The neural mechanisms underlying bumblebee visual learning and memory

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Abstract

Learning and memory offer animals the ability to modify their behavior in response to changes in the environment. A main target of neuroscience is to understand mechanisms underlying learning, memory formation and memory maintenance. Honeybees and bumblebees exhibit remarkable learning and memory abilities with a small brain, which makes them popular models for studying the neurobiological basis of learning and memory. However, almost all of previous molecular level research on bees' learning and memory has focused on the olfactory domain. Our understanding of the neurobiological basis underlying bee visual learning and memory is limited. In this thesis, I explore how synaptic organization and gene expression change in the context of visual learning.

In Chapter 2, I investigate the effects of color learning and experience on synaptic connectivity and find that color learning result in an increase of the density of synaptic complexes (microglomeruli; MG), while exposure to color information may play a large role in experience-dependent changes in microglomerular density increase. In addition, microglomerular surface area increases as a result of long-term memory formation. In Chapter 3, I investigate the correlations between synaptic organizations and individual performance and the results show that bees with a higher density of microglomeruli in visual association areas of the brain are predisposed to faster learning and better long-term memory during a visual discrimination task. In Chapter 4, I explore the genes involved in visual learning and memory by transcriptome sequencing and I show the unique gene expression patterns at different times after visual learning.

In summary, my findings shed light on the relationship between synaptic connections and visual learning and memory in bees at the group and individual level and show new candidate genes involved in visual learning, which provide new avenue for future study.

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Chapter 1

Introduction

Behavioral plasticity, especially learning and memory, enable animals to modify their behaviors in response to changing conditions and allow individuals to adapt to the environment. Behavioral plasticity is typically underpinned by neural plasticity. A central goal of neuroscience is to understand the neural plasticity and neural mechanisms underlying learning and memory. Scientists have used a variety of model organisms to explore the neural mechanisms of learning and memory, trying to answer the question 'what changes in the brain when we learn and how our brain encodes memory' (Bailey et al., 1996; Kandel, 2005; McGuire et al., 2005; Schwarzel and Muller, 2006; Holtmaat and Svoboda, 2009; Lövdén et al., 2013; Kolb and Gibb, 2014; Berry and Nedivi, 2016; Fahrbach and Van Nest, 2016).

Many cellular and molecular mechanisms of learning and memory are conserved between invertebrates and vertebrates (Kandel, 2001; Benfenati, 2007; Kolb and Gibb, 2014). Insects have relatively small brains and some species display rich behavioral repertoires, such as sophisticated navigation, communication and a variety of cognitive abilities (Menzel and Giurfa, 2006; Chittka and Niven, 2009; Wystrach and Graham, 2012). They are therefore useful models to study neural plasticity. For example, the molecular basis of olfactory learning and memory in *Drosophila melanogaster* has been widely studied. Neural circuits underpinning olfactory memory have been identified using memory-defective mutant flies (Dubnau et al., 2003; Davis, 2005; McGuire et al., 2005; Keene and Waddell, 2007). In addition to fruit flies, eusocial insects, especially bees and ants, have

also been extensively studied due to their elaborate social structures, remarkable communication systems and outstanding learning and memory capabilities. In this introduction, I review the current state of knowledge about the neural mechanisms of learning and memory, and how it is mediated by synaptic plasticity across animals. I subsequently focus on neuroplasticity in bees and other insects, and how my thesis work was conducted with the aim to deepen our understanding of the molecular mechanisms of learning and memory in the visual domain.

1.1 The neural mechanisms underlying learning and memory

Synaptic plasticity is regarded as the fundamental mechanism for learning and memory storage (Bliss and Lømo, 1973; Milner et al., 1998; Kandel, 2001). Types of memory are classified depending on their duration, the broadest classification being short-term and long-term memory. Short-time memory lasts from seconds to hours, mediated by modification of pre-existing proteins and pre-existing synaptic connections. Long-term memory lasts for days, weeks or years, some depending on the animal's longevity. The formation of long-term memory requires the activation of gene expression, new protein synthesis and the formation of new synaptic connections (DeZazzo and Tully, 1995; Bailey et al., 1996; Kandel, 2001). Synaptic plasticity including structural and functional plasticity has been studied at many levels ranging from proteins and other molecules, neuronal morphology to detailed behavioral paradigms (Benfenati, 2007; Butz et al., 2009; Caroni et al., 2012; Kolb and Gibb, 2014; Berry and Nedivi, 2016).

1.1.1 Signal transduction between neurons

Synapses are the contact points between neurons which can transmit electrical or chemical signals from one neuron to another. Most synapses in the nervous system are chemical synapses and neurotransmitters are stored in synaptic vesicles within the presynaptic terminals. A synapse typically includes a presynaptic active zone with synaptic vesicles, a synaptic cleft and a postsynaptic site with neurotransmitter receptors (Harris et al., 1992). In brief, signal transduction between neurons works as follows: the electrical activity of the presynaptic neuron first activates voltage-gated calcium channels and causes the release of neurotransmitters, then the neurotransmitters bind to receptors on the postsynaptic membrane and trigger electrical or a secondary messenger response, which may excite or inhibit the postsynaptic neuron. Signal transduction efficiency in the nervous system is determined by synaptic strength, which depends on the number of vesicle release sites (active zone, a highly-specialized area on the presynaptic membrane), vesicle release probability and the number of stimulated receptors on the postsynaptic membrane. Many proteins and intracellular processes work together to modulate signal transduction (Benfenati et al., 1999; Benfenati, 2007).

1.1.2 Molecular bases of synaptic plasticity

Kandel and colleagues used the sea snail (*Aplysia*) to study the cellular mechanisms underlying learning and memory. They provided the first evidence that the second messenger molecule, cyclic AMP (cAMP), and ion channels can regulate the strength of synaptic transmission (Brunelli et al., 1976; Siegelbaum et al., 1982; Byrne and Kandel, 1996). It has been confirmed that many factors play significant roles in animals' learning

and memory, including neurotransmitters (such as acetylcholine (ACh), glutamate, serotonin and dopamine), second messengers (such as cAMP, Inositol trisphosphate IP3, Calcium), protein kinases, ion channels, and transcription factors like CREB (Bailey et al., 1996; Kandel, 2005; Schwarzel and Muller, 2006; Benito and Barco, 2010; Gauthier and Grünewald, 2012).

For instance, in the cAMP pathway, stimulation causes the increase of cAMP in presynaptic sites which activates PKA (cAMP-dependent protein kinase). PKA phosphorylates different substrates in synaptic terminals, such as proteins involved in exocytosis and potassium channels, leading to enhanced neurotransmitter release and synaptic strengthening. Short-term memory storage is associated with short-term synaptic strengthening. On the other hand, persistent cAMP increase leads to long-term synaptic plasticity, in which PKA is transported to the nucleus and phosphorylates transcription factor CREB. Then the phosphorylated CREB binds to a cAMP response element (CRE) in the promoters of target genes, which activates a set of immediate early genes. Thereupon, the immediate early genes act on a broader set of downstream genes, encoding proteins for synaptic modification. These transcription and translation proceeding in the cell are essential for the growth of new synaptic connections and long-term memory (Kandel, 2001, 2012; Benito and Barco, 2010) (Figure 1.1).
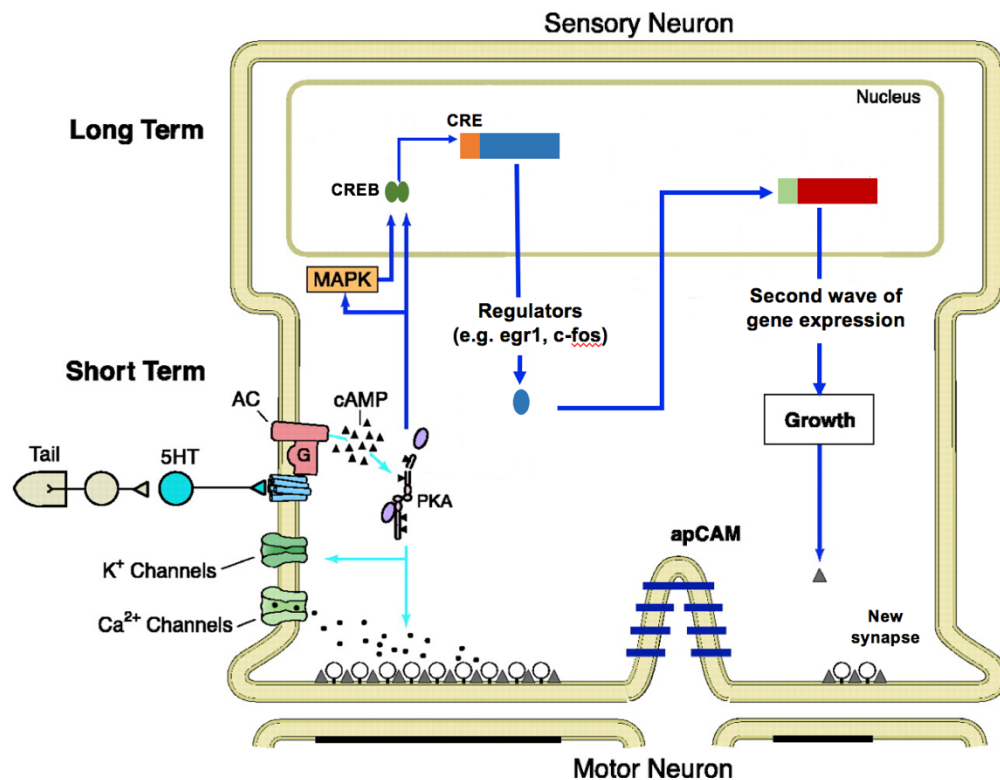
Figure 1.1. Schematic of the signaling pathway for synaptic plasticity and memory storage. This is the case for short- and long-term facilitation in *Aplysia* sensory neurons in gill-withdrawal reflex. Neuronal stimuli (such as serotonin release caused by tail shocks) trigger the activation of second messenger signaling cascades (e.g. cAMP or Ca^{2+} -dependent signaling). For the cAMP pathway, the cAMP-dependent protein kinase (PKA) is activated and phosphorylate different substrates (e.g. K^+ channels, exocytosis), which can enhance neurotransmitter release and strengthen synaptic connection (short-term synaptic plasticity). As cAMP increases persistently, PKA, together with mitogen-activated protein kinase (MAPK), is transported to the nucleus and phosphorylates transcription factor CREB. Further activated CREB triggers several immediate early genes and then downstream genes that lead to the growth of new synaptic connections (long-term synaptic plasticity). Modified after Kandel (2001).

Structural neural plasticity for long-term memory relies on transcription (Clayton, 2000; Kandel, 2001; Leslie and Nedivi, 2011; Benito and Barco, 2015). It has been found that at least two transcription waves are required for long-term memory formation, based on studies using inhibitors of transcription or protein synthesis in both vertebrates and

invertebrates (Bailey et al., 1996; Muller Igaz et al., 2002; Alberini, 2009; Lefer et al., 2013). Previous studies were mainly focused on individual genes to understand the role of genes in neural plasticity, especially the immediate early genes (IEGs). Such genes respond rapidly to a variety of cellular stimuli and play an important role in memory acquisition and consolidation (Flavell and Greenberg, 2008; Loebrich and Nedivi, 2009; Benito and Barco, 2015). Immediate early genes code for proteins including transcription factors (e.g. CREB, c-fos, c-jun), protein phosphatases, receptor subunits and cytoskeletal proteins. However, the relationship between transcription and synaptic modification is still unclear. Global gene expression changes and gene interactions should be examined to interpret the role of genes in neural plasticity. Transcriptional responses to specific conditions have been studied in the context of stress (Liu et al., 2008), social behaviors (Robinson et al., 2008), and long-term memory formation (Naeger et al., 2011; Wang et al., 2013; Qin et al., 2014). In Chapter 4, the genes involved in bee visual learning and memory formation are investigated using high throughput sequencing.

1.1.3 Learning and memory-related synaptic plasticity

Many studies from mammals, insects to mollusks have examined the association between behavioral plasticity and neural plasticity. Sensory experience, physical activity, and cognitive training can increase neuropil volume, reorganize presynaptic boutons and cause dendritic spines remodeling (Holtmaat and Svoboda, 2009; Xu et al., 2009; Lövdén et al., 2013; Kolb and Gibb, 2014; Scholz et al., 2015; Berry and Nedivi, 2016; Fahrbach and Van Nest, 2016). Experience and learning-induced brain structural changes have been found in human brains using imaging techniques (e.g. May, 2011; Lövdén et al., 2013). Studies on

rodents have revealed that the complex stimulation provided by an enriched environment can improve individuals' learning performance, and promote neuropil volumetric increase and dendritic branching and lengthening (Faherty et al., 2003; Leggio et al., 2005; Sale et al., 2009; Bednarek and Caroni, 2011; Scholz et al., 2015; Brenes et al., 2016). In addition, there are strong links between specific learning processes and the growth and pruning of specific synapses, based on different behavioral paradigms (Butz et al., 2009; Xu et al., 2009; Yang et al., 2009; Bednarek and Caroni, 2011; Caroni et al., 2012). For instance, learning novel motor skills can lead to the formation of postsynaptic dendritic spines and increase the efficacy of synapses in the motor cortex (Rioult-Pedotti et al., 2000; Xu et al., 2009), and different motor skills are encoded by different sets of synapses (Xu et al., 2009). Another example is fear learning and its extinction in mice, which results in the formation and elimination of spines on the same dendrites (Lai et al., 2012).

However, our understanding of the neural basis of learning and memory is still limited and many questions remain to be explored and answered, such as how specific learning types affect neural plasticity in different organisms, how genes or proteins regulate learning and memory processes globally, and what the neural basis for inter-individual cognitive variations is. In this thesis, I use the bumblebee (*Bombus terrestris*) to explore the neurobiological basis of visual learning and memory, and how it is related to synaptic complex morphology and gene-expression.

1.2 Bees as a model for understanding molecular mechanisms underlying learning and memory

Honeybees and bumblebees are popular models for studying mechanisms of learning and memory at both the individual and the population levels, because they have astounding cognitive abilities, very small brains and individual variation in cognitive performance (Muller & Chittka, 2012; Menzel & Giurfa, 2001; Raine & Chittka, 2012; Srinivasan, 2010).

1.2.1 Learning and memory in bees

Honeybees and bumblebees display a highly developed social structure and many complex behaviors, such as elaborate communication skills and excellent learning abilities. Younger honeybee workers (age 1-3 weeks) perform tasks such as brood care (nursing) inside the hive, while older honeybee workers (> 3weeks) perform foraging tasks for nectar and pollen outside the hive, which is called age-related division of labor (Winston, 1987). When foraging outside, bees experience a variety of stimuli and face substantial cognitive challenges (Fahrbach and Robinson, 1995; Spaethe et al., 2001; Raine et al., 2006). To forage successfully, bees have to learn and remember the odors, colors and shapes of flowers, and remember the locations of food resources and foraging routes. In the laboratory, bees can be trained to establish such associations as well, associating food with specific odors, colors, shapes and patterns, and even to learn simple forms of the concepts of sameness and difference, to count and learn tasks in a context-dependent way (i.e. bees can learn when and where to do a specific task) (Chittka and Geiger, 1995; Srinivasan et al., 1998; Menzel, 1999; Giurfa, 2007, 2013; Srinivasan, 2010).

Classical conditioning (Pavlovian conditioning) is extensively used to explore bees' associative learning and memory capabilities and their underlying neural basis. Proboscis extension response (PER) is a popular paradigm used in honeybees to study olfactory learning and its neural basis (Bitterman et al., 1983; Giurfa and Sandoz, 2012). Bees are immobilized in small tubes, and only the antennae and mouth parts (the proboscis) are free to move (Figure 1.2A). Hungry and harnessed bees are trained to associate a conditioned stimulus (CS, odor) with a rewarding unconditioned stimulus (US, sucrose) presented a few seconds afterwards. Bees extend their proboscis when their antennae are touched with sucrose solution. After conditioning, bees are able to respond to the conditioned stimulus with proboscis extension. For visual learning, a flight arena (such as Y-maze and rectangular flight arena) is often used to train free-flying bees to associate visual information with reward or punishment (Figure 1.2B,C) (Spaethe et al., 2001; Srinivasan, 2010; Whitney et al., 2016). Visual stimuli are presented on the wall or the floor of a flight arena and bees are trained to enter the arena to learn the stimuli. The setups vary according to different studying aims. With PER conditioning, bees' olfactory learning and memory have been well studied at both behavioral and molecular levels (Menzel and Giurfa, 2006; Gauthier and Grünewald, 2012; Menzel, 2012; Müller, 2012; Giurfa, 2013). With different visual training setups, behavioral experiments have revealed bees' remarkable visual learning and memory abilities. However, very little is known about the neural bases of visual cognitive abilities. Thus, my thesis will focus on bees' visual learning and memory and try to explore some of the neurobiological bases of it.
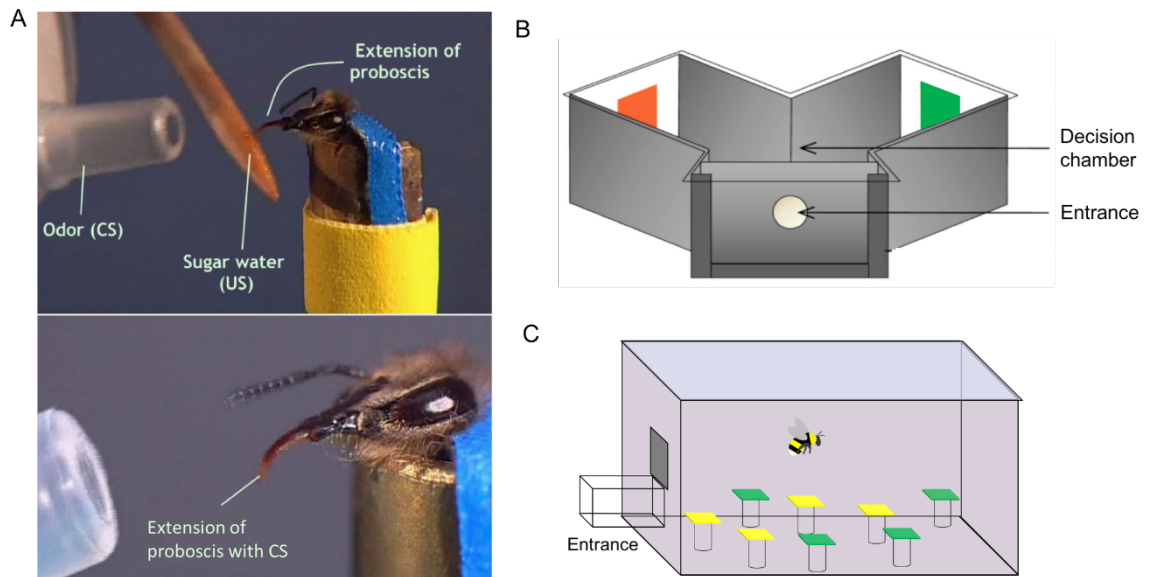
Figure 1.2. Typical training paradigms and apparatuses used for bees' olfactory and visual conditioning in a variety of studies. (**A**) Conditioning of the proboscis extension response (PER) in honeybees. A bee extends the proboscis in response to sucrose solution applied to the antennae. In PER, the harnessed bees are trained to associate a conditioned stimulus (typically an odor) with an unconditioned stimulus (sucrose solution) presented a few seconds later. After several trials, bees respond with proboscis extension when they are presented with odor alone. (**B**) The Y-maze apparatus for training bees to discriminate visual stimuli using a dual-choice paradigm. One of the stimuli offers sugar solution while the other does not. (**C**) A flight arena for training bees to associate visual stimuli with reward or punishment (e.g. quinine) in a free-flight multi-choice paradigm.

Bumblebees are the main insect model for studying individual variation in learning abilities (Chittka and Thomson, 1997; Thomson and Chittka, 2001; Chittka and Dyer, 2003; Raine and Chittka, 2008). They are able to discriminate different colors, shapes and patterns (Dyer and Chittka, 2004b; Muller and Chittka, 2012; Roper et al., 2017) and display consistent inter-individual differences in learning performance (Muller and Chittka, 2008, 2012). For color learning, previous studies have shown that bumblebees have innate color preference, that they can discriminate dissimilar colors easily, but can also learn very fine color discriminations by extended training (Dyer and Chittka, 2004a, 2004b; Raine and Chittka,

2005). Based on color learning, many other learning-related questions have been explored, such as the relationship between foraging speed and accuracy, the social learning ability in bumblebees, and decision making (Leadbeater and Chittka, 2007; Chittka et al., 2009; Perry et al., 2016). In this project, bumblebees (*Bombus terrestris*) are used as the experimental model and trained to forage in a flight arena and to learn associations between color and reward. The benefit of working with bumblebees is that, unlike honeybees, they forage comfortably in small flight arena, which allows complete control over their experience prior to foraging.

1.2.2 Bee brain and brain plasticity

The brain processes inputs from sensory organs and coordinates the behavioral output. The honeybee brain measures about 0.5 mm^3 and contains only ~1 million neurons compared to ~100 billion neurons in the human brain. This makes bees a useful model to explore how information can be efficiently processed, stored and retrieved under severe constraints on neuron numbers. Bees have more elaborate visual systems and larger mushroom bodies, compared with other popular model insects (e.g. fruit flies) (Menzel, 2012).

The mushroom body

The mushroom bodies of insect brains are high-level sensory integration centers that are involved in learning and memory (Heisenberg, 1998, 2003). Each mushroom body consists of cup-shaped calyces, peduncles and output lobes (α-lobes and β-lobes), which are formed mainly by intrinsic neurons, the Kenyon cells (Mobbs, 1982; Strausfeld, 2002; Fahrbach, 2006) (Figure 1.3A). The calyx is the main information input region within the mushroom body, which comprises the lip, collar and basal ring. The lip receives olfactory information

from the antennal lobe, the collar receives visual information from the optic lobe, and the basal ring receives both visual and olfactory information (Fahrbach, 2006).

Visual input from the optic lobes to the mushroom bodies is segregated into separate layers in the collar. Medulla neurons terminate in the outer region of the collar, where they segregate into five layers that receive alternating input from the dorsal or ventral medulla, respectively. Lobula neurons, conversely, terminate in the innermost layer of the collar (the sixth layer) (Figure 1.3B). Mushroom body visual input neurons are color sensitive (Ehmer and Gronenberg, 2002; Paulk and Gronenberg, 2008).
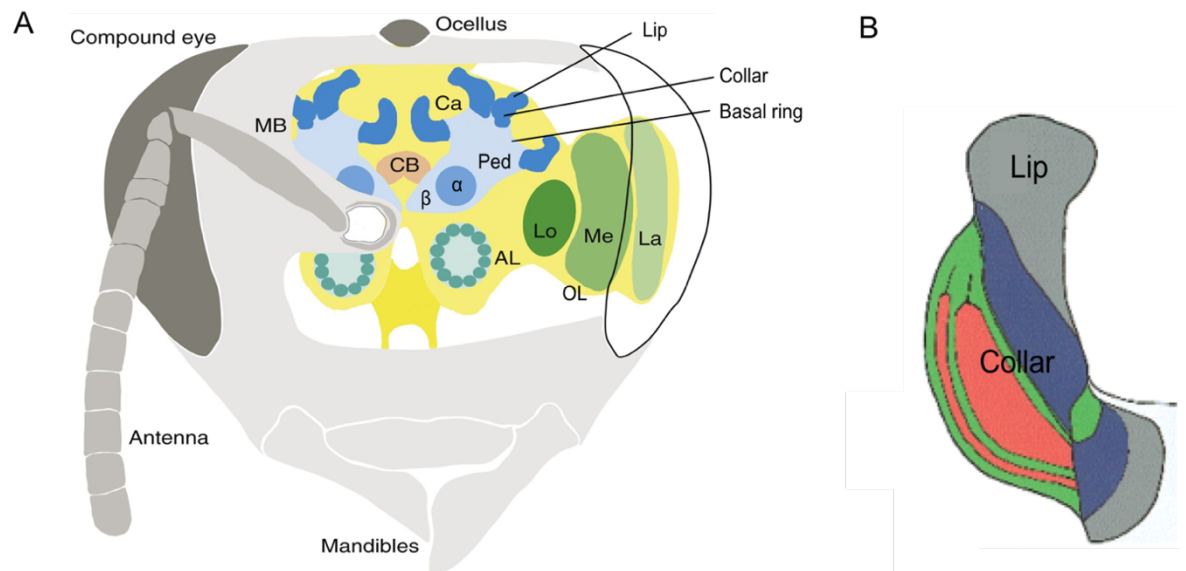
Figure 1.3. Schematic frontal view of the bee brain with head capsule removed and schematic calyx sections. (A) Bee brain. The mushroom body (MB) is the high-order sensory integration center and main site of learning and memory, which is comprised of cup-shaped calyces (Ca), peduncles (Ped) and information output lobes, α-lobes (α) and β-lobes (β). The calyx is subdivided into the lip (olfactory input region), collar (visual input region) and basal ring (both olfactory and visual input). Optic lobe (OL) consists of three layers, lamina (La), medulla (Me) and lobula (Lo). AL, antennal lobe; CB, central body. Modified after Chittka and Niven (2009). (B) Schematic calyx sections. Outer region of the collar receives visual input from the medulla (Red: from the dorsal medulla; Green: from the ventral medulla) and inner region of the collar receives visual input from the lobula (blue). Modified after Ehmer and Gronenberg (2002).

Microglomeruli: synaptic relays in the mushroom bodies

Within each of the three regions in the calyx, the neuronal connections are organized in synaptic complexes called microglomeruli, each consisting of a single presynaptic bouton from the axon terminal of a projection neuron surrounded by several postsynaptic dendrites of intrinsic neurons, called Kenyon cells (Yasuyama et al., 2002; Groh and Rossler, 2011) (Figure 1.4). A great number of synaptic connectivity patterns are attainable by spine or bouton growth (Chklovskii et al., 2004; Stepanyants and Chklovskii, 2005; Holtmaat and Svoboda, 2009).
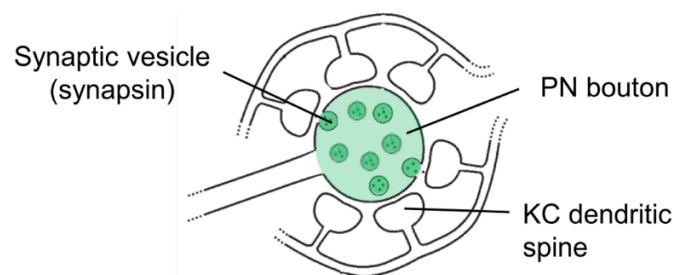
Figure 1.4. Schematic representation of one microglomerulus in the mushroom body calyx. A presynaptic projection neuron (PN) bouton is surrounded by Kenyon cell (KC) dendritic spines. Modified after Falibene et al. (2015).

A microglomeruli-detecting method has been developed for insect brains (Groh et al., 2012; Falibene et al., 2015). Each microglomerulus can be visualized by anti-synapsin I antibody via immunolabelling. The density of microglomeruli in previous studies was measured using optical sections (Hourcade et al., 2010; Groh et al., 2012). In general, three to five small circles or cuboids with certain size (such as 400 μm^2 or 1000 μm^3) were selected manually in the lip or collar regions of the calyx and the microglomeruli were counted in the defined regions. This allowed the calculation of microglomerular density per circle or

cuboid. The average microglomerular density of all the selected circles or cuboids represents the microglomerular density of the lip or collar. The volume of the lip or collar was measured directly by optical sections, so that the total number of microglomeruli could be estimated.

Synapsin is a presynaptic vesicle-associated protein shown to regulate new synapse formation (Ferreira et al., 1998) and associated with long-term memory formation (Morimoto et al., 1998; Sato et al., 2000; Hart et al., 2011). Identification of microglomeruli via immunolabelling has shown that age, age-dependent behavioral changes, temperature during pre-adult development, developmental changes and foraging activities of honeybees, bumblebees and ants lead to synaptic organizational and structural changes within the mushroom body calyces (Krofczik et al., 2008; Stieb et al., 2010, 2012; Groh et al., 2012; Fahrbach and Van Nest, 2016). In addition, the establishment of long-term memory is accompanied by synaptic plasticity within the mushroom body (Hourcade et al., 2010; Falibene et al., 2015). A summary of different factors that have varied effects on microglomerular organization in bees and ants is shown in Table 1.1. However, the existing works exploring how visual information affects the synaptic organization in the insect brain have been limited to simple light exposure or deprivation (Stieb et al., 2010, 2012; Scholl et al., 2014). In Chapter two, I examine how visual learning and visual experience in bumblebees affect microglomerular organization.

Table 1.1. Summary of different factors that show varied effects on microglomerular organization in bees and ants.

Factors	Microglomerullar density	Total number of microglomeruli	Calyx volume	Citation
Age (↑)	↑	↑	↑	Stieb et al., 2010; Falibene et al., 2015
Sensory stimulation	↓	↓	↑	Stieb et al., 2010, 2012; Scholl et al., 2014
Age-related behavioral changes (e.g. from nursing to foraging)	↓	↓	↑	Groh, et al., 2012 Stieb et al., 2010;
Long-term memory formation	↑	↑	− (keep constant)	Hourcade et al., 2010; Falibene et al., 2015

↑: Increase; ↓: Decrease.

1.3 Individual variation in learning ability and their underlying mechanism

Individual variation in general learning abilities is common and has been observed in many animals, from insects to humans (Süß et al., 2002; Conway et al., 2003; Matzel et al., 2003; Raine and Chittka, 2008; Kotrschal and Taborsky, 2010; Snell-Rood et al., 2011; Muller and Chittka, 2012). For example, some bumblebee individuals were found consistently better than others at discriminating stimuli across modalities (visual or olfactory) (Muller and Chittka, 2012). This variation affects animals' fitness and enables animals to respond flexibly to environmental variation in an adaptive manner (Dukas, 2008; Muller and Chittka, 2008; Hoedjes et al., 2011; Smid and Vet, 2016). Smid and Vet (2016) argue that insect learning and memory cannot be described as good or bad at individual, population or species level. Rather, the different learning or memory abilities allow them to optimally deal with the specific ecologies of their environments.

It has been suggested that individual variation in general cognitive ability correlated with brain size, working memory capacity, genetic background and sensory response sensitivity (Conway et al., 2003; Page et al., 2006; Dukas, 2008). However, nervous tissue size tells us little about why there are individual cognitive differences and the neural basis of this individual variation is still unknown. In my thesis, I examine the relationship between synaptic plasticity and individual learning ability and explore the neural mechanisms underlying individual cognitive differences.

1.4 Outline of thesis

Most molecular studies in bees are based on bees' olfactory learning and memory with PER conditioning and evaluated the learning/memory performance using tethered bees. In fact, the most impressive cognitive abilities of bees are in the visual domain, and bees need to be able to move freely in such tasks (Avarguès-Weber et al., 2010; Srinivasan, 2010). Thus, I choose to study the neurobiological basis of bees' visual learning and memory, from synaptic morphology and gene levels.

Chapter 2. Olfactory learning and memory formation can result in increases of synaptic complex (microglomeruli) density in honeybees and ants (Hourcade et al., 2010; Falibene et al., 2015). However, whether visual learning can increase synaptic complex (microglomeruli) density is still unclear. In Chapter 2, I aim to understand how the visual learning and experience relate to specific changes in the synaptic organization. Bees with different amount of visual learning and visual experience have been collected and the microglomeruli organization in bees' brain has been examined with immunolabelling and confocal microscope.

Chapter 3. The neural basis of individual differences in learning and memory ability is poorly understood. In Chapter three, I aim to understand how individual cognitive variations in color discrimination task correlate with the synaptic organization. A complex visual learning task is designed, and bees show variations for their learning speed and retention performance in this task. Then the microglomeruli organization in bees' brain is examined.

Chapter 4. Transcription is a molecular requisite for long-term synaptic plasticity and long-term memory formation. In Chapter 4, I analyze the dynamic transcriptional expression changes after learning which are required for long-term memory formation and find the visual learning and memory-related genes. Bees are sampled immediately and four hours after new color learning and the gene expressions in their brains are measured by transcriptome sequencing.

Overall, by these studies, my thesis aims to contribute to our knowledge on the presynaptic structural plasticity and gene regulations underlying learning and memory, and gain understanding about the molecular mechanisms of bee visual learning and long-term memory formation.

Chapter 2

Effects of visual learning and visual experience on synaptic organization in the mushroom body calyx of the bumblebee *(Bombus terrestris)*

Summary

Learning and experience have been linked to changes within the brain's neuronal architecture. But how particular types of learning and experiences relate to specific changes in synaptic connections is still poorly understood. In this study, I aimed to decipher how visual learning and visual experience affect synaptic plasticity in the brain of the bumblebee *Bombus terrestris*. Using behavioral paradigms, immunocytochemistry, confocal microscopy, and 3D-based quantification, my experiments show that visual learning can lead to microglomeruli density increase in the visual information input region of the mushroom body, i.e. the collar of the calyx region, but that simple exposure to color information may play a role in experience-dependent changes in microglomeruli density, and foraging activity alone may be responsible for changes in calyx volume.

2.1 Introduction

Neuronal modifications take place throughout life in many animals and allow individuals to respond properly to changes in their environment or new situations (Kolb and Whishaw, 1998; Stiles, 2000; Burke and Barnes, 2006). Studies in vertebrates and invertebrates have shown that brain plasticity is associated with many factors, such as age, circadian rhythm, enriched environments and memory formation (Kolb and Whishaw, 1998; Fahrbach, 2006; Sale et al., 2009, 2014; Greenough and Black, 2013; Frank and Cantera, 2014). Some insect species have remarkable learning ability and complex behavior, but relatively small brains, and have become good models to study brain plasticity. Synaptic plasticity within the insects' mushroom bodies (brain centers for sensory integration, learning and memory) have been widely investigated. Identification of microglomeruli via immunolabelling in ants and bees has shown that age, age-dependent behavioral changes, temperature during pre-adult development, developmental changes and foraging activities lead to synaptic organizational and structural changes within the mushroom body calyces, the olfactory and visual information input regions (Fahrbach and Van Nest, 2016).

2.1.1 Age-related effect on microglomerular organization

Age-related behavioral transition from nursing to foraging is accompanied by a significant calyx volume increase and a decreased microglomerular density, which has been demonstrated in both honeybees and ants (Stieb et al., 2010; Groh et al., 2012; Muenz et al., 2015). Groh et al. (2012) compared the microglomerular density and the ultrastructure of microglomeruli in the calyx lip and dense collar regions of 1-day- and 35-day-old honeybees. They found that the volumes of the calyx subdivisions increased significantly

while the microglomerular density in the lip and collar decreased significantly in 35-day-old foragers when compared with 1-day-old bees. The total number of microglomeruli in the calyx was less in 35-day-old foragers, which reveals that calyx volume increase is not accompanied by new microglomruli formation. Structural analysis showed that the bouton volume and surface area, the percentage of bouton with high vesicle densities and the number of postsynaptic sites increased in 35-day-old foragers compared with 1-day-old bees. Overall, their findings suggested that the volume increase of the mushroom body calyx and microglomerular density decrease during age-related behavioral transition is mainly caused by the outgrowth and connectivity of Kenyon cell dendrites, and the pruning of projection neuron boutons. In addition, Farris et al. (2001) examined the effect of age and foraging experience on dendritic morphology using Golgi impregnation. They had provided evidence that the length and branches of Kenyon cell dendrites were positively correlated with age and foraging experience and that the collar neuropil volume increases observed in expeirenced foragers and older bees were due to growth of Kenyon cell dendrites. The same calyx volume and microglomerular density changes can be seen in ants' behavior transition from inside brood care to outside foraging (Stieb et al., 2010). The effect of age, independent of behavior, on synaptic organization in the calyx has been examined in bees and ants, and aging can increase the microglomerular density, total number of microglomeruli, bouton volume and calyx volume (Krofczik et al., 2008; Stieb et al., 2010; Muenz et al., 2015).

2.1.2 Sensory and experience effect on microglomerular organization

Simple sensory stimulation, such as exposure to light or odor without formed associations, can cause microglomerular density and microglomeruli number to decrease, and calyx volume to increase. Stieb et al. (2010, 2012) and Scholl et al. (2014) studied the synaptic plasticity in the mushroom body calyx triggered by light exposure. Stieb and colleagues examined the microglomerular organization in two groups of ants. The control group was kept in dark and the light-exposed group was exposed to the sun (or an artificial light source) five times a day (45 minutes each time) for four days. They found that light exposure caused a significant decrease of microglomeruli number and microglomerular density in the collar, when compared with dark-reared ants. No differences were found between these two groups in the lip (the olfactory input region), which indicates modality-specific plasticity (Stieb et al., 2010, 2012). Similar results were found comparing dark-reared and light-exposed honeybees (Scholl et al., 2014). Falibene et al. (2015) compared ants that foraged on one plant species (one odor exposure) with those that foraged on ten different kinds of leaves (ten odors exposure) over three consecutive days. Ants exposed to ten odors displayed a significant reduction in microglomerular density in the non-dense lip region compared to ants exposed to only one odor. However, there were no significant differences in microglomerular density in the collar, or in lip and collar volume between the two groups. All these findings indicate that sensory stimulation can induce synaptic changes within the related sensory input region. However, how synaptic organization changes due to exposure to color information is unknown. One of my aims in this study is to examine the effect of color exposure on synaptic plasticity.

2.1.3 Long-term memory effect on microglomerular organization

The establishment of long term memory is accompanied by synaptic plasticity within the mushroom body of the insect brain (Kolb and Whishaw, 1998; Stiles, 2000; Hourcade et al., 2010; Falibene et al., 2015). Hourcade et al. (2010) trained bees to associate an odor with sucrose by proboscis extension reflex and compared microglomerular density in four groups of bees (Paired group: bees were trained to associated odor with sucrose; Unpaired group: bees were exposed to odor and sucrose separately; Naive group: bees were only placed in the setup without exposure to odor or sucrose; Paired ActD group: bees were trained to associated odor with sucrose and then were injected with ActD three hours after learning which could block long-term memory formation). Their results demonstrated that the microglomerular density in the lip was significantly higher in the paired group (long-term memory formation group) compared with other groups, while the lip volume remained constant. Similar results were found in ants; Falibene et al. (2015) trained ants to avoid one type of plant leaves that are naturally accepted by them, but are rejected when the leaves were treated with fungicide that is harmful to the ants' symbiotic fungus. This kind of long-term avoidance olfactory memory formation resulted in an increase of the microglomerular density in the lip, while the volume of this brain region stayed constant.

However, how visual learning affects brain plasticity is still poorly understood, as is the question of how particular types of learning and experiences relate to specific changes in synaptic connections. Bees' visual learning is important, since it enables them to form associations between visual information (such as flower colors and patterns) and food while foraging. Bees are able to discriminate various colors, shapes, and patterns in free flight (Lehrer et al., 1995; Dyer and Chittka, 2004c; Giurfa, 2007). Understanding of the synaptic

plasticity underneath bees' visual learning and memory formation can bring new insight to the mechanism of long-term memory formation. In this chapter, I examined the synaptic changes in the calyces of the MBs in response to different visual learning experiences of the bumblebee (*Bombus terrestris*). My findings show that visual learning can increase the microglomeruli density in the collar of the calyx, while color exposure (visual experience) alone may contribute to microglomeruli density increase. My results also provide robust evidence that collar region of the mushroom body calyx is involved in color learning and to the best of my knowledge this is the one of the first studies examining the effect of visual learning on microglomerular organization.

2.2 Materials and Methods

Animals and setup

Bumblebee colonies (*Bombus terrestris*) were purchased from Biobest Belgium NV (Westerlo, Belgium). All colonies were settled in wooden nest boxes ($40 \times 28 \times 11$ cm), which were connected to small flight arenas ($65 \times 45 \times 25$ cm) with a Perspex corridor ($25 \times 3.5 \times 3.5$ cm) (Figure 2.1). Small doors in the corridor allowed us to control which bees were able to enter the arena at any one time. Prior to training and experiments, bees were kept in the dark. Each day, newly emerged bees were marked with a number tag (Opalithplättchen, Warnholz & Bienenvoigt, Ellerau, Germany) glued (with superglue) to the top of the thorax to identify bees individually and to know their age. Bees were marked under red light, since red light is in the periphery of their visual spectrum and they can therefore see it only poorly (Chittka and Waser, 1997), to ensure visual color information

for bees was kept at a minimum. During experiments, the back chamber of the nest box was kept in the dark with a piece of cardboard while the front chamber of the nest box was open to light and illumination which was controlled with a 12 h day-night cycle (8:00 am - 8:00 pm) by a timer (Figure 2.1). All bees used in experiments were similar in age at time of collection (12.9 $\pm$ 0.2 days), i.e. end of experiment. Bumblebees were kept within the hive nest box without access to the arena until they were trained according to the experiment by using plastic doors in the corridor (Figure 2.1). 40% sucrose solution was pipetted directly into colony cells every day and no feeder was provided in the nest box to make sure bees had no foraging experience prior to pre-training.
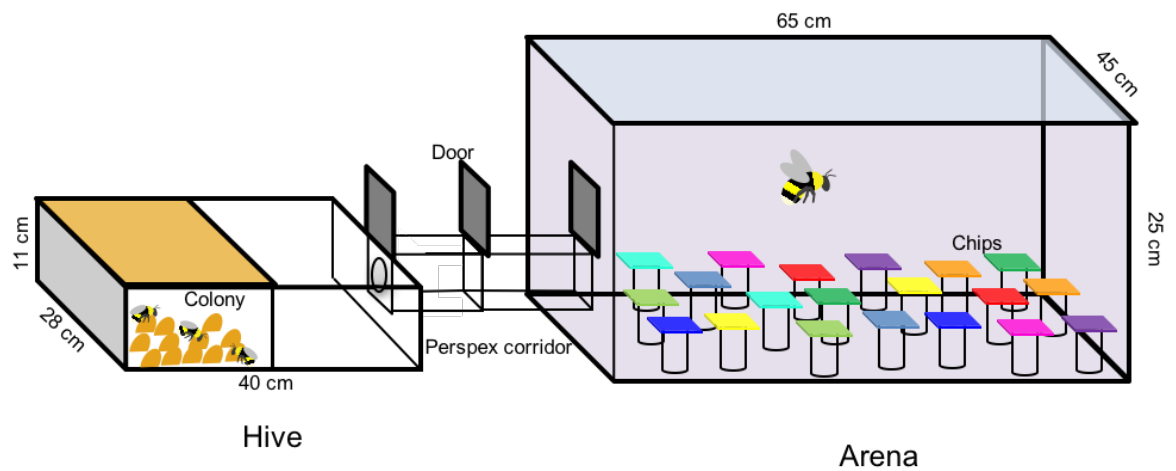
Figure 2.1. Experimental setup. Bees were housed in a bipartite wooden nest box (40 × 28 × 11 cm) which were connected to a small arena (65 × 45 × 25 cm) with a Perspex corridor. All bees were trained and tested in the same arena and on the same size Perspex chips (25 × 25 mm). The artificial flowers in the arena show the colored chips used in the 10 Color Learning group.

Pre-training

All bees except those in the No Color Learning group in Experiment 1 were first trained to land on colorless transparent chips (Perspex chips, 25 × 25 mm; artificial flowers) with 7 μl 40% sucrose solution. Flowers were arranged in a pseudorandom array of 10 flowers within the arena, each on top of a small glass vial. Bees successfully foraging from the transparent chips and returning to the colony 8-10 times on a regular basis (inter-trip interval within 5 minutes) were moved on to the training phase (Figure 2.2). Bees foraged together for most of their trips during pre-training (a trip is defined as the event between when a bee entered the arena from the nest to forage and when she returned to the nest to unload her collected crop). Worker (female) bumblebees of large size were selected visually, but their size was later quantified by measuring head width (maximal distance between the distal surfaces of the eyes measured in dorsal aspect; 4.2-5.1mm within a total range found to be 2.8-5.3mm (Hagen and Dupont, 2013)) as a proxy of body size, since head width is correlated with both body size and brain volume (e.g. Mares et al., 2005). Flowers were refilled during pre-training and training.

Training

Experiment 1. Age-matched bees (n = 42; 13.1 $\pm$ 0.3 days at end of experiment) were randomly assigned to three different groups. Bees in the **No Color Learning group** (no pre-training) were allowed to land and feed from one clear chip, then collected immediately landing on a second clear chip (Figure 2.2). This collection method was employed to confirm that bees were foragers and allowed us to ensure that no long-term memory of visual information could be formed and no synaptic changes would occur within the brain areas to be examined since time of first landing to collection was less than one minute. For

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the **2 Color Learning group**, bees foraged on 20 flowers, half of them (green) with 7 μl 40% sucrose solution, and the other half (yellow) with 7 μl saturated quinine (1.2 mg/ml H_2O) (Figure 2.2). The spectral reflectance of green and yellow is shown in Figure 2.3. Each bee was trained individually and had five foraging trips with an inter-trip interval of 10 min. For the **10 Color Learning group**, bees were trained individually to discriminate five different colored chips (25 × 25 mm) containing sucrose solution from five different colored chips containing bitter quinine solution (Figure 2.2). The spectral reflectance of the 10 color artificial flowers and color hexagon coordinates of the ten colors were shown in Figure 2.3. There were two flowers for each color and 20 flowers in total in the arena. All rewarding flowers contained 7 μl 40% sucrose solution and all unrewarding flowers contained 7 μl saturated quinine solution (1.2 mg/ml H_2O). Color loci nearest each other were split between rewarding and unrewarding (Figure 2.3A), so that the task would be more difficult. Flowers were moved to pseudorandom locations in the arena between trips to prevent bees from associating certain spatial locations with reward or color. Each bee had five foraging trips and the inter-trip interval was 10 min. Bees naturally returned to their nest to unload the collected sucrose solution once they filled their crop. Bees were confined to the nest for two days after training to prevent any further foraging experience. During this time, the colony was fed with 40% sucrose solution pipetted directly into their cells every day (approximately 10 mL). On day three, bees received a memory retention test on the same flower setting as in training, except that each flower contained 7 μl water (without sucrose). All bees in the 2 Color and 10 Color Learning groups were collected immediately after the memory retention test. Comparing these groups gave us three levels of learning (no color learning, simple color discrimination, complex color discrimination)

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to compare microglomerular density in the visual input region of the bee mushroom bodies after exposure to these tasks.

Experiment 2. Age matched bees (n = 37; 12.6 $\pm$ 0.3 days at end of experiment) were randomly assigned to three different groups. The **10 Color Learning group** experienced the same training procedure as the 10 Color Learning group in Experiment 1. Bees in the **Activity Control group** were trained to associate 20 clear chips with reward, so that they received the same foraging experience as the 10 Color Learning group, but without experiencing any colors (Figure 2.2). Bees in the **Color Control group** were trained to associate five clear chips with reward while 20 colored chips were in the arena at the same time (Figure 2.2). These 20 colored flowers contained no reward or water, and bees did not ever land on these colored chips, and therefore we know that bees did not learn any rewarding or punishing association with the colors. So that bees in this group received the same foraging experience and colors as the 10 Color Learning group, but without learning to discriminate any colors. All three groups of bees received the same training protocol, five foraging trips with 10 min inter-trip intervals. All bees were trained individually and collected immediately after the memory retention test conducted two days after training for immunolabelling.

In both experiments, flowers were cleaned with 70% ethanol in water between every trip to ensure no scent marks were being used to solve the task. Bees underwent five foraging trips with 10-minute inter-trip intervals (a paradigm that has been shown to cause long term memory formation in bees (Menzel et al., 2001). Inter-trip intervals were kept consistent because bees always attempted to come out prior to the 10-min time point and I would only need to prevent the bee from entering the arena using small doors in the corridor until the

10-minute interval had ended. Bees tended to return from their nest to the corridor each few minutes and therefore when a bee returned within minute before the 10-minute interval would end, the bee was prevented from leaving the corridor by closing the corridor doors until it was time for the next trip. Flower colors were chosen with relatively even distribution across the RGB spectrum. Flower spectral reflectance functions were measured in the laboratory using a spectrophotometer (Avantes AvaSpec-2048) with deuterium halogen source (AvaLight-DHS). The spectral reflectance and color information of the 10 colored flowers are shown in Figure 2.3. The color loci of stimuli were calculated in a hexagon color space (Chittka, 1992; Figure 2.3C) considering the spectral sensitivity functions of bumblebee photoreceptors (Skorupski et al., 2007). All the selected foragers (81 bees in total) were able to finish the trained task. Two of them could no longer fly in the retention test and therefore were excluded from my experiments (the analyses).
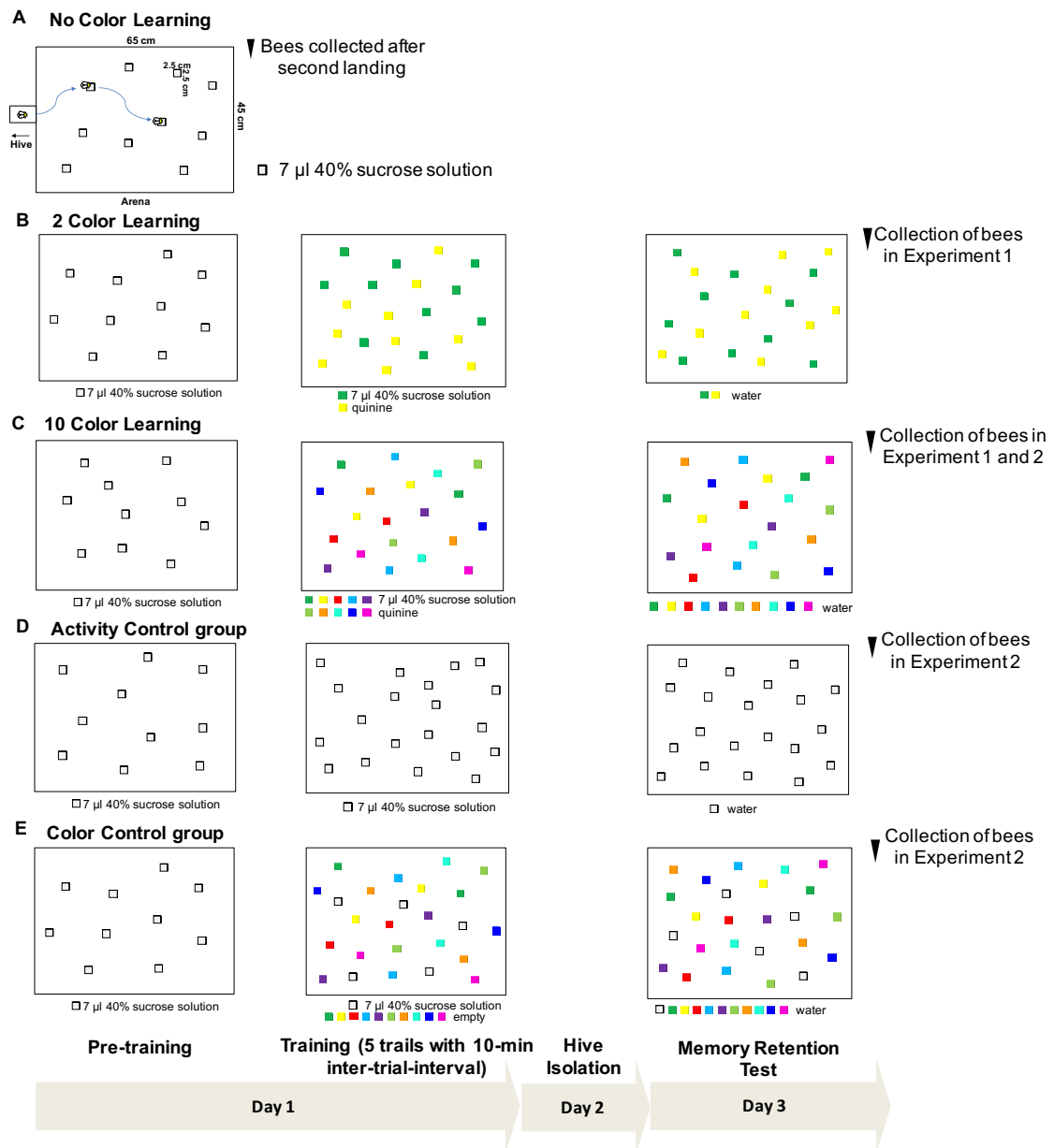
Figure 2.2. Training procedures. All bees were trained in the same arena (65 × 45 × 25 cm) and on the same size Perspex chips (25 × 25 mm). Before special training in each experiment, all bees except bees in no color learning group received the same pre-training procedure, in which bees were trained to forage on transparent chips with 7 μl 40% sucrose solution. (**A**) Bees in **No Color Learning group** in Experiment 1 were collected after their second landing in their first trip of the pre-training stage. These bees were foragers but with very limited foraging experience. After pre-training, (**B**) bees in **2 Color Learning group** in Experiment 1 were trained to discriminate two colored flowers; (**C**) bees in **10 Color Learning group** in Experiment 1 and 2 were trained to discriminate five rewarding (sucrose solution) colored flowers from five punishing (quinine) colored flowers; (**D**) bees in **Activity Control group** in Experiment 2 were trained to associate rewarding (sucrose) with transparent chips with the same training amount (5 foraging trips with 10-min inter-trip interval) in other groups; (**E**) bees in **Color Control**

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group in Experiment 2 were trained to associate rewarding (sucrose) with five transparent chips which were surrounded by 20 colored chips without any solution. Bees were left in the colony for two days without any further foraging experience. On day three, bees in each group received a retention test on each special flower settings and all flowers contained water. Bees were then collected immediately after retention test. The training phase in each group included 5 foraging trips with 10-min inter-trip interval which could lead to long-term memory formation.

A

Human Color		+	−	+ *	−	+ *	−	+	−	+	−
RGB values	242,242,242	255,1,1	255,152,1	243,255,1	152,255,1	1,255,61	1,255,213	1,182,255	61,1,255	182,1,255	255,1,213
Hexagon Loci (x,y)	-0.0819, 0.2193	-0.1916, 0.0392	0.0523, 0.0414	0.3316, -0.0407	0.4018, 0.0101	0.3790, 0.0238	0.3290, 0.0297	0.3025, 0.2003	0.2486, 0.2533	0.0026, 0.3127	-0.0685, 0.2628
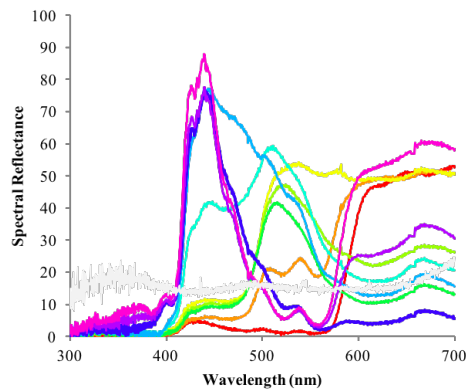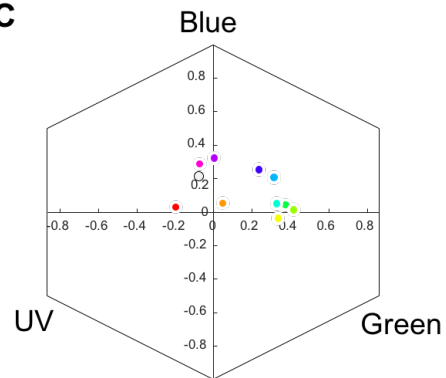
Figure 2.3. The information of colors used in experiments. (**A**) Human visual depiction of each of the colors used in experiments, with RGB values and bee vision hexagon loci. ✳ indicates the two colors used in the 2 Color Learning; + indicates the rewarding colors and − indicates the unrewarding colors used in 10 Color Learning. (**B**) Spectral reflectance plot of each of the colors used. (**C**) Loci of flower colors in bee color space, describing the range of colors a bee can see given their three photoreceptors sensitive to Blue, Green and UV light. Dots indicate each of the flower colors used in the experiments and are shown with the colors as they appeared to humans. The closer to the center the dot, the less saturated the color appears to the bee, and the closer to the edge, the more saturated the color appears. The closer the dots are together the more similar they look to a bee.

Quantification of microglomeruli in the mushroom body calyces

I established a methodology for immunolabelling of presynaptic terminals in whole-mount brains that enabled identification of microglomeruli, employing an antibody to the synaptic vesicle-associated protein synapsin I. My method combined the procedures from two previous studies (Ott, 2008; Groh et al., 2012), which enabled good immunostaining results where microglomeruli were clearly labelled with low background staining, very limited cell shrinkage and no cell fractionation. To check whether cell shrinkage or fractionation occurred during the preparation, brains which had been stained with synapsin were immunolabelled with DAPI, a fluorescent dye that binds to double-stranded DNA in the cell nuclei. Good nuclear morphology in the stained image indicates very limited shrinkage and no fractionation (Figure S2.1). Immediately after collection, bees were anesthetized with CO_2 by holding them a few centimeters above dry ice for five seconds. Up to five bees could be tested sequentially and collected each day of experiments. Once anesthetized, each bee was kept at -20℃ for approximately 10 min, enough time for the bee to be dead prior to dissection. Once the last of the tested bees had been placed at -20℃, the first bee was removed from -20℃, the head was then removed and head width was measured with Vernier calipers. Head width was used as a proxy of body size, since head width is correlated with both body size and brain volume (e.g. Mares et al., 2005). To dissect the bee brain, each bee's head was kept on ice and a rectangular window was cut in the head capsule to expose the brain. The semi-dissected heads were immediately immersed in ice-cold 4% formaldehyde and kept overnight at 4℃. The fixed head capsules were washed in phosphate buffer saline (PBS) twice and the brains were dissected out under PBS from the head capsule. After washes in 1% dimethyl sulfoxide (DMSO) in PBS (PBS/DMSO) (3×10

min) and in 0.2% Triton X-100 (Tx) in PBS/DMSO (PBS/DMSO/TX) (3×10 min), brains were permeabilized in 80% methanol/20% DMSO for two hours and then rehydrated through a methanol series (100% methanol, 1 hour; 90%, 70%, 50%, 30%, and 0% methanol in 0.1 M Tris buffer, pH 7.4, 10 min each). Prior to incubation in primary antibodies, brains were blocked in 5% normal goat serum (NGS; G9023-10ML, Sigma-Aldrich Company Ltd., Dorset, UK) in PBS/DMSO at 4℃ overnight. For synapsin immunolabelling, brains were incubated in a monoclonal mouse antibody against the Drosophila synaptic vesicle-associated protein synapsin I (SYNORF1, kindly provided by E. Buchner, University of Würzburg, Germany), diluted 1:10 in PBS/DMSO with 5% NGS for three days at 4℃. After several washes in PBS/DMSO/TX and then PBS/DMSO (one day), brains were incubated in Alexa Fluor 594–conjugated goat anti-mouse secondary antibody (115-585-062, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) (1:800) in 2.5% normal goat serum in PBS/DMSO for 2.5 days at 4℃. Brains were then washed in PBS/DMSO/TX (5×10 min) and PBS/DMSO (one day) and cleared in an ascending glycerol series (25%, 50%, 75% in PBS) until the brain sank to the bottom of the tube. Finally, the brains were stored in an anti-fade mounting medium prepared in accordance with the recipe (1:9 10X PBS:glycerol (ACS grade 99-100% purity) with 0.1 part 20% n-propyl gallate (Sigma P3130) added dropwise with rapid stirring) recommended by Jackson ImmunResearch Laboratories Inc (West Grove, PA). The whole brains were scanned using a laser-scanning confocal microscope (Leica SP5). For microglomeruli measurement, z-stacks were created by taking optical sections at 0.5 μm intervals with an x63 oil immersion objective at a resolution of 1,024 × 1,024 pixels. For calyx volume measurements, z-stacks were created by taking optical sections at 5 μm

intervals with an x20 oil immersion objective at a resolution of $1,024 \times 1,024$ pixels. Digital

images were processed using 3D reconstruction software Imaris 7.6 (Bitplane AG, Zürich,

Switzerland). Spheroidal structures of ~2.5μm were clearly visible at high magnification.

These microglomeruli represent distinct synaptic complexes in the calyx neuropil, each

comprising a central bouton from projection neuron axons surrounded by many KC

dendritic spines and processes from other extrinsic neurons (Yasuyama et al., 2002; Groh

and Rossler, 2011). Synapsin, which is associated with synaptic vesicles, stained the central

bouton of the microglomeruli. The microglomeruli counts for both collar and lip regions of

the calyces were determined by the Imaris 7.6 spot function. The spot function is created

by framing each specific region layer by layer through the 3D structure. The diameter range

of the microglomeruli was defined as being between 2.0 and 3.0 μm, set by measurement

of the microglomeruli through the Imaris function. Setting the diameter lower than this

range introduced background noise, and setting the diameter higher caused miscalculation

by overlapping parts. Results were visually confirmed to ensure that all defined

microglomeruli in this range were counted within each sampled section. Five cuboidal

volumes (7.8 μm × 7.8 μm × 7.8 μm) were manually selected in the lip or collar regions

and the synapsin-positive boutons were automatically counted in the defined regions

according to the diameter and staining intensity with background subtraction (Figure 2.4).

The average microglomerular density for each bee was then calculated by dividing the

average number of microglomeruli found in each cuboid by the volume of a cuboid,

474.552 μm^2. The five regions were dispersed uniformly throughout the entire lip or dense

collar region of the lateral calyx of one mushroom body of each bee. I chose, as others have,

to sample from only the dense collar region because this outer region of the collar receives

visual information from the optic lobe medulla (Ehmer and Gronenberg, 2002) and synapsin-positive microglomeruli are densely packed and homogenously distributed here (Stieb et al., 2010; Groh et al., 2012). The lip region is an oval-shaped structure on the top of each calyx branch and the collar region displays areas of dense and sparse staining. Note that measurements of entire calyx volume were used in my study because it was not possible to determine the boundary between collar and lip regions in each brain and therefore volume estimates of these regions would not be reliable. To ensure each cube was positioned distinctly within either the lip or the collar region, any area within 5 μm of these boundaries were avoided. Cubes were placed 1 μm away from the outer edge of the lip and collar. Counting and analyses were conducted blindly, as files were code-named by one individual and analyzed by a different individual. The number of microglomeruli in the lip and collar regions were averaged separately and the resulting means were used for later analyses. Using the Imaris 7.6 Surface function, the whole calyx was framed and the volume of the calyx was calculated directly from the surface determined.

To calculate the surface area of the presynaptic boutons, I used the Imaris 7.6 Surface function. Five cubes were manually selected in the lip and collar regions separately as described above. Within each of these cubes, the Imaris 7.6 Spot function was used to identify each microglomerulus. Subsequently, the Imaris 7.6 Surface function was applied to each selected cube and the surface area of each bouton was calculated according to staining intensity and background subtraction. The intensity threshold was set by Imaris automatically according to staining intensity and local contrast. The surface area was only recorded for those structures that the Imaris 7.6 Spot function had identified as microglomeruli.

Statistical analysis

GLMMs were used for each experiment to examine the effect of the different groups on microglomerular density, total calyx volume or surface area. Colony was not considered as a factor since only one colony was used for each experiment. Bee age and head width were random factors. Statistical tests were conducted with MATLAB (MathWorks, Natick, MA, USA). The significance level used was 5% in all analyses.
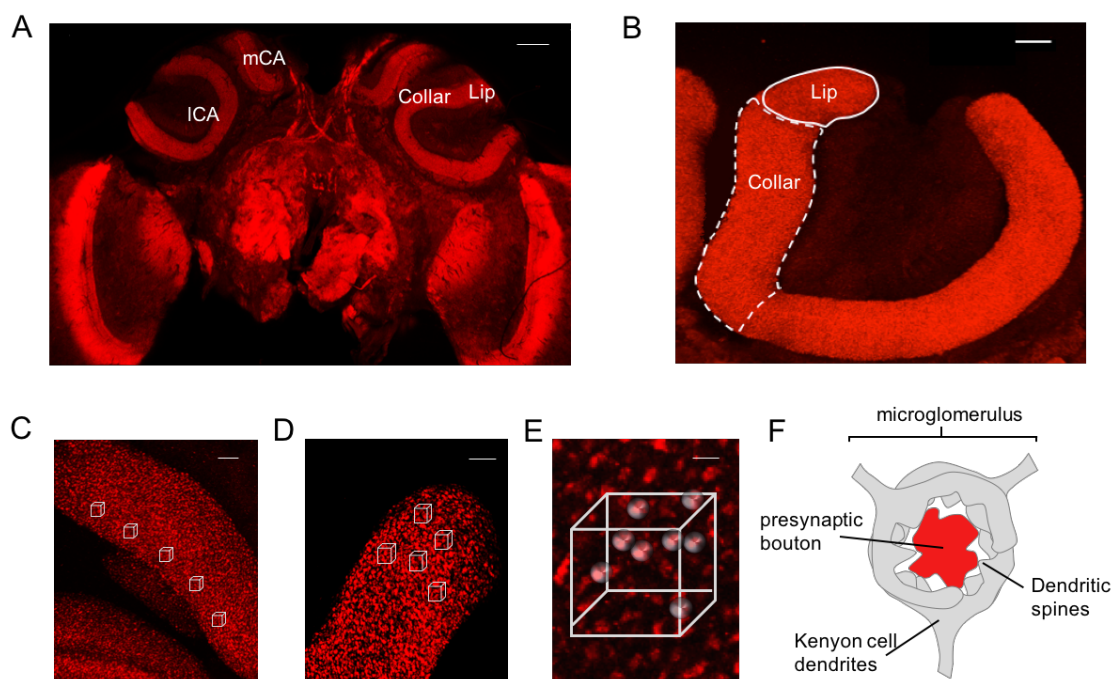
Figure 2.4. Quantification of microglomeruli. (**A**) Confocal section of the frontal view of an adult bumblebee's whole brain immunuolabelled with anti-synapsin (scale bar, 150 µm; lCA: lateral calyx, mCA: medial calyx). (**B**) 3D reconstruction of the right lateral calyx (scale bar, 50 µm; white solid line indicates lip region and white dotted line indicates collar region). (**C**) The left collar and (**D**) lip regions of right lateral calyx (scale bar, 20 µm), individual microglomeruli can be seen labelled with anti-synapsin. White outlines are example positions of selection cuboids (white). (**E**) Enlarged immunolabling view of an example cube. Each projection neuron bouton was visualized by spheres (gray) showing the position of each in a 7.8 µm × 7.8 µm × 7.8 µm cube. Scale bar, 2 µm. (**F**) Diagram of a microglomerular complex, including a presynaptic bouton from a projection neuron (red) and the postsynaptic endings of Kenyon cell neurons (gray).

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2.3 Results

2.3.1 The effects of visual learning on synaptic organization

To determine how visual learning affects synaptic organization, three groups of bees with different visual learning experiences (n = 42; No Color Learning, 2 Color Learning and 10 Color Learning; Experiment 1) were compared. Bees remembered the learned task in both two color learning (mean performance $\pm$ SD: 99 $\pm$ 2%; t-test: t = 93.24, df = 13, p < 0.0001, compared to chance expectation 50%) and ten color learning (mean performance $\pm$ SD: 91 $\pm$ 10%; t-test: t = 15.16, df = 12, p < 0.0001, compared to chance expectation 50%), but the retention performance was lower in ten color learning (t-test: t = -3.01, df = 25, p = 0.006) and bees took more trips to remember all five rewarding colors and avoid all five unrewarding colors, compared with two color learning (t-test: t = -4.61, df = 25, p = 1.02e-4). The microglomerular density within the collar region of the mushroom bodies of bees in the 10 Color Learning group was higher compared with the No Color Learning group (GLMM: p = 0.0156; Figure 2.5A and Table 2.1). No differences in microglomerular density were found in the lip (GLMM; Figure 2.5B and Table 2.1). However, the total calyx volume of the 10 Color Learning group bees was significantly different from No Color Learning (GLMM: p = 0.0011) and 2 Color Learning (GLMM: p = 0.0391) (Figure 2.5C and Table 2.1).

2.3.2 The effects of visual information on synaptic organization

The increases in both microglomerular density and calyx volume, in the 10 Color Learning group, could be due to greater foraging activity during training or increased visual experience or visual learning. To determine to what degree, in my paradigm, learning

induces changes in microglomerular density, I trained another three groups of bees in a similar paradigm (n = 37; Activity Control group, Color Control group, and Learning group; Experiment 2). All three groups of bees experienced similar amounts of foraging activity during training. Both the Color Control and Learning groups were exposed to similar amounts of color information. Only the Learning group experienced color learning and all bees remembered the learned color (mean performance ± SD: 82 ± 14%; t-test: t = 19.82, df = 11, p < 0.0001, compared to chance expectation 50%). Microglomerular density in the mushroom body collar of the Learning group was significantly higher than the Activity Control group (GLMM: p = 0.0143; Figure 2.5D and Table 2.2), suggesting that color learning increased microglomerular density, rather than any changes that might be caused by physical activity. However, there was no difference between microglomerular density in the mushroom body collar of the Learning group and the Color Control group (GLMM: p = 0.2895; Figure 2.5D and Table 2.2), suggesting that color information may play a significant role in synaptic plasticity observed during my visual learning paradigm. Again, no differences in microglomerular density were found across these three groups in the lip region (GLMM; Figure 2.5E and Table 2.2). Interestingly, the total calyx volume was not found to be different across groups (GLMM: p = 0.8658; Figure 2.5F and Table 2.2), suggesting that physical activity may be largely responsible for the calyx volumetric changes in my visual learning paradigm.
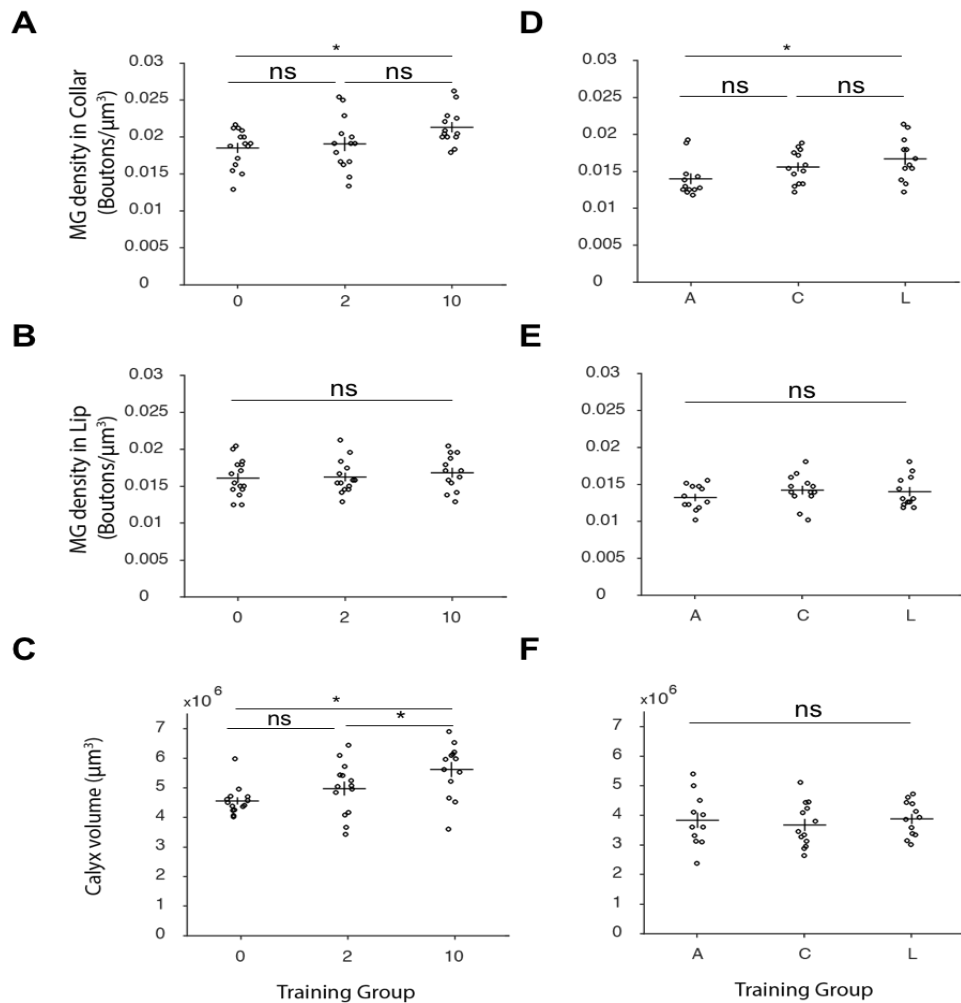
Figure 2.5. The effect of visual learning and visual information on synaptic organization. (**A-C**) The effect of different visual learning on synaptic organization (n = 42; 0: No Color Learning, n = 15; 2: 2 Color Learning, n = 14; 10: 10 Color Learning, n = 13; Experiment 1). A higher microglomerular density within the collar was found in the 10 Color Learning group compared to the No Color Learning group (**A**; GLMM; p = 0.0156; Table 2.1). No differences in lip microglomerular density were found across groups (**B**; GLMM; Table 2.1). Calyx volume of the 10 Color Learning group was significantly higher than in the No Color Learning group (**C**; GLMM; p = 0.0011; Table 2.1). (**D-F**) The role of color information on microglomerular density changes during color learning (n = 37; three groups: A: Activity Control group, n = 12; C: Color Control group, n = 13; L: Learning group, n = 12; Experiment 2). Microglomerular density in collar of the Learning group was significantly higher than in the Activity Control group (**D**; GLMM; p = 0.0143; Table 2.2). There were no differences across these three groups for microglomerular density in the lip (**E**; GLMM; Table 2.2) or calyx volume (**F**; GLMM; Table 2.2). Asterisks indicate significant differences (p < 0.05) and ns indicates not significant. Horizontal bars indicate mean. Vertical bars indicate standard error of the mean.

Table 2.1. Summary of generalized linear mixed models examining training condition factors in relation to microglomerular density (Collar or Lip) or Calyx Volume (Experiment 1).

Dependent variable	Fixed factors	df	Estimate	SE	F	P
Collar MG density	Intercept	1	0.0185	0.0008	595.64	0.0000
(No Color Learning)	2 Color Learning	2	0.0005	0.0011	0.25	0.6185
	10 Color Learning	2	0.0028	0.0011	6.40	**0.0156**
(2 Color Learning)	10 Color Learning	2	0.0023	0.0011	4.00	0.052

The dependent variable was the MG density in the collar. The training conditions were included as fixed factors. The reference conditions were the clear chip training (No Color Learning) and 2 Color Learning which were in bracket. The significant terms are highlighted in bold.

Dependent variable	Fixed factors	df	Estimate	SE	F	P
Lip MG density	Intercept	1	0.0157	$0.09e^{-3}$	277.55	0.0000
	Training condition	1	0.0004	$0.45e^{-6}$	0.6258	0.4336

The dependent variable was the MG density in the lip.

Dependent variable	Fixed factors	df	Estimate	SE	F	P
Calyx volume	Intercept	1	$4.56e^{6}$	$2.07e^{5}$	480.59	0.0000
(No Color Learning)	2 Color Learning	2	$0.42e^{6}$	$2.94e^{5}$	2.04	0.1614
	10 Color Learning	2	$1.06e^{6}$	$3.00e^{5}$	12.52	**0.0011**
(2 Color Learning)	**10 Color Learning**	2	$6.40e^{5}$	$3.00e^{5}$	4.58	**0.0391**

The dependent variable was the volume of the calyx. The training conditions were included as fixed factors. The reference conditions were the clear chip training (No Color Learning) and 2 Color Learning which were in bracket. The significant terms are highlighted in bold.

Table 2.2. Summary of generalized linear mixed models examining training condition factors in relation to microglomerular density (Collar or Lip) or Calyx Volume (Experiment 2).

Dependent variable	Fixed factors	df	Estimate	SE	F	P
Collar MG density	Intercept	1	0.0166	0.0007	506.72	0.0000
(Learning group)	**Activity Control**	2	-0.0027	0.0010	6.67	**0.0143**
	Color Control	2	-0.0011	0.0010	1.16	0.2895
(Activity Control)	Color Control	2	0.0016	0.0010	2.43	0.1286

The dependent variable was the MG density in the collar. The training conditions were included as fixed factors. The reference conditions were the Learning condition and Activity Control condition which were in bracket. The significant terms are highlighted in bold.

Dependent variable	Fixed factors	df	Estimate	SE	F	P
Lip MG density	Intercept	1	0.0129	$8.69e^{-4}$	220.13	0.0000
	Training condition	1	0.0004	$4.03e^{-3}$	0.97	0.3326

The dependent variable was the MG density in the lip.

Dependent variable	Fixed factors	df	Estimate	SE	F	P
Calyx volume	Intercept	1	$3.74e^{6}$	$3.36e^{5}$	123.72	0.0000
	Training Condition	2	$0.26e^{6}$	$1.54e^{5}$	0.03	0.8658

The dependent variable was the calyx volume.

2.3.3 Visual associative learning increases bouton surface area

Learning to discriminate colors must cause some changes to synaptic organization within the brain that are distinct from exposure to color information alone. The surface area of the presynaptic bouton is speculated to be an indicator for synaptic strength, assuming that a larger surface area equates to larger and/or more synaptic connections, resulting in greater synaptic transmission efficiency (Yeow and Peterson, 1991; Murthy et al., 2001). Therefore, using 3D reconstruction of boutons in both the collar and lip regions of the mushroom body calyces (Figure 2.6A-C), I investigated how the bouton surface area differed across the three training groups in Experiment 2. I found that the bouton surface area within the collar was significantly higher in the Learning group compared to both control groups (GLMM: $p < 0.001$; Figure 2.6D and Table 2.3). No significant differences were found across groups for bouton surface area in the lip region (GLMM: $p = 0.4754$; Figure 2.6E and Table 2.3). These results suggest that associative learning during a visual discrimination task may increase synaptic strength at the interfaces between visual projection neurons and the mushroom body intrinsic neurons (Kenyon Cells) of the bee brain.
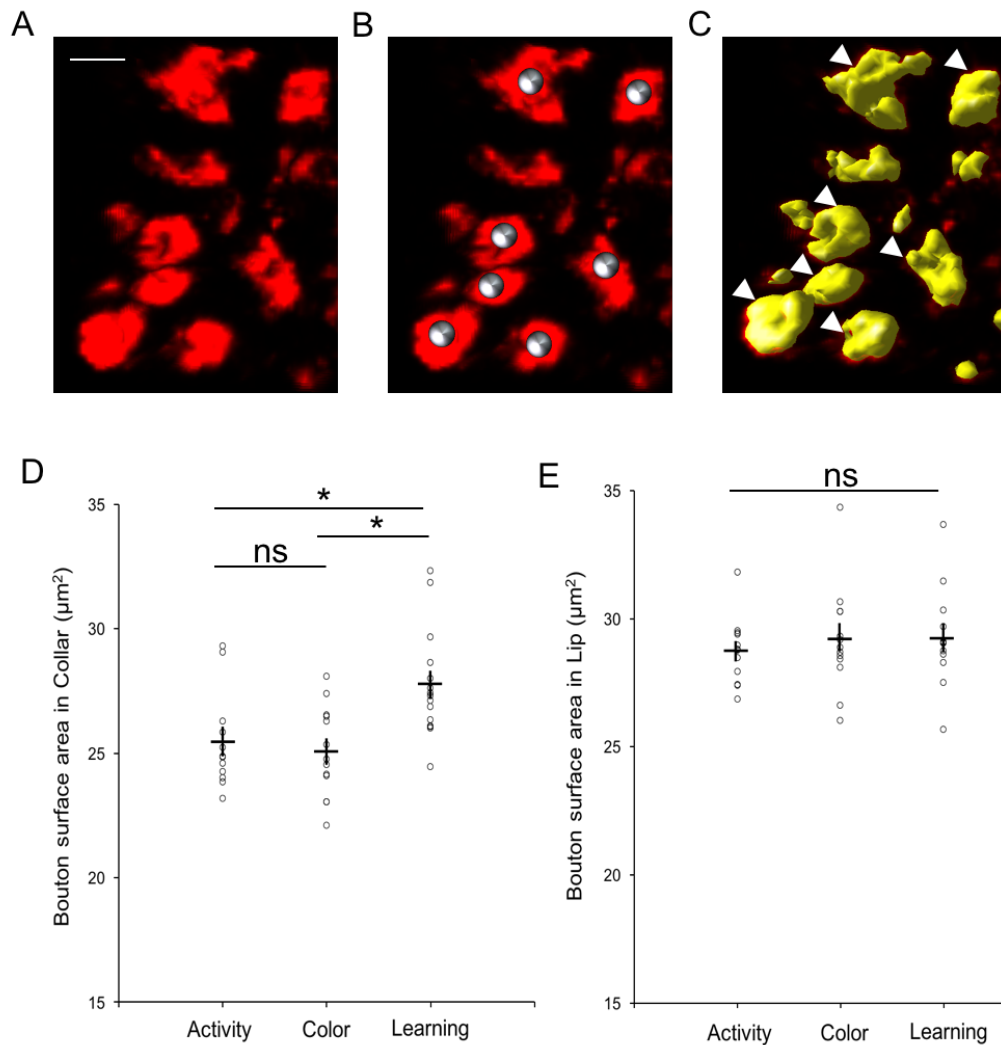
Figure 2.6. Effect of visual learning on microglomerular surface area. (A-C) Example 3D reconstructions of microglomeruli. (A) Raw image showing anti-synapsin immunostaining within the collar region. (B) Spheres identify microglomeruli using the Imaris Spot function (Materials and Methods). (C) Surface area reconstruction for presynaptic boutons using Imaris Surface function. Arrow heads indicate only those structures identified as microglomeruli in B (spheres). Scale bar in A-C, 2 μm. (D) Bouton surface area in the collar was significantly higher in the Learning group compared to both control groups (n = 37; Activity Control group, n = 12; Color Control group, n = 13; Learning group, n = 12; GLMM; Learning vs Activity: p < 0.001; Learning vs Color: p < 0.001; Table 2.3). (E) There were no significant differences across these three groups for bouton surface area in the lip region (n = 37; Activity Control group, n = 12; Color Control group, n = 13; Learning group, n = 12; GLMM; p = 0.4754; Table 2.3). Black circles indicate average bouton surface area measurements per bee. Asterisks indicate significant differences (p < 0.05) and ns indicates not significant. Black horizontal bars indicate mean. Black vertical bars indicate standard error of the mean.

Table 2.3. Summary of generalized linear mixed models examining training condition factors in relation to MG surface area (Collar or Lip) (Experiment 2).

Dependent variable	Fixed factors	df	Estimate	SE	F	P
Collar MG Surface Area	Intercept	1	27.71	0.5126	2922.48	0.0000
(Learning group)	**Activity Control**	2	-2.28	0.7689	8.76	**0.0053**
	Color Control	2	-2.66	0.7523	12.46	**0.0011**
(Activity Control)	Color Control	2	-0.38	0.7947	0.23	0.6374

The dependent variable was the surface area of MG within the collar. The training conditions were included as fixed factors. The reference conditions were the Learning condition and Activity Control condition which were in bracket. The significant terms are highlighted in bold.

Lip MG Surface Area	Intercept	1	28.53	0.7901	1303.93	0.0000
	Training condition	1	0.26	0.3664	0.52	0.4754

The dependent variable was the surface area of MG within the lip.

2.4 Discussion

Formation of long term memory accompanied by greater microglomerular density has been shown within the olfactory domain both in honeybees (Hourcade et al., 2010) and ants (Falibene et al., 2015). However, I show for the first time that changes in microglomerular organization can be induced via acquisition of visual memory, and both associative color learning and color exposure play roles in microglomerular density increase.

General findings

To assess how visual learning may affect synaptic organization, I examined how microglomerular density and calyx volume changed depending on the amount of color learning (10 colors; 2 colors; no colors). Microglomerular density in the calyx collar, as well as the volume of the entire calyx, of bees that had learned 10 colors was significantly higher than in bees that had received no training or had learned two colors, indicating that long-term visual memory formation may result in greater microlomerular density in the

collar and volumetric changes within the calyx.

In fact, the increase in microglomerular density and the calyx volume in the 10 colors learning group could have come from associative color learning, color stimulation or foraging activity. These were not controlled in Experiment 1. To explore the contribution of each of these factors to the observed microglomerular density and calyx volume increase, Experiment 2 was conducted. In the collar region, microglomerular density was significantly greater in the Learning group compared with Activity Control group, while no differences were present between any other two groups. These results suggest that although color learning (including associative color learning and color exposure) can increase microglomerular density, simple exposure to color information may play a significant role in the microglomerular density differences seen through color learning. Visual stimulation via simple light exposure is known to induce changes in microglomerular density. When ants and bees were exposed to light for three or four days, the microglomeruli density in the visual input region can be changed (Stieb et al., 2010, 2012; Scholl et al., 2014).

Comparisons with previous works

A recent study has similarly examined the relationship between visual discrimination learning and mushroom body calyx microglomerular density in honeybees (Sommerlandt et al., 2016). Sommerlandt and colleagues found no difference between honeybees trained with differential conditioning, with absolute conditioning, and stimulus-naïve bees. Age and experience of bees, which were not controlled for in their study, could very likely explain their results, because a large variation in age and experience across honeybees could

have overshadowed any changes in microglomerular density from learning a two-color discrimination task. In my study, the advantage of working with bumblebees was that both age and experience could easily be controlled for, mainly because bumblebees, unlike honeybees, can live and forage comfortably in a laboratory hive box and arena. Therefore, with bumblebees, I am able to know the individual age of every bumblebee and control precisely what experience each bee has prior to and during the experiments.

Structural plasticity can also manifest itself via more gross volumetric changes, especially in response to foraging experience (Ismail et al., 2006; Maleszka et al., 2009; Groh et al., 2012). I found that total calyx volume was significantly greater in the 10 Color Learning group. However, Hourcade et al., (2010) showed that long-term olfactory memory formation did not affect lip volume of honeybees after formation of odor memories. The apparent conflict of these results and mine may be explained by the difference in difficulty of tasks used (or perhaps the difference in sensory modalities). Hourcade and colleagues trained bees to only one odor, and used restrained bees, which limited both physical activity and all incoming sensory information. I used free-flying bumblebees exposed to ten different colors. In addition, others have shown that visual stimulation (exposure to light, sun or artificial light source, in five intervals of 45 min every two hours for at least three to four days) induces volume increases in the collar of the mushroom body calyx in ants (Stieb et al., 2010, 2012). It is possible that experiencing a greater number of different training stimuli during free-flight might be responsible for the volumetric changes I observed.

A plethora of studies have shown that increased number of environmental stimuli in which an animal interacts with (environmental enrichment) induces both structural and functional neural plasticity as well as improved learning and memory (Van Praag et al., 2000). It may

be that within my controlled environment of the lab, experience of 10 novel colors may represent an enriched environment able to induce significant structural reorganization in the visual regions of the brain.

Intriguingly, there were no significant differences for the total calyx volume among Learning, Color Control and Activity Control groups, in Experiment 2. My results indicate that physical activity may contribute to the total calyx volume changes in the 10 Color Learning group (Experiment 1). Alternatively, stimulation other than flower color (e.g. spatial, direction, olfactory, tactile information etc.) may contribute to differences in whole calyx volume, which might have overshadowed any changes due to learning or color information. In fact, natural foraging activity has been shown to induce differences in the volume of mushroom bodies (Farris et al., 2001; Maleszka et al., 2009).

The effect of visual learning on bouton surface area

The presynaptic bouton surface area tightly and positively correlates with several other synaptic structural elements, including number of synaptic vesicles, active zone area and postsynaptic density, and volume of postsynaptic spine (Yeow and Peterson, 1991; Murthy et al., 2001; Meyer et al., 2014). These anatomical changes lead to neurotransmitter release probability and are thought to be important for determining synaptic strength and efficiency (Yeow and Peterson, 1991; Murthy et al., 2001). I found that the bouton surface area within the collar was significantly higher in the Learning group compared with the two control groups (Experiment 2). Taking surface area of presynaptic boutons as a proxy of synaptic strength and efficiency, my results suggest that visual learning may lead to an increase in the synaptic strength between visual projection neurons and the mushroom body intrinsic

neurons of the bee brain. My findings suggest that exposure to color information and learning to discriminate multiple colors have distinct effects on synaptic organization. I speculate that a higher number of synapses may be a result of sensory information, readying an organism to learn faster and recall better by responding at a lower threshold (i.e. the enhanced connections between neurons enable the nervous system to respond quickly to a smaller number of stimuli). This lowered response threshold remains to be determined in bees; however, in support of this, an increased number of synapses in Drosophila sensory neurons causes a higher sensitivity to olfactory stimuli (Acebes and Ferrús, 2001). Learning then may cause an increase in presynaptic bouton surface area, indicating increased synaptic strengthening and efficiency (Yeow and Peterson, 1991; Murthy et al., 2001; Krofczik et al., 2008).

Some limitations of the method used in my study should be pointed out. The resolution of images taken by confocal microscopy may not be high enough to measure the bouton surface area precisely and the ultrastructure of microglomeruli, such as the number of presynaptic and postsynaptic sites, the size of synaptic sites and vesicle density in each bouton, cannot be seen with this method. Electron microscopy, with its higher resolution and magnification, makes it possible to investigate the ultrastructure of microglomeruli and should be used in the future to find out how associative memory formation changes synaptic ultrastructure. In addition, whether bouton surface area is a reliable indicator for synaptic strength and efficiency in bee brain is not established. Thus, the relationship between bouton surface area and other synaptic structures, such as the number of synaptic vesicles, active zone area and postsynaptic density, should be tested in bee brains in future work.

In summary, my results show that visual learning (associative color learning and color exposure) could induce changes in synapse density and morphology within visual information input region, but that exposure to color information may play a large role in experience-dependent changes in microglomerular density. Additionally, physical activity may come with experiences that contribute to the calyx volume changes. My findings give new insights into the mechanisms underlying visual learning and visual experience.

2.5 Supplementary information

Immunolabelling of cell nuclei

To examine whether there was cell shrinkage or fractionation occurred during synapsin immunolabelling, the synapsin-labelled brains were immunolabelled with DAPI, a fluorescent dye that binds to double-stranded DNA in the cell nuclei. Brains were incubated in 1µg/ml DAPI for five minutes and then were washed in PBS for three times. Please note that this experiment was conducted after that the synapsin stained brains have been scanned by confocal microscope. Good nuclear morphology in the stained image indicated very limited shrinkage and no fractionation happened in the preparation.
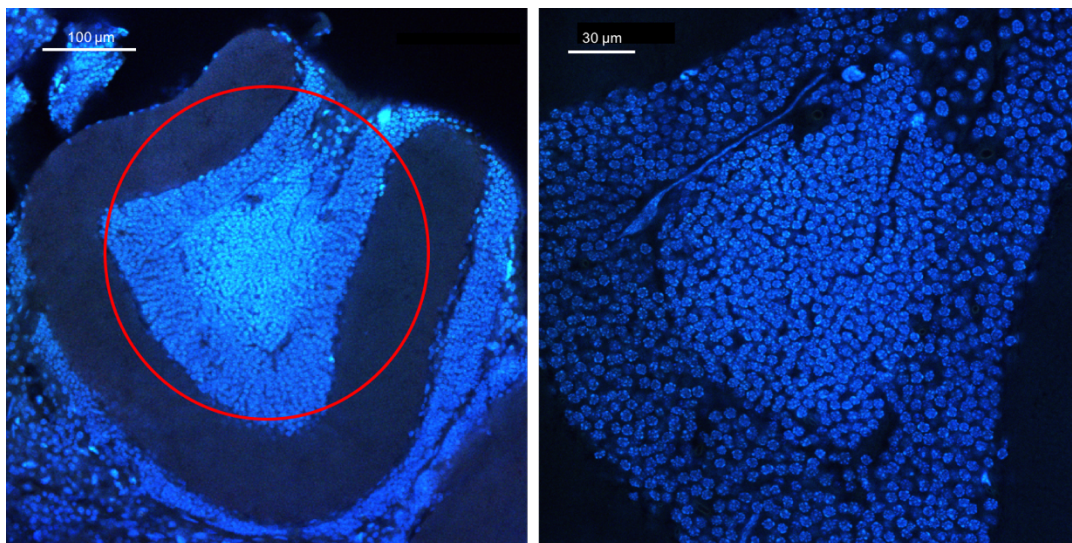
Figure S2.1. Cell nuclei in the right lateral calyx which were stained with DAPI. Left image: 3D reconstruction of the right lateral calyx immunuolabelled with DAPI, showing the nuclei (blue staining) in this region. Scale bar = 100 µm. Right image: Enlarged immunolablling view of the red circle in the left image. Good nuclear morphology in the image indicates no fractionation and very limited shrinkage happened in my immunolabelling method. Scale bar = 30 µm.

Chapter 3

The correlations between individual performance and synaptic connections in the mushroom body calyx of the bumblebee *(Bombus terrestris)*

Summary

Synaptic plasticity is considered to be the basis for learning and memory. However, the relationships between synaptic plasticity and individual differences in learning and memory are poorly understood. Here, using a visual discrimination paradigm, I explored how the organization of synaptic complexes (microglomeruli) within specific regions of the bumblebee (*Bombus terrestris*) brain relate to inter-individual differences in learning and memory performance. Using whole-brain immunolabelling to visualize synaptic organization, I found that bumblebees with a higher density of microglomeruli in the collar region (visual association areas) of the mushroom bodies of the bee brain learned faster (made fewer errors during training) and had better long-term memory (better retention of learned color/reward associations two days after training) during a visual discrimination task. Although my study does not provide a causal link between microglomerular density and performance, the observed positive correlations provide new insights into how neural structure may relate to inter-individual differences in cognitive ability and shed light on the neural underpinnings of learning and memory in a miniature brain.

3.1 Introduction

The search for the biological basis of learning and memory is as old as the field of cognitive science (Haier, 2011). Some studies in comparative cognition work suggest a positive correlation between general cognitive ability ('intelligence') and brain size (Snell-Rood et al., 2009; Kotrschal et al., 2013; Benson-Amram et al., 2016). However, the correlations found yield some inconsistencies (Healy and Rowe, 2007) and the overall size of nervous tissue tells us little about why there are individual cognitive differences (Chittka and Niven, 2009).

A mechanistic understanding of individual learning ability requires examining the underlying structures within the brain and how they change in relation to cognitive performance. Modifications of synaptic complexes are considered to be a basis for learning and memory (Bailey and Kandel, 1993; Kandel, 2001; Poo et al., 2016). Recent work and my study also showed that establishment of long term memory is accompanied by synaptic plasticity within the mushroom body of the insect brain for olfactory and visual learning (Hourcade et al., 2010; Falibene et al., 2015; my study in Chapter two). However, how inter-individual differences of animals' learning and memory performances are related to synaptic organization has been little explored (Fahrbach and Van Nest, 2016). Examining how individual cognitive abilities might relate to the underlying neural architecture will provide valuable information on the neural underpinnings of cognition in general. In the present study, I asked whether synaptic organization within the mushroom bodies of individuals correlate with their cognitive performance during the task.

The two color visual discrimination task is a classic training paradigm for studying bee learning and memory (Wittstock and Menzel, 1994; Lunau et al., 1996; Hill et al., 1997;

Leadbeater and Chittka, 2007; Ings et al., 2009). However, this is an easy task where performance variation between individuals is limited (i.e. colors are easily distinguishable); learning speed is consistently fast and memory retention is reliably good across individual bees given similar training. To study the neural correlates of differences in inter-individual learning ability, I thus designed a ten color learning paradigm, where bees had to distinguish five different rewarding colors from five different punishing colors (Figure 3.1). The rationale for this design was to make the visual task difficult enough to quantify differences in cognitive performance across individuals. Bees took more trips to remember all five rewarding colors and avoid all five unrewarding colors, compared with two color learning, and individual differences in memory retention varied enough to be examined, which has been shown in Chapter two.

Here, I show that individual differences in the ability to learn and recall visual information during a free-flight, "difficult" discrimination task are reflected in the microglomerular density of the mushroom body visual input region in the bumblebee brain.

3.2 Materials and Methods

Animals and setup

Animals and experimental setup are the same with that in Chapter two (please see details in Materials and Methods of Chapter two).

Pre-training

Pre-training is the same with that in Chapter two (please see details in Materials and Methods of Chapter two).

Training

Experiment 1. Bees were trained individually, utilizing acrylic doors in the corridor connecting the nest to the arena, to discriminate five different flowers (colored Perspex chips, 25 × 25 mm) containing sucrose solution from five different flowers containing bitter quinine solution (10 Color learning). The training procedures were the same with the 10 Color learning group in Chapter two (please see details in Materials and Methods of Chapter two; Figure 3.1). Each bee had five foraging trips and the inter-trip interval was 10 min. After training, bees were confined to the nest for two days to prevent any further foraging experience. During this time, the colony was fed with 40% sucrose solution pipetted directly into their cells every day (approximately 10 mL). On day three, bees received a memory retention test on the same flower setting as in training, except that each flower contained 7 μl water (without sucrose). All landings to flowers within three minutes of entering the arena were recorded. Age-matched bees (12.8 $\pm$ 0.4 days at end of experiment) were collected immediately after the retention test (three colonies) for immunolabelling.

Experiment 2. Training was performed exactly as in Experiment 1 and the 10 Color Learning group in Chapter two (please see detailed training procedures in Materials and Methods of Chapter two; Figure 3.1), but age-matched bees (n = 10; all 12 days at end of the experiment) were collected immediately after the final trip of the training on the first day for immunolabelling. Hours are required, after a behavioral experience, for new synapses to be formed, and for synapsin to increase to levels where microglomerular complexes are visible through immunolabelling (Morimoto et al., 1998; Nagerl et al., 2007; Zito et al., 2009; Hart et al., 2011). Therefore, collection of bees immediately after training

(< 50 minutes) ensured that no changes in pre-synaptic boutons could have taken place.

In both experiments, flowers were cleaned with 70% ethanol in water between every trip to ensure no scent marks were being used to solve the task. Bees underwent five foraging trips with 10-minute inter-trip intervals (a paradigm that has been shown to cause long term memory formation in bees (Menzel et al., 2001)). All the selected foragers (42 bees in total) were able to finish the trained task. One of them could no longer fly in the retention test and therefore was excluded from my experiments (the analyses).
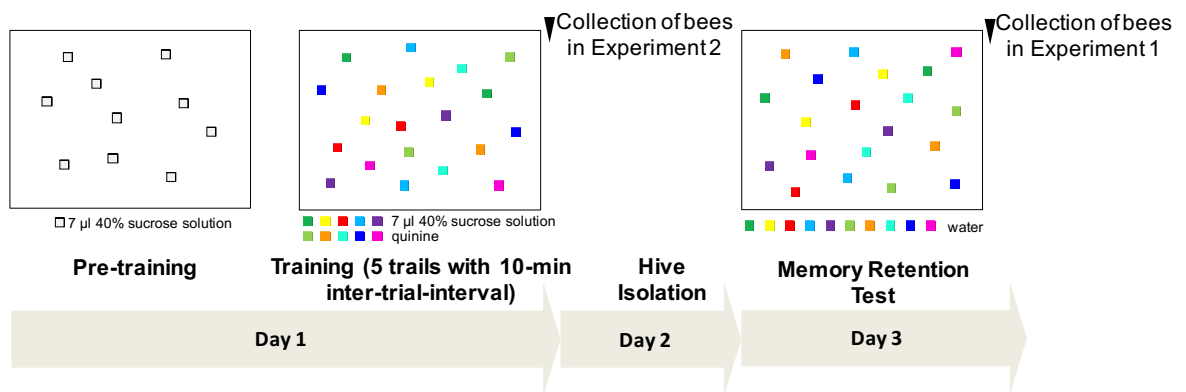
Figure 3.1. Training procedures in 10 Color Learning. All bees were trained in the same arena (65 × 45 × 25 cm) and on the same size Perspex chips (25 × 25 mm). Before training, all bees received the same pre-training procedure, in which bees were trained to forage on transparent chips with 7 μl 40% sucrose solution. After pre-training, bees were then trained to discriminate five rewarding (sucrose solution) colored flowers from five punishing (quinine) colored flowers. One group of bees was collected immediately after training (Experiment 2). The other group of bees were left in the colony for two days without any further foraging experience. On day three, this group of bees received a retention test and was collected immediately after that (Experiment 1).

Quantification of microglomeruli in the mushroom body calyces

The microglomeruli determination method is the same with that described in Chapter two (please see 'Quantification of microglomeruli in the mushroom body calyces' in Materials and Methods of Chapter two).

For memory retention assessment (Experiment 1), the proportion of landings on rewarding flowers in the retention test for each bee was calculated. A landing was defined as any time the bee was positioned on top of a chip and not flying for any amount of time. For learning speed (improvement on learning performance over trips) assessment (Experiment 2), a learning curve was obtained by fitting a first-order exponential decay function to the number of errors in each ten landings for each bee (Raine and Chittka, 2008) (Figure 3.2). An error was defined as any time the bee was positioned on top of a chip and not flying for any amount of time on an incorrect chip. The number of errors a bee made per number of choices were plotted and fitted to an exponential decay function with the equation $y = y_0 + Ae^{-x/t}$, where x is the number of flower choices the bee made since it entered the arena, and y is number of errors. The saturation performance level (y_0) is the errors made by a bee after finishing the learning process, i.e. when reaching a performance plateau (final asymptotic value of the y value). A is the curve amplitude (the maximum height of the curve above y_0). The decay constant (t) is a measure of learning speed: high values of t correspond to slow learning, whereas lower t values indicate faster learning. These t values were used for subsequent analysis.

Generalized linear mixed models (GLMM) were used to examine the effects of predictors on memory retention. Collar microglomerular density, lip microglomerular density and calyx volume were fixed factors, and age, head width, total number of landings and colony were random factors, for the predictor of memory retention. The same was done for learning speed except that colony and age were not included, since only one colony was used in this experiment and age was not included since all bees were 12 days old. A fully

standardized regression coefficient (Estimate; Table 3.1 and 3.2) was used to compare the

impacts of each factor. No correlation was found between colony (n = 3), age (10-16 days),

head width (4.2-5.1mm) or number of landings (54-124) and any of the predictors in any

of the experiments. Kolomogrov-Smirnov test was conducted to check the distribution of

landings to rewarding and unrewarding colors. Statistical tests were conducted with

MATLAB (MathWorks, Natick, MA, USA). The significance level used was 5% in all

analyses.
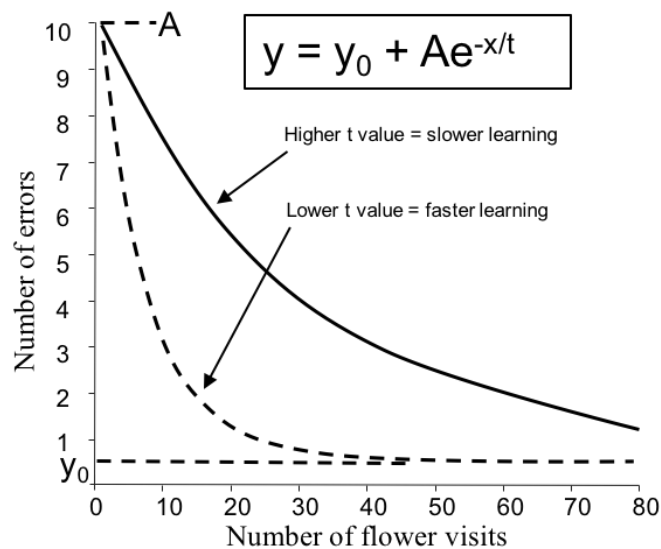
Figure 3.2. Learning speed of the bumblebee. Illustrational curves show the improvement on learning performance over time (i.e. learning speed), expressed as the decay constant (t) in the equation: $y = y_0 + Ae^{-x/t}$. x is the number of flower choices the bee made; y is number of errors the bee made; y_0 is the errors made by a bee after finishing the learning process, i.e. when reaching a performance plateau (final asymptotic value of the y value); A is the curve amplitude (the maximum height of the curve above y_0). High values of t correspond to slow learning, whereas low t values indicate fast learning. The dashed line represents the faster learning bee and the solid line represents slower learning bee.

3.3 Results

3.3.1 Memory retention positively correlates with microglomerular density

In Experiment 1, bees (n = 31) learned to land on five rewarding colors and to not land on five unrewarding colors (Materials and Methods; Figure 3.1). During the last 10 landings of training, bees' landings among rewarding colors were distributed uniformly (Kolomogrov-Smirnov test, p values for each bee varied from 0.2090 to 0.9996; Figure 3.3), showing no preference between any of the rewarding colors over the duration of the training. In addition, no color preference was found amongst unrewarding flower colors during training (Kolomogrov-Smirnov test, p values for each bee varied from 0.2090 to 0.6974; Figure 3.3). Two days after training, bees received a memory retention test on the same setup as in training. Bees were collected immediately after the retention test to examine synaptic density in the mushroom bodies (Materials and Methods). There were no significant correlations between learning speed and microglomerular density in collar and lip, and calyx volume (Figure 3.4A-C; Table 3.1). Only the density of microglomeruli in the collar region of the mushroom body calyx was significantly, and positively, correlated with memory retention (GLMM: $p < 0.0001$; Figure 3.4D; Table 3.1). Microglomeruli density in the lip region did not correlate with long term memory retention, nor did volume of the calyx (Figure 3.4E, F; Table 3.1). In addition, learning speed of individuals did not correlate with their memory retention (GLMM; Table 3.1).

3.3.2 Learning speed positively correlates with microglomerular density

To explore whether higher microglomerular density in the collar of bees results from visual learning, or whether it is already in place to allow for better performance, I trained another

group of bees (n = 10) on the same visual learning paradigm as Experiment 1 above, but each bee was collected immediately after training (Experiment 2, see Materials and Methods and Figure 3.1). As with memory performance, only microglomerular density in the collar correlated significantly, and positively, with learning speed (GLMM: p = 0.0064; Figure 3.4G; Table 3.2). Microglomeruli density in the lip region did not correlate with learning speed, nor did volume of the calyx (Figure 3.4H, I; Table 3.2).

These findings suggest that a higher microglomerular density within the mushroom body collar may predispose bees to better performance in both learning speed and long term memory retention in a visual learning task.
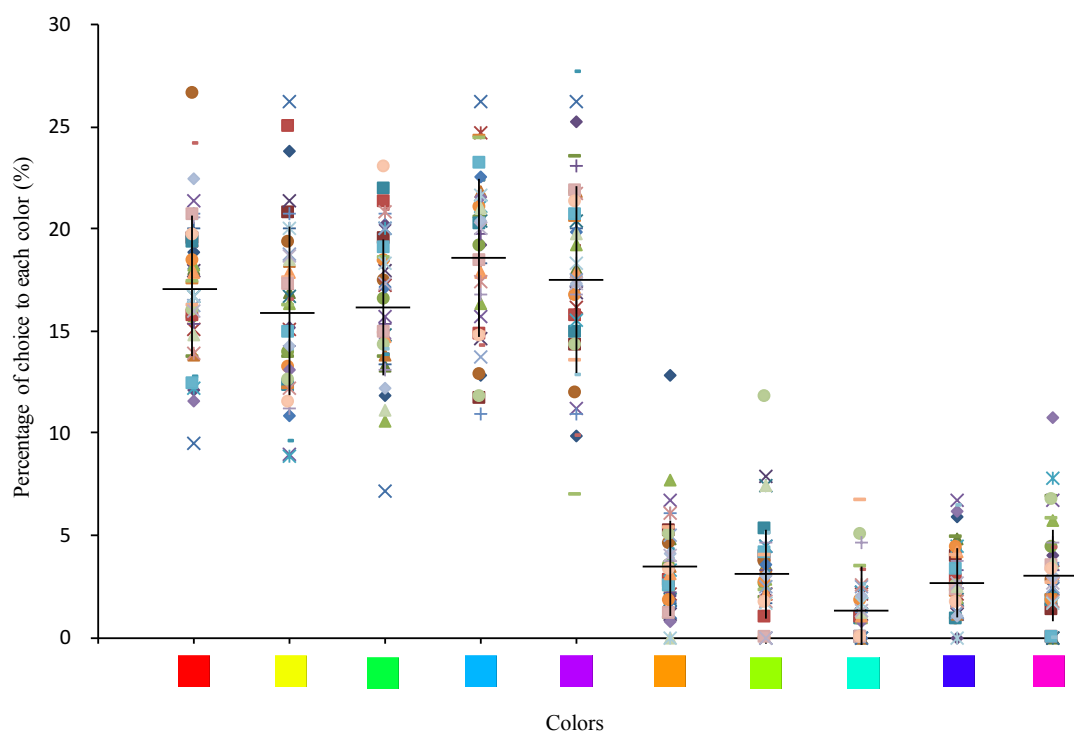
Figure 3.3. Distribution of landings among colors during training. During the last 10 landings of training, bees (n = 31) showed a relatively even distribution amongst rewarding flowers (Kolomogrov-Smirnov test; p values varied from 0.2090 to 0.9996) and similarly showed an even (low) distribution of choices amongst unrewarding flowers (Kolomogrov-Smirnov test; p values varied from 0.2090 to 0.6974) during training. Symbols indicate each individual bee. Black horizontal bars indicate mean. Black vertical bars indicate standard error of the mean.
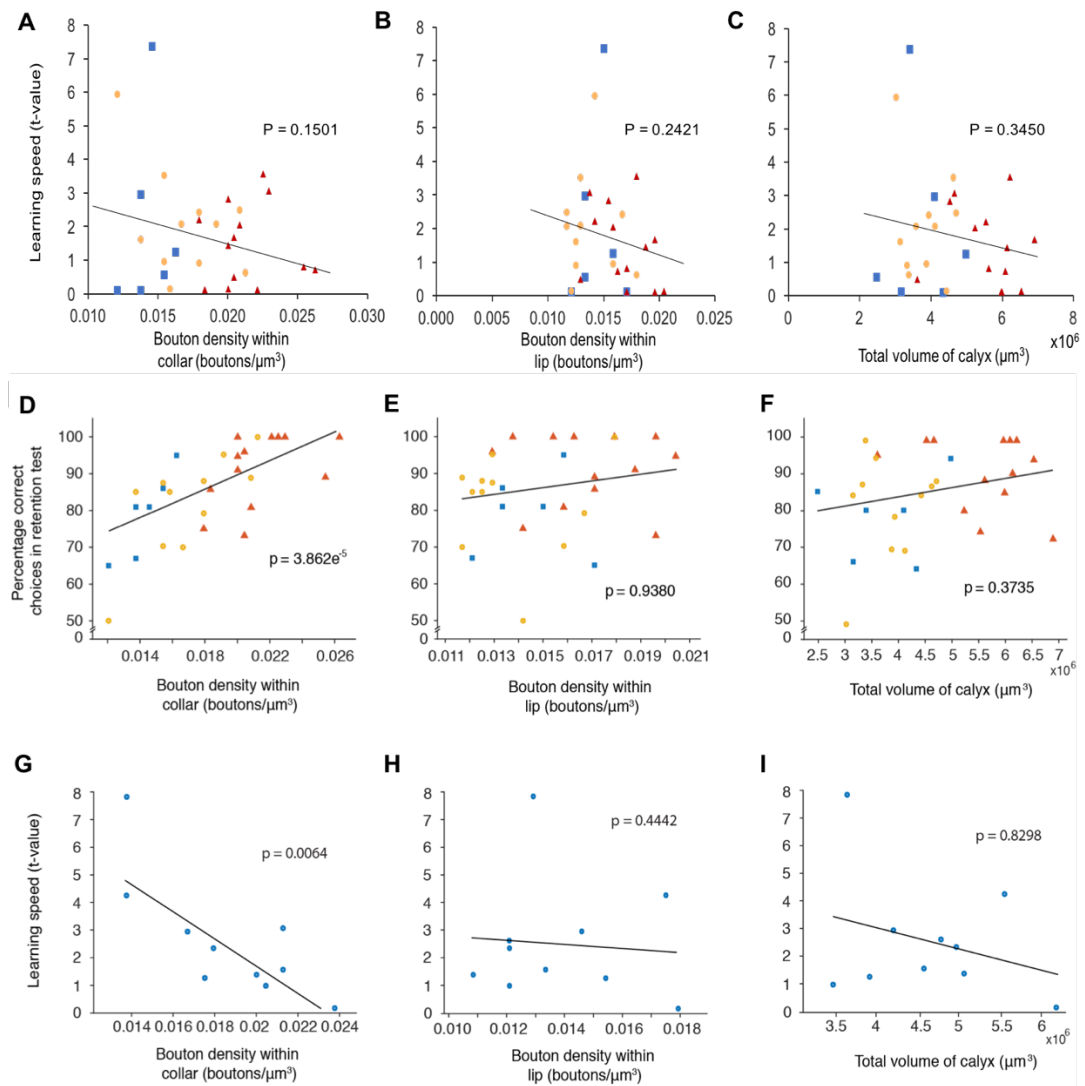
Figure 3.4. The relationship between microglomerular density in the collar and lip and calyx volume and memory retention and learning speed. There were no significant correlations between learning speed and microglomerular density in collar and lip, and calyx volume in Experiment 1 (**A-C**). Microglomerular density in the collar region of the calyces of the mushroom bodies correlates significantly with memory retention (**D**). No such correlation was found in the lip (**E**) or calyx volume (**F**). A-F symbols indicate different colonies (n = 31; colonies 1-3). There was a significant correlation between microglomerular density in the collar and learning speed during training in Experiment 2 (**G**). Again, no such correlation was found in the lip (**H**) or calyx volume (**I**). G-I, n = 10; colony 4. The t-value was the indicator for learning speed (see Materials and Methods). High t-values indicate slow learning, whereas low t-values indicate fast learning. Generalized linear mixed model analysis was conducted and the p-value of each factor is shown in each figure. Solid lines are lines of best fit on the means obtained from the generalized mixed model (Table 3.1 and 3.2).

Table 3.1. Summary of generalized linear mixed models examining learning speed and memory retention factors in relation to microglomerular density, and examining memory performance in relation to learning speed (Experiment 1).

Dependent variable	Fixed factors	df	Estimate	SE	F	P
Learning speed	Intercept	1	27.36	6.97	15.37	0.0006
	MG density in collar	1	-475.95	320.93	2.19	0.1501
	MG density in lip	1	-571.13	477.11	1.43	0.2421
	Total calyx volume	1	$1.16e^{-6}$	$1.21e^{-6}$	0.92	0.3450

The dependent variable was the t-value calculated for learning speed during training. The MG density in the collar, MG density in the lip, and the total calyx volume were included as fixed factors. Age, head width, number of landings and colony (N = 3) were included as random factors.

Dependent variable	Fixed factors	df	Estimate	SE	F	P
Memory retention	Intercept	1	43.55	11.01	15.65	0.0052
	MG density in collar	1	2699.40	545.63	24.48	**$3.862e^{-5}$**
	MG density in lip	1	67.11	854.12	0.01	0.9380
	Total calyx volume	1	$-1.90e^{-6}$	$2.10e^{-6}$	0.82	0.3735

The dependent variable was the percentage correct choices during the memory retention test. The MG density in the collar, MG density in the lip, and the total calyx volume were included as fixed factors. Age, head width, number of landings and colony (N = 3) were included as random factors. The significant terms are highlighted in bold.

Dependent variable	Fixed factors	df	Estimate	SE	F	P
Memory retention	Intercept	1	87.48	3.65	573.36	$3.4373e^{-20}$
	Learning Speed	1	-1.45	1.27	1.32	0.2604

The dependent variable was the percentage correct choices during the memory retention test. The t-value calculated for learning speed during training was included as a fixed factor. Colony (N = 3) was included as a random factor.

Table 3.2. Summary of generalized linear mixed models examining learning speed factors in relation to microglomerular density (Experiment 2).

Dependent variable	Fixed factors	df	Estimate	SE	F	P
Learning speed	Intercept	1	14.55	3.42	18.08	0.0054
	MG density in collar	1	-555.08	135.40	16.81	**0.0064**
	MG density in lip	1	-163.97	200.25	0.67	0.4442
	Total calyx volume	1	$-1.34e^{-7}$	$5.95e^{-7}$	0.05	0.8298

The dependent variable was the t-value calculated for learning speed during training. The MG density in the collar, MG density in the lip, and the total calyx volume were included as fixed factors. Age and colony were not included as random factors because all bees were 12 days old and from the same colony. Head width and number of landings were included as random factors. The significant terms are highlighted in bold.

3.4 Discussion

A mechanistic understanding of individual learning ability requires examining the underlying structures within the brain and how they change in relation to cognitive performance. To investigate the neural correlates underlying individual learning ability, I examined the synaptic organization in the brains of bees whose performance varied on a visual discrimination task. I found evidence to suggest that bees with higher density of synaptic complexes in their visual input region are predisposed to faster learning and better memory. Although not causal, the correlation I found provides, to my knowledge, the first evidence that synaptic organization plays an important role in determining differences in individual learning and memory ability of intact, freely behaving animals.

General findings

I examined how the variation in learning and memory performance across individual bees related to microglomerular density in the brain. The most exciting findings of my study is that the individual bees that performed well in the retention test (Experiment 1) and individual bees that learned quickly in training (Experiment 2) had a relatively high microglomerular density in the visual input region of the mushroom body calyx, compared to bees that performed poorer. Differences in synaptic organization was modality specific, as I found no correlation between microglomerular density in the lip region of the calyx and memory retention or learning speed. This was expected because the collar region of the calyx receives incoming visual information while the lip receives incoming olfactory information. Higher microglomerular density measured through anti-synapsin staining signifies higher density synapsin-positive presynaptic boutons, which indicates more

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functional synapses (Ferreira et al., 1998; Morimoto et al., 1998; Sato et al., 2000; Hart et al., 2011). My results suggest that a higher number of functional synapses present in the visual input region (calyx collar) of the brain may predispose bees to better visual learning and memory performance.

Can we determine to what extent learning contributed to memory performance through microglomerular changes? Learning speed of individuals tested in Experiment 1 and their memory retention did not correlate (GLMM; Table 3.1). This only suggests that learning likely causes varying degrees of changes in microglomerular density across individuals. The correlation (or lack of correlation) between these two measures are unhelpful for inferring the degree to which learning induced synaptic complex formation contributes to better memory performance. Higher microglomerular density however does seem to lead to faster learning speed and better memory retention.

Comparisons with previous works

The correlation found here between synaptic organization and visual learning performance is supported by recent findings on bees in the olfactory domain. In Haenicke's study (2015), bees were trained to associated an odor with sucrose solution in the proboscis extension reflex paradigm and odor responses in individual bouton in the calyx were recorded before and after training by Ca^+-imaging. The results showed that the training-induced neural plasticity (changes in odor response between before and after training) was positively correlated with the learning performance and short-term memory (Haenicke, 2015). These findings give a potential functional link to my findings, in that higher microglomerular density may increase total neural plasticity and then individuals can learn faster and

remember better.

Sommerlandt et al. (2016) examined the relationship between visual discrimination learning and mushroom body calyx microglomerular density in honeybees and similarly examined the correlation between learning performance and microglomerular density. They found a negative correlation between the performance of bees who learned through differential conditioning and their microglomerular density in the mushroom body calyx. Age and experience of bees were not controlled in their study, while both have been controlled very well in my study. The weak negative correlation between learning performance and microglomerular density, as surmised by Sommerlandt et al. (2016), could be due to variations in experience by the bees; pruning of synaptic connections with increased age and experience may correlate with better discriminative ability, or potentially less explorative behavior (Sommerlandt et al., 2016). Another similar study, also in honeybees, examined microglomerular density in the mushroom body calyces and performance on a two-color visual discrimination task (Van Nest et al., 2017). Van Nest et al. found no correlation between performance and microglomerular density. In my study, I found a statistically strong positive correlation between performance on a visual discrimination task and microglomeruli density in the mushroom body calyxes, and specifically in the visual input region (collar region). The reason for the difference in sign of correlation (with Van Nest 2017) and significance (with Sommerlandt 2016 and Van Nest 2017) may likely be due to the differences in controls and task difficulty. I controlled for both age and prior foraging experience and the task I used was arguably much more difficult, and allowed for more variation in performance. In addition, the difference in bee species (honeybees versus bumblebees) might be another reason for inconsistent findings.

Ecological Significance

Natural variations in learning speed and memory retention across bees exist commonly, and these variations are important for colony fitness and allow bees to respond flexibly to variable and complex environment (Muller and Chittka, 2008, 2012; Raine and Chittka, 2008). Bees need to explore new resources for food throughout the season as the floral patterns and food sources change over time, i.e. rather than making errors, some bees may be exploring alternative options (Raine and Chittka, 2008; Smid and Vet, 2016; Woodgate et al., 2016). Genetic diversity is associated with natural variations in learning and memory in insects (Raine and Chittka, 2012; Smid and Vet, 2016). The genetic differences across bee colonies/individuals and natural variations in microglomerular density across individual bees may predict bees' foraging performance, but they may also predict individual's exploratory behavior or other behavior yet untested. Future work should attempt to combine both measurements of cognitive performance and foraging behavior.

Chapter 4

Large-scale transcriptome changes following long-term visual memory formation in bumblebees, *Bombus terrestris*

Summary

The action of many genes is needed for stable long-term memory, but how the genome dynamically responds, transcriptionally, to learning and during memory formation has not been fully elucidated in many organisms. In this study, I use bumblebees (*Bombus terrestris*) to examine how visual learning and memory formation affect transcriptome profiles over time via high-throughput sequencing. Fifty-five genes were triggered immediately after bees were trained to associate reward with a single colored chip, and the upregulated genes were predominantly genes known to be involved in signal transduction. Eighty-one genes were activated four hours after learning a new color, the majority of which were related to transcription and translation, which suggests that the building of new proteins may be the predominant activity four hours after training. In addition, candidate genes (e.g. *Rab10*, *Shank1* and *Arhgap44*) involved in learning and long-term memory formation were identified in my study, which should be explored in future work.

4.1 Introduction

Learning and memory formation (as well as many other behaviors) are underpinned by the precisely timed, coordinated expression of many genes (Keene and Waddell, 2007; Robinson et al., 2008; Alberini, 2009; Zayed and Robinson, 2012; Hoedjes et al., 2015). The storage of information in long-term memory requires transcriptional and translational regulation in brains (Alberini and Kandel, 2015; Cho et al., 2015). Utilization of pharmacological approaches, in vertebrates and invertebrates, has helped determine that at least two waves of transcriptional activity are needed for long-term memory formation, one occurring immediately following the time of training and another occurring 3-6 hours after training (Bailey et al., 1996; Muller Igaz et al., 2002; Alberini, 2009; Lefer et al., 2013). The functions of specific genes for learning and memory formation has been established in a variety of model organisms, such as *Drosophila* and mouse (Keene and Waddell, 2007; Alberini, 2009; Hoedjes et al., 2015). Many of these genes are immediate early genes, which respond rapidly to a variety of cellular stimuli and belong to the first transcriptional wave. Immediate early genes encode transcription factors and other DNA-binding proteins, and the protein products of immediate-early genes act on a wider set of target genes that are responsible for synaptic reorganization, which is considered the basis of learning and memory (Kandel, 2001; Flavell and Greenberg, 2008; Alberini, 2009; Loebrich and Nedivi, 2009; Benito and Barco, 2015). However, our knowledge of the global gene expression changes in response to specific learning and memory is still limited.

High-throughput sequencing technology has made it possible to examine the genome-wide transcriptional response to specific behaviors, resulting in the identification of many known or novel transcripts involved in certain behaviors at high resolution and large scale. For

example, using this technology, the transcriptional responses to diseases in humans (Wu et al., 2012), to memory formation in specific learning task in rats (Cavallaro et al., 2002), to heavy metal stress and circadian clocks in *drosophila* (Yepiskoposyan et al., 2006; Hughes et al., 2012), and to social behaviors and specific learning in bees and other insects (Zayed and Robinson, 2012; Berens et al., 2017) have been examined.

Long-term memory formation of honeybees has been revealed to be associated with transcriptional changes (Wang et al., 2013; Cristino et al., 2014; Qin et al., 2014) by comparing gene expression between unconditioned and conditioned groups with high-throughput sequencing. For example, Qin and colleagues (2014) trained honeybees to discriminate two patterns (black/white gratings oriented at 45° and 135° to the horizontal) using a Y-maze. Gene expression in bee brains of the pattern-trained group and the untrained group was compared and a total of 388 differentially expressed genes were found. In all these studies, bees in the conditioned groups were collected two or three days after the first training trial when the bees had formed long-term memory. However, since some transcriptional changes responsible for long-term memory formation are initiated during or shortly after learning (Bailey et al., 1996; Alberini, 2009; Lefer et al., 2013), some genes involved in the bees' learning and memory formation processes might have been missed. In order to determine comprehensively which genes play a role in the process of long term memory formation, protocols need to be designed to focus on genes activated immediately or shortly following training.

In this study, I aimed to find the specific genes involved in the process of bees' visual long-term memory formation, which could provide future venues to work on, such as how the screened genes functions in the neural system and how the expression of the specific genes

affects long-term memory formation by changing neural plasticity. I also aimed to understand the dynamic gene expression changes shortly after associative visual learning, which could help our understanding of gene interaction that involves in learning and memory formation. Two time points (immediately and four hours after training) were selected to examine learning and memory-dependent genome-wide transcriptional regulation in the bumblebee (*Bombus terrestris*). Different gene expression patterns were supposed to be observed at these two time points, that is I hypothesized that different set of genes may regulate memory formation at different time shortly after learning. In brief, this study will provide valuable resources for future research on learning and memory.

4.2 Materials and Methods

Animals

Bumblebee (*Bombus terrestris*) colonies, with around 20 workers, were purchased from Biobest Belgium NV (Westerlo, Belgium). All colonies were housed in wooden nest boxes (40 × 28 × 11 cm), which were connected to small flight arenas (65 × 45 × 25 cm) through a Perspex corridor (25 × 3.5 × 3.5 cm). I manually controlled when individual bees entered the arena with small doors in the corridor. Bee identity was tracked with an individual number tag (Opalithplättchen, Warnholz & Bienenvoigt, Ellerau, Germany) glued to the top of the thorax. Bees were marked under red light, since red light is in the periphery of their visual spectrum and they can therefore see it only poorly (Chittka and Waser, 1997), to ensure visual color information for bees was kept at a minimum. During experiments, illumination in the lab was controlled with a 12 h day-night cycle (8:00 am - 8:00 pm).

Bees had no foraging experience until pre-training and all bees used in the experiments had similar age (11-13 days) at collection.

Behavioral procedures and bee sample collection

Pre-training. All bees were first trained to visit transparent Perspex chips (25×25 mm) with 7 μl 40% sucrose solution. Five chips were arranged in a pseudorandom array within the arena, each on top of a small glass vial. Only bees that successfully foraged from the transparent chips and returned to the colony 8-10 times with inter-trip interval under 5 minutes were included in the experiments.

Training. On Day 1, bees were trained individually to forage on only one transparent chip, which contained 100 μl 40% sucrose solution. Worker bumblebees of similar size were selected visually to ensure that all bees could consume 100 μl sucrose solution and filled their crop, and then they can naturally return to their nest to unload the collected sucrose solution. Each bee had five foraging trips and the inter-trip interval was 10 minutes. Inter-trip intervals were kept consistent because bees always attempted to leave the colony prior to the 10-min time point and I would only need to prevent the bee from entering the arena using small doors in the corridor until the 10-minute interval had ended. Bees tended to return from their nest to the corridor each few minutes and therefore when a bee returned within a minute or two from when the 10-minute interval would end, the bee was prevented from leaving the corridor by closing the corridor doors until it was time for the next trip. Chips were moved to pseudorandom locations in the arena between trips to prevent bees from associating certain spatial locations with reward or color. On Day 2, the bees received the same training as on Day 1. The two days' training allowed bees to get used to the environment in the arena, the artificial chips and reward levels. On Day 3, the bees were

divided into three groups (Figure 4.1). Group A received the same training on one transparent chip as Day 2 and were collected immediately after training. This group was used as the control group in the following experiments, i.e. bees in this group did not have new color learning experience (0-hour Control). Group B received the same training procedures as Day 2, except the transparent chip was replaced by a yellow chip, and the bees were collected immediately after training (0-hour Learning). Group C was trained the same as Group B, but was left in the hive for four hours without any further foraging experience, and was then collected (4-hour Learning). The training procedures limit the effect of the clear chip-reward association on the transcriptome. In this way, we could be more confident that we would see changes in gene expression due to the novel yellow color. Bees were collected in liquid nitrogen and stored at $-80^{\circ}C$ until dissection. On Day 3, Group C was trained in the morning while Group A and B were trained in the afternoon to make sure all bees were collected at the same time point of the day to avoid that any differences in gene expression might simply be the result of differences in the expression of circadian clock genes. For each of the three conditions (0-hour Control, 0-hour Learning, 4-hour Learning), 10-12 bees were collected from each of three separate colonies.

Retention test. Forty-five bees not used for sequencing were used to validate whether the training procedures actually lead to long-term memory formation. Bees were trained on one yellow chip (or one magenta chip) containing 100 µl 40% sucrose solution, and each bee had five foraging trips with 10 min inter-trip intervals. The retention test, conducted three days after training, required bees to forage among five yellow and five magenta chips each containing 100 µl water. Bees' landings over three minutes were recorded and the percentage of correct landings was calculated. A landing was defined as any time the bee

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was positioned on top of a chip and not flying for any amount of time.

Total RNA extraction, RNA-seq library construction and high-throughput sequencing

Whole brains were dissected out over dry ice and washed in cold phosphate buffer saline (PBS) to remove small pieces of hair or trachea. Ten to twelve bee brains were pooled in each sequencing sample for RNA extraction. There were three biological replicates for each of the three conditions (0-hour Control, 0-hour Learning, 4-hour Learning) (nine sequencing samples in total). Total RNA was extracted from whole brains using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quantity and integrity were measured using a 2100 Bioanalyzer (Agilent). The RNA concentration and the RNA integrity number (RIN) are shown in Table S4.1. RNA-seq libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina (New England Biolabs Inc., Ipswich, MA, USA). Qualification and quantification of the libraries were conducted by Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System separately. Finally, the libraries were paired-end sequenced using Illumina HiSeqTM 2000, which generates around 50 million paired-end 150 bp raw reads for each sample. Library construction and sequencing were conducted by BGI Genomics Co., Ltd. (Shenzhen, China).

Read mapping and gene expression calculation

Primary sequencing data produced by Illumina HiSeqTM 2000 were raw reads. Before data analysis, the reads with adapters, with more than 10% unknown bases and low quality reads were removed from raw reads. Quality control was performed on the remaining reads (clean reads) by drawing a base composition chart and a quality distribution chart, to ensure that

each sample possesses balanced base composition and high sequencing quality.

The cleaned reads were then aligned to the *Bombus terrestris* genome and reference genes (http://www.ncbi.nlm.nih.gov/genome/2739?genome_assembly_id=34093). Burrows-Wheeler Aligner (BWA) (Li and Durbin, 2009) was used to map clean reads to the reference genome and Bowtie (Langmead and Salzberg, 2012) was used to reference genes. The mapping ratio and the distribution of reads on bumblebee reference genes were calculated to evaluate the sequencing quality. Gene expression level was quantified by the RNA-Seq by Expectation Maximization (RSEM) software (Li and Dewey, 2011). Then the fragment (one pair of reads is considered a fragment in paired-end sequencing) counts were normalized to Fragments Per Kilobase of transcript per Million mapped reads (FPKM), which eliminated the influence of different gene length and discrepancy of the library size. FPKM values were used for gene expression analysis.

Identification of differentially-expressed genes and cluster analysis

One-way ANOVA was used to identify differentially expressed genes (DEGs) among the three experimental groups. Genes were considered differentially expressed with a p-value < 0.01 and |Fold change| ≥ 2. Principal component analysis (PCA) was performed on normalized FPKM values of the DEGs to detect global gene expression patterns in each sample. To find genes with similar expression patterns, hierarchical clustering analysis was conducted on the normalized FPKM values of DEGs. All statistical analysis was conducted with MATLAB 9.2 (MathWorks, Natick, MA, USA).

Gene ontology and pathway enrichment analysis of DEGs

Functional analysis of DEGs was obtained by performing gene ontology (GO) and KEGG

pathway enrichment analysis, which were conducted using a strict algorithm developed by BGI Genomics Co., Ltd. (Shenzhen, China) (see details in Wang et al. (2013)). The p-value was corrected through Bonferroni Correction (Abdi, 2007) and the corrected p-value threshold of 0.05 was used to detect significantly enriched GO terms and pathways. WEGO software (Ye et al., 2006) was used to do GO functional classification for DEGs to determine the distribution of gene functions.

Validation of differentially expressed genes

RNA samples that were the same as those used for RNA sequencing were used. cDNA was synthesized using BioRad iScript™ cDNA Synthesis Kit according to the manufacturer's instructions with about 1 µg RNA. Real-time PCR was performed with the BioRad CFX Connect qPCR machine and BioRad iTaq™ Universal SYBR® Green Supermix following steps recommended by the manufacturer. In brief, 20 µl PCR reaction was used and each reaction included 10 µl SYBR Green Supermix, 1 µl cDNA which was diluted 1:10, 0.8 µl of 10 pmol/µl (pM) forward and reverse primers and 7.4 µl ultra-pure water. Each 20 µl reaction per well was run in triplicate on 96-well plates. All primers used are listed in Table S4.2. A standard curve was used to calculate amplification efficiencies for each primer pair and a melting curve was also drawn to avoid nonspecific amplifications. Negative control (water) was always included. *Ef1α* was used as a reference gene (Tobback et al., 2011). The threshold cycle (CT) for each gene was read under the default parameters. Relative quantification of each gene expression was calculated by the Pfaffl method (Pfaffl, 2001) and Group A was the control group. One-way ANOVA was conducted to determine if there were significant differences ($p < 0.05$) across these three experimental groups.

4.3 Results

4.3.1 Retention test after color learning

Apart from the bees used for sequencing, extra bees in each colony were used to validate whether the training procedures led to long-term memory formation. Bumblebees were trained to associate one yellow chip with sucrose solution for five foraging trips with 10 min inter-trip intervals. Results of a retention test conducted three days after training showed that bees in all three colonies formed long-term memory after training (the ratio of correct landings in the retention test was significantly higher than chance, mean performance ± SD: 90 ± 15%; Figure 4.1B, C). In addition, I found that bees remembered the trained color in the retention test with high accuracy, which indicates that bees' performance was not strongly influenced by an innate preference for either of the colors used in my study (Figure 4.1B).
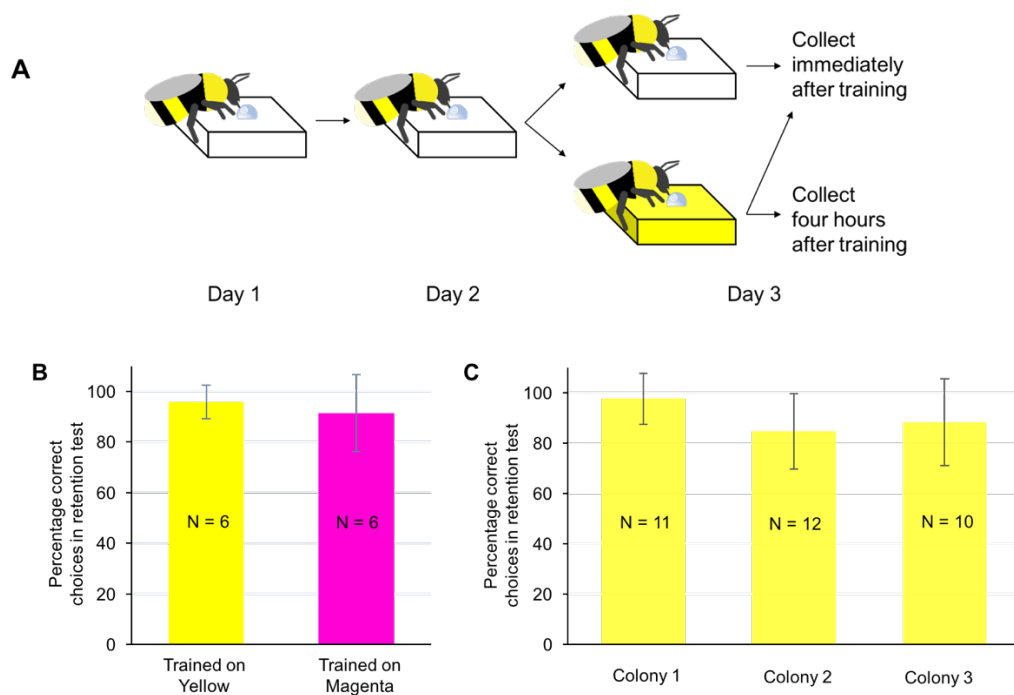

Figure 4.1. Results showing that long-term memory can been established with the training procedures used here. (**A**) Training procedures. Before training, all bees received the same pre-training

procedure, in which bees were trained to forage on five transparent chips with 7 μl 40% sucrose solution. Bees successfully foraged from the transparent chips and returned to the colony 8-10 times on a regular basis (inter-trip interval within 5 minutes) were moved on to the training phase. In the training, on day one and day two, bees were trained individually to forage on one transparent chip, which contained 100 μl 40% sucrose solution. On day three, bees were split into three groups; some bees were still trained to one transparent chip with 100 μl 40% sucrose solution and were collected immediately after training (0-hour Control); some bees were trained to one yellow chip with 100 μl 40% sucrose solution and were collected immediately after training (0-hour Learning); some bees were trained to one yellow chip with 100 μl 40% sucrose solution, and were left in the hive for four hours without any further foraging experience and then were collected (4-hour Learning). Each bee on each day had 5 trips with 10 min inter-trip interval. (**B**) Bees' memory performance in the behavioral experiment conducted to test whether long-term memory can be established in the training procedures. In this experiment, six bees were trained on one yellow chip contained 100 μl 40% sucrose solution and six bees were trained on one magenta chip contained 100 μl 40% sucrose solution. All bees had five foraging trips with 10 min inter-trip interval. A retention test was conducted three days after training, in which bees foraged among five yellow and five magenta chips and all chips contained 100 μl water. Bees' landings over three minutes were recorded. The bees remembered the rewarding color they were trained to associate, no matter which color (yellow or magenta) it was (t-test, Yellow: $t = 16.90$, $df = 5$, $p = 0.000$; Magenta: $t = 6.64$, $df = 5$, $p = 0.001$, compared to chance expectation 50%). The strength of preference did not differ between yellow and magenta (t-test, $t = 0.65$, $df = 10$, $p = 0.532$). (**C**) Bees' memory performance in the three experimental colonies used for sequencing. Bees in the three experimental colonies were trained on one yellow chip and a retention test was conducted three days after training (the same as in the above experiment) to confirm whether bees in each colony can form long-term memory after the training. My data showed that bees in all three colonies can form long-term memory (t-test, Colony 1: $t = 15.50$, $df = 10$, $p = 0.000$; Colony 2: $t = 8.05$, $df = 11$, $p = 0.000$; Colony 3: $t = 7.04$, $df = 9$, $p = 0.000$, compared to chance expectation 50%). The number within each bar indicates the number of bees tested. Vertical bars indicate standard deviation. Please note that the bees in each colony receiving the retention test were not used for RNA-seq sample collection.

4.3.2 Alignment of sequencing reads and quality assessment

RNA sequencing yielded an average of 40.5 million clean reads per sample (nine samples in total), occupying 81% of raw reads (Figure S4.1). An average of 73.3% of the clean reads were mapped to the *Bombus terrestris* reference genome and 72.8% of the clean reads were uniquely aligned. An average of 66.2% of the clean reads were mapped to *Bombus terrestris* reference genes while 35.1% of the clean reads were uniquely aligned (Table 4.1). The genome uniquely mapped reads were used for later gene expression analysis. A total number of 19,740 expressed genes were found in all samples, which is relatively similar to the number of genes that have been found in previous studies (Colgan et al., 2011; Harrison et al., 2015; Sadd et al., 2015); and of these, 86% were co-expressed among the three experimental groups (Figure 4.2A). Quality control on cleaned data suggests high sequencing quality and equivalent data characteristics across all RNAseq samples (Figure S4.2 and S4.3).

Table 4.1. Alignment of RNA-Seq reads to the bumblebee (*Bombus terrestris*) genome and reference genes.

Sample Name	Total Reads	Genome		Gene		Expressed Gene
		Total Mapped Reads	Unique Match	Total Mapped Reads	Unique Match	
A1	40722006 (100%)	31001346 (76.1%)	30828347 (75.7%)	27270084 (67.0%)	14358322 (35.3%)	16589
A2	40836060 (100%)	30567501 (74.9%)	30337986 (74.3%)	27671810 (67.8%)	14926440 (36.6%)	16145
A3	40027846 (100%)	29214232 (73.0%)	28998847 (72.5%)	26581182 (66.4%)	14119774 (35.3%)	16377
B1	41025478 (100%)	30167803 (73.5%)	29984447 (73.1%)	27048580 (65.9%)	14324660 (34.9%)	16524
B2	40866082 (100%)	29055426 (71.1%)	28844081 (70.6%)	26094806 (63.9%)	13860036 (33.9%)	16370
B3	40670906 (100%)	29895203 (73.5%)	29670668 (73.0%)	27272970 (67.1%)	14471884 (35.6%)	16430
C1	39965134 (100%)	29317201 (73.4%)	29141139 (72.9%)	26273690 (65.7%)	13822280 (34.6%)	16146
C2	40133868 (100%)	29659025 (73.9%)	29445244 (73.4%)	26489752 (66.0%)	14075364 (35.1%)	16559
C3	40481840 (100%)	28511125 (70.4%)	28278307 (69.9%)	26629568 (65.8%)	14036438 (34.7%)	16066

Paired-end clean reads (150 bp length) were mapped to the reference genome using BWA and mapped to reference genes using Bowtie. A: 0-hour Control; B: 0-hour Learning; C: 4-hour Learning.

4.3.3 Unique and temporal gene expression patterns required for long-term memory formation

Gene expression was compared between the three experimental groups. A total of 110 genes were significantly different in the overall ANOVA ($p < 0.01$, |Fold change| ≥ 2). PCA analysis revealed that samples in the three groups were separated into three non-overlapping clusters (Figure 4.2B). This result demonstrated the differences in gene expression pattern between the three experimental groups, which indicates gene expression patterns change temporally after new color learning. Hierarchical clustering analysis on samples also showed that the nine samples were separated by experimental treatment and the gene expression patterns in 0-hour Control and 0-hour Learning groups were closer (Figure 4.3). Hierarchical clustering analysis on the DEGs showed several different gene expression patterns and five of them were of particular interest (Figure 4.3). The largest cluster was Cluster 3 in which the DEGs were upregulated in 4-hour Learning group and 40% of the DEGs belong to this cluster.

The 110 DEGs were mapped to GO terms and 58 of them belong to the Biological process ontology (Figure S4.4). The GO terms with more than five DEGs were 'Cellular process' (GO:0009987), 'Localization' (GO:0051179), 'Metabolic process' (GO:0008152), 'Response to stimulus' (GO:0050896), 'Single-organism process' (GO:0044699), 'Binding' (GO:0005488) and 'Catalytic activity' (GO:0003824) (Figure S4.4). Pathway enrichment was conducted and seven of the top 20 enriched pathways (Table S4.3) were signaling pathways, including 'Estrogen signaling pathway' (ko04915), 'AMPK signaling pathway' (ko04152), 'Dopaminergic synapse' (ko04728), and 'Glucagon signaling pathway' (ko04922). Other pathways were related to transcription factors, protein synthesis (e.g.

'Protein processing in endoplasmic reticulum' (ko04141)) and energy generation (e.g. 'Fatty acid metabolism' (ko01212)). These findings suggest that the genome is highly responsive to new color learning; signaling, transcription factors, protein synthesis and energy generation are all involved in learning information processing and memory formation.
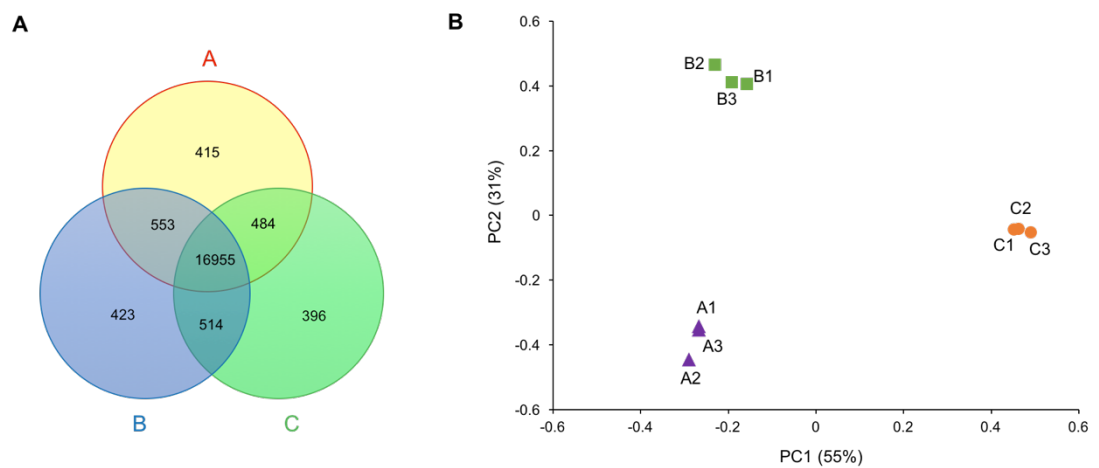
Figure 4.2. Gene expression differences associated with different learning and memory statuses. (**A**) The number of co-expressed and unique genes observed in the entire transcriptomes among the three experimental groups. 86% of genes (16955) were shared among the three experimental groups. (**B**) Scatterplot of PC1 and PC2 from a principal component analysis of all samples using the gene expression values for differentially-expressed genes. The symbols represent samples from different experimental groups. PC1 and PC2 contributed 55% and 31% of the total variance, respectively. Nine samples can be separated into three experimental groups, which indicates that each learning/memory state has its own specific gene expression pattern. A: 0-hour Control; B: 0-hour Learning; C: 4-hour Learning.

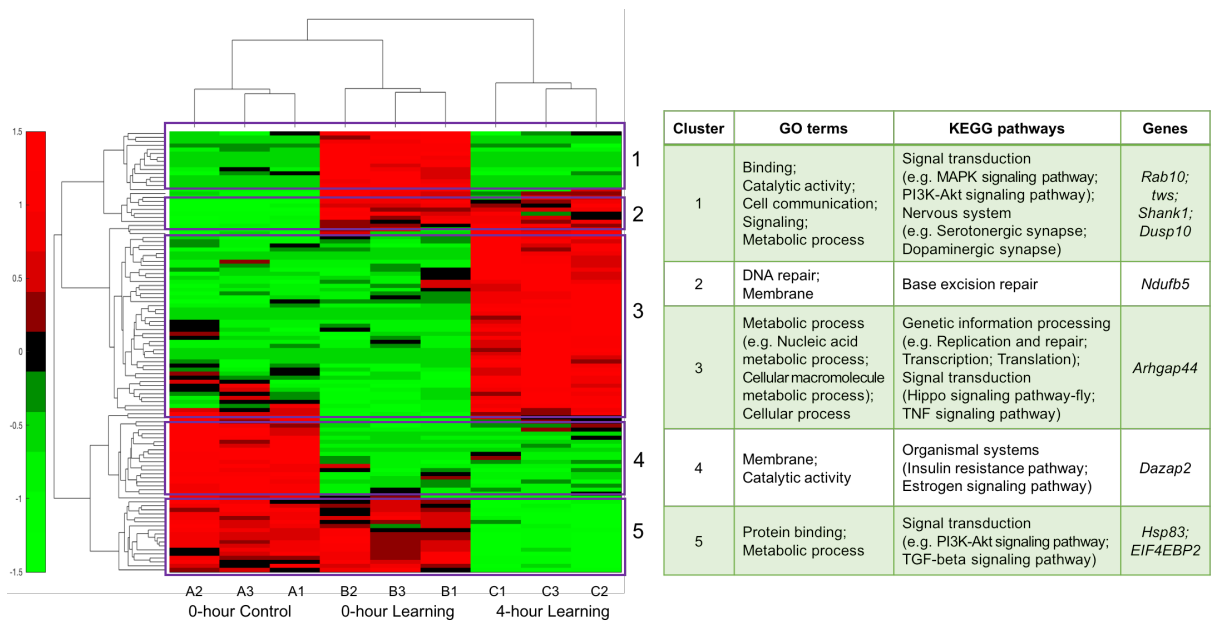| Cluster	GO terms	KEGG pathways	Genes
1	Binding; Catalytic activity; Cell communication; Signaling; Metabolic process	Signal transduction (e.g. MAPK signaling pathway; PI3K-Akt signaling pathway); Nervous system (e.g. Serotonergic synapse; Dopaminergic synapse)	*Rab10; tws; Shank1; Dusp10*
2	DNA repair; Membrane	Base excision repair	*Ndufb5*
3	Metabolic process (e.g. Nucleic acid metabolic process; Cellular macromolecule metabolic process); Cellular process	Genetic information processing (e.g. Replication and repair; Transcription; Translation); Signal transduction (Hippo signaling pathway-fly; TNF signaling pathway)	*Arhgap44*
4	Membrane; Catalytic activity	Organismal systems (Insulin resistance pathway; Estrogen signaling pathway)	*Dazap2*
5	Protein binding; Metabolic process	Signal transduction (e.g. PI3K-Akt signaling pathway; TGF-beta signaling pathway)	*Hsp83; EIF4EBP2*

Figure 4.3. Hierarchical clustering of brain gene expression levels in bees with different learning and memory statuses. Each column represents a sequencing sample and each row represents a gene. Gene expression values are color coded: red indicates higher expression and green indicates lower expression. The normalized gene expression values of 110 differentially expressed genes were used for hierarchical clustering. It is evident that samples in each experimental group can be clustered together and 0-hour Control group and 0-hour Learning group have more similar gene expression patterns. In addition, several gene expression patterns were found and five of them stood out, as highlighted with purple rectangles. The table on the right shows the main GO terms, KEGG pathways and interesting genes for each cluster.

4.3.4 New color learning triggers gene expression changes immediately after training

Compared with 0-hour Control group, 55 genes were differentially expressed in 0-hour Learning group (28 upregulated and 27 downregulated) induced by new color learning. Most of the upregulated genes kept high expression for a short time (less than four hours, Cluster 1). The GO terms found here were 'Binding' (GO:0005488), 'Catalytic activity' (GO:0003824), 'Cell communication' (GO:0007154), 'Signaling' (GO:0023052) and 'Metabolic process' (GO:0008152). The pathways were mainly fell into Signal transduction

category (such as 'AMPK signaling pathway' (ko04152), 'PI3K-Akt signaling pathway' (ko04151), 'MAPK signaling pathway' (ko04010)) and Nervous system category ('Serotonergic synapse' (ko04726), 'Glutamatergic synapse' (ko04724), 'Dopaminergic synapse' (ko04728)). PI3K-Akt signaling pathway and MAPK signaling pathway have both been found to be important in learning, long-term memory formation and synaptic plasticity (Man et al., 2003; Kelleher et al., 2004; Thomas and Huganir, 2004; Sui et al., 2008). Neurotransmitters (serotonin, glutamate and dopamine) have previously been shown to play important roles in associative learning (Bicker and Menzel, 1989; McEntee and Crook, 1993; Barron et al., 2010; Sitaraman et al., 2012; Waddell, 2013) and my results suggest that they also play a role in color learning in the bumblebee. The results also showed that a group of signaling-related genes (such as ras-related protein Rab-10 (*Rab10*); protein phosphatase PP2A 55 kDa regulatory subunit (*tws*); SH3 and multiple ankyrin repeat domains protein 1 (*Shank1*); dual specificity protein phosphatase 10 (*Dusp10*)) responded quickly (immediately after training) to new color learning and their expression dropped later.

Eight of the 28 upregulated genes kept at high expression level and the 27 downregulated genes kept at low expression level consistently after new color learning (Cluster 2 and 4), which suggests on-going changes. The significantly enriched pathway in Cluster 2 was 'Base excision repair' (ko03410), which may indicate the high transcriptional activity shortly after learning. Half of the enriched pathways in Cluster 4 belong to Organismal systems section, especially the Endocrine system, such as 'Insulin resistance pathway' (ko04931) and 'Estrogen signaling pathway' (ko04915), suggesting that the endocrine system becomes less active during learning.

4.3.5 Protein synthesis-related genes activated four hours after new color learning

Comparing the 0-hour Learning group with the 4-hour Learning group, 81 DEGs (46 upregulated and 35 downregulated in 4-hour Learning group) were found. The upregulated genes in 4-hour Learning group (Cluster 3) were enriched for genes related to 'Metabolic process' (GO:0008152) (e.g. 'Nucleic acid metabolic process' (GO:0090304), 'Cellular macromolecule metabolic process' (GO:0044260) and 'Organic substance metabolic process' (GO:0071704)) and 'Cellular process' (GO:0009987). Many pathways identified in this cluster fell into Genetic information processing category (Replication and repair, Transcription, Translation) and Signal transduction category (e.g. 'Hippo signaling pathway-fly' (ko04391) and 'TNF signaling pathway' (ko04668)). These results suggest that signaling- and protein synthesis-related genes are activated several hours after new color learning. The proteins synthesized here might be used for the reorganization of synaptic processes which can store the learned information (Bailey et al., 1996; Muller Igaz et al., 2002; Alberini, 2009; Lefer et al., 2013). The most interesting gene found here was rho GTPase-activating protein 44-like (*Arhgap44*), which is known to be involved in synaptic plasticity and can promote spine morphological changes associated with long-term potentiation (Nasu-Nishimura et al., 2006; Nakazawa et al., 2008; Tolias et al., 2011).

The 35 downregulated genes in the 4-hour Learning group (Cluster 1 and Cluster 5) were mainly involved in 'Protein binding' (GO:0005515), 'Cell communication' (GO:0007154), 'Signaling' (GO:0023052) and 'Metabolic process' (GO:0008152). Gene heat shock protein 83-like (*Hsp83*) was found in Cluster 5 and has been revealed to be associated with learning and can response to stimulus (Wang et al., 2013; Cristino et al., 2014; Qin et al., 2014). The most interesting gene found here was eukaryotic translation initiation factor 4E-

binding protein 2 (*EIF4EBP2*), which is a repressor of translation initiation and has been shown to be involved in synaptic plasticity, learning and memory formation (Banko, 2005; Gelinas et al., 2007).

4.3.6 Validation of DEGs by qPCR

SYBR Green Real-time PCR was performed to verify the DEGs identified in the RNAseq analysis. Based on gene expression and gene function, two DEGs (NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5, mitochondrial (*Ndufb5*) and *Bombus terrestris* DAZ-associated protein 2 (*Dazap2*)) and one gene with the highest expression in all samples but were not different across the three experimental groups (*Bombus terrestris* neurofilament heavy polypeptide (*Nefh*)) were selected for validation. Two of them (*Ndufb5* and *Nefh*) showed the same expression changing tendency with that in RNAseq (Figure 4.4). The protein coded by *Ndufb5* is a subunit of Complex I of the respiratory chain in mitochondria, which transfers electrons from NADH to ubiquinone. *Nefh* coding neurofilament heavy polypeptide has the highest expression in all samples. The protein encoded by *Nefh* is involved in the maintenance and maturation of neuronal calibre and axons, and may cause neural plasticity (Lee and Cleveland, 1996). The high expression of *Nefh* in all three experimental groups suggests that the neural system of bumblebee foragers maintains high plasticity during associative learning. The expression of *Dazap2* showed a change in the opposite direction in terms of qPCR and RNAseq (Figure 4.4), which may be due to experimental conditions, such as primer design and RNA sample storage.
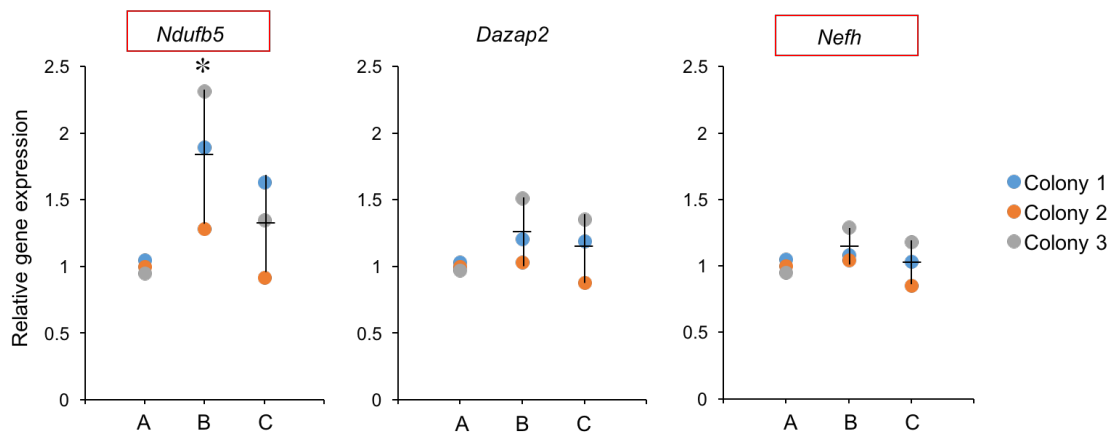
Figure 4.4. Expression of three genes measured by qPCR. Gene expression was relative to expression in Group A, which was normalized to 1. Symbols represent colonies used for test and the three dots in Group A were dispersed on the y-axis for clarity of presentation. The red box marks the genes which have been validated (the expression changes were consistent with the RNAseq results). The asterisk indicates a significant difference ($p < 0.05$) compared with Group A. Horizontal bars indicate mean. Vertical bars indicate standard error of the mean. A: 0-hour Control; B: 0-hour Learning; C: 4-hour Learning.

4.4 Discussion

In this study, I examined the dynamic gene expression changes after associative color learning. I found that gene expressions changed immediately after new color learning and different sets of genes were up- or down-regulated at different time points after learning (immediately and 4 hours after training), which may be responsible for long-term memory formation. In addition, many learning and memory-related candidate genes were found in this study and their particular roles in memory formation should be explored in the future.

The number of differentially-expressed genes associated with visual long-term memory formation

Transcriptomic expression patterns have been described for many different aspects of bee behaviors, such as division of labor, foraging experience, social behaviors, memory formation and brain lateralization during olfactory learning (Whitfield et al., 2003; Robinson et al., 2005, 2008; Sen Sarma et al., 2010; Lutz et al., 2012; Guo et al., 2016). For instance, thousands of differentially expressed genes were detected when comparing gene expression between bee nurses and foragers (Whitfield et al., 2003); 500 genes were found to be correlated with duration of foraging experience (Lutz et al., 2012); previous studies have also looked for learning/memory-related genes by comparing the learning group and the no-learning group and found 388 (visual learning; Qin et al., 2014), 259 (olfactory learning; Wang et al., 2013) and 77 (olfactory learning; Cristino et al., 2014) differentially expressed genes in honeybees. The number of differentially expressed genes found in my study was also relatively small. All these findings suggest that specific memory formation, compared to the behavioral and physiological transitions from one lifestyle to another, or the many environmental influences that come with foraging experience, involves a moderate number of genes. The number of genes related to bee visual learning and memory found in this study was much smaller than that found by Qin et al. (2014). This may be due to the fact that the two time points I tested were closer together and that I controlled for bees' foraging experience, reward level and the time of collection more strictly, each of which would reduce the number of DEGs.

The three studies in honeybee learning and memory have shown a general downregulation of protein-coding genes in the conditioned group (Wang et al., 2013; Cristino et al., 2014;

Qin et al., 2014), which was not found in this study. The differences should come from the different learning/memory states. The bees I used were collected immediately or 4 hours after training and they were in a state to process the learning information and store it in the brain (Bailey et al., 1996; Muller Igaz et al., 2002; Alberini, 2009; Lefer et al., 2013). Conversely, the bees in their studies were collected two or three days after the bees' first stimuli-reward association and they were in a state where long-term memory had already been formed and where there may not have been a need for synthesis of new proteins that used for neural plasticity. To the best of my knowledge, my study is the first to examine the transcriptomic changes shortly after learning or during memory formation in bees, i.e. when large transcriptomic changes are expected to be seen.

Genes and functional processes involved in visual long-term memory formation

The upregulated genes in the 0-hour Learning group, compared with the 0-hour Control group, were enriched for 'Binding' and 'Catalytic activity' (GO terms). Several genes encode enzymes, such as phosphatase, methyltransferase, glutaminyl-tRNA synthetase. Most of the detected pathways were in the category of Signal transduction and Nervous system (e.g. 'Serotonergic synapse', 'Glutamatergic synapse', and 'Dopaminergic synapse' pathways). In support of my findings, Hoedjes et al. (2015) have demonstrated the enrichment of signaling-related genes immediately after training in a wasp species that can form long-term memory after only one conditioning trial, such as SLIT-ROBO Rho GTPase-activating protein (*SRGAP1*) and Glutamate receptor subunit 1 (*GluR1*).

The synaptic-related genes *Rab10* and *Shank1* were detected here. The Rab family of proteins (GTPases) are responsible for vesicles formation, transport and fusion with membranes, and play significant roles in cognitive functions (D'Adamo et al., 2014). Rab10 regulates neuropeptide release from vesicles in the nematode *C. elegans* (Sasidharan et al., 2012) and is required for dendrite arborization and axon growth (Wang et al., 2011; Zou et al., 2015). The Shank family of proteins acts as scaffold proteins, which are required for the development and function of neuronal synapses. Shank1 regulates excitatory synaptic strength, promotes dendritic spine maturation and spine head enlargement, and enhances presynaptic function (Sala et al., 2001; Mao et al., 2015). One study reports that Shank1-deficient mice display enhanced performance in a spatial learning task, while their long-term memory retention in this task is impaired (Hung et al., 2008), which suggests an important role of Shank1 in memory formation. It is noteworthy that three genes (*Rab10*, *Hsp83*, LOC100642507 (DNA polymerase beta)) upregulated in the 0-hour Learning group belong to the GO term 'Response to stimulus'. The overexpression of these genes may enable organisms to respond properly to environmental stimuli at both the cellular and behavioral levels.

The genes that were upregulated in the 4-hour Learning group, compared with 0-hour Learning group, were enriched for GO terms involved in 'Metabolic processes' and 'Cellular processes'. The 'Metabolic processes' include 'Nucleic acid metabolic process', 'Cellular macromolecule metabolic process' and 'Organic substance metabolic process', which enable the process of transcription and translation. It seems that building of new proteins is the predominant activity during the four hours after training. Accordingly, the pathways identified here were in the categories of Transcription, Translation, Transport and

catabolism, and Signal transduction. Transcription is necessary for long-term memory formation and at least two transcriptional waves are required, the first occurs during or shortly after training and the second occurs 3-6 hours after training (Lefer et al., 2013). A small set of immediate-early genes is involved in the first wave and then their protein products trigger the expression of more target genes several hours later, which are responsible for synaptic reorganization through protein synthesis (Bailey et al., 1996; Stork and Welzl, 1999; Muller Igaz et al., 2002; Alberini, 2009; Lefer et al., 2013), which supports my findings. Cristino et al. (2014) report that the metabolic processes were downregulated after odor learning. The bees they used were collected after the second day of training (bees had 6 trials with 10 min inter-trial interval each day), when the bees had formed long-term memory. I speculate that some genes involved in the metabolic processes are kept at high expression level four hours after training and then their expression level decreases later.

The learning-related candidate genes found here included the upregulated gene *Arhgap44* (rho GTPase-activating protein), the downregulated gene *Hsp83* (heat shock protein 83-like) and *EIF4EBP2* (eukaryotic translation initiation factor 4E-binding protein 2) in the 4-hour Learning group. Rho GTPase-activating proteins, with their substrates Rho-family GTPases, regulate multiple processes in neuronal systems, such as axonal and dendritic growth, remodeling of spines and formation of synapses (Moon and Zheng, 2003; Nasu-Nishimura et al., 2006; Bustelo et al., 2007; Nakazawa et al., 2008; Tolias et al., 2011). Hoedjes et al. (2015) also detected the Rho signaling pathway by comparing gene expression in the heads of wasps at three different time points after odor conditioning. The family of heat shock proteins is produced by cells in response to stressful conditions (such

as heat shock and exposure to heavy metals) and the upregulation of heat shock proteins protect the cell from impairment (Pardue et al., 1992; Santoro, 2000). Several transcriptomic studies of honeybees show that heat shock proteins are associated with foraging activity (Whitfield et al., 2006; Williams et al., 2008; Sen Sarma et al., 2010; Toth et al., 2010; Lutz et al., 2012), and learning and memory formation (Wang et al., 2013; Cristino et al., 2014; Qin et al., 2014). The high expression of *Hsp83* in 0-hour Control and 0-hour Learning groups (bees were collected immediately after training) in my study may prepare the bees to respond properly in challenging foraging conditions and learning. The translation repressor EIF4EBP2 (eukaryotic translation initiation factor 4E-binding protein 2) has been shown to be involved in synaptic plasticity, learning and memory formation. *EIF4EBP2* knock-out mice exhibit a deficit in spatial learning and memory, and impaired fear-associative memory (Banko, 2005; Gelinas et al., 2007). The above results suggest that signaling- and protein synthesis-related genes are activated several hours after new color learning, which may modulate synaptic plasticity. Subsequently, the reorganization of synaptic structures can store the learned information.

In summary, my study shows for the first time the dynamic and temporal transcriptional expression patterns involved in long-term memory formation in bees following visual learning. Bioinformatical analyses showed that the genes triggered immediately by new color learning were associated with signal transduction; and the genes upregulated four hours after new color learning were related to transcription and translation, which suggests building of new proteins is the predominant activity four hours after training. I also suggest the candidate genes (e.g. *Rab10*, *Shank1* and *Arhgap44*) involved in bumblebee color learning, which should be validated and their functions in neural plasticity and learning and

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memory should be explored in detail in future work. Bumblebees accomplish a large number of complex behaviors (e.g. ball rolling, string pulling, decision making affected by emotion-like states) (Alem et al., 2016; Perry et al., 2016; Loukola et al., 2017), and the understanding of basic mechanisms of simple memory can contribute to utilize this powerful model to unravel the transcriptomic architecture of complex cognition in the future.
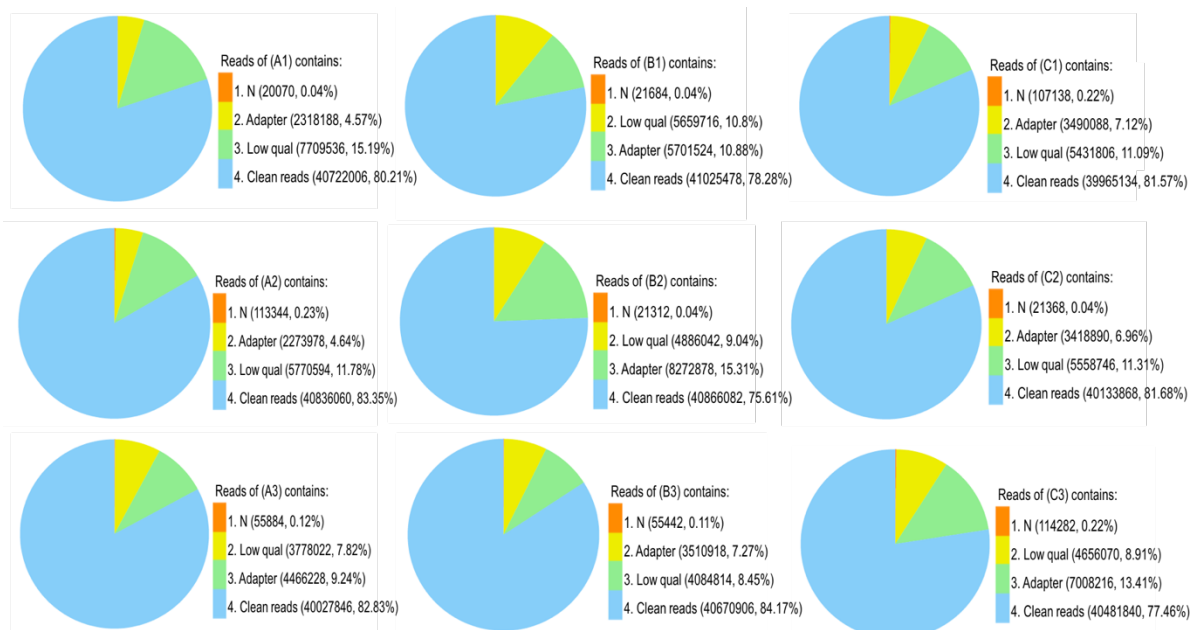
4.5 Supplementary information

Figure S4.1. Classification of raw reads for each sample. Before data analysis, the reads with more than 10% unknown nucleotides (N), with adapters (Adapter), and low quality reads (Low qual) were removed from raw reads. The remaining reads were the clean reads. The value indicates reads number and its ratio on raw reads. A: 0-hour Control; B: 0-hour Learning; C: 4-hour Learning.
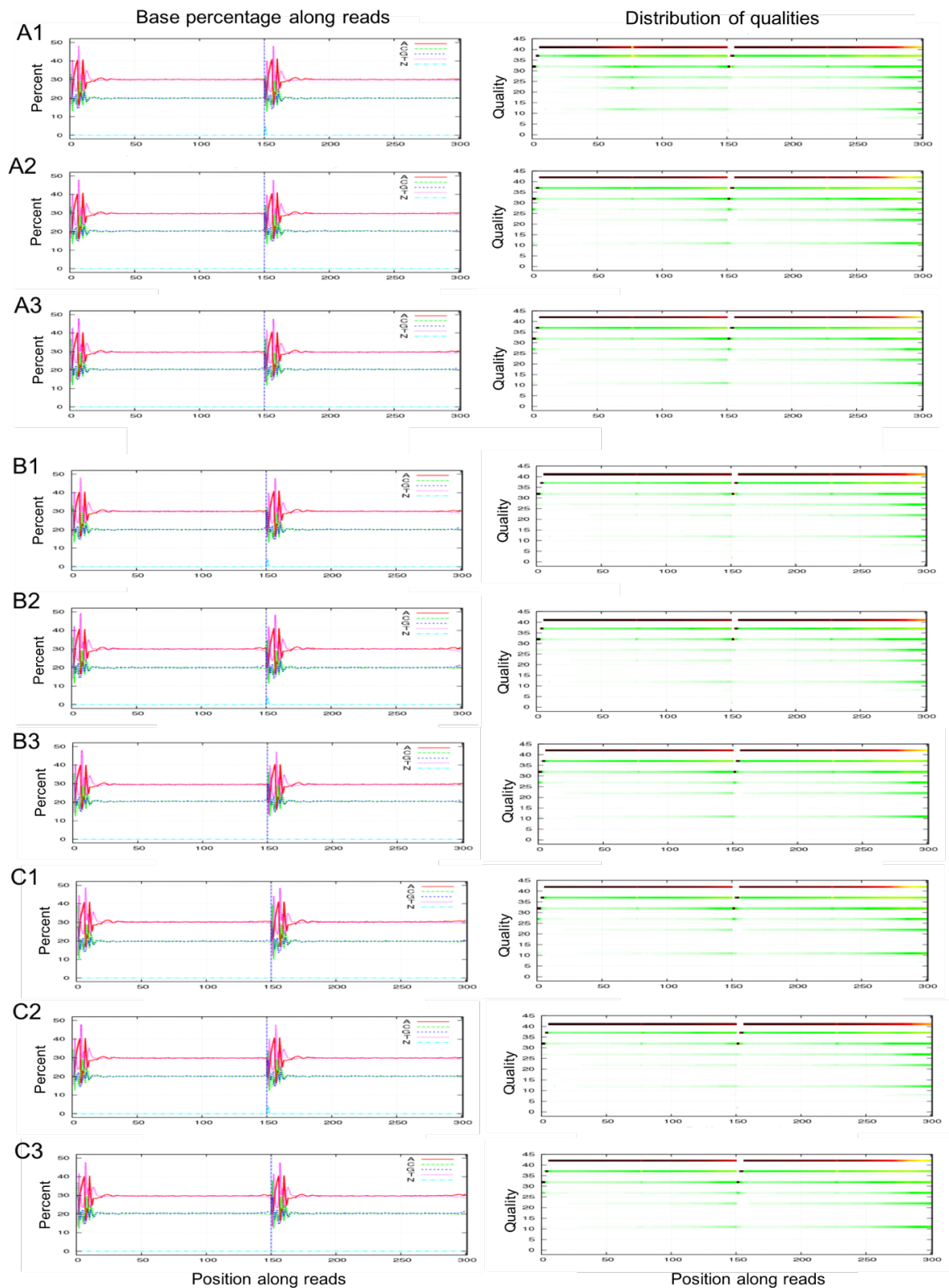
Figure S4.2. Quality assessment of sequencing paired-end clean reads. (**Left**) The nucleotide composition of RNA-Seq reads. The percentage of each nucleotide (Y-axis) is plotted against read length (X-axis; 1-150bp and 151-300bp represent the reads from end 1 and end 2 separately). Colors indicate different nucleotides (A = red; C = Green; G = Blue; T = Magenta; N: Light Blue). A curve should be overlapped with T curve, while G curve should be overlapped with C curve, which can be seen from all

my samples. If abnormal condition happens during sequencing, it may show an unbalanced composition. Note that the changing composition in the beginning of reads is common in RNA-Seq data. (**Right**) Quality distribution of bases along RNA-Seq reads. Each base quality (Y-axis) is plotted against read length (X-axis; 1-150bp and 151-300bp represent the reads from end 1 and end 2 separately). Quality score reflects the sequencing error rate, and the relationship between them is sequencing error rate 1%, 0.1% and 0.01% corresponds to quality score 20, 30 and 40. Most of base positions in my sequencing show good quality (score >20). A: 0-hour Control; B: 0-hour Learning; C: 4-hour Learning.
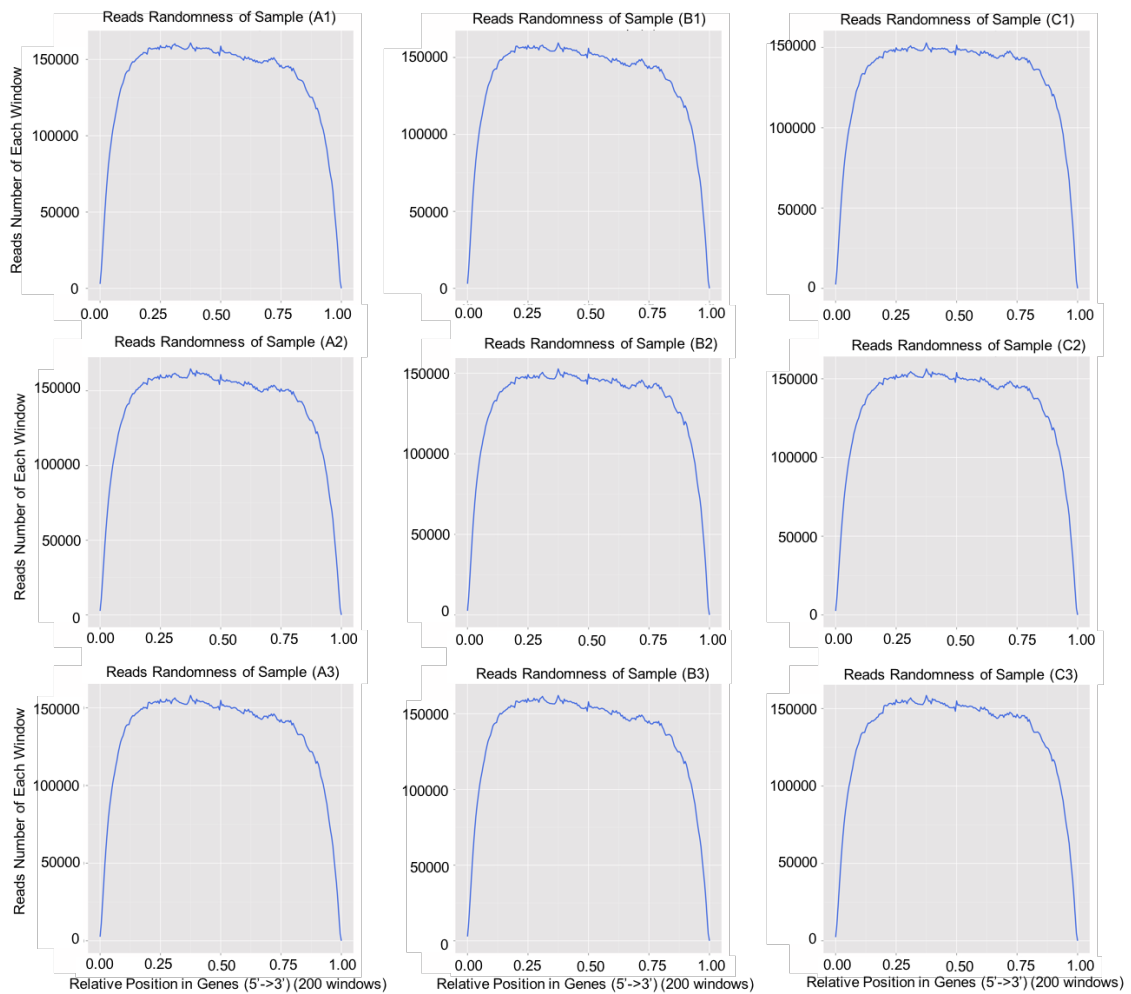

Figure S4.3. The distribution of reads on bumblebee (*Bombus terrestris*) reference genes. X-axis is the relative position in genes which is calculated as the ratio between read location and the gene length, and Y-axis is the number of reads. Reads should be evenly distributed on reference genes, otherwise it means the randomness is poor (i.e. reads prefer to specific gene region) which will affect following analyses. The read randomness in all my samples is good as shown in the figure (i.e. reads were evenly distributed on reference genes). A: 0-hour Control; B: 0-hour Learning; C: 4-hour Learning.
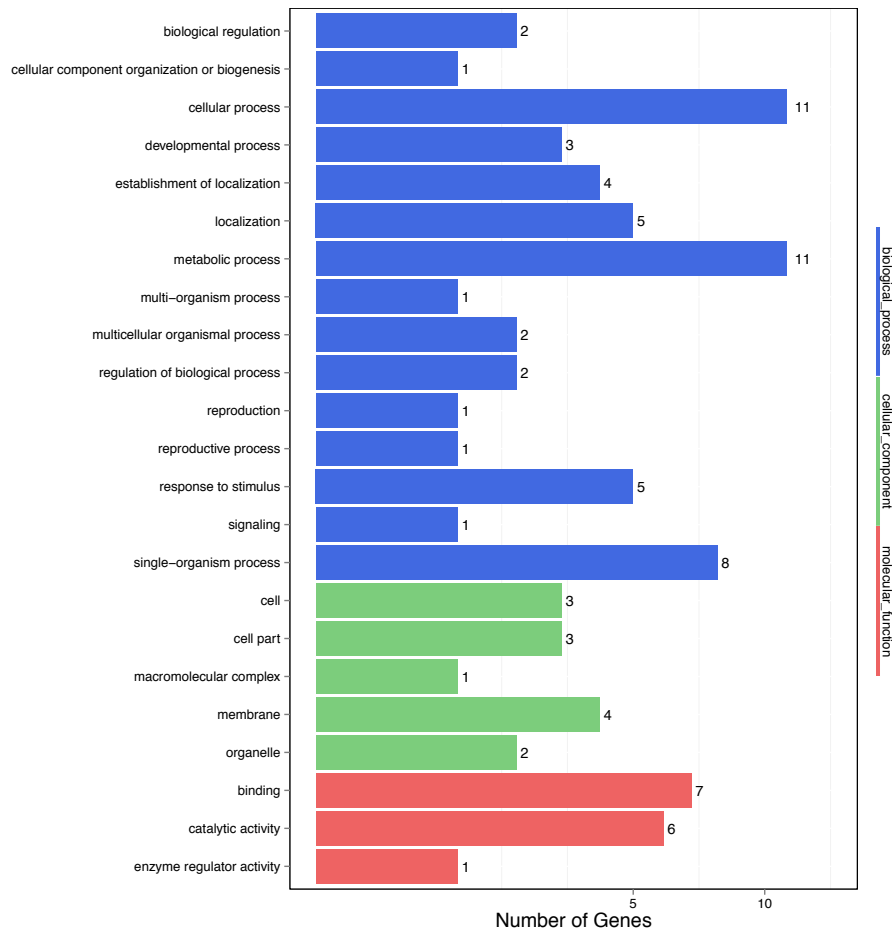
Figure S4.4. GO functional classification of differentially expressed genes (DEGs). The DEGs were annotated into three main categories: biological process, cellular component and molecular function. The number of genes in each GO term is displayed.

Table S4.1. The quality of RNA samples used for sequencing. RNA concentration and integrity were measured by Agilent 2100. Level A means the sample is qualified and the amount of sample satisfies two times of library construction or more. A: 0-hour Control; B: 0-hour Learning; C: 4-hour Learning.

Sample Name	Concentration (ng/µl)	Volume (µl)	Total Mass (µg)	RIN	Library Type	Test Result
A1	456	55	25.08	7.6	HiSeq Eukaryotic Transcriptome	Level A
B1	474	55	26.07	7.7	HiSeq Eukaryotic Transcriptome	Level A
C1	393	56	22.01	7.9	HiSeq Eukaryotic Transcriptome	Level A
A2	480	57	27.36	7.9	HiSeq Eukaryotic Transcriptome	Level A
B2	516	57	29.41	8	HiSeq Eukaryotic Transcriptome	Level A
C2	627	57	35.74	7.9	HiSeq Eukaryotic Transcriptome	Level A
A3	528	57	30.1	8	HiSeq Eukaryotic Transcriptome	Level A
B3	549	54	29.65	8	HiSeq Eukaryotic Transcriptome	Level A
C3	639	57	36.42	7.9	HiSeq Eukaryotic Transcriptome	Level A

Table S4.2. The primers used for qPCR.

Gene	Primers (Forward)	Primers (Reverse)
Ndufb5	GGAGGATATACAGGACCACG	GTCCGTGATAAGATAGAGGATGT
Dazap2	TGCCTAATGGATTCGATGCT	ATATAGCCACGCTGTGACTC
Nefh	TCCGAATCTCACGAAACTGT	CTGGGACTACAGCCTTAACA
Ef1α	AGAATGGACAAACCCGTGAG	CACAAATGCTACCGCAACAG

Table S4.3. The top 20 enriched pathways for the 110 DEGs.

Pathway	Pathway ID	Level 1	Level 2
Estrogen signaling pathway	ko04915	Organismal Systems	Endocrine system
Base excision repair	ko03410	Genetic Information Processing	Replication and repair
Antigen processing and presentation	ko04612	Organismal Systems	Immune system
AMPK signaling pathway	ko04152	Environmental Information Processing	Signal transduction
Fatty acid metabolism	ko01212	Metabolism	Global and overview maps
Viral carcinogenesis	ko05203	Human Diseases	Cancers: Overview
Protein processing in endoplasmic reticulum	ko04141	Genetic Information Processing	Folding, sorting and degradation
PI3K-Akt signaling pathway	ko04151	Environmental Information Processing	Signal transduction
Basal transcription factors	ko03022	Genetic Information Processing	Transcription
Fatty acid elongation	ko00062	Metabolism	Lipid metabolism
Nucleotide excision repair	ko03420	Genetic Information Processing	Replication and repair
Biosynthesis of unsaturated fatty acids	ko01040	Metabolism	Lipid metabolism
Insulin signaling pathway	ko04910	Organismal Systems	Endocrine system
NOD-like receptor signaling pathway	ko04621	Organismal Systems	Immune system
HTLV-I infection	ko05166	Human Diseases	Infectious diseases: Viral
Dopaminergic synapse	ko04728	Organismal Systems	Nervous system
Glucagon signaling pathway	ko04922	Organismal Systems	Endocrine system
Progesterone-mediated oocyte maturation	ko04914	Organismal Systems	Endocrine system
Prostate cancer	ko05215	Human Diseases	Cancers: Specific types
Endocytosis	ko04144	Cellular Processes	Transport and catabolism

Chapter 5

General Discussion and Conclusion

Unlike the molecular mechanisms underlying bees' olfactory learning (Hourcade et al., 2010; Gauthier and Grünewald, 2012; Menzel, 2012; Müller, 2012), little was known before this project about mechanisms underpinning bees' visual learning, although there is plentiful evidence showing their remarkable visual learning ability (Srinivasan et al., 1998; Menzel, 1999; Giurfa, 2007, 2013; Srinivasan, 2010). My thesis aims have been to study the neurobiological basis of bees' visual learning. The research was conducted at two levels, the synapse and gene expression levels. In addition, the neural basis of inter-individual differences in learning and memory ability, which is poorly explored in the literature, has also been investigated here. My thesis adds new knowledge to our understanding of the molecular mechanisms of learning and memory.

5.1 Visual learning and visual experience induced synaptic plasticity

It is widely accepted that synaptic plasticity is the fundamental mechanism of learning and memory (Bliss and Lømo, 1973; Milner et al., 1998; Kandel, 2001). There is accumulating evidence that experience or learning induces structurally synaptic plasticity (the appearance and disappearance of presynaptic boutons and dendritic spines, accompanied by synapse formation and elimination, respectively). The structural synaptic connectivity changes may lead to functional changes in animal brains (Holtmaat and Svoboda, 2009; Caroni et al., 2012).

In Chapter 2, the effect of visual learning and visual experience on synaptic plasticity was examined, where the microglomeruli (presynaptic boutons) were quantified in the mushroom body calyx. Microglomerular density was quantified in three groups with different amount of color learning (in 10 Color Learning task, bees were trained to associate five colors with reward and five different colors with punishment; in 2 Color Learning, bees were trained to associate one color with reward and another color with punishment; in 0 Color Learning, bees did not receive any training). Microglomerular density in the collar (the visual input region in the calyx) increased with the amount of color learning, which demonstrates that long-term visual memory formation boosts microglomerular density. To the best of my knowledge, this is the first study showing that changes in microglomerular organization can be induced via acquisition of visual memory. The result is consistent with findings for olfactory learning, showing that olfactory long-term memory formation increases microglomerular density in the lip, the olfactory input region in the calyx (Hourcade et al., 2010; Falibene et al., 2015). In addition, a volumetric increase of the whole calyx in the group that learned to associate many colors with reward was observed. The calyx volume increase in this study may be due to enriched visual stimulation or the foraging activity experienced in the training. It has been documented that sensory experience and foraging activity induce neuropil volumetric increases in the mushroom body of bees and ants (Farris et al., 2001; Maleszka et al., 2009; Stieb et al., 2010, 2012). In the following experiment, the effects of color associative learning, simple color stimulation and foraging activity on the observed microglomerular density and calyx volume changes were analyzed. Three groups of bees were compared: Activity Control group (bees were trained to associate reward with clear chips), Color Control group (bees

were trained to associate reward with clear chips which were surrounded by unrewarding colored chips), and Learning group (bees were trained to associate five colors with reward and five different colors with punishment). It was shown that simple color exposure resulted in an increase of the microglomerular density in the calyx collar and foraging activity played a significant role for the total calyx volume increase in the color learning group. Complex sensory stimuli in an enriched environment can induce structural and functional neural plasticity (Van Praag et al., 2000; Faherty et al., 2003; Leggio et al., 2005; Sale et al., 2009; Bednarek and Caroni, 2011; Hige et al., 2015; Brenes et al., 2016) and the 10 Color Learning may represent an enriched environment in my laboratory experiments.

I have also found that associative visual learning resulted in an increase of the surface area of presynaptic boutons. Changes in bouton size could reflect changes in synaptic strength (Schikorski and Stevens, 1997; Murthy et al., 2001). The presynaptic bouton surface area is tightly linked with several other synaptic components whose changes can alter neurotransmitter release and are thought to be important for determining synaptic strength and efficiency (Yeow and Peterson, 1991; Murthy et al., 2001; Meyer et al., 2014). Thus, my result indicates that associative learning may increase synaptic strength through changing presynaptic bouton morphology. However, the limitation should be noted that the relationship between presynaptic bouton surface area and the synaptic strength are speculative and have not been validated in bee brains. This should be examined in future work.

Long-term potentiation (LTP) and long-term depression (LTD) are two of major cellular mechanisms underpinning learning and memory formation (Bliss and Collingridge, 1993; Toni et al., 1999; Yuste and Bonhoeffer, 2001). In mammals, the induction of LTP in the

hippocampus results in the growth of new spines, stabilization and enlargement of dendritic spines, while LTD induction causes spine shrinkage and loss, and reduces the number of boutons associated with spines (Becker et al., 2008; Butz et al., 2009; Holtmaat and Svoboda, 2009). Similar to LTP, learning and memory formation (e.g. motor skill learning, spatial learning and fear learning) induce presynaptic bouton remodeling, the growth of new dendritic spines, spine maturation and stabilization by affecting spine's size and shape in rodents and birds (Holahan et al., 2006; Xu et al., 2009; Roberts et al., 2010; Caroni et al., 2012; Lai et al., 2012). The growth and loss of some axonal boutons and dendritic spines lead to synapse formation and elimination, and then the morphological changes of boutons and spines affect the synaptic strength and stabilization (Holtmaat and Svoboda, 2009; Caroni et al., 2012; Meyer et al., 2014). Based on these previous findings and my own results, I infer that exposure to the multi-color stimuli in the 10 Color Learning group (enriched environment) could result in an increase of the number of synapses first and then associative learning may enhance synaptic strength where the learned information is stored.

5.2 Memory retention and learning speed correlate with microglomerular density

There is evidence for inter-individual variation in learning abilities in a variety of animal species (Süß et al., 2002; Conway et al., 2003; Matzel et al., 2003; Raine and Chittka, 2008; Kotrschal and Taborsky, 2010; Snell-Rood et al., 2011; Muller and Chittka, 2012). This variation can affect animals' fitness and can enable animals to adapt to changing environments (Dukas, 2008; Muller and Chittka, 2008; Hoedjes et al., 2011; Smid and Vet, 2016).

The neurobiological mechanisms underlying individual variation in learning and memory have been explored in many animals and many neural correlates of cognitive ability have been discovered (Conway et al., 2003; Page et al., 2006; Dukas, 2008; Neubauer and Fink, 2009; Deary et al., 2010; Pietschnig et al., 2015). In humans, positive correlations have been found between individual intelligence and brain size, the size of a specific brain region (e.g. cerebral cortex, grey matter), working memory capacity, genetic background and neural efficiency (Gur et al., 1999; Thompson et al., 2001; Posthuma et al., 2002; Conway et al., 2003; Neubauer and Fink, 2009; Deary et al., 2010; Pietschnig et al., 2015). Positive correlations between intelligence and gray matter volume have been reported (Gur et al., 1999; Luders et al., 2009). Grey matter consists of neuronal cell bodies, dendrites and synapses. Increased grey matter might indicate an increase of numbers or the density of neurons or synapses, which could allow more efficient information processing and may lead to improved cognitive function (Luders et al., 2009). However, it is not easy to investigate the synaptic structures underlying human intelligence due to many limitations, e.g. ethical considerations and complexity of learning tasks.

In Chapter 3, I explored how the density of synaptic complexes (microglomeruli) within specific regions of the bumblebee brain relates to inter-individual differences in learning and memory performance on a visual discrimination task. The density of microglomeruli in the collar of the calyx (visual input region) was found to be positively correlated with learning speed in training and memory performance in the retention test, which was conducted two days after training. Higher microglomerular density, measured through anti-synapsin immunolabelling, signifies higher density of synapsin-positive presynaptic boutons, which indicates more functional synapses (Ferreira et al., 1998; Morimoto et al.,

1998; Sato et al., 2000; Hart et al., 2011). More active synapses mean higher neurotransmitter release probability which can promote signal transduction and strengthened neural connections (Schikorski and Stevens, 1997; De Paola et al., 2006). The observed structural and functional changes may lead to faster learning speed and better memory retention.

In Chapter 3, I also tried to answer the question whether higher microglomerular density in the collar of bees results from visual learning, or whether it is already in place to allow for better retention performance. Unfortunately, my study cannot provide causal links between microglomeruli density and retention performance. Visual learning can induce brain plasticity in visual representation areas in vertebrates (Sherry and Duff, 1996; Furmanski et al., 2004; Kolb et al., 2008; Sasaki et al., 2009; Lerch et al., 2011; Seitz, 2011). For example, spatial learning causes an increase in size of the hippocampus in food-storing birds, who rely on visual information from nearby landmarks to locate concealed caches (Sherry et al., 1992; Sherry and Duff, 1996); visual spatial learning affects dendritic arborization and spine density in rats (Kolb et al., 2008); studies on humans show that visual learning can increase primary visual cortex response to the trained stimuli and cause neural changes in the visual cortex (Furmanski et al., 2004; Sasaki et al., 2009). My own findings show that visual learning can increase microglomerular density, and that the density of the pre-existing microglomeruli is positively correlated with learning speed. I speculate that both the pre-existing and the learning-induced microglomeruli contribute to the final microglomerular density which correlates with memory performance. To the best of my knowledge, this is the first report of a positive correlation between synaptic complex density and individual bee learning and memory performance in a specific task. These new

findings improve our knowledge of how neural structure may relate to inter-individual differences in learning and memory.

5.3 The dynamic gene expression in bees' visual learning and memory formation

Transcription is necessary for structurally synaptic plasticity, learning and memory formation. In Chapter 4, I investigated the genes involved in bees' visual learning and memory, and examined their dynamic expression patterns at two time points (immediately and four hours after training) by high-throughput sequencing. The results showed that the upregulated genes triggered immediately after training related to signal transduction; the upregulated genes activated four hours after new color learning were associated with transcription and translation. These results are consistent with previous findings that two transcription waves are needed for long-term memory formation shortly after learning, and that the synaptic plasticity-related protein synthesis happens several hours after training (Bailey et al., 1996; Stork and Welzl, 1999; Muller Igaz et al., 2002; Alberini, 2009; Lefer et al., 2013).

Many learning and memory-related genes have been found and studied in a variety of animals (Keene and Waddell, 2007; Alberini, 2009; Hoedjes et al., 2015) and some of them were also detected in my study. For example, *Rab10*, *Shank1* and *Arhgap44* have been shown to be important for synaptic plasticity in rats (Sala et al., 2001; Moon and Zheng, 2003; Bustelo et al., 2007; Tolias et al., 2011; Wang et al., 2011; Mao et al., 2015; Zou et al., 2015) and all three genes were also found in this study, suggesting their roles in bees' color learning. However, the functions of these genes in bees' visual learning and long-term

memory formation should be validated by different methods (e.g. qRT-PCR, in situ hybridization and RNAi) in future work.

In conclusion, my thesis demonstrates that microglomerular organization in the mushroom body calyx of the bee brain plays a significant role in bee visual learning and memory formation, and in inter-individual learning differences. My work also reveals the dynamic and temporal transcriptomic patterns which are required for long-term memory formation and shows the candidate genes involved in learning and memory formation. Altogether, my work deepens our understanding of the neural basis of learning and memory. Future work is needed to investigate the molecular mechanism underlying inter-individual learning variations at other levels, such as the dendritic spine-related neural plasticity, the signaling cascade molecules and genes. Moreover, future studies should aim to combine the behavior, neural plasticity and gene expression changes together and understand their interplay.

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