Influence of Dopamine Transporter Knockout from α'β' Kenyon Cells on Associative Memory

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Introduction

Memory is the evolutionary and biological basis for decision making - a key process that has allowed organisms to survive by using past experience to influence judgement for the better (Klein et al., 2002). Learning to associate good and bad stimuli with positive and negative responses allows organisms to alter reflex responses into deliberate activity (Owald et al., 2015). The formation and retrieval of memory has been highly studied yet still remains largely unknown, and so a large interest in the research on this subject is on the physical pathways of memory function.

A key part of the memory making pathway is dopamine (DA). DA has several purposes in animals, including, but not limited to, learning and memory, movement, sleep, and motivation (Juárez Olguín et al., 2015; Ueno & Kume, 2014), making it a neurotransmitter of great interest. Additionally, DA is a catecholamine neurotransmitter (Schwaerzel et al., 2003), so it is also involved in wider body functions, such as blood flow and kidney activity (Jose et al., 2002). Once released from the synapse, DA diffuses into extrasynaptic space in order to bind to target receptors on other cells (Liu & Kaeser, 2019). The diffusion distance of DA is high compared to other neurotransmitters, and so it requires strong regulation in order to stop it from causing unwanted signalling in other areas of the brain (Cragg & Rice, 2004). For DA, this regulation is achieved by the dopamine transporter (DAT) - a transmembrane protein that controls the release out of and reintroduction of DA back into neurons (Cragg & Rice, 2004; Besada & Mortensen, 2025).

DAT takes in DA from extracellular space (Besada & Mortensen, 2025), hence influencing temporal, spatial, and voluminal DA dynamics (Cragg & Rice, 2004). Therefore, this allows regulation and reuse of DA in the brain. DAT is also prevalent in the control of DA spillover -- the diffusion of DA into wider extrasynaptic space - as the high diffusion distance of DA indicates that DAT is required to reuptake DA before it reaches neurons involved in different circuits to the target. Therefore, DAT is highly important in the control of signalling pathways in the brain. DAT is best known to be found in neurons where DA is also produced and released - dopaminergic neurons (DANs) (Chinta & Andersen, 2005; Croset et al., 2018). However, recently, single-cell transcriptomics has shown that DAT expression is also found in α'β' Kenyon cells (KCs) (Croset et al., 2018), which are non-dopaminergic neurons, and so do not make or release DA.

α'β' KCs are postsynaptic to dopamine neurons, and so it has been suggested that α'β' KCs may help to control how much of a DA signal is in the extracellular space and how long the signal remains (Croset et al., 2018). The position of α'β' KCs in the fruit fly brain is presented in Figure 1A. There are approximately 2000 KCs per brain hemisphere (Heisenberg, 1998) that get signals from antennal lobe projection neurons (PNs) (Hayashi et al., 2022; Aso, Sitaraman, et al., 2014). Most notably for this research, PNs relay odour information from the antennal lobes to the mushroom body (MB) (Turner et al., 2008; Christiansen et al., 2011). KCs are also the principal neurons of the mushroom body (Christiansen et al., 2011), and are considered the main neurons for olfactory learning and memory. 5 clusters are formed by

the grouping of axonal projections from the KCs due to their pathway - these lobes are α, α', β, β', and γ (Figure 1B). These divide KCs into 3 classes - αβ, α'β', and γ (Crittenden et al., 1998).

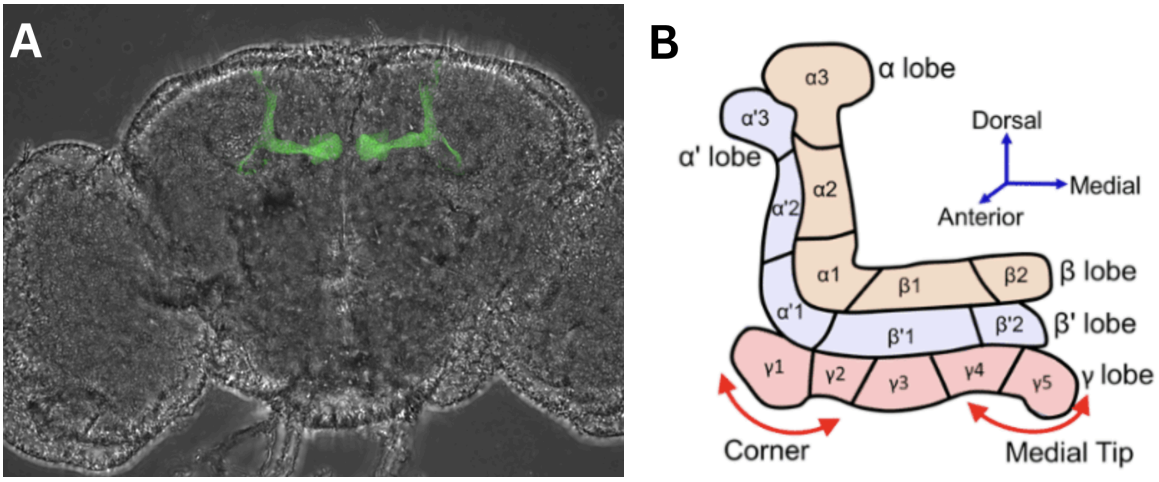

Figure 1: (A) α'β' KCs expression in the *Drosophila melanogaster* brain revealed by immunostaining. (B) Compartments of the MB (Lin, 2023).

Odour identities are represented by sparse populations of KCs in the mushroom body. The mushroom body is the center for learning and memory in insects (Aso, Sitaraman, et al., 2014), and where both appetitive and aversive memories are encoded (Perez-Orive, 2002; Zhang et al., 2008). It majorly consists of KCs, mushroom body output neurons (MBONs), and dopaminergic neurons (DANs) (Aso, Hattori, et al., 2014). MBONs are the outputs of the mushroom body, and their activity is necessary to recall memories and to send information from the MB to other parts of the insect brain (Aso, Sitaraman, et al., 2014). DANs are the main input modulatory neurons that are used to send neural reinforcement signals in the MB (Kato et al., 2023). Their modulation is required to pair stimuli with odour exposure - new odours can be assigned a valence, leading to increased attraction or avoidance to certain odours (or other stimuli) depending on whether the valence is positive or negative (Aso, Hattori, et al., 2014). Therefore, DANs are a key component of dopamine-dependent plasticity.

PAM-DANs are a cluster of DANs that are involved in giving rewarding reinforcement. Positive stimuli activate these neurons during associative learning. This stimulates them to release DA, which causes the connections between the synapses of KCs and aversion-promoting MBONs to weaken (Mohammad et al., 2024). The process allows flies to learn which odours are paired with a rewarding stimulus. Non-dopaminergic neurons do not release DA, unlike DANs, and most non-dopaminergic neurons do not have DAT expression. The fact that α'β' KCs are non-dopaminergic neurons, yet still contain DAT, is highly interesting to explore in the context of memory. KCs are essential in memory formation (Heisenberg, 2003) and so α'β' KCs' expression of DAT may influence learning and memory in flies.

The study organism chosen for this project was *Drosophila melanogaster* (commonly known as the fruit fly) - a popular organism used for neuroscience research. Their small brain, extremely fast reproductive and growth rate, and the simple methods that can be used to genetically modify them (Tito et al., 2016) make them an ideal model organism for use in this project. Additionally to this, *Drosophila* can be trained to associate different odours with positive (and negative) stimuli, making them an ideal subject for behaviour testing. Furthermore, the FLP/FRT system used in this project to genetically modify the fly is well researched and allows the knockout (KO) of a specified gene in designated tissues, without causing excessive cell death (Theodosiou & Xu, 1998). The approach for this project to use an FLP/FRT System to study the KO, rather than the knockdown, of DAT specifically from the α'β' KCs allows more specific and more accurate DAT removal to be achieved, hence making this method ideal for the project. This is because previous studies on this topic have either been not specific enough and so did not focus on α'β' KCs (Ueno & Kume, 2014), or were not accurate enough and so did not remove enough DAT from α'β' KCs (Karina Piotrowska, unpublished).

A previous study at the Croset Lab had found that the knockdown of DAT from α'β' KCs or all PAM-DANs using RNAi may improve associative appetitive memory (Karina Piotrowska, unpublished). This suggests that greater concentrations of extracellular DA improves memory, possibly by putting a higher valence to odours. However, RNAi failed to remove DAT from these cells completely, and hence the study may have been flawed. Additionally, existing literature shows overexpression and inhibition of DAT in mushroom bodies impacts memory in flies (Ueno & Kume, 2014; Zhang et al., 2008). Other literature also shows that DA drives learning using the DAT in KCs, and the impact of DAT on memory in the brain generally has also been researched (Ueno & Kume, 2014). Yet, there is no research on the effects of removing DAT from α'β' KCs, aside from what has been done at Croset Lab.

This project was centered on the hypothesis that DAT KO in α'β' KCs increases immediate sugar-rewarded olfactory memory performance. This is likely because the excess DA in extracellular space would enhance the rewarding signal for MBONs, leading to improved learning. Additionally, previous studies show a similar trend.

Therefore, this project aims to explain the function of DAT in α'β' KCs in learning and memory. This project will support our understanding of neurotransmitter modulation and the impact of dopamine transport on learning and memory. This study is also important in the understanding of DA transport and the transmission of neurotransmitters and it builds on understanding about the control of DA transport. DAT's function to reuptake DA from extracellular space is well known, however, this study aims to improve understanding about DAT function as a method of neurotransmitter control. This study also builds on the understanding of DA spillover as the removal of DAT would impact the distance that DA can travel.

The findings from this work could advance mechanistic understanding of synaptic transmission, and, more precisely, improve understanding of DA transmission and the

processes to control it. These findings could also help improve general understanding of learning and memory in other organisms - due to the use of DA in learning and memory systems across a wide range of animals, greater understanding of DA transport in flies improves the understanding of the mechanism in many other organisms. Results could also lead to more targeted memory disorder research in humans due to remarkable similarities between fly and human brains. There are many prevalent disorders affecting memory that are caused by dysfunctional DA transport, such as depression (Belujon & Grace, 2017), dementia (Zhou et al., 2023), ADHD (Volkow et al., 2009), bipolar disorder (Nepal et al., 2023), and addiction (Wise & Robble, 2020), and so, results pertaining to this investigation could influence clinical work that works to mitigate these disorders. Additionally, many drugs of abuse and some medications often target DAT (Nepal et al., 2023), so expansion of knowledge about DAT is also prevalent in drug research. Finally, this study can be used to improve computational models for DA transport in the mushroom body, as well as the computational neural activity model of the *Drosophila* brain.

— — — — — — — — — — — Methodology — — — — — — — — — — —

The experimental method consisted of three main sections:

1. Preparation of the fly stocks
2. Imaging
3. Behaviour testing.

Fly stocks

DAT cKO generated by Well Genetics; Gal4 lines from Bloomington.
Control genotypes:

- w; DAT cKO; UAS-FLP/DAT cKO - FLP control
- w; DAT cKO/w; DAT cKO; R58E02-Gal4 - PAM DAN driver control
- w; DAT cKO/w; DAT cKO; VT030604 - KC driver control

Experimental genotypes:

- w; DAT cKO; UAS-FLPD5.PEST/DAT cKO; R58E02-Gal4 - DAT KO in PAM-DANs
- w; DAT cKO; UAS-FLPD5.PEST/DAT cKO; VT030604-Gal4 - DAT KO in α’β’ KCs

cKO Expression was then tested under a fluorescence microscope, as shown in Figure 2.

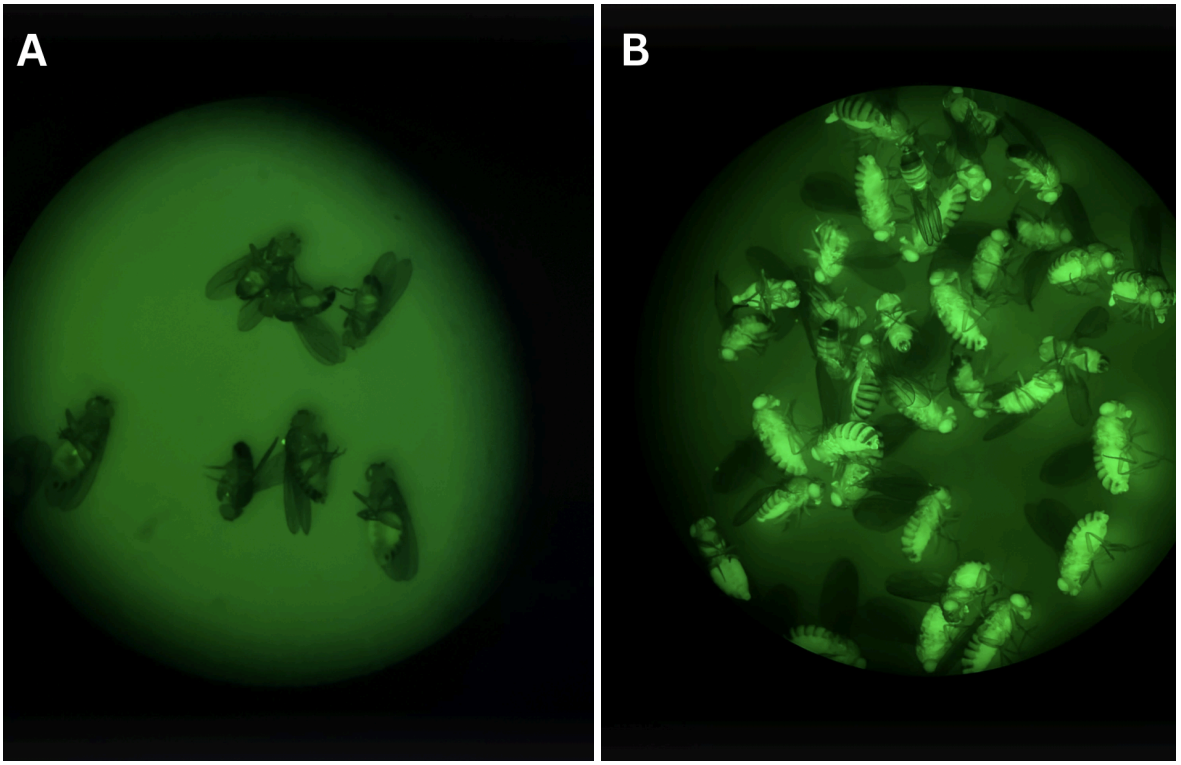

Figure 2: *Drosophila* as seen under a fluorescence microscope with genotypes of (A) wild type (w1118) and (B) DAT cKO.

The expression of the GFP tag in Figure 2B shows that the knock-in cassette used to make the DAT cKO line is present in the strain. Figure 2A shows no GFP expression, which confirms that the green fluorescence from the GFP tag is caused by the knock-in. Therefore, the cKO is being correctly expressed in DAT cKO flies.

Virgin females with the desired genotype were anaesthetised using CO_2 and introduced to males with the desired genotype. Stock bottles with standard cornmeal agar food were kept at 25^{o}C and 60% humidity with a 12 hour day/night cycle.

Imaging

Preparation

Dissections were performed using an altered version of the protocol discussed by Tito et al. (2016). Alterations to the protocol included: anesthetizing the adult flies with ice, and not dipping the flies in ethanol; removing the proboscis in order to get a better visual and route for forceps before moving onto step 4; removing the brain from the rest of the body at the end of the process.
The fly brains were then collected into an eppendorf tube and washed, immunostained, and fixed using Vectashield. The brains were arranged on a microscope slide, making sure not to crush the brains under the cover slip.

Imaging

Images of the fly brains were taken using an imaging microscope. The genotypes used for imaging were wild-type and PAM DANs KO.

Behaviour testing

Preparation:

Sets of 100 flies of each genotype were put into separate food vials each containing 4ml standard cornmeal agar food for 48 hours. Each of these sets of flies were then transferred into starvation vials each containing 4ml of 1% agar for 20 to 24 hours.

Training and testing:

A T-maze was used to test appetitive associative conditioning - a type of learning where a signal is paired with a positive event. The T-maze allows the flies to be trained to learn which odour is a “good” odour paired with a positive stimulus and make a future decision based on their training. This is the most suitable method of study as it has been optimised extensively in prior memory research.

The protocol used is shown as a diagram in Figure 2. The fruit flies were first given 30 seconds to acclimate to their surroundings. Then, they were exposed to two odours, each for 2 minutes: one paired with a positive stimulus (sugar coated filter paper) (CS+) and one paired with a neutral stimulus (dry filter paper) (CS-). The “positive” odour was switched in each repeat to maintain a controlled study and make sure that neither odour was preferential to the flies, and the flies were given 1 minute between the CS- and CS+. Finally, to test their memory, the flies were given a choice of the two odours and had 2 minutes to approach one or the other. Better memory would be visible through a greater proportion of flies approaching the CS+ odour. This protocol was repeated several times with each of the strains.

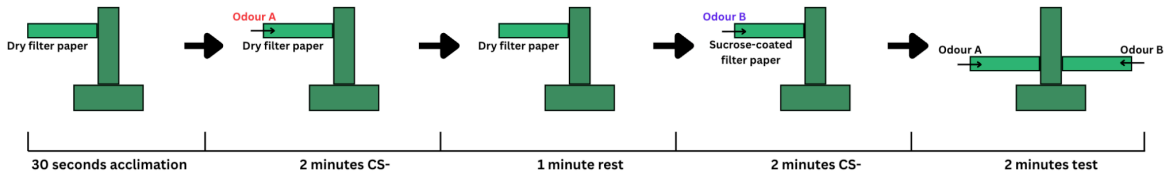

Figure 3: Diagram showing protocol used during behaviour training and testing.

Statistical Analysis:

Using the data collected from the T-maze, the Performance Index (PI) was found for each vial of flies using the following formula:

$$PI = \frac{\text{(number of flies in CS+ tube) - (number of flies in CS- tube)}}{\text{(number of flies in CS+ tube) + (number of flies in CS- tube)}}$$

Two PIs of the same genotype were always averaged to find a more reliable value for the PI. The mean PI of each genotype was found, and the standard error mean for each mean was calculated. The data gave a relatively normal distribution and variances were homoscedastic (Levene's test, Sig. = 0.371), so significance testing was completed using a One-Way ANOVA.

Results

cKO effectively suppresses DAT expression

Immunostaining using an anti-DAT antibody was used to prove that the cKO functioned properly. Figure 4 shows that the DAT cKO functioned properly and removed the DAT from PAM-DANs, greatly reducing the amount of DAT in the MB.

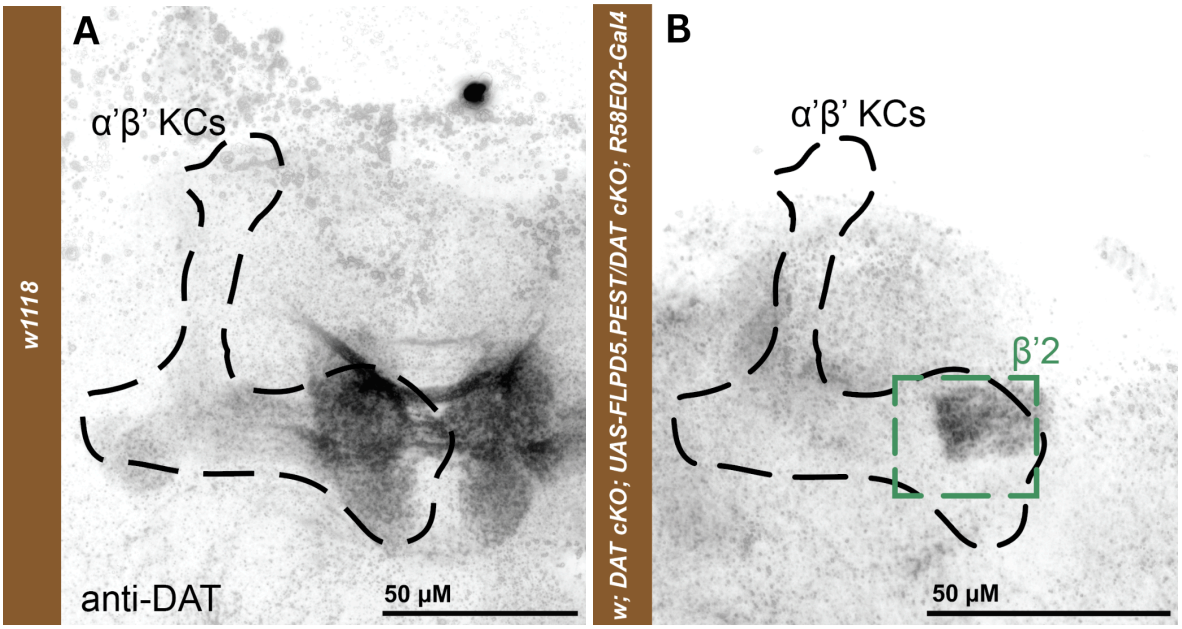

Figure 4: Immunohistochemistry of a *Drosophila* brains showing DAT localisation. (A) DAT presence in the MB in wild-type flies. (B) DAT localisation in DAT cKO in PAM-DANs flies, which likely shows that most DAT in α'β' KCs is innervated in the β'2 compartment.

Interestingly, the remaining DAT in α'β' KCs was mostly concentrated in the β'2 compartment, which suggests that the effect of DAT in the α'β' KCs likely stems from this section.

Behavioural testing did not show difference in memory performance

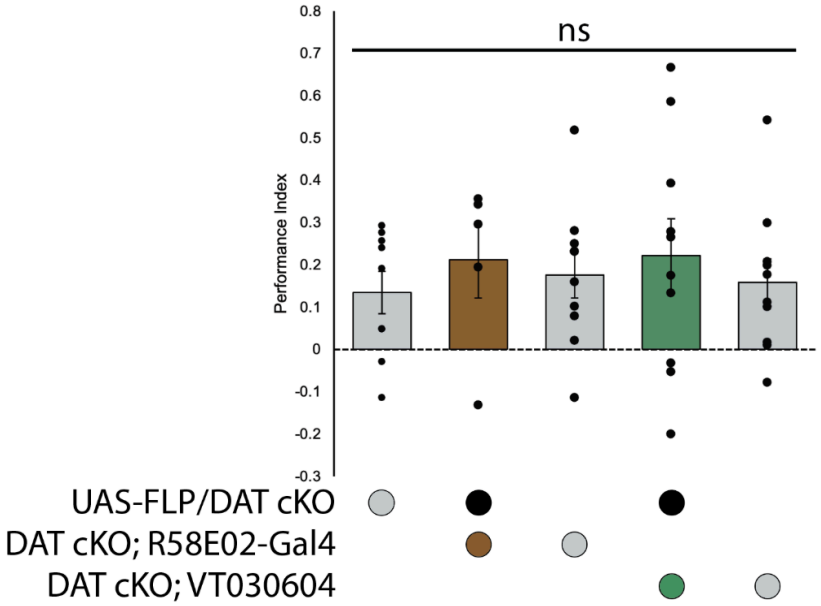

Figure 5: Behavioural assay results for appetitive learning - graph shows performance index values and the standard error mean for the three control and two experimental genotypes. Statistical significance using One-Way ANOVA was performed to compare all genotype groups with each other.

There is no evidence to suggest that the mean PI differed among the controls and the two experimental genotypes (ANOVA; $F = 0.280$, $P = 0.889 > 0.05$). The means were: FLP control, 0.1345368; DAT KO in PAM-DANs, 0.2115294; PAM DAN driver control, 0.1761285; DAT KO in α'β' KCs, 0.221291; KC driver control, 0.1586539.

— — — — — — — — — — — Discussion — — — — — — — — — — —

The immunostaining images show that there is a clear decrease of DAT from the wild-type to the DAT KO in PAM-DANs genotype. Interestingly, most leftover DAT from the cKO is localised in the β'2 compartment, which implies that DAT in α'β' KCs may be mainly expressed in that compartment. Also, due to DA's known role in appetitive memory (Ueno & Kume, 2014) and the MB's role in learning and memory (Aso, Sitaraman, et al., 2014), suggests that the compartment may have an important role for appetitive memory. This is further supported by the unusual convergence of multiple DAN subsets to the β'2 compartment (Li et al., 2020). One of these DAN subsets - PAM-β'2mp - is involved in memory retrieval during hunger. Therefore, the regulation of the DA sphere of influence by

DAT in the β’2 compartment by limiting spillover and preserving the specificity of the signal would influence hunger-mediated memory.

The results of the behavioural assay demonstrate that there was no significant difference between the memory performance of control flies and flies with DAT KO in α’β’ KCs. There was also no significant difference between memory performance of control flies and flies with DAT KO in PAM-DANs. Therefore, individually knocking out DAT in α’β’ KCs and PAM-DANs does not increase immediate sugar-rewarded olfactory memory performance. This is contrasting to the impaired memory performance revealed following the complete KO of DAT in *Drosophila* brains (Kume, 2005). Therefore, it is possible that neither the KO of DAT in PAM-DANs or in α’β’ KCs is enough to significantly impair memory as the learning and memory is maintained by DAT from the other source.

Due to limited time, less repeats could be completed as would have been preferred to generate more reliable data. Additionally, due to the KO of DAT making less flies survive than the control flies, the fly strains for the experimental genotypes did not generate many flies in the experimental protocol’s time-frame. Therefore, less repeats could be done on the experimental genotypes. More behaviour training using the protocol listed in this report would increase the amount of data available and help improve reliability of the experiment. As well as this, we were unable to prove that the conditional KO removed all the DAT from α’β’ KCs. This was due to a lack of flies for immunostaining imaging. Therefore, it may be possible that there was still DAT remaining in α’β’ KCs, which may have skewed the results. Future experiments could image the brains from the strain of flies that have the DAT KO in α’β’ KCs in order to confirm that the protocol has worked. Additionally, even though *Drosophila* have many conserved similarities to other insects and their markers can be extended to other animals, this study only looked at this one species and the functionality function of DAT in α’β’ KCs in learning and memory might be altered in other species. However, *Drosophila* are still a very useful species for behavioural research and so these results could be used as a way to make predictions on other organisms and similar neural circuits.

Ultimately, the next step would be to repeat the experiment and generate a much larger dataset, for the reasons explained above. Additionally, it may be useful to complete the protocol using long-term appetitive memory rather than short-term. This would allow us to see the effect of the removal of DAT on learning and memory over a longer period of time. It may also be interesting to look at the effect of α’β’ KC DAT KO using aversive rather than appetitive memory. Dopamine is likewise used during aversive learning and memory to respond to negative stimuli (Lopez & Lerner, 2024). Therefore, it would be useful to see the effect of DAT KO on aversive memory, for example by pairing an odour with an electric shock, in addition to the effect on appetitive memory. It would also be interesting to see the impact of making changes to the condition of the study organism - for example, by using older flies.

Overall, the DAT KO from α'β' KCs has no influence on appetitive associative memory. This is likely due to the surrounding presence of DAT in PAM-DANs maintaining memory performance. Therefore, it would be interesting to investigate this research question from a different perspective, for example by looking at the effect of the DAT KO from α'β' KCs on aversive associative memory, or by removing DAT from PAM-DANs and α'β' KC simultaneously, in order to confirm this hypothesis.

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