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Dilp2 neurosecretory cells regulate behaviour and metabolism through a CREB dependent pathway in *Drosophila*

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Abstract

Metabolic pathways, such as the insulin signaling pathway, that control energy homeostasis are highly conserved through evolution. In *Drosophila melanogaster* a subset of neuroendocrine-cells in the pars intercerebralis region of the brain are known to produce *Drosophila* Insulin Peptides (DILPs), which play the same role as mammalian insulin in glucose homeostasis. Previous studies suggest that octopamine signaling converges on the CREB transcription factor and its co-activator TORC (CRTC) in the pars intercerebralis, to regulate metabolism as well as aggression and mating behaviours via DILPs. The activity of TORC (CRTC) is regulated by kinases such as PKA and SIK2.

A variety of behavioural and metabolic assays were used to examine the role of the genes involved in the CREB pathway in Dilp2 producing cells. Our experiments demonstrate that knocking down the CREB, TORC (CRTC), PKA and SIK2 genes in Dilp2 producing cells significantly increases the flies' sensitivity to starvation. Furthermore, CREB knockdown flies show a significant increase in total lipid content of the body.
The results of mating assay indicate that Dilp2 producing cells in *Drosophila* control mating behaviour through a pathway including the CREB transcription factor and PKA. We suggest that PKA activation results in transcription of the genes under the control of the CREB binding promoter. Expression of these genes, namely gluconeogenic genes, suppresses the mating behaviour in *Drosophila*. The aggression assay implied that octopamine regulates aggressive behaviour of the flies by binding to its receptors (OAMB) on Dilp2 producing cells and suppresses aggressive behaviours, however, regulation of aggression is not related to the expression of the CREB transcription factor.

List of abbreviations

AKH	Adipokinetic Hormone
cAMP	cyclic Adenosine Monophosphate
CREB	cAMP Response Element-Binding Protein
CRTC	CREB Regulated Transcription Co-activator
DILPs	*Drosophila* Insulin-like Peptides
DN	Dominant-Negative Protein
dsRNA	double-stranded RNA
HIF	High intensity fighting
IPCs	Insulin Producing Cells
LIF	Low intensity fighting
MB	male-male Mating Behaviours
OAMB	Octopamine Receptor in Mushroom Bodies
PKA	Protein kinase A
RISC	RNA Induced Silencing Comple
RNAi	RNA-mediated interference
SIK2	Salt-Inducible Kinase 2
siRNA	small interfering RNA
TORC	Tansducer of Regulated CREB activity
UAS	Upstream Activating Sequences

Introduction

Homeostasis and metabolism are governed by the brain

Homeostasis is the ability to maintain a stable interior condition enabling organism to survive in a changing environment. It is a property that all organisms share; from simple unicellular bacteria to humans. There are self-regulating processes in every biological system that help it to stay in a dynamic equilibrium with its environment including the processes that control the balance between energy intake and energy expenditure. Due to the importance of maintaining an internal stability, metabolism is highly controlled via various mechanisms by different organs in the body using lipids and carbohydrates[1].

Lipids are a compact source of energy stored in cells and are used in conditions where the organism is lacking its primary source of energy, glucose. In mammals, keeping circulating glucose at a stable level is a vital part of energy homeostasis, a process which is maintained mainly by two pancreatic hormones, insulin and glucagon. This balance is tightly governed by the brain. The brain controls energy homeostasis by a subdued orchestration between body organs through different mechanisms. At a different level, the brain controls energy homeostasis by adjusting behaviours such as feeding, aggression or courtship. For instance, if starved, animals show more aggressive behavoiurs. Biogenic amines such as octopamine, dopamine and serotonin are among the neurotransmitters that have been shown to play roles in regulating both insulin signaling and aggression[2].

Different parts of the brain are involved in energy and glucose homeostasis, however, the hypothalamus plays the central role in this orchestration[3]. In insects such as *Drosophila*, the Pars intercerebralis is a brain region that plays a similar role as vertebrate hypothalamus in controlling

metabolic homeostasis. Pars intercerebralis is a neurosecretory center which conveys its effect on metabolic pathways via the *Drosophila* insulin-like peptides (DILPs) and the adipokinetic hormone (AKH) [4-6].

Energy homeostasis and CREB pathway

In mammals, food intake elevates the levels of circulating glucose and leads to secretion of insulin from beta cells of the pancreas. Insulin facilitates glucose uptake and as a result, energy will be stored in muscle and fat cells in the form of glycogen and triglycerides [7]. Another pancreatic hormone, glucagon, which has the opposite role of insulin, is secreted in response to low levels of circulating glucose. Glucagon binds to its receptor and activates adenylate cyclase which increases the level of cyclic adenosine monophosphate (cAMP) in the cell. Increased levels of cAMP lead to activation of another enzyme called protein kinase A (PKA) resulting in expression of gluconeogenic genes in the cell through a key molecule called cAMP responsive transcription factor (CREB) [8-9]. CREB transcription factor needs a co-activator to be able to activate the gluconeogenic program of the cell. This co-activator is a protein called transducer of regulated CREB activity (TORC) or CREB regulated transcription co-activator (CRTC) and the activity of it is regulated via phosphorylation.[10]

The regulatory role of TORC (CRTC) is dependent on its subcellular localization which is determined by its phosphorylation status. In high glucose condition TORC (CRTC) is phosphorylated on Ser171 and is kept in the cytoplasm by attachment of a scaffolding protein called 14-3-3 so it cannot activate CREB and gene expression. TORC (CRTC) is phosphorylated by activity of a salt-inducible kinase, SIK2 which can be inhibited by PKA. In low glucose condition, activated PKA inhibits SIK2 and it cannot further phosphorylate TORC (CRTC). In

parallel, high concentrations of cAMP and Ca^{2+} in the cell that are the result of glucagon receptor activation, triggers dephosphorylation of TORC (CRTC) by activity of Calcineurin/PP2B (Cn) enzyme. Dephosphorylation of TORC (CRTC) at Ser171 releases it from 14-3-3 sequestration and it can enter the nucleolus and activate CREB and the gluconeogenic program (Figure 1)[11].

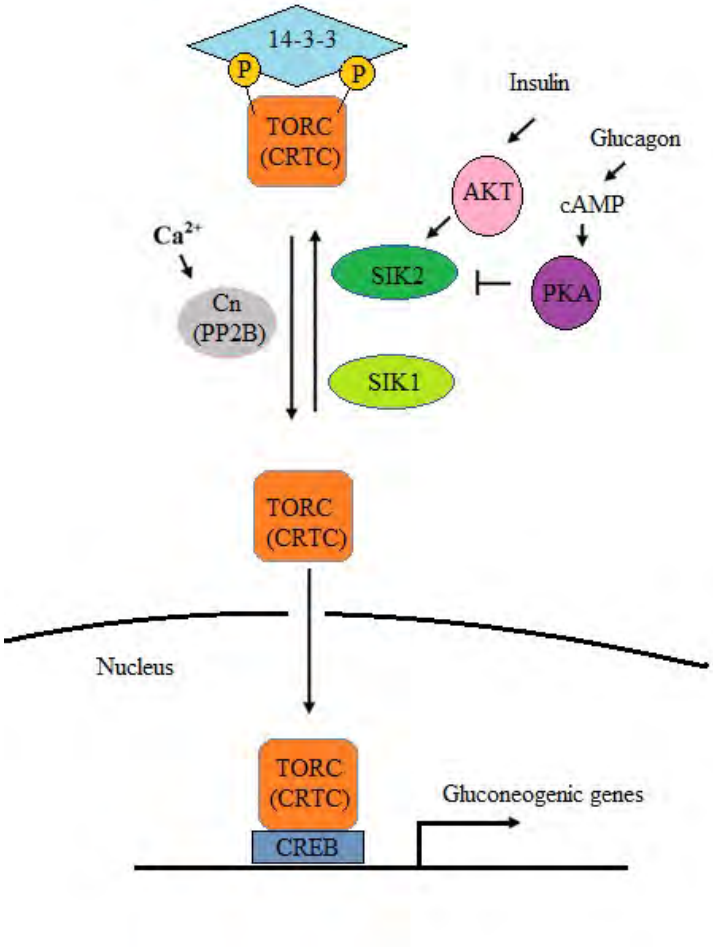

Figure 1. CREB dependant pathway involved in glucose homeostasis. The key step in regulation of this pathway is localization of TORC (CRTC) into nucleus which is determined by its phosphorylation status. When the level of glucose is high in the cell, TORC (CRTC) is phosphorylated by SIK2 kinase and attached to a scaffolding protein 14-3-3 which prevents it from interring nucleus. Low glucose conditions increases cAMP concentration in the cell which in turn activates PKA and elevates Ca^{2+} concentration in cytoplasm. PKA then phosphorylates SIK2 and prevents its kinase activity. Calcineurin/PP2B (Cn) that is a Ca^{2+} dependent enzyme dephosphorylates TORC (CRTC) enabling it to enter the nucleus and bind to CREB. Upon TORC (CRTC) binding, CREB transcription factor starts transcription of gluconeogenic genes which elevates circulating glucose levels in return. (Hietakangas V. and Cohen S.M., 2008)[1]

Previous studies have shown that in both mammals and flies molecular mechanisms involved in regulation of TORC (CRTC) activity, which maintain the energy balance, are highly conserved. The major difference between flies and mammals in the CREB pathway is the place of action. In mammals, TORC (CRTC) is mainly expressed in the liver to control gluconeogenesis but in flies, TORC (CRTC) is mainly expressed in the brain. There is also a difference in the site of insulin producing cells between mammal and flies. In mammals, β-cells located in the pancreas form the location of insulin production, whereas in flies a subset of neurosecretory cells in pars intercerebralis is the center of insulin production[1,12].

Insulin-like peptides (ILPs)

The insulin signaling pathway is highly conserved through evolution in animals and plays an important role in growth, development, reproduction, metabolism and regulation of life span[4,13-14]. In *Drosophila*, metabolic homeostasis is maintained by the *Drosophila* insulin-like peptides (DILPs) and the adipokinetic hormone (AKH), which seem to have the same roles as mammalian insulin and glucagon, respectively. DILPs have the same role as insulin in controlling uptake of carbohydrates and decreasing the blood sugar levels. On the other hand, AKH with the same role as glucagon increases blood sugar by controlling glycolysis in the body fat[4-5,15-18]. Seven DILPs (DILP1 – 7) have been identified in *Drosophila* with three of them, DILP2, 3 and 5 being produced in the insulin producing cells (IPCs) of the fly brain[13,19].

Drosophila a favourable model organism

The fruit fly *Drosophila melanogaster* has been widely used as a model organism for more than 100 years in different fields of biology from evolutionary to molecular biology. As a consequence of TH Morgan and his students' studies, Drosophila became a strong model in

genetic studies and helped biologists unravel some key aspects of metabolic and genetic control of conserved pathways which provides us with new insights into complex mechanisms that were not clear when studying complex model organisms. Some general advantages which make *Drosophila* a favourable model organism are; a) due to its small size, maintaining large populations of them requires small space. b) its life cycle is short, about 12 days when kept at 25℃, which makes large-scale studies over several generations easy to conduct in short time. c) fly maintenance is inexpensive compared to other model organism such as mice, rats, etc. and d) females and males are clearly distinguishable and virgins are easy to isolate.

Sequencing the fly genome was completed in the year 2000, and it was revealed that it contains around 14000 genes and homologues to 75% of genes known to be involved in human diseases in the human genome[20-21]. The most basic metabolic functions found in vertebrates are also present in *Drosophila*. Functions involved in energy homeostasis like keeping circulating glucose at a stable level and storage of excessive energy in the form of glycogen and lipid to be used in the time of stress or starvation[22-24]. In addition, organs that are involved in metabolism, food intake and energy homeostasis in humans have analogues in *Drosophila*. A main difference between *Drosophila* and mammalian metabolism is that, *Drosophila* cannot synthesize cholesterol which makes them cholesterol auxotrophs[25].

Targeted gene expression in *Drosophila* using GAL4/UAS system

A further advantage of *Drosophila* is the presence of a variety of genetic tools. The GAL4/UAS system is designed for targeted gene expression which enables scientists to activate any cloned gene in a tissue/ cell specific manner[26]. GAL4 which was first identified in the yeast *Saccharomyces cerevisia,* is a regulator of other genes and encodes for a protein[27-28]. GAL4

controls the expression of other genes by binding to a specific DNA sequence made of four related 17 base pair (bp) sites which are called Upstream Activating Sequences (UAS) and are analogous to an enhancer element found in multicellular eukariyots[29]. Fischer *et al.* demonstrated in 1988 that in *Drosophila,* expression of a reporter gene which is under the UAS control can be stimulated by GAL4 expression[30-32].

GAL4/UAS system in *Drosophila* is based on separation of GAL4 and UAS parts into two different parental lines called driver and responder lines. In the driver line, the GAL4 gene is placed under the control of a native promoter. Therefore, GAL4 is expressed in those tissues/cells where the native promoter is active. There is no transcription of the target gene in the parental responder line (under the UAS element control) due to the lack of GAL4 expression. This lack of expression enables scientists to design responder lines for lethal or toxic genes and also for genes that reduce viability when expressed[33]. After crossing the driver line to the responder line, progenies express both GAL4 and the target gene under the control of the UAS element which leads to translation of the target gene in those tissues that express GAL4 (Figure 2).

Although the GAL4/UAS system was designed primarily to analyze gain of function phenotypes, a combination of it with the RNA-mediated interference (RNAi) method provides a strong tool for analyzing loss of function phenotypes. To maintain a targeted gene knock down, a construct is designed which expresses an RNA molecule forming double-stranded RNA (dsRNA) and is mediating gene-specific RNAi[34]. The designed construct produces an anti-sense RNA molecule which is complementary to the mRNA of the target gene. This RNA then folds into a stem-loop construct which is recognized and cleaved by an enzyme called dicer into short fragments (<30 n) known as small interfering RNA (siRNA). siRNA is then unwound by a protein complex

called RNA Induced Silencing Complex (RISC) which degrades one of the strands and uses another strand (complimentary to mRNA) to recognize the mRNA of the target gene and cut it. This break in mRNA results in its degradation by exonuleases in the cell[35-36]. Another method for maintaining the gene knockdowns is producing a dominant-negative or dominant-interfering version of the target protein. In this method, the translated protein carries a mutation which disrupt its function, interferes with the normal function of simultaneously expressed wild-type protein[33,37].

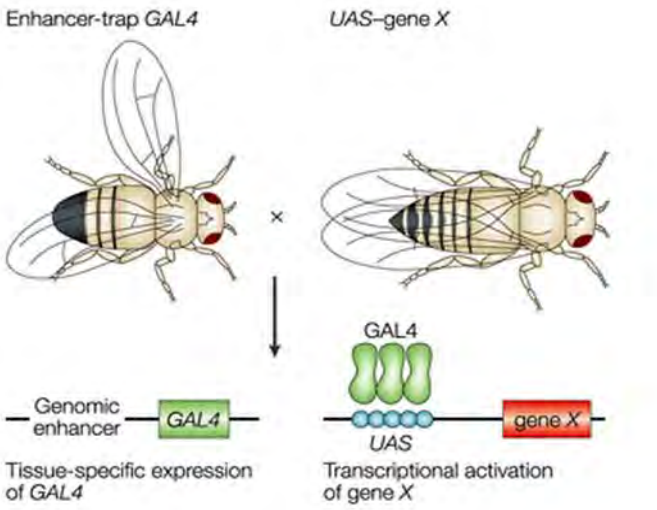

Figure 2. Targeted gene expression in *Drosophila* using the GAL4/UAS system. In the parental driver line the GAL4 gene is randomly inserted into the genome which leads to GAL4 expression from different genomic enhancers. In parental responder line target gene (gene x) is subcloned behind the UAS element. Gene x is silenced in parental responder line due to lack of GAL4 expression. To activate the expression of gene x in a tissue/ cell specific manner, the driver line (enhancer-trap GAL4) is crossed to the responder line (UAS-gene x). In the progeny of this cross, gene x is expressed as a result of UAS activation by GAL4 binding. (Picture is adopted from St Johnston D., 2002)[38]

Project hypothesis

A study in *Caenorhabditis elegans* shows that octopamine (invertebrate norepinephrine orthologue) signaling regulates feeding behaviour through the transcription factor CREB[39]. In

Drosophila, although CREB is not known to be controlled by octopamine, feeding behaviour was shown to be regulated by CREB and its co-activator TORC (CRTC) [40-41]. Furthermore, in *Drosophila*, octopamine was shown to regulate sleep behaviour via CREB regulation by means of serine/threonine kinase PKA[40-41]. Some preliminary results of studies in our group show that feeding flies with an octopamine agonist, Chlordimeform, increases lipid content of the flies. (Philip Geoergen, unpublished results). Putting together all these data, it can be hypothesized that octopamine signaling converges on TORC (CRTC) and CREB in the *Drosophila* hypothalamus, known as the *Pars intercerebralis*, to regulate various behaviours such as aggression and feeding.via DILPs produced in the neurosecretory cells of the *Pars intercerebralis*. In the proposed *Drosophila* model (Figure 3), increased octopamine signaling would inhibit the activity of SIK2, via its phosphorylation by PKA. SIK2 inhibition would allow TORC (CRTC) to be dephosphorylated by protein phosphotase 2B (PP2B). CRTC would then translocate into the nucleus to activate CREB, the result of which can be assessed by an increase in certain behaviours such as aggression.

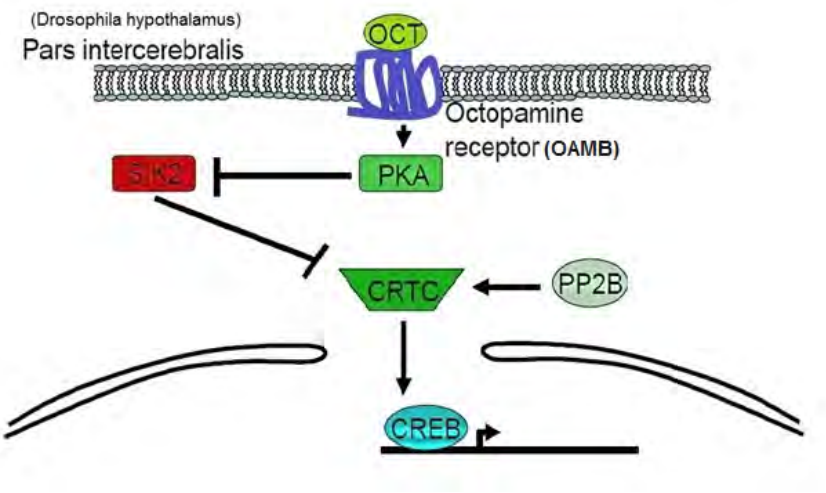

Figure 3. Schematic diagram of proposed octopamine signaling pathway in the *Pars intercerebralis.* Octopamine binding to its receptor would activate PKA to phosphorylate and inhibit SIK2. Inhibition of SIK2 would allow CRTC to be dephosphorylated by PP2B. CRTC would translocate to the nucleus and activate CREB.

Project aim

In this project we examine our hypothesis by knocking down particular genes involved in CREB pathway in Dilp2 neurosecretory cells of the Pars intercerebralis region of the brain (Figure 4). To knock down the target genes in Dilp2 cells, we use *Dilp2*-GAL4 driver line which produces the GAL4 protein specifically in Dilp2 producing cells, a subset of neurosecretory cells known as insulin producing cells (IPCs). The reason for choosing Dilp2 producing cells among other IPCs is that Dilp2 with 35% overall homology is most closely related to mature insulin peptide in human[42]. By crossing the *Dilp2*-GAL4 driver line to the UAS responder line which produces RNAi or dominant negative protein against the target gene, we would be able to knock down that gene in Dilp2 producing cells.

We examine the effect of those genes by carrying out metabolic and behavioural assays. In metabolic assays, namely starvation assay and lipid extraction, we consider the effect of four genes in the pathway including CREB, CRTC, PKA and SIK2. In behavioural assays, namely mating and aggressive behaviours, we examine the effect of CREB by knocking down its gene using both RNAi and dominant negative methods and also by over expressing the gene. We further examine the effect of PKA and OAMB (octopamine receptor) on aggressive and mating behaviours.

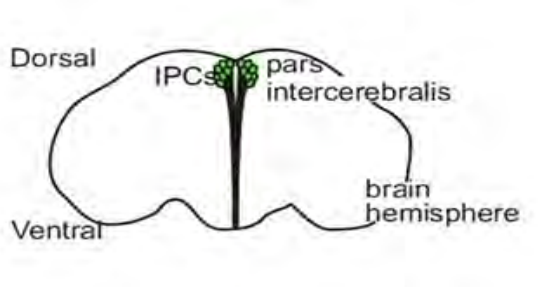

Figure 4. A schematic picture of the of the adult *Drosophila* brain showing insulin producing cells (IPCs) in the *pars intercerebralis* region (picture is adopted from Haselton AT. et al.,2010)[19]

Materials and methods

Fly husbandry

Flies were kept at 25℃ under 12:12 hours light:dark cycles, unless otherwise specified. The flies were fed standard fly food (Jazz-Mix, Fisher Scientific) consisting of brown sugar, corn meal, yeast, agar, benzoic acid, methyl paraben and propionic acid with additional dry yeast to increase food's yeast content up to 10%. The following fly strains were used: *w1118*, *yw*, CSORC (wild type), UAS-*CREB*^{RNAi}, UAS-*CRTC*^{RNAi}, UAS-*PKA*^{RNAi}, UAS-*SIK2*^{RNAi}, UAS-CREB^{OE} and UAS-CREB^{DN} were from Bloomington *Drosophila* stock center (BDSC; Indiana University, Bloomington, IN). UAS-OAMB^{RNAi} and *Dilp2*-GAL4 lines were donated by Professor Dick Nässel, Stockholms universitet. All the behavioural experiments were done within 3 hours after lights on in a closed covered chamber for maximum reduction of external noise and light.

Genetic crosses

For experimental crosses, virgin females from the *Dilp2*-GAL4 driver line were collected and crossed to UAS reporter lines. When 2nd and 3rd instar larvae started to crawl up the bottle walls, bottles were transferred to 29°C to activate the GAL4/ UAS system at optimum temperature. Two different strains were used to make control crosses in behavioural and metabolic assays. For metabolic assays (Starvation assay and lipid extraction) virgin females from *yw* line were collected and crossed to the same UAS reporter lines as experimental crosses following the procedure explained above. For behavioural assays (Mating and aggression assays) virgin females from *w1118* line were collected and crossed to the same UAS reporter lines as experimental crosses following by the procedure explained above.

Starvation assay

Newly eclosed males from first generation of offspring (F1 generation) were collected and aged at 29°C incubator for 5 to 7 days. Groups of 20 flies were put in vials containing 6 ml of 1% agarose gel which provides the flies with water and humidity but no food, and kept at 25°. The number of dead flies was checked every 12 hours and a survival curve was constructed. For each genotype 200 flies in 10 replicates were subjected to the experiment.

Lipid extraction

Newly eclosed males from F1 generation were collected and aged at 29°C for 5 to 7 days. To determine the lipid content, groups of 30 males were put into glass vials dried at 65°C for 1 hour. Flies were then weighed to obtain dry weight. Lipids were extracted by adding 10ml of diethyl ether (Sigma-Aldrich, Germany) to the dried flies followed by incubation period of 24 hours at room temperature. Diethyl ether (Sigma-Aldrich, Germany) was discarded then and flies were dried for 1 hour in 65°C. Flies were weighed again to obtain the lean dry weight. The difference between dry weight and lean dry weight was calculated as the total lipid content of the flies. For each genotype 300 flies in 10 replicates were subjected to the experiment.

Mating assay

Virgin males from F1 generation were collected and kept separately at 29°C for 5 to 7 days. Virgin CSORC (wt) females were used as mating partners and aged for 2-3 days at 25°C. 12 well

plates containing 6 ml of 1% agarose gel in each well were prepared to be used as a platform. Virgin females and males were then transferred to empty vials and anesthetized using ice water. One virgin male was put in the well together with one virgin female and the lid was placed back. When flies started to move filming started and they were recorded for 20 minutes using an HD camera (Panasonic, HC-V700).

In mating assay the time lag between the moment that flies are subjected to each other and the moment that male shows the first mating behaviour is scored and designated as the latency of mating behaviour. Once male started to show mating behaviour, the time of different mating behaviours was scored and the courtship index was determined. Courtship index (CI) is used to represent overall courtship enthusiasm of the male calculated as duration of courtship behaviours (sec) divided by total observation time (sec).

Aggression assay

Virgin males from the F1 generation were collected and kept in isolation at 29°C for 5 to 7 days. 12 well plates containing 6 ml of 1% agarose gel in each well were prepared to use as a platform. Males were anesthetized using ice water. Two virgin males were placed in the same well separate from each other and the lid was closed. The flies were filmed with an HD camera (Panasonic, HC-V700) for 30 minutes from the moment they started to move.

In this assay 6 different aggressive behaviours phenotypes were scored based on the scale defined by Chen et al.[43]. In addition to that, 5 different male-male mating behaviours (MB) were scored to obtain a complete range of male-male interaction in this assay. Aggressive behaviours were divided into two main groups; 1) High intensity fighting (HIF) including lunging, where a

fly rears up on hind legs and snaps down on the other, wing threat, where one fly quickly raises both wings to a 45° angle towards its opponent, and fencing, where both flies face each other, extend legs forward and push the opponent, and. 2) Low intensity fighting (LIF) included wing flick where one fly quickly flicks, with its wing for a very short time, and pushing where one fly extends one leg and pushes the opponent. Mating behaviour phenotypes are the same as those already described in the mating assay section.

Statistical analysis

To analyze the starvation assay results the log rank test, a nonparametric test that compares the survival curves of two samples was performed using online service at the address: http://bioinf.wehi.edu.au/software/russell/logrank/.

Statistical analysis for lipid extraction, aggression and mating assays was performed using the Minitab 14 and Graph Pad Prism 5.0 software package. To compare two groups of data a t-test or a Mann-Whitney Test (the nonparametric test equal to t-test) were performed and graphs were obtained using Graph Pad Prism 5.0 or Microsoft Office Excel 2007.

Results

Starvation assay

Starvation assays was performed on knockdown flies to examine the possible role of four genes, CREB, CRTC, PKA and SIK2 in fly metabolism. In all experiments knockdown flies showed a significant reduction in survival rate in comparison to control crosses (Figures 5). A starvation

assay on the *Dilp2*-GAL4 line as a negative back ground control was performed which also showed a reduction in survival rate and the plot was placed among experimental crosses rather than controls (Figure 6).

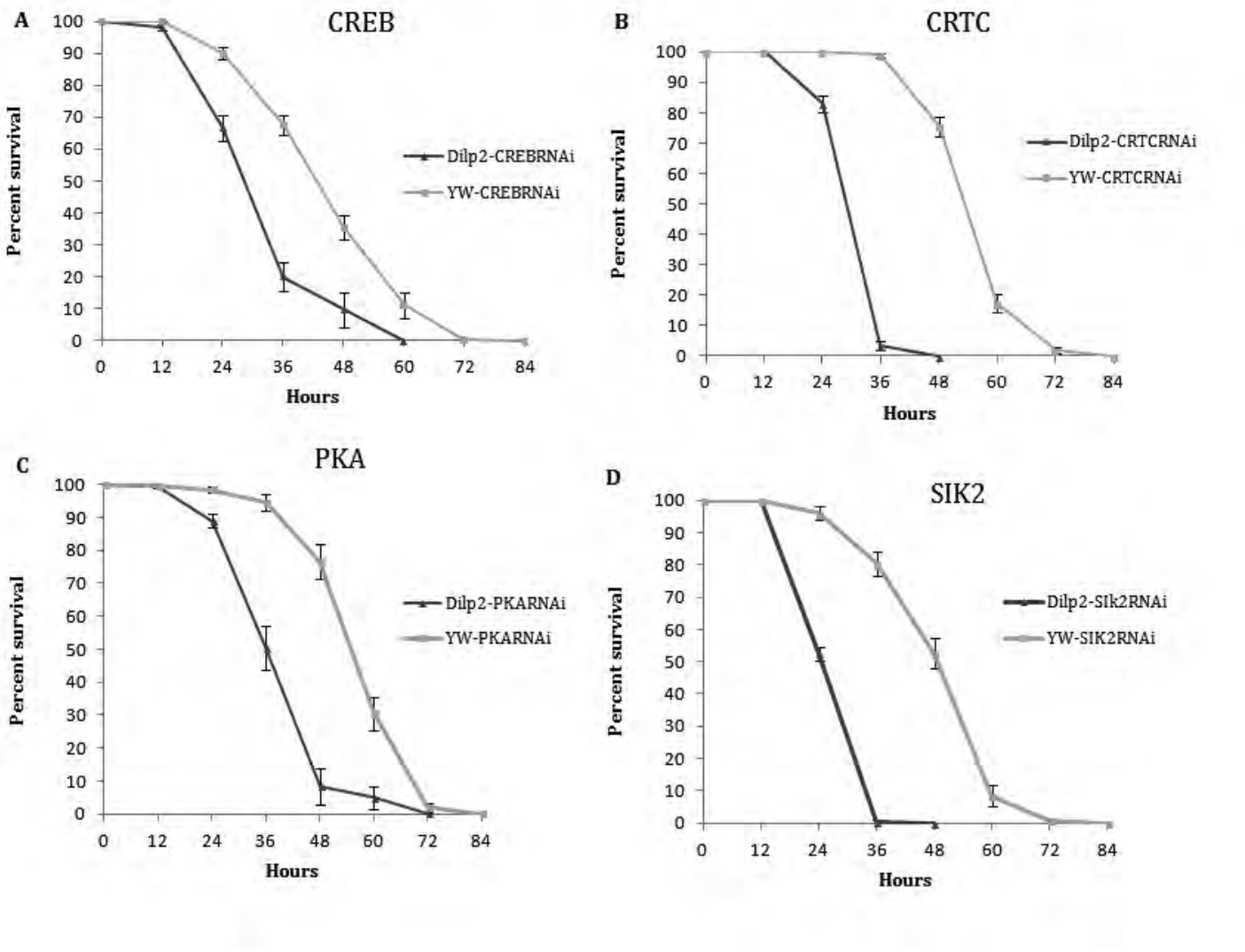

Figure 5. Knocking down four genes CREB, CRTC, PKA and SIK2, downstream of the octopamine pathway in Dilp2 producing cells of pars intercerebralis increases sensitivity to starvation in *Drosophila.* We tested the role of genes involved in CREB related octopamin pathway by knocking down those genes in Dilp2 producing cells using the *Dilp2*-GAL4 driver crossed to the UAS responder, producing RNAi against the target genes. To obtain controls, we crossed the responder lines to the yw strain, which produces the same genetic background as experimental crosses. **A.** knocking down CREB significantly increases the sensitivity to starvation (P-Value< 0.001, n=200-240) **B.** knocking down CRTC significantly increases the sensitivity to starvation (P-Value< 0.001, n=200) **C.** knocking down PKA significantly increases the sensitivity to starvation (P-Value< 0.001, n=200) and **D.** knocking down SIK2 significantly increases the sensitivity to starvation (P-Value< 0.001, n=200).

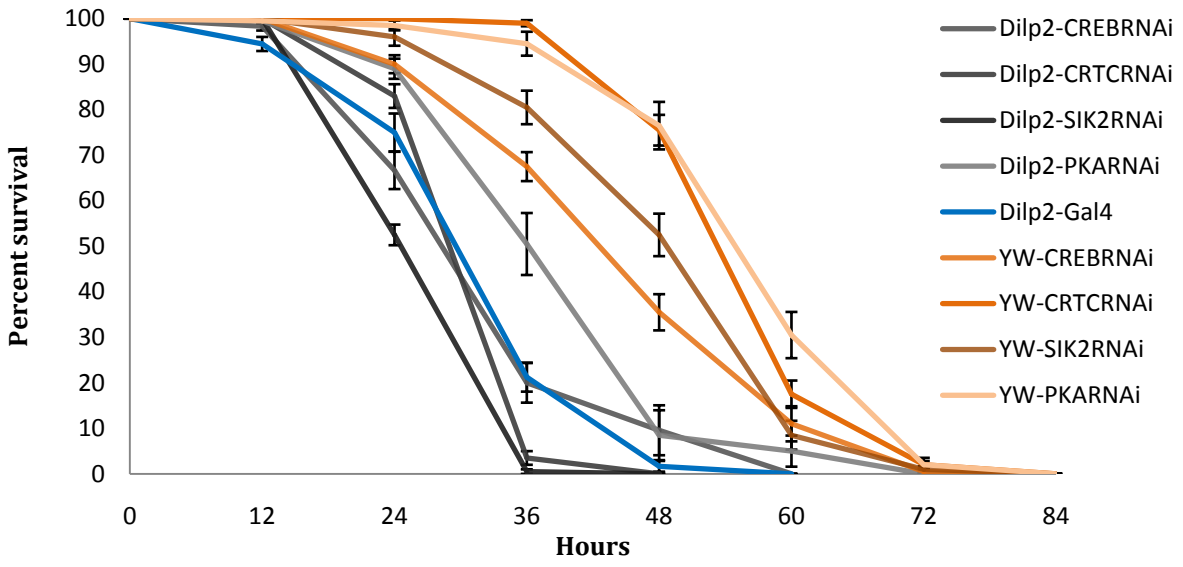

Figure 6. Starvation assay on the *Dilp2*-GAL4 driver line as a background control shows decreased resistance to starvation in comparison to other control crosses. The blue line which represents the survival curve of *Dilp2*-GAL4 places among the survival curves of experimental crosses (the gray lines) rather than control crosses (the orange lines).

Lipid extraction

Lipid extraction was performed to determine the average total lipid content of the flies. Among knockdown flies for four genes CREB, CRTC, PKA and SIK2, only CREB knockdowns showed a significant difference from their control cross ($*p < 0.05$) (Figure 7 to 10).

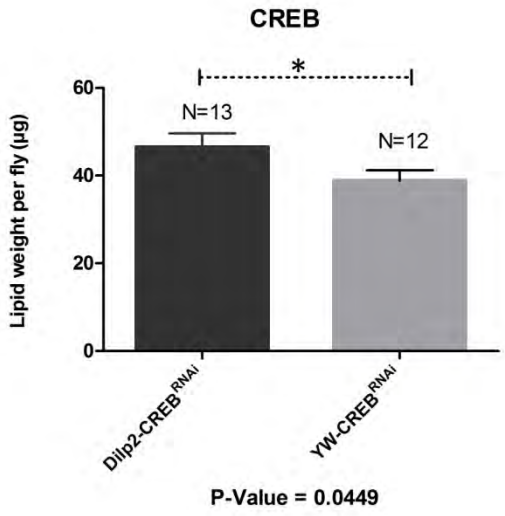

Figure7. CREB knockdown flies show significant increase in total lipid content compared to control flies (P-Value <0.05). The *Dilp2*-GAL4 driver line was crossed to the UAS-CREB^{RNAi} responder line to produce RNAi against CREB which knocks this gene down in Dilp2 producing cells in pars intercerebralis region. For control crosses, the yw strain was crossed to the UAS-CREB^{RNAi} responder line which results in progeny with the same background as experimental crosses. For *Dilp2*-CREB^{RNAi,} mean= 46.53 (SEM ± 3.078) and for yw-CREB^{RNAi} mean= 38.89 (SEM ± 2.333).

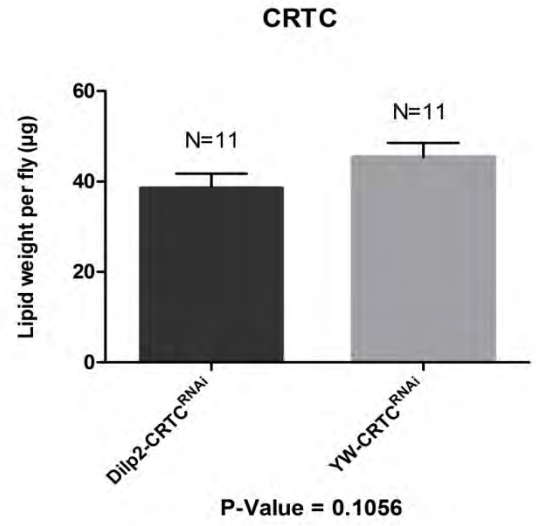

Figure 8. CRTC knockdown flies show no significant change in total lipid content of the body in comparison to control crosses (P-Value > 0.05). The *Dilp2*-GAL4 driver line was crossed to the UAS-CRTC^{RNAi} responder line to produce RNAi against CRTC which knocks this gene down in Dilp2 producing cells in pars intercerebralis. For control crosses, the yw strain was crossed to the UAS-CRTC^{RNAi} responder line which results in progeny with the same background as experimental crosses. For *Dilp2*-CRTC^{RNAi}, mean= 38.48 (SEM ± 3.252) and for yw-CRTC^{RNAi} mean= 45.39 (SEM ± 3.135).

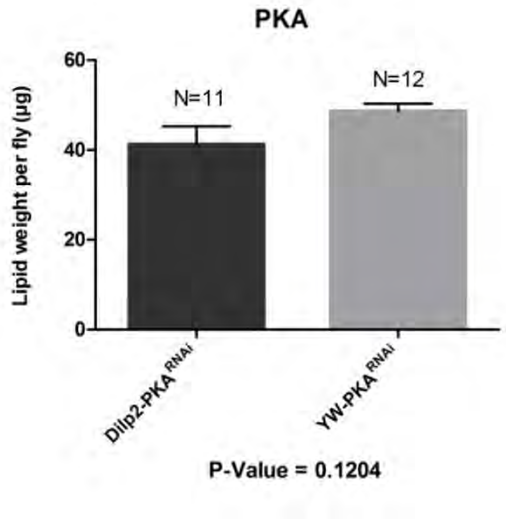

Figure 9. PKA knockdown flies show no significant change in total lipid content in comparison to control cross (P-Value > 0.05). The *Dilp2*-GAL4 driver line was crossed to the UAS-PKA^{RNAi} responder line to produce RNAi against PKA which knocks this gene down in Dilp2 producing cells in pars intercerebralis. For control crosses, the yw strain was crossed to the UAS-PKA^{RNAi} responder line which results in progeny with the same background as experimental crosses. For *Dilp2*-PKA^{RNAi} mean= 41.21 (SEM ± 4.052) and for yw-PKA^{RNAi} mean= 48.61(SEM ± 1.665).

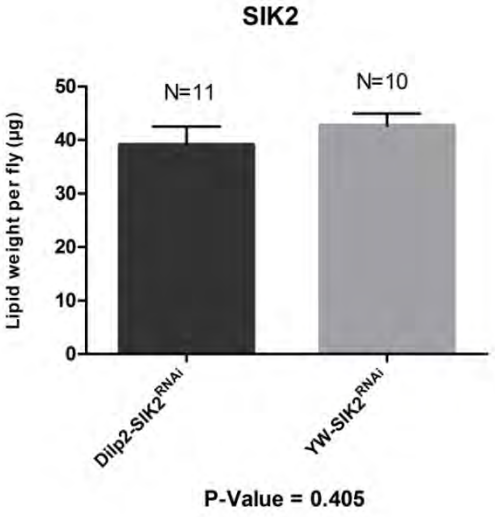

Figure 10. SIK2 knockdown flies show no significant change in total lipid content of the body in comparison to control crosses (P-Value > 0.05). The *Dilp2*-GAL4 driver line was crossed to the UAS-SIK2^{RNAi} responder line to produce RNAi against SIK2 which knocks this gene down in Dilp2 producing cells in pars intercerebralis. For control crosses, the yw strain was crossed to the UAS-SIK2^{RNAi} responder line which results in progeny with the same background as experimental crosses. For *Dilp2*-SIK2^{RNAi} mean= 39.09 (SEM ± 3.426) and for yw-SIK2^{RNAi} mean= 42.67 (SEM± 2.266).

Mating assay

The mating assay was performed on knockdown flies for three genes involved in the CREB pathway, CREB, PKA and OAMB, to investigate the hypothetical role of these genes in mating behaviour. For knocking down the CREB gene in Dilp2 producing cells, two different responder lines, UAS-CREB^{RNAi} and UAS-CREB^{DN}, were crossed to *Dilp2*-GAL4 driver line in order to investigate the accuracy of these two methods in knocking down the target gene and examine if the results confirm each other. In addition, the mating assay was also performed on CREB over expressing flies to examine if opposite results to the knock down experiment can be observed. To obtain control crosses, same UAS responder lines were crossed to the *w*1118 line which provided us with flies with similar genetic background to experimental crosses without any changes in their gene expression. Data were analyzed to obtain latency of mating behaviour and the percentage of courtship index (CI). The results of latency of courtship behaviour showed significant decrease in experimental crosses compared to control crosses in RNAi-mediated CREB knockdowns (Fig. 12). For CRTC knockdown flies, P-value equals to 0.086 which is not less than 0.05 to conclude that there is a significant difference between experimental and control flies, however, it is quite close to 0.05.Hence, we are able to conclude that there is a tendency for increase in latency of courtship behaviour in CRTC knockdown flies (Fig.13). The results of dominant negative protein-mediated CREB knock down flies, PKA and OAMB knockdown flies show no significant deference between experimental and control groups (Fig.11, Fig.14 and Fig.15).The results of courtship index showed significant difference between experimental and control crosses in RNAi mediated CREB and PKA knock down flies (Fig.17 and Fig.19). The results of dominant negative protein-mediated CREB knockdown, CREB overexpressed, PKA

and OAMB knockdown flies show no significant difference in experimental crosses compared to control crosses (Fig.16, Fig.18 and Fig.20). Asterisks show significance of difference (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

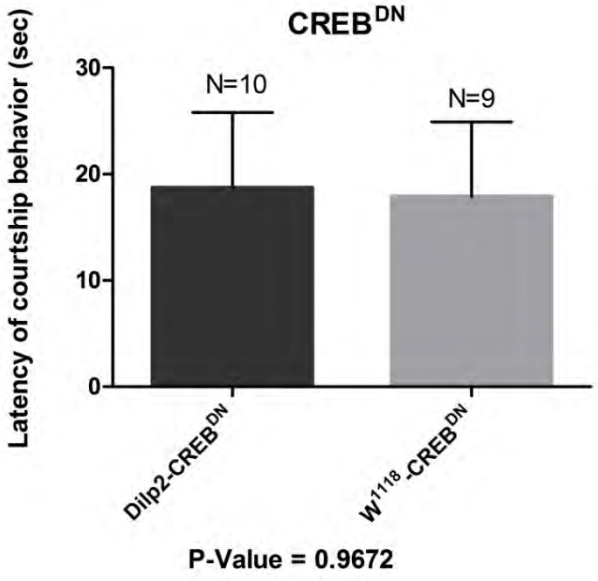

Figure 11. Dominant negative protein-mediated CREB knockdown flies show no significant changes in latency of courtship behaviour in comparison to control flies (P-Value > 0.05). The *Dilp2*-GAL4 driver line was crossed to the UAS-CREB^{DN} responder line to produce dominant negative protein against CREB which knocks this gene down in Dilp2 producing cells in pars intercerebralis. For control cross, the *w1118* strain was crossed to the same responder line, which results in progeny with the same genetic background as experimental cross. For *Dilp2*-CREB^{DN} mean=18.70 (SEM± 7.103) and for *w*1118 mean= 17.89 (SEM ± 7.035).

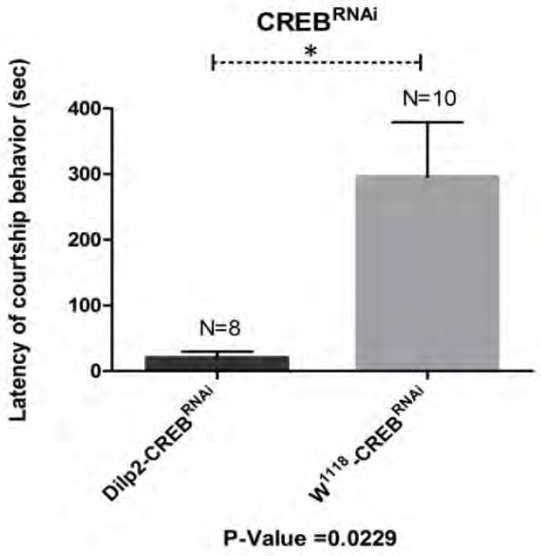

Figure 12. RNAi-mediated CREB knockdown flies show significant decrease in latency of courtship behaviour in comparison to control flies (P-Value <0.05). The *Dilp2*-GAL4 driver line was crossed to the UAS-CREB^{RNAi} responder line to produce RNAi against CREB which knocks this gene down in Dilp2 producing cells in pars intercerebralis. For control cross, the *w1118* strain was crossed to the same responder line which results in progeny with the same genetic background as experimental cross. For *Dilp2*-CREB^{RNAi} mean= 20.38 (SEM ± 9.526) and for *w*1118-CREB^{RNAi} mean= 295.7 (SEM ± 82.98).

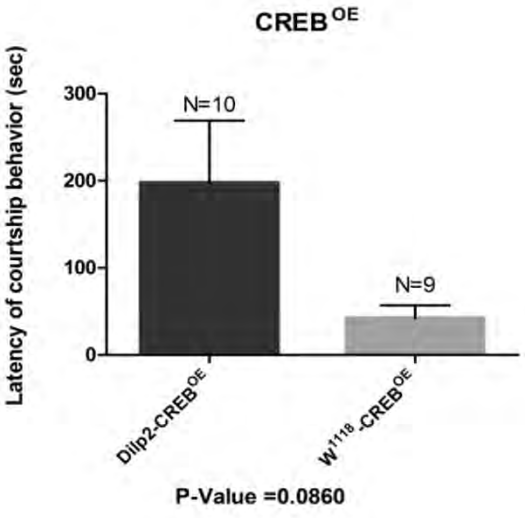

Figure 13. CREB overexpressed flies show an increase in latency of courtship behaviour but fail to reach statistical significance (P-Value > 0.05). The *Dilp2*-GAL4 driver line was crossed to the UAS-CREB^{OE} responder line to obtain over expression of CREB in Dilp2 producing cells in pars intercerebralis. For control cross, the *w1118* strain was crossed to the same responder line which results in progeny with the same genetic background as experimental cross. For *Dilp2*-CREB^{OE} mean= 197.5(SEM ± 71.74) and for *w*1118-CREB^{OE} mean= 42.33(SEM ± 14.91).

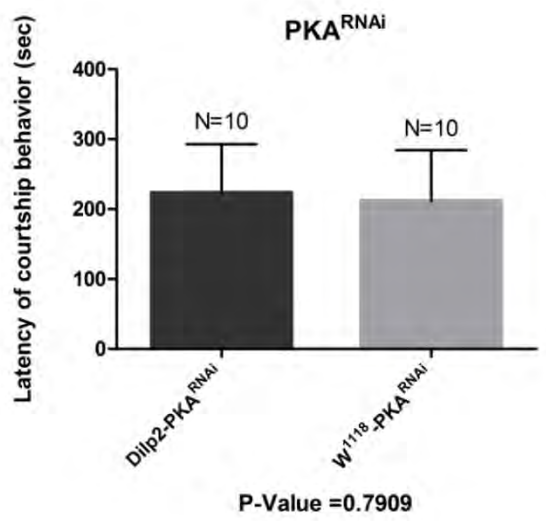

Figure 14. PKA knockdown flies show no changes in latency of courtship behaviour compared to control flies (P-Value > 0.05). The *Dilp2*-GAL4 driver line was crossed to the UAS-PKA^{RNAi} responder line to produce RNAi against PKA which knocks this gene down in Dilp2 producing cells in pars intercerebralis. For control cross, the *w1118* strain was crossed to the same responder line which results in progeny with the same genetic background as experimental cross. For *Dilp2*-PKA^{RNAi} mean= 223.4(SEM ± 69.37) and for *w*1118-PKA^{RNAi} mean= 211.8 (SEM ± 72.34).

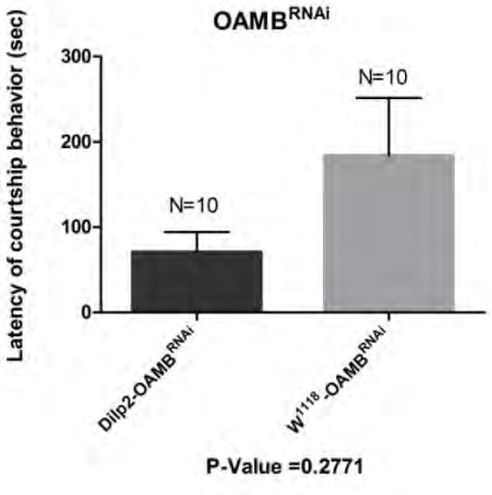

Figure 15. OAMB knockdown flies show no significant changes in latency of courtship behaviour compared to control flies (P-Value >0.05). The *Dilp2*-GAL4 driver line was crossed to the UAS-OAMB^{RNAi} responder line to produce RNAi against OAMB which knocks this gene down in Dilp2 producing cells in pars intercerebralis. For control cross, the *w1118* strain was crossed to the same responder line which results in progeny with the same genetic background as experimental cross. For *Dilp2*-OAMB^{RNAi} mean= 70.73 (SEM± 23.56) and for *w*1118-OAMB^{RNAi} mean= 183.5 (SEM ± 67.76).

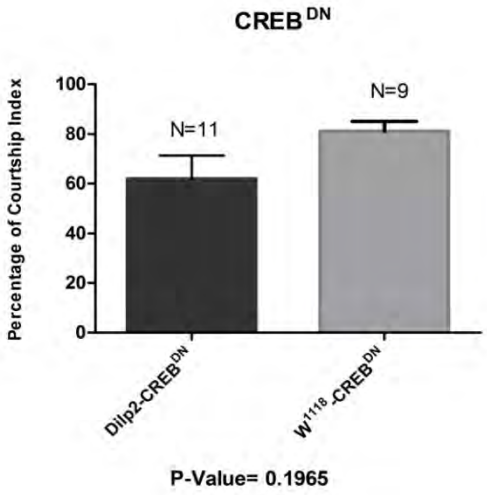

Figure 16. . Dominant negative protein-mediated CREB knockdown flies show no significant changes in percentage of CI in comparison to control flies (P-Value > 0.05). The *Dilp2*-GAL4 driver line was crossed to the UAS-CREB^{DN} responder line to produce dominant negative protein against CREB which knocks this gene down in Dilp2 producing cells in pars intercerebralis. For control cross, the *w1118* strain was crossed to the same responder line which results in progeny with the same background as experimental cross. For *Dilp2*-CREB^{DN} mean= 61.94 (SEM ± 9.386) and for *w*1118 -CREB^{DN} mean= 81.08 (SEM ± 3.972).

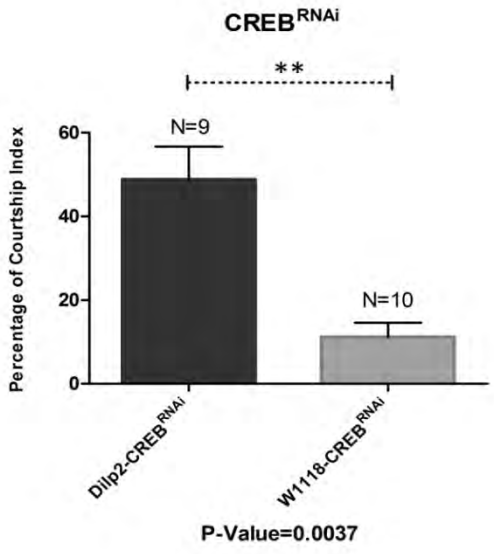

Figure 17. RNAi-mediated CREB knockdown flies show a significant increase in the percentage of CI compared to control flies (P-Value <0.05). The *Dilp2*-GAL4 driver line was crossed to the UAS-CREB^{RNAi} responder line to produce RNAi against CREB which knocks this gene down in Dilp2 producing cells in pars intercerebralis. For control cross, the *w1118* strain was crossed to the same responder line which results in progeny with the same genetic background as experimental cross. For *Dilp2*-CREB^{RNAi} mean= 48.80 (SEM ± 7.904) and for *w*1118-CREB^{RNAi} mean= 11.15 (SEM ± 3.426).

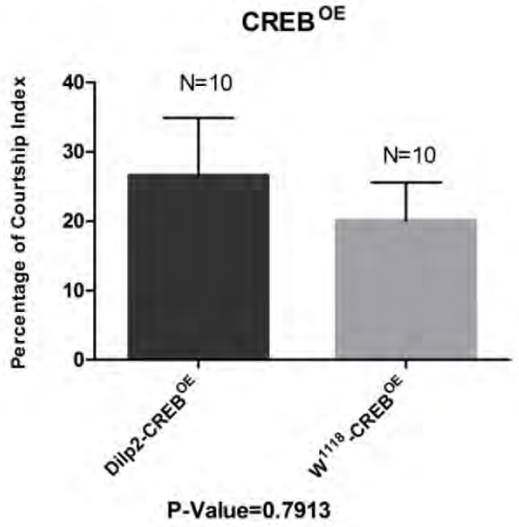

Figure 18. CREB overexpressing flies show no significant changes in percentage of CI compared to control flies (P-Value > 0.05). The *Dilp2*-GAL4 driver line was crossed to the UAS-CREB^{OE} responder line to obtain over expression of CREB gene in Dilp2 producing cells in pars intercerebralis. For control cross, the *w1118* strain was crossed to the same responder line which results in progeny with the same genetic background as experimental cross. For *Dilp2*-CREB^{OE} mean= 26.59 (SEM ± 8.305) and for *w*1118-CREB^{OE} mean= 20.07 (SEM ± 5.527).

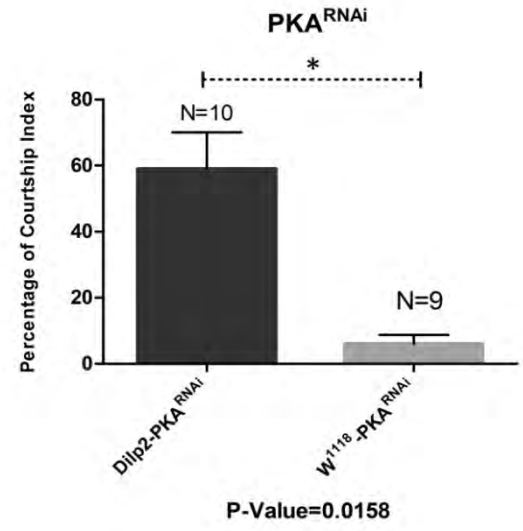

Figure 19. PKA knockdown flies show a significant increase in percentage of CI compared to control flies (P-Value < 0.05). The *Dilp2*-GAL4 driver line was crossed to the UAS-PKA^{RNAi} responder line to produce RNAi against PKA which knocks this gene down in Dilp2 producing cells in pars intercerebralis. For control cross, the *w1118* strain was crossed to the same responder line which results in progeny with the same genetic background as experimental cross. For *Dilp2*-PKA^{RNAi} mean= 58.97 (SEM ± 11.14) and for *w*1118-PKA^{RNAi} mean= 6.005 (SEM ± 2.672).

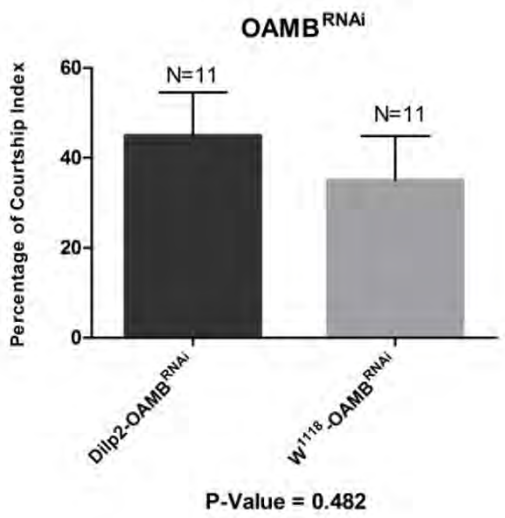

Figure 20. OAMB knockdown flies show no significant changes in percentage of CI compared to control flies (P-Value >0.05). The *Dilp2*-GAL4 driver line was crossed to the UAS-OAMB^{RNAi} responder line to produce RNAi against OAMB which knocks this gene down in Dilp2 producing cells in pars intercerebralis. For control crosses, the *w1118* strain was crossed to the same responder line which results in progeny with the same background as experimental crosses. For *Dilp2*-OAMB^{RNAi} mean= 44.90 (SEM ± 9.673) and for *w*1118-OAMB^{RNAi} mean= 34.99 (SEM ± 9.879).

Aggression assay

The aggression assay was performed on knockdown flies for three genes involved in the CREB pathway, CREB, PKA and OAMB, to investigate the hypothetical role of these genes in aggression behaviour of the flies. As mentioned previously, CREB was knocked down using two different responder lines and in addition an overexpression cross was tested. Data were first analyzed for total number of aggressive and mating behaviours which two males show towards each other (Fig.21). There was a significant increase in total number of behaviours of experimental crosse compared to control cross in OAMB knockdown flies (Fig. 21, E). In CREB overexpressed, CREB, PKA and OAMB knockdown flies no significant changes could be detected in experimental creooses compared to contril crosses (Fig.21, A, B, C, D). Data were further analyzed for three different categories of behaviours that males usually show towards each other; High Intensity Fighting (HIF), Low Intensity Fighting (LIF) and male-male Mating Behaviour (MB). In RNAi mediated-CREB knockdown flies a significant increase in HIF in experimental crosses compared to control crosses can be seen, however, there are no significant changes in LIF and MB (Fig.23). In PKA knockdown flies a significant decrease in LIF in experimental crosses compared to control crosses can be seen, however, there are no significant changes in HIF and MB (Fig.25). In dominant negative protein-mediated CREB knockdown, CREB overexpressed, and OAMB knockdown flies no significant changes in any of HIF, LIF and MB could be seen (Fig.22, 24 and 26).

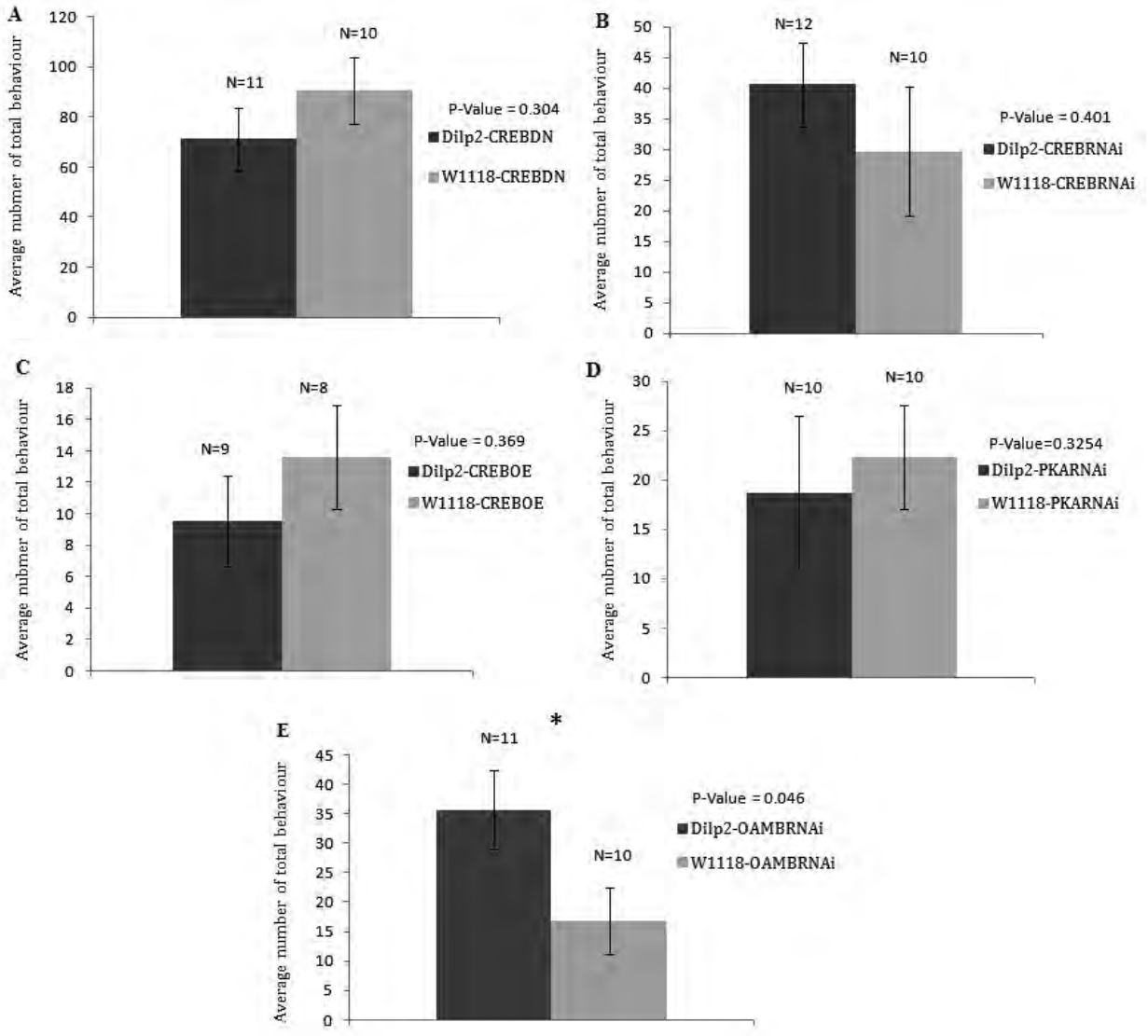

Figure21. The effect of changes in expression of the three genes CREB, PKA and OAMB in Dilp2 producing cells on aggressive and male-male courtship behaviours (the number of total behaviours).**A&B.** Both RNAi and dominant negative protein-mediated CREB knockdown flies show no significant changes in the number of their behaviours compared to control flies (P-Value > 0.05).**C.** Overexpressed CREB flies show no significant difference in the number of behaviours (P-Value > 0.05).**D.** PKA knockdown flies show no significant changes in the number of behaviours compared to control flies (P-Value > 0.05).**E.** OAMB knockdown flies show a significant increase in the number of behaviours compared to control flies (P-Value < 0.05).

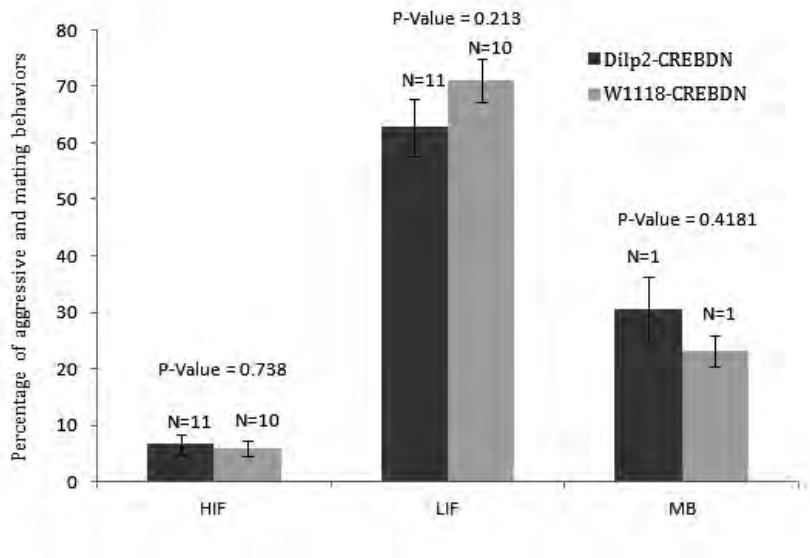

Figure 22. The effect of dominant negative protein-mediated CREB knockdown on High Intensity Fighting (HIF), Low Intensity Fighting (LIF) and male-male Mating Behaviour (MB) in *Drosophila.*CREB knockdown flies show no significant change in their aggressive or male-male mating behaviours compared to control flies (P-Value > 0.05).

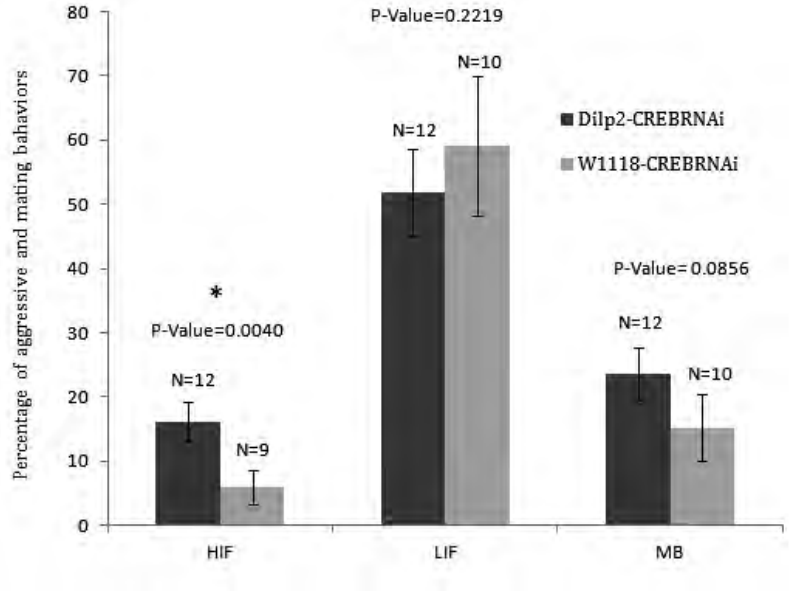

Figure 23. The effect of RNAi-mediated CREB knockdown on High Intensity Fighting (HIF), Low Intensity Fighting (LIF) and male-male Mating Behaviour (MB) in *Drosophila.* CREB knockdown flies show a significant increase in HIF behaviour compared to control flies (P-Value < 0.05). There is no significant change in LIF (P-Value > 0.05).There is an increase in MB but it did not reach statistical significance (P-Value > 0.05).

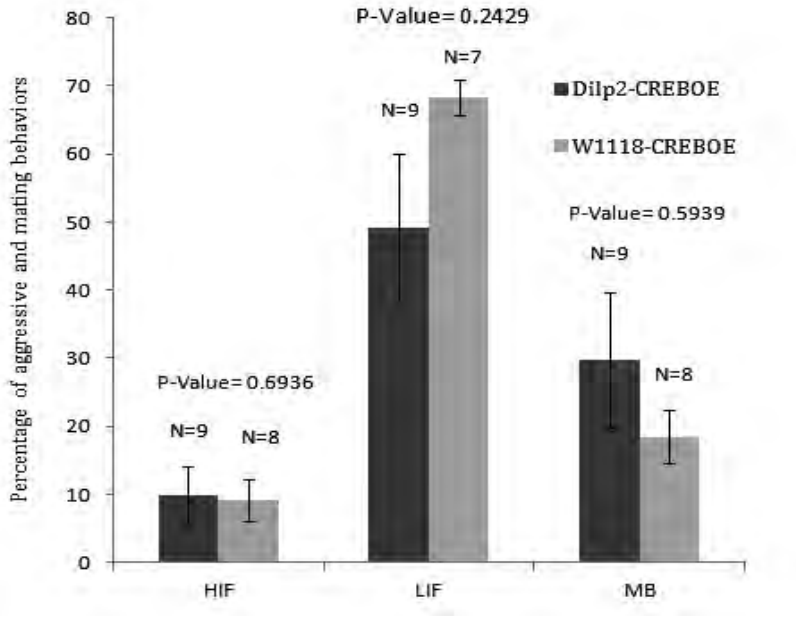

Figure 24. The effect of CREB overexpression on High Intensity Fighting (HIF), Low Intensity Fighting (LIF) and male-male Mating Behaviour (MB) in *Drosophila.* CREB overexpression flies show no significant change in their aggressive or male-male mating behaviours compared to control flies (P-Value > 0.05).

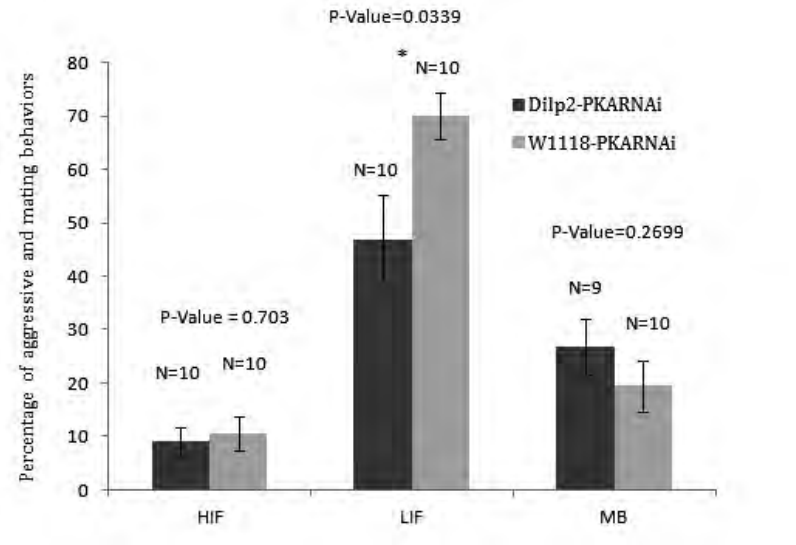

Figure 25. The effect of knocking down PKA on High Intensity Fighting (HIF), Low Intensity Fighting (LIF) and male-male Mating Behaviour (MB) in *Drosophila.* PKA knockdown flies show no significant change in their HIF behaviour compared to control flies (P-Value > 0.05) but there is a significant decrease in LIF (P-Value < 0.05). There is no significant change in MB (P-Value > 0.05).

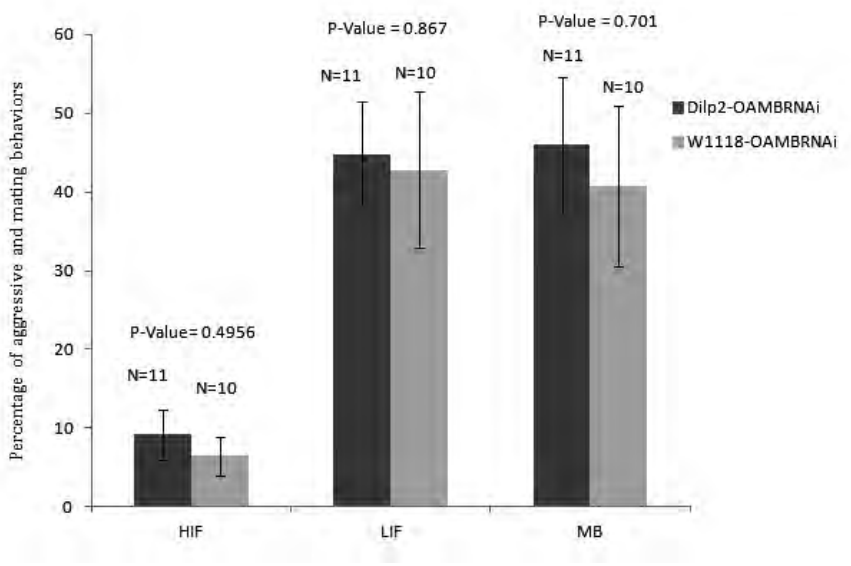

Figure 26. The effect of knocking down OAMB on High Intensity Fighting (HIF), Low Intensity Fighting (LIF) and male-male Mating Behaviour (MB) in *Drosophila.* OAMB knockdown flies show no significant change in their behaviour compared to control flies (P-Value > 0.05).

Discussion

In *Drosophila,* metabolic homeostasis is regulated by DILPs and AKH which have the same effect as mammalian insulin and glucagon pancreatic hormones[4-5]. The insulin signaling pathway is highly conserved through evolution due to its pivotal role in maintaining homeostasis but the site of insulin production differs between mammals and *Drosophila*. In *Drosophila* a subset of neuroendocrine cells called IPCs in the pars intercerebralis form the center of DILP production[1,4-6]. Among seven DILPs that have been identified in Drosophila, Dilp2, 3 and 5 are produced by IPCs and Dilp2 is most closely related to the human insulin peptide with 35% overall homology[13,19,42]. There are several proteins involved in the pathway that leads to expression of the genes related to metabolic homeostasis. Among those proteins, CREB is a transcription factor that binds upstream to genes involved in the gluconeogenic program of the

cell and activates their transcription. CREB activates downstream gene expression only when it is binds to its co-activator, TORC (CRTC). The function of TORC (CRTC) is depending on its localization in the cell which is regulated by phosphorylation/dephosphoeylation. SIK2 is a kinase that phosphorylates TORC (CRTC) and prevents it from entering the nucleus. SIK2 activity can be blocked by another kinase PKA which is activated by cAMP [1]. The molecular mechanisms of TORC (CRTC) phosphorylation which regulate its activity seems to be highly conserved in both flies and mammals[41].

In this experiment we knocked down the following genes; CREB, TORC (CRTC), PKA and SIK2 in Dilp2 producing cells using the UAS/GAL4-RNAi system to investigate the effect of these genes in the metabolism of the fly.The results from starvation assay reveals that the sensitivity to starvation increased in all knockdowns (CREB, TORC (CRTC), PKA and SIK2) significantly compared to control flies. This result suggests that there are strong correlations between the expression of these four genes and the metabolism of the flies. This increased sensitivity could result from disrupting the expression of gluconeogenesis-related genes. Flies with dysfunctions in gluconeogenesis can not compensate for the dropping levels of circulating glucose during starvation. Hence, they show a significant reduction in the survival rate compared to control flies with intact ability for gluconeogenesis.

Although the results of experimental and control crosses suggest strong effects of CREB, TORC (CRTC), PKA and SIK2 knockdowns on increased sensitivity to starvation, the result of the starvation assay on the *Dilp2*-GAL4 driver suggest that we should consider other possibilities. The *Dilp2*_GAL4 driver also shows increased sensitivity to starvation compared to other control crosses. These results give rise to the probability that the increased sensitivity to starvation seen in the CREB, TORC (CRTC), PKA and SIK2 knockdowns might be due to molecular

malfunctioning of the driver line that makes a molecular deficiency effecting metabolism in all the experimental crosses that have been made using *Dilp2*-GAL4 driver line. Furthermore, we should consider the possibility that molecular malfunctions of the *Dilp2*-GAL4 driver might arise just in homozygotic flies and it might not affect heterozygotic flies. Given that, the first generation of knockdown progenies might not have this malfunction and increased sensitivity to starvation could be just a result of gene knockdown. This can be investigated further by setting up a new control crosses between the *Dilp2*-GAL4 driver line and the *yw* strain to examine the effect of heterozygosity on sensitivity to starvation.

Measuring the total lipid content of flies in TORC (CRTC), PKA and SIK2 knockdowns showed no significant change in knockdown flies compared to control flies. The results for CREB knockdown flies show a significant increase in total lipid content of the knockdown flies compared to control flies. This increase in lipid content can be an indication of central role of CREB in the regulation of gluconeogenesis driven by dilp2 neurocecretory cells. Knocking down the CREB suppresses the expression of genes involved in gluconeogenesis. As a result, flies store the excessive energy in the form of lipids but they are not able to use this stored energy later on which in turn results in accumulation of lipid content. This lipid storage however, cannot help the knockdown flies to survive longer during starvation periods due to lack of functional gluconeogenesis.

Octopamine, a biogenic amine which is the invertebrate norepinephrine orthologue, was described as a regulator of feeding behaviour via transcription factor CREB in *C. elegans* [39]. Previous studies in *Drosophila* have shown that octopamine regulates sleep behaviour through a pathway involving CREB and PKA[40-41]. In addition to those studies, our own group's preliminary results indicates that feeding flies with an octopamine agonist (Chlordimeform)

increases lipid content of their bodies (Geoergen P. et al unpublished data). Having those in mind, we hypothesized that octopamine acts on the *Drosophila* hypothalamus, known as the *Pars intercerebralis*, to regulate various behaviours such as aggression, mating and feeding via a CREB dependent pathway. Thus we knocked down CREB in Dilp2 producing cells using two different responder lines, CREB-RNAi and CREB-DN. We also over expressed CREB in Dilp2 producing cells to examine if it causes the opposite phenotypes or not. In addition, the genes for the octopamin receptor (OAMB) and PKA were knocked down using UAS-RNAi responder lines.

The results from the mating behaviour indicate that CREB RNAi-mediated knockdown flies get engaged significantly more in mating behaviour. CREB RNAi-mediated knockdown males recognize the presence of a female significantly faster than control males and spend more time performing mating behaviours. In addition to that, CREB overexpressing flies demonstrate a tendency to have delayed recognition of female's presence. Although the results for CREB overexpression are not significantly different from controls in latency of courtship behaviour, the P-Value is interestingly small ($P = 0.086$) implying a tendency for increased latency. The results also indicate that in PKA knockdown flies the courtship index is significantly increased compared to control flies. CREB dominant negative protein-mediated knockdown flies show no significant change in their mating behaviour compared to controls. A possible explanation could be the efficiency of the method. The reason for this difference between two CREB knockdowns might be that UAS-CREB-DN responder line does not block the gene expression as efficiently as UAS-CREB-RNAi line.

From the mating assay results it can be concluded that Dilp2 producing cells in *Drosophila* control mating behaviour through a pathway including the CREB transcription factor and protein

kinase A (PKA). Considering the known role of PKA phospholylation in regulating CREB activity, we suggest that PKA activation results in transcription of the genes under the control of CREB binding promoter. Expression of these genes, namely gluconeogenic genes, suppresses mating behaviour in *Drosophila*. An explanation for this control is that, a low energy/ low glucose condition is sensed by Dilp2 producing cells in the brain resulting in an elevation of the cAMP concentration. Increased cAMP concentration activates PKA which leads to expression of gluconeogenic genes under the control of CREB binding promoter. Expression of these gluconeogenic genes not only results in bringing the circulating glucose concentration back to the normal levels, but also suppresses mating behaviours which need excessive energy which therefore are not favourable for animal survival under low energy conditions.

The results from the aggression assay indicate that knocking down or over expressing CREB has no significant effect on aggressive behaviour. Knocking down the PKA gene leads to a significant decrease in low intensity fighting but it shows no significant effect on total number of aggressive and male-male courtship behaviours. On the other hand, knocking down the octopamine receptor in Dilp2 producing cells significantly increases the number of total aggressive and male-male courtship behaviours but it does not change the percentage of high intensity or low intensity fighting and male-male courtship behaviours significantly. It can be concluded that octopamine regulates aggressive behaviour of the flies by binding to its receptors (OAMB) on Dilp2 producing cells and suppressing aggressive behaviours. Octopamine-mediated regulation of aggression is not related to CREB transcription factor expression, however.

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