Manipulation of Synaptic Pruning via Ecdysone Signaling Pathway and It's Effects on Complex Learning

by

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Submitted to the Graduate School of Natural Science and Engineering
In partial fulfillment of the requirement for the degree of
Master of Science

Sabanci University
December 2022

ABSTRACT

Manipulation of Synaptic Pruning via the Ecdysone Signaling Pathway and It's Effects on Complex Learning

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Molecular Biology Genetics and Bioengineering, M.Sc. Thesis, December 2022

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Keywords: Critical Period, Ecdysone Signaling Pathway, HR38, HR39, Reversal Learning, Synapse Elimination, Synaptic Pruning

Critical periods are important milestones in the maturation of an organism. It is characterized by hormonal fluctuations and an outburst of dendritic branching and axonal growth, followed by an experience dependent pruning of weaker synapses. In honey bees there is a critical period during the transition from an in-hive bee to a forager. *Drosophila* studies show that signaling via EcR and Usp heterodimer is necessary for synaptic pruning to take place. Both HR39 and HR38 inhibit or suppress this pathway. Expression of HR38 gradually increases as the bee ages and gains experience; concurrently, synaptic bouton numbers increase with age and experience. Increased synaptic bouton numbers are associated with a decrease in reversal learning assay performance. In addition, HR38 expression peaks after obtaining a sucrose reward during foraging. Because of this, HR38 is theorized to play a role in synapse stabilization as well. I aimed to induce synaptic pruning in experienced old foragers by performing a HR39/HR38 double knockdown via siRNAs. I observed decreased synaptic bouton numbers in the MB of the honeybee brain. These treated bees' acquisition of extinction during reversal learning was not affected, but the consolidation

of the extinction memory was blocked. I suspect that either the HR39/HR38 double knockdown directly disrupts molecular mechanisms of extinction learning or the reduction of the synaptic boutons leads to allocation of resources such that excitatory memory formed after pruning was better reinforced compared to extinction memory formed later

ÖZET

Sinaptik Budamanın Ecdysone Sinyal Yolağı Üzerinden Manipülasyonu ve Bunun Kompleks Öğrenime Etkisi

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Moleküler Biyoloji Genetik ve Biyomühendislik, YÜKSEK LİSANS TEZİ, Aralık 2022

Tez Danışmanı: Doç. Dr. Christopher Louis Mayack

Anahtar Kelimeler: Ecdysone Sinyal Yolağı, HR38, HR39, Kritik Evre, Sinaps Elenmesi, Sinaptik Budama, Tersini Öğrenme,

Kritik evreler, bir organizmanın olgunlaşmasında önemli kilometre taşlarıdır. Hormonal dalgalanmalar ve dendritik dallanma ve aksonal büyüme patlaması ve ardından daha zayıf sinapsların deneyime bağlı budaması ile karakterize edilir. Bal arılarında bu kritik evre, kovan içi arılıktan toplayıcı arıya geçiş sırasında gerçekleşir. Hormonal sinyal kaskadları, kritik evre ve toplayıcı geçişi sırasındaki değişikliklere aracılık eder. *Drosophila* çalışmaları, sinaptik budamanın gerçekleşmesi için EcR ve Usp heterodimer yoluyla sinyal göndermenin gerekli olduğunu göstermektedir. Hem HR39 hem de HR38, bu yolu inhibe eder veya baskılar. HR38'in gen anlatımı, arı yaşlandıkça ve deneyim kazandıkça kademeli olarak artar, aynı zamanda sinaptik buton sayıları da yaş ve deneyimle birlikte kademeli olarak artar. Artan sinaptik buton sayıları, tersini öğrenme testi performansında bir azalma ile ilişkilidir. Ek olarak, yiyecek arama sırasında sükroz ödülü aldıktan sonra HR38 gen anlatımı zirve yapar. Bu nedenle, HR38'in sinaps stabilizasyonunda da rol oynadığı teorize edilmiştir. siRNA'lar aracılığıyla bir HR39/HR38 çifte knockdown gerçekleştirerek deneyimli eski

toplayıcılarda sinaptik budama başlatmayı amaçladım. Bal arısı beyninin MB'sinde azalmış sinaptik buton sayıları gözlemledim. Bu tedavi edilen arıların tersini öğrenme sırasında sönme hafızasının edinimini etkilenmedi, ancak sönme hafızasının sağlamlaşması engellendi. Ya HR39/HR38 çift devre dışı bırakmanın, yok olma öğreniminin moleküler mekanizmalarını doğrudan bozduğundan ya da sinaptik butonların azaltılmasının, budama sonrasında oluşan eksitatör hafızanın daha sonra oluşan sönme hafızasına kıyasla daha iyi güçlendirildiği şekilde kaynakların tahsisine yol açtığından şüpheleniyorum.

ACKNOWLEDGEMENTS

I am thankful to my family and friends who stood by me during the making of this study. Ceyco, Zeynep, Sezgin, Asuman and Seden hardships and obstacles are easier to persevere with your good company. My dearest Esra, thanks for existing.

I give my most sincere and utmost thanks to Asst. Prof. Christopher Louis Mayack for his endless support in this long, hard fought, scientific adventure. I am thankful to members of our lab, Zaeema Khan, Büşra Elif Kıvrak, Emre Can Günaydın, their help was crucial for the completion of this project. I am thankful my students, Efe Cağferoğlu, Melis Konar and Özge Yalçın, best students a MA can have. I am thankful to Prof. Batu Erman for his mentorship and much needed advices. I am thankful to Prof. Emrah Eroğlu for his guidance in microscopy. I am thankful to Prof. Olgu Enis Tok for his help and guidance in histology. I also would like to thank my jury members, Prof. Levent Öztürk and Prof. Guntima Suwannapong for their valuable time and feedback.

Lastly and most importantly. I would like to give my special thanks to my grandfather, a man no polluted stream of time can tarnish, what a person should strive to be.

Dedicated to my grandfather, a free spirit.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

MB: Mushroom Body

GABA: γ-aminobutyric acid

siRNA: Small interfering RNA

RNAi: RNA interference

PER: Proboscis extension response

ddaC: Dendritic arborization neurons

EcR: Ecdysone receptor

EcR-B1: Ecdysone receptor B1

TGF-β: Transforming growth factor beta

ftz: fushi tarazu

ftz-f1: ftz transcription factor 1

Hr39: Hormone receptor like in 39

Hr38: Hormone receptor like in 38

USP: Ultraspiracle

Sox14: SRY-box transcription factor 14

Hdc: Headcase

MICAL: Microtubule Associated Monooxygenase, Calponin And LIM Domain Containing1

CUL1: Cullin1

DIAP1: Death Associated Inhibitor of Apoptosis 1

Egr: Early growth response

JH: Juvenile Hormone

GCM (Hr39 expressor): Glial Cells Missing

IEG: Immediate Early Genes

qPCR: Quantitative Polymerase Chain Reaction

RP49: Ribosomal Protein 49

SYNORF-1: Antibody against synapsin

PFA: Paraformaldehyde

PBS: Phosphate-buffered saline

PBST: Phosphate-buffered saline / Triton-X

NGS: Normal Goat Serum

DAPI: 4',6-diamidino-2-phenylindole

US: Unconditioned stimulus

CS: Conditioned stimulus

LTM: Long Term Memory

STM: Short Term Memory

LTD: Long Term Depression

NR4A: Nuclear Receptor 4A

NR5A: Nuclear Receptor 5A

1. INTRODUCTION

The dysregulation of synaptic pruning has been recently linked to the onset of Alzheimer's and Parkinson's disease as well as autism, schizophrenia, bipolar, and attention deficit hyperactivity disorders (Brucato & Benjamin, 2020; Graves & Surmeier, 2019; Kim et al., 2017; Tang et al., 2014). Therefore, it is suspected that synaptic pruning plays a central role in the functioning of cognitive tasks such as learning and memory. Synaptic pruning occurs mainly during adolescent stage of development after the brain experiences rapid growth of neurons, during 2-4 years of age in humans (Chechik et al., 1999). This overgrowth of neurons and neuronal connections are trimmed in order to increase the transmission efficiency across the most important connections and eliminate weaker connections. Frequently used synapses are reinforced and become stronger, while the less used ones become weaker and pruned (Changeux & Danchin, 1976). The human brain increases in size 5-fold by adulthood, reaching a final size of 86 billion neurons; this number of neurons remains the same, but the volume of synaptic connections decreases due to synaptic pruning (Azevedo et al., 2009). Initially synaptic pruning in humans was thought to be occurring mainly after birth until the mid-20s and mostly at night when the brain has been shown to undergo major re-organization and memory consolidation; however, it has been recently shown to continue into adulthood (Petanjek et al., 2011).

1.1. The role of synaptic pruning in learning and memory

Learning and memory require changes in existing neural networks and establishment of new neural networks in the brain (Kennedy, 2016). Synaptic plasticity is crucial for modifications at the synapses that connect neurons into networks (Kennedy, 2016). Learning and memory at the neuronal level involves synaptic plasticity in which the strength of excitatory synapses is altered, in response to synapse activity based on the environmental inputs into the brain. The constant rearrangement of synaptic connections is from a mosaic of three cell types: excitatory neurons, that use glutamate as their transmitter, inhibitory neurons that use γ-

aminobutyric acid (GABA) as their transmitter, and glial cells in which connections are being strengthened or weakened over time. These are the main drivers behind this process (Kennedy, 2016). Memories are formed when specific groups of neurons are reactivated, and dendritic spines of neurons communicate with other brain cells through synapses. Reinforcing plasticity mechanisms such as strengthening the synapse and long-term potentiation has been linked to memory formation (Afroz et al., 2016). However, more recently regressive mechanisms such as pruning is also widely thought to be an essential element mechanism for learning and memory, especially when it comes to learning new tasks and updating previously known information (Craik & Bialystok, 2006). The importance of axon and dendritic pruning is evident during synaptic remodeling events, which require precise spatial and temporal control, and this is achieved by a range of molecular mechanisms, some of which are still unknown. Recently, however, GABA receptors have been shown to trigger synaptic pruning at puberty in the mouse hippocampus, a brain area involved in learning and memory (Afroz et al., 2016). In general, disruption of the molecular mechanisms has been linked to abnormal pruning and brain dysfunction (Riccomagno & Kolodkin, 2015).

1.2. The honey bee as a model organism for synaptic pruning research

Honey bees are a useful and practical model organism for studying physiology, neurobiology, and behavior. The honey bee is a practical model, because it is amenable to genetic manipulation (its genome is sequenced) and RNA interference (RNAi) and CRISPR cas-9 techniques can be used to knockdown or knockout, respectively, specific genes, avoiding the need to maintain transgenic honey bee lines, which is expensive and not practical (Huang et al., 2018; Wang et al., 2012). The honey bee also has defined behavioral outputs to measure cognitive abilities. For example, learning and memory can be measured using the well-established proboscis extension response (PER) assay. There are numerous studies that pair

an odor (a conditioned stimulus) with a sucrose solution food reward (an unconditioned stimulus) and record whether or not a proboscis extension response (PER) occurs in anticipation for the delivery of the food reward (Paldi et al., 2003; Shafir et al., 1999, 2005, 2008). The assay is inexpensive and easily performed in the laboratory, under controlled conditions, in a short amount of time. Honey bees have a simple, but a true brain that is organized with specialized functional regions, which results in an ideal model system for understanding neural mechanisms which underlie learning and memory (Menzel, 1999, 2001, 2012). Honey bees are also fairly large insects, making it possible to inject substances into specific tissues like the brain and this facilitates the manipulation and quantification of synaptic connections. Lastly, the honey bee is also inexpensive to maintain such a large sample sizes can be obtained to analyze behavior and link significant differences from multiple treatments of RNAi knockdown (Guo et al., 2018).

Honey bees are powerful model organisms for studying learning and memory, they can learn reliably and fast that typically leads to a stable memory (Bitterman et al., 1983; Hussaini et al., 2007; Menzel & Müller, 1996). At the same time, honey bees display different learning and memory capabilities depending on their age, caste, and experience (Figure 1). The queen lays her fertilized eggs to the bottom of the cells, this is followed by the larval stage, nutrition is provided in the cell, and then they develop into the pupal (puparium) stage. At the puparium stage they are in their cells capped by the nurse bees, morphological changes start to form the final shape. After the puparium stage, the honey bees emerge from their cell, at this stage they are called newly emerged bees, when they are less than 24 h old. After the newly emerged stage honey bees transition into nurse bees when they are about 1-4 weeks old and perform in-hive chores such as removal of dead bees, feeding the larvae, and capping of honeycomb cells. Nurse bees have poorer learning compared to both young and old foragers. After the nurse bee stage, the honey bees transition to foraging duties and become foraging bees, this transition is marked by hormonal cascades and orientation flights as they go from spending most of their time from inside the hive to outside the hive.

Foragers show better performance in learning and memory tasks in comparison to nurses and newly emerged bees; also, young foragers display better learning and memory performance compared to older and more experienced foragers, which is thought to be due to senescence (Behrends et al., 2007; Cabirol et al., 2018; Scheiner & Amdam, 2009; Tolfsen et al., 2011). Similar to the human brain development, when honey bees transition to foraging, they experience an increase in the volume of the mushroom body region of the brain (Dobrin et al., 2011; Farris et al., 2001; Muenz et al., 2015), which is the higher order processing center that is involved in learning and memory (Menzel, 2012). Further supporting the similarity in mechanistic processes involved in synaptic pruning, dendritic arborization plays a role in the reorganization of this region (Dobrin et al., 2011; Farris et al., 2001; Muenz et al., 2015). Although young foragers have larger mushroom bodies, the number of synaptic boutons in this region is lower compared to newly emerged and nurse bees, and this also includes older more experienced foragers (Cabirol et al., 2018; Muenz et al., 2015). This indicates that some of the synapses are eliminated in young foraging bees (foragers) from synaptic pruning during the transition from being an in-hive bee to a foraging bee, this large transformation is accompanied by a critical period for their brain development (Figure 2). In addition, the number of synaptic boutons increases with foraging experience, which explains the increased bouton numbers in older foragers, and there is a negative association between synaptic bouton numbers and honey bees capability to update an existing learned association with new information (Cabirol et al., 2018). As the bee gets older and more experienced, gradual changes to behavior and physiology become more apparent. In terms of learning performance young foragers outperform old foragers, however more experienced foragers have larger MBs than young foragers (Behrends et al., 2007; Cabirol et al., 2018; Farris et al., 2001; Ismail et al., 2006; Scheiner & Amdam, 2009).

Aforementioned critical period coincides with the transition from nursing to foraging. During transitioning from nurse bee to forager stage an outburst of dendritic arborizations and axonal growth ensues from a hormonal cascade that marks this transition (Scholl et al., 2014; Ueno et al., 2015; Velarde et al., 2009). These early stage young foragers experience a novel environment during their orientation flight; this accompanies many physiological changes

that affect their behavior (Fahrbach et al., 1998; Farris et al., 2001; Ismail et al., 2006; Scholl et al., 2014; Withers et al., 1993). Both age and experience induce physiological and behavioral changes; as the forager ages and gains more experience, these gradual changes accumulate and there is a drastic difference in the physiology and behavior between the old and young forager bees (Behrends et al., 2007; Cabirol et al., 2018; Farris et al., 2001; Muenz et al., 2015; Tolfsen et al., 2011). One of the physiological changes is axonal pruning by microtubule disassembly that follows the growth outburst, and after that, new synaptic boutons are made as forager gains more experience (Cabirol et al., 2018; Muenz et al., 2015; Scholl et al., 2014; Williams & Truman, 2005). The excess dendritic arbors are pruned after the growth outburst, much like axonal synaptic boutons, new dendritic arborizations occur as the forager ages and gains more experience(Cabirol et al., 2018; Muenz et al., 2015; Scholl et al., 2014; Williams & Truman, 2005).

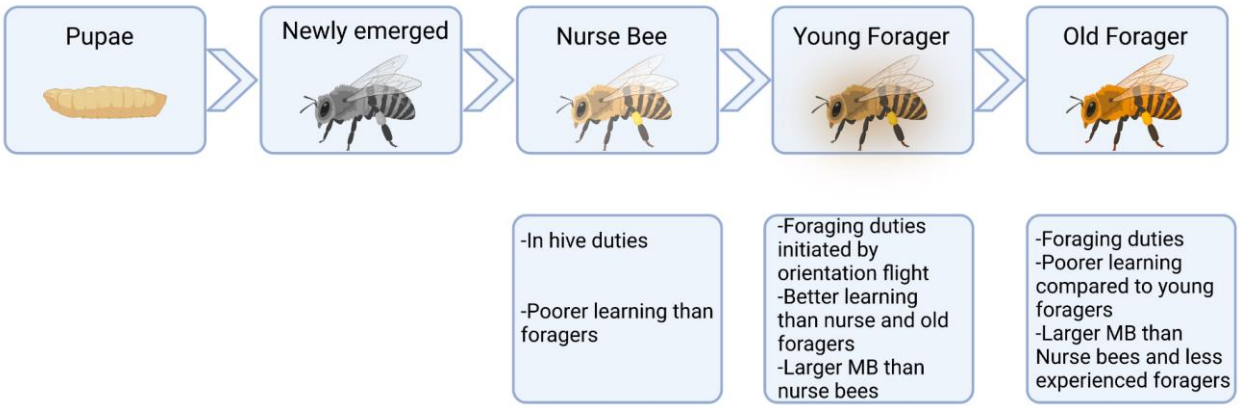

Figure 1. Physiological and behavioral differences of the honey bee at different stages of development.

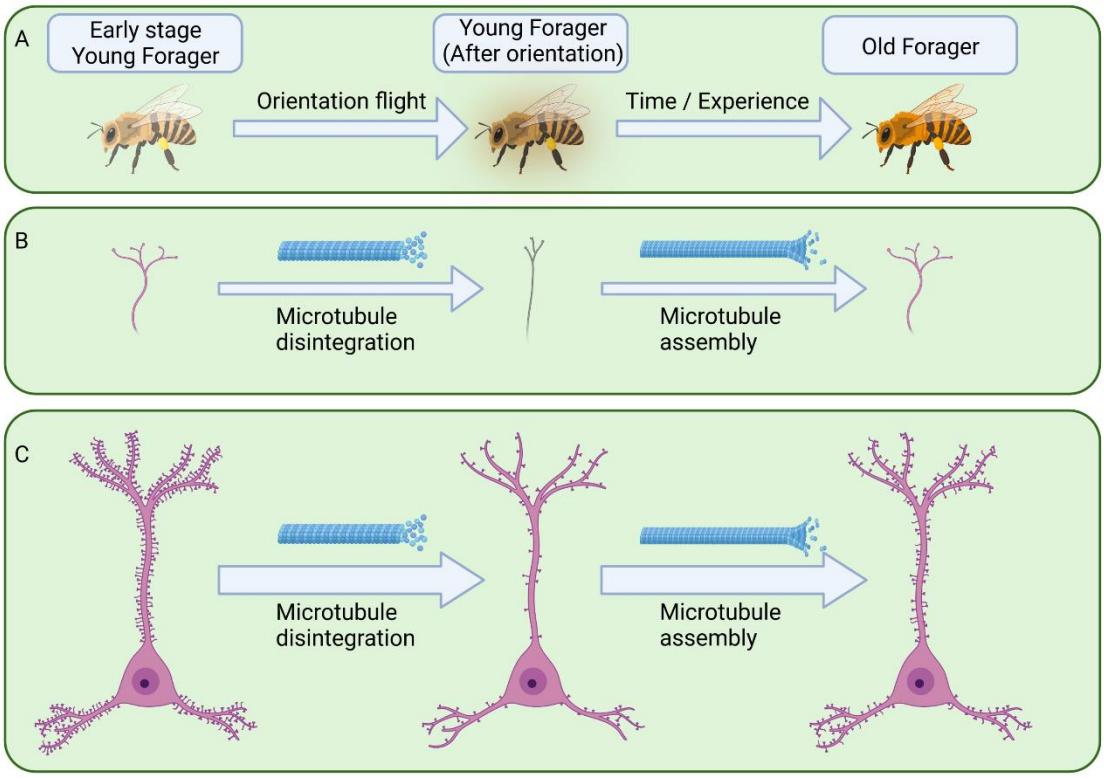

Figure 2. Timeline of synaptic pruning during the forager stage. Foragers go through orientation flights during the critical period, after the critical period many physiological changes occur with age and experience as well (A). During the critical period axonal pruning is observed, a characteristic of this type of pruning is microtubule disintegration, with age and experience axons start to branch again (B). Dendritic branches are pruned during critical periods as well, with age and experience dendrites branch again at a slower rate.

1.3. Potential molecular pathways directly involved in honey bee synaptic pruning

A key pathway regarding the developmental pruning in *Drosophila melanogaster* is the ecdysone signaling pathway, which controls pruning of MB axons and dendrite pruning of dendritic arborization (ddaC) neurons during metamorphosis (Figure 3). The pathway is initiated by Glial signaling via the Myoglianin activation of the TGF-β complex (Awasaki et al., 2011). Ecdysone receptor B1 (EcR-B1) is one of the most important receptors in this pathway, as it is required for both MB axon pruning and ddaC dendrite pruning (Lee et al., 1999, 2000; Schubiger et al., 1998; X. M. Yu et al., 2013). EcR-B1 activation can be

accomplished by TGF- β signaling (Zheng et al., 2003), and also nuclear receptor ftz-f1. Activation of EcR-B1 by ftz-f1 can be repressed by another nuclear receptor Hr39, which results in the inhibition of pruning by this pathway (Boulanger et al., 2011). Activated EcR-B1 heterodimerizes with ultraspiracle (USP), the EcR-B1/USP heterodimer is then activated by binding of ecdysone and regulates the expression of downstream genes required for axon pruning of gamma neurons in MB and ddaC neurons (Kuo et al., 2005; Lee et al., 2000). Specifically, heterodimerization of EcR with USP and binding of ligand Ecdysone, leads to activation of Sox14, Hdc, and other unknown transcription factors (Kirilly et al., 2009; Loncle & Williams, 2012). These transcription factors in turn activate MICAL and Cullin1, MICAL and Cullin1 facilitate the expression of the Ubiquitin proteasome system, which facilitates the degradation of Akt and DIAP1, and these have inhibitory effects on pruning (Kirilly et al., 2009; Kuo et al., 2006; Wong et al., 2013). Adapted from (F. Yu & Schuldiner, 2014).

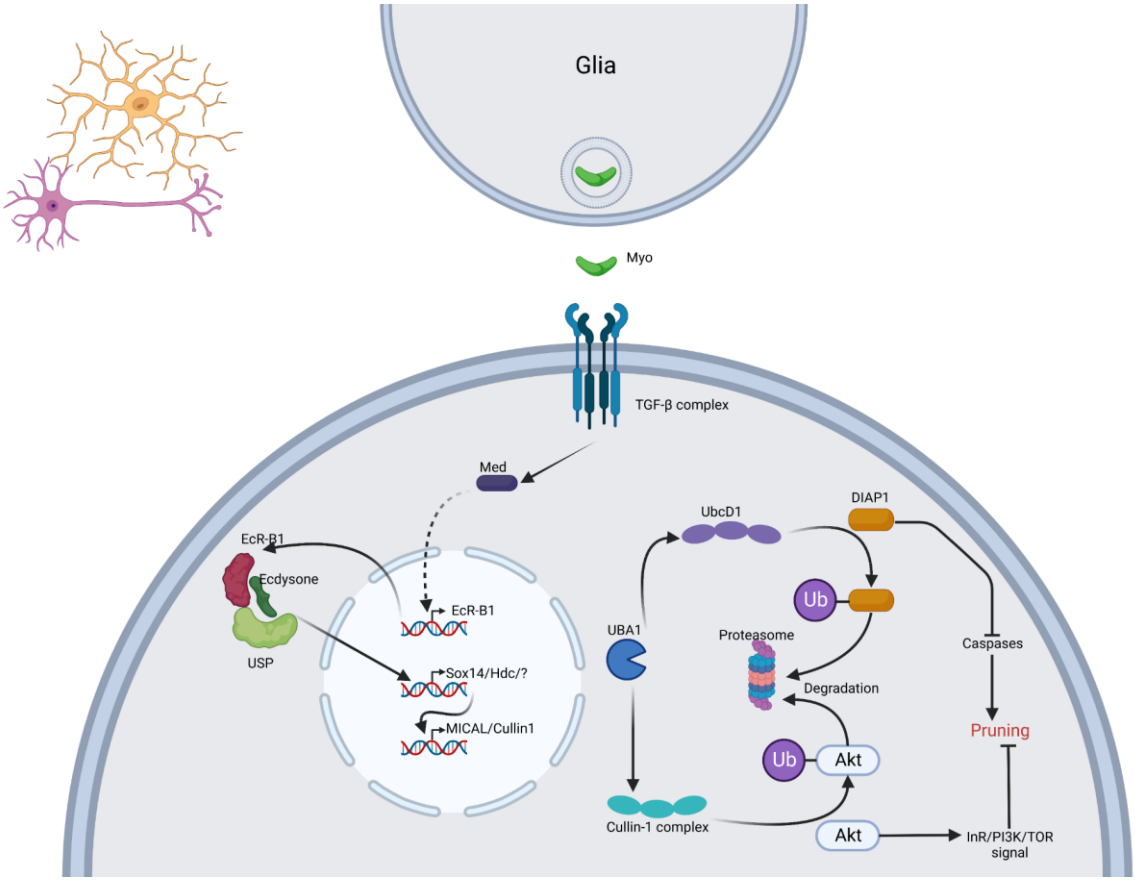

Figure 3. A representation of a *Drosophila melanogaster* dendritic arbor pruning pathway.

This pathway is found in honey bees as well. The ecdysone receptor (EcR-B1) is one of the most important receptors in this pathway, as it is required for mushroom body pruning (Figure 4) (Lee et al., 1999; X. M. Yu et al., 2013). EcR-B1 signaling can be inhibited by Hr39 (also called ftz-f1 beta), and Hormone-like Receptor 38 (Hr38) is known to repress EcR-B1 activity, this results in inhibition of pruning by this pathway (Boulanger et al., 2011; Zhu et al., 2000). Hr38 expression is increased in the mushroom bodies of the foraging bees (Yamazaki et al., 2006). Furthermore, the feeding reward during foraging activates the expression of Hr38 and its downstream genes more robustly, and this response is thought to be involved in learning and memory (Singh et al., 2018). Therefore, if we were to knockdown both Hr38 and Hr39 then we would expect an increase in synaptic pruning, and also expect that the synaptic connections will return to baseline levels after the knockdown effect diminishes. In honey bees Hr38 is upregulated by Egr, which is upregulated in foragers and triggers orientation flights in conjunction with exposure to a novel environment, also its expression increases in association with behavioral maturation (Khamis et al., 2015; Lutz & Robinson, 2013; Singh et al., 2018). Therefore, we suspect that Hr38 and Hr39 are also associated with the aging and development process in honey bees. In summary, Hr38 and Hr39 appear to be central regulators of the synaptic pruning pathway, making them an ideal target for RNAi knockdown.

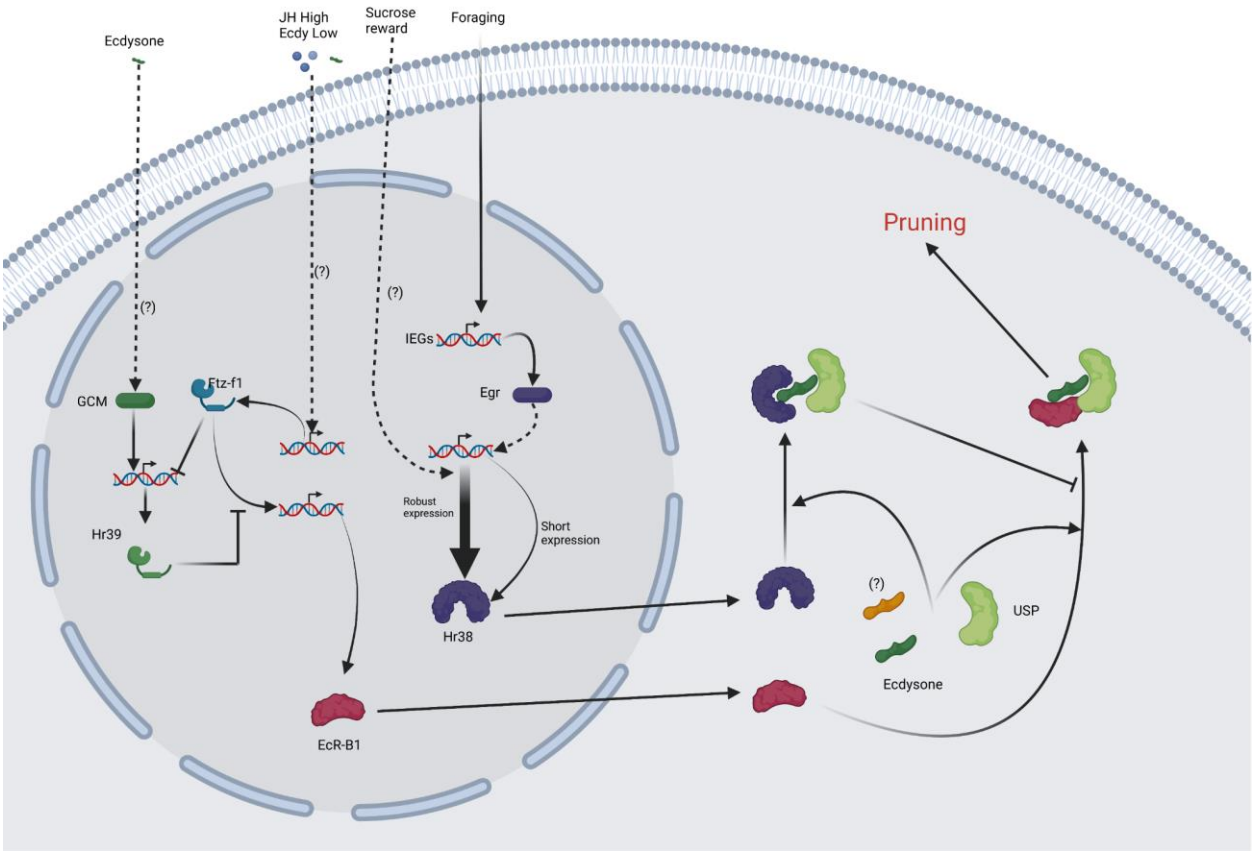

Figure 4. A representation of the Hr38-EcR-USP pathway in the Mushroom Body Kenyon cells of the honey bee brain.

Honey bees also produce ecdysteroids during metamorphosis; however, three days post emergence ecdysone titers in their hemolymph decrease after emergence and then remain low for the rest of their adult life (Hartfelder et al., 2002; Robinson et al., 1992). Even though ecdysone titers in the hemolymph are low in adult bees, EcR-USP and ftz-f1 are still expressed in the Kenyon cells of the adult bees during their behavioral maturation (Velarde et al., 2006, 2009). Both EcR and USP are upregulated in forager's brains compared to nurse bees (Khamis et al., 2015); in fact, EcR is upregulated in an experience-dependent manner in foragers after continuous foraging (Singh et al., 2018). Furthermore, recent studies show that ecdysone injections to the adult and forager brain elucidate behavioral changes, and also suggest that hypopharyngeal glands of the adult worker bees might produce ecdysteroids which are released to the brain (Geddes et al., 2013; Ueno et al., 2015). Regardless, expression of these nuclear receptors in the adult bees are at the same location, and the

experience-induced expression of EcR in the forager's brain suggests that this pathway may have an alternative function or may be activated by an alternative mechanism other than ecdysone signaling.

Another important component of the ecdysteroid signaling pathway is experience-dependent plasticity, in which synaptic pruning may play a role in synaptic remodeling that occurs during behavioral plasticity. Another candidate downstream target of Egr, is Hormone-like Receptor 38 (Hr38). Hr38 is an immediate early gene (IEG) that is shown to be a marker of neuronal activation in *Drosophila* (Chen et al., 2016) and silkworms (Fujita et al., 2013). In honey bees Hr38 is expressed differentially in Kenyon cells of the mushroom body of the forager brains (Yamazaki et al., 2006). Singh et al. (2018) demonstrated that both Hr38's and Egr's expression is induced by foraging; however, this expression is short-lived when foragers were presented with a known feeder plate without a food reward, while in the presence of a food reward, this expression was more robust. This suggests that Hr38 might play an important role in learning and memory reconsolidation. Hr38, like EcR, heterodimerizes with USP and responds to ecdysone signaling, however, Hr38/USP heterodimer activates transcription of targets that are distinct from the EcR/USP heterodimer and thus represents an alternative ecdysteroid signaling pathway to EcR (Baker et al., 2003). Hr38 also competes with EcR for USP heterodimerization as Hr38 disrupts EcR/USP transactivation (Zhu et al., 2000).

To summarize the pathway, a combination of hormonal cascades starts during the foraging transition, and the foraging experience further triggers this pathway. Expression of Ftz-f1 is triggered by a high titer of Juvenile Hormone (JH) combined with a low titer of Ecdysone in the hemolymph (Mello et al., 2019). Ftz-f1 triggers the expression of EcR, and Hr39 competes with Ftz-f1 and inhibits the expression of EcR (Boulanger et al., 2011). Hr39 is expressed due to the action of GCM that is activated from an Ecdysone pulse (Cattenoz et al., 2016). Neural activity from foraging triggers the expression of IEGs, one of which is Egr, Egr then upregulates the expression of Hr38 (Lutz & Robinson, 2013; Singh et al., 2018). Without the sucrose reward, this expression is short-lived, however in the presence of a sucrose reward, Hr38 expression is modified to last longer, and the expression of it is more

robust (Singh et al., 2018). Hr38 and EcR are capable of heterodimerization with USP; thus, both compete for USP heterodimerization (Baker et al., 2003). At this stage, there are no detectable Ecdysone titers in the hemolymph, however, the pathway is still active, implying that there may be another unknown ligand for these receptors (Geddes et al., 2013; Hartfelder et al., 2002; Robinson et al., 1992; Velarde et al., 2006; Yamazaki et al., 2006). Heterodimerization of USP with EcR leads to activation of pruning, while heterodimerization with Hr38 does not lead to this outcome (Baker et al., 2003; Boulanger et al., 2012; F. Yu & Schuldiner, 2014; Zhu et al., 2000).

Egr, Ecr, USP, and Hr38 are all transcription factors that are expressed in the same population of neurons, the Kenyon cells within the mushroom body of honey bee forager (Lutz & Robinson, 2013; Takeuchi et al., 2007; Velarde et al., 2006; Yamazaki et al., 2006). Egr is scattered throughout the Kenyon cell population, while Ecr and Hr38 expression is relatively higher in class 1 small Kenyon cell subpopulations (Takeuchi et al., 2007; Yamazaki et al., 2006), whereas USP expression is evenly distributed in younger foragers, but it is expressed at relatively low levels in the inner compact Kenyon cell subpopulations of the older foragers. However, it is still expressed adequately in outer Kenyon cell subpopulations (Velarde et al., 2006). Colocalization of competing Ecr and Hr38 with matching patterns and levels of expression and their colocalization with USP supports the notion that this pathway is still functional with or without Ecdysone signaling. The unmatching pattern of expression between USP and Hr38/Ecr is quite intriguing as it may point to another level of regulation of this pathway since these receptors are still colocalized. This shift in the expression pattern may explain the experience/age-dependent behavioral changes and why we don't see the same dramatic pruning event during the orientation flights later in life. Also, this expression pattern may allow both pathways to be active simultaneously in some neuron populations while their activation can be competitive in another population of neurons. We therefore would like to target Hr38 and Hr39 for knockdowns, specifically in the mushroom body region of the brain as a way to be able to manipulate the level of synaptic pruning, while controlling for the age of the bee to learn more about its role in enhancing learning and memory of the honey bee.

1.4. Thesis aims

A number of pathways related to synaptic pruning have been identified in the honey bee making it an amenable model organism for establishing more direct links between synaptic pruning and learning and memory. It has been hypothesized, based on the mechanisms identified in the previous literature, that the trimming of unused synapses via synaptic pruning increases the efficiency of synaptic transmission, thereby improving learning and memory (Chechik et al., 1999; Lichtman & Colman, 2000; Luo & O'Leary, 2005). However, this has never been explicitly tested as it requires manipulating the synaptic pruning levels in the brain and then experimentally investigating its effects on learning and memory. Synaptic pruning is also dynamic and associated with activity, aging, and development (Cabirol et al., 2017; Muenz et al., 2015; Scholl et al., 2014; Stieb et al., 2010). We, therefore, we propose using the honey bee as a model organism to manipulate the synaptic pruning levels using RNAi knockdowns in the brain to understand its role in synaptic formations in the mushroom body of the brain, learning and memory, and its function in relation to aging.

2. Methods

2.1. Section 1: Experimental *Setup*

2.1.1. Mark and recapture method for obtaining bees of known age (for all Ips)

Brood frames from three source colonies were collected and brought back to the laboratory. Newly emerged bees from the frames were harvested within 24 hours of emerging. These bees were individually painted and marked on the thorax with Testors paint. These marked bees were reintroduced back to a source colony for collection later on. After four weeks of reintroduction, the marked bees were collected as forager bees; previous work has shown the bees will typically be foragers at this time according to their age polyethism (Seeley, 1982).

2.1.2. Collecting marked forager bees for experimental setup

Bees were collected by placing a wire mesh over the hive's entrance to prevent the returning foraging bees from entering the hive. Using 20 ml glass liquid scintillation vials (Sigma-Aldrich, St. Louis, Missouri, United States), one bee was captured at a time and then immediately placed on ice for immobilization. A total of 50 bees were collected per experimental trial. The immobilized forager bees were then harnessed using a plastic drinking straw and 1 mm width duct tape strips. The bee was immobilized except for the head region so the proboscis could freely extend. Each bee was then fed *ad libitum* with 50% sucrose solution using a 10 µl micropipette. They were then randomly divided into two groups, either a gene expression or a PER behavioral assay group, each consisting of 25 bees.

2.2. Section 2: Validation of RNAi double knockdown effectiveness of Hr38 and Hr39

After 30 min of acclimation, the bees were set aside as the gene expression group was then placed in the following treatment groups: Non injected, Water sham negative control, or a Hr38 Hr39 double knockdown siRNA group. A subgroup of bees was randomly assigned to

be sacrificed at 2, 6, 12, and 24 hours after siRNAi injection for qPCR gene expression analysis.

2.2.1. siRNAi design

The siRNAi for the Hr38 and Hr39 honey bee gene targets were self-designed using Invitrogen's Block-iT™ RNAi Designer 5 (Table 1). Invitrogen's Block-iT™ RNAi Designer 5 uses standard and proprietary algorithms based on individual base composition to design and synthesize RNAi using Invitrogen's proprietary Stealth technology. Off-target effects were accounted for by using a stringent 8 mismatch criteria and with 3 of these being at the end of the sequence (Guo et al., 2018).

Table 1. List of siRNA designed for Hr38 and Hr39. The scrambled siRNA negative control is not shown as this is standardized and commercially available.

	Position	*Sequence*	*GC%*
Hr39			
1	*2223*	*AGAGCAUGAUCUCUCAUCUCUUCUG*	*44*
2	*2555*	*AAGCCAAGCUGUCUAGCCUGUUCUA*	*48*
Hr38			
1	*1434*	*AGUAAUCCAAGCUUGCUUGAUCGGG*	*48*
2	*2069*	*UAGACUACCGACGAACAUGGUCUCG*	*52*

2.2.2. Delivery of siRNAi into the brain

The heads of harnessed bees were fixed using a dental wax collar. SiRNAi ocellar tract injections were performed utilizing a 10 µl Hamilton syringe equipped with a 33 gauge needle for delivery to the whole brain of the honey bee. We used a microscapel and dissecting microscope to remove the ocelli lens and then inserted the syringe needle through the ocellar retina into the head capsule of the bee to a depth of 50 µm. We then injected 200 nl of solution (Søvik et al., 2016). The injections were performed underneath a Zeiss stereoscope and the Hamilton syringe was held in a WPI right handed micromanipulator (World Precision Instruments, United States). After the injection, we fed them *ad libitum* daily and maintained them at 25° C in an incubator with 60% Relative Humidity for one week.

2.2.3. qPCR gene expression analysis

At 2, 6, 12, and 24 hr post-injection, the pre-selected bees were flash frozen in liquid nitrogen, the heads were removed, and then these were stored in the -80°C freezer until further analysis. Previous studies have shown that siRNAi can result in gene expression knockdown as soon as a few hours after injection, and typically the peak of the activity is 2 - 4 hr after injection (Guo et al., 2018). Therefore, we expect to have diminishing knockdown levels from the RNAi injection after 6 hr.

The primers used for the qPCR gene expression analysis are shown in **Table 2**. For total RNA extraction, EcoPURE Total RNA kit (EcoTECH Biotechnology, Turkey) was used. For the qPCR analysis, each bee's head was ground by a sterile pestle. Then 300 µl of EcoPURE Lysis/Binding Buffer was added to ground tissue. The mixture was transferred to a 1,5 ml microcentrifuge tube and mixed thoroughly by vortexing for 10 seconds. 3 µl of β-mercaptoethanol was added to the mix to inhibit the RNase activity.

The mixture was spun for 10 min in a microcentrifuge at 12,298 G (10,000 rpm)to pellet the debris. Then the supernatant was transferred to another 1.5 ml microcentrifuge tube, an equal amount of absolute ethanol (96-100%) was added to the supernatant, and the mix was vortexed for 10 s. An EcoPURE column was inserted into a collection tube, and the mixture was transferred to the column. The mixture was centrifuged at 12,298 G (10,000 rpm) for 30 s at room temperature. Flow through was discarded, and 300 µl EcoPURE Wash buffer 1 was added to the column. The buffer was centrifuged at 12,298 G (10,000 rpm) for 30 s at room temperature again. The flow through was discarded and 500 µl EcoPURE Wash buffer 2 was added to the column. The buffer was centrifuged at 10,000 rpm for 2 min at room temperature this time. The flow through was discarded and 200 µl of EcoPURE Wash buffer 2 was added to the column. To remove any residual wash buffer the buffer was centrifuged at 10,000 rpm for 2 min at room temperature. The column was transferred to a sterile 1.5 ml microcentrifuge tube, and 50 µl of EcoPURE Elution buffer was added to the column. After 5 minutes of

incubation at room temperature the solution was centrifuged at 10,000 rpm for 2 minat room temperature. The column was discarded and purified RNA was stored at -80°C.

Table 2. Primers for assessing the gene expression of the 2 gene targets in order to manipulate the synaptic pruning levels in the honey bee brain.

Gene	***Primer***	***Sequence***	***Product Length***	***Reference***
Hr38	*F*	*AGA GCC CCT TCG GAT GTA GT*	*78 bp*	*Self-designed*
	R	*GTA GGC TTC CAG CAT CCG TT*		
Hr39	*F*	*CGT CAG CAC CGT CTG GTA TT*	*148 bp*	*Self-designed*
	R	*TCG TCT GTA GCA ACG TGT CC*		

Ribosomal protein 49 (RP49) was used as a reference gene since it has been shown as a stable housekeeping gene in *Apis mellifera* (Lourenço et al., 2008). qPCR assays were carried out with a final volume of 20 µL. BrightGreen 2X qPCR MasterMix-No Dye (Applied Biological Materials, Canada) kits which are suitable for our LightCycler 480 Real-Time PCR system (Roche Life Sciences, Germany) were used. For one reaction, 10 µl of 2X master mix, 0.6 µl of reverse and forward primer (0.3 µm final concentration each), 2.4 µl of template RNA (240 ng in total) and 6.4 µl of sterile water was added. The qPCR thermocycler program was as follows: 10 min at 95° C, followed by 50 cycles of 15 s at 95° C, 30 s at a melting temperature of 62° C and 30 s at 72° C. We confirmed the specificity of each primer set using a melt curve analysis and all samples were run in technical duplicates. Each target's relative gene expression was measured against the reference gene RP49 using the -ΔΔCT method. We set aside a total of 5 additional bees from each treatment from this time point for immunohistochemistry staining as described in **section 1**. These were used for a phenotypic

quantitative visual confirmation of the effects of the siRNAi on the number of synaptic connections in the honey bee brain.

2.3. Section 3 Phenotypic confirmation of synaptic pruning manipulation

In order to visualize the number of synaptic boutons in the honey bee mushroom body of the brain, we are using a modified version of a previously established immunohistochemistry protocol that targets the SYNORF-1 protein located both in the pre-and post-synaptic areas of the neuron (Groh et al., 2012; Pasch et al., 2011). Briefly, we decapitated the honey bee head and immediately dissected a fresh brain out under the brain Ringer's solution (130 mM NaCl, 5 mM KCl, 4 mM MgCl2, 5 mM CaCl2, 15 mM Hepes, 25 mM glucose, 160 mM sucrose, pH 7.2). This was performed by fixing the bee head capsule to a wax dissecting plate with insect pins. Using a probe and micro scissors, the brain was removed from the head capsule. Dissected brains were then placed in a 1.5 ml microcentrifuge tube protected from light and fixed with a 4% PFA solution overnight at 4° C. The brains were rinsed with PBS (1x) for 10 min and then embedded in low melting point agarose (5%). Afterward the brains were sliced with a Leica 1,000s vibratome at room temperature into 50 or 100 µm thick cross sections. The sections were rinsed with PBS (1X) 3 times (for 10 min each) and permeabilized using 1% PBST (Triton X in PBS) for 10 min. Then the sections were blocked using 2% NGS in 0.5% PBST for 1 hr. The brain sections are incubated overnight at 4° C using the anti-SYNORF-1 primary antibodies at a dilution of 1:10 with 2% NGS and 0.5% PBST. After this, the brain sections were washed with PBS (1x), 3 times, for 10 min. A secondary antibody for SYNORF-1 at a dilution of 1:250 with 2% NGS and 0.5% PBST was conducted for 2 hr at room temperature, and once again, the brain sections were washed with PBS (1x), 3 times, for 10 min. Lastly, the brain sections were stained with 0.4 U of phalloidin to visualize the actin filaments and DAPI to visualize the nuclei of the neurons, with 0.5% PBST, for 20 min. After carrying out the staining, the brain slices were placed on the microscope slide and were mounted with methyl salicylate mounting media. The coverslips of the slides were then sealed with nail polish.

2.3.1 Confocal image analysis for the quantification of synaptic connections in the bee brain

From the confocal images obtained, synaptic counts and quantification were performed manually from the same mushroom body region of the bee brain, as previous research has shown that manual counts from a trained eye are more accurate than visual automated counting systems (Rössler et al., 2017). The manual counts and image processing was based on Haase & Cabirol 2019(Cabirol & Haase, 2019). First, utilizing Amira 3d software, images were put through a blind deconvolution step. Then the images were resampled to a final voxel size of 0.1 x 0.1 x 0.1 μm. Three random sub-sections of the mushroom body, with a volume of 1,000 μm^3, under 63x magnification, were selected as representative areas for quantitative counts. The number of synaptic boutons were counted in these regions by placing landmarks on identified microglomeruli and using Amira 3d's built in landmark counter.

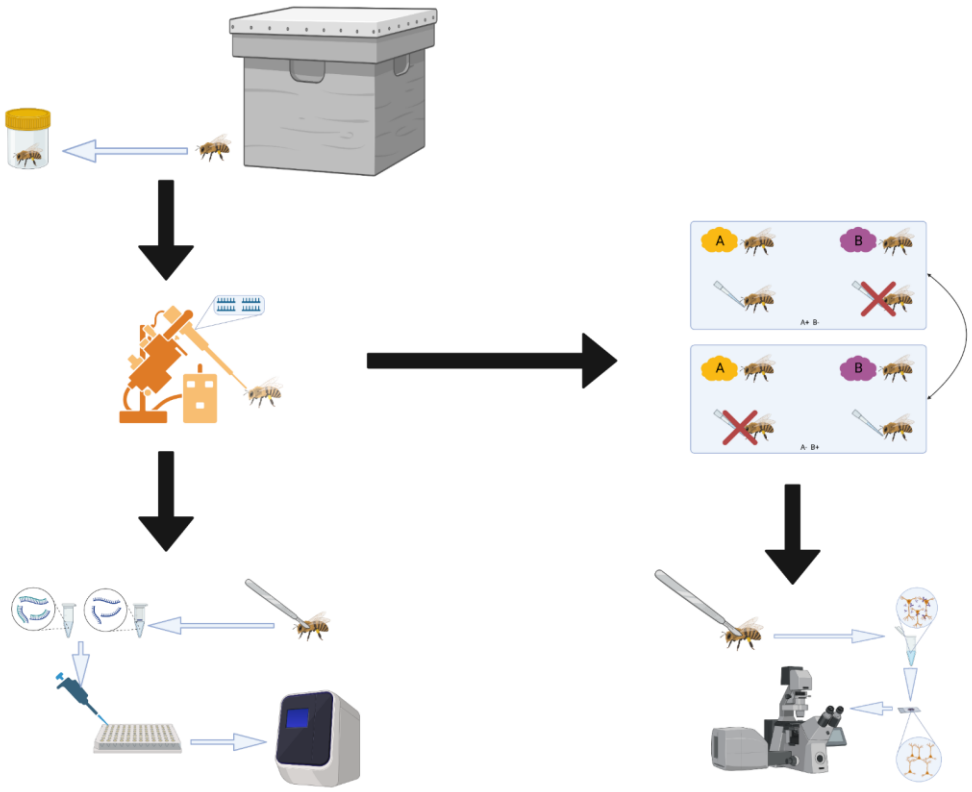

Figure 5.

General experimental procedure. Briefly, I paint marked forager bees that were then collected from the hives and designated siRNA's are injected to their brains. Half of the bees are frozen at different time points and put through RNA extraction and cDNA synthesis, then these samples are put through qPCR to confirm knockdown. The other half are subjected to a reversal learning assay. These bees will be decapitated and dissected to extract the brain, the brains will be sliced and stained. Stained samples will be viewed with confocal microscopy to count synaptic bouton numbers.

2.4. Section 4: Determining the role of synaptic pruning in learning and memory

2.4.1. Testing the effects of synaptic pruning on learning and memory

2.4.1.1. Proboscis Extension Retention assay setup

A total of 25 bees at a time were harnessed, as in **section 1**. After harnessing, these bees were fed *ad libitum* with 50% sucrose solution to ensure the highest survivarate possible. Brain

injections of the siRNA's treatment, as in **section 2** were carried out**.** The honey bees were then subjected to a reversal learning assay to assess their learning and memory abilities 18 hr after injection. The harnessed honey bees were placed in a custom Proboscis Extension Response chamber to be used for classical conditioning, where a honey bee individual learns to associate a 1 ul 50% sucrose solution reward (unconditioned stimulus) with a puff of odor A (Lavender oil) (conditioned stimulus). However, when exposed to odor B (Grapefruit oil) the bee did not receive any food reward. The odor was delivered using a Proboscis Extension Response assay apparatus customized for classical conditioning. The delivery system contains an air pump controlled by a valve that is connected to a glass cartridge that contains filter paper soaked with 5 µl of the undiluted odor solution. These cartridges are prepared ahead of time and sealed with parafilm so that the filter paper does not dry out. At the beginning of a trial, a button is pushed, which is wired to an Arduino UNO microcontroller board that is programmed to open the air valve for a duration of 6 s, which will bring the air across the filter paper within the cartridge that is directed towards the harnessed honey bee, which stands about 1 cm away from the bee head. While the odor is being delivered, the food reward, 1 ul of 50% sucrose solution, is given to the bee 3 s afterwards, using a toothpick, after the start of the odor delivery.

2.4.1.2 Reversal learning assay

The reversal learning assay was performed following Menzel and Hadar et al. (2010), with minor modifications. Two odors: lavender oil and grapefruit oil, were used as conditioned stimuli (CS), 50% sucrose was used as the unconditioned stimulus (US)(Hadar & Menzel, 2010). The CS that is paired with the delivery of US is termed CS+ as it is rewarded, while CS with the absence of the US is CS- as it is unrewarded. The reversal learning assay was performed one day after injections, the bees were subjected to 18 hours of starvation to ensure they were motivated and responded to the sucrose reward. The bees were carried to the PER box with a fan and a fume hood vacuum so that there was no stagnant odor in the box. The setup also has a delivery system containing an air pump controlled by a valve connected to a glass cartridge containing filter paper soaked with 5 µl of the undiluted odor solution. The

bees were subjected to a pseudo-randomized order of odor delivery of odor A and B with an intertrial time of 10 min, the order was: ABBABAABABBA. The bee was placed in the middle of the box in front of an empty glass syringe cartridge, the bee was then allowed to acclimate for 10s, then the airflow was switched over to the odor-charged cartridge. The bee was presented with an odor for s, during the last second the bee is then allowed to feed from a toothpick submerged in 50 % sucrose, totaling to a duration of seven seconds for the learning trial, the bee acclimated to being in front of the odor delivery system for 1.5 second before and after the odor delivery for a total time interval of 10 seconds. Each bee was subjected to six learning trials of each odor. On the first day the odor A was the CS+ and B was the CS-, then on the next day for reversal learning, this process was repeated, with the CS+ being B and the CS- being odor A. On the third day after harnessing, a memory retention assay was performed to assess the reversal learning performance of the previously trained bees.

3. Results

3.1. qPCR

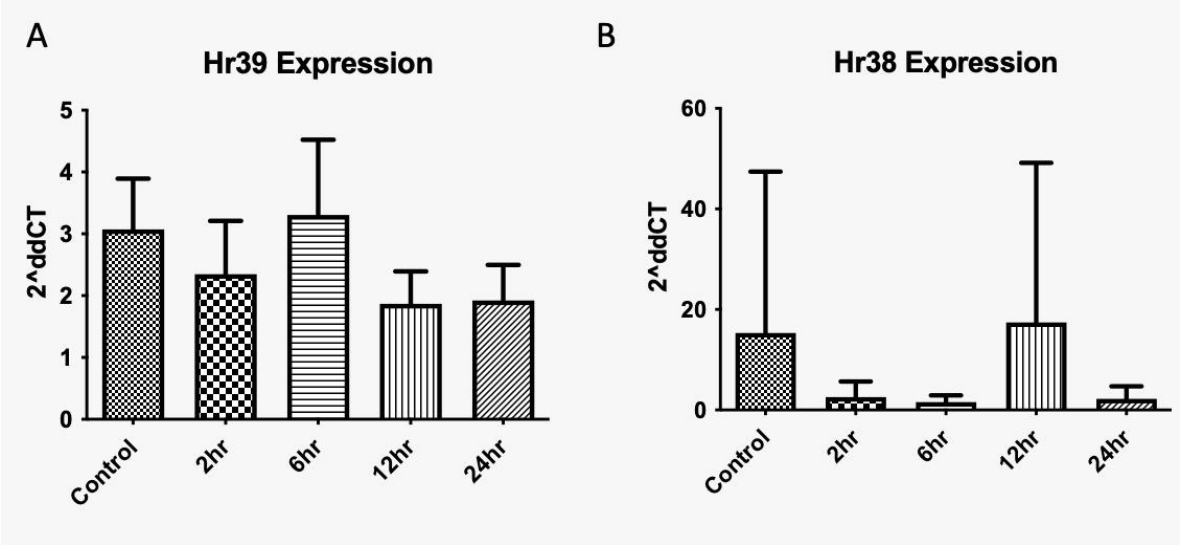

Figure 6. Expression analysis of ΔΔCT values of the Hr39 (A) and Hr38 (B) double knockdown siRNA treatment. Honey bees were injected and then sacrificed for gene expression analysis at 2, 6, 12, and 24 hours after injection. Each target was normalized to the RP49 reference gene.

ΔΔCT values of Hr39 of the treatment group at time point 2 hr 2.35 6 hr 3.31, 12 hr 1.87, and 24 hr 0.3539 while the control had a gene expression value of 3.0733. There were no significant differences found in the expression levels over time(Kruskal-Wallis test; p-value = 0.86) (Figure 6 A). ΔΔCT values of Hr38 of the treatment group at time point 2 hr 2.59 6hr 1,60 12 hr 17.40, and 24hr 2.24, while control was 15.32. There was no significant differences found of gene expression levels across time (Kruskal-Wallis test; p-value = 0.43)(Figure 6 B).

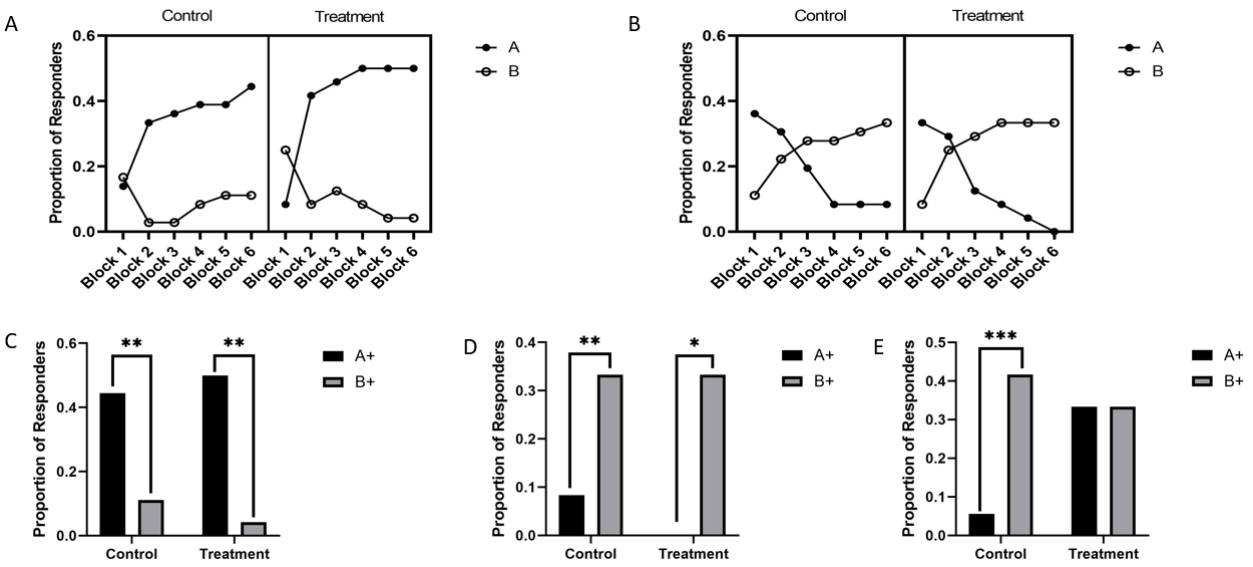

Figure 7. Reversal learning assay trials, The proportion of bees from control and treatment groups that displayed a Proboscis Extension Response (PER) towards odor A and odor B on day 1 (A). The proportion of bees from control and treatment groups that displayed PER towards odor A and B on day 2 (B). The proportion of bees from control and treatment groups that displayed PER towards odor A and B on Block 6 of day 1 (C). The proportion of bees from control and treatment groups that displayed PER towards odor A and B on Block 6 of day 2 (D). The proportion of bees from control and treatment groups that displayed PER towards odor A and B in the retention (memory) test on day 3 (E).

3.2. Reversal Learning Assay

Reversal learning assay revealed that both control (McNemar's Chi squared = 8.64, p-value = 0.003) and Hr38 and Hr39 knockdown treated (McNemar's Chi squared = 9.09, p-value = 0.002) bees were able to learn to respond to odor A while ignoring odor B on day 1 (Figure 7A and C). Both control (McNemar's Chi squared = 7.11, p-value = 0.007) and treated (McNemar's Chi squared = 6.12, p-value = 0.01) bees were capable of adapting to reversed conditions on day 2 as well (Figure 7B and D). However, there was no significant difference between responsiveness to odor A and B for the treatment group on day 3 (McNemar's Chi squared = 0, p-value = 1) (Figure 7E), whereas the difference was significant for the control group (McNemar's Chi squared = 11.07, p-value = 0.0008).

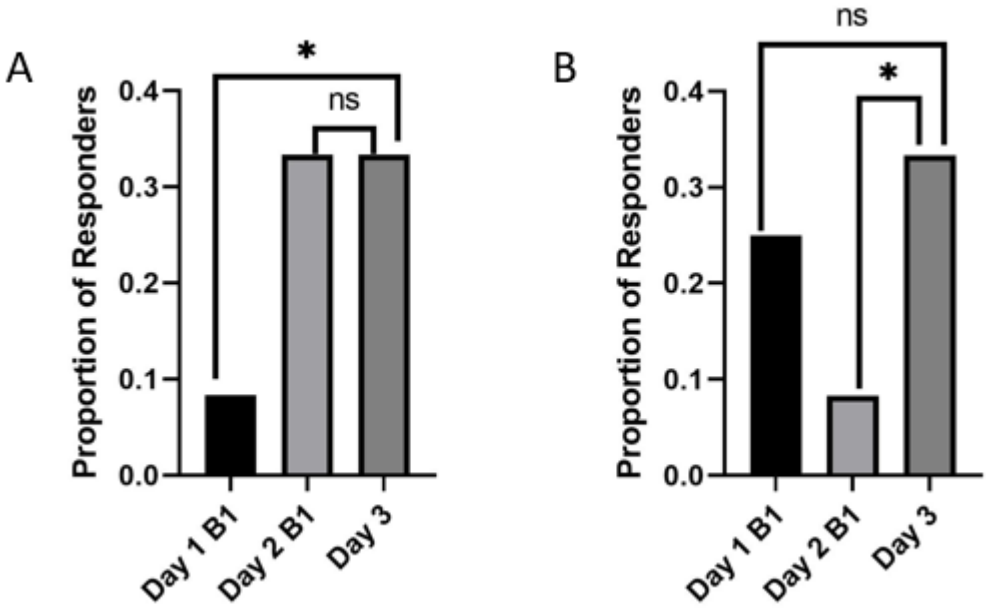

Figure 8. Treatment group response to odors From day 1 to day 3. The proportion of bees from the treatment group that displayed PER to odor A at day 1 block 1, day 2 block 1 and day 3 (A). The proportion of bees from the treatment group that displayed PER to odor B at day 1 block 1, day 2 block 1 and day 3 (B).

Further analysis on the performance of the treatment group throughout the assay was conducted. A portion of bees that displayed PER to odor A at day 1 block 1 was around 0.0833 whereas it was 0.3333 for day 3 and this difference was significant (McNemar's Chi squared = 4.16, p-value = 0.04) (Figure 8A). A portion of bees that displayed PER to odor B at day 2 block 1 was around 0.0833 whereas it was 0.3333 for day 3 and this difference was found to be significant (McNemar's Chi squared = 4.16, p-value = 0.04) (Figure 8B).

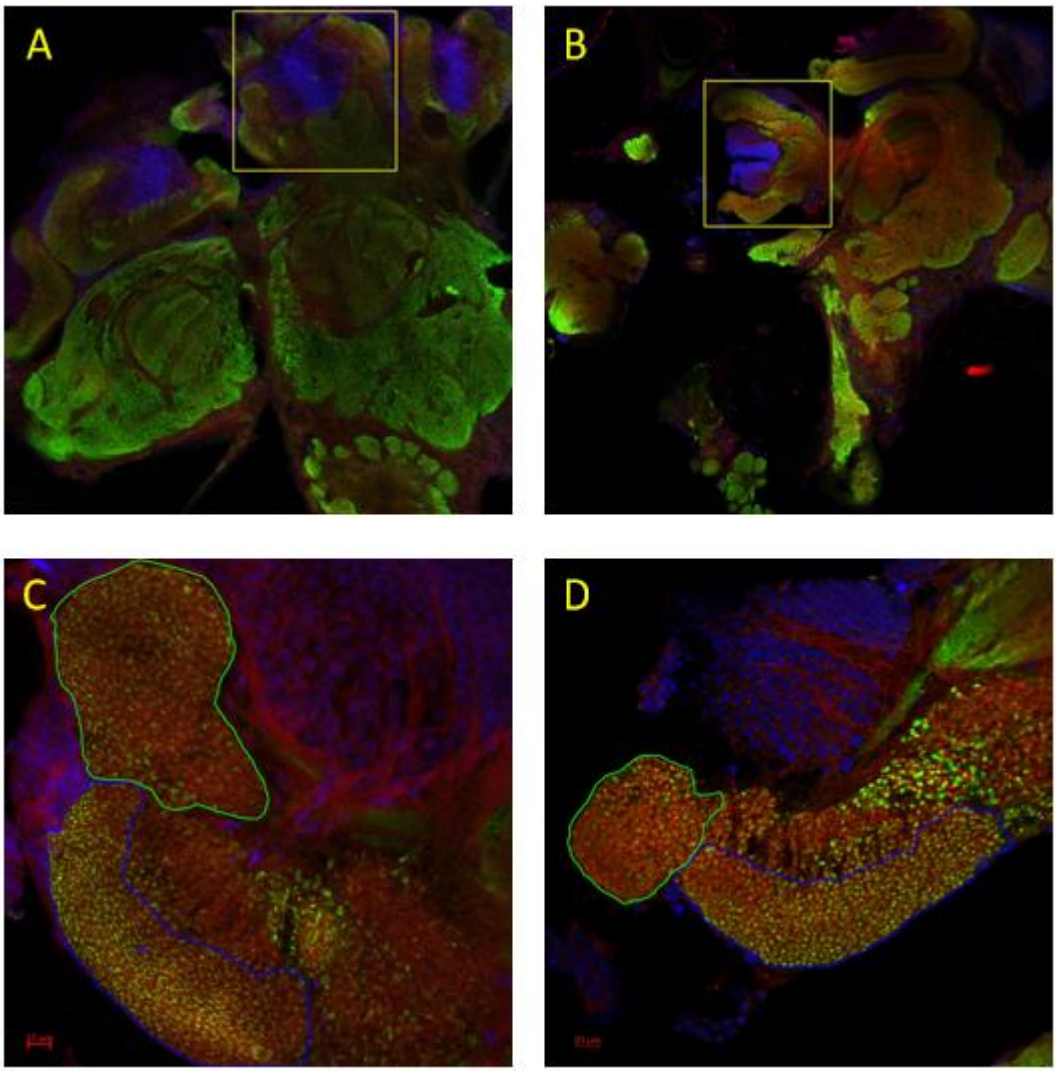

Figure 9. Synaptic bouton counts of the medial calyx of the Mushroom body. Z-Stack image from the control group taken with 10X magnification, one of the Medial Calyces is marked with a yellow rectangle (A). A Z-Stack image from the treatment group taken with 10X magnification, the Medial Calyce is marked with a yellow rectangle (B). A Z-Stack image of one of the calyces from the control group zoomed in on the Mushroom Body with 63X magnification; the lip region was encircled by a green marking, while the dense collar region was encircled by a blue marking (C). A Z-Stack image of one of the calyces from the treatment group with 63X magnification, the lip region was encircled by a green marking, while the dense collar region was encircled by a blue marking (D).

3.3. Synaptic Bouton Counts

The counts were made from the medial calyx region of the honey bee mushroom body. An image of the honey bee brain is shown in Figure 8 at 10X magnification (Figure 9A, 9B); a part of the mushroom body and its calyces were marked (Figure 9A, 9B). Although the brain taken from the treatment group had its other half missing, one of the medial calyces was intact (Figure 9B). Images of the medial calyces from the control and treatment groups are shown in Figures 9C and 9D, respectively. The lip and dense collar regions were surrounded by green and blue markers, respectively (Figure 9C, 9D).

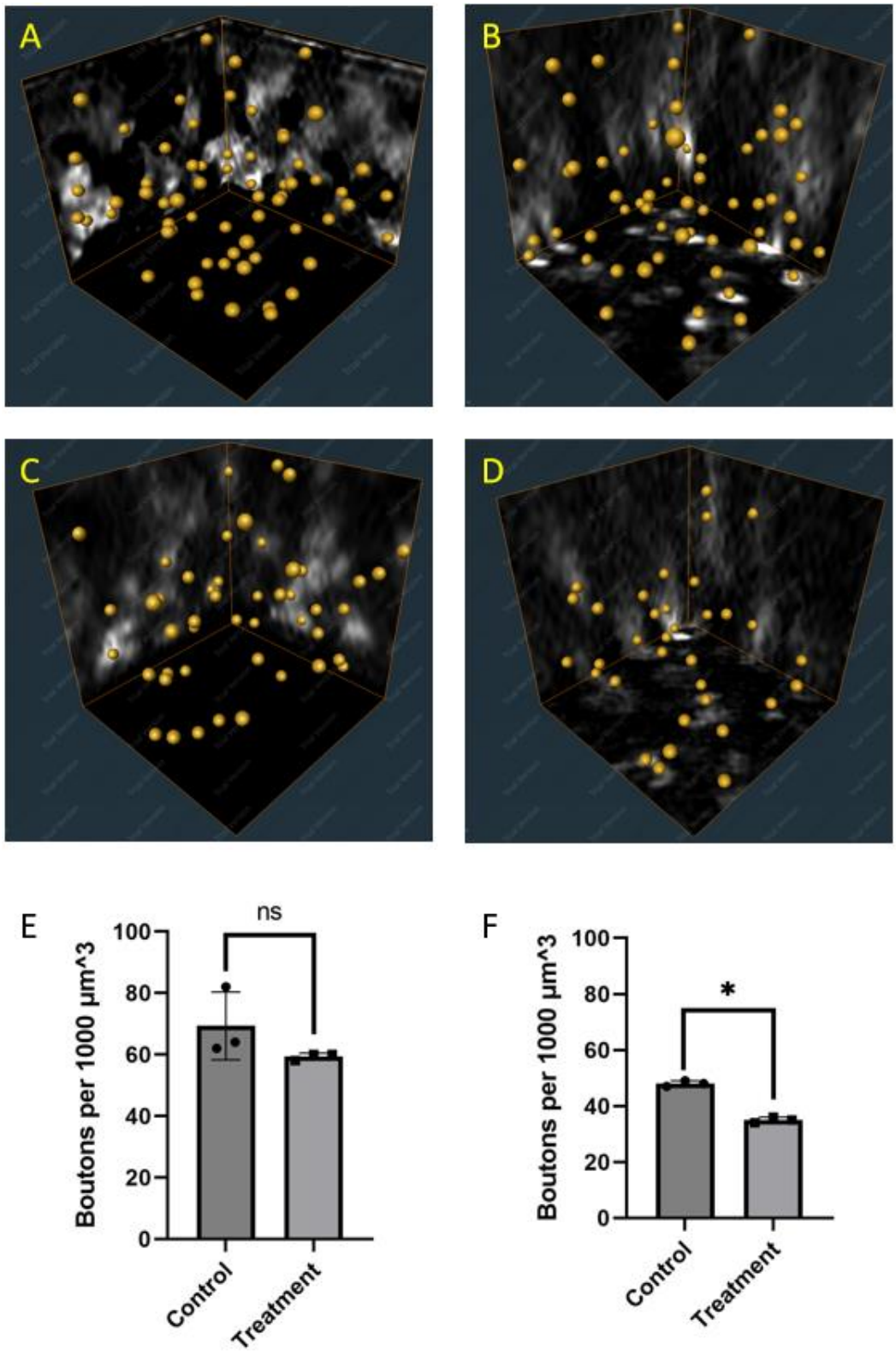

Figure 10. Synaptic Bouton counts, A 3-D region of interest from the control group dense collar region of the medial calyx where the bouton counts were made from (A). One of the

region of interest from the treatment group dense collar region of the medial calyx, where the bouton counts were made from (B). One of the regions of interest from the control group lip region of the medial calyx, where the bouton counts were made from (C). One of the regions of interest from the treatment group lip region of the medial calyx, where the bouton counts were made from (D). Averages of bouton counts from the dense collar region of the medial calyx were compared among control and treatment groups (E). Averages of bouton counts from the lip region of the medial calyx was compared among control and treatment groups (F).

The counts were made from three regions of interest, each from the lip and dense collar regions (Figure 9C, 9D). Each region of interest had a volume of 1000 μm^3. Representative images from the region of interest and landmarks that mark the boutons from the control dense region, control lip region, treatment dense region, and treatment lip region are shown in Figures 10A, 10C, 10B, and 10D, respectively. Counts were summed up and analyzed between control and treatment groups; there were no significant differences in synaptic bouton counts for the dense collar region between the control and treatment groups\(Figure 10E) (Chi-squared = 2.33, p-value = 0.12), while there were significantly lower synaptic boutons in the treatment group for the lip region (Figure 10F) (Chi squared = 5.79, p-value = 0.01).

4. Discussion

Retention test results at the end of the reversal learning assay indicate that control group bees performed better in the formation of LTM for odor B and extinction of odor A in the retention test after 24 hours of reversal (Figure 7E), in contrast to the proportion of bees that succeeded in formation of LTM for odor B in the treatment group was slightly lower and inhibitory learning or extinction for odor A was blocked in the treatment group (Figure 7E). Although the treatment group displayed somewhat poorer results compared to the control group in the retention test, the treatment group displayed faster and more robust acquisition of odor A at day 1 and odor B on day 2 of the reversal learning test in terms of excitatory learning (Figure 7A, B). Despite the better acquisition by the treatment group, the end results of day 1 and day 2 were comparable for both groups as both displayed the desired results in the initial and reversal phase (Figure 7C, D). The proportion of responders to odor A and non-responders to odor B was slightly better in the treatment group at block 6 of day 1, and non-responders to A was slightly better as well at day 2, block 6, however, the proportion of the responders to B was the same with control (Figure 7C, D). These results indicate that the LTM formation for excitatory learning is intact, but the extinction of LTM was impaired due to the double knockdown of Hr38 and Hr39. Results also suggest that the Hr38 and Hr39 double knockdown may have slightly improved acquisition and short-term memory.

A closer inspection of responses of the treatment group throughout the first blocks of the assay supports the notion that extinction during the reversal phase is impaired for odor A (Figure 8A). However, there is a clear drop in responses to odor B between day 1 and day 2 block 1 (Figure 8B), this successful long term depression (LTD) of responses to odor B indicates that inhibitory learning is intact. Furthermore, in the reversal phase, the treatment group successfully reverses this inhibition, and a significant increase in the PER towards odor B and the formation of LTM is observed (Figure 8B). This suggests extinction and inhibitory learning is not completely impaired as it is still successful against a novel stimulus, but the extinction of an established excitatory memory does not translate into long-term memory due to our treatment.

To discern the physiological differences, we inspected images from the medial calyx of the mushroom bodies of the control and the treated bees (Figure 9). Synaptic boutons were counted from three different regions of interest in the lip and dense collar regions (Figure 10A-D). Counts from both the dense collar and lip region were lower in treated bees compared to control; for the lip region it was significantly so (Figure 10E, F). Results confirm that we were able to induce a large-scale pruning as intended. Lower numbers of boutons correlate with better reversal learning performance (Cabirol et al., 2018), yet this was not the case for our experiments. Here it should be considered that this association holds for bees on their natural course of development, whereas our treatment does not exactly reflect it. During the critical period, we first observe a drastic increase in mushroom body volume due to dendritic branching, after which most of these excess synapses are eliminated with a large-scale pruning, after this the synaptic bouton numbers gradually increase in an experience-dependent manner (Farris et al., 2001; Ismail et al., 2006). In honey bees the expression of Egr, which expresses Hr38, increases with behavioral maturation, meaning that as the honey bee forages more, the expression increases gradually (Khamis et al., 2015; Lutz & Robinson, 2013; Singh et al., 2018). Since the bees in our experiment were old foragers who had plenty of foraging experience, the Egr expression would already be increased compared to a less experienced younger bee. This means that after the pruning induced by the knockdown, we would see a sudden extreme increase in Hr38 expression, and thus the block on pruning would be maintained after the knockdown wears off. The fluctuations we see in the expression analysis of Hr38 and Hr39 after knockdown supports this notion (Figure 6). Thus, the plasticity observed in bees with lower bouton numbers may be due to the difference in the intensity of the block on pruning; in other words, lower intensity of the block on pruning and a slow incremental increase of this block could cause better performance in reversal learning assays. This would explain why we were not able to see better reversal learning performance in the treatment group despite a lower number of synaptic boutons.

Another effect of our treatment is the apparent block on long-term extinction memory. Studies in vertebrates point out that extinction is another form of learning, and there

is an establishment of extinction memory as opposed to the forgetting of an existing memory (Garelick & Storm, 2005; Myers & Davis, 2002). Similarly, studies in *Drosophila* also suggest that extinction learning relies on different molecular mechanisms in comparison to excitatory aversive learning (Qin & Dubnau, 2010; Schwaerzel et al., 2002). In honey bees, evidence suggests excitatory learning and extinction learning are two separate processes as well (Ben-Shahar et al., 2000; Ferguson et al., 2001; Menzel, 2012). In fact, in honey bees the blocking of transcription with emetine leads to the blocking of either excitatory learning or extinction learning, depending if it is a summer or winter bee (Menzel, 2012). Taken in conjunction with the aforementioned studies, this supports the notion that the transition of STM to LTM may lead to a separation at the network level for excitatory and inhibitory memories, and this process may be controlled differently for excitatory and inhibitory memories by hormonal factors as suggested in Menzel et al., (2012). Although our treatment does not affect hormones, it affects Hr38, a receptor of the Ecdysone hormone. This suggests that our treatment might have blocked the consolidation of extinction memory. However, during our treatment's effect, we see a fluctuation at 12 hours, which wanes at 24 hours (Figure 6), so there is a chance that memory consolidation that occurs after learning to be unaffected from the double knockdown directly. Alternatively, our treatment might have amplified the consolidation of excitatory memory of odor A on day 1. To add another dimension, we can consider the large-scale pruning caused by our treatment, which resulted in a lower number of boutons in the treatment group (Figure 10F). This might have caused a better allocation of resources to the remaining boutons and newly established connections, which would be in line with the homeostatic control of plasticity. In this case, the excitatory memory of odor A on day 1 would have persisted and might have overcome the extinction memory.

Here we report, for the first time, the induction of large-scale synaptic pruning outside of a critical period. The Hr38/Hr39 double knockdown successfully reduced the synaptic bouton number in the lip region of the honeybee and the bouton numbers in the dense collar region were slightly reduced as well. The reversal learning performance of the bees did not correlate positively with a lower number of boutons as reported previously in the literature (Cabirol et al., 2018). This suggests that other supporting mechanisms may be behind the

plasticity observed in younger foragers. In addition, the treatment effect peaked at 12 hr and started to wear off after that, such a transient effect might not be not enough to cause an impact as the expression of Egr and thus Hr38 continues to increase incrementally in an experience dependent manner throughout the lifespan of the bee (Khamis et al., 2015; Lutz & Robinson, 2013; Singh et al., 2018) as opposed to a sudden decrease and peak of expression. Double knockdown of Hr38/Hr39 also affects the transition of STMs to LTMs, however, it is not exactly clear if it affects excitatory or inhibitory learning or both in this case. To elucidate the effects of this knockdown on learning a more in-depth study spanning all age and experience groups would be beneficial. A knockdown of mammalian homologues of Hr38 and Hr39, NR4A and NR5A, respectively, may reveal more insights into mechanisms of synaptic pruning in vertebrates.

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