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Contributions of PPL2 dopaminergic neurons to
olfactory representations in *Drosophila*
melanogaster

Katie Marquand

Supervised by Dr Tamara Boto and Professor Mani Ramaswami

A thesis submitted to Trinity College Dublin, the University of Dublin, in accordance
with the requirements of the degree of Doctor of Philosophy.

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Katie Marquand, 30th November 2025

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Contents

1	Introduction	1
1.1	Sensory representation	1
1.1.1	Generalisation and discrimination	3
1.1.2	Sparse coding	7
1.2	<i>Drosophila</i> as a model organism	10
1.2.1	The <i>Drosophila</i> olfactory system	10
1.2.2	Genetic tools	20
1.2.3	The connectome	22
1.3	The mushroom body calyx	26
1.3.1	Calyx organisation	26
1.3.2	Microglomerular architecture of PN-KC synapses	28
1.3.3	Inhibition from APL	30
1.3.4	Structural plasticity of microglomeruli	31
1.4	Dopamine systems in <i>Drosophila</i>	33
1.4.1	Dopamine receptors	34
1.4.2	Internal state dependent dopamine	40
1.4.3	PPL2 dopaminergic neurons	41
1.5	Aims and objectives	42
2	Materials and Methods	45
2.1	Fly lines and husbandry	45
2.2	Immunohistochemistry	45
2.3	Neuron activity reporting	48
2.4	Y-maze behavioural assay	50
2.5	<i>In vivo</i> functional imaging	52

2.6	Connectomics analysis	54
2.6.1	Circuit analysis	54
2.6.2	Microglomeruli analysis	55
3	Results	59
3.1	Two neurons from the PPL2 cluster innervate the calyx	59
3.2	PPL201 and PPL202 innervate olfactory related brain regions	62
3.3	PPL201 and PPL202 connect with different neuron types across the brain	65
3.4	Synapse distribution in the calyx differs between brains	66
3.5	APL and KC γ are the main neuron partners of PPL201 and PPL202 in the calyx	70
3.5.1	PPL201 and PPL202 receive information from KCs, APL and PNs in the calyx	70
3.5.2	PPL201 and PPL202 receive information from PNs, and LH local interneurons in the lateral horn	72
3.5.3	PPL201 and PPL202 send information to APL and KCs in the MB calyx	72
3.5.4	PPL201 and PPL202 send information to PNs and LH local interneurons in the lateral horn	73
3.6	Identification of microglomeruli structures	75
3.6.1	The spatial distribution of microglomeruli is different across datasets	76
3.6.2	The distribution of microglomeruli across PNs and KCs is similar across datasets	78
3.7	Properties of microglomeruli containing PPL2 synapses	79
3.7.1	Equivalent PN types have different microglomeruli structures across datasets	79
3.7.2	Spatial distribution of microglomeruli containing PPL2 matches PPL2 skeletons	80
3.7.3	Microglomeruli that contain PPL2 synapses are larger than microglomeruli that do not	82
3.7.4	Microglomeruli that contain PPL2 synapses also contain more unique neuron types than microglomeruli that do not contain PPL2	83

3.7.5	Microglomeruli that contain PPL2 are larger than microglomeruli that do not, with a restrictive definition	85
3.8	Properties of PPL2 synapses located within microglomeruli	87
3.8.1	PPL2 pre-synaptic zones are located mostly in microglomeruli	87
3.8.2	The top PPL2 synaptic connections have synapses both inside and outside microglomeruli	88
3.9	Knockdown of three dopamine receptors differently affect odor-evoked responses in KCs	89
3.9.1	Knockdown of Dop1R1 in 247-Gal4 targeted KCs alters response dynamics	90
3.9.2	Knockdown of Dop1R2 in 247-Gal4 targeted KCs increases response to a familiar odor	92
3.9.3	Knockdown of Dop2R results in robust decrease of 247-KC response in female flies	93
3.10	Knockdown of dopamine receptors differently affect subsets of the KC population	94
3.10.1	Knockdown of D1-like receptors have no effect on response of either γ - or $\alpha\beta$ -KCs in females	96
3.10.2	Knockdown of Dop2R has opposing effects on the response of γ - and $\alpha\beta$ -KCs	98
3.11	Characterisation of different Gal4 driver lines for PPL2 targeting	99
3.11.1	PPL2 cluster provides dopaminergic innervations to the calyx	100
3.11.2	oc2-Gal4 driver targets PPL2 cluster and glia	101
3.11.3	Different split-Gal4 drivers target subpopulations of the PPL2 cluster	103
3.12	Silencing of PPL2 dopaminergic neurons has no effect on KC activity	106
3.13	Exploratory analysis of internal state effect on KC activity	107
3.13.1	KC odor-evoked activity is not affected by age	108
3.13.2	KC odor-evoked response dynamics are changed by mating status	110
3.13.3	KC odor-evoked response increases in the calyx following a period of starvation	112
3.14	Knockdown of D1-like and D2-like differently affect odor-evoked responses in γ -KCs of starved flies	113

3.14.1 Dop1R1 knockdown does not affect starvation induced increase in γ -KC response	114
3.14.2 Dop2R knockdown abolishes starvation induced increase in γ -KC response	114
3.15 Measuring activity of PPL2 neurons under starvation conditions	116
3.15.1 Odor-evoked calcium imaging of PPL2 responses to different odours is inconclusive	118
3.16 Measuring calcium activity of PPL2 neurons in freely behaving flies . .	119
3.17 PPL2 activation during naïve olfactory behaviour	121
3.17.1 PPL2 activation increases approach behaviour at low concentrations	122
3.17.2 PPL2 activation increases avoidance behaviour at high concentrations	123
4 Discussion	127
4.1 Summary of results	127
4.2 Discussion	129
4.2.1 Limitations of connectomic analysis	129
4.2.2 Comparing FAFB and hemibrain data	133
4.2.3 PPL201 and PPL202 are involved in modulating olfactory behaviour via connections with the MB calyx	134
4.2.4 PPL2 modulation of KC odor-evoked responses	138
4.2.5 A model for PPL2 starvation-mediated affects	139
4.3 Future Experiments	141
Appendix	171

List of Figures

1.1	Schematic of the <i>Drosophila</i> olfactory circuit	12
1.2	Schematic of the <i>Drosophila</i> mushroom body	20
1.3	The Gal4/UAS system and variants	23
3.1	PPL201 and PPL202 have substantial synapses located within the calyx	61
3.2	Brain region connectivity of PPL2 neurons	63
3.3	Comparison of PPL201 morphology between datasets	68
3.4	Comparison of PPL202 morphology between datasets	69
3.5	Upstream connections to PPL2 within the calyx	71
3.6	Downstream connections of PPL2 within the calyx	74
3.7	Analysis of identified microglomeruli.	77
3.8	Comparison of microglomeruli with or without PPL2 synapses	81
3.9	Testing a restrictive definition of microglomeruli boundaries	83
3.10	Proportion of PPL201 synapses located in microglomeruli	85
3.11	Proportion of PPL202 synapses located in microglomeruli	88
3.12	Dopamine receptor knockdowns affect the odor-evoked response of KCs.	91
3.13	Comparison of response 1 and response 2 for Dop1R2 knockdowns in 247-KCs.	92
3.14	Dop2R knockdown in male 247-Gal4 flies	94
3.15	Dopamine receptor knockdowns affect the odor-evoked response of γ -KCs.	95
3.16	Dop1R1 knockdown in male 5HTR1B-Gal4 flies	96
3.17	Dopamine receptor knockdowns affect the odor-evoked response of $\alpha\beta$ - KCs.	97
3.18	Characterisation of PPL2-Gal4	101
3.19	Characterisation of oc2-GAL4 driver	102
3.20	Characterisation of split-Gal4 lines	103

3.21	Characterisation of PPL2-split Gal4 driver	104
3.22	Effect of silencing PPL2 neurons on odor-evoked KC activity.	107
3.23	KCs odor-evoked response under different internal conditions	109
3.24	KC odor-evoked response in different compartments of the MB	111
3.25	Dopamine receptor knockdowns in starved or fed γ -KCs	115
3.26	PPL2 odour-evoked response in the calyx under starvation conditions	117
3.27	PPL2 activity in behaving flies	120
3.28	Preliminary data of PPL2 activity in behaving flies	121
3.29	Naïve olfactory behaviour with PPL2 activation	125
4.1	5HTR1B-Gal4 starvation induced increase in response varies by day	140
A.1	Dop1R1 knockdown in starved or fed KC γ	171
A.2	KC odor-evoked response in the calyx under starvation conditions	171
A.3	Comparison of y-maze performance between males and females	172
A.4	Comparison of preference index when the odour was located in the right or left arm of the y-maze	173
A.5	Naïve olfactory behaviour with new Gal4 control	173
A.6	Percentage of flies that made a choice at each concentration	174

List of Tables

2.1	Table of genetic lines	46
2.2	Table of reagents	47
3.1	Summary of different neuron metrics across datasets and PPL2 types .	66
3.2	Summary of dopamine receptor knockdown results	98
3.3	GAL4 driver lines and their GFP positive PPL2 neurons	99

Abbreviations

ACA	Accessory Calyx	LH	Lateral Horn
AD	Activation Domain	LTM	Long Term Memory
APL	Anterior Paired Lateral	MB	Mushroom Body
ARM	Anaesthesia Resistant Memory	MBON	Mushroom Body Output Neuron
Brp	Bruchpilot	oc	Ocelliless
CA	Calyx	OR	Odorant Receptor
Ca ²⁺	Calcium	ORN	Olfactory Receptor Neuron
cAMP	Cyclic adenosine monophosphate	otd	Orthodenticle
CREB	cAMP-response element binding	PAM	Protocerebral Anterior Medial
CS	Conditioned Stimulus	PN	Projection Neuron
DAN	Dopaminergic Neuron	PPL1	Protocerebral Posterior Lateral 1
DAT	Dopamine transporter	PPL2	Protocerebral Posterior Lateral 2
DBD	DNA-Binding Domain	SLP	Superior Lateral Protocerebrum
Ddc	Dopa decarboxylase	SNP	Superior Neuropils
EB	Ethyl Butyrate	TH	Tyrosine Hydroxylase
GFP	Green Fluorescent Protein	UAS	Upstream Activating Sequences
GPCR	G-protein-coupled receptor	US	Unconditioned Stimulus
KC	Kenyon Cell	Vmat	Vesicular monoamine transporter

Abstract

Animal responses to the same stimulus vary with experience, motivation, hunger, age, and other internal or external conditions. This raises the question of how the brain encodes stimulus identity while allowing flexible behavioural outputs. In this thesis, we use the species *Drosophila melanogaster* to investigate how dopamine modulates internal olfactory representations.

Drosophila is an ideal model for studying olfactory coding due to its well-mapped circuitry, small neuron number, and capacity for complex behaviour. Although anatomically distinct from vertebrate brains, flies share conserved neuromodulators and functional organisation. Here, we focus on sparse olfactory representations in Kenyon Cells (KCs), whose processes form the Mushroom Body (MB), a centre for olfactory learning and memory. This work examines the PPL2 dopaminergic neuron cluster which innervates the MB calyx, the entry point for olfactory inputs, positioning it to modulate odour representations upstream of memory formation.

Connectome analysis reveals KCs and the inhibitory neuron APL as the main postsynaptic targets of PPL2. Synapse location analysis suggests that PPL2 forms a presynaptic component of microglomeruli in the calyx, indicating a modulatory role within these complex synaptic structures. Additionally, calcium imaging experiments with dopamine receptor knockdown suggest that dopamine acts via Dop2R to differentially modulate KC subsets. Preliminary evidence indicates that KC responses vary with hunger state, potentially mediated by dopamine. Finally, a naïve olfactory behavioural assay shows that PPL2 activation enhances responses to odour regardless of valence.

Overall, these findings support a role for PPL2 dopaminergic input in shaping olfactory processing by modulating distinct KC populations. We identify key circuit elements for future study and provide initial evidence for how PPL2 activity influences both neural representation and behaviour.

Lay Abstract

Animals are constantly receiving different sensory input which guides their behaviour, but they don't respond in fixed, automatic ways. A food odour that entices you when you are hungry might be ignored just after you have eaten. How can the same input produce such different behavioural outcomes? The internal representation of the stimuli in the brain must be flexible depending on the animal's current needs and previous experience.

In this thesis I use fruit flies to explore how the brain creates and modifies these internal representations of odours. Fruit flies are useful for this kind of research because their brains are small and well-mapped, yet they still display surprisingly complex behaviours. We look specifically at how dopamine helps to reshape the way odours are processed. Recordings of brain activity show that removing dopamine input can change how strongly neurons respond to an odour, and behaviour experiments show that activating dopamine neurons alters the flies' response. Early results also suggest that internal states, such as whether the fly is hungry, can also shift these odour responses.

Overall, this research provides new insight into how the brain stays flexible, allowing the same odour to lead to different actions depending on context. Understanding this in a simple animal helps reveal general principles of how the brain interprets the world in an adaptive way.

Aims and Hypothesis

The overall aim of this thesis is to investigate the role of PPL2 dopaminergic modulation on olfactory representations in the mushroom body calyx of the species *Drosophila melanogaster*. Specific objectives are as follows:

1. Uncover the PPL2 circuitry. Using connectome analysis we aim to uncover the pre- and post-synaptic targets of PPL2, and we will use this information to guide our experiments.
2. Test how dopamine input can modulate olfactory representations. Using calcium imaging techniques and dopamine receptor knockdown in post-synaptic partners we will examine how dopamine input affects the olfactory circuit, and through which receptors.
3. Investigate the potential of PPL2 as a salience encoding neuron. Using a combination of imaging techniques and naïve behaviour we will investigate how dopamine input affects downstream targets under different internal conditions such as starvation, age and mating status.

These can be broken down into testable hypotheses that align with our aims:

1. PPL2 neurons are strongly connected to olfactory related neurons within the calyx and contribute to microglomeruli structures.
2. PPL2 will act to alter KC odour-evoked activity either directly, or indirectly via inhibitory modulatory neurons that strongly innervate the calyx, such as APL.
3. Different internal states will change the activity of PPL2 to odours, which in turn will modulate KC activity. Artificial activation of PPL2 will increase naïve behavioural response to odours regardless of valence.

Value of Research

Understanding the neural mechanisms that underlie flexible response to sensory stimuli is a fundamental problem in neuroscience. Although understanding of how representations are modulated in the context of memory is moving forward, there are few studies that investigate sensory representation upstream of memory formation. Due to this gap in knowledge current computational models of learning and memory do not include signals of internal state that might alter how a stimulus is perceived and therefore how a memory is formed. *Drosophila melanogaster* provide an ideal model organism to investigate sensory representation due to its small brain size, and thus reduced number of neurons, and the genetic tools that enable us to target specific neurons and express genes of interest. Although, the insect brain looks very different to ours, there are brain areas similar in function and the same neuromodulators and neurotransmitters are present. Therefore, the value of this research is twofold:

1. Impact on knowledge.

Sensory representations are an important aspect of not only learning and memory but also innate behaviour. Understanding how these representations are changed with internal or external contexts provides us with more knowledge regarding how we process the sensory world around us.

2. Impact on healthcare

Changes in sensory perception are common in neurological disorders such as autism spectrum disorder, panic disorders, anxiety, and different types of dementia. Progress in understanding the molecular mechanisms that cause plasticity, or modulate representations in the brain could provide avenues for further investigation in human's with these disorders.

Outputs

1. **Marquand, K.**, Roselli, C., Cervantes-Sandoval, I., & Boto, T. (2023). **Sleep benefits different stages of memory in *Drosophila*.** *Frontiers in Physiology*, 14, 33.

Chapter 1

Introduction

1.1 Sensory representation

Animals are constantly being bombarded with an array of different information from the environment, including visual inputs, tactile sensations, tastes, odours, and sounds. Some of these signals are essential to inform the animal's decisions; for example, odour plumes can lead an animal to a food source [Cardé, 2021], a bitter taste can indicate something dangerous to avoid [Kim et al., 2016], or a mating call can indicate a potential nearby mate [Podos, 2013]. This list of examples is obviously non-exhaustive and the different types of information carried in sensory inputs is vast [Fuller and Endler, 2018]. However, a lot of information is irrelevant noise and if the brain attempted to process everything equally, it would quickly become overwhelmed. Therefore, it is critical for the animal's sensory system to extract important elements, and filter out the noise in order to properly process the most relevant information [Heekeren et al., 2008, Romo and de Lafuente, 2013, van Hateren, 1992]. As information flows through successive sensory processing stages, from detection to higher-order integration, neural circuits transform and reformat these signals into representations optimised for each level's task, from preserving information, to efficient storage, and ultimately to formats that enable flexible behavioural decision-making.

At the detection level a lot of sensory information simply goes undetected if an animal lacks the physiological capacity to do so. For example, humans are insensitive to the Earth's magnetic field, although this is a sensory cue vital to other, typically migratory, animals such as birds [Wiltschko and Wiltschko, 2019, Mouritsen and Ritz, 2005].

A particular animal might also be tuned to specific ranges of information which are more relevant, for example the ability of insects to detect the ultraviolet range of light [Kevan et al., 2001, Lubbock, 1882]. These adaptations have occurred over evolutionary timescales in order for each animal to perceive cues in their environment relevant for survival [Briscoe and Chittka, 2001, Oteiza and Baldwin, 2021, Valencia-Montoya et al., 2024]. However, sensory adaptation also occurs on shorter timescales, from milliseconds to days, to filter sensory inputs [Wark et al., 2007, Jafari and Alenius, 2021]. Imagine you enter a room with a strong odour, at first you clearly notice and respond to the smell, however, over time the odour seems to fade until you can't even smell it any more [Moncrieff, 1956]. This occurs due to olfactory receptors decreasing their response to the familiar, constant odour, essentially filtering out the consistent background stimulus and allowing your brain to focus on processing what is important [Kurahashi and Menini, 1997, Pellegrino et al., 2017, Jafari and Alenius, 2021]. Habituation to a signal leads to the same behavioural result as sensory adaptation, a reduced response to a constant, familiar signal, however habituation occurs at the brain level as opposed to at the receptor level [Pellegrino et al., 2017, Jafari and Alenius, 2021, Rankin et al., 2009, Schmid et al., 2015]. Habituation is a type of learning that involves the suppression of awareness of a frequent stimulus. For example, the constant ticking of a clock can be easily ignored but, since the auditory receptors are still responding, a shift in attention will bring the ticking back to the forefront [Öhman and Lader, 1972, Picton et al., 1976, Rankin et al., 2009]. This allows the brain to stop paying attention to a repetitive stimulus but allows quick re-detection if the stimulus changes suddenly. Selective attention is the brain's top-down filtering system, allowing the animal to consciously prioritise certain inputs [Wickens, 2021]. There are many other mechanisms like predictive coding [Ramaswami, 2014], salience filtering [Parr and Friston, 2019], or motivational filtering [Matteucci et al., 2022, Ruff, 2013] which are altered by the internal state of the animal and allow for flexibility in deciding what is important information to process.

While filtering ensures only the most important and relevant information reaches higher brain processing centres, this alone is not sufficient for effective stimulus pro-

cessing. The sensory world, and the organism’s relationship to it, is constantly changing. Thus, even once information has been selected, the way it is represented must remain flexible. Certain stimuli may elicit an instinctual response that is hard-wired through evolution, such as the defensive or escape response shown by many species to a looming visual stimulus [Ball and Tronick, 1971, Dunn et al., 2016, Zacarias et al., 2018, Yilmaz and Meister, 2013]. However, many stimuli may need a different response depending on the context, previous experience, or internal state of the animal [Flavell et al., 2022]. This flexibility in behaviour is essential for choosing the best action in new scenarios and for survival. Therefore, investigating the structure of these internal sensory representations and how these are modulated under different conditions to support adaptive behaviour is critical.

1.1.1 Generalisation and discrimination

An essential feature of a sensory representation is to encode stimulus identity in a way that optimises for both generalisation and discrimination of stimuli. Generalisation is crucial for efficient learning, to be able to apply past experience to new situations which are not exactly the same, but are similar [Hilgard and Marquis, 1961]. By contrast, discrimination is needed to distinguish between similar stimuli that need to elicit different responses [Phillmore, 2008]. A balance between generalisation and discrimination is needed to respond to stimuli appropriately, and these responses can be altered by context, experience, and internal state of the animal. Over-generalisation can lead to inappropriate stimuli responses and even disorders. Post-traumatic stress disorder, generalised anxiety, or panic disorders are associated with an over-generalisation of fear contexts, leading to fear responses in safe environments [Lissek and Grillon, 2015, Lissek et al., 2010, Laufer et al., 2016]. On the other hand, extreme discrimination with no generalisation leads to the inability to recognise patterns between similar stimuli, and therefore having to learn separately about each instance of the vast array of stimuli. A common symptom of semantic dementia, a class of dementia associated with degeneration of the frontal and temporal lobes, is the patient’s ability to use familiar

objects in their own home but failure to generalise this knowledge to be able to use different examples of the same objects [Snowden et al., 1994, Bozeat et al., 2002, Snowden et al., 1995, Davies et al., 2005]. In general, healthy humans are good at discrimination and generalisation tasks, however it is not known how the brain manages this flexibility. The balance between generalisation and discrimination is also an open problem in machine learning, where brain inspired neural networks have not yet reached the robustness of the human brain in generalisation tasks [Geirhos et al., 2018, Bober-Irizar and Banerjee, 2024].

The phenomenon of generalisation was first observed by Pavlov during his classical associative conditioning experiments [Pavlov and Anrep, 1927]. These experiments, and most associative learning paradigms since, involved the temporal pairing of a neutral stimulus (conditioned stimulus, CS), with some reinforcement (unconditioned stimulus, US). The US should have some perceived value by itself that induces an innate, unconditioned response, for example a negative electric shock inducing a freeze response, or a positive food reward inducing salivation. When the CS and US are repeatedly paired the subject animal learns to associate the two, such that when the CS is presented by itself the animal displays a conditioned response that is the same as the innate response to the US to reflect the newly acquired predictive property of the CS. In Pavlov’s experiments the CS was a 1000Hz tone, and the US was a food reward leading his dog to salivate at the sound of the tone. However, Pavlov noticed that similar sounding tones of different frequencies would spontaneously acquire the predictive properties of the CS after conditioning. Meaning that the dog would salivate in response to tones of frequencies other than the 1000Hz despite them not being used for training. Thus his subjects were generalising their learnt behaviour to similar stimuli.

Pavlovian associative conditioning, one of the simplest and well conserved forms of learning, has been shown in many species including insects such as bees, cockroaches, locusts, ants and *Drosophila melanogaster*, also known as the fruit fly [Simões et al., 2013, Tully and Quinn, 1985, Menzel, 1993, Busto et al., 2010, Hawkins and Byrne,

2015, Pavlov and Anrep, 1927]. A typical olfactory associative learning paradigm for *Drosophila* involves the pairing of a CS^+ with some reinforcement (US), and a second odor, CS^- , that is presented without the US [Tully and Quinn, 1985]. This training may be repeated depending on the protocol, and then followed by a testing phase which involves a choice assay between the CS^+ and CS^- [Tully et al., 1994]. Usually these experiments are performed with a population of *Drosophila* and the strength of memory is measured by the proportion of flies that make the correct choice, i.e. approach or avoid the CS^+ depending on the valence of reinforcement used [Tully and Quinn, 1985]. Although insects have much simpler, and smaller brains compared to mammals, this learning assay can be modified to study more complex types of learning such as discrimination or generalisation [Barth et al., 2014], associations with compounds [Young et al., 2011], reversal learning [Quinn et al., 1974], second-order associations [Tabone and de Belle, 2011], and many others.

Although insects rely primarily on odor to navigate their surroundings, associative learning can be seen for a range of modalities including vision, sound, and taste [Schnaitmann et al., 2010, Zhang et al., 2005, Menda et al., 2011, Masek and Keene, 2016]. Different modalities can be used in conjunction with each other to strengthen memories, with visual and odor bimodal training leading to stronger memory than either stimulus alone [Okroy et al., 2023]. Bumble bees can even recognise objects in the dark by touch when the object has previously been seen but not touched, showing a much more complex ability in sensory integration which requires modality-independent representations [Solvi et al., 2020].

In an olfactory discrimination task *Drosophila* are tasked with choosing between the CS^+ , associated with a reinforcement, and a second cue that could be similar to, or distinct from the CS [Modi et al., 2023]. The more perceptually similar the second odor is to the CS^+ , for example a chemically similar compound, or using odor mixtures containing A, the more difficult the task becomes [Chen and Gerber, 2014, Campbell et al., 2013, Parnas et al., 2013, Lin et al., 2014]. In a generalisation task the choice is between an odor that is similar to the CS^+ and another odor that is different. In

this phase neither odor is the trained odor, therefore the score relies on the ability to generalise the association to the similar odor [Modi et al., 2023, Campbell et al., 2013]. Negative patterning discrimination is a more complex, non-linear discrimination task where stimulus components A and B are both reinforced individually, while stimulus mixture AB is non-reinforced. The test phase consists of the choice between either A or B and AB. A positive result would mean the animal is able to discriminate between the reinforced component and the mixture. Mammals such as rats [Harris et al., 2008, Delamater et al., 1999], rabbits [Bellingham et al., 1985, Kehoe and Graham, 1988], and humans [Livesey et al., 2011] have demonstrated the ability to perform this type of discrimination. This complex behaviour has also been demonstrated in bees [Deisig et al., 2001] and although it was thought that *Drosophila* were not capable of this task [Young et al., 2011], evidence suggests that after multiple training cycles they can also discriminate odor mixtures elements in this way [Durrieu et al., 2020].

Elemental theory

Theoretically, it was proposed that generalisation occurred when two stimuli were perceptually close to each other, and discrimination occurred when they were perceptually far [Lashley and Wade, 1946]. The question remains, how is the stimulus represented internally? Kenneth Spence formalised the theory of elemental association which proposed that stimuli can be conceptualised as a series of different elements, and the similarity between two stimuli is determined by the amount of overlapping elements [Spence, 1936, Spence, 1937]. Therefore, the response to a stimuli depends on the associations formed to each individual element. This theory was developed by Konorski who postulated that an encounter with each stimuli would activate a dispersed population of units in the brain, and this population of activated units can overlap leading to generalisation between these two stimuli [Konorski, 1948]. How this elemental theory is applied to Pavlovian conditioning in practise was demonstrated using aversive conditioning to a combination of flavours in rats [Mackintosh et al., 1991]. Here, an aversion conditioned to saline, did not generalise to sucrose, but one conditioned to saline-lemon did generalise to sucrose-lemon. In this case, the overlapping component

of lemon is thought to be the element of the stimuli causing this generalisation.

Although the elemental theory of learning has been popular during the last decades of neuroscience research and can explain numerous aspects of learning and stimulus perception, it cannot explain everything. For example, negative patterning discrimination cannot be captured by this theory, since an association odor elements A and B would activate the same neural units as odor mixture AB, leading to the same behavioural outcome and inability to discriminate [Durrieu et al., 2020, Young et al., 2011]. Another theory based on this idea of overlapping units is the configural theory, where the activated units represent the specific configuration of stimulus elements [Rudy and Sutherland, 1995, George and Pearce, 2012]. This means that odor elements A, B and the mixture AB will each activate distinct neural populations, although they may have some overlap to aid in generalisation. While both theories provide useful frameworks for understanding how stimuli might be represented in the brain, neither can fully account for all observed learning phenomena, suggesting the need for a flexible representation in the brain [Young et al., 2011].

1.1.2 Sparse coding

A widely researched sensory encoding strategy, reminiscent of the elemental theory of learning is sparse coding [Olshausen and Field, 2004]. Sparse representations of stimuli in the brain are characterised by distributed responses across a set of neurons where each neuron responds to few stimuli and each stimuli causes few neurons to respond. This neural architecture increases separation between representations, therefore increasing discriminability, and maximising storage capacity in an energy efficient manner. It is therefore not surprising that physiological evidence of this encoding strategy has been found in brain areas processing various sensory modalities and across species.

In the mammalian hippocampus, an area largely associated with memory and learning, sparse neural activity is thought to be the basis of memory storage [Guan et al., 2016, Tonegawa et al., 2015]. In an episodic memory recall task, where humans were

asked to discriminate between learned and novel words, each recognised word elicited a response in a small percentage of recorded neurons, while any one neuron only responded strongly to a small percentage of recognised words [Wixted et al., 2014]. Similarly, spatial memories of rodents are encoded by highly selective place cells in the hippocampus [O’Keefe and Dostrovsky, 1971]. In mice exposed to an enriched environment during development, the resulting increased sparsity of hippocampal neuron activation in response to a novel environment was correlated with increased habituation speed to the novel environment [Bilkey et al., 2017]. This suggests that the degree of sparsity in sensory representations can have a great impact on behavioural output and that the degree of sparsity can be altered throughout the animals lifetime, allowing flexibility in sensory representations. In fact, mathematical models of randomly connected neuron networks have shown that the degree of sparseness is responsible for controlling the generalisation-discrimination trade-off [Barak et al., 2013].

Sparse neuron activity is also found across the mammalian cortex, forming representations of numerous sensory modalities. Neurons in the auditory cortex of rats respond sparsely to a range of auditory stimuli including simple tones, white noise or more complex sounds such as animal noises, or complex artificial sounds [Hromádka et al., 2008, Kang and Kanold, 2024]. This sparse encoding strategy is also seen in the auditory cortex of marmosets, allowing for pitch discrimination of harmonic sounds [Kadia and Wang, 2003, Terashima et al., 2013, Bendor and Wang, 2005]. It has been demonstrated in macaques that natural scenes produce more sparse responses in the visual cortex compared to simple scenes containing only the classical receptive field [Vinje and Gallant, 2000, Vinje and Gallant, 2002]. This suggests that context can increase sparsity in visual neuron responses. Lastly, odor representations in the piriform cortex of rats are also sparse, with each tested odor eliciting a response in approximately 10% of recorded cells, and around 80% of the recorded cells only responding strongly to one odor [Poo and Isaacson, 2009]. Sparse encoding of odor stimuli is well established across insect species including locusts [Gupta and Stopfer, 2014, Perez-Orive et al., 2002, Finelli et al., 2008], bees [Szyszka et al., 2005, Carcaud et al., 2015], and *Drosophila* [Honegger et al., 2011, Lin et al., 2014]. We will discuss the neural ar-

chitecture used by *Drosophila* to achieve sparse representations of odors in more detail in Section 1.2.1.

Despite the large evolutionary distance between the organisms mentioned, the sparse sensory encoding strategy is common across species, suggesting that it is efficient, robust, and flexible to allow for an array of complex behaviours. In fact, complex discrimination behaviours such as negative patterning could be explained by a computational model based on the sparse connectivity in the honey bee olfactory system [Peng and Chittka, 2017]. Sparse representations are able to be altered by external or internal stimuli, with auditory representations in rodents changing depending on levels of inhibition which increase during anaesthesia [Hromádka et al., 2008, Gaese and Ostwald, 2001, Feigin et al., 2021]. Sparseness in auditory cortex can be reduced by suppressing inhibitory neurons, therefore representations of stimuli can be dynamically regulated by modulating the excitatory/inhibitory balance [Liang et al., 2019]. Even in locusts it has been shown that inhibition from the giant GABAergic neuron provides a negative feedback loop required to maintain the level of sparsity [Papadopoulou et al., 2011]. In the mammalian hippocampus, computational network modelling evidence suggests that inhibition from granule cells in the dentate gyrus region responsible for maintaining sparseness, since blocking granule cells leads to increased excitability and performance impairments in a pattern separation task [Chavlis et al., 2017]. Complementary to these inhibitory signals, dopamine release in the medial prefrontal cortex during learning has been shown to increase discrimination learning ability, suggesting a role for dopamine signalling in these sensory representation circuits [Popescu et al., 2016].

The *Drosophila* olfactory system provides an ideal model to investigate modulation of sensory representations since the presence of sparse representations is well established, the neural circuitry is well-characterised and contains dense inhibitory and dopaminergic networks.

1.2 *Drosophila* as a model organism

The species *Drosophila melanogaster* has been used for over one hundred years as a model organism in biomedical sciences [Kohler, 1994]. *Drosophila* offer a highly tractable and compact nervous system along with a complex repertoire of behaviour, low cost, and high throughput. The availability of a complete connectome and a multitude of genetic tools for manipulating neural populations means *Drosophila* is an ideal system to study the link between circuit architecture, function, and behaviour. Importantly, many circuit motifs, neurotransmitters, and behaviours found in *Drosophila* are conserved across other species, including mammals, making insights from this model organism broadly applicable in sensory neuroscience.

1.2.1 The *Drosophila* olfactory system

Olfaction is a primary sense for insects including *Drosophila*, with a range of critical behaviours such as foraging, courtship, and egg laying relying on the detection different odours [Vosshall, 2000, Carlson, 1996, Busto et al., 2010]. Although the mammalian sensory landscape tends to prioritise audio or visual cues, odour provides fruit flies with reliable access to ecologically relevant information. The olfactory circuit is therefore well characterised not only in terms of anatomical structure but also as a model for associative conditioning (Figure 1.1). Since the insect olfactory circuit shows many similarities to the mammalian system, in terms of information coding strategy, anatomical structure, and circuit motifs, *Drosophila* provides us with a generalisable model to investigate fundamental principles of sensory coding and neuromodulation.

Odour detection and the antennal lobe

Odour detection begins with the olfactory sensory neurons (OSNs), which are housed in the small sensory hairs, sensilla, which cover the surface of the antennae and maxillary palps of insects or in the olfactory epithelia of vertebrates [Buck and Axel, 1991, Carl-

son, 2001, De Bruyne et al., 1999, Vosshall et al., 2000]. Typically, in *Drosophila* each OSN expresses one type of olfactory receptor (OR) along with the co-receptor OR83b [Vosshall et al., 2000, Vosshall et al., 1999]. Each type of OSN converges onto the same subregion, or glomerulus, of the antennal lobe or vertebrate homolog, the olfactory bulb [Vosshall et al., 2000, Laissue et al., 1999, Gao and Chess, 1999, Stocker et al., 1990]. In *Drosophila* there are two classes of receptors identified in the sensilla, the odourant receptors, and the ionotropic receptors (IRs) [Benton et al., 2009, Mombaerts, 1999, Rytz et al., 2013]. Although both families are chemosensors in the olfactory system, they differ in structural and functional characteristics [Gomez-Diaz et al., 2018, Rytz et al., 2013]. While ORs seem to be used exclusively in olfaction, highly tuned to esters and alcohols [Hallem et al., 2004, Hallem and Carlson, 2006, Benton et al., 2007], IRs have been reported to function for other sensory modalities including taste [Hussain et al., 2016, Zhang et al., 2013b, Lee et al., 2017, Croset et al., 2016, Tauber et al., 2017], hygrosensation [Enjin et al., 2016, Knecht et al., 2016, Chu et al., 2024, Knecht et al., 2017, Tang et al., 2024], and temperature sensing [Knecht et al., 2016, Ni et al., 2016]. In terms of olfactory function, IRs are highly tuned to amines and acids but are more narrowly tuned to only a few compounds compared to ORs [Benton et al., 2009, Min et al., 2013]. Overall, there are about 50 classes of OSNs, each expressing a different olfactory receptor, which map to about 50 antennal lobe glomeruli since each glomeruli receives information from one class of OSN [Laissue et al., 1999, Bates et al., 2020b, Fishilevich and Vosshall, 2005, Couto et al., 2005, Schlegel et al., 2021]. Within the antennal lobe there are two other main types of neurons, projection neurons (PNs) which send olfactory information to higher order brain areas, and local neurons (LNs), which connect glomeruli to each other.

PNs are the output neuron type of the antennal lobe, sending olfactory information to the lateral horn, an area associated with innate behaviours, and the mushroom body, the learning and memory center for insects [Bates et al., 2020b, Wilson, 2013] (more detail on these structures in the following sections). This OSN-PN connection acts as a convergence point in the olfactory pathway since each glomerulus receives information from around 50 individual OSNs which synapse onto, on average, 3 PNs

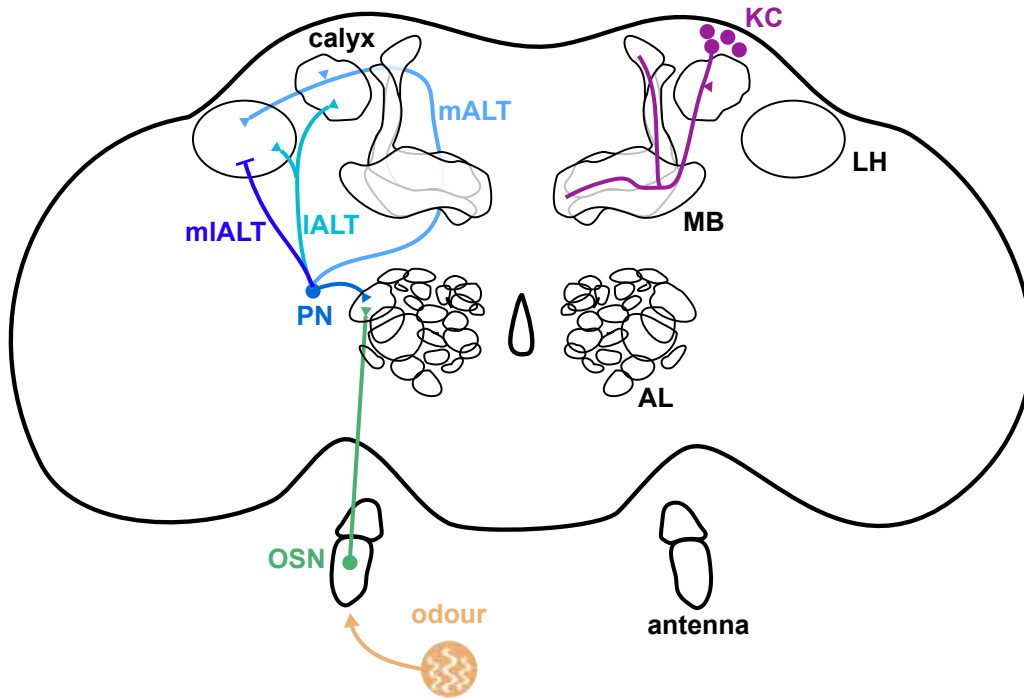


Figure 1.1: Schematic of the *Drosophila* olfactory circuit

Diagram showing the neural pathway of odour information in the *Drosophila* brain. Odour (orange) is detected by olfactory sensory neurons (green) in the antenna which send axons to specific glomeruli in the antennal lobe. There, projection neurons send information to higher order brain areas via three tracts (blues). These higher order brain areas are the lateral horn, and the mushroom body. Projection neurons send information to the mushroom body calyx where they synapse onto kenyon cells (purple). These kenyon cells project axons that bifurcate to form the lobes of the mushroom body. OSN: olfactory sensory neuron, PN: projection neuron, mIALT: mediolateral antennal lobe tract, lALT: lateral antennal lobe tract, mALT: medial antennal lobe tract, AL: antennal lobe, MB: mushroom body, LH: lateral horn, KC: kenyon cell.

[Vosshall et al., 2000]. Most PNs have dendrites that innervate a single glomerulus, and therefore receive information from OSNs of the same type [Wong et al., 2002]. However, some PNs may be multiglomerular, with dendrites innervating two, or sometimes even all antennal glomeruli [Stocker et al., 1990, Tanaka et al., 2012a, Lai et al., 2008]. There is some evidence of labeled line connectivity throughout this section of the olfactory pathway, i.e. specialised glomeruli which provide a specific pathway for special, ecologically relevant odours such as pheromones [Agarwal and Isacoff, 2011], carbon dioxide [Suh et al., 2004], or harmful microbes such as geosmin [Stensmyr et al., 2012]. However, the odourant landscape for *Drosophila* is incredibly vast, and most relevant odours contain a mixture of chemicals which may bind to different types of odourant receptors. Since there are many more odours than odourant receptor types it has been proposed that the odour code in the antennal lobe is combinatorial, so

odour identity is encoded in the combination of OSNs that are activated [Malnic et al., 1999, Schubert et al., 2014].

PNs also receive input from a heterogeneous population of LNs, which have varying morphologies, neurotransmitter types, and connectivity [Das et al., 2011, Chou et al., 2010, Lai et al., 2008, Seki et al., 2010]. These neurons can be inhibitory [Olsen and Wilson, 2008, Root et al., 2008, Nagel and Wilson, 2016, Okada et al., 2009] or excitatory [Olsen et al., 2007, Shang et al., 2007], and provide inter- and intra-glomerular connectivity which is vital for early olfactory processing [Schenk and Gaudry, 2023, Wilson and Laurent, 2005]. In the antennal lobe, lateral inhibitory input to a glomerulus scales with total OSN activity, this provides a gain control such that when total OSN input is weak, OSN-PN connections remain strong, however when OSN input is strong GABAergic interneurons prevent the oversaturation of PN response [Olsen and Wilson, 2008]. This inter-glomerular communication also maintains contrast between PN responses to increase the signal-to-noise ratio in PN odour coding and discriminability between odours [Olsen and Wilson, 2008, Root et al., 2008, Olsen et al., 2010] while intra-glomerular inhibition acts to regulate spontaneous activity within a glomerulus [Schenk and Gaudry, 2023]. Excitatory connectivity between glomeruli is important to boost PN signals, perhaps aiding in the processing of faint odour inputs [Shang et al., 2007].

There is also evidence of dopaminergic [Parnas and Avishai, 2025, Chou et al., 2010, Inagaki et al., 2012], serotonergic [Sizemore and Dacks, 2016, Zhang and Gaudry, 2016, Dacks et al., 2009], and octopaminergic [Busch et al., 2009] input to the antennal lobes which may modulate PN responses in an odour-specific manner under different physiological conditions. Overall, early olfactory processing in the antennal lobe leads to a higher signal-to-noise ratio and separated representations in PN olfactory code which can be sent to third order brain areas for further signal processing that leads to behaviour [Masse et al., 2009].

There are three main tracts by which the PN axons leave the antennal lobe, the medial (mALT), mediolateral (mlALT), and lateral (lALT) antennal lobe tracts (Figure

1.1) [Stocker et al., 1990, Tanaka et al., 2012a, Marin et al., 2002, Tanaka et al., 2012b].

The mALT is the largest of the three and contains mostly cholinergic, uniglomerular PNs which first innervate the MB and then extend into the LH [Marin et al., 2002, Tanaka et al., 2004]. The PNs following the mlALT tract bypass the MB completely to innervate the LH [Marin et al., 2002, Tanaka et al., 2012a]. These PNs consist mostly of GABAergic, multiglomerular PNs which provide an inhibitory input to the LH [Wilson and Laurent, 2005, Okada et al., 2009]. Lastly, PNs of the lALT tract can be uni- or multiglomerular and branch to innervate both the MB and LH [Tanaka et al., 2012a].

Lateral Horn

The lateral horn (LH) consists of approximately 1,400 neurons and more than 165 cell types, making it a very complex and diverse neuropil [Fisek and Wilson, 2014, Frechter et al., 2019, Schlegel et al., 2021]. The structure does not have clearly defined physical boundaries and is instead defined by the location of PN axonal terminals [Fisek and Wilson, 2014, Jefferis et al., 2007, Marin et al., 2002, Wong et al., 2002, Tanaka et al., 2004]. These PN-LH connections are stereotyped across individuals, and zonal in nature, suggesting that different areas of the LH respond to different types of odours [Fisek and Wilson, 2014, Frechter et al., 2019]. Similar to the antennal lobe, the LH is composed of input neurons (mainly PNs carrying olfactory information), local neurons (mainly inhibitory), and output neurons that transmit information to the next layer of olfactory processing, the superior lateral protocerebrum (SLP) [Frechter et al., 2019, Dolan et al., 2019]. Although the input neurons mainly provide olfactory information, there also exists input from visual, mechanosensory, gustatory, and thermosensory areas that innervate a restricted ventral area of the LH [Dolan et al., 2019]. Local neurons broadly innervate the entire LH, providing lateral inhibition which increases odour specificity of LH responses [Dolan et al., 2019, Fisek and Wilson, 2014]. Along with the inhibitory GABA-ergic local neurons, there also exists glutamatergic and cholinergic populations of local neurons which suggests lateral excitation may also occur in the LH. Interestingly, there are three LHONs that have been identified to project bilat-

erally, connecting the two brain hemispheres and suggested to provide a coordination mechanism to control behaviour [Dolan et al., 2019]. LH neurons can receive two types of PN input, either from several glomeruli, or from only one glomerulus [Fişek and Wilson, 2014]. This connectivity layout supports both a labeled line coding strategy for specific odours and also the ability to categorise similar odours. Indeed, there is evidence of spatial segregation of valence information that is received from inhibitory PNs and also PNs carrying information about pheromones innervated a different area of the LH compared to PNs related to food odours [Strutz et al., 2014, Datta et al., 2008, Luo et al., 2010, Min et al., 2013, Wang et al., 2003b, Jefferis et al., 2007, Liang et al., 2013, Parnas et al., 2013].

One innate odour pathway that has been extensively studied in the LH is the response to 1-*cis*-vaccenyl acetate (cVA), a male pheromone that elicits sex specific responses [Ruta et al., 2010, Jefferis et al., 2007, Datta et al., 2008]. OSNs and PNs that respond to cVA express *fruitless* (*fru*), a gene that regulates sex specific neural development [Ryner et al., 1996]. These *fru*⁺ PNs that terminate in the LH have significantly increased axonal branching in the LH in males compared to females, meaning that the LH is a sexually dimorphic structure in order to support the appropriate responses [Datta et al., 2008, Cachero et al., 2010, Ruta et al., 2010, Kurtovic et al., 2007, Berg et al., 2025]. Some LHONs themselves also express *fru* and have sex specific innervation patterns, for example the LHONs that receive input from cVA responding PNs [Kohl et al., 2013, Ruta et al., 2010, Cachero et al., 2010]. In addition to pheromone cues, there have been specific LH neurons have been implicated in other innate behaviours such as egg-laying reduction behaviour induced by geosmin detection [Huoviala et al., 2018, Stensmyr et al., 2012] and aversion to carbon dioxide [Varela et al., 2019].

Although the LH is associated with innate behaviours, and much evidence suggests a hardwired circuit, it is important to note that innate responses can change depending on the context and so odour representations in the LH still have to remain flexible. We have already seen that the LH is sexually dimorphic, but responses are also modulated by internal state of the fly, such as hunger, or odour concentration [Lerner et al., 2020].

The LH is innervated by modulatory neurons such as the serotonergic deutocerebral neuron (CSD), and also the PPL2 dopaminergic cluster (discussed in detail in section 1.4.3), although it is not currently known the effects of this modulation [Li et al., 2020a, Coates et al., 2020, Sizemore and Dacks, 2016, Zhang and Gaudry, 2016].

The LH is one of two third order olfactory processing centers, the other being the mushroom body (MB), an area strongly associated with learning and memory. These two structures are not isolated from each other, receiving inputs from the same PNs via the mALT and lALT tracts, but also through direct connections [Bates et al., 2020b, Li et al., 2020a]. This suggests that learned and innate olfactory information could be integrated to modulate and update instincts by previous experience, or prevent experience overriding critical hardwired behaviours.

Mushroom Body

The MB is a key area in *Drosophila* and other insects, known to be an integration zone for sensory information [Gruntman and Turner, 2013]. In contrast to the LH, the MB is the ‘learning and memory centre’ of the brain and has well established roles in olfactory [Heisenberg et al., 1985, McGuire et al., 2001], gustatory [Masek and Keene, 2016, Kirkhart and Scott, 2015], visual [Liu et al., 1999] and courtship memory [McBride et al., 1999]. Interestingly, precise chemical ablation of the MB during development leads to adult flies that show normal behaviour in most respects, except that they are unable to perform in aversive olfactory associative conditioning tasks [De Belle and Heisenberg, 1994]. This deficit was shown to not be due to reduced sensitivity to odour, or to locomotor effects, providing strong evidence that the MB is the centre for associative learning. A huge amount of work has demonstrated that the MB is required for different stages of olfactory associative conditioning [Krashes et al., 2007, Dubnau et al., 2001, McGuire et al., 2001] and there is also evidence of a role in integrating not only different sensory modalities, but also the internal state, such as motivation or satiety, of the animal [Cohn et al., 2015, Krashes et al., 2009, Lewis et al., 2015, Tsao et al., 2018]. Therefore, sensory representations in the MB are important

in order to ensure a balance between specific and generalised associative memories.

Each MB hemisphere contains approximately 2,000 intrinsic Kenyon cells (KCs) that receive olfactory information from PNs [Aso et al., 2009, Technau and Heisenberg, 1982]. The PN-to-KC connections are found in the MB calyx, the input zone of the MB, and the sparse activity of the KC population provides an encoding of odour identity [Lin et al., 2014, Campbell et al., 2013]. The MB calyx anatomy and function is discussed in detail in section 1.3 since it is the main region of interest of this thesis, here we introduce the circuitry of the MB lobes.

The axons of KCs extend and bifurcate to form the 5 MB lobes; the vertical lobes, α , α' , and the horizontal lobes, β , β' , and γ . KCs can be categorised into 3 main neuron subtypes based on which lobes their axons form; α/β , α'/β' , or γ [Crittenden et al., 1998, Aso et al., 2009]. These types can be further divided depending on which specific layer of the MB lobe the KC axon occupies [Aso et al., 2014a, Tanaka et al., 2008, Li et al., 2020a]. The γ lobe can be divided into the main (m) and dorsal (d) layers, the α'/β' into the middle (m) and anterior-posterior layers (ap) and the α/β into the posterior (p), core (c), and surface (s) layers. Of these, 5 types have dendrites located within the calyx; α/β -c and -s, α'/β' -m and -ap, and γ -m while γ -d and α/β -p dendrites are found exclusively in the accessory calyces. [Aso et al., 2014a]. These anatomical distinctions are coupled with functional differences between KC subtypes. Broadly, $\text{KC}\gamma$ neurons are required for short-term memory formation [Blum et al., 2009, Zars et al., 2000, Xie et al., 2013], α'/β' are responsible for memory consolidation [Keene et al., 2006, Krashes et al., 2007, Cervantes-Sandoval et al., 2013], and the α/β neurons are required for memory retrieval and long-term memory [Krashes et al., 2007, Dubnau et al., 2001, McGuire et al., 2001, Pascual and Pr  at, 2001, Akalal et al., 2006, Yu et al., 2006]. As well as being necessary for different stages of memory, the different KC types also support different types of memory. For example, α/β -s neurons are required during retrieval of both aversive and appetitive memory, whereas α/β -c are only required for appetitive memory [Perisse et al., 2013].

KC axons form *en passant* synapses with mushroom body output neurons (MBONs)

throughout the MB lobes to provide cholinergic input [Barnstedt et al., 2016]. There are 20 typical MBON types that exclusively have dendrites in the MB lobes, and 14 atypical MBONs types that also extend dendrites outside of the MB lobes, for example directly contacting descending neurons that drive motor action [Li et al., 2020a, Rubin and Aso, 2024, Tanaka et al., 2008, Aso et al., 2014a, Aso et al., 2014b]. The MBON dendrites tile the lobes to create 15 distinct compartments, which are the functional units of the MB [Aso et al., 2014b, Cohn et al., 2015]. MBONs receive olfactory information from KCs and project to many downstream brain regions such as the LH, SMP, and crepine to drive appropriate response behaviour to an odour stimulus [Li et al., 2020a, Dolan et al., 2019]. In fact, optogenetically stimulating different populations of MBONs themselves can directly induce either approach or avoidance behaviour to odours in untrained flies [Aso et al., 2014b, Cohn et al., 2015].

Also innervating the MB are 21 types of dopaminergic neurons (DANs), organised into three clusters [Mao and Davis, 2009, Aso et al., 2014a]. The protocerebral posterior lateral 1 (PPL1) cluster innervates the vertical lobes (α and α') and respond to aversive stimuli [Aso et al., 2014a]. Stimulation of the PPL1 cluster is sufficient to induce an aversive memory when paired with an odour in the absence of an external reinforcement signal, suggesting a role for encoding punishment [Riemensperger et al., 2005, Aso et al., 2010, Schroll et al., 2006, Claridge-Chang et al., 2009]. On the other hand, the protocerebral anterior medial (PAM) dopaminergic cluster innervates the horizontal lobes (β , β' , and γ) and respond to appetitive reward stimuli such as sucrose [Yamagata et al., 2015]. PAM neurons are necessary and sufficient for forming appetitive memories [Liu et al., 2012a]. Together these dopaminergic neurons convey the valence of a stimulus, or how attractive or aversive the stimulus is (Figure 1.2 A). During learning, PPL1 and PAM dopaminergic neurons act to modulate the strength of KC-MBON synapses in a MB compartment specific manner [Hige et al., 2015a, Oswald and Waddell, 2015]. For example, PPL1 neurons will respond to an electric shock and the coincidence of DAN activity and KC activity weakens the synaptic connections between active KCs and the approach driving MBONs. Since aversive MBONs are unaffected this leads to behavioural avoidance of the conditioned odour [Oswald and

Waddell, 2015, Perisse et al., 2016, Hige et al., 2015a, Gkanias et al., 2022]. The opposite is also true; a reward activated PAM neuron acts on KC-MBON connections that encode avoidance behaviour, weakening this connection. Dopamine induced pre-synaptic depression in KCs is a well-established mechanism of associative plasticity, but it has been demonstrated that post-synaptic mechanisms also contribute [Pribbenow et al., 2022]. Anatomical studies have shown direct connections between dopaminergic pre-synapses and MBONs and post-synaptic calcium transients in MBONs can change in response to activation of DANs paired with application of acetylcholine, circumventing KC output [Pribbenow et al., 2022]. The third cluster of dopaminergic neurons, protocerebral posterior lateral 2 (PPL2), do not project to the MB lobes and instead innervate the calyx (discussed in detail in section 1.4.3) [Mao and Davis, 2009, Boto et al., 2019, Li et al., 2020a, Zheng et al., 2018].

In addition to dopamine, there are other neuromodulatory inputs to the MB, including serotonergic input via the DPM neuron acting on both MBONs and KCs to modulate long-term water memory and anaesthesia resistant memory (ARM), respectively [Lee et al., 2021, Lee et al., 2011]. Five types of octopaminergic (OA) neurons, the functional analog of noradrenaline, have been reported to innervate the MB calyx and lobes with roles in signaling the presence of a sweet reward, often acting upstream of dopamine [Sinakevitch and Strausfeld, 2006, Monastirioti et al., 1995, Aso et al., 2014a, Busch et al., 2009, Burke et al., 2012, Schroll et al., 2006]. One of these neurons is APL, the anterior paired lateral neuron, which extensively and specifically innervates the entire MB and has been demonstrated to contain GABA as a co-transmitter [Liu and Davis, 2009, Wu et al., 2013]. APL is an important inhibitory input in the MB calyx that maintains the sparseness of KC activity (see section 1.3) [Lin et al., 2014, Prisco et al., 2021].

Overall, the *Drosophila* olfactory system provides us with a model where the sensory processing pipeline is well characterised, transforming the odour representation at each step. Throughout this circuitry there remains the flexibility for the odour representation to be altered by modulatory signals.

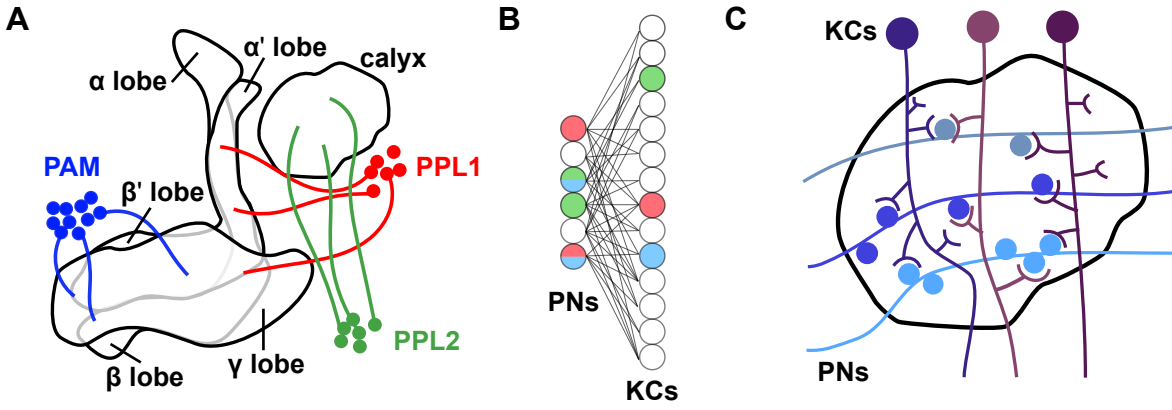


Figure 1.2: Schematic of the *Drosophila* mushroom body
 (A) Schematic of the mushroom body lobes and calyx along with their dopaminergic inputs. PPL1 neurons (red) innervate the vertical lobes, α and α' , plus the heel of the γ lobe. PAM neurons (blue) innervate the horizontal lobes, β , β' and the γ lobes. PPL2 dopaminergic neurons (green) innervate the calyx. PAM: protocerebral anterior medial, PPL1: protocerebral posterior lateral 1, PPL2: protocerebral posterior lateral 2. (B) The expansion layer circuit motif of projection neuron (PN) to kenyon cell (KC) connections. Each colour (red, green and blue) represents the neural activation by a different stimulus. The small number of PNs leads to overlapping activation patterns which can be separated by the expansion layer connectivity. The large amount of KCs then have reduced overlapping activation for each stimulus. (C) Simplified schematic of the mushroom body calyx showing the PN-to-KC connections forming the microglomerular synaptic structures. The KCs (kenyon cells, purples) form claws that enwrap PN (projection neuron, blues) boutons to form the microglomeruli.

1.2.2 Genetic tools

Neural circuit research benefits from *Drosophila* as a model organism since the nervous system is much simpler than mammals, with only around 100,000 neurons in the adult brain compared to the approximately 86 billion in the human brain [Goriely, 2025, Zheng et al., 2018]. Therefore, dedicated neural circuits that control behaviour involve smaller numbers of neurons, they are individually morphologically identifiable, and manipulation of a single or small groups of neurons are likely to have a bigger affect on behavioural output [Alekseyenko et al., 2013]. A range of incredible genetic tools can be used to target specific sub populations of neurons and control the expression of genes both spatially and temporally. One such tool for targeted gene expression is the Gal4-UAS system (Figure 1.3) [Brand and Perrimon, 1993, Fischer et al., 1988]. The Gal4 gene was identified in the yeast *Saccharomyces cerevisiae* and encodes a protein which functions as a transcription activator. The Gal4 protein binds to an Upstream Activating Sequences (UAS) site, which induces the transcription of the effector gene

under the control of the UAS [Duffy, 2002]. This system has been used in *Drosophila* to provide temporal and spatial control of gene expression. In this system, a gene of interest (the responder) is controlled by the UAS element. Since the Gal4 element is required for transcription to take place, the responder fly line does not express the gene as it remains in a silent state in the absence of Gal4. In another fly (the driver line) the Gal4 element is expressed in the cells of interest so that when the responder and driver lines are mated, the resulting progeny expresses the gene of interest (Figure 1.3 A) [Brand and Perrimon, 1993]. The Gal4 element is expressed in the driver line in a particular pattern to control where the gene of interest will be expressed. This can be under the control of a native *Drosophila* enhancer, or a plasmid with a designed enhancer can be inserted into the *Drosophila* genome. This allows precise targeted expression of the gene, since, for example, using a native enhancer which is present in only one cell type will allow Gal4 expression only in that one cell type.

This system can be extended in a number of ways to increase the specificity of targeted gene expression. For example, the Gal4 inhibitor Gal80, can be used in combination with the Gal4/UAS system to silence gene expression in a subset of cells (Figure 1.3 C) [Lee and Luo, 1999]. This system is often used so that a Gal4 is expressed in subset A and Gal80 in subset B creating a system where any neurons that fall within the overlap of the two drivers are silenced due to the Gal80 inhibition of Gal4. This can increase the specificity of a Gal4 by removing unwanted neurons from the driver expression. This system can also be used with a temperature sensitive Gal80 molecule to gain temporal control on gene expression (Figure 1.3 D) [McGuire et al., 2004]. In this case, at the permissive temperature the Gal80 is active, inhibiting Gal4 transcriptional activity, however, above a certain temperature (restrictive temperature, 29/30°C) Gal80 becomes inactive and the gene of interest is expressed. This is particularly useful for ensuring normal development and only manipulating gene expression in adult flies.

The development of the split-Gal4 allows for increased spatial resolution for targeted gene expression (Figure 1.3 B). The Gal4 protein can be split into two distinct protein

domains; the DNA-binding domain (DBD) and the activation domain (AD) [Ma and Ptashne, 1987, Keegan et al., 1986]. Both of these domains are required for the Gal4 to be active, and incapable of promoting gene expression when separated. This feature can be exploited and each domain can be targeted to different cells under control of distinct enhancers. This means that both Gal4 domains are expressed in cells where both enhancers are active and thus Gal4 activity is reconstituted in these cells only [Luan et al., 2006]. Libraries of Gal4 and split-Gal4 lines are publically available to researchers, making it easy to identify driver lines that target the neuron population of interest [Meissner et al., 2025, Jenett et al., 2012, Meissner et al., 2023].

Other binary gene expression systems exist, for example the LexA/LexAop [Lai and Lee, 2006] or QF/QUAS systems [Potter et al., 2010, Potter and Luo, 2011]. These work on the same principle as Gal4/UAS and combining these systems in the same fly can be a powerful method to express different genes in multiple subsets of neurons, allowing manipulation of separate cells independently.

1.2.3 The connectome

A connectome is a complete map of the brain, including a list of neurons and the synapses between them. This provides a wealth of information to analyse neural circuits in detail and select targets for in vivo experiments. The idea of connectomics, or developing a map of neural circuitry has been around for decades, with the first full nervous system of the nematode *C. elegans* being reconstructed in 1986 [White et al., 1986]. This reconstruction consists of electron microscopy images of serial sections of the nematode, and subsequent identification of 300 neurons, and 5,000 chemical synapses connecting them [White et al., 1986]. Despite the animal’s small size and relative simplicity, this project was a 15 year undertaking and the data acquired has several shortcomings such as the inability to identify neurotransmitters, and the thickness of the sections (75nm) truncating some neurons [White et al., 1986]. However, recent advances in microscopy and computational processing power has made it possible to develop connectomes much easier. In particular, connectivity maps of *Drosophila* 1st instar larva

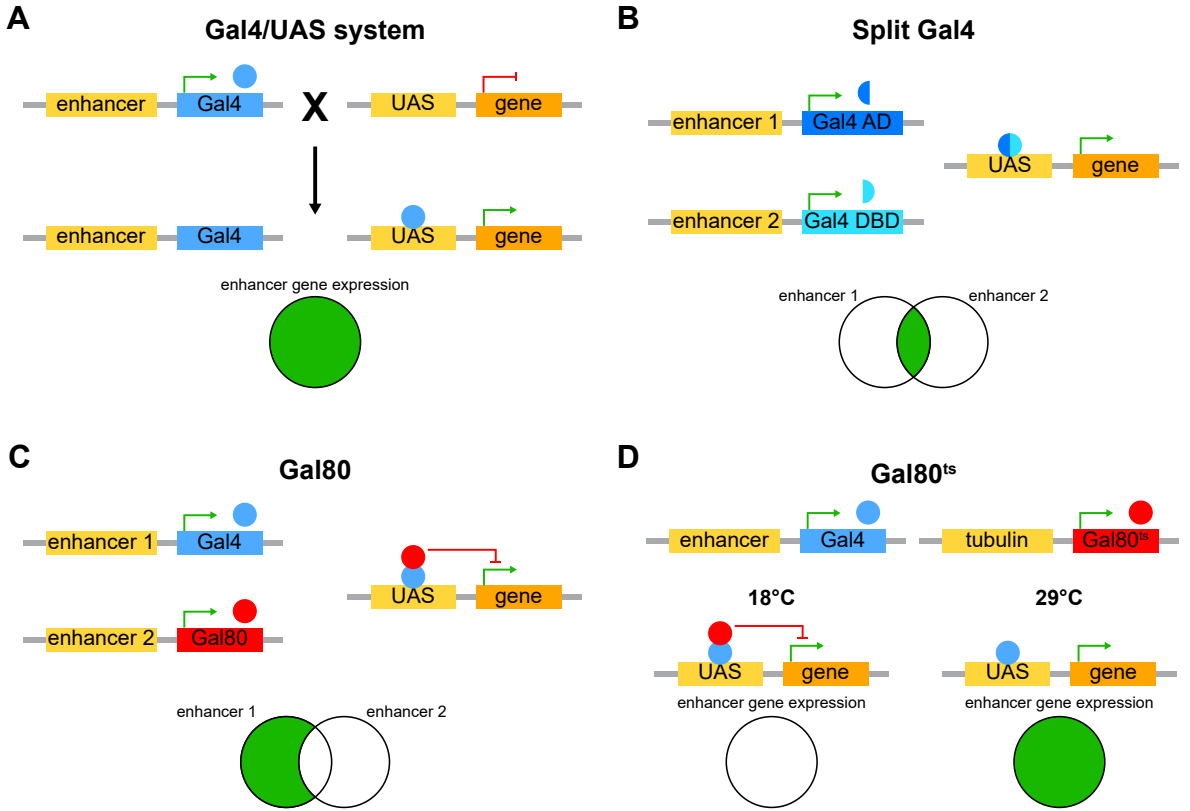


Figure 1.3: The Gal4/UAS system and variants

(A) Gal4/UAS. A specific enhancer (yellow) is upstream of the insertion site of transcription factor Gal4 (blue) that is exclusively expressed in cells where this promoter is active. UAS is inserted before a gene of interest. Offspring containing both Gal4 and UAS components express the gene of interest only in the presence of Gal4. Venn diagram showing a single green circle representing gene expression where there is also enhancer gene expression. (B) Split Gal4. Two enhancers are used to express the activation domain (AD, dark blue) or DNA binding domain (DBD, light blue) of the Gal4. The gene of interest is only expressed in cells where both Gal4 components are present. Venn diagram represents gene expression (green) only at the intersection of enhancer 1 expression and enhancer 2 expression. (C) Gal80. The Gal4 (blue) inhibitor Gal80 (red) can be used to suppress gene expression. Venn diagram represents gene expression (green) only where there is enhancer 1 expression and no enhancer 2 expression. (D) Gal80^{ts}. A temperature sensitive Gal80 inhibits Gal4 at the permissive temperature, but allows gene expression at the restrictive temperature, allowing temporal control of Gal4/UAS induced gene expression. Venn diagrams showing gene expression (green) only at the restrictive temperature of 29°C.

circuitry including the olfactory [Eichler et al., 2017, Saumweber et al., 2018, Eschbach et al., 2020, Berck et al., 2016], feeding [Schlegel et al., 2016, Miroshnikow et al., 2018], motor [Zarin et al., 2019], and visual [Larderet et al., 2017] circuits are available, plus the connectome of the full larval nervous system [Ohyama et al., 2015, Winding et al., 2023]. The speed of reconstruction of these maps for small subjects such as larva and *C. elegans* has improved such that multiple full connectomes from many individuals can be rapidly developed for comparative analysis across different developmental stages or different sexes [Valdes-Aleman et al., 2019, Witvliet et al., 2021, Cook et al.,

2019, Gerhard et al., 2017]. Even larger, and more complex circuitry is being tackled, with recent advancements in mapping the mouse visual cortex and retina [Consortium, 2025, Helmstaedter et al., 2013, Kim et al., 2014].

For *Drosophila*, multiple full connectomes of both the brain and ventral nerve cord (VNC) have been released in the past decade, providing a wealth of data on connectivity patterns but also easy to use interfaces and programmatically accessible databases to query the information [Dorkenwald et al., 2024, Schlegel et al., 2024, Zheng et al., 2018, Scheffer et al., 2020, Clements et al., 2020, Matsliah et al., 2023]. Currently, the publically available datasets are: FAFB: full adult female fly brain [Dorkenwald et al., 2024, Schlegel et al., 2024], BANC: brain and nerve cord of a female adult [Bates et al., 2025], MANC: male adult nerve cord [Takemura et al., 2023, Cheong et al., 2023], and MCNS: male adult central nervous system (brain) [Berg et al., 2025]. These datasets are available on codex, the connectome data explorer developed to simplify analysis and visualisation of the immense amounts of data [Matsliah et al., 2023]. Along with these full brain/nerve cord reconstructions there is also the hemibrain dataset, a partial reconstruction of the adult female brain available to query through the neuprint interface [Scheffer et al., 2020, Plaza et al., 2022, Clements et al., 2020]. This thesis utilises the hemibrain and the FAFB datasets for a comparative analysis of PPL2 connectivity and synapse localisation.

The FAFB dataset contains around 140,000 neurons and over 70 million synaptic connections between them for a 7 day old female fly [Zheng et al., 2018, Dorkenwald et al., 2024, Schlegel et al., 2024] whereas the partial hemibrain contains full reconstruction of 25,000 neurons and 20 million synapses plus partially reconstructed neurons that are truncated by the imaging volume from a 5 day old female fly [Scheffer et al., 2020]. Additionally, the two datasets were acquired at different resolution and with different acquisition methods, FAFB using serial section electron microscopy (ssTEM) with a resolution of $4 \times 4 \times 40 \text{ nm}^3$ versus the hemibrain imaging via focused ion beam scanning electron microscopy (FIB-SEM) with a resolution of $8 \times 8 \times 8 \text{ nm}^3$ [Zheng et al., 2018, Scheffer et al., 2020, Briggman and Bock, 2012]. After acquisition, images were

aligned, neurons automatically segmented, cell types assigned, synapses automatically identified, and neurotransmitter predictions made [Dorkenwald et al., 2025, Dorkenwald et al., 2022, Heinrich et al., 2018, Buhmann et al., 2021, Yu et al., 2025, Huang et al., 2018, Costa et al., 2016, Eckstein et al., 2024]. A comparative analysis of these two datasets at the time of FAFB’s release showed that Of 8,453 cell types, 3,643 were previously identified in the hemibrain, and 4,581 are new types, mostly from brain regions outside the hemibrain volume [Schlegel et al., 2024]. Synapse counts are correlated between the two datasets, and the core connectome is largely conserved with differences in synapse count of up to 30% being explainable by technical noise [Schlegel et al., 2024]. The FAFB data, and the other Flywire datasets found on Codex, rely heavily on community efforts for proofreading, annotations, neuron reconstructions, and analysis [Matsliah et al., 2023, Dorkenwald et al., 2022]. There are over 9,000 annotations submitted by members of the Flywire community, including corrections to neurotransmitter predictions, genes of interest known to be expressed by particular neurons, cell-type labelling and there have even been citizen scientist data analysis competitions to accelerate findings [Dorkenwald et al., 2022, Matsliah et al., 2023, Dorkenwald et al., 2025, Dorkenwald et al., 2024].

Overall, these datasets provide invaluable information on neural circuitry including precise synapse locations, cell types, neurotransmitter predictions, neuron morphology, and the ability for comparative analysis. Connectomes have helped accelerate scientific progress by providing targets for analysis and a basis for computational models. For example, analysis and computational modelling based on the full connectome of *C. elegans* motor circuit suggested that mechano-sensory feedback was an essential component in the circuit for forward motion, which was then confirmed experimentally [White et al., 1986, Wen et al., 2012, Bryden and Cohen, 2008]. However, it is important to remember that these connectomes are a snapshot of an individual brain at one point in time. Neural circuits are not static, and are constantly being shaped by modulatory signals as a consequence of previous experience, internal state, or environmental context.

1.3 The mushroom body calyx

Having established *Drosophila* as a powerful model for dissecting neural circuit function, with powerful genetic tools and synaptic level connectome data, we now focus on the main topic of this thesis. The MB sits at the interface between innate and learned olfactory processing, receiving sensory input from PNs and transforming it into sparse, high-dimensional representations that underpin learning and memory. This makes the MB an ideal system to investigate how neuromodulatory mechanisms enable flexible and adaptive coding. In particular, the MB calyx, the entry point for olfactory information into the MB, provides a tractable model for studying how defined neuromodulatory inputs, such as those from the dopaminergic PPL2 cluster, shape sensory representations. Understanding how these modulatory signals influence the PN-to-KC synapse offers crucial insight into how the fly brain dynamically reconfigures its sensory networks according to experience, internal state, and behavioural context.

1.3.1 Calyx organisation

In the calyx, KCs receive olfactory information from the ~ 50 types of PNs arriving from the antennal lobe via the mALT or lALT tracts and collectively represent the odour identity [Marin et al., 2002, Tanaka et al., 2004]. While the axons of each type of KCs are extremely organised in layers to give rise to the different lobes of the MB, the dendrites are less structured. During development, there are four neuroblasts that each give rise to every type of KC and create four loosely defined territories in the calyx which KC dendrites occupy [Leiss et al., 2009, Aso et al., 2014a, Zhu et al., 2003]. KC- γ dendrites mostly make up the calyx core, while KC- α/β and KC- α'/β' dendrites are found in the periphery [Leiss et al., 2009, Aso et al., 2014a, Zhu et al., 2003].

The PN-to-KC connection is a key point in the olfactory circuit, forming what is known as an ‘expansion layer’ motif which is important for creating a sparse, combinatorial, and distributed representation of an odour among the KC population [Turner

et al., 2008, Honegger et al., 2011, Campbell et al., 2013, Perez-Orive et al., 2002]. The expansion layer circuit motif consists of a small population of neurons sending information to a much larger population of neurons in a combinatorial manner (Figure 1.2 B) [Luo, 2021]. In the case of the *Drosophila* olfactory system, each KC receives input from on average 7 PNs and responds only when input from multiple of these channels is present [Caron et al., 2013, Leiss et al., 2009, Honegger et al., 2011, Gruntman and Turner, 2013, Ahmed et al., 2023]. This leads to a sparse encoding of odour identity across the population of KCs, where only 5-10% of the population responds to each odour, and each individual KC only responds to a small portion of odours [Honegger et al., 2011, Turner et al., 2008, Jortner et al., 2007]. This circuit motif and subsequent coding strategy is beneficial for energy efficiency, memory storage capacity, and pattern separation of complex inputs [Olshausen and Field, 2004, Babadi and Sompolinsky, 2014, Litwin-Kumar et al., 2017]. Therefore, it is not surprising that we see sparse neuronal activity across a variety of sensory systems and brain regions such as the primate visual system [Vinje and Gallant, 2000], rodent auditory cortex [DeWeese et al., 2003], somatosensory cortex [Hafner et al., 2004], hippocampus [Thompson and Best, 1989, Cayco-Gajic and Silver, 2019], and the cerebellum [Litwin-Kumar et al., 2017, Marr, 1969, Cayco-Gajic and Silver, 2019]. In fact, this expansion network architecture is also the basis of many ‘brain-inspired’ neural network machine learning algorithms [Recanatesi et al., 2019, Coop and Arel, 2013, Chen et al., 2024]. In the *Drosophila* olfactory circuit this sparse encoding of odour information is crucial for stimulus discrimination, due to the non-overlapping representations [Parnas et al., 2013, Lin et al., 2014]. This dimensionality expansion allows discrimination between many more odours than the number of OSNs available and enhances the memory capacity by allowing more stimulus-specific associations, reminiscent of the elemental theory of learning [Lin et al., 2014, Groschner and Miesenböck, 2019].

While most inputs to the calyx are olfactory, about 20% of sensory inputs are from non-olfactory centers, placing the MB calyx as a multimodal integration zone [Tanaka et al., 2008, Zheng et al., 2018, Scheffer et al., 2020]. The ventral, dorsal, and lateral accessory calyces are three subregions which do not receive olfactory information but

instead are a site of convergence for non-olfactory inputs [Yagi et al., 2016]. Visual information innervates specific KCs, the $KC\gamma$ -d and $KC\alpha/\beta$ -p sub-types, in the ventral and dorsal accessory calyces respectively [Li et al., 2020b, Butcher et al., 2012, Vogt et al., 2016]. Similarly to the olfactory information, KCs receiving visual input also produce a sparse, combinatorial encoding of information [Ganguly et al., 2024]. The lateral accessory calyx is innervated by $KC\alpha'/\beta'$ -ap and is a hub for thermo- and hygrosensory inputs, although these can also innervate olfactory KCs in the main calyx [Marin et al., 2020, Caron et al., 2013]. Lastly, the calyx receives input from gustatory centers to both the dorsal accessory calyx and the main calyx [Kirkhart and Scott, 2015].

1.3.2 Microglomerular architecture of PN-KC synapses

PN-to-KC connections form large, complex synaptic structures called microglomeruli [Yasuyama et al., 2002]. Microglomeruli consist of a single PN that forms a large pre-synaptic bouton which is enwrapped by claw-like dendrites from several KCs which can be of any type [Baltruschat et al., 2021, Leiss et al., 2009] (Figure 1.2 C). Microglomerular structures can be found in the MB calyx across insect species including honey bees [Ganeshina and Menzel, 2001], crickets [Frambach et al., 2004], ants [Steiger, 1967], and cockroaches [Groh and Rössler, 2011] although the calyx size and morphology varies between species [Farris, 2005]. In *Drosophila*, microglomeruli are relatively large, but less distinct, less circular, and more unevenly distributed compared to social insects such as bees, and ants [Groh and Rössler, 2011]. PN boutons that form the core of the microglomeruli are synaptically dense and can be one of three forms: unilobed, which consist of one round bouton, clustered, consisting of multiple round terminals, or elongated, with several boutons in a beads-on-string arrangement [Butcher et al., 2012]. These morphological types can be found on the same PN, although the functional differences are currently not known. KC claws also have different morphological types, and can be simple, a single claw or complex, comprising of many claw-like processes [Butcher et al., 2012, Lee et al., 1999]. Each claw enwraps a single PN bouton,

approximately 11 claws wrap each bouton, and each KC has on average 6 claws, although claws are distributed non-uniformly across KC types with KC γ possessing more claws on average [Butcher et al., 2012, Li et al., 2020a, Caron et al., 2013, Baltruschat et al., 2021]. There have been different estimates of the total number of boutons in the *Drosophila* calyx, ranging from 786 to 1,590. The differences depend on the method used to count the boutons, with 1,115 as a mathematical estimate based on the number of PNs and distribution of boutons [Turner et al., 2008]. More conservative estimates have been made in the range of 786 ± 73 by counting actin-rich rings labeled with GFP although this number doubled when using a different labeling method, using LimK-GFP, a regulator of actin dynamics overexpression in KCs [Leiss et al., 2009, Frambach et al., 2004].

In contrast to the LH, the PN-to-KC connections are not stereotyped across individuals and inputs to a particular KC are seemingly random [Caron et al., 2013]. Characterisation of PN inputs to individual KCs through multiple methods including electrophysiological recordings of KC odour responses, dye tracing of the inputs, and larval and adult EM studies have provided evidence of these unstructured connections [Murthy et al., 2008, Caron et al., 2013, Eichler et al., 2017, Zheng et al., 2018]. However, it was found that the connections, although unstructured, are not completely random. PN types innervating different antennal lobe glomeruli can have a different number of neurons, and a different number of boutons per PN, creating connection biases towards PNs that are more represented [Li et al., 2020a, Turner et al., 2008, Caron et al., 2013]. Furthermore, PNs representing different ecologically relevant odour categories, e.g. food, or pheromones, occupy different areas of the calyx [Tanaka et al., 2004, Zheng et al., 2022, Li et al., 2020a]. KCs have been shown to randomly sample inputs from local PNs, leading to spatial segregation of categorical odour inputs [Zheng et al., 2022, Tanaka et al., 2004, Choi et al., 2022]. The input biases are not uniform across KC types, however, and it has been shown that although KC- α/β and KC- α'/β' have a bias towards inputs from food-related PNs, KC- γ neurons do not show a bias towards a particular ecological type of PN [Chan et al., 2025].

These PN-to-KC connections described above are the main neurons that make up the MB calyx, forming the core of the microglomeruli units and encoding odour information in a sparse manner to aid in stimulus pattern separation [Olshausen and Field, 2004, Lin et al., 2014]. However, sensory inputs are not the only type of input to the calyx, there are other types of neurons in this area to modulate connections, bridge to the LH, or provide inhibitory signals.

1.3.3 Inhibition from APL

The APL neuron is a GABAergic neuron which extensively and specifically innervates the entire MB, including all lobes and calyx, providing inhibitory feedback to KCs, suppressing their activity [Prisco et al., 2021, Lin et al., 2014, Lei et al., 2013, Tanaka et al., 2008, Liu and Davis, 2009, Wu et al., 2013]. Therefore, as KCs become more active in response to an odour, APL can suppress the firing, enhancing the contrast between active and inactive KCs. In terms of the sparse sensory encoding of odours, APL ensures that only a small subset of KCs respond, while others are inhibited [Lin et al., 2014, Olshausen and Field, 2004]. Each hemisphere contains a single, large APL neuron with pre- and post-synaptic terminals distributed throughout the entire neuron skeleton, with no anatomical distinction between axon and dendrite [Tanaka et al., 2008, Liu and Davis, 2009, Wu et al., 2013]. This is contrasting to in the *Drosophila* larval brain, where the APL neuron receives input from the MB lobes, and sends feedback to the calyx [Masuda-Nakagawa et al., 2014]. APL is both pre- and post- synaptic to PNs and KCs in the calyx, and is known to contribute to the microglomeruli synaptic structures [Yasuyama et al., 2002, Wu et al., 2013, Baltruschat et al., 2021, Prisco et al., 2021]. KC odour activity in the microglomeruli is normalised via an APL-KC negative feedback loop, and the spatial localisation of APL inhibition restricts affects to different MB compartments or specific microglomeruli, depending on where APL was stimulated [Amin et al., 2020, Prisco et al., 2021, Inada et al., 2017]. Therefore, APL is in the prime position to provide localised inhibition to sparsen KC activity as is also seen in the locust analog, the giant GABAergic neuron [Lin et al.,

2014, Lei et al., 2013, Amin et al., 2020, Prisco et al., 2021, Papadopoulou et al., 2011]. In fact, silencing APL results in a decrease in sparsity among the KC population, coupled with the fly losing its ability to discriminate between similar odours due to the increased overlap in sensory representation [Lin et al., 2014].

This APL-KC negative feedback loop is complemented by the existence of inhibitory KC-KC connections [Bielopolski et al., 2019]. The development of detailed connectomes of the MB have revealed a large amount of KC-KC reciprocal synapses [Takemura et al., 2017, Li et al., 2020a, Zheng et al., 2018]. KC pre-synaptic zones are mostly spatially segregated from PN boutons, suggesting that they are not a part of the microglomeruli structures, but can synapse onto other KCs, APL, PN axons and other neurons [Christiansen et al., 2011].

While the APL neuron provides a dominant source of inhibition that maintains sparse and selective KC responses, it operates within a broader network of modulatory and feedback pathways that shape activity within the calyx. Several other neuron types, and neurotransmitters, also innervate this region and contribute to fine-tuning olfactory processing. For example, octopamine is co-released by APL and other octopamine specific neurons innervate the calyx [Wu et al., 2013, Busch et al., 2009, Wong et al., 2021], whereas the MB-C1 interneurons provide additional inhibitory input directly within microglomeruli [Baltruschat et al., 2021]. Moreover, dopaminergic neurons from the PPL2 cluster project to the calyx, where they are positioned to influence both KC and PN terminals (see section 1.4.3) [Li et al., 2020a, Mao and Davis, 2009, Zheng et al., 2018, Boto et al., 2019]. Together, these interconnected modulatory systems give the calyx the capacity for dynamic adjustment of sensory representations.

1.3.4 Structural plasticity of microglomeruli

A huge amount of structural plasticity takes place during development as neural circuits are sculpted, synapses created and pruned, and new neurons are born [Tavosanis, 2012]. This neural development can be influenced by external factors such as enrich-

ment of sensory input, temperature, or stress [McEwen, 2010]. In the MB calyx, PN bouton and microglomeruli connections are controlled by the KC population during development [Elkahlah et al., 2020, Ahmed et al., 2023]. When the population of KCs is decreased via neuroblast ablation or increased in *mushroom body defect* mutants, the PNs bidirectionally alter the number of boutons in order to maintain an optimal number of PN inputs onto each KC and therefore maintain a sparse distribution of activity for odour encoding [Elkahlah et al., 2020, Ahmed et al., 2023]. This developmental plasticity allows the calyx microcircuits to be computationally robust, having minimal effect on the odour representation. However, explicitly altering the number of claws per KC results in the fly losing the ability to properly discriminate between odours [Ahmed et al., 2023]. In the case of an increased number of claws, each KC becomes less selective of odours it will respond to, therefore losing sparsity as more KCs respond to each odour [Ahmed et al., 2023, Lin et al., 2014]. On the other hand, a decreased number of claws leads to the opposite effect, where less KCs receive enough input to overcome their high firing rate, meaning the population-level responses are too sparse leading to delayed behavioural responses [Ahmed et al., 2023]. This developmental plasticity suggests that the KC population controls the PN-to-KC connectivity in the calyx, and that the number of PN inputs to KCs is important for maintaining a useful sparse odour representation.

Structural plasticity does not only occur during development, circuits can be re-wired and altered during the course of an animals lifetime [Marzola et al., 2023]. In honey bees there are age-related increases in calyx size and dendritic complexity while dendritic spine density remains constant, this changes occur even in the absence of social interaction or foraging activity [Farris et al., 2001, Withers et al., 1995, Fahrbach et al., 1998]. Similarly, *Drosophila* ageing is coupled with an increase in size of pre-synaptic active zones in the calyx [Gupta et al., 2016]. Activity, or lack of activity of particular PNs also specifically alters the structure of the associated microglomeruli [Kremer et al., 2010, Pech et al., 2015, Baltruschat et al., 2021]. Prolonged deprivation of PN synaptic input to KCs leads to increased microglomeruli number and enlarged pre and postsynaptic elements in microglomeruli associated with the silenced PN [Kre-

mer et al., 2010]. This structural change is similar to changes that happen in the antennal lobe, where prolonged exposure to an odour leads to odour specific changes in glomeruli volume, increased inhibitory input to glomeruli, decreased PN activity and therefore decreased response to the pre-exposed odour [Devaud et al., 2001, Devaud et al., 2003, Sachse et al., 2007]. The microglomeruli in the calyx also undergo structural changes during associative conditioning which results in additional synaptic connections [Baltruschat et al., 2021, Hourcade et al., 2010]. During memory consolidation we can see an increase in the number of PN boutons, which are functional, synaptically connected to KCs, and respond in an odour-specific manner [Baltruschat et al., 2021].

Overall, the MB calyx is a site of extensive neural plasticity, both at the structural and synaptic levels, but it is a subregion of the MB that remains relatively unexplored. Modulatory inputs by dopaminergic neurons have the potential to update sensory representations based on external or internal states, upstream of the site of associative memory.

1.4 Dopamine systems in *Drosophila*

The classical role for dopamine is to encode the reward prediction error that drives associative learning. This depends on recognising a reward and updating the value of the stimulus based on the unexpected reward. This difference between the reward received and the expected reward is termed the reward prediction error (RPE) and is used as a learning signal to update the expectation. Rescorla and Wagner [Rescorla, 1972] formulated this idea mathematically in the Rescorla-Wagner rule which suggests that the strength of association between a stimuli and its associated reward is updated in proportion to the RPE. In this way, the prediction error drives learning. Numerous observations of animal behaviour seem to show that some sort of reinforcement learning is happening in the brain, and recordings of neural activity during rewarding behaviours also supports this idea [Ljungberg et al., 1992, Pizzagalli et al., 2008]. However, recent

research has revealed a more complex role for dopamine signals, responding to both reward and punishment in associative learning paradigms [Lerner et al., 2015, Zweifel et al., 2011, Matsumoto et al., 2016]. In fact, some dopamine neurons may even encode the innate value of odours, responding and providing a continuous value update signal during exploration without explicit external reinforcement [Kato et al., 2023]. Dopamine signalling is not always excitatory either, it can modulate circuits in diverse ways depending on cell type, receptor, and internal state.

1.4.1 Dopamine receptors

The role of dopamine as a reward signal is well established, but the release of dopamine on neural circuits modulates their activity in diverse ways depending on the dopamine receptors receiving the signal. The mammalian brain expresses 5 different types of dopamine receptors which can be split into 2 categories: excitatory D1 receptors, D1 and D5, and inhibitory D2 receptors, D2, D3 and D4 [Civelli et al., 1993, Niznik and Van Tol, 1992]. This means that dopamine can bidirectionally effect downstream circuits.

In the brains of *Drosophila* there are four types of dopamine receptors: Dop1R1 (aka DUMB) and Dop1R2 (aka DAMB) are classified as D1-like, Dop2R is D2-like, and there is one unique dopamine/ecdysteroid receptor, DopEcR [Feng et al., 1996, Gotzes et al., 1994, Han et al., 1996, Hearn et al., 2002, Srivastava et al., 2005]. Dopamine receptors in *Drosophila*, like in mammals, are G-protein coupled receptors, or GPCRs [Hanlon and Andrew, 2015]. This means that the receptor is coupled with an intracellular heterotrimeric G-protein, made up of α , β , and γ subunits [Hanlon and Andrew, 2015, Syrovatkina et al., 2016]. When the ligand, in this case dopamine, binds to the receptor it induces conformational changes in the GPCR which allow the α subunit to dissociate from the β and γ subunits [Syrovatkina et al., 2016]. Both the $G\alpha$ subunit and $G\beta\gamma$ dimer signal to various cellular pathways depending on the family of G-protein [Syrovatkina et al., 2016]. For example, one of the best described pathways either activated or inhibited by different families of the $G\alpha$ subunit is the adenylyl

cyclase/cyclic adenosine monophosphate (cAMP) pathway [Cooper et al., 1995, Davis et al., 1995]. Flies defective in cAMP metabolism, such as *dunce* and *rutabaga* mutants, are impaired in learning and/or memory in associative conditioning tasks, suggesting the strong role for cAMP signalling in these tasks [Davis et al., 1995, Martín et al., 2001, Zhong et al., 1992]. KCs in the *Drosophila* MB have also been shown to express all four of these dopamine receptor types [Deng et al., 2019, Kondo et al., 2020], and throughout the brain the expression is highly overlapping, unlike the spatial segregation of the D1- and D2-like receptors in the mammalian brain [Gerfen and Surmeier, 2011, Croset et al., 2018, Kondo et al., 2020, Hiramatsu et al., 2025].

Dop1R1

Dop1R1 is highly expressed in the MB and strongly couples the $G_{\alpha s}$ subunit [Kim et al., 2003, Himmelreich et al., 2017, Sugamori et al., 1995]. In the cAMP pathway, the activated $G_{\alpha s}$ subunit binds to and activates the enzyme adenylyl cyclase (AC), which catalyses the conversion of ATP to cAMP. In turn, increases of cAMP may lead to the activation of cyclic nucleotide-gated ion channels, exchange proteins activated by cAMP (EPAC), and the enzyme protein kinase A (PKA) [Davis et al., 1995, Cooper et al., 1995]. PKA is the most well understood effector of cAMP. cAMP binds to regulatory subunits of PKA, freeing the PKA catalytic subunits which then phosphorylate many targets, e.g. voltage-gated calcium channels, or transcription factors such as CREB which is crucial for long-term memory formation [Horiuchi et al., 2008, Miyashita et al., 2018].

Within the MB, Dop1R1 is strongly expressed in the γ and $\alpha\beta$ lobes as well as the calyx [Deng et al., 2019, Kondo et al., 2020, Hiramatsu et al., 2025]. This receptor is required for aversive and appetitive olfactory learning, with expression of Dop1R1 in KCs being sufficient to rescue the learning deficits of *dumb* mutants with abnormal Dop1R1 expression, in both aversive and appetitive learning paradigms [Kim et al., 2007, Sun et al., 2020]. Dopamine activity in the MB lobes is thought to act as a coincidence detector in associative learning, acting in concert with KC odor-evoked activity

dependent Ca^{2+} influx to increase cAMP and PKA in order to generate plasticity in KC Ca^{2+} responses [Tomchik and Davis, 2009, Gervasi et al., 2010]. This dopamine induced plasticity is compartmentalised in the MB lobes, with the γ lobes showing the most sensitivity to elevations of cAMP [Boto et al., 2014, Cohn et al., 2015]. In fact, Dop1R1 activity in KC γ only is sufficient to support all temporal stages of aversive associative memory, suggesting that the KC γ are recruited first to the memory trace, followed by a relaying of information to KC $\alpha\beta$ during consolidation [Qin et al., 2012, Boto et al., 2014, Blum et al., 2009]. This evidence points towards Dop1R1 as the central coincidence detector for associative learning in the MB lobes, providing spatially specific dopamine inputs from PPL1 or PAM neurons to induce plasticity in segregated compartments [Qin et al., 2012, Boto et al., 2014, Hige et al., 2015b, Hige et al., 2015a]. Dop1R1 in KC γ is also required for male courtship conditioning, inducing lasting changes in the processing of cVA [Keleman et al., 2012]. This provides evidence that dopamine signalling via Dop1R1 is important for different types of learning in the MB.

Beyond associative learning, caffeine-induced wakefulness requires Dop1R1 in the MBs [Andretic et al., 2008] and loss of function mutants enhance startle-induced arousal while increasing sleep [Lebestky et al., 2009]. Moreover, it has been found that knock-down of Dop1R1 in a subset of clock neurons results in reduced cAMP, and decreased daytime sleep, suggesting a complex role for dopamine signalling via Dop1R1 in sleep regulation across multiple brain regions including the MB and the central complex [Fernandez-Chiappe et al., 2020]. Sleep deprivation is associated with learning deficits, and also an overall transcriptional downregulation of Dop1R1 in the brain [Seugnet et al., 2008]. This sleep-loss induced performance loss can be rescued with specific expression of Dop1R1 in the MBs, emphasising the core role of Dop1R1 in MB learning and memory [Seugnet et al., 2008]. Additionally, loss of Dop1R1 signalling in the MBs results in hyperactive flies that ignore food [Landayan et al., 2018], knockdown specifically in KC- γ induces increased motor output in larva [Silva et al., 2020], and Dop1R1 signalling is involved in selection of goal-directed behaviours in the ellipsoid body (EB), working in parallel with Dop1R2 to affect the excitability state of EB neu-

rons [Frighetto et al., 2022]. This evidence suggests a range of behaviours that are controlled by Dop1R1 signalling across different areas of the brain.

Overall, Dop1R1 acts as the major excitatory dopamine receptor in the fly brain, translating dopamine release into increased cAMP and enhanced Ca^{2+} , controlling a range of behaviours including learning and memory [Seugnet et al., 2008, Kim et al., 2007, Berry et al., 2012, Himmelreich et al., 2017], sleep and arousal [Seugnet et al., 2008, Andretic et al., 2008, Lebestky et al., 2009], goal-directed behaviours [Frighetto et al., 2022] and locomotion [Silva et al., 2020].

Dop1R2

The second D1-like receptor, Dop1R2, is interesting since it can couple two different G-proteins, $\text{G}\alpha\text{s}$, and $\text{G}\alpha\text{q}$ [Himmelreich et al., 2017]. While Dop1R2 couples $\text{G}\alpha\text{s}$ at high concentrations of dopamine to elevate cAMP, $\text{G}\alpha\text{q}$ activates a signalling cascade that results in the release of intracellular calcium stores, directly increasing Ca^{2+} levels at lower dopamine concentrations [Himmelreich et al., 2017].

This receptor is mainly expressed in the MB, with particularly strong immunoreactivity in the α and β lobes compared to the γ lobe, pedunculus, or calyx regions [Han et al., 1996, Deng et al., 2019]. Dop1R2 has been implicated in regulating active forgetting in the MB [Berry et al., 2012, Berry et al., 2018]. *damb* mutants exhibit significantly enhanced aversive memory retention up to 24hours, the time point at which memory in control flies is completely forgotten [Berry et al., 2012]. Specifically, knockdown of $\text{G}\alpha\text{q}$ subunit in the MB inhibits forgetting, identifying the role of the Dop1R2/ $\text{G}\alpha\text{q}$ pathway in forgetting [Himmelreich et al., 2017]. There is also a role for Dop1R2 in KCs for memory stabilisation [Sun et al., 2020, Placais et al., 2017]. Knockdown of Dop1R2 in KCs results in intact short-term but impaired long-term appetitive memory [Sun et al., 2020, Musso et al., 2015]. Specifically, it was found that Dop1R2 stabilises long-term appetitive memory via a mitogen-activated protein kinase (MAPK) pathway in KCs [Sun et al., 2020]. Flip-out of Dop1R2 specifically in the $\alpha\beta$ and $\alpha'\beta'$ but not γ lobes impairs long-term memory, implicating this re-

ceptor in later forms of memory [Kaldun et al., 2025, Blum et al., 2009]. The role of Dop1R2 complements Dop1R1 to allow for adaptive behaviour when the temporal relationship of odor cues and dopaminergic reinforcement change [Handler et al., 2019]. While Dop1R1 is required for the depression of KC-MBON synapses when an odor is followed by the presentation of a reward (forward pairing), on the other hand Dop1R2 is responsible for KC-MBON potentiation during backwards pairing, where the reward comes before the odor [Handler et al., 2019]. This synaptic plasticity leads to opposing behavioural output dependent on the temporal dynamics of the association [Handler et al., 2019, Jacob and Waddell, 2020, Tanimoto et al., 2004].

Outside of the MB, dopamine activity in the superior medial protocerebrum acts through Dop1R2 to regulate male mating drive [Zhang et al., 2016]. Interestingly, ubiquitous Dop1R2 knockdown leads to critical developmental problems, with a reduced number of flies emerging from pupa and those that did displaying cuticle and wing abnormalities [Regna et al., 2016].

Overall, Dop1R2 expression is highly overlapping with Dop1R1 in KCs [Croset et al., 2016] and complements the function of Dop1R1 by allowing dopamine to regulate KC-MBON signalling in opposing ways [Handler et al., 2019]. While Dop1R1 activity seems to be involved in all temporal stages on memory, Dop1R2 functions to affect long-term memory, either in the stabilisation or forgetting of memories [Berry et al., 2018, Sun et al., 2020].

Dop2R

Dop2R is *Drosophila*'s sole D2-like receptor that inhibits AC via G α i/o, thus reducing cAMP levels [Hearn et al., 2002]. Dop2R is expressed in all lobes of the MB, however is less abundant in KCs compared to the other dopamine receptors [Deng et al., 2019, Croset et al., 2018]. However, Dop2R is highly expressed in dopamine neurons themselves, with PAM neurons expressing both Dop1R1 and Dop2R while PPL1 express Dop2R only [Croset et al., 2018, Hiramatsu et al., 2025]. Dop2R is expressed pre-synaptically in these neurons to act as autoreceptors, activating at high concentra-

tions of dopamine to regulate dopamine release through negative feedback [Vickrey and Venton, 2011, Shin and Venton, 2022, Hiramatsu et al., 2025]. Knockdown of Dop2R in KCs or DANs results in impairments in appetitive and aversive olfactory memory in both adults and larva, suggesting a key role in memory [Qi and Lee, 2014, Qi et al., 2025]. In the adult, the loss of Go signalling in the $\alpha\beta$ or γ lobes leads to impaired aversive short-term memory, showing that inhibition of these areas is required for memory acquisition [Zhang and Roman, 2013, Ferris et al., 2006, Madalan et al., 2012].

Dop2R has been shown to regulate the GABAergic systems in the MB, for example working alongside Dop1R1, both expressed in a GABAergic MBON, to provide feedback inhibition that regulates consolidation of appetitive memory [Pavlovsky et al., 2018]. Another key GABAergic neuron innervating the MB is the APL neuron [Prisco et al., 2021, Liu and Davis, 2009]. APL is suppressed after learning and this can be traced to Dop2R mediated inhibition, suggesting an important role for dopamine regulation of GABAergic signals, releasing the inhibition from KCs [Liu and Davis, 2009, Zhou et al., 2019].

Apart from learning and memory, Dop2R has been implicated in other behaviours such as promoting female egg-laying [Lyman et al., 2023] and hormonal metabolism [Karpova et al., 2011, Karpova et al., 2010], male courtship [Love et al., 2023], and locomotion [Draper et al., 2007]. In summary, Dop2R provides a counter to Dop1R1 mediated excitation, preventing cAMP accumulation and providing a negative feedback mechanism [Vickrey and Venton, 2011, Pavlovsky et al., 2018]. Dop2R has a clear role in olfactory memory, and particularly is associated with disinhibition mechanisms of GABAergic neurons [Zhou et al., 2019, Pavlovsky et al., 2018].

DopEcR

DopEcR is a unique receptor that binds both dopamine and the major steroid hormones in insects, ecdysone and 20E, allowing it to integrate neuromodulatory and hormonal cues [Srivastava et al., 2005]. Although the G-protein subunits coupled to DopEcR have not yet been described, it has been determined that activation of the receptor by

dopamine can increase cAMP levels to activate the PI3K pathway, whereas ecdysteroid binding can activate the MAPK pathway, or inhibit dopamine stimulated cAMP levels [Srivastava et al., 2005, Ishimoto et al., 2013]. It is expressed strongly in the $\alpha\beta$ lobes, γ lobe, and calyx [Deng et al., 2019, Ishimoto et al., 2013, Aranda et al., 2017].

DopEcR expression in KCs is required for courtship memory, with expression of DopEcR in the $\alpha\beta$ lobes was sufficient to rescue deficits in DopEcR mutants [Ishimoto et al., 2013]. When dopamine synthesis was blocked in flies, courtship memory was impaired, however feeding of 20E hormone rescued this deficit, suggesting that ecdysone signalling via DopEcR can provide a compensatory mechanism to increase cAMP levels [Ishimoto et al., 2013]. The evidence of DopEcR’s role in courtship conditioning and in affecting the cAMP levels in the MB suggest a role in associative conditioning, however this has not been investigated in depth so far.

The majority of literature surrounding DopEcR implicates this receptor in physiological and behavioural response to stressors that increase ecdysone levels such as starvation [Inagaki et al., 2012], heat [Petruccelli et al., 2020], courtship rejection [Ishimoto et al., 2013], and alcohol [Petruccelli et al., 2016, Rauschenbach et al., 2000]. For example, in the MB, KCs show nicotine-induced Ca^{2+} responses, which are enhanced in the calyx and KC cell bodies with ecdysone application in a DopEcR-dependent manner [Lark et al., 2017]. Overall, DopEcR provides a unique receptor that allows internal-state dependent hormones to regulate neural circuits.

1.4.2 Internal state dependent dopamine

We know that dopamine input to the lobes of the MB integrates external valence information, e.g. from an electric shock, with the odour information to update the odour representation in an associative conditioning paradigm. However, learning from a previous exposure to an odour is not the only way sensory representations are modulated. For example, the mating status, age, satiety, or motivations of the animal can alter odour representations to change the behavioural output in accordance to the internal

state.

Beyond external sensory input, the mushroom body (MB) integrates information about the animal’s internal state, allowing learned odour representations to adapt to motivational and physiological context. Hunger, satiety, mating status, age, and arousal state all influence how sensory information is processed and how reinforcement signals shape memory formation [Inagaki et al., 2012, Krashes et al., 2009, Lin, 2023, Vogt et al., 2021, Corrales-Carvajal et al., 2016]. For instance, hunger enhances appetitive learning and can even reverse aversive memory expression through activation of specific protocerebral anterior medial (PAM) dopaminergic neurons that signal nutritional value [Krashes et al., 2009, Ko et al., 2015, Senapati et al., 2019]. Starvation increases dopaminergic drive onto MB compartments associated with approach-promoting MBONs, biasing odour-guided behavior toward food-related cues [Perisse et al., 2016, Placais et al., 2017, Landayan et al., 2018]. Conversely, satiation recruits inhibitory feedback that suppresses memory expression [Placais and Preat, 2013]. The MB also receives input from neurons releasing neuropeptides such as neuropeptide F (NPF), the *Drosophila* homolog of mammalian neuropeptide Y, which conveys information about motivational state and reward sensitivity [Chung et al., 2017, Krashes et al., 2009]. NPF acts as a co-transmitter in a subset of KCs and can directly modulate dopaminergic neurons, enhancing reward signalling when flies are hungry or sexually deprived [Crocker et al., 2016, Johard et al., 2008]. With age and experience, MB dopamine signalling and synaptic plasticity also undergo remodelling, contributing to changes in learning capacity and memory persistence [Tamura et al., 2003, White et al., 2010]. Collectively, these mechanisms ensure that the MB remains a flexible integration hub, dynamically weighting sensory and reinforcement signals according to the fly’s internal state to optimize behavioural outcomes.

1.4.3 PPL2 dopaminergic neurons

The only dopaminergic neurons innervating the calyx are from the cluster PPL2, the activation of which has been shown to increase the calcium responses to odour in the

MB γ lobe [Boto et al., 2019]. The PPL2 cluster responds to odour and to electric shock but activation is not sufficient to induce any memory when paired with an odour, contrasting with both PPL1 and PAM which induce aversive and approach memory, respectively [Boto et al., 2019, Riemensperger et al., 2005, Liu et al., 2012a, Aso et al., 2010, Aso et al., 2012]. However, PPL2 activation does affect memory strength. When PPL2 activation was paired with a shock and odour presentation, this lead to an increased behavioural response, which is interpreted as a stronger aversive memory [Boto et al., 2019]. One member of the PPL2 cluster, PPL201 has been shown to be suppressed by repeated odour presentation, and the suppression is accelerated by increasing the odour pulse rate [Marquis and Wilson, 2022]. PPL2 activation also drives an increase in behavioural response in innate behaviours such as increasing the salience of sucrose before egg-laying [Yang et al., 2015], or restoring courtship behaviour in aged males [Kuo et al., 2015]. Together this gives evidence that PPL2 has a strong role in olfactory related behaviours, but the circuits and mechanisms behind this are still unknown.

1.5 Aims and objectives

The overall aim of this thesis is to investigate the role of PPL2 dopaminergic modulation of olfactory representations in the mushroom body calyx of the species *Drosophila melanogaster*. The influence of PPL2 on the olfactory circuit, particularly how the dopaminergic input to the calyx shapes neural representations of odour stimuli and consequently how this effects olfactory behaviour is explored. Although dopaminergic modulation in the mushroom body lobes and the role in associative conditioning has been extensively studied, the function of neuromodulatory inputs to the calyx is left relatively unexplored. As the input structure of the mushroom body, and the region where olfactory information is distributed to a sparse code among Kenyon cells, modulatory input to this region has the potential to alter the olfactory representation upstream of memory formation. Therefore, understanding how PPL2 neurons interact with synaptic structures of the calyx and how this dopaminergic input influences

odour representations is key to furthering our knowledge of how sensory information can allow for flexible behaviours.

Specifically, the combination of connectomic analysis, in vivo functional imaging, and behavioural experiments provide a detailed view of the PPL2 circuitry, with a focus on KCs as a post-synaptic target.

The thesis results are organised as follows:

1. Connectomic analysis of PPL2. Using the publically available connectome datasets the morphology of each PPL2 neuron is described, including key brain areas innervated. The pre- and post-synaptic partners of PPL2 are identified and information from each dataset compared. This analysis provides the neuronal targets for further functional experiments.
2. Synaptic organisation of PPL2 within the calyx. Precise information on PPL2 synapse locations is used to determine the level of connectivity within or outside calyx microglomeruli structures. This provides an anatomical framework to describe how PPL2 could influence the olfactory circuit with synaptic resolution.
3. Dopamine effect on KC activity. Systematic knockdown of dopamine receptors in KC subsets is performed and the odour-evoked calcium activity is recorded to determine which dopamine receptors are involved in PPL2 modulation of KCs.
4. Targeting PPL2 neurons for manipulation. Available Gal4 drivers for genetic access to PPL2 are characterised, establishing the specificity of these tools for subsequent behavioural and imaging experiments.
5. PPL2 as salience encoding neurons. Exploratory experiments investigating activity of PPL2 and KCs under different physiological conditions such as age, mating status, and hunger. The effect of starvation on baseline PPL2 activity in freely behaving flies is measured using CalexA, and preliminary data revealing a starvation effect on Kenyon cell activity is presented. Lastly, naïve olfactory behaviour while activating PPL2 neurons provides preliminary evidence for PPL2 activity translating to behavioural output.

Finally, I discuss how the anatomical, functional, and behavioural evidence together advances our understanding of how PPL2 neurons contribute to olfactory representation in the calyx.

Chapter 2

Materials and Methods

2.1 Fly lines and husbandry

Fly strains used throughout this project are detailed in the table below. The source of each fly line is specified in the ‘Reference’ column, and lines that were generated in our lab are also specified. Flies were housed in standard food vials and maintained on a 12:12hr light:dark cycle. Flies were kept at 25°C and 60% relative humidity unless otherwise specified.

2.2 Immunohistochemistry

For characterisation of Gal4 drivers and visualisation of expression patterns male flies from the Gal4 line were crossed with females from either UAS-mCD8::GFP or UAS-GFP::nls and processed according to a previously published protocol [[Boto et al., 2019](#), [Jenett et al., 2012](#)]. Specifically, 3-5 day old progeny flies were briefly anaesthetised on ice before brains and/or VNC were dissected in ice cold S2 media. Brains were fixed in 1% paraformaldehyde (PFA) overnight at 4°C and on rotation. Dissected brains were washed three times for 1 hour per wash in PAT3 (PBS, 0.5% TritonX-100, and 0.5% bovine serum albumin) and placed on a nutating mixer at room temperature. Brains were then incubated for 1.5 hours at room temperature with a blocking solution (3% normal goat serum, NGS, in PAT3 buffer). Brains were incubated with the primary antibodies in the blocking solution for 3 hours at room temperature followed by 4°C overnight. The primary antibodies used were: rabbit anti-GFP (1:1000), mouse anti-

Genotype	Chromosome	Reference
TH-C'-Gal4, f-Gal80	III	[Liu et al., 2012b]
TH-Gal80 ; TH-C', f-Gal80	II,III	[Xie et al., 2018]
Oc2-Gal4	II	[Blanco et al., 2011]
Repo-gal80	II	[Xiong et al., 1994]
Oc2-Gal4, Repo-Gal80	II	Boto Lab
TH-D'-Gal4	II	[Liu et al., 2012b]
Vmat AD ; DAT DBD	II,III	[Xie et al., 2018]
Vmat AD ; TH-C DBD	II,III	[Xie et al., 2018]
UAS-dTrpA1	II	BL:26263
UAS-GFP::nls	III	BL:4776
UAS-mCD8::GFP	II	BL:32186
247-GCaMP3	III	[Boto et al., 2014]
247-Gal4	III	BL:50742
5HTR1B-Gal4	II	BL:27636
C739-Gal4	II	BL:7362
UAS-GCaMP6m	II	BL:42748
UAS-GCaMP6m	III	BL:42750
UAS-Dop1R1 _{RNAi}	III	BL:55239
UAS-Dop1R2 _{RNAi}	III	BL:65997
UAS-Dop2R _{RNAi}	II	BL:50621
UAS-GCaMP6m ; UAS-Dop1R1 _{RNAi}	II,III	Boto Lab
UAS-GCaMP6m ; UAS-Dop1R2 _{RNAi}	II,III	Boto Lab
UAS-Dop2R _{RNAi} ; UAS-GCaMP6m	II,III	Boto Lab
<i>w^{CS10}</i>		Ronald Davis Lab
<i>w¹¹¹⁸</i>		Alex Keene Lab
ple AD ; R42H01 DBD	II,III	BL:602072
UAS-kir2.1, Gal80 ^{ts} ; MB-GCaMP3	II,III	Boto Lab
p65 AD ; Gal4 DBD	II,III	BL:79603
lexAop-CD8-GFP ; UAS-LexA-VP16-NFAT,	II,III	BL:66542
LexAop rCD2-GFP		
ple-Gal4, UAS-Gal80 ^{ts}	II,III	Boto Lab
UAS-jGCaMP7b	III	BL:79029

Table 2.1: Table of genetic lines

Reagent	Source	Identifier
Antibodies		
Rabbit anti-GFP	ThermoFisher	Cat # A11122; RRID: AB_221569
Mouse anti-nc82	DSHB	AB_2314866
Goat anti rabbit - Alexa 488	ThermoFisher	Cat # A11008; RRID: AB_143165
Goat anti mouse - Alexa 633	ThermoFisher	Cat # A21052; RRID: AB_2535719
Software		
Fiji	[Schindelin et al., 2012]	https://www.imagej.net
Python 3.9.7	Python Software Foundation	https://www.python.org
SciPy	[Virtanen et al., 2020]	https://www.scipy.org
neuPrint	[Plaza et al., 2022]	https://neuprint.janelia.org
Codex	[Matsliah et al., 2023]	https://codex.flywire.ai
ZEISS Zen Black	Carl Zeiss	RRID:SCR_018163
Deposited data		
<i>Drosophila</i> hemibrain v1.2.1	[Scheffer et al., 2020]	https://neuprint.janelia.org
<i>Drosophila</i> FAFB v783	[Dorkenwald et al., 2024, Schlegel et al., 2024]	

Table 2.2: Table of reagents

nc82 (1:50), and mouse anti-TH (1:200). See Table 2.2 for details on reagents. The nc82 antibody recognises the *Drosophila* protein Bruchpilot (Brp) which is a component of the synaptic active zone. This can therefore be used as a marker for presynapses and a helpful method to visualise the entire brain and its structures. The next day the samples were washed with PAT3 three times for 1 hour each at room temperature and then incubated with secondary antibodies in the blocking solution. This incubation lasted 3 hours at room temperature followed by 5 day at 4°C and wrapped in tinfoil to prevent bleaching from light. Secondary antibodies used were: anti-mouse AlexaFluor 633 (1:400) and anti-rabbit AlexaFluor 488 (1:800). After this incubation the brains underwent a final round of washes, liquid was removed and brains were covered in vectashield medium and kept at 4°C overnight. Finally, whole brains were mounted in vectashield media for imaging. Images were obtained by a Zeiss LCM 880 confocal microscope and analysed using Fiji image processing software.

2.3 Neuron activity reporting

To assess long-term neural activity in freely behaving flies the technique CalexA (calcium-dependent nuclear import of LexA) is used [Masuyama et al., 2012]. This technique utilises the nuclear factor of activated T cells (NFAT) which, upon prolonged depolarisation of a neuron, is translocated from the cytoplasm to the nucleus. The CalexA fly line (lexAop-CD8-GFP ; UAS-LexA-VP16-NFAT, LexAop rCD2-GFP) contains a modified NFAT, LexA-VP16-NFAT, under control of a UAS so that it can be targeted to any population of cells using the Gal4/UAS system. Upon sustained calcium activity the NFAT is translocated to the nucleus where expression of the GFP reporter gene, under control of the LexA operator, LexAop, is induced. This system results in accumulation of GFP after depolarisation, giving GFP intensity as a read-out of neural activity.

Male flies from either ple AD ; R42H01 DBD (PPL2-split), for spatial restriction, or TH-Gal4, Gal80^{ts}, for temporal restriction, were crossed with females from the CalexA

fly line. 0-3d old driver>CalexA flies were collected and kept on standard food at 25°C for 2 days. Half the flies were then transferred to a vial containing 0.75% agar for food deprivation. Experiments involving temperature sensitive Gal80 were kept at 29°C during the starvation period to allow for CalexA expression. Flies were starved for approximately 48hours, until around 5% of flies in starvation vials had died.

4-7d day old flies (both male and female) were briefly anaesthetised on ice before brains were dissected in ice cold PBS. Following dissection brains were fixed for 20 minutes in 4% PFA solution at room temperature. Brains were then washed, consisting of four 20 minute washes in 0.5% PBT (0.5% TritonX-100 in PBS). Brains were blocked overnight at 4°C in 10% NGS in PBT. Next, the brains were incubated in primary antibodies with blocking solution for 2 days at 4°C (protocol adapted from [Meschi et al., 2024]). The primary antibodies used were rabbit anti-GFP (1:1000) and either mouse anti-nc82 (1:50) or mouse anti-TH (1:200). Brains were washed before incubation in secondary antibodies for 2 days at 4°C. Secondary antibodies used were anti-mouse AlexaFLuor 633 and anti-rabbit AlexaFluor 488. The brains underwent a final set of washes before being left overnight at 4°C, upright in a few drops of vectashield. The following day brains were mounted on imaging slides and imaged using a 20X objective. Image stacks of cell bodies were collected with 0.5µm steps and identical laser power and scan settings across all brains within an experiment.

Images were then analysed using FIJI. For each brain one hemisphere is analysed, chosen based on the visibility and least overlapping cell bodies. An individual ROI was drawn around each cell and only the slices that cell appeared were used for the analysis. The mean fluorescence intensity of the 488 channel was divided by the mean fluorescence intensity of the 633 channel for each cell to get the normalised GFP intensity per cell body.

2.4 Y-maze behavioural assay

0-5 day old flies (both males and females) were collected from each genotype for behavioural experiments. Flies were first anaesthetised on a CO₂ pad and then groups of 50-80 flies per vial, separated by sex, were collected. Flies were given at least 24 hours to recover from anaesthesia before being transferred to starvation vials. Starvation vials contained approximately 5ml of 0.75% agar solution which is without nutritional value but provides the flies with sufficient water. Flies were starved for 24 hours before naïve behaviour assessment.

Naïve behaviour was assessed with 2-7 day old flies using the Y-maze assay [Mohanadasan et al., 2020]. Y-maze parts were 3D printed (3D Printing Ireland ltd.) and consist of a loading vial, a central Y connector, and 3 vial connectors. Y-maze arms were made by wrapping a clear plastic vial in black electrical tape to block out any light. Behavioural assessments were performed using up to 24 Y-mazes in tandem. The Y-mazes are held in an upright position throughout testing and take advantage of the flies natural negative geotactic instinct. Flies climb up the middle Y connector and at the decision point choose to either go into the left arm or the right arm. The flies walk through an eppendorf tube with the end cut to create a 1mm gap. This acts as a trap so flies can enter the Y-maze arm but cannot go back. In this way, once flies have made a decision they are stuck with that decision.

Ethyl Butyrate (EB) was diluted in paraffin oil to obtain the desired concentration. Concentrations in the range of 10^{-6} - 10^{-2} were tested. To reach a concentration of 10^{-6} , 10^{-4} dilutions were further serially diluted. Paraffin oil was used as an odorless control to place in the other Y-maze arm.

Filter paper was cut into squares (1cm x 1cm) and placed at the bottom of each Y-maze arm. 20 μ l of either EB or paraffin oil was pipetted onto the filter paper and Y-maze arms were connected to the central Y connector immediately to avoid outside odor diffusion. Each Y-maze was set up with one arm containing paraffin oil and one

arm containing EB. The left and right positions of each odor were alternated for each maze to eliminate any direction bias. Y-mazes were left to odorise for 20 minutes. Meanwhile, experimental flies were placed in an incubator at the desired temperature (22°C for the temperature control, 32°C for dTrpA1) for 15 minutes with the lights on. Once the Y-mazes had odorised, flies were transferred from their starvation vials into the loading vial of the Y-maze. Y-mazes were then transferred to the incubator at the experimental temperature (22°C or 32°C) with the lights off, and left for 30 minutes for the flies to make their decisions. The lights are turned off to further eliminate any bias occurring from light direction, and the order of genotypes is interleaved and changed for each repetition to eliminate biases that may occur due to position or inconsistencies in environment (temperature, humidity, etc.).

After 30 minutes of decision making, the Y-mazes are transferred to a -80°C freezer for 15 minutes to cull the flies. The number of flies in each Y-maze arm, and the number of flies that have remained in the loading vial are counted and the preference index for each maze is calculated using the following formula:

$$PI = \frac{\text{number of flies in odor arm} - \text{number of flies in paraffin oil arm}}{\text{number of flies in odor arm} + \text{number of flies in paraffin oil arm}} \quad (2.1)$$

After the experiment, Y-mazes were rinsed and left to dry for at least 48 hours to remove traces of any odors. Y-maze arms containing odors were kept consistent, so an arm containing EB will always contain EB in future experiments, to avoid contamination and small traces of odor being left behind.

Statistical analysis of behavioural data was performed using standard functions of the SciPy scientific computing python package. A one-way analysis of variance (ANOVA) was used to compare the mean preference index of our three groups (experimental, Gal4 control, UAS control). ANOVA was followed by a post-hoc Sidak multiple comparison correction. All error bars represent standard error of the mean.

Y-maze experiments involving *PPL2>TrpA* were partially run by undergraduate student Benjamin Adams.

2.5 *In vivo* functional imaging

To image the odor-evoked response of neurons, males from different Gal4 drivers (247-Gal4, 5HTR1B-Gal4, C739-Gal4, PPL2-split) were crossed with females from flies expressing the genetically encoded calcium indicator GCaMP. For dopamine receptor knockdown experiments, the knockdown is achieved by RNA interference (RNAi) where the target mRNA is degraded, preventing protein translation.

Single flies were briefly cold anaesthetised before being transferred to a custom recording chamber as described in [Boto and Tomchik, 2024]. The fly was immobilised by applying myristic acid to the proboscis and eyes. A small window was opened in the cuticle with a syringe needle to allow optical access to the brain, and covered with sugar-free saline (103mM NaCl, 3mM KCl, 5mM HEPES, 1.5mM CaCl₂, 4mM MgCl₂.6H₂O, 26mM NaHCO₃, 1mM NaH₂PO₄.H₂O, 10mM trehalose, 7mM sucrose, 10mM glucose) to prevent drying of the brain, avoid activating sugar-sensitive neurons, and preserve starvation states (Section 3.13). For age and mating status experiments, a version of saline with sugar was used (103mM NaCl, 3mM KCl, 5mM HEPES, 1.5mM CaCl₂, 4mM MgCl₂.6H₂O, 26mM NaHCO₃, 1mM NaH₂PO₄.H₂O). Tracheae was gently removed using forceps so the brain was completely exposed. The chamber was then placed on the microscope stage for imaging.

1 μ l of pure odorants (ethyl butyrate, Sigma-Aldrich E15701, or ammonium hydroxide, Sigma-Aldrich 221228) were pipetted onto filter paper, placed in a scintillation vial and integrated into a odor control circuit. The odors were delivered through a stainless steel pipette mounted 1 cm anterior to the fly’s head and controlled via the CS-55 stimulus controller. The flies were subject to a continuous flow of air and upon a button press the odor line was opened for 3 seconds, integrating the odor flow into the main continuous flow line. Flies were subject to 5 minutes of air flow before image acquisition to allow time to recover from anaesthesia and the dissection. Two 1 minute recording sessions were completed per fly, separated 5 minutes of continuous air flow. First a 15 second baseline was acquired with continuous airflow followed by 3 seconds

of odor stimulus. Continuous air flow was 0.8L/min and the odor pulse flow rate was 0.2L/min making a total airflow at time of stimulus presentation 1L/min.

Images were captured using a 20X water immersion Zeiss objective at a rate of 10Hz and a frame size of 256x256. A single z position containing the widest section of the ROI (calyx, or mushroom body lobes) in both hemispheres was monitored and one hemisphere was chosen for analysis based on the quality of image and signal. ROIs were drawn manually at the peak of fluorescence to capture the entire ROI area and the mean fluorescence of pixels within that ROI per frame was saved directly from ZEN black software. Images were discarded if there was excessive movement, no visible response, or poor image quality. Imaging analysis was performed using custom python scripts. The mean fluorescence tables were read and data was first corrected for any bleaching causing a constant decrease in signal over time. The signal was smoothed and the baseline (F_0) calculated as the mean fluorescence of 10 seconds before the odor stimulus (between 5s and 14s). The change from baseline (ΔF) is calculated for each frame as the fluorescence, F , minus F_0 . Finally, the normalised response is achieved by $\Delta F/F_0$.

The response magnitude was calculated by subtracting the response baseline from the maximum fluorescence value within the response window (15s-18s). The area under the curve was calculated as the sum of fluorescent values between 16s and 30s. Finally, the time to peak is calculated as the time taken after onset of odor stimulus (15s) to reach the maximum fluorescence magnitude. Mean fluorescence traces are plotted as $\Delta F/F_0$ over time (seconds) and the standard error of the mean (SEM) is shown as shaded area.

Statistical analysis was performed using Graphpad Prism version 10.5.0. First the D'Agostino & Pearson, Anderson-Darling, and Shapiro-Wilk tests for normality were performed. If at least two out of the three tests resulted in 'Yes' for normality then the data was treated as normally distributed, else it was treated as non-parametric. All calcium imaging data was non-parametric. For data involving more than two groups a Kruskal-Wallis test followed by Dunn's multiple comparisons post-hoc test

was performed. For comparison of two independent groups, the Mann-Whitney test was used.

2.6 Connectomics analysis

2.6.1 Circuit analysis

The connectomic analysis presented in this thesis makes use of the NAVis (Neuron Analysis and Visualization) Python library [Bates et al., 2020a]. NAVis provides the data types needed to represent neurons and functions such as `navis.plot2d()` for neuron and neuropil visualisation.

Hemibrain v1.2.1 data was accessed via custom python code, using the `neuprint-python` API [Clements et al., 2020, Plaza et al., 2022, Scheffer et al., 2020]. Neuron skeletons were obtained using the `fetch_skeletons()` function with the following bodyIds; PPL201: 328533761, PPL202: 5813071348, PPL203: 294436967, and PPL204: 542039038 (Figure 3.1 A). Upstream and downstream neuron connectivity was obtained using the `neuprint fetch_adjacencies()` function, which provides a per ROI synapse count for each neuron pair (Figures 3.1 C, 3.2 A and B, 3.5 C and D, 3.6 C and D). For precise information on synapse location the `neuprint fetch_synapse_connections()` was used to extract coordinates of individual synapses (Figures 3.3 F and 3.4 F). In order to compare spatial and morphological information across datasets, the hemibrain data was transformed to FAFB coordinate space using the `navis-flybrains` python library. Transformations between the hemibrain (JRCFIB2018Fraw) template, and the FAFB (FAFB) template also requires the Computational Morphometry Toolkit (CMTK) (<http://nitrc.org/projects/cmtk>). Prior to plotting, the `flybrains xform_brain(data, source='JRCFIB2018Fraw', target='FAFB')` function was used to transform the hemibrain coordinate system to FAFB space. FAFB brain meshes were used for visualisations (Figures 3.3 and 3.4 [Bates et al., 2020a, Bogovic et al., 2020, Buhmann et al., 2021, Zheng et al., 2018, Xu

et al., 2020].

FAFB v783 and BANC v626 data was downloaded from codex.flywire.ai [Matsliah et al., 2023]. Specifically, the `consolidated_cell_types`, `cell_stats`, `connections_princeton_no_threshold`, `fafb_v783_princeton_synapse_table`, and `connections_princeton` (BANC) .csv files were used [Heinrich et al., 2018, Buhmann et al., 2021, Yu et al., 2025]. Data up to date as of July 25th 2025. Analysis was performed using custom python code and the `fafbseg` python tools built on top of NAVis. Neuron skeletons were obtained by downloading the relevant .swc files from Codex. Neuron ids used were; PPL201: 720575940625926693, 720575940621226405, PPL202: 720575940619676224, 720575940626022074, PPL203: 720575940618130833, 720575940646852660 and PPL204: 720575940630913221, 720575940615405718 (Figure 3.1 A). For comparative analysis between hemibrain and FAFB data only the right-hand hemisphere neurons were used (PPL201: 720575940621226405, PPL202: 720575940626022074). Upstream and downstream neuron connectivity was extracted from `fafb_connections_princeton_no_threshold.csv` which provides a per ROI synapse count for each neuron pair (Figures 3.1 C, 3.2 A and B, 3.5 A and B, 3.6 A and B). For precise information on synapse location the file `fafb_v783_princeton_synapse_table.csv` provides coordinates and information for individual synapses (Figures 3.3 D and 3.4 D).

Heatmaps, graphs and neuron skeleton images were generated using a combination of `navis`, `seaborn`, `matplotlib`, and `plotly` data visualisation packages.

2.6.2 Microglomeruli analysis

Analysis of PPL2 synapse localisation is completed with custom Python code. The synapse location data is extracted from the FAFB and hemibrain datasets as described above.

First, all synapses from PNs to KCs are identified based on the `cell_type` attribute. For each PN, these synapses are clustered using the DBSCAN algorithm with parameters

`eps=900`, `min_samples=5` from the `sklearn` python module [Ester et al., 1996]. This algorithm clusters points based on distance, where `eps` defines the maximum distance between points for those points to be considered in the same cluster, and `min_points` defines the minimum number of points needed per cluster. All coordinates and distance measures are calculated in nm. The output of this algorithm assigns each point either to a cluster or to ‘noise’. This means we can cluster all PN- KC synapses without knowing how many clusters there will be. Finally, the maximum and minimum x, y, and z coordinates of each cluster are identified and a bounding box, the smallest rectangular box encompassing all the points, is assigned to each cluster. This bounding box defines the microglomeruli volume and each microglomeruli is assigned an identifier. Therefore, each PN has many associated microglomeruli which each contain many PN- KC synapses (Figure 3.7 A).

The more restrictive definition of the microglomeruli volume (Figure 3.9 A) uses the `ConvexHull()` function from the `scipy` python library to calculate the smallest convex set that contains all of the points [Barber et al., 1996].

Next, each synapse located within the MB calyx is checked for whether it is located within a microglomeruli structure (Figure 3.8). This is done simply by checking if the coordinates fall within the bounding box or convex hull we have defined for each microglomeruli. If a synapse is located inside more than one microglomeruli (there may be overlapping volumes) the candidate microglomeruli are ranked based on the following:

- If the PN associated with the microglomeruli is also the pre-synaptic component of the synapse then that microglomeruli is more likely to be assigned.
- The distance from the synapse to each candidate microglomeruli is measured and the closer microglomeruli is more likely to be assigned.

The pre/post synaptic index for each microglomeruli group (Figure 3.8 F and J) was calculated first by identifying every unique neuron which has a pre-synapse in the microglomeruli and every unique neuron which has a post-synapse in the microglomeruli.

Then, the index is calculated by:

$$index = \frac{\text{number pre} - \text{number post}}{\text{number pre} + \text{number post}} \quad (2.2)$$

. This gives a result between 1 and -1 where a microglomeruli with more unique pre-synaptic neurons is greater than 0, and a microglomeruli with more unique post-synaptic neurons is less than 0.

Statistical analysis was performed using Graphpad Prism version 10.5.0. First the D'Agostino & Pearson, Anderson-Darling, and Shapiro-Wilk tests for normality were performed to confirm non-parametric data. Then a Kruskal-Wallis test followed by Dunn's multiple comparisons post-hoc test was used.

Chapter 3

Results

The main aim of this project is to investigate the role of PPL2 dopaminergic neurons in olfactory processing in the adult *Drosophila* brain. This work contributes to the wider research surrounding sensory representation. This chapter presents the results and main findings of the project; the PPL2 circuitry and connectivity is analysed, including individual synapse location. The effect of dopamine receptor knockdown in downstream targets to odor-evoked response were imaged. Lastly, we present preliminary evidence of an internal state dependant role for PPL2.

3.1 Two neurons from the PPL2 cluster innervate the calyx

To understand the influence of the PPL2 cluster on olfactory representation in the calyx it is important to first establish the extent of innervations in the MB. The PPL2 cluster is comprised of on average 7 dopaminergic neurons per hemisphere [Mao and Davis, 2009, Boto et al., 2019]. The soma of each PPL2 neuron is located in the posterior lateral region, forming the cluster, but the morphological structure of each neuron is very different. Neurons from the same cluster can have different connectivity patterns, branch to different areas of the brain, and thus likely have different functions, or encode different information. The neurons can be classified into neuron types based off their morphologies. Both the FAFB and hemibrain datasets identify four different types of PPL2 neurons, namely PPL201, PPL202, PPL203, and PPL204 (Figure 3.1 A). The FAFB dataset contains one of each of these types per hemisphere, and the

hemibrain dataset identifies one of each type in the right hand hemisphere available. The BANC v626, and MCNS v0.9 datasets currently have also identified the same four PPL2 types, but the BANC data has also partially reconstructed the skeletons of two individual PPL201 neurons in the left hemisphere, suggesting that individual brains may have more neurons of a certain type in the cluster and even within the same brain the cluster may not be symmetrical. All PPL2 neuron types are unipolar, with a single neurite that extends dorsally from the cell body and branches with no clear morphological distinction between axon and dendrite (Figure 3.1 A). The four neuron types have unique branching patterns, PPL201 and PPL203 branch to the dorsal-most brain regions with PPL201 extending medially to the midline without crossing hemispheres. PPL202 and PPL204 appear to contain processes that extend beyond the brains midline, innervating the contralateral hemisphere. PPL204 in particular crosses the midline to extensively innervate both the contra- and ipsi-lateral antler (ATL) region, an area thought to be involved in encoding wind direction [Suver et al., 2019]. In fact, the PPL204 neuron extends so far into the left hemisphere in the hemibrain dataset that it is truncated by the boundaries of image data collected in this dataset.

To establish which neurons of the cluster could be involved in modulating olfactory representation we looked at whether these neurons innervate the MB calyx, and how many synapses associated with these neurons could be found in the calyx neuropil. Since the hemibrain data only includes neurons with cell bodies in the right hemisphere of the brain, we only analyse the right-hand side neurons from the FAFB dataset to get the best comparison. Two neurons, PPL201 and PPL202, have a substantial number of both pre- and post-synapses within the calyx (Figure 3.1 B & C). PPL201 has a total of 850, 576 pre- and 274 post-, synapses located in the MB calyx identified in the FAFB dataset (Figure 3.1 B) and a total of 976, 719 pre- and 257 post-, synapses identified in the hemibrain dataset (Figure 3.1 C). PPL202 seems to much more extensively innervate the calyx with a higher total of 1,569, 1,295 pre- and 476 post-, synapses identified in the FAFB dataset and a total of 2,823, 2,275 pre- and 548 post-, synapses in the calyx identified in the hemibrain dataset. PPL203 and PPL204 also have a small amount of synapses located within the MB calyx, but we don't consider these

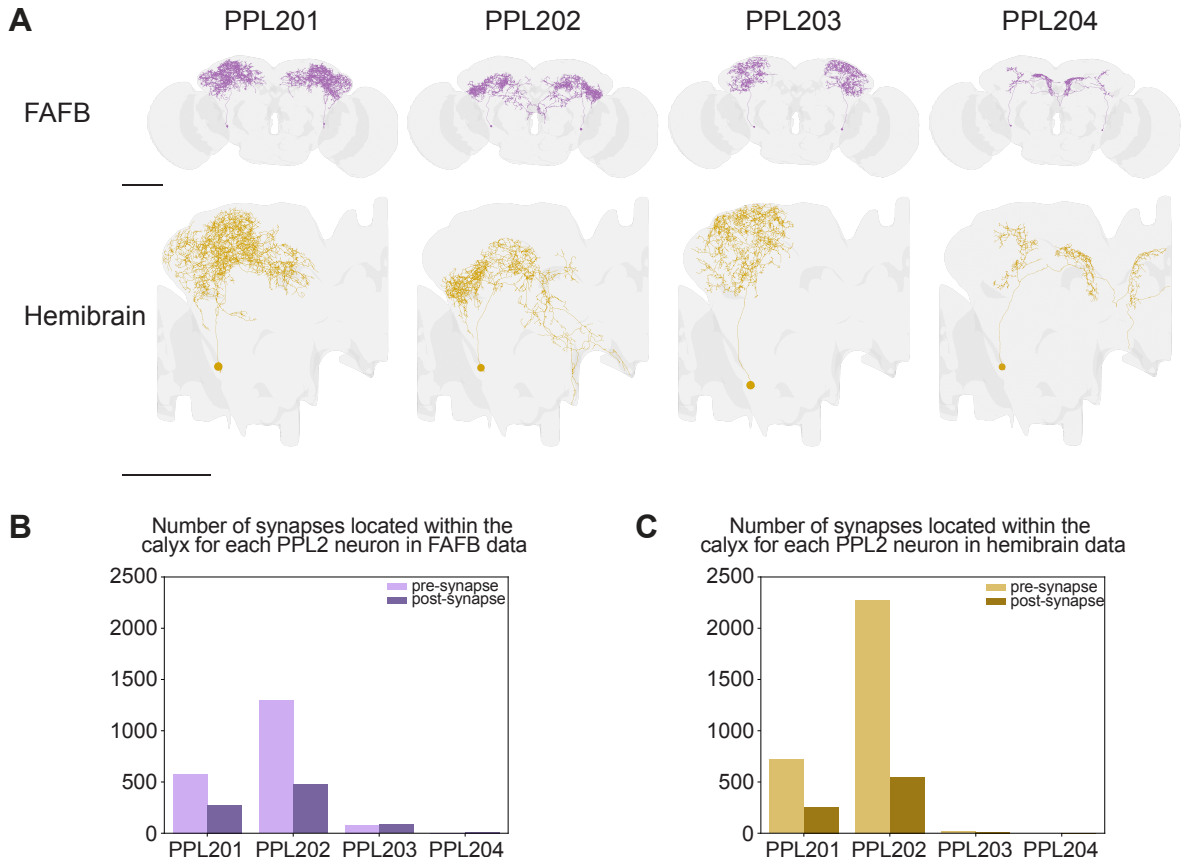


Figure 3.1: Only PPL201 and PPL202 have substantial synapses located within the calyx (A) Images of each of the PPL2 neurons identified in the FAFB (top) and hemibrain (bottom) datasets. Scale bar: 100µm. (B) Number of pre-and post-synaptic compartments located within the calyx for each PPL2 neuron in the FAFB dataset. (C) Number of pre-and post-synaptic compartments located within the calyx for each PPL2 neuron in the hemibrain dataset.

significant (PPL203: FAFB=168, hemibrain=30, PPL204: FAFB=17, hemibrain=3). Data from the BANC dataset was also analysed, giving a total of 85 synapses identified for PPL202, 5 synapses for PPL201, and no synapses within the calyx for the other 2 identified PPL2 types. This data corroborates the trend that only PPL201 and PPL202 innervate the calyx, although since the dataset is newly released and not fully complete we do not use this dataset for further analysis.

Across the two datasets analysed there are differences between the exact number of synapses identified, especially for PPL202 pre-synapses. However, there are a lot of things that are consistent across datasets, firstly the neuron types that are identified as innervating the calyx, PPL201 and PPL202, the number of pre-synapses identified being higher than the number of post-synapses, and that PPL202 has a higher number of synapses in the calyx compared to PPL201. Together, this suggests that across

brains PPL201 and PPL202 are consistently sending more information than they are receiving within the calyx.

Overall, since we have identified PPL201 and PPL202 as extensively innervating the mushroom body calyx, these are the best candidates from this cluster to be modulating olfactory representations in the calyx. Therefore the connectomic analysis going forward focuses on these two neuron types only.

3.2 PPL201 and PPL202 innervate olfactory related brain regions

Having identified PPL201 and PPL202 as our neurons of interest we characterise their connectivity patterns at the level of brain regions by quantifying the proportion of upstream and downstream synapses within each brain region (Figure 3.2).

Both PPL201 and PPL202 send and receive information from olfactory related brain regions, but with different preference (Figure 3.2 A). The largest proportion of downstream connectivity, making up 17-30% of the total number of synapses, from PPL201 is located in the superior lateral protocerebrum (SLP), an area which is also a target for MBONs and thought to be a convergence zone for associative conditioning related signals [Ito et al., 1998, Aso et al., 2014a]. However, the SLP is only a small portion of PPL202 downstream targets, making up less than 5% of total synapses, this matches the morphological data (Figure 3.1 A) since PPL202 does not extend to innervate the entire SLP. Other major downstream regions from PPL201 include the LH (10-15%) and MB calyx (4-5%), two major olfactory processing areas. These regions are also targeted by PPL202, with 36-40% of total synapses split between the two. The regions receiving the next highest proportion of synapses from PPL202 are the posterior lateral protocerebrum (PLP), a visual processing center [Wu et al., 2016], and the superior clamp (SCL), a convergence zone for visual and olfactory information [Yu et al., 2013]. PPL202, especially as shown in the hemibrain dataset, has a higher proportion

3.2. PPL201 AND PPL202 INNERVATE OLFACTORY RELATED BRAIN REGIONS

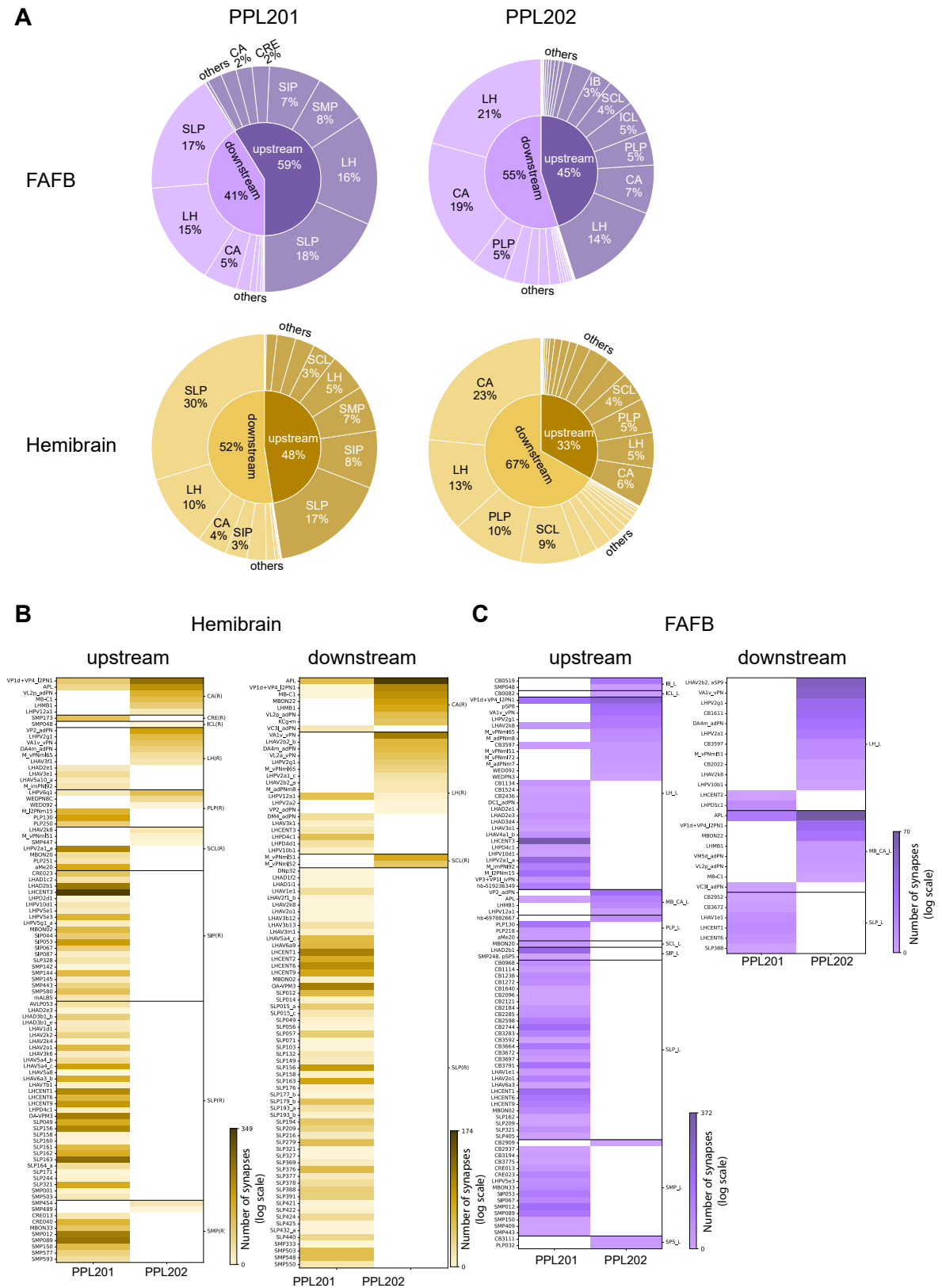


Figure 3.2: Brain region connectivity of PPL2 neurons
(A) Percentage of upstream and downstream synapses per brain region for PPL201 (left) and PPL202 (right). Data from FAFB dataset (top) and hemibrain dataset (bottom).
(continues on next page)

of downstream connections compared to upstream, meaning that overall this neuron is sending information through more synapses than it is receiving (see Table 3.1 for exact

counts).

In terms of upstream connectivity, the regions sending information to PPL2, PPL201 receives a large proportion of information from the SLP (17-18%), superior intermediate protocerebrum (SIP), and the superior medial protocerebrum (SMP) which together form a large area known as the superior neuropils (SNP). Upstream connectivity is also seen in the LH, but only at 5% in the hemibrain, and 16% in the FAFB datasets. The MB calyx makes up an even smaller proportion of upstream synapses, with less than 3% of total synapses found upstream in this area. PPL202, on the other hand, has the largest proportion of synapses upstream being located in either the LH or MB calyx, with 11-21% of synapses split between these two regions. Overall, the FAFB dataset has identified a larger proportion of LH connectivity for both PPL201 and PPL202 compared to the connectivity seen in the hemibrain dataset. Other regions sending information to PPL202 include the PLP and SCL.

For both PPL201, and PPL202 we see the same regions both upstream and downstream, suggesting reciprocal connectivity, and indeed there are a large proportion of reciprocal neuron partners that can be identified throughout the brain (Table 3.1). PPL202 seems to have more downstream synapses than upstream, whereas PPL201 is more evenly split, and infact, the FAFB data shows PPL201 having a higher proportion of upstream synapses (59%).

Overall, PPL201 connectivity is very specific to areas related to olfactory processing: MB, LH and sub-areas of the SNP. However, PPL202 targets both olfactory centers (MB and LH) and also areas associated with sensory integration, especially with visual information, such as the PLP.

Figure 3.2 (previous page): (B&C) Neuron types that are upstream or downstream of PPL201/202 anywhere in the brain. Colour gradient represents the relative strength of connection between the two neurons, strength of connection is equal to the number of synapses. Hemibrain dataset (B), FAFB dataset (C). SLP: Superior lateral protocerebrum, LH: Lateral Horn, CA: MB calyx, SMP: Superior medial protocerebrum, SIP: Superior intermediate protocerebrum, PLP: Posterior lateral cerebrum, ICL: Inferior clamp, SCL: Superior clamp, IB: Inferior bridge. CRE: crepine.

3.3 PPL201 and PPL202 connect with different neuron types across the brain

Figures 3.2 B and C show the neuron type connectivity across the whole brain with each region indicated. For neuron types with more than one individual neuron the number of synapses are summed. Since there are a large proportion of neuron-to-neuron connections that have a very small number of synapses, and these may not be as reliable in the dataset [Schlegel et al., 2024] the number of synapses involved in a connection is thresholded such that only those with more than 10 are included here. The number of synapses involved in a connection is used as a proxy for total connection strength. Some neurons, for example LHCENT3 connect with PPL2 by making distributed synapses across many brain areas. In these cases, all synapses are summed and the connection is labelled with the neuropil in which the most synapses are made.

It is clear in these plots (Figures 3.2 B and C) that PPL201 and PPL202 largely connect to different types of neurons within each neuropil. For example, both FAFB and hemibrain datasets highlight that in the SMP the two PPL2 neurons receive information from different types of SMP neurons and this pattern is consistent across each brain region with only a few neuron types connecting to both PPL2 neurons. We look in more detail at the neurons connected in olfactory related brain regions in section. Across the entire hemibrain, PPL201 receives the most information from LHCENT3, a LH interneuron, and sends information to OA-VPM3, with the most synapses for these connections located in the SIP and SLP, respectively. In the FAFB data this is not consistent, with OA-VPM3 not making an appearance as a major downstream connection. Instead, APL, a major inhibitory neuron associated olfactory representations, receives the highest amount of synapses from PPL201, which are located in the calyx. The data for PPL202 is consistent across datasets, receiving the most synapses from PN VP1d+VP4.l2PN1 and sending the most information to APL, both within the MB calyx. PPL201 creates synapses with more neuron types overall compared with PPL202.

Since PPL201 has a longer cable length and more synapses both neurons end up with similar synaptic densities within the same dataset (Table 3.1). Across datasets, the hemibrain identifies more synapses overall, leading to an increased synaptic density but this is consistent across both neurons.

Overall, although there is overlap between PPL201 and PPL202 in terms of the region level connectivity, at the neuron type level PPL201 and PPL202 do not send or receive information from the same types of cells across the brain.

Metric	PPL201 (FAFB)	PPL201 (hemibrain)	PPL202 (FAFB)	PPL202 (hemibrain)
Cable length (μm)	19,149	20,369	11,721	11,729
Synapses	11,886	17,028	6,849	9,702
Pre-synaptic sites	4,896	8,933	3,760	6,505
Post-synaptic sites	6,990	8,095	3,089	3,226
Synaptic density (synapses/ μm)	0.62	0.83	0.58	0.83
Input neurons	1,862	1,974	1,000	1,066
Output neurons	2,395	3,162	1,937	2,694
Reciprocal neurons	1,015	1,229	492	672
Calyx metric				
Cable length (μm)	1,109	799	2,615	2,342
Number of synapses	850	985	1,771	2,832
Pre-synaptic sites	576	724	1,295	2,279
Post-synaptic sites	274	261	476	553
Synaptic density (synapses/ μm)	0.77	1.23	0.68	1.21
Input neurons	188	151	225	215
Output neurons	424	480	749	958
Reciprocal neurons	65	67	108	161

Table 3.1: Summary of different neuron metrics across datasets and PPL2 types

3.4 Synapse distribution in the calyx differs between brains

To investigate in more detail how the morphology and innervations differ across brains, and specifically in the mushroom body calyx, we can visualise the neuron skeletons from FAFB and hemibrain together in the FAFB coordinate space (Figures 3.3 & 3.4 A, B, C, D). The general morphology of the PPL2 neurons are consistent across datasets with

large amounts of overlap between the skeletons. PPL201 in the hemibrain dataset is $1,220\mu\text{m}$ longer than in the FAFB dataset, the extra length may be attributed to an extra branch towards the midline (Table 3.1). The morphologies of these two PPL2 neurons reveal extensive innervations to numerous brain regions in the dorsal region, with PPL201 extending across the entire posterior-anterior axis, and PPL202 remaining mainly posterior, with no clear division into axon and dendrite (Figure 3.1 A & C). PPL201 is broader than PPL202, covering a wider area of the brain in total whereas PPL202's branches seem to be more restricted. The entire LH is innervated by PPL201, whereas PPL202 branches are only present in a small area of the inferior LH.

PPL201 innervations in the calyx are sparse, and the area covered differs between the two datasets (Figure 3.3 B & D). Although the hemibrain PPL201 has a longer total cable length, the cable that is located in the calyx is less than the FAFB dataset and is located more anterior. The shorter cable length is, however, paired with a higher number of synapses in the calyx which leads to a high synaptic density (Table 3.1). In the FAFB dataset the PPL201 post-synapses are distributed evenly throughout the calyx in all directions, whereas the pre-synapses are located slightly more towards the center in all axis directions (Figure 3.3 E). The hemibrain PPL201 neuron follows the same pattern in the medial-lateral and anterior-posterior directions, however, the pre-synapses are distributed more clustered towards the dorsal area of the calyx (Figure 3.3 F).

Compared to PPL201, PPL202 has a total shorter cable length, but has a longer cable length within the calyx. PPL202 has less total synapses but more synapses in the calyx compared to PPL201, suggesting that PPL202 is a more calyx focused neuron, whereas PPL201 is more broadly connected. The innervations in the calyx are much more extensive compared to PPL201, and across datasets the cable length in this brain region is comparable, although the number of synapses identified is much larger in the hemibrain dataset (Table 3.1). In the FAFB dataset the PPL201 post-synapses are distributed evenly throughout the calyx in all directions, whereas the pre-synapses are located slightly more towards the center in all axis directions (Figure 3.4 E). The

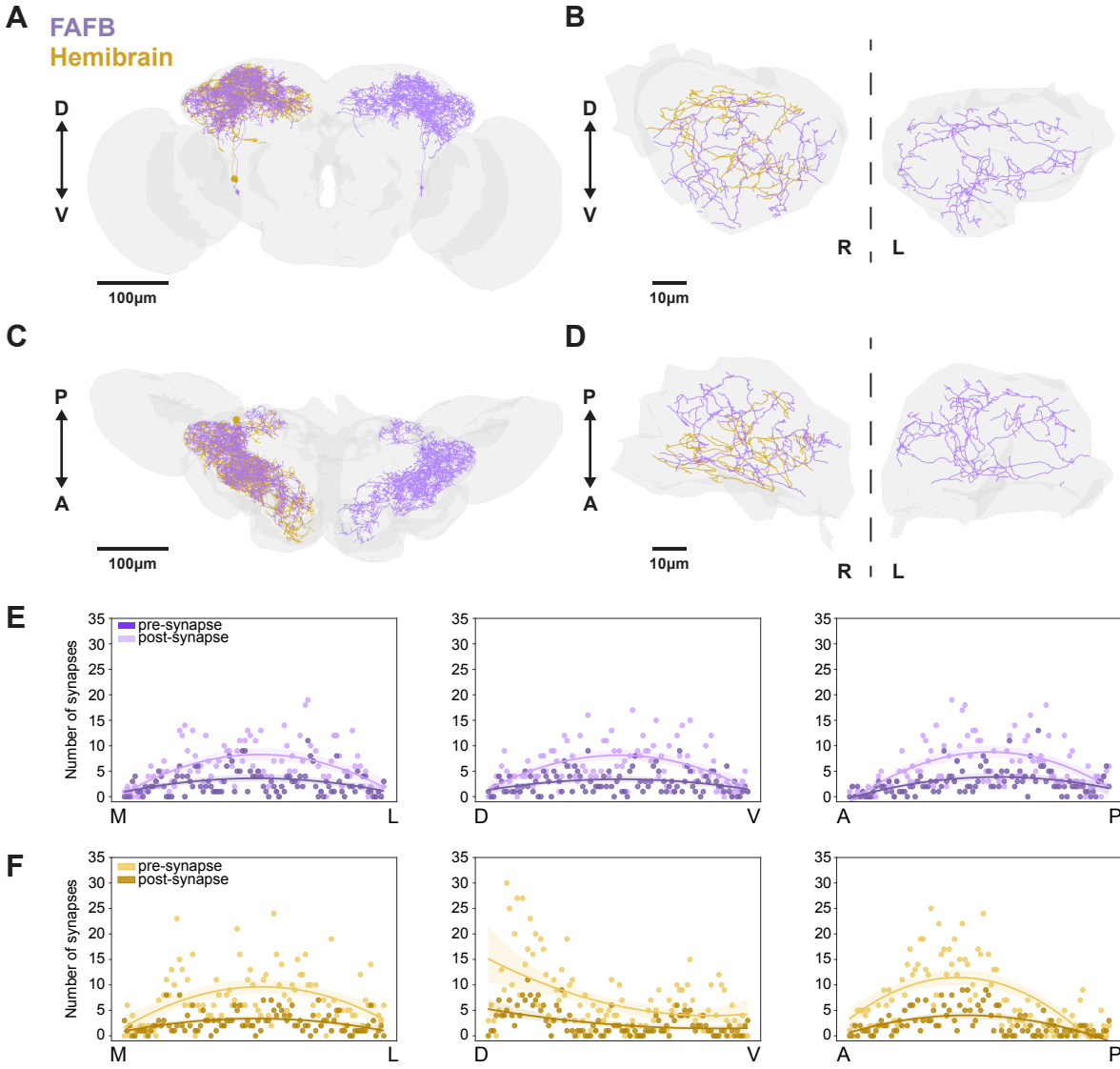


Figure 3.3: Comparison of PPL201 morphology between datasets

Images showing neurons identified as PPL201 from FAFB (purple) and hemibrain (orange) datasets transformed to FLYWIRE space. (A) Frontal view of the whole brain showing full morphology of neurons from the two datasets. D: dorsal, V: ventral. Scale bar: 100µm. (B) Frontal view of calyx innervations of PPL201. D: dorsal, V: ventral, R: right, L: left. Scale bar: 10µm. (C) Posterior-anterior axis view of the whole brain showing full morphology of PPL201 neurons. P: posterior, A: anterior. Scale bar: 100µm. (D) Posterior-anterior axis view of calyx innervations of PPL201. P: posterior, A: anterior, R: right, L: left. Scale bar: 10µm. (E & F) Plots showing the number of synapses distributed across the medio-lateral (left), dorso-ventral (center) and antero-posterior (right). The axis was divided into 100 equal bins representing relative position, and the number of synapses per bin is shown. E shows data from the FAFB set, and F from the hemibrain.

hemibrain PPL201 neuron follows the same pattern in the medial-lateral and anterior-posterior directions, however, the pre-synapses are distributed more clustered towards the dorsal area of the calyx (Figure 3.4 F).

In total, the calyx innervations of PPL201 and PPL202 differ across brains, with neuron cable spanning different areas of the calyx and synapses distributed more evenly

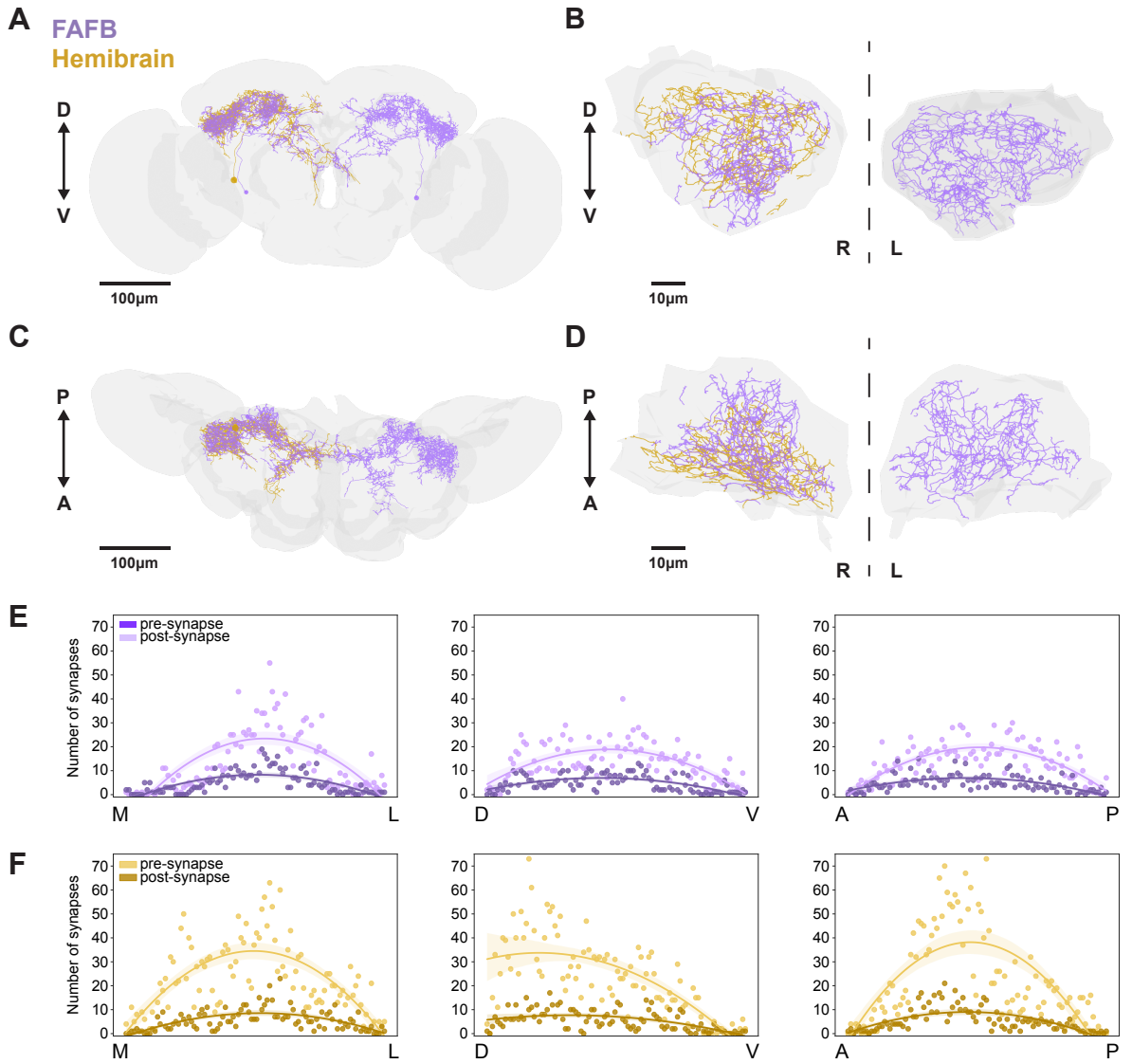


Figure 3.4: Comparison of PPL202 morphology between datasets

Images showing neurons identified as PPL202 from FAFB (purple) and hemibrain (orange) datasets transformed to FLYWIRE space. (A) Frontal view of the whole brain showing full morphology of neurons from the two datasets. D: dorsal, V: ventral. Scale bar: 100µm. (B) Frontal view of calyx innervations of PPL202. D: dorsal, V: ventral, R: right, L: left. Scale bar: 10µm. (C) Posterior-anterior axis view of the whole brain showing full morphology of PPL202 neurons. P: posterior, A: anterior. Scale bar: 100µm. (D) Posterior-anterior axis view of calyx innervations of PPL202. P: posterior, A: anterior, R: right, L: left. Scale bar: 10µm. (E & F) Plots showing the number of synapses distributed across the medio-lateral (left), dorso-ventral (center) and antero-posterior (right). The axis was divided into 100 equal bins representing relative position, and the number of synapses per bin is shown. E shows data from the FAFB set, and F from the hemibrain.

in the FAFB data versus more concentrated in the center of the calyx in the hemibrain.

3.5 APL and KC γ are the main neuron partners of PPL201 and PPL202 in the calyx

Next, we look in more detail at the cell type level connections in olfactory centers, the MB calyx and LH. Figure 3.5 shows the upstream connections, i.e. neuron types that are sending input to PPL201 and PPL202 and 3.6 shows the downstream connections, i.e. neuron types that are receiving information from PPL201 and PPL202. The connections are filtered to only include those involving more than 10 synapses, and the synapse counts for cell types with more than one neuron are then summed in order to exclude connections with a very low number of synapses. In the calyx, the analysis of connections to KCs are separated, since there is a very large population of KCs, each may only have connections with PPL2 via one or two synapses so the thresholding does not include them. Therefore, to analyse the extent of KC connections all synapses to the same type of KC is summed before thresholding to include only connections with more than 10 synapses.

3.5.1 PPL201 and PPL202 receive information from KCs, APL and PNs in the calyx

PPL201 and PPL202 receive information from different types of neurons in the calyx, and PPL202 receives information from more types of neurons (Figure 3.5 A & C). The only common upstream neuron types between PPL201 and PPL202 are APL and VP1d+VP4_l2PN1, an individual multiglomerular PN. PPL201 also receives information from one more neuron type, LHCENT3. This LHCENT3 neuron provides the highest upstream synapse count onto PPL201 across the entire brain (Figure 3.2 B & C), although the majority of these synapses are located in regions other than the calyx, such as the LH and SIP. PPL202 also receives input from another PN, VP2_adPN, a single uniglomerular projection neuron, and two LH associated neurons, LHMB1, and LHPV12a1 which both form connections between the LH and the MB. Interestingly,

3.5. APL AND KC_γ ARE THE MAIN NEURON PARTNERS OF PPL201 AND PPL202 IN THE CALYX

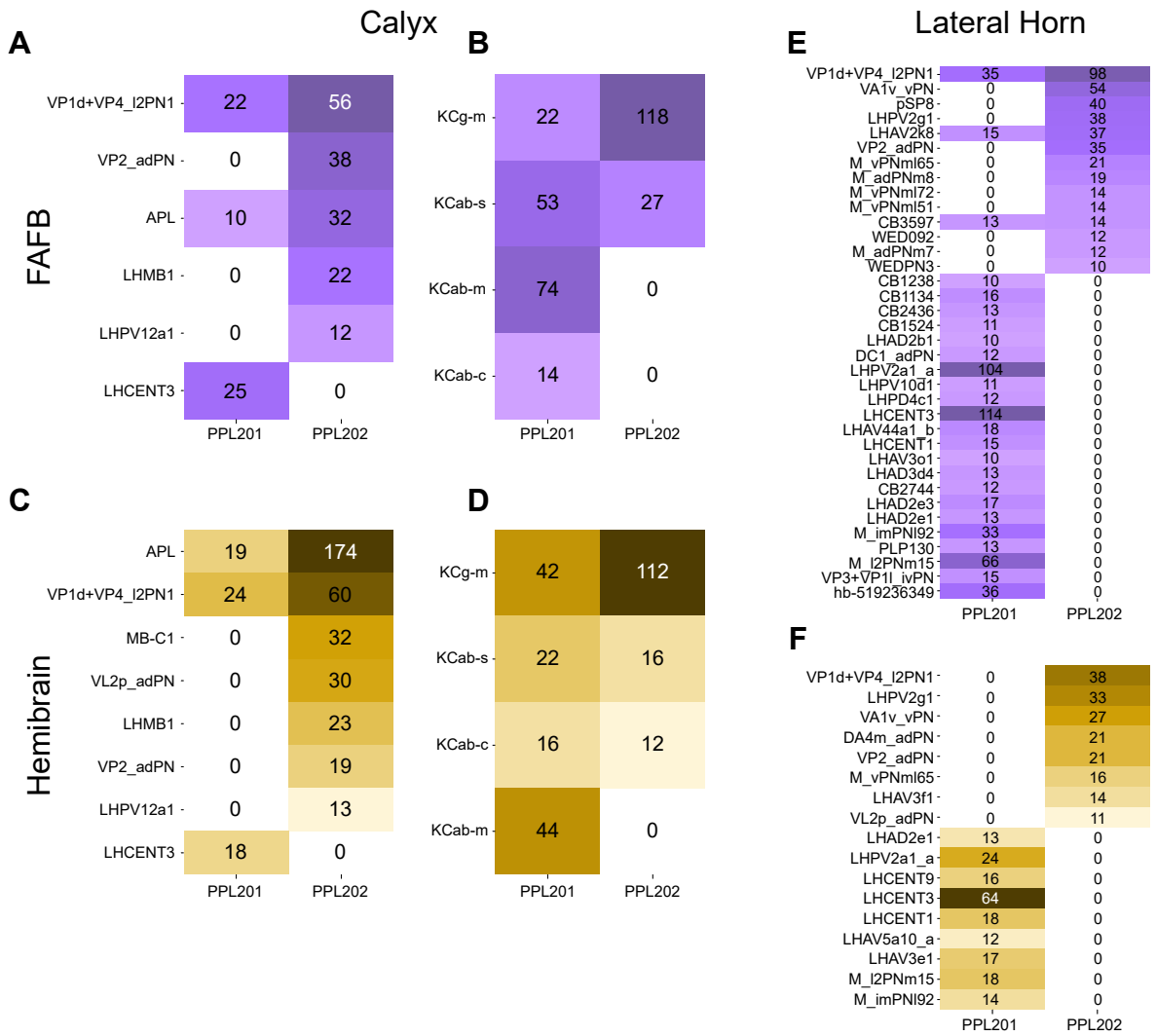


Figure 3.5: Upstream connections to PPL2 within the calyx. Heatmaps showing the pre-synaptic neurons and number of synapses located within the calyx from the FAFB (A and B) and hemibrain (C and D) datasets. A and C show neurons not including Kenyon Cells, B and D show Kenyon Cells only. (E and F) Heatmaps showing the pre-synaptic neurons and number of synapses located within the lateral horn from the FAFB (E) and hemibrain (F) dataset. For all heatmaps only neurons with 10 or more synapses are included. Number of synapses represents the sum of all neurons of that type

LHPV12a1 innervations span the MB and LH in both hemispheres with one soma per hemisphere. In the right hand calyx, PPL202 receives input from both LHPV12a1 neurons, although the neuron with their soma in the left hand side provides much fewer input synapses. The hemibrain dataset also includes input from MB-C1 which is a type of neuron that includes 2 cells per hemisphere and, again, connects the LH and MB.

As well as PN, PPL2 also receive olfactory input from KCs. There are three main subtypes of KC, KC_γ , $KC_{\alpha\beta}$, and $KC_{\alpha'\beta'}$ but these can be further divided into

sub-types based on exact morphology. $KC-\alpha'/\beta'$ are not represented in this heatmap (Figure 3.5 A & C) since from a total of approximately 283 (FAFB data, 337 hemibrain data) $KC-\alpha'/\beta'$ neurons, the total input synapses to PPL2 is less than 10. $KC-\gamma$ -main neurons make up the highest number of upstream synapses from KCs, although each individual KC only sends information via one or two synapses. In the FAFB dataset there is one individual KC which has a total of 9 synapses with PPL. In general, the KC input comes from a large population of KCs with very few synapses each. However, only less than 20% of all KCs are sending information to PPL2. For example, in the FAFB dataset there are a total of 1,069 $KC-\gamma$ -m neurons identified, but only 20, and 99 send information to PPL201 and PPL202, respectively. So, even though connections with KCs makes up a large proportion of input to PPL2 neurons, the connections per KC are weak, and only a small proportion of KCs send information to PPL2.

3.5.2 PPL201 and PPL202 receive information from PNs, and LH local interneurons in the lateral horn

In the LH, PPL201 and PPL202 receive information from different types of neurons, with the only overlap being identified in the FAFB dataset with VP1d+VP4.l2PN1, a PN that also synapses with PPL2 in the calyx, LHAV2k8, an LH output neuron, and CB3597, a multiglomerular olfactory PN. LHCENT3 is identified in both datasets as the highest input to PPL201, and this neuron synapses onto PPL201 in multiple regions of the brain, providing input from the most synapses across all connections to PPL201.

3.5.3 PPL201 and PPL202 send information to APL and KCs in the MB calyx

PPL201 has a much smaller number of post-synaptic partners compared to PPL202 in the calyx and only one neuron is consistent across datasets for receiving information from both PPL2 neurons, and that is APL (Figure 3.6). APL is also the top out-

put neuron in terms of synapse count for both PPL2 neurons in the calyx but also for PPL202 across the entire brain. The hemibrain dataset also identifies MB-C1, and VP1d+VP4.l2PN1 as receiving input from both PPL2 neurons. Interestingly, the VP1d+VP4.l2PN1 olfactory PN also receives a high level of input from both PPL2 neurons, creating a feedback loop. There are a number of other neurons that are reciprocal with PPL2 neurons, meaning they form both pre- and post-synaptic connections, including inhibitory neurons APL, LHMB1, LHPV12a1, MB-C1, and olfactory projection neuron VL2p_adPN.

PPL2 neurons also send information to KCs, forming reciprocal connections with $KC\gamma$ and $KC\alpha\beta$. However, although $KC\alpha'\beta'$ were not a significant upstream connection, here 3 subtypes, $KC\alpha'\beta'$ -ap1, $KC\alpha'\beta'$ -ap2, and $KC\alpha'\beta'$ -m, are identified as significant downstream targets. $KC\gamma$ -m are identified as the top downstream neuron, but contrasting to the number of upstream neurons, there are approximately 40% of all $KC\gamma$ neurons that receive input from PPL202. $KC\gamma$ -m also appears in figure 3.6 C, left hand column with a total of 23 synapses with PPL202. This is due to 2 individual KCs with 11 and 12 synapses identified, which is much higher than the usual one or two synapses per KC. Consistent across the two datasets is that PPL202 sends information to $KC\gamma$ -d but PPL201 does not. These KCs have their dendrites located in the MB accessory calyx, a structure that receives visual input from the PLP.

3.5.4 PPL201 and PPL202 send information to PNs and LH local interneurons in the lateral horn

In the lateral horn there is zero overlap between PPL201 and PPL202 outputs, suggesting they are modulating different circuits in this area. PPL201 has more post-synaptic targets identified in the hemibrain dataset, with LHCENT2, a centrifugal LH neuron, being the only consistent target across datasets. The hemibrain dataset also identifies other centrifugal neurons and LHPV12a1, which is also an upstream connection of PPL202 in the calyx, creating a circuit connecting the calyx and LH where LHPV12a1 sends information to PPL202 in the calyx and receives information from PPL201 in

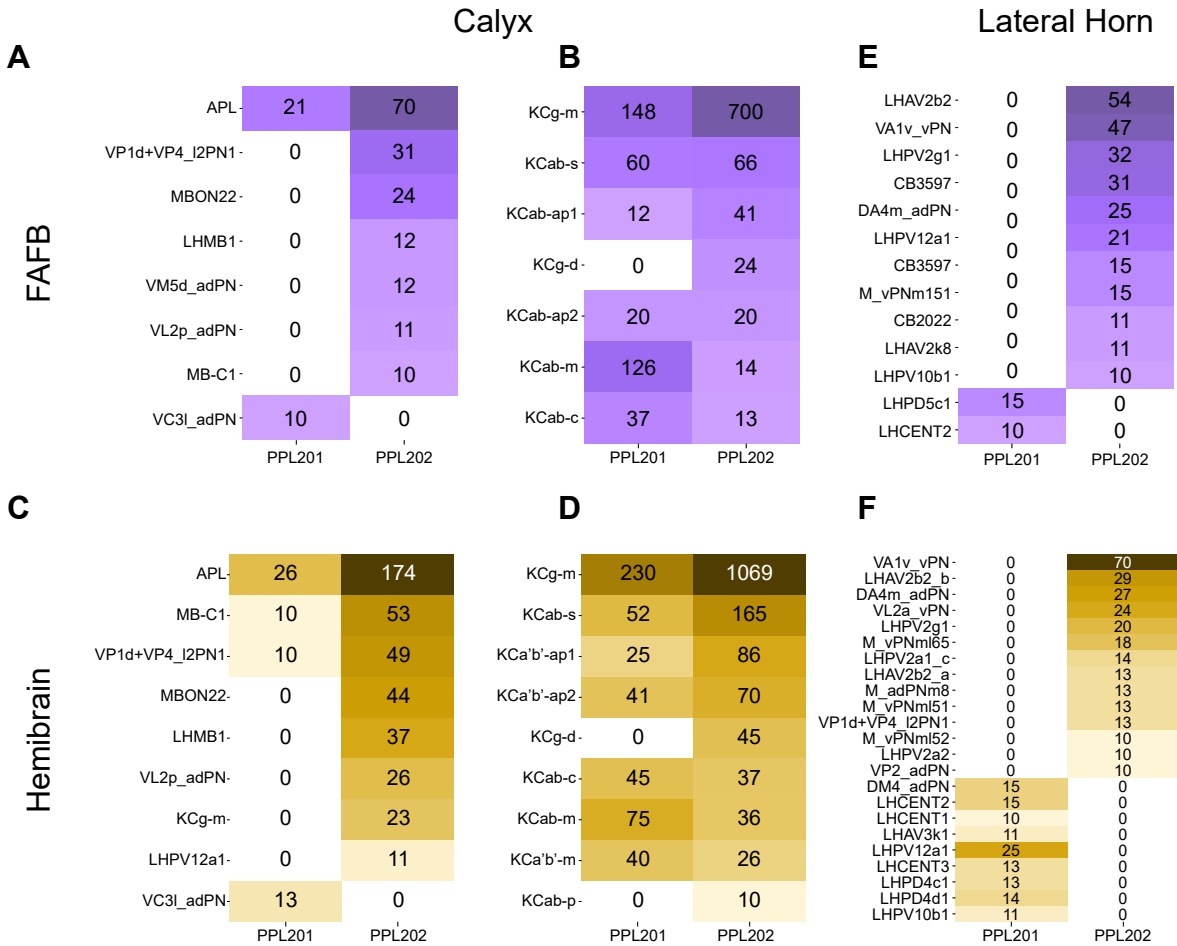


Figure 3.6: Downstream connections of PPL2 within the calyx

Heatmaps showing the post-synaptic neurons and number of synapses located within the calyx from the FAFB (A and B) and hemibrain (C and D) datasets. A and C show neurons not including Kenyon Cells, B and D show Kenyon Cells only. (E and F) Heatmaps showing the post-synaptic neurons and number of synapses located within the lateral horn from the FAFB (E) and hemibrain (F) dataset. For all heatmaps only neurons with 10 or more synapses are included. Number of synapses represents the sum of all neurons of that type.

the lateral horn.

PPL202, on the other hand, has more neuron types identified as downstream targets in both datasets (Figure 3.6 B & D). The top output type identified in FAFB is LHAV2b2, a group of six LH output neurons, and in the hemibrain it is an olfactory PN, VA1v_vPN, which is also pre-synaptic to PPL202. VP1d+VP4_l2PN1 seems to be one of the strongest PPL2 connections, being identified both upstream and downstream of PPL202 in both the calyx and lateral horn.

In summary, across datasets the same neuron types are identified as the top connections to and from PPL201 or PPL202 although the precise synapse counts differ between the datasets slightly altering the overall rankings. Upstream from PPL2 in

the calyx the major connections are APL, KC γ -m, and VP1d+VP4_l2PN1. Downstream, the same neuron types are identified as the main connections, suggesting that PPL2 forms many reciprocal synapses. Lastly, PPL202 connects strongly with more neuron types in the calyx compared to PPL201 with the only common connectivity between the two types being with various subtypes of KC, APL, VP1d+VP4_l2PN1, and MB-C1.

3.6 Identification of microglomeruli structures

Within the calyx connections between PNs and KCs form synaptic structures called microglomeruli. These structures consist of one large pre-synaptic PN bouton and many post-synaptic KCs whose dendrites wrap around the bouton in a claw-like shape. The synaptically dense structures can be thought of as units of olfactory representation since individual microglomeruli can undergo structural plasticity following an olfactory learning event [Baltruschat et al., 2021]. The previous results have shown that PPL2 neurons strongly connect with both PNs and KCs within the calyx. We hypothesise that sparse PPL2 innervations in the calyx could be targeting specific microglomeruli, in order modulate specific units. The other neuron strongly connected to PPL2, APL, has been shown to be a participant in the microglomeruli structures, providing localised inhibition [Baltruschat et al., 2021, Amin et al., 2020]. Therefore, we aim to investigate whether PPL2 synapses are also located within the microglomeruli structures to contribute to these microcircuits.

Using the FAFB and hemibrain datasets we can extract information on the coordinates of all the synapses that are located within the calyx. For each PN, the PN-to-KC synapses are located and sorted into clusters such that synapses that are close together are sorted into the same cluster. A volume is then defined which encompasses all of the synapses in the same cluster and this volume is what we define as a microglomeruli (see Chapter 2 for details). Two examples of PNs and their associated microglomeruli are given in Figure 3.7 A. The number and size of microglomeruli identified varies

per PN and the methods used work better on PNs with few microglomeruli which are further apart. VP1m_l2PN is a PN with extensive innervations in the calyx where 12 microglomeruli were identified in the FAFB dataset, and 14 identified in the hemibrain dataset. Some of these identified microglomeruli are small and close together, making the boundaries between them difficult to distinguish and the defined volumes overlapping. In contrast, VM5d_adPN has 2 well defined, and clearly distinguishable microglomeruli present in both FAFB and hemibrain.

3.6.1 The spatial distribution of microglomeruli is different across datasets

Overall, there are a total of 702 microglomeruli across 138 PNs in the FAFB dataset, and 682 microglomeruli between 126 PNs in the hemibrain dataset. The central locations of each microglomeruli is shown in Figure 3.7 B, where each circle represents one microglomeruli, the size of the circle represents the relative size of that microglomeruli in terms of number of post-synaptic KC synapses, and the colour shows the anterior-posterior axis depth. In both datasets the location of microglomeruli forms a lateral-medial gradient, with structures on the medial side located more anterior (Figure 3.7 B). Towards the center of the calyx are the most posterior positioned microglomeruli. In the hemibrain dataset, the microglomeruli seem more clustered to the dorsal region of the calyx, and a more sparse and smaller in the ventral region. However, the FAFB dataset has a more even distribution of microglomeruli across the dorsal-ventral axis. Although there are small microglomeruli distributed throughout the calyx, in both FAFB and hemibrain there is an isolated cluster of extremely small microglomeruli towards the dorsolateral portion of the calyx.

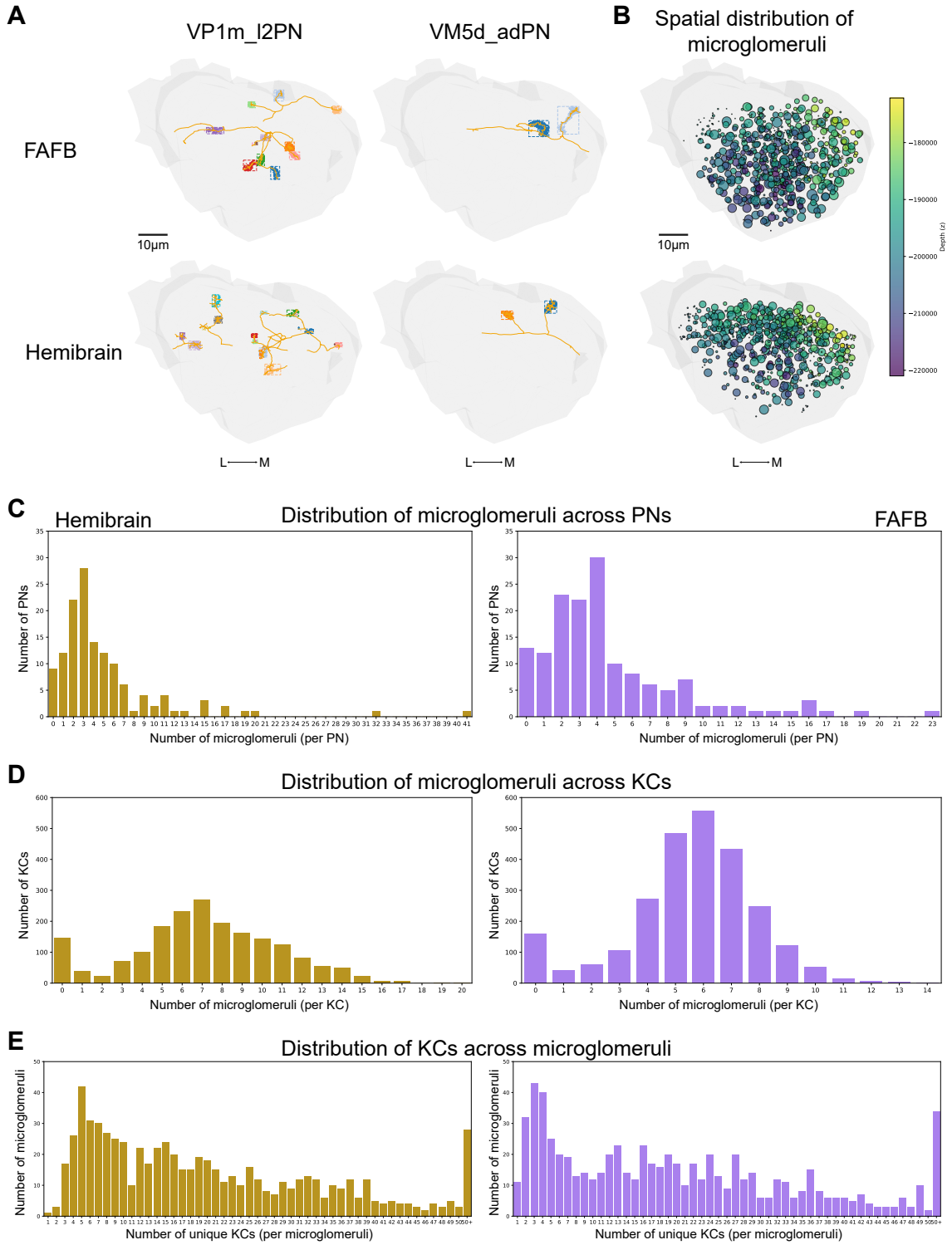


Figure 3.7: Analysis of identified microglomeruli.

(A) Example images showing PN skeleton and identified microglomeruli structures for that neuron. Every PN→KC post-synaptic coordinate is plotted and the points forming the same microglomeruli are plotted in the same colour. The microglomeruli bounding box is shown with a dashed rectangle. (B) Spatial distribution of identified microglomeruli. Each microglomeruli identified is represented by one circle, the size represents the number of KC synapses post-synaptic to the parent PN in that microglomeruli. The colour indicates the z-axis depth. (A&B) Top row: FAFB data, bottom row: hemibrain data. All coordinates have been transformed to FAFB space for plotting, and the right-hand side FAFB calyx mesh is used for visualisation. L: lateral; M: medial. Scale bar: 10 μ m. Depth scale: nm. (*continues on next page*)

3.6.2 The distribution of microglomeruli across PNs and KCs is similar across datasets

In order to compare the identified microglomeruli and assess the consistency of our method across datasets, the distribution of microglomeruli per PN, microglomeruli per KC, and KC per microglomeruli were quantified (Figure 3.7 C-E). The distribution of microglomeruli per PN can be thought of as the number of boutons per PN (Figure 3.7 C). Some PNs do not have any microglomeruli associated with them, these are likely PNs that follow the mALT tract, passing through the calyx but not branching to form any boutons. The highest number of microglomeruli identified for a single PN was 41, in the hemibrain data, and 23 in the FAFB data. These high extremes seem to be outliers, and the overall distribution forms a positively skewed normal distribution in both datasets. The average number of microglomeruli per PN is 5.1 in the hemibrain data, and 4.6 in the FAFB data.

The distribution of microglomeruli across KCs corresponds to the number of claws each KC has, since each claw is assumed to enwrap a different bouton and therefore contribute to different microglomeruli (Figure 3.7 D). The average number of microglomeruli innervated by a KC in the hemibrain dataset is 7.2, versus 5.6 in the FAFB dataset. In both datasets there are KCs that contribute to zero microglomeruli, and the maximum number of claws for a KC is 17 for the hemibrain, and 13 in FAFB.

Lastly, the average number of unique KCs per microglomeruli in the hemibrain dataset is 20.2, and 20.5 in FAFB (Figure 3.7 E). This distribution has a large variance, from 1 KC to over 50 unique KCs contributing to a single microglomeruli.

Figure 3.7 (previous page): (C) Distribution the number of microglomeruli identified per PN (D) Distribution of the number of microglomeruli a synapse is identified as being within, per KC. (E) Distribution of the number of unique KCs that have synapses identified as within the microglomeruli, per microglomeruli. (C-E) Hemibrain data is shown in the left column, FAFB data is shown in the right column.

3.7 Properties of microglomeruli containing PPL2 synapses

After identifying the microglomeruli volumes we investigate the differences between microglomeruli containing PPL2 synapses and those without PPL2 synapses. In order to determine whether or not a synapse is classed as within a microglomeruli the coordinates of the synapses are tested and if they fall within the microglomeruli volume that synapse is said to be contributing to that microglomeruli. If the synapse falls into more than one volume the distance to other synapses in the microglomeruli and the PN identity is also considered (see Chapter 2 for details).

3.7.1 Equivalent PN types have different microglomeruli structures across datasets

Figure 3.8 A shows two examples of PNs that have associated microglomeruli containing PPL2 synapses (PPL201 in red, and PPL202 in blue). These synapses are not necessarily partnered with the PN, or the KCs that are used to define the microglomeruli but the synapses identified tend to be closer to the center of the defined volume, placed along the PN skeleton which is what you would expect if the synapse is involved in these synaptic structures. The VP1d+VP4_l2PN1 neuron is a PN that is one of the major pre- and post-synaptic connections to both PPL201 and PPL202 within the calyx (Figures 3.5 and 3.6 A & C). There are 19 microglomeruli identified in FAFB, and 12 in the hemibrain data for this neuron, and the skeleton has a different branching pattern in each brain, extending along the dorsal-ventral axis in FAFB, but more laterally in the hemibrain. These differences make it more difficult to compare ‘equivalent’ microglomeruli. The other example, VM5d_adPN, has 2 identified microglomeruli in both datasets, this type of neuron has 4 instances in the FAFB dataset, and 8 instances in the hemibrain dataset with varying numbers of microglomeruli for each. These two instances of VM5d_adPN were chosen as examples since they have

the same number of microglomeruli as each other, and a similar morphology within the calyx. However, the PPL2 synapses identified in the associated microglomeruli are very different, with the hemibrain only identifying PPL201 synapses within these microglomeruli and FAFB identifying a majority of PPL202 synapses. This suggests that neurons of the same type with similar morphology and overall connectivity can have different microglomeruli structures containing different types of neurons.

3.7.2 Spatial distribution of microglomeruli containing PPL2 matches PPL2 skeletons

The total number of microglomeruli containing any PPL2 synapse is 292 out of 702 in the FAFB data, and 239 out of 682 in the hemibrain data (Figure 3.8 C & G). This breaks down to 161 with PPL202, 71 with PPL201, and 60 with both from the FAFB data, and 153 with PPL202, 37 with PPL201, and 49 with both from the hemibrain dataset. This corresponds to 58% (FAFB) and 65% (hemibrain) of identified microglomeruli containing no PPL2 synapses. Since PPL2 skeletons, especially PPL201, within the calyx are quite sparse it would make sense that these neurons do not innervate all of the microglomeruli (Figures 3.3 & 3.4 B & D). The PPL2 skeletons differ in the two connectomes (Figure 3.3 & 3.4) therefore, it is not surprising that the spatial distribution of PPL2 containing microglomeruli is also different between the two datasets (Figure 3.8 B). In the FAFB data we see that microglomeruli containing PPL201 synapses are closer to the perimeter of the neuropil, while microglomeruli containing PPL202 dominates the center, matching the skeleton innervations. In the hemibrain dataset the microglomeruli containing PPL2 also follow the skeleton innervations, densely packed on the dorsal edge of the calyx and in the center but more sparse as you move away from the center of the calyx along the medial-lateral axis. This also matches the synaptic distributions shown in Figures 3.3 and 3.4 E & F where we saw that most PPL2 synapses are located towards the center of the calyx, and more dorsal in the case of the hemibrain data. In general, microglomeruli containing PPL202 seem to contain more PPL2 synapses than those

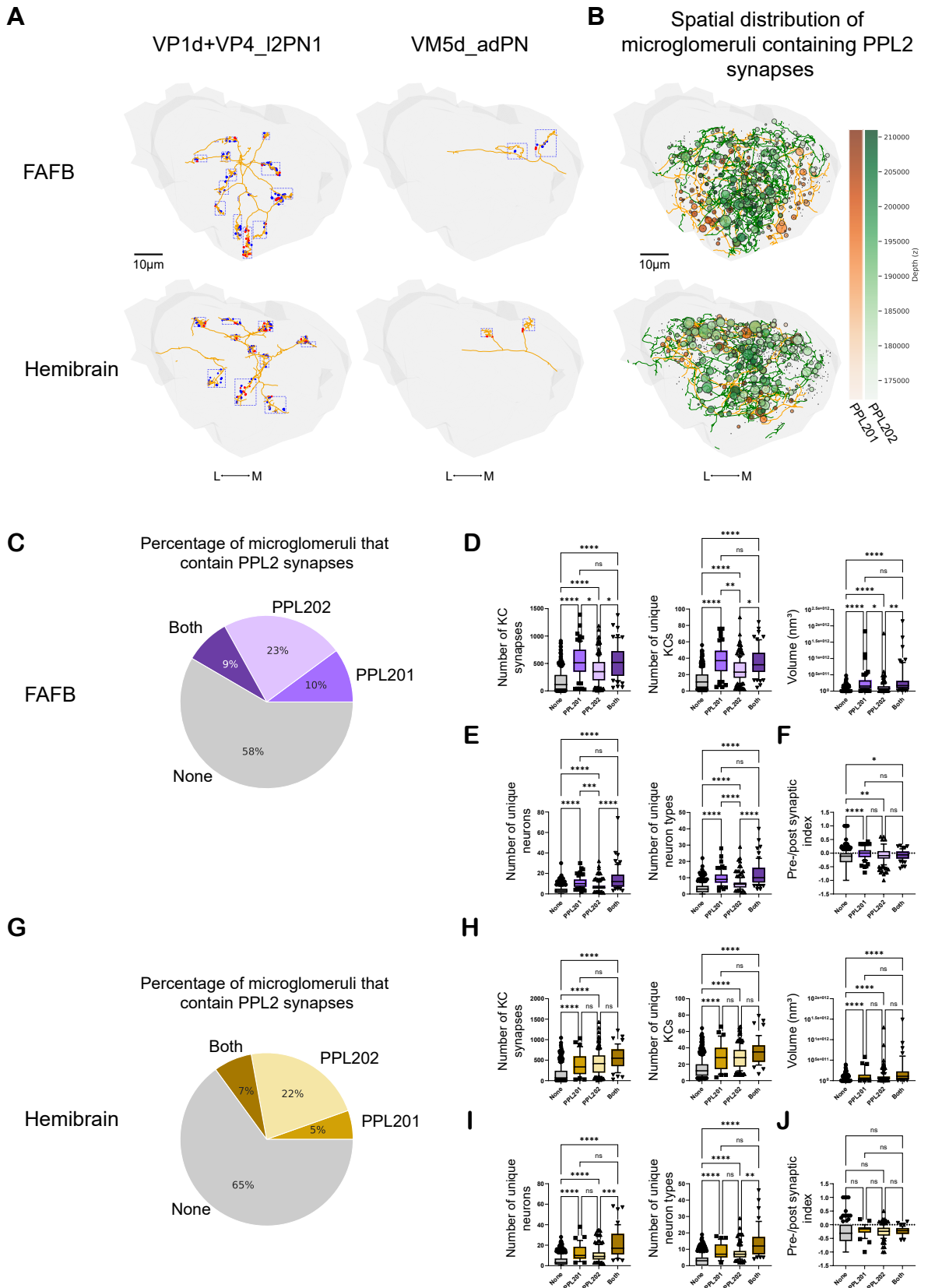


Figure 3.8: Comparison of microglomeruli with or without PPL2 synapses
(A) Example images of PN skeletons showing each microglomeruli and PPL2 synapses within those microglomeruli. Dashed rectangles represent the dimensions of the identified microglomeruli. Each point represents one synapse, red:PPL201, blue:PPL202. (B) Spatial distribution of microglomeruli identified to contain at least one PPL2 synapse. Each circle represents one microglomeruli, the size represents the number of PPL2 synapses in that microglomeruli. PPL2 skeletons are shown. The colour of circles indicates the z-axis depth, orange: PPL201, green: PPL202. (*continues on next page*)

containing PPL201 and the microglomeruli containing PPL2 synapses are not restricted to one section of the PPL2 neurons, they are distributed across the entire skeleton.

3.7.3 Microglomeruli that contain PPL2 synapses are larger than microglomeruli that do not

To investigate any differences between microglomeruli that contain PPL2 synapses and those that do not, we quantify three metrics which correlate to microglomeruli size (Figures 3.8 D and H). First, the number of PN-to-KC post-synapses which were used to define the microglomeruli are counted. Next, the number of unique individual KCs that contribute PN-to-KC post-synapses are counted, and lastly the volume of the 3D bounding-box used to define the microglomeruli is calculated. With all three metrics, in both FAFB and hemibrain data, the microglomeruli which do not contain any PPL2 synapses are significantly lower, meaning that they are on average smaller than microglomeruli that do contain at least one PPL2 synapse. In the FAFB data, there is also a significant difference between microglomeruli containing PPL201 synapses only, versus PPL202 synapses only, with those containing PPL201 being significantly larger across all three metrics (Figure 3.8 D). Furthermore, microglomeruli containing both PPL201 and PPL202 synapses are significantly larger than those that contain PPL202 only, but are not statistically different to microglomeruli containing PPL201

Figure 3.8 (previous page): (A&B) Top row: FAFB data, bottom row: hemibrain data. All coordinates have been transformed to FAFB space for plotting. The right-hand side FAFB calyx mesh is used for visualisation. L: lateral; M: medial. Scale bar: 10 μ m. Depth scale: nm. (C-E) FAFB data analysis. (C) Percentage of microglomeruli that contain PPL2 synapses. (D) Comparing metrics related to size of the microglomeruli across groups containing different PPL2 synapses. (E) Comparing metrics related to diversity of neurons in the microglomeruli. (F) Comparing the ratio of pre- to post- synaptic neurons. A positive score means there are more pre-synaptic neurons, a negative score means there are more post-synaptic neurons in the microglomeruli (number of microglomeruli: None = 410, PPL201 = 71, PPL202 = 161, Both = 60). (G) Same as (C) for hemibrain data. (H) Same as (D) for hemibrain data. (I) Same as (E) for hemibrain data. (J) Same as (F) for hemibrain data. (G-J) number of microglomeruli: None = 443, PPL201 = 37, PPL202 = 153, Both = 49. All statistics: Kruskal-Wallis test with Dunn's multiple comparisons correction, ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

each microglomerulus with both pre- and post-synapses. The number of unique neurons and the number of unique neuron types are quantified. In the FAFB data we see that the number of unique neurons and neuron types in microglomeruli without PPL2 synapses is significantly lower than the three other groups (Figure 3.8 E). Microglomeruli containing PPL202 synapses only have significantly less unique neurons and neuron types compared to microglomeruli containing PPL201 only or both PPL201 and PPL202. However, there is no significant differences between microglomeruli containing PPL201 and microglomeruli containing both PPL201 and PPL202 in either metric. In the hemibrain data, the trends are similar in that microglomeruli with no PPL2 synapses are less diverse than those with PPL2 synapses, i.e. they have significantly less different neuron types or individual neurons (Figure 3.8 H). However, the hemibrain data has no significant differences between microglomeruli containing PPL201 versus PPL202 synapses. Microglomeruli containing both, however, have significantly more unique neurons and neuron types.

Establishing the number of pre- and post-synapses within a microglomeruli is important to determine if the synaptic structure mainly consists of post-synaptic targets to the core PN pre-synaptic bouton, becoming a transmitter structure where olfactory information is sent to many different neurons, or if the structure consists of pre-synapses that provide feedback in a more complex microcircuit. The pre-/post-synaptic index is calculated based on the number of unique neurons that have pre- and post-synapses within the microglomeruli, where an index of zero means equal pre- and post-synaptic neurons, a positive index indicates more pre-synaptic, and a negative index indicates more post-synaptic neurons (see Chapter 2 for details). In the FAFB data, microglomeruli without PPL2 synapses have a significantly lower pre-/post-synaptic index, indicating that they have more post-synaptic neurons (Figure 3.8 F). The three groups which contain different types of PPL2 synapses are not significantly different, and the means of each are close to zero, showing that they tend to have equal numbers of pre- and post-synaptic neurons. The hemibrain dataset, however, does not show any significant differences between any of the groups (Figure 3.8 J). The mean index for each group is below zero, indicating a larger number of post-synaptic neurons.

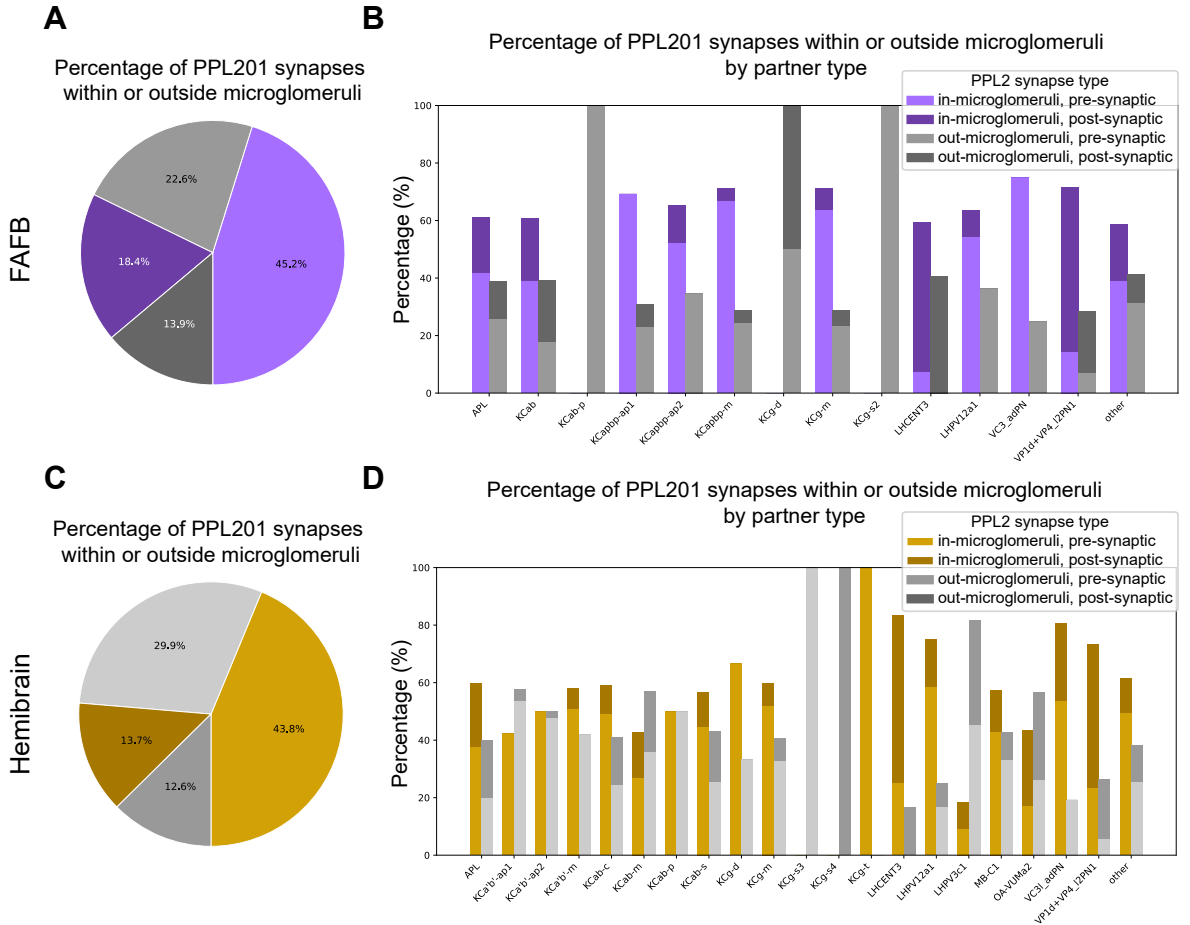


Figure 3.10: Proportion of PPL201 synapses located in microglomeruli (A) Pie chart showing the percentage of PPL201 synapses located inside or outside microglomeruli for FAFB dataset. (B) Bar chart showing the percentage of PPL201 synapses inside or outside microglomeruli per partner neuron type. Data normalised by number of synapses for PPL201-neuron type pair. Purple: in microglomeruli, light: pre-synapse, dark: post-synapse; grey: outside microglomeruli, light: pre-synapse, dark: post-synapse. (C & D) same as A & B for the hemibrain data. Yellow: in microglomeruli, light: pre-synapse, dark: post-synapse; grey: outside microglomeruli, light: pre-synapse, dark: post-synapse.

3.7.5 Microglomeruli that contain PPL2 are larger than microglomeruli that do not, with a restrictive definition

We have shown that with our definition of microglomeruli, the microglomeruli that contain PPL2 synapses are significantly larger than microglomeruli that do not contain PPL2 synapses. However, this difference could arise for a few different reasons. Firstly, the result could be biologically true and there is some reason that PPL2 synapses innervate larger microglomeruli, second the result is a statistical consequence of larger microglomeruli, i.e. a larger microglomeruli will have more neurons innervating it, and

therefore is more likely to contain a PPL2 synapse. Or thirdly, this result is an artifact of our definition of the microglomeruli volume (each of these options are discussed in further detail in Section 4.2.1). The boundaries used for the microglomeruli volume are calculated by using the maximum PN-to-KC synapse coordinate in each direction to give a cuboid bounding box. This method tends to overestimate the volume of the structure, especially when the shape of the cluster of synapses is on a diagonal to the coordinate system, and the error tends to increase with the size of the volume. Therefore, to establish whether our differences between microglomeruli are due to these method errors, a restrictive definition of the microglomeruli volume is described in the FAFB dataset (Figure 3.9 A). This restrictive definition uses the convex hull of the synapse cluster (see Chapter 2 for details) which results in a volume that is much tighter to the edges of the synapse cluster. Using this restrictive definition, the number of microglomeruli that do not contain any PPL2 synapses increases from 410 to 584, a total of 83% of all microglomeruli (Figure 3.9 B). There are 30, 84, and 5 total microglomeruli containing PPL201 only, PPL202 only, or both, respectively. The number of microglomeruli containing either PPL201 or PPL202 only are roughly halved compared to the more permissive volume definition. Despite the differences in the number of microglomeruli in each group, we see the same trends regarding the size of these synaptic structures (Figure 3.9 C). In each metric quantified, the number of PN-to-KC post-synapses, the number of unique KCs, and the total volume of the microglomeruli, there are statistically significant increases for all three groups that contain PPL2 synapses compared to the microglomeruli that do not contain PPL2. Therefore, the result that microglomeruli containing PPL2 are significantly larger stands even with this restrictive volume definition. There are no statistical differences between the groups containing PPL201 only, PPL202 only, or both in any metric but this could be due to the low number of microglomeruli that fall into these groups.

Overall, microglomeruli that contain PPL2 synapses are larger, and more diverse than those that do not contain PPL2 synapses. This finding stands if we use a restrictive definition for the microglomeruli volume that underestimates the amount of microglomeruli that contain PPL2 synapses.

3.8 Properties of PPL2 synapses located within microglomeruli

Next, a PPL2 synapse-centric analysis approach is taken, analysing the differences between synapses that are part of microglomeruli structures, and those that are located outside of the microglomeruli.

3.8.1 PPL2 pre-synaptic zones are located mostly in microglomeruli

PPL201 has a total of 850 (FAFB), or 976 (hemibrain) synapses located within the MB calyx, while PPL202 has 1,771 (FAFB), or 2,823 (hemibrain) (Table 3.1). In both cases, more than 50% of PPL2 synapses involve PPL2 as the pre-synaptic partner, meaning that PPL2 neurons are sending information to more synapses than they are receiving from. The vast majority of PPL2 pre-synapses are found within microglomeruli, and this group of synapses, pre-synapses that are within microglomeruli, make up almost 50% of all PPL2 synapses (Figures 3.10 A & C, and 3.11 A & C). The number of pre-synapses that are inside microglomeruli are almost double the amount of pre-synapses that are found outside of microglomeruli, suggesting that both PPL201 and PPL202 preferentially send information via the microglomeruli structures.

In the case of PPL2 post-synapses, however, there is an even split between synapses that are inside or outside of microglomeruli. For PPL201 in the hemibrain data there are 123 post-synapses found outside microglomeruli and 134 inside while PPL202 has 223 outside and 325 inside. This is similar to what is found in the FAFB data, with PPL201 having 118 outside, and 156 inside and PPL202 has 168 outside, and 308 inside microglomeruli. Overall, PPL2 pre-synapses seem to preferentially be found within microglomeruli, whereas post-synapses are equally likely to be found inside or outside the microglomeruli structures.

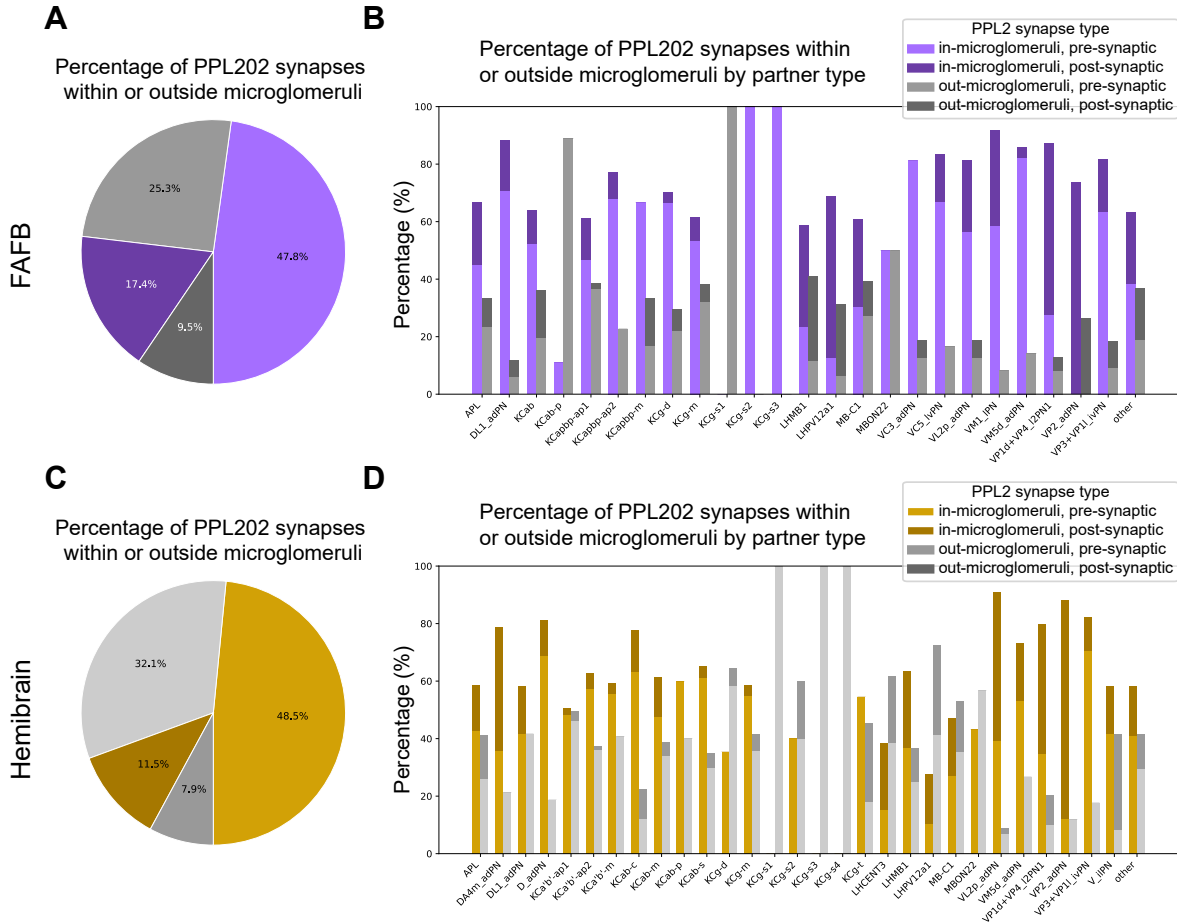


Figure 3.11: Proportion of PPL202 synapses located in microglomeruli

(A) Pie chart showing the percentage of PPL202 synapses located inside or outside microglomeruli for FAFB dataset. (B) Bar chart showing the percentage of PPL202 synapses inside or outside microglomeruli per partner neuron type. Data normalised by number of synapses for PPL202-neuron type pair. Purple: in microglomeruli, light: pre-synapse, dark: post-synapse; grey: outside microglomeruli, light: pre-synapse, dark: post-synapse. (C & D) same as A & B for the hemibrain data. Yellow: in microglomeruli, light: pre-synapse, dark: post-synapse; grey: outside microglomeruli, light: pre-synapse, dark: post-synapse.

3.8.2 The top PPL2 synaptic connections have synapses both inside and outside microglomeruli

Each synapse has a synaptic partner that is either up- or downstream to PPL2. An interesting question is to understand which types of partner neurons are in or outside of microglomeruli, are there any patterns in certain connections which are always inside or vice versa? To visualise this information took the PPL2 synaptic connections with more than 10 total synapses located within the calyx (as listed in figures 3.5 and 3.6 A & C) and plotted the proportion that are located inside or outside of microglomeruli

per partner neuron type (Figure 3.10 and 3.11 B & D). For almost every neuron partner type there are more total synapses located within microglomeruli. Notable exceptions to this are some KC subtypes, for example connections with $KC\gamma$ -s are more likely to be outside of microglomeruli. PPL202 connections with PNs tend to be majority inside of microglomeruli for both the pre- and post-synapses with this type, providing a modulatory feedback system within the microglomeruli microcircuits.

In summary, our method of identifying microglomeruli is sound, with metrics such as average number of claws per KC, average number of boutons per PN and total microglomeruli are within a reasonable range compared to the current literature. PPL2 pre-synapses are largely located within microglomeruli, suggesting the ability of PPL2 to provide local dopamine input to these micro-circuits. Approximately half of PPL2 post-synapses are located within microglomeruli, providing a potential feedback mechanism within the structure. Microglomeruli containing PPL2 synapses are significantly larger in volume, and in terms of the number of KCs post-synaptic to the PN core, than microglomeruli without any PPL2 synapses. This effect is robust to a more restrictive definition of the microglomeruli volume, suggesting this is not an artifact of our microglomeruli model.

Having established the circuitry PPL2 is involved in, we now investigate the functional role of dopaminergic input in the calyx, with a particular focus on KC odor-evoked responses.

3.9 Knockdown of three dopamine receptors differently affect odor-evoked responses in KCs

Connectomic analysis of PPL2 revealed KCs as a major post-synaptic target, with a large portion of synapses located within microglomeruli in the calyx. We hypothesise that PPL2 input in the calyx will modulate KC responses to odor and therefore, this section will investigate the effect of decreasing dopaminergic input on the odor-evoked

response of KCs and by which receptor dopamine is acting through.

To address this question a GCaMP reporter was expressed in subsets of the KC population and fluorescence levels in the calyx measured following a short exposure to ethyl butyrate (full details of imaging protocol are given in Section 2). The fluorescence level of GCaMP is proportional to calcium activity of the cell, which can be used as a proxy of neural activity. Additionally, three different types of dopamine receptor were systematically knocked down via RNA interference in subsets of the KC population, and the control fly lines did not express the UAS-RNA_i. For all calcium imaging experiments female flies (unless otherwise stated) of age 3-5d old were used and each fly underwent two recording sessions, each consisting of a 3 second odor puff and separated by 5 minutes of air. The two sets of data are referred to as response 1 and response 2 (Figure 3.12 A). The responses were quantified using two metrics; the area under the curve captures changes in response dynamics that do not necessarily lead to more cell activity, and the peak magnitude of the response captures changes in maximum level of cell activity (Figure 3.12 C).

We first used a broad KC driver, 247-gal4 [Zars et al., 2000], to target GCaMP6m and receptor-RNAis to a large population of cells. This driver strongly targets both γ and $\alpha\beta$ subtypes of KCs and weakly targets $\alpha'\beta'$ cells (Figure 3.12 A and B).

3.9.1 Knockdown of Dop1R1 in 247-Gal4 targeted KCs alters response dynamics

First, knockdown of Dop1R1, an excitatory D1-like receptor leads to altered odor-evoked response dynamics in the calyx, as indicated by a significant decrease in the area under the curve of knockdown flies, as compared to the genetic controls (Figure 3.12 C and D). Although the peak magnitude of the response is not significantly altered by Dop1R1 knockdown there is a trend towards a decrease (response 1 $p=0.1335$, response 2 $p=0.0688$), which can also be seen in the fluorescence traces. The difference between these two metrics, paired with the fluorescence traces, suggests that Dop1R1

3.9. KNOCKDOWN OF THREE DOPAMINE RECEPTORS DIFFERENTLY AFFECT ODOR-EVOKED RESPONSES IN KCS

knockdown results in a shortened odor-evoked response.

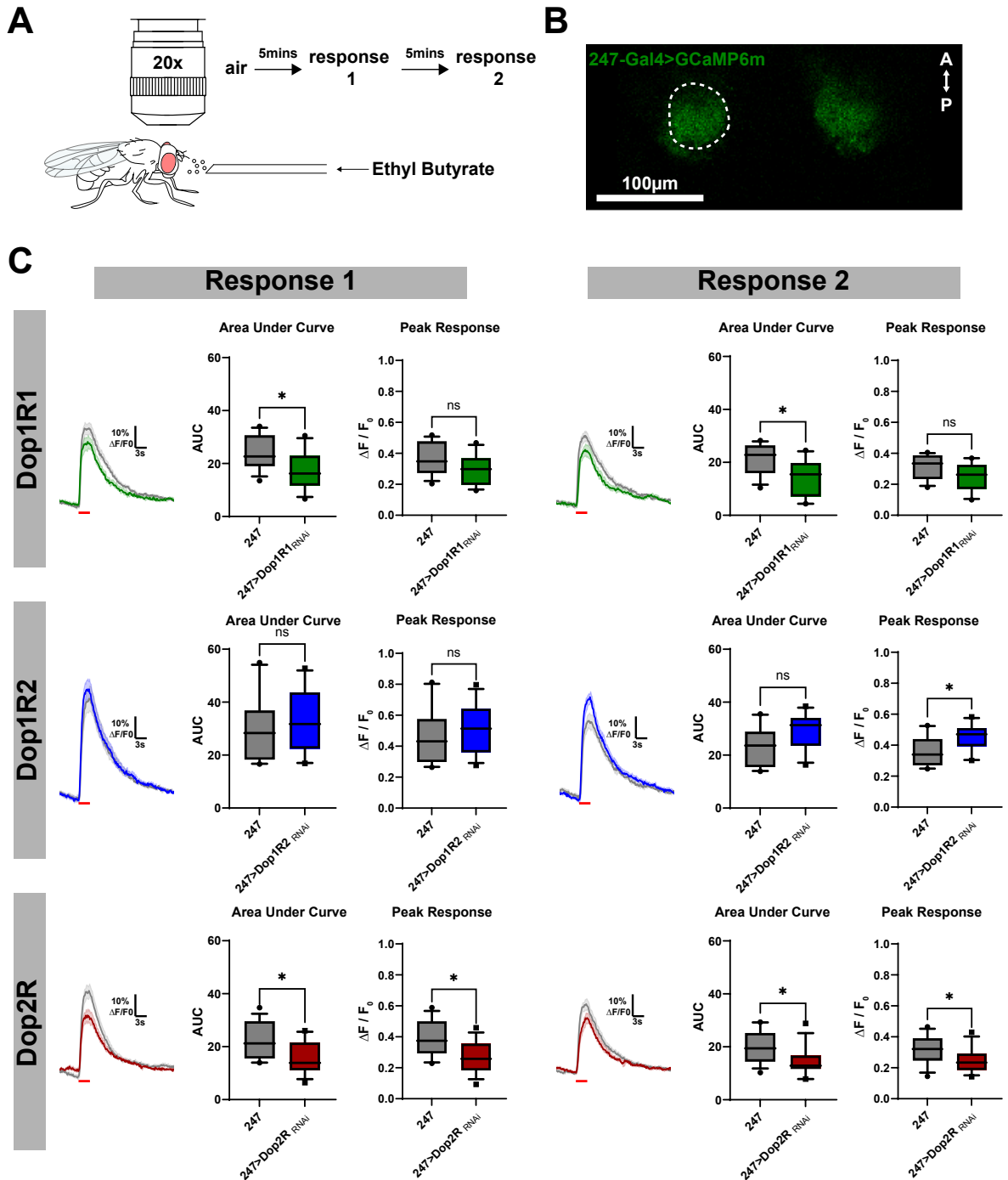


Figure 3.12: Dopamine receptor knockdowns affect the odor-evoked response of KCs. (A) Schematic of the mushroom body highlighting the subset of KCs targeted by 247-Gal4. (B) Representative image of odor-evoked calcium response in an 247-Gal4>GCaMP6m fly. A region of interest was drawn around the MB calyx. A, anterior; P, posterior. (C) Odor-evoked responses of 247-KCs following dopamine receptor knockdown. Left column, first response to novel odor; right column, second response following 5 minutes of constant air flow after the first response. First row, knockdown of Dop1R1 (n = 11-12; second row, knockdown of Dop1R2 (n = 10-11); third row, knockdown of Dop2R (n = 13-14). Each set of data consists of: (left) GCaMP6m fluorescence across time (mean $\pm$ SEM), the red bar on the x-axis indicates odor delivery duration. (Middle) quantification of area under the curve, and (right) quantification of the peak calcium response. Odor used was ethyl butyrate. Mann-Whitney test, *p<0.05.

3.9.2 Knockdown of Dop1R2 in 247-Gal4 targeted KCs increases response to a familiar odor

Drosophila express a second D1-like receptor, named Dop1R2. Knockdown of this receptor in 247-KCs leads to no significant differences in either area under curve or peak response in the response to the first exposure of ethyl butyrate (Figure 3.12 C, row 2). However, the second response, which follows response 1 after 5 minutes of constant air flow, shows a significant increase in the peak response magnitude and a trend towards increase in area under the curve ($p=0.0986$). This suggests that dopamine input acting through Dop1R2 may have different effects on KC responses depending on whether the odor has been experienced previously.

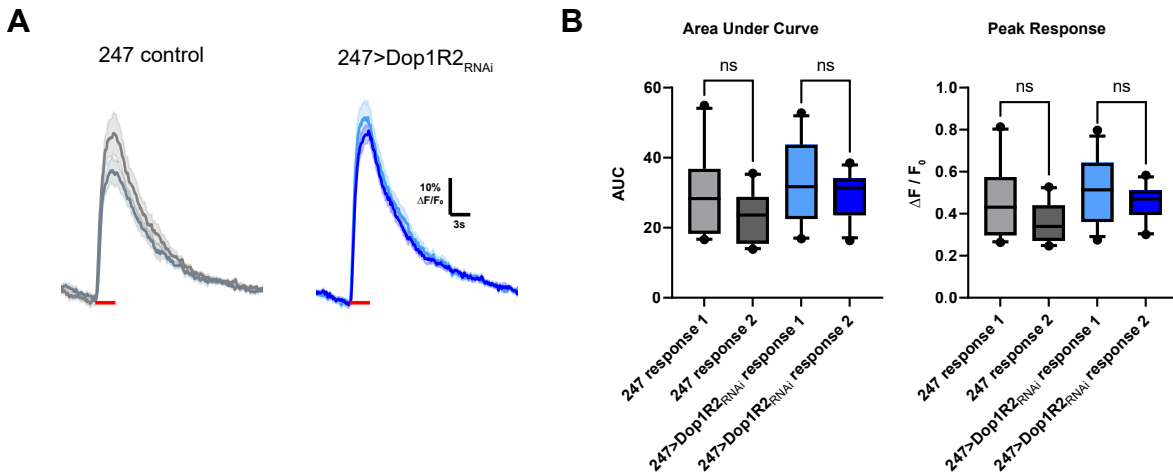


Figure 3.13: Comparison of response 1 and response 2 for Dop1R2 knockdowns in 247-KCs.

(A) Fluorescence traces across time (mean $\pm$ SEM). Red bar on the x-axis indicates odor delivery duration. (B) Quantifications of area under the curve and peak magnitude of the response. Odor used was ethyl butyrate. $n=10-11$. Kruskal-Wallis test with Dunn's multiple comparisons correction, ns $p>0.05$.

To explore this possibility we compared the first and second responses for both the control and experimental knockdown flies (Figure 3.13). Here, we do not see any significant differences between the two responses for either experiment group, however, observationally there does seem to be a trend towards the control group decreasing their calcium activity in the second response, although this is not statistically significant. It is important to point out that the experimental setup was not designed to investigate the response to novel versus familiar odours, and is therefore not optimised

for this type of analysis. Nevertheless, this preliminary analysis gives an indication that Dop1R2 may be acting to modulate the response to odours that have been previously experienced.

3.9.3 Knockdown of Dop2R results in robust decrease of 247-KC response in female flies

Lastly, the knockdown of inhibitory D2-like receptor, Dop2R, leads to a decrease in overall KC odor-evoked activity, as seen by significant decreases in both area under the curve, and peak magnitude of the response across both response 1 and 2 (Figure 3.12 C, row 3).

Dop2R is differentially expressed in male and female flies, and has critical roles in sexually dimorphic behaviours such as female egg-laying [Lyman et al., 2023], and male courtship [Love et al., 2023]. Therefore, we investigated whether knockdown of Dop2R in male flies would have a different result. Indeed, we do not see the same robust decrease in calcium activity, instead we do not see any significant differences between the control and knockdown male flies (Figure 3.14). This indicates sexually dimorphic modulation of KC odor-evoked responses by dopamine which could lead to control of different behavioural outputs in response to the same odor.

Overall, knockdown of each dopamine receptor has a different effect on ethyl butyrate-evoked KC activity in the MB calyx. Dop1R1 knockdown results in a significant decrease in the area of the response curve, alluding to a change in response dynamics. Dop1R2 knockdown effects the response to the second odor exposure only, decreasing the peak magnitude, potentially influencing KC responses to familiar odors. Finally, knockdown of Dop2R results in a robust decrease in calcium activity in female flies, but does not effect the response of KCs in males. Together, this suggests that dopaminergic input to the KCs acts through all three receptors to modulate KC activity in different ways. All dopamine receptor knockdown results are summarised in table 3.2.

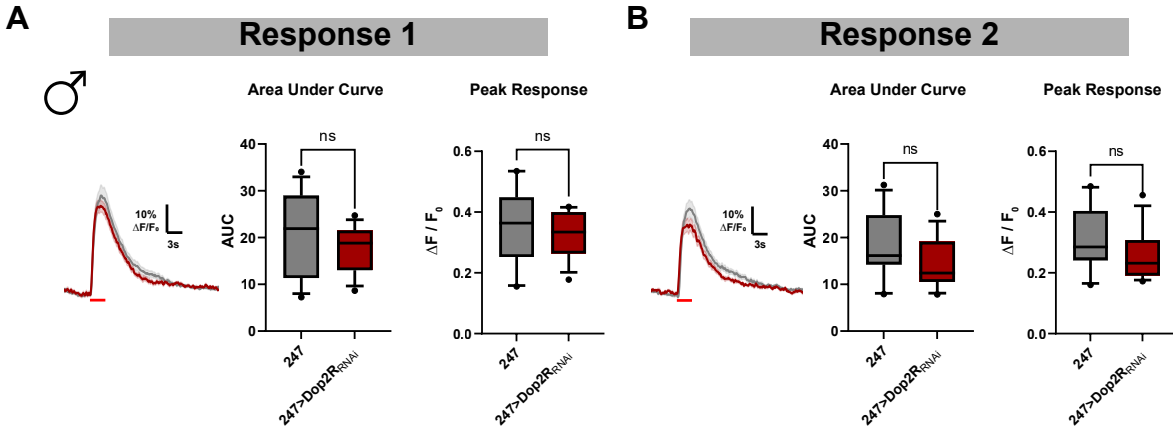


Figure 3.14: Dop2R knockdown in male 247-Gal4 flies.

(A & B) Odor-evoked responses of male 247-Gal4 flies expressing GCaMP6m following Dop2R knockdown. (Left) GCaMP6m fluorescence across time (mean $\pm$ SEM), the red bar on the x-axis indicates odor delivery duration. (Middle) quantification of area under the curve, and (right) quantification of the peak calcium response. (A) First response to novel odor, (B) second response after 5 minutes of constant air flow following the first response. Odor used was ethyl butyrate. $n=12$. Mann-Whitney test, ns $p>0.05$.

3.10 Knockdown of dopamine receptors differently affect subsets of the KC population

The 247-Gal4 targets a large population of KCs, including cells from all three main subtypes, γ , $\alpha\beta$, and $\alpha'\beta'$. However, these subtypes have distinct innervation patterns, projecting their axons to form different MB lobes, different inputs, receiving biased input from PNs and different levels of dopaminergic input from PPL2, and, most importantly, different functions [Aso et al., 2014a, Tanaka et al., 2008, Li et al., 2020a, Aso et al., 2009]. Therefore, it is crucial to separate the KC subtypes in order to get an understanding of how dopaminergic input affects the olfactory circuit. We target two sub populations of KCs, the γ -KCs and $\alpha\beta$ -KCs using 5HTR1B-Gal4 and C739-Gal4 respectively (Figures 3.15 A and 3.17 A) [Shyu et al., 2017, Wu et al., 2023, Akalal et al., 2006, Armstrong et al., 1998].

3.10. KNOCKDOWN OF DOPAMINE RECEPTORS DIFFERENTLY AFFECT SUBSETS OF THE KC POPULATION

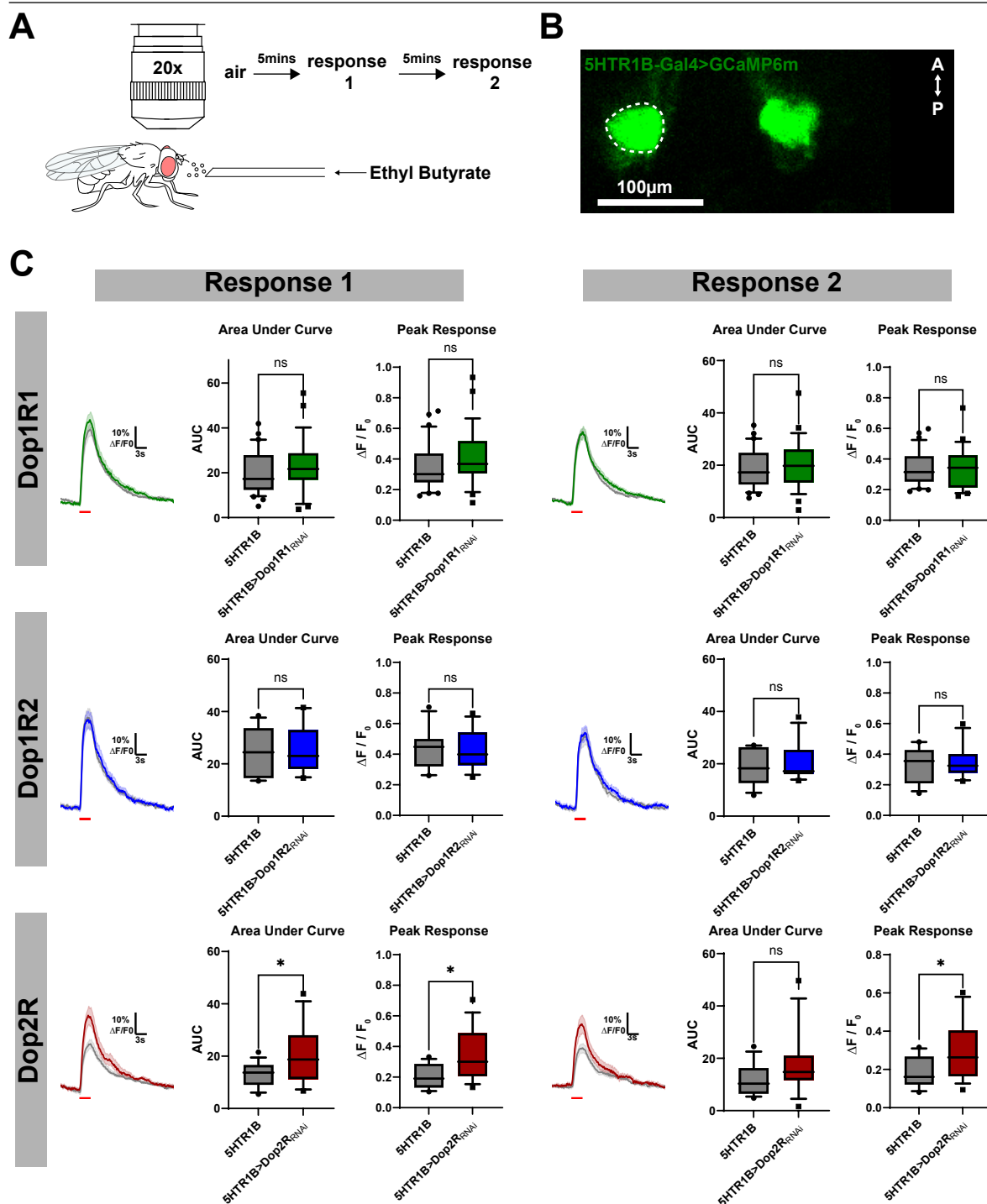


Figure 3.15: Dopamine receptor knockdowns affect the odor-evoked response of γ -KCs. (A) Schematic of the mushroom body highlighting the subset of KCs targeted by 5HTR1B-Gal4. (B) Representative image of odor-evoked calcium response in an 5HTR1B-Gal4>GCaMP6m fly. A region of interest was drawn around the MB calyx. A, anterior; P, posterior. (C) Odor-evoked responses of 5HTR1B-KCs following dopamine receptor knockdown. Left column, first response to novel odor; right column, second response following 5 minutes of constant air flow after the first response. First row, knockdown of Dop1R1 (n = 26-31; second row, knockdown of Dop1R2 (n = 11); third row, knockdown of Dop2R (n = 14). Each set of data consists of: (left) GCaMP6m fluorescence across time (mean $\pm$ SEM), the red bar on the x-axis indicates odor delivery duration. (Middle) quantification of area under the curve, and (right) quantification of the peak calcium response. Odor used was ethyl butyrate. Mann-Whitney test, *p<0.05.

3.10.1 Knockdown of D1-like receptors have no effect on response of either γ - or $\alpha\beta$ -KCs in females

Knockdown of Dop1R1 and Dop1R2 in either 5HTR1B-KCs or C739-KCs do not show any significant differences across any of our metrics (Figures 3.15 and 3.17 C, rows 1 and 2).

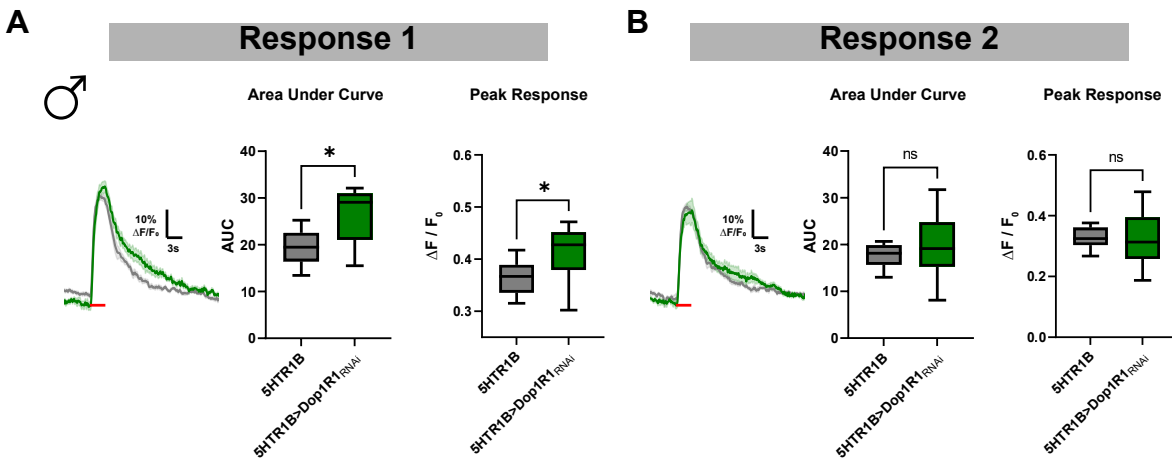


Figure 3.16: Dop1R1 knockdown in male 5HTR1B-Gal4 flies.

(A & B) Odor-evoked responses of male 247-Gal4 flies expressing GCaMP6m following Dop2R knockdown. (Left) GCaMP6m fluorescence across time (mean $\pm$ SEM), the red bar on the x-axis indicates odor delivery duration. (Middle) quantification of area under the curve, and (right) quantification of the peak calcium response. (A) First response to novel odor, (B) second response after 5 minutes of constant air flow following the first response. Odor used was ethyl butyrate. $n=9$. Mann-Whitney test, $*p<0.05$.

To investigate any sexually dimorphic results we also performed a knockdown of Dop1R1 in male flies expressing 5HTR1B-Gal4. Here, we see the knockdown flies have an increase in the odor-evoked response to a novel odor (Figure 3.16 A). Looking at the fluorescence trace it seems as though there is a change in response dynamics with the Dop1R1 knockdown leading to a response with a slower decay to baseline. There are no significant differences in the second response (Figure 3.16 B), suggesting that in males Dop1R1 may have a role in modulating responses to novel odors.

3.10. KNOCKDOWN OF DOPAMINE RECEPTORS DIFFERENTLY AFFECT SUBSETS OF THE KC POPULATION

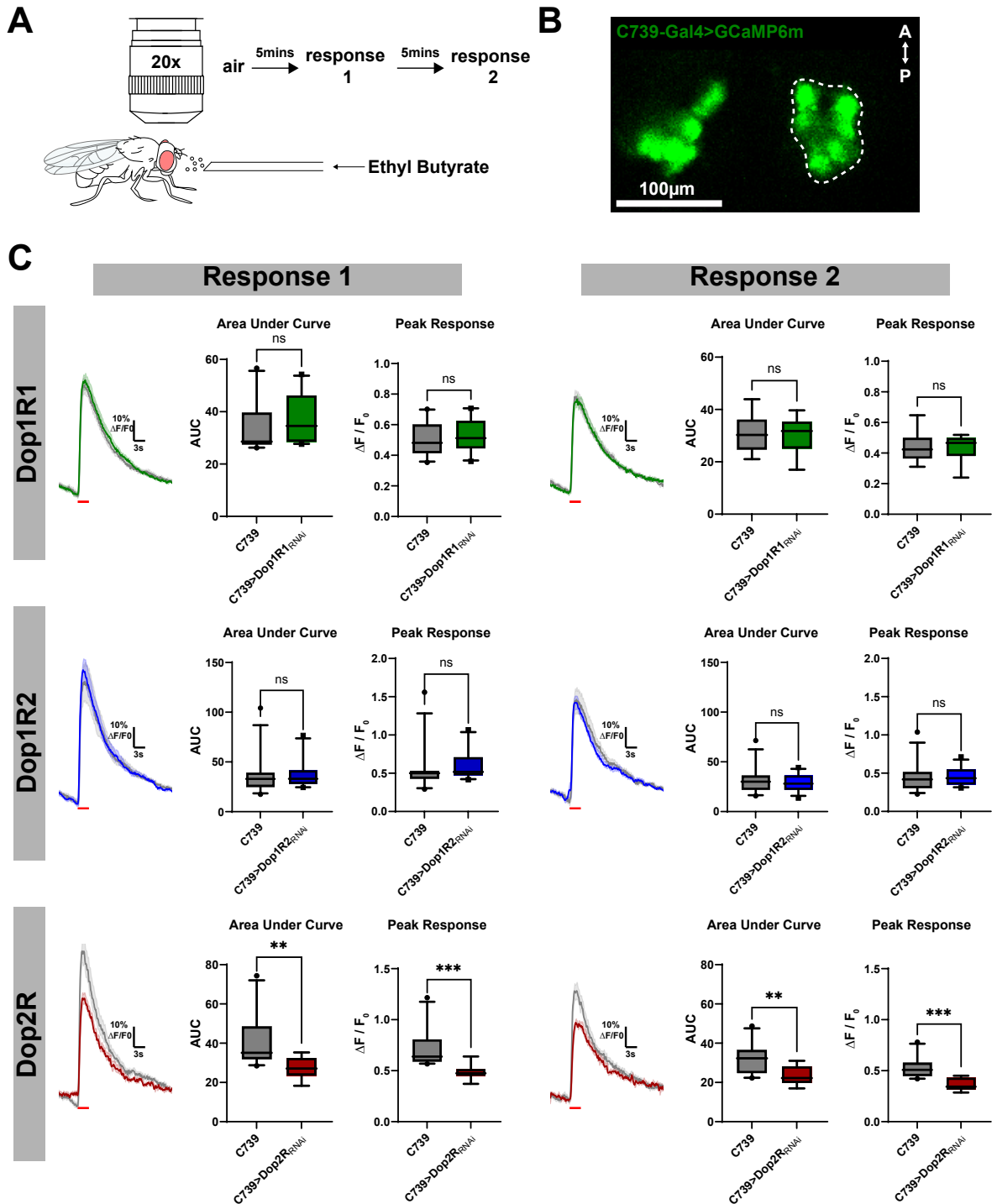


Figure 3.17: Dopamine receptor knockdowns affect the odor-evoked response of $\alpha\beta$ -KCs. (A) Schematic of the mushroom body highlighting the subset of KCs targeted by C739-Gal4. (B) Representative image of odor-evoked calcium response in an C739-Gal4>GCaMP6m fly. A region of interest was drawn around the MB calyx. A, anterior; P, posterior. (C) Odor-evoked responses of C739-KCs following dopamine receptor knockdown. Left column, first response to novel odor; right column, second response following 5 minutes of constant air flow after the first response. First row, knockdown of Dop1R1 ($n = 9$; second row, knockdown of Dop1R2 ($n = 10-12$); third row, knockdown of Dop2R ($n = 9-10$). Each set of data consists of: (left) GCaMP6m fluorescence across time (mean $\pm$ SEM), the red bar on the x-axis indicates odor delivery duration. (Middle) quantification of area under the curve, and (right) quantification of the peak calcium response. Odor used was ethyl butyrate. Mann-Whitney test, ** $p < 0.01$, *** $p < 0.001$.

3.10.2 Knockdown of Dop2R has opposing effects on the response of γ - and $\alpha\beta$ -KCs

Knockdown of Dop2R in γ -KCs targeted by 5HTR1B-Gal4 leads to a significant increase in odor-evoked calcium response (Figure 3.15 C, row 3). For the first response this result is robust, with significant increases in both the area under curve and peak magnitude metrics. In the second response the peak magnitude gives a significant increase with the knockdown, however, the area under curve is trending towards a significant increase ($p=0.1139$). This suggests a change in response dynamic between the first and second responses.

Conversely, knockdown of Dop2R in C739-Gal4 targeted $\alpha\beta$ -KCs results in a robust decrease in calcium response across all the quantified metrics. This suggests that dopamine input to the different subsets of KCs has a different function and modulates KC activity in opposing directions.

Overall, the knockdown of different dopamine receptors affect the two KC subgroups we have investigated differently. Dop1R1 and Dop1R2 knockdown in females does not significantly change the odor evoked response for either γ - or $\alpha\beta$ -KCs. However, in the γ -KCs of males we see a significant increase in response to novel odors with knockdown of Dop1R1. Lastly, knockdown of Dop2R gives the most robust effect, increasing responses in γ -KCs, and decreasing responses in $\alpha\beta$ -KCs. These results, along with the results of receptor knockdowns using a broad KC driver are summarised in table 3.2

	Dop1R1	Dop1R2	Dop2R
247-Gal4 (all KCs)	decrease (area only)	increase (second response only)	decrease (males no change)
5HTR1B-Gal4 (γ -KCs)	no change (males increase first response only)	no change	increase
C739-Gal4 ($\alpha\beta$ -KCs)	no change	no change	decrease

Table 3.2: Summary of dopamine receptor knockdown results

3.11 Characterisation of different Gal4 driver lines for PPL2 targeting

As well as dopaminergic input from PPL2 in the calyx, KCs also receive input from two other dopamine clusters to their axons in the MB lobes. Inputs and modulation in the MB are known to be highly compartmentalised [Boto et al., 2014, Hige et al., 2015b, Hige et al., 2015a] and therefore it is unlikely for dopaminergic inputs to the lobes to back-propagate to modulate activity in the calyx. However, it is still important target PPL2 directly to determine exactly how PPL2 input is affecting KCs overall. Here, we analyse the expression patterns of numerous Gal4 driver lines that target the PPL2 cluster.

Although there seem to be numerous Gal4 driver lines for our purpose, these lines are not perfect and it is very difficult to find a driver that targets your neurons of interest and no other cells. For our experiments it is necessary that our driver targets the correct PPL2 neuron types that innervate the calyx, and that we do not target any neurons that may have an affect on olfactory response such as PNs, or other neurons that innervate olfactory related areas.

Driver	PPL2 neurons in left hemisphere	PPL2 neurons in right hemisphere	Brains Counted	Calyx?	Figures
PPL2-Gal4	5.28±0.19	5.28±0.19	12	y	Figure 3.18 A
TH-Gal80;PPL2-Gal4	0	0	12	n	Figure 3.18 B
ΔTH-D ¹ -Gal4	0	0	6	n	Figure 3.18 C
Vmat AD;DAT DBD	3.5±0.29	3.75±0.25	4	n	Figure 3.20 A
Vmat AD;TH-C DBD	4.5±0.5	3.5±0.5	3	n	Figure 3.20 B
oc2-Gal4	4±0.26	3.5±0.5	7	y	Figure 3.19 A
oc2-Gal4, repo-Gal80	4±0.82	4.7±0.41	10	y	Figure 3.19 C
PPL2-split Gal4	2.4±0.23	2.6±0.13	16	y	Figure 3.21 A

Table 3.3: GAL4 driver lines and their GFP positive PPL2 neurons
Different GAL4 drivers investigated. GFP positive PPL2 neurons were counted and the mean calculated for each hemisphere (mean ± SEM). Presence of calyx innervation was determined and indicated by a yes (y) or no (n). Figures showing the PPL2 neuron location and an inset of the calyx for each driver indicated.

To test the available drivers, we express green fluorescent protein (GFP) in each of our lines. We use either a UAS-GFP::nls, that localises GFP to the nucleus of target cells, but has leaky expression, meaning we can visualise innervations of these cells, or

a UAS-mcD8::GFP, a membrane-targeted marker that strongly labels neural processes (See Chapter 2: Materials and Methods for the full immunostaining protocol). For each driver, the number of neurons from the PPL2 cluster that are targeted are counted, observations of the innervations in the calyx, or any off-target neurons, are noted. A summary table of results can be found in Table 3.3.

3.11.1 PPL2 cluster provides dopaminergic innervations to the calyx

One driver that has been reported for targeting the PPL2 cluster is the Δ TH-C'-Gal4 combined with the f-Gal80 repressor, which we will now refer to as PPL2-Gal4 [Liu et al., 2012b, Xie et al., 2018, Boto et al., 2019]. This driver targets almost all PPL2 neurons, 5.28 ± 0.19 in each hemisphere (Table 3.3) out of a total of around 7 neurons per hemisphere [Mao and Davis, 2009, Boto et al., 2019]. GFP is visible within the calyx in claw-like shapes (Figure 3.18 A and B) but the driver also targets non-PPL2 neurons elsewhere in the brain as well as members of the PPL2 cluster that do not innervate the calyx.

In order to determine whether the calyx innervation is resulting from the PPL2 neurons, this driver is combined with a TH-Gal80. Gal80 is a Gal4 inhibitor (see Section 1.2.2), and TH (Tyrosine Hydroxylase) is used as a dopamine marker. Therefore, using this combination of drivers the Gal4 is inhibited in dopaminergic neurons, removing GFP expression from these cells. Using this genetic combination the PPL2 cluster is no longer labelled (Table 3.3) and there are no GFP labelled processes visible in the calyx (Figure 3.18 C). This suggests that the calyx innervation we saw from the PPL2-Gal4 was coming from dopaminergic neurons, likely our PPL2 neurons.

To check the calyx innervations are not originating from another dopaminergic cluster, PPL1, the driver Δ TH-D'-Gal4 is used, which specifically targets this cluster of neurons and does not target PPL2 [Xie et al., 2018] (Figure 3.18 D, Table 3.3). When GFP is expressed using this driver, there are no GFP innervations visible in the calyx.

3.11. CHARACTERISATION OF DIFFERENT GAL4 DRIVER LINES FOR PPL2 TARGETING

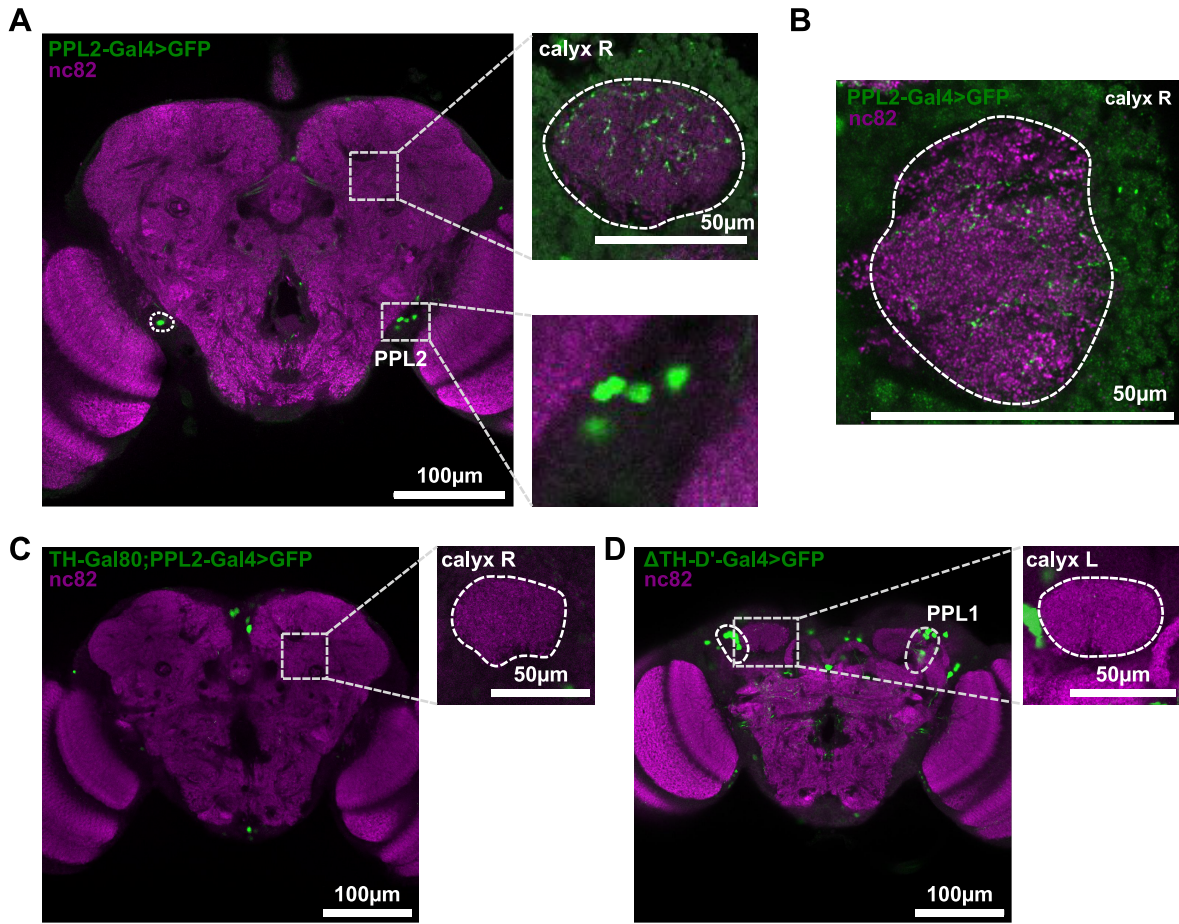


Figure 3.18: Characterisation of PPL2-Gal4

(A) (Left) Representative image of anti-GFP (green) and anti-nc82 (magenta) immunostaining of the brain of a PPL2-Gal4 > UAS-GFP::nls fly. (Right) Representative images showing innervation in the calyx (top) and GFP positive PPL2 cell bodies (bottom). (B) Airy scan image of anti-GFP (green) and anti-nc82 (magenta) immunostaining of the calyx of a PPL2-Gal4 > UAS-GFP::nls fly. (C) (Left) Representative image of anti-GFP (green) and anti-nc82 (magenta) immunostaining of the brain of a TH-Gal80;PPL2-Gal4 > UAS-GFP::nls fly. (Right) Representative image showing absence of calyx innervation. (D) (Left) Representative image of anti-GFP (green) and anti-nc82 (magenta) immunostaining of the brain of a Δ TH-D'-Gal4 > UAS-GFP::nls fly. Location of PPL1 cluster is shown. (Right) Representative image showing absence of calyx innervation.

This further supports the notion that the dopaminergic calyx innervation is specific to the PPL2 cluster.

3.11.2 oc2-Gal4 driver targets PPL2 cluster and glia

Ocelliless (*oc*) is a hypomorphic mutation of the *orthodenticle* (*otd*) gene, which plays a crucial role in specifying cell identity and position during development [Finkelstein et al., 1990]. *otd* is expressed in PPL2 neurons throughout development and is required

for these neurons survival [Blanco et al., 2011]. We can therefore use the oc2-Gal4 driver to target PPL2 neurons, and indeed this driver targets 3.5 ± 0.5 neurons from the dopaminergic cluster in the right hemisphere (Table 3.3, Figure 3.19 A). We can also see GFP positive innervations within the calyx, meaning it is likely PPL201 and/or PPL202 that are targeted by this driver (Figure 3.19 A, right).

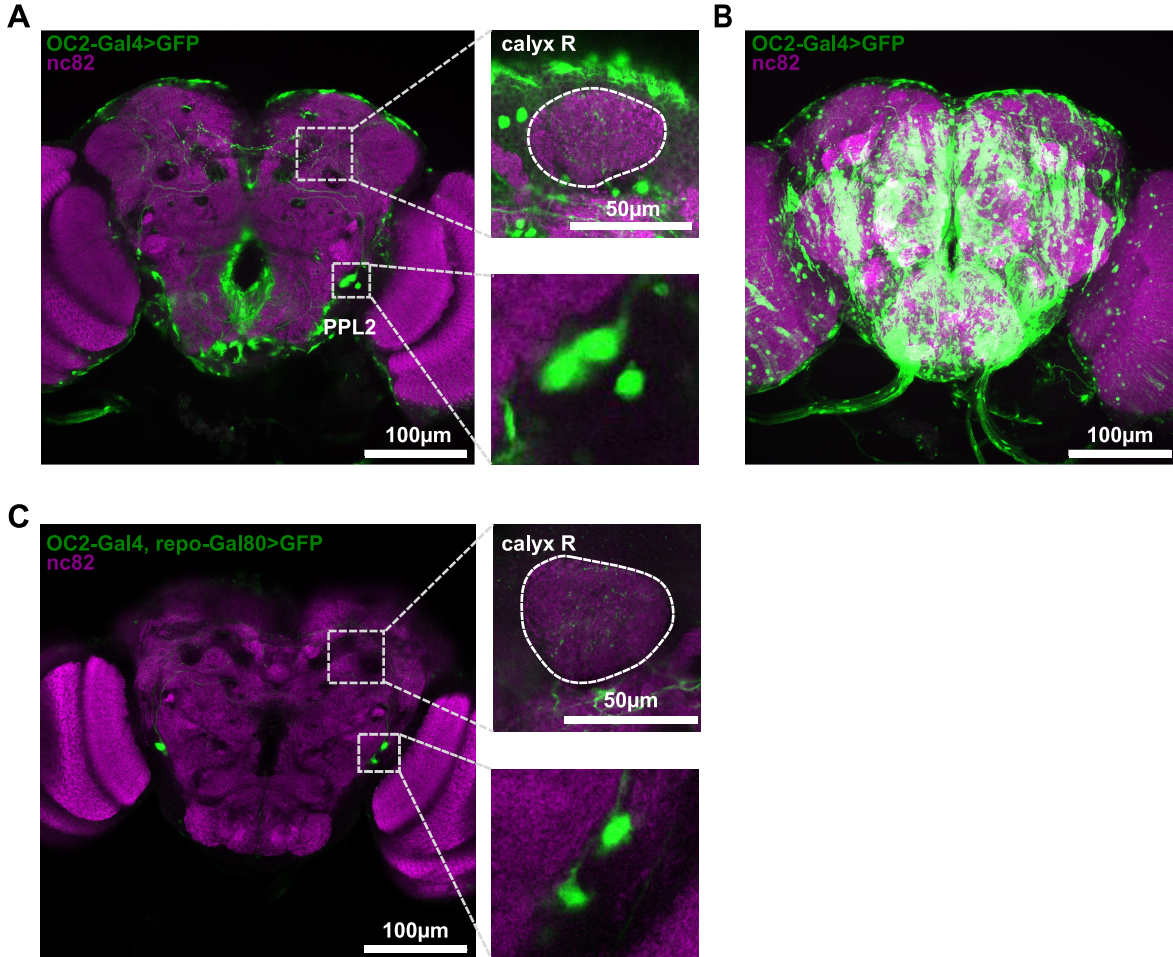


Figure 3.19: Characterisation of oc2-GAL4 driver
 (A) (Left) Representative image of anti-GFP (green) and anti-nc82 (magenta) immunostaining of the brain of an oc2-GAL4 > UAS-GFP::nls fly. GFP positive PPL2 neurons are indicated. (Right, top) Representative image showing innervation in the calyx. (Right, bottom) GFP positive PPL2 cell bodies. (B) Maximum intensity projection of anti-GFP (green) and anti-nc82 (magenta) immunostaining of an oc2-GAL4 > UAS-GFP::nls fly. (C) (Left) Representative image of anti-GFP (green) and anti-nc82 (magenta) immunostaining of the brain of an oc2-GAL4, repo-Gal80 > UAS-GFP::nls fly. GFP positive PPL2 neurons are indicated. (Right, top) Representative image showing innervation in the calyx. (Right, bottom) GFP positive PPL2 cell bodies.

However, this driver also has a lot of off target expression throughout the brain in glial cells. (Figure 3.19 B). In *Drosophila*, the 'blood-brain barrier' is formed by glia, meaning the brain is ensheathed in a layer of these cells [Limmer et al., 2014]. This makes it difficult to visually identify individual neurons that are targeted by this driver

3.11. CHARACTERISATION OF DIFFERENT GAL4 DRIVER LINES FOR PPL2 TARGETING

and we also don't want to affect this large population of cells with our manipulations. Therefore, the glia expression in this driver needs to be removed if this driver is to be used in experiments. By combining the oc2-Gal4 with a Gal80 that specifically targets glia, we can inhibit Gal4 expression in these cells. *Repo* is a gene that is not expressed in other cell types and is commonly used as a pan-glial marker [Xiong et al., 1994, Delandre et al., 2025]. Therefore, we can use a repo-gal80 to inhibit gal4 in glia.

Figure 3.19 C shows an immunostaining showing the resulting oc2-gal4, repo-Gal80 expression pattern. The pattern of GFP positive cells surrounding the periphery of the brain has been removed, but PPL2 cell bodies can still be seen. We still target on average 4 PPL2 neurons per hemisphere and the calyx innervations can still be seen (Table 3.3, Figure 3.19 C, right). This suggests that PPL201 and/or PPL202 targeting has not been lost by introducing the Gal80 and this driver is much more usable.

3.11.3 Different split-Gal4 drivers target subpopulations of the PPL2 cluster

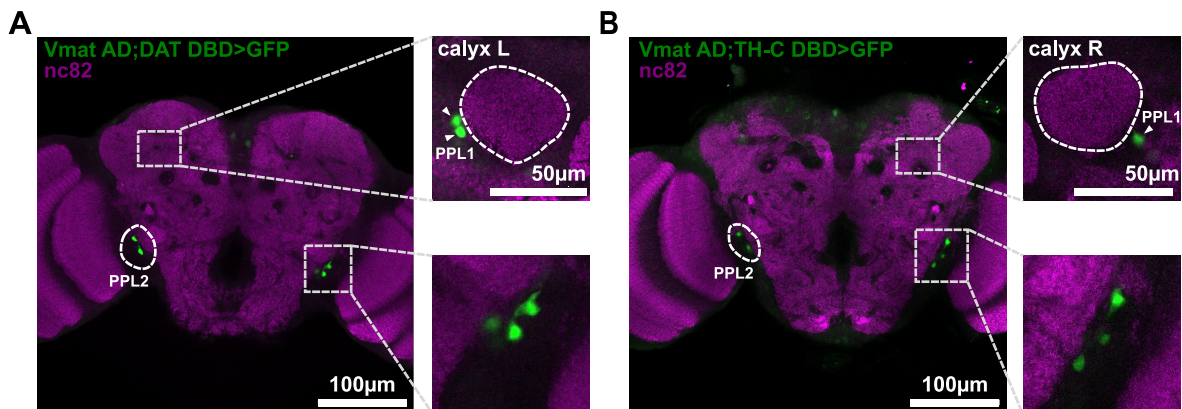


Figure 3.20: Characterisation of split-Gal4 lines

(A) (Left) Representative image of anti-GFP (green) and anti-nc82 (magenta) immunostaining of the brain of a *Vmat AD;DAT DBD > UAS-GFP::nls* fly. GFP positive PPL2 neurons are indicated. (Right, top) Representative image showing absence of calyx innervation. Arrowheads indicate GFP positive neurons of the PPL1 cluster. (Right, bottom) Zoomed image of GFP positive PPL2 neurons. (B) (Left) Representative image of anti-GFP (green) and anti-nc82 (magenta) immunostaining of the brain of a *Vmat AD;TH-C DBD > UAS-GFP::nls* fly. GFP positive PPL2 neurons are indicated. (Right, top) Representative image showing absence of calyx innervation. Arrowheads indicate GFP positive neurons of the PPL1 cluster. (Right, bottom) Zoomed image of GFP positive PPL2 neurons.

Another way of achieving more specific drivers is using the split Gal4 method (see Section 1.2.2). Briefly, the split Gal4s use two different enhancers to separately drive the Gal4 activation domain (AD) and DNA-binding domain (DBD). Since both of these domains are required for Gal4 to be active, gene expression will only be seen in cells where both the AD and DBD are present. Here, we characterise three different split Gal4s that are all based on TH expression.

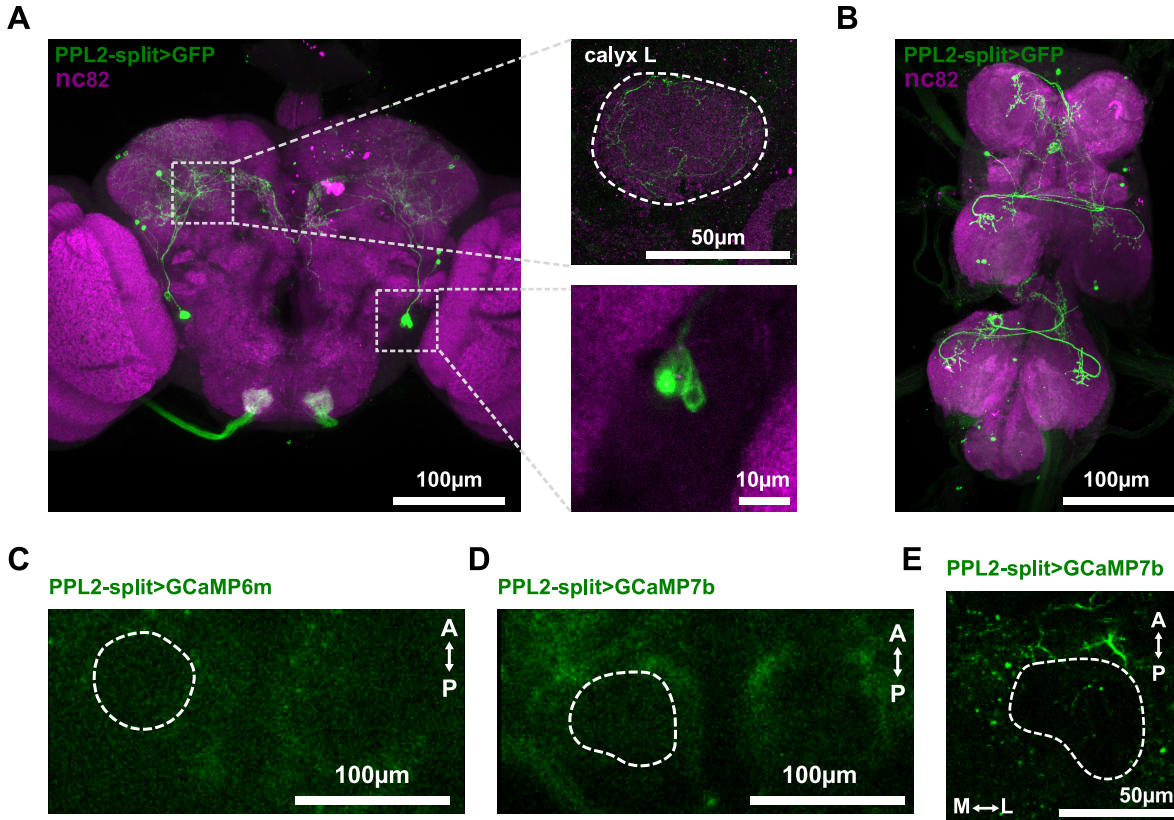


Figure 3.21: Characterisation of PPL2-split Gal4 driver

(A) (Left) Maximum intensity projection representative image of anti-GFP (green) and anti-nc82 (magenta) immunostaining of the brain of a ple-AD ; R42H01-DBD > UAS-mcD8::GFP fly. (Right) Maximum intensity projection airyscan image showing innervation in the calyx (top). Representative image showing PPL2 cell bodies (bottom) (B) Maximum intensity projection representative image of anti-GFP (green) and anti-nc82 (magenta) immunostaining of the ventral nerve cord (VNC) of a ple-AD ; R42H01-DBD > UAS-mcD8::GFP fly. (C-E) Representative image of calcium imaging when expressing GCaMP6m (C), GCaMP7b (D), or GCaMP7b with two-photon imaging (E) in ple-AD ; R42H01-DBD flies. A, anterior; P, posterior; M, medial; L, lateral.

The first expresses the Gal4 AD under control of *Vmat*, the vesicular monoamine transporter, which, in flies, is expressed in all dopaminergic, serotonergic, and octopaminergic neurons [Chang et al., 2006, Greer et al., 2005]. The Gal4 DBD is expressed under control of the dopamine transporter *DAT*, which mediates dopamine reuptake and is therefore expressed in dopamine neurons. 3.75 ± 0.25 neurons of the

3.11. CHARACTERISATION OF DIFFERENT GAL4 DRIVER LINES FOR PPL2 TARGETING

PPL2 cluster are targeted in the right hemisphere (Table 3.3, Figure 3.20 A). However, other dopaminergic neurons from the PPL1 cluster are also targeted. Since PPL1 are known to have a role in olfactory behaviour and innervate the MB lobes, we cannot use a driver that targets these since it would be impossible to attribute any affect to PPL2 over PPL1. Additionally, there are no visible GFP innervations in the calyx, suggesting that of the PPL2 neurons targeted, it does not include PPL201 or PPL202.

The next split Gal4 driver also uses the *Vmat* enhancer to drive Gal4 AD. However, the Gal4 DBD is expressed under control of *TH-C*. *TH-C* targets a subset of *TH* positive dopaminergic neurons. This driver was created by using different enhancer fragments of the TH regulatory region in order to target smaller subsets of neurons that express *TH* [Xie et al., 2018]. The *Vmat AD ; TH-C DBD* driver targets, on average, 3.5 ± 0.5 PPL2 neurons (Table 3.3, Figure 3.20). However, there is one PPL1 neuron that is also targeted, and there are no visible innervations in the calyx. Therefore, this driver is not suitable for experiments manipulating PPL2 to determine effects on olfactory behaviour.

Lastly, we express GFP using the *ple AD ; R42H01 DBD* split Gal4, which we from here on refer to as PPL2 split-Gal4 (Figure 3.21 A). The gene *ple* is another name for the gene *TH*. This results in a driver that is very specific for only a few PPL2 neurons, targeting 2.6 ± 0.13 in the right hemisphere (Table 3.3). We see GFP positive innervations in the calyx, particularly around the perimeter of the structure, suggesting that despite targeting only a few PPL2 neurons, we are including at least one of PPL201 or PPL202. Indeed, if we compare the GFP positive innervation patterns in the brain to the FAFB or hemibrain neuron skeletons (Figure 3.1 A & B) the patterns look similar to PPL201 and PPL202. It also appears that PPL204 is targeted, with strong GFP positive innervations in the antler regions. There are also GFP positive neurons found in the ventral nerve cord (VNC) (Figure 3.21 B).

Overall, the *PPL2-Gal4*, *oc2-Gal4*, *repo-Gal80*, and *PPL2-split Gal4* drivers provide a specific, targeted expression in PPL2 neurons that innervate the calyx. These drivers do not target other dopaminergic subsets such as PPL1 that would interfere with manip-

ulations to determine olfactory response effects. *PPL2-Gal4* targets the most neurons in the cluster, while *PPL2-split Gal4* is the most specific, only targeting approximately 3 neurons. Therefore, for experimental PPL2 manipulations we use these drivers.

3.12 Silencing of PPL2 dopaminergic neurons has no effect on KC activity

Firstly, to determine the overall affect of removing dopaminergic input from the calyx on 247-KC odour-evoked activity, we express *kir2.1* using the *PPL2-split Gal4* driver. *Kir2.1* is an inward rectifier potassium channel which, when overexpressed in a neuron using the *Gal4/UAS* system, effectively silences the cell. Since our results have shown that knockdown of either *Dop1R1* or *Dop2R* in 247-Gal4 targeted KCs both result in a decreased response to novel odor, and *Dop1R2* knockdown has no effect, we hypothesise that silencing of PPL2 and thus removal of dopaminergic input to the calyx will result in an overall decrease in KC activity to novel odours. For the second response, we have shown that knockdown of *Dop1R2* increases the magnitude of response, *Dop2R* knockdown decreases magnitude of response and *Dop1R1* knockdown has no effect. Therefore, we can expect that silencing of PPL2 will have no effect on the magnitude of response to a familiar odor in KCs. Although this hypothesis is assuming total knockdown by all RNAis and the weight from each KC subset is equivalent.

To silence PPL2 neurons we use *UAS-kir2.1* driven by *PPL2-split Gal4* to hyperpolarise the cells [Baines et al., 2001]. To avoid any possible developmental effect from silencing these neurons during development we also express a temperature sensitive *Gal80* and raise flies at the permissive temperature of 18°C to inhibit *Gal4* mediated expression of *kir2.1* (see Figure 1.3) [McGuire et al., 2004]. Flies were then transferred to the restrictive temperature of 29°C for at least 48 hours before imaging to allow for *UAS* expression (Figure 3.22 A). Silencing of PPL2 neurons did not show any significant differences from genetic controls in either quantified metric, area under curve or peak magnitude of response, across either response measured. The area under the

on the perceived salience.

To explore this hypothesis we first investigate KC odor-evoked responses under different internal conditions, chosen to alter the perceived salience of the odour stimulus. Since PPL2 has been shown to affect age- [Kuo et al., 2015], mating status- [Yang et al., 2015, Kuo et al., 2015], and satiety- [Sayin et al., 2019] dependant behaviours, these are the internal states we decided to investigate. The first steps to exploring a potential dopamine-mediated affect on an internal state is to identify an internal state that by itself shows KC odor-evoked differences. Then, by manipulating dopamine we can observe how PPL2 alters this affect. Therefore, we first image KC odor-evoked activity under different internal conditions to find a candidate for further investigation.

3.13.1 KC odor-evoked activity is not affected by age

First, we investigate the affect of ageing on KC odor-evoked responses in the calyx. Female flies were allowed to age to one of three age groups: 3-5d old are considered young adult flies with fully developed brains, robust memory capabilities and full activity levels [Iliadi and Boulianne, 2010], 12-15d old flies are typically considered middle aged and start showing signs of cognitive decline such as short term memory deficits [Tonoki and Davis, 2012], and finally flies above 30d old are considered old-aged since this age is associated with severe deficits in behaviours such as spontaneous locomotion and olfactory memory [Simon et al., 2006, König and Gerber, 2022]. The calcium imaging protocol (Figure 3.23 A) is the same as previously discussed in section 3.9 and details can be found in methods section 2. For these experiments flies expressing GCaMP3 directly under control of the 247 enhancer without the need of a Gal4 were used to minimise the amount of crosses needed (Figure 3.23 B).

As *Drosophila* age, these associated behavioural deficits, especially decline in olfactory memory, may indicate a change in sensory processing and olfactory representation. However, there were no significant differences in KC odor-evoked activity in the calyx between any of our groups (Figure 3.23 C).

3.13. EXPLORATORY ANALYSIS OF INTERNAL STATE EFFECT ON KC ACTIVITY

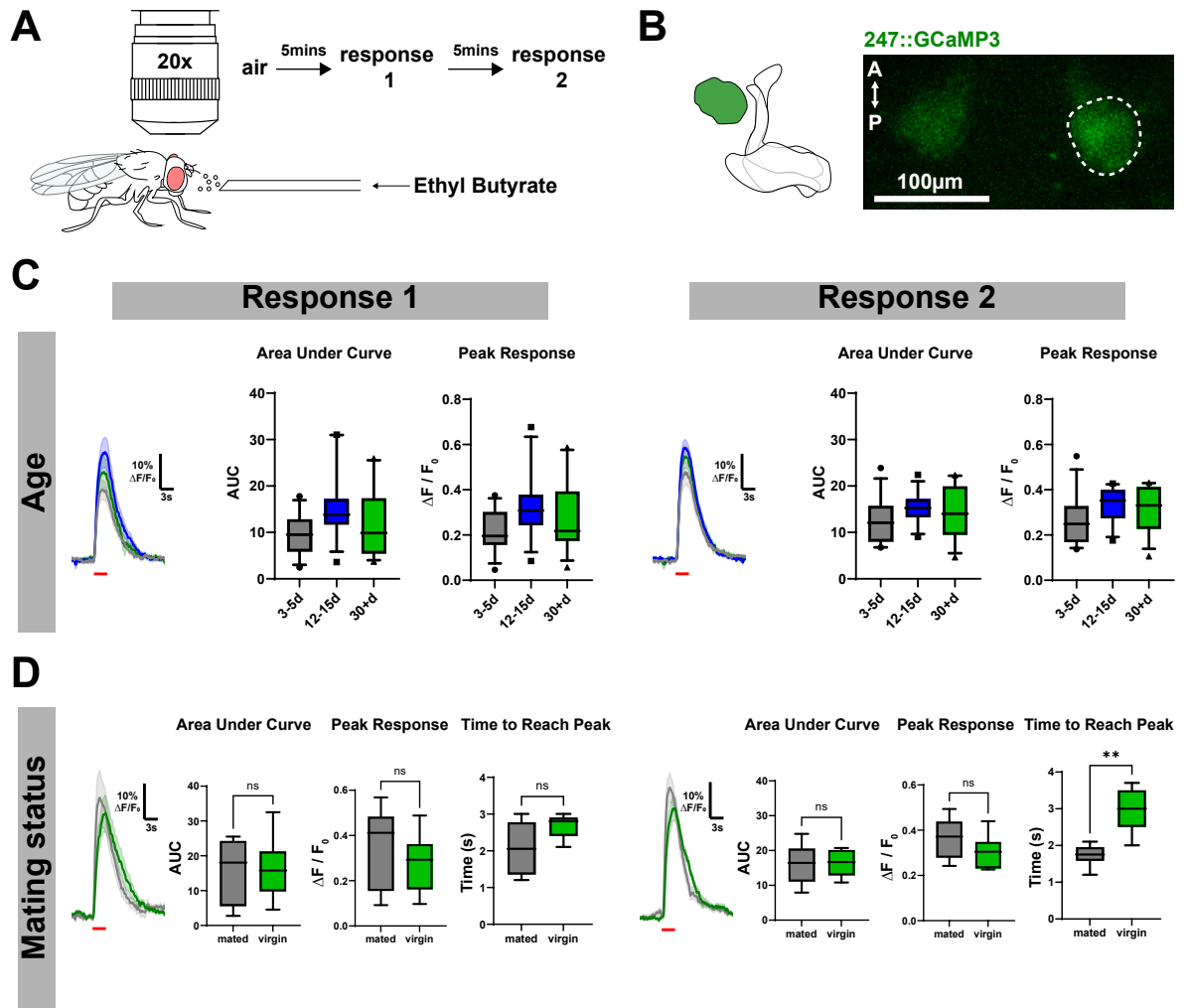


Figure 3.23: KCs odor-evoked response under different internal conditions (A) Experimental design. (B) Schematic showing the area of the MB imaged and representative image of odor-evoked calcium response in an *247::GCaMP3* fly. A region of interest was drawn around the MB calyx. A, anterior; P, posterior. (C) Odor-evoked responses of 247-KCs in flies of different ages. Left column, first response to novel odor; right column, second response following 5 minutes of constant air flow after the first response. Each set of data consists of: (left) GCaMP3 fluorescence across time (mean $\pm$ SEM), the red bar on the x-axis indicates odor delivery duration. (Middle) quantification of area under the curve, and (right) quantification of the peak calcium response. Odor used was ethyl butyrate. $n=12$ Mann-Whitney test, ns $p>0.05$. (D) Odor-evoked responses of 247-KCs in mated or virgin flies. Left column, first response to novel odor; right column, second response following 5 minutes of constant air flow after the first response. Each set of data consists of: (left) GCaMP3 fluorescence across time (mean $\pm$ SEM), the red bar on the x-axis indicates odor delivery duration. (middle-left) quantification of area under the curve, (middle-right) quantification of the peak calcium response, and (right) quantification of time taken to reach the peak response. Odor used was ethyl butyrate. $n=6-7$ Mann-Whitney test, ns $p>0.05$, ** $p<0.01$.

3.13.2 KC odor-evoked response dynamics are changed by mating status

Next, we investigate the effect of mating status, that is if the fly is a virgin or mated, on odor evoked KC activity. After mating, a female fly undergoes numerous physiological changes including increased egg production, decreased receptivity to male courtship attempts, changes in metabolism, and many others. The priorities of the fly changes drastically from finding a mate, to finding a suitable egg-laying substrate. Therefore, we expect the odor-evoked response of KCs to also change with mating status.

Virgin females were selected <6 hours after eclosion and aged for 3-5days separated from their male counterparts. Meanwhile, another group of females were housed in mixed sex vials for 3-5days to ensure mating. We do not see any differences between mated and virgin females in either the area under the curve or the peak magnitude of response (Figure 3.23 D). However, looking at the fluorescence plots it is clear that the two traces are not the same. Here, we introduce another metric to compare our responses, the time taken to reach the peak magnitude (details on how this is calculated in section 2). Using this metric the first response has a trend toward an increase in time taken ($p=0.1906$). In the second response, there is a very significant increase in time taken to reach the peak in virgins compared to mated females (Figure 3.23 D). This means that in virgin flies the KCs take longer to reach the peak of the response after the onset of an odour. It is important to clarify that our experimental setup was not optimised for this type of analysis, the odor was delivered by a manual button press which may lead to human error in timing of odor delivery and thus slight error in the calculation of time taken to reach peak response.

3.13. EXPLORATORY ANALYSIS OF INTERNAL STATE EFFECT ON KC ACTIVITY

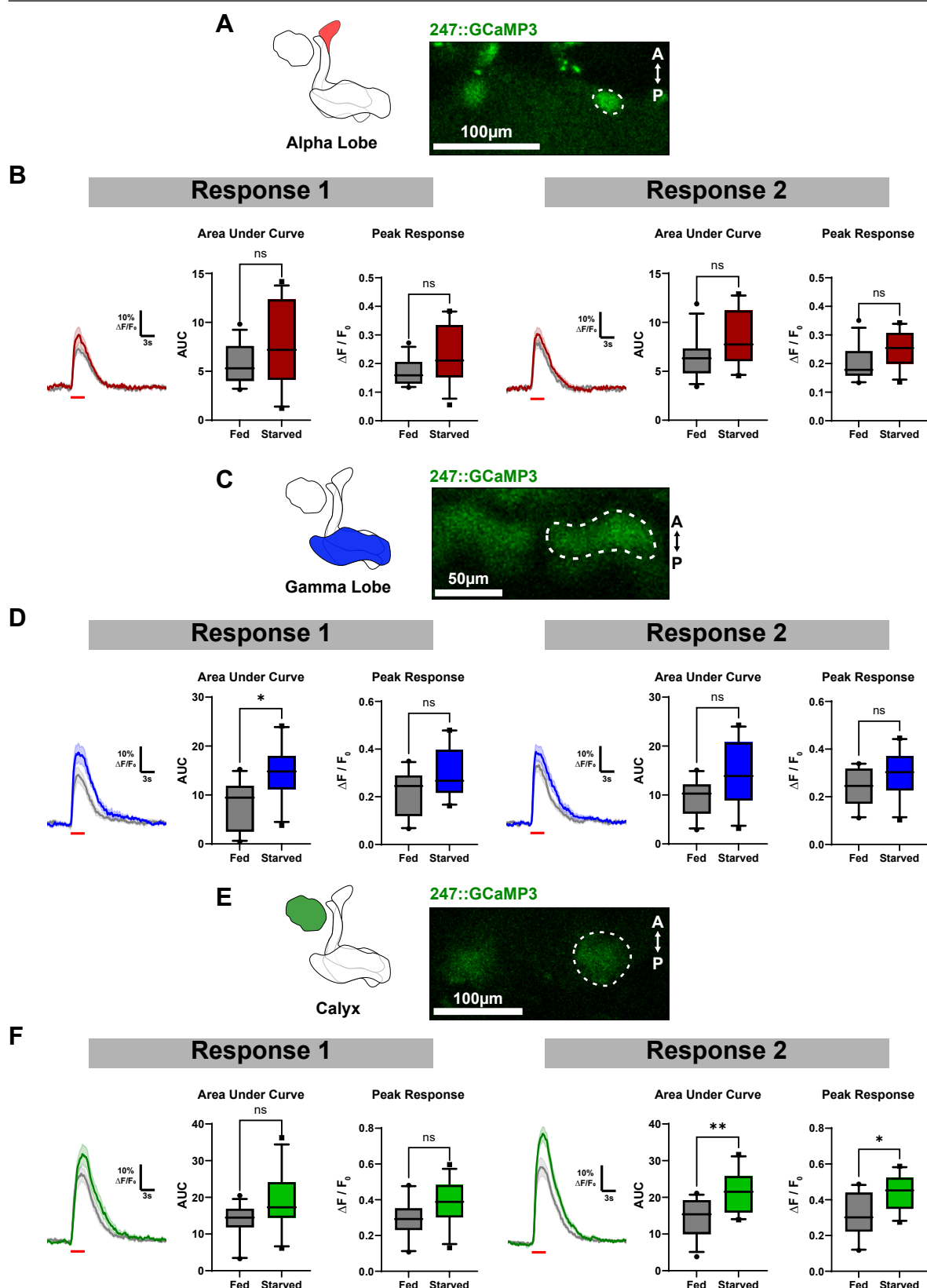


Figure 3.24: KC odor-evoked response in different compartments of the MB
 (A) Schematic showing the area of the MB imaged and representative image of odor-evoked calcium response in an 247::GCaMP3 fly. A region of interest was drawn around the tip of the MB alpha lobe. A, anterior; P, posterior. (B) Odor-evoked responses of 247-KCs in flies under different starvation states. Left column, first response to novel odor; right column, second response following 5 minutes of constant air flow after the first response. Each set of data consists of: (left) GCaMP3 fluorescence across time (mean $\pm$ SEM), the red bar on the x-axis indicates odor delivery duration. (*continues on next page*)

3.13.3 KC odor-evoked response increases in the calyx following a period of starvation

Finally, we consider the effect of starvation on KC activity. Hunger is known to affect multiple behaviours such as increasing foraging behaviour [Tsao et al., 2018], decreasing aversion to noxious stimuli [Ohashi and Sakai, 2018, Plaçais and Preat, 2013], and hunger provides the needed motivation to form and express appetitive olfactory memories [Ko et al., 2015, Krashes et al., 2009, Gruber et al., 2013]. Therefore, the salience of stimuli may be altered by starvation state in order to produce the appropriate behavioural output.

Flies were starved in a vial containing 0.75% agar solution, providing them with water but no nutrition, for approximately 40-45 hours prior to imaging and the control group did not undergo any food deprivation (see section 2 for details). We imaged KC responses in three areas of the MB: imaging KC axons in the tip of the alpha lobe (Figure 3.24 A) and the gamma lobe (Figure 3.24 C), and imaging the KC dendrites in the calyx (Figure 3.24 E).

Firstly, starvation does not affect the KC response in the alpha lobe, since we find no significant differences between our fed and starved groups (Figure 3.24 B). In the gamma lobe, there is an increase in the area under the curve in the first response, but no significant differences in the second response (Figure 3.24 D). The first response also is trending towards an increase in peak response ($p=0.1655$) and the fluorescence trace gives an indication that there is an increase in the response. A change in area under the curve but not in peak response indicates a change in response dynamic, making the response longer in duration.

Figure 3.24 (previous page): (middle) quantification of area under the curve, and (right) quantification of the peak calcium response. Odor used was ethyl butyrate. $n=12$ Mann-Whitney test, ns $p>0.05$. (C-D) Same as A-B but imaging in the MB gamma lobe. $n=10$ Mann-Whitney test, ns $p>0.05$, * $p<0.05$. (E-F) Same as A-B but imaging in the MB calyx. $n=12$ Mann-Whitney test, ns $p>0.05$, * $p<0.05$, ** $p<0.01$.

Lastly, in the calyx we see a significant increase in KC odor-evoked response to the second odor presentation (Figure 3.24 F). The first response is also strongly trending towards a starvation induced increase in response (AUC: $p=0.0887$, peak response: $p=0.0887$). The fluorescence traces show a clear increased response in the hungry flies, with a 1.5x increase in the median peak response after starvation.

In summary, KC odour-evoked responses are modulated by some internal states. While the responses were not affected by age in the age range we have tested, mating status did alter the response dynamics with KC responses in female virgins taking almost double the amount of time to reach the peak of the response after odour presentation. Lastly, there is a starvation induced increase in KC responses specifically in the dendrites located in the calyx. There is also some evidence of this starvation induced increase in response in the axons of γ -KCs. The next question is therefore is this effect in the calyx modulated by PPL2 dopaminergic input? Therefore, we chose to further investigate the starvation effect and the potential role of dopamine.

3.14 Knockdown of D1-like and D2-like differently affect odor-evoked responses in γ -KCs of starved flies

To investigate the affect of PPL2 dopaminergic input on the starvation induced increase in KC activity we express GCaMP6m in KCs while knocking down the expression of dopamine receptors, effectively removing dopamine input. We use the same imaging protocol as described previously, 3 seconds of ethyl butyrate exposure followed by a second response, separated by 5 minutes of air flow. For these experiments we use the 5HTR1B-Gal4 driver to target specifically the KC γ subtype and imaged in the calyx (Figure 3.25). We choose this subgroup of neurons since plasticity following odour-exposure paired with PPL2 activity in KC γ has been previously reported [Boto et al., 2019]. Plus we have shown that PPL2 has the strongest connection with KC γ in

the calyx, and therefore is a good candidate for showing a strong dopamine mediated affect. Lastly, the previous results showing the starvation affect show a trend towards an increase in odor-evoked response in the gamma lobe, suggesting that the increase in the calyx may be due to KC γ .

3.14.1 Dop1R1 knockdown does not affect starvation induced increase in γ -KC response

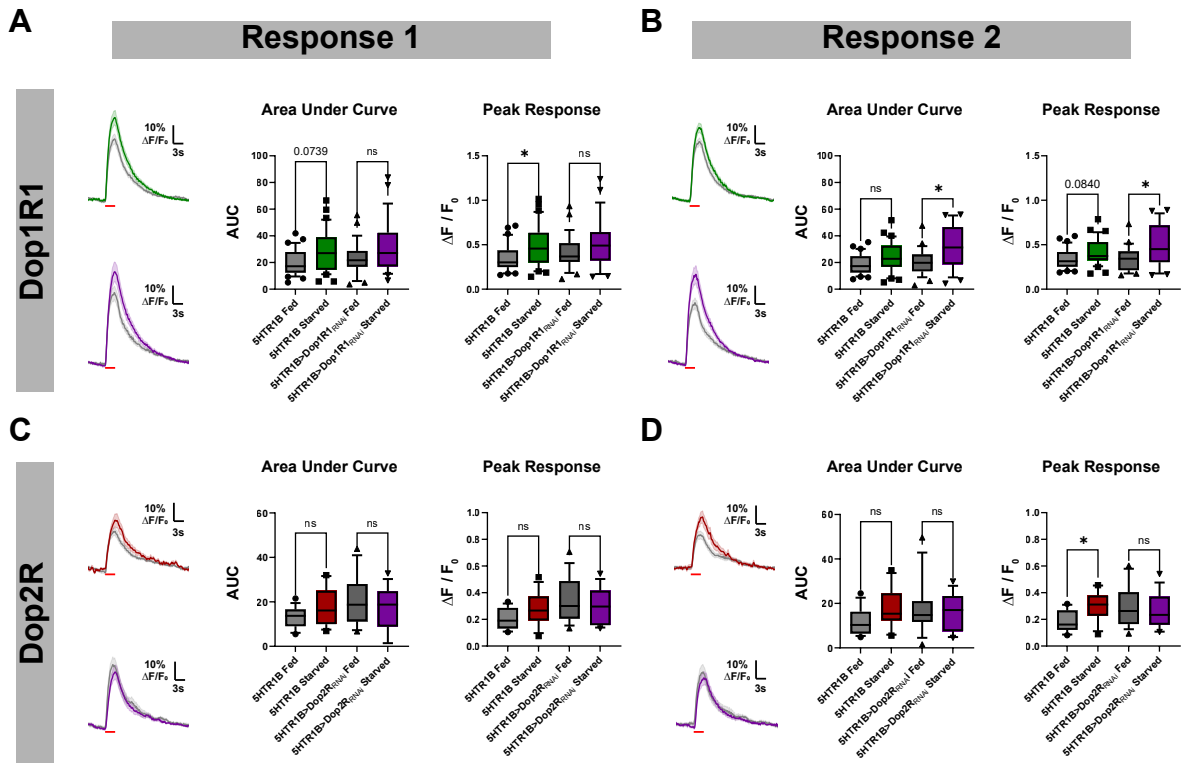
After a period of 40-45 hours of food deprivation we imaged the response to ethyl butyrate exposure in female flies expressing Dop1R1-RNAi (Figure 3.25 A & B). In the control flies, without dopamine knockdown, we see an increase in the peak response in the first response and a trend towards increase in the area under the curve ($p=0.0739$, Figure 3.25 A). The second response shows a trend towards an increase in peak response ($p=0.0840$) but there is no significant increase in the area under the curve (Figure 3.25 B). This indicates that the starvation induced increase in KC response is still present when you image the dendrites of KC γ only.

Introducing the Dop1R1 knockdown we do not see any significant differences in the first response (Figure 3.25 A). However, in the second response there is a significant increase in the odor-evoked response of starved flies for both the area under the curve, and the peak response (Figure 3.25 B). Considering that we did not see any differences in odor-evoked response between controls and Dop1R1 knockdown without starvation, these results suggest that Dop1R1 knockdown does not affect the starvation induced increase.

3.14.2 Dop2R knockdown abolishes starvation induced increase in γ -KC response

In our controls, without Dop2R knockdown, there is a significant increase in the peak response of the second response (Figure 3.25 D) but no significant differences in the

3.14. KNOCKDOWN OF D1-LIKE AND D2-LIKE DIFFERENTLY AFFECT ODOR-EVOKED RESPONSES IN γ -KCS OF STARVED FLIES



first response (Figure 3.25 C). The Dop2R knockdown groups show no significant differences across any quantified metric in either response and the fluorescence traces show no observational differences (Figure 3.25 C & D). Therefore, the starvation induced increase has been abolished with Dop2R knockdown.

In Section 3.10.2 we showed that knockdown of Dop2R lead to an increased odor-evoked response in $KC\gamma$. Therefore, in the knockdown group starvation does not further increase the response, suggesting that fed flies without Dop2R respond to ethyl butyrate as if they were starved flies. This data implicates Dop2R as mediating a starvation induced response, perhaps providing $KC\gamma$ with inhibition in the fed state.

In summary, the starvation induced increase of $KC\gamma$ is unaffected by knockdown of Dop1R1, but is abolished by knockdown of Dop2R, with the fed flies then responding

more similar to starved flies. In this section we assume the starvation induced increase in response is present, although the evidence using 5HTR1B>GCaMP6m is not as strong as with 247-GCaMP3. Replication of the effect was also inconsistent. A second replicate of the 5HTR1B-Gal4 driven Dop1R1 knockdown failed to recapture the starvation induced increase, with no significant differences in any comparison (Appendix A.1). The fluorescence traces also showed no indication of an increase in response. We then replicated the imaging experiment using the 247-GCaMP3 driver. Here, the starved flies showed an increase in response, with a significant difference in the peak of the first response and clear differences in fluorescence trace (Appendix A.2). Although this dataset has a small sample size, this indicates that the starvation effect is replicable using 247-GCaMP3, but not 5HTR1B>GCaMP6m. We discuss the possible causes of this and the reliability of this effect in Section 4.2.5.

3.15 Measuring activity of PPL2 neurons under starvation conditions

Having established that dopamine input into the calyx seems to have an affect on the starvation induced increase in response of KC γ , the next step is to image the response of PPL2 itself to understand how the activity changes under starvation conditions. The imaging and starvation protocol used was the same as previously described; 42-45 hours of food deprivation, two odour responses measured separated by 5 minutes of air.

Since *PPL2-split Gal4* gave us the most specific targeting to the PPL2 neurons we are interested in, we use this driver to express GCaMP (Figure 3.21). However, since PPL2 innervations in the calyx are very sparse, it is difficult to visually identify the calyx during imaging. First, we tested GCaMP6m since this is the GCaMP version we have used throughout the imaging experiments (Figure 3.21 C). Here, the calyx is identified as a globular structure of decreased GFP signal, surrounded by higher GFP signal from the SNP area which has dense PPL2 innervation but this is very difficult

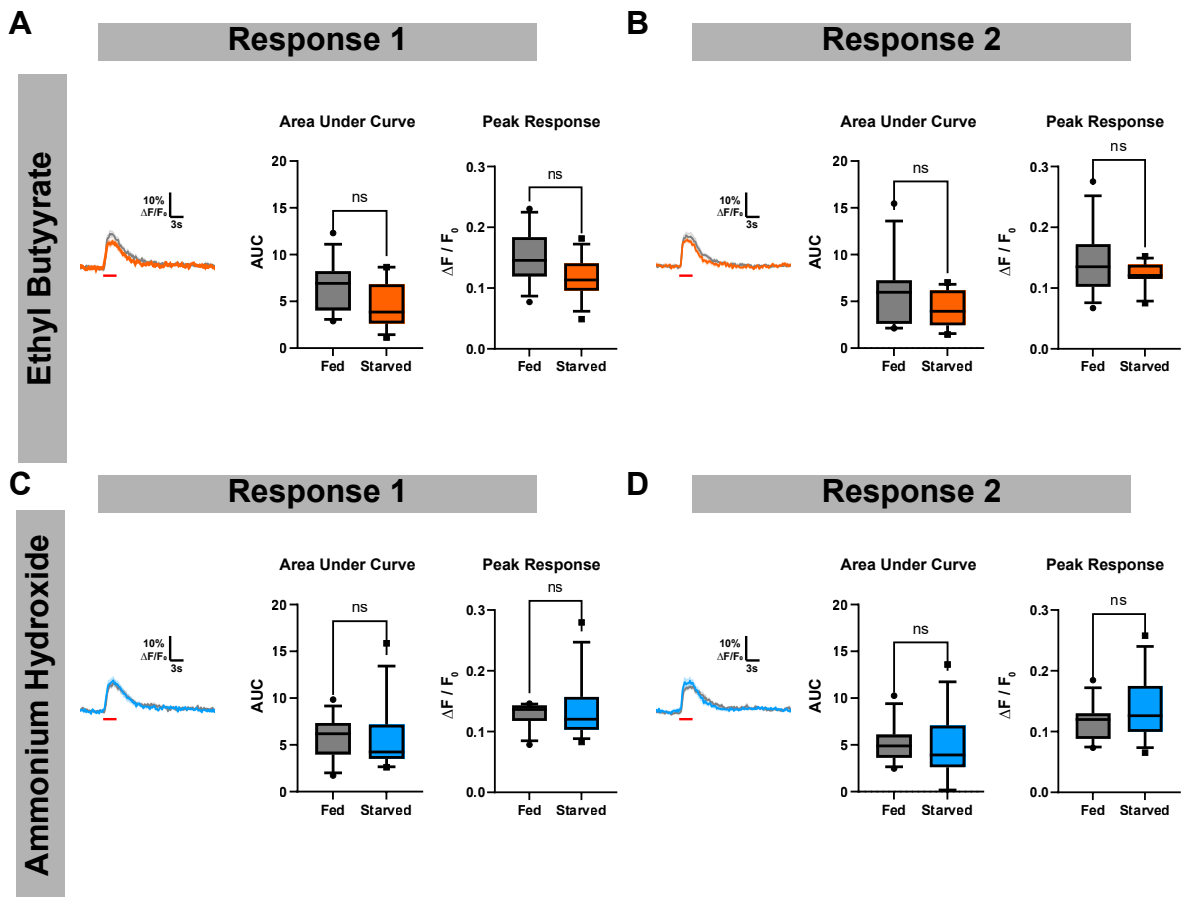


Figure 3.26: PPL2 odour-evoked response in the calyx under starvation conditions. (A&B) Odour-evoked response of PPL2-split Gal4 > GCaMP7b flies following a period of starvation to the first odour stimulus. (A) (Left) GCaMP7b fluorescence across time (mean $\pm$ SEM), the red bar on the x-axis indicates odour delivery duration. (Middle) quantification of area under the curve, and (right) quantification of the peak calcium response. (B) Same as A but the second response to the odour stimulus after 5 minutes of constant air. Odour used was ethyl butyrate. $n=12$. Mann-Whitney test, ns $p > 0.05$. (C&D) The same as in A & B but the odour used was 30% ammonium hydroxide. $n=12$. Mann-Whitney test, ns $p > 0.05$.

to identify (Figure 3.1 A & B). GCaMP7b ('bright') is optimised for imaging in dim structures, neurites and neuropils [Dana et al., 2019]. Therefore, we tested this GCaMP version since it seemed to better suit our needs of imaging the sparse neurites in the calyx 3.21 D & E). Figure 3.21 D shows the imaging view, compared to the results with GCaMP6m PPL2 processes are brighter and more defined, although the calyx itself is still extremely dim. Anatomical markers such as the PPL2 innervations in the SNP, seen in the anterolateral region of the imaging plane, is used as a landmark to identify the calyx. Using two-photon microscopy in conjunction with GCaMP7b gives a higher resolution view of calyx innervations, revealing the sparseness and allowing easier identification of the calyx [Helmchen and Denk, 2005]. The trade-off with two

photon is that only one hemisphere could be imaged at a time and the fluorescence data, i.e. the peak responses, were similar to the data from using standard confocal with GCaMP7b. Therefore, for PPL2 imaging experiments the flies used were *PPL2-split Gal4>GCaMP7b* imaged under standard confocal.

3.15.1 Odor-evoked calcium imaging of PPL2 responses to different odours is inconclusive

First we measured the response to ethyl butyrate presentation, the same odour we have been using throughout these results. There are no significant differences between fed and starved flies in either response (Figure 3.26. However, the first response is trending towards a decrease (AUC: $p=0.1005$, peak response: $p=0.0887$).

The responses to ethyl butyrate are very small, averaging around a 15% increase in fed flies, which is around half of the increases we see in KCs. This could be due to the sparse calyx innervations, where most of the region of interest we are measuring is not increasing at all, homogenising the results. To see if we could increase the response using a different odour, ammonium hydroxide was used. Ammonium hydroxide was chosen since PPL2 receives strong input from the PN VP1d+VP4.l2PN1 which has been shown to receive information from OSNs that respond to ammonia [Silbering et al., 2016] (Figure 3.5). However, results imaging PPL2 calyx response to ammonia did not reveal any significant differences between fed and starved flies (Figure 3.26 C & D). Additionally, the percentage increase in fluorescence still averaged around 15%, suggesting that ammonium hydroxide presentation did not lead to a stronger PPL2 response.

Overall, the results indicate a possible decrease in PPL2 response to ethyl butyrate under starvation conditions, although this is not statistically significant. Response to ammonium hydroxide shows no differences between starved and fed flies, suggesting that PPL2 responses may be odour specific. However, the imaging setup used was not completely optimised for PPL2 imaging, the calyx region was difficult to identify

and sparse innervations mean the fluorescence may be homogenised when analysing the entire region together. Therefore, a different technique to read PPL2 activity was needed.

3.16 Measuring calcium activity of PPL2 neurons in freely behaving flies

Since functional imaging of PPL2 under starvation conditions did not yield conclusive results due to calcium imaging not being fully optimised, we used CalexA to quantify long-term calcium activity in freely behaving flies [Masuyama et al., 2012]. In this technique, neurons expressing the CalexA construct accumulate GFP in a calcium activity dependent manner. Therefore, the brains of these flies can be stained for GFP, and the relative fluorescence intensity can be measured to indicate differences in basal activity (see Chapter 2 for details).

Firstly, to spatially restrict CalexA expression to only the PPL2 neurons that innervate the calyx, we used *PPL2-split Gal4*. Flies were food deprived for approximately 48 hours before brains were dissected and stained (Figure 3.27 A). During the staining protocol we used an anti-TH antibody in order to stain all dopamine neurons. This staining was used to normalise the GFP fluorescence of each individual neuron separately to remove any influence of uneven GFP staining, for example from how deep the neurons are in the brain (see Chapter 2 for details, Figure 3.27 B). In this case we did not see any differences between fed and starved flies in the GFP fluorescence level, suggesting that baseline PPL2 activity is not affected by starvation. However, perhaps this is due to CalexA being saturated from the period prior to starvation.

Therefore, in order to temporally restrict CalexA activity to the starvation period only, we expressed a temperature sensitive Gal80 in combination with TH-Gal4 [McGuire et al., 2004]. Using this method we could raise the flies at a low temperature of 18°C so that Gal80 is active and inhibits Gal4, and then during the 48 hours of

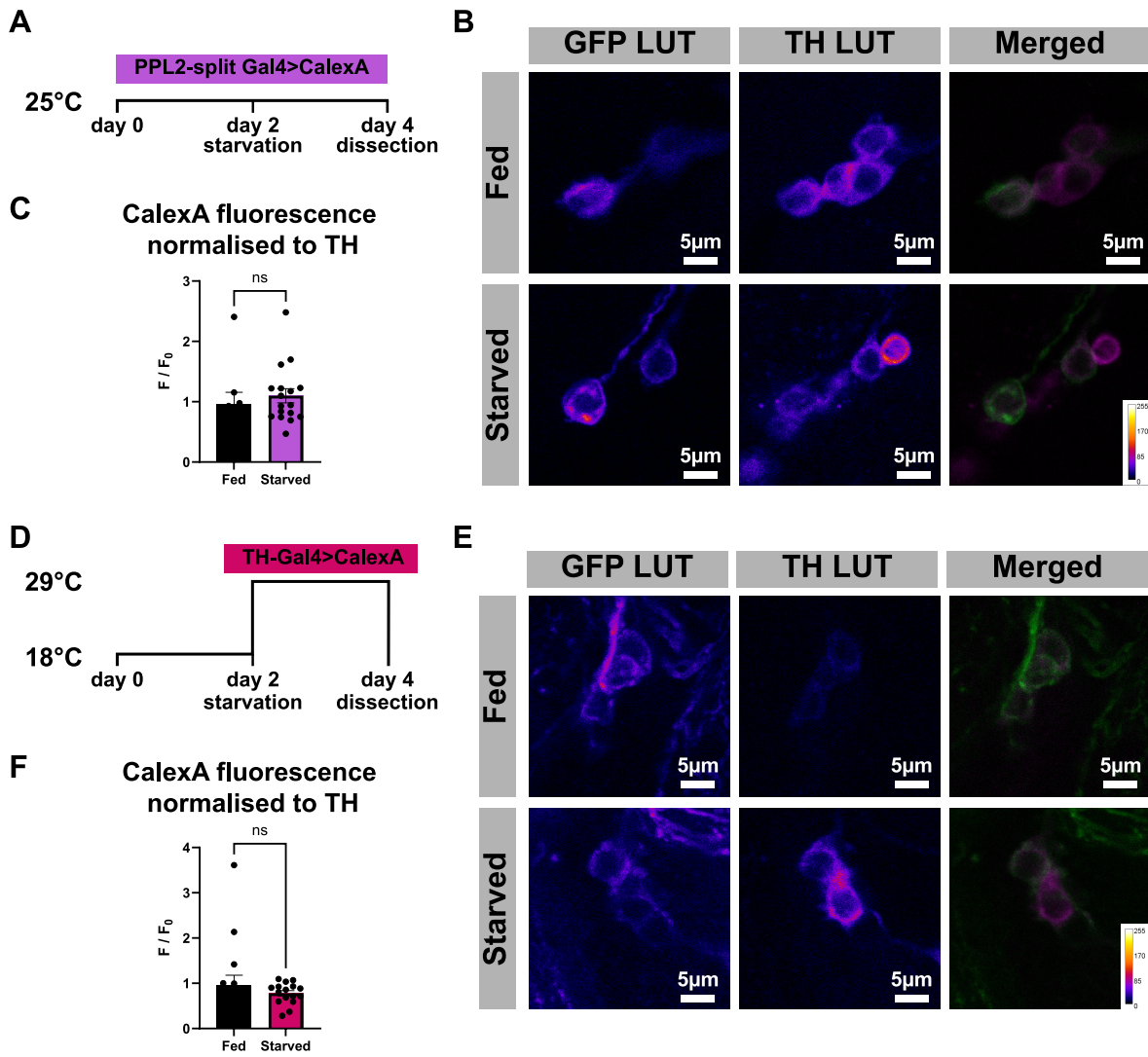


Figure 3.27: PPL2 activity in behaving flies.

(A) Experimental timeline for expressing CalexA using PPL2-split Gal4. (B) Representative images showing the fluorescence levels in each channel imaged. (C) Quantification of fluorescence level. Fluorescence of GFP is normalised against TH fluorescence. $n = 9-17$ where each n is one cell. Mann-Whitney test, ns $p > 0.05$. (D) Experimental timeline for temperature controlled expression of CalexA using the TH-Gal4,tub-Gal80^{ts} driver. (E) Representative images showing the fluorescence levels in each channel imaged. (C) Quantification of fluorescence level. Fluorescence of GFP is normalised against TH fluorescence. $n = 15-16$ where each n is one cell. Mann-Whitney test, ns $p > 0.05$.

starvation increase the temperature to 29°C so that Gal80 no longer functions (see Section 1.2.2). Again, we stained with TH to provide a signal to normalise against and imaged the PPL2 cluster (Figure 3.27 E). Here, we also see no significant differences in calcium accumulation between fed and starved flies.

Starvation causes a lot of changes throughout the brain, including decreases in protein synthesis in *Drosophila* larva [Nagarajan and Grewal, 2014]. If the tyrosine hydroxylase levels in the dopamine neurons is decreased by starvation then we may not

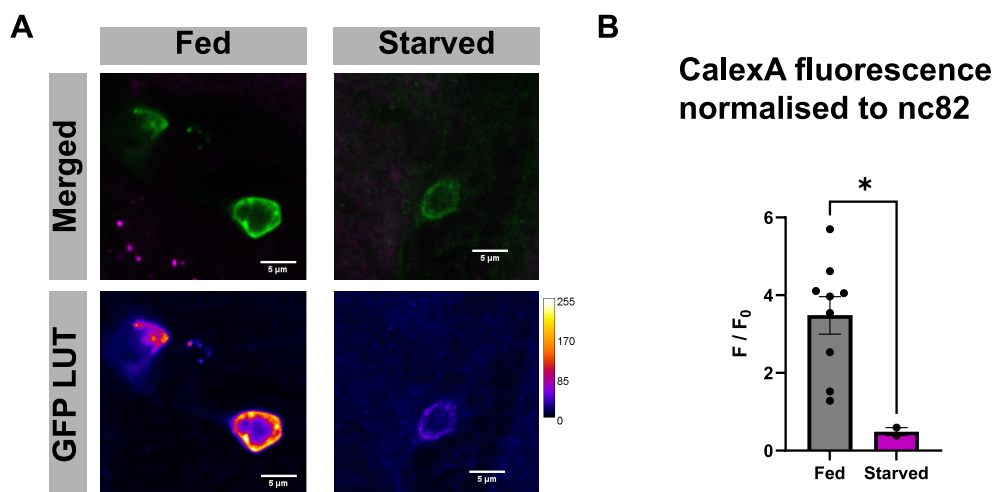


Figure 3.28: Preliminary data of PPL2 activity in behaving flies.

(A) Representative images showing the fluorescence levels of the GFP channel. (B) Quantification of fluorescence level. Fluorescence of GFP is normalised against a section of nc82 fluorescence. $n = 2-9$ where each n is one cell. Mann-Whitney test, $*p < 0.05$.

be able to see any decreases in GFP fluorescence using this method. Therefore, we expressed CalexA driven by *PPL2-split Gal4* and used an nc82 staining for the normalisation (Figure 3.28 A). Here, we see a significant decrease in the relative GFP fluorescence levels between fed and starved flies, however this is very preliminary data with a very small sample size (Figure 3.28 B).

Overall, we have shown that there is some evidence to suggest that basal PPL2 activity is decreased in starved flies, however this data is extremely preliminary. Further experiments to validate a starvation induced decrease in TH levels, and to confirm the decrease in PPL2 activity are needed.

3.17 PPL2 activation during naïve olfactory behaviour

Lastly, we use a behavioural method to determine the possible effect of PPL2 activation on naïve olfaction. We hypothesise that if PPL2 neurons are responsible for encoding salience of a stimulus then behavioural responses will be increased with PPL2 activation regardless of stimulus valence. This means that if the flies are averse to an odour, they will become more averse upon PPL2 activation during this behaviour. Similarly,

if flies are attracted to an odour and exhibit approach behaviour, this will be enhanced with PPL2 activity. Assessment of approach/avoidance behaviour to a range of concentrations of ethyl butyrate was performed using the Y-maze assay (see Chapter 2). In this assay, flies were given the choice between two arms of a maze, one containing the odour, and the other an odourless control. After 30 minutes to make a choice between the arms, the preference index is calculated, with a positive score indicating approach behaviour, and a negative score indicating avoidance. Flies had no previous experience with the odour, but were starved for 40-45 hours prior to the experiments to provide motivation to make a choice. Activation of PPL2 neurons was achieved by expression of the temperature sensitive ion channel dTrpA1. Using this channel, neurons can be activated by an increase of temperature, at low temperatures the channel is closed but at increased temperatures the channel opens, inducing depolarisation of the cell (experimental timeline shown in Figure 3.29 B). We used both the *PPL2-Gal4* and the *oc2-Gal4,repo-Gal80* driver lines for these experiments (Figure 3.29 A & H).

The behavioural experiments were run at two temperatures, 22°C as a temperature control, and 32°C to induce the activation of PPL2. At the control temperature there were no differences between our *PPL2-Gal4>TrpA1* experimental line and the two genetic controls, and these flies showed a typical concentration response curve (Figure 3.29 C). As the concentration increases a typical response curve follows the pattern of the odour first being undetectable, leading to a random chance of entering each arm, followed by a period of approach behaviour as the flies are curious about the odour, and then as the concentration gets stronger there is a period of avoidance.

3.17.1 PPL2 activation increases approach behaviour at low concentrations

At 32°C, PPL2 neurons are activated. These experiments were run with both males and females, there were no differences between these groups using *PPL2-Gal4* (Appendix A.3) so they were pooled in this data. There were also no differences found between the directionality of the odour (Appendix A.4) showing that the flies did not have a

bias towards choosing the left or the right.

At low concentrations, for example at 0.0001% we see a significant increase between the experimental fly and at least one of the controls (Figure 3.29 D & I). In the case of *PPL2-Gal4* this is more robust, as our experimental fly shows enhanced approach behaviour compared to both genetic controls at 0.0001% concentration of odour (Figure 3.29 E). Using *oc2-Gal4,repo-Gal80* we see an increase compared to the Gal4 control but not the UAS control at 0.0001% concentration (Figure 3.29 J).

3.17.2 PPL2 activation increases avoidance behaviour at high concentrations

At high concentrations, such as 0.01% and 0.5% we see significant differences between our experimental and at least one genetic control (Figure 3.29 D & I). With the *PPL2-Gal4* driver at 0.01% concentration there is a significant decrease in preference index compared to the UAS control, indicating more avoidance behaviour (Figure 3.29 F). In fact, at this concentration point the controls have a positive preference index, indicating approach behaviour, while the flies with PPL2 activation show avoidance behaviour. At the very high concentration of 0.1% the flies behaviour becomes slightly erratic, possibly due to the over saturation of the odour within the maze, or the increased toxicity of a highly concentrated odour. Here, the *PPL2-Gal4* experimental group increase their preference index into the approach range again, while the UAS control avoids the odor. At 0.5% concentration of ethyl butyrate using the *oc2-Gal4,repo-Gal80* driver the experimental group shows a decreased preference index compared to Gal4 controls.

Looking at the concentration curves in figure 3.29 D and I, the behaviour of the Gal4 control in particular seems strange. This is clear in figure 3.29 D where the experimental line and the UAS control show the shape of a typical response curve, while the Gal4 control seems to have a preference index constantly near zero, indicating that the flies cannot smell, or have some impairment that makes them indifferent

towards the odour. Analysing the percentage of flies that made a choice to enter the maze at each concentration we can see that the Gal4 control has significantly more flies entering compared to the UAS control and experimental groups (Appendix A.6). Moreover, as the concentration increases the percentage of flies entering the maze for the experimental and UAS control decreases, whereas the Gal4 flies remains high, suggesting that the increased odour concentration is not detected. Previous reports have indicated that accumulation of Gal4 protein can lead to issues including cell death [Rezával et al., 2007, Kramer and Staveley, 2003]. Therefore we tried expressing *UAS-GFP* under control of our Gal4 as a genetic control, to give the Gal4 protein a UAS to bind that should have minimal affects on neural function. However, the flies here also seemed to be anosmic (Figure A.5).

Overall, our behavioural results give some suggestion that PPL2 activation via *PPL2-Gal4 > dTrpA1* enhances naïve behavioural response to ethyl butyrate regardless of valence. The experimental flies of this genotype show statistically significant differences compared to the UAS control at certain concentrations of odour, increasing approach behaviour at low concentrations, and increasing avoidance at higher concentrations. Unfortunately, due to an issue with the Gal4 control this data is preliminary but hints at an exciting possibility that PPL2 encodes the salience of an odour.

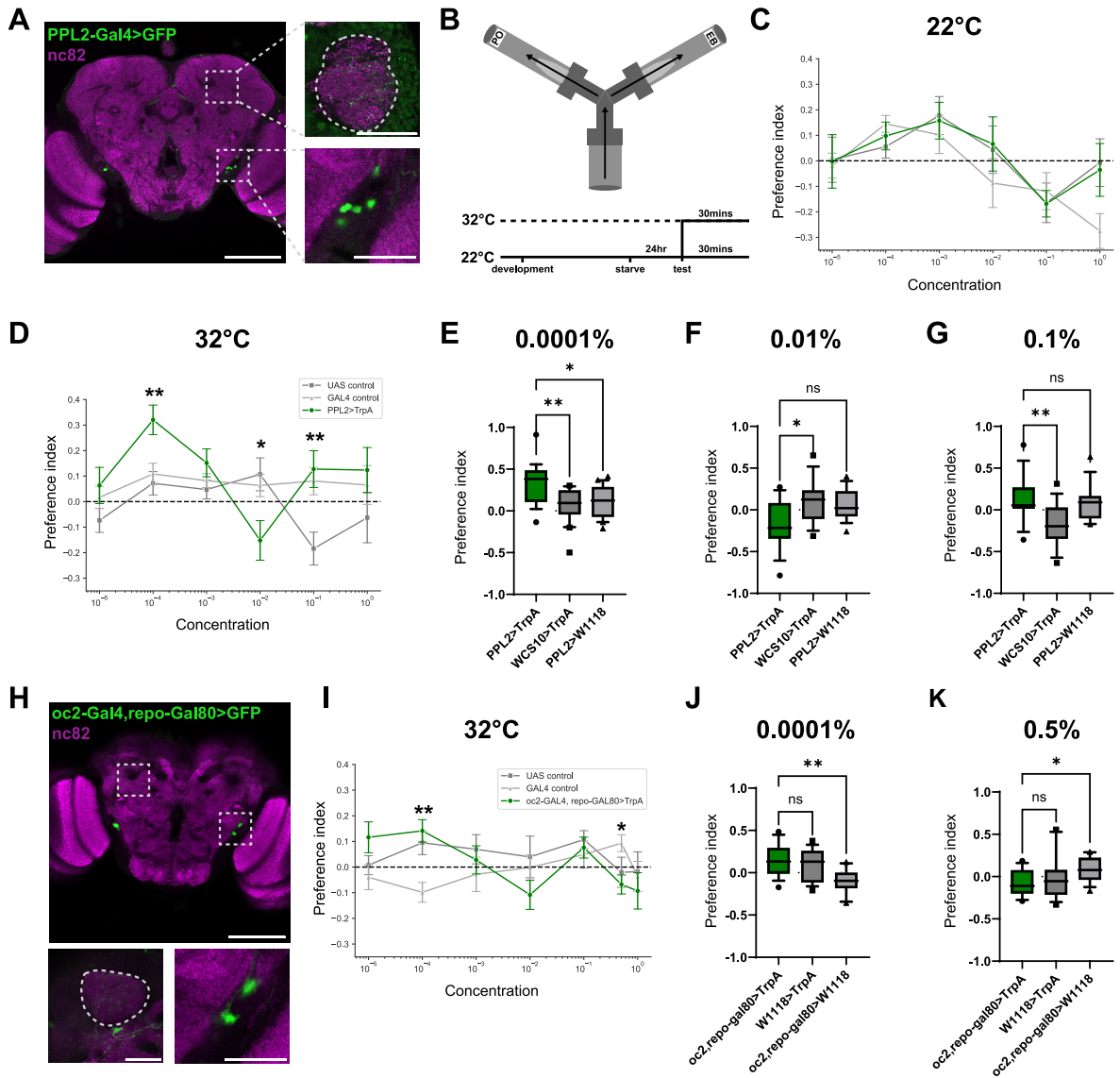


Figure 3.29: Naïve olfactory behaviour with PPL2 activation

(A) (Left) Representative image of anti-GFP (green) and anti-nc82 (magenta) immunostaining of the brain of a PPL2-GAL4>UAS-GFP::nls fly. Scale bar, 100µm. (Right) Representative image of innervations in the calyx, and cell bodies. Scale bar, 30µm

(B) Experimental timeline. Flies are bred and maintained at 22°C to avoid thermogenetically activating PPL2 neurons during development. Olfactory experiment is taken place at either 22°C, as a temperature control or 32°C to activate PPL2 neurons. (C & D) Concentration curves showing the change in behavioural response, measured by the preference index, across a range of concentrations of the odor ethyl butyrate at 22°C (n=10-16) (C) and 32°C (n=12-26) (D). Kruskal-Wallis test, *p<0.05, **p<0.01. Error bars represent the standard error of the mean. (E, F & G) Preference index at specific concentrations. (E) n=18-20. (F) n=14-17. (G) n=14-16. Kruskal-Wallis test followed by Dunn's multiple comparisons correction. ns p>0.05, *p<0.05, **p<0.01. (H) (Top) Representative image of anti-GFP (green) and anti-nc82 (magenta) immunostaining of the brain of an Oc2-GAL4, repo-GAL80>UAS-GFP::nls fly. Scale bar, 100µm. (Bottom) Representative image of innervations in the calyx, and cell bodies. Scale bar, 30µm. (I) Concentration curves showing the change in behavioural response, measured by the preference index, across a range of concentrations of the odor ethyl butyrate at 32°C. n=10-19. Kruskal-Wallis test, *p<0.05, **p<0.01. Error bars represent the standard error of the mean. (J & K) Preference index at specific concentrations. (J) n=10-18. (K) n=17-19. Kruskal-Wallis test followed by Dunn's multiple comparisons correction. ns p>0.05, *p<0.05, **p<0.01. All y-maze experiments had ≥50 flies per maze, and 20% flies made a choice between the arms. n numbers stated represent the number of mazes per condition.

Chapter 4

Discussion

4.1 Summary of results

This project uses the species *Drosophila melanogaster* to investigate the role of dopaminergic input to the MB calyx from the PPL2 cluster on olfactory processing. The MB is the center of olfactory learning and memory, and the calyx is formed by the dendritic arbors of Kenyon Cells [Bates et al., 2020b, Wilson, 2013]. These neurons are sparsely activated by odours to form an internal representation of the stimulus [Lin et al., 2014, Campbell et al., 2013]. This information is then sent to MBONs in the MB lobes, where plastic KC-MBON connections shape memories [Hige et al., 2015a, Oswald and Waddell, 2015]. While there are numerous studies focusing on the dopaminergic modulation in the MB lobes the calyx is much less explored. The PPL2 cluster provides the sole dopaminergic input to the calyx, positioning these neurons to modulate olfactory representations upstream of memory formation [Mao and Davis, 2009, Boto et al., 2019].

The main results presented in this thesis are summarised as follows:

1. PPL2 neurons innervating the calyx primarily target KC γ and APL. Using the publically available connectome datasets, FAFB and hemibrain, two PPL2 neurons innervating the calyx were identified. Analysis of their pre- and post-synaptic targets within the calyx revealed APL, KCs, particularly KC γ and PNs as the primary connections.
2. PPL2 provide a pre-synaptic component to microglomeruli synaptic structures.

Analysis of PPL2 synapse locations showed that PPL2 pre-synapses are more likely to be located within a microglomeruli than outside. Microglomeruli containing PPL2 synapses tended to be larger than microglomeruli that did not contain PPL2 synapses.

3. Knockdown of dopamine receptors in subsets of KCs affects the odour-evoked response in the calyx. Knockdown of each of Dop1R1, Dop1R2, and Dop2R had some affect on the odour-evoked response KCs in female flies. We showed that knockdowns of Dop1R1 or Dop2R using a broad KC driver resulted in a decrease in response, while knockdown of Dop1R2 resulted in an increase in the response to a familiar odour. Dop2R knockdown also resulted in robust changes in KC subsets, increasing the response in $KC\gamma$ neurons, but decreasing the response in $KC\alpha\beta$.
4. A preliminary investigation of internal state mediated odour responses revealed a starvation induced increase in odour-evoked KC response specifically in the calyx. This starvation induced increase can be abolished following Dop2R knockdown in $KC\gamma$, with fed and starved flies both responding with an increased calcium activity compared to fed controls.
5. Very preliminary data of PPL2 activity recording points towards a potential decrease in basal PPL2 activity during starvation conditions.
6. The first steps in identifying a behavioural affect of PPL2 activity are taken. Preliminary data from a naïve olfactory assay suggest that PPL2 activity increases behavioural response to an odour.

4.2 Discussion

4.2.1 Limitations of connectomic analysis

Datasets only include chemical synapses

Despite the connectomic datasets and electron microscopy reconstructions of the full brain being an invaluable resource for investigating synaptic connectivity, there are limitations and drawbacks that need to be considered. Firstly, only chemical synapses are considered here, meaning we are missing information about the locations of electrical synapses [Buhmann et al., 2021, Yu et al., 2025, Heinrich et al., 2018]. Gap junctions have been shown to be present in about 10% of KCs (although this number is probably an under estimate) throughout the MB lobes and calyx, connecting these neurons to each other and also other neurons in the circuit [Liu et al., 2016]. Electrical coupling between PNs of the same type, and PNs with LNs, in the antennal lobe has also been shown to reduce noise, increase the robustness of KC odor representations, and promote sensitivity [Zhang et al., 2013a, Yaksi and Wilson, 2010, Huang et al., 2010, Kazama and Wilson, 2009]. The neuron APL also forms gap junctions with the dorsal paired medial (DPM) neuron, a serotonergic neuron that densely innervates the MB lobes and has a role in regulating the coincidence time window in associative learning [Okray et al., 2023, Zeng et al., 2023, Keene et al., 2004]. These gap junctions are necessary for proper formation of anesthesia-sensitive memory in an aversive conditioning paradigm, shown by impaired performance after RNA_i knockdown of gap junction associated genes *inx6* and *inx7* in APL and DPM [Wu et al., 2011]. Therefore, gap junctions in *Drosophila* and in particular in the MB, between neurons in the olfactory circuit that we have shown to be major targets of PPL2 chemical signalling, play an important role in memory and should not be discarded.

Similarly, we do not take into consideration here extra-synaptic dopamine release sites and volume transmission [Saitoe et al., 2022]. In *Drosophila* dopamine neurons

signal via ‘broad transmission’, where dopamine is released from many release sites and target neurons non-specifically [Saitoe et al., 2022]. This means that dopamine release cannot necessarily be constrained by synapse-synapse communication and can affect many downstream targets that may not be captured when analysing synapse based connectivity. This may be especially important when considering dense structures structures that involve many synapses within a small volume, like the microglomeruli. PN boutons are reported to be approximately $2\text{-}7\mu\text{m}$ in diameter [Yasuyama et al., 2002], which, assuming spherical would give approximately a $4\text{-}179.6\mu\text{m}^3$ volume. Electron microscopy data analysis found a volume range of $1.3\mu\text{m}^3$ to $39.9\mu\text{m}^3$ [Yang et al., 2022]. The data presented in figure 3.8 identifies microglomeruli throughout the calyx with an average volume of $103.6\mu\text{m}^3$. Therefore, dopamine release into the microglomeruli may affect more components than those with identifies post-synapses with PPL2. PPL2 pre-synapses are largely found within microglomeruli structures, suggesting that this could be a mechanism by which dopamine modulates many neurons in a specific microglomeruli, perhaps inducing plasticity in these microcircuits that is required for memory.

Synapse identification limitations

There are known limitations in the machine learning algorithms used to identify synapses in these datasets [Yu et al., 2025, Heinrich et al., 2018, Buhmann et al., 2021]. Challenges include detection of axo-axonic synapses, complex polyadic synapses [Meinertzhagen and O’neil, 1991] and autapses [Takemura et al., 2015], which are all known to be present in the *Drosophila* neural network [Yu et al., 2025, Heinrich et al., 2018, Buhmann et al., 2021]. The FAFB data recently updated the synapse prediction algorithm to improve on these shortcomings although there are still reported problems with identification of autapses, and only one pre-synapse is assigned per post-synaptic terminal, meaning many-to-one polyadic synapses are not detected [Yu et al., 2025]. Overall, this updated algorithm detected more synapses than the original algorithm, increasing from 54,492,922 to 76,944,499 [Buhmann et al., 2021, Yu et al., 2025]. In particular, the synapses within the MB calyx increase by 75%, with the number of

identified KC- γ post-synapses increasing by 114% [Yu et al., 2025].

Synapse count differences between FAFB and hemibrain

Between FAFB and hemibrain datasets, we have seen that broad trends within PPL2 connectivity are consistent, but specific synapse counts differ. The question is, how do we know which specifics matter? Throughout this chapter we use a thresholding approach to only include connections with more than 10 synapses. This decision was due to a few factors: 1. Connections with more synapses are more reliable within the datasets [Schlegel et al., 2024]. As the synapse count between a pair of neurons decreases, the variability across datasets increases, meaning there is an increase in noise and possibly false positive identification. 2. There is an assumption that more synapses means that there is a stronger connection between these two neurons and therefore we are including only the strongest connections. This, however, is not necessarily the case. Although the number of synapses is a good proxy for synaptic contact area, and therefore strength in larva *Drosophila*, while synapse size does not vary across different types of connections [Barnes et al., 2022], the synapse size could still be taken into account since in mammalian neocortex there is a correlation between synapse size and strength [Holler et al., 2021]. Another point to make is that the synapses may be distributed along different areas of the neuron, and many synapses may not mean a stronger connection if these synapses do not fire synchronously. Therefore, by filtering out connections with less synapses we may be losing information on the total connectivity of PPL2.

Differences between the two datasets can either be due to biological variability or to technical noise but due to the current low n number it is difficult to distinguish which differences are real. Network comparisons between FAFB and hemibrain have shown that about 50% of the datasets are different (prior to updated FAFB synapse detection) [Schlegel et al., 2024]. The total synapse counts between neuron pairs are highly correlated between datasets, but the weaker the connection the less likely it is to be consistent. For example, it has been shown that 30% of differences can be

attributed to technical noise [Schlegel et al., 2024]. Since the connections we have seen between PPL2 and APL are connections with a high number of synapses, these are more likely to be reliable, meaning the difference in connectivity seen between the two brains (Figures 3.5 and 3.6 A&C) are probably due to real biological differences.

Microglomeruli size is overestimated

The model used in this thesis to identify microglomeruli is based on the PN-KC synapse connection. A clustering algorithm based on proximity is used to group the synapses into clusters that are then labelled as individual microglomeruli. However, PN boutons in the fly brain have been identified to have different bouton shapes that can be identified in the electron microscopy imaging data but are not taken into account here [Yang et al., 2022, Butcher et al., 2012]. For example, one shape of bouton consists of a 'beads-on-a-string' shape, which creates an elongated structure. Whether this type of bouton would contribute to multiple microglomeruli structures is unclear, and in the data presented here KC post-synapses that are close in proximity along this bouton will be clustered together. The maximum distance two synapses can be from each other in our model is $0.9\mu\text{m}$ which may be too large considering our average microglomeruli volume is much larger than reported estimates ($39.9\mu\text{m}^3$ vs $103.6\mu\text{m}^3$) [Yang et al., 2022]. The method to obtain the volume of microglomeruli does overestimate since we are using the bounding box containing all KC post-synapses, which will always create a rectangular volume in the orientation of the axis. This means that if the synapses fall in a cluster on a diagonal relative to the x,y axes then the bounding box will be much larger than the ground truth. Figure 3.9 shows analysis using a more restrictive definition of the volume, the convex hull of the synapse points. This more restrictive definition gives an average of $26.5\mu\text{m}^3$ which is closer to reported values. However, the computational cost of this definition was too high to use for all analyses.

Considering the overestimation of the size of microglomeruli, it is not surprising that this model then seems to underestimate the total number of microglomeruli present in the calyx. Reported estimations of the number of microglomeruli within the calyx range

from 786 to 1,590 [Turner et al., 2008, Leiss et al., 2009, Frambach et al., 2004]. Here, we identified 702 in the FAFB dataset and 682 in the hemibrain dataset. However, it is known that microglomeruli in the calyx undergo structural plasticity and the total number in the calyx can vary depending on experience or age [Baltruschat et al., 2021, Hourcade et al., 2010, Groh et al., 2006, Stieb et al., 2010]. Therefore, we would not expect the number of microglomeruli to be a fixed number across individuals, although it is not currently known by how much these numbers can vary.

4.2.2 Comparing FAFB and hemibrain data

Within the MB there are reported differences in the numbers of KCs. In particular, the population of KC γ -m neurons is almost doubled in FAFB compared to the hemibrain. This has been attributed to the possibility of larval protein starvation in the FAFB fly that was used for this data, which is known to specifically increase the number of KC γ -m neurons during development [Lin et al., 2013]. Although there is a large difference in cell counts, KC γ -m still consistently makes and receives 32% and 45% of all KC pre- and post-synapses. Therefore, individual KC γ -m cells receive fewer inputs and make fewer outputs than hemibrain. This could explain our differences in synapse counts for PPL2-to-KC γ -m connections. Since KCs only receive 1-2 synapses each from PPL2 and low counts are more likely to be missed by synapse detection, there are more false negatives in the FAFB dataset. In the hemibrain dataset we see 2 individual KC γ -m that receive upwards of 10 synapses each from PPL201 whereas in the FAFB data the highest number of synapses from PPL201 onto a singular KC is 8. This could be explained by the increased number of KCs in FAFB, meaning the input needs to be more spread across a larger population. The report of larval starvation in the FAFB fly is also speculation, ultimately we do not have much information about the previous experiences of the flies used to create these datasets and so we don't know how these experiences have altered the connectivity throughout the life of the fly.

It is important to point out the age difference between the flies used for the datasets, FAFB used a 5-day old female, versus a 7-day old. Also, we only have available

full datasets for female flies, which limits our analysis of potential dimorphic circuits. *fruitless* is a gene that regulates sex specific neural development [Ryner et al., 1996] and is known to be expressed in $KC\gamma$ and $KC\alpha\beta$ subtypes, suggesting that there may be some sex specific differences [Billeter and Goodwin, 2004, Palmateer et al., 2023]. A recent analysis of the newly released male CNS connectome excludes KCs since the population of *fru*⁺ could not be disambiguated [Deutsch et al., 2025]. Berg et al. [Berg et al., 2025] also report an upcoming paper in which no obvious sex-specific differences are found within olfactory input to the MB (Pleijzier et al., ms in prep). Despite this, it is highly likely that each brain is a snowflake, and there will never be two connectomes with the exact same connectivity. This can be due to previous experience, genetics, environment, random connectivity like that has been found in the optic lobe [Cornean et al., 2024]. This likelihood means that general trends may be more important than definitive number of synapses, but we cannot solely rely on connectome analysis to inform us, there must be experimental evidence to support connectomics to determine the population trends.

4.2.3 PPL201 and PPL202 are involved in modulating olfactory behaviour via connections with the MB calyx

Our connectome analysis revealed PPL201 and PPL202 as two dopaminergic neurons innervating the calyx. This is supported by previous data by Mao and Davis who also identified 2 subtypes of PPL2 neurons and no other dopaminergic neurons innervating the calyx by examining TH-Gal4 expression and anti-TH immunoreactivity [Mao and Davis, 2009]. However, there is some confusion regarding the nomenclature used to describe individual PPL2 neurons. The dopaminergic neuron innervating the calyx first described by Tanaka et al. was named the MB-C1 (mushroom body calyx 1) neuron [Tanaka et al., 2008]. It was then discovered that there are actually two dopaminergic neurons from the PPL2 cluster innervating the calyx, and one of these is the same neuron previously described, named MB-C1 [Mao and Davis, 2009]. This MB-C1 neuron is most likely the PPL202 neuron we have described since the innervations to

the inferior portion of the LH matches the innervation pattern for MB-C1 [Tanaka et al., 2008, Mao and Davis, 2009]. At some point the nomenclature for these neurons has changed and now MB-C1 refers to the GABAergic interneuron that innervates the entire calyx and PPL201 and PPL202 are used for the two PPL2 neurons innervating the calyx.

The two PPL2 neurons also innervate other brain areas involved in olfaction and that are also targeted by MBONs, such as the SLP and superior intermediate protocerebrum (SIP) [Aso et al., 2014a]. This supports the hypothesis that PPL2 has a role in olfaction. PPL2 has been shown to respond to odor [Boto et al., 2019], and we have shown that the output targets of PPL2 in the calyx are all olfaction related neurons. PPL201 and PPL202 have 4 neurons in common as their output targets, and 3 of these are GABAergic inhibitory neurons; APL, MB-C1, and LHPV12a1. Of these 3, synaptic connections to APL and MB-C1 are located within the MB calyx. Airyscan images of PPL2 innervations in the calyx (Figures 3.18 and 3.21) show sparse, innervations surrounding the periphery of the calyx, forming claw like shapes reminiscent of the claw-like dendritic terminals of the KCs [Baltruschat et al., 2021].

APL

We have shown that APL is a key output target in the MB calyx for both PPL201 and PPL202. APL plays a role in olfactory learning since it has been shown that the calcium response after conditioning is reduced, specifically to the conditioned odor [Liu and Davis, 2009, Zhou et al., 2019]. This decrease in activity upon learning is not unexpected since APL is an inhibitory GABAergic neuron, and the decrease in activity allows the connected KCs to increase activity [Boto et al., 2014]. The question remains how does this decrease occur? Perhaps PPL2 is providing signals to modify the circuit since the salience would increase after learning. The *Drosophila* dopamine 2-like receptor, D2R, is an inhibitory receptor expressed in APL. Knockdown of this receptor in APL has been shown to impair learning, suggesting that dopamine acts through Dop2R to inhibit APL, in turn reducing inhibition on KCs which is

necessary during learning [Hearn et al., 2002, Zhou et al., 2019]. It has also been shown that dopaminergic neurons that are activated by electric shock act on APL via D2R to reduce GABA inhibition but the origin of this dopaminergic activity is still up for debate [Zhou et al., 2019]. PPL1 in particular is one candidate that has been implicated in mediating PN-KC plasticity, strengthening the connection during aversive olfactory memory formation [Qiao et al., 2022]. This type of plasticity at the input to the mushroom body would be a good candidate to be a salience encoding signal; a higher salience would strengthen the PN-KC connection, allowing the KC to reach its firing threshold quicker and thus leading to stronger memory formation. However, this is not consistent with evidence from the literature showing that PPL1 does not innervate the mushroom body input site, the calyx [Mao and Davis, 2009, Tanaka et al., 2008, Takemura et al., 2017, Li et al., 2020a]. On the other hand, we do know that PPL2 both responds to electric shock [Boto et al., 2019] and innervates the calyx so perhaps the PPL2 dopaminergic activity representing salience acts through APL via D2R receptors to restrain APL inhibition, allowing KCs to fire and strengthening memory.

KC- γ

Another neuron population that is a major output target from PPL201 and PPL202 is the KC- γ neuron type, particularly KC γ -m. These KCs are implicated in aversive conditioning and activation of PPL2 neurons paired with electric shock leads to significant Ca^{2+} plasticity in the MB γ lobe [Boto et al., 2019]. The plasticity between PN-KCs in the calyx was also investigated specifically in KC- γ neurons, and these KCs were found to express significantly more Dop2R receptors than α/β or α'/β' KCs, the knockdown of which impairs aversive LTM [Qiao et al., 2022, Scholz-Kornehl and Schwärzel, 2016]. In our data, the knockdown of Dop2R in KC- γ leads to an increase in odour-evoked response. This suggests that dopamine acts through Dop2R to inhibit these neurons, therefore when this inhibition is removed KC- γ increase their calcium activity. KC- γ are also the most numerous KC type, so it may be expected that knockdown of Dop2R in all KCs would mirror the result from KC- γ alone. However, we see that knockdown

of Dop2R using a broad KC driver results in a decrease in activity, matching the results of using a $\alpha\beta$ driver. This decrease in response following knockdown of an inhibitory driver could be due to a compensatory mechanism, perhaps through an increase in APL inhibition acting on these KC subtypes. The imaging of $\alpha'\beta'$ neurons was outside the scope of this project, but the activity of these neurons together with $\alpha\beta$ could overpower the effect of KC- γ such that the overall resulting effect we see is that knockdown of Dop2R decreases total KC activity.

PNs

Olfactory information from the antennal lobe is received by PPL2 via a range of PNs that innervate different glomeruli. A lot of these connections are reciprocal, providing a feedback to PNs. The main PN providing both upstream and downstream connections is a multiglomerular PN, VP1d+VP4_l2PN1. This PN receives information from OSNs which innervate the VP1 and VP4 glomeruli and express the IR40a and IR95a receptors [Li et al., 2022, Knecht et al., 2016, Benton et al., 2009]. These OSNs have been shown to respond to cooling [Enjin et al., 2016], dry air [Frank et al., 2017, Marin et al., 2020, Knecht et al., 2016], and ammonia [Silbering et al., 2016], and are therefore considered non-olfactory PNs with an established role in hygro- and thermo-sensation. Other thermosensory PNs also include VL2p_adPN which receives information from OSNs expressing the IR31a receptor, an acid sensing receptor, specifically 2-oxopentanoic acid [Silbering et al., 2011, Hamada et al., 2008]. VC3l_adPN also responds to ammonia and other amines [Min et al., 2013], while VM5d_adPN is innervated by Or46a, Or67a or Or98a-expressing OSNs which respond to volatile, aversive chemicals [Dweck et al., 2018, Mansourian et al., 2016, Grabe et al., 2016].

In the lateral horn one of the main downstream targets for PPL202 is the VA1v_vPN [Horne et al., 2018]. VA1v is a sexually dimorphic fruitless-positive glomerulus responsive to fly odour [Sakurai et al., 2013], that signals the sex pheromones cis-vaccenyl acetate and methyl laurate [Dweck et al., 2013, Kurtovic et al., 2007], and is significantly larger in male flies than in females [Stockinger et al., 2005]. Activation of PPL2

has been shown to increase sexual activity in aged males, which indicates a role in processing pheromone odors. DM4 responds to attractive stimuli [Mohamed et al., 2019] and DA4 is innervated by Or43a expressing OSNs, responding to several food related odors [Wang et al., 2003a].

In starved flies, we assume that the salience of food-related odours is increased. Imaging PPL2 activity in the calyx of starved flies in response to either ethyl butyrate or ammonia stimulation showed a odour specific difference in response. To ethyl butyrate, the PPL2 response trended towards a decrease in the starved flies, while responses to ammonium hydroxide showed no differences. Both of these odours are food related, with ethyl butyrate providing a fruity pineapple aroma, and ammonia being released by the breakdown of proteins. Ethyl butyrate is attractive at low concentrations but will eventually become aversive, and ammonia is also attractive since it indicates the presence of protein rich food [Min et al., 2013]. However, it has been shown that *Drosophila* display attraction to ammonia in a T-maze assay regardless of hunger state, while other odours, such as apple cider vinegar, required a period of starvation to display strong attraction behaviour [Min et al., 2013]. This suggests that the starvation mediated increase in salience does not apply to ammonia, ammonia is always salient to the fly. Therefore, if PPL2 encodes salience, the PPL2 response to ammonia will not change with starvation, while the response to ethyl butyrate does. Overall, this data indicates odour-specific PPL2 responses under starvation conditions.

4.2.4 PPL2 modulation of KC odor-evoked responses

Dopamine can act through different receptors to differently modulate circuits, providing both excitatory and inhibitory signals. We investigated how the knockdown of each dopamine receptor affected the ethyl-butyrate evoked response of KC populations. Knockdown of Dop2R gives the most robust effect in each of the three populations we tested, decreasing the response in KCs targeted by 247 and C739 ($\alpha\beta$ -KCs) but increasing the response in γ -KCs. We discussed above the possibilities for the affect in all KCs to not match the γ -KCs. It is also interesting that we do not see any changes

in γ - or $\alpha\beta$ -KCs with knockdowns of either D1-like receptor, but we do see differences using a broad KC driver. This could be due to different strengths of each driver, or it could be due to the affect of $\alpha'\beta'$ -KCs that we did not image. Both $\alpha\beta$ - and $\alpha'\beta'$ -KCs but not γ -KCs show bias towards connectivity with food related PNs [Chan et al., 2025] so perhaps the PN inputs activated by ethyl butyrate are more strongly connected to these KC types, biasing the total KC affect.

Dop1R2 knockdown gives an interesting affect in the total KC population, showing an increase in the second odour response only. This indicates that dopamine acts through Dop1R2 to modulate responses to familiar odours. It has been reported that the odour-evoked response in PPL201 decreases upon repeated stimulation [Marquis and Wilson, 2022]. Together this could suggest that the change in dopamine upon repeated stimulation acts through Dop1R2 to reduce KC excitability, and when Dop1R2 is knocked down this decrease does not occur, perhaps since dopamine is taken up by another excitatory receptor instead.

4.2.5 A model for PPL2 starvation-mediated affects

Our results suggest that KCs increase their response to ethyl butyrate under starvation conditions. However, this affect was not very reliable to reproduce across experiments. Figure 4.1 shows data from figure 3.25 split by day and only presenting the control flies. Here, we can see that some days, for example day 4, day 8 and day 9, show a clear starvation induced increase in response. However, other days, such as day 10, or day 11 show a completely reversed affect. All variables were kept constant as much as possible, such as the food recipe, freshness of food, timing of starvation, amount of flies housed together etc. There have been recent reports in honey bees suggesting that sNPF signaling is different between bees born in the winter versus the summer [da Silva et al., 2025]. For example, learning in winter bees is already very high and is not enhanced by sNPF, however in summer bees sNPF signaling can significantly enhance learning and memory even in fed bees. Although, the physiological differences between winter and summer bees are more extreme than those in *Drosophila* developed at different

temperatures, there could be NPF signalling differences depending on temperature or season in *Drosophila* that means the starvation affect is not always present. We were able to replicate the starvation affect using the 247-GCaMP3 driver, but not using GCaMP6m expressed used 247-Gal4 or 5HTR1B-Gal4. This could be due to differences in kinetics between the two calcium indicators. GCaMP6m is more sensitive to calcium, meaning it produces a higher $\Delta F/F$ for the same calcium transient and is more likely to saturate with large increases in calcium, which would result in not seeing the full range of calcium increases.

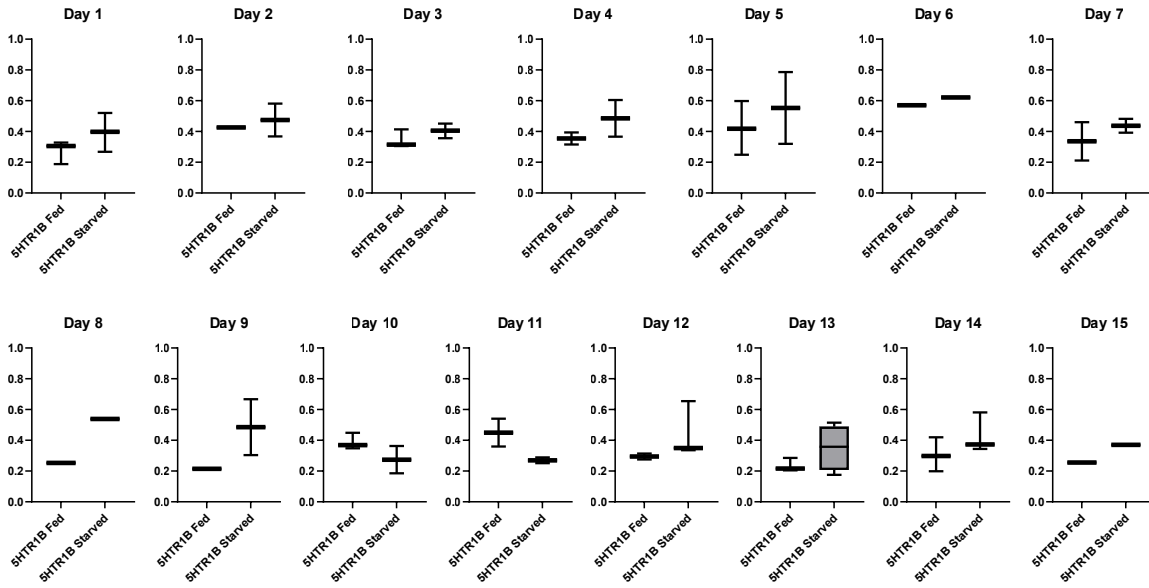


Figure 4.1: 5HTR1B-Gal4 starvation induced increase in response varies by day
Peak of the second response per day of 5HTR1B-Gal4 starved vs fed experiments. Total $n = 31-33$.

CalexA experiment results differ depending on how the GFP signal is normalised, using a background nc82 signal, or an anti-TH signal to individually normalise each cell. When using the anti-TH signal this leads to no differences between fed and starved flies, however the nc82 normalisation method leads to a decrease in calcium accumulation in starved flies. Starvation has been shown to decrease protein synthesis in *Drosophila* larva [Nagarajan and Grewal, 2014]. Although there is no evidence of TH levels decreasing with starvation in PPL1 or PAM neurons [Lee et al., 2025], this paper only looked at 24 hours of starvation. It is possible that after extreme lengths of starvation, like the 48 hours used here, that TH levels begin to decay. If this happens, then we would be unable to see the decrease in PPL2 calcium activity

with this method. There have been reported decreases in PPL1 CalexA accumulation due to global hunger-induced inhibition via NPF signals [Meschi et al., 2024, Krashes et al., 2009, Plaçais and Preat, 2013].

Overall, our current working model for PPL2 mediated starvation signaling is that: during fed conditions, tonic PPL2 dopamine activity inhibits γ -KCs via Dop2R. When the fly is starved, this baseline PPL2 activity decreases, releasing γ -KCs to respond to the odor, leading to the increase in odor-evoked response in γ -KCs and also in KCs overall. This possibly increases learning and memory capabilities by increasing the efficiency of KC responses to a learnt odor [Boto et al., 2019]. Since imaging experiments were done in the calyx, it is not known whether the increases or decreases in KC activity were due to an increase in response of the individual neurons, or a recruitment of more KCs to the odor representation. If it is the latter, then it is possible that decreased PPL2 activity will result in increased generalisation since recruiting more KCs to the odor representation will increase the overlap with other representations. Since PPL2 pre-synapses tend to be within microglomeruli, its possible that the PPL2 starvation effect is modulating specific microglomeruli, perhaps to prime them for the plasticity occurs during learning.

4.3 Future Experiments

Although a large portion of this data is exploratory or preliminary analysis, it gives a clear direction for future research to investigate the role of PPL2 in modulating olfactory representation. Future experiments that would be needed to further this investigation are:

1. Experimental confirmation of PPL2 innervations in the microglomeruli. Imaging of microglomeruli structures to confirm the connectome analysis will be crucial. It would be interesting to expose *Drosophila* to a particular odour and image specifically the odour-related microglomeruli to determine if PPL2 is recruited to microglomeruli due to previous exposure or if PPL2 is bias towards specific PNs.

The differences before and after learning would also be interesting to see if the newly formed microglomeruli recruit PPL2.

2. Starvation has been shown to alter levels of dopamine receptors Dop1R1 and Dop2R in PAM and PPL1 neurons [[Hiramatsu et al., 2025](#)]. A full analysis of precise dopamine receptor localisation in PPL2, APL, and KCs is needed plus how these receptor levels change under different internal states, particularly starvation.
3. Sexual dimorphic affects. Some of our data hints at potential sexually dimorphic circuits controlling KC odour responses. Repeating these receptor knockdown and starvation experiments with males is needed to get a full picture. Additionally, more odours will need to be tested, perhaps ammonium hydroxide will not produce the starvation-induced increase in response in KCs.
4. Activation of PPL2 during KC calcium imaging. Activation of PPL2 is needed to address the role of PPL2 on modulating KC activity.
5. Investigation of the role of APL. This thesis focused on one of the major post-synaptic targets of PPL2, KCs, but the other main post-synaptic target APL has not yet been investigated. APL also expressed dopamine receptors, and has a large role in maintaining KC sparse activity. APL is also a member of microglomeruli subunits so it is possible that dopamine acts through APL to modulate other components in the calyx.

Bibliography

- [Agarwal and Isacoff, 2011] Agarwal, G. and Isacoff, E. (2011). Specializations of a pheromonal glomerulus in the drosophila olfactory system. *Journal of Neurophysiology*, 105(4):1711–1721.
- [Ahmed et al., 2023] Ahmed, M., Rajagopalan, A. E., Pan, Y., Li, Y., Williams, D. L., Pedersen, E. A., Thakral, M., Previero, A., Close, K. C., Christoforou, C. P., et al. (2023). Input density tunes kenyon cell sensory responses in the drosophila mushroom body. *Current Biology*, 33(13):2742–2760.
- [Akmal et al., 2006] Akmal, D.-B. G., Wilson, C. F., Zong, L., Tanaka, N. K., Ito, K., and Davis, R. L. (2006). Roles for drosophila mushroom body neurons in olfactory learning and memory. *Learning & memory*, 13(5):659–668.
- [Alekseyenko et al., 2013] Alekseyenko, O. V., Chan, Y.-B., Li, R., and Kravitz, E. A. (2013). Single dopaminergic neurons that modulate aggression in drosophila. *Proceedings of the National Academy of Sciences*, 110(15):6151–6156.
- [Amin et al., 2020] Amin, H., Apostolopoulou, A. A., Suárez-Grimalt, R., Vrontou, E., and Lin, A. C. (2020). Localized inhibition in the drosophila mushroom body. *Elife*, 9:e56954.
- [Andretic et al., 2008] Andretic, R., Kim, Y.-C., Jones, F. S., Han, K.-A., and Greenspan, R. J. (2008). Drosophila d1 dopamine receptor mediates caffeine-induced arousal. *Proceedings of the National Academy of Sciences*, 105(51):20392–20397.
- [Aranda et al., 2017] Aranda, G. P., Hinojos, S. J., Sabandal, P. R., Evans, P. D., and Han, K.-A. (2017). Behavioral sensitization to the disinhibition effect of ethanol requires the dopamine/ecdyson receptor in drosophila. *Frontiers in Systems Neuroscience*, 11:56.
- [Armstrong et al., 1998] Armstrong, J. D., de Belle, J. S., Wang, Z., and Kaiser, K. (1998). Metamorphosis of the mushroom bodies; large-scale rearrangements of the neural substrates for associative learning and memory in drosophila. *Learning & Memory*, 5(1):102–114.
- [Aso et al., 2009] Aso, Y., Grübel, K., Busch, S., Friedrich, A. B., Siwanowicz, I., and Tanimoto, H. (2009). The mushroom body of adult drosophila characterized by gal4 drivers. *Journal of neurogenetics*, 23(1-2):156–172.
- [Aso et al., 2014a] Aso, Y., Hattori, D., Yu, Y., Johnston, R. M., Iyer, N. A., Ngo, T.-T., Dionne, H., Abbott, L., Axel, R., Tanimoto, H., et al. (2014a). The neuronal architecture of the mushroom body provides a logic for associative learning. *elife*, 3:e04577.
- [Aso et al., 2012] Aso, Y., Herb, A., Ogueta, M., Siwanowicz, I., Templier, T., Friedrich, A. B., Ito, K., Scholz, H., and Tanimoto, H. (2012). Three dopamine pathways induce aversive odor memories with different stability. *PLoS genetics*, 8(7):e1002768.
- [Aso et al., 2014b] Aso, Y., Sitaraman, D., Ichinose, T., Kaun, K. R., Vogt, K., Belliard-Guérin, G., Plaçais, P.-Y., Robie, A. A., Yamagata, N., Schnaitmann, C., et al. (2014b). Mushroom body output neurons encode valence and guide memory-based action selection in drosophila. *elife*, 3:e04580.
- [Aso et al., 2010] Aso, Y., Siwanowicz, I., Bräcker, L., Ito, K., Kitamoto, T., and Tanimoto, H. (2010). Specific dopaminergic neurons for the formation of labile aversive memory. *Current biology*, 20(16):1445–1451.
- [Babadi and Sompolinsky, 2014] Babadi, B. and Sompolinsky, H. (2014). Sparseness and expansion in sensory representations. *Neuron*, 83(5):1213–1226.
- [Baines et al., 2001] Baines, R. A., Uhler, J. P., Thompson, A., Sweeney, S. T., and Bate, M. (2001). Altered electrical properties in drosophilaneurons developing without synaptic transmission. *Journal of Neuroscience*, 21(5):1523–1531.
- [Ball and Tronick, 1971] Ball, W. and Tronick, E. (1971). Infant responses to impending collision: Optical and real. *Science*, 171(3973):818–820.
- [Baltruschat et al., 2021] Baltruschat, L., Prisco, L., Ranft, P., Lauritzen, J. S., Fiala, A., Bock, D. D., and Tavosanis, G. (2021). Circuit reorganization in the drosophila mushroom body calyx accompanies memory consolidation. *Cell reports*, 34(11):108871.

-
- [Barak et al., 2013] Barak, O., Rigotti, M., and Fusi, S. (2013). The sparseness of mixed selectivity neurons controls the generalization–discrimination trade-off. *Journal of Neuroscience*, 33(9):3844–3856.
- [Barber et al., 1996] Barber, C. B., Dobkin, D. P., and Huhdanpaa, H. (1996). The quickhull algorithm for convex hulls. *ACM Transactions on Mathematical Software (TOMS)*, 22(4):469–483.
- [Barnes et al., 2022] Barnes, C. L., Bonn  ry, D., and Cardona, A. (2022). Synaptic counts approximate synaptic contact area in drosophila. *Plos one*, 17(4):e0266064.
- [Barnstedt et al., 2016] Barnstedt, O., Oswald, D., Felsenberg, J., Brain, R., Moszynski, J.-P., Talbot, C. B., Perrat, P. N., and Waddell, S. (2016). Memory-relevant mushroom body output synapses are cholinergic. *Neuron*, 89(6):1237–1247.
- [Barth et al., 2014] Barth, J., Dipt, S., Pech, U., Hermann, M., Riemensperger, T., and Fiala, A. (2014). Differential associative training enhances olfactory acuity in drosophila melanogaster. *Journal of Neuroscience*, 34(5):1819–1837.
- [Bates et al., 2020a] Bates, A. S., Manton, J. D., Jagannathan, S. R., Costa, M., Schlegel, P., Rohlfing, T., and Jefferis, G. S. (2020a). The natverse, a versatile toolbox for combining and analysing neuroanatomical data. *Elife*, 9:e53350.
- [Bates et al., 2025] Bates, A. S., Phelps, J. S., Kim, M., Yang, H. H., Matsliah, A., Ajabi, Z., Perlman, E., Delgado, K. M., Osman, M. A. M., Salmon, C. K., et al. (2025). Distributed control circuits across a brain-and-cord connectome. *bioRxiv*, pages 2025–07.
- [Bates et al., 2020b] Bates, A. S., Schlegel, P., Roberts, R. J., Drummond, N., Tamimi, I. F., Turnbull, R., Zhao, X., Marin, E. C., Popovici, P. D., Dhawan, S., et al. (2020b). Complete connectomic reconstruction of olfactory projection neurons in the fly brain. *Current Biology*, 30(16):3183–3199.
- [Bellingham et al., 1985] Bellingham, W. P., Gillette-Bellingham, K., and Kehoe, E. J. (1985). Summation and configuration in patterning schedules with the rat and rabbit. *Animal Learning & Behavior*, 13(2):152–164.
- [Bendor and Wang, 2005] Bendor, D. and Wang, X. (2005). The neuronal representation of pitch in primate auditory cortex. *Nature*, 436(7054):1161–1165.
- [Benton et al., 2009] Benton, R., Vannice, K. S., Gomez-Diaz, C., and Vosshall, L. B. (2009). Variant ionotropic glutamate receptors as chemosensory receptors in drosophila. *Cell*, 136(1):149–162.
- [Benton et al., 2007] Benton, R., Vannice, K. S., and Vosshall, L. B. (2007). An essential role for a cd36-related receptor in pheromone detection in drosophila. *Nature*, 450(7167):289–293.
- [Berck et al., 2016] Berck, M. E., Khandelwal, A., Claus, L., Hernandez-Nunez, L., Si, G., Tabone, C. J., Li, F., Truman, J. W., Fetter, R. D., Louis, M., et al. (2016). The wiring diagram of a glomerular olfactory system. *Elife*, 5:e14859.
- [Berg et al., 2025] Berg, S., Beckett, I. R., Costa, M., Schlegel, P., Januszewski, M., Marin, E. C., Nern, A., Preibisch, S., Qiu, W., Takemura, S.-y., et al. (2025). Sexual dimorphism in the complete connectome of the drosophila male central nervous system. *bioRxiv*, pages 2025–10.
- [Berry et al., 2012] Berry, J. A., Cervantes-Sandoval, I., Nicholas, E. P., and Davis, R. L. (2012). Dopamine is required for learning and forgetting in drosophila. *Neuron*, 74(3):530–542.
- [Berry et al., 2018] Berry, J. A., Phan, A., and Davis, R. L. (2018). Dopamine neurons mediate learning and forgetting through bidirectional modulation of a memory trace. *Cell Reports*, 25(3):651–662.
- [Bielopolski et al., 2019] Bielopolski, N., Amin, H., Apostolopoulou, A. A., Rozenfeld, E., Lerner, H., Huetteroth, W., Lin, A. C., and Parnas, M. (2019). Inhibitory muscarinic acetylcholine receptors enhance aversive olfactory learning in adult drosophila. *Elife*, 8:e48264.
- [Bilkey et al., 2017] Bilkey, D. K., Cheyne, K. R., Eckert, M. J., Lu, X., Chowdhury, S., Worley, P. F., Crandall, J. E., and Abraham, W. C. (2017). Exposure to complex environments results in more sparse representations of space in the hippocampus. *Hippocampus*, 27(11):1178–1191.
- [Billeter and Goodwin, 2004] Billeter, J.-C. and Goodwin, S. F. (2004). Characterization of drosophila fruitless-gal4 transgenes reveals expression in male-specific fruitless neurons and innervation of male reproductive structures. *Journal of Comparative Neurology*, 475(2):270–287.
-

- [Blanco et al., 2011] Blanco, J., Pandey, R., Wasser, M., and Udolph, G. (2011). Orthodenticle is necessary for survival of a cluster of clonally related dopaminergic neurons in the drosophila larval and adult brain. *Neural development*, 6(1):1–14.
- [Blum et al., 2009] Blum, A. L., Li, W., Cressy, M., and Dubnau, J. (2009). Short-and long-term memory in drosophila require camp signaling in distinct neuron types. *Current Biology*, 19(16):1341–1350.
- [Bober-Irizar and Banerjee, 2024] Bober-Irizar, M. and Banerjee, S. (2024). Neural networks for abstraction and reasoning. *Scientific Reports*, 14(1):27823.
- [Bogovic et al., 2020] Bogovic, J. A., Otsuna, H., Heinrich, L., Ito, M., Jeter, J., Meissner, G., Nern, A., Colonell, J., Malkesman, O., Ito, K., et al. (2020). An unbiased template of the drosophila brain and ventral nerve cord. *Plos one*, 15(12):e0236495.
- [Boto et al., 2014] Boto, T., Louis, T., Jindachomthong, K., Jalink, K., and Tomchik, S. M. (2014). Dopaminergic modulation of camp drives nonlinear plasticity across the drosophila mushroom body lobes. *Current biology*, 24(8):822–831.
- [Boto et al., 2019] Boto, T., Stahl, A., Zhang, X., Louis, T., and Tomchik, S. M. (2019). Independent contributions of discrete dopaminergic circuits to cellular plasticity, memory strength, and valence in drosophila. *Cell reports*, 27(7):2014–2021.
- [Boto and Tomchik, 2024] Boto, T. and Tomchik, S. M. (2024). Functional imaging of learning-induced plasticity in the central nervous system with genetically encoded reporters in drosophila. *Cold Spring Harbor Protocols*, 2024(6):pdb-top107799.
- [Bozeat et al., 2002] Bozeat, S., Ralph, M. A. L., Patterson, K., and Hodges, J. R. (2002). The influence of personal familiarity and context on object use in semantic dementia. *Neurocase*, 8(1-2):127–134.
- [Brand and Perrimon, 1993] Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *development*, 118(2):401–415.
- [Briggman and Bock, 2012] Briggman, K. L. and Bock, D. D. (2012). Volume electron microscopy for neuronal circuit reconstruction. *Current opinion in neurobiology*, 22(1):154–161.
- [Briscoe and Chittka, 2001] Briscoe, A. D. and Chittka, L. (2001). The evolution of color vision in insects. *Annual review of entomology*, 46(1):471–510.
- [Bryden and Cohen, 2008] Bryden, J. and Cohen, N. (2008). Neural control of caenorhabditis elegans forward locomotion: the role of sensory feedback. *Biological cybernetics*, 98(4):339–351.
- [Buck and Axel, 1991] Buck, L. and Axel, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell*, 65(1):175–187.
- [Buhmann et al., 2021] Buhmann, J., Sheridan, A., Malin-Mayor, C., Schlegel, P., Gerhard, S., Kazimiers, T., Krause, R., Nguyen, T. M., Heinrich, L., Lee, W.-C. A., et al. (2021). Automatic detection of synaptic partners in a whole-brain drosophila electron microscopy data set. *Nature methods*, 18(7):771–774.
- [Burke et al., 2012] Burke, C. J., Huetteroth, W., Oswald, D., Perisse, E., Krashes, M. J., Das, G., Gohl, D., Silies, M., Certel, S., and Waddell, S. (2012). Layered reward signalling through octopamine and dopamine in drosophila. *Nature*, 492(7429):433–437.
- [Busch et al., 2009] Busch, S., Selcho, M., Ito, K., and Tanimoto, H. (2009). A map of octopaminergic neurons in the drosophila brain. *Journal of Comparative Neurology*, 513(6):643–667.
- [Busto et al., 2010] Busto, G. U., Cervantes-Sandoval, I., and Davis, R. L. (2010). Olfactory learning in drosophila. *Physiology*, 25(6):338–346.
- [Butcher et al., 2012] Butcher, N. J., Friedrich, A. B., Lu, Z., Tanimoto, H., and Meinertzhagen, I. A. (2012). Different classes of input and output neurons reveal new features in microglomeruli of the adult drosophila mushroom body calyx. *Journal of Comparative Neurology*, 520(10):2185–2201.
- [Cachero et al., 2010] Cachero, S., Ostrovsky, A. D., Jai, Y. Y., Dickson, B. J., and Jefferis, G. S. (2010). Sexual dimorphism in the fly brain. *Current biology*, 20(18):1589–1601.
- [Campbell et al., 2013] Campbell, R. A., Honegger, K. S., Qin, H., Li, W., Demir, E., and Turner, G. C. (2013). Imaging a population code for odor identity in the drosophila mushroom body. *Journal of Neuroscience*, 33(25):10568–10581.

-
- [Carcaud et al., 2015] Carcaud, J., Giurfa, M., and Sandoz, J.-C. (2015). Differential combinatorial coding of pheromones in two olfactory subsystems of the honey bee brain. *Journal of Neuroscience*, 35(10):4157–4167.
- [Cardé, 2021] Cardé, R. T. (2021). Navigation along windborne plumes of pheromone and resource-linked odors. *Annual Review of Entomology*, 66(1):317–336.
- [Carlson, 1996] Carlson, J. R. (1996). Olfaction in drosophila: from odor to behavior. *Trends in Genetics*, 12(5):175–180.
- [Carlson, 2001] Carlson, J. R. (2001). Functional expression of a drosophila odor receptor. *Proceedings of the National Academy of Sciences*, 98(16):8936–8937.
- [Caron et al., 2013] Caron, S. J., Ruta, V., Abbott, L. F., and Axel, R. (2013). Random convergence of olfactory inputs in the drosophila mushroom body. *Nature*, 497(7447):113–117.
- [Cayco-Gajic and Silver, 2019] Cayco-Gajic, N. A. and Silver, R. A. (2019). Re-evaluating circuit mechanisms underlying pattern separation. *Neuron*, 101(4):584–602.
- [Cervantes-Sandoval et al., 2013] Cervantes-Sandoval, I., Martin-Peña, A., Berry, J. A., and Davis, R. L. (2013). System-like consolidation of olfactory memories in drosophila. *Journal of Neuroscience*, 33(23):9846–9854.
- [Chan et al., 2025] Chan, I. C. W., Kalle Kossio, F. Y., and Tavosanis, G. (2025). Odour representations supporting ethology-relevant categorisation and discrimination in the drosophila mushroom body. *bioRxiv*, pages 2025–01.
- [Chang et al., 2006] Chang, H., Grygoruk, A., Brooks, E., Ackerson, L., Maidment, N., Bainton, R., and Krantz, D. (2006). Overexpression of the drosophila vesicular monoamine transporter increases motor activity and courtship but decreases the behavioral response to cocaine. *Molecular psychiatry*, 11(1):99–113.
- [Chavlis et al., 2017] Chavlis, S., Petrantonis, P. C., and Poirazi, P. (2017). Dendrites of dentate gyrus granule cells contribute to pattern separation by controlling sparsity. *Hippocampus*, 27(1):89–110.
- [Chen et al., 2024] Chen, F., Li, S., Han, J., Ren, F., and Yang, Z. (2024). Review of lightweight deep convolutional neural networks: F. chen et al. *Archives of Computational Methods in Engineering*, 31(4):1915–1937.
- [Chen and Gerber, 2014] Chen, Y.-c. and Gerber, B. (2014). Generalization and discrimination tasks yield concordant measures of perceived distance between odours and their binary mixtures in larval drosophila. *Journal of Experimental Biology*, 217(12):2071–2077.
- [Cheong et al., 2023] Cheong, H., Eichler, K., Stürner, T., Asinof, S., Champion, A., Marin, E., Oram, T., Sumathipala, M., Venkatasubramanian, L., Namiki, S., et al. (2023). Transforming descending input into behavior: The organization of premotor circuits in the drosophilamale adult nerve cord connectome. *(No Title)*.
- [Choi et al., 2022] Choi, K., Kim, W. K., and Hyeon, C. (2022). Olfactory responses of drosophila are encoded in the organization of projection neurons. *Elife*, 11:e77748.
- [Chou et al., 2010] Chou, Y.-H., Spletter, M. L., Yaksi, E., Leong, J. C., Wilson, R. I., and Luo, L. (2010). Diversity and wiring variability of olfactory local interneurons in the drosophila antennal lobe. *Nature neuroscience*, 13(4):439–449.
- [Christiansen et al., 2011] Christiansen, F., Zube, C., Andlauer, T. F., Wichmann, C., Fouquet, W., Oswald, D., Mertel, S., Leiss, F., Tavosanis, G., Luna, A. J. F., et al. (2011). Presynapses in kenyon cell dendrites in the mushroom body calyx of drosophila. *Journal of Neuroscience*, 31(26):9696–9707.
- [Chu et al., 2024] Chu, L.-A., Tai, C.-Y., and Chiang, A.-S. (2024). Thirst-driven hygro-sensory suppression promotes water seeking in drosophila. *Proceedings of the National Academy of Sciences*, 121(34):e2404454121.
- [Chung et al., 2017] Chung, B. Y., Ro, J., Hutter, S. A., Miller, K. M., Guduguntla, L. S., Kondo, S., and Pletcher, S. D. (2017). Drosophila neuropeptide f signaling independently regulates feeding and sleep-wake behavior. *Cell reports*, 19(12):2441–2450.
-

- [Civelli et al., 1993] Civelli, O., Bunzow, J. R., and Grandy, D. K. (1993). Molecular diversity of the dopamine receptors. *Annual review of pharmacology and toxicology*, 33(1):281–307.
- [Claridge-Chang et al., 2009] Claridge-Chang, A., Roorda, R. D., Vrontou, E., Sjulson, L., Li, H., Hirsh, J., and Miesenböck, G. (2009). Writing memories with light-addressable reinforcement circuitry. *Cell*, 139(2):405–415.
- [Clements et al., 2020] Clements, J., Dolafi, T., Umayam, L., Neubarth, N. L., Berg, S., Scheffer, L. K., and Plaza, S. M. (2020). Neu print: Analysis tools for em connectomics. *BioRxiv*, pages 2020–01.
- [Coates et al., 2020] Coates, K. E., Calle-Schuler, S. A., Helmick, L. M., Knotts, V. L., Martik, B. N., Salman, F., Warner, L. T., Valla, S. V., Bock, D. D., and Dacks, A. M. (2020). The wiring logic of an identified serotonergic neuron that spans sensory networks. *Journal of Neuroscience*, 40(33):6309–6327.
- [Cohn et al., 2015] Cohn, R., Morantte, I., and Ruta, V. (2015). Coordinated and compartmentalized neuromodulation shapes sensory processing in drosophila. *Cell*, 163(7):1742–1755.
- [Consortium, 2025] Consortium, T. M. (2025). Functional connectomics spanning multiple areas of mouse visual cortex. *Nature*, 640(8058):435–447.
- [Cook et al., 2019] Cook, S. J., Jarrell, T. A., Brittin, C. A., Wang, Y., Bloniarz, A. E., Yakovlev, M. A., Nguyen, K. C., Tang, L. T.-H., Bayer, E. A., Duerr, J. S., et al. (2019). Whole-animal connectomes of both caenorhabditis elegans sexes. *Nature*, 571(7763):63–71.
- [Coop and Arel, 2013] Coop, R. and Arel, I. (2013). Mitigation of catastrophic forgetting in recurrent neural networks using a fixed expansion layer. In *The 2013 International Joint Conference on Neural Networks (IJCNN)*, pages 1–7. IEEE.
- [Cooper et al., 1995] Cooper, D. M., Mons, N., and Karpen, J. W. (1995). Adenylyl cyclases and the interaction between calcium and camp signalling. *Nature*, 374(6521):421–424.
- [Cornean et al., 2024] Cornean, J., Molina-Obando, S., Gür, B., Bast, A., Ramos-Traslosheros, G., Chojetzki, J., Lorsch, L., Ioannidou, M., Taneja, R., Schnaitmann, C., et al. (2024). Heterogeneity of synaptic connectivity in the fly visual system. *Nature Communications*, 15(1):1570.
- [Corrales-Carvajal et al., 2016] Corrales-Carvajal, V. M., Faisal, A. A., and Ribeiro, C. (2016). Internal states drive nutrient homeostasis by modulating exploration-exploitation trade-off. *Elife*, 5:e19920.
- [Costa et al., 2016] Costa, M., Manton, J. D., Ostrovsky, A. D., Prohaska, S., and Jefferis, G. S. (2016). Nblast: rapid, sensitive comparison of neuronal structure and construction of neuron family databases. *Neuron*, 91(2):293–311.
- [Couto et al., 2005] Couto, A., Alenius, M., and Dickson, B. J. (2005). Molecular, anatomical, and functional organization of the drosophila olfactory system. *Current Biology*, 15(17):1535–1547.
- [Crittenden et al., 1998] Crittenden, J. R., Skoulakis, E. M., Han, K.-A., Kalderon, D., and Davis, R. L. (1998). Tripartite mushroom body architecture revealed by antigenic markers. *Learning & memory*, 5(1):38–51.
- [Crocker et al., 2016] Crocker, A., Guan, X.-J., Murphy, C. T., and Murthy, M. (2016). Cell-type-specific transcriptome analysis in the drosophila mushroom body reveals memory-related changes in gene expression. *Cell reports*, 15(7):1580–1596.
- [Croset et al., 2016] Croset, V., Schleyer, M., Arguello, J. R., Gerber, B., and Benton, R. (2016). A molecular and neuronal basis for amino acid sensing in the drosophila larva. *Scientific reports*, 6(1):34871.
- [Croset et al., 2018] Croset, V., Treiber, C. D., and Waddell, S. (2018). Cellular diversity in the drosophila midbrain revealed by single-cell transcriptomics. *Elife*, 7:e34550.
- [da Silva et al., 2025] da Silva, R. C., Baracchi, D., Ricciardi, G., Giurfa, M., and de Brito Sanchez, M. G. (2025). Seasonal and nutritional modulation of honeybee olfactory learning by the short neuropeptide f. *Proceedings B*, 292(2051):20250655.
- [Dacks et al., 2009] Dacks, A. M., Green, D. S., Root, C. M., Nighorn, A. J., and Wang, J. W. (2009). Serotonin modulates olfactory processing in the antennal lobe of drosophila. *Journal of neurogenetics*, 23(4):366–377.

- [Dana et al., 2019] Dana, H., Sun, Y., Mohar, B., Hulse, B. K., Kerlin, A. M., Hasseman, J. P., Tsegaye, G., Tsang, A., Wong, A., Patel, R., et al. (2019). High-performance calcium sensors for imaging activity in neuronal populations and microcompartments. *Nature methods*, 16(7):649–657.
- [Das et al., 2011] Das, A., Chiang, A., Davla, S., Priya, R., Reichert, H., Vijayraghavan, K., and Rodrigues, V. (2011). Identification and analysis of a glutamatergic local interneuron lineage in the adult drosophila olfactory system. *Neural systems & circuits*, 1(1):4.
- [Datta et al., 2008] Datta, S. R., Vasconcelos, M. L., Ruta, V., Luo, S., Wong, A., Demir, E., Flores, J., Balonze, K., Dickson, B. J., and Axel, R. (2008). The drosophila pheromone cva activates a sexually dimorphic neural circuit. *Nature*, 452(7186):473–477.
- [Davies et al., 2005] Davies, R. R., Hodges, J. R., Kril, J. J., Patterson, K., Halliday, G. M., and Xuereb, J. H. (2005). The pathological basis of semantic dementia. *Brain*, 128(9):1984–1995.
- [Davis et al., 1995] Davis, R. L., Cherry, J., Dauwalder, B., Han, P.-L., and Skoulakis, E. (1995). The cyclic amp system and drosophila learning. *Molecular and cellular biochemistry*, 149(1):271–278.
- [De Belle and Heisenberg, 1994] De Belle, J. S. and Heisenberg, M. (1994). Associative odor learning in drosophila abolished by chemical ablation of mushroom bodies. *Science*, 263(5147):692–695.
- [De Bruyne et al., 1999] De Bruyne, M., Clyne, P. J., and Carlson, J. R. (1999). Odor coding in a model olfactory organ: The drosophila maxillary palp. *Journal of Neuroscience*, 19(11):4520–4532.
- [Deisig et al., 2001] Deisig, N., Lachnit, H., Giurfa, M., and Hellstern, F. (2001). Configural olfactory learning in honeybees: negative and positive patterning discrimination. *Learning & Memory*, 8(2):70–78.
- [Delamater et al., 1999] Delamater, A. R., Sosa, W., and Katz, M. (1999). Elemental and configural processes in patterning discrimination learning. *The Quarterly Journal of Experimental Psychology Section B*, 52(2b):97–124.
- [Delandre et al., 2025] Delandre, C., McMullen, J. P., and Marshall, O. J. (2025). Dynamic changes in neuronal and glial gal4 driver expression during drosophila aging. *Genetics*, 229(3):iyaf014.
- [Deng et al., 2019] Deng, B., Li, Q., Liu, X., Cao, Y., Li, B., Qian, Y., Xu, R., Mao, R., Zhou, E., Zhang, W., et al. (2019). Chemoconnectomics: mapping chemical transmission in drosophila. *Neuron*, 101(5):876–893.
- [Deutsch et al., 2025] Deutsch, D., Matsliah, A., Wang, K., Dorkenwald, S., Mondal, A., Burke, A., Hebditch, J., Gager, J., Yu, S.-C., Sterling, A., et al. (2025). Sexually-dimorphic neurons in the drosophila whole-brain connectome. *bioRxiv*, pages 2025–06.
- [Devaud et al., 2001] Devaud, J.-M., Acebes, A., and Ferrús, A. (2001). Odor exposure causes central adaptation and morphological changes in selected olfactory glomeruli in drosophila. *Journal of Neuroscience*, 21(16):6274–6282.
- [Devaud et al., 2003] Devaud, J.-M., Acebes, A., Ramaswami, M., and Ferrús, A. (2003). Structural and functional changes in the olfactory pathway of adult drosophila take place at a critical age. *Journal of neurobiology*, 56(1):13–23.
- [DeWeese et al., 2003] DeWeese, M. R., Wehr, M., and Zador, A. M. (2003). Binary spiking in auditory cortex. *Journal of Neuroscience*, 23(21):7940–7949.
- [Dolan et al., 2019] Dolan, M.-J., Frechter, S., Bates, A. S., Dan, C., Huoviala, P., Roberts, R. J., Schlegel, P., Dhawan, S., Tabano, R., Dionne, H., et al. (2019). Neurogenetic dissection of the drosophila lateral horn reveals major outputs, diverse behavioural functions, and interactions with the mushroom body. *Elife*, 8:e43079.
- [Dorkenwald et al., 2024] Dorkenwald, S., Matsliah, A., Sterling, A. R., Schlegel, P., Yu, S.-C., McKellar, C. E., Lin, A., Costa, M., Eichler, K., Yin, Y., et al. (2024). Neuronal wiring diagram of an adult brain. *Nature*, 634(8032):124–138.
- [Dorkenwald et al., 2022] Dorkenwald, S., McKellar, C. E., Macrina, T., Kemnitz, N., Lee, K., Lu, R., Wu, J., Popovych, S., Mitchell, E., Nehoran, B., et al. (2022). Flywire: online community for whole-brain connectomics. *Nature methods*, 19(1):119–128.
- [Dorkenwald et al., 2025] Dorkenwald, S., Schneider-Mizell, C. M., Brittain, D., Halageri, A., Jordan, C., Kemnitz, N., Castro, M. A., Silversmith, W., Maitin-Shephard, J., Troidl, J., et al. (2025). Cave: Connectome annotation versioning engine. *Nature Methods*, pages 1–9.

- [Draper et al., 2007] Draper, I., Kurshan, P. T., McBride, E., Jackson, F. R., and Kopin, A. S. (2007). Locomotor activity is regulated by d2-like receptors in drosophila: an anatomic and functional analysis. *Developmental neurobiology*, 67(3):378–393.
- [Dubnau et al., 2001] Dubnau, J., Grady, L., Kitamoto, T., and Tully, T. (2001). Disruption of neurotransmission in drosophila mushroom body blocks retrieval but not acquisition of memory. *Nature*, 411(6836):476–480.
- [Duffy, 2002] Duffy, J. B. (2002). Gal4 system in drosophila: a fly geneticist’s swiss army knife. *genesis*, 34(1-2):1–15.
- [Dunn et al., 2016] Dunn, T. W., Gebhardt, C., Naumann, E. A., Riegler, C., Ahrens, M. B., Engert, F., and Del Bene, F. (2016). Neural circuits underlying visually evoked escapes in larval zebrafish. *Neuron*, 89(3):613–628.
- [Durrieu et al., 2020] Durrieu, M., Wystrach, A., Arrufat, P., Giurfa, M., and Isabel, G. (2020). Fruit flies can learn non-elemental olfactory discriminations. *Proceedings of the Royal Society B*, 287(1938):20201234.
- [Dweck et al., 2013] Dweck, H. K., Ebrahim, S. A., Kromann, S., Bown, D., Hillbur, Y., Sachse, S., Hansson, B. S., and Stensmyr, M. C. (2013). Olfactory preference for egg laying on citrus substrates in drosophila. *Current Biology*, 23(24):2472–2480.
- [Dweck et al., 2018] Dweck, H. K., Ebrahim, S. A., Retzke, T., Grabe, V., Weißflog, J., Svatoš, A., Hansson, B. S., and Knaden, M. (2018). The olfactory logic behind fruit odor preferences in larval and adult drosophila. *Cell reports*, 23(8):2524–2531.
- [Eckstein et al., 2024] Eckstein, N., Bates, A. S., Champion, A., Du, M., Yin, Y., Schlegel, P., Lu, A. K.-Y., Rymer, T., Finley-May, S., Paterson, T., et al. (2024). Neurotransmitter classification from electron microscopy images at synaptic sites in drosophila melanogaster. *Cell*, 187(10):2574–2594.
- [Eichler et al., 2017] Eichler, K., Li, F., Litwin-Kumar, A., Park, Y., Andrade, I., Schneider-Mizell, C. M., Saumweber, T., Huser, A., Eschbach, C., Gerber, B., et al. (2017). The complete connectome of a learning and memory centre in an insect brain. *Nature*, 548(7666):175–182.
- [Elkahlah et al., 2020] Elkahlah, N. A., Rogow, J. A., Ahmed, M., and Clowney, E. J. (2020). Presynaptic developmental plasticity allows robust sparse wiring of the drosophila mushroom body. *Elife*, 9:e52278.
- [Enjin et al., 2016] Enjin, A., Zaharieva, E. E., Frank, D. D., Mansourian, S., Suh, G. S., Gallio, M., and Stensmyr, M. C. (2016). Humidity sensing in drosophila. *Current Biology*, 26(10):1352–1358.
- [Eschbach et al., 2020] Eschbach, C., Fushiki, A., Winding, M., Schneider-Mizell, C. M., Shao, M., Arruda, R., Eichler, K., Valdes-Aleman, J., Ohyama, T., Thum, A. S., et al. (2020). Recurrent architecture for adaptive regulation of learning in the insect brain. *Nature neuroscience*, 23(4):544–555.
- [Ester et al., 1996] Ester, M., Kriegel, H.-P., Sander, J., Xu, X., et al. (1996). A density-based algorithm for discovering clusters in large spatial databases with noise. In *kdd*, volume 96, pages 226–231.
- [Fahrbach et al., 1998] Fahrbach, S. E., Moore, D., Capaldi, E. A., Farris, S. M., and Robinson, G. E. (1998). Experience-expectant plasticity in the mushroom bodies of the honeybee. *Learning & Memory*, 5(1):115–123.
- [Farris, 2005] Farris, S. M. (2005). Evolution of insect mushroom bodies: old clues, new insights. *Arthropod Structure & Development*, 34(3):211–234.
- [Farris et al., 2001] Farris, S. M., Robinson, G. E., and Fahrbach, S. E. (2001). Experience-and age-related outgrowth of intrinsic neurons in the mushroom bodies of the adult worker honeybee. *Journal of Neuroscience*, 21(16):6395–6404.
- [Feigin et al., 2021] Feigin, L., Tasaka, G., Maor, I., and Mizrahi, A. (2021). Sparse coding in temporal association cortex improves complex sound discriminability. *Journal of Neuroscience*, 41(33):7048–7064.
- [Feng et al., 1996] Feng, G., Hannan, F., Reale, V., Hon, Y. Y., Kousky, C. T., Evans, P. D., and Hall, L. M. (1996). Cloning and functional characterization of a novel dopamine receptor from drosophila melanogaster. *Journal of Neuroscience*, 16(12):3925–3933.

- [Fernandez-Chiappe et al., 2020] Fernandez-Chiappe, F., Hermann-Luibl, C., Peteranderl, A., Reinhard, N., Senthilan, P. R., Hieke, M., Selcho, M., Yoshii, T., Shafer, O. T., Muraro, N. I., et al. (2020). Dopamine signaling in wake-promoting clock neurons is not required for the normal regulation of sleep in drosophila. *Journal of Neuroscience*, 40(50):9617–9633.
- [Ferris et al., 2006] Ferris, J., Ge, H., Liu, L., and Roman, G. (2006). G (o) signaling is required for drosophila associative learning. *Nature neuroscience*, 9(8):1036–1040.
- [Finelli et al., 2008] Finelli, L. A., Haney, S., Bazhenov, M., Stopfer, M., and Sejnowski, T. J. (2008). Synaptic learning rules and sparse coding in a model sensory system. *PLoS computational biology*, 4(4):e1000062.
- [Finkelstein et al., 1990] Finkelstein, R., Smouse, D., Capaci, T. M., Spradling, A. C., and Perri-mon, N. (1990). The orthodenticle gene encodes a novel homeo domain protein involved in the development of the drosophila nervous system and ocellar visual structures. *Genes & development*, 4(9):1516–1527.
- [Fischer et al., 1988] Fischer, J. A., Giniger, E., Maniatis, T., and Ptashne, M. (1988). Gal4 activates transcription in drosophila. *Nature*, 332(6167):853–856.
- [Fişek and Wilson, 2014] Fişek, M. and Wilson, R. I. (2014). Stereotyped connectivity and computations in higher-order olfactory neurons. *Nature neuroscience*, 17(2):280–288.
- [Fishilevich and Vosshall, 2005] Fishilevich, E. and Vosshall, L. B. (2005). Genetic and functional subdivision of the drosophila antennal lobe. *Current Biology*, 15(17):1548–1553.
- [Flavell et al., 2022] Flavell, S. W., Gogolla, N., Lovett-Barron, M., and Zelikowsky, M. (2022). The emergence and influence of internal states. *Neuron*, 110(16):2545–2570.
- [Frambach et al., 2004] Frambach, I., Rössler, W., Winkler, M., and Schürmann, F.-W. (2004). F-actin at identified synapses in the mushroom body neuropil of the insect brain. *Journal of Comparative Neurology*, 475(3):303–314.
- [Frank et al., 2017] Frank, D. D., Enjin, A., Jouandet, G. C., Zaharieva, E. E., Para, A., Stensmyr, M. C., and Gallio, M. (2017). Early integration of temperature and humidity stimuli in the drosophila brain. *Current Biology*, 27(15):2381–2388.
- [Frechter et al., 2019] Frechter, S., Bates, A. S., Tootoonian, S., Dolan, M.-J., Manton, J., Jamasb, A. R., Kohl, J., Bock, D., and Jefferis, G. (2019). Functional and anatomical specificity in a higher olfactory centre. *elife*, 8:e44590.
- [Frighetto et al., 2022] Frighetto, G., Zordan, M. A., Castiello, U., Megighian, A., and Martin, J.-R. (2022). Dopamine modulation of drosophila ellipsoid body neurons, a nod to the mammalian basal ganglia. *Frontiers in physiology*, 13:849142.
- [Fuller and Endler, 2018] Fuller, R. C. and Endler, J. A. (2018). A perspective on sensory drive. *Current zoology*, 64(4):465–470.
- [Gaese and Ostwald, 2001] Gaese, B. H. and Ostwald, J. (2001). Anesthesia changes frequency tuning of neurons in the rat primary auditory cortex. *Journal of neurophysiology*, 86(2):1062–1066.
- [Ganeshina and Menzel, 2001] Ganeshina, O. and Menzel, R. (2001). Gaba-immunoreactive neurons in the mushroom bodies of the honeybee: An electron microscopic study. *Journal of Comparative Neurology*, 437(3):335–349.
- [Ganguly et al., 2024] Ganguly, I., Heckman, E. L., Litwin-Kumar, A., Clowney, E. J., and Behnia, R. (2024). Diversity of visual inputs to kenyon cells of the drosophila mushroom body. *Nature Communications*, 15(1):5698.
- [Gao and Chess, 1999] Gao, Q. and Chess, A. (1999). Identification of candidate drosophila olfactory receptors from genomic dna sequence. *Genomics*, 60(1):31–39.
- [Geirhos et al., 2018] Geirhos, R., Temme, C. R., Rauber, J., Schütt, H. H., Bethge, M., and Wichmann, F. A. (2018). Generalisation in humans and deep neural networks. *Advances in neural information processing systems*, 31.
- [George and Pearce, 2012] George, D. N. and Pearce, J. M. (2012). A configural theory of attention and associative learning. *Learning & Behavior*, 40(3):241–254.
- [Gerfen and Surmeier, 2011] Gerfen, C. R. and Surmeier, D. J. (2011). Modulation of striatal projection systems by dopamine. *Annual review of neuroscience*, 34(1):441–466.

- [Gerhard et al., 2017] Gerhard, S., Andrade, I., Fetter, R. D., Cardona, A., and Schneider-Mizell, C. M. (2017). Conserved neural circuit structure across drosophila larval development revealed by comparative connectomics. *Elife*, 6:e29089.
- [Gervasi et al., 2010] Gervasi, N., Tchénio, P., and Preat, T. (2010). Pka dynamics in a drosophila learning center: coincidence detection by rutabaga adenylyl cyclase and spatial regulation by dunce phosphodiesterase. *Neuron*, 65(4):516–529.
- [Gkaniyas et al., 2022] Gkaniyas, E., McCurdy, L. Y., Nitabach, M. N., and Webb, B. (2022). An incentive circuit for memory dynamics in the mushroom body of drosophila melanogaster. *Elife*, 11:e75611.
- [Gomez-Diaz et al., 2018] Gomez-Diaz, C., Martin, F., Garcia-Fernandez, J. M., and Alcorta, E. (2018). The two main olfactory receptor families in drosophila, ors and irs: A comparative approach. *Frontiers in Cellular Neuroscience*, 12:253.
- [Goriely, 2025] Goriely, A. (2025). Eighty-six billion and counting: do we know the number of neurons in the human brain? *Brain*, 148(3):689–691.
- [Gotzes et al., 1994] Gotzes, F., Balfanz, S., and Baumann, A. (1994). Primary structure and functional characterization of a drosophila dopamine receptor with high homology to human d1/5 receptors. *Receptors & channels*, 2(2):131–141.
- [Grabe et al., 2016] Grabe, V., Baschwitz, A., Dweck, H. K., Lavista-Llanos, S., Hansson, B. S., and Sachse, S. (2016). Elucidating the neuronal architecture of olfactory glomeruli in the drosophila antennal lobe. *Cell reports*, 16(12):3401–3413.
- [Greer et al., 2005] Greer, C. L., Grygoruk, A., Patton, D. E., Ley, B., Romero-Calderon, R., Chang, H.-Y., Houshyar, R., Bainton, R. J., Diantonio, A., and Krantz, D. E. (2005). A splice variant of the drosophila vesicular monoamine transporter contains a conserved trafficking domain and functions in the storage of dopamine, serotonin, and octopamine. *Journal of neurobiology*, 64(3):239–258.
- [Groh et al., 2006] Groh, C., Ahrens, D., and Rössler, W. (2006). Environment-and age-dependent plasticity of synaptic complexes in the mushroom bodies of honeybee queens. *Brain Behavior and Evolution*, 68(1):1–14.
- [Groh and Rössler, 2011] Groh, C. and Rössler, W. (2011). Comparison of microglomerular structures in the mushroom body calyx of neopteran insects. *Arthropod structure & development*, 40(4):358–367.
- [Groschner and Miesenböck, 2019] Groschner, L. N. and Miesenböck, G. (2019). Mechanisms of sensory discrimination: insights from drosophila olfaction. *Annual Review of Biophysics*, 48(1):209–229.
- [Gruber et al., 2013] Gruber, F., Knapek, S., Fujita, M., Matsuo, K., Bräcker, L., Shinzato, N., Siwanowicz, I., Tanimura, T., and Tanimoto, H. (2013). Suppression of conditioned odor approach by feeding is independent of taste and nutritional value in drosophila. *Current Biology*, 23(6):507–514.
- [Gruntman and Turner, 2013] Gruntman, E. and Turner, G. C. (2013). Integration of the olfactory code across dendritic claws of single mushroom body neurons. *Nature neuroscience*, 16(12):1821–1829.
- [Guan et al., 2016] Guan, J.-S., Jiang, J., Xie, H., and Liu, K.-Y. (2016). How does the sparse memory “engram” neurons encode the memory of a spatial-temporal event? *Frontiers in neural circuits*, 10:61.
- [Gupta and Stopfer, 2014] Gupta, N. and Stopfer, M. (2014). A temporal channel for information in sparse sensory coding. *Current Biology*, 24(19):2247–2256.
- [Gupta et al., 2016] Gupta, V. K., Pech, U., Bhukel, A., Fulterer, A., Ender, A., Mauermann, S. F., Andlauer, T. F., Antwi-Adjei, E., Beuschel, C., Thriene, K., et al. (2016). Spermidine suppresses age-associated memory impairment by preventing adverse increase of presynaptic active zone size and release. *PLoS biology*, 14(9):e1002563.
- [Hafner et al., 2004] Hafner, V. V., Fend, M., König, P., Körding, K. P., and Paul, K. (2004). Predicting properties of the rat somatosensory system by sparse coding. *Neural information processing letters and reviews*, 4(1):11–18.

-
- [Hallem and Carlson, 2006] Hallem, E. A. and Carlson, J. R. (2006). Coding of odors by a receptor repertoire. *Cell*, 125(1):143–160.
- [Hallem et al., 2004] Hallem, E. A., Ho, M. G., and Carlson, J. R. (2004). The molecular basis of odor coding in the drosophila antenna. *Cell*, 117(7):965–979.
- [Hamada et al., 2008] Hamada, F. N., Rosenzweig, M., Kang, K., Pulver, S. R., Ghezzi, A., Jegla, T. J., and Garrity, P. A. (2008). An internal thermal sensor controlling temperature preference in drosophila. *Nature*, 454(7201):217–220.
- [Han et al., 1996] Han, K.-A., Millar, N. S., Grotewiel, M. S., and Davis, R. L. (1996). Damb, a novel dopamine receptor expressed specifically in drosophila mushroom bodies. *Neuron*, 16(6):1127–1135.
- [Handler et al., 2019] Handler, A., Graham, T. G., Cohn, R., Morante, I., Siliciano, A. F., Zeng, J., Li, Y., and Ruta, V. (2019). Distinct dopamine receptor pathways underlie the temporal sensitivity of associative learning. *Cell*, 178(1):60–75.
- [Hanlon and Andrew, 2015] Hanlon, C. D. and Andrew, D. J. (2015). Outside-in signaling—a brief review of gpcr signaling with a focus on the drosophila gpcr family. *Journal of cell science*, 128(19):3533–3542.
- [Harris et al., 2008] Harris, J. A., Livesey, E. J., Gharaei, S., and Westbrook, R. F. (2008). Negative patterning is easier than a biconditional discrimination. *Journal of Experimental Psychology: Animal Behavior Processes*, 34(4):494.
- [Hawkins and Byrne, 2015] Hawkins, R. D. and Byrne, J. H. (2015). Associative learning in invertebrates. *Cold Spring Harbor Perspectives in Biology*, 7(5):a021709.
- [Hearn et al., 2002] Hearn, M. G., Ren, Y., McBride, E. W., Reveillaud, I., Beinborn, M., and Kopin, A. S. (2002). A drosophila dopamine 2-like receptor: Molecular characterization and identification of multiple alternatively spliced variants. *Proceedings of the National Academy of Sciences*, 99(22):14554–14559.
- [Heekeren et al., 2008] Heekeren, H. R., Marrett, S., and Ungerleider, L. G. (2008). The neural systems that mediate human perceptual decision making. *Nature reviews neuroscience*, 9(6):467–479.
- [Heinrich et al., 2018] Heinrich, L., Funke, J., Pape, C., Nunez-Iglesias, J., and Saalfeld, S. (2018). Synaptic cleft segmentation in non-isotropic volume electron microscopy of the complete drosophila brain. In *International Conference on Medical Image Computing and Computer-Assisted Intervention*, pages 317–325. Springer.
- [Heisenberg et al., 1985] Heisenberg, M., Borst, A., Wagner, S., and Byers, D. (1985). Drosophila mushroom body mutants are deficient in olfactory learning. *Journal of neurogenetics*, 2(1):1–30.
- [Helmchen and Denk, 2005] Helmchen, F. and Denk, W. (2005). Deep tissue two-photon microscopy. *Nature methods*, 2(12):932–940.
- [Helmstaedter et al., 2013] Helmstaedter, M., Briggman, K. L., Turaga, S. C., Jain, V., Seung, H. S., and Denk, W. (2013). Connectomic reconstruction of the inner plexiform layer in the mouse retina. *Nature*, 500(7461):168–174.
- [Hige et al., 2015a] Hige, T., Aso, Y., Modi, M. N., Rubin, G. M., and Turner, G. C. (2015a). Heterosynaptic plasticity underlies aversive olfactory learning in drosophila. *Neuron*, 88(5):985–998.
- [Hige et al., 2015b] Hige, T., Aso, Y., Rubin, G. M., and Turner, G. C. (2015b). Plasticity-driven individualization of olfactory coding in mushroom body output neurons. *Nature*, 526(7572):258–262.
- [Hilgard and Marquis, 1961] Hilgard, E. R. and Marquis, D. G. (1961). Generalization.
- [Himmelreich et al., 2017] Himmelreich, S., Masuho, I., Berry, J. A., MacMullen, C., Skamangas, N. K., Martemyanov, K. A., and Davis, R. L. (2017). Dopamine receptor damb signals via gq to mediate forgetting in drosophila. *Cell reports*, 21(8):2074–2081.
- [Hiramatsu et al., 2025] Hiramatsu, S., Saito, K., Kondo, S., Katow, H., Yamagata, N., Wu, C.-F., and Tanimoto, H. (2025). Synaptic enrichment and dynamic regulation of the two opposing dopamine receptors within the same neurons. *ELife*, 13:RP98358.
- [Holler et al., 2021] Holler, S., Köstinger, G., Martin, K. A., Schuhknecht, G. F., and Stratford, K. J. (2021). Structure and function of a neocortical synapse. *Nature*, 591(7848):111–116.
-

- [Honegger et al., 2011] Honegger, K. S., Campbell, R. A., and Turner, G. C. (2011). Cellular-resolution population imaging reveals robust sparse coding in the drosophila mushroom body. *Journal of neuroscience*, 31(33):11772–11785.
- [Horiuchi et al., 2008] Horiuchi, J., Yamazaki, D., Naganos, S., Aigaki, T., and Saitoe, M. (2008). Protein kinase a inhibits a consolidated form of memory in drosophila. *Proceedings of the National Academy of Sciences*, 105(52):20976–20981.
- [Horne et al., 2018] Horne, J. A., Langille, C., McLin, S., Wiederman, M., Lu, Z., Xu, C. S., Plaza, S. M., Scheffer, L. K., Hess, H. F., and Meinertzhagen, I. A. (2018). A resource for the drosophila antennal lobe provided by the connectome of glomerulus valv. *Elife*, 7:e37550.
- [Hourcade et al., 2010] Hourcade, B., Muenz, T. S., Sandoz, J.-C., Rössler, W., and Devaud, J.-M. (2010). Long-term memory leads to synaptic reorganization in the mushroom bodies: a memory trace in the insect brain? *Journal of Neuroscience*, 30(18):6461–6465.
- [Hromádka et al., 2008] Hromádka, T., DeWeese, M. R., and Zador, A. M. (2008). Sparse representation of sounds in the unanesthetized auditory cortex. *PLoS biology*, 6(1):e16.
- [Huang et al., 2018] Huang, G. B., Scheffer, L. K., and Plaza, S. M. (2018). Fully-automatic synapse prediction and validation on a large data set. *Frontiers in neural circuits*, 12:87.
- [Huang et al., 2010] Huang, J., Zhang, W., Qiao, W., Hu, A., and Wang, Z. (2010). Functional connectivity and selective odor responses of excitatory local interneurons in drosophila antennal lobe. *Neuron*, 67(6):1021–1033.
- [Huoviala et al., 2018] Huoviala, P., Dolan, M.-J., Love, F. M., Myers, P., Frechter, S., Namiki, S., Pettersson, L., Roberts, R. J., Turnbull, R., Mitrevica, Z., et al. (2018). Neural circuit basis of aversive odour processing in drosophila from sensory input to descending output. *BioRxiv*, page 394403.
- [Hussain et al., 2016] Hussain, A., Zhang, M., Üçpınar, H. K., Svensson, T., Quillery, E., Gompel, N., Ignell, R., and Grunwald Kadow, I. C. (2016). Ionotropic chemosensory receptors mediate the taste and smell of polyamines. *PLoS biology*, 14(5):e1002454.
- [Iliadi and Boulianne, 2010] Iliadi, K. G. and Boulianne, G. L. (2010). Age-related behavioral changes in drosophila. *Annals of the new York Academy of Sciences*, 1197(1):9–18.
- [Inada et al., 2017] Inada, K., Tsuchimoto, Y., and Kazama, H. (2017). Origins of cell-type-specific olfactory processing in the drosophila mushroom body circuit. *Neuron*, 95(2):357–367.
- [Inagaki et al., 2012] Inagaki, H. K., De-Leon, S. B.-T., Wong, A. M., Jagadish, S., Ishimoto, H., Barnea, G., Kitamoto, T., Axel, R., and Anderson, D. J. (2012). Visualizing neuromodulation in vivo: Tango-mapping of dopamine signaling reveals appetite control of sugar sensing. *Cell*, 148(3):583–595.
- [Ishimoto et al., 2013] Ishimoto, H., Wang, Z., Rao, Y., Wu, C.-F., and Kitamoto, T. (2013). A novel role for ecdysone in drosophila conditioned behavior: linking gpcr-mediated non-canonical steroid action to camp signaling in the adult brain. *PLoS genetics*, 9(10):e1003843.
- [Ito et al., 1998] Ito, K., Suzuki, K., Estes, P., Ramaswami, M., Yamamoto, D., and Strausfeld, N. J. (1998). The organization of extrinsic neurons and their implications in the functional roles of the mushroom bodies in drosophila melanogaster meigen. *Learning & memory*, 5(1):52–77.
- [Jacob and Waddell, 2020] Jacob, P. F. and Waddell, S. (2020). Spaced training forms complementary long-term memories of opposite valence in drosophila. *Neuron*, 106(6):977–991.
- [Jafari and Alenius, 2021] Jafari, S. and Alenius, M. (2021). Odor response adaptation in drosophila—a continuous individualization process. *Cell and Tissue Research*, 383(1):143–148.
- [Jefferis et al., 2007] Jefferis, G. S., Potter, C. J., Chan, A. M., Marin, E. C., Rohlffing, T., Maurer, C. R., and Luo, L. (2007). Comprehensive maps of drosophila higher olfactory centers: spatially segregated fruit and pheromone representation. *Cell*, 128(6):1187–1203.
- [Jenett et al., 2012] Jenett, A., Rubin, G. M., Ngo, T.-T., Shepherd, D., Murphy, C., Dionne, H., Pfeiffer, B. D., Cavallaro, A., Hall, D., Jeter, J., et al. (2012). A gal4-driver line resource for drosophila neurobiology. *Cell reports*, 2(4):991–1001.

- [Johard et al., 2008] Johard, H. A., Enell, L. E., Gustafsson, E., Trifilieff, P., Veenstra, J. A., and Nässel, D. R. (2008). Intrinsic neurons of drosophila mushroom bodies express short neuropeptide f: relations to extrinsic neurons expressing different neurotransmitters. *Journal of Comparative Neurology*, 507(4):1479–1496.
- [Jortner et al., 2007] Jortner, R. A., Farivar, S. S., and Laurent, G. (2007). A simple connectivity scheme for sparse coding in an olfactory system. *Journal of Neuroscience*, 27(7):1659–1669.
- [Kadia and Wang, 2003] Kadia, S. C. and Wang, X. (2003). Spectral integration in a1 of awake primates: neurons with single-and multi-peaked tuning characteristics. *Journal of neurophysiology*, 89(3):1603–1622.
- [Kaldun et al., 2025] Kaldun, J. C., Calia, E., Mukunda, G. C. B., Fritsch, C., Komarov, N., and Sprecher, S. G. (2025). A temporally restricted function of the dopamine receptor dop1r2 during memory formation. *Elife*, 13:RP99368.
- [Kang and Kanold, 2024] Kang, H. and Kanold, P. O. (2024). Sparse representation of neurons for encoding complex sounds in the auditory cortex. *Progress in Neurobiology*, 241:102661.
- [Karpova et al., 2011] Karpova, E., Gruntenko, N., Shumnaya, L., Romanova, I., and Rauschenbach, I. Y. (2011). The role of d2-like receptors in the regulation of juvenile hormone metabolism in drosophila females in normal state and at increased dopamine level. In *Doklady. Biochemistry and Biophysics*, volume 439, page 155. Springer Nature BV.
- [Karpova et al., 2010] Karpova, E., Rauschenbach, I. Y., Shchumnaya, L., and Gruntenko, N. (2010). Activation of d2-like dopamine receptors inhibits young hormone degradation in female drosophila. In *Doklady Biological Sciences*, volume 432, page 187. Springer Nature BV.
- [Kato et al., 2023] Kato, A., Ohta, K., Okanoya, K., and Kazama, H. (2023). Dopaminergic neurons dynamically update sensory values during olfactory maneuver. *Cell reports*, 42(10).
- [Kazama and Wilson, 2009] Kazama, H. and Wilson, R. I. (2009). Origins of correlated activity in an olfactory circuit. *Nature neuroscience*, 12(9):1136–1144.
- [Keegan et al., 1986] Keegan, L., Gill, G., and Ptashne, M. (1986). Separation of dna binding from the transcription-activating function of a eukaryotic regulatory protein. *Science*, 231(4739):699–704.
- [Keene et al., 2006] Keene, A. C., Krashes, M. J., Leung, B., Bernard, J. A., and Waddell, S. (2006). Drosophila dorsal paired medial neurons provide a general mechanism for memory consolidation. *Current Biology*, 16(15):1524–1530.
- [Keene et al., 2004] Keene, A. C., Stratmann, M., Keller, A., Perrat, P. N., Vosshall, L. B., and Waddell, S. (2004). Diverse odor-conditioned memories require uniquely timed dorsal paired medial neuron output. *Neuron*, 44(3):521–533.
- [Kehoe and Graham, 1988] Kehoe, E. J. and Graham, P. (1988). Summation and configuration: Stimulus compounding and negative patterning in the rabbit. *Journal of Experimental Psychology: Animal Behavior Processes*, 14(3):320.
- [Keleman et al., 2012] Keleman, K., Vrontou, E., Krüttner, S., Yu, J. Y., Kurtovic-Kozaric, A., and Dickson, B. J. (2012). Dopamine neurons modulate pheromone responses in drosophila courtship learning. *Nature*, 489(7414):145–149.
- [Kevan et al., 2001] Kevan, P. G., Chittka, L., and Dyer, A. G. (2001). Limits to the salience of ultra-violet: lessons from colour vision in bees and birds. *Journal of Experimental Biology*, 204(14):2571–2580.
- [Kim et al., 2016] Kim, H., Choi, M. S., Kang, K., and Kwon, J. Y. (2016). Behavioral analysis of bitter taste perception in drosophila larvae. *Chemical senses*, 41(1):85–94.
- [Kim et al., 2014] Kim, J. S., Greene, M. J., Zlateski, A., Lee, K., Richardson, M., Turaga, S. C., Purcaro, M., Balkam, M., Robinson, A., Behabadi, B. F., et al. (2014). Space-time wiring specificity supports direction selectivity in the retina. *Nature*, 509(7500):331–336.
- [Kim et al., 2007] Kim, Y.-C., Lee, H.-G., and Han, K.-A. (2007). D1 dopamine receptor *dda1* is required in the mushroom body neurons for aversive and appetitive learning in drosophila. *Journal of Neuroscience*, 27(29):7640–7647.
- [Kim et al., 2003] Kim, Y.-C., Lee, H.-G., Seong, C.-S., and Han, K.-A. (2003). Expression of a d1 dopamine receptor *dda1/dmdop1* in the central nervous system of drosophila melanogaster. *Gene Expression Patterns*, 3(2):237–245.

- [Kirkhart and Scott, 2015] Kirkhart, C. and Scott, K. (2015). Gustatory learning and processing in the drosophila mushroom bodies. *Journal of neuroscience*, 35(15):5950–5958.
- [Knecht et al., 2017] Knecht, Z. A., Silbering, A. F., Cruz, J., Yang, L., Croset, V., Benton, R., and Garrity, P. A. (2017). Ionotropic receptor-dependent moist and dry cells control hygrosensation in drosophila. *Elife*, 6:e26654.
- [Knecht et al., 2016] Knecht, Z. A., Silbering, A. F., Ni, L., Klein, M., Budelli, G., Bell, R., Abuin, L., Ferrer, A. J., Samuel, A. D., Benton, R., et al. (2016). Distinct combinations of variant ionotropic glutamate receptors mediate thermosensation and hygrosensation in drosophila. *Elife*, 5:e17879.
- [Ko et al., 2015] Ko, K. I., Root, C. M., Lindsay, S. A., Zaninovich, O. A., Shepherd, A. K., Wasserman, S. A., Kim, S. M., and Wang, J. W. (2015). Starvation promotes concerted modulation of appetitive olfactory behavior via parallel neuromodulatory circuits. *Elife*, 4:e08298.
- [Kohl et al., 2013] Kohl, J., Ostrovsky, A. D., Frechter, S., and Jefferis, G. S. (2013). A bidirectional circuit switch reroutes pheromone signals in male and female brains. *Cell*, 155(7):1610–1623.
- [Kohler, 1994] Kohler, R. E. (1994). *Lords of the fly: Drosophila genetics and the experimental life*. University of Chicago Press.
- [Kondo et al., 2020] Kondo, S., Takahashi, T., Yamagata, N., Imanishi, Y., Katow, H., Hiramatsu, S., Lynn, K., Abe, A., Kumaraswamy, A., and Tanimoto, H. (2020). Neurochemical organization of the drosophila brain visualized by endogenously tagged neurotransmitter receptors. *Cell Reports*, 30(1):284–297.
- [König and Gerber, 2022] König, C. and Gerber, B. (2022). Age-related decrease in appetitive associative memory in fruit flies. *Journal of Experimental Biology*, 225(21):jeb244915.
- [Konorski, 1948] Konorski, J. (1948). *Conditioned reflexes and neuron organization*. CUP Archive.
- [Kramer and Staveley, 2003] Kramer, J. M. and Staveley, B. E. (2003). Gal4 causes developmental defects and apoptosis when expressed in the developing eye of drosophila melanogaster. *Genet Mol Res*, 2(1):43–47.
- [Krashes et al., 2009] Krashes, M. J., DasGupta, S., Vreede, A., White, B., Armstrong, J. D., and Waddell, S. (2009). A neural circuit mechanism integrating motivational state with memory expression in drosophila. *Cell*, 139(2):416–427.
- [Krashes et al., 2007] Krashes, M. J., Keene, A. C., Leung, B., Armstrong, J. D., and Waddell, S. (2007). Sequential use of mushroom body neuron subsets during drosophila odor memory processing. *Neuron*, 53(1):103–115.
- [Kremer et al., 2010] Kremer, M. C., Christiansen, F., Leiss, F., Paehler, M., Knapek, S., Andlauer, T. F., Förstner, F., Kloppenburg, P., Sigrist, S. J., and Tavosanis, G. (2010). Structural long-term changes at mushroom body input synapses. *Current Biology*, 20(21):1938–1944.
- [Kuo et al., 2015] Kuo, S.-Y., Wu, C.-L., Hsieh, M.-Y., Lin, C.-T., Wen, R.-K., Chen, L.-C., Chen, Y.-H., Yu, Y.-W., Wang, H.-D., Su, Y.-J., et al. (2015). Ppl2ab neurons restore sexual responses in aged drosophila males through dopamine. *Nature Communications*, 6(1):7490.
- [Kurahashi and Menini, 1997] Kurahashi, T. and Menini, A. (1997). Mechanism of odorant adaptation in the olfactory receptor cell. *Nature*, 385(6618):725–729.
- [Kurtovic et al., 2007] Kurtovic, A., Widmer, A., and Dickson, B. J. (2007). A single class of olfactory neurons mediates behavioural responses to a drosophila sex pheromone. *Nature*, 446(7135):542–546.
- [Lai et al., 2008] Lai, S.-L., Awasaki, T., Ito, K., and Lee, T. (2008). Clonal analysis of drosophila antennal lobe neurons: diverse neuronal architectures in the lateral neuroblast lineage. *Biologists*.
- [Lai and Lee, 2006] Lai, S.-L. and Lee, T. (2006). Genetic mosaic with dual binary transcriptional systems in drosophila. *Nature neuroscience*, 9(5):703–709.
- [Laissue et al., 1999] Laissue, P., Reiter, C., Hiesinger, P., Halter, S., Fischbach, K., and Stocker, R. (1999). Three-dimensional reconstruction of the antennal lobe in drosophila melanogaster. *Journal of Comparative Neurology*, 405(4):543–552.
- [Landayan et al., 2018] Landayan, D., Feldman, D. S., and Wolf, F. W. (2018). Satiation state-dependent dopaminergic control of foraging in drosophila. *Scientific reports*, 8(1):5777.

-
- [Larderet et al., 2017] Larderet, I., Fritsch, P. M., Gendre, N., Neagu-Maier, G. L., Fetter, R. D., Schneider-Mizell, C. M., Truman, J. W., Zlatić, M., Cardona, A., and Sprecher, S. G. (2017). Organization of the drosophila larval visual circuit. *Elife*, 6:e28387.
- [Lark et al., 2017] Lark, A., Kitamoto, T., and Martin, J.-R. (2017). Modulation of neuronal activity in the drosophila mushroom body by dopecr, a unique dual receptor for ecdysone and dopamine. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1864(10):1578–1588.
- [Lashley and Wade, 1946] Lashley, K. S. and Wade, M. (1946). The pavlovian theory of generalization. *Psychological Review*, 53(2):72.
- [Laufer et al., 2016] Laufer, O., Israeli, D., and Paz, R. (2016). Behavioral and neural mechanisms of overgeneralization in anxiety. *Current Biology*, 26(6):713–722.
- [Lebestky et al., 2009] Lebestky, T., Chang, J.-S. C., Dankert, H., Zelnik, L., Kim, Y.-C., Han, K.-A., Wolf, F. W., Perona, P., and Anderson, D. J. (2009). Two different forms of arousal in drosophila are oppositely regulated by the dopamine d1 receptor ortholog dopr via distinct neural circuits. *Neuron*, 64(4):522–536.
- [Lee et al., 2017] Lee, M. J., Sung, H. Y., Jo, H., Kim, H.-W., Choi, M. S., Kwon, J. Y., and Kang, K. (2017). Ionotropic receptor 76b is required for gustatory aversion to excessive na⁺ in drosophila. *Molecules and cells*, 40(10):787–795.
- [Lee et al., 2025] Lee, P.-S., Chiang, M.-H., Wu, T., and Wu, C.-L. (2025). Systematic analysis of mushroom body-innervating dopaminergic neuron activity in different physiological states in drosophila. *Biomedical Journal*, page 100907.
- [Lee et al., 2011] Lee, P.-T., Lin, H.-W., Chang, Y.-H., Fu, T.-F., Dubnau, J., Hirsh, J., Lee, T., and Chiang, A.-S. (2011). Serotonin–mushroom body circuit modulating the formation of anesthesia-resistant memory in drosophila. *Proceedings of the National Academy of Sciences*, 108(33):13794–13799.
- [Lee et al., 1999] Lee, T., Lee, A., and Luo, L. (1999). Development of the drosophila mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. *Development*, 126(18):4065–4076.
- [Lee and Luo, 1999] Lee, T. and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron*, 22(3):451–461.
- [Lee et al., 2021] Lee, W.-P., Chiang, M.-H., Chang, L.-Y., Shyu, W.-H., Chiu, T.-H., Fu, T.-F., Wu, T., and Wu, C.-L. (2021). Serotonin signals modulate mushroom body output neurons for sustaining water-reward long-term memory in drosophila. *Frontiers in Cell and Developmental Biology*, 9:755574.
- [Lei et al., 2013] Lei, Z., Chen, K., Li, H., Liu, H., and Guo, A. (2013). The gaba system regulates the sparse coding of odors in the mushroom bodies of drosophila. *Biochemical and Biophysical Research Communications*, 436(1):35–40.
- [Leiss et al., 2009] Leiss, F., Groh, C., Butcher, N. J., Meinertzhagen, I. A., and Tavosanis, G. (2009). Synaptic organization in the adult drosophila mushroom body calyx. *Journal of Comparative Neurology*, 517(6):808–824.
- [Lerner et al., 2020] Lerner, H., Rozenfeld, E., Rozenman, B., Huetteroth, W., and Parnas, M. (2020). Differential role for a defined lateral horn neuron subset in naïve odor valence in drosophila. *Scientific reports*, 10(1):6147.
- [Lerner et al., 2015] Lerner, T. N., Shilyansky, C., Davidson, T. J., Evans, K. E., Beier, K. T., Zalcusky, K. A., Crow, A. K., Malenka, R. C., Luo, L., Tomer, R., et al. (2015). Intact-brain analyses reveal distinct information carried by snc dopamine subcircuits. *Cell*, 162(3):635–647.
- [Lewis et al., 2015] Lewis, L. P., Siju, K., Aso, Y., Friedrich, A. B., Bulteel, A. J., Rubin, G. M., and Kadow, I. C. G. (2015). A higher brain circuit for immediate integration of conflicting sensory information in drosophila. *Current biology*, 25(17):2203–2214.
- [Li et al., 2020a] Li, F., Lindsey, J. W., Marin, E. C., Otto, N., Dreher, M., Dempsey, G., Stark, I., Bates, A. S., Pleijzier, M. W., Schlegel, P., et al. (2020a). The connectome of the adult drosophila mushroom body provides insights into function. *Elife*, 9:e62576.
- [Li et al., 2020b] Li, J., Mahoney, B. D., Jacob, M. S., and Caron, S. J. C. (2020b). Visual input into the drosophila melanogaster mushroom body. *Cell reports*, 32(11).
-

- [Li et al., 2022] Li, S., Li, B., Gao, L., Wang, J., and Yan, Z. (2022). Humidity response in drosophila olfactory sensory neurons requires the mechanosensitive channel *tmem63*. *Nature communications*, 13(1):3814.
- [Liang et al., 2019] Liang, F., Li, H., Chou, X.-l., Zhou, M., Zhang, N. K., Xiao, Z., Zhang, K. K., Tao, H. W., and Zhang, L. I. (2019). Sparse representation in awake auditory cortex: cell-type dependence, synaptic mechanisms, developmental emergence, and modulation. *Cerebral Cortex*, 29(9):3796–3812.
- [Liang et al., 2013] Liang, L., Li, Y., Potter, C. J., Yizhar, O., Deisseroth, K., Tsien, R. W., and Luo, L. (2013). Gabaergic projection neurons route selective olfactory inputs to specific higher-order neurons. *Neuron*, 79(5):917–931.
- [Limmer et al., 2014] Limmer, S., Weiler, A., Volkenhoff, A., Babatz, F., and Klämbt, C. (2014). The drosophila blood-brain barrier: development and function of a glial endothelium. *Frontiers in neuroscience*, 8:365.
- [Lin et al., 2014] Lin, A. C., Bygrave, A. M., De Calignon, A., Lee, T., and Miesenböck, G. (2014). Sparse, decorrelated odor coding in the mushroom body enhances learned odor discrimination. *Nature neuroscience*, 17(4):559–568.
- [Lin, 2023] Lin, S. (2023). Internal-state-dependent modulation of olfactory responses: a tale of dopamine neurons in the adult drosophila mushroom body. *Current Opinion in Insect Science*, 59:101104.
- [Lin et al., 2013] Lin, S., Marin, E. C., Yang, C.-P., Kao, C.-F., Apenteng, B. A., Huang, Y., O’Connor, M. B., Truman, J. W., and Lee, T. (2013). Extremes of lineage plasticity in the drosophila brain. *Current Biology*, 23(19):1908–1913.
- [Lissek and Grillon, 2015] Lissek, S. and Grillon, C. (2015). Overgeneralization of conditioned fear in the anxiety disorders. *Zeitschrift für Psychologie/journal of Psychology*.
- [Lissek et al., 2010] Lissek, S., Rabin, S., Heller, R. E., Lukenbaugh, D., Geraci, M., Pine, D. S., and Grillon, C. (2010). Overgeneralization of conditioned fear as a pathogenic marker of panic disorder. *American Journal of Psychiatry*, 167(1):47–55.
- [Litwin-Kumar et al., 2017] Litwin-Kumar, A., Harris, K. D., Axel, R., Sompolinsky, H., and Abbott, L. (2017). Optimal degrees of synaptic connectivity. *Neuron*, 93(5):1153–1164.
- [Liu et al., 2012a] Liu, C., Plaçais, P.-Y., Yamagata, N., Pfeiffer, B. D., Aso, Y., Friedrich, A. B., Siwanowicz, I., Rubin, G. M., Preat, T., and Tanimoto, H. (2012a). A subset of dopamine neurons signals reward for odour memory in drosophila. *Nature*, 488(7412):512–516.
- [Liu et al., 1999] Liu, L., Wolf, R., Ernst, R., and Heisenberg, M. (1999). Context generalization in drosophila visual learning requires the mushroom bodies. *Nature*, 400(6746):753–756.
- [Liu et al., 2012b] Liu, Q., Liu, S., Kodama, L., Driscoll, M. R., and Wu, M. N. (2012b). Two dopaminergic neurons signal to the dorsal fan-shaped body to promote wakefulness in drosophila. *Current Biology*, 22(22):2114–2123.
- [Liu et al., 2016] Liu, Q., Yang, X., Tian, J., Gao, Z., Wang, M., Li, Y., and Guo, A. (2016). Gap junction networks in mushroom bodies participate in visual learning and memory in drosophila. *Elife*, 5:e13238.
- [Liu and Davis, 2009] Liu, X. and Davis, R. L. (2009). The gabaergic anterior paired lateral neuron suppresses and is suppressed by olfactory learning. *Nature neuroscience*, 12(1):53–59.
- [Livesey et al., 2011] Livesey, E. J., Thorwart, A., and Harris, J. A. (2011). Comparing positive and negative patterning in human learning. *Quarterly Journal of Experimental Psychology*, 64(12):2316–2333.
- [Ljungberg et al., 1992] Ljungberg, T., Apicella, P., and Schultz, W. (1992). Responses of monkey dopamine neurons during learning of behavioral reactions. *Journal of neurophysiology*, 67(1):145–163.
- [Love et al., 2023] Love, C. R., Gautam, S., Lama, C., Le, N. H., and Dauwalder, B. (2023). The drosophila dopamine 2-like receptor *d2r* (*dop2r*) is required in the blood brain barrier for male courtship. *Genes, Brain and Behavior*, 22(1):e12836.

-
- [Luan et al., 2006] Luan, H., Peabody, N. C., Vinson, C. R., and White, B. H. (2006). Refined spatial manipulation of neuronal function by combinatorial restriction of transgene expression. *Neuron*, 52(3):425–436.
- [Lubbock, 1882] Lubbock, J. (1882). On the sense of color among some of the lower animals. *Zoological Journal of the Linnean Society*, 16(90):121–127.
- [Luo, 2021] Luo, L. (2021). Architectures of neuronal circuits. *Science*, 373(6559):eabg7285.
- [Luo et al., 2010] Luo, S. X., Axel, R., and Abbott, L. (2010). Generating sparse and selective third-order responses in the olfactory system of the fly. *Proceedings of the National Academy of Sciences*, 107(23):10713–10718.
- [Lyman et al., 2023] Lyman, R. F., Lyman, R. A., Yamamoto, A., Huang, W., Harbison, S. T., Zhou, S., Anholt, R. R., and Mackay, T. F. (2023). Natural genetic variation in a dopamine receptor is associated with variation in female fertility in drosophila melanogaster. *Proceedings of the Royal Society B*, 290(1996):20230375.
- [Ma and Ptashne, 1987] Ma, J. and Ptashne, M. (1987). Deletion analysis of gal4 defines two transcriptional activating segments. *Cell*, 48(5):847–853.
- [Mackintosh et al., 1991] Mackintosh, N., Kaye, H., and Bennett, C. (1991). Perceptual learning in flavour aversion conditioning. *The Quarterly Journal of Experimental Psychology Section B*, 43(3b):297–322.
- [Madalan et al., 2012] Madalan, A., Yang, X., Ferris, J., Zhang, S., and Roman, G. (2012). G (o) activation is required for both appetitive and aversive memory acquisition in drosophila. *Learning & memory*, 19(1):26–34.
- [Malnic et al., 1999] Malnic, B., Hirono, J., Sato, T., and Buck, L. B. (1999). Combinatorial receptor codes for odors. *Cell*, 96(5):713–723.
- [Mansourian et al., 2016] Mansourian, S., Corcoran, J., Enjin, A., Löfstedt, C., Dacke, M., and Stensmyr, M. C. (2016). Fecal-derived phenol induces egg-laying aversion in drosophila. *Current Biology*, 26(20):2762–2769.
- [Mao and Davis, 2009] Mao, Z. and Davis, R. L. (2009). Eight different types of dopaminergic neurons innervate the drosophila mushroom body neuropil: anatomical and physiological heterogeneity. *Frontiers in neural circuits*, page 5.
- [Marin et al., 2020] Marin, E. C., Büld, L., Theiss, M., Sarkissian, T., Roberts, R. J., Turnbull, R., Tamimi, I. F., Pleijzier, M. W., Laursen, W. J., Drummond, N., et al. (2020). Connectomics analysis reveals first-, second-, and third-order thermosensory and hygro-sensory neurons in the adult drosophila brain. *Current Biology*, 30(16):3167–3182.
- [Marin et al., 2002] Marin, E. C., Jefferis, G. S., Komiyama, T., Zhu, H., and Luo, L. (2002). Representation of the glomerular olfactory map in the drosophila brain. *Cell*, 109(2):243–255.
- [Marquis and Wilson, 2022] Marquis, M. and Wilson, R. I. (2022). Locomotor and olfactory responses in dopamine neurons of the drosophila superior-lateral brain. *Current Biology*, 32(24):5406–5414.
- [Marr, 1969] Marr, D. (1969). A theory of cerebellar cortex. *The Journal of physiology*, 202(2):437–470.
- [Martín et al., 2001] Martín, F., Charro, M., and Alcorta, E. (2001). Mutations affecting the camp transduction pathway modify olfaction in drosophila. *Journal of Comparative Physiology A*, 187(5):359–370.
- [Marzola et al., 2023] Marzola, P., Melzer, T., Pavesi, E., Gil-Mohapel, J., and Brocardo, P. S. (2023). Exploring the role of neuroplasticity in development, aging, and neurodegeneration. *Brain sciences*, 13(12):1610.
- [Masek and Keene, 2016] Masek, P. and Keene, A. C. (2016). Gustatory processing and taste memory in drosophila. *Journal of neurogenetics*, 30(2):112–121.
- [Masse et al., 2009] Masse, N. Y., Turner, G. C., and Jefferis, G. S. (2009). Olfactory information processing in drosophila. *Current Biology*, 19(16):R700–R713.
- [Masuda-Nakagawa et al., 2014] Masuda-Nakagawa, L. M., Ito, K., Awasaki, T., and O’Kane, C. J. (2014). A single gabaergic neuron mediates feedback of odor-evoked signals in the mushroom body of larval drosophila. *Frontiers in Neural Circuits*, 8:35.
-

- [Masuyama et al., 2012] Masuyama, K., Zhang, Y., Rao, Y., and Wang, J. W. (2012). Mapping neural circuits with activity-dependent nuclear import of a transcription factor. *Journal of neurogenetics*, 26(1):89–102.
- [Matsliah et al., 2023] Matsliah, A., Sterling, A., Dorkenwald, S., Kuehner, K., Morey, R., Seung, H., and Murthy, M. (2023). Codex: connectome data explorer. *Preprint at [https://doi.org/10.13140/RG.2\(35928.67844\)](https://doi.org/10.13140/RG.2(35928.67844))*.
- [Matsumoto et al., 2016] Matsumoto, H., Tian, J., Uchida, N., and Watabe-Uchida, M. (2016). Mid-brain dopamine neurons signal aversion in a reward-context-dependent manner. *Elife*, 5:e17328.
- [Matteucci et al., 2022] Matteucci, G., Guyoton, M., Mayrhofer, J. M., Auffret, M., Foustoukos, G., Petersen, C. C., and El-Boustani, S. (2022). Cortical sensory processing across motivational states during goal-directed behavior. *Neuron*, 110(24):4176–4193.
- [McBride et al., 1999] McBride, S. M., Giuliani, G., Choi, C., Krause, P., Correale, D., Watson, K., Baker, G., and Siwicki, K. K. (1999). Mushroom body ablation impairs short-term memory and long-term memory of courtship conditioning in drosophila melanogaster. *Neuron*, 24(4):967–977.
- [McEwen, 2010] McEwen, B. S. (2010). Stress, sex, and neural adaptation to a changing environment: mechanisms of neuronal remodeling. *Annals of the New York Academy of Sciences*, 1204:38–59.
- [McGuire et al., 2001] McGuire, S. E., Le, P. T., and Davis, R. L. (2001). The role of drosophila mushroom body signaling in olfactory memory. *Science*, 293(5533):1330–1333.
- [McGuire et al., 2004] McGuire, S. E., Mao, Z., and Davis, R. L. (2004). Spatiotemporal gene expression targeting with the target and gene-switch systems in drosophila. *Science’s STKE*, 2004(220):pl6–pl6.
- [Meinertzhagen and O’neil, 1991] Meinertzhagen, I. A. and O’neil, S. (1991). Synaptic organization of columnar elements in the lamina of the wild type in drosophila melanogaster. *Journal of comparative neurology*, 305(2):232–263.
- [Meissner et al., 2023] Meissner, G. W., Nern, A., Dorman, Z., DePasquale, G. M., Forster, K., Gibney, T., Hausenfluck, J. H., He, Y., Iyer, N. A., Jeter, J., et al. (2023). A searchable image resource of drosophila gal4 driver expression patterns with single neuron resolution. *Elife*, 12:e80660.
- [Meissner et al., 2025] Meissner, G. W., Vannan, A., Jeter, J., Close, K., DePasquale, G. M., Dorman, Z., Forster, K., Beringer, J. A., Gibney, T., Hausenfluck, J. H., et al. (2025). A split-gal4 driver line resource for drosophila neuron types. *elife*, 13:RP98405.
- [Menda et al., 2011] Menda, G., Bar, H. Y., Arthur, B. J., Rivlin, P. K., Wytenbach, R. A., Strawderman, R. L., and Hoy, R. R. (2011). Classical conditioning through auditory stimuli in drosophila: methods and models. *Journal of Experimental Biology*, 214(17):2864–2870.
- [Menzel, 1993] Menzel, R. (1993). Associative learning in honey bees. *Apidologie*, 24(3):157–168.
- [Meschi et al., 2024] Meschi, E., Duquenoy, L., Otto, N., Dempsey, G., and Waddell, S. (2024). Compensatory enhancement of input maintains aversive dopaminergic reinforcement in hungry drosophila. *Neuron*, 112(14):2315–2332.
- [Min et al., 2013] Min, S., Ai, M., Shin, S. A., and Suh, G. S. (2013). Dedicated olfactory neurons mediating attraction behavior to ammonia and amines in drosophila. *Proceedings of the National Academy of Sciences*, 110(14):E1321–E1329.
- [Miroshnikow et al., 2018] Miroshnikow, A., Schlegel, P., Schoofs, A., Hueckesfeld, S., Li, F., Schneider-Mizell, C. M., Fetter, R. D., Truman, J. W., Cardona, A., and Pankratz, M. J. (2018). Convergence of monosynaptic and polysynaptic sensory paths onto common motor outputs in a drosophila feeding connectome. *Elife*, 7:e40247.
- [Miyashita et al., 2018] Miyashita, T., Kikuchi, E., Horiuchi, J., and Saitoe, M. (2018). Long-term memory engram cells are established by c-fos/creb transcriptional cycling. *Cell Reports*, 25(10):2716–2728.
- [Modi et al., 2023] Modi, M. N., Rajagopalan, A. E., Rouault, H., Aso, Y., and Turner, G. C. (2023). Flexible specificity of memory in drosophila depends on a comparison between choices. *Elife*, 12:e80923.
- [Mohamed et al., 2019] Mohamed, A. A., Retzke, T., Das Chakraborty, S., Fabian, B., Hansson, B. S., Knaden, M., and Sachse, S. (2019). Odor mixtures of opposing valence unveil inter-glomerular crosstalk in the drosophila antennal lobe. *Nature Communications*, 10(1):1201.

-
- [Mohandasan et al., 2020] Mohandasan, R., Iqbal, F. M., Thakare, M., Sridharan, M., and Das, G. (2020). Enhanced olfactory memory performance in trap-design y-mazes allows the study of novel memory phenotypes in drosophila. *bioRxiv*, pages 2020–11.
- [Mombaerts, 1999] Mombaerts, P. (1999). Seven-transmembrane proteins as odorant and chemosensory receptors. *Science*, 286(5440):707–711.
- [Monastirioti et al., 1995] Monastirioti, M., Gorczyca, M., Rapus, J., Eckert, M., White, K., and Budnik, V. (1995). Octopamine immunoreactivity in the fruit fly drosophila melanogaster. *Journal of Comparative Neurology*, 356(2):275–287.
- [Moncrieff, 1956] Moncrieff, R. W. (1956). Olfactory adaptation and odour likeness. *The Journal of physiology*, 133(2):301.
- [Mouritsen and Ritz, 2005] Mouritsen, H. and Ritz, T. (2005). Magnetoreception and its use in bird navigation. *Current opinion in neurobiology*, 15(4):406–414.
- [Murthy et al., 2008] Murthy, M., Fiete, I., and Laurent, G. (2008). Testing odor response stereotypy in the drosophila mushroom body. *Neuron*, 59(6):1009–1023.
- [Musso et al., 2015] Musso, P.-Y., Tchenio, P., and Preat, T. (2015). Delayed dopamine signaling of energy level builds appetitive long-term memory in drosophila. *Cell reports*, 10(7):1023–1031.
- [Nagarajan and Grewal, 2014] Nagarajan, S. and Grewal, S. S. (2014). An investigation of nutrient-dependent mrna translation in drosophila larvae. *Biology open*, 3(11):1020–1031.
- [Nagel and Wilson, 2016] Nagel, K. I. and Wilson, R. I. (2016). Mechanisms underlying population response dynamics in inhibitory interneurons of the drosophila antennal lobe. *Journal of Neuroscience*, 36(15):4325–4338.
- [Ni et al., 2016] Ni, L., Klein, M., Svec, K. V., Budelli, G., Chang, E. C., Ferrer, A. J., Benton, R., Samuel, A. D., and Garrity, P. A. (2016). The ionotropic receptors ir21a and ir25a mediate cool sensing in drosophila. *elife*, 5:e13254.
- [Niznik and Van Tol, 1992] Niznik, H. B. and Van Tol, H. H. (1992). Dopamine receptor genes: new tools for molecular psychiatry. *Journal of psychiatry and neuroscience*, 17(4):158.
- [Ohashi and Sakai, 2018] Ohashi, H. and Sakai, T. (2018). Leucokinin signaling regulates hunger-driven reduction of behavioral responses to noxious heat in drosophila. *Biochemical and biophysical research communications*, 499(2):221–226.
- [Öhman and Lader, 1972] Öhman, A. and Lader, M. (1972). Selective attention and “habituation” of the auditory averaged evoked response in humans. *Physiology & behavior*, 8(1):79–85.
- [Ohyama et al., 2015] Ohyama, T., Schneider-Mizell, C. M., Fetter, R. D., Aleman, J. V., Francoville, R., Rivera-Alba, M., Mensh, B. D., Branson, K. M., Simpson, J. H., Truman, J. W., et al. (2015). A multilevel multimodal circuit enhances action selection in drosophila. *Nature*, 520(7549):633–639.
- [Okada et al., 2009] Okada, R., Awasaki, T., and Ito, K. (2009). Gamma-aminobutyric acid (gaba)-mediated neural connections in the drosophila antennal lobe. *Journal of Comparative Neurology*, 514(1):74–91.
- [O’Keefe and Dostrovsky, 1971] O’Keefe, J. and Dostrovsky, J. (1971). The hippocampus as a spatial map: preliminary evidence from unit activity in the freely-moving rat. *Brain research*.
- [Okray et al., 2023] Okray, Z., Jacob, P. F., Stern, C., Desmond, K., Otto, N., Talbot, C. B., Vargas-Gutierrez, P., and Waddell, S. (2023). Multisensory learning binds neurons into a cross-modal memory engram. *Nature*, 617(7962):777–784.
- [Olsen et al., 2007] Olsen, S. R., Bhandawat, V., and Wilson, R. I. (2007). Excitatory interactions between olfactory processing channels in the drosophila antennal lobe. *Neuron*, 54(1):89–103.
- [Olsen et al., 2010] Olsen, S. R., Bhandawat, V., and Wilson, R. I. (2010). Divisive normalization in olfactory population codes. *Neuron*, 66(2):287–299.
- [Olsen and Wilson, 2008] Olsen, S. R. and Wilson, R. I. (2008). Lateral presynaptic inhibition mediates gain control in an olfactory circuit. *Nature*, 452(7190):956–960.
- [Olshausen and Field, 2004] Olshausen, B. A. and Field, D. J. (2004). Sparse coding of sensory inputs. *Current opinion in neurobiology*, 14(4):481–487.
-

- [Oteiza and Baldwin, 2021] Oteiza, P. and Baldwin, M. W. (2021). Evolution of sensory systems. *Current Opinion in Neurobiology*, 71:52–59.
- [Owald and Waddell, 2015] Oswald, D. and Waddell, S. (2015). Olfactory learning skews mushroom body output pathways to steer behavioral choice in drosophila. *Current opinion in neurobiology*, 35:178–184.
- [Palmateer et al., 2023] Palmateer, C. M., Artikis, C., Brovero, S. G., Friedman, B., Gresham, A., and Arbeitman, M. N. (2023). Single-cell transcriptome profiles of drosophila fruitless-expressing neurons from both sexes. *Elife*, 12:e78511.
- [Papadopoulou et al., 2011] Papadopoulou, M., Cassenaer, S., Nowotny, T., and Laurent, G. (2011). Normalization for sparse encoding of odors by a wide-field interneuron. *Science*, 332(6030):721–725.
- [Parnas and Avishai, 2025] Parnas, M. and Avishai, G. (2025). Differential dopaminergic modulation of the antennal lobe of drosophila melanogaster. *preprint*.
- [Parnas et al., 2013] Parnas, M., Lin, A. C., Huetteroth, W., and Miesenböck, G. (2013). Odor discrimination in drosophila: from neural population codes to behavior. *Neuron*, 79(5):932–944.
- [Parr and Friston, 2019] Parr, T. and Friston, K. J. (2019). Attention or salience? *Current opinion in psychology*, 29:1–5.
- [Pascual and Pr  at, 2001] Pascual, A. and Pr  at, T. (2001). Localization of long-term memory within the drosophila mushroom body. *Science*, 294(5544):1115–1117.
- [Pavlov and Anrep, 1927] Pavlov, I. P. and Anrep, G. V. (1927). *Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex*, volume 142. london: oxford University Press.
- [Pavlovsky et al., 2018] Pavlovsky, A., Schor, J., Placais, P.-Y., and Preat, T. (2018). A gabaergic feedback shapes dopaminergic input on the drosophila mushroom body to promote appetitive long-term memory. *Current Biology*, 28(11):1783–1793.
- [Pech et al., 2015] Pech, U., Revelo, N. H., Seitz, K. J., Rizzoli, S. O., and Fiala, A. (2015). Optical dissection of experience-dependent pre-and postsynaptic plasticity in the drosophila brain. *Cell reports*, 10(12):2083–2095.
- [Pellegrino et al., 2017] Pellegrino, R., Sinding, C., De Wijk, R., and Hummel, T. (2017). Habituation and adaptation to odors in humans. *Physiology & behavior*, 177:13–19.
- [Peng and Chittka, 2017] Peng, F. and Chittka, L. (2017). A simple computational model of the bee mushroom body can explain seemingly complex forms of olfactory learning and memory. *Current Biology*, 27(2):224–230.
- [Perez-Orive et al., 2002] Perez-Orive, J., Mazor, O., Turner, G. C., Cassenaer, S., Wilson, R. I., and Laurent, G. (2002). Oscillations and sparsening of odor representations in the mushroom body. *Science*, 297(5580):359–365.
- [Perisse et al., 2016] Perisse, E., Oswald, D., Barnstedt, O., Talbot, C. B., Huetteroth, W., and Waddell, S. (2016). Aversive learning and appetitive motivation toggle feed-forward inhibition in the drosophila mushroom body. *Neuron*, 90(5):1086–1099.
- [Perisse et al., 2013] Perisse, E., Yin, Y., Lin, A. C., Lin, S., Huetteroth, W., and Waddell, S. (2013). Different kenyon cell populations drive learned approach and avoidance in drosophila. *Neuron*, 79(5):945–956.
- [Petrucelli et al., 2020] Petrucelli, E., Lark, A., Mrkvicka, J. A., and Kitamoto, T. (2020). Significance of dopecr, a g-protein coupled dopamine/ecdysteroid receptor, in physiological and behavioral response to stressors. *Journal of neurogenetics*, 34(1):55–68.
- [Petrucelli et al., 2016] Petrucelli, E., Li, Q., Rao, Y., and Kitamoto, T. (2016). The unique dopamine/ecdysteroid receptor modulates ethanol-induced sedation in drosophila. *Journal of Neuroscience*, 36:4647–4657.
- [Phillmore, 2008] Phillmore, L. S. (2008). Discrimination: From behaviour to brain. *Behavioural Processes*, 77(2):285–297.
- [Picton et al., 1976] Picton, T. W., Hillyard, S. A., and Galambos, R. (1976). Habituation and attention in the auditory system. In *Auditory system: Clinical and special topics*, pages 343–389. Springer.

-
- [Pizzagalli et al., 2008] Pizzagalli, D. A., Evins, A. E., Schetter, E. C., Frank, M. J., Pajtas, P. E., Santesso, D. L., and Culhane, M. (2008). Single dose of a dopamine agonist impairs reinforcement learning in humans: behavioral evidence from a laboratory-based measure of reward responsiveness. *Psychopharmacology*, 196(2):221–232.
- [Placais et al., 2017] Placais, P.-Y., de Treder, É., Scheunemann, L., Trannoy, S., Goguel, V., Han, K.-A., Isabel, G., and Preat, T. (2017). Upregulated energy metabolism in the drosophila mushroom body is the trigger for long-term memory. *Nature communications*, 8(1):15510.
- [Plaças and Preat, 2013] Plaças, P.-Y. and Preat, T. (2013). To favor survival under food shortage, the brain disables costly memory. *Science*, 339(6118):440–442.
- [Plaza et al., 2022] Plaza, S. M., Clements, J., Dolafi, T., Umayam, L., Neubarth, N. N., Scheffer, L. K., and Berg, S. (2022). neuprint: an open access tool for em connectomics. *Frontiers in Neuroinformatics*, 16.
- [Podos, 2013] Podos, J. (2013). Sexual selection and the evolution of vocal mating signals: lessons from neotropical songbirds. In *Sexual Selection*, pages 341–363. Elsevier.
- [Poo and Isaacson, 2009] Poo, C. and Isaacson, J. S. (2009). Odor representations in olfactory cortex: “sparse” coding, global inhibition, and oscillations. *Neuron*, 62(6):850–861.
- [Popescu et al., 2016] Popescu, A. T., Zhou, M. R., and Poo, M.-m. (2016). Phasic dopamine release in the medial prefrontal cortex enhances stimulus discrimination. *Proceedings of the National Academy of Sciences*, 113(22):E3169–E3176.
- [Potter and Luo, 2011] Potter, C. J. and Luo, L. (2011). Using the q system in drosophila melanogaster. *Nature protocols*, 6(8):1105–1120.
- [Potter et al., 2010] Potter, C. J., Tasic, B., Russler, E. V., Liang, L., and Luo, L. (2010). The q system: a repressible binary system for transgene expression, lineage tracing, and mosaic analysis. *Cell*, 141(3):536–548.
- [Pribbenow et al., 2022] Pribbenow, C., Chen, Y.-c., Heim, M.-M., Laber, D., Reubold, S., Reynolds, E., Balles, I., Fernández-d V Alquicira, T., Suárez-Grimalt, R., Scheunemann, L., et al. (2022). Postsynaptic plasticity of cholinergic synapses underlies the induction and expression of appetitive and familiarity memories in drosophila. *Elife*, 11:e80445.
- [Prisco et al., 2021] Prisco, L., Deimel, S. H., Yeliseyeva, H., Fiala, A., and Tavosanis, G. (2021). The anterior paired lateral neuron normalizes odour-evoked activity in the drosophila mushroom body calyx. *Elife*, 10:e74172.
- [Qi and Lee, 2014] Qi, C. and Lee, D. (2014). Pre-and postsynaptic role of dopamine d2 receptor dd2r in drosophila olfactory associative learning. *Biology*, 3(4):831–845.
- [Qi et al., 2025] Qi, C., Qian, C., Steijvers, E., Colvin, R. A., and Lee, D. (2025). A pair of dopaminergic neurons dan-c1 mediate drosophila larval aversive olfactory learning through d2-like receptors. *Elife*, 13:RP100890.
- [Qiao et al., 2022] Qiao, J., Yang, S., Geng, H., Yung, W.-H., and Ke, Y. (2022). Input-timing-dependent plasticity at incoming synapses of the mushroom body facilitates olfactory learning in drosophila. *Current Biology*, 32(22):4869–4880.
- [Qin et al., 2012] Qin, H., Cressy, M., Li, W., Coravos, J. S., Izzi, S. A., and Dubnau, J. (2012). Gamma neurons mediate dopaminergic input during aversive olfactory memory formation in drosophila. *Current Biology*, 22(7):608–614.
- [Quinn et al., 1974] Quinn, W. G., Harris, W. A., and Benzer, S. (1974). Conditioned behavior in drosophila melanogaster. *Proceedings of the National Academy of Sciences*, 71(3):708–712.
- [Ramaswami, 2014] Ramaswami, M. (2014). Network plasticity in adaptive filtering and behavioral habituation. *Neuron*, 82(6):1216–1229.
- [Rankin et al., 2009] Rankin, C. H., Abrams, T., Barry, R. J., Bhatnagar, S., Clayton, D. F., Colombo, J., Coppola, G., Geyer, M. A., Glanzman, D. L., Marsland, S., et al. (2009). Habituation revisited: an updated and revised description of the behavioral characteristics of habituation. *Neurobiology of learning and memory*, 92(2):135–138.
- [Rauschenbach et al., 2000] Rauschenbach, I. Y., Sukhanova, M. Z., Hirashima, A., Sutsugu, E., and Kuano, E. (2000). Role of the ecdysteroid system in the regulation of drosophila reproduction under environmental stress. *Doklady Biological Sciences*, 375:641–643.
-

- [Recanatesi et al., 2019] Recanatesi, S., Farrell, M., Advani, M., Moore, T., Lajoie, G., and Shear-Brown, E. (2019). Dimensionality compression and expansion in deep neural networks. *arXiv preprint arXiv:1906.00443*.
- [Regna et al., 2016] Regna, K., Kurshan, P. T., Harwood, B. N., Jenkins, A. M., Lai, C.-Q., Muskavitch, M. A., Kopin, A. S., and Draper, I. (2016). A critical role for the drosophila dopamine d1-like receptor dop1r2 at the onset of metamorphosis. *BMC developmental biology*, 16(1):15.
- [Rescorla, 1972] Rescorla, R. A. (1972). A theory of pavlovian conditioning: Variations in the effectiveness of reinforcement and nonreinforcement. *Current research and theory*, pages 64–99.
- [Rezával et al., 2007] Rezával, C., Werbach, S., and Ceriani, M. F. (2007). Neuronal death in drosophila triggered by gal4 accumulation. *European journal of neuroscience*, 25(3):683–694.
- [Riemensperger et al., 2005] Riemensperger, T., Völler, T., Stock, P., Buchner, E., and Fiala, A. (2005). Punishment prediction by dopaminergic neurons in drosophila. *Current biology*, 15(21):1953–1960.
- [Romo and de Lafuente, 2013] Romo, R. and de Lafuente, V. (2013). Conversion of sensory signals into perceptual decisions. *Progress in neurobiology*, 103:41–75.
- [Root et al., 2008] Root, C. M., Masuyama, K., Green, D. S., Enell, L. E., Nässel, D. R., Lee, C.-H., and Wang, J. W. (2008). A presynaptic gain control mechanism fine-tunes olfactory behavior. *Neuron*, 59(2):311–321.
- [Rubin and Aso, 2024] Rubin, G. M. and Aso, Y. (2024). New genetic tools for mushroom body output neurons in drosophila. *Elife*, 12:RP90523.
- [Rudy and Sutherland, 1995] Rudy, J. W. and Sutherland, R. J. (1995). Configural association theory and the hippocampal formation: an appraisal and reconfiguration. *Hippocampus*, 5(5):375–389.
- [Ruff, 2013] Ruff, C. C. (2013). Sensory processing: who’s in (top-down) control? *Annals of the New York Academy of Sciences*, 1296(1):88–107.
- [Ruta et al., 2010] Ruta, V., Datta, S. R., Vasconcelos, M. L., Freeland, J., Looger, L. L., and Axel, R. (2010). A dimorphic pheromone circuit in drosophila from sensory input to descending output. *Nature*, 468(7324):686–690.
- [Ryner et al., 1996] Ryner, L. C., Goodwin, S. F., Castrillon, D. H., Anand, A., Villella, A., Baker, B. S., Hall, J. C., Taylor, B. J., and Wasserman, S. A. (1996). Control of male sexual behavior and sexual orientation in drosophila by the fruitless gene. *Cell*, 87(6):1079–1089.
- [Rytz et al., 2013] Rytz, R., Croset, V., and Benton, R. (2013). Ionotropic receptors (irs): chemosensory ionotropic glutamate receptors in drosophila and beyond. *Insect biochemistry and molecular biology*, 43(9):888–897.
- [Sachse et al., 2007] Sachse, S., Rueckert, E., Keller, A., Okada, R., Tanaka, N. K., Ito, K., and Vosshall, L. B. (2007). Activity-dependent plasticity in an olfactory circuit. *Neuron*, 56(5):838–850.
- [Saitoe et al., 2022] Saitoe, M., Naganos, S., Miyashita, T., Matsuno, M., and Ueno, K. (2022). A non-canonical on-demand dopaminergic transmission underlying olfactory aversive learning. *Neuroscience Research*, 178:1–9.
- [Sakurai et al., 2013] Sakurai, A., Koganezawa, M., Yasunaga, K.-i., Emoto, K., and Yamamoto, D. (2013). Select interneuron clusters determine female sexual receptivity in drosophila. *Nature communications*, 4(1):1825.
- [Saumweber et al., 2018] Saumweber, T., Rohwedder, A., Schleyer, M., Eichler, K., Chen, Y.-c., Aso, Y., Cardona, A., Eschbach, C., Kobler, O., Voigt, A., et al. (2018). Functional architecture of reward learning in mushroom body extrinsic neurons of larval drosophila. *Nature communications*, 9(1):1104.
- [Sayin et al., 2019] Sayin, S., De Backer, J.-F., Siju, K. P., Wosniack, M. E., Lewis, L. P., Frisch, L.-M., Gansen, B., Schlegel, P., Edmondson-Stait, A., Sharifi, N., et al. (2019). A neural circuit arbitrates between persistence and withdrawal in hungry drosophila. *Neuron*, 104(3):544–558.
- [Scheffer et al., 2020] Scheffer, L. K., Xu, C. S., Januszewski, M., Lu, Z., Takemura, S.-y., Hayworth, K. J., Huang, G. B., Shinomiya, K., Maitlin-Shepard, J., Berg, S., et al. (2020). A connectome and analysis of the adult drosophila central brain. *Elife*, 9:e57443.

-
- [Schenk and Gaudry, 2023] Schenk, J. E. and Gaudry, Q. (2023). Nonspiking interneurons in the drosophila antennal lobe exhibit spatially restricted activity. *Eneuro*, 10(1).
- [Schindelin et al., 2012] Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nature methods*, 9(7):676–682.
- [Schlegel et al., 2021] Schlegel, P., Bates, A. S., Stürner, T., Jagannathan, S. R., Drummond, N., Hsu, J., Serratos Capdevila, L., Javier, A., Marin, E. C., Barth-Maron, A., et al. (2021). Information flow, cell types and stereotypy in a full olfactory connectome. *Elife*, 10:e66018.
- [Schlegel et al., 2016] Schlegel, P., Texada, M. J., Miroshnikow, A., Schoofs, A., Hückesfeld, S., Peters, M., Schneider-Mizell, C. M., Lacin, H., Li, F., Fetter, R. D., et al. (2016). Synaptic transmission parallels neuromodulation in a central food-intake circuit. *Elife*, 5:e16799.
- [Schlegel et al., 2024] Schlegel, P., Yin, Y., Bates, A. S., Dorkenwald, S., Eichler, K., Brooks, P., Han, D. S., Gkantia, M., Dos Santos, M., Munnely, E. J., et al. (2024). Whole-brain annotation and multi-connectome cell typing of drosophila. *Nature*, 634(8032):139–152.
- [Schmid et al., 2015] Schmid, S., Wilson, D. A., and Rankin, C. H. (2015). Habituation mechanisms and their importance for cognitive function.
- [Schnaitmann et al., 2010] Schnaitmann, C., Vogt, K., Triphan, T., and Tanimoto, H. (2010). Appetitive and aversive visual learning in freely moving drosophila. *Frontiers in behavioral neuroscience*, 4:1008.
- [Scholz-Kornehl and Schwärzel, 2016] Scholz-Kornehl, S. and Schwärzel, M. (2016). Circuit analysis of a drosophila dopamine type 2 receptor that supports anesthesia-resistant memory. *Journal of Neuroscience*, 36(30):7936–7945.
- [Schroll et al., 2006] Schroll, C., Riemensperger, T., Bucher, D., Ehmer, J., Völler, T., Erbguth, K., Gerber, B., Hendel, T., Nagel, G., Buchner, E., et al. (2006). Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in drosophila larvae. *Current biology*, 16(17):1741–1747.
- [Schubert et al., 2014] Schubert, M., Hansson, B. S., and Sachse, S. (2014). The banana code—natural blend processing in the olfactory circuitry of drosophila melanogaster. *Frontiers in physiology*, 5:59.
- [Seki et al., 2010] Seki, Y., Rybak, J., Wicher, D., Sachse, S., and Hansson, B. S. (2010). Physiological and morphological characterization of local interneurons in the drosophila antennal lobe. *Journal of neurophysiology*, 104(2):1007–1019.
- [Senapati et al., 2019] Senapati, B., Tsao, C.-H., Juan, Y.-A., Chiu, T.-H., Wu, C.-L., Waddell, S., and Lin, S. (2019). A neural mechanism for deprivation state-specific expression of relevant memories in drosophila. *Nature neuroscience*, 22(12):2029–2039.
- [Seugnet et al., 2008] Seugnet, L., Suzuki, Y., Vine, L., Gottschalk, L., and Shaw, P. J. (2008). D1 receptor activation in the mushroom bodies rescues sleep-loss-induced learning impairments in drosophila. *Current Biology*, 18(15):1110–1117.
- [Shang et al., 2007] Shang, Y., Claridge-Chang, A., Sjulson, L., Pypaert, M., and Miesenböck, G. (2007). Excitatory local circuits and their implications for olfactory processing in the fly antennal lobe. *Cell*, 128(3):601–612.
- [Shin and Venton, 2022] Shin, M. and Venton, B. J. (2022). Fast-scan cyclic voltammetry (fscv) reveals behaviorally evoked dopamine release by sugar feeding in the adult drosophila mushroom body. *Angewandte Chemie*, 134(44):e202207399.
- [Shyu et al., 2017] Shyu, W.-H., Chiu, T.-H., Chiang, M.-H., Cheng, Y.-C., Tsai, Y.-L., Fu, T.-F., Wu, T., and Wu, C.-L. (2017). Neural circuits for long-term water-reward memory processing in thirsty drosophila. *Nature communications*, 8(1):15230.
- [Silbering et al., 2016] Silbering, A. F., Bell, R., MÄ1/4nch, D., Cruchet, S., Gomez-Diaz, C., Laudes, T., Galizia, C. G., and Benton, R. (2016). Ir40a neurons are not deet detectors. *Nature*, 534(7608):E5–E7.
- [Silbering et al., 2011] Silbering, A. F., Rytz, R., Grosjean, Y., Abuin, L., Ramdya, P., Jefferis, G. S., and Benton, R. (2011). Complementary function and integrated wiring of the evolutionarily distinct drosophila olfactory subsystems. *Journal of Neuroscience*, 31(38):13357–13375.
-

- [Silva et al., 2020] Silva, B., Hidalgo, S., and Campusano, J. M. (2020). Dop1r1, a type 1 dopaminergic receptor expressed in mushroom bodies, modulates drosophila larval locomotion. *Plos one*, 15(2):e0229671.
- [Simões et al., 2013] Simões, P. M., Niven, J. E., and Ott, S. R. (2013). Phenotypic transformation affects associative learning in the desert locust. *Current Biology*, 23(23):2407–2412.
- [Simon et al., 2006] Simon, A. F., Liang, D. T., and Krantz, D. E. (2006). Differential decline in behavioral performance of drosophila melanogaster with age. *Mechanisms of ageing and development*, 127(7):647–651.
- [Sinakevitch and Strausfeld, 2006] Sinakevitch, I. and Strausfeld, N. J. (2006). Comparison of octopamine-like immunoreactivity in the brains of the fruit fly and blow fly. *Journal of Comparative Neurology*, 494(3):460–475.
- [Sizemore and Dacks, 2016] Sizemore, T. R. and Dacks, A. M. (2016). Serotonergic modulation differentially targets distinct network elements within the antennal lobe of drosophila melanogaster. *Scientific reports*, 6(1):37119.
- [Snowden et al., 1994] Snowden, J., Griffiths, H., and Neary, D. (1994). Semantic dementia: Autobiographical contribution to preservation of meaning. *Cognitive neuropsychology*, 11(3):265–288.
- [Snowden et al., 1995] Snowden, J. S., Griffiths, H. L., and Neary, D. (1995). Autobiographical experience and word meaning. *Memory*, 3(3-4):225–246.
- [Solvi et al., 2020] Solvi, C., Gutierrez Al-Khudhairy, S., and Chittka, L. (2020). Bumble bees display cross-modal object recognition between visual and tactile senses. *Science*, 367(6480):910–912.
- [Spence, 1936] Spence, K. W. (1936). The nature of discrimination learning in animals. *Psychological review*, 43(5):427.
- [Spence, 1937] Spence, K. W. (1937). The differential response in animals to stimuli varying within a single dimension. *Psychological Review*, 44(5):430.
- [Srivastava et al., 2005] Srivastava, D. P., Esther, J. Y., Kennedy, K., Chatwin, H., Reale, V., Hamon, M., Smith, T., and Evans, P. D. (2005). Rapid, nongenomic responses to ecdysteroids and catecholamines mediated by a novel drosophila g-protein-coupled receptor. *Journal of Neuroscience*, 25(26):6145–6155.
- [Steiger, 1967] Steiger, U. (1967). Über den feinbau des neuropils im corpus pedunculatum der waldameise: elektronenoptische untersuchungen. *Zeitschrift für Zellforschung und mikroskopische Anatomie*, 81(4):511–536.
- [Stensmyr et al., 2012] Stensmyr, M. C., Dweck, H. K., Farhan, A., Ibba, I., Strutz, A., Mukunda, L., Linz, J., Grabe, V., Steck, K., Lavista-Llanos, S., et al. (2012). A conserved dedicated olfactory circuit for detecting harmful microbes in drosophila. *Cell*, 151(6):1345–1357.
- [Stieb et al., 2010] Stieb, S. M., Muenz, T. S., Wehner, R., and Rössler, W. (2010). Visual experience and age affect synaptic organization in the mushroom bodies of the desert ant cataglyphis fortis. *Developmental Neurobiology*, 70(6):408–423.
- [Stocker et al., 1990] Stocker, R., Lienhard, M., Borst, A., and Fischbach, K. (1990). Neuronal architecture of the antennal lobe in drosophila melanogaster. *Cell and tissue research*, 262:9–34.
- [Stockinger et al., 2005] Stockinger, P., Kvitsiani, D., Rotkopf, S., Tirián, L., and Dickson, B. J. (2005). Neural circuitry that governs drosophila male courtship behavior. *Cell*, 121(5):795–807.
- [Strutz et al., 2014] Strutz, A., Soelter, J., Baschwitz, A., Farhan, A., Grabe, V., Rybak, J., Knaden, M., Schmuker, M., Hansson, B. S., and Sachse, S. (2014). Decoding odor quality and intensity in the drosophila brain. *Elife*, 3:e04147.
- [Sugamori et al., 1995] Sugamori, K. S., Demchyshyn, L. L., McConkey, F., Forte, M. A., and Niznik, H. B. (1995). A primordial dopamine d1-like adenylyl cyclase-linked receptor from drosophila melanogaster displaying poor affinity for benzazepines. *FEBS letters*, 362(2):131–138.
- [Suh et al., 2004] Suh, G. S., Wong, A. M., Hergarden, A. C., Wang, J. W., Simon, A. F., Benzer, S., Axel, R., and Anderson, D. J. (2004). A single population of olfactory sensory neurons mediates an innate avoidance behaviour in drosophila. *Nature*, 431(7010):854–859.

- [Sun et al., 2020] Sun, H., Nishioka, T., Hiramatsu, S., Kondo, S., Amano, M., Kaibuchi, K., Ichinose, T., and Tanimoto, H. (2020). Dopamine receptor dop1r2 stabilizes appetitive olfactory memory through the raf/mapk pathway in drosophila. *Journal of Neuroscience*, 40(14):2935–2942.
- [Suver et al., 2019] Suver, M. P., Matheson, A. M., Sarkar, S., Damiata, M., Schoppik, D., and Nagel, K. I. (2019). Encoding of wind direction by central neurons in drosophila. *Neuron*, 102(4):828–842.
- [Syrovatkina et al., 2016] Syrovatkina, V., Alegre, K. O., Dey, R., and Huang, X.-Y. (2016). Regulation, signaling, and physiological functions of g-proteins. *Journal of molecular biology*, 428(19):3850–3868.
- [Szyszka et al., 2005] Szyszka, P., Ditzen, M., Galkin, A., Galizia, C. G., and Menzel, R. (2005). Sparsening and temporal sharpening of olfactory representations in the honeybee mushroom bodies. *Journal of neurophysiology*, 94(5):3303–3313.
- [Tabone and de Belle, 2011] Tabone, C. J. and de Belle, J. S. (2011). Second-order conditioning in drosophila. *Learning & memory*, 18(4):250–253.
- [Takemura et al., 2017] Takemura, S.-y., Aso, Y., Hige, T., Wong, A., Lu, Z., Xu, C. S., Rivlin, P. K., Hess, H., Zhao, T., Parag, T., et al. (2017). A connectome of a learning and memory center in the adult drosophila brain. *Elife*, 6:e26975.
- [Takemura et al., 2023] Takemura, S.-y., Hayworth, K. J., Huang, G. B., Januszewski, M., Lu, Z., Marin, E. C., Preibisch, S., Xu, C. S., Bogovic, J., Champion, A. S., et al. (2023). A connectome of the male drosophila ventral nerve cord. *BioRxiv*, pages 2023–06.
- [Takemura et al., 2015] Takemura, S.-y., Xu, C. S., Lu, Z., Rivlin, P. K., Parag, T., Olbris, D. J., Plaza, S., Zhao, T., Katz, W. T., Umayam, L., et al. (2015). Synaptic circuits and their variations within different columns in the visual system of drosophila. *Proceedings of the National Academy of Sciences*, 112(44):13711–13716.
- [Tamura et al., 2003] Tamura, T., Chiang, A.-S., Ito, N., Liu, H.-P., Horiuchi, J., Tully, T., and Saitoe, M. (2003). Aging specifically impairs amnesiac-dependent memory in drosophila. *Neuron*, 40(5):1003–1011.
- [Tanaka et al., 2004] Tanaka, N. K., Awasaki, T., Shimada, T., and Ito, K. (2004). Integration of chemosensory pathways in the drosophila second-order olfactory centers. *Current biology*, 14(6):449–457.
- [Tanaka et al., 2012a] Tanaka, N. K., Endo, K., and Ito, K. (2012a). Organization of antennal lobe-associated neurons in adult drosophila melanogaster brain. *Journal of Comparative Neurology*, 520(18):4067–4130.
- [Tanaka et al., 2012b] Tanaka, N. K., Suzuki, E., Dye, L., Ejima, A., and Stopfer, M. (2012b). Dye fills reveal additional olfactory tracts in the protocerebrum of wild-type drosophila. *Journal of Comparative Neurology*, 520(18):4131–4140.
- [Tanaka et al., 2008] Tanaka, N. K., Tanimoto, H., and Ito, K. (2008). Neuronal assemblies of the drosophila mushroom body. *Journal of Comparative Neurology*, 508(5):711–755.
- [Tang et al., 2024] Tang, R., Busby, R., Laursen, W. J., T. Keane, G., and Garrity, P. A. (2024). Functional dissection of mosquito humidity sensing reveals distinct dry and moist cell contributions to blood feeding and oviposition. *Proceedings of the National Academy of Sciences*, 121(35):e2407394121.
- [Tanimoto et al., 2004] Tanimoto, H., Heisenberg, M., and Gerber, B. (2004). Event timing turns punishment to reward. *Nature*, 430(7003):983–983.
- [Tauber et al., 2017] Tauber, J. M., Brown, E. B., Li, Y., Yurgel, M. E., Masek, P., and Keene, A. C. (2017). A subset of sweet-sensing neurons identified by ir56d are necessary and sufficient for fatty acid taste. *PLoS genetics*, 13(11):e1007059.
- [Tavosanis, 2012] Tavosanis, G. (2012). Dendritic structural plasticity. *Developmental neurobiology*, 72(1):73–86.
- [Technau and Heisenberg, 1982] Technau, G. and Heisenberg, M. (1982). Neural reorganization during metamorphosis of the corpora pedunculata in drosophila melanogaster. *Nature*, 295(5848):405–407.

- [Terashima et al., 2013] Terashima, H., Hosoya, H., Tani, T., Ichinohe, N., and Okada, M. (2013). Sparse coding of harmonic vocalization in monkey auditory cortex. *Neurocomputing*, 103:14–21.
- [Thompson and Best, 1989] Thompson, L. T. and Best, P. (1989). Place cells and silent cells in the hippocampus of freely-behaving rats. *Journal of Neuroscience*, 9(7):2382–2390.
- [Tomchik and Davis, 2009] Tomchik, S. M. and Davis, R. L. (2009). Dynamics of learning-related camp signaling and stimulus integration in the drosophila olfactory pathway. *Neuron*, 64(4):510–521.
- [Tonegawa et al., 2015] Tonegawa, S., Pignatelli, M., Roy, D. S., and Ryan, T. J. (2015). Memory engram storage and retrieval. *Current opinion in neurobiology*, 35:101–109.
- [Tonoki and Davis, 2012] Tonoki, A. and Davis, R. L. (2012). Aging impairs intermediate-term behavioral memory by disrupting the dorsal paired medial neuron memory trace. *Proceedings of the National Academy of Sciences*, 109(16):6319–6324.
- [Tsao et al., 2018] Tsao, C.-H., Chen, C.-C., Lin, C.-H., Yang, H.-Y., and Lin, S. (2018). Drosophila mushroom bodies integrate hunger and satiety signals to control innate food-seeking behavior. *Elife*, 7:e35264.
- [Tully et al., 1994] Tully, T., Preat, T., Boynton, S., and Del Vecchio, M. (1994). Genetic dissection of consolidated memory in drosophila. *Cell*, 79(1):35–47.
- [Tully and Quinn, 1985] Tully, T. and Quinn, W. G. (1985). Classical conditioning and retention in normal and mutant drosophila melanogaster. *Journal of Comparative Physiology A*, 157(2):263–277.
- [Turner et al., 2008] Turner, G. C., Bazhenov, M., and Laurent, G. (2008). Olfactory representations by drosophila mushroom body neurons. *Journal of neurophysiology*, 99(2):734–746.
- [Valdes-Aleman et al., 2019] Valdes-Aleman, J., Fetter, R. D., Sales, E. C., Doe, C. Q., Landgraf, M., Cardona, A., and Zlatić, M. (2019). Synaptic specificity is collectively determined by partner identity, location and activity. *bioRxiv*, page 697763.
- [Valencia-Montoya et al., 2024] Valencia-Montoya, W. A., Pierce, N. E., and Bellono, N. W. (2024). Evolution of sensory receptors. *Annual review of cell and developmental biology*, 40.
- [van Hateren, 1992] van Hateren, J. H. (1992). A theory of maximizing sensory information. *Biological cybernetics*, 68(1):23–29.
- [Varela et al., 2019] Varela, N., Gaspar, M., Dias, S., and Vasconcelos, M. L. (2019). Avoidance response to co2 in the lateral horn. *PLoS biology*, 17(1):e2006749.
- [Vickrey and Venton, 2011] Vickrey, T. L. and Venton, B. J. (2011). Drosophila dopamine2-like receptors function as autoreceptors. *ACS chemical neuroscience*, 2(12):723–729.
- [Vinje and Gallant, 2000] Vinje, W. E. and Gallant, J. L. (2000). Sparse coding and decorrelation in primary visual cortex during natural vision. *Science*, 287(5456):1273–1276.
- [Vinje and Gallant, 2002] Vinje, W. E. and Gallant, J. L. (2002). Natural stimulation of the nonclassical receptive field increases information transmission efficiency in v1. *Journal of Neuroscience*, 22(7):2904–2915.
- [Virtanen et al., 2020] Virtanen, P., Gommers, R., Oliphant, T. E., Haberland, M., Reddy, T., Cournapeau, D., Burovski, E., Peterson, P., Weckesser, W., Bright, J., et al. (2020). Scipy 1.0: fundamental algorithms for scientific computing in python. *Nature methods*, 17(3):261–272.
- [Vogt et al., 2016] Vogt, K., Aso, Y., Hige, T., Knapik, S., Ichinose, T., Friedrich, A. B., Turner, G. C., Rubin, G. M., and Tanimoto, H. (2016). Direct neural pathways convey distinct visual information to drosophila mushroom bodies. *elife*, 5:e14009.
- [Vogt et al., 2021] Vogt, K., Zimmerman, D. M., Schlichting, M., Hernandez-Nunez, L., Qin, S., Malacon, K., Rosbash, M., Pehlevan, C., Cardona, A., and Samuel, A. D. (2021). Internal state configures olfactory behavior and early sensory processing in drosophila larvae. *Science advances*, 7(1):eabd6900.
- [Vosshall, 2000] Vosshall, L. B. (2000). Olfaction in drosophila. *Current opinion in neurobiology*, 10(4):498–503.
- [Vosshall et al., 1999] Vosshall, L. B., Amrein, H., Morozov, P. S., Rzhetsky, A., and Axel, R. (1999). A spatial map of olfactory receptor expression in the drosophila antenna. *Cell*, 96(5):725–736.

- [Vosshall et al., 2000] Vosshall, L. B., Wong, A. M., and Axel, R. (2000). An olfactory sensory map in the fly brain. *Cell*, 102(2):147–159.
- [Wang et al., 2003a] Wang, J. W., Wong, A. M., Flores, J., Vosshall, L. B., and Axel, R. (2003a). Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. *Cell*, 112(2):271–282.
- [Wang et al., 2003b] Wang, Y., Chiang, A.-S., Xia, S., Kitamoto, T., Tully, T., and Zhong, Y. (2003b). Blockade of neurotransmission in drosophila mushroom bodies impairs odor attraction, but not repulsion. *Current Biology*, 13(21):1900–1904.
- [Wark et al., 2007] Wark, B., Lundstrom, B. N., and Fairhall, A. (2007). Sensory adaptation. *Current opinion in neurobiology*, 17(4):423–429.
- [Wen et al., 2012] Wen, Q., Po, M. D., Hulme, E., Chen, S., Liu, X., Kwok, S. W., Gershow, M., Leifer, A. M., Butler, V., Fang-Yen, C., et al. (2012). Proprioceptive coupling within motor neurons drives c. elegans forward locomotion. *neuron*, 76(4):750–761.
- [White et al., 1986] White, J., Southgate, E., Thomson, J., and Brenner, S. (1986). The structure of the nervous system of the nematode caenorhabditis elegans. *Philos Trans R Soc Lond B Biol Sci*, 314(1165):1–340.
- [White et al., 2010] White, K. E., Humphrey, D. M., and Hirth, F. (2010). The dopaminergic system in the aging brain of drosophila. *Frontiers in neuroscience*, 4:205.
- [Wickens, 2021] Wickens, C. (2021). Attention: Theory, principles, models and applications. *International Journal of Human-Computer Interaction*, 37(5):403–417.
- [Wilson, 2013] Wilson, R. I. (2013). Early olfactory processing in drosophila: mechanisms and principles. *Annual review of neuroscience*, 36(1):217–241.
- [Wilson and Laurent, 2005] Wilson, R. I. and Laurent, G. (2005). Role of gabaergic inhibition in shaping odor-evoked spatiotemporal patterns in the drosophila antennal lobe. *Journal of Neuroscience*, 25(40):9069–9079.
- [Wiltshcko and Wiltshcko, 2019] Wiltshcko, R. and Wiltshcko, W. (2019). Magnetoreception in birds. *Journal of the Royal Society Interface*, 16(158):20190295.
- [Winding et al., 2023] Winding, M., Pedigo, B. D., Barnes, C. L., Patsolic, H. G., Park, Y., Kazimiers, T., Fushiki, A., Andrade, I. V., Khandelwal, A., Valdes-Aleman, J., et al. (2023). The connectome of an insect brain. *Science*, 379(6636):eadd9330.
- [Withers et al., 1995] Withers, G., Fahrbach, S., and Robinson, G. (1995). Effects of experience and juvenile hormone on the organization of the mushroom bodies of honey bees. *Journal of neurobiology*, 26(1):130–144.
- [Witvliet et al., 2021] Witvliet, D., Mulcahy, B., Mitchell, J. K., Meirovitch, Y., Berger, D. R., Wu, Y., Liu, Y., Koh, W. X., Parvathala, R., Holmyard, D., et al. (2021). Connectomes across development reveal principles of brain maturation. *Nature*, 596(7871):257–261.
- [Wixted et al., 2014] Wixted, J. T., Squire, L. R., Jang, Y., Papesh, M. H., Goldinger, S. D., Kuhn, J. R., Smith, K. A., Treiman, D. M., and Steinmetz, P. N. (2014). Sparse and distributed coding of episodic memory in neurons of the human hippocampus. *Proceedings of the National Academy of Sciences*, 111(26):9621–9626.
- [Wong et al., 2002] Wong, A. M., Wang, J. W., and Axel, R. (2002). Spatial representation of the glomerular map in the drosophila protocerebrum. *Cell*, 109(2):229–241.
- [Wong et al., 2021] Wong, J. H., Wan, B. A., Bland, T., Montagnese, M., McLachlan, A. D., O’Kane, C. J., Zhang, S. W., and Masuda-Nakagawa, L. M. (2021). Octopaminergic neurons have multiple targets in drosophila larval mushroom body calyx and can modulate behavioral odor discrimination. *Learning & memory*, 28(2):53–71.
- [Wu et al., 2011] Wu, C.-L., Shih, M.-F. M., Lai, J. S.-Y., Yang, H.-T., Turner, G. C., Chen, L., and Chiang, A.-S. (2011). Heterotypic gap junctions between two neurons in the drosophila brain are critical for memory. *Current Biology*, 21(10):848–854.
- [Wu et al., 2013] Wu, C.-L., Shih, M.-F. M., Lee, P.-T., and Chiang, A.-S. (2013). An octopamine-mushroom body circuit modulates the formation of anesthesia-resistant memory in drosophila. *Current Biology*, 23(23):2346–2354.

- [Wu et al., 2016] Wu, M., Nern, A., Williamson, W. R., Morimoto, M. M., Reiser, M. B., Card, G. M., and Rubin, G. M. (2016). Visual projection neurons in the drosophila lobula link feature detection to distinct behavioral programs. *Elife*, 5:e21022.
- [Wu et al., 2023] Wu, M.-S., Liao, T.-W., Wu, C.-Y., Hsieh, T.-H., Kuo, P.-C., Li, Y.-C., Cheng, K.-C., and Chiang, H.-C. (2023). Aversive conditioning information transmission in drosophila. *Cell reports*, 42(10).
- [Xie et al., 2018] Xie, T., Ho, M. C., Liu, Q., Horiuchi, W., Lin, C.-C., Task, D., Luan, H., White, B. H., Potter, C. J., and Wu, M. N. (2018). A genetic toolkit for dissecting dopamine circuit function in drosophila. *Cell reports*, 23(2):652–665.
- [Xie et al., 2013] Xie, Z., Huang, C., Ci, B., Wang, L., and Zhong, Y. (2013). Requirement of the combination of mushroom body γ lobe and α/β lobes for the retrieval of both aversive and appetitive early memories in drosophila. *Learning & Memory*, 20(9):474–481.
- [Xiong et al., 1994] Xiong, W.-C., Okano, H., Patel, N. H., Blendy, J. A., and Montell, C. (1994). repo encodes a glial-specific homeo domain protein required in the drosophila nervous system. *Genes & development*, 8(8):981–994.
- [Xu et al., 2020] Xu, C. S., Januszewski, M., Lu, Z., Takemura, S.-y., Hayworth, K. J., Huang, G., Shinomiya, K., Maitin-Shepard, J., Ackerman, D., Berg, S., et al. (2020). A connectome of the adult drosophila central brain. *BioRxiv*, pages 2020–01.
- [Yagi et al., 2016] Yagi, R., Mabuchi, Y., Mizunami, M., and Tanaka, N. K. (2016). Convergence of multimodal sensory pathways to the mushroom body calyx in drosophila melanogaster. *Scientific Reports*, 6(1):29481.
- [Yaksi and Wilson, 2010] Yaksi, E. and Wilson, R. I. (2010). Electrical coupling between olfactory glomeruli. *Neuron*, 67(6):1034–1047.
- [Yamagata et al., 2015] Yamagata, N., Ichinose, T., Aso, Y., Plaçais, P.-Y., Friedrich, A. B., Sima, R. J., Preat, T., Rubin, G. M., and Tanimoto, H. (2015). Distinct dopamine neurons mediate reward signals for short-and long-term memories. *Proceedings of the National Academy of Sciences*, 112(2):578–583.
- [Yang et al., 2015] Yang, C.-H., He, R., and Stern, U. (2015). Behavioral and circuit basis of sucrose rejection by drosophila females in a simple decision-making task. *Journal of Neuroscience*, 35(4):1396–1410.
- [Yang et al., 2022] Yang, K., Liu, T., Wang, Z., Liu, J., Shen, Y., Pan, X., Wen, R., Xie, H., Ruan, Z., Tan, Z., et al. (2022). Classifying drosophila olfactory projection neuron boutons by quantitative analysis of electron microscopic reconstruction. *iScience*, 25(5).
- [Yasuyama et al., 2002] Yasuyama, K., Meinertzhagen, I. A., and Schürmann, F.-W. (2002). Synaptic organization of the mushroom body calyx in drosophila melanogaster. *Journal of Comparative Neurology*, 445(3):211–226.
- [Yilmaz and Meister, 2013] Yilmaz, M. and Meister, M. (2013). Rapid innate defensive responses of mice to looming visual stimuli. *Current Biology*, 23(20):2011–2015.
- [Young et al., 2011] Young, J., Wessnitzer, J., Armstrong, J., and Webb, B. (2011). Elemental and non-elemental olfactory learning in drosophila. *Neurobiology of learning and memory*, 96(2):339–352.
- [Yu et al., 2006] Yu, D., Akalal, D.-B. G., and Davis, R. L. (2006). Drosophila α/β mushroom body neurons form a branch-specific, long-term cellular memory trace after spaced olfactory conditioning. *Neuron*, 52(5):845–855.
- [Yu et al., 2013] Yu, H.-H., Awasaki, T., Schroeder, M. D., Long, F., Yang, J. S., He, Y., Ding, P., Kao, J.-C., Wu, G. Y.-Y., Peng, H., et al. (2013). Clonal development and organization of the adult drosophila central brain. *Current Biology*, 23(8):633–643.
- [Yu et al., 2025] Yu, S.-c., Bae, A., Matsliah, A., Dorkenwald, S., Gager, J., Hebditch, J., Silverman, B., Willie, K. P., Willie, R., Burke, A. T., et al. (2025). New synapse detection in the whole-brain connectome of drosophila. *bioRxiv*, pages 2025–07.
- [Zacarias et al., 2018] Zacarias, R., Namiki, S., Card, G. M., Vasconcelos, M. L., and Moita, M. A. (2018). Speed dependent descending control of freezing behavior in drosophila melanogaster. *Nature communications*, 9(1):3697.

- [Zarin et al., 2019] Zarin, A. A., Mark, B., Cardona, A., Litwin-Kumar, A., and Doe, C. Q. (2019). A multilayer circuit architecture for the generation of distinct locomotor behaviors in drosophila. *Elife*, 8:e51781.
- [Zars et al., 2000] Zars, T., Fischer, M., Schulz, R., and Heisenberg, M. (2000). Localization of a short-term memory in drosophila. *Science*, 288(5466):672–675.
- [Zeng et al., 2023] Zeng, J., Li, X., Zhang, R., Lv, M., Wang, Y., Tan, K., Xia, X., Wan, J., Jing, M., Zhang, X., et al. (2023). Local 5-HT signaling bi-directionally regulates the coincidence time window for associative learning. *Neuron*, 111(7):1118–1135.
- [Zhang et al., 2013a] Zhang, D., Li, Y., Wu, S., and Rasch, M. J. (2013a). Design principles of the sparse coding network and the role of “sister cells” in the olfactory system of drosophila. *Frontiers in Computational Neuroscience*, 7:141.
- [Zhang et al., 2005] Zhang, S., Bock, F., Si, A., Tautz, J., and Srinivasan, M. V. (2005). Visual working memory in decision making by honey bees. *Proceedings of the National Academy of Sciences*, 102(14):5250–5255.
- [Zhang and Roman, 2013] Zhang, S. and Roman, G. (2013). Presynaptic inhibition of gamma lobe neurons is required for olfactory learning in drosophila. *Current Biology*, 23(24):2519–2527.
- [Zhang et al., 2016] Zhang, S. X., Rogulja, D., and Crickmore, M. A. (2016). Dopaminergic circuitry underlying mating drive. *Neuron*, 91(1):168–181.
- [Zhang and Gaudry, 2016] Zhang, X. and Gaudry, Q. (2016). Functional integration of a serotonergic neuron in the drosophila antennal lobe. *Elife*, 5:e16836.
- [Zhang et al., 2013b] Zhang, Y. V., Ni, J., and Montell, C. (2013b). The molecular basis for attractive salt-taste coding in drosophila. *Science*, 340(6138):1334–1338.
- [Zheng et al., 2018] Zheng, Z., Lauritzen, J. S., Perlman, E., Robinson, C. G., Nichols, M., Milkie, D., Torrens, O., Price, J., Fisher, C. B., Sharifi, N., et al. (2018). A complete electron microscopy volume of the brain of adult drosophila melanogaster. *Cell*, 174(3):730–743.
- [Zheng et al., 2022] Zheng, Z., Li, F., Fisher, C., Iqbal, A., Sharifi, N., Calle-Schuler, S., Hsu, J., Masoodpanah, N., Kmecova, L., Kazimiers, T., Perlman, E., Nichols, M., Li, P., Jain, V., and Bock, D. (2022). Structured sampling of olfactory input by the fly mushroom body. *Current Biology*.
- [Zhong et al., 1992] Zhong, Y., Budnik, V., and Wu, C.-F. (1992). Synaptic plasticity in drosophila memory and hyperexcitable mutants: role of camp cascade. *Journal of Neuroscience*, 12(2):644–651.
- [Zhou et al., 2019] Zhou, M., Chen, N., Tian, J., Zeng, J., Zhang, Y., Zhang, X., Guo, J., Sun, J., Li, Y., Guo, A., et al. (2019). Suppression of gabaergic neurons through d2-like receptor secures efficient conditioning in drosophila aversive olfactory learning. *Proceedings of the National Academy of Sciences*, 116(11):5118–5125.
- [Zhu et al., 2003] Zhu, S., Chiang, A.-S., and Lee, T. (2003). Development of the drosophila mushroom bodies: elaboration, remodeling and spatial organization of dendrites in the calyx. *Development*.
- [Zweifel et al., 2011] Zweifel, L. S., Fadok, J. P., Argilli, E., Garelick, M. G., Jones, G. L., Dickerson, T. M., Allen, J. M., Mizumori, S. J., Bonci, A., and Palmiter, R. D. (2011). Activation of dopamine neurons is critical for aversive conditioning and prevention of generalized anxiety. *Nature neuroscience*, 14(5):620–626.

Appendix

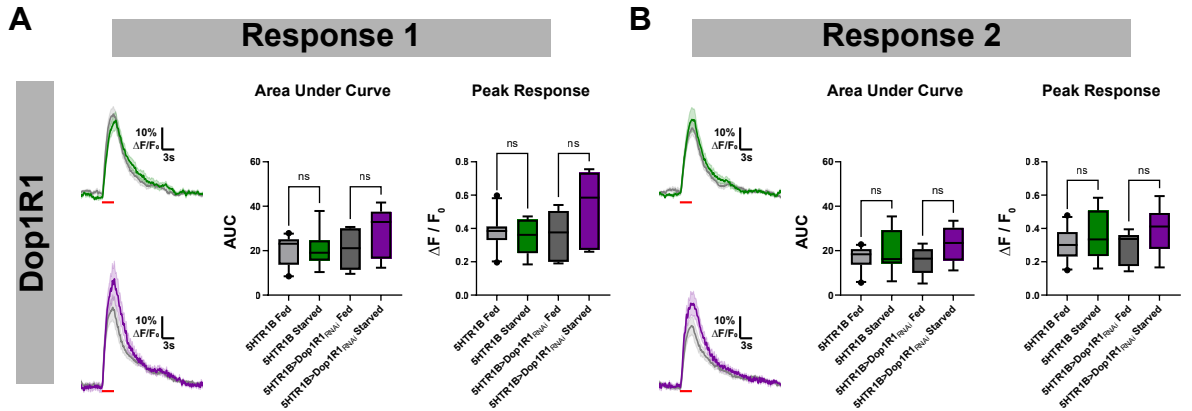


Figure A.1: Dop1R1 knockdown in starved or fed KC γ
 (A) Odour-evoked response of starved or fed 5HTR1B-GCaMP6m flies following Dop1R1 knockdown. (Left) GCaMP6m fluorescence across time comparing fed versus starved flies in the control (top) or knockdown (bottom) groups. Mean $\pm$ SEM, the red bar on the x-axis indicates odor delivery duration. (Middle) quantification of area under the curve, and (right) quantification of the peak calcium response. (B) Same as A but the second response to the odor stimulus after 5 minutes of constant air. Odor used was ethyl butyrate. $n=7-10$. Kruskal-Wallis test followed by Dunn's multiple comparisons correction, ns $p>0.05$.

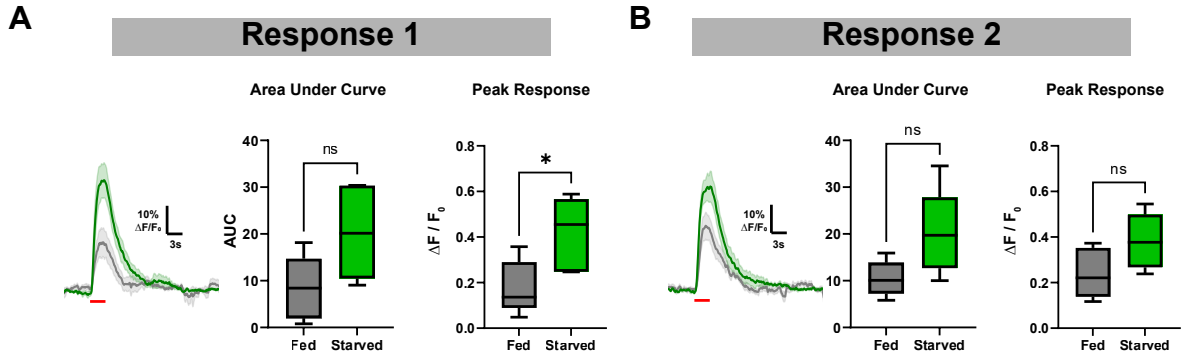


Figure A.2: KC odor-evoked response in the calyx under starvation conditions
 (A) Odour-evoked response of 247-GCaMP3 flies following a period of starvation to the first odor stimulus. (Left) GCaMP3 fluorescence across time (mean $\pm$ SEM), the red bar on the x-axis indicates odor delivery duration. (Middle) quantification of area under the curve, and (right) quantification of the peak calcium response. (B) Same as A but the second response to the odor stimulus after 5 minutes of constant air. Odor used was ethyl butyrate. $n=5$ Mann-Whitney test, ns $p>0.05$, * $p<0.05$.

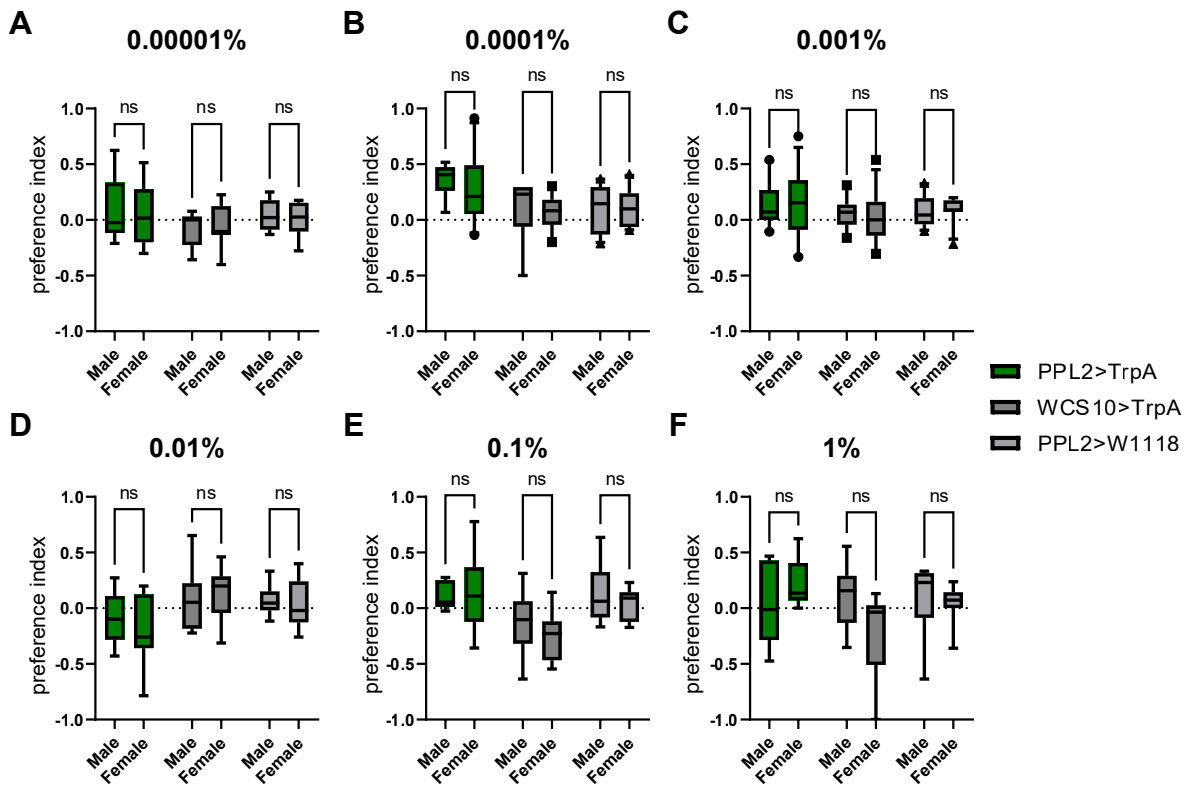


Figure A.3: Comparison of y-maze performance between males and females. (A-F) Preference index at specific concentrations split by sex (A) n=7-8. (B) n=10-13. (C) n=11-13. (D) n=6-9. (E) n=6-8. (F) n=6-8. 2-way ANOVA followed by Sidak's multiple comparisons correction, ns p>0.05. Each maze contained ≥ 50 flies, and $\geq 20\%$ of flies made a choice. n represents number of mazes per condition.

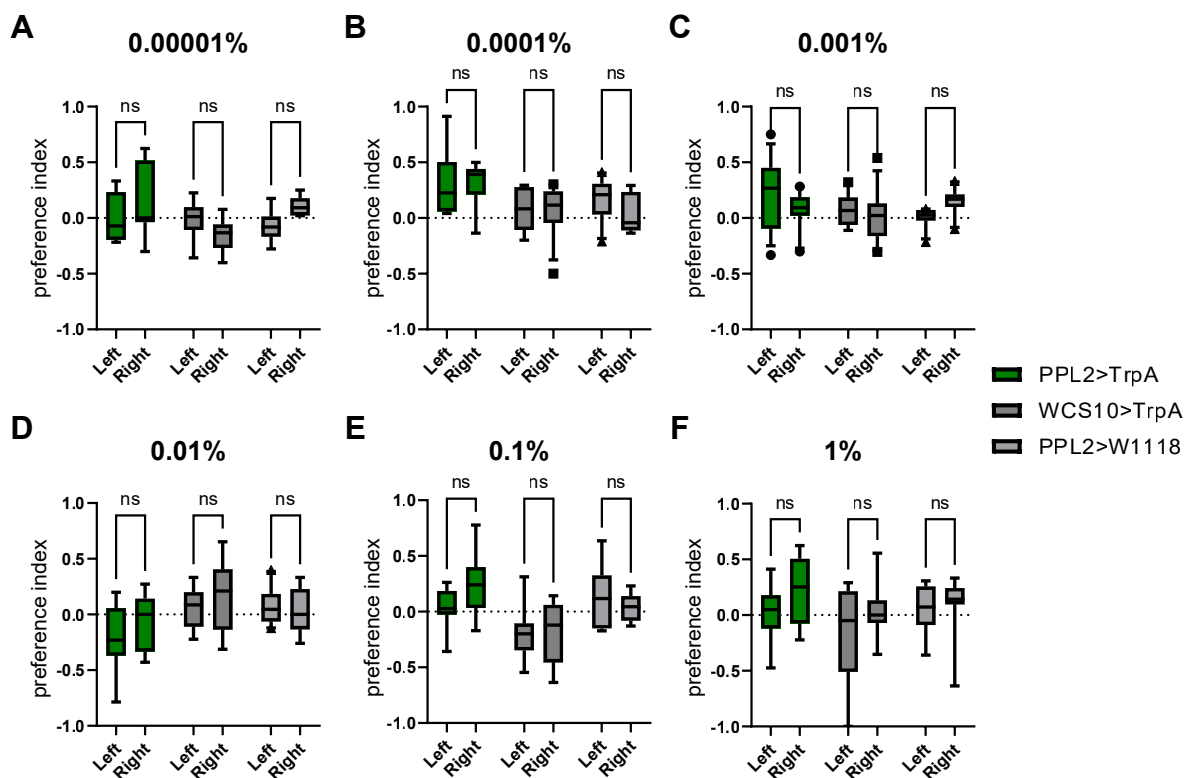


Figure A.4: Comparison of preference index when the odour was located in the right or left arm of the y-maze.

(A-F) Preference index at specific concentrations. The preference index of 'Left' indicates the preference index when the odour was in the left arm of the maze, and vice-versa for 'Right'. (A) n=6-8. (B) n=8-12. (C) n=10-14. (D) n=7-10. (E) n=7-8. (F) n=6-8. 2-way ANOVA followed by Sidak's multiple comparisons correction, ns p>0.05. Each maze contained ≥ 50 flies, and $\geq 20\%$ of flies made a choice. n represents number of mazes per condition.

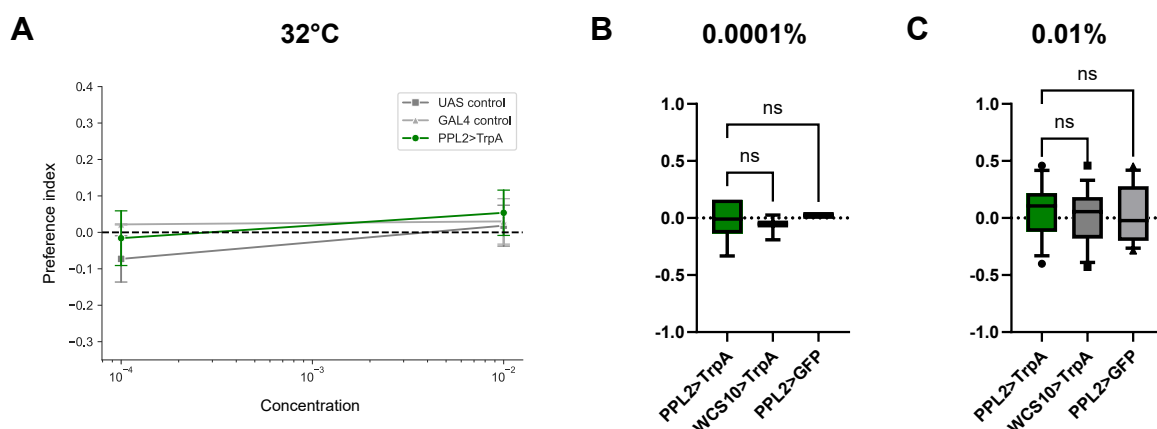


Figure A.5: Naive olfactory behaviour with new Gal4 control.

(A) Concentration curve showing the change in behavioural response, measured by the preference index, across a range of concentrations of the odour ethyl butyrate at 32°C. n=2-18. (B & C) Preference index at specific concentrations. (B) n=2-6. (C) n= 15-18. Kruskal-Wallis test followed by Dunn's multiple comparisons correction. ns p>0.05. Each maze contained ≥ 50 flies, and $\geq 20\%$ of flies made a choice. n represents number of mazes per condition.

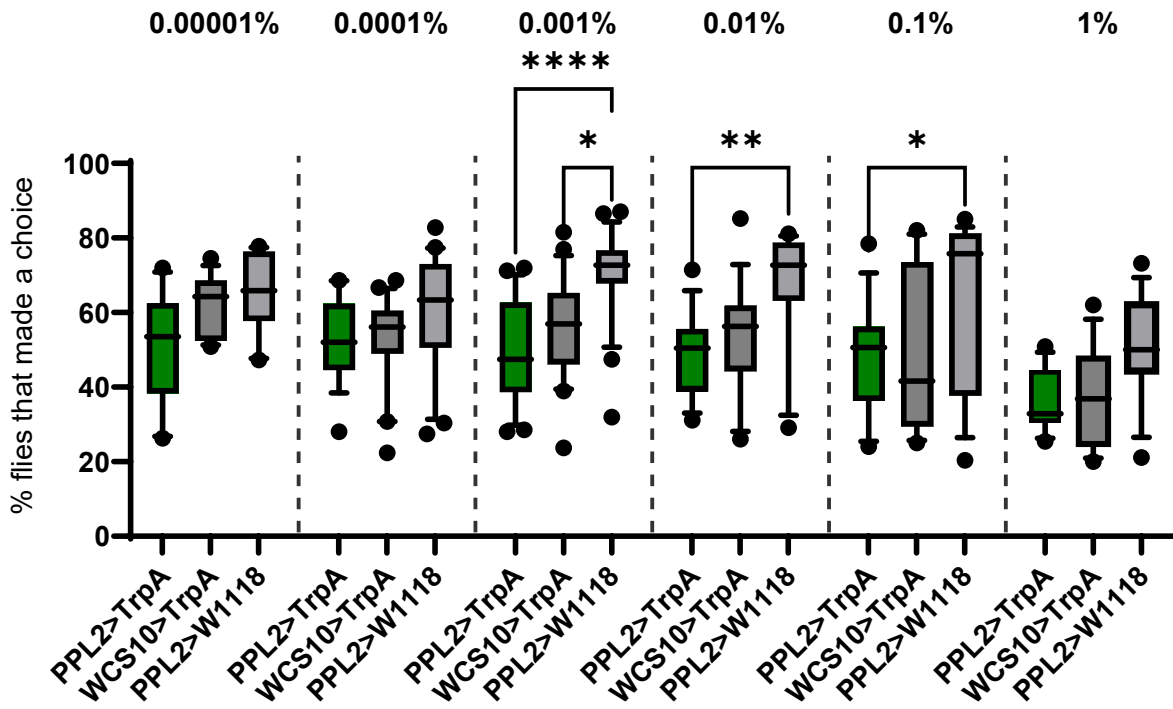


Figure A.6: Percentage of flies that made a choice at each concentration.

The percentage of flies that entered the y-maze and were counted as having made a choice in one of the arms. $n=12-26$. 2-way ANOVA followed by Sidak's multiple comparisons correction, $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$. Each maze contained ≥ 50 flies, and $\geq 20\%$ of flies made a choice. n represents number of mazes per condition.