The potential neuronal role of the activity-regulated gene Hr38 and dopamine-induced synaptic plasticity within long-term memory

Patrick. C. Hughes

Durham University, Department of Biosciences

The Laidlaw Undergraduate Leadership and Research Scholarship

Abstract:

Synaptic plasticity is a core mechanism responsible for neuronal functions such as long-term memory and learning, as when humans and animals learn, the synaptic plasticity within neuronal networks are responding to specific cues which elicit various changes in behavior[2]. Additionally, recent findings have suggested that activity-regulated genes (ARGs), such as *Hormone receptor-like in 38* (Hr38), and dopamine-driven synaptic plasticity are equally crucial for long-term memory (LTM) and memory consolidation, as well as the re-evaluation of learned information and the integration of various 'internal states'[1,2]. Surprisingly, ARGs are poorly studied in well-used model organisms like Drosophila, thus, in the hopes of further understanding the neuronal mechanisms behind LTM, here we stimulated Drosophila neurons with appetitive conditioning to induce dopamine-driven plasticity, and then used the GAL4-UAS system, along with interference RNAs (RNAis), to knock-down and subsequently try to deduce the role of Hr38 within LTM[1].

Introduction:

Groundbreaking work during the 1960s demonstrated that synaptic plasticity is heavily involved in development via manipulation of sensory environments, and later studies continuing this work then showed that said plasticity was actually not limited to periods of development but instead persists with everyday brain functions[4]. To clarify, synaptic plasticity, which is also termed activity-dependent synaptic plasticity, is the process by which perpetuated activity results in changes for the strength of connections between neurons[2]. Theoretically, the activity in question leads to improved memory and learning capabilities by altering neuronal connections and firing within the hippocampus, but more interestingly, if the activity occurs concurrently with a positive or negative outcome/'valence' it is thought that the activity acts as a neutral stimulus which is then associated with the subsequent outcome[2]. This, in turn, is termed associative or olfactory association, depending on what neutral stimuli is used, and, theoretically, when paired with a positive valence, dopaminergic neuros (DANs) induce dopamine secretion for modifying neuronal connections and consolidating memory, something which is coined dopamine-driven synaptic plasticity[2].

In addition to synaptic plasticity, activity-regulated genes (ARGs) also play important roles for neuronal functions including long-term memory (LTM), however, unlike synaptic plasticity, the basic understanding of ARGs is essentially incomplete, especially within model organisms such as Drosophila[4]. Thus, in an attempt to further understand the neuronal mechanisms behind LTM, the aim was to manipulate and deduce the role of one ARG of particular interest, *Hormone receptor-like in 38* (Hr38), which is the only ARG identified within Drosophila and is believed to be involved with the memory consolidation and LTM of Drosophila via the process of appetitive conditioning (olfactory association with a positive valence)[1].

Materials and methods:

To obtain accurate results that depict the activity of Hr38, and thus allude to a greater understanding of LTM, various materials are required, the majority of which are explained below in company with the protocols followed for this report and include:

1. Drosophila and the GAL4-UAS system:

Drosophila is used as a model organism, or more specifically an organism which can easily possess and phenotypically visualize a gene of interest (GOI), such as Hr38, as it possesses various 'practical advantages'[7]. For example, Drosophila are easy and cheap to store to facilitate high-throughput experiments, they have a short development period of 10 days that allows 'pedigrees' from several generations ahead to be easily planed and produced in a few weeks/months, they can be manipulated and observed with relative ease as they require no ethical clearing to be manipulated and therefore can be studied live, and crucially they possess multiple physiological similarities with higher organisms, such as humans, as represented by figure 1[5,7]. All of which makes experiments centered on flies key to focused experiments then carried out on higher vertebrates and mammalian models[7].

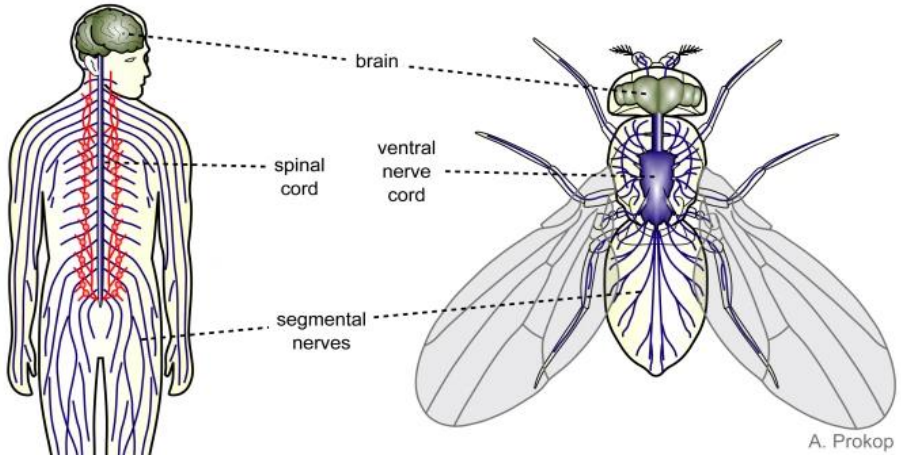


Figure 1[5]. Diagrammatic organization of both human and Drosophila based nervous systems. Source: Organs | droso4schools (wordpress.com) (Manchester Fly Facility)

As depicted above, both humans and flies share commonalities via their nervous systems and corresponding nerve cells/neurons[3]. For example, both have sensory neurons that lie outside the central nervous system for assisting vision, smell, taste, hearing, mechanical resistance (etc.), both have a subdivision of the CNS into the spinal cord and the brain, and many functional mechanisms centered on neurodegeneration and development were originally discovered in flies[5].

The final key reason why Drosophila are used for the referred LTM testing is due to the GAL4-UAS system. In laymen's terms, this system is a biochemical method co-opted from yeast to 'knockdown'/downregulate/'turn off' GOIs, including Hr38[7]. The GAL4 portion of the system itself is a transcriptional activator that binds to the UAS enhancers found within DNA to encode a GOI, Hr38, and RNAi (RNA interference, which is a conserved biological process focused on the suppression of gene expression via double-stranded RNA)[7]. This system works incredibly well in Drosophila and is often accompanied by temperature sensitive GAL80, to inhibit and control when a GOI is 'knocked down', and/or balancers which preserve a GOI within Drosophila progeny.

Finally, these new flies purchased and supplied to the laboratory are initially quarantined for a few generations/weeks to ensure that any undetected diseases or parasites in said Drosophila are excluded from upcoming testing[7]. Afterwards, stocks of Drosophila are contained within small vials of larval food made up of yeast, glucose, and flour, for easy transfer to fresh vials every 4 days and incubation at 18-25 degrees Celsius for regulated production of progeny and a 10-day larval development period.

2. Memory assays and the T-maze:

Prior to the beginning of this project, the discussed Drosophila had already completed the initial quarantine phase and the first cross of progeny containing Hr38, termed cross 0, had been produced in advance by Dr Vincent Croset and James Evans, allowing the LTM testing to be conducted faster than initially expected. To be specific, this is advantageous as Drosophila are not applicable to the LTM testing till they are 10 days old or have completed larval development and eclosed, and this is usually preceded by a few days of collecting virgin female Drosophila and segregating them from males to ensure the progeny produced contain the GAL4-UAS system with Hr38/the GOI, as otherwise Hr38 cannot be downregulated, and false results would be produced. Thus, having these initials steps completed for cross 0 saved at least 10 days.

Moreover, the LTM testing is typically divided into appetitive or aversive learning, with the former focusing on associating odors with sugar/nutritional rewards, while the latter focuses on electric shocks and associated odors. However, as inferred, time is a variable to consider, and as a result, here, the less-time consuming appetitive conditioning was preferred and chosen instead of aversive.

Appetitive conditioning relies primarily on olfactory conditioning with a neutral stimulus and a positive valence/nutritional reward, and the Drosophila being starved 24 hours prior to conditioning. For example, here, we used 2 odors, Indole-3-acetic acid (IAA) and 4-methylcyclohexanol (MCH), and during training associated with them with either water paper, a neutral stimulus, or sugar paper, a positive valence/reward. Moreover, after associating IAA or MCH with sugar paper, testing is then performed 24 hours later, and wild type Drosophila, Drosophila not containing Hr38 RNAi, are expected to prefer the sugar odor over the opposing one.

To elaborate, 2 days prior to training/conditioning, the adult progeny are ‘flipped’ into food vials for 24 hours to ensure all flies are fully fed. 1 day prior, the same progeny are flipped into agar starvation tubes, which essentially only contains deionized water for hydration and allows the flies to be starved and in turn make them eager enough to associate IAA or MCH with sugar, a nutritional reward that provides the energy needed for the protein synthesis within LTM during and after training. At the same time both the water and sugar paper are produced by covering filter paper with either deionized water or sucrose respectively and leaving them to dry for 24 hours as this prevents the paper from constricting the flies upon the day of training. The training and testing themselves are conducted within a behavior room kept at 60% humidity, 50 Pa of pressure, and 23 degrees Celsius with T-mazes, a device depicted by figure 2, as these are believed to be the optimum conditions for Drosophila to associate odors via olfactory association and to be tested for said LTM in an unbiased manner. Additionally, IAA and MCH are connected to test tubes via silicone tubing and consist of a ratio of 8µl odor: 8ml mineral oil.

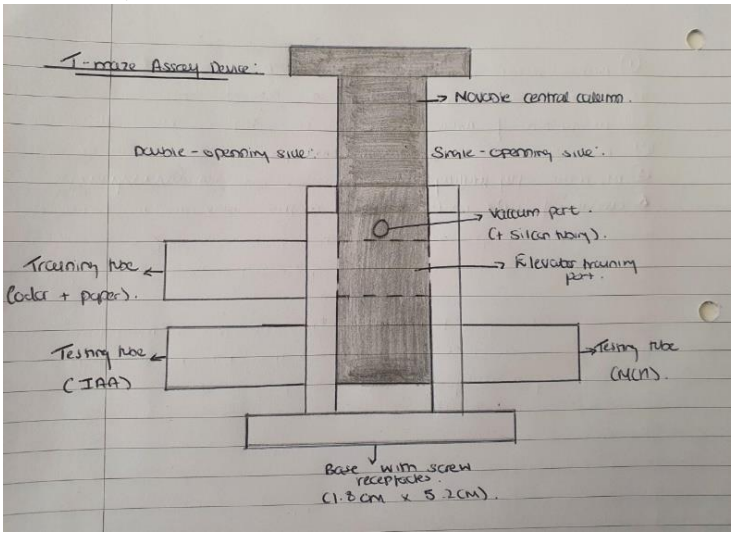


Figure 2. Diagrammatic illustration of T-maze memory assay device.

A T-maze consists of three plastic columns with a lift centralized to the middle column. Additionally, the most left column contains 2 compartments each for test tubes that respectively allows training and testing as the top compartment is used for training/associating IAA or MCH with sugar paper, and the bottom is used as the location for the sugar test tube which is connected to IAA or MCH by silicone tubing during testing. This is also the same for the further right column but for water instead of sugar. Finally, the central column also moves up and down to transport the Drosophila from the training test tube to the lower ‘testing test tubes’ where both MCH and IAA are being pumped in. Additionally, a photograph of the physical T-maze used for my training and testing is within the appendix of this report (figure S1).

During both training and testing you are performing 2 assays at once via 2 T-mazes with 2 parental controls as well: Hr38UAS x CS (no GAL4, genotype 2) and R19GAL4 x CS (no UAS, genotype 3) so you know Hr38UAS RNAi x R19B03 GAL4 (genotype 1) is working properly via a 50/50 unbiased split for both odors while the other 2 genotypes/controls prefer sugar. This is also followed up by other sugar preference and activity/avoidance tests to ensure results are reliable as they can be.

Finally, the steps for training and testing include:

a. Ensure the room is at 50 Pa of pressure, 23 degrees Celsius and 60% humidity.
b. Flip flies into a training test tube containing water paper for association with neutral stimulus/water = 2 minutes with MCH or IAA in the top compartment of the T-maze.
c. During this, rewater the filter paper within the original agar tube to ensure the flies are hydrated and increase their chance of survival during the next starvation period.
d. After 2 minutes, flip flies into a 2nd training test tube containing sugar paper and allow a 45 second break.
e. Place the second tube into the top compartment for association with sugar/positive valence = 2 minutes with opposing odor.
f. Repeat a-f for all progeny, with the same tubes, and then flip flies back into the rewatered agar tubes and starve/incubate for another 24 hours.
g. 24 hours later, begin testing by flipping a vial of flies into a 'clean' testing test tube.
h. Next lift the middle column of the T-maze and then bash flies into the lift of said T-maze. Also, at the same time push the lift down half-way to prevent flies escaping back into the test tube.
i. Attach both sugar and water test tubes into the lower compartments of the T-maze (the most left and right columns) with both MCH and IAA being connected to one tube each.
j. Turn off any lights, as flies are phototaxic, and push the lift fully down to the base of the T-maze for 2 minutes to allow flies to migrate to one tube. The controls should migrate to the sugar-related tube, while Hr38 RNAi (genotype 1) flies should act unbiased.
k. After 2 minutes, lift the lift back up to starting position, as this stops the flies form moving between the tubes in the lower compartment, and turn any lights back on. Then place flies into corresponding collection tubes, flies in the bottom left tube = 'A' collection tubes, and the bottom right tube='B' collection tubes.
l. Repeat h-l for all progeny with the same testing test tubes, and then freeze flies for counting.

Despite the straightforward protocol listed above, there is one caveat that must be discussed prior to moving onto to the next protocol focused on RNA extraction. Within this project, both training and testing act as memory assays that indicate whether the Hr38 crosses/progeny of Drosophila had their Hr38 downregulated and whether this downregulation in turn influenced memory consolidation and LTM, which shall be discussed later. However, to support this indication, both RNA extraction and qPCR are needed for quantifying Hr38 expression. Thus, during training there is allocated vials of Drosophila that are then frozen in liquid nitrogen at specific time points post-training. These time points include 0 hours after training, 2 hours after and 4 hours after and this subdivision were frozen so quickly to allow us to quantify Hr38 expression and physically see whether Hr38 was downregulated in comparison to more 'stable housekeeping' (HK) genes, including GADPH and SdhA, that rarely fluctuate in the majority of cells. In addition to freezing this subdivision, the remaining flies post-testing are then frozen at –4 degrees Celsius for approximately an hour and are counted to analyze the aforementioned indication centered on Hr38.

Thus, the following protocols are centered on flies frozen in liquid nitrogen and how, via RNA extraction and quantitative Polymerase Chain Reaction (qPCR), their genes are displayed for discussion/analysis, and, for clarity, they are respectively done with the RNA extraction/cDNA synthesis kits from biolabs with a PCR machine, and a qPCR machine:

3. RNA extraction/cDNA synthesis with New England Biolab cDNA kits:
    a. Clean work surface with RNA inhibitor spray as this protects very sensitive RNA from RNases. Also work with gloves for the same reason.
    b. Transfer flies from the agar tubes to 15 ml flacon tubes to freeze in liquid nitrogen, and upon freezing shake the tubes to remove Drosophila heads.
    c. Transfer the heads into 50µl of protection buffer in an Eppendorf tube and squish their brains with sterile morsel.
    d. Add another 250µl of buffer post-squishing and place samples at –80 degrees Celsius to preserve RNA, as RNA is temperature sensitive and degrades quickly at room temperature. Spin down prior to cover squished brains with protective reagents.
    e. After preservation, add 30µl of proteinase k and 15µl of proteinase k reaction buffer, and incubate at 55 degrees Celsius for 5 minutes.
    f. Vortex sample and centrifuge (spin down) at 16,000g for 2 minutes. Then transfer supernatant to a clean 1.5ml Eppendorf tube.
    g. Add 345µl of RNA lysis buffer, vortex and transfer supernatant to gDNA removal column.
    h. Centrifuge for 30 seconds, keep the supernatant, and add 690µl of ethanol to the supernatant.
    i. Transfer 800µl of mix to a RNA purification column, spin for 30 seconds, discard supernatant, and redo the step if any mix from step h is left over.
    j. Add 500µl of RNA wash buffer and spin for 30 seconds, discard supernatant.
    k. Pipette 80µl of DNase 1 onto column and keep at room temperature for 15 minutes.
    l. Add 500µl of RNA priming buffer and spin for 30 seconds, discard supernatant.
    m. Repeat step j twice, however upon second repeat spin down for 2 minutes instead of 30 seconds.
    n. Place column into a 1.5 ml collection tube and add 25µl nuclease-free H_2O. Spin for 30 seconds.
    o. Spin down one more time for 1 minute to remove ethanol traces.
    p. Place samples on ice and measure RNA concentration with a nanodrop machine.
    q. Once measured, create a 2x reverse transcription (RT) mix and transfer equal volumes of the mix into RNA samples and use a PCR machine to convert reagents into cDNA. The reagents for said mix and cDNA synthesis are located in the appendix (figure S2).

4. QPCR (quantitative Polymerase Chain Reaction) :

QPCR is a common approach for measuring the expression of target genes in a wide range of samples from many species, for example cultured cells from mammals[3]. To quantify gene expression, cDNA must be utilized with qPCR instead of RNA, hence step q of the RNA extraction protocol. The only other material required is a qPCR plate to hold the cDNA of interest[3].

A plate has 8 rows and 12 columns, and contains 3 technical repeats (3 samples of the same cDNA). Additionally, 3 biological repeats, or 3 repeats of the same gene, is also desired, and thus qPCR is normally performed with 3 plates, making 9 samples per gene of interest. An example of a plate used in our project is also located in the appendix (figure S3).

The target genes of interest for our qPCR included Hr38, and other genes that are either regulated by Hr38 or regulate Hr38 itself, for clarity these include: Nckx30c, Hsc703, Fntb, Aqz, SF2, Dikar, Tau, Wdb, Mrp33, NAChRaplha5, GluRia, GADPH, Mpcp2, and SdhA.

The last three targets are HK genes that act as positive controls, as HK expression shouldn't change drastically, and therefore they're essentially used to normalize data and see whether target genes are upregulated or downregulated in comparison to HK genes.

Once qPCR has been completed for each plate, the measurements of each sample is exported onto a pen drive for analysis and the following results. Finally, for clarity, the protocol for qPCR is also stated below along with figure 3, which is used to summarize the 'sequential' stages of both the prior RNA extraction and qPCR protocols[3].

a. Add cDNA mix to wells of a qPCR plate (8.4µl per well).
b. Add primer and SybGreen mix to the same wells (11.6µl per well) and pipette/mix samples.
- Interestingly, SybGreen mix intercalates between the minor grooves of double stranded DNA (dsDNA) and thus can bind to single stranded DNA (ssDNA) and RNA.
c. Seal the plate with sticky plastic and a ruler to ensure any reagents will not spill out of plate during qPCR.
d. Spin down the plate at 1000rpm for 1 minute.
e. Run the plate within a qPCR machine, which approximately takes 51 minutes per plate.

- During the run, the amplification curve is split into 3 phases, termed initiation, exponential, and plateau.
(Amplification = baseline/background recordings
Exponential = increase in fluorescence SybGreen mix and surpass minimum amount of qPCR cycles to become visible.
Plateau = when 20-30 cycles have been reached and fluorescence runs out)

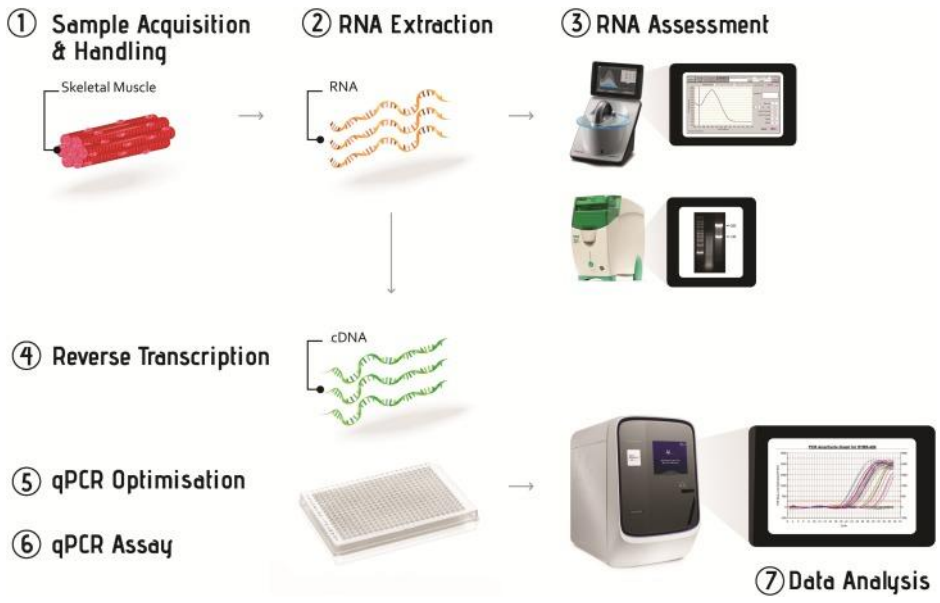


Figure 3[3]. Summary of RNA extraction and qPCR workflow.

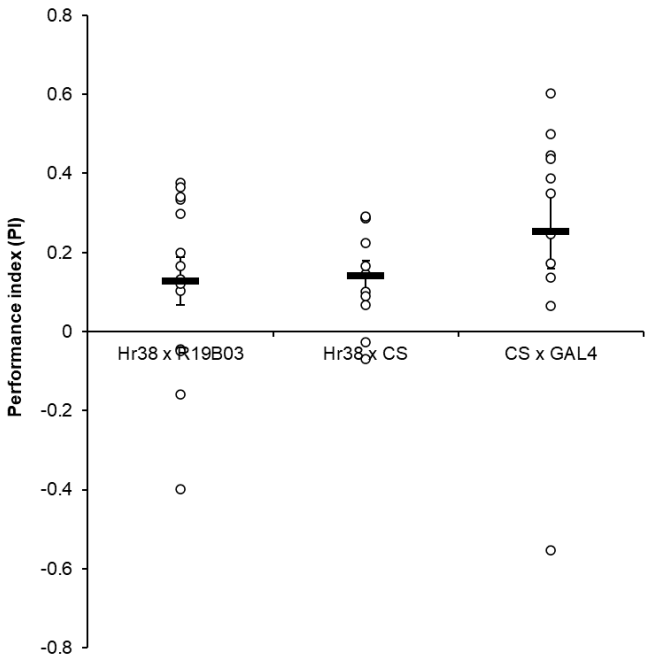
This figure is used primarily to depict the stages involved in the post-testing stages.
Steps 1-4 = RNA extraction protocol, 5-6 =qPCR protocol.

Results:

From the prior six weeks, the following results from both the memory assay and qPCR-based protocols were produced:

1. Memory assay – results:

A.



B.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	14	1.782977	0.127356	0.051056
Column 2	11	1.563257	0.142114	0.015481
Column 3	11	2.788973	0.253543	0.098442

ANOVA

ce of Varia	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between G	0.110781	2	0.05539	1.013824	0.373851	3.284918
Within Gro	1.802959	33	0.054635			
Total	1.91374	35				

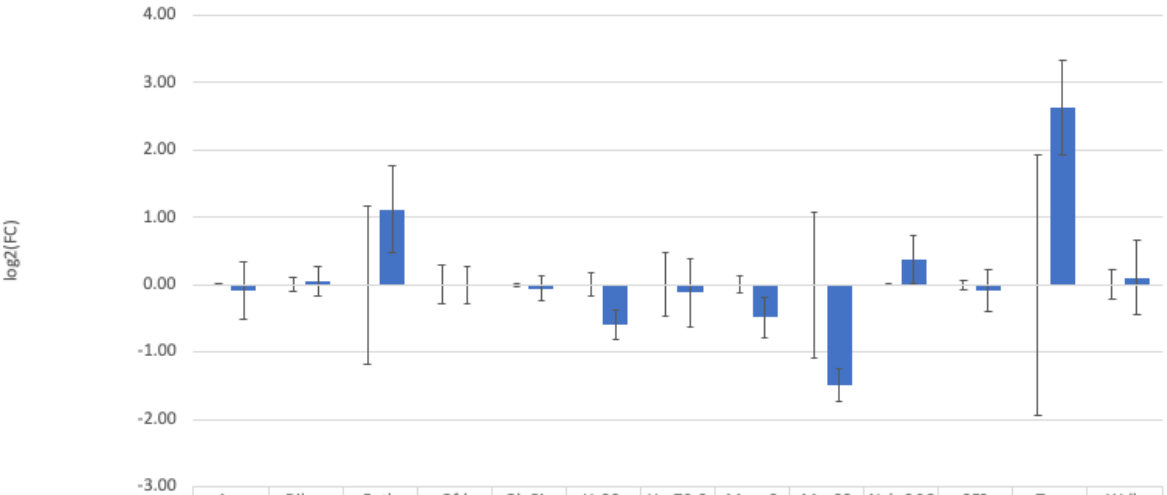
Figure 4. Memory assay data (08/08/2022 - 09/09/2022)
Figure 4a. Scatterplot depicting memory assay data.
Figure 4b. One-way Anova of memory assay data.

Both figure 4a and 4b focused on the 3 genotypes of Drosophila used during the memory assay protocols:

1. Hr38 RNAi x R19B03 GAL4 (Hr38 knockdown –expected 50/50 split between odors/sugar and water)
2. Hr38 RNAi x CS (parental control 1 – no knockdown, expected preference for sugar, however **proved difficult to handle**)
3. R19B03 GAL4 x CS (parental control 2 – no knockdown, expected preference for sugar)

Unfortunately, as displayed by figure 4b, none of the results proved to be significantly different ($p>0.05$), and thus difficult to interpret whether Hr38 is involved in memory or not.

2. qPCR data – results:



	Aqz	Dikar	Fntb	Gfrl	GluRia	Hr38	Hsc70-3	Mpcp2	Mrp33	Nckx30C	SF2	Tau	Wdb
CS X Hr38 RNAi	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MB-Gal4 X Hr38 RNAi	-0.09	0.04	1.12	-0.01	-0.06	-0.59	-0.12	-0.49	-1.49	0.36	-0.09	2.63	0.10

Figure 5. Expression test for Hr38 and other target genes.

Figure 5 represents the data obtained from the qPCR utilized to quantify the expression of Hr38. Interestingly, Hr38 is not so strongly downregulated as expected, while for the other target genes, I had to remove both biological replicates number 3 as the values were completely off and expression values were extraordinarily high, thus there were only 2 replicates for each genotype. Also, only SdhA was used as a HK as some values were missing for GAPDH, and unfortunately there was no good data for nAChRalpha5.

Discussion/conclusions:

From the results, a few things centered around Hr38 and LTM can be extrapolated. Firstly, from the memory assays, both parental controls, genotype 2 and 3, consistently preferred sugar over water, indicating that olfactory association/appetitive conditioning works, and the Drosophila progeny had their LTM functioning by associating IAA and MCH with both the nutritional reward of sugar over the more neutral water. However, as stated genotype 2, Hr38 UAS x CS, proved difficult to handle and there was consistently less progeny for genotype 2 than both genotype 1 and 3, Hr38UAS RNAi x R19B03 GAL4 and R19B03 GAL4 x CS. Potentially this suggests that the UAS system is 'leaky' and as a result there was impaired development and LTM during the development phase, something which occurred sooner than intended, but this does remain to be proven. Additionally, despite the positive result, the memory assay results were also not significant ($p= 0.373851/p>0.05$), and thus it cannot be stated with confidence that Hr38 is involved in LTM periodically and instead remains ambiguous as samples of genotype 1 only performed an expected unbiased preference between IAA and MCH occasionally (figure 4).

Moreover, from the qPCR expression tests (figure 5), it is demonstrated that Hr38 is decreased in genotype 1 samples, showing that the RNAi line used in the project worked successfully by downregulating Hr38 as expected. Furthermore, it was seen that by downregulating Hr38, the RNAi in turn caused downstream fluctuations in the levels of expression of the other target genes, including a decrease in Mrp33 and an increase in Tau, implying a respective decrease in mitochondrial RNA processing and an accumulation of tau, which possibly relates to an increased chance of developing tauopathies, such as Alzheimer's[6]. However, as noted, some of the qPCR samples obtained were quite poor in quality and had to be removed from calculations as a result, thus, in the future, I would wish to re-attempt the qPCR protocol and expression tests to obtain more accurate results on the expression of Hr38 and its downstream effects on tau and the other target genes involved.

In relation to future work, despite the lack of significance, I would also like to use another GAL4 line to investigate whether Hr38 is involved in other parts of the Drosophila mushroom body, the centre of learning and memory for Drosophila, and other functions relating to memory. This could also be done in co-ordination with using another Hr38 line to disprove or prove the results displayed in figure 4 and in turn disprove or prove Hr38's involvement with LTM specifically. Finally, I would also wish to perform more memory assays based on female Drosophila and male Drosophila separately to investigate the sex-specific differences with Hr38 and LTM, as Hr38 is believed to be upregulated in males when compared to females.

Overall, despite the room for expansion, it can be said that greater knowledge on neuronal mechanisms and networks was achieved as I managed to show that appetitive conditioning does indeed induce olfactory association and LTM in wild-type Drosophila, and while it was not possible to deduce the role of Hr38, it can be suggested that Hr38 is involved with memory as dysregulation of Hr38 fluctuated the expression of other genes downstream, possibly leading to impaired functions that would be normally be performed at least in conjuncture with LTM. However, further work will be required to clarify this suggestion.

Acknowledgments:

It has not escaped my attention that this project could not be completed without the funding provided by the Laidlaw Scholarship and the much-needed assistance from the members of lab 20 within the Department of Biosciences. In particular, I am indebted to Vincent Croset for acting as my supervisor and providing me a place in the department, and crucially I am indebted to James Evans for providing guidance on the protocols and equipment, and countless answers to questions surrounding the project itself. Thank you.

Appendix/Supplementary materials:

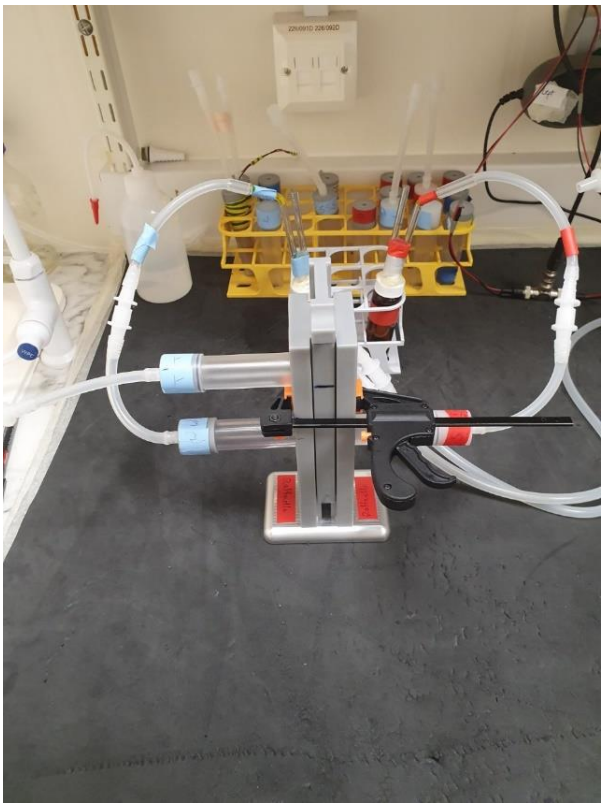


Figure S1. Photograph of T-maze:

The T-maze displayed here is the real-life version of the same one represented by figure 2 and is one of the two that is used by members of lab 20 in the Department of Biosciences at Durham University. For clarity its model name is Raphael and is shown here with both IAA (blue) and MCH (red) test tubes connected via silicon tubing as described above.

Reagent	Volume for 1 sample	n samples + 10% extra for pipetting error n*1.1 =
Nuclease-free H2O	4.2 uL	
10X RT Buffer	2 uL	
10X RT Random Primers*	2 uL	
25X dNTP Mix (100 mM)	0.8 uL	
MultiScribe™ Reverse Transcriptase	1 uL	
Total for RT mix	10 uL	
RNA!	10 uL	
Total with RT mix and RNA	20 uL	

Figure S2. cDNA RT mix reagents.

The materials listed are the reagents required to convert RNA into cDNA for qPCR and the expression tests performed after cDNA is synthesized. Each one is compiled into a 'master mix' that reduces time for pipetting and pipetting error that occurs during this protocol, increasing the chance of obtaining more accurate results later.

	UAS Hr38 RNAi x R19B03-GAL4 - 4 h						UAS Hr38 RNAi x CS (control) - 4 h					
	1	2	3	4	5	6	7	8	9	10	11	12
A	Hr38	Hr38	Hr38	Nckx30C	Nckx30C	Nckx30C	Hr38	Hr38	Hr38	Nckx30C	Nckx30C	Nckx30C
B	Hsc70-3	Hsc70-3	Hsc70-3	Fntb	Fntb	Fntb	Hsc70-3	Hsc70-3	Hsc70-3	Fntb	Fntb	Fntb
C	Aqz	Aqz	Aqz	SF2	SF2	SF2	Aqz	Aqz	Aqz	SF2	SF2	SF2
D	Dikar	Dikar	Dikar	Tau	Tau	Tau	Dikar	Dikar	Dikar	Tau	Tau	Tau
E	Wdb	Wdb	Wdb	Mrp33	Mrp33	Mrp33	Wdb	Wdb	Wdb	Mrp33	Mrp33	Mrp33
F	nAChRalpha5	nAChRalpha5	nAChRalpha5	Gfrl	Gfrl	Gfrl	nAChRalpha5	nAChRalpha5	nAChRalpha5	Gfrl	Gfrl	Gfrl
G	GluRia	GluRia	GluRia	GADPH	GADPH	GADPH	GluRia	GluRia	GluRia	GADPH	GADPH	GADPH
H	Mpcp2	Mpcp2	Mpcp2	SdhA	SdhA	SdhA	Mpcp2	Mpcp2	Mpcp2	SdhA	SdhA	SdhA

Figure S3. qPCR plate example.

Finally, figure S3 represents one of the plates used for qPCR, which in turn produced figure 5. In particular this was one of 3 plates used to investigate the expression of Hr38 and other target genes at the aforementioned 4-hour time point after training. This plate specifically (plate 1) contains UAS-Hr38 RNAi x R19B03 (Columns 1-6) and UAS-Hr38 RNAi x CS (control, columns 7-12) and was collected from genotype 1 flies (Hr38 RNAi knockdown) and genotype 2 flies (parental control) respectively to quantify Hr38 expression and subsequently compare the samples to see if Hr38 expression was in fact knockdown or not.

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