Addressing the role of central plasticity for olfaction using a novel model system *Apis dorsata*

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by

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DECLARATION

I, **M. Sandhya**, hereby declare that this thesis entitled, **"Addressing the role of central plasticity for olfaction using a novel model system *Apis dorsata*"** submitted by me under the guidance and supervision of **Dr. Joby Joseph** is a bonafide research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma. I also declare that this is a bonafide work which is free from plagiarism.

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CERTIFICATE

This is to certify that the thesis entitled **'Addressing the role of central plasticity for olfaction using a novel model system *Apis dorsata*'** submitted by **Ms. M. Sandhya** bearing registration no **13CCPC01** in partial fulfilment of the requirements for the award of Doctor of Philosophy in Cognitive Sciences is a bonafide work carried out by her under my supervision and guidance.

The thesis has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

(Dr. Joby Joseph)
Research Supervisor

(Prof. Ramesh K. Mishra)
Head of the Department/Centre

(Prof. Prakash Babu)
Dean of the School

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(Dr. Joby Joseph)
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Dedicated to

To my Parents,

Sri. M. Vijaya Kumar Reddy and Smt. Leela
For all the LOVE, Trust and privileges

"Miss You"

Samyukta and Nandann, this is for you

Joby Sir, this is because of you. I OWE this to you

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List of Abbreviations

AN	Antennal Nerve
AL	Antennal Lobe
CS	Conditional Stimulus
EPL	External Plexiform Layer
IPSP	Inhibitory post synaptic potential
GABA	gamma-amino butyric acid
KC	Kenyon Cell
ITI	Inter Trial Interval
lALT	lateral Antennal lobe tract
LN	Local Neuron
LH	Lateral Horn
LTM	Long Term Memory
LTH	Long Term Habituation
mALT	medial Antennal lobe tract
mlALT	mediolateral Antennal lobe tract
MB	Mushroom Body
mPN	multiglomerular projection neuron
MTM	Mid-term Memory
NGS	normal goat serum
NO	Nitric oxide
OB	Olfactory Bulb
OR	olfactory receptors
ORN	olfactory Receptor Neuron
PBS	phosphate buffered saline

PFA	paraformaldehyde
PER	proboscis extension response
PN	Projection Neuron
PBS	phosphate buffer solution
PCT	Picrotoxin
PKA	protein kinase A
PKC	protein kinase C
uPN	Uniglomerular projection neuron
US	Unconditioned Stimulus
UR	Unconditioned Response
SER	Sting Extension Response
STM	Short Term Memory
STH	Short Term Habituation
VUM mx1	Ventral Unpaired Medial Neuron of the Maxillary Neuromere 1
ODA	9-oxo-(*E*)-2-decenoic acid

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Chapter 1

Introduction

"Stopping before the narrow garage, he sniffed the fumes from Paradise with great sensory pleasure, the protruding hairs in his nostrils analyzing, cataloging, categorizing, and classifying the distinct odors of the hot dog, mustard, and lubricant."

A Confederacy of Dunces by John Kennedy Toole, 1980

1.1 Sensory coding and olfaction

In the representationalist view of brain function, the states of the brain correspond to mental or behavioral states. In sensory systems, the representations are contributing to sensory perception. In sensory systems of organisms, in many cases, we see representations of the features of the stimulus in the outside world inside the brain in terms of the activity of the neurons. Some sensory systems like the visual system are more accessible to us to investigate as we have concrete ways of representing the stimulus mathematically, and we can identify features of the stimuli that are getting represented in the brain. The olfactory stimuli are multi-dimensional in the sense of chain lengths and groups, and we do not yet have a good way of representing them to make sense across organisms. We know that in all organisms where we have been able to record from the olfactory system, the system transforms the olfactory stimulus input of odorants into spatiotemporal neural activity, which we consider as odor representations. First, odor molecules activate dedicated receptors and associated

proteins, activating the subset of receptor cells expressing that receptor in a combinatorial way, with possibly odor-specific transients in their response. The receptor cells convey information to structures in the brain, where odor representations are spatiotemporally transformed, allowing the extraction of relevant information (Kay and Stopfer 2006). The behavioral decision is taken based on this processing.

A large number of model systems have been used to investigate the olfactory systems to understand the principles underlying olfactory processing. Each model system affords possibly different strengths. In this thesis, I characterize a novel model system, *Apis dorsata,* the rock bee or the giant honey bee, and use this model system to get insight into the role of mechanisms involved in generation of oscillations, in the olfactory processing and memory, a long-standing puzzle.

1.2 Model systems in Neuroscience research

Karl Von Frisch (1886-1982), a Nobel Laureate described honey bees as "magic well; for discoveries in biology, because the more is drawn from it, the more is to draw." A Model system is a species used to study a particular aspect of a biological phenomenon where the results can be extrapolated to other organisms. Some species can be used as model systems because all organisms share common ancestors, and many of the metabolic pathways, developmental stages, genetic material, etc., are conserved.

Though primates and rodents are extensively used for research in neuroscience since the 19th – century, invertebrates have also been used as model organisms and aided tremendously in increasing our understanding of mechanisms in neuroscience (Hodgkin and Huxley 1952). They provide advantages like small size, ease of breeding in a laboratory situation, shorter life span, suitability to study many generations, amenability to genetic manipulations, etc.

A classic example is the use of the squid by Hodgkin and Huxley (1952) to study the generation of action potentials. The squid proved to be ideal because of the large size of the axon and the presence of fewer types of ion channels on the membrane, thereby decreasing the complexity. The gill withdrawal response of sea slug, *Aplysia punctata* has contributed to breakthroughs in neuronal basis involved in processes like sensitization, habituation, and classical conditioning (Kandel 1976). The nematode *Caenorhabditis elegans* is preferred as a model organism due to its small size, consisting of around a thousand cells out of which 302 are neurons. The entire neuronal circuitry and the complete genome of *C. elegans* have been mapped (White et al. 1986). It is widely used to study the cellular pathways in the action of neurotransmitters and the working of psychiatric drugs. The diversity of these organisms and the uniqueness of each in addressing very different specific questions points to the need for choosing organisms suited for the problem of interest.

1.2.1 Insect model systems in neuroscience

Drosophila melanogaster is extensively used as a model system in genetic studies. Apart from having a short life span, it has fewer chromosomes compared to humans. Its entire genome is sequenced and annotated, with around 60% of its genes being similar to humans. Chromosomal theory of inheritance (Morgan 1916), X-ray effects on the rate of mutation (Muller 1928) have been studied using Drosophila as a model system. Drosophila has been pivotal in our understanding of sleep (Shaw et al. 2000; Huber et al. 2004), circadian rhythms (Doubey and Sehgal 2017), epileptic seizures (Parker et al. 2011), the action of neurotransmitters (Perisse et al. 2016), labeled line coding of innate behavior (Dolan et al. 2019), etc.,. However it has not been great with studies on the role of oscillations or other features of local field potentials (LFP) owing to its small size.

The locust *Schistocerca americana* is used in studies concerning olfactory coding, oscillatory synchronization (Mac Leod and Laurent 1996; Bazhenov et al. 2001), noise, and baseline activity in the sensory systems (Joseph et al. 2012), vision (Gabbiani et al.2002). *Bombax mori*, the silkworm, that originated in China, has its whole genome sequenced and is used to study the effects of drugs and pesticides (due to its high sensitivity) (Nwibo et al. 2015), and in genetics (Jingade et al. 2011; Zhou et al. 2015). *Manduca sexta*, the tobacco hornworm is used to throw light on timing in olfactory coding on learning and memory (Koenig et al. 2015; Ito et al. 2008; Delahunt 2017). They have been very good for doing electrophysiolgy and have been used to look at olfactory coding in different parts of the olfactory pathway. The bumblebee *Bombus* spp. has been used as a model system to learn about evolution, behavior, and ecology (Woodard et al. 2015). They have been used to study learning and memory, visual and olfactory, but not as much for the physiology underlying it. Honey bee is advantageous as it affords behavioral as well as electrophysiological manipulations.

1.3 Honey bee as a model organism

Honey bees exhibit a rich repertoire of behavior despite their small size. They are social insects and forage large distances in search of food. This requires bees to be able to navigate large distances using visual cues, remember the source and communicate about the location to their nest mates. Bees are shown to possess color vision (Menzel and Backhaus 1991), an ability to distinguish patterns and shapes (Srinivasan et al. 1996; Srinivasan 2010), and a well-developed olfactory system (Guerrieri et al. 2005).

Early investigations by Karl Von Frisch on honey bees opened the doors for the current development in neuro ethological research. Honey bees are the first invertebrates known to possess color vision, detect ultraviolet light, and have dance communication. They communicate information about food sources, nesting sites etc., through what is known as the

waggle dance. The speed and angle of the dance with respect to the vertical, reveals information about the distance of the food source and its relative direction to the sun.

This diverse and rich behavioral repertoire of honey bee with a small neuron number makes it a very valuable system. It is amenable to studies conducted in a restricted and stressful laboratory situation and allows in vivo observation of the neural circuitry. The nervous system of the honey bee consists of a brain and ganglia present in the abdomen and thorax. The bee brain measures around 0.4 - 0.6 mm^3 and has around 960,000 (Witthoft 1967) neurons, and the brain size is large compared to many other insect species. The brain is 30-50 times larger than the drosophila brain (Giurfa 2007). This makes it convenient not only to study the neural circuity but also to access the brain electrophysiologically and study the individual neurons associated with a particular behavior.

Bees are extensively used as a model system to study areas like learning and memory (Menzel and Erber 1978; Menzel 1985), circadian rhythms (Ada Eban-Rothschild and Bloch 2010), aging (Behrends and Scheiner 2010), the action of neurotransmitters (Hosler et al. 2000), DNA methylation (Foret et al. 2012; Li et al. 2017), etc.,.

1.3.1 Honey bee as a model to study learning and memory

Honey bees are extensively used to study the nuances of learning and memory. Learning is said to have occurred when an animal's response to a stimulus has changed after an experience. Learning and memory are crucial for the survival of honey bees as they are required to navigate large distances, remember the location and quality of flower patches and the way back to the hive. Associative learning equips an organism to predict the relations between contingent events they come across and develop adaptive behaviors accordingly. Animals exhibit this form of learning across species. Two forms of associative learning, viz. classical conditioning and operant conditioning, are widely recognized. Pavlov (1927), while

experimenting with salivation in dogs, found that ringing a bell before presenting food made the dog salivate to the ringing of the bell even without food presentation. The dog had learned to associate a previously neutral stimulus (conditioned stimulus, CS) with a reward (unconditioned stimulus, US) and elicit an unconditioned response (UR). This is called classical conditioning. In operant conditioning (Skinner 1938), the animal modifies its behavior based on whether that behavior or set of actions was rewarded or not.

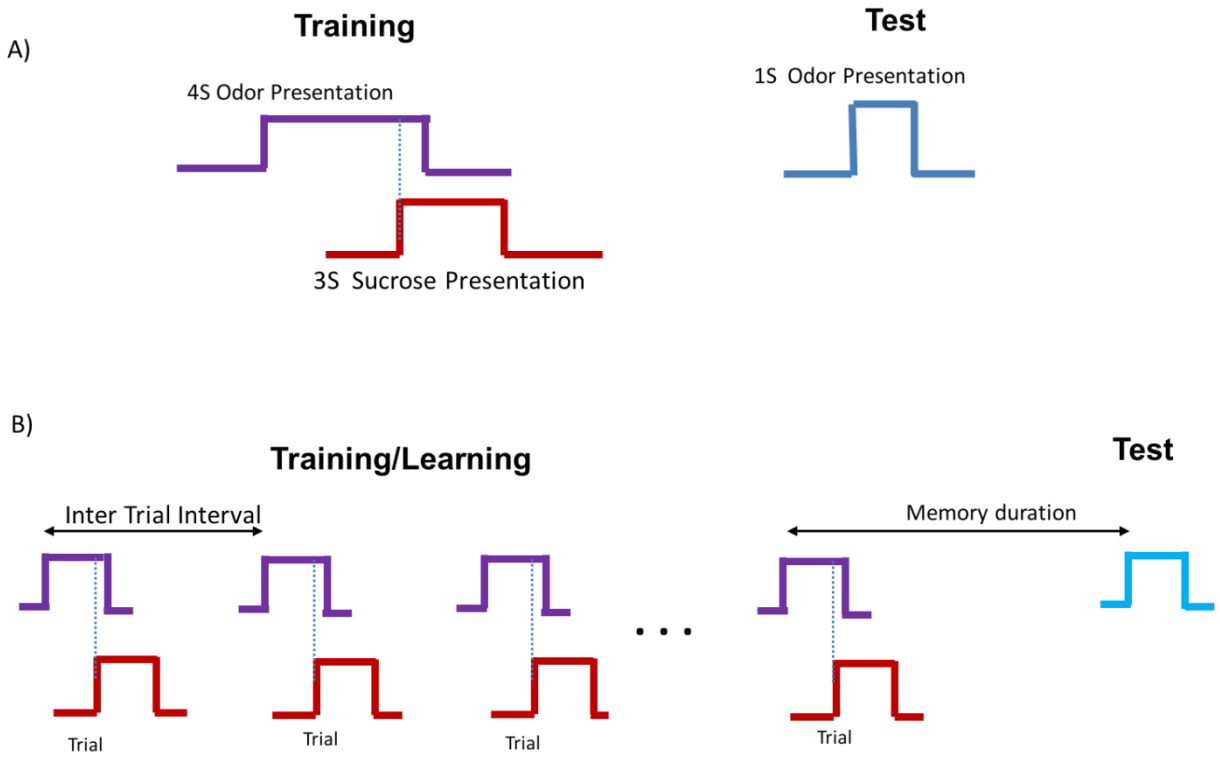


Fig 1.1 PER conditioning protocol: A) Sequence of stimuli in a single pairing or testing. B) Sequence of pairings and testing.

A simple form of classical conditioning/associative learning is found in bees (Giurfa 2007). Absolute conditioning, where stimulus A alone is reinforced, and differential conditioning, where one stimulus (A+) is reinforced, and another stimulus (B-) is not, are the two examples of associative learning. These are called elemental learning because the stimuli used are

elemental. An example of a complex form of associative learning, non-elemental learning called negative patterning, is also reported in bees (Deisig et al. 2001). Here two single stimuli (A+), (B+) are rewarded, whereas their compound (AB-) is unrewarded. Other forms of non-elemental learning like contextual learning (Collett et al. 1997), categorization, and rational discrimination are also found in bees (Mansur et al. 2018).

Unlike in other species like Drosophila (Tully and Quinn 1985), where en-masse training is often required to make effective conclusion, honey bee offers a unique possibility of implementing training protocols individually. This individual training helps in adapting neurobiological approaches to study the neuronal circuitry involved.

The main protocols used to study learning and memory in honey bees include

a) Conditioning of the free-flying bees to approach a visual target: free-flying bees are trained to feed on a feeder voluntarily and are conditioned to visual stimuli like color, shape and patterns.

b) Olfactory conditioning of the proboscis extension response (PER) where olfactory stimuli (CS) are used to condition bees (Kuwabara 1957; Takeda 1961; Bitterman et al. 1983, Giurfa and Sandoz 2012). Individual bees are restrained in tubes in such a way that the antennae and proboscis are accessible. An odor (CS) is forward paired with a sucrose (US) reward presented to the antennae and proboscis. After one or more training trials, the bees exhibit a UR (unconditioned response) of extending the proboscis to the odor alone. Thus, this is a classical conditioning paradigm (Bitterman et al. 1983). The protocol consists of rewarded training trials and unrewarded test trials. During the training trials, the extension of proboscis before the presentation of sucrose is considered as acquisition. If the bee extends proboscis during the test trial, it is said to have learned.

c) Mechanosensory conditioning of the PER: There are three protocols in this conditioning. In one protocol, the bee is rewarded with sucrose solution if the frequency with which the antennae touch an object crosses a threshold (Kisch and Erber 1999). Thus, this is an example of operant conditioning protocol. In another procedure, the bee is rewarded when its antennae scan the surface of an object to determine its texture (Erber et al. 1998; Scheiner 1999). In this case it is not clear wether it involves operant or classical conditioning In a third protocol, the sucrose reward is delivered upon touching one or both antennae following classical conditioning protocol (Giurfa and Malun 2004). In these three protocols, the bee's eyes are painted to prevent interference due to visual cues.

d) Olfactory conditioning of the sting extension response: This is an aversive conditioning protocol in bees (Vergoz et al. 2007). A restrained bee is administered an electric shock paired with an odor. The bee exhibits an unconditioned response of extending the sting or the sting extension response (SER) (Carcaud et al. 2009; Tedjakumala and Giurfa 2013). Training and test trials are recorded similar to appetitive PER conditioning. Bee exhibiting SER is considered to have learned.

1.3.2 Neural basis of olfactory learning and memory

The honey bee is amenable to studying the neural circuitry involved in appetitive olfactory learning online through electrophysiological and optical studies. The cuticle can be cut open, and electrodes can be placed in intended locations, and the neuronal activity can be recorded in vivo. The bee's CS and US pathways are well-identified allowing an integrative study of the underlying neural circuits.

The CS neural pathway starts at the olfactory receptor neurons (ORNs) present on the antennal sensillae. They innervate the olfactory glomeruli present in the antennal lobe (AL). Here the ORNs synapse on to the projection neurons (PNs), and local neurons (LNs). In the

combinatorial coding view, the odor is represented by patterns of glomerular activity of the AL (Joerges et al. 1997; Galizia and Menzel 2000). These activity patterns are bilaterally symmetrical and conserved across individuals (Gailiza et al. 1998, 1999). There is evidence that odor mixtures are represented either as a sum of the individual odors or as the dominant component (Deisig et al. 2006). As the number of components increases, inhibitory interactions are seen to be more (Joerges et al. 1997; Deisig et al. 2006). Faber (1999) found an increase in the glomerular activity pattern for a rewarded odor but not for an unrewarded odor. This kind of combinatorial odor coding is observed in the mushroom bodies (MB) also (Faber and Menzel 2001), the activity of Kenyon cells (KCs), the intrinsic cells of the mushroom body are sparser (Szyszka et al. 2005).

Recording from neurons intracellularly in *Schistocerca americana* (Laurent et al. 1993), *Manduca sexta* (Ito et al. 2008), and *Apis mellifera* (Stopfer et al.1997) has shown that the projection neurons of the AL show multiphasic temporally patterned activity to constant odor pulse. In the temporal coding view, this pattern of activity also has to be taken into account. Thus, which PNs are coactive, and when, forms part of the code, and this expands the coding space. The oscillatory synchronization of the PN spikes is another aspect of the PN responses observed and thought to be involved in sparse coding in the memory centers. Though both these temporal coding aspects of the input to the mushroom body, oscillations and temporal patterning are consistently observed across species, the evidence that they are decoded by the Kenyon cells or the extrinsic neurons of the mushroom body to perform any function is still not clear.

The US pathway comprises the specialized receptors for sucrose on the sensilla, tarsi, and the mouthparts (Whitehead and Larsen 1976; de Brito Sanchez et al. 2005), which innervate the sub esophageal ganglia (Altman and Kien 1987). VUMmx1 neuron or the “ventral unpaired medial neuron of the maxillary neuromere 1”, responds with “long-lasting action potentials

for sucrose presentation to the proboscis and the antenna and is necessary and sufficient to substitute for the sucrose reward" (Hammer 1993). It innervates the AL, lip region of the MB calyces, and the lateral horn (LH) (Hammer 1993, 1997; Schroter et al. 2006) symmetrically. The forward pairing of stimulation of VUMmx1 with odor as CS, can substitute for sucrose reward (Hammer 1993, 1997) in classical PER conditioning. VUMmx1 is an octopaminergic (Kreissl 1994) neuron. Menzel and Hammer (1998) found that octopamine application to the AL or MBs forward paired with olfactory stimuli produced olfactory conditioning similar to that produced by pairing with a sucrose reward. This proves octopamine signaling through VUMmx1 is necessary and sufficient to substitute sugar reward in bees. When a bee is differently conditioned to two odors, VUMmx1 responds to the CS+ but not to CS-. Subsequently, the response to CS + acquires the characters of the response to the US. If a new CS is paired with the previous CS+, this old CS+ can act as reward and cause PER conditioned response to CS. Thus mechanism involving VUMmx1 provides the cellular basis for second-order conditioning. The activity of VUMmx1 resembles the representation by reward dopaminergic neurons of substantia nigra and ventral striatum in mammals (Schultz et al. 1997).

Learning is said to have occurred when information is stored, i.e. memory storage, and it is possible to access this memory, i.e. memory retrieval (Hammer 1994). In bee, different phases of memory are identified viz. short term memory (STM), mid-term memory (MTM), and long term memory (LTM). In olfactory PER conditioning, the very first conditioning trial leads to the formation of STM. A significant part of early or eSTM comprises sensitization due to reward presentation and increases general response to stimuli (Hammer 1994). The late or lSTM develops quickly after pairing (Menzel et al.1999; Eisenhardt 2006). A specific associative memory develops in a few minutes after pairing (Menzel1990; Hammer and

Menzel 1995). These memories are independent of transcription or translation (Menzel et al. 2001; Eisenhardt 2006).

After three CS-US pairing, MTM, early or eLTM and late or lLTM form parallelly. MTM is active 1-6 h after acquisition. It is independent of transcription and translation (Eisenhardt 2006). eLTM forms 1-2 days after conditioning and is translation dependent and can be blocked by anisomycin, whereas lLTM forms after 3-4 days, is dependent on both translation and transcription (Friedrich et al. 2004; Eisenhardt 2006) and can be blocked by actinomycin and anisomycin.

2-3 mins after the learning trial is a period when nonspecific memory decreases, but specific associative memory is still to be formed. Memory during this stage is susceptible to amnesic treatment (Menzel et al. 1974; Erber et al.1980; Menzel1999). For extinction trials that were spaced 1min, 2min, and 10min intervals, memory was least at 2min intervals (Menzel1999). In another experiment, an inter-trail interval (ITI) of 3 mins was less effective in producing LTM than shorter or longer ITIs (Gerber et al. 1998). 3 mins ITI had the lowest LTM after four days compared to 1min and 20 mins ITI (Menzel 1999). These experiments showed that memory 2-3 mins after learning was susceptible to interference. This process of memory consolidation was found to be similar to that in mammals (Kamin 1968).

Interference with PKC affects the formation of MTM but does not affect the LTM. Blocking of protein kinase A (PKA) affected LTM formation but not MTM and STM (Schwarzel and Muller 2006; Eisenhardt 2006). From these results it is suggested that the conversion of STM to MTM is mediated by protein kinase C (PKC), whereas the conversion of STM to LTM is mediated by cyclic AMP-dependent protein kinase A (PKA). The formation of eLTM required releasing the neurotransmitter glutamate in the MBs (Lucatelli et al. 2005) and LTM formation increases the synaptic density in the lip region (Hourcade et al. 2010). The amount of

starvation also affected LTM. Bees starved for 18 hours developed LTM and not the ones, starved for only 4 hours (Friedrich et al. 2004; Eisenhardt 2006). PKA activity was found to be lower in animals starved for 4 hours compared to 18 hours. Different PKA mechanisms appear to control eLTM, and lLTM as an experimental increase of PKA rescued lLTM but not eLTM (Schwarzel and Muller 2006; Eisenhardt 2006). How the temporal coding aspects of the PN population response, temporal patterning and oscillations, interact with these molecular mechanisms, some of which are happening in the AL, is not understood.

1.3.3 Honey bee as a model system to study effect of ITI on learning and memory

It is reported that learning trials with longer spacing between trials produce a better learning rate and long-term memory than closely spaced trials (Ebbinghaus 1885; Jost1897; Menzel et al. 2001). Ebbinghaus observed that "Given equal associative strength, the older the memory trace at the time of learning repetition, the less forgetting over the long term." The spacing effect is interpreted based on the theory of memory dynamics (Squire 1987). Every learning trial is supposed to aid in memory formation by consolidation and forgetting (Menzel et al. 2001). It has been shown in many model systems that memory formation is affected by the interval between the trials (Carew1972; Tully 1994).

Menzel et al. (2001) studied the result of massed and spaced trials on acquisition and retention of memory and found that spaced trials (10 min ITI) produced better acquisition and long-term memory in PER conditioning of bees than massed trials (30secs ITI). The result remained the same for different trial numbers and a variety of CS. For massed trials, retention for eLTM was higher than lLTM and vice versa for spaced trials. Protein synthesis blockade with Actinomycin D reduced the eLTM in spaced and not massed conditioning, and lLTM was decreased in both massed and spaced conditioning. The decrease in acquisition in spaced conditioning is attributed to interference to the consolidation of memory or due to

competition to short-term storage (Hintzman 1974; Gerber et al. 1998) due to short intervals between trials in massed conditioning. Habituation to the CS and saturation of the US may also affect acquisition in massed conditioning but not to a large extent. On the other hand, spaced trials result in better LTM as more time is available for memory consolidation, and other contextual stimuli may also play a role (Menzel 2001)**.**

Retrograde amnesia caused by the cooling of different brain areas after acquisition aided in identifying regions necessary for memory formation (Menzel 1964). Cooling of the AL immediately after conditioning produced retrograde amnesia in the ipsilateral side. Cooling MBs 3-4 mins after conditioning hampered memory retrieval in both sides of the brain (Erber 1980). This time period indicates that AL could be involved in eSTM whereas MBs could be involved in lSTM (Menzel 1999).

There are plasticity mechanisms affecting coding as read out in the field potential and intracellular recordings in the olfactory pathway (Stopfer and Laurent 1999). How these interact or correlate with the molecular mechanisms mentioned above is not known.

1.3.4 Honey bee as a model system to study olfaction

Olfaction is very important for honey bees for successful foraging, nest mate recognition, communication, and defense. The visual system is more accessible for us to investigate as we have a concrete way of representing the stimulus mathematically, and we can identify features of the stimuli that are getting represented in the brain. The olfactory stimuli are multi-dimensional and involve many characteristics. It comprises the transformation of the chemical input of odorants into spatiotemporal neural activity in the organism's brain, leading to a perceptual odor representation. First, the features of odor molecules are detected by dedicated receptors and associated proteins; the signal is then transduced, activating a subset of receptor cells in a combinatorial way. The receptor cells convey information to

structures in the brain, where odor representation occurs, allowing extraction of relevant information (Kay and Stopfer 2006). The behavioral decision is taken based on this processing.

The honey bee olfactory system is evolved to detect a large number of odors as evident from results of odor conditioning experiments from different labs. Bees extensively use pheromones for communication. The queen's mandibular glands release a pheromone through which the queen communicates with the individuals of the hive. It was initially assumed that queen pheromone was a single compound, 9-oxo-(*E*)-2-decenoic acid (ODA) (Barbier and Lederer, 1960; Pain 1961) later on methyl *p*-hydroxybenzoate (HOB) and 4-hydroxy-3-methyoxyphenylethanol (HVA) (Slessor et al.1988) were also found to be main constituents. Queen pheromone attracts the drones to participate in the mating flight. The queen also produces Queen Retinue Pheromone, which attracts the workers to groom the queen and inhibits the development of ovaries of workers (Hoover et al. 2003). The absence of this pheromone encourages the workers to rear new queen or causes some workers to develop ovaries ((de Groot and Voogd 1954; Slessor 2005). The workers secrete nasanov pheromone from the surface of their abdomen, which aids in swarming (Free 1987; Winston 1987). When a worker stings, alarm pheromone is released from the sting glands, which recruits the other members to attack the threat (Free 1987).

During foraging, olfaction also plays an essential role in the detection of floral and other odors. The scent of a flower may give information about the nectar and pollen content of the flower. Bees also associate non-flower odors with a reward if encountered (Finkelstein et al. 2018). Honey bees have good odor discrimination abilities and can sense the subtle differences in flower volatiles (Wright et al. 2002; Smith and Menzel 1989; Bhagavan and Smith 1997; Laska et al. 1999; Wright and Smith 2005). The ability of a bee to discriminate odors is found to increase with the odor concentration. Bees are also known to generalize

similar odors. This generalization is based on the odors' features like carbon chain length, functional group, etc. A Study involving different functional groups and carbon chain lengths found that generalization is not necessarily symmetrical. Generalization from odor 1 to odor 2 may not be the same as a generalization from odor 2 to odor 1 (Guerrieri et al. 2005). It is also found that bees trained to an odor mixture recognized a component based on the salience of the component (Laloi et al. 2000).

1.3.5 Mammalian vs insect olfaction

The mechanism of olfactory processing shares commonalities between mammals and insects. The basic architecture and computation is very similar but there are differences in the molecular mechanisms mediating the processing. The odorant molecules are perceived by olfactory receptor neurons (ORNs) which are highly species dependent. Vertebrates have G-protein coupled receptors, whereas the concensus is that insects, including honey bees, have receptors that act as sites for binding odor molecules and gated ion channels. Thus the tansduciton and signaling occurs through metabotropic channels in mammals and ionotropic channels in insects (Kaupp 2010; Sinakevitch et al. 2017). ORNs converge on second-order neurons in the olfactory bulb (OB) in humans and the AL in honey bees. Humans have 350 odor receptors and 5500 glomeruli in the OB, whereas the honey bee has 170 odor receptors, and the AL has around 160 glomeruli. In humans, each glomerulus is innervated by eight mitral cells and in honey bees by 5-6 projection neurons (PNs) (Sinakevitch et al. 2017). Parallel pathways for olfactory processing are present in humans and honey bees.

In honey bees, PNs innervate the higher olfactory centers MBs and lateral horn (LH), similar to mitral and tufted cells in mammals innervating the piriform cortex (Sinakevitch et al. 2017). In mammals, links between olfactory and auditory pathways are known (Belkin et al. 1997), and in insects, including honeybees, olfactory and visual inputs are processed in the

MBs (Mobbs 1982). Periglomerular cells provide intraglomerular inhibition, and glomerular cells provide interglomerular inhibition in mammalian systems (Shipley and Ennis 1997). In insects, in general, local neurons (LNs) perform this role (Wilson and Laurent 2005). The glomerular core of the insects where LNs inhibit the activity of PNs and other LNs is comparable to the external plexiform layer (EPL) in mammalian systems. In mammalian systems, the olfactory tract connects the OB with higher brain areas, piriform cortex, and anterior olfactory nucleus (Sarnat and Yu 2016), similar to the median and lateral antennal lobe tracts (ALTs) in honey bees (Kirschner et al. 2006). Due to all the similarities described above, the study of insect olfaction can throw light on aspects of mammalian olfactory processing.

1.3.6 Olfactory system of honey bee vs other insects

Drosophila offers advantages like having a simpler olfactory system and available genetic and molecular tools, which can aid in achieving a better understanding of the honey bee olfactory system. The two insects have very similar schemes in the olfactory circuit with few variations. Drosophila has 62 olfactory receptor proteins (Vosshall and Stocker 2007) coded by 60 genes (Larson et al. 2004) related to the G- protein-coupled receptor family compared to 170 OR genes and receptors in honey bee (Robertson and Warner 2006). Drosophila has ORNs even on the maxillary palps in addition to the antenna whereas honey bee has ORNs only in the antenna (Gaudry et al. 2013). In *drosophila*, the 1300 ORNs converge on to specific glomeruli out of the 54 glomeruli present, bilaterally through antennal nerve tracts (Jefferis et al. 2001; Grabe et al. 2015). PNs of the AL innervate the higher olfactory centers the MBs, and the lateral horn through three antennal lobe tracts similar to the honey bee. The PNs in drosophila have been categorized into 11 classes, while the honey bee has only two types (Tanaka et al. 2012). Around 3 PNs innervate a glomerulus in drosophila against 5-6 PNs in honey bees (Mosca and Luo 2014). LNs in drosophila sometimes innervate glomeruli

of AL of both sides, and there are about 100 identified types of LNs (Chou et al. 2010). The Mushroom bodies in drosophila have only one calyx (Power 1943) and comprise around 2500 intrinsic cells called Kenyon cells (Schürmann 1987) compared to the 170,000 Kenyon cells in the double calyx of honey bee.

1.4 Honey bee classification and distribution

Honey bees are arthropods that belong to the class Insecta and order Hymenoptera, (hymen meaning membrane, and pteron meaning wing in Greek), genus *Apis,* and tribe Apini. Along with bees, sawflies, ants, and wasps belong to Hymenoptera. Hymenopterans are characterized by complete metamorphosis in the life cycle and the development of male offspring from unfertilized eggs. Many eusocial species of insects belong to Hymenoptera.

Apis is known to have emerged 30-40 million years ago around the time when flowering plants appeared in Southeast Asia (Arias and Shepherd 2005). Bees evolved from feeding on insect larvae to feeding on pollen and nectar. They acquired morphological features like pollen baskets, proboscis, and colonies to adapt for pollen and nectar collection. Honey bees are estimated to have originated during the Cretaceous period in the Gondwana region, i.e. the areas constituting India, Australia, Africa, Antarctica, and South America. The dry tropical weather in this region was conducive to the evolution of flowering plants with colorful patterns and nectar to attract bees. During the Oligocene period, during extreme cold, the European bee became extinct, whereas the Asian bees thrived. As Europe warmed during the Pleistocene age, *Apis mellifera* or the European bee emerged there, while all other species are sympatric to the tropical climate and originated from Asia (Shepherd and Meixner 2003). Open nesting bees could have appeared before cavity-nesting ones in tropical regions. Open nesting bees are considered to be basal based on the complexity of queen mandibular pheromone (Plettner et al. 1997; Arias and Shepherd 2005).

Currently, the genus is divided into ten species based on DNA markers (Tanaka et al. 2001; Arias and Shepherd 2005; Raiffudin 2007). The cavity-nesting *A. mellifera, A. cerana, A. nuluensis, A. koschevnikovi, A. nigrocincta.* The dwarf honey bees' *A. florea, A. andreniformis,* and giant honey bees' *A. dorsata, A. binghami, A. laboriosa.* These three groups are also divided as *Micrapis* Ashmead consisting of *A. florea, A. andreniformis* and *Megapis* Ashmead consisting of *A. dorsata, A. binghami, A. laboriosa,* and *A. indica* (Engel1988, 1999). *A. laboriosa* is the largest bee species (Sakagami et al. 1980; Arias and Shepherd 2005) and is found in high altitudes of the Himalayas and can measure up to 3 cms or 1.2 inches in length. *A. andreniformis* is the smallest bee species and is found in south Asia (Gupta 2014). The species *A. mellifera* and *A. cerana* are domesticated. A. florea is considered to be the most basal of all the species. *A. dorsata*, *A. mellifera* and *A. cerana* share common ancestry with *A. florea* (Arias and Shepherd 2005). *A. mellifera* and *A. cerana* have diverged from each other more recently, about one million years ago.

A honey bee hive consists of three castes, the male drones, sterile female workers, and the fertile female queen. Drones are haploid and develop when the queen's eggs are unfertilized or from eggs laid by workers. They are distinguishable from workers by large eyes, body size, and absence of sting. They do not perform any function in the hive and aid in fertilizing the queen. The workers perform all the activities of the hive. Young workers initially participate in cleaning cells. One-week-old adults nurse the larvae and maintain the hive. Foragers are recruited by three weeks. There are nectar foragers, water foragers, and pollen foragers who change their roles based on demand. However, the individual has a propensity to restrict itself to a particular job (Pankiw and Page 2000). The average life span of a bee is around five to six weeks (Galizia et al. 2011).

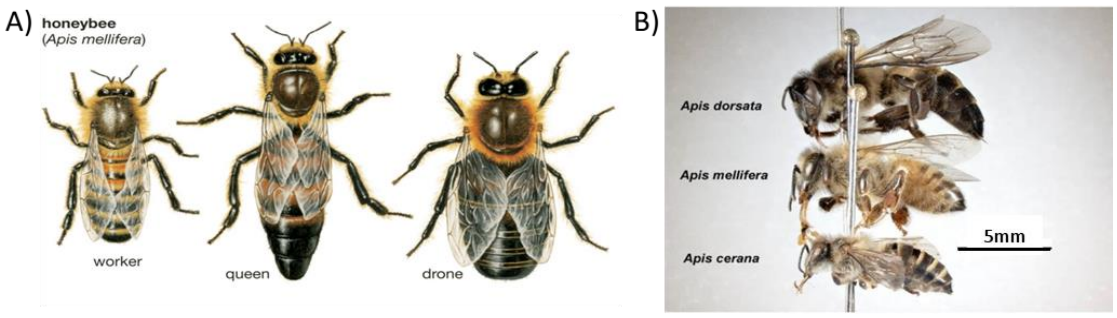


Fig 1.2 A) Picture of different castes of A. mellifera. B) Various honey bee species found around the globe Adapted from Encyclopedia Britannica 2012 Image credit; Ken Walker\Museum Victoria

The queen alone is fertile in the colony and is responsible for laying eggs. The queen collects the sperm during mating in spermatheca and controls whether an egg is fertilized or not (Rubinsky 2010).

1.4.1 *A. dorsata* nesting characteristics

A. dorsata is referred to as the giant bee or the rock bee. It is an open nesting bee and makes its hives on trees or cliffs at the height of 3 m above the ground (Gupta 2014). Nests might also be in groups on a single tree or rooftops. Honey is stored at the top region of the hive. At the next level, pollen and brood are maintained. The hive's bottom consists of the hive entrance from where the foragers land and take off (Akratanakul 1986). *A. dorsata* differs from the other species in two aspects. They have the ability to forage on moonlit nights (Dyer 1985; Somanathan et al. 2009) and migrate large distances up to 200 km in search of food sources (Koeniger and Koeniger 1980; Venkatesh1989; Ahmad 1989).

They are one of the most aggressive honey bee species and exhibit shimmering mechanisms to ward off predators (Seeley and Seeley 1982; Weihmann 2014). Hundreds of bees move their abdomen in an upward direction causing a Mexican wave-like motion. This activity

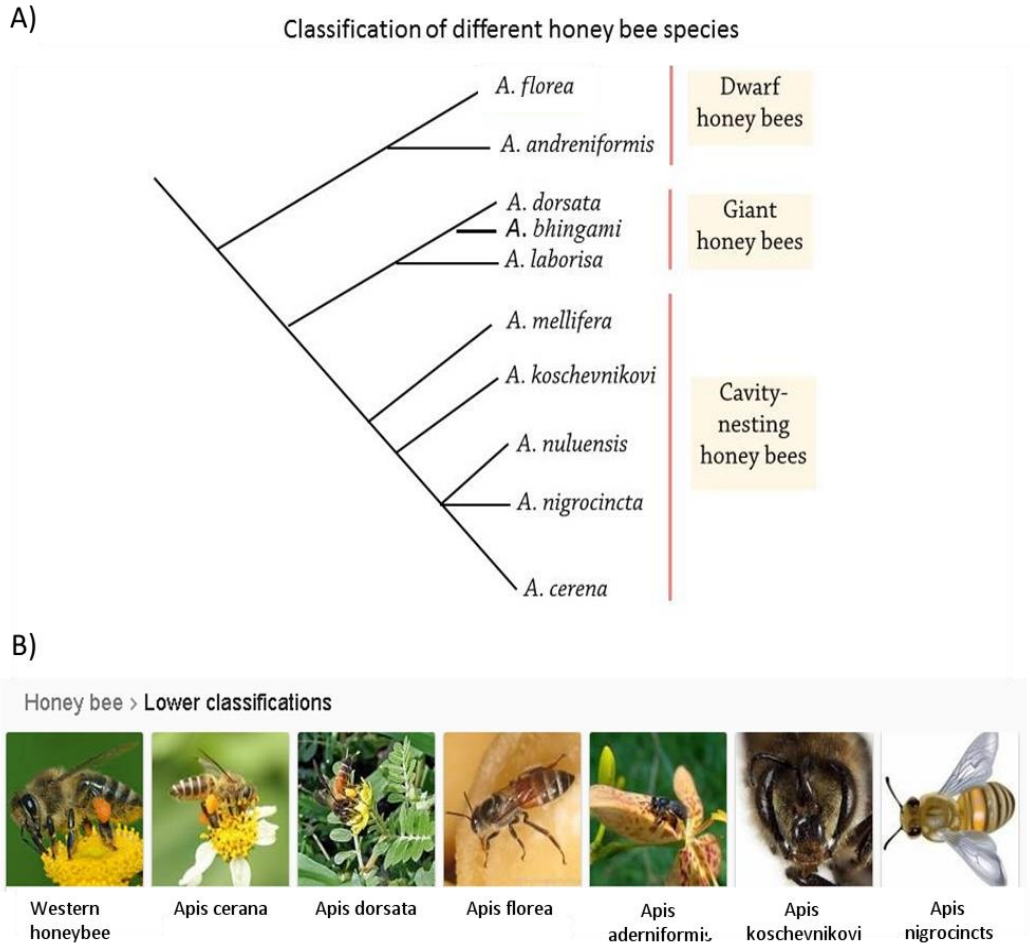


Fig1.3 A) The classification of honey bee species B) Different species of Apis.

Adapted from Oldryod and Wongsiri 2006

helps alert the hive bees and prevents predatory birds, mammals, and wasps from attacking the hive. Four subspecies are recognized in *A. dorsata,* namely *A. dorsata binghami, A. dorsata breviligula, A. dorsata dorsata* and *A. dorsata laboriosa* (Arias and Shepherd 2005). *A. d. laboriosa* was first considered a separate species by Mc Evoy and Underwood (1988), but Ruttner (1988) opposed the division as the male genitalia of *A. dorsata* and *A. laboriosa* did not show any differences. The two, only differ in housekeeping and swarming behavior.

Fig.1. 4 Picture of an *Apis dorsata* open nesting hive

A. laboriosa is called the Himalayan giant honey bee and is found in China, Laos, Myanmar and northeast India. *A. d. breviligula* is called the Philippines giant honey bee found in the Philippines and has a shorter tongue than *A. d. dorsata* and does not migrate (Radloff et al. 2010). *A. d. binghami* is called the Indonesian giant honey bee, found in Malaysia and Indonesia. There are up to a maximum of 5 hives on a nest tree, unlike *A. d. dorsata*, which can form up to a hundred colonies (Hepburn et al. 2004). It has a longer tongue and a longer wing compared to *A. d. dorsata* and is black in color with white stripes (Lo et al. 2010; Radloff et al. 2010). *A. d. dorsata* is called the Indian honey bee. It is found in India. The workers are brownish with orange stripes.

1.4.2 Differences between *A. mellifera* and *A. dorsata* natural history

A. mellifera and *A. dorsata* primarily differ from each other in their nesting habit. *A. dorsata* is an open nesting species, whereas *A. mellifera* is cavity-nesting. This condition causes

increased exposure to predators in *A. dorsata* and could be a reason why it is one of the most aggressive species of honey bees. The reluctance of this species to inhabit closed spaces prevents domestication. *A. mellifera* is cavity-nesting and is widely domesticated around the globe, and is considered less aggressive. *A. mellifera* is found in varied climatic regions. It is native to Europe, western Asia and Africa but has been introduced in many parts of the globe (Winston et al. 1991). *A. dorsata* is sympatric in tropical regions and is native to South Asia. Unlike *A. mellifera,* that forages only in the day, *A. dorsata* is known to forage at twilight and on moonlit nights (Dyer 1985; Somanathan et al., 2009) and is known to forage large distances. *A. dorsata* is a bigger species with a body size ranging from 17-20 mm (Hepburn & Radloff 2011) with a mean head width of 4 mm and bodyweight of 71 mg. *A. mellifera* is 10-15mm long with a mean head width of 3.5 mm and bodyweight of 49 mg (Gowda 2016). In *A. mellifera,* the drones are bigger than the workers measuring around 15-17 mm long. *A. dorsata* the workers and drones do not exhibit size variation. *A. mellifera* though smaller in size, has a brain size similar to *A. dorsata,* unlike other species. *A. mellifera* has a mean brain volume of 1.54 ± 0.07 mm3 versus *A. dorsata,* which has a volume of 1.56 ± 0.06 mm3 (Gowda 2016).

1.4.3 Comparison of Olfactory systems of *A. mellifera* and *A. dorsata*

The olfactory system of *A. mellifera* is extensively studied and well characterized. In *A. mellifera,* the odor detection is carried out by nearly 60,000 olfactory receptor neurons (ORNs) present in the sensilla of the antennae (Esslen and Kaisling 1976; Kropf et al. 2014). ORNs from either side innervate the ipsilateral antennal lobe (AL) through the T1–4 tracts of the antennal nerve (AN) (Suzuki 1975; Mobbs 1982; Galizia et al. 1999; Abel et al. 2001; Kirschner et al. 2006). In the AL, ORNs synapse on to around 800 projection neurons (PNs) (Bicker et al. 1993; Hammer 1997; Galizia 2008) and about 4000 local neurons (LNs) (Witthöft 1967; Sachse and Galizia 2006; Galizia 2008; Galizia and Rossler 2010) in the

glomeruli, the functional unit of the AL (Hildebrand and Shepherd 1997; Anton and Homberg 1999; Hansson and Anton 2000). PNs innervate the higher olfactory centers, the lateral horn (LH), and the mushroom body (MB)

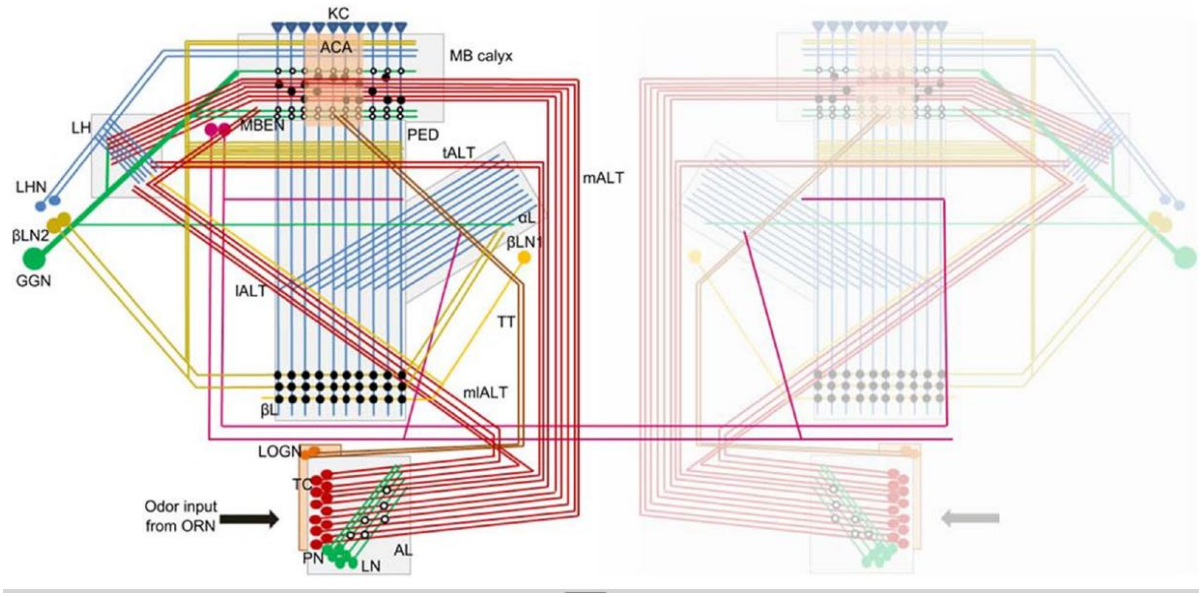


Fig 1.5 Schematic showing the olfactory system of locust (Adapted from Singh and Joseph 2019). Schematic over view of *Heiroglyphus banian* olfactory circuit from the second-order (AL) to the fourth-order (β-lobe and GGN).

through five antennal lobe tracts (ALTs). In the MB, the PNs synapse on to 170,000 Kenyon cells (KCs) (Mobbs 1982; Abel et al. 2001; Muller et al. 2002; Kirschner et al. 2006; Rossler and Brill 2013). The dendrites of the KCs innervate the calyces, and the axons constitute the mushroom body pedunculus. Subsequently, the axons of the KCs bifurcate and the branches innervate the vertical lobe and the medial lobe. Olfactory input is received by the lip region and inner half of the basal ring of the MB calyces (Mobbs 1982). MB extrinsic neurons and centrifugal neurons connect the pedunculus and lobes to the protocerebrum and AL, respectively (Mobbs 1982; Rybak and Menzel 1983).

The demand on the olfactory systems of both the species may differ due to the differences explained above viz increased exposure to predators, migratory habits, foraging in moonlight, and difference in habitats. In our study, we characterized the olfactory system of *A. dorsata* and compared it with the well-studied system of *A. mellifera* to look for similarities and possible differences.

In chapter three, the AL architecture of *A. dorsata*, was characterized and the glomerular number was established using tract tracing from the antenna. A digital atlas of AL glomeruli of *A. dorsata* was constructed keeping the available atlas of *A. mellifera* as a reference (Arnold et al. 1985; Flanagan and Mercer 1989; Galizia et al. 1999). This atlas can aid in identifying specific glomeruli during intracellular studies and glomerular identification in optical studies. A 3D reconstruction of the AL is also made available.

In chapter four, the physiological properties of AL interneurons were examined using electrophysiology. Using tract-tracing methods the innervation of AL interneurons to the higher olfactory centers was labeled. The GABAergic innervation in the AL was identified, and the cell bodies were located. The 5 AL tracts that innervate MBs and LH were identified. The mushroom body architecture was identified by tracing, and the MBs were reconstructed using Fiji software.

The AL of *A. mellifera* is larger than *A. dorsata* (Gowda 2014) despite *A. mellifera* being smaller in size. Glomerular volume is known to be influenced by foraging behavior. Bees that experienced restricted foraging developed lesser AL volume than bees that foraged freely (Jernigan et al. 2019). We tried to estimate the efficacy of the odor perception of *A. dorsata* compared to *A. mellifera* by using PER conditioning and comparing the learning rates of both species. We subjected *A. dorsata* to PER learning protocol using different odors and

established the rate of olfactory learning and ability to distinguish trained from untrained odors and established *A. dorsata* as a good model system to study olfactory learning.

1.5 Neural coding

Having established *A. dorsata* as a comparable model system to *A. mellifera*, in chapter 4, we used *A. dorsata* to look at issues in odor coding**.** How odor stimulus features are encoded, memories are formed in the brain, and how these memories influence different behaviors are open questions in neuroscience. According to the neural doctrine, neurons, which are the fundamental units of information processing in the brain, represent transient information in the state of the membrane potential as that is the key determinant of the rate of neurotransmitter release from the neuron. In neurons having action potentials, the spikes/action potentials form the key unit of representation. For some cells having only calcium spikes, the depolarization itself is gauged to be the representation. Across modalities, information about the perceived stimulus is believed to be coded in the rate of spikes (Adrian 1928). Experimentally it was shown in frog leg muscle that the amount of pressure applied to the leg proportionally affected the rate of spiking of a stretch receptor.

In most scenarios, it is assumed that postsynaptic neurons integrate the spikes over a time window. But in many instances, the nervous system responds faster than the time it takes to integrate spike trains. Neurons of the primary cortex of primates are known to have a response time of 100 ms (Oram and Perrett 1992). In a categorization decision task, humans are known to have a response time of 150 ms (Thorpe et al. 1996). After accounting for delays in, at least 10 synapses, between the retina and infratemporal cortex, this time window is not sufficient to integrate more than 2 spikes. This leads to the hypothesis that integration happens over time, over a pool of neurons rather than a single neuron, i.e. spatial or identity coding.

In the spatial coding view of olfaction, each odor is encoded by a spatial code comprised of the spatially distributed activity pattern of the glomeruli activated by that odor (Galizia et al. 1999). Stereotyped maps of active glomeruli are available for many odors as a result of various studies. These maps are found to be bilaterally symmetric and species-specific (Galizia et al. 1999). This hypothesis enables coding for both the intensity and features of the odor like the chemical group and carbon chain length. It is possible that very large number of odors can be distinctly combinatorially coded in this way due to the many glomeruli present (Mori et al. 2006). But that works only in the ideal scenario where the signal-to-noise ratio is very large.

A special case of spatial coding is labeled line coding. In this picture there are specific receptors activated by specific ligands that eventually drive specific behavior/perception. This view fits well with innate behaviors. It is likely that both labeled line for innate behavior and a spatiotemporal code for learned behavior exist. This view is supported by the results (Kobayakawa et al. 2007) that. in mice if a particular set of glomeruli required for innate avoidance response is disrupted, then the innate behavior is abolished. However, the same odor stimulus that caused the innate avoidance can still form associative memory.

In the temporal coding view, the code is based on the relative timing of the neural response with each other or with some standard reference like a rhythm or a clock. Moreover, it is required that the variation in the temporal pattern on responses is faster than the stimulus that it is encoding (Dayan and Abbott 2001). This does not preclude spatial variations as that appears as a vector of activity varying over time. Thus, in general, ensembles of neurons distributed spatially represent odors by their temporal pattern of activity. Two main models that explain temporal coding's role in olfaction are Hopfield's latency coding model and Laurent's slow evolving decorrelation model (Perl et al. 2020). According to Hopfield's latency coding model, a change in concentration results in a shift in spike timing of multiple

neurons thereby leading to no change in the relative timing across neurons. This aids in concentration invariant odor identification. According to this model, “time is an integral part of the odor code and there by two odors activating a similar set of glomeruli but at different times should give rise to two distinct percepts” (Perl et al. 2020). This model assumes an underlying time reference like the breath cycle in mammals. So it is not clear if this can work in insect systems where such underlying ongoing rhythm is not present.

In phase coding, time is measured in relation to oscillation cycles like internal gamma oscillations. In latency coding, it is measured relative to the onset of inhalation, and in relative time coding, time is measured in relation to the activation time of other neurons. Later versions of this model propose that early components of activated glomeruli encode odors, and those activated later aid in differentiating similar odors (Wilson et al. 2017).

The Laurent slow evolving decorrelation model states that “populations of neurons exhibit synchronized oscillatory activity, but each neuron only transiently participates in this population activity” (Laurent 2002). Populations of neurons participating in the oscillatory cycle evolve over time resulting in an odor representation that is increasingly decorrelated, i.e. even similar odors generating similar responses in the PNs slowly drift apart thereby during the course of the response reducing overlap in the representation of similar odors, thus aiding in better odor discrimination over time. Experimental evidence showing that odor representations become more decorrelated and increasingly sparser over time (Friedrich and Laurent 2004; Gupta and Stopfer 2014) and impairment of odor discrimination by the abolition of beta oscillations generated in the antennal lobe support this theory (Stopfer et al. 1997).

All the features of the temporal code proposed above only specify the necessary features of the neural responses that encode them. The ultimate proof that it is a code can only be given

if we show that a system exists that looks at this code and interprets it. This proof is still lacking. This is not to say that we don't know neuronal subsystems that do not integrate information from the stimulus over time. We have to distinguish that kind of decoding from decoding the temporal code generated by the system.

1.5.1 Role of Oscillations in temporal coding

While recording local field potential (LFP) from higher brain areas like MBs, they exhibit typical sinusoid waves during odor presentation, which hitherto were absent. Oscillations were first observed while recording LFP by Adrian (1942), in the hedgehog, when it inhaled an odorant in an anesthetized state. They were also observed in rodents (Boeijinga and Da Silva 1989), olfactory cortex of vertebrates (Freeman 1978), fish (Friedrich et al. 2004) and insects like locust (Laurent and Naraghi 1994) Moth (Wu et al. 1995), honey bee (Stopfer et al. 1998). These oscillations can occur in theta (1-12 Hz), beta (15-30 Hz) or gamma (40-100 Hz) Frequency bands (Kay et al. 2009). Theta oscillations, also called respiratory oscillations, have been recorded from the hippocampal and other regions of mammals such as rats, bats, dogs, humans etc. They are implicated in navigation and learning and memory (Buzsaki et al. 2013). Gamma oscillations are the best-studied odor-evoked oscillations, which are observed in both waking and sleeping states. They were observed in the neocortex (Grey et al. 1989), amygdala (Halgren et al. 1977), hippocampus (Buzsaki et al. 1983), olfactory bulb (Adrian 1942), and other brain regions. They occur within a single respiratory cycle. Though in insects and fish, the oscillatory frequency is lower, arund 20 Hz, it is considered analogous to the gamma oscillations, in mammals. In waking rats (15-20 Hz), beta oscillations are observed in the entorhinal cortex, hippocampus, and OB during odor exposure. In the honey bee, 30 Hz oscillations were observed in the LFP of the mushroom body (Stopfer et al. 1997b)

1.5.2 How are oscillations produced in the LFP of MB calyx of insects?

Glomeruli are the functional units of odor processing in the AL of insects, particularly in *A. mellifera* they receive input from around 375 ORN axons expressing the same receptors and are innervated by around 1300 LNs and 30-80 PNs (Galizia 2001; Menzel and Rybak 2010). A variety of synaptic contacts occur within the glomerulus. The ORNs synapse on to LNs and PNs, PNs synapse on to LNs, and LNs synapse on to other LNs, PNs, and ORN dendrites (Gascuel and Masson 1991; Tanaka et al. 2011). PNs receive excitatory input from the ORNs and are inhibited by LNs. LNs receive input from PNs and ORNs. LNs have shorter latencies, thereby having a fast inhibitory effect on the PNs (Krofczik et al. 2009). PN responses result from excitation from the ORNs and inhibition from the LNs via GABAergic synapses (Sun et al. 1993). This circuit is similar to a recurrent inhibition network. When this network receives input from the ORNs, it rapidly generates alternating cycles of inhibition and excitation (Laurent and Davidowitz 1994). LNs arborizations are widely distributed in the AL and facilitate inhibition. Thus the AL circuitry generates oscillatory synchrony of PN spikes. . Blocking the inhibitory synapse between LN and PN resulted in the subsequent abolishing of the synchronization of the AL interneurons (MacLeod and Laurent 1996). Paired recordings from LN and PN revealed that the relative phases of PN and LN are consistent with this model **(Fig 1.5).**

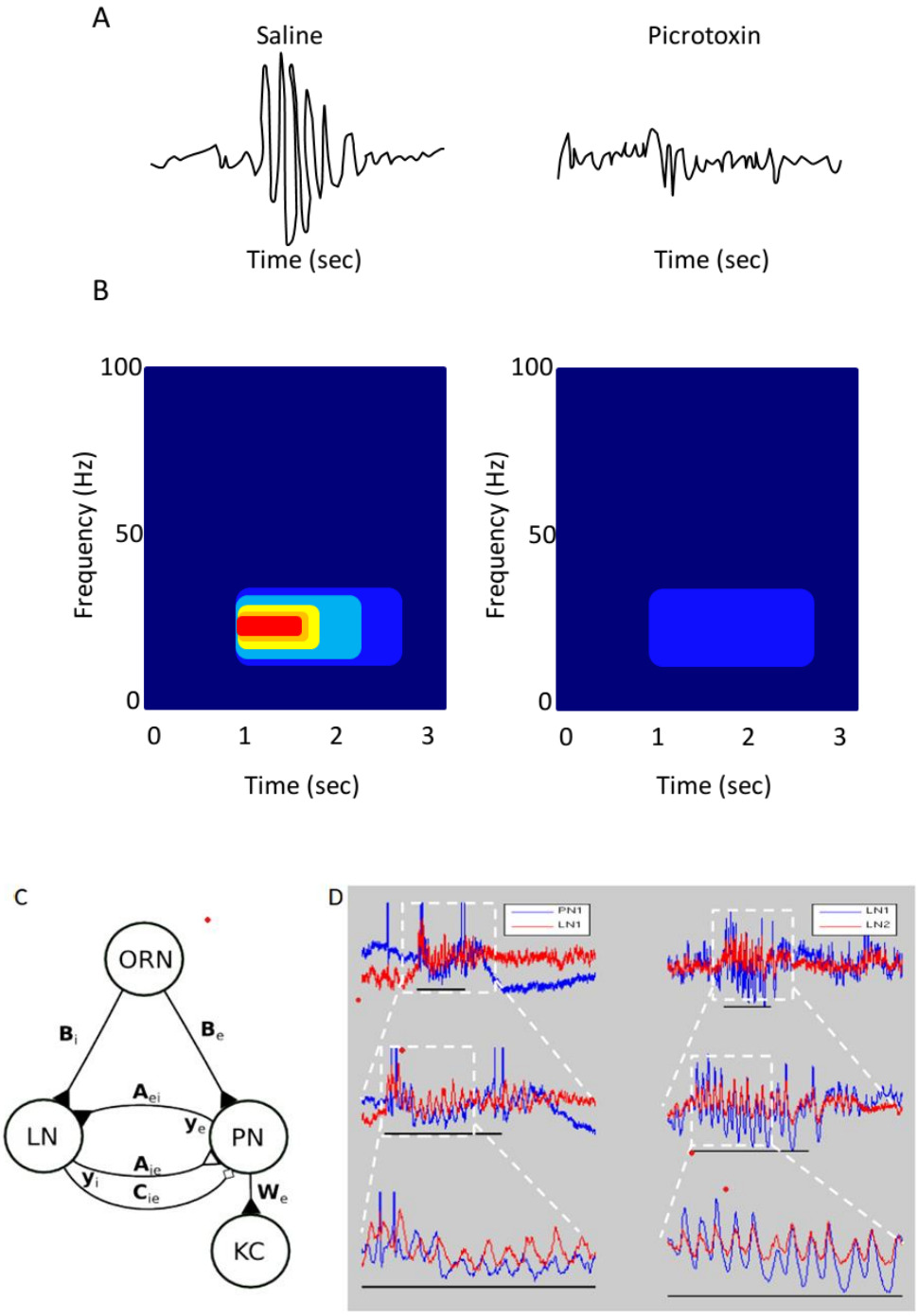


Fig 1.6 Picrotoxin abolishes 20-30 Hz oscillations recorded from the mushroom body. A) Presentation of odor resulted in regular, large-amplitude oscillations initially (left) but after PCT perfusion, no oscillations were observed 8 min after application (right). B), Spectrogram around the odor presentation indicating power in the 20-30 Hz band during odor response vanishing after application of picrotoxin. A) The ORN-LN-PN connections. ORNs are connected to LNs and PNs. LNs have an inhibitory connection with the PNs and other LNs and PNs form excitatory synapses onto LNs and KCs (Bhavana Penmetcha and Joby Joseph, unpublished). B) Phase relationship of LN and PN membrane potentials in *Hieroglyphus banian* during odor response showing that PN membrane depolarization leads the LN

depolarization and PN hyperpolarization lags LN depolarization. Adapted from Singh and Joseph 2019 and Stopfer and Laurent 1999

To ascertain that the MB LFP oscillations are driven by AL input, Laurent and Davidowitz (1994) recorded from different regions of the MB of the locust, *Schistocerca americana.* They found that the oscillations observed from those areas were highly correlated without phase lag, indicating an absence of traveling waves. Further, intracellular recording from the PNs revealed that the membrane potential oscillations of PNs due to odor application correlated with the MB LFP. PN spikes coincided with the ascending phase of the LFP, whereas the IPSPs preceded the rest phase. LNs were also found to oscillate in phase with the MB LFP, and the peak of the graded potential of LNs coincided with the peak of LFP. In honey bees, the MB LFP was found to lag behind the membrane potential oscillations of the AL neurons (Stopfer et al. 1997b).

1.5.3 Role of oscillations in odor discrimination

Waldrop et al. (1987) had shown that the application of picrotoxin (PCT), a GABA mediated chloride channel antagonist, to an insect AL interfered with the action of the LNs. MacLeod and Laurent (1996) found that in *Schistocerca americana*, PCT injection but not saline abolished the oscillations in the MB LFP and the IPSPs of the AL neurons. PCT specifically blocked the IPSPs but did not affect the spatiotemporal activity patterns of the PNs. Though the PNs continued firing their specific responses to odorants, they ceased to fire synchronously as the LN activity was blocked. This treatment by and large did not affect the odor-specific slow temporal patterns of the PNs, even if the patterns consisted of sustained periods of inhibition. This may be either due to the involvement of different receptors or transmitters like histamine or due to metabotropic GABA receptor channels. Stopfer et al. (1997a) showed that even in honey bees, PCT had a similar effect as in locusts, suggesting

that neural synchrony in honey bees too is mediated by $GABA_A$ type receptor. They employed a behavioral assay where bees were trained with an aliphatic alcohol, either hexanol or octanol and tested after one hour with the same odor and a similar odor (alcohol of different carbon chain length) and a dissimilar odor geraniol. The control bees, which had their cuticle opened, and saline applied, could distinguish the trained odor from similar and dissimilar odors. The PCT-treated bees did not differ in their learning ability and ability to distinguish the dissimilar odorant but were found to be specifically deficient in discriminating similar odorants. Hence they concluded that oscillatory synchronization is essential for fine odor discrimination. To further establish this, PCT treatment was done before training, before testing and both these times. All the PCT-trained bees in the three conditions performed poorly in discriminating similar odors showing that poor performance of PCT-treated bees is not due to degradation of selectivity at the level of AL neurons but due to some $GABA_A$ link phenomenon. Stopfer et al. (1997a) concluded that this is due to the desynchronization of neural ensembles.

1.5.4 Central plasticity in the AL and mechanisms (habituation, oscillation buildup, timescales)

Olfactory plasticity refers to changes in response to odors due to past experience. During olfaction, an animal encounters repeating stimuli. This repetition of stimuli is afforded by media breaking up continuous odor plumes to discrete filaments and by sniffing activity of mammals and antennal flicking of insects. Stopfer and Laurent (1999) applied a series of identical odor puffs for one second at 0.1 Hz frequency to one locust antenna and simultaneously recorded from the ipsilateral LNs, PNs, and LFP of mushroom body. When a novel stimulus was applied, it initially elicited strong PN and LN response without any periodic subthreshold or spiking activity, and the LFP waveform had little power at 20 Hz.

Repetition of stimuli produced two effects viz decrease in PN spikes and 20 ± 5 Hz periodic activity in LNs, PNs, and LFP.

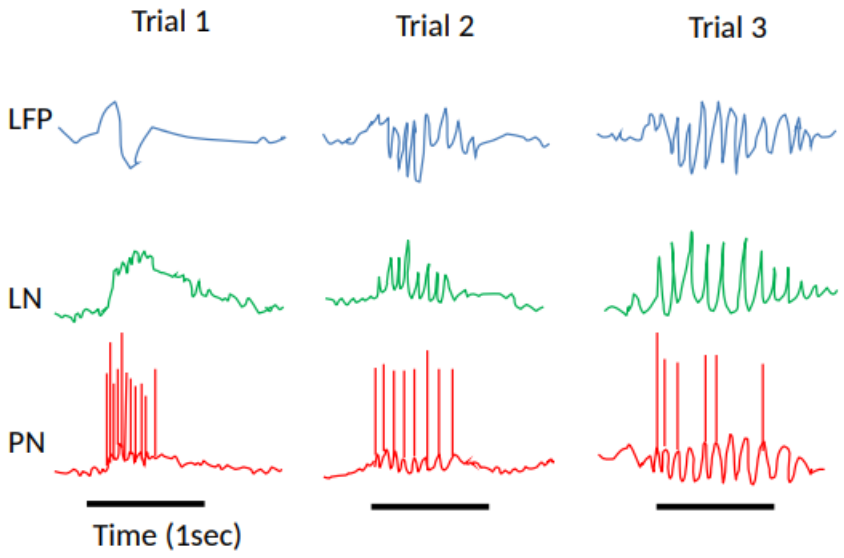


Fig.1.7 PN, LN, and MB LFP change with repeated stimulation. **a.** Traces from the LFP in the MB, and intracellular recording from LNs and PNs. **b**, the PN spikes were not synchronized to the LFP in the earlier trials. PN spikes are synchronized in the later trials.

The power of these LFP oscillations reached a maximum by the 8th trial and did not increase further. Also, the coherence between LN membrane potential and PN spikes with the LFP waveform increased. They found this change in response pattern was independent of inter trial interval and the duration of odor pulse. They found this state change persisted for many minutes without intervening exposure to the same odor. They also found that an interval of 12 min without odor exposure resets the system to its naïve state. If an animal is put through the same protocol of repeated trials, after 15 min the response change pattern showed similar evolution as the first time. This plasticity was found to be odor-specific. Exposure to a novel odor between sequences of stimuli with a given odor did not affect animals' response to the given odor. But exposure to an odor produced oscillations in the very first presentation

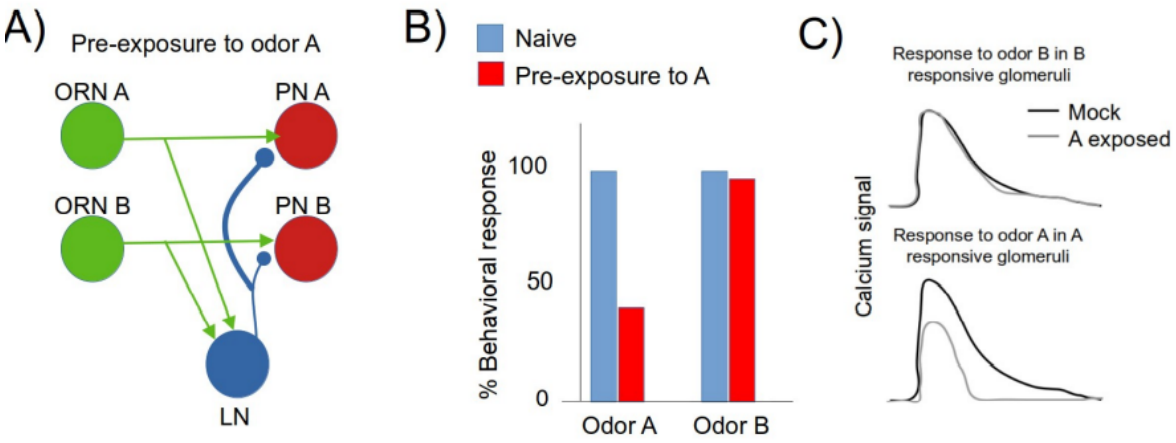


Fig 1.8 Network Plasticity in adaptive filtering and behavioral Habituation. A) Nework of the AL that shows the ORN-LN-PN network and the odor-pathway specific enhancement of synapse of the LN (synapse on to PN A) that mediates habituation to odor A. ORN-X and and PN-X denotes ORN and PN responding to odor X. B) Behavioral response to odors A and B before and after exposure, showing decreased response only to A after exposure to A. C) Calcium influx to PN A decreases after habituation to A consistent with decreased depolarization in PN A after habituation

(Adapted from Sudhakaran et al. 2012; Das et al. 2011)

of a similar odor. To determine whether this effect is central or peripheral, they closed a part of an antenna and applied odor only to a specific part of an antenna. Later application of odor to the initially closed part of the antenna produced oscillations in the AL which is trained, indicating that this plasticity is a central phenomenon.

Another phenomenon occurring due to repeated exposure to unrewarded stimuli is 'habituation.' It's a form of implicit, associative learning where there is a reduction in behavioral response due to repeated exposure to an unrewarded stimulus (Thompson and spencer 1966). This is thought to enhance the animal's ability to focus on novel stimuli by filtering out constant input (Rankin et al. 2009). Depending on the duration of exposure and

time taken to recover from habituation, habituation can be long-term habituation (LTH) or short-term habituation (STH). A behavioral phenomenon exhibiting habituation to short exposure to odors is the olfactory jump response. This response habituates with repeated presentation of the odor (Asztalos et al. 2007). Habituation by prolonged exposure to an odor has been reported in drosophila. They exhibit habituation by decreasing their movement after prolonged exposure towards an otherwise attractive order (Das et al. 2011).

It has been established that cAMP-dependent inhibitory potentiation of the LN-PN synapse underlies both STH and LTH in y-maze assay following habituation to odor. Co-release of GABA and glutamate from LNs and enhanced GABAergic inhibition by the LNs onto PNs, aided by glutamate and GABA receptors on PNs were found to be crucial for habituation. Consistent with the enhanced inhibition of the PNs, PN activity is reduced following habituation which manifests as reduced olfactory avoidance behavior in habituated flies (Das et al. 2011). Sudakaran et al. 2012 showed that recurrent feedback from the PNs leading to sustained activation of the LNs was functional in inducing the inhibitory potentiation of LN-PN synapse.

Oscillations are generated in the AL in a similar way using LN to PN GABAergic inhibition, and there is a buildup of oscillations with repeated presentation of odor implying increased LN to PN inhibition. It is possible that habituation and oscillations recruit similar mechanisms or they may be even two facets of the same phenomenon.

In the repeated presentation of odor stimuli during PER conditioning, the habituation mechanism, oscillation mechanism, and classical association mechanisms may interact. In chapter five we attempted to test the effect of mechanisms underlying oscillations/habituation on odor discrimination by training bees with different inter-trial intervals (ITI) and concentrations, where there are different rates of central plasticity that leads to oscillation

build up/habituation. We recorded from the MB calyx to measure the LFP responses and their change, in conditions used for PER conditioning.

Chapter 2

Materials and methods

2.1 Tracing the antennal lobe

Apis dorsata workers were collected either from the flowers while foraging or directly from the hives. They were anesthetized at 4° C by cooling them for a few minutes. Cooled bees were mounted in plastic tubes and held with adhesive tape stuck at the neck region. To fill the antennal lobe, the scapus of the antenna was cut, and a crystal of dye, Dextran Biotin (3000MW, lysine fixable BDA 3000; Molecular Probes) or Dextran tetramethylrhodamine (3000MW, anionic lysine fixable D3308; Molecular Probes) was inserted into the antenna. A pulled glass capillary was used to insert the dye. Vaseline was applied to seal the antenna to prevent desiccation. The animals were left overnight in a moist chamber to facilitate dye transport. The next day head capsule was opened and fixed using a drop of PFA (paraformaldehyde). The brains were taken out carefully so that the antennal lobes are intact. The brains were fixed overnight in 4% PFA at 4° C. In samples where dextran biotin was used, the brains were washed in 3% Triton X-100 and incubated in Streptavidin Alexa Fluor 633 conjugate (S21375, Invitrogen) in 3% Triton X-100 for four days. Later the brains were washed thrice in PBS (phosphate buffered saline), 20 mins per wash, dehydrated in ascending alcohol series (30%, 50%, 70%, 80%, and 90% and 100%, 20 mins each) and cleared in methyl salicylate. Single concavity slides were used for mounting the brains.

Processed brains were scanned with a laser scanning confocal microscope (Leica TCS SP2. or Leica Carl Zeiss model LSCM NLO 710, Germany). An excitation wavelength of 568nm

was used for dextran tetramethylrhodamine, and 633nm was used for Alexa Fluor 633. An objective lens (oil immersion) with a 0.80 mm working distance was used. Images were acquired at a resolution of 512 x512 or 1024 x 1024 pixels with 20 X or 10X objectives.

2.2 Establishing the glomerular number and making the Digital Atlas

The scanned images were checked to see whether all the glomerular margins are clearly visible. Out of the 49 filled samples, three samples in which all the glomeruli are filled completely were selected for counting. The images were processed using ImageJ (NIH, lifeline Fiji version 5.1) (Schindelin et al. 2012). Firstly, the antennal nerve (AN) tracts were identified, and glomeruli innervated by each tract were located. First primary glomeruli, the ones which are invariant and can be easily identified, were located. Based on their position relative to the primary glomeruli, the secondary glomeruli were identified. Using segmentation editor, the outline of the individual glomeruli was marked in a few sections. Interpolation was used to mark the outline of the remaining sections. Marked glomeruli were added to a labels file and saved as an Amira file. Glomeruli innervated by each tract were labeled with different colors. Glomeruli innervated by the T1 tract were marked in red, T2 in yellow, T3a in light blue, T3b in dark blue, T3c in purple and T4 in green. Tracts were also labeled as explained above. A 3D reconstruction of the labeled glomeruli was done using the tool “Show color surfaces.”

To make a digital Atlas, alternate sections of the Amira file were transferred to PowerPoint, and each glomerulus was named following nomenclature by Galizia et al. (1999). T1 glomeruli were numbered with prefix A, T2 with B, T3 with C, and T 4 with D**.**

2.3 Glomerular Volume

To measure glomerular volume, the confocal stacks of dextran fills of the antennal lobe were used. Boundaries of the glomeruli innervated by each tract were marked out using segmentation editor and each type of glomeruli was labelled differently. From the voxel volumes obtained from the confocal image metadata, the image stack was read using a custom script in Matlab and the volume of glomeruli was calculated.

2.4 Tracing the Antennal lobe Tracts

Honey bees (n=30) collected from the hives in the university campus were cooled in a refrigerator at 4°C for a short duration. They were mounted in plastic tubes, and the neck region was held using adhesive tape. The head was immobilized with paraffin wax. The cuticle was cut open to expose the brain. After removing the trachea and glands, a glass electrode containing dye, dextran Tetramethylrhodamine was inserted into the antennal lobe and left for a few seconds. The brain was later covered with the cuticle that was previously cut and kept aside for 3-4 hours. Later the brain was dissected and fixed in 4% PFA overnight at 4°C.

The fixed brains were rinsed thrice in PBS (20 mins each) and were dehydrated in ascending ethanol series and mounted on concavity glass slides in methyl salicylate for imaging. The whole-brain mounts were imaged using a confocal microscope with an excitation wavelength of 568 nm, at 1024x1024 pixels resolution with a 20 X objective. Out of the images obtained we selected the one in which all the tracts could be visualized. This image was transferred to the PowerPoint and the tracts were traced in detail to make a schematic representation.

2.5 Mushroom body Reconstruction

Dextran Tetramethylrhodamine was injected into the AL of the honey bee brain, which filled the lip of the mushroom body calyx due to axonal innervation. After extraction and

processing as described in the previous section, the brain was imaged with a confocal microscope. The collar, pedunculus, and vertical lobe were visualized by auto fluorescence. The image was reconstructed using a plugin called segmentation editor in ImageJ.

2.6 Anti GABA Immunohistochemistry

Antennal lobes were filled as explained previously. The brains in which ALs could be clearly visualized were selected for processing with anti-GABA antibody (Sigma A2052). The brains were incubated for one hour in a solution of 3% Triton X-100 in PBS and 10% normal goat serum (NGS). After an hour anti-GABA antibody was added to the solution at 1:1000 concentration and left for four days with intermittent shaking. The solution was discarded and the tissue was rinsed in 3% Triton X-100 thrice (20 mins each). After 24 hours it was again incubated in a 3% Triton X-100 and 10% NGS solution for one hour, followed by incubation in anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488 (Invitrogen, A-11008) for four days. The tissue was rinsed thrice in PBS (20 mins each) and dehydrated in ascending alcohol series. It was cleared in methyl salicylate and mounted on concavity slides.

Processed brains were scanned with a laser scanning confocal microscope (Leica TCS SP2, Leica Microsystems, or Carl Zeiss LSCM NLO 710, Germany). An excitation wavelength of 568 nm was used for dextran tetramethylrhodamine, 488 nm was used for Alexa Fluor 488. Images were acquired at a resolution of 1024x1024 pixels with 10 X objective.

2.7 Response of Antennal lobe neurons to odors

The bees were collected and mounted in plastic tubes, as mentioned previously. A wax cup was made around the neck of the bee to hold saline. The cuticle was cut open to access the brain. Glands and sheath were removed carefully, and the brain was perfused with bee saline

(37 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.4 mM KH2PO4, Ph 7.2) (Kirschner et al. 2006). A chlorided-silver wire was placed in saline to serve as the reference electrode. Neuronal activity of antennal lobe neurons was recorded using sharp microelectrodes (70-100MΩ) filled with 200 mM KCl, using Axoclamp 900A (Molecular Devices) and amplified ten times. The signal was low pass filtered at 4 kHz. Microelectrodes were pulled using a horizontal puller (Sutter Instruments Co. USA Model P.97). The antennae was in the constant stream of airflow of 1l/min, and the odorants were delivered for a duration of 1s at a rate of 1l/min switched by a solenoid valve, switched by a relay controlled by pCLAMP and digitizer (1440A, Molecular Devices). Odors Hexanol, Octanoic acid, Nonanol, Geraniol (Sigma Aldrich) were used for odor stimulation and were stored in 30 ml bottles. The odor was removed from the setup using an air suction vent placed behind the animal. The data were digitized at 10 kHz using a digitizer (1440A, Molecular Devices) and analyzed using a custom program written in MATLAB (Mathworks, Natick, MA).

2.8 Olfactory PER conditioning

A. dorsata and *A. mellifera* bees were collected from their respective hives in the evening and were harnessed in plastic tubes with adhesive tape on their neck region. They were fed 1M sucrose solution (Sigma Aldrich) ad libitum and stored in a humid, dark chamber. The following day the bees were tested for proboscis extension response (PER), and ones that exhibited robust PER were alone selected for training (Bitterman et al. 1983; Giurfa and Sandoz 2012; Matsumoto et al. 2012; Menzel et al. 2001).

During training, the bees were placed in front of an odor delivery system with an air suction vent located behind them. Before the actual trial bees were left for 25 secs in the training area to reduce contextual stimuli. A computer program controlled the odor delivery. The bees were stimulated for 4 sec with 2-Octanol (Sigma Aldrich). A constant air flow was

maintained over the antennae at the rate of 2 l/min. The odor was delivered for duration of 4s at a rate of 1 l/min going into the bottle. Sugar reward was presented to the bee after the 3rd second, with a toothpick dipped in 1M sucrose. The antennae of the bees were touched first; later the proboscis was touched to allow the bees to feed. The timing of onset of odor and sucrose stimulation was indicated by red, and green LED indicators placed such that the bees cannot see them. After training, the bees were left undisturbed for 25 secs before being removed from the training location. Bees (N=56) were trained with 10min, 3 min, and 30-sec inter-trial intervals (ITI). *A. mellifera* bees (N=20) were trained with 10 min ITI to compare the learning curve between *A. dorsata* and *A. mellifera*. To test the odor discrimination, two groups of *A. dorsata* bees (N=29) were trained, one group with Hexanol and the other with Geraniol (Sigma Aldrich) as conditioned odor (CS), with 10 min ITI. The above odors were chosen as they were found to be discriminated well from each other in *Apis mellifera* (Smith and Menzel 1989). All the bees were tested 60 mins after the acquisition for retention of memory. Bees that did not have robust PER were not included in the testing. Cochran's Q test was used to check the effect of trials on the acquisition. Data were analyzed using MATLAB (Mathworks, Natick, MA)**.**

2.9 Learning Assay for odor discrimination task

Apis dorsata foragers were collected while foraging, from the flowers in the university campus. After cooling at 4°c the bees were harnessed in plastic tubes using adhesive tape. They were given a drop of sucrose to recover from the shock and left for two hours. Before starting the experiment the bees were checked for PER by touching the antenna with a tooth pick dipped in sucrose and only bees exhibiting robust PER were selected for the assay. The bees were divided in to twelve equal groups. There were three concentrations i.e 100%, 10%, and 1% (the dilutions were obtained in mineral oil) of the odors 1-Hexanol and 1-Octanol and

two different ITI s, 2 min and10 min. A constant air flow was maintained over the antennae at the rate of 2 l/min. The odor was delivered for duration of 4s at a rate of 1 l/min going into the bottle. Odor was removed from the setup using air suction vent placed behind the animal. Before the CS was applied the bee was placed in front of odor delivery system for 25 secs. The training protocol consisted of stimulating the bee with the CS (100%, 10%, or 1% Hexanol / 100%, 10%, or 1% Octanol) for 4 secs. The bee is presented with sucrose reward 3 secs after the start of odor delivery by initially stimulating the antenna and later allowing the bee to feed from a tooth pick dipped in sucrose. There were 6 training trials either 10 min or 2 min apart. The timing of odor delivery and sucrose delivery was indicated by colored LEDs placed such that the bees cannot see them and controlled by a computer program. The bees were tested for PER by touching the antenna with a toothpick dipped in sucrose solution and the ones that showed robust PER were selected for testing 60 mins after training. The bees trained with hexanol/octanol were tested with trained odor (hexanol/octanol), similar odor (octanol for hexanol trained bees/hexanol for octanol trained bees) of the same concentration as the trained odor and novel odor (100% Geraniol). The order of testing with trained odor, similar odor and novel odor was randomized. The response of the bee was marked as one for extension of proboscis and zero for no extension of proboscis. As Hexanol trained bees and octanol trained bees showed similar acquisition, both the sets were pooled for analysis. The data was plotted using MS Excel and MATLAB.

A separate set of 20 bees were trained using the above mentioned protocol with 100% geraniol as CS. They were tested one hour after conditioning with geraniol and hexanol and octanol (as different odors) to test wether the bees can detect and discriminate geraniol.

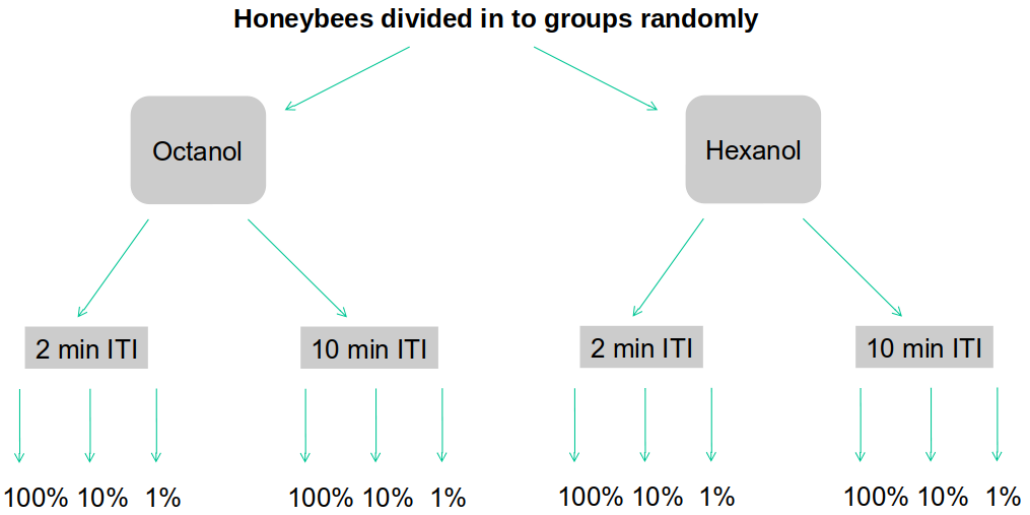


Fig 2.1 Experimental design for olfactory conditioning. There are totally 12 groups. 6 groups are trained with hexanol as CS. 6 groups were trained with octanol as CS. Within the 6 groups 3 groups were trained with 2 min ITI and 3 groups with 10 min ITI. With In the 3 groups each group was trained with 100%, 10% or 1% odor concentration.

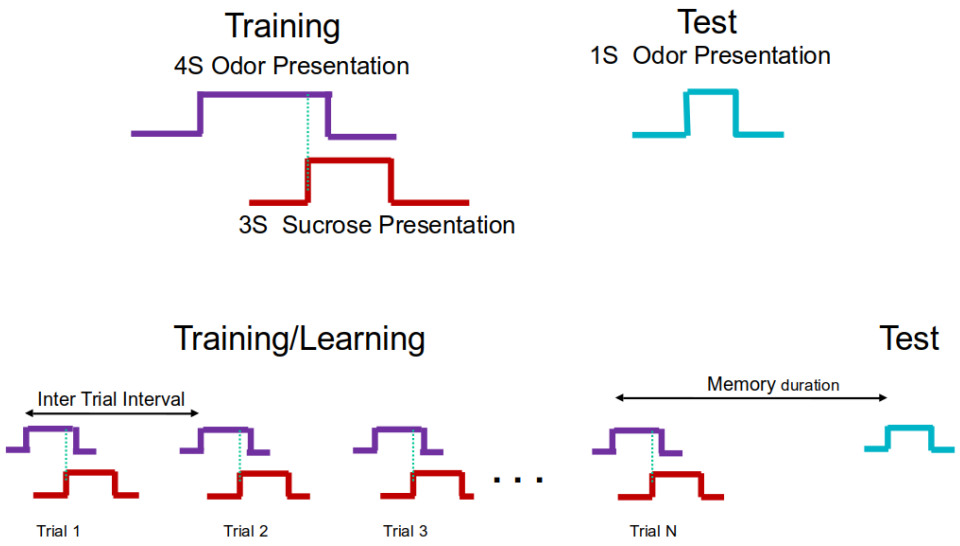


Fig 2.2 Schematic showing the protocol for olfactory conditioning and testing that was used. Each bee is presented with an odor stimulus for 4 secs followed by a sucrose reward for 3 secs with one second overlap. There were 6 conditioning trials. There was a test trial after 60 mins. In the test, the bees were tested with trained odor, similar odor and different odor, in a random order.

2.10 Recording mushroom body LFP

Foragers were collected while foraging and mounted in plastic tubes after anesthetizing by cooling at 4ºc. The neck region was plastered using a thin strip of adhesive tape. A wax cup was made around the head to hold the saline. The antennae were held in place either with plastic tubes or thin strips of adhesive tape at the base. The cuticle was cut open to expose the brain. The trachea and hypo pharyngeal glands were removed gently. The brain was perfused

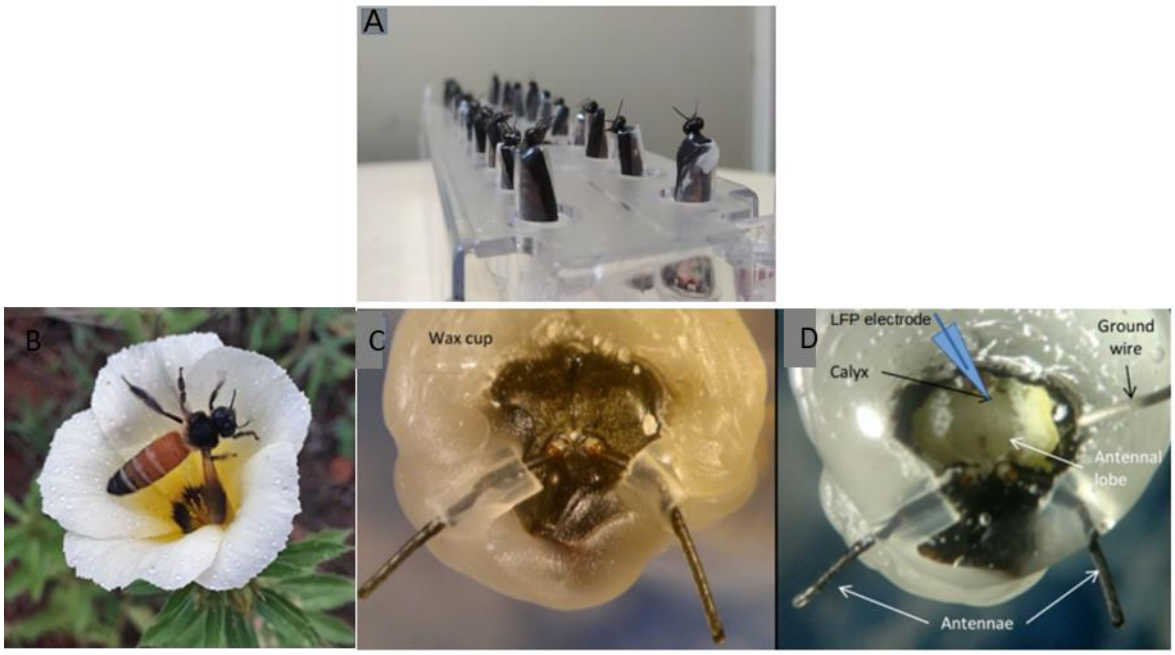


Fig 2.3 A) Harnessed bees are kept in a holder ready for training. B) Individual bee C) wax cup was built around the head capsule to hold saline.The antennae were held in place using tubes and cuticle was cut open to expose the brain. D) Brain exposed and positions of ground wire and LFP electrode indicated.

with bee insect saline (37 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.4 mM KH2PO4, Ph 7.2) (Kirschner et al. 2006). A chlorided-silver wire placed in saline served as the ground. A blunt microelectrode (1-10MΩ) filled with saline was placed in the calyx region of the MB to record the LFP. Microelectrodes 1mm OD, 0.5mm ID were pulled using horizontal puller (Sutter instruments Co. USA Model P.97). The MB LFP was recorded by inserting the micro

electrode to the calyx region of the MB. The signal was amplified (1000 X) using Axoclamp 900A (Molecular Devices) and was filtered between 0.1Hz and 80Hz while recording. The data acquisition was carried out using Axon instruments AD1440 at 10 kHz, which also controlled the odor delivery valve. Constant flowrate and odor flowrate for LFP recordings were identical to that of the training condition. To see the oveall trends with odor concentration and ITI, Hexanol (Sigma Aldrich) at 100%, 10% and 1% (diluted in mineral oil) was used for odor stimulation. LFP of MB was recorded by stimulating the animal with

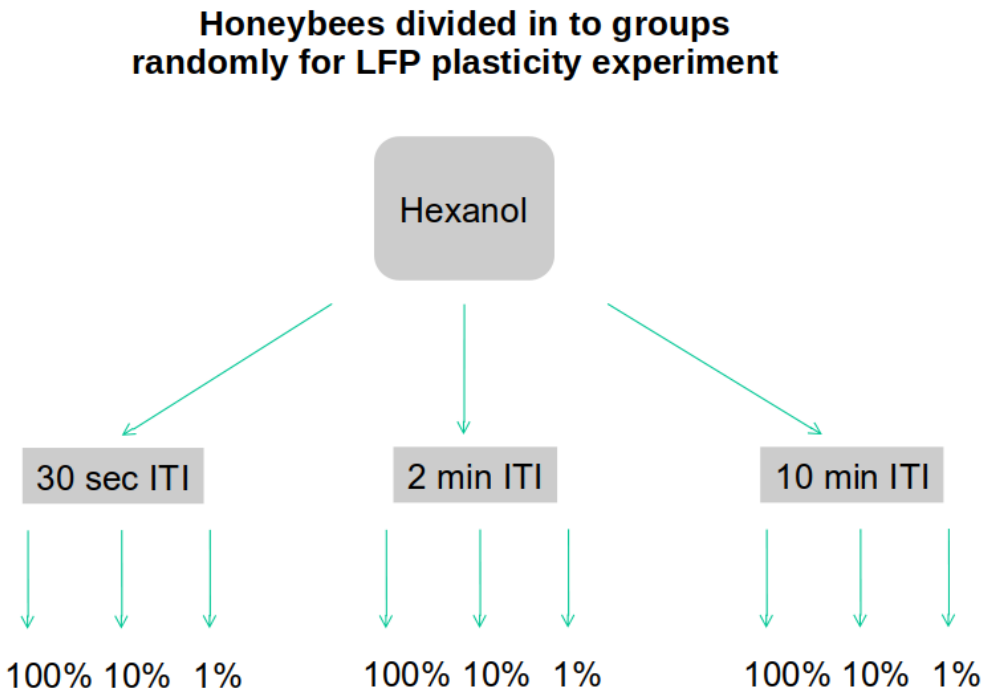


Fig 2.4 Experimental Design for recording deflection of MB LFP. The LFP in MB was recorded with an ITI of 30 secs, 2 mins and 10 mins. For each ITI all three odor concentrations of 100%, 10% and 1 % were used for measurements.

the above mentioned concentrations of hexanol for intervals of 30secs, 2 mins and 10 mins. A time gap of 10 mins was maintained between two different recordings. We included 30 sec ITI to check if the oscillatory build up was higher at this ITI. Five sets of recordings were obtained for all the above conditions.

When testing specifically for difference between 100% and 10% concentrations for 2 min ITI a time gap of 15 mins was kept between the pairs of trials to allow for full recovery from the previous trial pair (N = 10 bees). For calculating the change in deflection from first trial to second trial, the LFP of the second trial was subtracted from the first trial and absolute value summed up. For calculating spectrum, fft function in matlab was used and magnitude squared spectrum was calculated. Spectral power was calculated as area under the curve in respective spectral bands.

2.11 Simulations

Simulation of the antennal lobe network and LFP generation was carried out in matlab. The simulations followed the basic schemes in (Bazenov et al. 2005; Ito et al. 2009) Both PNs and LNs were modeled as Izhikevich regular spiking neurons (Izhikevich 2003) with parameters a = 0.02, b = 0.2, c = -65, d = 8. The network had 100 PNs and 30 LNs. Synapses were shaped as alpha functions and currents proportional to the synaptic strengths were injected into the post synaptic neuron when presynaptic neuron generated action potential. There were synapses with two different timescales. We used, one alpha function (τ = 7 msec) to represent fast $GABA_A$ type and nicotinic type acetyl choline receptors and another slow alpha function to represent GABAв type (τ = 100msec). PNs were connected to LNs via excitatory fast synapses with weights chosen to be random with uniform distribution. Half the LNs were connected to PNs via inhibitory slow synapses initiated with synaptic strength having uniform distribution. The other half of LNs were connected to PNs via inhibitory fast synapses whose strength increased with the plasticity mechanism that results in buildup of oscillations and concurrently caused decrease in deflection. The plasticity mechanism was that if the LN fired then LN to PN strength increased in magnitude. The LN to PN synaptic strength was made to decay at a constant rate to capture the recovery from this plasticity if

odor was not presented. Because the purpose of the simulation was to show that: as oscillations build up, the deflection component decreases, these strengths were scaled to capture the change in two trials.

The ORNs were modeled using first order kinetics with receptors that have free, active and inactive states. Three first order difference equations for the reactions shown below captured their behavior.

The distribution rates of reactions were adjusted to have a small fraction of the receptor types have stronger binding rates to a particular odorant, while the others have weak binding rates.

$$free \underset{a_2}{\overset{a_1}{\rightleftharpoons}} active$$
$$active \underset{b_2}{\overset{b_1}{\rightleftharpoons}} inactive$$
$$inactive \underset{c_2}{\overset{c_1}{\rightleftharpoons}} free$$

This kind of model, rather than a shape specifying model, (Bazenov et al. 2005; Ito et al. 2009) automatically increases the number of strongly active receptor types when the ligand concentration is increased. LFP deflection and difference in LFP deflection were calculated on LFP low pass filtered at 5Hz.

2.12 Analysis

Cochran's Q test was done to determine the nature of response to similar and dissimilar odors in the behavioral experiment. MB LFP was analyzed using custom written program in Matlab. Mean of 200 msec data before the odor onset was subtracted to detrend the data. 1 sec of data was collected during and before the odor application for response and baseline respectively. Anova was used to compare MB LFP in the different test conditions and paired t- test was used to compare the MB LFP responses to 100% and 10% odor concentrations in 2 min ITI condition.

Chapter 3

Characterization of the antennal lobe anatomy of *Apis dorsata* and comparison with *Apis mellifera*

3.1 Introduction

In honey bees, olfaction is crucial for activities like foraging, brood recognition, communication, mating and defense. The odor molecules are detected by ORNs present in the sensilla of the antennae (Esslen and Kaisling 1976; Kropf et al. 2014). ORNs from one side innervate a single corresponding antennal lobe (AL) through the T1-4 tracts of the antennal nerve (AN) (Suzuki 1975; Mobbs 1982; Galizia et al. 1999; Abel et al. 2001; Kirschner et al. 2006). ORNs synapse with the AL interneurons, PNs and LNs in structures called glomeruli which are the morpho-functional units of the AL (Hildebrand and Shepherd 1997; Anton and Homberg 1999; Hansson and Anton 2000). They are usually egg-shaped or spherical, or pyramid-shaped and surrounded by a discontinuous layer of glial cell processes (Arnold et al. 1985). They have a thick cortical layer in the upper region of the glomeruli, where the ORNs synapse with the AL interneurons (Arnold et al. 1985). The glomerular number and arrangement in different species varies based on their phylogenetic distance and is species-specific (Anton and Homberg 1999; Hansson and Anton 2000; Masante Roca et al. 2005; Galizia 2008; Lin et al. 2018).

Drosophila melanogaster has 43 glomeruli (Laissue et al. 1999), *Manduca sexta* has around 63 (Rospars and Hildebrand 1992), *Helicoverpa armigera* has 79 (Zhao et al. 2016), *Bombyx mori* (Kazawa et al. 2009), and *Spodoptera litteralis* (Couto et al. 2009) have around 60 glomeruli. In Hymenoptera, *Neodiprion autumnalis,* has about 44 glomeruli (Dacks et al. 2011). Desert ants of the genus *Cataglyphis* have around 198-249 glomeruli, wood ants of genus *Formica* have 370 glomeruli (Stieb et al. 2011). Carpenter ant, *Camponotus japonicas* has 430 glomeruli (Nishikawa et al. 2008). In the genus *Apis, Apis mellifera* has around 156-166 glomeruli. There are also grasshoppers in Orthoptera that do not have well-defined glomeruli in this sense but have micro glomeruli.

In most species we know of, each glomerulus receives input from all ORNs expressing a single receptor type. So the count of glomeruli can act as a constraint in searching for receptor genes in the DNA sequence database (Karpe et al. 2016).

In the AL, odor information from the ORNs is processed in the glomeruli, where they evoke odor-cell specific, temporally patterned responses or the spatio-temporal odor code (Laurent 1997, Galizia and Menzel 2000). These responses are characteristic of each odorant and highly reproducible (Galizia et al. 1999; Carlsson et al. 2002; Stopfer et al. 2003). To understand this olfactory code, it is essential to understand the glomerular arrangement. The glomerular arrangement is stereotypical and invariable across individuals, so that it is possible to create an atlas of the glomerular arrangement in the AL. Atlases are helpful to understand the olfactory code by identification of individual glomeruli in optical studies and to study PN innervation in intracellular recordings.

Digital atlases are available for species like *Drosophila melanogaster* (Laissue et al. 1999; Grabe et al. 2015), *Lobesia botrana* (Mosante Roca et al. 2005), *Bactrocera dorsalis* (Lin et

al. 2018). *Heliothis virescens* (Berg et al. 2002), *Cotesia glomerata* (Smid et al. 2003) and *Apis mellifera* (Flanagan and Mercer 1989; Galizia et al. 1999).

Flanagan and Mercer 1989 divided the antennal lobe of *Apis mellifera* into four major regions based on the antennal nerve innervation. The largest region was the T3 region, innervated by the T3 tract of the AN, which contained between 72-77 glomeruli. T1 region innervated by the T1 tract was the next largest with 66-69 glomeruli. The T2 region had a narrow band of seven glomeruli innervated by the T2 tract. T4 tract innervated 7 glomeruli in the rear of the AL. They used landmarks like lateral passage and the crescent tract to identify glomeruli. Galizia et al. (1999) used confocal microscopy to obtain optical sections of the AL. They compared the glomerular arrangement with the atlas of Flanagan and Mercer and established the glomerular identity.

A. dorsata and *A. mellifera* differ in their body size, nesting habits, and foraging pattern. *A. dorsata* is an open nesting species, so, it may have increased exposure to predators and unlike *A. mellifera*, it exhibits mechanisms like shimmering to ward off predators. *A. dorsata* is the only honey bee species that occasionally forages on moonlit nights. Though there are differences in the visual system that have been characterized it is also likely that olfactory cues are essential for this behavior. Even evolutionarily, *A. dorsata* evolved earlier compared to *A. mellifera* (Engel 1997). So, it becomes interesting to check the olfactory system of both species to look for similarities and possible differences.

In this chapter, we characterized the antennal lobe of *A. dorsata* workers, established the glomerular number, and prepared a digital atlas of the AL glomeruli, based on the available atlas of *A. mellifera* (Arnold et al. 1985; Flanagan and Mercer 1989; Galizia et al. 1999). We found both species to be similar in glomerular count and arrangement. The antennal nerve (AN) tracts innervating the AL were also similar in their innervation pattern in both species.

We created a 3D reconstruction of the AL and made it available. This reconstruction can aid in the identification of individual AL interneurons during intracellular recording and during optical studies. We measured the glomerular volume of *A. dorsata. The* glomerular volume of *A. mellifera* was found to be more compared to *A. dorsata* even though its body size is smaller compared to *A. dorsata*.

2.2 Results

2.2.1 AN tracts innervating the antennal lobe

We mass filled the antenna of *A.dorsata* bees, which lit up the AN tracts and the ORN innervation in the AL. ORNs innervated the AL through the T1-4 tracts of the AN. The other two tracts, T5 and T6, bypassed the AL. T5 entered the dorsal lobe, and T6 innervated the protocerebrum **(Fig 3.1).**

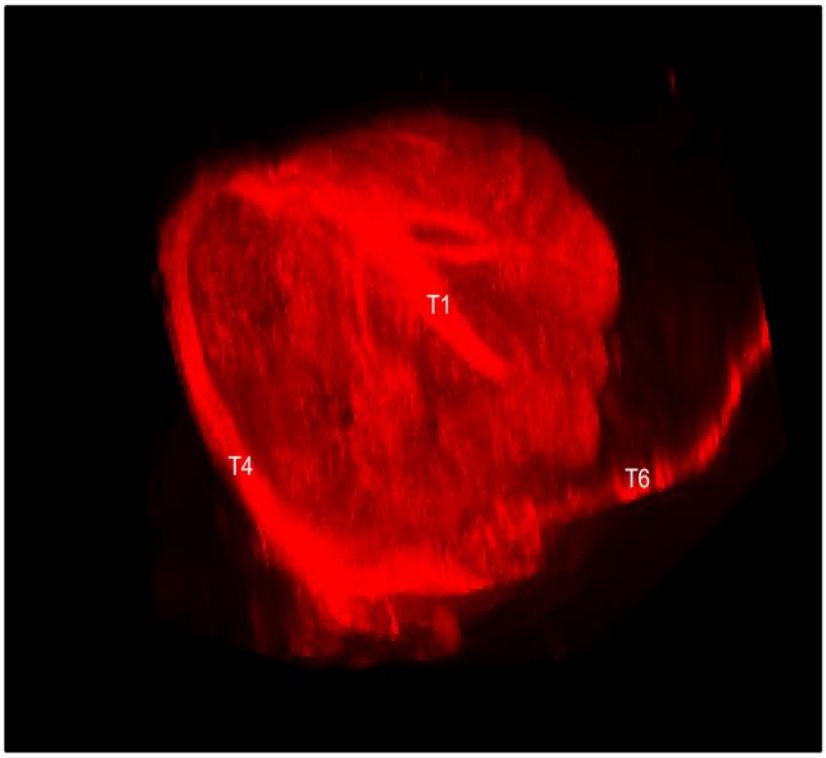


Fig 3.1 A transverse section of the AL showing the tracts T1, T4 as well as T6. T6 is seen projecting to the ventral protocerebrum bypassing the AL.

T1 tract innervated 70.3 ± 0.5 (Mean and SD) dorsal anterior glomeruli in the three samples that were analyzed. These glomeruli had a mean glomerular volume of 21.4 μm³. This tract innervated some of the most voluminous glomeruli of the AL **(Fig 3.2).**

The T2 tract has two branches T2-1 and T2-2. T2-1 innervated a single glomerulus that is inwardly oriented, and T2-2 innervated six medial glomeruli, and these glomeruli had a mean glomerular volume of 24.3 μm³.

T3 tract innervated 80.33 ± 0.5 (Mean and SD) ventro-posterior glomeruli that had a mean glomerular volume of 16.4 μm³ and has three divisions namely T3a, T3b and T3c. T3b glomeruli were located separately from the rest, caudal-dorsal to the T1 tract, and they were smaller in size than the other glomeruli. They have ORN innervation in the core region as well as in the cortex. For the above reasons, T3b glomeruli in *A. mellifera* were termed 'lobule' by Arnold et al. (1985) (Kirschner et al. 2006). We found around 12-16 T3b glomeruli in the three samples that were analyzed. T4 tract innervated seven posterior glomeruli. These glomeruli are the most voluminous of all the AL glomeruli and had a mean glomerular volume of 40.6 μm³. The total glomerular counts were 164, 165, and 165 in the three individuals that were counted. The glomerular number of *A. dorsata* was found to be similar to that of *A. mellifera* which had between 156 and 166 glomeruli in previous studies (Flanagan and Mercer 1989; Arnold et al. 1985) **(Table 2)**.

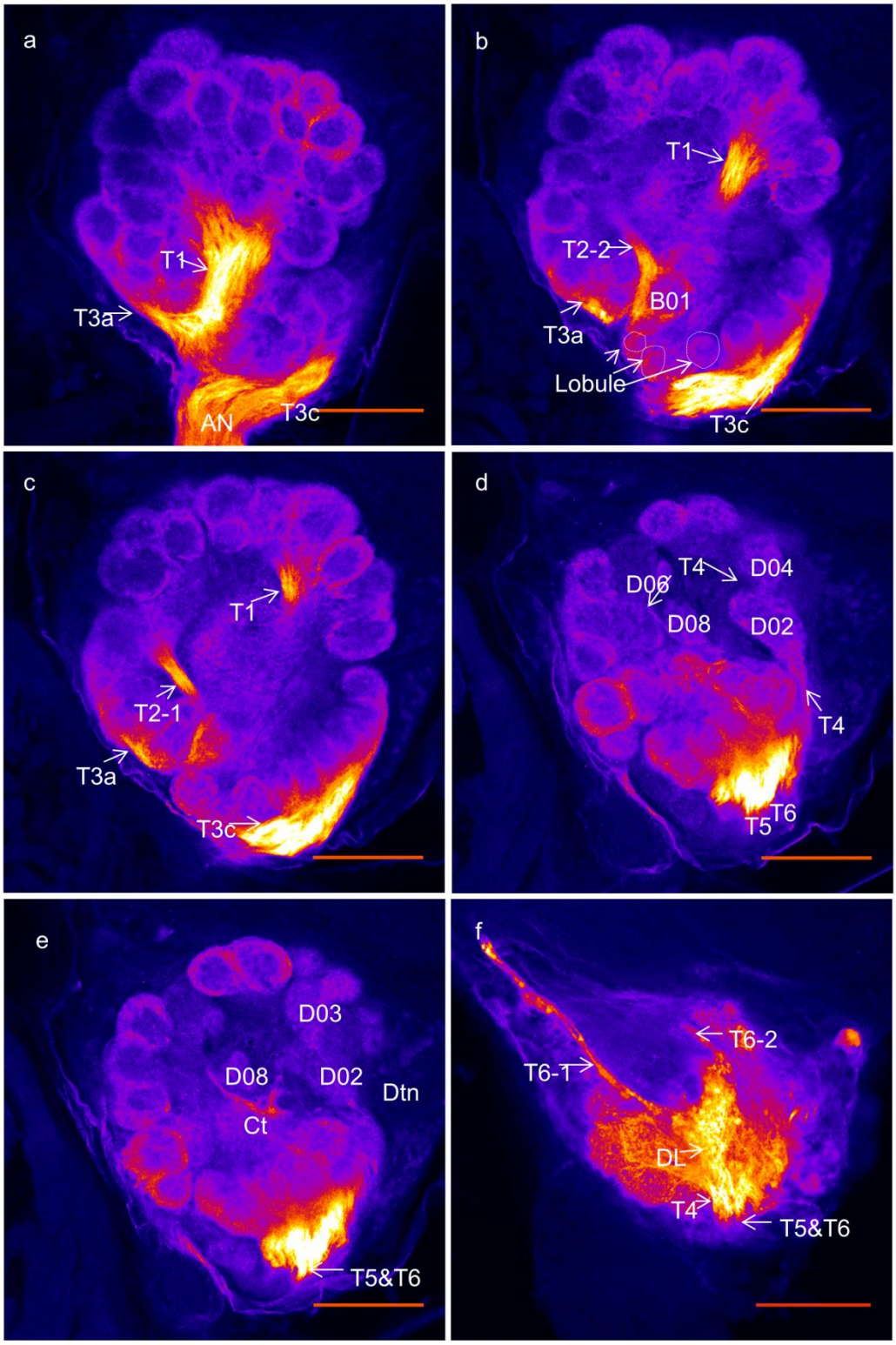


Fig 3.2 Optical sections of the AL show the AN tracts innervating the glomeruli. **a**. AN can

be seen bifurcating in to T1, T3a and T3b. **b.** T2-2 tract can be seen that innervates one medial glomerulus. T3c can be visualized; lobule innervated by T3b is circled. **c.** T2-1 which innervates 6 glomeruli can be seen. **d.** T4 tract can be seen. This tract further divides into 7 tracts and can be seen innervating 7 posterior glomeruli. **e.** Crescent tract (ct) originating between D03 and D02 can be identified. **f.** T5 ending in the dorsal lobe and T6 innervating the protocerebrum can be seen. Scale bar = 100 µm.

Table 1

Name of the study	Number of T1 glomeruli	Number of T2 glomeruli	Number of T3 glomeruli	Number of T 4 glomeruli	Total number of glomeruli
Arnold et al. 1985	71	6	81(n= 1) 82 (n =3)	7	166
Flanagan and Mercer 1989	66-69	7	72-77	7	156
Kirschner et al. 2006	72	7	77	7	163

Table 1 The total number of glomeruli and glomeruli innervated by different AN tracts in various studies done on *A. mellifera*

Table 2

Sample name	No. of T1 glomeruli	No. of T 2 glomeruli	No of T3 glomeruli	No. of T4 glomeruli	Total No. of glomeruli
A	71	7	80	7	165
B	70	7	80	7	164
C	70	7	81	7	165

Table 2. The number of glomeruli innervated by the T1-T4 tracts of the AN and the total number of glomeruli present in each sample. A, B, C represent three samples from different individuals in which the glomerular number was determined. The number of glomeruli innervated by each AN tract and the total number of glomeruli are represented.

3.2.2 Glomerular volumes of *A. dorsata*

The volume of glomeruli varied from 13.4 x 10^3 µm³ to 45.0 x 10^3 µm³ in glomeruli innervated by different tracts **(Table 3)**. T4 glomeruli had the largest mean glomerular volume with a mean volume of 40.6 x 10^3 µm³, which accounted for 8.64% of the total volume. T2 glomeruli had the next highest mean glomerular volume of 24.3 x 10^3 µm³ and accounted for

Table 3

Mean glomerular voume of glomeruli innervated by different AN tracts of A. dorsata	Animal A	Animal B	Animal C	Mean glomerular voume of A. dorsata (x 10^3µm³)	Mean glomerular volume of A. mellifera (x 10^3µm³)
T1	21.6	22.4	20.2	21.4	29.7
T2	25.9	27.1	19.9	24.3	34.8
T3	18.4	17.5	13.4	16.4	19.8
T4	45	39.6	37.2	40.6	57.6
Total glomerular volume	3487.2	3465.2	2899.1	3283.8	4314.0

Table3 Comparison of glomerular volumes of glomeruli innervated by T1-4 tracts and the total glomerular volume measured in *A. dorsata* in this study compared with *A.mellifera* (Arnold et al. 1985)

5.17% of the total volume. T1 glomeruli had a mean volume of 21.4 x 10^3 µm³ and accounted for 45.61% of the total volume. T3 glomeruli had the least glomerular volume of 16.4 x 10^3 µm³ and accounted for 39.9% of the total glomerular volume.

3.2.3 The glomerular arrangement in the AL of *A. dorsata*

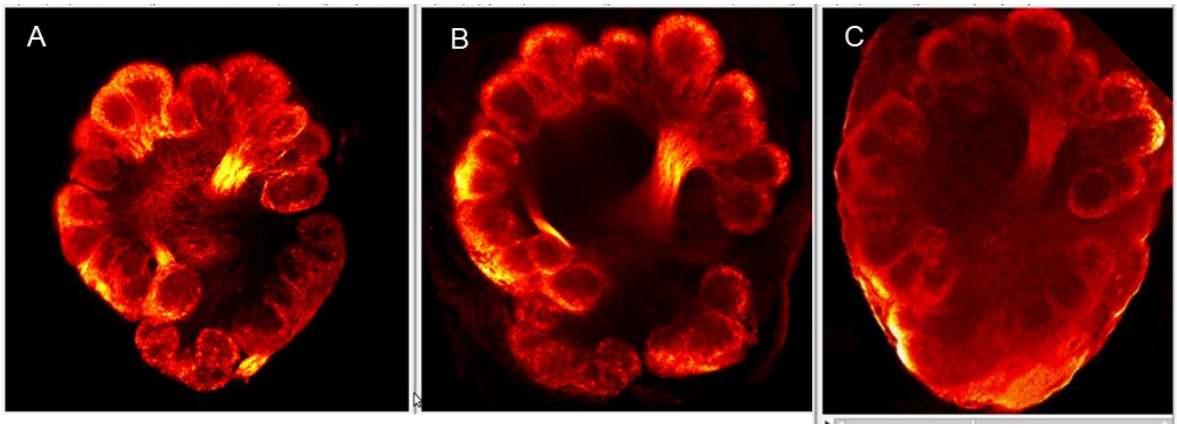


Fig 3.3 Optical sections of AL of *A. dorsata* compared with A. mellifera AL. The first two samples (A &B) are ALs from two individuals of *A. dorsata*. C is the optical section of *A. mellifera.*

Glomerular identification was made based on the approach established by Rospars and Hildebrand (1992) and Galizia et al. (1999). First, the Primary glomeruli, which are invariant and could be identified based on their shape, size, and location in relation to prominent landmarks like the lateral passage, were identified. In the next step, secondary glomeruli, the glomeruli which can be identified based on their position relative to the primary glomeruli, were identified. We first identified T1 glomeruli A17, A33, and A41, as they are three big primary glomeruli located in the anterior part of the AL and, could be easily identified **(Fig 3.4 A)**. At the lateral passage, we could easily identify the primary glomeruli A4, A7, A11, and A22. Though we found A44 to be the biggest T1 glomerulus in most cases, we found A40, A39, A70, and A46 to be large **(Fig 3.4 B)**.

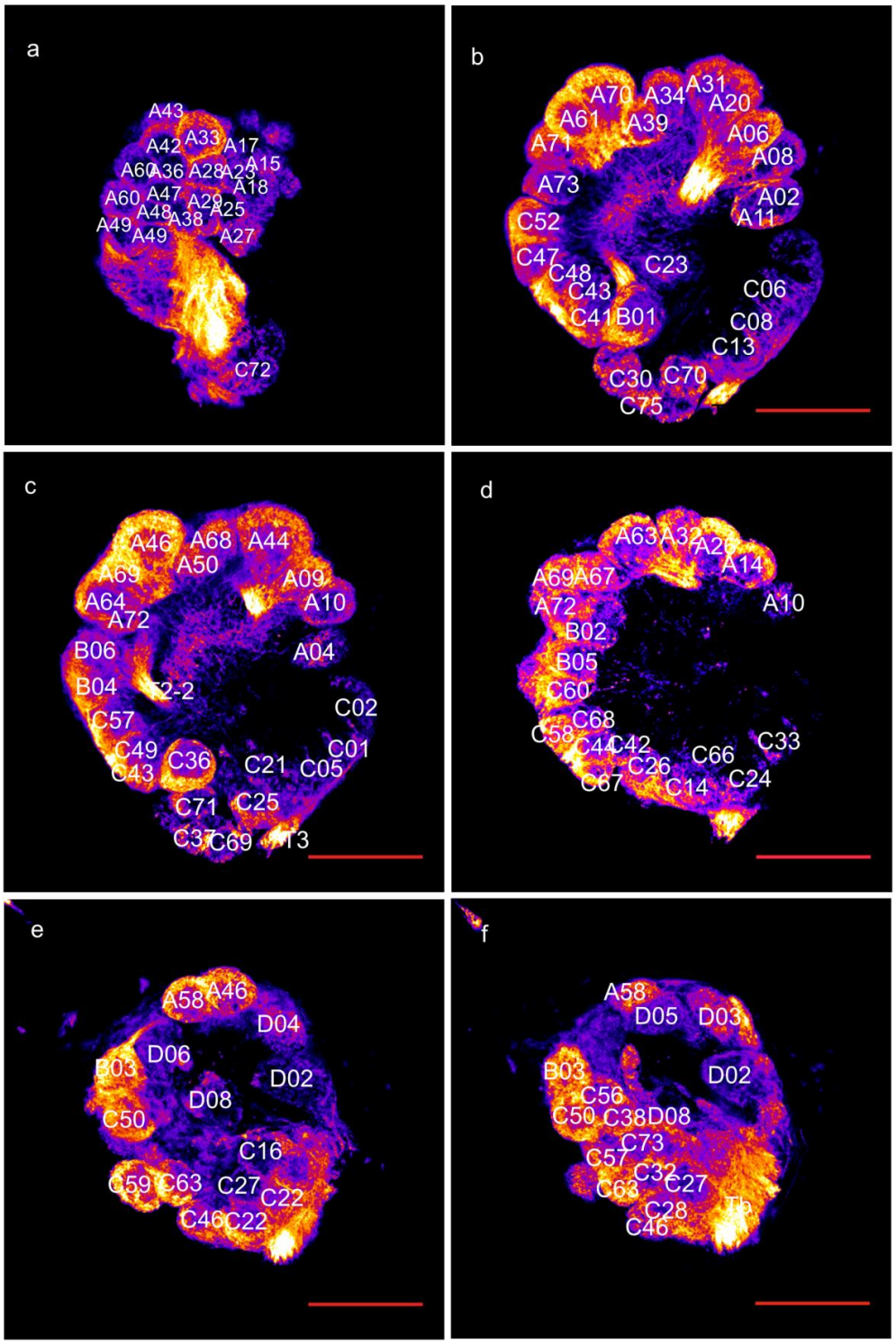


Fig 3.4 Optical sections of the AL at different depths showing primary and secondary glomeruli and their labels. **a**. The 3 primary glomeruli A32, A17, and A33 are visible. The C23 glomerulus which is Primary and invariant and present at the start of T1 and T2 tract is

seen. The largest T1 glomerulus C44 and C71, C37, and C69 are visible. **d.** B02 and B05 the biggest T2 glomerulus, are visible. **e.** A big, primary glomerulus A 41 which is closest to the T4 cluster can be seen. **f.** A 08, A 02, A04, and A06 can be seen.

At the Rostral region, A14, A41, and A58 were identified due to their proximity to the T4 cluster. B01 is an inwardly oriented glomerulus of the T2 cluster and was easily identified. The remaining six T2 glomeruli could be identified due to their closeness to B01 **(Fig 3.4 B)**. In the T3 cluster, C23 was identified unambiguously as it is located at the origin of the T1 tract. C73 could be identified due to its proximity to the T4 glomeruli. C18, C45, C29, C31 are the most dorsal of the T3a glomeruli **(Fig 3.4 B&C)**. C51 is the most ventrally located T3c glomeruli. T3b glomeruli were unique. They are smallest in size compared to the rest and found ventral to the T1 bundle at the point of entry into the AL. They varied in number in the three samples which were counted. Maximum variation in number was found in these glomeruli. T4 cluster glomeruli were the most voluminous of the AL glomeruli located at AL's posterior end. They were different from the rest in size and ORN innervation pattern. D02 and D03 could be identified due to their proximity to the crescent track. The glomerulus D08 first reported in *A. mellifera* by Kirschner et al. (2006) could be identified by its spheroidal shape in all three of the analyzed AL's **(Fig. 3.4 E&F)**.

Similar to that reported by Galizia et al. (1999), we found some glomeruli 'missing' and some glomeruli in 'double' in the counted samples. In the samples analyzed, few glomeruli (about 10) were missing, while some (around 5) were found to be in duplicate. For example, in sample 1, glomeruli A5, A35, A37, A40, A51, A55, A66, and C23 were missing, whereas A49, A60, A62, C22, C34, and C66 were found in double. In sample 2, glomeruli A16, A54, A59, A71, and C39 were missing, and A27, A62, C6, C45, C52, and C55 were double. More variation was observed in individuals collected from different hives in glomerular size and position than individuals collected from the same hive. In Fig. 3.5, A and B represent two

sides (left and right) of the same individual and, C and D represent individuals from different hives. It can be observed that among individuals collected from different hives there are slight variations in the position of glomeruli. When we studied both the AL's of an individual, we found the glomeruli in the right and left AL to be identical in number and spatial arrangement, and this suggests that the variability observed among individuals is real and is not an artifact of tissue processing or imaging **(Fig.3.5 A and B)**.

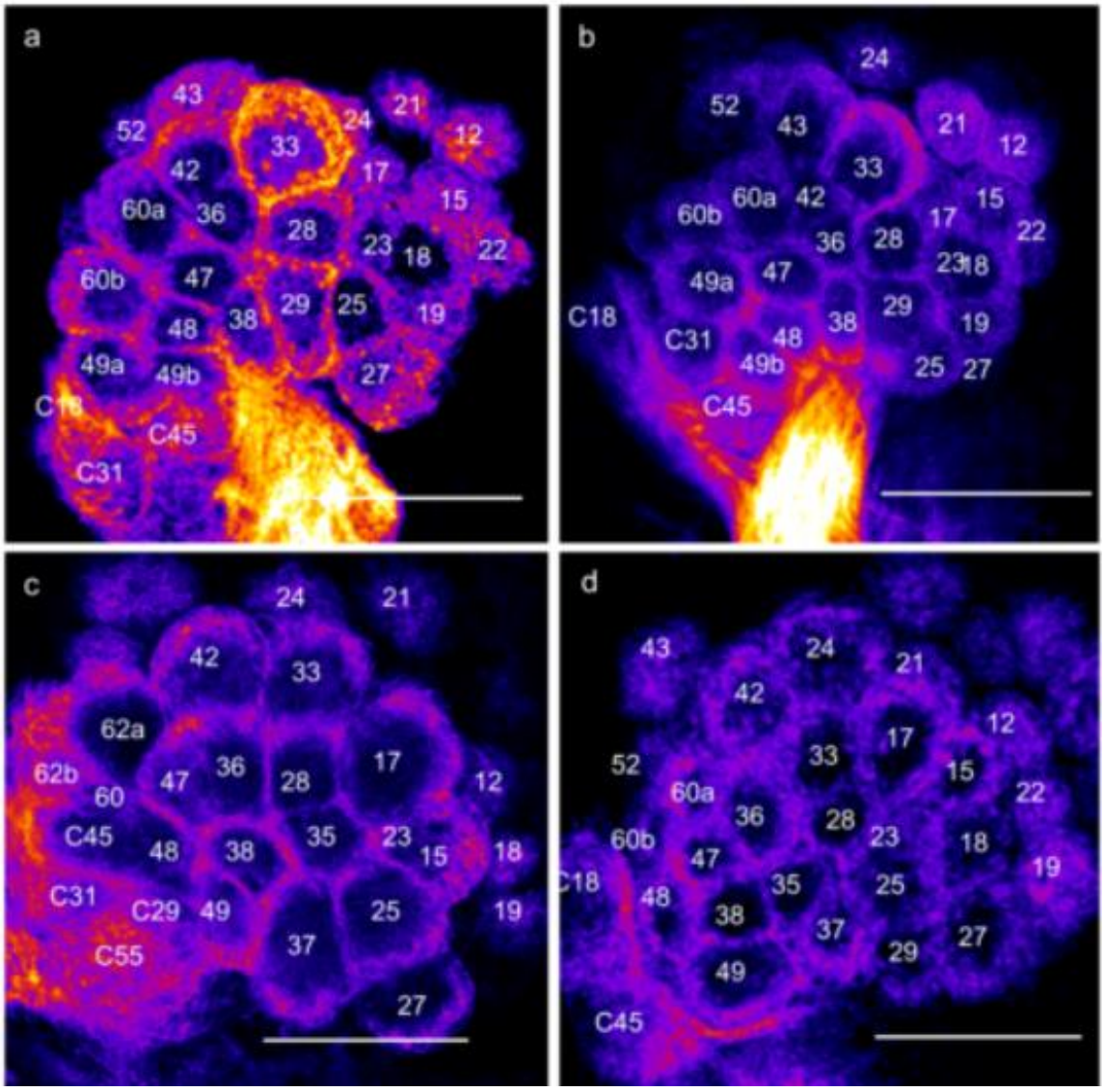


Fig 3.5 Inter-individual differences in glomerular organization. A, B) Optical sections of the right and the left ALs of an individual. It can be noted that they are identical in the

glomerular arrangement. The T1 tract glomeruli are indicated by numbers alone, without the prefix A. Glomeruli A35 and A37 are missing and A60 and A49 are double in both A and B. C, D) ALs of individuals from different hives. Slight variations in the position of the glomeruli between A and B, C, and D can be seen. In C, it can be noted that glomerulus A62 is double and in D, A60 is double. Scale =100 µ

3.2.4 3D reconstruction of the AL

Different views of the 3D model of the AL of *A. dorsata* are shown in Fig.3.6. Each division was given a different color. T1 tract glomeruli were colored red, and those innervated by the T2 tract are yellow. T3a tract glomeruli are in light blue, while those innervated by the T3b tract are colored purple and the T3c tract glomeruli are denoted in dark blue. T4 tract glomeruli were represented in green.

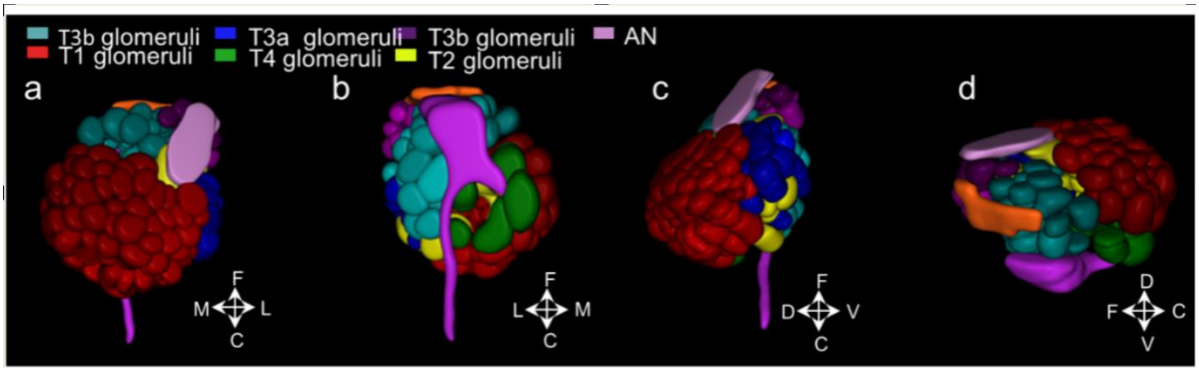
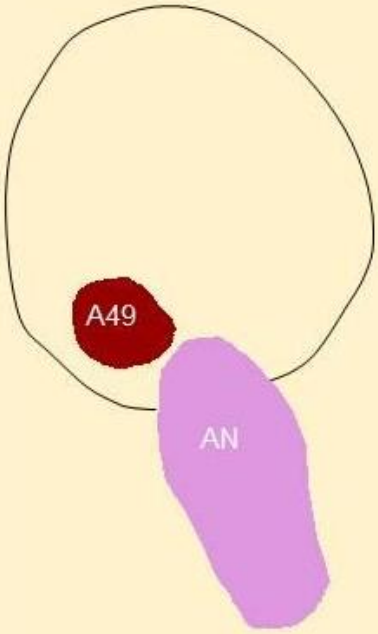


Fig 3.6 3D model of the AL illustrating the arrangement of the glomeruli. **a.** AN can be seen entering the AL in the Dorsal view. **b.** Ventral view shows T6 nerve bypassing the AL. **c.** lateral side of the AL can be seen in the side view. **d.** The side view shows the medial side of the AL.

3.2.5 Digital Atlas of AL of *A. dorsata*

The digital atlas represents each of the glomerulus at different depths in the optical sections and glomeruli have been identified and numbered based on the existing *A. mellifera* digital atlas (Arnold et al. 1985; Flanagan and Mercer 1989; Galizia et al. 1999).

Digital Atlas of *Apis dorsata* based on the established atlases of *Apis mellifera*



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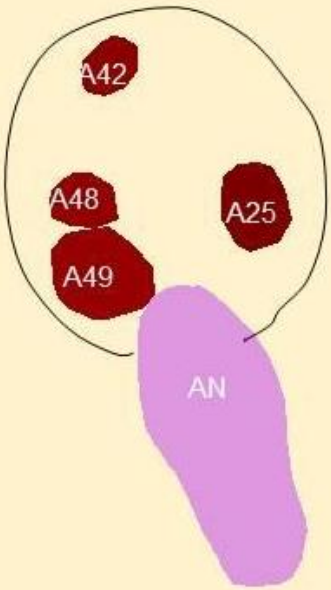

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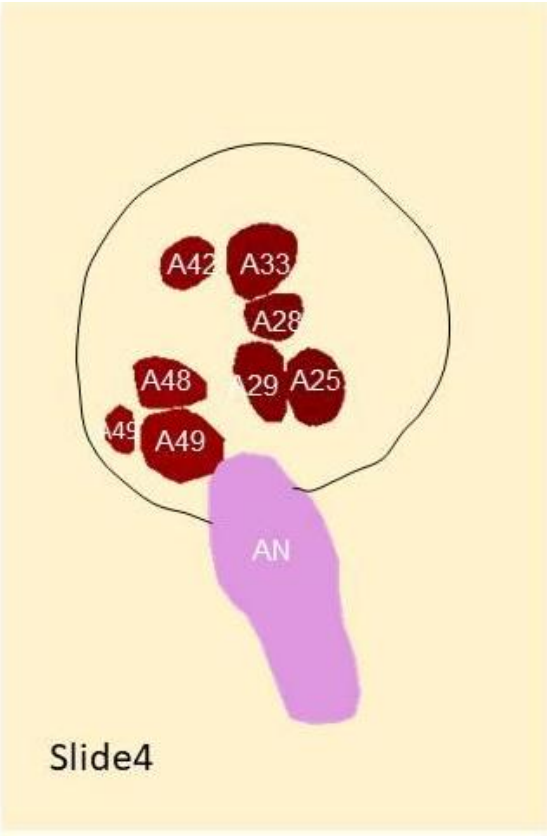

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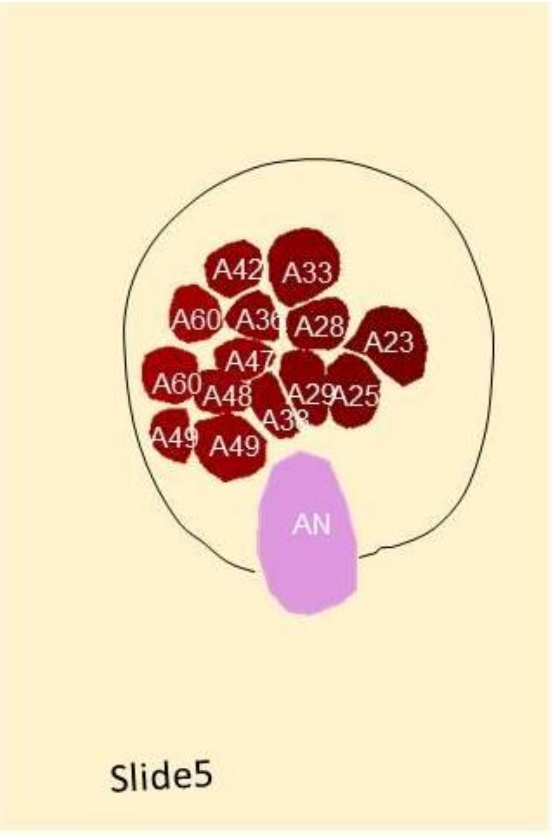

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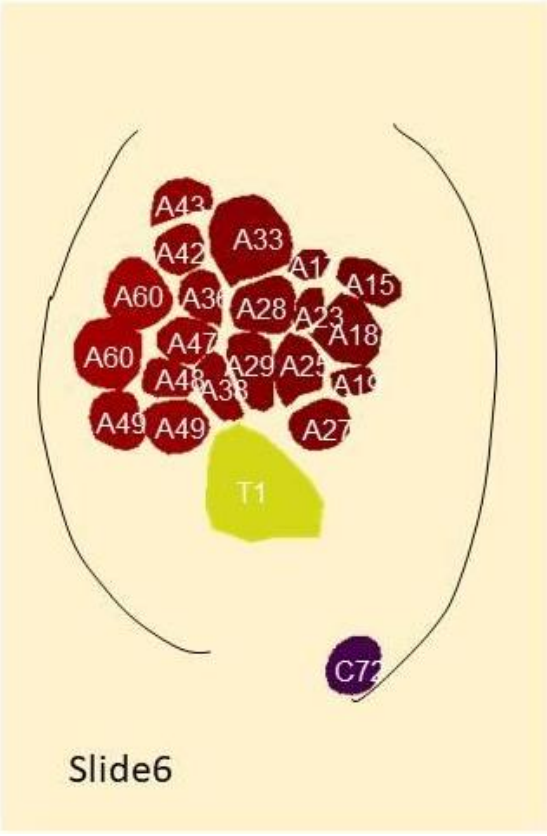

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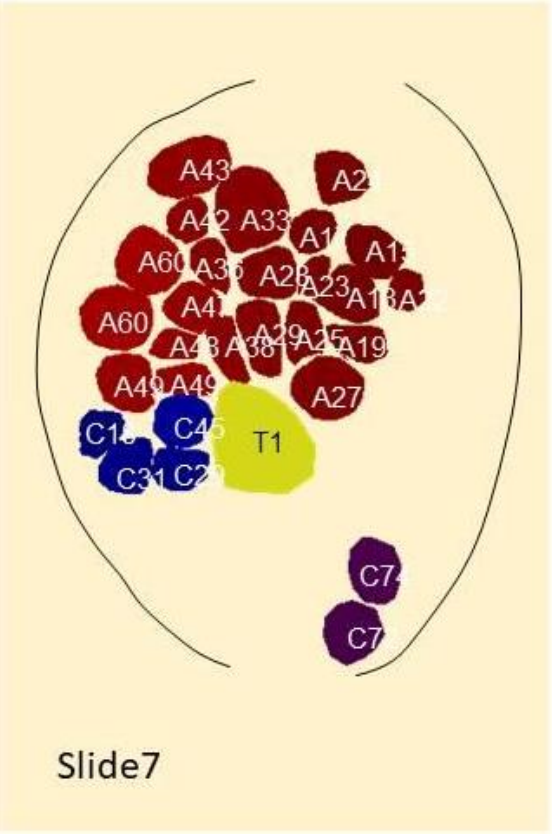

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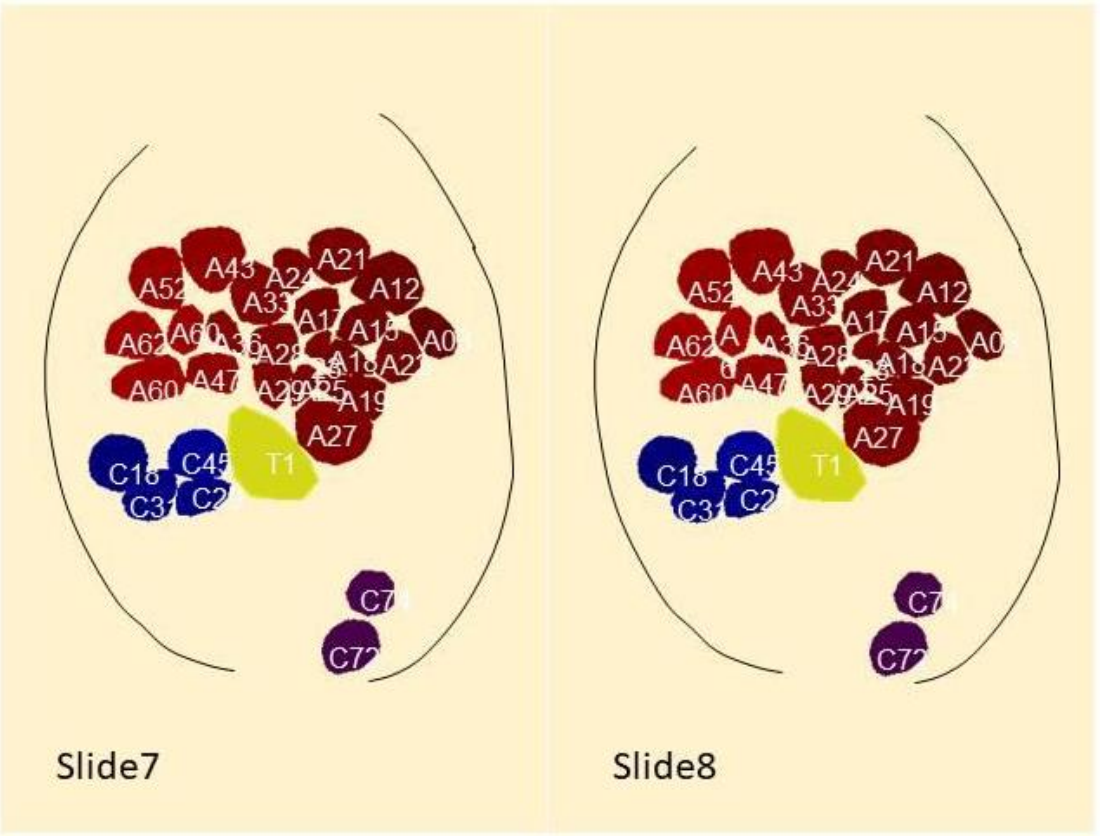
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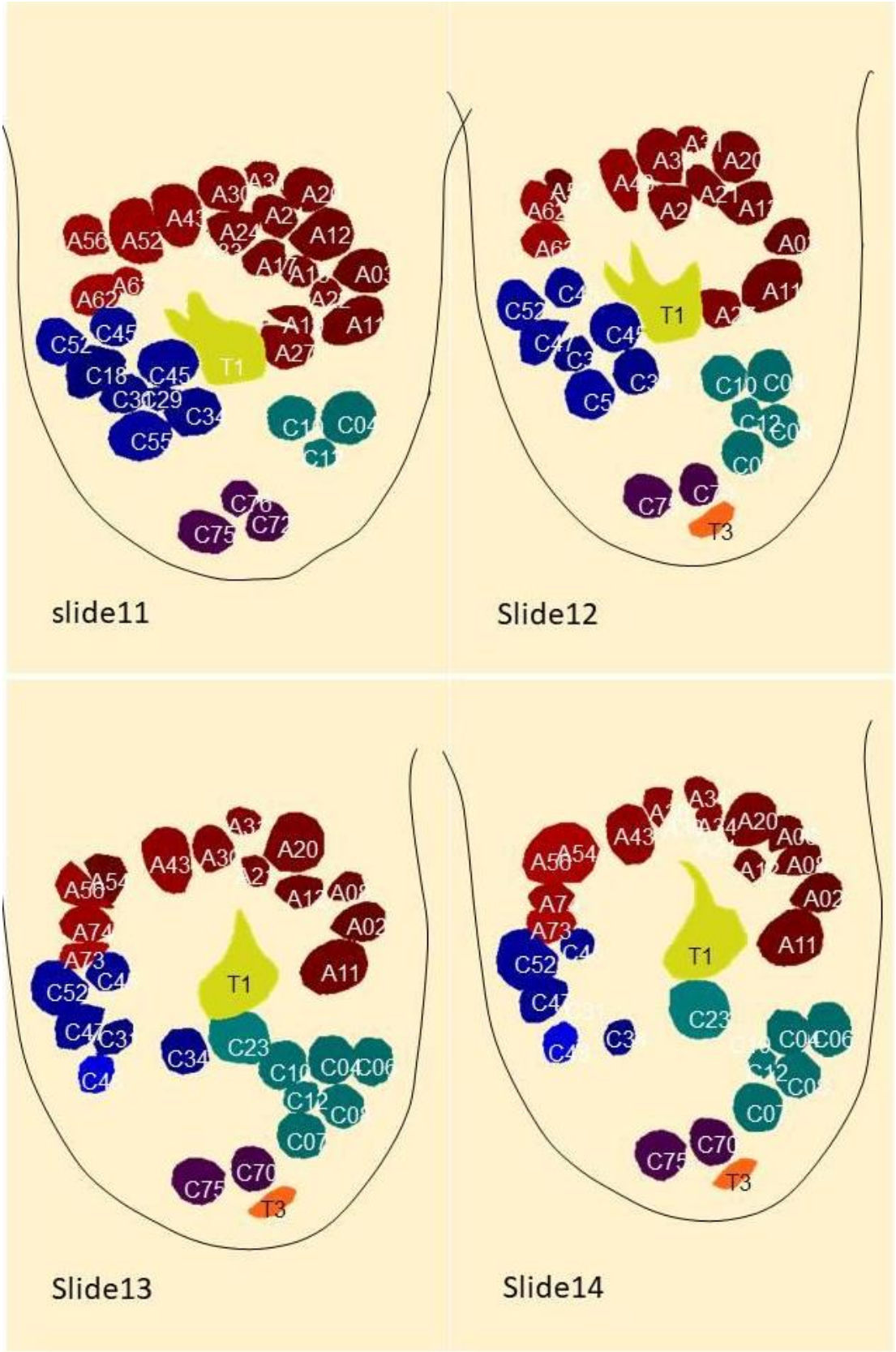
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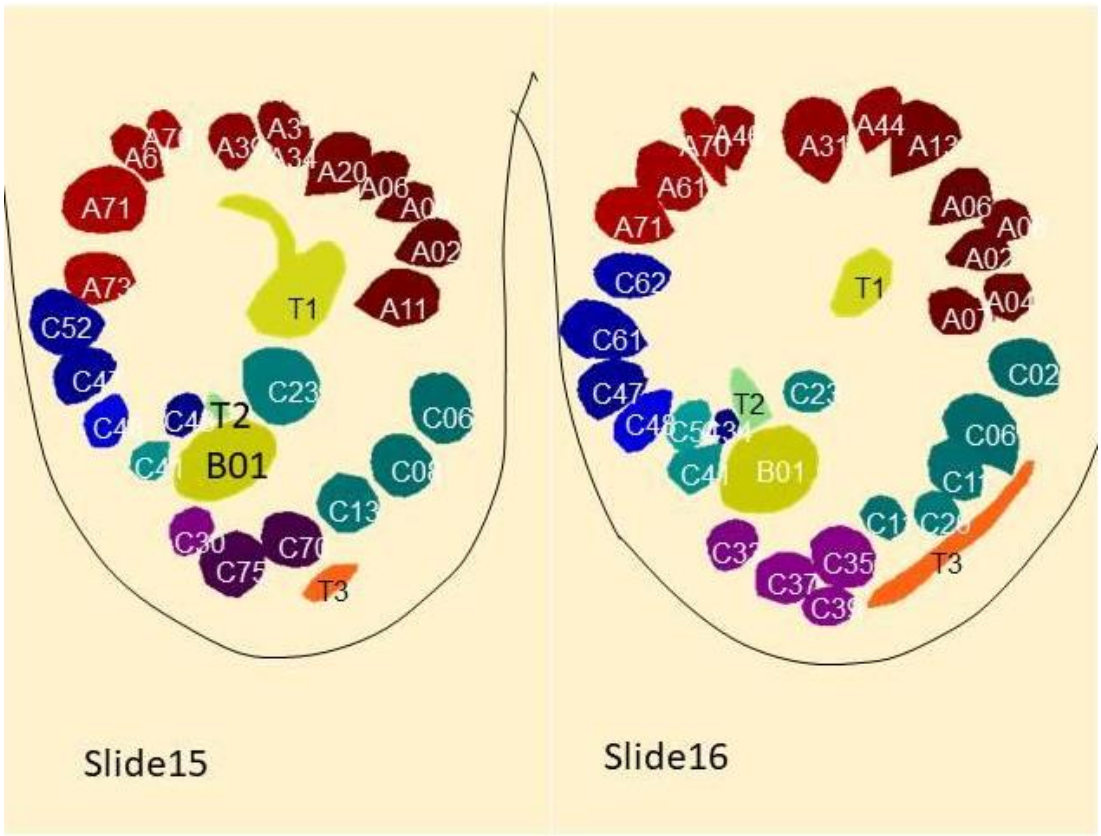

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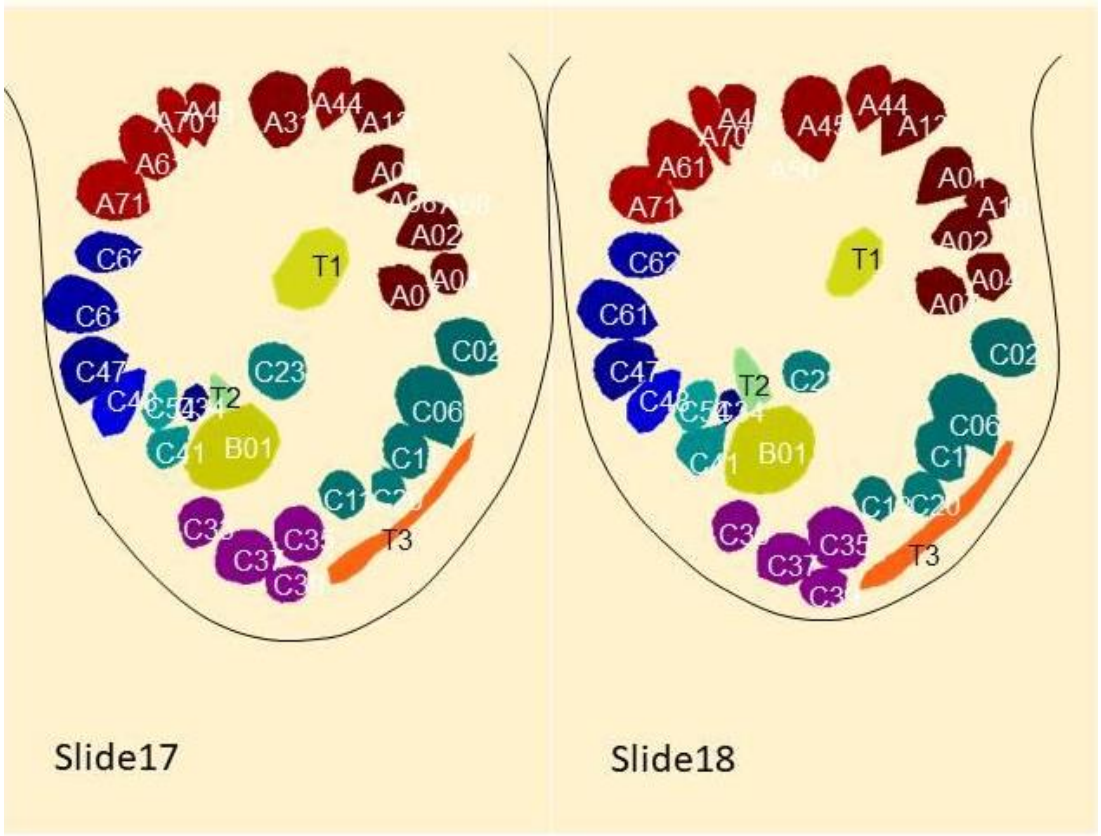

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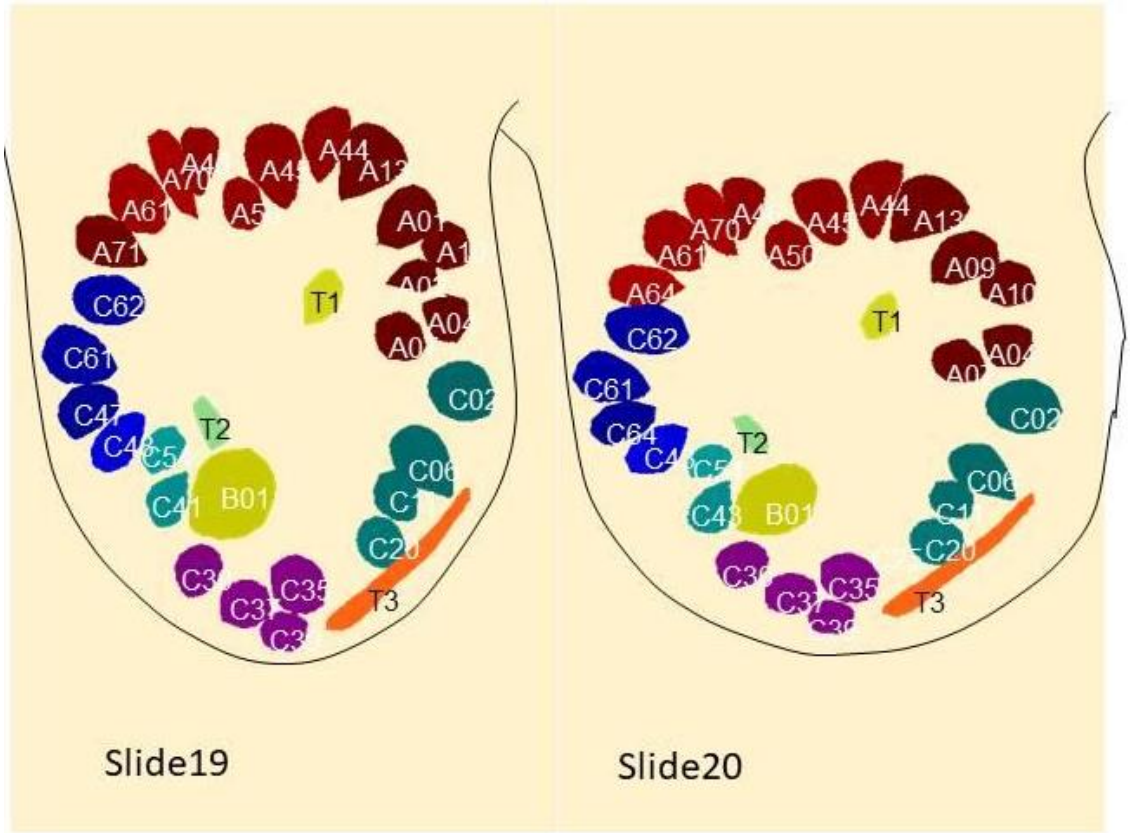

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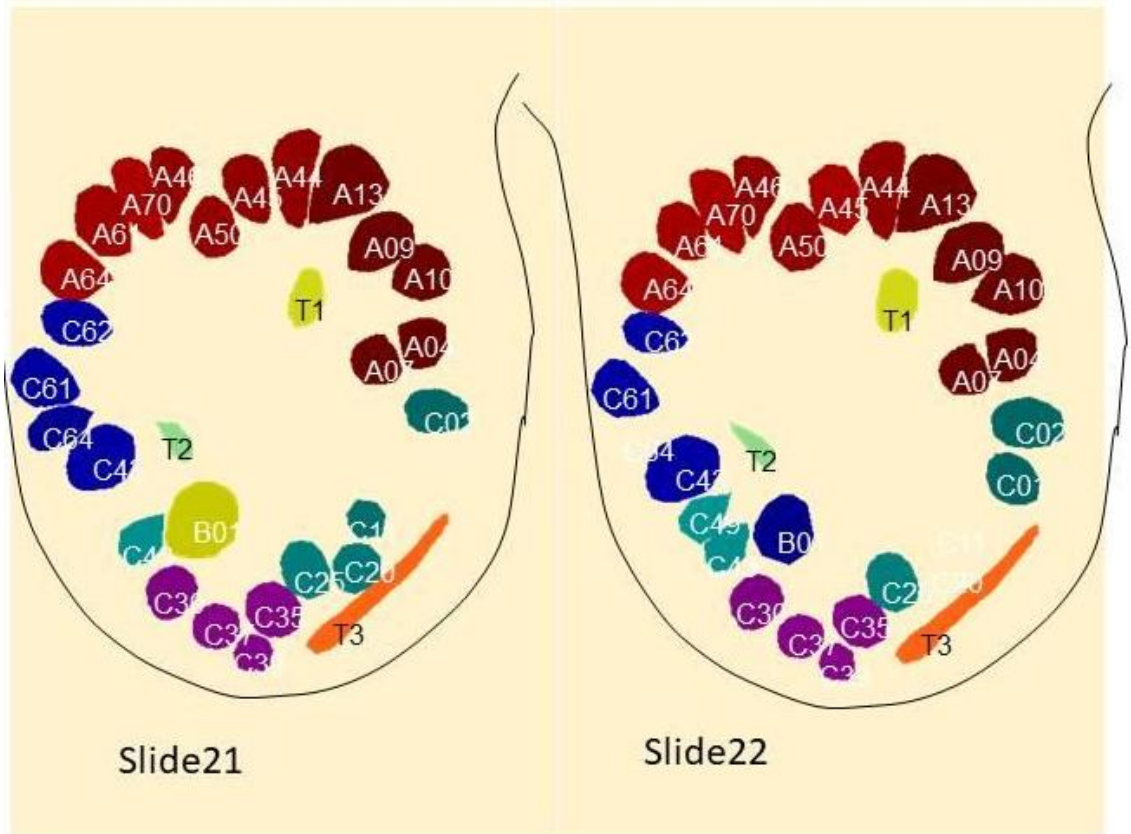

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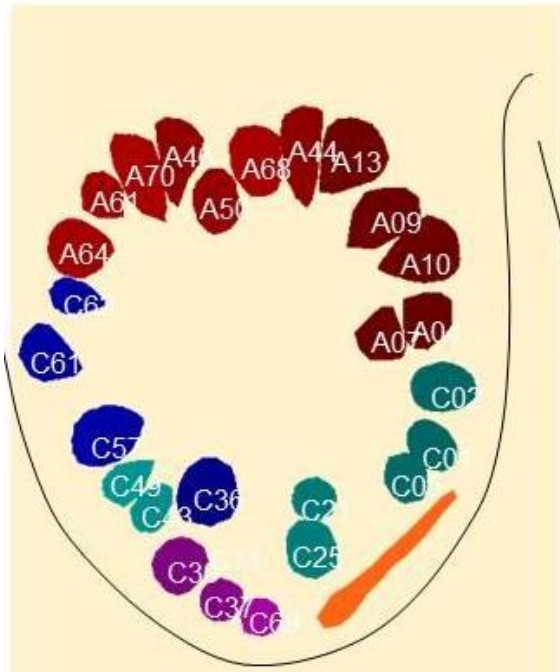

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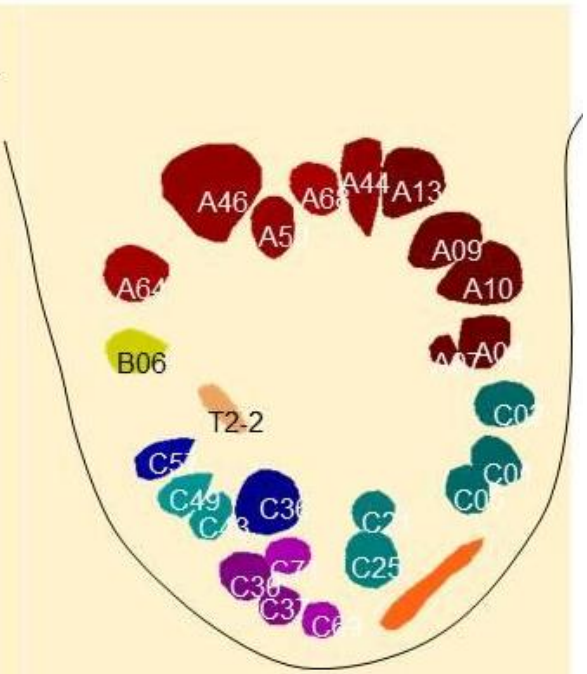

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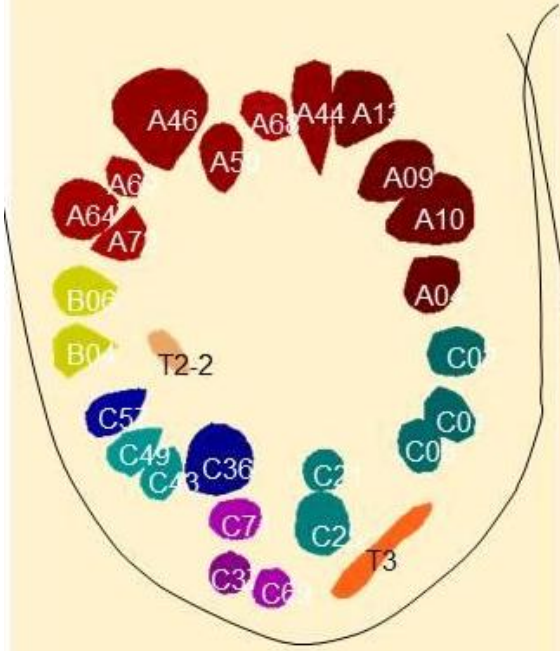

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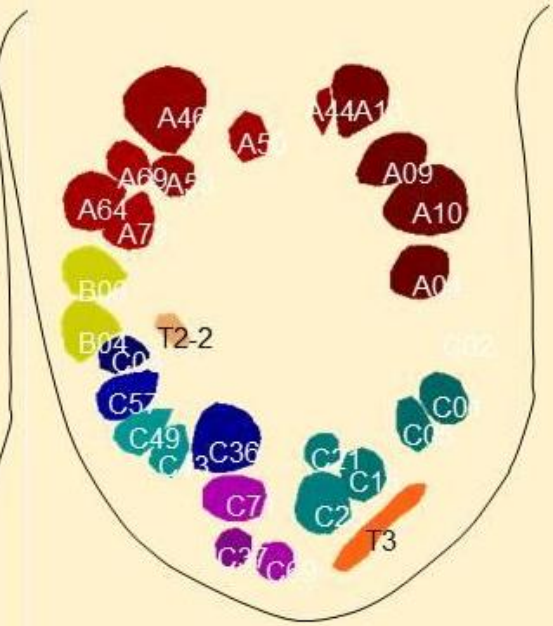

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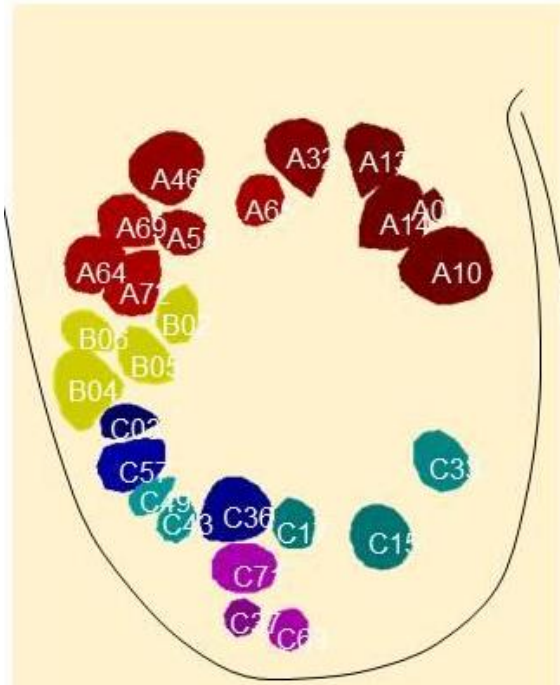

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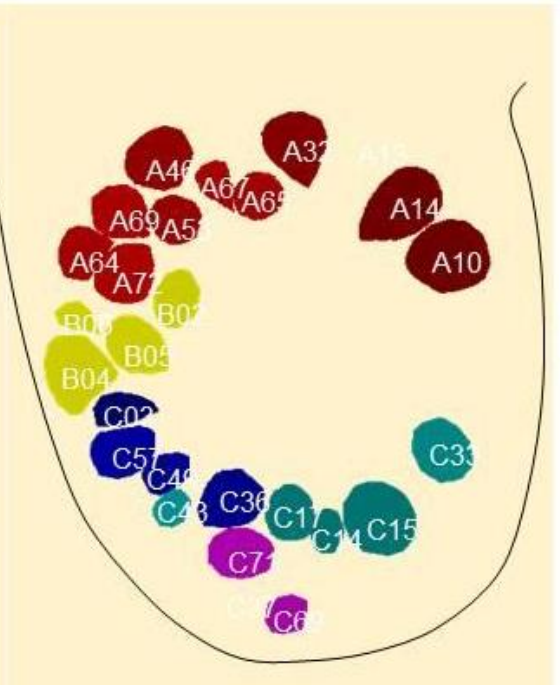

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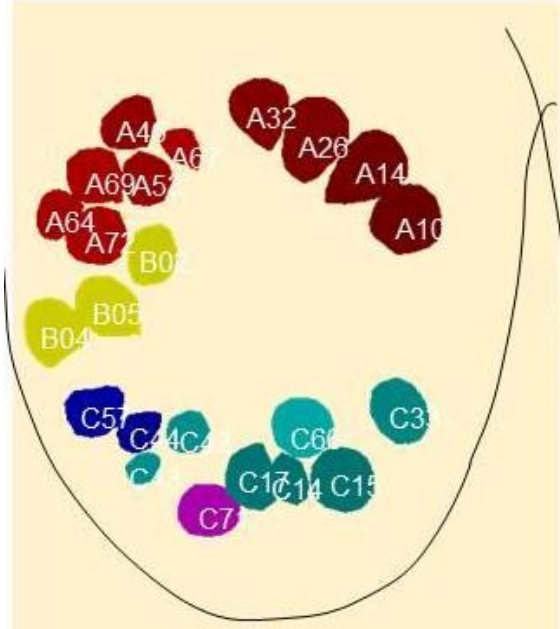

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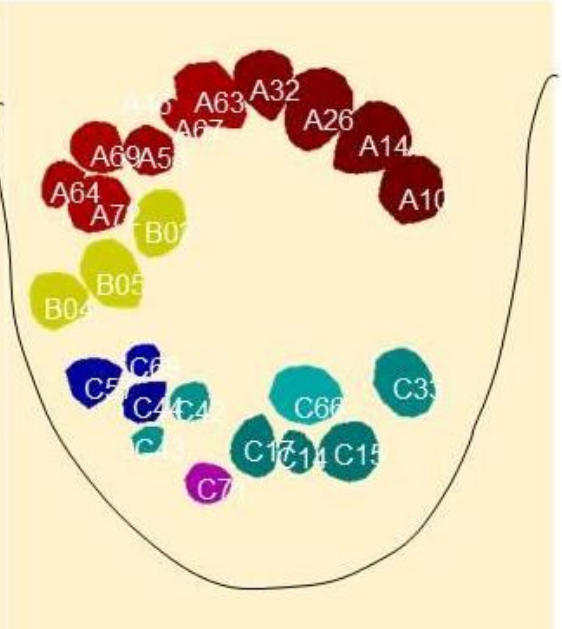

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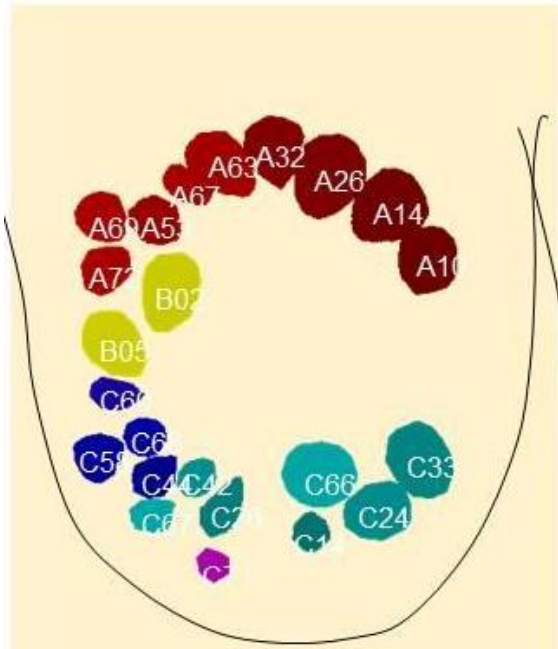

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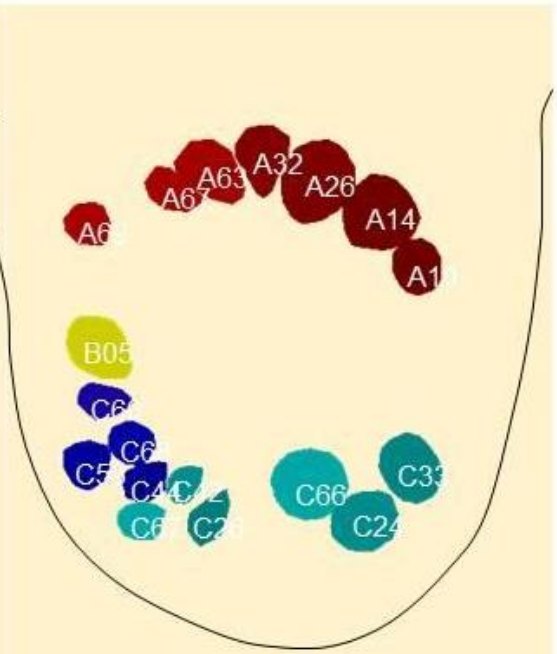

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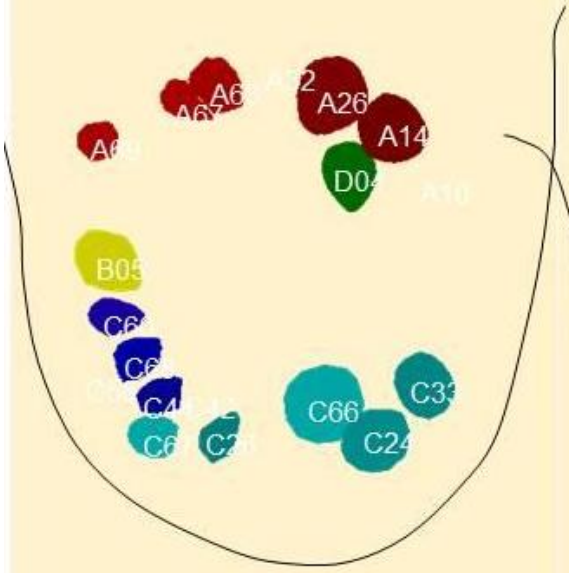

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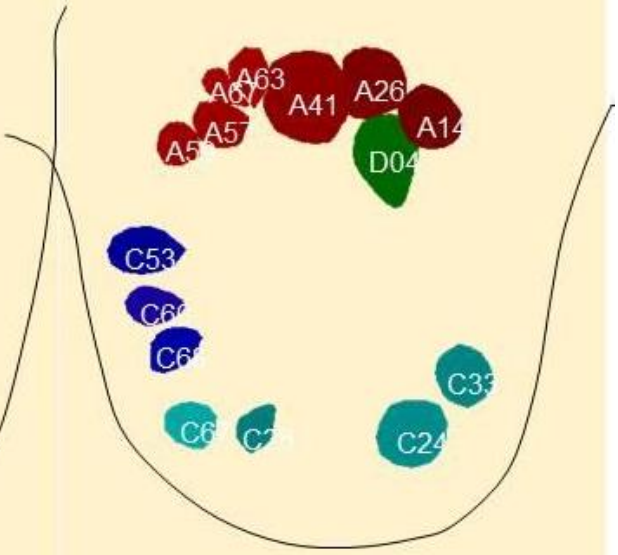

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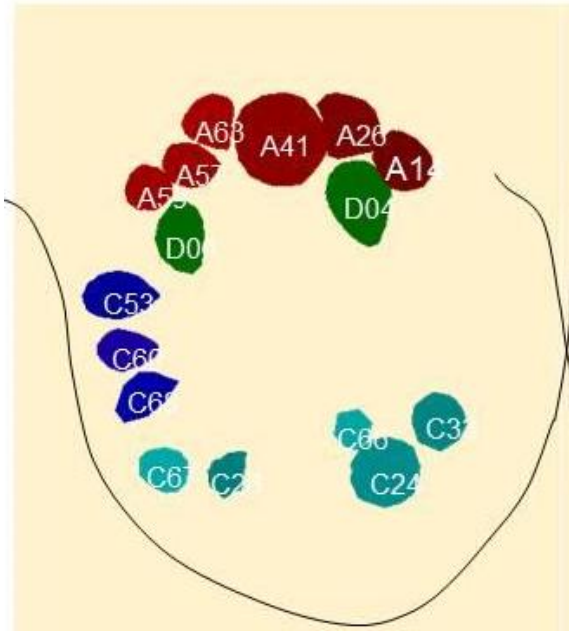

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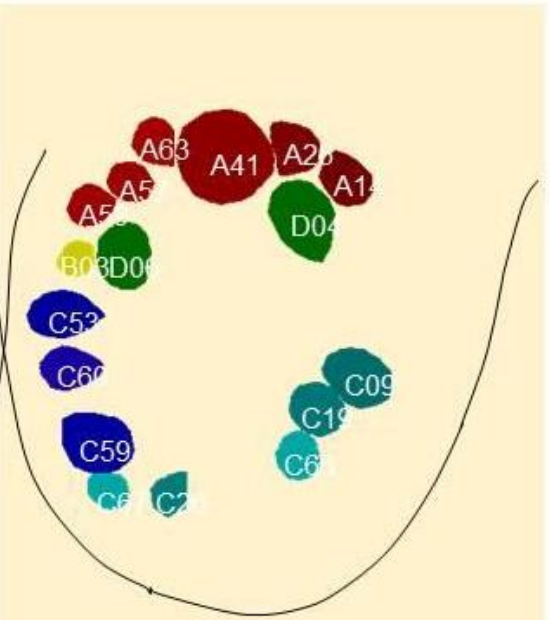

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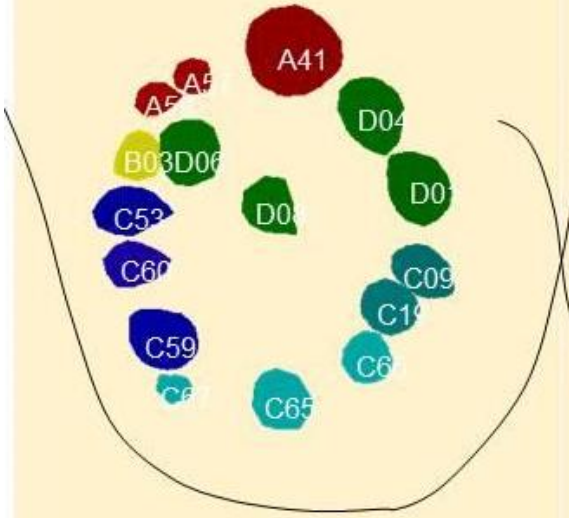

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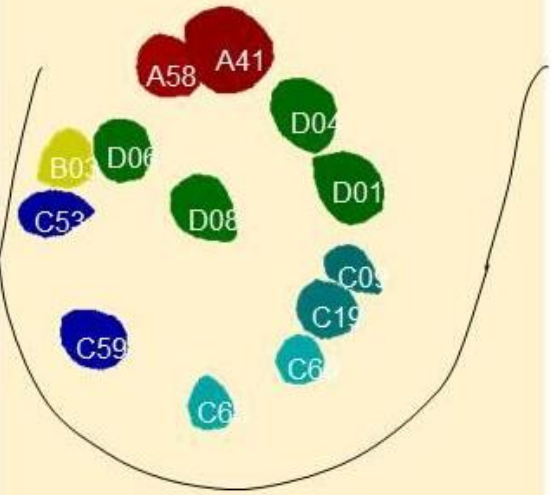

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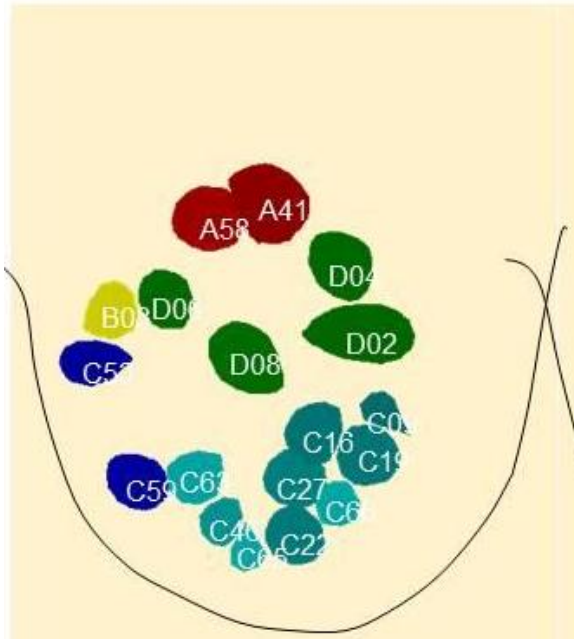

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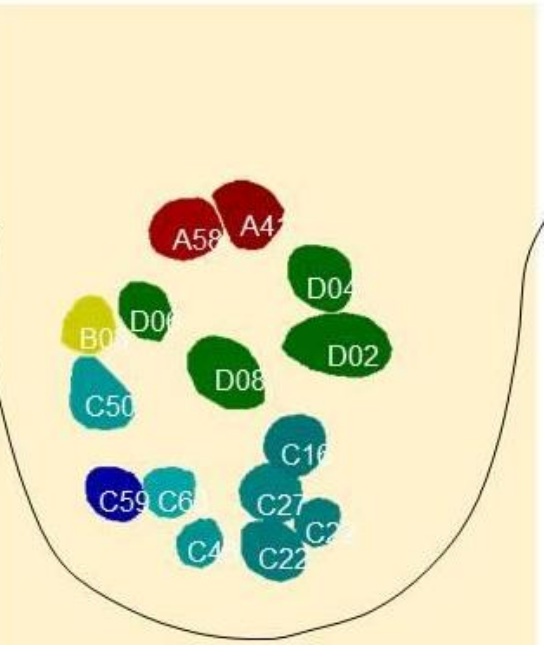

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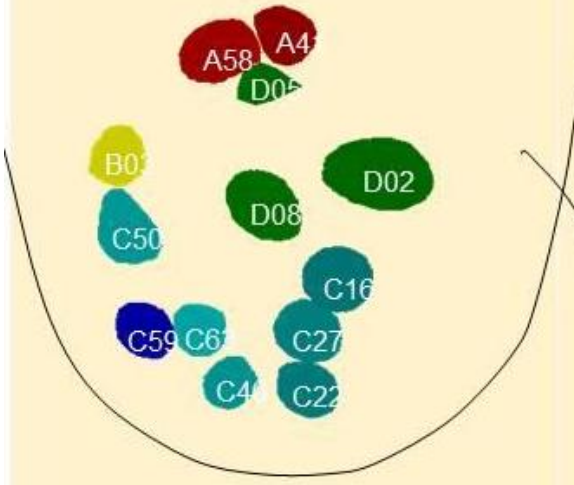

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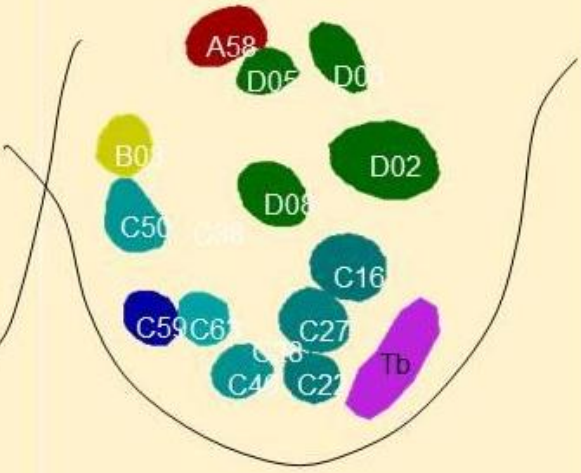

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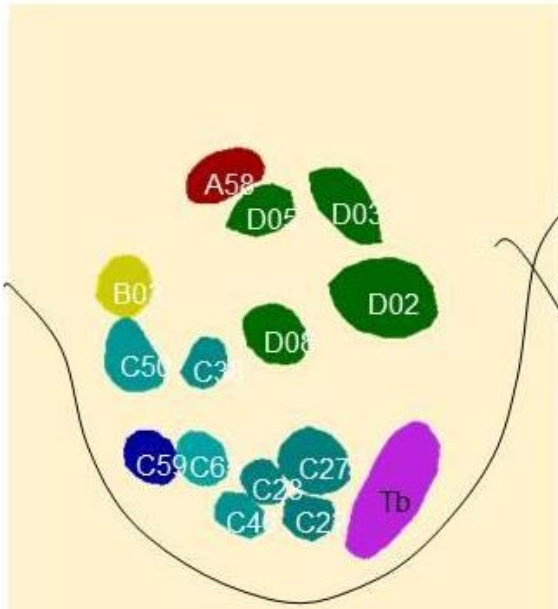

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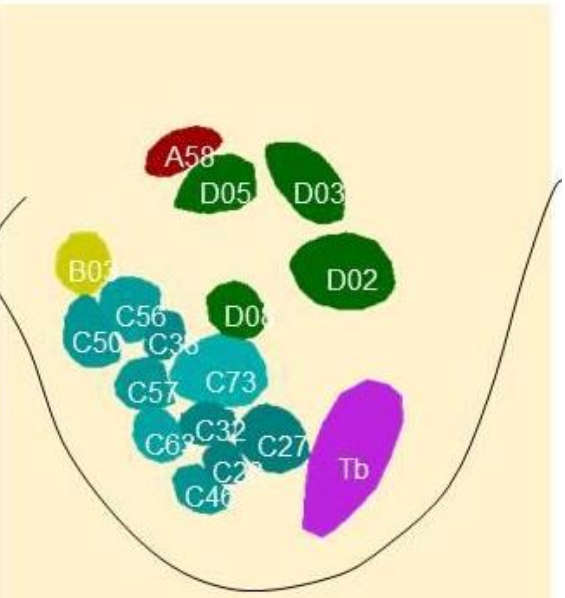

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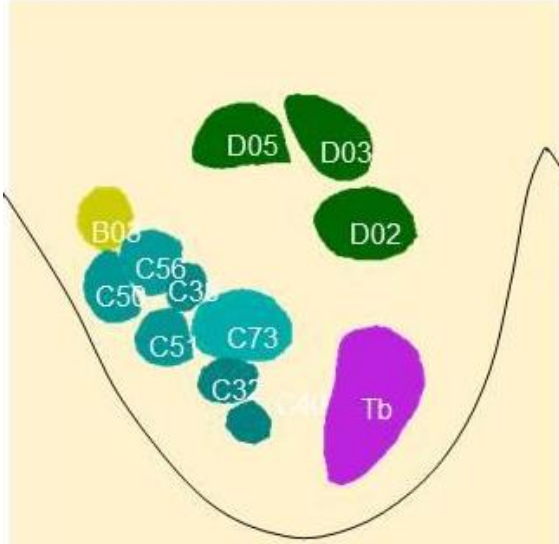

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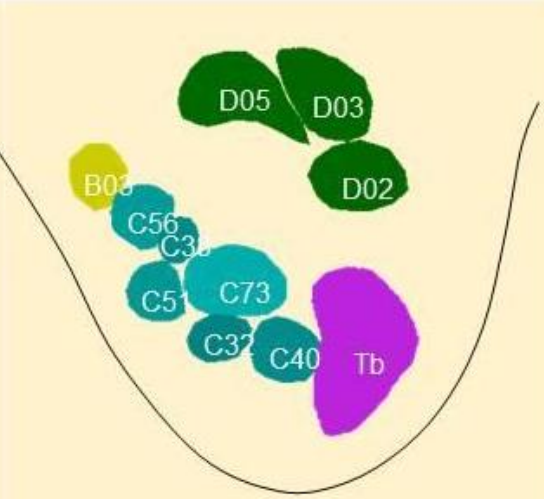

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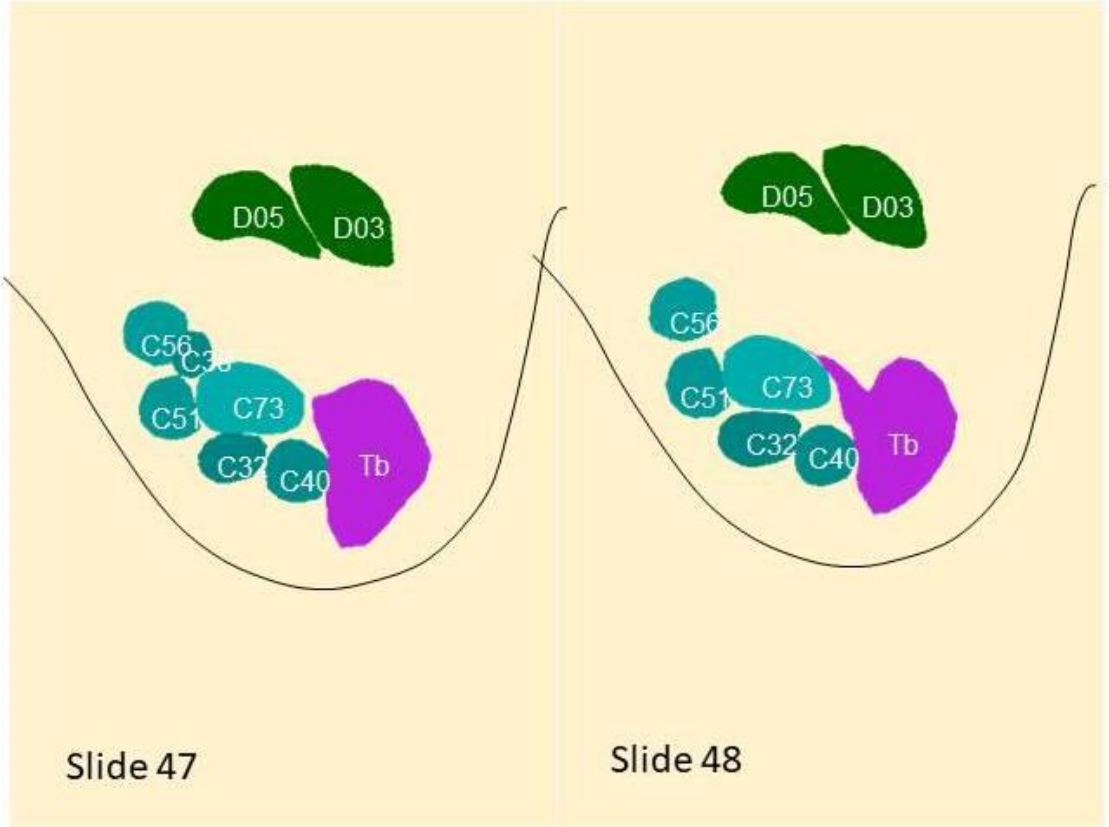
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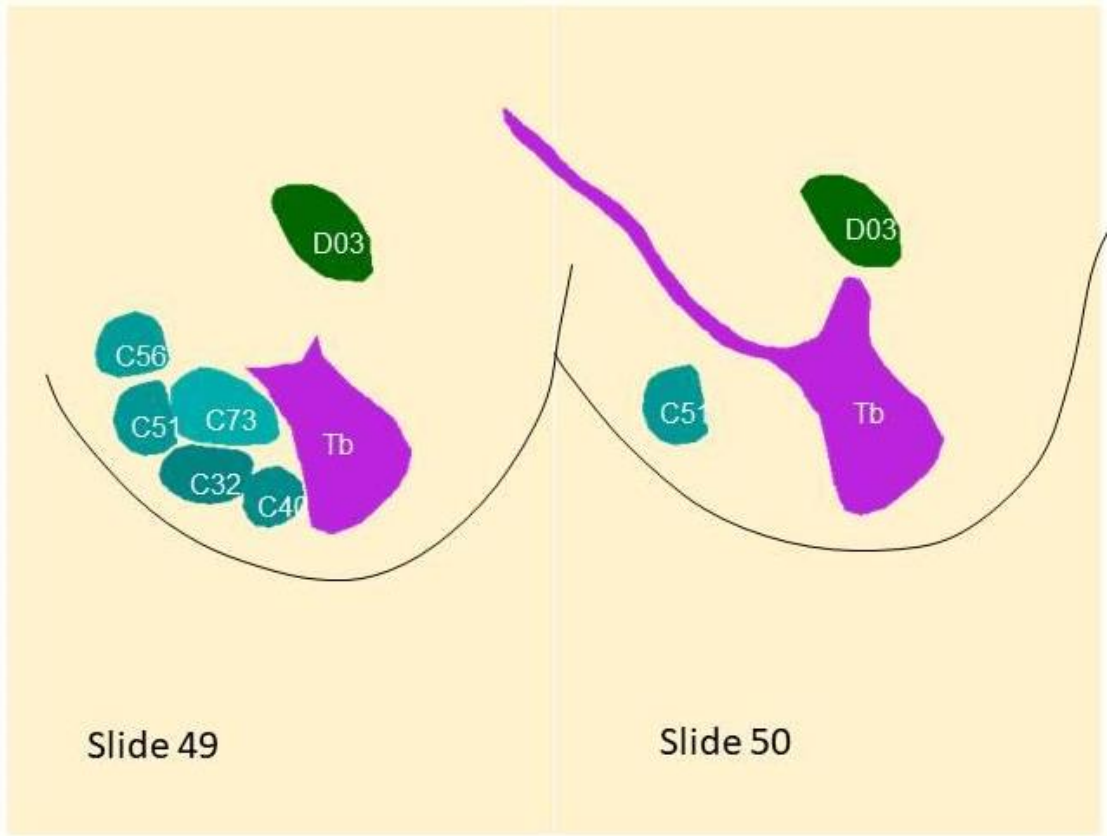
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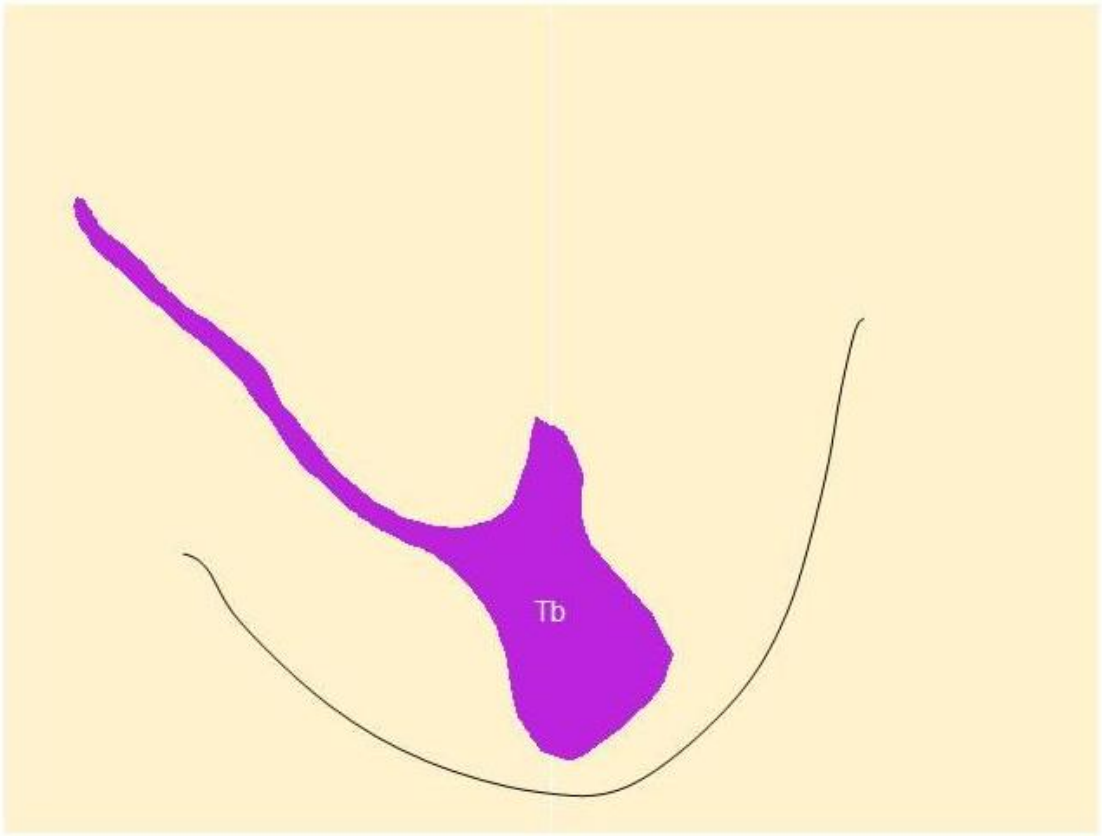

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3.3 Discussion

In this chapter, we characterized the glomerular architecture of *A. dorsata*, established glomerular number, measured glomerular volume, and compared the glomerular organization with *A. mellifera*. Kumar et al. (2014) had characterized the antenna of *A.dorsata*. They found the scape of the antenna had one type of sensilla and two types on the pedicel. Sensilla trichodea type I, II, III, IV, sensilla placodea and sensilla campaniformia were found on the flagellum (Kumar et al. 2014). *A. dorsata* drones were found to have 7300 pore plate sensilla which is half the number compared to *A. mellifera* (Brockman and Brückner 2003).

In *A. dorsata*, the ORNs are present in the antennal sensilla and innervate the glomeruli of the ipsilateral AL like in the other honey bee species unilaterally and unlike *Drosophila*, where the ORNs are present in the antennae and the maxillary palps (Gaudry et al. 2013) and in the hawk moth Manduka sexta where the CO2 receptors are present on the maxillary palps (Thom et al. 2004). In *A. dorsata*, The ORNs innervated the AL via T1-T4 AN tracts like in the other *Apis* species, whereas ant species like *Camponotus japonicus* have seven AN tracts (Nakanishi et al. 2010) and Periplanata americana has ten AN tracts (Watanabe et al. 2012). The arrangement of these tracts in space and thickness was very similar to *A. mellifera* (Mobbs 1982). The glomerular arrangement and number in *A. dorsata* were also found to be similar to *A. mellifera* in all three counted samples.

Generally, glomerular numbers correlate with the number of variety of chemically mediated behaviors the organism exhibits. In ants, it is observed that species that depended more on olfactory cues like trail pheromone have a high glomerular number (Kleineidam and Rössler, 2009; Stieb et al. 2011). It is also observed that in species depending more on visual cues than olfactory cues, the glomerular number is reduced, like that observed in

Cataglyphis, *Gigantiops destructor* (Hölldobler and Wilson 1990; Beugnon et al. 2001), *Formica pratensis* (Goll 1967), and *Harpegnathos saltator* (Hoyer et al. 2005).

A. dorsata forages in the night ocassionally, whereas *A. mellifera* forages only during the day. Given this difference in foraging habit, we still did not observe any difference in glomerular number; this could be explained by the larger facet diameter and acceptance angle of the ommatidia and the larger diameter of the ocelli in *A. dorsata*.

In orthoptera, there is a progression from basal suborder Ensifera to suborder Caelifera in antennal lobe morphology. In the lower orthopterans, like *Tettigonia viridissima*, the AL is similar to other insects, with about 50 glomeruli, In *Gryllus bimaculatus*, each glomerulus has divided into several microglomeruli, whereas *Schistocerca gregaria* has nearly a thousand microglomeruli (Hansson 2011). In the genus *Apis, the* glomerular number does not scale with evolutionary divergence. Drones of evolutionarily distant species like basal *A. florea* (122.4 ± 0.4) and more recent *A. mellifera* (116.5 ± 0.6) have a similar number of isomorphic glomeruli (Bastin et al. 2018). *A. dorsata* drones were reported to have (125.5 ± 0.85) glomeruli, slightly higher than the number in *A. mellifera* (Bastin et al. 2018). *A. mellifera* and *A. florea* have the same number of OR genes implicating they have the same number of glomeruli (Karpe et al. 2016).

The glomerular count is a good constraint for searching for olfactory receptor genes as most of the glomeruli receive inputs from all receptor neurons expressing a single receptor type and all receptor neurons expressing a receptor type innervate single glomerulus (Robertson and Wanner 2006; Karpe et al. 2016). As expected from this line of argument, both *A. florea* and *A. mellifera* were found to have a similar number of olfactory receptor (OR) genes (Karpe et al. 2016) that equals their glomerular counts. *A. dorsata* has a similar number of

glomeruli as *A. mellifera*. It might also have a similar number of OR genes as reported in *A. mellifera* and *A. florea*.

While constructing glomerular maps it was found that some glomeruli in the counted individuals, were missing, and some were present in duplicate. We say that some glomeruli are missing when an expected glomerulus could not be found at a location, and we say that there are duplicates when an additional glomerulus is located at a specific location, and we are not certain which one in the pair is the one we can map to in *A. mellifera*. But these duplicates were not consistent in the individuals we counted, so we mentioned them as duplicates in the same convention adopted by Galizia et al. (1999).

Even if the glomerular maps aligned perfectly in the two species, this information is insufficient to infer that the corresponding glomeruli are innervated by ORNs expressing the same receptor type. Karpe et al. (2016) have compared the putative genes for the olfactory receptor (ORs) in *A. florea* to that of the identified genes in *A. mellifera* and have shown that most of the genes are orthologous except for certain species-specific genes, like that for receptors for cuticular hydrocarbons. Given the overall similarity between the two species, it is likely that one will find a similar extent of orthologous genes between *A. mellifera* and *A. dorsata*. If the ORNs expressing orthologous receptor types innervate the homologous glomeruli in the two species, which is a likely scenario, we will likely find similar responses in the PNs innervating these homologous glomeruli. Another factor that would shape these responses is the local neurons in the AL. We do not have evidence of the similarity of the innervation of LNs as of now. Even in species as divergent as rats and mice, some glomeruli placed in identical locations respond to odors similarly (Soucy et al. 2009). Thus, there may be a great deal of similarity in the response properties of the glomeruli of *A. dorsata* and *A. mellifera*.

The overall brain volume in *A. mellifera* (1.54 ± 0.07 mm^3) was found to be comparable to that of *A. dorsata* (1.56 ± 0.07 mm^3) as reported in Gowda (2016). *A. dorsata* has a greater body weight (71.08 ± 4.37 mg) compared to *A. mellifera* (49.06 ± 5.57 mg) (Gowda 2016). But, we found *A. dorsata* brain structurally sturdier than *A. mellifera* brain, as evidenced by the easiness to keep it intact while carrying out electrophysiology.

Glomerular volumes were found to be different in *A. mellifera* and *A. dorsata*. Glomeruli of T1-T4 tracts in *A. mellifera* had a greater volume than the glomeruli of *A. dorsata*. It is known that environmental selection factors such as food and oviposition site-related odors affect glomerular volume. In *Drosophila sechellia*, the two glomeruli (DM2 and VM5d) where the ORNs for noni-fruit, the primary food source, innervate are 200% larger in both sexes compared to *Drosophila melanogaster* (Dekkar et al. 2006). In leaf-cutting ants, one glomerulus in workers and three glomeruli in drones, which process components of trail-pheromone (Hansson 2011), are enlarged. In *Manduca sexta,* females possess two enlarged glomeruli, specific to some of the host plant volatiles and are assumed to affect behaviors involved in the location and selection of suitable oviposition sites (King et al. 2000). However, there is no plausible explanation for a higher glomerular volume of *A. mellifera* when compared to *A. dorsata*. However, glomeruli of the T4 cluster were the biggest in the AL of both the species (Flanagan and Mercer 1989). This could be because T4 glomeruli were found to process input from other sensory modalities like the taste, temperature, and humidity (Nishino et al. 2009; Zwaka et al. 2016), whereas glomeruli in the clusters innervated by T1–T3 tracts get only olfactory input (Abel et al. 2001; Müller et al. 2002).

3.4 Summary

A. dorsata and *A. mellifera* differ in body size, habitat, nesting habit and foraging behavior. *A. dorsata* is prevalent in South Asia, whereas *A. mellifera* is naturalized in most parts of the

world. *A. dorsata* is an open nesting bee with increased exposure to predators than the cave nesting *A. mellifera*. *A. dorsata* is also known to forage on moonlit nights. Given the above differences, we aimed to look at the differences in the olfactory system at the level of the antennal lobe. We found in *A. dorsata*, ORNs innervate the primary olfactory center, the AL through T1-T4 tracts of AN, and are similar in form and thickness to the tracts in *A. mellifera.* The glomeruli innervated by different tracts were similar in number and arrangement. We counted around 164 glomeruli in *A. dorsata. A. mellifera* was found to have around 166 (Arnold et al. 1985), 156 (Flanagan and Mercer 1989), and 163 (Galizia et al. 1989) in different studies. We created the digital atlas of *A. dorsata* AL and reconstructed the AL.

Though *A. dorsata* is larger in size than *A. mellifera,* the brains are of comparable size in both the species. *A. mellifera* had greater mean glomerular volumes than *A. dorsata* for the glomeruli innervated by all the AN tracts. In both the species, T4 glomeruli had the largest mean glomerular volume.

Chapter 4

Characterization of Olfactory pathway of *Apis dorsata*

4.1Introduction

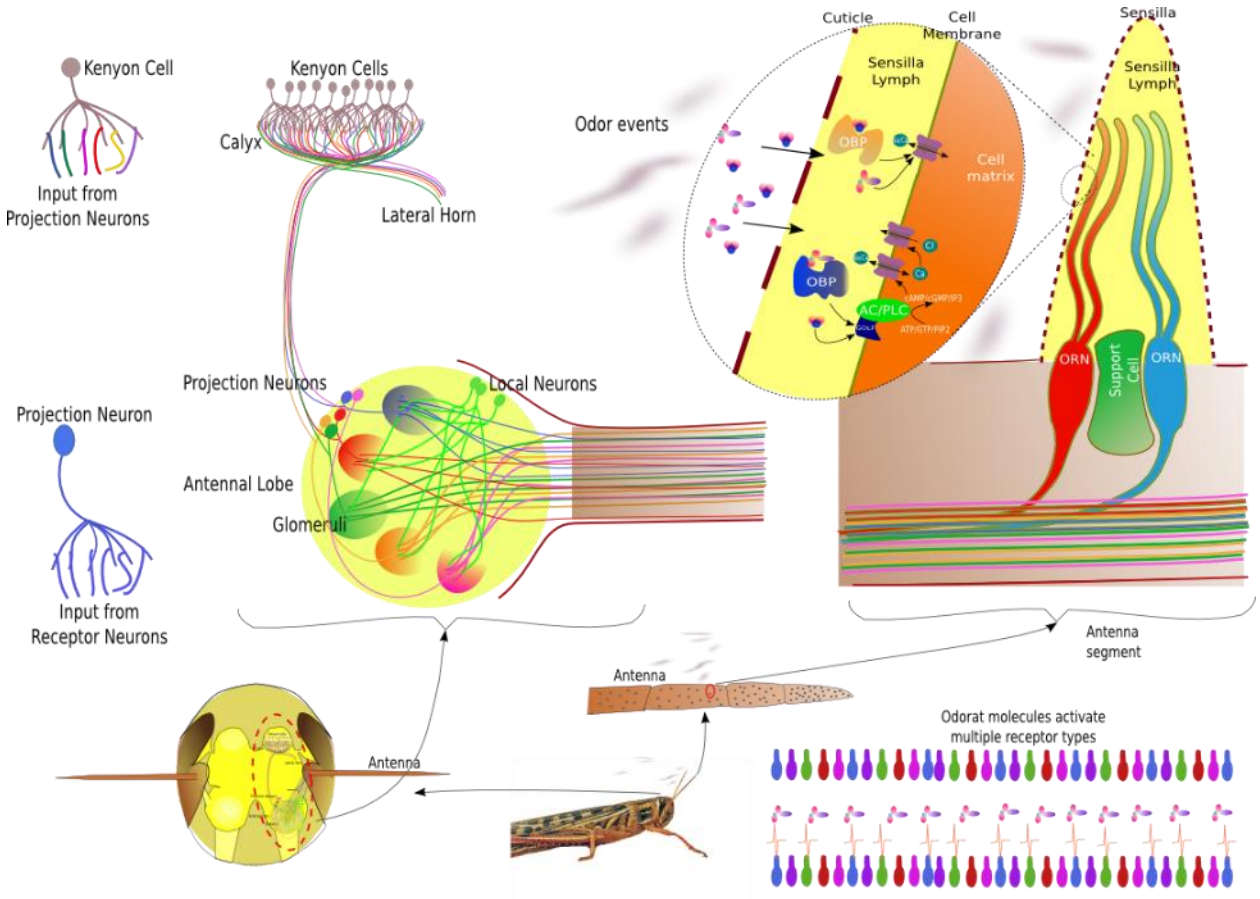

Fig 4.1 The olfactory pathway in insects (here shown for grasshopper, adapted from Joby Joseph). The odor molecules are received by the sensilla present in the antenna and the input reaches the antennal lobe which is the primary olfactory center. The output neurons of the AL transfer the input to higher brain regions, the mushroom body, and the lateral horn.

The olfactory pathway in honey bees begins at the periphery, i.e. at the antenna. Here around 60,000 ORNs that express specific olfactory receptors are present beneath cuticular structures, the sensilla (Kaissling 1987). Among the different types of sensilla, sensilla placodea or the pore plate sensilla are important olfactory sensilla in the honey bee. Each sensillum is innervated by around 5-35 ORNs (Esslen and Kassling 1976). The sensillum cavity is filled with sensillum lymph that acts as a medium through which the odorant molecules reach the dendrites of the ORNs.

The axons of the ORNs course through the AN and innervate the AL. Here, they synapse with the interneurons of the AL in the spheroidal structures, the glomeruli. The majority of ORNs are known to use the excitatory neurotransmitter Acetylcholine (Bicker 1999) based on the presence of the enzyme Choline acetyltransferase, which is used in the synthesis of Acetylcholine (Kreissl and Bicker 1989, Bicker 1999). Nitric oxide (NO) is detected in the AL of honey bees, but the exact areas releasing it are not known. NO is associated with olfactory habituation (Muller and Hildebrandt 2002). In adult honeybees blocking NO activity disrupted olfactory discrimination in ways similar to blocking chloride channels (Muller and Hildebrandt 2002).

4.1.1 Local Neurons

The AL interneurons are predominantly two types, the projection neurons (PN) and the local neurons (LN). PNs innervate the higher olfactory centers, the mushroom bodies (MBs) and the lateral horn (LH). The LNs, however, are restricted to the AL alone and branch only within the AL. Two morphological types of LNs, the homogeneous LNs (homo LNs), which innervate most if not all of the AL glomeruli and the heterogenous LNs (hetero LNs) that innervate unequally only a small number of glomeruli are found in the honey bee AL (Flanagan and Mercer 1989b). Hetero LNs have dense innervation in one glomerulus and

sparse innervation in several glomeruli. In the densely innervated glomerulus they innervate both the core and cortex and only the core of the sparsely innervated glomeruli. The hetero LNs are found to be the dominant LNs in the bee (Galizia 2008) inferred from the fact that the frequency of recording from hetero LNs is more than that from homo LNs.

Bees are generally found to have around 4000 LNs (Witthoft 1967). Honeybees have by far the highest number of LNs. LN number is counted based on the total number of 7350 deuto cerebral neurons in each side (Witthoft 1967) including the AL and dorsal lobe. AL is found to have 4750 neurons, of which the majority are LNs and PNs. PNs are counted to be 800, which fixes the number of LNs at around 4000 (Galizia 2008).

LNs in insects are predominantly GABAergic (Hoskins et al.1986; Malun 1991). In *A. Mellifera*, around 750 LNs are GABAergic (Schafer and Bicker 1986). Not much is known about LNs which are non-GABAergic. Approximately 35 LNs in honeybee stained for Histamine neurotransmitter (Bornhauser and Meyer 1997). In the AL, histamine is found to act as an inhibitory neuro transmitter.

4.1.2 Projection neurons

PNs are either Uniglomerular (uPN), branching in a single glomerulus or multiglomerular (mPN), branching in several if not all glomeruli. Each glomerulus receives input from around 5-6 uniglomerular PNs (Rybak 2012). Honey bees are estimated to have 800 PNs (Hammer 1997). uPNs are found to have acetylcholine as the transmitter (Kreissl and Bicker 1989), and mPNs are shown to be GABAergic (Schafer and Bicker 1986).

VUMmx1 neurons that are Octopaminergic innervate the AL. The excitation of VUM and octopamine application has been shown to be necessary and sufficient to elicit olfactory associative learning in bees (Hammer 1993; Hammer and Menzel 1998).

Dopamine, serotonin, and tyramine-positive fibers have also been found in the AL. Their function is not yet determined, but evidence points that dopamine is involved in aversive learning (Gauthier and Grünewald 2012).

4.1.3 Mushroom body architecture

MBs are paired structures present on either side of the midline of the protocerebrum. They have three major divisions, the cup-shaped calyx, the pedunculus, and the lobes. The MBs are composed of intrinsic neurons named Kenyon cells (KC) (Kenyon 1896). In *A. mellifera*, they are around 170,000 KCs in each hemisphere, with small stomata packed densely in the calyces (Witthoft 1967; Mobbs 1982). Their dendrites densely arborize the calyces, and the axons run along the pedunculus and divide and send branches to the vertical lobe and medial lobe (Mobbs 1982; Strausfeld et al. 2000; Farris et al. 2005).

MB Calyces can be further divided into a lip that receives olfactory input, a collar that receives visual input, and a basal ring that receives both olfactory and visual input. The calyces are the input areas of the Kenyon cells, whereas the lobes are the output regions (Strausfeld et al. 2003). They synapse with the PNs and form microglomeruli in the calyces. KCs are divided into Ka and Kb subtypes. They further have four types of specializations namely dense spiny, sparse spiny, clawed, small field lumpy. The lip of the calyx is innervated by dense spiny Ka type, collar by dense spiny Kb and clawed Ka, and the basal ring by sparse spiny Kb and clawed Ka and Kb (Mobbs 1982). KC axons from the lip, collar, and basal ring project as separate bands into the medial and vertical lobes (Galizia 2008). Some KC sub-populations are glutamatergic (Bicker et al. 1988).

The MB microglomeruli are also innervated by the GABAergic MB feedback neurons that feedback from the lobes to the calyces (Bicker et al. 1985). Around 400 extrinsic neurons connect the pedunculus and lobes to the protocerebrum (Mobbs 1982; Rybak and Menzel

1983). PE1 neuron is a single neuron present in both sides of the brain which connects the pedunculus with the LH (Rybak and Menzel 1983; Brandt et al. 2005) and has been shown to play a role in learning-related plasticity (Mauelshagen 1993; Menzel and Manz 2005; Okada et al. 2007). Centrifugal neurons connect the MBs to the AL (Kirschner et al. 2006).

PNs innervate the higher olfactory centers through five AL output tracts, namely the medial antennal lobe tract (m-ALT), lateral antennal lobe tract (l-ALT), and three mediolateral antennal lobe tracts (ml-ALT) (Bicker 1993; Abel 2001; Muller et al. 2002; Kirschner et al. 2006; Zwaka et al. 2016). m-ALT and l-ALT are made up of axons of uPNs (Rybak and Eichmueller 1993; Rybak 2012), whereas the ml-ALT is made of axons of mPNs (Fonta et al. 1993).

m-ALT neurons receive input from T2, T3, and T4 glomeruli of the AL and travel dorsally and innervate first the ipsilateral MB and then the lateral horn (LH). The l-ALT neurons receive input from the T1 glomeruli and a few T2 glomeruli (Mobbs 1982; Abel et al. 2001; Kirschner et al. 2006). The l-ALT travels ventromedially first to the LH and then to the MB calyces. ml-ALTs receives input from mPNs. ml-ALTs branch off from the m-ALT at different depths, travel transversely, and innervate the lateral protocerebrum (Kirschner et al. 2006). Around 300 l-ALT somata were found around the AL rim along the l-ALT hemisphere, and m-ALT PNs somata were counted to be about 400 and found around the m-ALT hemisphere (Kirschner et al. 2006).

The m-ALT and the l-ALT run parallelly around the distal end of the pedunculus. From the pedunculus, the PNs send collaterals to the MB calyces and form the inner ring tract (IRT) (Mobbs 1982). Axon collaterals of PNs innervate only the inner half of the basal ring and terminate in the lip region. m-ALT and l-ALT PNs differ in the pattern of innervations in the basal ring and lip regions (Kirschner et al. 2006; Zube at al. 2008; Nishikawa et al. 2012;

Rossler and Brill 2013). PNs of the m-ALT innervate the periphery of the basal-ring, which is multimodal, whereas l-ALT PNs innervate the central part, and both the innervations remain segregated. The basal ring is further divided into the outer basal ring, which receives visual input; the middle basal ring, which receives input from the m-ALT PNs; and the central basal ring that receives input from l-ALT PNs (Gronenberg 2001). In the lip, the central core of the lip region is innervated by l-ALT and m-ALT PNs, it is dominated by l-ALT PNs, and the outer cortex is innervated only by m-ALT PNs.

The LH can be divided into four compartments based on the innervations by the three ALTs. Compartment one is innervated by m-ALT PNs, compartment two is innervated by PNs of l-ALT, m-ALT, and ml-ALT 2 & 3, but less densely compared to the other compartments. Compartment three is densely innervated by l-ALT and ml-ALT 2 & 3. Compartment 4 is predominantly innervated by ml-ALT 1 PNs (Kirschner et al. 2006). LH is implicated in innate behavior, multimodal integration, bilateral coding, and concentration coding.

The PNs of the ml-ACT 2 & 3 divide the lateral protocerebral lobe (LPL) into three compartments, the ring neuropil, triangle and the lateral bridge (Abel et al. 2001; Kirschner et al. 2006).

l-ALT PNs were found to code odorants with broader odorant-tuning profiles when compared to m-ALT PNs. Additionally, odorant information was conveyed faster by l-ALT PNs than m-ALT PNs (Müller et al. 2002). Regarding the neurotransmitter the m-ALT PNs are cholinergic (Kreissl and Bicker 1989) and l-ALT PNs exhibit taurine-immunoreactivity (Schäfer et al. 1988; Kreissl and Bicker 1989). Few m-ALT and ml-ALT PNs were found to be GABAergic (Schäfer and Bicker 1986).

4.1.4 LN physiology

In honey bees, LNs exhibit action potentials (Sun et al. 1993, Galizia and Kimmerle 2004) unlike locusts where LNs are non-spiking (Laurent and Davidowitz 1994). In spiking LNs, it is shown that in resting animal they are tonically active and when stimulated with odors, they respond with a decrease or increase from the baseline firing rate. Intracellular recording from LNs of bees exhibits multiple spike amplitudes this indicates that LNs may have multiple spike initiation zones, or they could be electrically coupled (Galizia 2008), or the multiple spike heights could be artifacts caused by the electrical connections created by the electrode (Galizia and Kimmerle 2004).

Homo-LNs and Hetero-LNs show distinct odor-response properties. Hetero-LNs respond to odors associated with the dominant glomerulus implying that the dominant glomerulus is their input site (Galizia and Kimmerle 2004). Homo-LNs exhibited broad response profile in drosophila, responding to most odors, with activity spread across their entire arborization (Wilson et al. 2004).

4.1.5 PN physiology

In honey bees, PN responses are measured either electrophysiologically (Hansson et al. 1991; Abel et al.2001; Muller et al. 2002; Galizia and Kimmerle 2004; Sachse and Galizia 2002) or by optical methods by filling the PNs with calcium dyes (Sachse and Galizia 2002). PNs exhibit spontaneous activity and show either excitatory or inhibitory responses to odors (Christensen et al. 1998; Sachse and Galizia 2002; Wilson and Laurent 2005; Shang et al. 2007).

LNs affect the activity of PNs in two different ways. They cause fast inhibition by GABA acting on the ionotropic channels and on a slower timescale continuing inhibitions even after stimulus offset (Christensen 1998). It has been shown that the fast GABA-mediated ionic currents synchronize PN activity and cause odor-evoked oscillations in the range of 20–30 Hz

(MacLeod and Laurent 1996; Wehr and Laurent 1996) in the local field potential (LFP) of the MB. LNs also have a disinhibitory effect on the PNs. In an arrangement of ORN-LN-LN-PN, when the ORN is active, it excites the LN, which inhibits the other LN, thereby releasing the PN from inhibition and causing it to spike (Av-ron and Rospars 1995).

4.1.6 KC physiology

KCs show low or zero baseline activity. They have sparse and highly specific response to stimuli (Laurent and Naroghi 1994; Stopfer et al. 2003; Joseph et al. 2012).

4.1.7 Olfactory coding in the honey bee

The olfactory system is highly conserved in evolution. In insects, mice, and humans, ORNs express olfactory receptors (OR), which convert the olfactory information to neural activity. The ORs converge on to the glomerulus of the AL. The number of glomeruli in a species is nearly equal to the number of ORs (Galizia et al. 1999; Karpe et al. 2016). Glomeruli are arranged based on an odotopic map, where each glomerulus receives input from ORNs which express the same OR. Before the information is passed to the higher olfactory centers by the PNs, the LNs perform gain control on the ORN signals by their GABAergic inhibition. Homo LNs facilitate gain control by setting the background activity of the glomeruli close to the threshold and optimizing sensitivity. Hetero LNs are thought to aid in enhancing contrast across glomeruli. The few excitatory LNs help distribute info across glomeruli boundaries and assist in glomerulus amplification of information (Galizia 2008).

Neural information is coded using the dimensions of time and space. Particular odors activate particular ORs, which in turn activate a specific subset of glomeruli resulting in a spatial code. Each odor activates around 10-15% of the PNs (Laurent et al. 2001). It is also found that encoding of information also has a temporal aspect. Along with which neurons are

active, the precise timing of their activity also encodes information. The temporal aspects involve two interlocked phenomena: "the transient and periodic synchronization of active PNs and the evolution of the odor-coding assemblies along an odor-specific trajectory during an odor response." In this hypothesis, the oscillation can be seen as a "clock at whose rate the spatial representation is updated during a single odor response" (Laurent et al. 1996; Laurent et al. 1996b; Wehr and Laurent 1996). In this chapter we studied odor-cell specific temporal responses of *A. dorsata* by recording from the AL interneurons.

4.1.8 Olfactory conditioning of proboscis extension response in *A. dorsata*

A. mellifera has been used extensively in studies involving olfactory conditioning of the proboscis extension response (PER). It has been established as a good model system for studies on learning and memory. It is found that Eusocial animals are better at PER conditioning compared to solitary species (McCabe et al. 2007). Studies testing amenability to olfactory conditioning have been carried out on other species of Hymenoptera like *tragonisca angustula* (Mc Cabe and Farina 2010), *Megachile rotundata, and Megachile pugnata Osmia lignaria* (Vorel and Pitts-Singer 2010), *Melipona tellaris* (Abramson et al. 1999), showed them to be poor performers. Species like *Trigona hockingsi* (Frasnelli et al. 2011) fared moderately. Good PER conditioning performance was noticed in the species *Trigona carbonaria* (Frasnelli et al. 2011).

Species belonging to the same genus showed differences in olfactory PER conditioning. In the genus *Bombus, B. terrestris* and *B. occidentalis* showed good conditioning, whereas eight other *Bombus* species showed poor performance (Laloi et al. 1999). *Osmia carnuta* (Anfora et al. 2010) showed good response to PER conditioning, whereas *O. linaria* did not show PER conditioning. Differences have been observed at the level of subspecies also (Abramson et al. 1997).

Studies on other species in the genus *Apis* showed them to be good performers. Kaspi and Shafir (2011) showed *A. florea* to be amenable to olfactory conditioning and performs on par with *A. mellifera,* which is by far shown to be the best performer. The species *A. cerana* was studied and also found to be a good performer in olfactory conditioning.

We subjected *A. dorsata* to an olfactory conditioning protocol similar to that used in A. *mellifera* (Bitterman et al. 1982) to compare both *A. dorsata* and *A. mellifera,* given the differences in their nesting and foraging activities.

4.2 Results

4.2.1 GABAergic innervations of the AL with respect to the ORN terminals

Dextran fills from the antennal nerve showed ORN terminals in the periphery of the glomeruli. Immunohistochemistry with the anti-GABA antibody revealed that the periphery of the glomeruli innervated by the ORNs was GABA-negative, whereas the inner core was GABA-positive **(Fig 4.1 c)**. Dextran injection in the AL made the glomeruli of the AL visible **(Fig.4.1.a)**. The cell bodies were mostly located on the lateral side of the AL. Immunohistochemistry with the anti-GABA antibody also showed that GABA-positive cell clusters of putative LNs were on the lateral side of the AL **(Fig.4.1e)**.

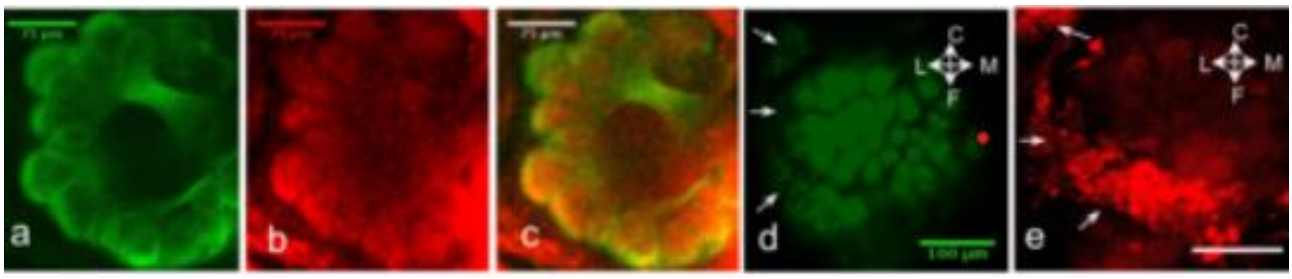

Fig 4.1 Visualization of Dextran fill of AL shows GABA positivity in the centre and ORN innervation in the periphery. **a.** Dextran fill from the antenna shows ORN innervations in the periphery. **b.** The inside of the glomeruli shows regions of GABA positivity. **c.** Merge of a and b showing GABA positivity in the core region. **d.** Cell bodies of the neurons of the AL

are majorly on the lateral side. **e.** GABA-positive cell clusters of the AL are located on the lateral side. Scale bar = 100 μm.

4.2.2 Antennal lobe tracts of *A. dorsata*

The anterograde mass fills from AL revealed that the efferent tracts from the AL of *A. dorsata* were similar to *A. mellifera* (Mobbs 1982; Abel et al. 2001; Muller et al. 2002; Kirschner et al. 2006; Rossler and Brill 2013; Zwaka et al. 2016). In *A. dorsata*, axons of the PNs project to the protocerebrum via five antennal lobe tracts, the medial (m-ALT), lateral (l-ALT), and mediolateral tracts (ml-ALTs 1, 2, and 3) **(Fig. 4.2)**. The m-ALT travels dorsally to the MB calyces, where it sends out collaterals to the lips of both the calyces and then it travels ventrolaterally to the LH. The l-ALT leaves the AL and innervates the LH first, and then projects to the MB calyces. The three ml-ALTs branch off from the m-ALT at different depths. ml-ALT 1 branches first from the m-ALT and innervates the LH. It does not have branches. ml-ALT 2 bifurcates into two: one branch goes to the LH, and the other branch projects to the ring neuropil of the vertical lobe. ml-ALT 3 is found to be made up of a network of four tracts; each subtracts branches off from m-ALT at different positions and projects towards the lateral protocerebral lobe (LPL). ml-ALT 3a and ml-ALT 3b project towards the base of the calyx, ml-ALT 3c innervates the LH, and ml-ALT 3d bends before branching into two branches, which join the l-ALT **(Fig 4.2)**.

A thin tract was also found to innervate the contralateral AL, similar to what has been reported in *A. mellifera* (Arnold et. al. 1985).

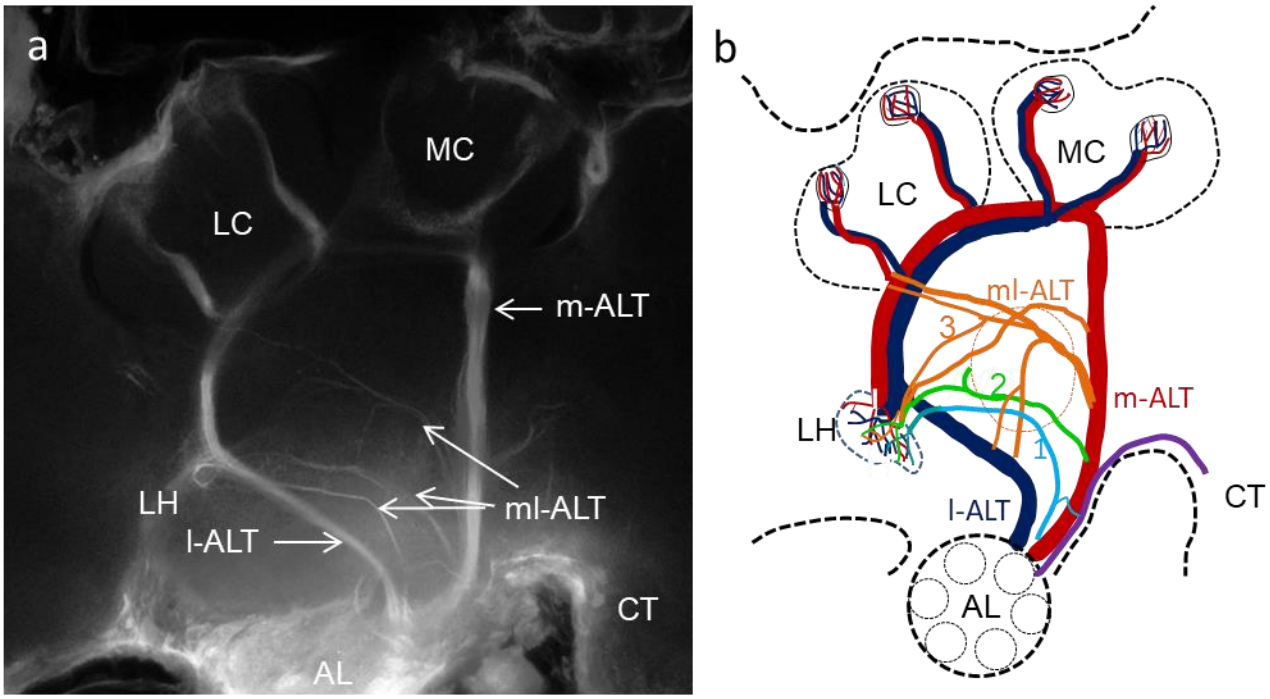

Fig 4.2 PNs innervate the higher brain areas through their axons which form the ALTs. a. Dextran fill from the AL illustrated the 5 AlTs innervating the MB and the LH. b. Schematic showing the ALTs. m-ALT innervates the MB calyces first and the LH later. l-ALT innervates the LH first and later innervates the MB calyces. e. ml-ALT1 innervates the LH without branching. ml-ALT 2 has two branches: one branch runs to the LH and the other innervates the vertical lobe. ml-ALT 3 consists of 4 subtracts. ml-ALT 3-a and ml-ALT 3-b run to the base of the calyx, ml-ALT 3-c projects to the LH, and ml-ALT 3-d bends and terminates on the l-ALT. Few axons can be seen going to the contralateral antennal lobe (CT). LC lateral calyx; MC median calyx; CT contralateral AL

4.2.3 Neurons of the antennal lobe respond in an odor cell-specific way

Temporally patterned responses are features of the responses of the AL neurons that are thought to encode odor identity and concentration using a temporal code. This phenomenon has been reported in multiple model species like *Schistocerca americana* (Laurent et al. 1996; Stopfer et al. 2003), *Manduca sexta* (Ito et al. 2008), and *A. mellifera* (Nawrot 2012). Laurent

et al. (1996) showed that in locusts, odors are represented by Spatio-temporal patterns of responding neurons and that each neuron responded differently to different odors and different neurons responded differently to the same odor. The response of a neuron to the same odor remained constant with repeated trials. We recorded intracellularly from the AL interneurons to see if the neurons in the antennal lobe of *A. dorsata* exhibited such response properties. Each cell was different from the others in terms of duration of response and temporal structure. Cells had a baseline firing rate of 1 to 2 spikes per second and exhibited varied responses when presented with different odors. Some cells were inhibited by an odor. For example, in Fig 4. 3 a, the cell was inhibited by nonanol, whereas hexanol produced excitation. Different cells exhibited different temporally patterned responses to a single odor.

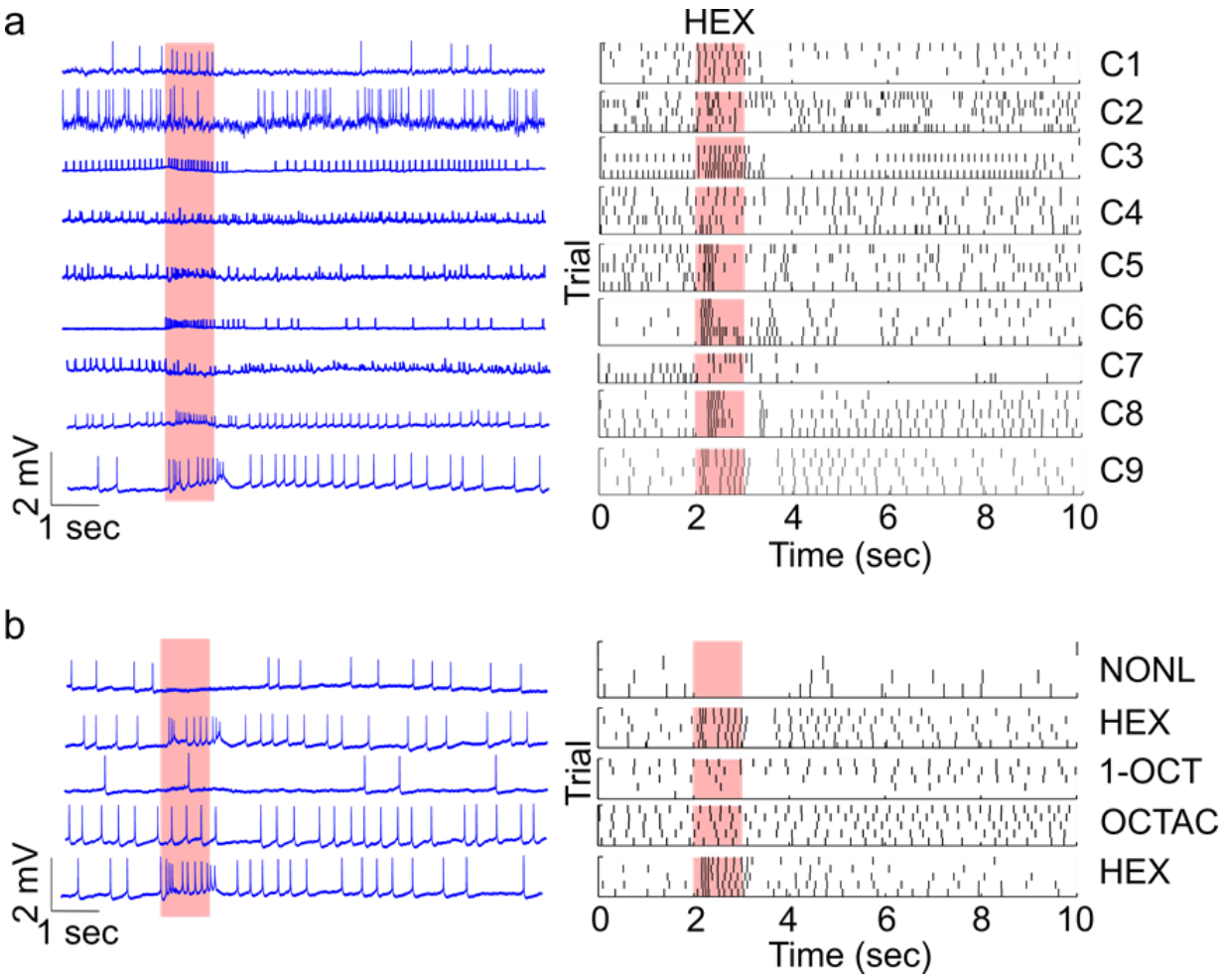

Fig 4.3 Different cells of the AL exhibit different temporally patterned responses to the same odor, and the same cell has different types of temporally patterned responses for different odors. **a.** Responses of different neurons (C1–C9) of the AL to hexanol (100%). b Responses of a single cell to four different odors. The odor hexanol was repeated to show the invariability of odor responses. NONL: nonanol; HEX: 1-hexanol; 1-OCT: octanol; OCTAC: octanoic acid

Figure 4.3 b shows the responses of different cells to the odor hexanol. It is observed that these cells often had a spontaneous spiking activity even when an odor stimulus was not applied. Response to odor hexanol in repeated trials is shown in the fig 4.3 b to illustrate constancy of response patterns in repeated trials.

4.2.4 Mushroom body reconstruction

A reconstruction of the mushroom body was carried out using dextran fills. The MB consisted of double calyx; namely lateral calyx (LC) and medial calyx (MC), pedunculus, and the lobes. The local field potential (LFP) of the MB was recorded for different odors using a blunt electrode.

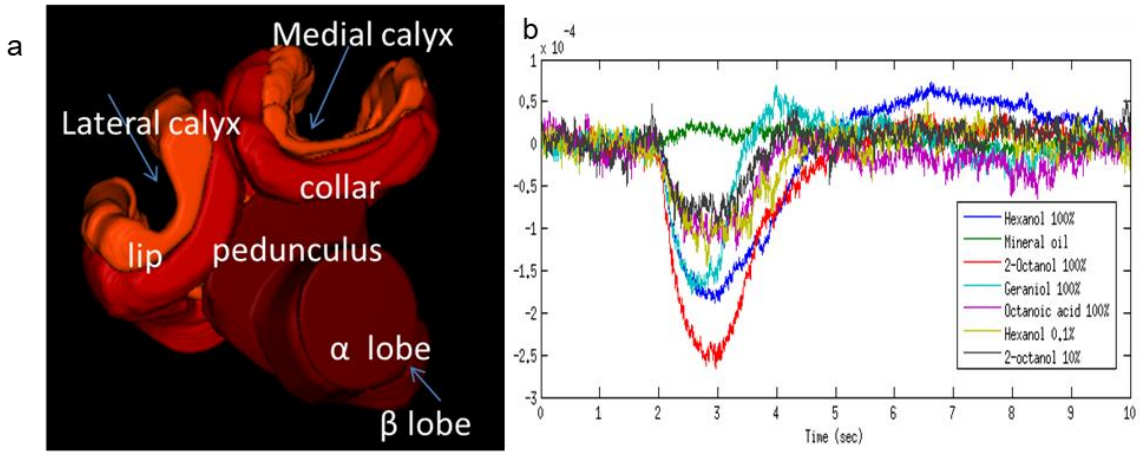

Fig 4.5 a. Mushroom body reconstruction in *A. dorsata* showing lateral calyx, medial calyx, pedunculus, α-lobe and β-lobe. b. The waveform showing the response in the LFP from the MB calyx to different odors. Response to each odor is represented by a different color.

4.2.5 *A. dorsata* shows robust olfactory PER conditioning

We tested the amenability of *A. dorsata* to olfactory conditioning. These bees had very low PER (less than 4%) to untrained odor. After training using olfactory conditioning protocol (Bitterman et al. 1983; Menzel 1993; Matsumoto et al. 2012), we found that odor-evoked PER scores increased up to 92% with trial number. They were amenable to both massed and spaced training and showed a significant increase in PER scores for 30-s, 3-min, and 10-min inter-trial intervals **(Fig. 4.6 a)** (Cochran's Q values 46.9, 41.09, 49.2; df=6; p<0.001).

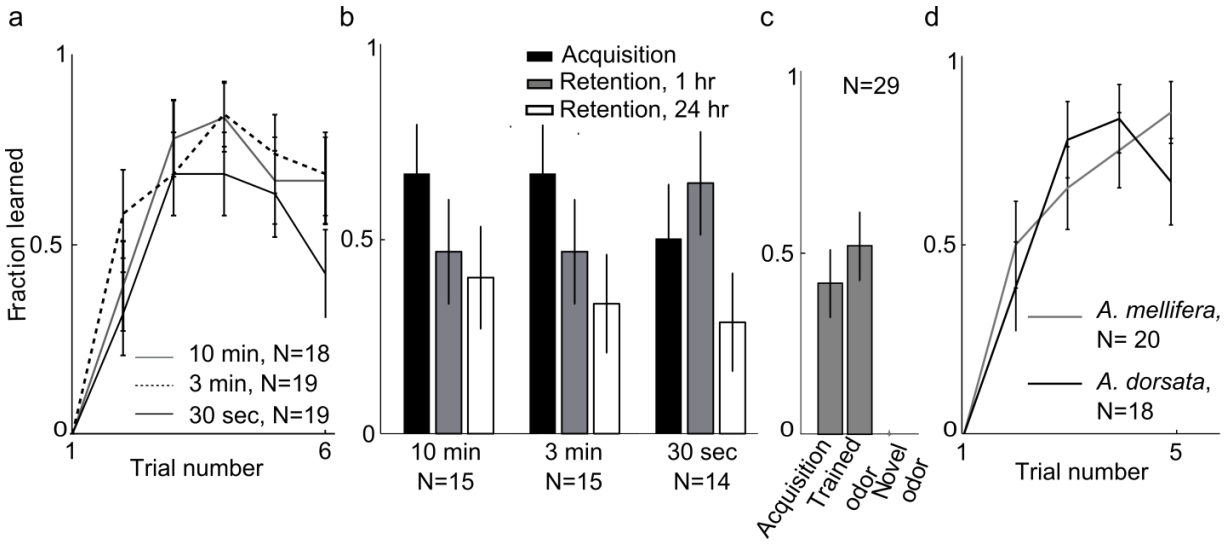

Fig 4.6 *A. dorsata* learns and retains memory in the PER olfactory conditioning paradigm. **a.** The acquisition for the three different ITI s viz 10 min, 3 min, 30 sec. Y- axis represents fraction learned and X–axis represents trial number. **b.** Memory tested 1 hour after training and 24 h after training and acquisition after the 6th trial are represented in the bar graph. 10 min ITI produced better long-term memory and 30-sec ITI produced better memory 1 hour after training. **c.** bees trained with hexanol/geraniol (CS) and tested with novel odor geraniol/hexanol showed no response to the novel odor and 50% response to CS. **d.** Learning curves for *A. dorsata* and *A. mellifera* for 5 training trials show similar acquisition.

They exhibited both short-term and long-term memory. They had retention of 57, 47, and 52%, respectively, one hour after conditioning and retention of 28, 33, and 42% 24 hrs after conditioning for the 3 ITIs viz 10 min, 3 min and 30 sec **(Fig. 4.6 b)**. They were able to distinguish a trained odor from a novel untrained odor. Bees trained with either hexanol or geraniol as the CS with six conditioning trials showed no response to the novel odor (the untrained between the two) and 50% response to CS 1 h after conditioning **(Fig. 4.6 c)**. Figure 4.6 d compares the learning rate of *A. mellifera* with that of *A. dorsata.* Learning rates are broadly similar for the two species. We cannot draw a firmer conclusion about the relative performance of these two species given that the body sizes are different, and thus the reward of an identical quantity of 30% sucrose may not be equivalent in the two.

4.3 Discussion

In *A.dorsata,* the ORNs innervated only the cortex region except in the glomeruli innervated by T3 and T4b tracts. This pattern is similar to what has been observed in *A. mellifera* (Arnold et al. 1985; Galizia et al. 1999). In *Neodiprion ventralis* and *Neodiprion autumnalis,* the ORNs innervate the entire glomerulus (Dacks and Nighorn 2011). In moths, ORNs innervate only a distal portion of the glomeruli (Oland et al. 1990). In humans, the ORNs innervate only the cortex (Sinakevitch et al. 2018).

We also found the cortex of the glomerulus with ORN innervation to be non-GABAergic. However, the inner core, made of the LN and PN innervation, was found to be GABAergic. We further need to establish which subsets of PNs and LNs have dense GABAergic innervation. In the case of *A. mellifera,* ~750 out of 4000 of the LNs were found to be GABAergic (Schafer and Bicker 1986), and around 17 multiglomerular PNs, which leave the AL through ml-ALT-1 and m-ALT-2, were also found to be GABAergic (Sinakevitch et al. 2013). GABA was found to be the major transmitter in LNs of many Hymenopterans like

moths (Hoskins et al. 1986) and honeybees (Schäfer and Bicker 1986), unlike drosophila, where one-third of the LNs are glutamatergic (Liu and Wilson 2013). In human OB the granule cells, which are equivalent to LNs, are mostly found to be GABAergic (Ohm et al. 1990).

A cluster of LN/PN somata on the lateral AL side with a thick bundle of neurites entering the AL that we report in workers was also reported in the drones of *A. dorsata, A. mellifera, A. cerana*, and *A. florea* (Bastin et al. 2018)

4.3.1 AL tracts

AL filling showed that the PN axons leave the AL via the 5 ALTs similar to other Hymenopterans (Mobbs 1982; Galizia et al. 1999; Abel et al. 2001; Kirschner et al. 2006). In the primitive Apterygota, there is only one ALT (Strausfeld et al. 2009), whereas Orthoptera (Anton et al. 2002) and Coleoptera (Farris 2008) have two, and Diptera (Marin et al. 2001) and Lepidoptera (Homberg et al. 1988) have three ALTs.

In *A. mellifera*, the m-ALT and l-ALT contain axons of uniglomerular PNs (Bicker et al. 1993; Abel et al. 2001; Brandt et al. 2005) primarily whereas ml-ALT contains mostly axons of multiglomerular PNs (Fonta et al. 1993). Further, l-ALT PNs belong to the T1 cluster, and m-ALT PNs majority belong to the T3 cluster. In drosophila, the uPNs project to the higher olfactory regions MBs and LH via m-ALT, whereas multiglomerular PNs project via the ml-ALT and l-ALT (Jefferis et al. 2001; Tanaka et al. 2012; Wang et al. 2014). These tracts form parallel pathways in processing olfactory information.

Parallel pathways are an essential feature in many sensory systems in vertebrates and invertebrates. Parallel processing is present in the auditory, visual, and olfactory systems. In the vertebrate visual system, the magno and parvo cellular pathways form parallel pathways

to the primary visual cortex (Callaway 2005). These pathways carry information about color and spatiotemporal aspects, respectively. In insects, mushroom bodies receive input via a segregated pathway from the optic lobe (Ribi and Scheel 1981; Strausfeld et al. 2006). In both vertebrate and invertebrate auditory systems, parallel pathways are known to code various parameters of sensory input to support processing of speed and accuracy (Knudsen et al. 1993; Nassi and Callaway 2009; Helversen and Helversen 1995).

Parallel pathways are found in both vertebrate and insect olfactory systems. The uPN pathway innervating MB and LH seems to have evolved uniquely in Hymenoptera (Galiziza and Rossler 2010). Rossler and Zube (2011) study has shown the presence of a dual olfactory pathway to the MB in ants, social bees, solitary wasps, and a species of plant-eating sawfly. They concluded that dual pathway could have evolved in basal plant-eating sawflies by observing that the l-ALT is small in some species and absent in some species of sawflies (Symphyta). A division of AL into two nearly equal hemilobes similar to honey bee is found in *Componotus floridanus* (Zube et al. 2008; Zube and Rössler 2008).

People have questioned whether the olfactory system in honey bees serves two integrated streams or processes parallelly (Galizia and Rössler 2010; Nawrot 2012). Müller et al. (2002) observed that l-ALT PNs have broader odorant tuning profiles when compared to m-ALT PNs. They also found that l-ALT PNs conveyed odor information faster compared to m-APT PNs. They concluded that similar odors may be coded by the PNs of the two pathways using different strategies to process different properties of the same stimulus. However, it was found that the input received by both the ALT PNs was redundant through calcium imaging studies (Carcaud et al. 2012; Galizia et al. 2012).

Brill et al. (2013) recorded from many PNs belonging to both the tracts simultaneously and found that l-ALT PNs have ~14 ms shorter latencies and have broader odorant–response

profiles compared to m-ALT PNs, which had longer latencies and were more odor specific. It was concluded that l-ALT PNs are more broadly tuned and deliver temporal information faster, while m-ALT PNs carry more specific odor identity information. These studies provide evidence for the parallel processing of information in *Apis*. Though these studies are not yet done in *A. dorsata,* as the basic anatomy of the AL tracts is similar to *A. mellifera,* one may conclude that the parallel processing could also be happening in *A. dorsata*.

4.3.2 Mushroom body architecture

The first study on insect MB was conducted by Dujardin in 1850, and he showed that enlarged MBs were characteristic of sociality. The MBs are enlarged and complex in insects belonging to Lepidoptera, Coleopteran, and Dictyoptera, along with Hymenoptera (Strausfeld et al. 2009). A study on the entire Hymenoptera has shown that large MBs evolved in the aculeatae of Hymenoptera prior to the arrival of sociality (Withers et al. 2008; Farris and Schulmeister 2011). The size and complexity of hymenopteran MB are well illustrated by *A. mellifera. A. mellifera* MBs contain 340000 KCs (Whitthoft 1967), which account for one-third of the total neurons in the brain (Menzel 2012). Meanwhile, the MB of solitary drosophila is single and has only 2500 KCs that make up 2% of the total neurons. In *Apis*, the MBs are structurally subdivided to process information from different modalities (Gronenberg 2001) which is not found in Drosophila (Tanaka et al. 2004). The MBs of Apis are multi-modal, whereas those of aquatic whirligig beetles (Coleoptera) and the dragonflies (Odonata) are entirely dedicated to vision (Lin and Strausfeld, 2012). The MBs of *A. dorsata* also resemble A. *mellifera* in architecture on par with other Hymenopterans.

Dye injections in the calyx of *A. dorsata* showed that axons of the KCs form parallel tracts and stay compartmentalized in the pedunculus. Mobbs et al. (1982) had demonstrated in

A.mellifera by taking horizontal sections of the MB that the KC projections form discrete rays. This has been confirmed by us in *A. dorsata.*

Sandoz and Menzel (2001) showed that in *A. mellifera*, during olfactory PER conditioning; memory for a trained odor can be transferred and could be tested on the other side of the brain three hours after training. We tested this behaviorally and anatomically in *A. dorsata* (Vijay kumar et al. 2019). Our behavioral experiments did not show any transfer of memory to the contralateral side. Dextran injections in the areas that probably can have anatomical connections like AL and MB showed a small group of axons connecting the two ALs (also shown in *A. mellifera* by Mobbs (1982)), but no connections from calyces to contralateral medial lobes or AL could be found. We conclude that there is no transfer of olfactory information to the contralateral side and that each side learns and retrieves information independently.

4.3.4 Olfactory PER conditioning

The proboscis extension reaction evoked by antennal or tarsal contact in flies, butterflies, and honey bees with sucrose solution was demonstrated by Minnich (1921, 1932) and was used as a means of conditioning honey bees by Frings (1944). Nearly 60% of harnessed *A. dorsata* bees collected from the hive showed PER response during the motivation test, whereas 90% *A.mellifera* bees and *A. dorsata* foragers collected from flowers showed PER response. *A. florea* showed 55% PER response in a study conducted by Kaspi and Shabir (2012). *A. dorsata* had around 76% correct responses by the end of the 7^{th} trial, whereas *A. mellifera* showed around 80% correct responses. *Bombus terrestris* showed 56% correct responses by the 7^{th} trial and 60% after the 10^{th} trial (Sommerlandt et al. 2014).

We used three different ITIs to condition bees, 30s ITI is considered as massed training and known to produce lesser long-term memory. 10 min ITI is shown to produce better long-term

memory due to protein synthesis in *A. mellifera* (Menzel et al. 2001). Our results are in accordance with the results obtained in *A. mellifera. A. dorsata* bees performed better in discrimination tasks compared to *A. mellifera.* This could be due to the higher PER response rate of *A. mellifera* compared to *A. dorsata.*

4.4 Summary

We characterized the olfactory system of *A. dorsata* and compared it with the well-studied *A. mellifera.* Dextran fill of the AL showed GABA negative ORN innervation in the periphery and GABA positive innervations in the core region. The PN tracts innervated the higher brain areas via five antennal lobe tracts viz m-ALT, l-ALT and three ml-ALTs. m-ALT innervated the calyces first and later arborized the LH, whereas l-ALT projected first to the LH and later innervated the calyces. ml-ALTs mostly innervated the LH.

Electrophysiological study of the AL interneurons revealed their spatiotemporal response pattern. Various cells responded differently to different odors, inhibited by some and excited by some others. Different cells exhibited different temporally patterned responses to a single odor and had constancy of response patterns in repeated trials. *A.dorsata* showed robust olfactory PER learning. They showed good retention and novel odor discrimination. We found all the above aspects of the olfactory system of *A. dorsata* comparable to *A. mellifera.*

Chapter 5

Interaction of slow plasticity, mushroom body LFP oscillations and classical conditioning

5.1 Introduction

5.1.1 Honey bee odor discrimination

Karl von Frisch (1919) from his experiments found that honey bees could discriminate 28 out of 32 pairs of odorants tested. He also observed that when these odorants were diluted there was a limit of dilution at which the bees fail to discriminate. Takeda (1961) and Vareschi (1961) showed that bees trained to an odor can partially respond to other odors using proboscis extension reflex (PER). Vareschi found that when he grouped odorants in to groups, bees were able to discriminate odorants belonging to different groups but not ones in the same group. This study established that PER of bees can be used to measure odor similarity of odorants.

Smith and Menzel (1989) conducted a study using response in the electromyogram of the M17 muscle of honey bee as a readout of PER to estimate odor discrimination of odorants having different functional groups. They found the response rates to be similar to different odorants belonging to the same class like aldehydes, ketones etc. Generalization between odorants was proportional to the similarity in their molecular structure. Marfaing (1989) in their study using PER of bees found that bees trained at higher concentrations performed poorly when tested at lower concentration of the odors and vice versa. In another study it was found that the concentration of the trained odor affected the generalization.

5.1.2 Oscillatory synchronization in the mushroom body LFP of honey bee

When odor (not air alone) was puffed on to the antenna of a honey bee, the LFP of the MB exhibited 30 Hz oscillations that lasted for 0.5–1 s for a stimulus of 1 s duration (Stopfer et al. 1997). Intracellular recording of AL interneurons also responded to odors with membrane potential oscillations (Stopfer et al. 1997). The phase of the MB LFP oscillations in their recordings lagged behind membrane potential oscillations of AL neurons. In locust, the MB LFP oscillations are driven by the activity of the AL neurons **(Fig 5.1)**. Intracellular recording of the antennal lobe neurons showed membrane potential oscillations that maintained a fixed phase in relation to the MB LFP. PN spikes preceded the peaks of the MB LFP whereas IPSPs coincided with the descending phase of the LFP (Laurent and Davidowitz 1994). LNs were also found to have membrane potential oscillations and the peak of their depolarizing waves coincided consistently with a single phase of the MB field potential oscillations indicating that they may underlie the PN inhibitory post synaptic potentials (IPSPs) **(Fig 5.1)**. The phase of PN and LN oscillations was independent of the odorant indicating that odor quality may not be encoded in the phase of firing of the AL neurons (Laurent and Davidowitz 1994).

In locust LNs were found to inhibit the PN spikes and this action was mediated by GABA (Laurent 1996). Previous studies have shown that picrotoxin (PCT) blocks the GABA-gated chloride channels (Homberg et al. 1987; Waldrop and Hildebrand 1998). It was found in honey bee, that application of PCT to the entire brain or the AL abolished the 30 Hz oscillation 8 mins after application (Stopfer et al. 1997). The application of PCT was found to hamper discrimination of similar odors in honey bee **(Fig 5.2)**. The ability to associate

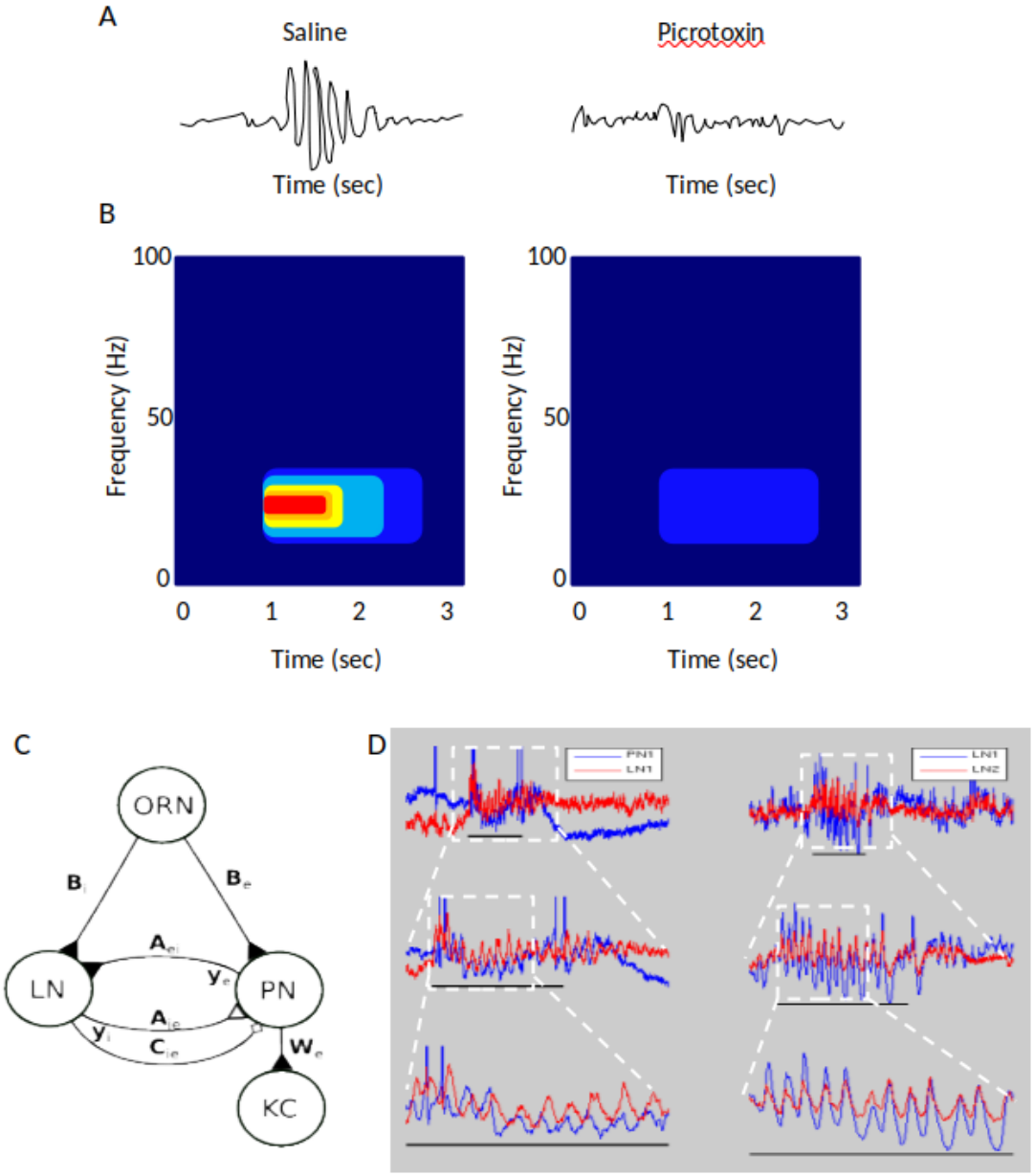

Fig 5.1: Oscillations in the mushroom body calyx are abolished by PCT application in *A. mellifera*. A) Oscillations in saline and after PCT application. B) Spectrogram around the odor presentation indicating power in the 20-30 Hz band during odor response vanishing after application of PCT. C) The ORN-LN-PN connections .ORNs are connected to LNs and PNs. LNs have an inhibitory connection with the PNs and other LNs and PNs form excitatory synapses onto LNs and KCs (Bhavana Penmetcha and Joby Joseph, unpublished). D) The expected phase relationships can be seen in the sub threshold membrane potential of PN and LN in *Hieroglyphus banian* antennal lobe preparation. PN membrane depolarization leads the LN depolarization and PN hyperpolarization lags LN depolarization.

an odorant to a sucrose reward and the ability to discriminate between dissimilar odors was not affected (Stopfer et al. 1997). From this it was concluded that oscillations were crucial for fine discrimination of odors. Abolishing oscillations affected the fine discrimination of odors in *A. mellifera.*

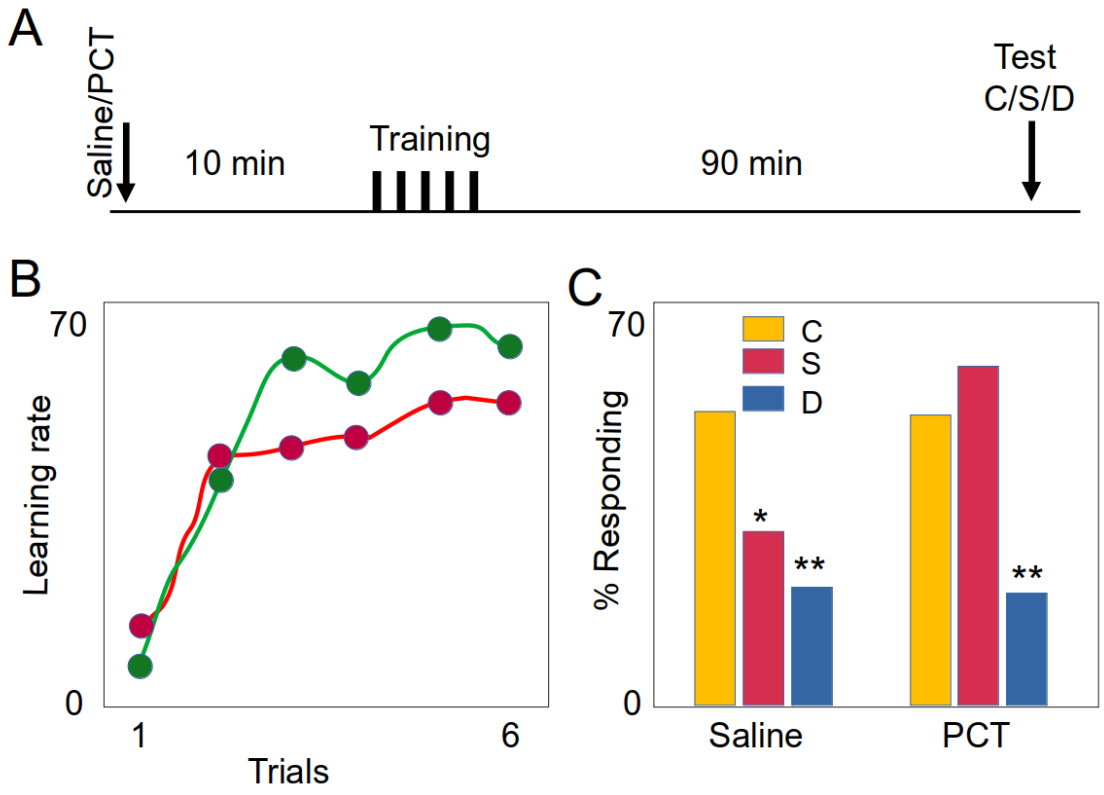

Fig 5.2 PCT perfusion prior to training affects fine discrimination of odors. A. Experiment protocol for testing fine discrimination with and without oscillations during training. B. The acquisition curves for bees treated with saline (green) and PCT (red) with 10 min ITI are similar. C. Responses to novel odor are significantly different from responses to trained odor in both saline and PCT treated bees. Responses to similar odor were not significantly different from responses to trained odor in bees treated with PCT unlike bees treated with saline. (Adapted from Stopfer et al. 1997)
C trained odor; S similar odor; D dissimilar novel odor

3.1.3 Effect of odor concentration on oscillatory synchronization

Stopfer et al. (2003) studied how odors are encoded across concentrations using locust olfactory system. They evaluated the effect of odorant concentration on oscillatory

synchronization of the AL and phase of firing of PNs. They recorded an oscillatory frequency of 20-30 Hz from the MB LFP which did not change with odor concentration but there was an increase in the LFP power with increase in concentration **(Fig 5.3)**. Extracellular recordings from the MB showed an increase in LFP power with increase in odor concentration **(Fig 5.3 B)**. This was attributed to increased synchrony of firing PNs and increased efficacy of LN modulation. Intracellular and extracellular recording from PNs revealed no change in mean phase of PN spikes during odor response due to increase in odorant concentrations of the same odor.

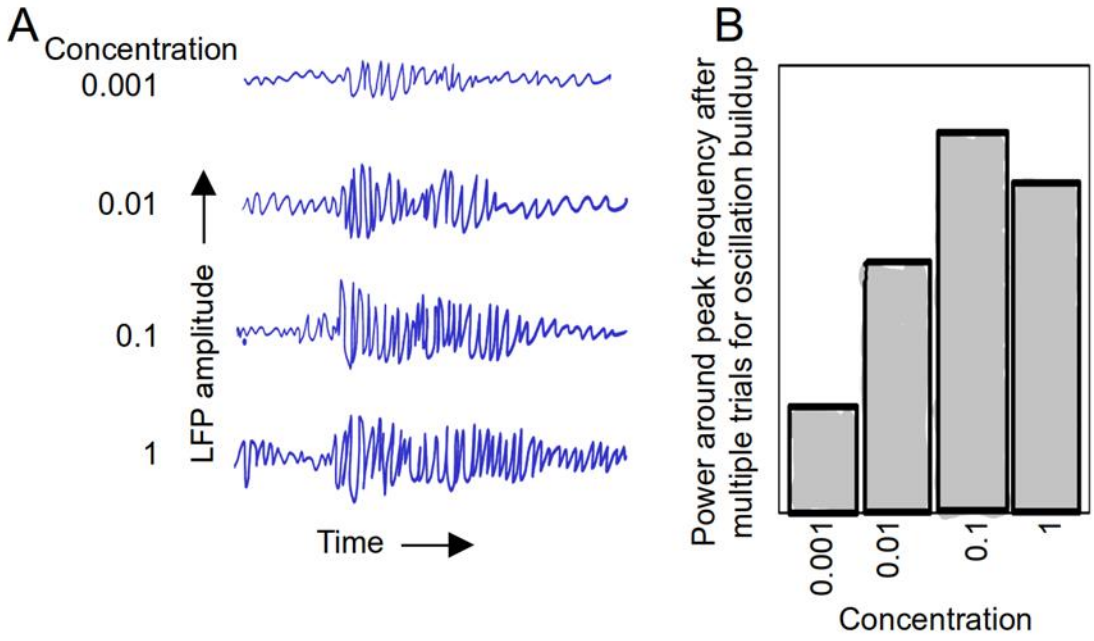

Fig 5.3 An increase in odor concentration produced an increase in oscillation strength (at the flowrates at which the experiments were conducted) and slightly decreased at the highest concentration. A) Raw traces of LFP waveform recorded from the locust MB for different concentrations of the same odorant (filtered, 5-55Hz). B) Power of MB LFP oscillation increases in strength with increasing concentration of the same odorant (Adapted from Stopfer et al. 2003)

The afferent input to the AL increased with concentration (Wachowiak et al. 2002). But, mean output of the AL (population firing rate) was found not to vary significantly with

increase in concentration, by Stopfer et al. (2003). This, they attributed to a possible adaptive gain control mechanism in the AL due to increased modulatory effect of LNs and increased synchronization of PN activity (Backer 2002; Stopfer et al. 2003). However it is not clear why synchronization per se should contribute to gain control. Moreover because of the result from the disruption of fine discrimination by PCT application, that also disrupted oscillation in bees (Stopfer et al. 1997) it is suggested that oscillations are essential for this independent of gain control. It is not clear if system is carrying out gaincontrol via LN inhibition, then it can do so without generating oscillations at all. It is also not clear why, if oscillations were a measure of performance in some memory mechanism, it would be concentration and odor dependent?

Stopfer et al (2003) attributed the increase in LFP power to an increase in oscillatory synchrony due to increased strength of inhibition with increase in odorant concentration. Euclidian distances of time matched vectors of PN responses suggested more overlap between responses for different concentrations of same odor than responses of PN sets activated by different odors. Based on this evidence, Stopfer et al. (2003) proposed that AL conveys the information about odor intensity through spatio-temporal code, which did not require oscillations or synchronization as a mechanism.

5.1.4 Effect of repeated trials on odor processing

Stopfer and Laurent (1999) tested the effect of repeated trials on LN-PN responses and MB LFP oscillations. Simultaneous recording from LNs, PNs and MB LFP showed that during the very first trial PN activity was strong with PN firing rates more than 20-30 Hz and without any periodic sub threshold activity. The MB LFP had very little power at 20 Hz. After repeating the trial, by second trial it was observed that PN spike rate went

down, 20 ± 5 Hz periodic activity was visible in LN, PN and MB LFP. The coherence between PN spike time and MB LFP waveform and LN membrane potential and MB LFP increased by 4-5 times. However, concomitant with the increase in oscillatory power, the PN spike rates decreased as well, as expected to happen with a mechanism that requires increased GABA inhibition **(Fig 5.4).**

Singh and Joseph (2019) reported that concomitant with the increase in oscillatory power around 20 Hz band with repeated presentation of odor, the low frequency component (less than 5 Hz) that corresponds to the deflection in the LFP decreases in power, demonstrating that there are other phenomenon correlated to the oscillation buildup.

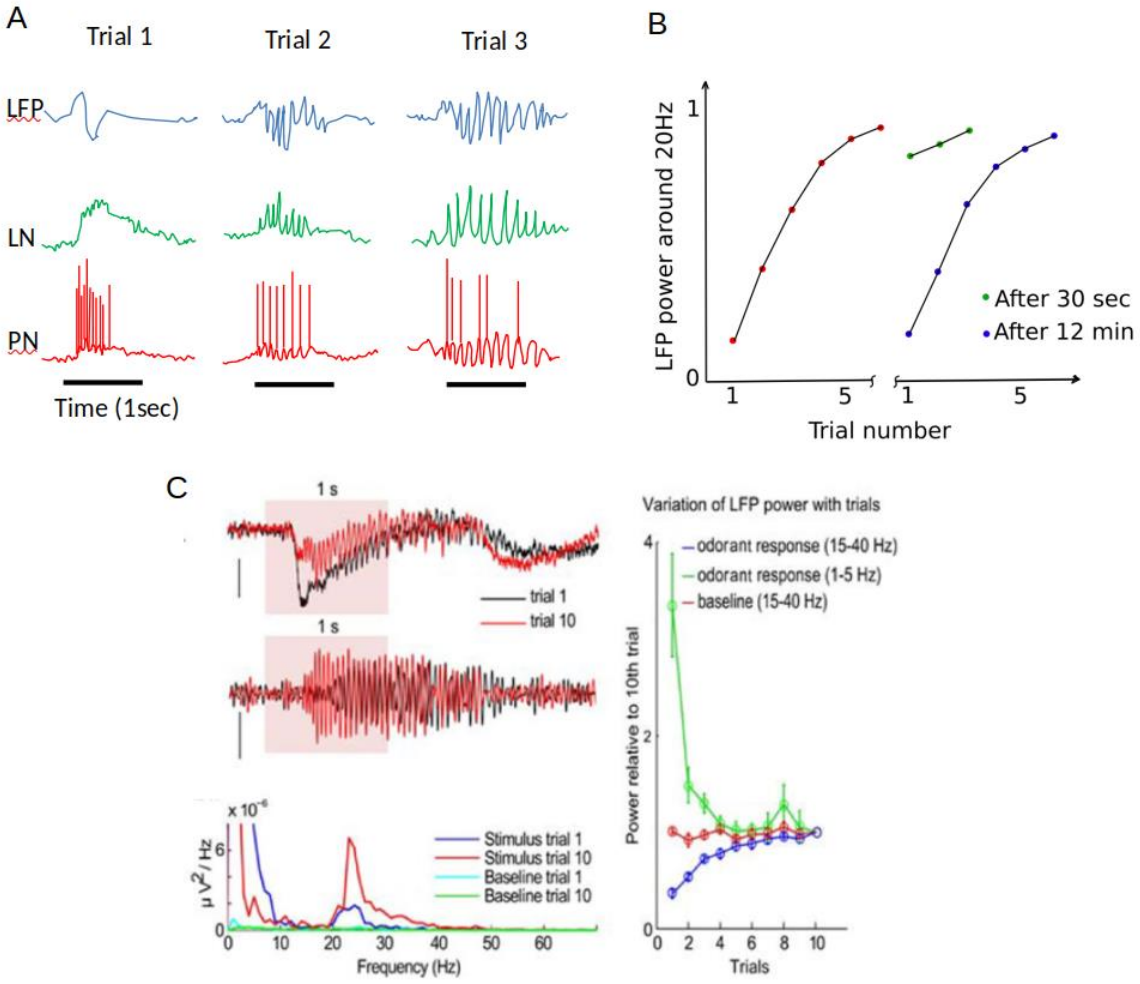

Fig 5.4 Oscillations in the MB LFP develop over repeated trials of odor presentation and decay if odor is not presented, consistent with some of the characteristics of habituation like phenomena. A) Consistent with the mechanism of generation of LFP in MB calyx, subthreshold activity in PN and LN shows coherent oscillations. MB LFP recordings show increased oscillations by 10^{th} trial compared to first trial. PN spike rate reduced with repeated odor presentations and coherence between LN, PN spike time and MB LFP waveform increased. B) When odor was delivered 10 times at an interval of 10 seconds, it leads to an increase in the power of oscillatons around the 20Hz. When odor presentation was stopped and later resumed after a time gap of Δt, the LFP power in the first trial had more power if the Δt was of shorter duration. (Adapted from Stopfer and Laurent 1999) C) Along with increase in oscillations, the deflection in the LFP decreased when the oscillatory power around 20Hz band increased with repeated presentation of odor. This low frequency component (less than 5Hz) corresponds to the deflection in the LFP (Adapted from Singh and Joseph 2019).

Stopfer et al. (1999) also reported that, if left alone, the system went back to naïve state in 12 mins if there is no further presentation of odorant. Interrupting the trials with a trial of a novel odor did not disturb the response to the familiar odor. To test whether this effect in the repeated trials is due to receptor adaptation, they initially applied odor to one half of the antenna using a plastic barrier in the middle of the antenna, through which the antenna was threaded. Later a test pulse of the same odor was applied to the naïve side and the oscillation strength that had developed, persisted. Thus it was shown that the effect is due to stimulus specific changes in the antennal lobe and not dependent on receptor adaptation. It was also reported in (Perez-Orive et al. 2004) that inhibiting $GABA_A$ receptor via picrotoxin and concomitant abolition of oscillations, also increased KC firing rates. The lower frequency component of MB LFP (in the 3-9 Hz band) increased. This is also consistent with the mean input from PNs to the KCs increasing when $GABA_A$ is blocked.

From all these observations about the inhibitory connections mediating negative feedback and their plasticity in the primary olfactory center the following question arises. If the system is having adaptive negative feedback ($GABA_A$ LNs and its plasticity), is it conceivable to implement it without giving rise to oscillations?

5.1.5 Role of GABAergic interneurons in olfactory habituation

Habituation is a form of implicit learning in which repeated exposure to stimulus that is not reinforced results in reduction in behavioral response (Thompson and Spencer 1966). Habituation allows an organism to focus on novel features of the environment by filtering constant stimuli. Features like spontaneous recovery, dishabituation and stimulus generalization differentiate habituation from receptor adaptation. In Drosophila repeated stimulation of the bristles leads to both habituation and sensory adaptation. Habituation takes time scale of minutes to recover whereas sensory adaptation recovers in seconds time scale. Das et al. (2011) showed that there is a habituation phenomenon in *Drosophila* olfactory system arising due to plasticity in the antennal lobe. Their study using Drosophila larvae established that odorant selective short term habituation is not mediated by odorant receptors and can occur downstream of ORNs. They showed that this habituation was evident both at the behavioral level (as decrease in response to the habituated odorant) and physiological level in the AL (as decrease in calcium influx). Moreover (Sudhakaran et al. 2012) also showed that this plasticity is mediated via LNs and $GABA_A$ receptors in the AL **(Fig 1.8)**.

5.1.6 Interaction of mechanisms underlying habituation or oscillatory synchrony due to repeated odor presentation and odor concentration, while undergoing olfactory conditioning

The above observations reported in grasshoppers, drosophila and honey bee systems suggest the following. Increasing oscillatory power/strength is favored by conditions of increased concentration and repeated exposure of odor stimuli. It is known that increased sensory drive necessitates gain control for systems with limited dynamic range. So it is not surprising that there is mechanism mediated by inhibitory feedback currents (like $GABA_A$) that reduces gain for systems which otherwise have limited dynamic range.

Under a given sensory drive (say concentration for a given odorant) repetition of sensory drive is a situation demanding habituation. This requires again a decrease in gain mediated by increase in negative feedback (say increasing $GABA_A$ synaptic strength), an adaptive gain control process.

In either of these cases, in discrete event systems like network of neurons, when negative feedback is applied it is possible that oscillations can occur, irrespective of its use in coding (Li and Hopfield 1989). From the above results it is also clear that state of the $GABA_A$ feedback and consequently oscillations and output of the primary olfactory center in the organism can be varied with odor concentration as well as interval between the odor stimuli.

Does decrease of deflection component (low frequency) of LFP decrease concomitant with increasing oscillation power in honey bee, like in grasshopper? Does a model that incorporates $GABA_A$ negative feedback and its plasticity for adaptive gain control, automatically gives rise to change in deflection component of the LFP and concurrent oscillations? How do these factors interact when the honey bee is undergoing PER conditioning, affecting its performance in olfactory discrimination task? What is the nature of plasticity in the olfactory pathway under these stimulus conditions? These are addressed in this chapter.

5.2 Results

5.2.1 *Apis dorsata* can be conditioned with geraniol as the CS in the PER paradigm

To check whether bees can detect the odor geraniol, twenty bees were trained with geraniol with 10 min ITI and the bees showed good acquisition **(Fig 5.5 A).** When tested with trained

odor (geraniol) and novel odors hexanol/octanol, bees exhibited 50 % response to geraniol one hour after training and showed zero response to novel odors, hexanol/octanol **(Fig 5.5 B).**

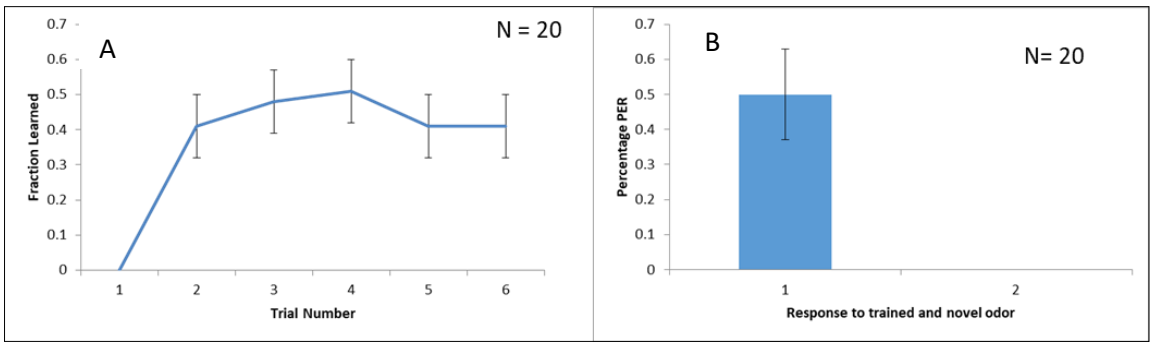

Fig 5.5 A) Learning curve for geraniol shows acquisition in *Apis dorsata* **B)** Response of bees to trained odor (geraniol) and untrained novel odor (hexanol/octanol), one hour after training.

PER: proboscis extension response

5.2.2 Discrimination of similar odor and novel odor under different odor concentrations and ITI

Learning and discrimination performance tested one hour after training for the bees trained in the 12 training conditions **(Fig 2.1)** namely, 2 min ITI and 10 min ITI in combination with 1%, 10% and 100% odor concentration of odors Hexanol or Octanol is presented in Fig 5.6. The hexanol and octanol trained bees were pooled for analysis as they did not show difference in performance. For all the six conditions, the response of the bees to the trained odor was not significantly different (Cochran's Q test, Q = 2.2589, N = 30, df = 5, p =0.8123). Response to similar odor, i.e., hexanol of the same concentration as octanol for octanol trained bees and octanol of the same concentration as hexanol for hexanol trained bees was significantly different (Cochran's Q, Q = 16.954 ,N = 30, df = 5, p= 0.0046) . The response to dissimilar novel odor, i.e., 100% geraniol, for all training conditions was significantly different (Cochran's Q, Q = 12.307, N = 30, df = 5, p = 0.0308). The response to similar odor of the bees trained with 100% concentration of an odor and 2 min ITI differed

from the response to similar odor of bees trained with 10% concentration of odor and 2 min (Cochran's Q, Q = 8, N = 30, df =1 , p = 0.0047) **(Fig 5.6),** where as the response to the trained odor in these two conditions was not different (Cochran's Q, Q = 0, N = 30, df = 1 , p = 1).

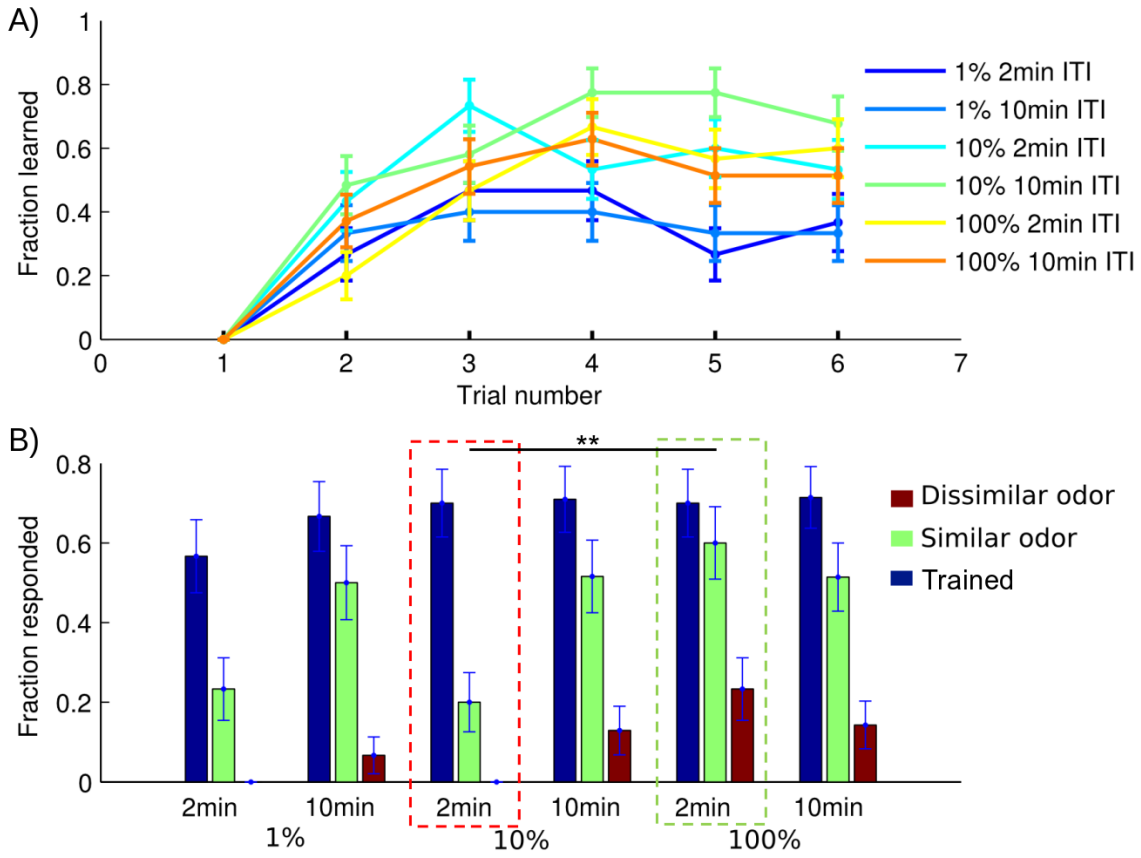

Fig 5.6 Responses to similar odor and dissimilar odor in different training conditions. **A)** Learning curves for the six training conditions. Trial number is represented on the X-axis and fraction learned on the Y- axis. **B)** Response rate to trained, similar and dissimilar odor. In all the conditions the response of bees to trained odor was not significantly different whereas the response to similar odor and dissimilar odor was significantly different. Response of bees to similar odor in bees trained with 100% concentration of odor and 2 min ITI differed significantly from response of bees trained with 10% odor concentration and 2 min ITI. (N= 30 for all groups)

5.2.3 MB LFP exhibited oscillation build up in response to repeated presentation of odor

In general it was not easy to see LFP oscillations in our preparations, though LFP deflections were present consistently. In those preparations that showed evident oscillations, oscillations built up due to repeated odor presentations (**Fig 5.7 A**). The LFP showed ~25 Hz oscillations during odor presentation from the second trial onwards. The raw data showed a deflection in the first trial and subsequent oscillatory buildup in the later trials. The same data when filtered at 15-40 Hz shows no oscillations in the first trial and oscillations developing in the later trials akin to the data we find in other published works **(Fig 5.7 B)**. The power in 15- 40 Hz band of the LFP, increased with trial number whereas power in 1-5 Hz band of the LFP decreased

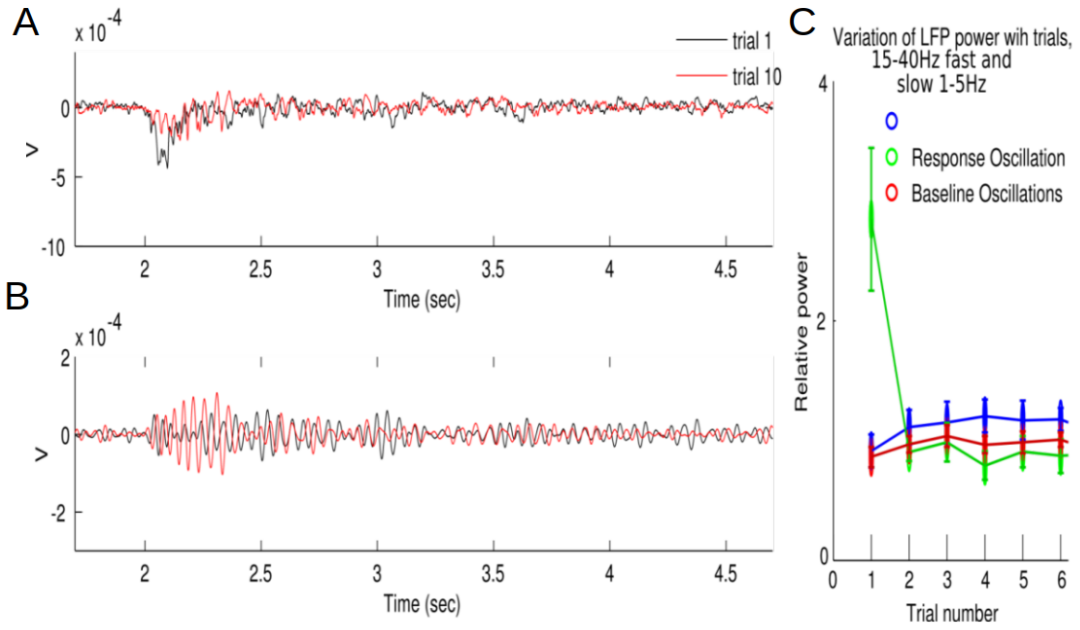

Fig 5.7 Oscillations build up in MB LFP with repeated odor presentation. **A)** Raw traces of MB LFP comparing first and 10th trial of odor presentation showing increase in oscillations while deflection is decreasing. **B)** The data in A is filtered at 15–40 Hz to emphasize the oscillatory response of MB LFP to odor. **C)** Oscillatory power (15- 40 Hz) and low frequency deflection (1–5 Hz) component of MB LFP across trials compared to the baseline. It shows same features as in *Heiroglyphus banian* MB LFP **(Fig.5.4 C)** indicating that behavioral

phenomena that is attributed to the presence of oscillations could be because of change in deflection or gain.

with trial number **(Fig 5.7 C)**. This is consistent with the observation in *Heiroglyphus banian* (Singh and Joseph 2019). More over this consistent observation in both the species is in agreement with a single mechanism that increases $GABA_A$ synaptic strengths in the AL with repeated trials giving rise to both oscillations build up and a concurrent decrease in deflection component. In our experiment in honeybees, the change in deflection was a more consistent phenomenon than build up of oscillations.

5.2.4 A model of the antennal lobe that exhibits buildup of oscillations with repeated trials shows concurrent decrease in deflection component of the MB LFP

Adaptive gain control to be implemented in the AL mediated by inhibitory synapse would require that $GABA_A$ synapses from LN to PN should increase in strength with repetition and more so with concentration. This is an identical requirement that is required to capture oscillation build up in earlier models of AL. Therefore can /does this plasticity mechanism give rise to both the phenomena: the decrease in deflection and increase in oscillations, if the antennal lobe is modeled? Though a complete understanding of the network of the AL is yet to be arrived at, we took into account the consensus on connectivity of AL based on experiments and earlier models (Bazenov et al. 2005; Ito et al. 2009).

The results from the simulations are shown in figure Fig 5.8. The model exhibits oscillations and it captures the phenomena of LFP buildup. As seen in the (**Fig 5.8 A**) comparison between the traces of first and second trials show increased oscillations in the second trial. When the same raw LFP trace is low pass filtered at 5Hz to compare the deflection we see that deflection has decreased when the oscillations increased. In **Fig 5.8 C** It can be seen that when the power in the oscillations is quantified, it increases with concentration. When

quantified, the change in deflection from the first to second trial increases with concentration for a given ITI. These results show that as one would expect, a $GABA_A$ mechanism that

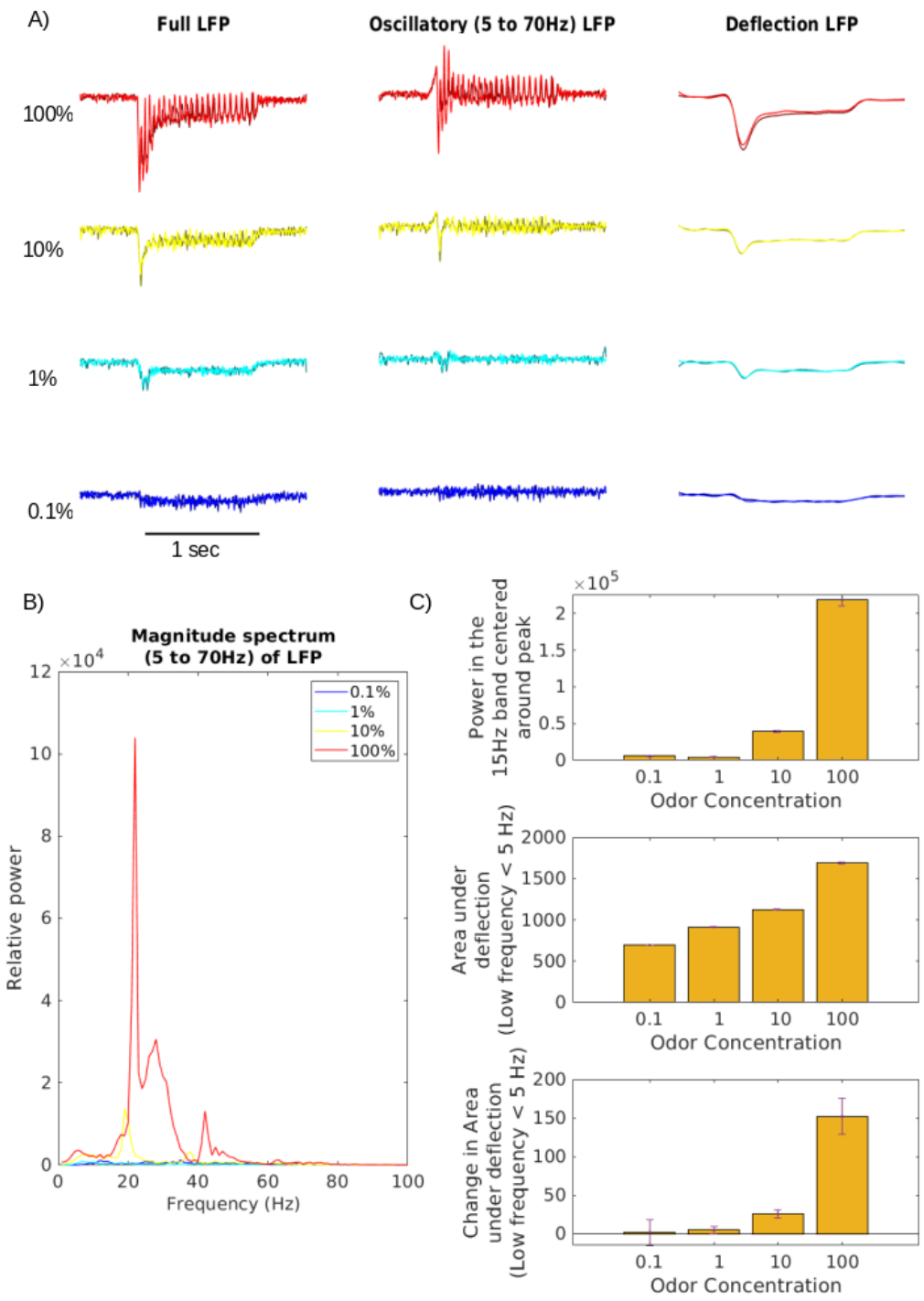

Fig 5.8 In a model of the olfactory pathway, with repeated trials, the oscillations build up in MB LFP and deflection component decrease, occurred concurrently. **A)** Traces of LFP filtered in different ways indicating that the model captures the features of LFP changes with repeated trials seen in experiments **B)** The spectrum of the LFP in the 5-70 Hz band with different concentrations shows increase in power and some shift in frequency with

concentration. **C)** Change in nature of LFP averaged over 10 trials for different concentrations of odor. Mean and standard error of mean, showing same trends as in experiments, thus indicating that behavioral phenomena that are attributed to the presence of oscillations may also be due to change in deflection or gain.

increases the oscillations with repeated presentation will decrease the deflection component even if we did not explicitly introduce another mechanism. Thus the decrease in deflection component with repeated trials seen in the physiology experiments measuring LFP can stem from this same mechanism i.e. the increase in $GABA_A$ synaptic strengths that increases oscillation strength. This gives us confidence to use change in deflection as a measure, or proxy of phenomena underlying the buildup of oscillations.

5.2.5 MB LFP deflection component in two consecutive trials measured in *Apis dorsata*

Deflection in the MB LFP is observed due to odor presentation as described before. The recording of the MB LFP deflection for each trial for all the 9 conditions (30 sec, 2 min and 10 min ITI and 100%, 10% and 1% odor concentration) across animals are shown in **(Fig 5.9).** The first trials are represented in blue, Subsequent trials in red. In the short ITI conditions, it is very evident that the deflection for the first trial is more than the second trial. To see the change in deflection from first trial to second trial clearly, their differences are plotted in **Fig 5.10**. There is a clear trend, the second trial had more decrease at shorter ITI and the changes were prominent for higher concentrations. For reasons still not clear, the larger ITI had an increase for second trial though this is consistent with the trend. The mean change in area of deflection component for the 9 combinations is quantified **(Fig 5.11 A)** and the mean change in deflection was significantly different in all the 9 conditions (Anova, N=5, df=36, p=0.0055). They show a clear trend, the second trial had more decrease at shorter ITI and the changes were prominent for higher concentrations. The area under the curve for mean

change in deflection for all the conditions is plotted in Fig 5.11 B. The area under the curve increased with concentration and was more for smaller ITIs. The mean deflection itself increased with concentration **(Fig 5.11 C).**

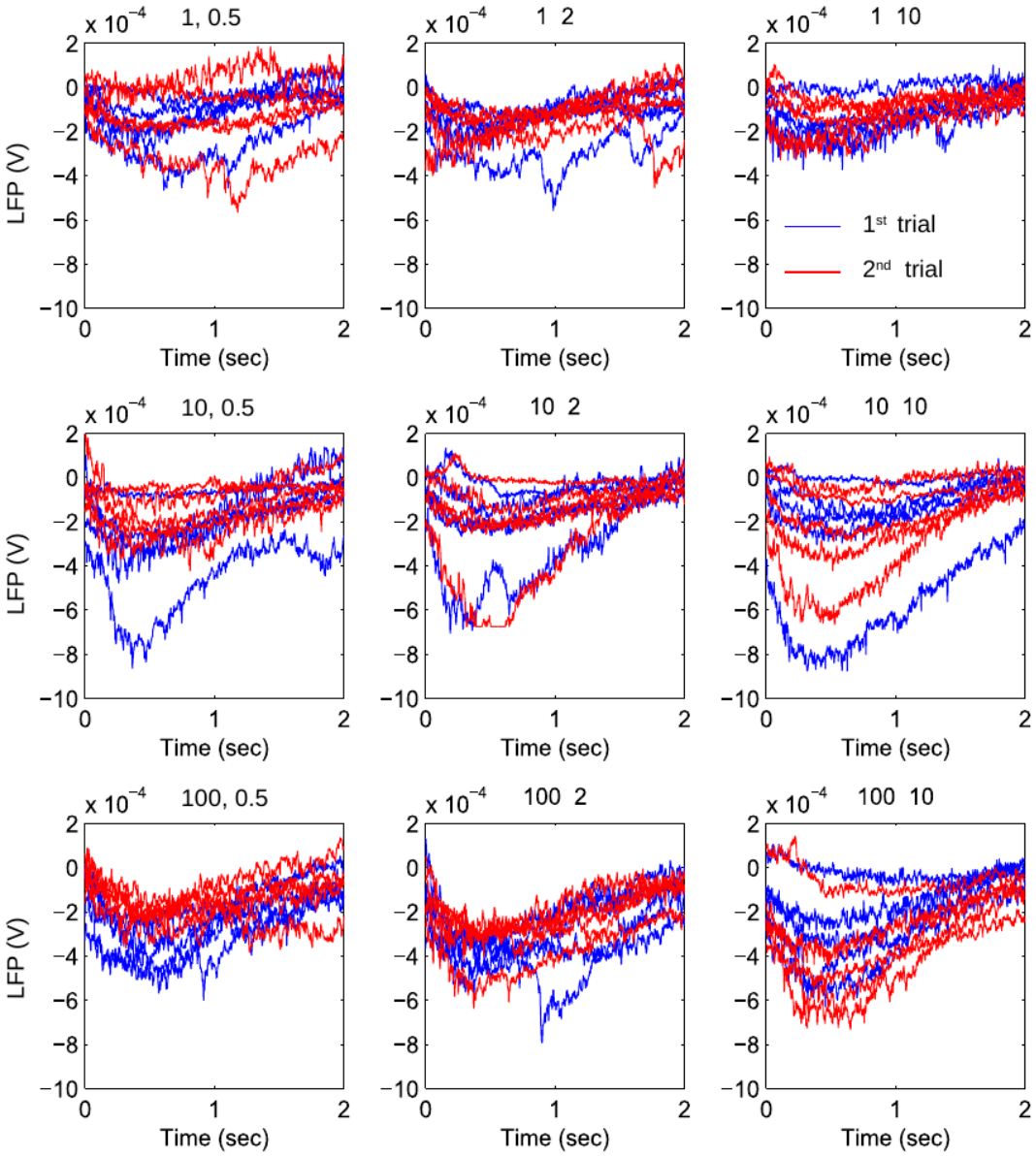

Fig 5.9 Deflection of the MB LFP due to odor presentation, (first trial in blue) and (second trial in red). The first row contains the responses for 1% odor concentration, second row for 10%, and third row for 100%. The first column represents 30 min ITI, second 2 min ITI and third 10 min ITI. N = 5 (data pooled from different bees). The decrease in deflection in the second trail is very evident in shorter inter trial intervals.

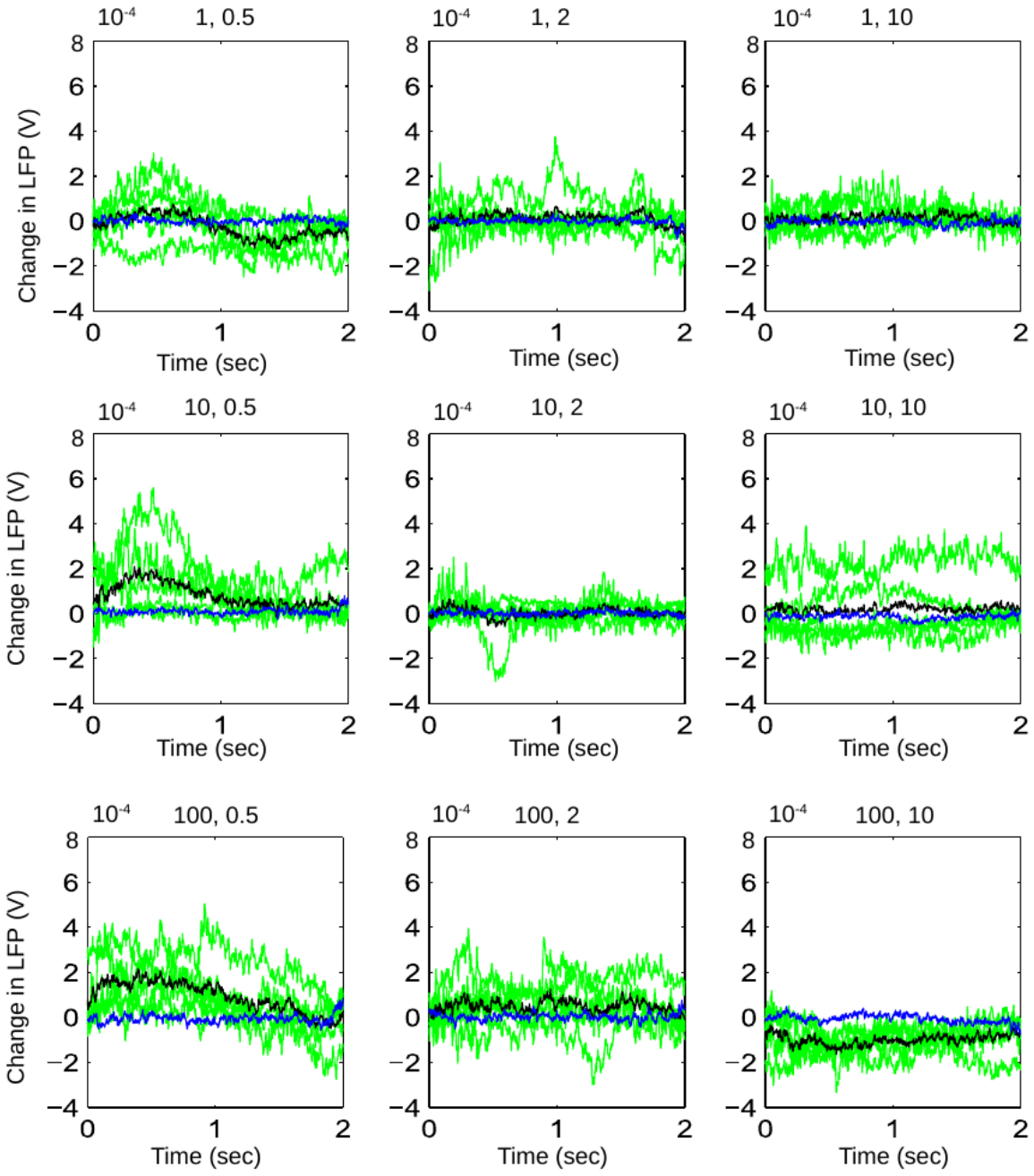

Fig 5.10 Change in deflection of MB LFP odor response between first trial and second trial (green) in various training conditions. Mean change in deflection (black) and mean base line before odor stimulus (blue). The condition of odor concentration and ITI are indicated above each subplot. The first row contains the responses for 1% odor concentration, second row for 10%, and third row for 100%. The first column represents 30 min ITI, second 2 min ITI and third 10 min ITI. (N=5, data pooled from different bees)

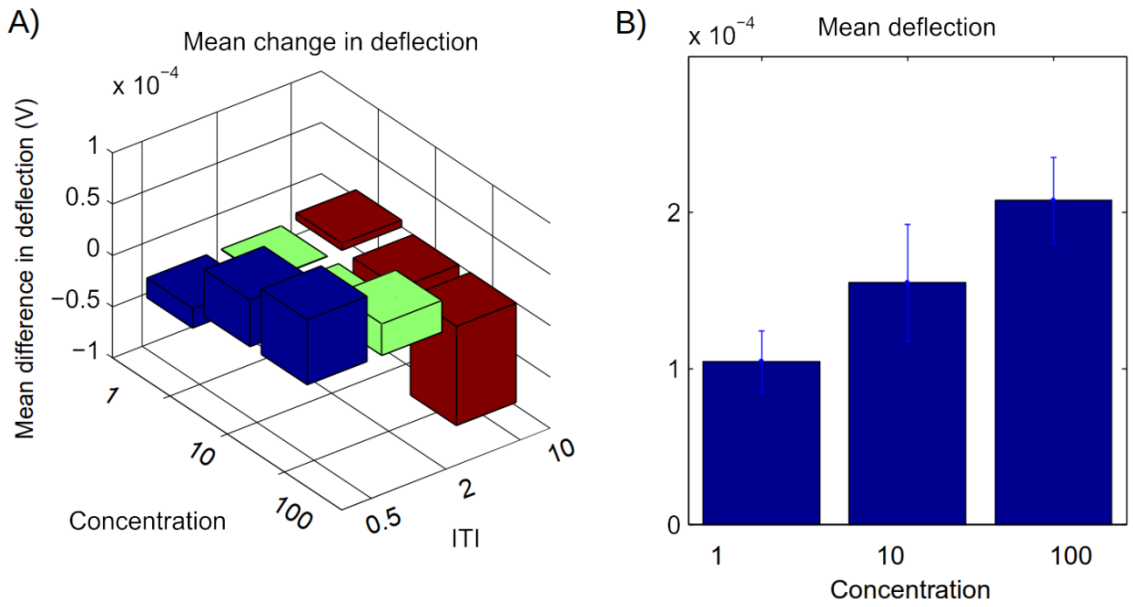

Fig 5.11 A) The bars represent the mean change in deflection between the first trial and second trial and the mean change in deflection was significantly different in the nine training conditions (Anova, N=5, df=36, p=0.0055). **B)** The mean deflection increased with concentration.

5.2.6 Comparison of change in deflection between the conditions that showed good discrimination vs one that showed greater oscillation build up

In the experiments quantifying fine discrimination performance at different concentrations and ITI, it was observed that the 10% odor concentration 2min ITI had the best performance and on the other hand 100% odor concentration 2min ITI condition had significantly lower discrimination performance compared to 10% odor concentration 2min ITI but it had the maximum change in deflection or oscillation build up. So, LFP measurement was carried out in a set of bees where LFP change was measured in both these conditions in the same bees, so as to enable pairwise comparison. The comparison between the odor response for 100% and 10% odor concentrations with 2 min ITI was done with 15 min time gap between each set of recordings to allow for recovery from plasticity. The raw traces of the first (blue) and second (red) trials are shown in **(Fig 5.12 A).** The difference in deflection between the first and second trial are shown in **(Fig 5.12 B).** The mean deflection was more for 100% odor

concentration condition **(Fig 5.12 C).** The mean deflection change for the first trial and second trial **(Fig 5.12 D)** was significantly higher for 100% odor concentration condition than 10% odor concentration condition (Paired t-test N = 10, p=0.040).

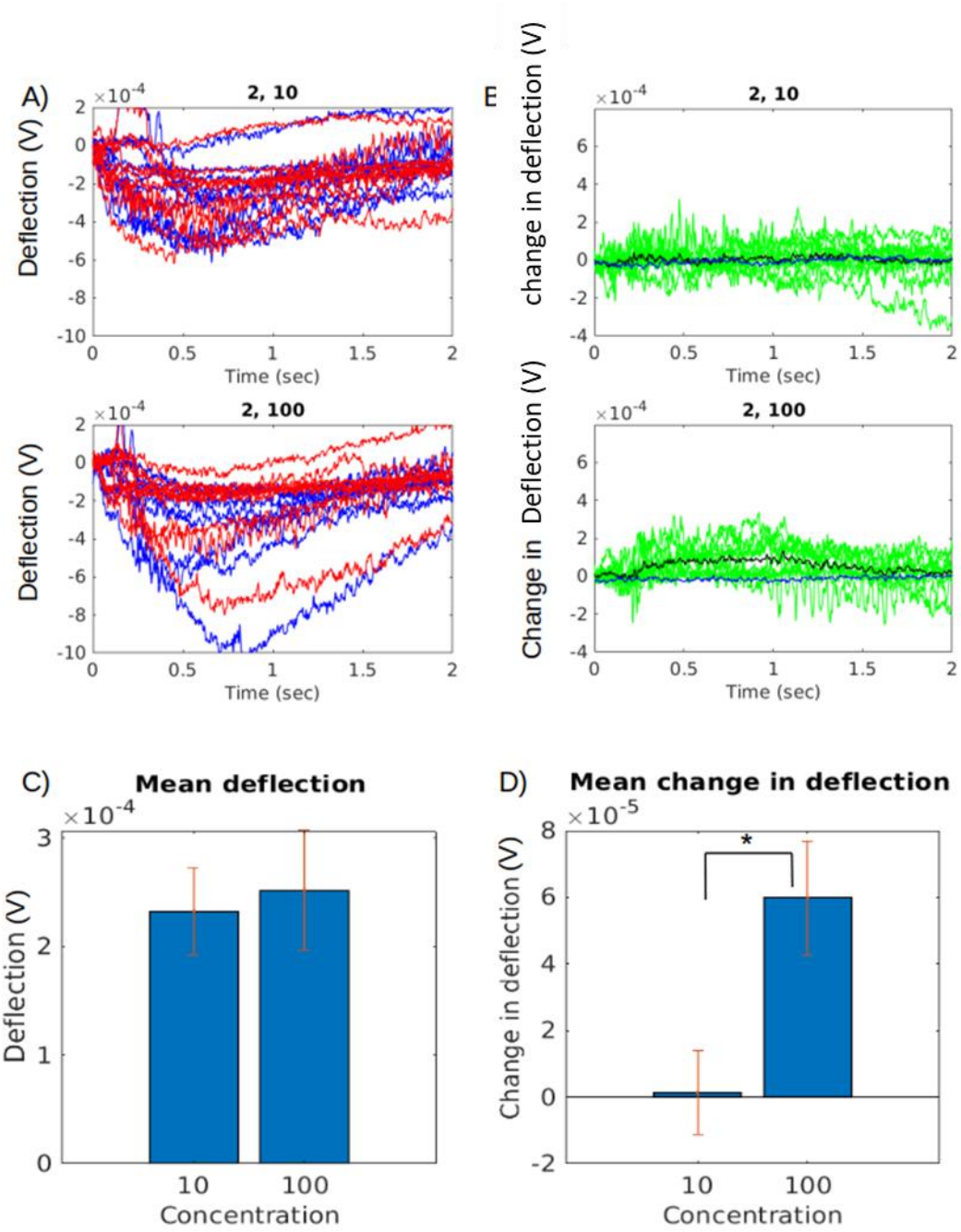

Fig 5.12 Change in deflection of MB LFP for the conditions that showed most discrimination performance versus condition that had more change in deflection. A) Deflection of the MB LFP due to odor presentation, (first trial in blue) and (second trial in red). B) Comparison of

the waveforms representing the difference of deflection between the first trial and second trial in the two training conditions i.e., 10 % odor concentration and 100% odor concentration with 2 min ITI. The condition of concentration and ITI is indicated above the plots. The green trace is the difference between the first trial and the second trial. Black is the mean difference between the first and second trial and blue is the pre-stimulus mean. C) Mean deflection in 10% and 100% odor concentration conditions. D) Mean difference in deflection between first and second trial (Paired t-test N = 10, p=0.040) in both the conditions

shows that that change was significantly larger for 100% odor concentration than 10% odor concentration (Paired t-test N = 10, p=0.040).

5.2.7 Consolidation of results obtained in behavioural paradigm and MB LFP measurement

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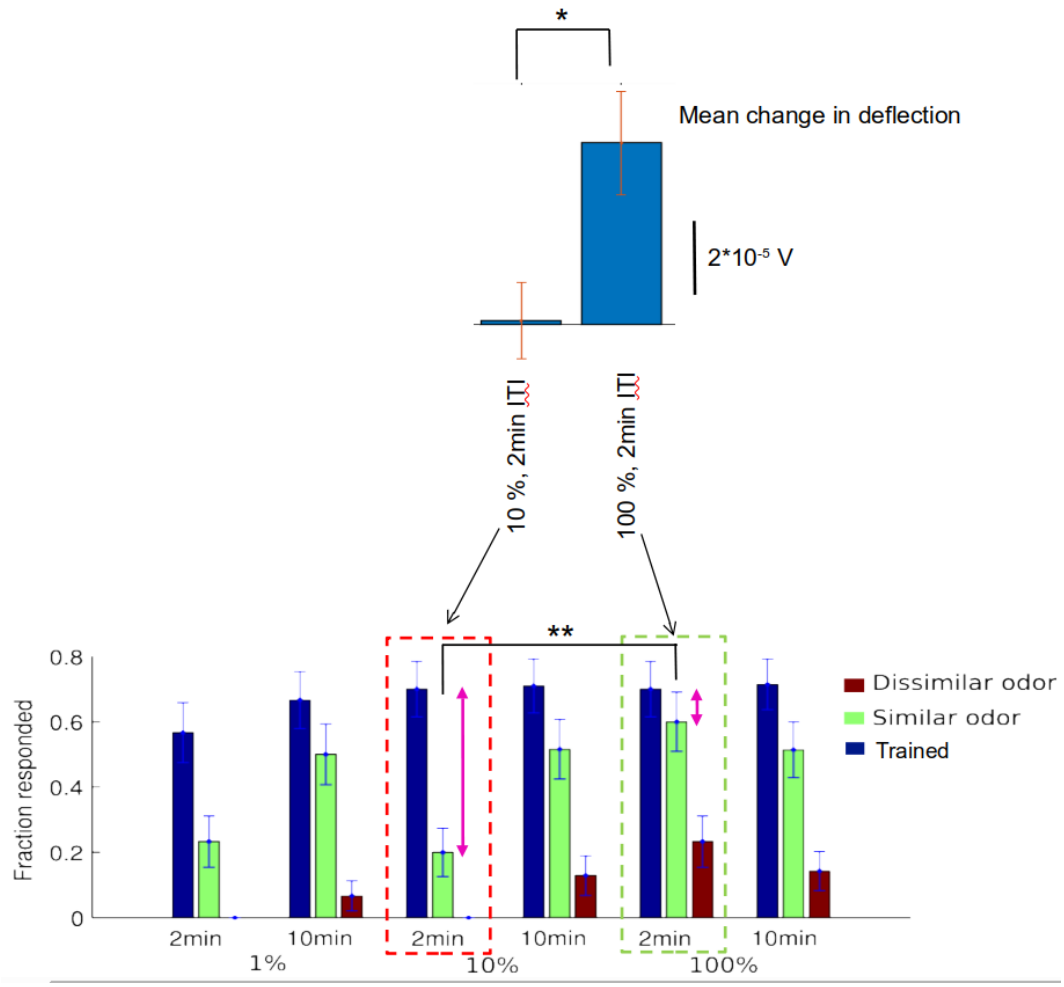

Fig 5.13 The consolidated figure showing the mean change in deflection for 2 min ITI 100% odor concentration and 2 min ITI 10% odor concentration and the corresponding performance

in odor discrimination. The bees trained with 2 min ITI and 10% odor concentration showed significantly higher discrimination than bees trained with 2 min ITI 100% odor concentration whereas the mean change in deflection was higher for 2 min ITI 100% odor concentration condition compared to 2 min ITI 10% odor concentration condition.

Our results show that better similar odor discrimination occured in bees trained with 10% odor concentration and 2 min ITI whereas the measurement of MB LFP indicated that the mean change in deflection from the first trial to second trial which also reflects oscillation build up is more in bees exposed to 100% odor concentration and 2 min ITI **(Fig 5.13).** This would imply better discrimination was possible when there was no requirement for larger $GABA_A$ synaptic strength change, (ie 10%) compared to a condition requiring greater $GABA_A$ synaptic strength change, (ie 100%) Viewed from adaptive gain control point of view this would mean that better discrimination is possible if concentration is such that adaptive gain control does not have to take place, the linear response regime of operation in such systems.

5.4 Discussion

Singh and Joseph (2019) have demonstrated that in *Hieroglyphus banian*, the low frequency component or the deflection observed in MB LFP recordings decreases with repeated odor presentations while the high frequency component (15-40 Hz) increases with repeated trials. This is confirmed in *Apis dorsata* by us. Because the build up of oscillations recorded from the MB LFP in *Apis dorsata* was not as reliable as change in deflection, we used the measure of deflection change as a surrogate to the $GABA_A$ mediated increase in oscillation strength. A condition where the mean change in deflection is high can signify high increase in oscillatory power and higher gain control.

In summary this chapter provides evidence for the following

1) The deflection component (low frequency) of LFP decreases concomitant with increasing oscillatory power in *Apis dosrsata*.

2) In a model of the AL if $GABA_A$ negative feedback from LN to PN, and its plasticity for adaptive gain control is implemented, it automatically gives rise to increase in oscillations. The effect of the adaptive gain control is visible as change in deflection strength.

3) The combination of odor concentration and ITI affect the bees' performance in olfactory discrimination task.

4) Mesasuring for the nature of $GABA_A$ plasticity underlying gain control in two conditions of ITI-concentration combination (100% and 10% with 2 min ITI), it is seen that condition requiring lesser gain control is better for fine discrimination. So, we have a counter example where the AL mechanisms favouring increasing oscillations were higher in one condition (100% with 2 min ITI) but the discrimination was higher in the other condition (10% with 2 min ITI).

Oscillations and their role in olfactory discrimination

There are a number of views on how oscillations help odor perception. Stopfer et al. (1997) showed that in behavioral experiment disruption of oscillations using picrotoxin compromised discriminability of similar orders thus resulting in weaker memory. Backer (2002) checked to see whether oscillations aided in decorrelation of PN responses and resulted in neuronal assemblies to be different in successive cycles, but found absolute difference in spike count between successive 50 msec windows slightly lower in control compared to when picrotoxin was injected and also showed synchronized spikes are not more informative than unsynchronized spikes. A final hypothesis states even if

synchronized spikes are not intrinsically more informative they may be read out preferentially by the decoding algorithm employed by cells down stream of PNs like the β-lobe neurons (Mac Leod et al. 1998; Perez-Orive et al. 2004). But, there is no satisfactory constructive theory about the role of oscillations in odor discrimination as of now.

We propose an alternate theory that the system does better in terms of discrimination when it has to do less gain control. At low odor concentrations the system cannot perform well due to low signal strength and at very high concentrations the system has to do gain control and cannot perform as well as when it does not have to do adaptive gain control, the linear regime. There are existing theories, on gain control enabling efficient encoding (Shows up as Webber Fechner law in many sensory systems). In models it is seen that if we incorporate gain control in discrete event systems like neuronal network, invariably oscillations show up (Li and Hopfield 1989), even if it does not serve any coding purpose. So, the self-consistent picture of beta-gamma rhythms and the associated plasticity in the AL/OB is that it is a byproduct of adaptive gain control. This is not to say that oscillations do not play any role. But to show that they play a role we need a constructive theory and a demonstration of it.

From all these observations about the inhibitory connections mediating negative feedback and their plasticity in the the particular case of primary olfactory center the following question arises. If the system is requiring adaptive negative feedback ($GABA_A$ LNs and their synaptic plasticity in the case of olfactory pathway), is it conceivable to implement it without giving rise to oscillations?

5.4 Summary

Stopfer et al. (1997) showed that 30 Hz oscillations are present in the MB LFP of honey bees due to odor presentation to the antenna and in a behavioural experiment using PER conditioning of *A. mellifera* they showed that abolishing oscillations using picrotoxin compromised fine discrimination of odors. Studies (Stopfer and Laurent 1999; Stopfer et al. 2003) showed that repeated trials of odor presentation increased power of oscillations in the MB LFP in *Schistocera Americana,* and these oscillations by and large increase with odor concentration. Consistent with results from Singh and Joseph (2019), *A. dorsata* also showed decrease in deflection component of the LFP when oscillation component increased with repeated presentation of an odor. Oscillation build up was not as reliable as change in deflection from trial to trial. In a model built by us based on experiments and earlier models by Bazenov et al. (2005) and Ito et al. (2009), we found measurement of change in deflection as an equivalent measure of build-up of oscillations, consistent with the above experiments.

Based on these results, an experiment was designed, to vary the amount of oscillations present in MB LFP by varying inter-trial interval (ITI) and odor concentration of the CS in an olfactory PER conditioning paradigm.

We trained *A. dorsata* bees in six training conditions with varying ITIs, and odor concentration and in our experiments bees trained with 2 min ITI and 10% odor concentration showed best discrimination of similar odors.

We measured the change in deflection of the MB LFP for consecutive trials for all the training conditions and found the change in deflection, which is a measure of oscillatory build up to be maximum for bees tested with 2 min ITI and 100% odor concentration. So, we have an example where discrimination was higher while plasticity favouring oscillation build up was lower.

Our results are consistent with the view in which $GABA_A$ plasticity as mechanism for adaptive gain control with oscillations as a by-product. Thus, we propose that the system does better in terms of discrimination when it has to do less gain control. In low concentrations the system cannot perform well due to low signal strength and at very high concentration the system has to do gain control and cannot perform as well as when it does not have to do gain control.

Chapter 6

Conclusions

In *A. mellifera* it was shown that oscillations were necessary for fine discrimination of odors. The study tried to check this using *A. dorsata* which is a sturdier species and native to India. The olfactory systems of both the species were compared given the differences in body size, nesting habit, and their habitat. It was found that *A. dorsata* had a similar glomerular number and arrangement, ALT innervation compared to *A. mellifera*. Neurotransmitter GABA which is involved in the generation of oscillations was found to be present in *A. dorsata* AL and MB LFP of *A. dorsata* exhibited 30 Hz oscillations. *A. dorsata* proved to be amenable to olfactory PER condition and was shown to learn with different ITIs. It could distinguish trained odor from novel odor. So, *A. dorsata* was used as a model system to study oscillations and central plasticity.

In the experiment, bees were trained with different ITIs and odor concentrations so as to vary the amount of oscillations present. It showed bees trained with 2 min ITI and 10% odor concentration have the best discrimination. The measurement of oscillations or the change of deflection of MB LFP from the first trial to the second trial showed the highest change for 2 min ITI and 100% odor concentration. So it was showed that the system can do better odor discrimination in a condition with lesser oscillations. These results point to a view that oscillations in the AL may be a byproduct of input-dependent adaptive gain control in the AL.

Findings of the Thesis (Summarized)

- *A. dorsata* has around 165 glomeruli which is similar to the glomerular number of *A. mellifera*
- *A. dorsata* has a total glomerular volume of 3283.8 x 10^3 μm^3, which is less than that observed in *A. mellifera.*
- AL interneurons respond to odors with spatio-temporally patterned responses.
- PNs innervate the higher olfactory centers MB and LH through 5 ALTs, similar to *A. mellifera*.
- MB LFP of *A. dorsata* exhibits 30 Hz oscillations in response to odor presentation.
- MB LFP deflections decrease with an increase in 30 Hz oscillatory power, consistent with an increase in GABAergic inhibition in the AL.
- *A. dorsata* is amenable to olfactory PER conditioning and has learning rates similar to *A. mellifera.*
- *A. dorsata* is amenable to massed and spaced conditioning, exhibits long-term and short-term memory, and can distinguish trained odor from novel odor.
- The discrimination ability was dependent on ITI and the concentration of the odor. It was best at intermediate concentration.
- Change in LFP deflection was dependent on ITI and concentration as expected with plasticity in the AL.
- *A. dorsata* bees could discriminate trained odor from a similar odor better in a condition where the deflection component of LFP changed less than a condition where it changed more. This would correspond to the condition of less $GABA_A$ plasticity and less oscillation build-up.

Application of the research

- The glomerular number of *A. dorsata* established by this study has been used by Karpe et al. (2021) to identify the olfactory receptor genes in *A. dorsata.*

- *A. dorsata* can be used as a model system to study olfaction as the olfactory system is well characterized.
- *A. dorsata* can be used in studies involving olfactory conditioning. Vijay kumar et al. (2019) investigated the lateral transfer of olfactory learnt information using PER conditioning of *A. dorsata*
- The digital Atlas can aid in identification of odor code and innervation patterns of local and projection neurons.

Recommendations for future Research

- Identification of the different glomeruli activated by a particular odor can be done by optical studies and compared with other closely related species.
- Though we did not encounter any non-spiking inter neurons it will be interesting to fill local neurons and study their morphology.
- In olfactory PER conditioning there is a reward component. It would be interesting to study the oscillations during reward presentation.

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REGULAR ARTICLE

Characterization of the olfactory system of the giant honey bee, *Apis dorsata*

Sandhya Mogily[1] · Meenakshi VijayKumar[1] · Sunil Kumar Sethy[2] · Joby Joseph[1]

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Abstract
Apis dorsata is an open-nesting, undomesticated, giant honey bee found in southern Asia. We characterized a number of aspects of olfactory system of *Apis dorsata* and compared it with the well-characterized, western honeybee, *Apis mellifera*, a domesticated, cavity-nesting species. *A. dorsata* differs from *A. mellifera* in nesting behavior, foraging activity, and defense mechanisms. Hence, there can be different demands on its olfactory system. We elucidated the glomerular organization of *A. dorsata* by creating a digital atlas for the antennal lobe and visualized the antennal lobe tracts and localized their innervations. We showed that the neurites of Kenyon cells with cell bodies located in a neighborhood in calyx retain their relative neighborhoods in the pedunculus and the vertical lobe forming a columnar organization in the mushroom body. The vertical lobe and the calyx of the mushroom body were found to be innervated by extrinsic neurons with cell bodies in the lateral protocerebrum. We found that the species was amenable to olfactory conditioning and showed good learning and memory retention at 24 h after training. It was also amenable to massed and spaced conditioning and could distinguish trained odor from an untrained novel odor. We found that all the above mentioned features in *A. dorsata* are very similar to those in *A. mellifera*. We thereby establish *A. dorsata* as a good model system, strikingly similar to *A. mellifera* despite the differences in their nesting and foraging behavior.

Keywords *Apis dorsata* · Olfactory system · Digital atlas · Mushroom body · Olfactory conditioning

Introduction

Honey bees were described as magic well for discoveries in biology by Karl Von Frisch. The Western honey bee, *Apis mellifera*, is well established as a model system to investigate various fundamental scientific questions at the behavioral, neural, and molecular levels. The olfactory conditioning paradigm in bees is extensively used for research in learning and memory (Menzel and Erber 1978; Menzel 1993; Menzel and Muller 1996; Giurfa 2007) as features and mechanisms of learning and memory in bees are found to have similarities to those in mammals and humans (Squire 1987; Menzel et al. 1996; Menzel 2012).

In *A. mellifera*, the odor molecules are detected by around 60,000 olfactory receptor neurons (ORNs) present in sensilla located on the antennae (Esslen and Kaisling 1976; Kropf et al. 2014). ORNs from each side innervate the ipsilateral antennal lobe (AL), the primary olfactory center, through the T1–4 tracts of the antennal nerve (AN) (Suzuki 1975; Mobbs 1982; Galizia et al. 1999; Abel et al. 2001; Kirschner et al. 2006). In the AL of *A. mellifera*, ORNs synapse on to around 800 projection neurons (PNs) (Bicker et al. 1993; Hammer 1997; Galizia 2008) and approximately 4000 local neurons (LNs) (Witthöft 1967; Sachse and Galizia 2006; Galizia 2008; Galizia and Rossler 2010) in dense spheroidal structures called glomeruli, the morpho-functional unit of the AL (Hildebrand and Shepherd 1997; Anton and Homberg 1999; Hansson and Anton 2000). PNs project to the higher olfactory centers, lateral horn (LH), and the mushroom body (MB) through five antennal lobe tracts (ALTs). In the MB, the PNs synapse on approximately 180,000 Kenyon cells (KCs) (Mobbs 1982; Abel et al. 2001; Muller et al. 2002; Kirschner et al. 2006; Rossler and Brill 2013; Zwaka et al.

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RESEARCH ARTICLE

Evidence for absence of bilateral transfer of olfactory learned information in *Apis dorsata* and *Apis mellifera*

Meenakshi Vijaykumar[1,2], Sandhya Mogily[1], Aparna Dutta-Gupta[2] and Joby Joseph[1,*]

ABSTRACT

The capacity and condition under which the lateral transfer of olfactory memory is possible in insects is still debated. Here, we present evidence in two species of honeybees, *Apis mellifera* and *Apis dorsata*, consistent with the lack of ability to transfer olfactory associative memory in a proboscis extension response (PER) associative conditioning paradigm, where the untrained antenna is blocked by an insulating coat. We show that the olfactory system on each side of the bee can learn and retrieve information independently and the retrieval using the antenna on the side contralateral to the trained one is not affected by the training. Using the setup in which the memory on the contralateral side has been reported at 3 h after training, we see that the memory is available on the contralateral side immediately after training. In the same setup, coating the antenna with an insulator on the training side does not prevent learning, pointing to a possible insufficiency of the block of odor stimuli in this setup. Moreover, the behavior of the bee as a whole can be predicted if the sides are assumed to learn and store independently, and the organism as a whole is able to retrieve the memory if either of the sides have the memory.

KEY WORDS: PER conditioning, Mushroom body, Olfactory coding, Insect olfaction, Learning and memory

INTRODUCTION

Lateral transfer of information helps environmental stimuli acquired and learned on one side to become accessible to both lobes of a bilateral brain (Aboitiz and Montiel, 2003; Gazzaniga, 2000). This helps to maximize the computational ability of the brain by allowing each side of the brain to co-opt the other for joint decision-making or to avoid duplicity of storage for efficient use of the substrate (Aboitiz and Montiel, 2003; Gazzaniga, 2000, 2014). Information transfer across the midline has also been theorized to be the basis of unified consciousness (Barron and Klein, 2016) and its importance has been highlighted in split-brain patients (Gazzaniga, 2014). In higher mammals, this crucial function is carried out by the corpus callosum, a tissue present in eutherian mammals alone (Aboitiz and Montiel, 2003; Gazzaniga, 2000, 2014; Suárez et al., 2014). The formation of the corpus callosum has been suggested to be an evolutionary innovation (Mihrshahi, 2006), highlighting the importance of developing and evolving the process of transfer of information as an evolutionarily stable strategy. Does this evolutionary jump have correlates in invertebrates such as insects? Despite their primitive nature, insects are known to be able to perform complex tasks with their rather simple brains consisting of a few 100,000 neurons. Insects such as ants, wasps and honeybees, belonging to the order Hymenoptera, can perform complex tasks involving locating food sources, nesting sites and foraging back and forth between the food source and nest, which would require coordination of a range of modalities (Hansson and Stensmyr, 2011; Kaupp, 2010; Matsumoto et al., 2012; Roper et al., 2017; Sanes and Zipursky, 2010; Su et al., 2009).

In free-flying bees (Masuhr and Menzel, 1972), it was reported that side-specific olfactory conditioning does not transfer to the contralateral side. It was later reported that in *Apis mellifera*, if the bee is trained in the proboscis extension response (PER) to associate an odor with reward when a stimulus is applied to only one antenna, the odor memory can be retrieved by applying trained odor to the untrained contralateral antenna, 3 h after training (Sandoz and Menzel, 2001). In these experiments, a wall was used to separate the two antennae and deliver the odor in a side-specific manner, arguing that blocking the antenna using a coating influences the context of training and impairs transfer. In their study, 3 h post training, up to 50% of the bees responded by extending their proboscis when the learned odor and not a novel odor was applied only to the contralateral antenna, suggesting the presence of a commissure relaying encoded odor-specific memory between sides. Guo et al. (2016) reported changes on a molecular level in the contralateral side of *A. mellifera* after training even if the contralateral side was isolated by coating the antenna. That study, however, did not show transfer using behavior, compared with controls trained with both sides closed. This group used silicon paste to block one antenna while training the exposed antenna to an odor. Post 24 h transcriptomic analysis was carried out and the results showed an upregulation in memory and learning-related genes on the untrained side of the brain, indicating a possible lateral transfer of this learned information and memory. The above experiments and works pointed to the possible presence of a commissure dedicated to the relaying of olfactory learned information from one brain lobe to the other. If this is true, then recording the activity of the neurons in this commissure would also provide insight into the nature of olfactory code, an exciting prospect.

Work in our laboratory recently showed the presence of bilateral extrinsic neurons of the mushroom body calyx in a species of grasshopper, *Hieroglyphus banian* (Singh and Joseph, 2018 preprint). In addition, a cluster of lateral horn neurons in *Schistocerca americana* have been shown to have a bilateral innervation (Gupta and Stopfer, 2012). Thus, there are very few possible substrates for lateral transfer of olfactory memory in insects and none reported in Hymenoptera. We attempted to look for the neuronal basis of the phenomenon of bilateral transfer of information in a species of honey bee native to South East Asia, *Apis dorsata*, also referred to as the giant honey bee or the rock bee, which is one of the crucial pollinators in the region. In our

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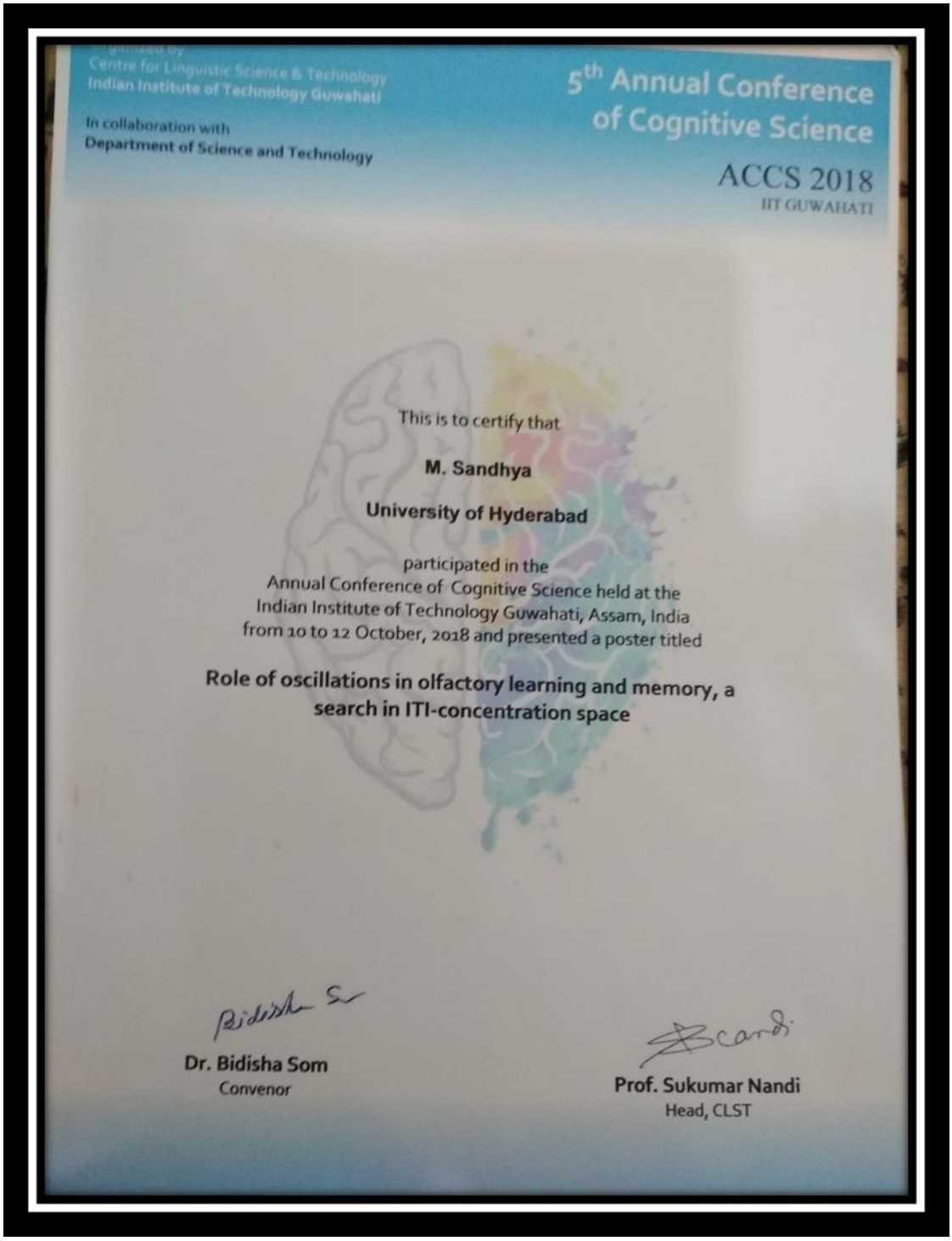

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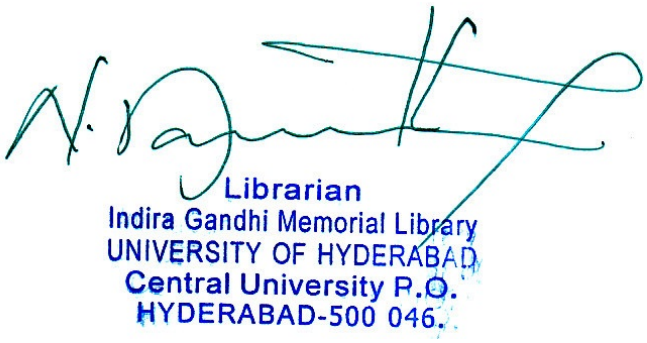

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