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Department of Microbiology
Master´s thesis • 30 hec • Second cycle, A2E
Biotechnology - Master's programme • Examensarbete/Sveriges lantbruksuniversitet,
Institutionen för mikrobiologi, 2014:2 • ISSN 1101-8151
Uppsala 2014

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Credits: 30 hec
Level: Second cycle, A2E
Course title: Independent project/Degree project in Biology - Master's thesis
Course code: EX0565
Programme/education: Biotechnology - Master's programme

Place of publication: Uppsala
Year of publication: 2014
Title of series: Examensarbete/Sveriges lantbruksuniversitet, Institutionen för mikrobiologi
no: 2014:2
ISSN: 1101-8151
Online publication: http://stud.epsilon.slu.se

Keywords: Dopamine, Octopamine, Octopaminergic Neurons, CREB(cAMP response element binding protein), DopR1 (Dopamine receptor 1) and DopR2 (dopamine receptor 2), *Yw* flies (genetic markers), UAS/GALV4 system, Tdc2 neurons (Tyrosine decarboylase 2)

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Abstract

Dopamine and octopamine, a possible analogue of noradrenaline, are among a few neurotransmitters which play a crucial role in several physiological processes. Dopamine participates in learning, memory and in reward, whereas, octopamine participates in egg laying, fight and flight response and also exhorts aggressive behaviour. Researchers had demonstrated that dopaminergic signalling can act antagonistic to octopamine in *Caenorhabditis elegans*. When *C. elegans* was fed, it exhibit sleep and reward emotions, due to the production of Dopamine. Conversely, when animals were subjected to fasting, they have produced octopamine, by suppressing dopamine signalling, and this lead to CREB activation, which eventually resulted in longevity *of C. elegans* (Suo et al.2009). These observations illustrate the need to investigate the dopamine and octopamine interactions and their effects on longevity in another model organism, *Drosophila melanogaster*. To carry out my investigation, two dopamine receptors (DopR1 and DopR2) in octopaminergic neurons (neurons where their primary transmitter is octopamine) were knocked down and the insect's social behaviour as well as longevity was monitored. Based upon my study, I conclude that the cessation of dopamine receptors may not result in longevity of *D. melanogaster,* instead the starvation resistance of DopR2 knockdown flies was reduced when compared to DopR1 knockdown flies and wild type flies. However, it was observed that DopR2 knockdown flies showed increased aggressive behaviour, decreased male-male courtship and reduced activity when compared to wild type flies.

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1. Introduction

Drosophila melanogaster, also called the 'fruit fly', has provided us with unparalleled insights in terms of understanding the basic fundamental structure and functions of genes and proteins. Due to its short life cycle, one can study a gene of interest and its functions by knocking it out and investigating the phenotype of the resulting mutant. Although the number of genes in *Drosophila* is lower than in humans, many of them are homologous to human genes (Banfi et al., 1996), thus making it possible to study complex problems like Alzheimer's, Parkinson's disease (Iijima et al., 2004). Hence *Drosophila* is called the "jack of all trades".

1.1 Dopamine and dopamine receptor

Dopamine is a monoamine, synthesized from the amino acid tyrosine by aromatic L amino acid decarboxylase (Luca et al., 2003). Dopamine is a neurotransmitter and plays a major role in social and physiological relations. It is involved in reward, cognition, punishment, sleep, memory and pulse. (Di Chiara and Bassareo, 2007, Draper et al., 2007). Physiological actions of Dopamine is mediated by five closely related G-protein coupled receptors (Beulieu and Gainetdinov, 2011) and these G coupled receptors are classified into two different families; D1-like family and D2-like family. The D1-like family is further sub classified into D1 and D5 receptors, and D2 like family is sub divided into D2, D3, D4 receptors, (Jackson and Westlind-Danielsson, 1994, Jaber et al., 1997)

These receptors have the ability to modulate adenylyl cyclase(AC) and can stimulate the formation of cyclic adenosine 3',5'-monophosphate (cAMP) (Jaber et al., 1997). They are classified into two different families (D1 and D2) based on their ability to modulate AC as well as cAMP production. D1 like receptors are known to activate AC, which increases production of cAMP. D2 like receptors are responsible for inhibition of AC (Beaulieu and Gainetdinov, 2011, Jaber et al., 1997). D1- like family receptors not only modulates AC but they also play a moderate role in locomotion and they are located postsynaptically of dopamine receptive cells (Missale et al., 1998). Unlike D1 class receptors, D2 class receptors inhibit AC and are also found to express both postsynaptically and

presynaptically on dopamine target neurons and are involved in much more complex roles than D1 class receptors as they are present postsynaptically and presynaptically (Missale et al., 1998). Dopamine receptors also alter the calcium levels via stimulation of phosphatidylinositol hydrolysis with the help of phospholipase. They also have an active role in regulating sodium ions exchange (Beaulieu and Gainetdinov, 2011).

1.2 Synthesis of dopamine

Dopamine is synthesized from amino acid tyrosine into precursor L-DOPA by the enzyme tyrosine hydroxylase, the enzyme dopa decarboxylase converts L-DOPA molecule into dopamine (Cole et al., 2005). This enzyme is also believed to be responsible for longevity of *Drosophila* (Luca et al., 2003). Immunocytochemical studies on *Drosophila* nervous system revealed that the enzyme tyrosine hydroxylase and neuro transmitter dopamine have the same distribution pattern (Friggi-Grelin et al., 2003).

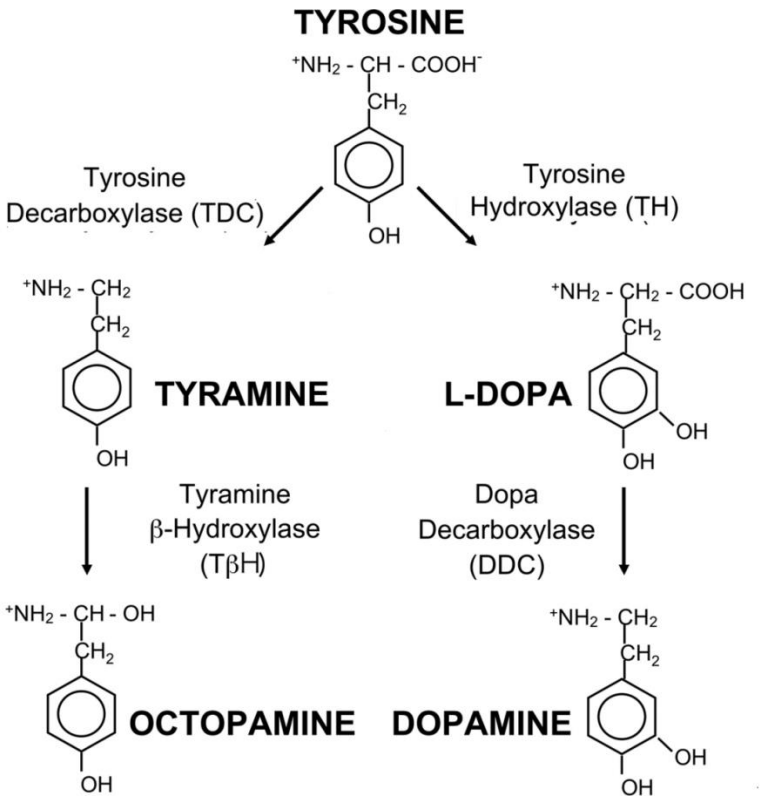

Figure 1. Dopamine and octopamine synthesis: Synthesis of both dopamine and octopamine starts from the amino acid tyrosine. In Octopamine synthesis tyrosine is decarboxylased to tyramine with tyrosine decarboxylase and then converted to octopamine with hydroxylase. In Dopamine pathway, tyrosine is converted to DOPA by tyrosine hydroxylase and then to dopamine by dopa carboxylase. Modified from (Cole et al., 2005).

1.3 Octopamine

Octopamine (OA), a possible analogue of noradrenaline, is one of the biogenic amines which play a crucial role in several physiological processes. OA, a neurotransmitter derived from tyrosine plays crucial role in regulating sensory functions in *Drosophil*a as well as in egg laying, sterility in females, flight, fights and aggression (Simon et al., 2009). Octopamine is involved in modulation of the skeletal muscles functions, visceral muscles functions (Orchard, I, 1987) peripheral target organs including fat body, oviduct, heart, and sensory organs, and gregarization in insects.

OA is present in high concentrations in the central and peripheral nervous systems of most invertebrate species, including insects, where it plays a multifunctional role. OA is a sympathomimetic amine and known as a false neurotransmitter because it can be stored in vesicles replacing endogenous classical amines such as norepinephrine, dopamine, and serotonin (Farooqui, 2012).

1.4 Synthesis of octopamine

Octopamine is a monoamine similar to mammalian nor-adrenaline, which is synthesized from the amino acid tyrosine. By decarboxylation, tyrosine is converted to tyramine by the enzyme tyrosine decarboxylase and the hydroxylation to octopamine with the help of tyramine beta hydroxylase (Figure 1). At least two different types of receptors are present in octopaminergic neurons OA1 and OA2 and the OA1 receptor upon activation increases intracellular level of calcium ions and OA 2 type receptor upon activation stimulates adenylyl cyclase and hence thereby increasing the amount of cyclic adenosine 3',5'-monophosphate (cAMP) (Balfanz et al., 2005).

1.5 Dopamine suppressing octopamine signalling

Dopamine is released when the animals are fed, and in Caenorhabditis elegans, dopamine signalling is activated just by tactile perception of food as the dopaminergic neurons in C. elegans are mechano-sensory (Sulston et al., 1975). In C. elegans, it was also observed that the activation of dopamine signalling due to feeding had led to a decrease of octopamine signalling. Conversely, when animals were fasted they increased octopamine signalling and this activated CREB (cAMP response element binding protein) (Suo et al., 2006) and

ultimately would play role in C. elegans life span. CREB is a signal activated transcription factor that, after phosphorylation, activates expression of genes from promoter region containing cAMP response element enhancer, and takes part in cell survival (Mayr and Montminy, 2001). In C. elegans, researchers in order to study the interaction between dopamine and octopamine have constructed dopamine synthesis mutant animals and these dopamine signalling mutants have spontaneously activated CREB (Suo et al., 2006).

1.6 UAS/GAL4 RNAi system

GAL4 encodes a protein of 881 amino acids in yeast, *Saccharomyces cerevisiae,* as a regulator of genes (Duffy 2002). *GAL4* regulates transcription of genes by binding to four related 17 base pair sites and these sites define an upstream activator sequence (UAS), which is analogous to an enhancer. This ability of *GAL4* and UAS expression lead scientists to study various gene expressions in *Drosophila* (Fischer et al., 1988). The discovery that expression of the *S. cerevisiae GAL4* gene in *D. melanogaster* does not result in deleterious effects helped Brand and Perrimon to develop the *GAL4*/UAS system for targeted gene expression in this organism (Brand and Perrimon, 1993). In this system, expression of target gene (also called responder) is controlled by the UAS element, because transcription of the responder needs the presence of *GAL4* and without the *GAL4*, the responder will be in silent state (Brand and Perrimon, 1993). To activate the transcription of the gene of interest, the responder lines are mated with *GAL4* lines, and these *GAL4* lines are also called driver lines (Duffy, 2002). Improved *GAL4*/UAS technology has later revealed better understanding on how to handle the driver lines or *GAL4* lines. *GAL4* lines are temperature dependent and in *Drosophila* fly the activity of *GAL4* expression is minimal at 16°C while 29°C provides maximal effects on fertility and maximal *GAL4* activity (Duffy, 2002). Hence just by altering the temperature the expression levels of gene of interest can be increased or decreased (Duffy 2002). This *GAL4*/UAS system was initially used to investigate the function of genes and their effects on phenotypes with altered gene expression, but recently the same technology has also been used in RNAi technology. Using this RNAi technology, researchers are able to silence or knockout a specific gene of interest. This is done by connecting an inverted sequence of the gene of interest to the UAS element and crossing the resulting lines to *GAL4* (driver lines) flies (Figure 3). This crossing will result

in formation of double stranded RNA (dsRNA) molecules (Giordano et al., 2002). Fly's immune system recognizes these dsRNA molecules as virus genetic material and will disintegrate them, thus achieving gene knockdown.

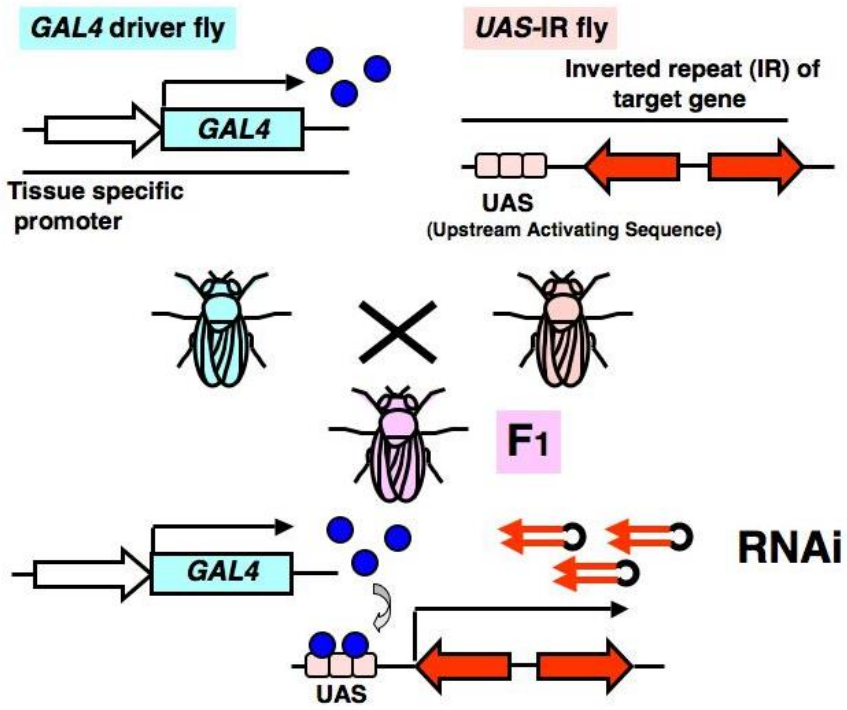

Figure 2. The driver fly has a transgene containing the yeast transcriptional factor GAL4. The responder fly has an inverted repeat of the target gene tagged to the UAS element. Crossing of these flies will result in F1 generation containing dsRNA of the target gene, which will be disintegrated by fly's immune system, thus achieving successful gene knockdown (Taniguchi. N, 2008) (Image obtained from 'Experimental glycoscience Glycobiology book with permission from Springer Japan publications).

2. Aim

In *C. elegans,* it was shown that when the organisms were fed they produce dopamine signalling and would reduce the octopamine signalling. Conversely, when starved, octopamine signalling was increased and dopamine signalling was reduced, this lead to the CREB activation and ultimately affecting longevity in *C. elegans*. My experiment was carried out to investigate whether the dopamine signalling would regulate the octopamine signalling and affect *Drosophila melanogaster's* longevity and social behaviour. To study the dopamine role in octopamine signalling, I have used UAS-*GAL4* RNAi system (Duffy, 2002) to knockdown dopamine receptors, DopR1 and DopR2 specifically at Tdc2 expression site , which is responsible for octopamine synthesis. The knockdown effect of DopR1 and DopR2 on octopamine was predicted by analysing behavioural phenotype.

3. Materials and Methods

3.1 Fly stocks

For this experiment I have used wild type flies, CSORC flies which were created by crossing the two available wild type flies Canton-s and Oregon- R. Tdc2 (w^{*};p{PUAS-Tdc2.c}) flies were crossed with Elav-*GAL4* flies to achieve driver flies. These driver flies were crossed with *yw* and w^{1118} to get control flies. Knockdown flies; Uas DopR1^{RNAi} and Uas DopR2^{RNAi}. See table 1 for all the strains and *Drosophila* species used in this experiment. All these flies were ordered from Bloomington stock centre Indiana USA.

3.2 Crosses

The wild type flies were created by crossing the two wild type flies available- the Canton-s and Oregon- R (CSORC). The other line was *yw* (yellow body and white eye) and was used as a genetic marker. The experimental flies were DopR1^{RNAi} (*yw;*UAS-DopR1^{RNAi}) and DopR2^{RNAi} (*yw;UAS*-DopR2^{RNAi}). To make driver flies, the Tdc2 flies were crossed with *GAL4* flies (w; p{tdc2-*GAL4*}). To get flies with knockdown Dopamine receptor 1, driver flies; Tdc2-*GAL4* flies were crossed with UAS-DopR1^{RNAi} (resulting in Tdc2-*GAL4*;UAS-DopR1^{RNAI}) and to make flies with dopamine receptor 2 knockdown, the Tdc2-*GAL4* flies were crossed with UAS-DopR2^{RNAi} (resulting in Tdc2-*GAL4*;UAS-DopR2^{RNAi}). To make control flies for starvation studies, Tdc2-*GAL4* flies were crossed with *yw (*Tdc2-*GAL4*;*yw*). Then another control flies were made for the aggression studies, to make these flies, Tdc2-*GAL4* flies (w^{*};p{PUAS-Tdc2.c}) were crossed with w^{1118} (Tdc2-*GAL4*;w^{1118}) flies. *yw,* w^{1118} ,G*AL4* flies, UAS-DopR2^{RNAi} ,UAS-DopR1^{RNAi} flies were all stored at 29°C in larval stage.

3.3 Starvation

In this experiment starvation was performed on flies to understand how the cessation of dopamine receptors will affect the fly's starvation resistance, and how it differs from the wild type flies. In order to achieve better understanding about the dopamine receptors, twenty virgin dopamine receptor 1 knockdown flies (DopR1) and twenty virgin dopamine receptor 2 knockdown flies (DopR2) were collected.

Virgin flies can be easily distinguished from adult flies as they have a dark spot on their abdomen. For collecting the virgin flies, the flies were first anesthetized with carbon dioxide gas. When the flies were asleep, flies with a dark spot on their abdomen were carefully isolated and transferred to a new chamber. After collecting the virgins, all twenty virgin flies of DopR1 and DopR2 were stored in two separate chambers. These flies were now aged up to 5-7 days. While ageing all the experimental flies and control flies were fed with standard fly food till the experiment was conducted on them.

While virgin flies were ageing, new agarose vials were prepared. After ageing, the flies were again anesthetized with carbon dioxide gas and carefully transferred into the newly prepared agarose vials, which were then closed with paraffin tape. Now after setting up experimental conditions, the fly's starvation resistance was monitored. At every 12 hour intervals the dead flies were counted and noted.

3.4 Aggression behaviour

Aggression assays were performed because biogenic amines are responsible for animal's aggression (Edwards et al., 2006) and here in my experimental flies the dopamine receptor, which controls the biogenic amine levels, which is believed to be responsible for aggression, is knocked down (Zhou et al., 2008). By conducting this assay it has exposed how fly's behaviour is affected when dopamine receptors were knocked down. When *Drosophila* is showing aggressive behaviour, it exhibits certain offensive characteristics like 1) high intensity fights and 2) low intensity fights.

High intensity fights: In high intensity fight experiment four different territorial fights were monitored (Johnson et al., 2009).

A) Wing threat: Where one fly moves its body in parallel to the other and lifts one wing and vibrates it

B) Fencing: where one fly moves his body in parallel to the other fly and both extend their legs to one another and then fence.

C) Lunging: where an aggressive fly approaches quickly towards non-aggressive fly and quickly pounces or gives head butts.

D) Boxing: Where both the flies come into fight with their front legs lifted up, standing on hind legs and then they box often retreating to ground and again stand on hind legs and box.

Low intensity fights: Low intensity fights are wing flicks and pushing, where one fly orients towards the other and flicks its wings, pushing; where one fly moves in parallel to the other and pushes it away.

In order to perform these aggression assays, a cylindrical behavioural chamber with 2 cm by 2.5 cm (height * diameter) was filled with 1% agarose up to 1.5 cm in height in order to provide appropriate humidity to flies. New knockdown virgin male flies and control virgin male flies were collected and incubated at 25°C for 5-7 days. Then while performing the assay, two flies, one knockdown fly and one control fly were anesthetized under the influence of carbon dioxide and then transferred to behavioural chamber. A Panasonic HDC-SD90 camera was used to record the fly's activity. Each session of activity was recorded for 20 minutes and ten replicates were conducted.

3.5 Male-Male courtship behaviour

Male-male courtship behaviour studies were performed on 5 days old virgin males, flies were collected similar to previous experiments, stored and aged up to 5 days and then males which were collected to test were transferred into a vial, later a second male was introduced into the same vial, and their courtship was recorded using camera, to analyse their interactions wherein only their acceptance behaviours were considered (tapping, abdomen bending, circling, licking). Courtship index was calculated and it was measured by total time taken by the fly to mate with other fly, and latency was measured by counting the time taken by the fly to initiate the courtship, but here in this experiment only the courtship behaviour patterns like tapping, winging out, abdomen bending, circling and licking were considered. These courtship tests were performed on Tdc2 *GAL4* controls, DopR1 and DopR2 knockdowns. Each ten replicates were investigated.

3.6 Speed and distance

To find out the *Drosophila's* locomotion and distance covered, the software CTRAX (ctrax.sourceforge.net/install.html) was used. This software can follow the locomotion of multiple insects. To perform this experiment 6 adult flies were collected. These flies were fed with normal standard fly food and stored in 250 ml bottles at 25°C on a 12h: 12h light-dark period, transitions between light and dark are immediate. While performing the experiment the flies were transferred to agar petri dishes, and their locomotion was recorded by using a HD camera (Panasonic HDC-SD90).

Flies were subdued by placing them on ice first for 2 min and then transferred to a petridish, where they were able to move, walk or run but not to fly. A HD camera (Panasonic HDC-SD90) was placed above the dish to record their activity. But before the flies were recorded, the flies were first left to acclimatize to the experimental setup and then recorded for 30 min. The recorded trajectories were analyzed by CTRAX software and the distances of flies' movements were calibrated based on the diameter off the petri dish. Then tracked data was transferred to MATLAB, which was used to calculate the activity of the flies by the distance they travelled per frame. Based on the image analysis, a standard threshold was established below which flies were moving but not walking and above which flies are walking, and the threshold which shows walking was given score of 1 and below threshold was awarded a score of 0.

3.7. Statistical Analysis

Mean and standard deviation from all replicates of each experiment was evaluated using Excel (Microsoft). Survival curves were analyzed using the log-rank test. One-way analysis of variance (ANOVA) was performed with appropriate post hoc test for multiple comparisons. A p-value of less than 0.05 was considered as statistically significant.

4. Results

To study behavioural changes in fruit flies, dopamine receptors (DopR1 and DopR2) were knocked down using UAS-*GAL4* system. To achieve desired knock outs following crosses were made.

i) Tdc2-*GAL4*xUAS-DopR1^{RNAi} (to knockdown DopR1)

ii) Tdc2-*GAL4*xUAS-DopR2^{RNAi} (to knockdown DopR2)

iii) CSORC (wild type)

IV) Control Tdc2-*GAL4*xw1118

4.1 Effect of starvation

Twenty virgin males, control flies (Tdc2_*yw*) and experimental flies (Dopamine knockdowns-DopR1^{RNAi} and DopR2^{RNAi}) were aged for 5-7 days and then transferred to agar vials and the flies were starved to death. The resulting dead flies were counted at every 12 hour interval. Flies which have Dopamine receptor 1 (DopR1) knocked down were seen to have similar survival rate compared to control flies. But unlike DopR1, the DopR2 has shown its effect on flies, these flies started to die from 24 hour and almost all flies were dead by 48 hours (Figure 3). The resistance was analysed by log rank test analysis software (http://bioinf.wehi.edu.au/software/russell/logrank/). The surviving time of DopR1 flies was similar to control flies (p=0.871), but the surviving time of DopR2 knockdown flies was reduced by 24 hours when compared to control flies (p=0.00129) and DopR1 knockdown flies (0.00165).

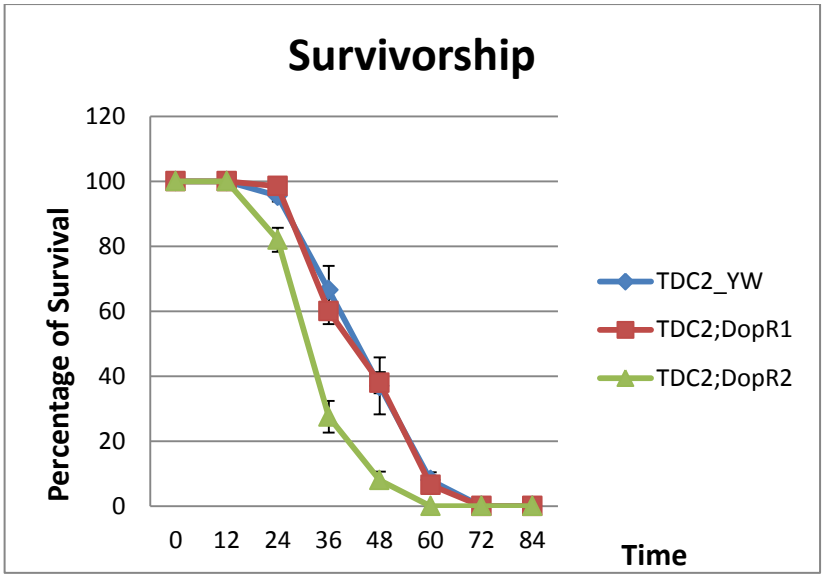

Figure 3. Effect OF DopR1 and DopR2 on starvation: Twenty flies of control and experimental flies were aged to 7 days and starved to death. The numbers of dead flies at 12 hours interval was noted and plotted against starvation time. Here a graph for DopR1 knockdown in Tdc2 neurons is shown with square shapes DopR2 knockdown in Tdc2 neurons as green colour and control blue colour is shown.

4.2 Aggression studies

A. High Intensity Fight

Aggression is natural among animals which helps in survival fitness. Behaviour studies were conducted on DopR1 and DopR2 knockdown flies respectively and compared with control flies. The aggression in the DopR1 flies was higher when compared to controls but not significant; whereas in the DopR2 knockdown flies the activity was significantly higher. In the total high intensity behaviour graph (Figure 4 (A)), DopR2 knockdown flies showed high intensity aggression ($p=0.0015$) when compared to DopR1 knockdown flies, control flies and wild type flies. But in the individual behaviours like wing threat ($p=0.66$) or fencing ($p=0.50$) no significant differences between control and wild types flies were observed Figure 4 (B). However there was significant difference in lunging ($p=0.05$), and chasing ($p=0.03$) and difference was higher compared to control flies, DopR1 and wild type flies.

Figure 4 (A)

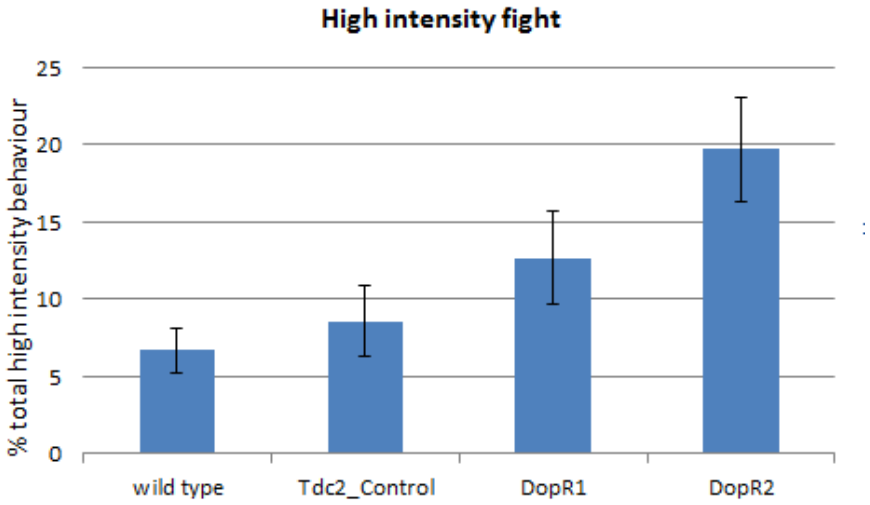

Figure 4 (B)

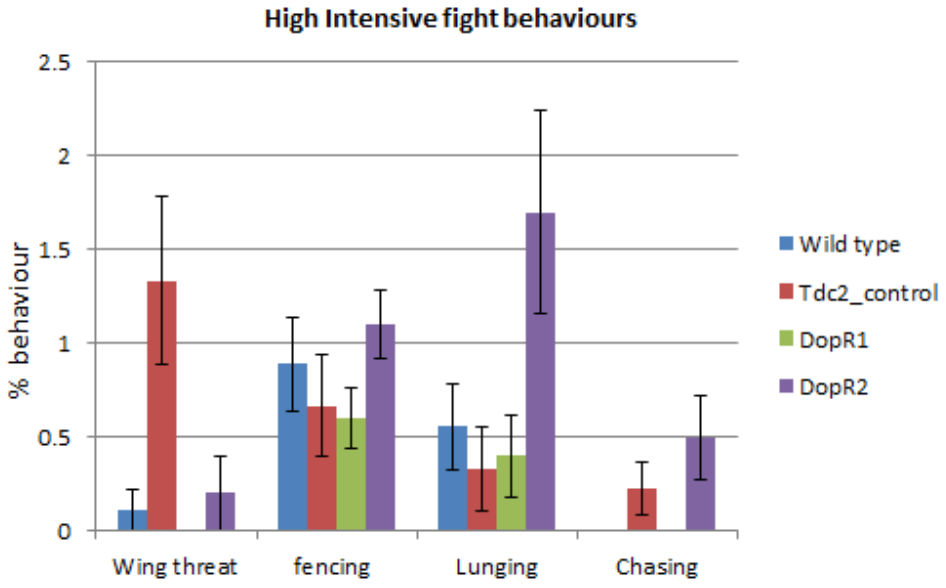

Figure 4 (A) & 4 (B). Increased octopamine signalling by inhibiting dopamine receptors affected *Drosophila's* high intensity fights. The aggression assay was conducted in behavioural chamber on isolated male virgin flies and aged them for 5 to 7. Different fly behaviours were observed and percentage behaviours were plotted against strains with mean and SEM.) 4(A): percentage high intensive fight. 4(B) different behaviours of high intensity fights wing threat (p=0.66), fencing (0.*50*), lunging (0.05) and chasing (0.03). Graphs represent results of DopR1 and DopR2 knockdown in Tdc2 expressing neurons.

B. Low Intensity Fight

Similar to high intensity fights low intensity aggression behaviour assay was also conducted. Virgin males were collected, aged for 5-7 days and transferred to behavioural chambers and the activity was recorded with a Panasonic HDC-SD90 camera. In low intensity fights only wing flick and pushing were considered. The score was given by counting the number of times fly flicks its wing on other fly and number of times the fly pushes the other fly. Using the score obtained from wing flick and pushing, a graph of wing flick and pushing against total percentage was made. The obtained graphs are shown in Fig (Figure 5 (A)) for total low intensity fight and (Figure 5 (B)) for individual low intensity behaviour. In low intensity assay graph, there was decreased wing flick activity ($p=<0.0001$) in DopR2 flies when compared to control and wild type flies. But when total percentage of low intensity activity is considered there seems to be no difference between control flies, wild type flies, DopR1 and DopR2 flies.

Figure 5 (A)

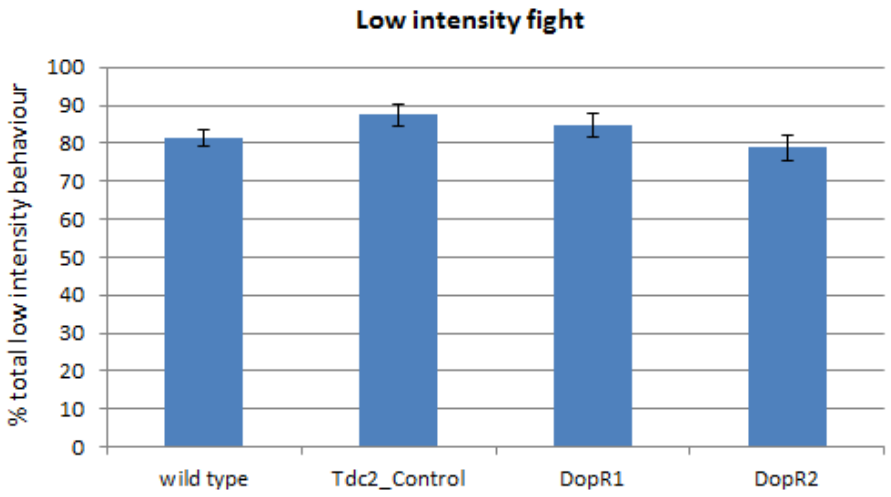

Figure 5 (B)

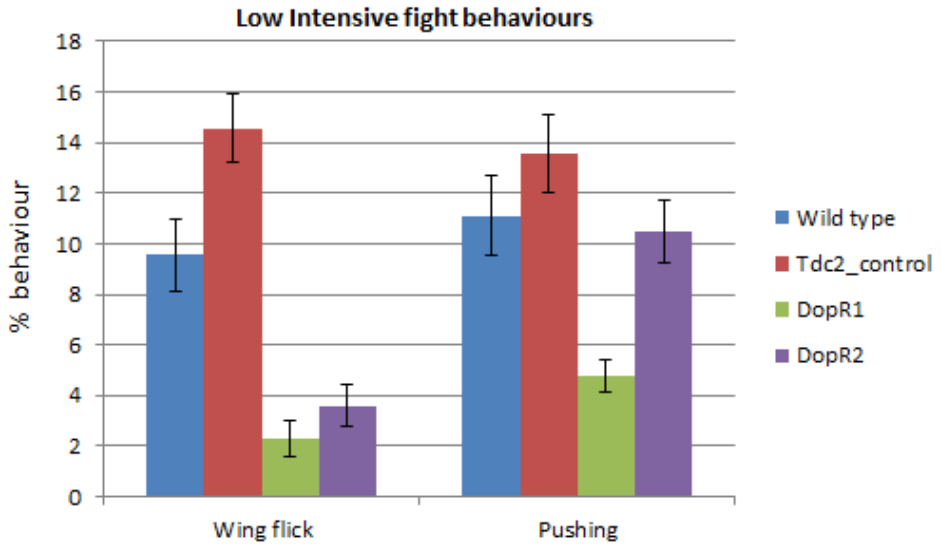

Fig 5(A) and 5(B). Increased octopamine signalling by inhibiting dopamine receptors did not increase *Drosophila's* low intensity fights compared to controls. Wing flick and pushing behaviours in low intensity fight was studied in controls (wild type, TDC2_control) and dopamine knockdown (TDC2; DopR1, TDC2; DopR2) flies, in both the assays, ten replicates were conducted, containing two 5-7 days old male virgin flies for each session of recording.

4.3 Male-Male Courtship behaviour

Male-male courtship behaviour study was conducted on DopR1, DopR2 knockdown flies, Tdc2 control flies and wild type flies. Regarding individual courtship behaviour (Figure 6 (B)) shows that in DopR2 knockdown flies, abdomen bend and circling was completely absent and activity was only seen in "one wing out", but still significantly less than wild type and control flies. Even in DopR1 knockdown flies the male-male courtship behaviour like abdomen bend and circling were absent compared to wild type and control flies. There was no significant difference between wild type flies and DopR1 and DopR2 flies, especially in licking and tapping. But according to (Figure 6 (A)), male-male courtship interests were clearly reduced in both knockdowns, DopR2 and DopR2, when compared to wildtype flies and Tdc2 control flies. This reduction in male-male courtship could be due to the increase in octopamine, and this increase could be due to the knockdown of Dopamine receptors (DopR1 and DopR2). In a study on Drosophila males (Certel et al., 2010) it has been shown that with the decrease of octopamine levels, the male-male courtship was increased.

Therefore it can be said that the octopamine levels in brain play a huge role in male-male courtship behaviour in Drosophila. The total male-male courtship behaviour in DopR2 (p=0.0001) was much lower than wild type flies, Tdc2 control flies and DopR1 knockdown flies. According to Male-male courtship assay, DopR2 seems to play a bigger role in deciding male-male courtship behaviours.

Figure 6 (A)

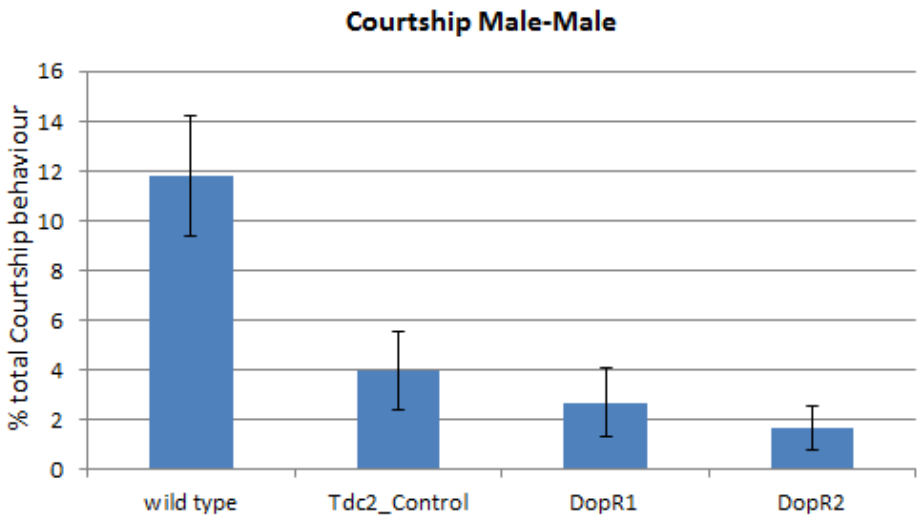

Figure 6 (A). Increased octopamine signalling had affected male-male courtship behaviour. The Mating assay was conducted between 5 to 7 days old isolated male with virgin female fly. Different mating behaviours were observed and percentage behaviours were plotted against strains with mean and SEM. DopR1 and DopR2 knockdown in Tdc2 neuron.

Figure 6 (B)

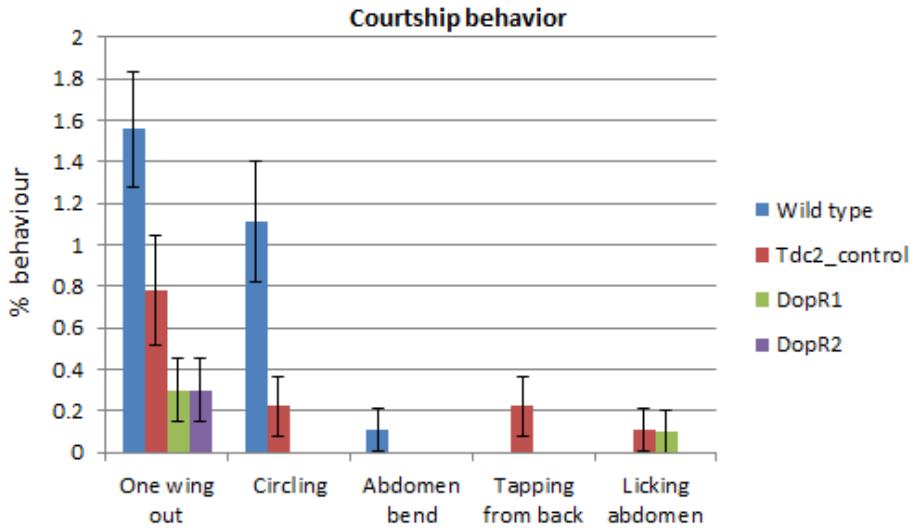

Figure 6 (B). Increased octopamine signalling effected fly's individual courtship behaviour. Male-Male courtship behaviour study was carried out by investigating the actions of one wing out, circling, abdomen bend, tapping from back, licking abdomen in control and dopamine knockdown flies and the observed values were plotted. One wing out (p=0.0010), circling (p=0.0008), abdomen bend (p=0.33), tapping from back (p=1.00), licking abdomen (p=1.000). Anova and posthoc test was performed for multiple comparisions.

4.4 Activity of flies

Activity and aggression are basic requirements for animal's survival, thus the total activity was measured. In order to measure total activity, *Drosophila's* movements like walking were recorded for a minimum of 30 minutes. Activity was determined at the percentage of time male spent activity walking over the period of 30 minutes. Cleaning, self grooming and licking itself was not considered. Figure 7 shows that activity of the dopamine receptor knockdown flies of both genotypes were less than controls and wild types. This may be because DopR1 and DopR2 control the flies' locomotion (Andersen et al., 1990) and thus knockdown of these receptors may be the reason for reduced activity among knockdown flies.

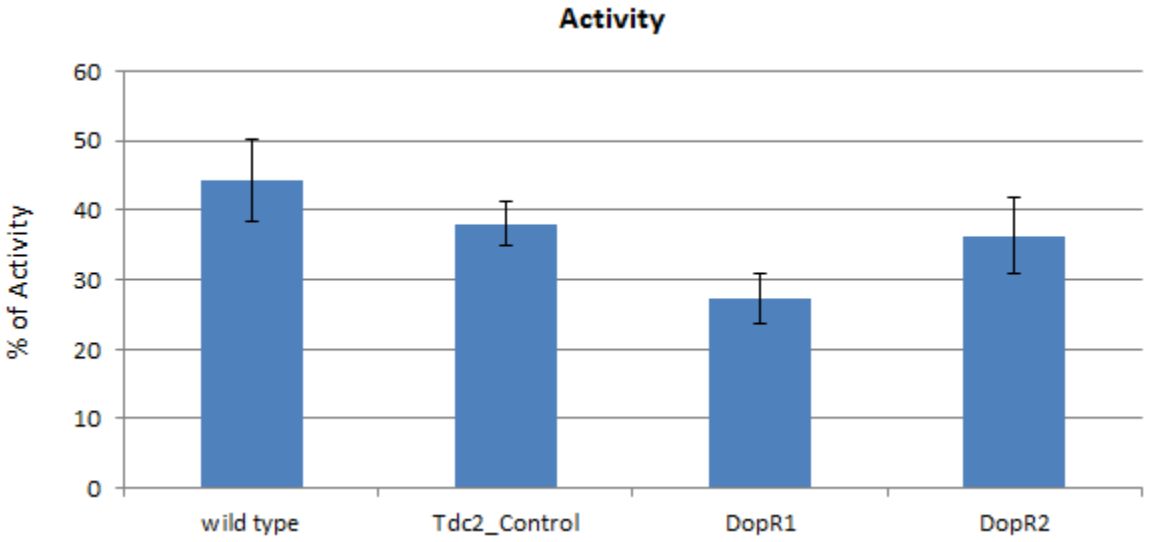

Figure 7. Increased octopamine had not increased activity of *Drosophila.* The graph 6.4 shows the total activity study on wild type flies, controls (TDC2_control) and dopamine knockdown (TDC2; DopR1, TDC2; DopR2) flies conducted by recording flies movements by camera. The overall activity percentage was not higher in DopR2 (p=0.28) and DopR1 flies when compared to control.

4.5 Speed and distance study

The rate of change in speed and distance was investigated and the observed values of respective flies were calculated and plotted. There was no significant change in speed among all DopR1 (p=0.09) flies when compared to control flies (Figure 8). But there was a change in speed among DopR2 (p=0.08) flies compared to control flies, but the obtained p value did not suggest any significant difference. There was no change in distance or top speed in DopR1 when compared to control flies, but in DopR2 (p=0.02) mutant flies, change was observed in speed when compared to DopR1 and control flies (Figure 8).

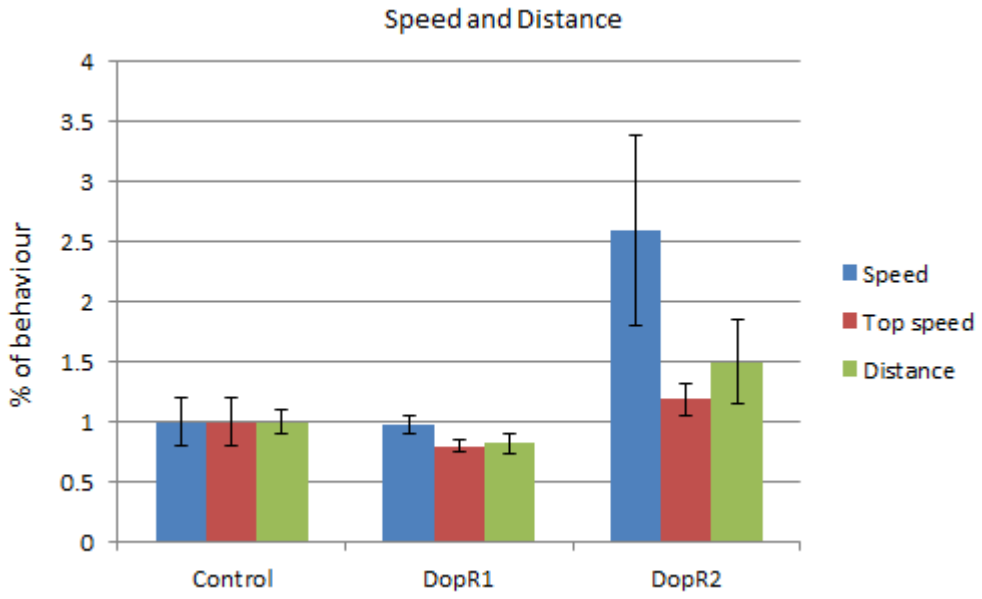

Figure 8. Influence of increased octopamine levels on speed and distance. CTRAX AND MATLAB were used to measure both speed and distance of walking of 5-7 days old male for each genotype. Males were put in behavioural assay chamber and observed for 30 mintutes. Control flies (Tdc2-*GAL4*) and dopamine knockdown (TDC2; DopR1 (p=0.09)) , TDC2; DopR2(p=0.08)) flies DopR2(p=0.02) compared to DopR1 and control. ANOVA and posthoc test was performed for comparisons).

There was a slight change in top speed in between DopR1 and DopR2 (p=0.02) knockdown flies. This result may suggest that the octopamine will help the insects in flight and may also help in production of energy required for flight (Orchard et al., 1993), but the flies could not cover long distance because the high levels of octopamine would have burned the energy in a short period of time resulting in the flies getting exhausted quickly.

5. Discussion

Octopamine may influence the survival rate in Drosophila

It is known that hormones control social behaviour and play a considerable role in metabolism, sleep and addiction. Among many hormones, dopamine and octopamine have considerable role in social behaviour. The result obtained from starvation assay shows that the survival rate of DopR2 knocked down flies was decreased by 24 hours, compared to control and DopR1 knock down flies. In *C. elegans*, octopamine signalling along with serotonin and mianserin signalling had led to the activation of CREB. Ultimately, then the activation of CREB was shown to extend longevity in *C. elegans* (Suo et al., 2006). In contrast to this finding, starvation experiment indicated that an increase in octopamine may be responsible for early death of *Drosophila*. Similarly according to satoshi suo report, dopamine signalling suppresses octopamine signalling, In this experiment increase in octopamine signalling could be due to the diminished dopamine signalling (Suo et al., 2009). One of the possible reasons for the reduced starvation resistance of DopR2 knockout flies (Figure 3) might be due to the increased octopamine in neurons. The D2 class receptors are involved in inhibition of AC and the other possible reason for the reduced starvation resistance in DopR2 knockdown flies could be due to the increased AC.

Knockdown of DopR1 and DopR2 had reduced male-male courtship.

Courtship results show that DopR1 and DopR2 receptors are involved in courtship behaviour regulation. DopR1 and DopR2 mutant flies showed decreased male–male courtship, this may be due to decreased amount of receptors and thus diminished dopamine signalling due to the receptor knock down (Liu et al., 2008). A recent report suggests that altered neurotransmitter concentration in synaptic cleft could induce male–male courtship behaviour, possibly as a result of changed sensitivity of postsynaptic receptors towards the neurotransmitter (Chen et al., 2012). Due to the insensitivity to dopamine it can be speculated that male flies display male–male courtship behaviour. Recently, it has been reported that Dopamine influence male-female courtship (Alekseyenko et al., 2010) and Octopamine is believed to decrease male-male courtship and increase aggression behaviour (Certel et al., 2010)

It was assumed before, that Male-male courtship behaviour is due to incapability of flies in distinguishing female and male targets But Chen et al (Chen et al., 2012) reported that DopR1 mutant showed the male-male court ship behaviour and there was a huge reduction in male-male courtship. This could be also due to high levels of octopamine (Certel et al., 2010).

Increased octopamine levels effects Drosophila melanogaster's aggression.

Due to the knockout of DopR2 receptor, increased aggressive behavioural changes were observed in high intensity fights. This increased aggression could be due to the up regulation of octopamine levels. Hence there was increased aggressive behaviour and also reduced male-male courtship behaviour. Aggressive behaviour results show that octopamine levels could be playing a major role in regulating aggression and social interactions in *Drosophila* (Certel et al., 2010).

Tyramine beta hydroxylase (TBH) is the enzyme responsible for the conversion of tyramine to octopamine. So the octopamine synthesis corresponds to the expression level of TBH. The DopR2 knockdown flies showed a partial behavioural phenotypes of null mutants, i.e. increased in aggression, decreased in male-male courtship behaviours compared to DopR1 knockdown flies (Baier et al., 2002, Zhou et al., 2008). This behavioural variation between DopR1 and DopR2 knockdown flies suggests that regulation of octopamine was different at these two receptors. Thus DopR1 knockdown flies were little passive compared to DopR2 knockdown flies due to less octopamine production. (Hoyer et al., 2008).

Knockdown of DopR1 and DopR2 receptors reduced *Drosophila's* activity percent.

DopR1 receptor is involved in moderate locomotion of *Drosophila* and DopR2 is also involved in much complex movements (Beaulieu and Gainetdinov, 2011), thus if these receptors were knocked down they will affect overall activity of the flies. This was shown in Figure 7, the total activity percent of the knockdown flies were reduced when compared to controls and wild type flies.

Increased levels of octopamine may influence flight and metabolism in *Drosophila*.

In speed and distance (Figure 8) assay, distance covered by dopamine receptor knockdown flies and control flies were similar and no significant differences were observed. However there was increased speed in DopR2 knockdown flies and this could be due to an increased octopamine level. Orchard et al reported that, in locusts high levels of octopamine will make the insects to burn high energy in first few minutes and make insects to fly rapidly and causes it to burn the stored fat easily (Orchard et al., 1993). Thus even in Drosophila, octopamine may be causing flies to reach high speeds and making the flies to burn energy very rapidly. Another effect of high levels octopamine is to affect the metabolism in flies by altering insulin signalling on lipid accumulation in flies and mammals.

6. Conclusion

In conclusion, this study provided the insights on octopamine and dopamine role in *Drosophila* behaviour. Cessation of dopamine receptors had effect on *Drosophila's* longevity, aggression and courtship. To study mechanisms behind receptors knockdown effect on longevity and social behaviour further molecular studies are needed.

7. Acknowledgement

I would like to thank my supervisor Prof. Helgi Schiöth, Head of the department of Neuroscience, Uppsala University, for providing with the opportunity to work in his group. I would like to thank my co-supervisor Dr. Michael J Williams, Senior research fellow, for his support and assistance.

Furthermore I would like to give my special gratitude to my course co-ordinator, Prof. Volkmar Passoth, Department of microbiology, for his extensive support and suggestions, especially helping me in writing this report.

Finally I would like to thank Prof. Taniguchi and Springer Japan publications for giving me permission to use an image in this report.

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9. Appendix

Table 1. *Drosophila s*trains and species used in the experiments.

Fly line	species	Genotype	Company
CSORC	*D. melanogaster*	Wild type	BSC
Tdc2	*D. melanogaster*	w^{*};p{PUAS-Tdc2.c}	BSC
W1118	*D. melanogaster*	Wild type	BSC
yw	*D. melanogaster*	Wild type	BSC
Elav-*GAL4*	*D. melanogaster*	P{GawB}elav^{c155}	BSC
Uas-DopR1	*D. melanogaster*	UAS-DopR1^{RNAi}	BSC
Uas-DopR2	*D. melanogaster*	UAS-DopR2^{RNAi}	BSC

*BSC= Bloomington stock centre.

Table 2. Mean and SEM of starvation assay.

Mean*			SEM		
Tdc2 Control	**DopR1**	**DopR2**	**Tdc2 Control**	**DopR1**	**DopR2**
100	**100**	**100**	**0**	**0**	**0**
100	**100**	**100**	**0**	**0**	**0**
95.5	**98.5**	**82**	**1.7**	**1.1**	**3.7**
66.5	**60**	**27.5**	**7.5**	**3.9**	**4.8**
37	**38**	**8**	**8.8**	**3.3**	**2.6**
8	**6.5**	**0**	**2.4**	**2.8**	**0**
0	**0**	**0**	**0**	**0**	**0**

***= Number of flies dead**

Table 3. Mean and SEM of individual High intensity fight behaviours

	Mean *				SEM			
Strain/Behaviour	**Wild type**	**Tdc2_control**	**DopR1**	**DopR2**	**Wild type**	**Tdc2_control**	**DopR1**	**DopR2**
Wing threat	**0.11**	**1.33**	**0**	**0.2**	**0.1**	**0.44**	**0**	**0.2**
Fencing	**0.88**	**0.66**	**0.6**	**1.1**	**0.2**	**0.27**	**0.16**	**0.17**
Lunging	**0.55**	**0.33**	**0.4**	**1.7**	**0.2**	**0.22**	**0.22**	**0.53**
Chasing	**0**	**0.22**	**0**	**0.5**	**0.0**	**0.13**	**0**	**0.22**

***=Number of times.**

Table 4. Mean and SEM of individual Low intensity fight behaviour

Mean*					SEM			
Strain/Behaviour	**Wild type**	**Tdc2_control**	**DopR1**	**DopR2**	**Wild type**	**Tdc2_control**	**DopR1**	**DopR2**
Wing flick	**9.55**	**14.55**	**2.3**	**3.6**	**1.4**	**1.33**	**0.71**	**0.8**
Pushing	**11.11**	**13.55**	**4.8**	**10.5**	**1.6**	**1.55**	**0.64**	**0.12**

***= Number of times.**

Table 5. Mean and SEM of individual Courtship behaviours

	Mean				SEM			
Strain/Behaviour	**Wild type**	**Tdc2_control**	**DopR1**	**DopR2**	**Wild type**	**Tdc2_control**	**DopR1**	**DopR2**
One wing out	**9.55**	**14.55**	**2.3**	**3.6**	**1.4**	**1.33**	**0.71**	**0.8**
Circling	**11.11**	**13.55**	**4.8**	**10.5**	**1.6**	**1.55**	**0.64**	**0.12**
Abdomen bend	**0.55**	**0.33**	**0.4**	**1.7**	**0.2**	**0.22**	**0.22**	**0.53**
Tapping from back	**0**	**0.22**	**0**	**0.5**	**0.0**	**0.13**	**0**	**0.22**
Licking abdomen	**0**	**0.11**	**0.1**	**0**		**0.0**	**0.10**	**0.1**

***= Number of times.**

Table 6. Mean and SEM of individual Speed and Distance behaviours

	Mean*			SEM		
	Control	**DopR1**	**DopR2**	**Control**	**DopR**	**DopR2**
Speed	**1**	**0.98**	**2.6**	**0.196131**	**0.08**	**0.79**
Top speed	**1**	**0.8**	**1.19**	**0.2**	**0.05**	**0.13**
Distance	**1**	**0.82**	**1.5**	**0.1**	**0.08**	**0.35**

***= The difference of the length (mm) of the fly moved from one frame to another frame**