How does the Dopamine Transporter regulate memory in *Drosophila melanogaster*?

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ABSTRACT

The dopamine transporter (DAT) is a target of both recreational and therapeutic psychostimulants and is involved in conditions including depression and ADHD. Expressed primarily in dopaminergic neurons (DANs), DAT mediates dopamine reuptake from synapses, modulating extracellular dopamine levels. Recently, single-cell transcriptomics has uncovered unexpected DAT expression in a subset of Kenyon cells (KCs), which encode olfactory information and innervate the mushroom body, a structure in the fly brain essential for olfactory learning and memory. Although KCs are not dopaminergic, the synapses they form with mushroom body output neurons are modulated by subsets of reward or punishment DANs across individual mushroom body compartments. Here we show that knocking down DAT in reward DANs or in KCs increases appetitive memory, which suggests a joint action of both synaptic partners to modulate dopamine dynamics. Interestingly, DAT knockdown in punishment DANs had an adverse effect on aversive olfactory memory performance. We attribute this result to increased forgetting, which relies on the same dopaminergic neurons as aversive learning, highlighting the importance of dopamine transport to balance the acquisition and elimination of memories. Our results also indicate that post-synaptic DAT in KCs contributes to preventing dopamine spillover when functionally distinct dopaminergic synapses share the same mushroom body compartment.

INTRODUCTION

Drosophila melanogaster, commonly known as the fruit fly, has been extensively studied in scientific research, particularly in the fields of genetics, developmental biology, neuroscience, and evolutionary biology. It is considered a model organism for several reasons, such as a short life cycle, with a generation time of just about two weeks. This rapid reproduction allows researchers to study multiple generations quickly, facilitating genetic studies, and observations of developmental processes. Moreover, a single pair of fruit flies can produce hundreds of offspring, providing ample material for genetic experiments and screening. *Drosophila* melanogaster has a well-characterized and relatively small genome, which makes it an excellent model for studying genetics. Many genes in fruit flies have homologs in other organisms, including humans. Studying these genes in *Drosophila* can provide insights into their functions in more complex organisms. Many fundamental developmental processes, such as cell signalling, pattern formation, and organ development, are highly conserved between *Drosophila* and other animals, including humans. Fruit flies are easy to maintain in the laboratory, require little space, and have low maintenance costs compared to larger animal models. This accessibility makes them a cost-effective choice for research. Furthermore, despite having a simple nervous system, *Drosophila* displays a large range of easily quantifiable behaviours, making it an excellent model for studying neurobiology (Rubin et al., 2000). This study has used fruit flies to understand the genetic basis of learning and memory.

The dopamine transporter (DAT) is a protein responsible for regulating the levels of the neurotransmitter dopamine in the synaptic cleft, the small gap between neurons where neurotransmission occurs. DAT plays a crucial role in terminating the action of dopamine by facilitating its reuptake from the synaptic cleft back into the presynaptic neuron. This reuptake process is essential for the precise control of dopamine signalling in the brain. DAT is expressed in dopaminergic neurons, some of which innervate the mushroom body (MB) structure of the fly brain. (Yamamoto et al., 2014) The MB is a critical structure involved in learning and memory processes in flies. It is innervated by Kenyon cells, which sparsely respond to various sensory cues, especially olfactory and visual, and activate MB output neurons (MBONs) that convey valence information to other brain regions. Distinct groups of Dopaminergic neurons innervate individual compartments of the mushroom body in response to various rewards or punishments. The precise timing and amount of dopamine release are tightly regulated to modulate KC-MBON connections and implement short- or long-term memories. DAT is generally thought to help maintain this regulation by controlling the timing, concentration, and diffusion of dopamine in the intercellular space. (Ueno et al., 2014)

Recently, transcriptomics data on both single-cells and isolated nuclei has identified unexpected expression of DAT in the α′β′ subset of KCs, which are post-synaptic to DANs (Croset et al., 2018, Shih et al., 2018, Park et al., 2022). In this project, we use a behavioural readout to explore whether post-synaptic DAT contributes to dopamine reuptake in the MB. In addition to investigating the role of postsynaptic DAT in memory, we also use learning paradigms to understand how DAT controls dopamine spillover. This phenomenon refers to the diffusion of dopamine from a synapse into neighbouring regions. Whereas most compartments of the mushroom body are innervated by a single type of dopaminergic neurons, one of them (β'2) receives input from two distinct groups of dopaminergic neurons. Therefore, we anticipate that precise control of dopamine levels might be crucial to prevent excessive spillover between dopaminergic synapses within this compartment.

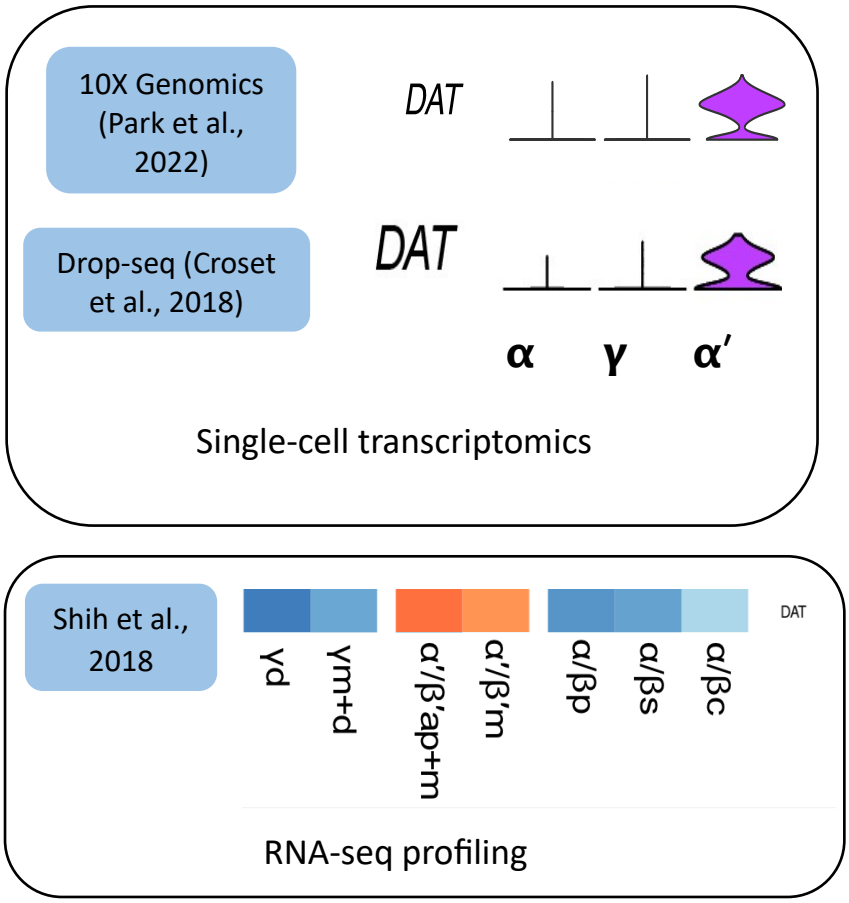

Figure 1: Recent studies have discovered DAT expression in the post-synaptic neurons - ***α′β′*** *Kenyon Cells, through RNA sequencing and single-cell transcriptomics.*

By manipulating DAT expression in *Drosophila*, this study was able to gain a better understanding of its normal function and how it contributes to dopamine regulation. This knowledge is crucial for identifying potential therapeutic targets and strategies for conditions where dopamine dysregulation is a key feature. Our work provides mechanistic insights into how alterations in DAT expression affect neural circuits, synaptic plasticity, and behaviour. These insights can help inform our understanding of the pathophysiology of conditions like Parkinson's and ADHD.

METHODS AND MATERIALS

Fly Stocks

Virgin females were anaesthetized and collected using CO2 before being exposed to males with the desired genotype. Adult flies were removed from the vial 3-4 days after introduction before the offspring hatched. Stock bottles were maintained in 75ml bottles and were kept in an incubator at 25°Cat 60% humidity with a

12-hour day/night cycle. Stocks were flipped every 4 days into new bottles. Vials with 4ml of food were used for short-term maintenance of stocks.

Immediate short-term memory assay

Sugar (Arabinose or Sucrose) and Water paper were made 24 hours before the experiment. On the day of the experiment, odours (Isoamyl acetate/IAA and 4-methylcyclohexanol/MCH) were diluted 1:1000 in mineral oil. Unless mentioned otherwise, experiments took place at 23ºC with 60% humidity.

During appetitive training, flies were exposed to CS+ for 2 minutes, then 30 seconds of air, and then CS- for another 2 minutes. Subsequent odour preference was then tested in the lower end of the T-Maze for 2 minutes. Flies are then frozen before counting.

During aversive training, flies were exposed to CS+ for 1 minute, then 45 seconds of air, and then CS- for 1 minute. The rest of the steps were repeated as above.

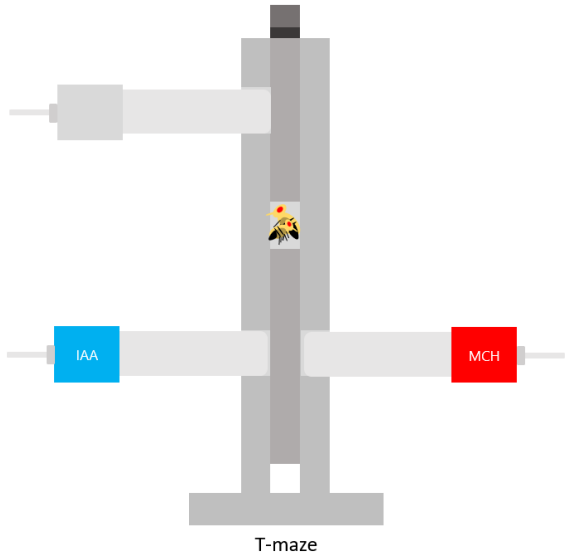

Figure 2: An illustration of the T-Maze instrument used for Appetitive and Aversive STM.

Methylphenidate Feeding

Flies were fed 5 mM MPH mixed with 10% Sucrose for 24 hours prior to the experiment using the DIETs apparatus.

Data Analysis and Statistics

The Predictive Index (PI) score is calculated by taking the average of [(CS+) – (CS-)]/Total No. of Flies for two oppositely conditioned odours.

Samples were analysed using a two-tailed t-test, either paired or unpaired depending on the data type, or a one-way ANOVA for 3+ samples. A value of $p < 0.05$ was taken as significant. All statistical analyses were conducted using the GraphPad online calculator (Graphpad., 2021).

RESULTS

DAT knockdown in PAM DANs with Sucrose improves Immediate Appetitive Short-Term Memory

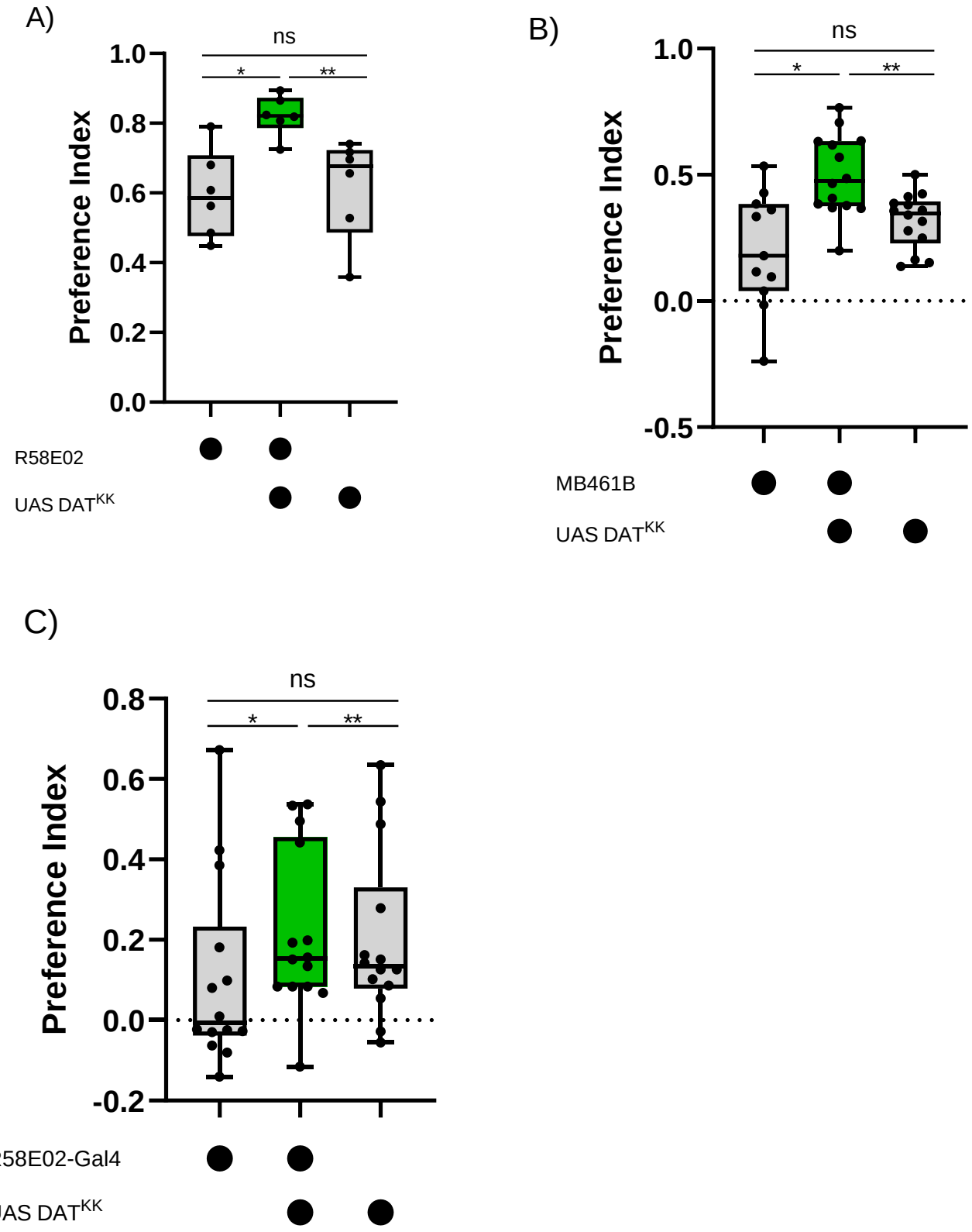

Figure 3: Immediate Appetitive Short-Term Memory tests on Drosophila post knockdown of DAT. A) DAT knocked down in PAM-DANs of flies trained with Sucrose. P>0.05, one-way ANOVA test. B) DAT knocked down in α'β' Kenyon Cells of flies trained with Sucrose. P>0.05, one-way ANOVA test C) DAT knocked down in PAM-DANs of flies trained with Arabinose. P>0.05, one-way ANOVA test.

We first tested the role of DAT in short-term appetitive memory retrieval. Fig. 3A shows that knocking down DAT in reward DANs (PAM-DANs) increased memory performance. This suggests that likely increased DA levels at the synapse might contribute to stronger immediate appetitive memory formation. Interestingly, we obtained a similar effect when knocking down DAT in α'β' KCs neurons (Fig. 3B). We conclude that both pre-and post-synaptic DAT contribute to regulating dopamine levels in synapses between PAM-DANs and their postsynaptic partners. (Cognigni et al., 2017) To test whether this increased memory performance was specific to memories that are further consolidated, we trained flies with DAT knocked down from PAM-DANs using arabinose, a sweet but non-nutritious sugar that only triggers short-term memories (Burke et al., 2011). In this case, we did not observe any increase in memory performance (Fig. 3C), suggesting that DAT specifically controls memories that are in the process of being consolidated into long-term memory.

DAT knockdown in PPL1 DANs worsens Immediate Aversive Short-Term memory

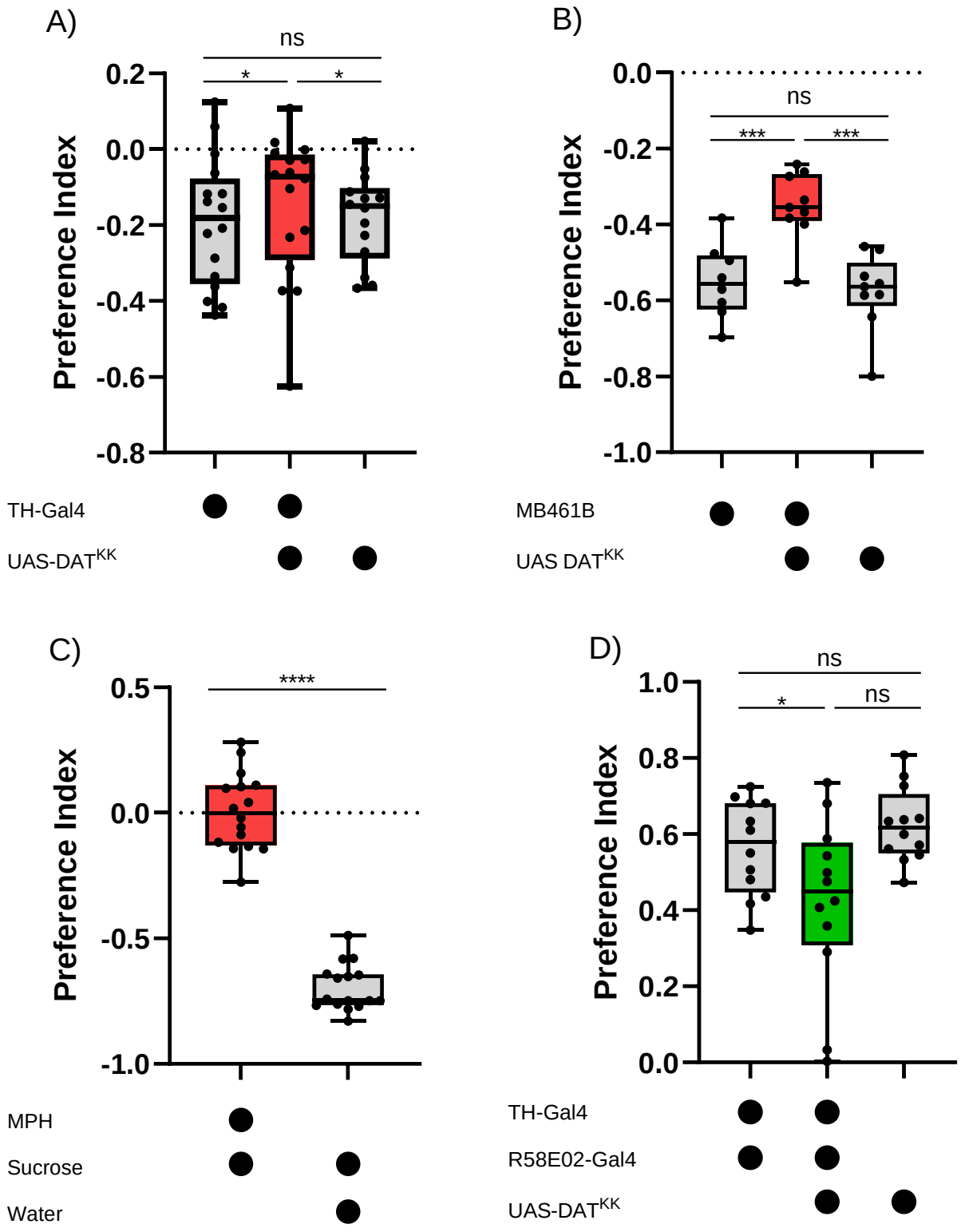

Figure 4: Immediate Aversive Short-term Memory tests on Drosophila post knockdown of DAT. A) DAT knocked down in PPL1 DANs. p>0.05, one-way ANOVA test. B) DAT knocked down in α'β' Kenyon Cells. p>0.05, one-way ANOVA test. C) DAT blocked in test flies using Methylphenidate and compared with control flies. p>0.05, two-tailed t-test. D) DAT knocked down in both PAM DANs and PPL1 DANs, trained appetitively. p>0.05, one-way ANOVA test.

To investigate the function of DAT on aversive memory, we first knocked down DAT in punishment dopamine neurons (PPL1 DANs). Instead of the memory increase described above, we observed a decrease in memory (Fig. 4A). We then tested the effects of DAT knockdown in α′β′ Kenyon Cells and found a

similar reduction in memory performance (Fig. 4B). To further validate this data, we fed with 5 mM of methylphenidate (MPH), a drug that blocks DAT, for 24 hours prior to aversive training. The results depicted in Fig. 4C show that these flies could not retrieve memory, suggesting that blocking DAT provides the same effects as DAT knock down. (Zhang et al., 2008) We hypothesise that the opposing effect of DAT in appetitive versus aversive memory could be due to the fact that PPL1 neurons also trigger forgetting (Berry et al., 2012). Our results suggest that increased forgetting due to DAT knock down might overpower memory formation and thus, lead to weaker memory scores. To test this, we knocked down DAT in both reward and punishment DANs and trained these animals with appetitive conditioning. We find that these animals did not perform differently than controls, which indicates that even the increased memory due to PAM-DANs knock down might have been compensated for by knockdown of DAT in forgetting DANs.

DAT knockdown in α′β′ KCs causes DA Spillover

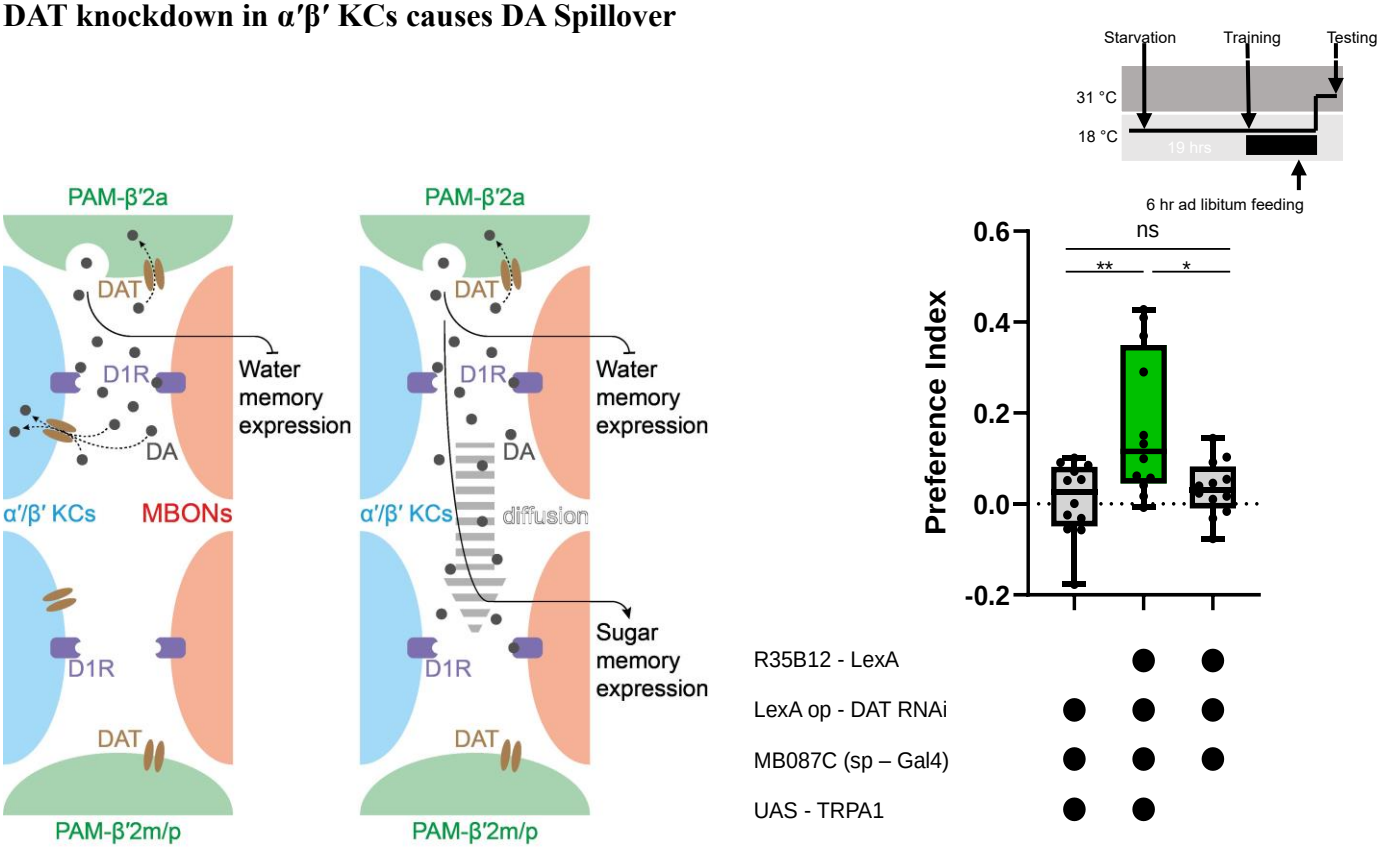

Figure 5: The DA spillover effects caused by DAT knockdown in α′β′ KCs. (Left) A schematic explaining the mechanism of DA spillover between the synaptic clefts of PAM-β′2a neurons and the PAM-β′2m/p neurons due to a DAT knockdown. (Right) Data from the appetitive memory test performed comparing results of flies that have both DAT knocked down and PAM- β′2a neurons activated to the flies that lack either of the two.

The β′2 compartment of the mushroom body is the only one in the MB that is innervated by two different types of DANs, which have specific functions during memory retrieval. (Cragg et al., 2004) PAM-β′2a inhibit the expression of water memories in non-thirsty animals, whereas PAM-β′2mp activates the expression of sugar memories in hungry animals (Senapati et al., 2019). To test whether post-synaptic DAT contributes to preventing DA diffusion between PAM-β′2a and PAM-β′2mp synapses, we knocked down DAT in in α′β′ KCs and trained flies with sugar and then fed them for 6 hours. We then used thermogenetics to activate PAM-β′2a neurons during memory retrieval. As flies were sated during retrieval, controls with only DAT knocked down or only PAM-β′2a neurons artificially activated did not approach the sugar-paired odour. However, flies that had both DAT knocked down and PAM-β′2a neurons activated did express the memory, therefore behaving similarly to animals with PAM-β′2mp activation. We conclude that in the

absence of post-synaptic DAT, DA released from PAM-β′2a may have been allowed to spill over onto PAM-β′2mp synapses.

DISCUSSION

In addition to its expression in dopaminergic neurons, DAT is also expressed in the α′β′ Kenyon Cells of the *Drosophila* brain. To our knowledge, our work represents the first demonstration of DAT activity in post-synaptic neurons. We found that DAT is required in the α′β′ Kenyon Cells to co-reuptake dopamine during both appetitive and aversive memory formation. Interestingly, our results also show that DAT is essential to regulate the balance between learning and forgetting. This is particularly important because it implies that the regulation of dopamine levels and signalling activity in both pre- and post-synaptic neurons in the brain have a significant impact on the acquisition of information and the degree of memory retrieval. The dopamine-driven plasticity and activity in feedback and feedforward connections between distinct parts of the mushroom body neural network is significant for memory formation and expression. These computational patterns have been demonstrated to be important for olfactory memory consolidation, internal state integration, re-evaluation, and updating of learnt information. The frequently recurring circuit structure, as well as the extended requirement for activity in parts of these underlying networks, indicating that self-sustaining and precisely timed activity is a basic aspect of network computations in the insect brain. These mechanisms work together to allow flies to constantly modify the content of their learned knowledge and direct their behaviour in a way that best represents learnt expectations and satisfies their most pressing current requirements.

We also find that post-synaptic DAT plays a role in controlling spillover within MB compartments. We speculate that this could be a way to optimise efficiency: by allowing two distinct classes of DANs to innervate the same MB compartment, post-synaptic DAT expression reduces the need for additional MB compartments. Therefore, whereas we cannot exclude post-synaptic DAT expression outside of the *Insecta* class, we hypothesise that this could be a specific feature of smaller brains, allowing them to remain compact while maintaining their physiology and accurate execution of their behavioural functions.

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