Conditional knockout strategy to quantify dopamine transporter function in memory

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Introduction

Dopamine, a neurotransmitter crucially involved in both learning [1] and reward [2], is released into the brain by dopaminergic neurones (DANs) during synaptic transmission. During neurotransmission, the synaptic vesicles containing DA molecules fuse to the cell membrane of the neurone and secrete the DA molecules into the synaptic cleft. This local spike in DA concentration around the synapse diffuses into extracellular space, allowing DA molecules to bind to nearby DA receptors. The magnitude and range of these extracellular DA transients is controlled by the dopamine transporter (DAT) [3], a trans-membrane protein that actively transports secreted DA across the cell membrane of neurones, allowing the molecule to either be repackaged into synaptic vesicles for future release, or metabolised by the cell.

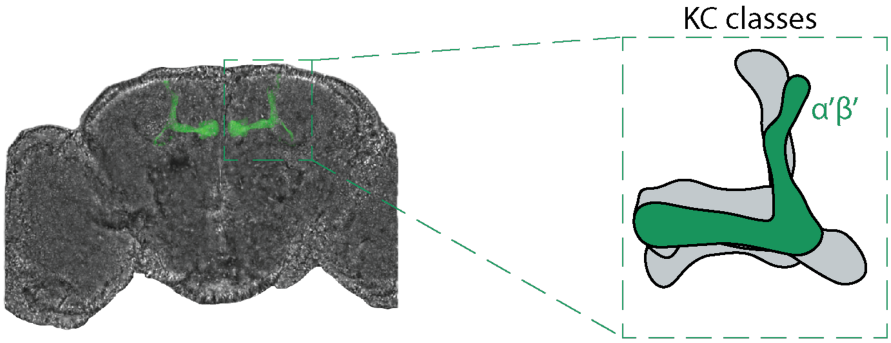

Figure 1. Visualisation in green of the α'β' KCs in mushroom body using GFP.

In Drosophila, the mushroom body, a bilaterally symmetrical paired structure found within the protocerebrum, functions as the associative centre of the brain [4]. Kenyon Cells (KCs) are the principal neurones that make up the MB. KCs receive input that encodes olfactory information from antennal lobe projection neurones (PNs). Different odours active different distinct populations of KCs, so that the unique pattern of KCs activation is what encodes the identity of the odour [5]. The axonal projections of the KCs are organised into 5 spatially distinct lobes: α, α', β, β', and γ. The MB can be further subdivided into at least 15 sub-components, each innervated by the dendrites of one or more distinct type of mushroom body output neurone (MBON). The information outputted by MBONs conveys the behavioural response triggered by a stimulus to various neuropils outside the MB [6].

The MB mediates appetitive learning. The reinforcement signals that modulate the valence of stimuli are inputted into MB through three specific clusters of DANs, which each project axonal arbours into specific lobes. Protocerebral anterior medial dopaminergic neurones (PAM-DANs) generally innervate the base of the vertical α lobe and the horizontal β, β' and γ lobes [7]. These modulatory neurones confer a positive valence to stimuli through DA transmission, thus producing an appetitive memory. The PAM-DANs, which have axon terminals located close to KC-to-MBON synapses, release DA, which induces long-term synaptic depression in KC-to-MBON synapses that produce aversive responses. Upon

future presentation, the conditioned odour would be more likely to activate appetitive MBONs rather than the chemically depressed aversive MBONs, producing an appetitive response.

The interface between DA and memory is well established. Aberrant DA dynamics are known to produce profound neurological consequences in humans. Most psychostimulants, such as cocaine and methamphetamine, operate by increasing the extracellular concentration of DA in the brain, thus artificially leveraging neural reward circuitry to produce euphoria [8]. This is often done by disabling DA transport through the inhibition or reversal of DAT function. Long-term psychostimulant abuse is strongly associated with a permanent decline in memory performance [9]. Moreover, atypical extracellular DA concentration is a characteristic mechanistic feature of ADHD, a widespread neurodevelopmental difference [10]. Impaired working memory is a frequent symptom associated with ADHD [11].

DAT has widely been regarded as a specific marker for dopaminergic neurones. However, single-cell transcriptomic data has shown that non-dopaminergic KCs in the $\alpha'\beta'$ lobe of the mushroom body express DAT. The implications of this unexpected expression on associative memory have not been explored. To quantify the effect, we leveraged conditional mutants with DAT knocked out in either α' KCs or DANs.

Materials and Methods

Drosophila husbandry. Flies were raised on standard cornmeal food under a 12:12 light:dark cycle at 60% humidity and 25°C unless otherwise stated.

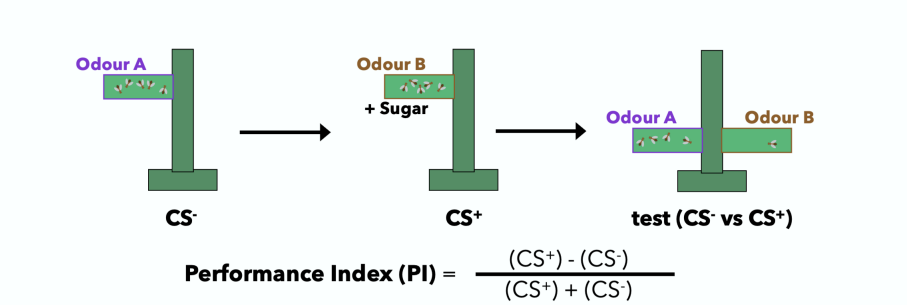

Figure 2. Schematic of the appetitive memory assay used in this study.

Appetitive memory assay. Appetitive Memory Assay. The two conditioning odours used were 8μL isoamyl acetate (Sigma-Aldrich 24900822) in 8mL mineral oil (Sigma-Aldrich 330760) and 8μL 4-methylcyclohexanol (Sigma-Aldrich 153095) in 8mL mineral oil. Cohorts of 40-80 mixed sex flies were starved in a tube with 4μL of solidified 1% agar and one 20 x 60 mm piece of filter paper for 18-20 hours before conditioning. During conditioning training, one odourant was presented with a dry filter paper (unconditioned odour, CS-) for 2 min, before a 30 sec break, and presentation of a second odourant with filter paper coated with dry sucrose (conditioned odour, CS+). That forms one half-replicate for this genotype, and a full replicate is derived by swapping the odours and repeating the above with a new cohort. The flies were tested immediately after training. Testing consisted of exposing the trained cohort to a T-maze junction with each odour being pumped through a branch of the maze, and counting the number of flies in each branch after 2 min. Airflow was maintained at approximately 2L/min and monitored regularly through experiments. All experiments were performed at 19-22°C and 50-70% humidity, and testing was conducted in in pitch-black conditions. Performance index (PI) was calculated in the same way as in Krashes and Waddell [12], as the number of flies approaching the conditioned

odour minus the number of flies going in the opposite direction, divided by the total number of flies. A single PI value is the average score from the test with both conditioned odour combinations.

Immunostainings. The flies were anaesthetisied on ice, then dissected in PBST (PBS + 0.5% Triton-X). Samples were then fixed in 4% paraformaldehyde in PBST for 50 min at RT. Samples were then underwent four 10 min washes in PBST with rotation at RT and afterwards were blocked for 90 min in PBST with 5% Normal Goat Serum at RT. Samples were then incubated in primary antibody solution for 48 hours with rotation at 4°C. The primary anti-body solution consisted of a 1:1000 ratio of anti-DAT (from the lab of Eric Gouaux) to blocking solution. After incubation, samples underwent four 10 minutes washes with PBST at RT with rotation in the same manner as above. The samples were then incubated for 72hrs at 4°C with rotation. The secondary anti-body solution consisted of a 1:1000 of Alexa Fluor 568 anti-mouse (Invitrogen, A11031) to blocking solution.

Results

Immunohistology

Our mutants were generated in the lab. In our mutants exon 2, which contains the start codon, of the *dat* gene is flanked by two Flippase Recognition Target (FRT) sites. Flippase recombinase (FLP) binds to FRT sites and triggers the excision of the DNA between the FRT sites via recombination. We conditionally expressed FLP in the $\alpha'\beta'$ KCs using the VT030604-GAL4 driver and in the PAM-DANs using the R58E02-GAL4 driver, thus upon excision DAT expression would theoretically be abolished in those regions. Moreover, we inserted a ubiquitin-GFP cassette alongside exon 2 that allowed us to screen for the mutants using fluorescence microscopy.

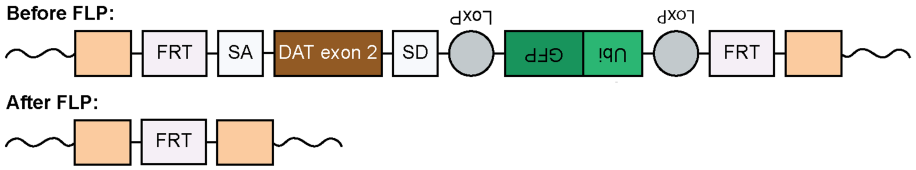

Figure 3. Simplified diagram showing the edited portion of exon 2 before and after FLP excision.

We used immunofluorescent staining to compare the extent of DAT expression between flies that had DAT conditionally knocked out of reward DANs and wild-type flies. We see that the faint expression of DAT in the vertical portion of the $\alpha'\beta'$ lobe is hardly affected by the knockout. Immunorectivtiy in the horizontal lobe, however, is significantly lower than in our wild-type control. Despite the reduced presence of DAT in the horizontal lobe, we see residual expression of DAT in the $\beta'2$ compartment. This residual expression could represent DAT expression in $\alpha'\beta'$ KCs. These images provide the first evidence of DAT's presence in non-dopaminergic neurones and reveal the subcellular location of DAT in $\alpha'\beta'$ KCs. Among MB compartments, the $\beta'2$ compartment has the highest variety of dopaminergic subtype innervation, being innervated by 3 different DAN subtypes: PAM-β'2a, PAM- β'2p and PAM- β'2m . This relative variety may suggest that KC-MBON synapses within the $\beta'2$ compartment may be prone to interference generated by nearby dopaminergic signalling originating from different DAN subtypes. Thus, increased DA transport within this region may serve to limit potential crosstalk by limiting the sphere of influence of DA transients. For example, the PAM- β'2mp DAN subtype has been shown to mediate sugar-reward memories in hungry flies while the PAM- β'2a subtype inhibits thirst in water-sated flies . Cross-synaptic interference between these neurones could cause inaccurate representations of the fly's internal state, and thus crosstalk would have to be limited to preserve specificity within these circuits.

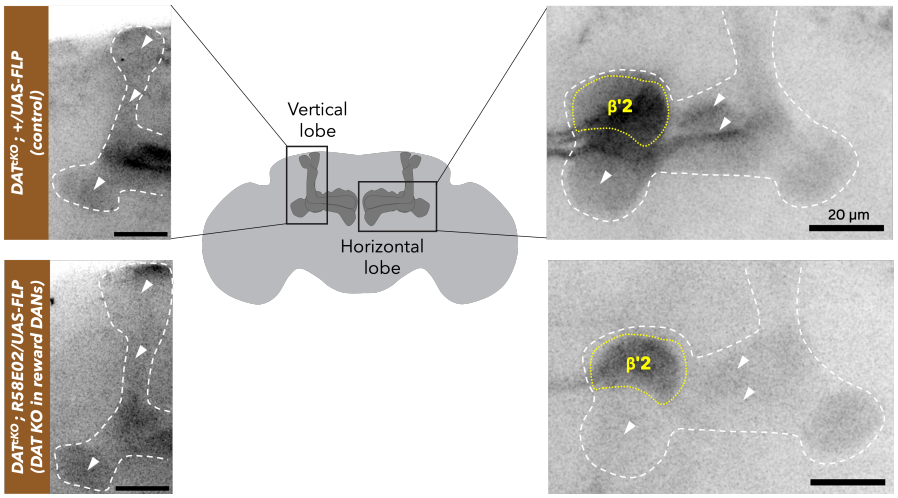

Figure 4. Left panels show the immunoreactivity pattern in the vertical lobe of the $\alpha'\beta'$ KCs of wild-type flies and the reward DAN DAT knockout mutant. Right panels show the horizontal lobes in the same two genotypes. The dotted line shows the outline of the MB, and arrows show areas with significantly different levels of immunoreactivity.

Behaviour

Finally, we conducted behaviour experiments to show how the knockout of DAT affected the mutant's ability to form immediate appetitive memories. We used our olfactory T-maze assay to compare both the PAM-DAN knockouts and the $\alpha'\beta'$ KC knockouts against control genotypes. Our behavioural data suggests that the presence of DAT in PAM-DANs or KCs doesn't significantly impact the strength of appetitive sugar memories. In contrast to whole-brain knockouts such as the *fmn* mutant which display decreased appetitive sugar memory scores. This suggests possible functional redundancy between these cell types where DAT expression in either group is sufficient to maintain the memory phenotype. This could mean that DAT expression in either cell type is sufficient to suppress interfering crosstalk between cell-types.

Discussion

We've showed that our conditional knockout strategy allows us to achieve near complete DAT suppression in cell-specific manner. Thus, this same strategy could possibly be leveraged to knockout DAT expression in other neurones.

We also see that the DAT in $\alpha'\beta'$ KCs is localised to the $\beta'2$ compartment. We do not know why DAT is localised to this specific compartment, and future investigation would be required to understand how this localisation relates to high variety of DANs innervating this region.

Finally given that we only tested mutants with DAT knocked out in either cell type, it would be interesting to quantify how both appetitive sugar memory would be affected by DAT knockout in both PAM-DANs and KCs. It would be fascinating to see whether the simultaneous PAM-DAN and KC knockout would display the same phenotype as our DAT null mutants. Moreover, we only tested the mutants against immediate appetitive sugar memory, therefore it would be illuminating to characterise the mutants' long-term memory phenotype. And given the high functional variety of DANs that input into the the $\beta'2$ compartment, characterising the phenotypes of other types of memory, such as aversive memory or appetitive water memory, would allow us to quantify how DAT expression affects this variety.

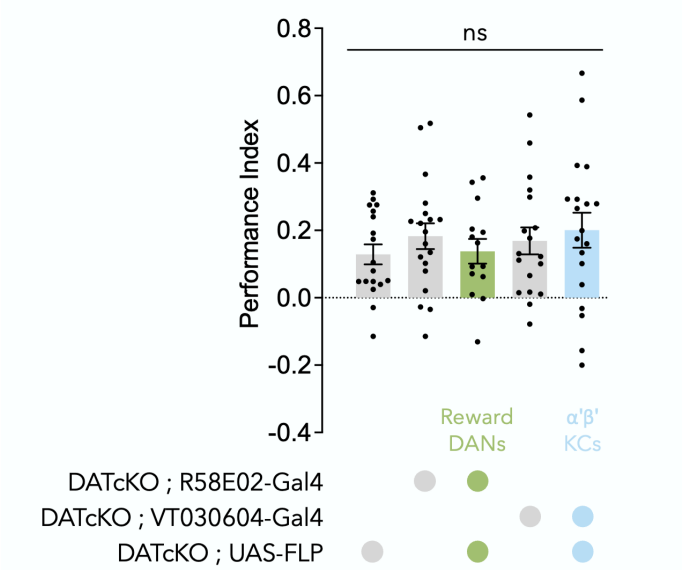

Figure 5. A bar plot showing our the results of our behavioural experiments. We see that knocking out DAT in either cell type did not significantly alter memory performance. Individual data points correspond to independent behavioural experiments.

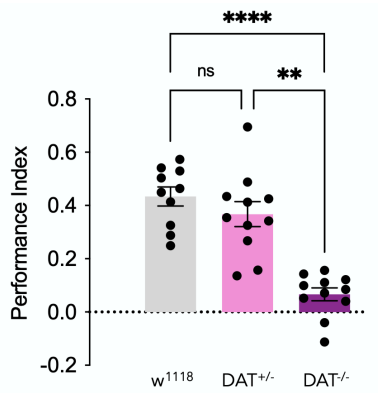

Figure 6. A bar plot comparing appetitive memory scores of wild-type flies, our DAT conditional mutant flies and *fmn* mutant flies. Individual data points correspond to independent behavioural experiments.

Acknowledgments

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