Trace Eyeblink Conditioning

Headbolt

Procedures for conditioning for headbolt implanted animals was previously described in Chapter Two. Prior to behavioral training, mice were handled for three days for five minutes/day to habituate mice to restraint and the experimenter. After three days of handling, mice were habituated to head-fixation on a moveable cylinder apparatus for the length of a training session without the presentation of stimuli. Training began two days following habituation. Training consisted of one session per day for ten days (Figure 3b). Conditioned animals received a 65±2 dB tone (250ms, 2kHz) conditioned stimulus (CS) paired with a 35±5 PSI corneal airpuff (30-40 ms) unconditioned stimulus (US) (Figure 3b). The CS and US were separated by a 250 ms trace interval in which no stimuli were presented. Each conditioning session consisted of 50 paired CS/US trials with a random 35-55 second inter-trial interval (Figure 3b). Custom routines in

LabVIEW (National Instruments) were used for stimulus presentation procedures, data collection, storage, and analysis (routines available upon request). Tone intensity was calibrated with a sound meter, placed where the mouse would be, at the start of each day of training. Air pressure was calibrated with a manometer (Thermo Fisher Scientific) secured at the output of a 0.5-inch 16-gauge blunted needle before each training session. Animals were visually monitored during training through a camera (Logitech C270) attached to the frame of the cylinder apparatus (Figure 4). Trials were not presented when the animal was visibly moving.

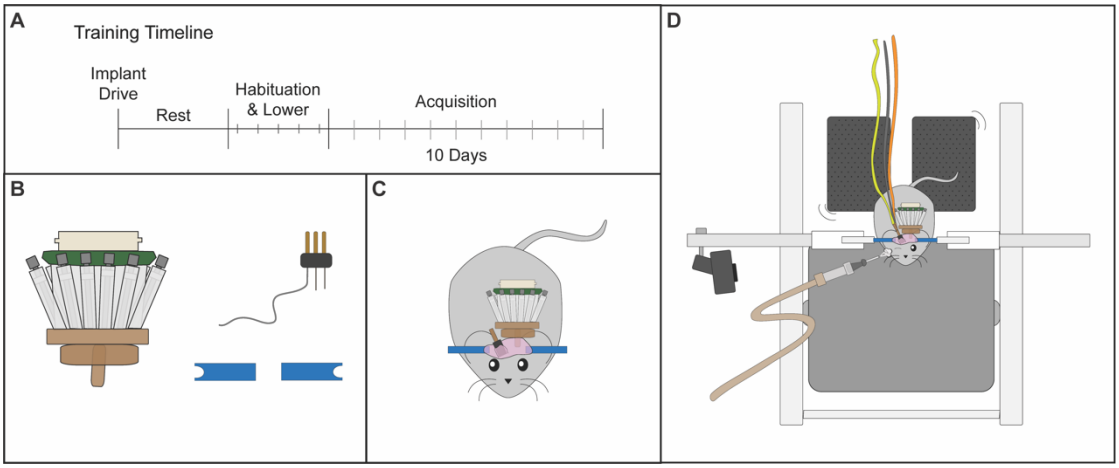

Figure 11 Microdrive Animal Setup. (A) Experimental timeline for Microdrive and Dummy Drive animals. (B) Microdrive illustration and modified headbolt.

Microdrive and Dummy Drive

Prior to behavioral training, mice were habituated to the head-fixed apparatus for forty minutes/day for five days. During these habituation sessions, tetrodes were lowered into the Entorhinal Cortex for Microdrive animals (Figure 11a). Conditioning training consisted of one session per day for ten days (Figure 11a). Conditioned animals received a 65±2 dB tone (250ms, 2kHz) conditioned stimulus (CS) paired with a 35±5 PSI corneal airpuff (30-40 ms) unconditioned stimulus (US) separated by a 250 ms trace interval (Figure 3b). Each conditioning

session consisted of 50 paired CS/US trials with a random 35-55 second inter-trial interval (Figure 3b). Stimulus presentation and calibration was performed similarly to the headbolt animal group. Trials were automatically paused by the LABVIEW software when EMG baseline exceeded 0.25V. Trials were restarted when the EMG baseline was below 0.25V for two consecutive seconds. Animals were visually monitored during training through a camera (Logitech C270) attached to the frame of the cylinder apparatus (Figure 11d).

Data Analysis

EMG signal output was amplified (x5,000) and filtered (100Hz-5kHz), then digitized at 3kHz and stored by computer. For analysis, EMG data were rectified and integrated with a 10ms time constant. A conditioned response (CR) was defined as increased EMG activity lasting at least 15ms with an amplitude at least 4 standard deviations above the mean baseline activity. Baseline activity was the average EMG activity starting 250ms before CS onset. Trials were excluded if baseline activity was 2 standard deviations above the mean baseline activity for the session. CR onset was calculated in reference to the start of the tone CS. An adaptive CR was defined as a CR that was present in the 200ms before US onset. Animals that reached at least 60% adaptive CRs were considered to have learned the task. The number of trials to 8 consecutive CRs was also used as a measure of learning.

Data were analyzed with Two-Way ANOVA or Mixed-Effects Analysis, One-Way ANOVA, and post-hoc Tukey's Multiple comparisons test, when appropriate (Prism v8) (Table 3). The probability level of $p < 0.05$ was used as an indicator of statistical significance. Data are expressed with standard error of the mean. Statistical tests did not include data from habituation, except for direct habituation comparison. Mice were excluded from analysis due to poor health, poor EMG signal, or failure to learn delay conditioning (intact female n=2; male n=4; ovx=1).

Delay conditioning is non-hippocampal dependent, where the stimuli overlap (Figure 1). Failure to learn delay conditioning indicates a possible brainstem/cerebellar deficit (Cheng et al., 2008; Heiney et al., 2014; Yang et al., 2015).

Corticosterone Measurement

Fecal matter was collected from the cages of mice prior to surgery, following habituation week, training week 1 (session 1-5) and training week 2 (sessions 6-10). This matter was stored in 2ml Eppendorf tubes in the -80C freezer following collection. Corticosterone was measured through DetectX Corticosterone Enzyme Immunoassay Kit (Arbor Assay: K014). Corticosterone was extracted according to Arbor Assay's Steroid Solid Extraction Protocol. Fecal matter was dried and powdered with a mortar and pestle (DOT Scientific: JMD050). 0.2 grams of powdered sample was weighed out and 1mL of ethanol was added per 0.1 gm of solid and sealed in a 2ml tube. Samples were vigorously shaken with a vortex for 30 minutes. Samples were centrifuged at 5000 rpm at 4C for 15 minutes. Supernatant was reserved in a clean tube. Supernatant was evaporated in a SpeedVac and dissolved in 100 µl of ethanol. 25µL of the concentrated extract was then added to 475µL Assay Buffer. Samples were run on the DetectX Corticosterone Enzyme Immunoassay Kit in the 50 µL format according to the Arbor Assay Protocol. Optical density of the Enzyme Linked Immunosorbent Assay (ELISA) was measured at 450 nm using a Synergy HTX multi-mode microplate reader (Biotek) and compared to a standard curve to determine the final concentration. Results were calculated through the online tool MyAssays (MyAssays Ltd). Corticosterone levels were compared through Two-Way ANOVA (Prism V8).

Western Blot

For western blot and immunohistology analysis, mice were transcardially perfused with ice cold 0.1M PBS. For the perfusion, animals were injected with ketamine/xylazine cocktail (91.95mg/ml ketamine, 8.05mg/ml xylazine). The brains were extracted and sagittally hemisected in 0.1 M PBS. One half of the brain was flash frozen in dry ice and ethanol, while the other hemisphere was drop-fixed in 4% PFA. Brain regions were homogenized in homogenization buffer (4 mM HEPES, 0.32 M sucrose, 0.1 mM MgCl2) containing the following protease inhibitors: aprotinin, leupeptin, AEBSF, benzamidine, PMSF, and pepstatin A. A bead based Precellys 24 homogenizer was used. 300 µl of homogenization buffer was used for the hippocampus. Protein concentration was then determined by BCA assay (Thermo Scientific, Cat# 23225) per manufacturer's instructions, and optical density (OD) at 562 nm was read on a Synergy HTX multi-mode microplate reader (Biotek) and compared with the respective standard curve.

To prepare the samples for Western blot, each sample was mixed with 6 X SDS sample buffer at a 5:1 ratio. The mixtures were boiled at 95°C for 5 minutes. 50 µg of sample was separated by SDS-PAGE using a 4-12% Tris-Glycine Gel (Thermo Scientific, Cat# XP00120BOX). Gels were run at ~100 V for ~2 hours, then were wet transferred to a 0.2 µm nitrocellulose membrane. Membranes were subsequently washed with Tris-buffered saline with 0.1% Tween® (TBST) and blocked with Odyssey Blocking Buffer (LI-COR, Cat# 92740003) in PBS for 1 hour then incubated overnight with primary antibody. The next day membranes were washed and incubated in secondary antibody for 1 hour at RT. Blots were imaged on an Odyssey CLx (Li-Cor).

Immunohistochemistry

The hemisected half of the brain that was drop fixed in 4% PFA was used for immunohistochemistry. After brains were dehydrated in 30% sucrose and mounted in Tissue Tek® Optimal Cutting Temperature (OCT) Compound, brains were sliced on a freezing cryostat. Sections were serially washed with PBS, 0.01 M glycine, 0.2% Triton-X, blocked with 10% horse serum, and incubated with primary antibody overnight. The next day sections were washed and blocked before they were incubated with secondary antibody overnight. Finally, after adding DAPI for a nuclear stain, slides were mounted with Fluoromount-G and imaged on a Nikon A1R confocal microscope with 10X objectives. For analysis, max projected images were stitched together and imported to Fiji.

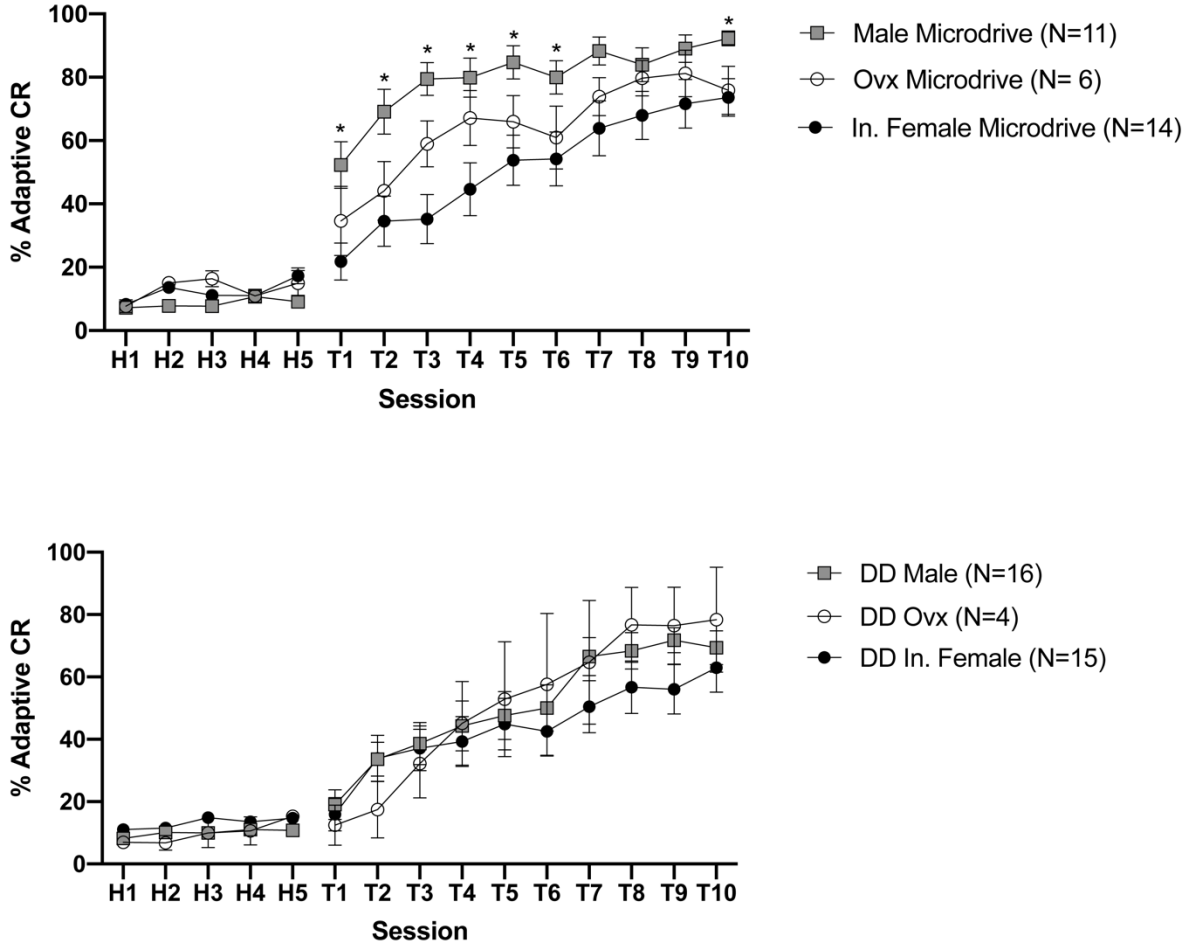

*Figure 12. Percent Adaptive CR for Microdrive and Dummy Drive Animals. * Tukey's Multiple Comparison p <0.05, Microdrive Male vs Microdrive In. Female. Mean ± SEM*

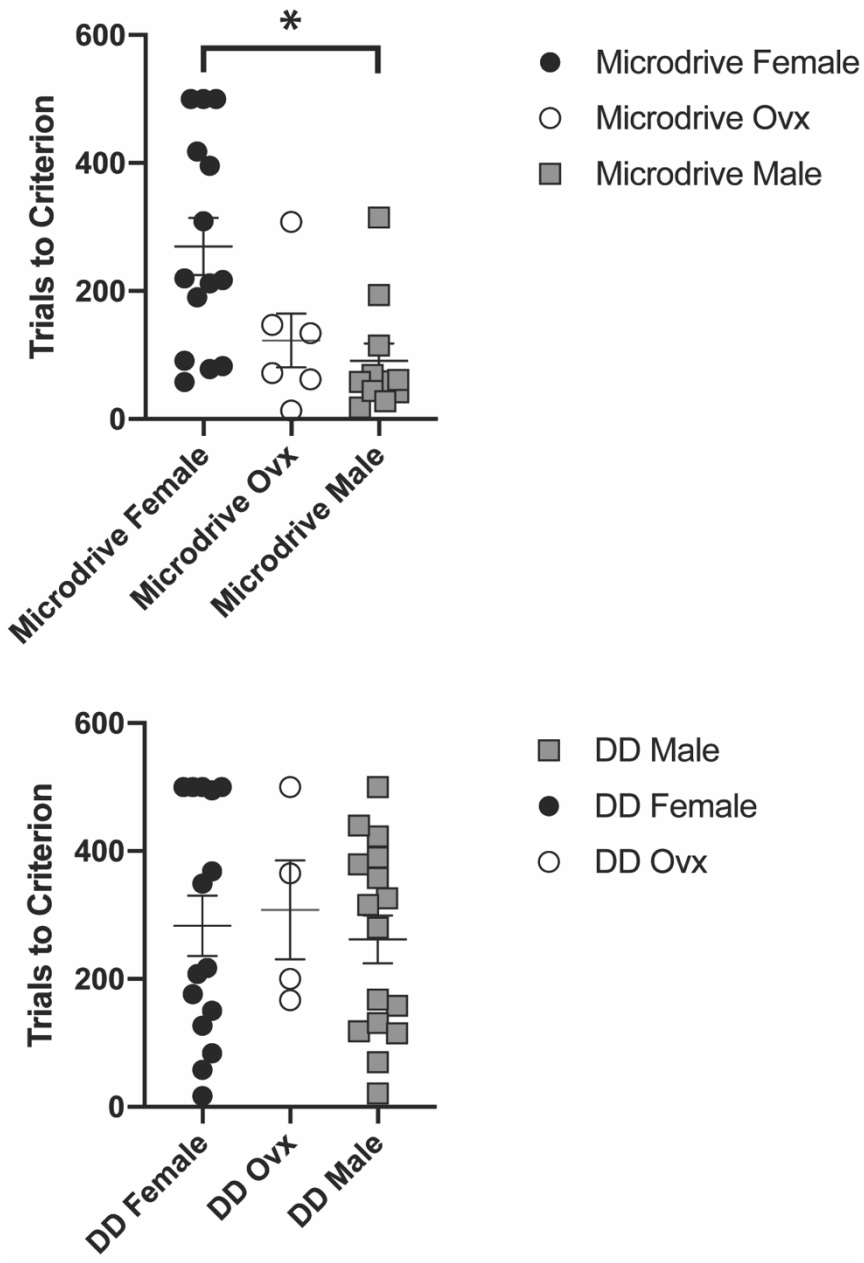

*Figure 13 Trials to 8 Consecutive CRs for Microdrive and Dummy Drive Animals. * Tukey's Multiple Comparison p <0.05, Microdrive Male vs Microdrive In. Female. Mean ± SEM. Microdrive Males n= 11 , Intact Females n= 14, Ovariectomized Females n= 6. Dummy Drive Males n= 16 , Intact Females n= 15, Ovariectomized Females n= 4.*

RESULTS

Eyeblink Conditioning

Microdrive

Implanted male, intact female, and ovx mice acquired tEBC over the course of 10 training sessions [$F(1.752, 49.07)= 26.39$, ($p= <0.0001$)]. Sex differences were observed in the rate of acquisition of tEBC in chronically implanted mice [$F(2,28)= 6.275$, $p= 0.0056$] (Figure 12)(Table 2). Male mice implanted with a chronic microdrive acquired tEBC significantly faster than intact female microdrive mice Tukey's Multiple Comparison tests, T1, [Tukey's Multiple Comparisons Test, T1, intact female vs male ($p= 0.0108$), T2, intact female vs male ($p=0.0094$), T3, intact female vs male ($p=0.003$), T4, intact female vs male ($p=0.0072$), T5, intact female vs male ($p=0.01$), T6, intact female vs male ($p=0.0452$), T10, intact female vs male ($p=0.0239$)] (Figure 12). However, ovariectomized females did not perform significantly different from either intact female or male mice [Tukey's Multiple Comparisons Test, T1, intact female vs ovx ($p= 0.5759$), ovx vs male ($p=0.4093$), T2, intact female vs ovx ($p= 0.7183$), ovx vs male ($p=0.1267$), T3, intact female vs ovx ($p= 0.0968$), ovx vs male ($p=0.1041$), T4, intact female vs ovx ($p= 0.1853$), ovx vs male ($p=0.4843$), T5, intact female vs ovx ($p= 0.5512$), ovx vs male ($p=0.1907$), T6, intact female vs ovx ($p= 0.865$), ovx vs male ($p=0.2669$), T7, intact female vs ovx ($p= 0.6208$), ovx vs male ($p=0.1787$), T8, intact female vs ovx ($p= 0.4405$), ovx vs male ($p=0.8481$), T9, intact female vs ovx ($p= 0.6472$), ovx vs male ($p=0.6435$), T10, intact female vs ovx ($p= 0.9701$), ovx vs male ($p=0.1752$)] (Figure 12) (Table 2).

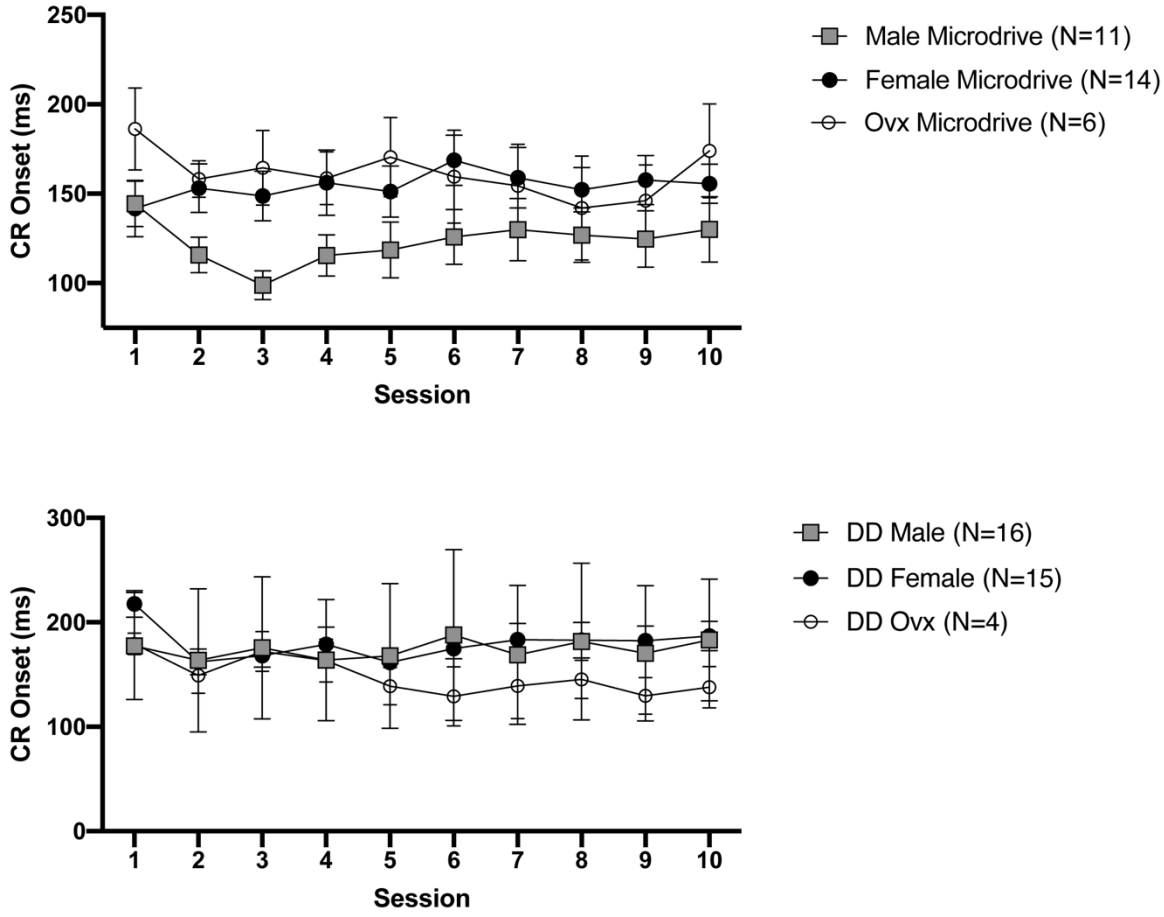

Figure 14 CR Onset for Microdrive and Dummy Drive Mice. Mean ± SEM.

Number of trials to reach 8 consecutive CRs was also used as a measurement of learning rate. Animals that failed to reach 8 consecutive CRs by the end of 10 training sessions were scored as 500 trials, the total number of conditioning trials. Male microdrive mice reached 8 consecutive CRs significantly faster than intact female male [$F(2,28)= 6.187$, $p= 0.006$, Tukey's Multiple Comparisons Test, Microdrive Male vs. Microdrive Female ($p= 0.0067$)] There were no significant differences between ovariectomized females and intact females or males [Tukey's Multiple Comparisons Test, Microdrive Male vs. Microdrive Ovx ($p= 0.8872$), Microdrive Female vs. Microdrive Ovx ($p=0.0778$)] (Figure 12).

There was no significant difference between the sexes in onset time of the CR of microdrive implanted animals [F(2,28)= 2.47, p=0.1028] (Figure). Onset time of the CR also did not vary significantly across sessions [F(2.070,57.73)= 1.0250, p= 0.3672].

Dummy Drive

The weight of the implanted microdrive alone did not lead to sex differences in acquisition. All animals with dummy drive implants acquired tEBC at similar rates (F(2,32)= 0.3988, p= 0.6744) (Figure 12). Additionally, across all groups, dummy drive animals were able to acquire tEBC over the ten sessions (F(1.578, 50.49)=29.9,p=<0.0001).

There were no significant differences between the sexes in trials to reach 8 consecutive CRs (F(2,32)=0.146, p=0.8648) (Figure 13). Sex did not significantly affect the onset time of CRs (F(2.395,76.65)=1.379, p=0.2579) (Figure 14) . Across all sessions, onset time did not significantly vary (F(18,288)=0.8857, p=0.5965).

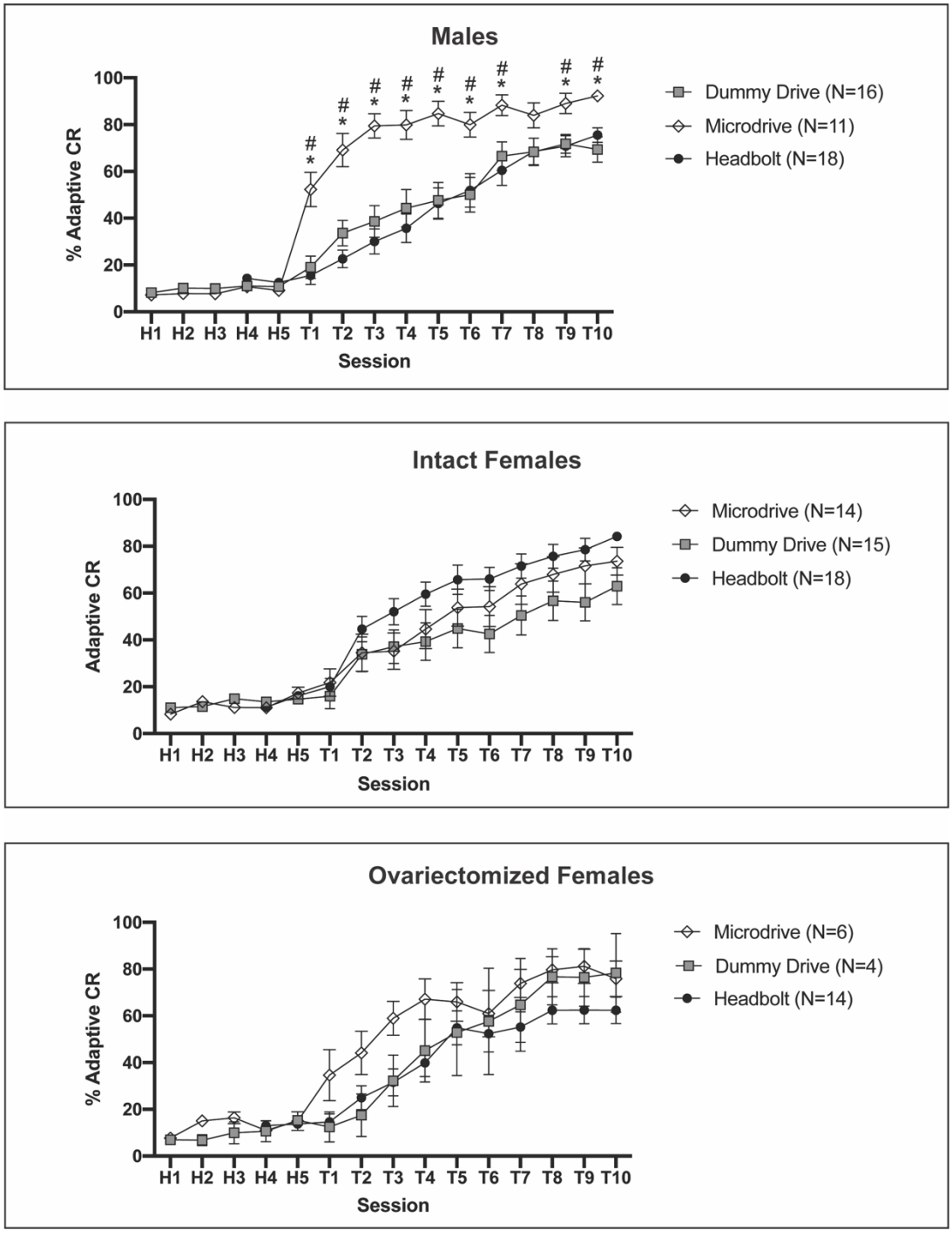

*Figure 15 Percent Adaptive CR by Sex. (Top) Male Comparison # Tukey's Multiple Comparison $p < 0.05$ Male Micro vs Male DD, * Tukey's Multiple Comparison $p < 0.05$ Male Micro vs Male Headbolt. (Center) Intact Female Comparison (Bottom) Ovx Female Comparison. Mean ± SEM.*

Males

Male mice acquired tEBC at significantly different rates depending on the surgical procedure they received (F (2,42) =12.42, p=<0.0001) (Figure 15). Male microdrive mice learned tEBC significantly faster than both dummy drive and headbolt implanted males [Tukey's Multiple Comparisons Test, T1, Microdrive Male vs. DD Male, p=0.0036, Microdrive Male vs. Headbolt Male, p=0.0013, T2, Microdrive Male vs. DD Male, p=0.002, Microdrive Male vs. Headbolt Male, p=<0.0001, T3, Microdrive Male vs. DD Male, p=0.0002, Microdrive Male vs. Headbolt Male, p=<0.0001, T4, Microdrive Male vs. DD Male, p=0.0049, Microdrive Male vs. Headbolt Male, p=<0.0001, T5, Microdrive Male vs. DD Male, p=0.0015, Microdrive Male vs. Headbolt Male, p=0.0003, T6, Microdrive Male vs. DD Male, p=0.0082, Microdrive Male vs. Headbolt Male, p=0.0105, T7, Microdrive Male vs. DD Male, p=0.02, Microdrive Male vs. Headbolt Male, p=0.0037, T9, Microdrive Male vs. DD Male, p=0.0203, Microdrive Male vs. Headbolt Male, p=0.02, T9, Microdrive Male vs. DD Male, p=0.0203, Microdrive Male vs. Headbolt Male, p=0.02, T10, Microdrive Male vs. DD Male, p=0.0027, Microdrive Male vs. Headbolt Male, p=0.0006](Figure 15). Across all surgical groups, male mice acquired tEBC (F(1.831,76.89)=52.18, p=<0.0001)(Figure 15). There was a significant interaction between surgical procedure group and session (F(18,378)= 2.704,p=0.0002).

Surgical procedure also had a significant effect on the number of trials male mice required to reach 8 consecutive CRs (F(2,42)=9.19020, p=0.0005)(Figure 16). Male microdrive animals reached 8 consecutive CRs in significantly fewer trials compared to dummy drive males

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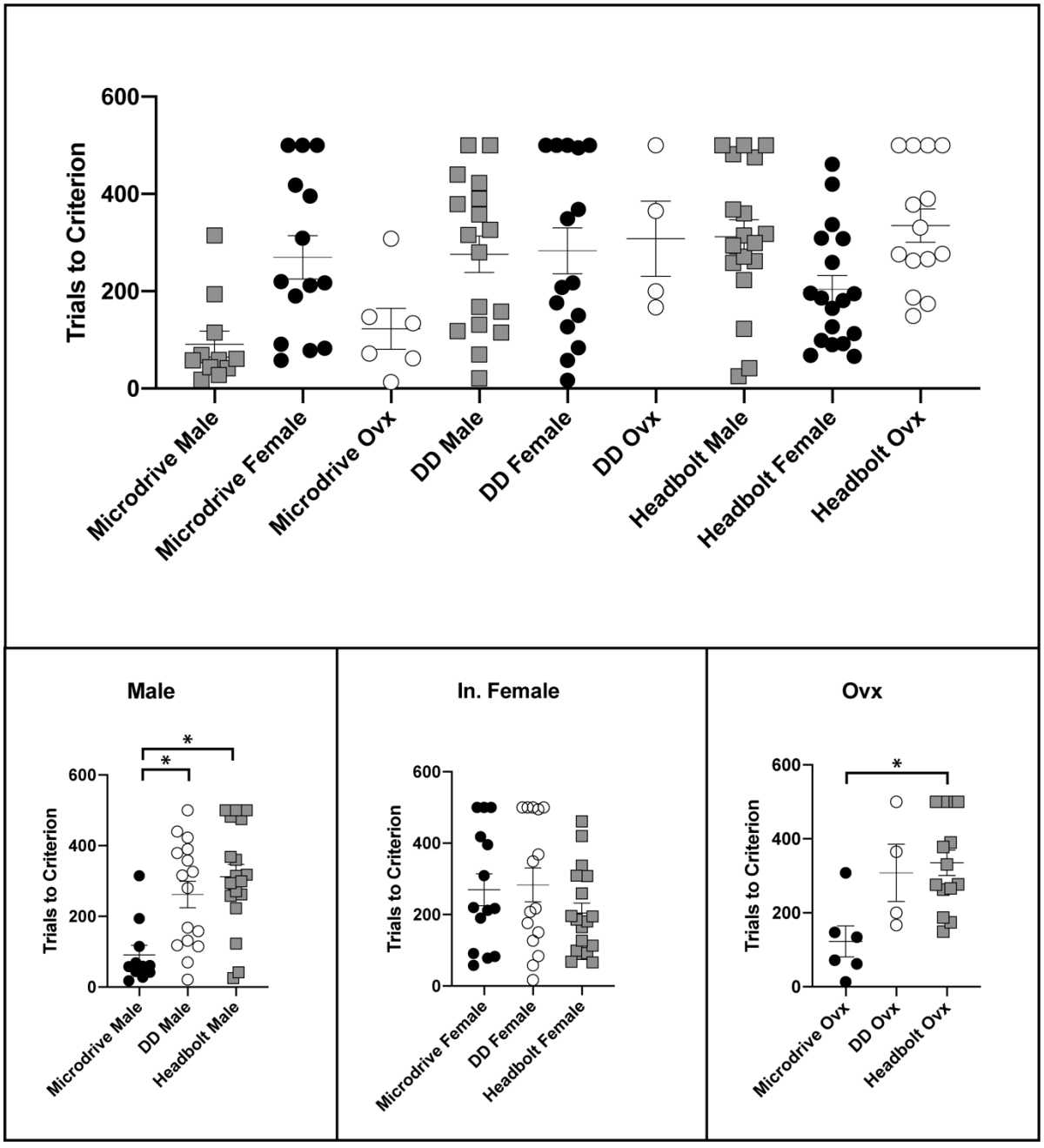

*Figure 16 Trials to 8 Consecutive CRs. All animals (top), males (bottom left), intact females (bottom center), ovariectomized females (bottom right). Mean ± SEM shown. * Tukey's Multiple Comparison Test $p < 0.05$*

and headbolt males (Tukey's Multiple Comparisons Test, Microdrive Male vs. DD Male p=0.0075, Microdrive Male vs. Headbolt Male, p=0.0004) (Figure 16).

The effect of surgical procedure on tEBC was also observed through onset time of the conditioned response($F(2,42)=4.906, p=0.0122$) (Figure 17). Microdrive males have an earlier CR onset time compared to both dummy drive and headbolt implanted animals [Tukey's Multiple Comparisons test, T1, Microdrive Male vs. Headbolt Male, p=0.0291, T2, Microdrive Male vs. DD Male, p=0.0597, Microdrive Male vs. Headbolt Male, p=0.0006, T3, Microdrive Male vs. DD Male, p=0.0015, Microdrive Male vs. Headbolt Male, p=0.0002, T4, Microdrive Male vs. DD Male, p=0.0388, Microdrive Male vs. Headbolt Male, p=0.063].

Intact Females

There is a trend towards significance of surgical procedure on acquisition of tEBC in intact females ($F(2,44)=2.704$, $p=0.0781$) (Figure 15). Intact female microdrive and dummy drive animals tended to learn more slowly compared to headbolt implanted animals (Figure 15). Across all surgical groups, animals acquired tEBC ($F(1.533,67.47)= 53.62$, $p=<0.0001$).

Surgical group did not significantly affect the number of trials required for intact females to reach 8 consecutive CRs ($F(2,44)=1.2230, p=0.3041$)(Figure 16). Surgical procedure also did not significantly impact the timing of the CR of intact females ($F(18,395)=1.382$, $p=0.1363$)(Figure 17). However, there was an effect of session on CR onset across all intact female groups ($F(2,44)=4.542, p=0.0161$).

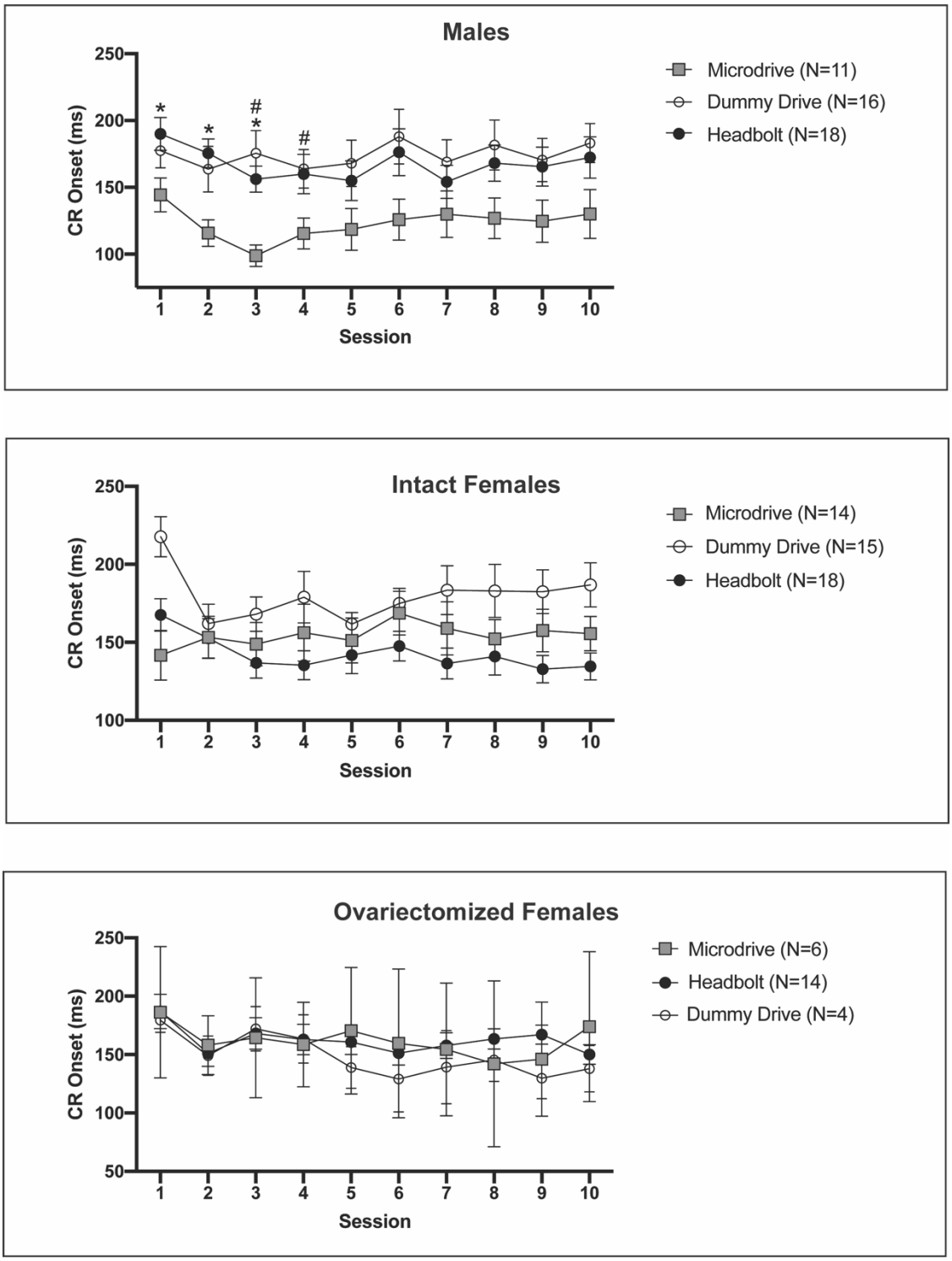

*Figure 17. CR Onset by Sex. Male (Top), Intact Female (Middle), Ovariectomized Females (Bottom). Mean ± SEM shown. * Tukey's Multiple Comparison Test $p < 0.05$ Male Microdrive vs Headbolt, # Tukey's Multiple Comparison Test $p < 0.05$ Male Microdrive vs Dummy Drive.*

Ovariectomized Females

Surgical group did not significantly affect the acquisition of adaptive CRs in tEBC in ovariectomized female mice (F(2,21)=2.28, p=0.127)(Figure 15). Across all groups, animals learned tEBC by the final session (F(2.008,42.16)=24.32,p=<0.001).

However, there was a significant effect of surgical group on number of trials to reach 8 consecutive CRs (F(2,21)=6.0250, p=0.0085)(Figure 16). Ovariectomized female microdrive mice required significantly fewer trials to reach 8 consecutive CRs (Tukey's Multiple Comparisons test, DD Ovx vs. Microdrive Ovx, p=0.0836, Microdrive Ovx vs. Headbolt Ovx, p=0.0068, DD Ovx vs. Headbolt Ovx, p=0.9252)(Figure 17).

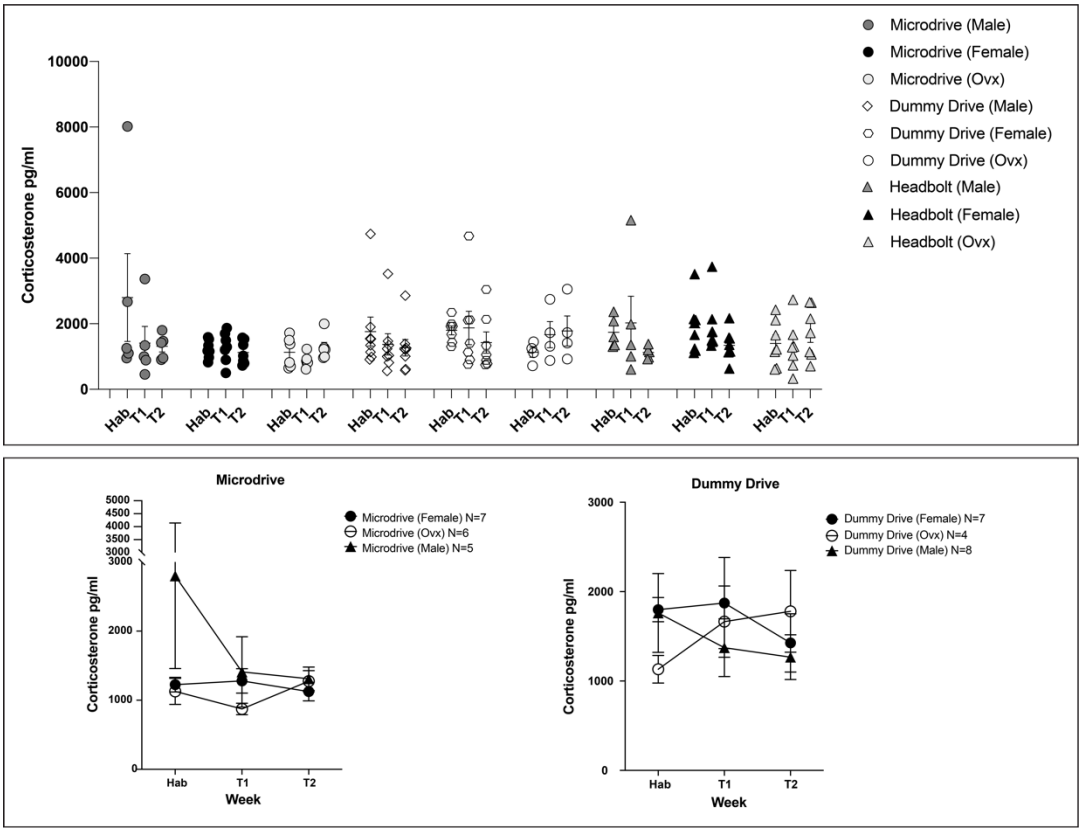

Figure 18 Corticosterone Levels Analysis. Across all animals (top), Microdrive animals (bottom left) and Dummy Drive animals (bottom right). Mean ± SEM.

Corticosterone Levels

All Subjects

Across all animals, there was a significant effect of session on corticosterone levels ($F(1.837,62.47)=3.64$, $p=0.0355$). Fecal matter collected during habituation week was significantly higher than fecal matter collected from cages during the second week of training (Tukey's Multiple Comparisons test, Hab vs T2, $p=0.0385$)(Figure 18).

Microdrive/ Dummy Drive

Sex and session did not have a significant effect on corticosterone levels of microdrive animals [Sex: $F(2,15)=2.989$, $p=0.0808$, Session: $F(1.181,17.71)=1.368$,$p=0.2647$](Figure 18). Furthermore, corticosterone levels did not significantly change with sex or session in Dummy Drive animals [Sex: $F(2,16)=0.3016$, $p=0.7437$, Session: $F(1.781,28.49)=0.1202$,$p=0.8653$].

Male/Intact Female/Ovariectomized Female

There was no significant effect of surgical group or session on corticosterone levels of male mice [Group: $F(2,15)=0.3727$, $p=0.6951$, Session: $F(1.579,23.68)=1.928$,$p=0.1736$](Figure 19). Levels of corticosterone also did not significantly change in intact female mice with surgical procedure or session [Group: $F(2,19)=2.501$, $p=0.1086$, Session: $F(1.504,28.58)=2.302$, $p=0.1296$]. Ovariectomized females did not show significant changes in corticosterone levels with surgical group or session [Group: $F(2,14)=1.873$, $p=0.1902$, Session: $F(1.866,26.13)=1.873$, $p=0.1757$].

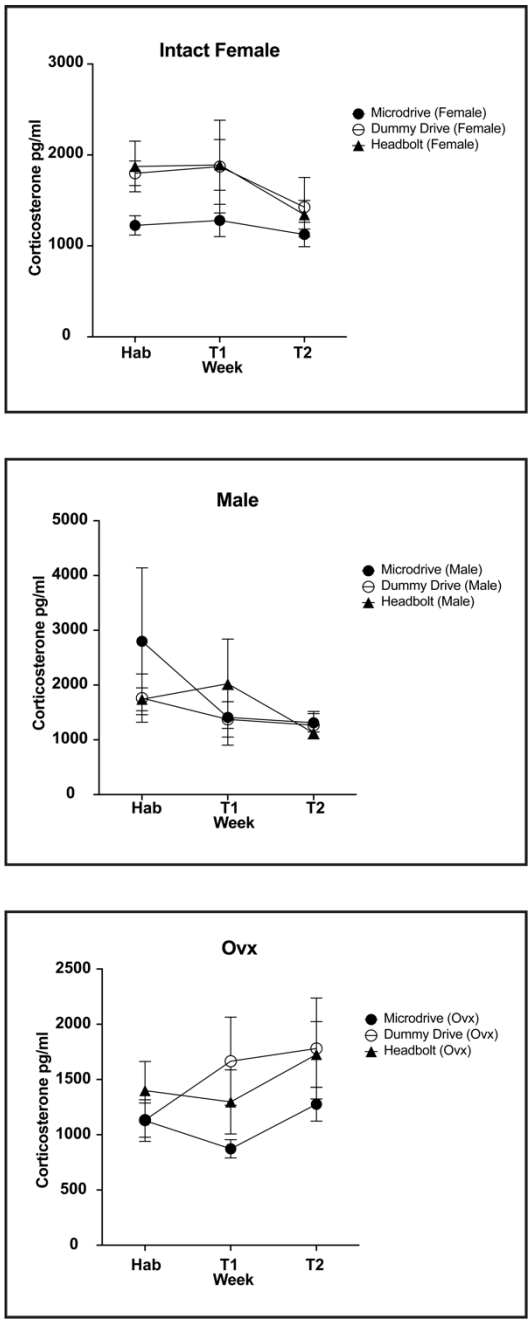

Figure 19 Corticosterone Level Analysis by Sex. Intact Females (top), Males (middle), Ovariectomized females (bottom). No significant effects of groups or session were observed in intact females, males or ovariectomized females, $p>0.05$. Mean ± SEM.

Western Blot

Preliminary Western Blot analysis did not show significant differences in neuroinflammatory or neurogenesis markers across sexes in the Dummy Drive and Microdrive groups (Figure 20). Dummy Drive [GFAP: $F(2,13)= 2.805$, $p= 0.6077$, DCX: $F(2,13)= 1.446$, $p=0.2711$, TGF-β: $F(2,13)= 0.1261$, $p=0.8826$, Iba1: $F(2,13)=3.779$, $p=0.0508$]. Microdrive analysis [GFAP: $F(2,12)= 0.1783$, $p=0.8389$, DCX: $F(2,12)= 0.2644$,$p=0.772$,TGF-β: $F(2,12)= 2.089$, $p=0.1665$, Iba1: $F(2,12)= 0.5193$, $p=0.6077$]. Headbolt animals showed significant differences in levels of TGF-β ($F(2,15)= 3.965$, $p=0.0415$). Ovx females had significantly higher levels of TGF-β compared to intact females, (Tukey's Multiple Comparisons Test, $p= 0.0398$).

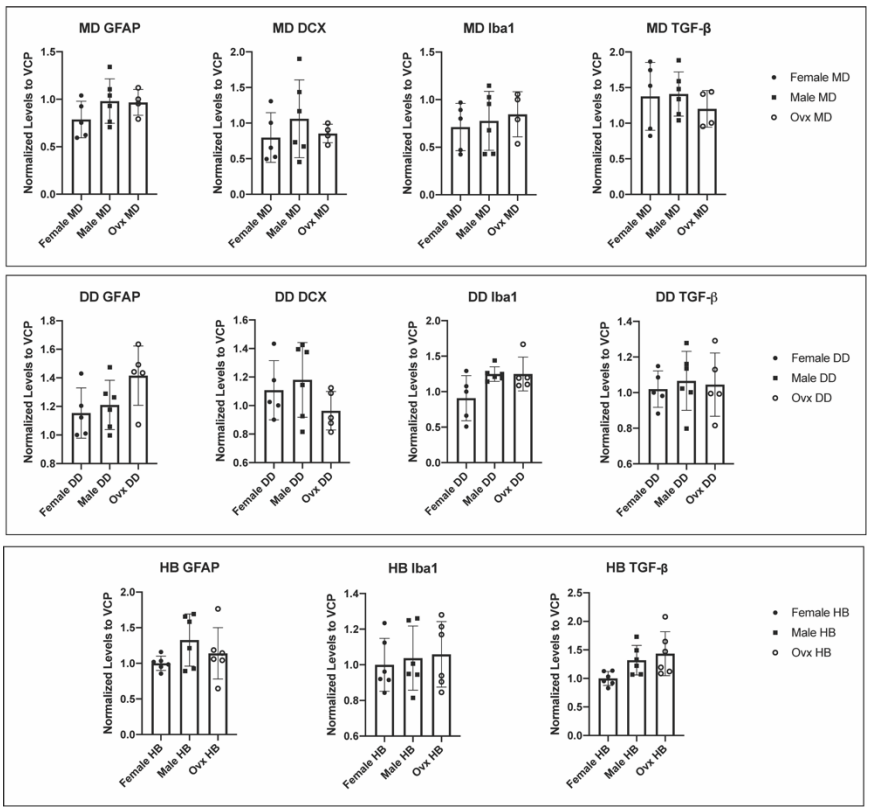

*Figure 20 Western Blot Analysis for Dummy Drive and Microdrive Animals. Mean ± SD. * Tukey's Multiple Comparison Test $p < 0.05$*

No other markers showed significant differences across sexes. Headbolts [GFAP: $F(2,15)= 1.756$, $p= 0.2065$, Iba1: $F(2,15)= 0.182$, $p=0.8354$].

When comparing across surgical groups, males, intact females and ovariectomized females showed no significant differences in levels of GFAP [Males: $F(2,13)= 1.587$, $p=0.2417$, In. Females: $F(2,13)= 1.686$, $p=0.2233$, Ovx: $F(2,13)=0.3382$, $p=0.7191$] (Figure 21). Males also did not show significant differences across surgical groups in DCX or TGF-β [DCX:$F(2,13)=1.008$, $p=0.3919$, TGF-β: $F(2,13)= 0.2235$,$p=0.8027$]. However, both intact females and ovariectomized females showed significant differences in TGF-β levels across surgical groups [Intact Female: $F(2,13)= 13.31$,$p=0.0007$, Ovx: $F(2,13)= 6.837$,$p=0.0094$]. Levels of TGF-β in intact females decreased between headbolt and microdrive as well as dummy drive and microdrive [Tukey's Multiple Comparisons: HB vs MD, $p= 0.0005$, MD vs DD, $p= 0.036$, HB vs DD, $p= 0.1065$]. TGF-β levels in ovariectomized females were significantly lower in microdrive compared to headbolt animals. [Tukey's Multiple Comparisons: HB vs MD, $p= 0.0098$, MD vs DD, $p= 0.4719$, HB vs DD, $p= 0.0548$]. Intact and ovariectomized females also showed significant differences in DCX between surgical groups [Intact Female: $F(2,13)= 4.963$, $p=0.025$, Ovx: $F(2,13)= 20.93$, $p=<0.0001$]. Intact female DCX levels decreased with chronic dummy drive implant [Tukey's Multiple Comparisons: HB vs DD, $p= 0.0199$, HB vs MD, $p= 0.2682$, MD vs DD, $p= 0.3469$]. Ovariectomized female DCX levels decreased with both microdrive and dummy drive implantation [Tukey's Multiple Comparisons: HB vs MD, $p= 0.0005$, HB vs DD, $p= 0.0002$, MD vs DD, $p= 0.4778$].

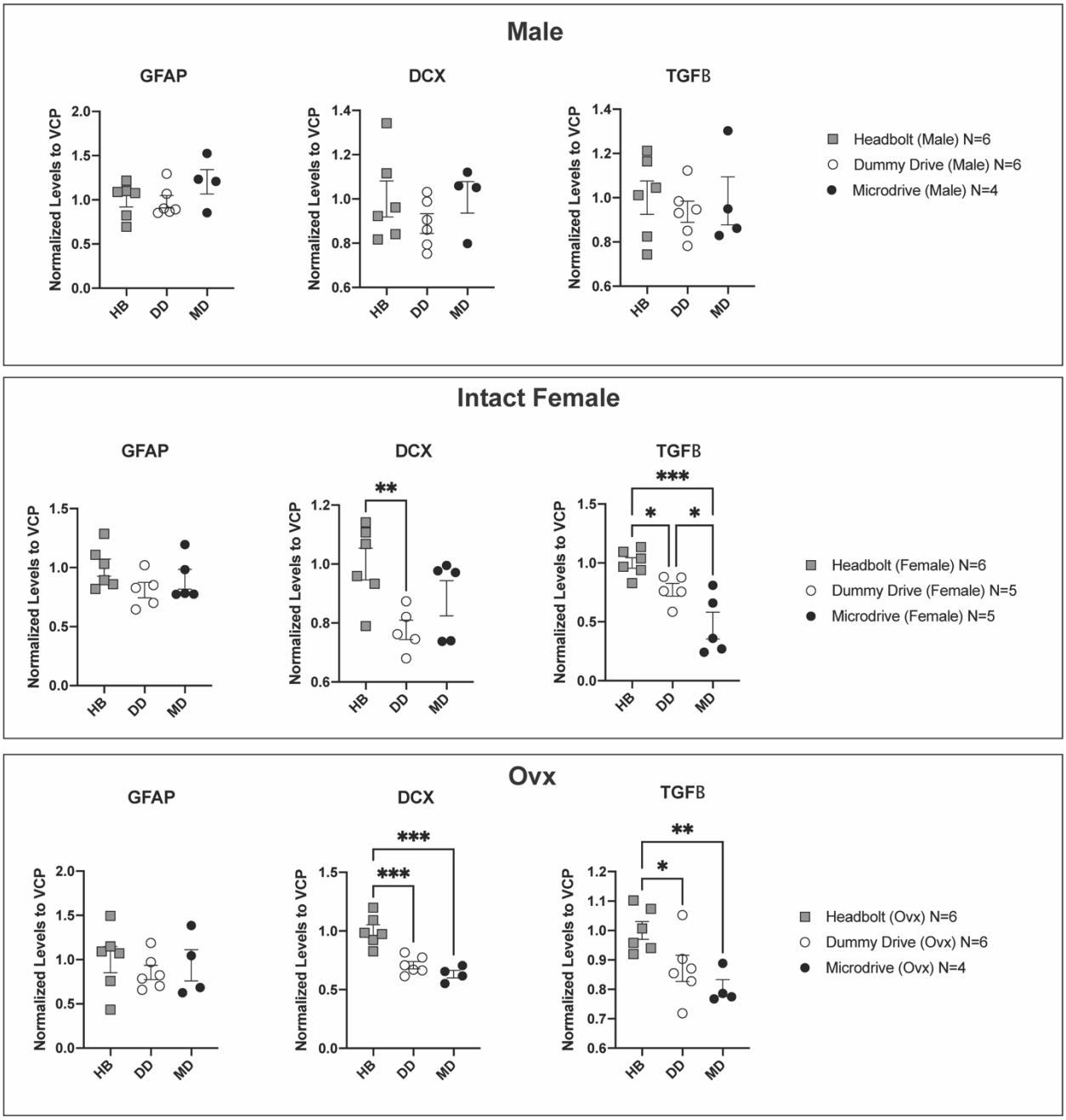

*Figure 21 Western Blot Analysis Comparison by Sex. (Top) Male Analysis. (Middle) Intact Female Analysis. (Bottom) Ovx Female Analysis. Mean ± SD. * Tukey's Multiple Comparison Test $p < 0.05$*

DISCUSSION:

Sexually dimorphic behaviors were observed in tEBC acquisition with surgical implants. In contrast to Chapter Two, where headbolt implanted intact females were shown to learn faster than male and ovariectomized females, implantation of a dummy drive or microdrive tended to delay learning (Figure 13). However, microdrive implanted males acquired tEBC at a significantly faster rate compared to dummy drive and headbolt implanted males (Figure 13). Additionally, microdrive implanted males learned faster than microdrive implanted females (Figure 12). Sexually dimorphic behaviors have previously been observed in stressed animals (Wentworth-Eidsaune et al., 2016; Wood and Shors, 1998), therefore we investigated the concentrations of corticosterone in conditioned animals to measure stress level. Corticosterone analysis from fecal matter collected from all animals showed a significant decrease in corticosterone from habituation week to training week two, suggesting animals habituate to the head-fixed apparatus over conditioning. However, there were no significant differences observed between surgical groups or sexes. As our analysis grouped fecal matter over the course of a week, it is plausible that sex and/or group differences had inadequate signal-to-noise. Furthermore, Shors found no significant differences in corticosterone levels between acutely stressed and conditioned rats when trunk blood was collected for corticosterone serum analysis (Shors et al., 1992). It is possible that surgery and tEBC alone leads to increased corticosterone that is not further increased by additional stress of the microdrive implant.

Stress may facilitate learning by leading to an increase in α-amino-3-hydroxy-5-methylisolazole-4-propionic acid (AMPA) binding, as seen in male Long-Evans rats (Tocco et al., 1991). When male Wistar rats were chronically stressed with seven stressors, subsequent corticosterone treatment led to significantly greater glucocorticoid receptor activation compared

to unstressed rats (Karst et al., 2005). Chronic stress due to the implant may lead to activation of glucocorticoid receptors during conditioning sessions. Stressed rats subsequently showed significantly larger amplitudes of AMPA-mediated currents (Karst et al., 2005). Furthermore, modulating AMPA receptors with 1-(1,3-benzodioxol-5-ylcarbonyl) perperidine facilitated acquisition of dEBC in male Sprague-Dawley rats (Shors et al., 1995). When acutely stressed with intermittent tail shock, stressed male rats had a significantly greater density of apical dendrites in CA1 compared to unstressed male rats and stressed and naïve females (Shors et al., 2001). These mechanisms may potentially underlie the facilitation observed in microdrive implanted males.

However, stress due to weight of the implant alone does not account for facilitation in acquisition of male microdrive animals. Microdrive males learned significantly faster than dummy drive males, which also received chronic implants. These differences in acquisition suggest an additional mechanism of facilitation due to additional surgical procedures including craniotomy and implanted tetrodes. These aspects of microdrive implantation may lead to a neuroinflammatory response that supports learning in males but not intact or ovariectomized females. Past studies have shown implantation of microelectrodes produces a reactive tissue response (Patrick et al., 2011; Polikov et al., 2005; Prasad et al., 2012a). This neuroinflammatory response may underlie the differences observed between groups.

The molecular and cellular response of glial cells evolves over several weeks in reaction to brain injury (Acaz-Fonseca et al., 2015; Burda and Sofroniew, 2014). Microglia move to the area of injury and produce signaling molecules like cytokines, chemokines, reactive oxygen species, and nitric oxide, which recruit peripheral immune cells and astrocytes (Acaz-Fonseca et al., 2015; Helmut et al., 2011; Kohman and Rhodes, 2013). In a resting state, microglia have fine

processes that survey the surrounding areas for damage and injury and participate in neurogenesis through phagocytosis (Kohman and Rhodes, 2013). When activated, microglia can support or impair adult neurogenesis depending on their activation state.

Classically activated microglia negatively affect hippocampal neurogenesis by releasing proinflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6)(Kohman and Rhodes, 2013). Survival of new neurons, proliferation, cell death and cell integration are affected by classically activated microglia (Belarbi et al., 2012; Ekdahl et al., 2003; Jakubs et al., 2008). However, these inflammatory cytokines including IL-1, IL-6 and tumor necrosis factor (TNF-α) have also been investigated for their roles in facilitation of learning and memory. Administration of low doses of IL-1β has been shown to facilitate acquisition of water maze (Gibertini, 1998). Low doses of IL-1β within contextual fear conditioning also improved recall of fear conditioning 48 hours later (Goshen et al., 2007). High doses of IL-1β facilitated acquisition of delay eyeblink conditioning in male Sprague-Dawley rats (Servatius and Beck, 2003). IL-1β stimulates corticotropin-releasing hormone (CRH) and administration of CRH also facilitates eyeblink acquisition (Servatius and Beck, 2003). IL-1β may facilitate acquisition in male microdrive animals by influencing signaling in brain regions associated with eyeblink conditioning like the nucleus of the tractus solitarius and amygdala (Servatius and Beck, 2003).

Surgical implantation of the microdrive may also serve as a neuroinflammatory priming event for male mice. This neuroinflammatory priming may lead to facilitation during subsequent stressful events, including acquisition of eyeblink conditioning (Frank et al., 2007). When male rats received chronic restraint stress and subsequent lipopolysaccharide (LPS) endotoxin injection, male rats showed exaggerated levels of proinflammatory cytokines IL-1β and TNF-α

compared to primed females (Bekhbat and Neigh, 2018; Munhoz et al., 2006). Additionally, repeated exposure to stress led to a sensitized cytokine response and increased hippocampal IL-1β in CD-1 male mice, but not female mice (Bekhbat and Neigh, 2018; Hudson et al., 2014). As IL-1β was previously found to facilitate eyeblink conditioning (Servatius and Beck, 2003), neuroinflammatory priming due to the implantation of tetrodes may underlie observed sexually divergent behavior in acquisition.

In contrast to the classical inflammatory pathway, activation of the "alternative" pathway could also underlie facilitation in acquisition in male mice. The alternative pathway is characterized by the expression of MHC II, arginase 1 (AG1), peroxisome proliferation activation receptor gamma (PPAR-γ), Ym1 (Chitinase 3-like 3) and mannose receptor (MRC1) (Colton, 2009; Kohman and Rhodes, 2013). Alternatively activated microglia express anti-inflammatory cytokines interleukin-10 (IL-10), transforming growth factor-β (TGF-β), as well as growth factors including NGF, insulin-like growth factor (IGF) and brain derived neural growth factor (BDNF) (Kohman and Rhodes, 2013). TGF-β has been suggested to play a role in neurogenesis, increasing the survival of new cells (Mathieu et al., 2010), and neuronal differentiation (Battista et al., 2006). Activation of the "alternative" M2 pathway promotes neuroprotective and regenerative processes and therefore may facilitate acquisition in microdrive implanted males (Colton, 2009; Kohman and Rhodes, 2013). In an investigation of cortical stab injury, males showed a higher density of microglia/macrophages with a nonreactive morphology compared to females, potentially suggesting their shift to the M2 "alternative" phenotype (Acaz-Fonseca et al., 2015). M2 phenotypes are associated with decreased phagocytosis, and protective microglia (Acaz-Fonseca et al., 2015; Glezer et al., 2007; Lai and Todd, 2008). This M2 phenotype may lead to increased neuronal survival. While levels of neuroinflammatory markers

were not significantly greater in Microdrive males compared to microdrive intact females and ovariectomized females (Figure 20), it is possible that together activation of enhanced neurogenesis through the alternative pathway and increase of IL-1β may lead to the facilitation of tEBC in microdrive males. Further investigation into additional markers, as well as IL-1β specifically, may help elucidate the underlying mechanisms.

Microdrive intact females are impaired in acquisition of tEBC compared to males, potentially due to the previously described sexually dimorphic neuroinflammatory responses. Intact female CD1 mice showed significantly fewer ionized calcium-binding adapter molecule 1 (Iba1) immunoreactive cells compared to males in response to stab wound injury (Acaz-Fonseca et al., 2015). Iba1 is a macrophage/microglia-specific calcium binding protein suggested to play a role in calcium homeostasis (Helmut et al., 2011; Imai and Kohsaka, 2002). This provides additional support that males may recruit more microglia in the alternative pathway in response to lesion compared to females. Further support for a potential role of neuroinflammation is provided by work from Wood et al 2001. Stress effects were not impacted by adrenalectomy in female rats, therefore, the stress-induced impairment in tEBC in females is not dependent on glucocorticoids and is mediated by additional mechanisms (Wood et al., 2001). Interestingly, adrenalectomy did impact the performance of male rats, further supporting sexually dimorphic mechanisms (Wood et al., 2001). Exposure to an acute stressor impaired eyeblink conditioning in female adult Sprague-Dawley rats, especially those in proestrus and diestrus, when estrogen levels are high (Shors et al., 1998; Wood et al., 2001).

Sex steroids including estrogen, progesterone and testosterone have known roles in the regulation of the immune system and may provide further explanation for observed sex differences in acquisition (Berkiks et al., 2019). For instance, estrogen may decrease the

activation, proliferation and migration of microglia to an injury site (Acosta-Martínez, 2020). Estradiol has been shown to increase peripheral immune response, while testosterone decreases these responses (Berkiks et al., 2019). Estradiol has been shown to affect HPA axis functioning by modulating mineralocorticoid and glucocorticoid receptors and directly enhancing CRH gene transcription (M. P. Carey et al., 1995; KITAY, 1963; Kudielka and Kirschbaum, 2005). Estradiol has also been shown to reduce habituation to a repeated stressor (Heck and Handa, 2019). Estradiol may underscore the sex differences observed in habituation to repeated restraint, where female rats habituated significantly more slowly than males (Galea et al., 1997a; Heck and Handa, 2019; Zavala et al., 2011). In acute stress studies, estradiol increased CRH and vasopressin mRNA levels in ovariectomized females (Lunga and Herbert, 2004). The influence of estradiol on the HPA axis may in part explain trends in differences between intact and ovariectomized microdrive females.

Stress and neuroinflammatory processes underlie sexually dimorphic behavior observed with surgical implantation of the microdrive. Observed sex differences in glucocorticoid receptors, neurogenesis and Iba1 support the notion that these systems shape the differences in acquisition of eyeblink conditioning.

Figure		Test	F-Value	P-Value	Standard Omega-Square	R-Squared	Mean Difference
	Adaptive CR	**Two-Way ANOVA**					
	Microdrive		18,252; 1.752,49.07; 2,28				
		Session x Sex	1.392	0.1353	1.982		
		Session	26.39	<0.0001	18.79		
		Sex	6.275	0.0056	17.39		
		Tukey's Multiple Comparisons Test					
		T1					
		Microdrive Male vs. Microdrive Female		0.0108			30.51
		Microdrive Male vs. Microdrive Ovx		0.4093			17.62
		Microdrive Female vs. Microdrive Ovx		0.5759			-12.89
		T2					
		Microdrive Male vs. Microdrive Female		0.0094			34.57
		Microdrive Male vs. Microdrive Ovx		0.1267			25
		Microdrive Female vs. Microdrive Ovx		0.7183			-9.567
		T3					
		Microdrive Male vs. Microdrive Female		0.0003			44.27
		Microdrive Male vs. Microdrive Ovx		0.1041			20.5
		Microdrive Female vs. Microdrive Ovx		0.0968			-23.78
		T4					
		Microdrive Male vs. Microdrive Female		0.0072			35.22
		Microdrive Male vs. Microdrive Ovx		0.4843			12.72
		Microdrive Female vs. Microdrive Ovx		0.1853			-22.5
		T5					
		Microdrive Male vs. Microdrive Female		0.01			30.97
		Microdrive Male vs. Microdrive Ovx		0.1907			18.77
		Microdrive Female vs. Microdrive Ovx		0.5512			-12.2
		T6					
		Microdrive Male vs. Microdrive Female		0.0452			25.77
		Microdrive Male vs. Microdrive Ovx		0.2669			19.01
		Microdrive Female vs. Microdrive Ovx		0.865			-6.752
		T7					
		Microdrive Male vs. Microdrive Female		0.0535			24.42
		Microdrive Male vs. Microdrive Ovx		0.1787			14.44
		Microdrive Female vs. Microdrive Ovx		0.6208			-9.984
		T8					
		Microdrive Male vs. Microdrive Female		0.2168			16.03
		Microdrive Male vs. Microdrive Ovx		0.8481			4.245
		Microdrive Female vs. Microdrive Ovx		0.4405			-11.78
		T9					
		Microdrive Male vs. Microdrive Female		0.1474			17.41
		Microdrive Male vs. Microdrive Ovx		0.6435			7.818
		Microdrive Female vs. Microdrive Ovx		0.6472			-9.588
		T10					
		Microdrive Male vs. Microdrive Female		0.0239			18.69
		Microdrive Male vs. Microdrive Ovx		0.1752			16.43
		Microdrive Female vs. Microdrive Ovx		0.9701			-2.258
	Dummy Drive		18, 288; 1.578, 50.49; 2, 32				
		Session x Sex	1.142	0.3107	1.596		
		Session	29.9	<0.0001	20.9		
		Sex	0.3988	0.6744	1.237		
	Adaptive CR	**Two-Way ANOVA**					
Figures 12 & 15	Male		18, 378; 1.831, 76.89; 2,42				
		Session x Group	2.704	0.0002	2.536		
		Session	52.18	<0.0001	24.46		
		Group	12.42	<0.0001	18.42		
		Tukey's Multiple Comparisons Test					
		T1					
		Microdrive Male vs. DD Male		0.0036			33.21
		Microdrive Male vs. Headbolt Male		0.0013			36.69
		DD Male vs. Headbolt Male		0.8396			3.487
		T2					
		Microdrive Male vs. DD Male		0.002			35.49
		Microdrive Male vs. Headbolt Male		<0.0001			46.51
		DD Male vs. Headbolt Male		0.2377			11.03
		T3					
		Microdrive Male vs. DD Male		0.0002			40.84
		Microdrive Male vs. Headbolt Male		<0.0001			49.41
		DD Male vs. Headbolt Male		0.5878			8.573
		T4					
		Microdrive Male vs. DD Male		0.0049			35.6
		Microdrive Male vs. Headbolt Male		<0.0001			44.11
		DD Male vs. Headbolt Male		0.6806			8.511
		T5					
		Microdrive Male vs. DD Male		0.0015			37.120
		Microdrive Male vs. Headbolt Male		0.0003			38.410
		DD Male vs. Headbolt Male		0.9912			1.294
		T6					
		Microdrive Male vs. DD Male		0.0082			29.92
		Microdrive Male vs. Headbolt Male		0.0105			28.11
		DD Male vs. Headbolt Male		0.9831			-1.814
		T7					
		Microdrive Male vs. DD Male		0.02			21.76
		Microdrive Male vs. Headbolt Male		0.0037			27.86
		DD Male vs. Headbolt Male		0.7704			6.095
		T8					
		Microdrive Male vs. DD Male		0.1397			15.59
		Microdrive Male vs. Headbolt Male		0.1386			15.44
		DD Male vs. Headbolt Male		0.9998			-0.1511
		T9					
		Microdrive Male vs. DD Male		0.0203			17.23
		Microdrive Male vs. Headbolt Male		0.02			18.22
		DD Male vs. Headbolt Male		0.9852			0.9891
		T10					
		Microdrive Male vs. DD Male		0.0027			22.93
		Microdrive Male vs. Headbolt Male		0.0006			16.72
		DD Male vs. Headbolt Male		0.5909			-6.217

	In. Female		18, 396; 1.533,67.47; 2,44				
		Session x Group	1.041	0.4125	0.9792		
		Session	53.62	<0.0001	25.23		
		Group	2.704	0.0781	5.737		
	Ovx		18,189; 2.008,42.16; 2,21				
		Session x Group	0.7589	0.7459	1.813		
		Session	24.32	<0.0001	29.05		
		Group	2.28	0.127	6.972		
	Consecutive 8 CRs	**Ordinary One-Way ANOVA**					
	Microdrive		2, 28				
		Sex	6.187	0.006		0.3065	
		Tukey's Multiple Comparisons Test					
		Microdrive Male vs. Microdrive Female		0.0067			-178.2
		Microdrive Male vs. Microdrive Ovx		0.8872			-31.48
		Microdrive Female vs. Microdrive Ovx		0.0778			146.8
	Dummy Drive		2, 32				
		Sex	0.146	0.8648		0.00904	
Figures 13 & 16	**Consecutive 8 CRs**	**Ordinary One-Way ANOVA**					
	Male		2,42				
		Group	9.1920	0.0005		0.3044	
		Tukey's Multiple Comparisons Test					
		Microdrive Male vs. DD Male		0.0075			-170.9
		Microdrive Male vs. Headbolt Male		0.0004			-220.8
		DD Male vs. Headbolt Male		0.5436			-49.88
	In. Female		2,44				
		Group	1.2230	0.3041		0.05268	
	Ovx		2,21				
		Group	6.0250	0.0085		0.3646	
		Tukey's Multiple Comparisons Test					
		DD Ovx vs. Microdrive Ovx		0.0836			185.3
		DD Ovx vs. Headbolt Ovx		0.9252			-27.07
		Microdrive Ovx vs. Headbolt Ovx		0.0068			-212.4
	Onset	**Mixed-Effects Analysis**					
	Microdrive		2.070, 57.73; 2,28; 18,251				
		Session	1.0250	0.3672			
		Sex	2.47	0.1028			
		Session x Sex	0.8901	0.5911			
		Two-Way ANOVA	18, 288; 2.395,76.65; 2,32				
	Dummy Drive	Session	0.8857	0.5965	2.294		
		Sex	1.379	0.2579	1.786		
		Session x Sex	0.827	0.4465	2.645		
	Onset	**Two-Way ANOVA**					
	Male		18,378; 2.032,85.35; 2,42				
		Session x Group	0.6161	0.8874	1.056		
		Session	2.065	0.1323	1.77		
		Group	4.906	0.0122	11.57		
		Tukey's Multiple Comparisons Test					
		T1					
		Microdrive Male vs. DD Male		0.1809			-33.14
		Microdrive Male vs. Headbolt Male		0.0291			-47.72
		DD Male vs. Headbolt Male		0.6826			-14.58
Figures 14 & 17		T2					
		Microdrive Male vs. DD Male		0.0597			-47.89
		Microdrive Male vs. Headbolt Male		0.0006			-61.45
		DD Male vs. Headbolt Male		0.777			-13.56
		T3					
		Microdrive Male vs. DD Male		0.0015			-76.8
		Microdrive Male vs. Headbolt Male		0.0002			-60.58
		DD Male vs. Headbolt Male		0.6916			16.23
		T4					
		Microdrive Male vs. DD Male		0.0388			-48.37
		Microdrive Male vs. Headbolt Male		0.063			-43.12
		DD Male vs. Headbolt Male		0.9632			5.257
	Intact Female	**Mixed Effects Analysis**	2.816, 123.6; 2,44; 18,395				
		Session x Group	1.839	0.1472			
		Session	4.542	0.0161			
		Group	1.382	0.1363			
	Ovx	**Two-Way ANOVA**	18, 189; 3.413, 71.68; 2, 21				
		Session x Group	0.5026	0.9548	2.482		
		Session	1.909	0.1282	4.713		
		Group	0.3376	0.7173	1.264		
	Corticosterone ELISA						
	All Subjects	**Two-Way ANOVA**	10, 68; 1.837, 62.47; 5,34				
		Session x Condition	0.6476	0.7678	5.139		
		Session	3.64	0.0355	5.776		
		Condition	0.8514	0.5233	3.983		
		Tukey's multiple comparisons test					
		Hab vs. T1		0.7171			190.3
		Hab vs. T2		0.0385			550.9
		T1 vs. T2		0.1305			360.6
	Microdrive	**Two-Way ANOVA**	4,30; 1.181,17.71; 2,15				
		Session x Sex	1.2	0.3313	9.004		
		Session	1.368	0.2647	5.134		
		Sex	2.989	0.0808	8.826		
Figures 18-19	Dummy Drive	**Two-Way ANOVA**	4,32; 1.781,28.49; 2,16				
		Session x Sex	0.7651	0.5558	5.261		
		Session	0.1201	0.8653	0.4128		
		Sex	0.3016	0.7437	1.403		

	Male	**Two-Way ANOVA**	4,30; 1.579,23.68; 2,15				
		Session x Group	0.6291	0.6455	4.819		
		Session	1.928	0.1736	7.385		
		Group	0.3727	0.6951	1.468		
	Intact Female	**Two-Way ANOVA**	4,38; 1.504,28.58; 2,19				
		Session x Group	0.2668	0.8974	1.274		
		Session	2.302	0.1296	5.494		
		Group	2.501	0.1086	9.922		
	Ovx	**Two-Way ANOVA**	4,28; 1.866,26.13; 2,14				
		Session x Group	0.5938	0.67	3.964		
		Session	1.873	0.1757	6.251		
		Group	1.873	0.1902	9.06		
	Western Blot	**Ordinary One-Way ANOVA**					
	Dummy Drive	DD GFAP	2,13				
		Sex	2.805	0.0971	0.3014		
		DD DCX	2,13				
		Sex	1.446	0.2711	0.182		
		DD TGF-β	2,13				
		Sex	0.1261	0.8826	0.01903		
		DD Iba1	2,13				
		Sex	3.779	0.0508	0.3676		
	Microdrive	**Ordinary One-Way ANOVA**					
		MD GFAP	2,12				
		Sex	0.1783	0.8389	0.02886		
		MD DCX	2,12				
Figure 20		Sex	0.2644	0.772	0.04221		
		MD TGF-β	2,12				
		Sex	2.089	0.1665	0.2583		
		MD Iba1	2,12				
		Sex	0.5193	0.6077	0.07966		
	Headbolt	**Ordinary One-Way ANOVA**					
		MD GFAP	2,15				
		Sex	1.756	0.2065	0.1897		
		MD Iba1	2,15				
		Sex	0.182	0.8354	0.02369		
		MD TGF-β	2,15				
		Sex	3.965	0.0415	0.3458		
		Tukey's multiple comparisons test					
		Female HB vs. Male HB		0.1466			-0.3193
		Female HB vs. Ovx HB		0.0398			-0.4339
		Male HB vs. Ovx HB		0.757			-0.1146
	Western Blot	**Ordinary One-Way ANOVA**					
	Male	GFAP	2,13				
		Group	1.587	0.2417	0.1963		
		DCX	2,13				
		Group	1.008	0.3919	0.1342		
		TGF-β	2,13				
		Group	0.2235	0.8027	0.03324		
	Intact Female	**Ordinary One-Way ANOVA**					
		GFAP	2,13				
		Group	1.686	0.2233	0.206		
		DCX	2,13				
		Group	4.963	0.025	0.4329		
		Tukey's multiple comparisons test					
		HB vs. DD		0.0199			0.2234
		HB vs. MD		0.2682			0.1159
		DD vs. MD		0.3469			-0.1075
		TGF-β	2,13				
		Group	13.31	0.0007	0.6719		
		Tukey's multiple comparisons test					
Figure 21		HB vs. DD		0.1065			0.2279
		HB vs. MD		0.0005			0.5317
		DD vs. MD		0.036			0.3038
	Ovx	**Ordinary One-Way ANOVA**					
		GFAP	2,13				
		Group	0.3382	0.7191	0.04946		
		DCX	2,13				
		Group	20.93	<0.0001	0.763		
		Tukey's multiple comparisons test					
		HB vs. DD		0.0005			0.2923
		HB vs. MD		0.0002			0.3679
		DD vs. MD		0.4778			0.07564
		TGF-β	2,13				
		Group	6.837	0.0094	0.5126		
		Tukey's multiple comparisons test					
		HB vs. DD		0.0548			0.1289
		HB vs. MD		0.0098			0.1959
		DD vs. MD		0.4719			0.067

Table 3. Statistical Table for Chapter Three

CHAPTER FOUR: LEARNING RELATED CHANGES IN THE LATERAL ENTORHINAL CORTEX DURING ACQUISITION OF ASSOCIATIVE MEMORY

BACKGROUND:

Although the role of the hippocampus in associative memory has been well established, less is known about the regions it shares reciprocal connections with, including the entorhinal cortex (EC) (Igarashi et al., 2014; Morrissey and Takehara-Nishiuchi, 2014). As a whole, the EC processes sensory information and relays this information to respective targets (Morrissey et al., 2012). The EC is divided into the medial entorhinal cortex (MEC) and lateral entorhinal cortex (LEC), based on projection patterns and cellular structure of these regions (Figure 21)(Neves et al., 2008). The MEC receives input from the parahippocampal cortex (postrhinal cortex in nonprimates) and innervates proximal CA1, while the LEC primarily receives input from the perirhinal cortex and projects to distal CA1 (Kitamura et al., 2014; Knierim et al., 2014; Neves et al., 2008; Wilson et al., 2013). LEC has been presumed to integrate sensory information for the hippocampus as LEC receives sensory information through the perirhinal cortex.

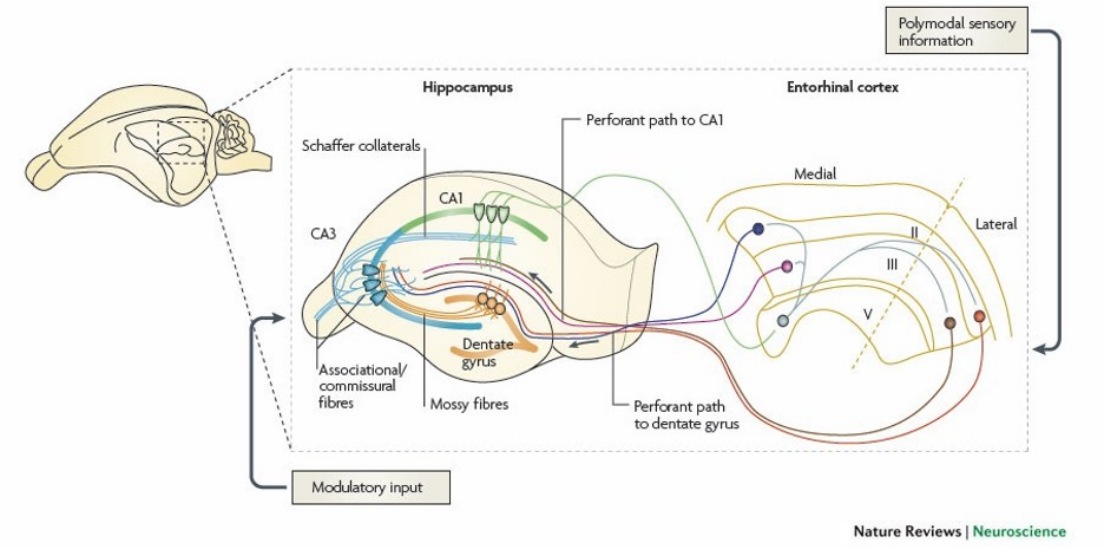

Figure 22 Entorhinal Circuit. From Neves et al 2008

The role of LEC in associative memory was of interest not only for its reciprocal connections with the hippocampus, but due to preliminary work in the region. Coherence in firing activity between distal CA1 and LEC, but not CA1 and MEC, was necessary for successful memory formation in an odor place match task (Igarashi et al., 2014). Furthermore, the importance of LEC to short and long-term memory of trace eyeblink conditioning was established through an inactivation study that used repeated injections of the GABAergic agonist muscimol into EC (Morrissey et al., 2012). Utilizing a Sim1:Cre mouse line, Ainge and colleagues showed fan cells of Layer II are necessary for episodic-like novel object-place-context memories (Vandrey et al., 2020). LEC has also been shown to provide object (Deshmukh and Knierim, 2011; Hargreaves et al., 2005) odor (Leitner et al., 2016; Wood et al., 1999; Xu and Wilson, 2012) and texture (Boisselier et al., 2014) information to the hippocampus. LEC is also connected to the medial prefrontal cortex (mPFC), and it is suggested that LEC acts to modulate information between hippocampus and mPFC (Chao et al., 2016). Disruption of the LEC-mPFC circuit, through NMDA injection, resulted in reduced memory of object-place relationships (Chao et al., 2016). These associative memory studies suggest LEC may play an important role in the acquisition of tEBC.

As superficial and deep layers of LEC project to and receive input from different sources, it is conceivable these layers may serve different roles in learning and memory. Cortical input converges in superficial layers while deep layers receive hippocampal output and relay this information back to the cortex. fMRI studies have shown that activity in the deep layers of the EC better predicts recall than activity in the superficial layers of the LEC during a visual encoding task, indicating LEC also has the potential for extra-hippocampal memory storage (Maass et al., 2014).

Physiological properties of LEC further support inquiry into the region as a site of associative memory formation and storage. Layer III and layer V LEC pyramidal neurons exhibit the unique property of persistent firing. Persistent firing is neuronal activity that extends long after its triggering stimulus ends (Lin et al 2020; Tahvaldari et al 2007). Persistent firing has been previously suggested to be a pivotal mechanism for associative memory (Fuster, 1973; Tahvildari et al., 2007). Fuster showed neurons in the prefrontal cortex maintained firing across a period of delay during a delayed response procedure (Fuster, 1973). Recent work from Carmen Lin showed the effects of learning and aging on persistent firing in pyramidal cells in LEC III (Lin et al 2020). In naive animals, persistent firing probability decreased in aged rats. However, in successfully conditioned animals, learning enhanced firing probability in both young and aged animals (Lin et al., 2020). Persistent firing may serve as the mechanism to bridge the gap between the CS and US allowing subjects to learn the association in tEBC.

The EC has been implicated in a number of neurological conditions that are accompanied by memory impairment (Coutureau and Di Scala, 2009). EC is one of the first regions to develop pathology of Alzheimer's disease and other non-Alzheimer's dementias (Braak et al., 2000; Coutureau and Di Scala, 2009) Cerebral blood volume functional magnetic resonance imaging (CBV-fMRI) determined that tau and amyloid precursor protein led to EC dysfunction that subsequently served as a source of dysfunction for other cortical regions (Khan et al., 2014). Functional connectivity between EC and medial prefrontal cortex was strongly reduced in AD (Berron et al., 2020). Connectivity between EC and Posterior Parietal Cortex was also strongly reduced in Apolipoprotein E (APOE) 4 carriers, who are at high-risk of developing AD (Coughlan et al., 2020). Layer II of LEC has been shown to be selectively sensitive to AD, where terminal zones of neurons atrophy early in disease (Stranahan and Mattson, 2010). Astrocytes in

EC were shown to decrease with age in a mouse model, suggesting reduced astroglial support for neural networks (Rodríguez et al., 2014). The entorhinal-hippocampal system has also shown increased activation of toll-like receptors in microglia linked to degeneration of neurons (Landreth and Reed-Geaghan, 2009; Okun et al., 2010; Stranahan and Mattson, 2010). EC is a region of interest in part due to these many connections to early AD pathology.

In addition to dementia, EC dysfunction may also be present in schizophrenia (Coutureau and Di Scala, 2009). MRI studies have shown decreased EC volume in diagnosed schizophrenics compared to control patients (Baiano et al., 2008). In addition, chondroitin sulfate proteoglycan (CSPG)-positive glial cells were significantly increased in Layer II EC in schizophrenia. Abnormalities in these extracellular matrix components have been hypothesized to play a critical role in pathology of schizophrenia (Pantazopoulos et al., 2010).

Neuronal loss in layer III of EC has also been implicated in temporal lobe epilepsy (Coutreau et al 2009; Du et al., 1993). Kuhn and colleagues saw spatial organization of EC activity was lost in patients with temporal lobe epilepsy compared to healthy controls (Kuhn et al., 2018). All $GABA_A$ subunits were decreased in epilepsy patients except in the α1 subunit (Stefanits et al., 2019). The entorhinal-hippocampal circuit is believed to be involved in seizure generation, through the reciprocal connection EC has with the hippocampus (Janz et al., 2017). Associations with numerous neurological and neuropathological disorders exemplifies the importance of LEC research. Therefore, examining LEC in the normal brain would provide a

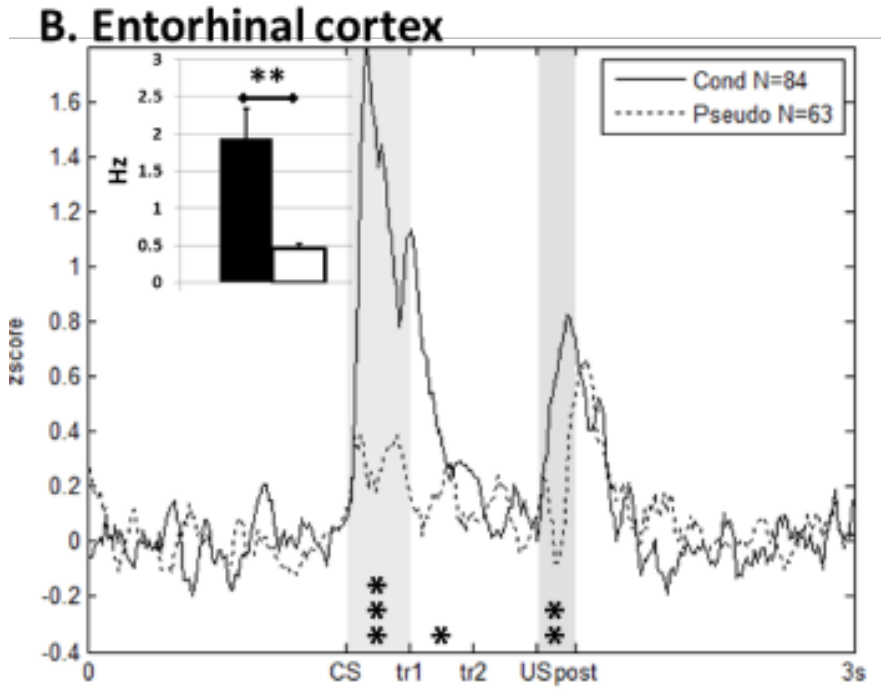

Figure 23 Entorhinal Firing from Suter et al 2019.

greater understanding of the entorhinal-hippocampal circuit that could be used to establish how the circuit is affected in neurodegenerative disorders.

Eugenie Suter obtained extracellular recordings from LEC of New Zealand white rabbits and found both rate-increasing and rate-decreasing neuron populations with acquisition of tEBC. With acquisition of tEBC, neurons showed an increase in magnitude and duration of the CS response (Suter et al., 2019). Neurons also increased in their baseline firing rates which was suspected to reflect the reductions in post-burst afterhyperpolarization (Suter et al 2019). Neurons in LEC showed an increase in firing during the trace period, though this activity did not bridge the trace interval (Figure 22). This activity supports the suggestion that persistent firing in LEC may underlie trace-bridging associations.

The below study aimed at expanding Eugenie's findings in LEC to mice and further investigating the role of persistent firing in tEBC *in vivo.* Additionally, we expanded the study to include intact female, male and ovariectomized female mice to investigate any potential sex differences in learning related changes in LEC. As described in chapter three, microdrive male mice learned significantly faster than intact females, and therefore it is possible that learning related changes would be observed in LEC in males before intact females. The study also proposed to investigate LFP activity in LEC, of particular interest was activity in the theta band (6-10Hz), as this oscillatory activity has been linked to memory (Buzsáki and Moser, 2013; Kragel et al., 2020; Lubenov and Siapas, 2009; Sosa et al., 2018; Tokuda et al., 2014).

Theta oscillations are prominent rhythms in the hippocampus, and activity that is theta-modulated has been believed to generate temporal associations (Deshmukh et al., 2010). Theta synchronization between LEC and mPFC increased during successful CRs in tEBC (Takehara-Nishiuchi et al., 2012). However, increased theta synchronization between LEC and hippocampus occurred throughout learning, regardless of CR expression (Takehara-Nishiuchi et al., 2012). LEC was found to have less theta modulation compared to MEC, however researchers noted LEC fires at a lower rate, and therefore the theta modulation may go undetected by autocorrelograms (Deshmukh et al., 2010). It was of interest to understand if these results found in male rats could be further confirmed in male and female mice.

METHODS:

Animals

All procedures were approved by and completed in accordance with the Northwestern University Animal Care and Use Committee guidelines. Experiments were performed with young adult (3-4 months) male, intact female and ovariectomized female C57BL/6J mice

obtained from Jackson Laboratory (Bar Harbor, Maine). Ovariectomy was performed by Jackson Laboratory at least two weeks prior to shipment. All mice were housed in Northwestern University temperature-controlled facilities in a 14-hour light: 10-hour dark cycle and fed ad lib. Mice were group housed at arrival and allowed to acclimate to Northwestern University facilities for a minimum of one week prior to surgery. After microdrive implantation, mice were housed individually.

Microdrive Implant Surgery

Microdrive Implant animals (Males n= 11, Intact Females n= 14, Ovariectomized Females n= 6) were implanted with a modified headbolt and an Omnetics connector Custom 7-Degree Neuralynx Halo-10-Mini Microdrive (n= 19) or custom 3D-printed microdrive (n= 12) (Figure 9). Microdrives consisted of eight independently moveable tetrodes and one ground wire. Each tetrode consisted of four .0007" tungsten wires (California Fine Wire: CFW0011845). Prior to implantation of the modified headbolt, the skull (bregma-lambda) was leveled. Two stainless-steel skull screws were implanted on either side of the coronal suture (0-80 Screw, 91772a049, McMaster Carr) for the headbolt ground. An additional stainless steel skull screw (00-90) was implanted above the cerebellum for the Microdrive ground wire (Figure 9). A craniotomy was made (Drill bit: Stoeling Co: 0.45mm: 514551) at AP: +3.3 ML: -3.2. Recordings were obtained from the left hemisphere, contralateral to the airpuff US. The exit tip of the Microdrive was coated in silicone lubricant and the Microdrive was lowered into place (Figure 9). The tips of the tungsten tetrodes extended past the exit tip of the Microdrive and were lowered marginally into the cortex at implantation. The microdrive was cemented in place with multiple coats of Metabond dental adhesive and dental cement. After the skin was placed naturally around the dental cement and secured, 3D printed head-fixation bars were cemented in place as well.

Animals recovered on a warm heating pad before being returned to their home cage. Chow was placed in a glass bowl (Amazon, B08KNTWCDD) at the bottom of the cage as the wire-top was removed to prevent damage to the implant.

Microdrive tEBC Training

Prior to behavioral training, mice were habituated to the head-fixed apparatus for forty minutes/day for five days. During these habituation sessions, tetrodes were lowered into the Entorhinal Cortex (Figure 11). Advancement of tetrodes was recorded according to the number of turns. Each turn was equivalent to lowering the tetrode 256 microns. Tetrode placement was determined by listening to the neuronal activity (at 200-2000Hz) during the advancement. Conditioning training consisted of one session per day for ten days (Figure 11). Conditioned animals received a 65±2 dB tone (250ms, 2kHz) conditioned stimulus (CS) paired with a 35±5 PSI corneal airpuff (30-40 ms) unconditioned stimulus (US) (Figure 11). Each conditioning session consisted of 50 paired CS/US trials with a random 35-55 second inter-trial interval (Figure 11). Trials were automatically paused by the LABVIEW software when EMG baseline exceeded 0.25V. Trials were restarted when the EMG baseline was below 0.25V for two consecutive seconds. Animals were visually monitored during training through a camera (Logitech C270) attached to the frame of the cylinder apparatus (Figure 11).

Single-Neuron Isolation and Data Analysis

Neurons were isolated using an adapted version of the MountainSort (MountainLab) software, which was modified in collaboration with Venus Nitinkumar Sherathiya. Mann-Whitney U tests were used to analyze significant differences between baseline as CS, US and during the trace period. For theta analysis, neural activity was bandpass filtered offline to the

theta rhythm (6–10 Hz) using a Butterworth filter. The Hilbert transform was applied to obtain the instantaneous phase and amplitude of the theta rhythm. For each neuron, each spike was assigned to a given phase of the theta cycle. Then, all theta cycles were superimposed to examine the firing probability of each neuron at a given phase of the theta cycle (20 bins).

Power spectra analysis was calculated as described in (Deshmukh et al., 2010), power spectra of the LFPs were determined using the fast Fourier transform (FFT). Power in the theta band (6–10 Hz) is referred to as absolute theta power. To analyze phase-resetting in theta, procedures described in (Hattori et al., 2015) were utilized. Circular statistics were calculated to obtain the mean resultant vector (MRV) of each animal per session, which is the mean phase of the signal. As a measure of the variability of the animals MRVs within a session, resultant vector lengths (RVL) were computed per session. A RVL of 1 indicates that the data sample is more concentrated around the MRV and a RVL of 0 indicates that the data is less concentrated around the MRV (Hattori et al., 2015).

Histology

The tetrode locations were confirmed post-mortem. Mice were anesthetized with ketamine/xylazine cocktail (91.95mg/ml ketamine, 8.05mg/ml xylazine), and electrolytic lesions were created with the NanoZ (Neuralynx). Lesion settings were -12 mA for 12 seconds. Mice were returned to their homecage for one week following lesions. Mice were intracardially perfused with 0.1 M phosphate-buffered saline (PBS) followed by 4% Paraformaldehyde (PFA). Mice were left intact for an hour following perfusion and then the brain was extracted and placed in 4% PFA overnight. Following this, brains were tripled-rinsed and placed in 0.01 PBS. Brains are sliced into 40-micron sections in the horizontal plane in 0.01PBS on a Leica VT1000s

vibratome (Leica Biosystems). Sections are then mounted on Fisherbrand SuperFrost Plus slides (Thermo Fisher Scientific: 12-550-15) in 20% Ethanol. Slides were heated to dry overnight.

Sections were Nissl stained with cresyl violet. Slides were imaged at 5x Objective on an Axioplan 2 microscope (Zeiss; Germany) with an ORCA-Flash 4.0 LT camera (Hamamatsu; Japan) using NEUROLUCIDA NEUROEXPLORER software (MBF Bioscience). Following image capture, images were compared with tetrode maps created prior to implantation in Adobe Illustrator. Tetrode maps along with the number of turns of each tetrode were used to determine the location of each tetrode in the brain.

RESULTS

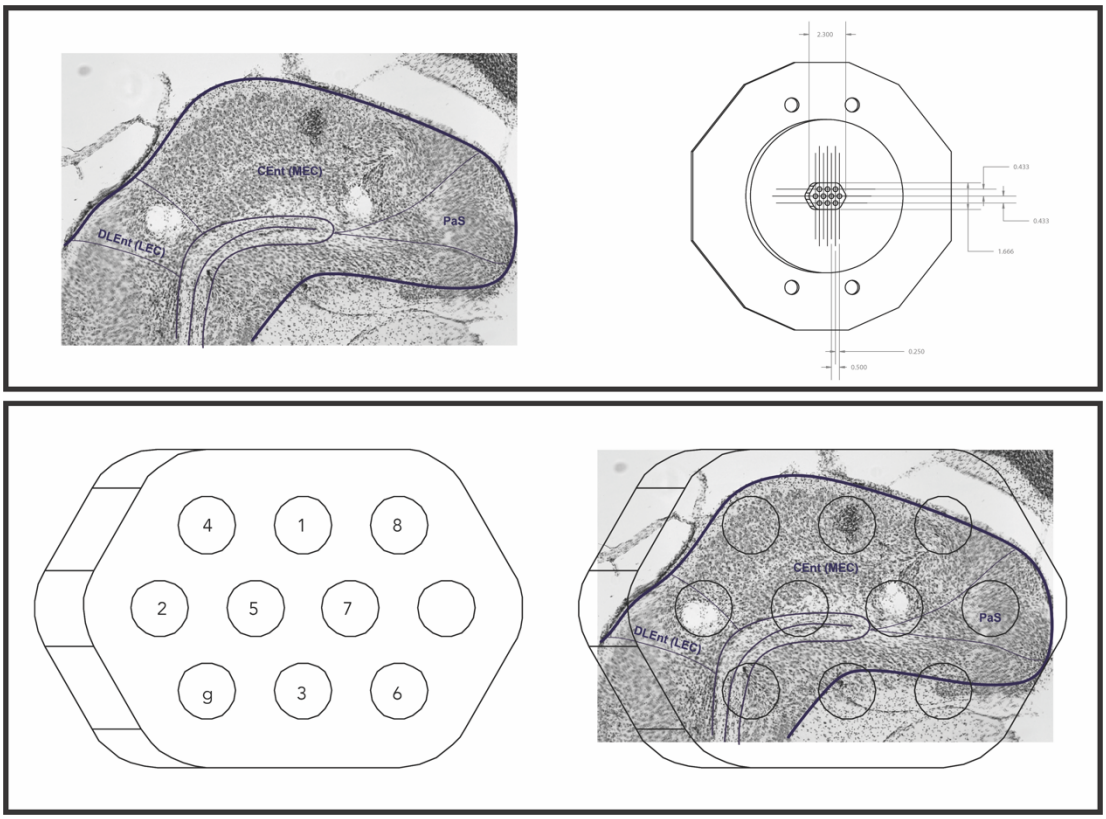

Figure 24 Tetrode Map. (Top left) Entorhinal portion of Horizontal Section, (Top Right) Neuralynx Exit Tip Sketch, (Bottom Left) Tetrode microdrive map made prior to implantation (Bottom Right) Overlapped microdrive map on horizontal section to determine tetrode location.

The following are preliminary results obtained from the microdrive animals utilized in Chapter Three.

Histology

These preliminary animals allowed for the development of a histology protocol to localize individual tetrodes in the brain (Figure 23). Utilizing the Neuralynx Halo-36 sketches, we created a map that can be overlaid on captured slice images to determine the location of each tetrode in the brain. Creating the map where each tetrode is labeled on the map prior to implantation was necessary for the localization process post-mortem.

Single-Unit Recording

Preliminary single-unit recording analyses showed multiple patterns of neuronal activity in response to the experimental stimuli. Cells significantly increased in activity in response to the CS and US (Figure 24). Additional cells increased in firing to only to the presentation of the US or the CS (Figure 24). Several cells show increased persistent activity during the trace period (Figure 24. Pilot recordings also showed several cells that decreased in firing during the trace period (Figure 24).

Analysis of theta shows pilot cells preferentially fire to different phases of theta (Figure 25). While some cells preferentially fired at the peak (top), others fired predominately at the trough of theta (middle) and additional cells were not theta dependent (bottom).

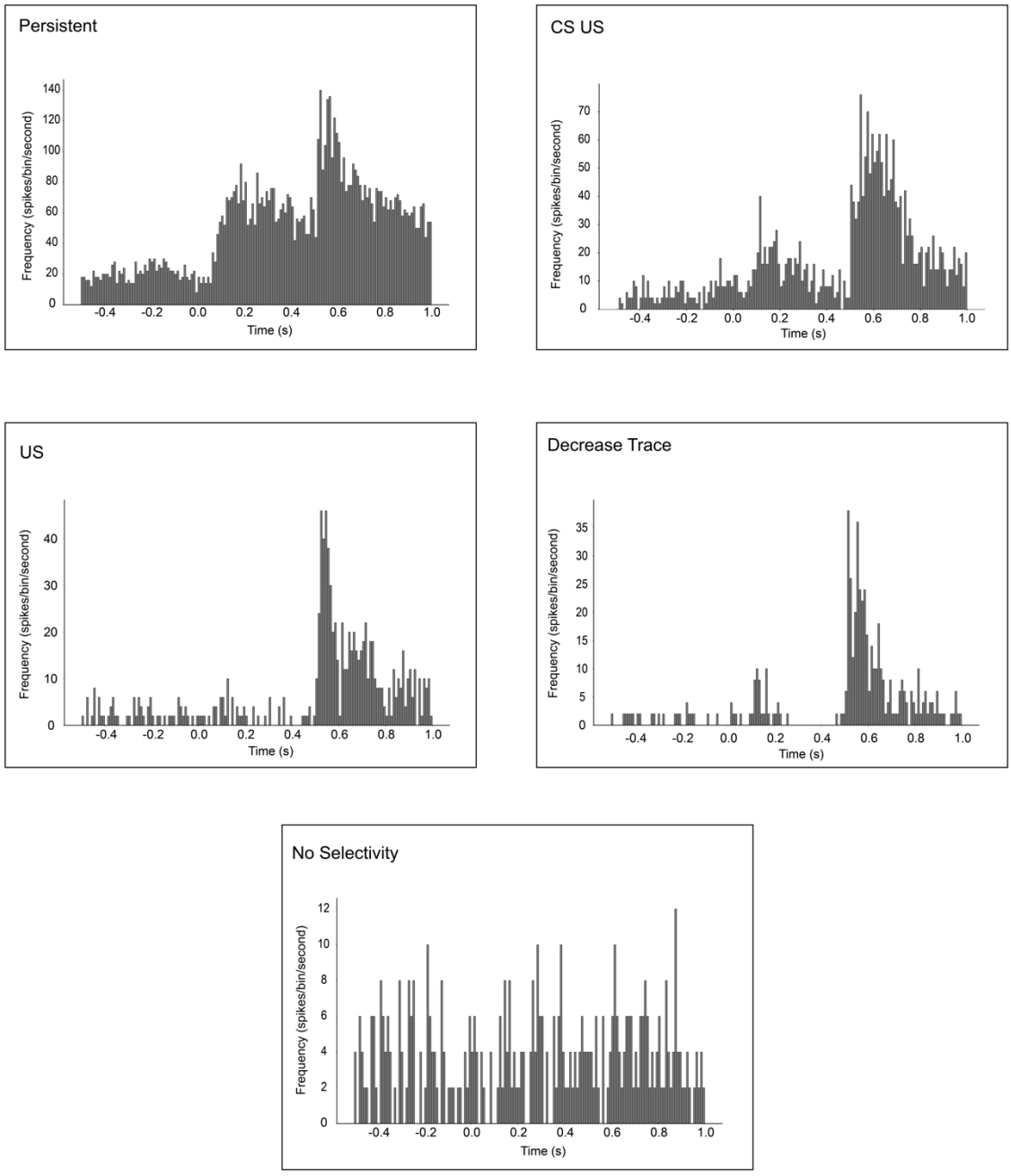

Figure 25 Single Unit Recording Example Histograms

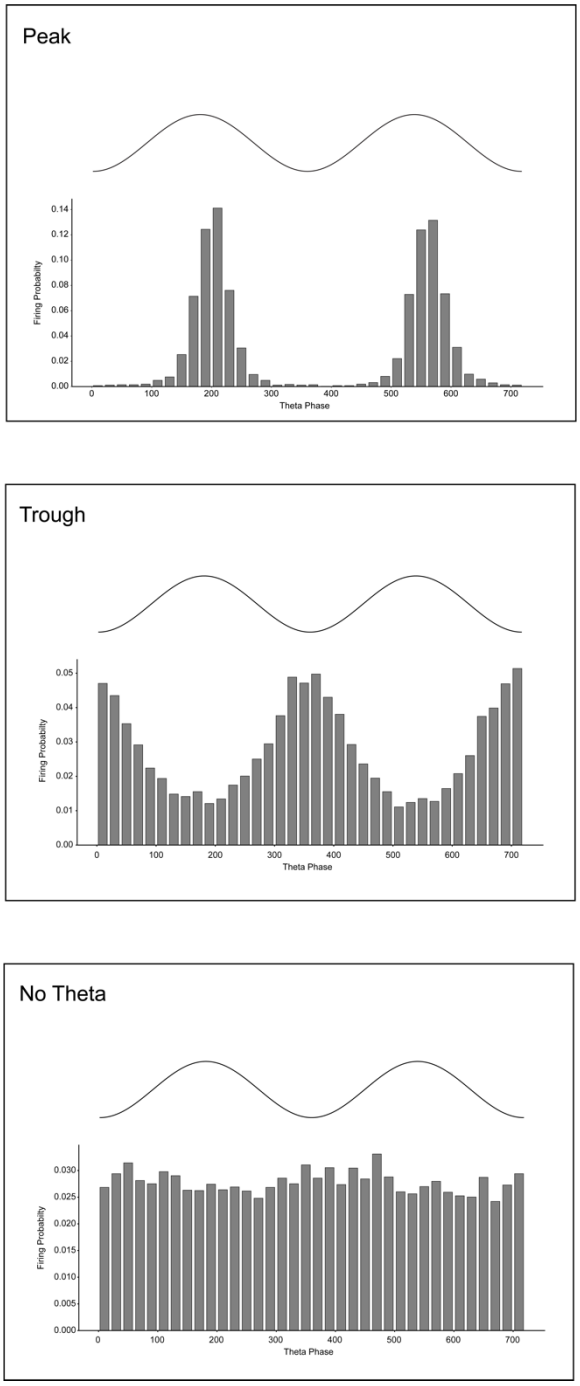

Figure 26 Preferential Firing to Theta. Pilot Recordings fire preferentially to different phases of theta, (Top) Peak Theta (Middle) Trough of Theta (Bottom) No Theta Preference.

LFP Recording

Pilot power spectra analysis shows example LFP recordings preferentially in the theta band between 5 and 7 Hz (Figure 26). Analysis of phase resetting shows that theta reset after the presentation of the CS and US and appeared to reset prior to the start of the US late in training (Figure 27).

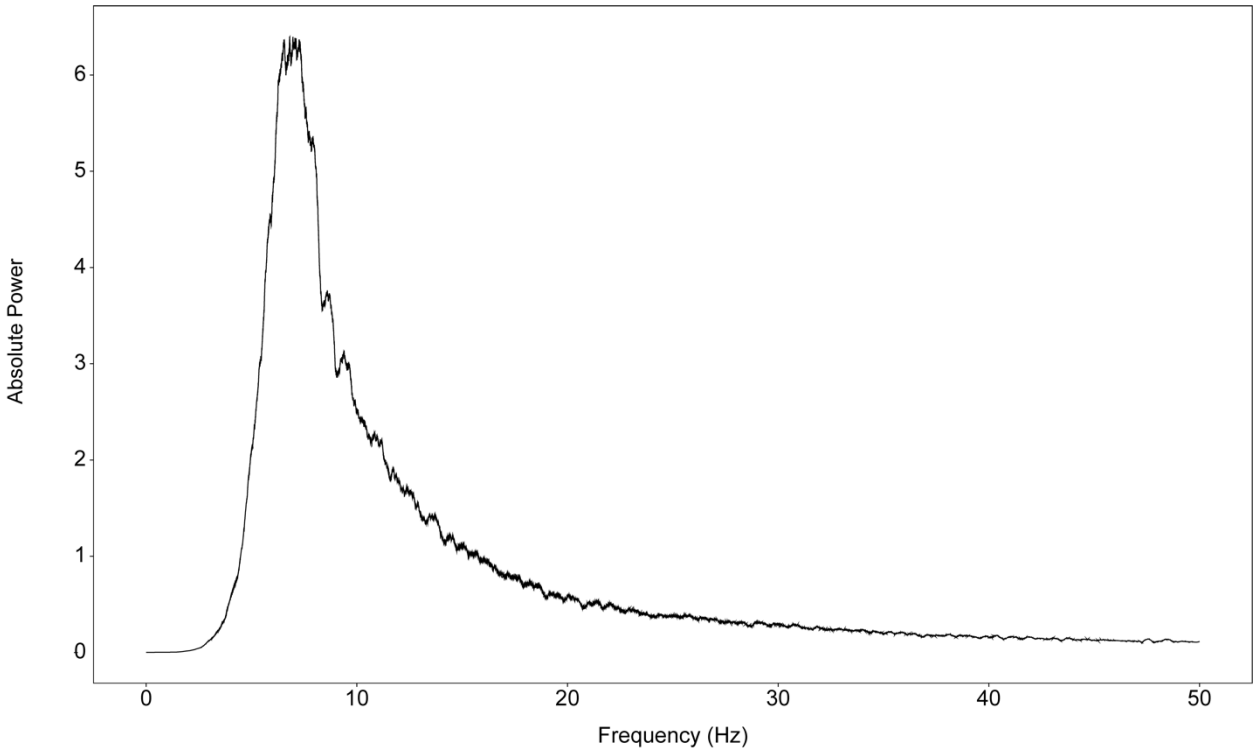

Figure 27. Absolute Power Spectra Analysis.

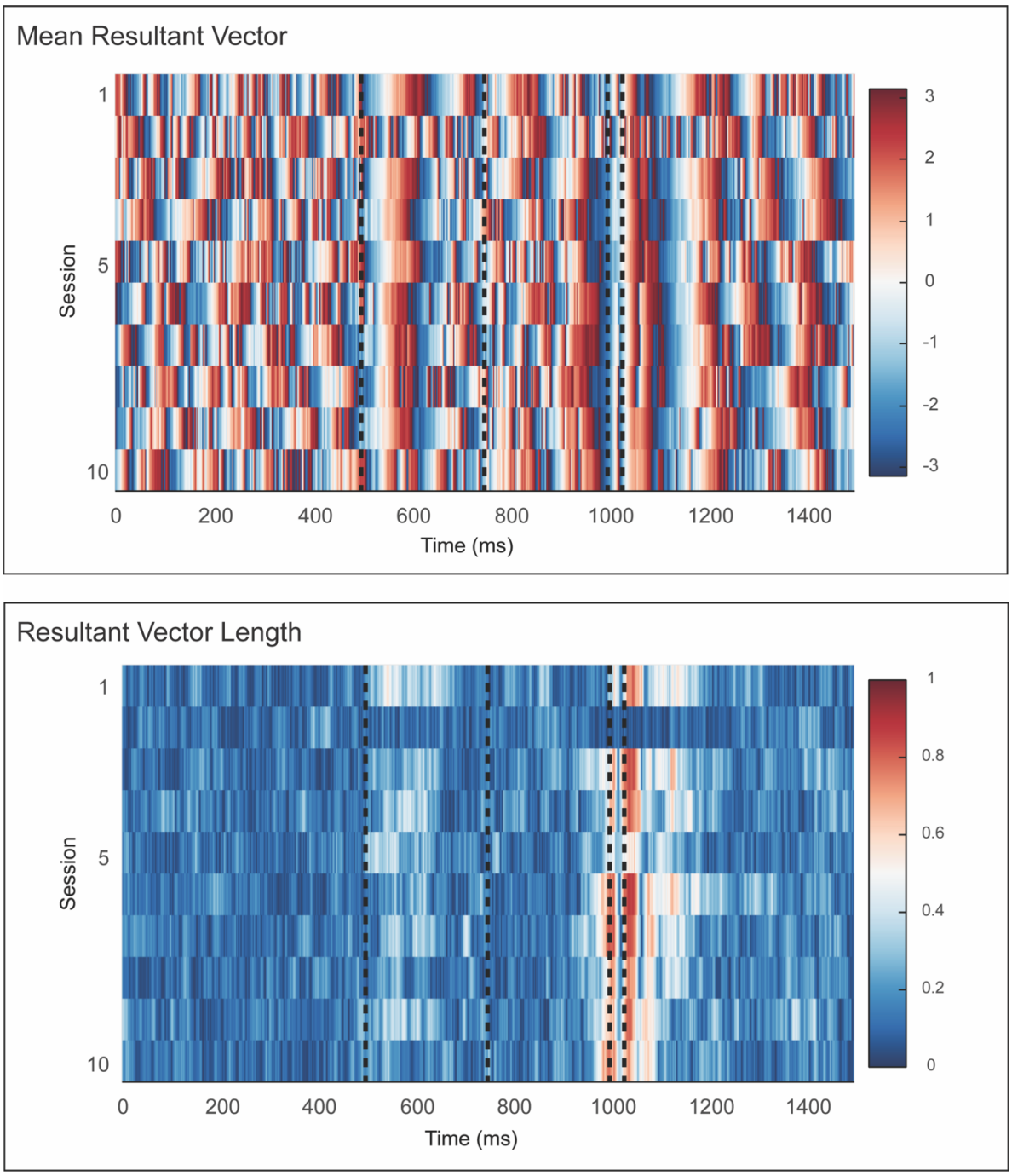

Figure 28 Phase resetting analysis. Tetrode 1, MD 12

DISCUSSION:

Pilot single-unit recordings show similar responses to the previous work of Eugenie Suter (Suter et al 2017). These preliminary results support previous findings that the Entorhinal Cortex may act as a center to relay and preprocess sensory information to the hippocampus, reacting to the CS and US and showing an increase in activity during the trace period late in training.

Some of the recorded cells show persistent firing throughout the trace period, which may act as a mechanism of memory maintenance to bridge the gap between the CS and US. It is believed persistent firing underlies associative memory because Fuster's recordings from prefrontal cortex of monkeys showed firing activity that bridged the delay gap in a delayed-response procedure (Fuster, 1973). However, subsequent studies have failed to show in-vivo recordings of persistent firing, though in-vitro studies have illustrated cells propensity to persistently fire in entorhinal cortex (Lin et al., 2020; Yoshida et al., 2008). Pilot recordings here show firing activity that bridges the gap between the CS and US (Figure 23), potentially supporting the role of persistent firing in behavior.

Preliminary LFP power spectra analysis suggests the importance of theta band frequencies in the recorded areas. As theta rhythms are heavily associated with learning and memory (Hattori et al., 2015; Igarashi et al., 2014; Sosa et al., 2018; Takehara-Nishiuchi et al., 2012), the importance of these frequencies to acquisition would be expected and will be further investigated. Example LFP recordings also showed theta resetting to the CS and US, with notable changes in later sessions, suggesting theta resetting may occur with acquisition of conditioned responses.

Future analysis aims to investigate differences between animals that did not successfully acquire trace or delay eyeblink conditioning. This analysis will allow us to differentiate between

learning-related changes and changes due to the stimulus presentation alone. Furthermore, we will investigate LFP changes in theta coherence with learning. Similar to analysis performed in (Takehara-Nishiuchi et al., 2012), we aim to investigate changes in local theta with conditioned responses compared to non-CR trials. While our preliminary recordings show individual cells fire during particular phases of theta, we are interested in seeing coherence with theta across the region.

CHAPTER FIVE: DISCUSSION

Sex as a Biological Variable

Biomedical research has largely neglected studying sex as a biological variable, though numerous biological differences have been reported between males and females (Beery and Zucker, 2011b; Galea et al., 1997b; Gould et al., 1990; Gresack and Frick, 2003). Underrepresentation of females in animal studies has been primarily due to misconceptions of perceived increased variability to estrous cycle (Beery, 2018; Mahmoud et al., 2016; Shansky, 2019), as well as increased costs and time (Shansky, 2019). When females are included in experiments, many researcher conduct experiments in males first and then compare these findings in females, perpetuating the notion that males are the standard that females may deviate from (Shansky, 2019).

Investigating sexual dimorphisms in the brain is necessary as these differences have important influences on neurological and psychiatric disorders and therapeutics (Zagni et al., 2016). Sex differences have been shown in a variety of mental illnesses and disorders, including higher frequency of Autism Spectrum Disorders and Attention Deficit and Hyperactivity Disorders in males (Zagni et al., 2016). Trauma-related disorders and depression and anxiety disorders also report sex differences in prevalence. Women are approximately twice as likely to suffer from anxiety disorders and have an increased likelihood of depression compared to men (Zender and Olshansky, 2009). Notably, women have a higher prevalence of Alzheimer's Disease and show an increased deterioration of cognition compared to elderly men (Li and Singh, 2014). Further investigation of sex differences in associative memory in mice is necessary in laying the foundation for future clinical research as preclinical research commonly utilizes transgenic rodent models. Therefore, we have focused on expanding our studies of acquisition of

trace eyeblink conditioning and single-unit recording to include intact and ovariectomized females.

Intact Females Acquire tEBC Faster than Males in Headbolt Behavior

In Chapter Two, we show intact female mice acquired tEBC faster than both male and ovariectomized females (Figure 6). As ovariectomy impaired acquisition, circulating hormones such as estrogen and progesterone are presumed necessary for this facilitation. Estrogen has been shown to increase density of dendritic spines in CA1, neurogenesis in dentate gyrus and synaptic plasticity in young female rodents (Drake et al., 2000; Frick et al., 2015; Gould et al., 1997, 1990; Woolley and McEwen, 1993). Sex differences were also not due to increased sensitization to the stimuli since, across sex, pseudoconditioned controls were not significantly different in their responses to the CS-alone or US-alone trials. These studies expanded on work in rats from Tracey Shors (Dalla et al., 2009; Dalla and Shors, 2009; Leuner et al., 2004) and illustrate that sex differences observed in acquisition in tEBC are not dependent on specific experimental parameters including conditioned stimuli, trial sequences or number of trials.

Sex Dependent Impact of Chronic Implantation on Acquisition

In Chapter Three we illustrate how experimental procedures and technology may impact behavior in a sex-dependent manner, as surgical implantation of a microdrive for single-unit recording significantly facilitated learning in males, but not intact females (Figure 13). In fact, implantation of the microdrive tended to impair acquisition in intact females (Figure 13). It is necessary to investigate the sexually dimorphic effects of experimental procedures as this factor will greatly affect the interpretation of results. While many previous *in-vivo* recording

experiments had been performed in a single sex (Hattori et al., 2015; Suter et al., 2013; Weiss et al., 1996), including both intact females and males showed starkly divergent behavioral patterns.

The weight of the implant alone was not sufficient to elicit sex-differences in acquisition, therefore additional considerations are necessary. As implantation of tetrodes into the entorhinal cortex may provoke a neuroinflammatory response (Groothuis et al., 2014; Polikov et al., 2005; Sankar et al., 2014), involvement of various inflammatory cytokines, microglia and signaling markers is plausible. Repeated stress experiments suggest males may develop neuroprotection and resilience to subsequent stressors, while intact females do not (Wellman et al., 2018). Implantation surgery may act as a priming event that invokes resilience in males. Materials and surgical procedures to further limit inflammation should be considered to avoid confounding variables in single-unit recording experiments (Prasad et al., 2012b).

Excitability as an Underlying Factor in Acquisition

In both Chapters Two and Three, acquisition of tEBC is facilitated by mechanisms that increase excitability. Estradiol increases excitability by enhancing glutamatergic transmission and decreasing GABAergic inhibition (Scharfman and MacLusky, 2006). In CA1 of the rat, estradiol induces structural changes, increasing the number of spine synapses, the density of spines and the shape of spines (Li et al., 2003; Lu et al., 2019; MacLusky et al., n.d.; Pozzo-Miller et al., 1999; Scharfman and MacLusky, 2006). Estradiol may also increase the effects of glutamate in CA1 at NMDA receptors (Rudick and Woolley, 2001; Scharfman and MacLusky, 2006; Smith and McMahon, 2005; Zamani et al., 2004). Estradiol also reduces slow afterhyperpolarization mediated by calcium-dependent potassium channels (Carrer et al., 2003; Kumar and Foster, 2002). Indirectly, estradiol influences excitability by increasing acetylcholine

release, influencing NMDA receptor binding and regulating BDNF (Cyr et al., 2001; Daniel et al., 2005; Scharfman and MacLusky, 2006; Smith and Cizza, 1996; Zamani et al., 2004).

Estradiol is not the only ovarian hormone that may affect excitability, progesterone may also facilitate acquisition in associative memory. Progesterone upregulates the expression of AMPA receptors, which enhances synaptic transmission in CA1 (Joshi et al., 2018; Kapur and Joshi, 2021). BDNF expression is also mediated by progesterone through the classical progesterone receptor (Kapur and Joshi, 2021; Su et al., 2012). Progesterone and its metabolites also regulate subunits of $GABA_A$ receptors, thereby altering the density of the receptor (Weiland and Orchinik, 1995). Together, circulating ovarian hormones may increase excitability and therefore improve acquisition of tEBC in intact females.

Stress has been shown to increase hippocampal excitability (Weiss et al., 2005). Acute restraint stress facilitated acquisition of tEBC in c57Bl6 male mice, temporarily increased corticosterone levels and increased excitability of CA1 pyramidal neurons of mice 1hr and 24hrs after receiving stress (Weiss et al., 2005). Stressed male mice had significantly reduced slow AHP and spike accommodation to injected current compared to naïve mice (Weiss et al., 2005). When animals are injected with drugs that produce similar biophysical changes in excitability, they learn tEBC at a faster rate, therefore facilitation of acquisition may be facilitated by these biophysical changes (Moyer and Disterhoft, 1994; Power et al., 2001; Weiss et al., 2000). Stress may increase hippocampal excitability through corticotropin-releasing factor (CRF), which has been shown to increase synaptic efficacy *in vivo* (Blank et al., 2003; Randesi et al., 2018; Wang, 1998). Chronic immobilization stress upregulated genes that code for CRF, while it downregulated genes that encode plasticity and kinase/signaling genes in male Sprague-Dawley

rats (Randesi et al., 2018).

Stress may lead to sexually dimorphic changes in excitability by increasing the calcium dependent afterhyperpolarization in intact females through voltage-sensitive calcium channels, and decreasing excitability (Joëls et al., 2007; Joëls and de Kloet, 1992; Joëls and Ronald de Kloet, 1994; Landfield et al., 1992). It is of interest to investigate changes in excitability in males, intact females and ovariectomized females with acquisition. These future studies may provide additional insight into the underlying mechanisms that facilitate acquisition with chronic implantation in males but impair learning in intact females.

Association with Sex Differences in Mental Health and Neurodegenerative Disorders

Sex differences are observed in incidence, manifestation, symptoms and therapeutic efficacy of neuropsychiatric disorders (Gobinath et al., 2015; Ma et al., 2019). For example, women are twice as likely to be affected by depression in their lifetime compared to males (Gobinath et al., 2015; Gutiérrez-Lobos et al., 2002). However, as previously noted, sex as a biological variable has been largely overlooked in preclinical research. Development of depression has been heavily linked to chronic stress; therefore, it is of interest to study sex-dependent effects of stress in both females and males. Stress resistance provided by ovarian hormones may be abolished with chronic stress due to dysregulation of glucocorticoid signaling (Bekhbat and Neigh, 2018). Sex-dependent differences in dendritic and synaptic plasticity may in part affect instances of neuropsychiatric disorders (Hyer et al., 2018). Many of the mechanisms discussed in Chapter Three that may potentially serve as the basis for sexually dimorphic acquisition have also been suggested as mechanisms for depression. For example, abnormal modulation of the HPA axis, BDNF levels, and gonadal hormones have been theorized to impact depression (Ma et al., 2019). Therefore, understanding the mechanisms underlying

sexually dimorphic behavioral changes in associative learning that occur with stress and neuroinflammation may provide further insight into phenomena including learned helplessness and depression.

Neuroinflammation has also been linked to neurodegenerative disorders including Alzheimer's Disease, Amyotrophic Lateral Sclerosis and Parkinson's disease (Kwon and Koh, 2020). Neuroinflammation protects the brain by promoting tissue repair, removing or inhibiting pathogens or removing debris (Kwon and Koh, 2020). However, with sustained activation, inflammatory responses can lead to neurodegenerative disease. Inflammatory responses contribute to the pathogenesis of Alzheimer's Disease, with cognitive-impaired patients containing higher levels of TNF-α, IL-1β and IL-6 (Kwon and Koh, 2020). BDNF is also implicated in Alzheimer's disease, certain polymorphisms have been linked to increased risk of the disease (Fisher et al., 2018). Sex differences in neuroinflammation affect neurodegeneration in a multitude of ways. For instance, microglial function has been linked to plaque-associated ApoE, with females containing the ε4 variant of the ApoE gene at a higher rate than males (Kodama and Gan, 2019) . Certain BDNF polymorphisms have been shown to increase Alzheimer's risk only in women, but not men (Fisher et al., 2018). Investigating the mechanisms that underlie sex-differences in associative learning may provide insight into these disorders.

Future Directions

Future studies should investigate the mechanisms underlying the sexually dimorphic behavior observed with chronic implantation. Performing a Tandem Mass Tag (TMT) Spectrometry screen would allow researchers to identify and quantify differences in protein between the experimental groups and sexes. The screen would also provide insight into

additional avenues for immunohistochemistry and western blot analysis as it is possible that neuroinflammation and neurogenesis do not underlie sex differences observed with chronic implantation.

If implantation of the chronic microdrive leads to lesions in the medial temporal lobe, this may lead to rewiring of the tEBC acquisition circuit. It is plausible that females and males rewire the circuit in different ways, leading to facilitation in males, but impairment in females. Implanting the microdrive into visual cortex, or another region of the brain, may elucidate the importance of location of the implanted wires in the observed changes in behavior.

Including both sexes in preclinical research opens additional avenues for discovery and promotes equivalent knowledge of both sexes. As there are numerous diseases and disorders with known sex-differences, it is critical to understand the mechanisms underlying these distinctions to provide better targeted therapeutics and treatments in the future.

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