

**Adrenergic signaling in the fruit fly *Drosophila*
melanogaster: establishment and analysis of models
for obesity and diabetes**

DISSERTATION

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List of abbreviations

aa	amino acids
aaRNA	aminoallyl ribonucleic acid interference
AKH	Adipokinetic Hormone
ATP	Adenosine Triphosphate
BODIPY	4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene
bp	base pair
Ca ²⁺	calcium ion
CAFE	Capillary Feeder
cAMP	cyclic adenosine monophosphate
CCA	Coupled Colorimetric Assay
cDNA	complementary deoxyribonucleic acid
cm	centimeter
CM	Concentrated Medium
CNS	Central Nervous System
CTCF	Corrected Total Cell Fluorescence
d	day
DA	Dopamine
DAG	Diacylglycerin
DAMS	<i>Drosophila</i> Activity Monitor system
DAPI	4',6-Diamidin-2-phenylindol
DNA	Deoxyribonucleotideic acid
DMSO	Dimethylsulfoxide
dNTPs	deoxyribonucleotide
dsRNA	double stranded RNA
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra acetic acid
EtOH	Ethanol

FB	Fat Body
FBS	Fetal Bovine Serum
g	gram
GFP	Green Fluorescence Protein
GPCR	G-Protein Coupled Receptor
GS	GeneSwitch
h	hour
HF	High Fat
HL3	Hemolymph-like buffer
HPLC	High Pressure Liquid Chromatography
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IIS	Insulin/IGF1-Signalling
ILP	Insulin-like Peptide
IPCs	Insulin Producing Cells
JNK	Jun amino-terminal kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
kb	kilobase pairs
L	Liter
LD	Light/Dark
L-DOPA	Dihydroxyphenylalanine
MB	Mushroom Bodies
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
ng	nanogram
OA	Octopamine
PBS	Phosphate Buffered Saline

PFA	Paraformaldehyde
PI	Pars Intercerebralis
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein Kinase A
qRT-PCR	quantitative Real-Time Polymerase Chain Reaction
RNAi	RNA interference
rpm	rounds per minute
RT	Room Temperature
RT-PCR	Reverse Transcription PCR
sec	second
S.E.M	Standard Error of the Mean
SSC	Saline Sodium Citrate
TA	Tyramine
TAG	Triacylglycerin
TARs	Tyramine receptors
Tdc2	Tyrosine decarboxylase 2
T β H	Tyrosine- β - Hydroxylase
UAS	Upstream Activating Sequence
VDRC	Vienna <i>Drosophila</i> Resource Center
ZT	Zeitgeber

1 Introduction

1.1 Overview of octopaminergic/tyraminerbic signaling systems and their receptors

Biogenic amines are ubiquitously found in the whole animal kingdom and exert functional diversity in numerous physiological processes. In insects, they have been detected not only in central, but also in peripheral and visceral nervous systems, and in neurohemal organs. Two monoamines, octopamine (OA) and tyramine (TA), are suggested to be biological counterparts of the mammalian adrenergic compounds norepinephrine and epinephrine that act as endogenous neurotransmitters, neuromodulators and neurohormones and that play important roles in various aspects of behavior and metabolism in almost all invertebrates studied so far (Roeder, 1999; Roeder et al., 2003). Originally, TA was thought to be just a transient molecule in the process of OA biosynthesis. However, increasing evidence has accumulated showing that TA acts as an independent neuroactive compound in various biological systems.

Biosynthesis of OA and TA starts from the same precursor, the amino acid tyrosine. As the first step, TA is synthesized by the action of the tyrosine decarboxylase (TDC), which is also the biological precursor for OA. In the same cells, the tyramine- β -hydroxylase (T β H) converts TA to OA by adding a hydroxyl group in the β -position. Thus, OA is the end product in the synthesis pathway (Roeder, 2005)(Fig. 1).

The occurrence of TA and OA in the same cells was the reason for a long lasting discussion if TA and OA act as independent transmitter compounds and if OA containing cells also release TA or not. For example, T β H deficient flies that lack OA contain much higher amounts of TA, which complicates the interpretation of results obtained with these mutant flies (Monastirioti et al., 1996). However, a study using TA-specific antibody identified a subset of tyraminerbic neurons distinguished from octopaminergic neurons in the larval brain, suggesting these cells are a major source of neuronal TA release (Nagaya et al., 2002). These data suggest that TA and OA act independently of each other, meaning that OA exerts its effect via specific OA receptors and TA through a different set of specific TA receptors.

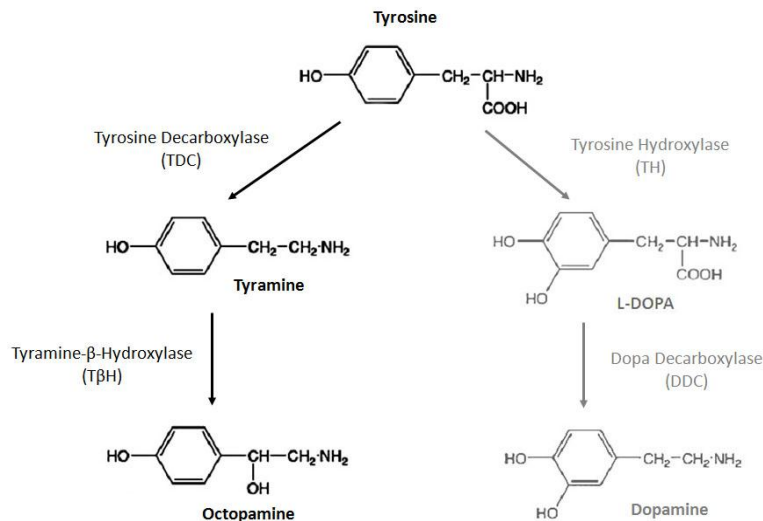


Figure 1. Biosynthesis of TA and OA. TA and OA are derivatives of the amino acid tyrosine. Tyrosine is converted to TA by action of the TDC, TA is then converted to OA by the TβH. Biosynthesis of dopamine starts with the same amino acid leading to dopamine via two successive enzymatic steps (gray). Modified from (Roeder, 2005).

Similar to adrenergic receptors in vertebrates, OA and TA receptors belong to the family of G-protein coupled receptors (GPCRs), capable of modulating different second messenger signal transduction systems (Evans and Maqueira, 2005). Four OA receptors (OAMB, Octβ1R (OA2), Octβ2R and Octβ3R) and three TA receptors (TyrR I, TyrR II, TyrR III) genes were identified in the *Drosophila* genome. OAMB, the first OA receptor that was identified, shows structural similarities to mammalian α-adrenergic receptors. It is crucial for ovulation via increasing Ca²⁺ levels in cells of the oviduct (Han et al., 1998; Lee et al., 2009) and for controlling sleep-awake behavior via modestly increases cAMP level (Crocker et al., 2010). The three other OA receptors share substantial similarities with mammalian β-adrenergic receptors that do not alter Ca²⁺ levels but strongly increase cAMP levels (Balfanz et al., 2005; Maqueira et al., 2005). TyrR I was identified almost 20 years ago as a receptor that can be activated by OA as well as by TA. Nevertheless, TA shows a substantially higher affinity than OA, which implies that it is a TA receptor (Arakawa et al., 1990). Although TyrR II is thought to be a fully specific receptor for TA, its activation does not induce any changes of cAMP levels. In contrast, TyrR III activated by TA binding decreases intracellular cAMP and also increases Ca²⁺ levels (Bayliss et al., 2013). Bayesian phylogenetic analysis of OA and TA receptors using the MUSCLE alignment

tool showed their relationship in *Drosophila*, *C.elegans* and human aminergic GPCRs (Fig. 2). The Oct β 1R, Oct β 2R and Oct β 3R cluster closely with β -adrenergic receptors, which were expected from earlier studies. However, this analysis implies that OAMB is not found within the vertebrate α 1-adrenergic receptor group; it is a sister group of other aminergic GPCRs (Beggs et al., 2011; El-Kholy et al., 2015). The TA receptors cluster into different groups unrelated to the “classical” OA receptors mentioned above. Two of the TA receptors, TyrR I and TyrR III, generate a unique cluster from others, whereas the TyrR II clusters together with the *C. elegans* ser-2 in close proximity to 5-HT receptors (El-Kholy et al., 2015).

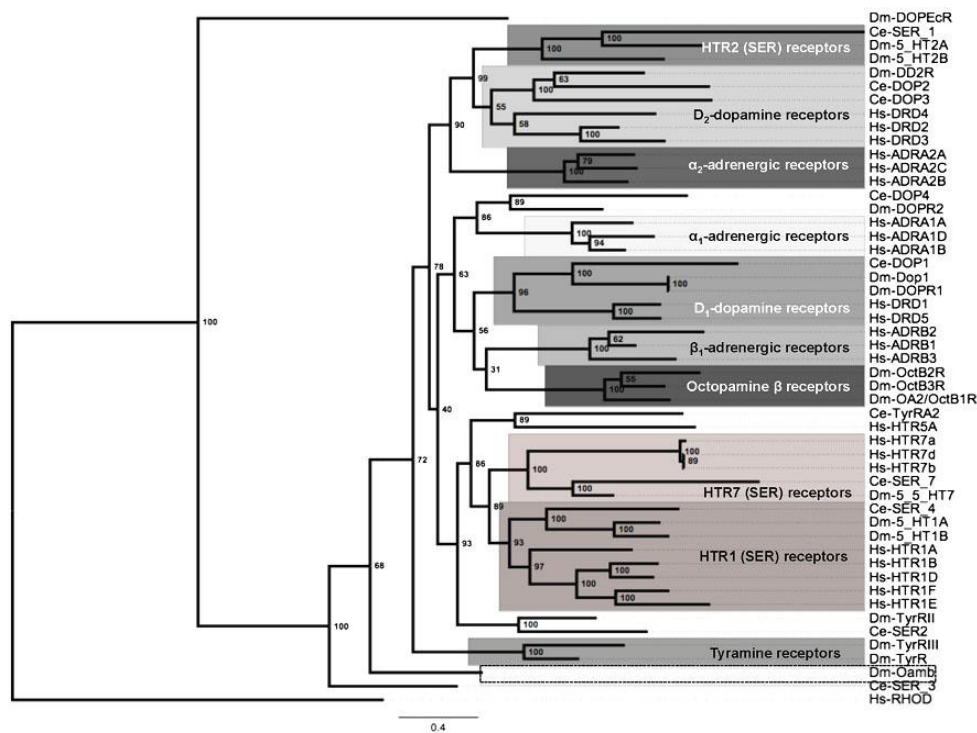


Figure 2. Bayesian phylogenetic analysis of bioamine GPCRs. The OA and TA receptors as well as other GPCRs from selected invertebrate and vertebrate were analyzed. Human rhodopsin is used as the outgroup. *Ce*, *Caenorhabditis elegans*; *Dm*, *Drosophila melanogaster*; *Hs*, human; *ADR*, adrenergic receptors; *HTR*, *SER*, serotonin receptors; *DRD*, *DOPR*, dopamine receptors. Adopted from (El-Kholy et al., 2015).

1.2 The functions of OA and TA in invertebrates

In invertebrates, OA and TA act as potent modulators that mediate diverse physiological processes not only in central nervous system (CNS) but also in

peripheral organs (Roeder, 2005). For example, OA is critical for eliciting a flash of light in the firefly lantern (Robertson and Carlson, 1976). Injection of OA into the circulation results in extendable postures, whereas injection of serotonin shows opposite behavior effects in lobster and crayfish (Livingstone et al., 1980; Glanzman and Krasne, 1983). The effects of OA on alteration of metabolism are elucidated in many organisms, which will be discussed in details below. In locusts, OA increases the rate, amplitude and speed of contraction and relaxation in skeletal and visceral muscles (Stevenson and Meuser, 1997). In *C.elegans*, OA and TA independently inhibit serotonin-stimulated aversive behaviors via separate receptors (Wragg et al., 2007). OA can activate cAMP response element-binding activity under starvation in *C.elegans* (Suo et al., 2006).

In *Drosophila*, OA has been found to increase the oviductal muscle contraction, and this process is critical for normal egg-laying in adult females (Lee et al., 2003). In contrast, TA shows an opposite effect on oviductal muscle contractions in locusts (Donini and Lange, 2004). The mechanisms of underlying the modulatory role of OA on ovulation are discussed below. Numerous studies showed that OA and TA also modulate the synaptic and neuronal intrinsic excitability in the periphery as well as in the CNS. OA increases the excitatory junction potential (EJP) in the neuromuscular junction, whereas TA decreases EJP amplitudes (Nagaya et al., 2002). TA and to a certain degree also OA are necessary for normal locomotor and rhythmic activity of both larvae and adult flies (Saraswati et al., 2004; Hardie et al., 2007). For learning and memory, OA and/or TA are involved in the formation of appetitive olfactory memories in adult (Schwaerzel et al., 2003) and larval flies (Schroll et al., 2006). Recently, diminished OA and TA neurotransmission was shown to substantially reduce flight initiation and maintenance in *Drosophila* (Brembs et al., 2007). In addition, octopaminergic neurons are involved in courtship, aggressive (Certel et al., 2007; Zhou et al., 2008), sleep-wake (Crocker and Sehgal, 2008; Crocker et al., 2010), social behavior, decision-making (Certel et al., 2010) and autoregulatory maintenance of synaptic and behavioral plasticity (Koon et al., 2011).

As pointed out above, increasing evidence shows that two biogenic amines, OA and TA, play important roles in controlling behaviors. Early study suggested that OA and

TA have opposite roles in regulation of locomotor activity in *Drosophila* larvae, and normal behavior producing dependent on a balance between the two signaling (Saraswati et al., 2004). Pharmacological studies also provided evidence that OA and TA influence different locomotor activity in honey bees (Fussnecker et al., 2006). In *C. elegans*, TA released from tyraminerpic neurons has behavioral roles that are independent of OA (Alkema et al., 2005). In adult *Drosophila*, flies deficient in only OA or both OA plus TA displayed different defects in behavioral phenotypes. These data imply that: (1) OA relaxes oviduct musculature promoting egg-ovulation, whereas TA contracts it (Cole et al., 2005); (2) TA undergoes effects on regulation of locomotor activity (Hardie et al., 2007); (3) OA participates the neural mechanisms for regulation of sleep-awake behavior (Crocker and Sehgal, 2008). Although an important role of OA is found and a subset of octopaminergic neurons are identified as being important for aggression, the functional significance of TA has not been elucidated (Zhou et al., 2008). Although much is known about the functions of OA and TA in physiological behavior, little is known about their effects on circadian systems. Recently, a genome-wide study of circadian changes in transcription within specific neural clock cells reveals that *Tdc2* is required within clock neurons for maintaining proper circadian rhythms. Furthermore, *Tdc2*^{R054} mutant flies have aberrant rhythmic activity (Huang et al., 2013).

In summary, OA and TA play physiological roles not only on almost every invertebrate group, but also on almost every sensory modality and peripheral organs.

1.3 Insulin/IGF signaling and its regulation

Studies in recent years revealed that insulin and IGF signaling (IIS) is evolutionary conserved between vertebrates and invertebrates and act as key effectors regulating lifespan, metabolism, growth, development, reproduction, stress resistance and feeding behaviors (Brogiolo et al., 2001; Baker and Thummel, 2007; Giannakou and Partridge, 2007). In *Drosophila*, eight insulin-like peptides (designated as dILP 1-8) and only one receptor (dInR) have been identified (Fernandez et al., 1995; Brogiolo et al., 2001; Garelli et al., 2012). Of these, three dILPs are highly expressed in a set of insulin producing cells (IPCs), which take the role of pancreatic beta cells. IPCs are a

subset of the *Pars intercerebralis* (PI) neurons and have a similar morphology with other PI neurons to produce distinct neurohormones such as neuropeptide-like precursor, SIFamide peptide and diuretic hormones 31 and 44 (Terhzaz et al., 2007; Park et al., 2008). These neuropeptides are all released into the circulation from the axon terminations of the tritocerebrum to the *corpora cardiaca/allata*, aorta and anterior gut structures (Rulifson et al., 2002; Cognigni et al., 2011). It is known that three different dILPs genes are co-expressed in IPCs, however, dILP2, 3 and 5 possess their own individual transcriptional regulation and target redundant functions among molecular evolution (Ikeya et al., 2002; Gronke et al., 2010). Ablation of IPCs or other genetic manipulations that diminished insulin signaling results in growth diapause, extended lifespan, increased resistance to oxidative and starvation stress, elevated carbohydrates and triacylglycerol storage, reduced fecundity and increased feeding under poor nutritional condition (Broughton et al., 2005).

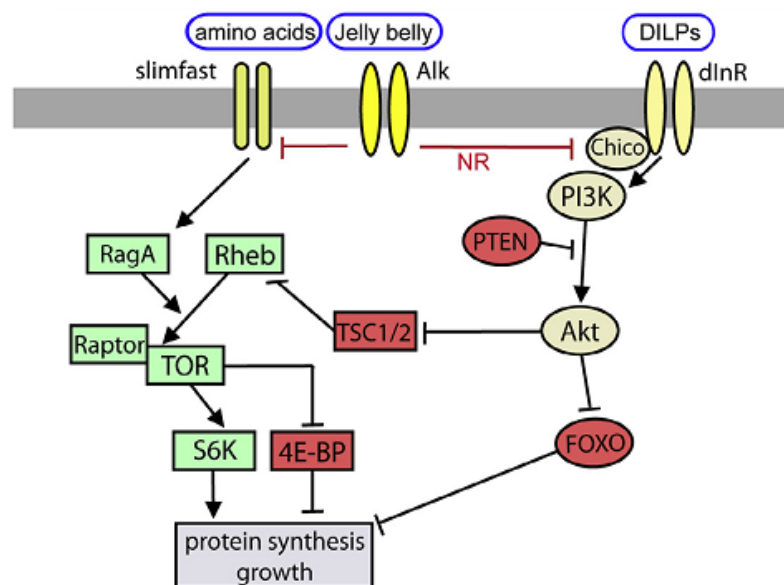


Figure 3. Scheme of the interaction between insulin/IGF and TOR signaling pathways. The two pathways are known to mediate protein synthesis and growth in response to nutrient availability, they intersect at the level of Akt (protein kinase B) and TSC1/2 (tuberous sclerosis tumor suppressor). The Alk (anaplastic lymphoma kinase) signaling pathway is only activated by nutritional restriction (NR) and inhibits two other pathways to alter size of the neuroendocrine cell bodies and axon terminations. Modified from (Cheng et al., 2011; Nässel et al. 2015).

The different insulin/IGF act effect via binding to the insulin receptor (InR) in order to regulate various physiological functions. InR is a dimeric glycosylated receptor

tyrosine kinase (RTK) that triggers signaling pathways targeting the activity of phosphatidylinositol-3-kinase (PI3K) and further downstream components to regulate a great variety of processes (Teleman, 2010). Especially in *Drosophila*, much progress has been made in order to understand the role of the insulin receptor signaling pathway and the interaction with other pathways (Fig. 3), as well as their role in growth, protein synthesis and metabolism (Saucedo et al., 2003; Cheng et al., 2011).

The functional roles of dILPs have been clarified based on a series of studies, however, the control of dILP production and release from IPCs by multiple factors has come into the research focus only recently. Apparently, dILPs production is regulated by neurohormones, neuropeptides, O-GlcNac and microRNA (Lee et al., 2008; Sekine et al., 2010; Varghese et al., 2010). A key trigger of dILPs release from IPCs appears to be cell autonomous nutrient intake, with many fat body derived nutritional signaling compounds mediating these effects. Unpaired 2 (Upd2), a protein with similarities with leptin molecule, activates the JAK/STAT signaling by regulating GABAergic neurons and thereby relieves the tonic inhibition to the IPCs (Rajan and Perrimon, 2012). The expression of dILP6 in the fat body inhibits dILP2 release from the brain and extends lifespan (Bai et al., 2012). Furthermore, the adiponectin receptor was identified in *Drosophila* IPCs and shown to be relevant for dILP2 release, indicated that it may sense to another adipokine signal from the fat body (Kwak et al., 2013). In addition, short neuropeptide F (sNPF) has been studied with respect to the role in regulation of growth, metabolism and lifespan, as well as for insulin production (Lee et al., 2008; Hong et al., 2012). Although sNPF and corazonin are co-expressed in the same neurons, corazonin seems to affect carbohydrate and lipid levels via acting in different ways on the IPCs (Kapan et al., 2012). Additionally, the GTPase nucleostemin 3 (NS3) is expressed in serotonergic neurons and serotonin 5-HT_{1A} receptors, on the other hand, are expressed on IPCs, making a direct connection between the two set of cells, which implicates an effect on insulin signaling mediated by these neurotransmitters (Kaplan et al., 2008; Luo et al., 2012). Recently, GABA and its metabotropic GABA_B receptor were shown to inhibit dILP production and release in response to metabolic stress (Enell et al., 2010). It was found that the fat

body-secreted factor Upd2, which is a functional homolog of leptin in mammals, activates the IPCs through relieving the inhibitory tone of the GABAergic neurons (Rajan and Perrimon, 2012). Another inhibitory neuropeptide, *Drosophila* tachykinin (DTK) acting via the receptor DTKR, was identified to regulate IPCs activity and consequently also the metabolism (Birse et al., 2011).

In addition, one of the OA receptors, OAMB, was found to be expressed in IPCs and was implicated to modulate IPC's activity, where it alters potassium channel activity via an increase of cAMP and protein kinase A (PKA). Moreover, a genetic knockdown of the OAMB receptor altered sleep:wake behavior in these flies (Crocker et al., 2010). Considerable evidence indicates that sleep modulation and metabolism (notably obesity) are highly correlated in mammals as well as in *Drosophila* (Horne, 2009; Spiegel et al., 2009), mutants in dILPs as well as those defective in the corresponding mutant exhibit decreased total sleep times. Overexpression of dILPs and dInR in the nervous system result in increased sleep times (Cong et al., 2015). In fact, OA signaling modulating metabolism was found in other insects (Roeder et al., 2003). It is known that OA stimulates release of lipids from isolated fat bodies in a dose-dependent manner in locusts and crickets (Fields and Woodring, 1991). OA together with synephrine, which is a highly potent OA receptor agonist, were examined for stimulatory effects on trehalose synthesis and glycogen phosphorylase activity in the fat body. In *Manduca sexta*, OA could enhance glucose and trehalose levels in the hemolymph (Ismail and Matsumura, 1992) and improve the glycogen phospholase activity in the fat body of larvae. The effects of OA on mobilization of carbohydrates are under feedback regulatory control and depend on the age of the insect because high sugar concentration in the hemolymph might inhibit OA-induced glycogen phosphorylase in larvae (Fields and Woodring, 1991). OA also has, as the hypertrehalosemic hormone (HTH), a hypertrehalosemic role on locust and cockroach fat body, however, injection of OA does not increase inositol trisphosphate (InsP3) synthesis in the trophocytes (Steele et al., 2001). Mobilization of energy reserves, which induced by OA release is considered to be a biological response to diverse stressors. In *Caenorhabditis elegans*, starvation activates the octopaminergic system through the cAMP response element-binding protein (CREB) dependent on

the activation of $G\alpha_q$ (Suo et al., 2006). Within the central nervous system (CNS), OA is known to modulate the most intensive stressful behavior namely the so-called fight or flight response (Roeder et al., 2003).

1.4 Modulation of circadian clock systems in *Drosophila*

Most organisms, including bacteria, plants and humans, have an internal circadian clock system to control and organize their daily rhythms with respect to the external stimuli. Circadian rhythms are physiological processes and behaviors that occurring 24h period. The entrainable system consists of three functional parts: 1) a neural circuit (the clock or pacemaker) that generates a 24 h oscillation plays a vital role in the circadian system, 2) input pathways that synchronize and reset the clock through external signals (such as light and temperature), and 3) output pathways that are driven by the clock system to generate overt rhythms on various physiological and behavioural processes (such as sleep-awake activity).

In both, mammals and insects, the circadian clocks are considered to act cell-autonomously that constructs with conserved genetic network, and this mechanism is modulated by more than three independent transcriptional feedback loops and other intracellular regulatory pathways (Stanewsky, 2003). Neuronal circuits are critical for generating circadian rhythms which has been elucidated in detail in *Drosophila*. In *Drosophila*, about 150 neurons organized in seven paired clusters (Sheeba, 2008; Lim and Allada, 2013) (Fig. 4). Three clusters are located laterally at the junction of optic lobe and central brain. They are distinguished from the small ventral lateral neurons (s-LN_vs), the large ventral lateral neurons (l-LN_vs) and the dorsal lateral neurons (LN_ds). Furthermore, three clusters of neurons are located in the dorsal region of the central brain that named the dorsal neurons (DN1, DN2, and DN3). The last cluster of neurons named the lateral posterior neurons (LPNs) is located on the lateral posterior side of the central brain. These seven distinct clusters of clock neurons form a combined network that together control the fly's rhythmic activity.

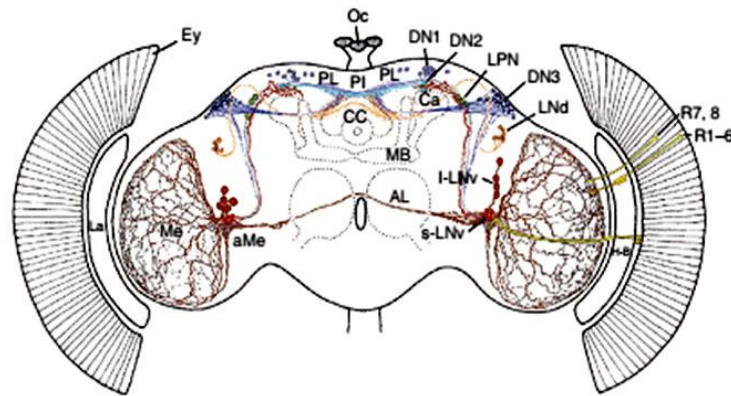


Figure 4. Scheme of circadian clock neurons in adult fly brain. Seven paired clusters of clock neurons are present in the fly's brain. Four clusters (I-LN_vs, s-LN_vs, LN_ds and LPNs) are located laterally; three clusters (DN1, DN2, and DN3) are located dorsally. The optic lobe transmits environmental signals to the I-LN_vs and s-LN_vs as well as possibly LN_ds, in which pass through dorsal clock neurons and access to downstream output neurons in the PI, and then integrate these signals to produce behavioral rhythms. The bilateral optic lobes are connecting through the posterior optic tract (POT).

Functional analysis of these clock neurons shows that s-LN_vs act as a dominant circadian oscillator that synchronize the clock cells (most the I-LN_vs) to drive the morning peak and a part of the evening peak activity via secreting the pigment dispersing factor (PDF), which is a neuropeptide transmitter expressed in LN_vs (Stoleru et al., 2005; Yoshii et al., 2009). Furthermore, I-LN_vs have been found to be involved in light-dependent arousal and phase shifting in the late-night (dawn) (Shang et al., 2008). Few studies of the roles on DN_s, currently the DN1s is known to integrate PDF and photic signaling producing appropriate daily behavior (Zhang et al., 2010b). DN2s show the principal role in regulation of temperature preference rhythm to influence overt rhythms (Kaneko et al., 2012). Additionally, non-PDF clock cells (DN_s, LN_ds or cryptochrome, CRY+ cells) seem to contribute to the evening peak of the rhythmic activity, flies with ablation of these cells displayed defects in dusk anticipation rather than in dawn anticipation in the presence of light:dark cycles (LD) (Murad et al., 2007; Stoleru et al., 2007).

Regarding the input factors relevant for entrainment of the clock circuits, light and temperature are the most important timing cues to synchronize the clock. Among them, several photoreceptors are crucial for light entrainment in some insects, such

as the compound eyes, the ocelli, the Hofbauer–Buchner eyelet in larvae, the blue light receptor molecule CRY1, rhodopsins and others (Chang, 2006). Temperature entrainment can produce phase shifts of circadian in response to the time of environmental cycles (Zimmerman et al., 1968). Recently, temperature entrainable oscillators have been reported in *Drosophila* brain. Clk⁺ neurons, DN1s, integrate light and temperature inputs to influence overt rhythmic activity (Zhang et al., 2010a) and DN2s circadian neural circuit specifically regulates temperature preference rhythm (Kaneko et al., 2012).

Increasing evidence showed that signals modulating the clock fall into the big category of neuropeptides, hormones and neurotransmitters. PDF is the best identified and the most important output molecule for the circadian system. Its receptor (PDFR) is expressed in a subset of clock cells and regions in the optic lobe and dorsal brain where PDF⁺ LN_s are known to project (Mertens et al., 2005). More recently, a screen of circadian-relevant neurons in *Drosophila* identified a circadian output circuit in subsets of the PI. Molecular analysis of specific PI neurons revealed a diuretic hormone peptide, DH44, as a circadian output molecule that is exclusively expressed in PI neurons and that is required for proper circadian rhythms (Cavanaugh et al., 2014).

1.5 The process of ovulation and fertilization

Ovulation is a very important step in the process of egg-laying. It comprises the process of releasing eggs from the ovary into the oviduct, the movement of the eggs down to the oviduct, and deposition of the eggs in the uterus preparing them for fertilization. Ovulation spontaneously occurs at a low level in adult virgins, however, copulation dramatically improves female ovulation rate during a time of 1.5 h after mating (Heifetz et al., 2000). Sperm storage is another important process for increasing female fecundity and fertility, which occurs in two types of tissues, seminal receptacle and spermathecae. The two types of storage tissues are different from each other in morphology and in sperm storage patterns. Between them, the seminal receptacle exerts a dominant role and stores most sperms. In contrast, few sperm are stored in the pairwise spermathecae. Although the seminal receptacle is

more efficient for sperm storage than the spermathecae, the latter one is a long-term storage organ and secretes substances, which are essential for sperm viability. The coordination of mature oocytes release from ovaries via ovulation with sperm release from storage tissues ensures successful fertilization, which is followed by egg-laying (Middleton et al., 2006).

In *Drosophila*, beyond mating itself, a number of seminal compounds are transferred from males to females after mating (Rubinstein and Wolfner, 2013). These proteins induce ovulation in females, thus representing the major signal that links mating and ovulation. These include seminal fluid proteins (Sfps), ejaculate components and peptides that play important roles in mediating female ovulation rate and sperm storage. For example, sex peptide (SP) increases egg-laying behavior and suppresses female receptivity, and is also essential for sperm release from storage in several days after mating (Avila et al., 2010).

In mammals, non-sperm components in the male ejaculate are produced in the testis, prostate and epididymis, containing hundreds of seminal fluid proteins which are vital for fertility. For example, SgI, SgII and fibronectin secreted by the seminal vesicles mix with other seminal fluid proteins of semen to generate ejaculate coagulation (Lilja et al., 1987). Within half an hour after ejaculation, the seminal clot is degraded by kallikrein-like proteases (KLKs) (Lilja et al., 1989). These processes are important to protect sperm from female immune system attacks and to promote immune tolerance that is essential for successful pregnancy (Robertson, 2005; Moldenhauer et al., 2009).

In addition, the entire female reproductive system is under strict hormonal control. Whereas classical hormones involved in the general control of reproductive processes such as the juvenile hormone and the ecdysteroids act on the longer time frame (Simonet et al., 2004), others directly control, for example, the transport of eggs to and within the oviduct. For the latter control system, two neuroactive compounds, namely, glutamate and OA execute this complex control system. Whereas glutamate induces oviduct contractions, OA inhibits these contractions and leads to relaxation (Rodriguez-Valentin et al., 2006).

The role of OA in controlling various aspects of female reproduction appears to be

slightly more complex as it controls various successively induced steps to enable laying of fertilized eggs. OA release increases the strength of peritoneal sheath contractions while the oviduct muscles are relaxed. This combined activity ensures that mature eggs are expelled from the ovaries and that the relaxed oviduct is able to accept the eggs and guide them to the uterus (Middleton et al., 2006). This effect is OA-specific. This complex modulatory system ensures that ovulation is induced only if sperm is available. Otherwise, unfertilized eggs would be laid, thereby reducing reproductive fitness dramatically. Thus, only in mated female that have sperm deposited in their sperm storage organs should trigger ovulation. Ovulin induces the increased peritoneal sheath contractions as well as the decreased tonus of the oviduct muscles in an indirect manner, obviously utilizing the endogenous octopaminergic system of the female fly (Rubinstein and Wolfner, 2013).

Octopaminergic neurons innervating oviduct muscle and epithelia are needed for ovulation and OAMB is essential for mediating the effects of OA on egg laying, meaning that OAMB-deficient flies phenocopy the OA-deficient ones regarding their female sterility (Lee et al., 2003). Restoring expression of the OAMB receptor in the oviduct of OAMB-deficient animals only, could save the egg-laying deficiency (Lee et al., 2009). Thus, OAMB expressed in the oviduct muscle seems to transmit the effects of OA on inducing ovulation in the fly, via activation of Ca^{2+} -signaling in epithelial cells.

1.6 Advantage of *Drosophila* as a model organism

The fruit fly *Drosophila melanogaster* has been used as a model organism for over 100 years and thousands of scientists around the world work with them. As with most of the long-established model organisms, it has a great number of advantages compared with other invertebrate models. The fruit fly has a very short life cycle, an inexpensive husbandry, and the presence of large polytene chromosomes allows genes to be mapped easily. Moreover, the utility of balancer chromosomes allows propagation of recessive, often lethal genes and induced mutations. Most of all, the fruit fly genome contains about 14,000 genes that share a high degree of homology with the corresponding human genes (Chen et al., 2008).

During the past century, enormous resources for public research have been accumulated. A large collection of *Drosophila* stocks including P-element insertions (Bellen et al., 2004), genomic gene-defined deficiencies (Parks et al., 2004; Thibault et al., 2004), induced mutations and RNA interference mediated gene-specific knockdown lines (Dietzl et al., 2007), are mostly available from the Bloomington Stock Centre or the Vienna *Drosophila* Resource Centre (VDRC). In addition, a comprehensive knowledge and information could be obtained in online Flybase, the most important well-annotated bioinformatics database for *Drosophila*.

Additionally, a unique advantage for using *Drosophila* as research model is the availability of powerful binary and ternary expression systems, such as GAL4/UAS system, LexA/LexAop system or QF/QUAS systems. It allows to ectopically overexpress or silence genes of interest in different spatial and temporal patterns. Among these systems, the GAL4/UAS expression system is the most widely used (Brand and Perrimon, 1993). In brief, the two essential components, GAL4 and UAS are separately inserted into two transgenic fly lines. The GAL4 gene is under the control of an endogenous gene promoter creating a driver line; a gene of interest is introduced under the control of upstream activating sequence (UAS) to create a responder line, and both units have no effect on the host organism per se. When the driver line is crossed with the responder line, their progeny has the gene of interest expressed in a specific cell or tissue result as defined by the GAL4 line (see Fig. 5). In addition to the accessible spatial control, many extremely versatile updates of GAL4/UAS system has been achieved using as temporal control, such as the introduction of GeneSwitch or TARGET systems, which is activated by a hormone induce or by a permissive temperature (Osterwalder et al., 2001; Suster et al., 2004). Due to these advantages, *Drosophila* has been studied extensively for investigating developmental and biological processes such as growth, cell death, metabolic disorders, learning and memory, sleep and awake, circadian rhythms and flight. Modeling of fruit fly physiological development has made important contributions to the understanding of developmental processes in humans.

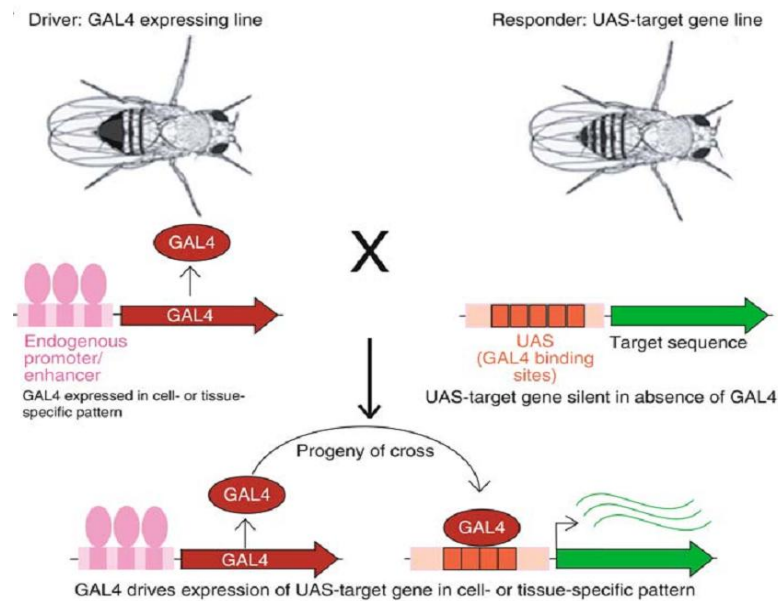


Figure 5. The schematic of GAL4/UAS system. While the GAL4 driver line crosses with UAS responder line, target gene is only expressed in a cell- or tissue-specific pattern in their progeny. Adopted from (Elliott et al., 2008).

1.7 Aims of the study

The major aim of this project was to study the roles of OA and TA signaling in regulation of metabolism and behavior in *Drosophila*, as well as the specific receptors that mediate the corresponding effects. My study comprises four major aspects.

The first part focussed on the roles of OA and TA in controlling production and release of dILPs from IPCs. The phenotypical peculiarities of flies deficient in OA and/or TA synthesis were studied especially according to metabolism, including lifespan, starvation resistance, lipids and carbohydrate levels. Genetic manipulation in order to increase the synthesis of OA or TA was also analyzed in detail. To elucidate which receptor mediates regulation of dILP release from IPCs, various fly lines defective in different receptors were studied.

The mechanisms mediating the metabolic effects were studied in the second part. I performed microarray analyses to identify transcriptional profiles regulated by OA in the adult *Drosophila* brain under starvation. A more detailed characterization of the regulated genes and their potential roles for the control of energy-demanding behaviors was studied.

The behavioral effects of OA and/or TA on circadian rhythms are described in the third part. To address this, the locomotor activity of flies deficient in OA and/or TA was compared and analyzed. Furthermore, an interesting finding that a tyramine receptor, TyrR I, is expressed in DH44 positive neurons in the PI. Manipulation of this receptor using RNAi-mediated knockdown was used to elucidate the role of this receptor for controlling rhythmic activity.

In contrast to the study that OA acts effects on ovulation through OAMB, I studied the role of Oct β 2R for ovulation but also for fertilization. For this, the expression pattern of Oct β 2R in the female reproductive organ was analyzed and a number of phenotypical defects of Oct β 2R mutant in ovulation and fertilization were quantified in females.

2 Materials and Methods

2.1 Materials

2.1.1 *Drosophila* strains and husbandry

Without otherwise statement, the fly stocks were raised on standard medium kept at 25°C with 50%-60% relative humidity under a 12 h light/dark cycle. Moreover, all assays utilized virgin females and unmated males at a density of 25-30 adults in each vial.

Table 1. Fly stock, genotype and donator.

stocks name	genotype	donator
w ¹¹¹⁸	w ¹¹¹⁸	Bloomington 5905
TβH ^{nM18}	w ¹¹¹⁸ ; TβH-M18, Fn7	Henrike Scholz
Tdc2 ^{RO54}	w ¹¹¹⁸ , Tdc2-RO54, Cyo	Jay Hirsh
Octβ2R	w ¹¹¹⁸ ; PBac[WH]Octβ2R ^{f05679}	Bloomington 18896
TyrR I	w*; PBac[GAL4D,EYFP]Oct-TyrR ^{PL00408}	Bloomington 19486
TβH-GAL4	w ¹¹¹⁸ ; P[GMR76G10-GAL4]attP2	Bloomington 46970
Tdc2-GAL4	w[1118]; P[w[+mC]=GMR15G03-GAL4]attP2	Bloomington 45806
Dilp2-GS-GAL4	w*; dilp2-geneswitch-Gal4	Osterwalder & Davis
nsyb-GAL4	w*; nsyb-Gal4	Julia Simpson
tubP-GAL4	y1 w*; P[tubP-GAL4]LL7/TM3, Sb1	Bloomington 5138
Mef2-GAL4	y[1] w[*]; P[w[+mC]=GAL4-Mef2.R]3	Bloomington 27390
Octβ2R-GAL4	w*; octβ2R-Gal4	BestGene Inc
TyrR I-GAL4	w*; TyrR I-Gal4	BestGene Inc
DH44-GAL4	w ¹¹¹⁸ ; P[Dh44-GAL4.TH]2M	Bloomington 51987
USA-GFP	w ¹¹¹⁸ ; P[UAS-Cameleon.2.1]82	Bloomington 6901
UAS-mCD8-GFP	w*; P(20XUAS-IVS-mCD8::GFP)attP2	Bloomington 32194
UAS-dTrpA1	w*; UAS-dTrpA1	gift
UAS-ChR2	w[*]; P[w[+mC]=UAS-ChR2.S]3	Bloomington 9681
UAS-Octβ2R-RNAi	yw*; UAS- Octβ2R ^{RNAi}	VDRC 104050
UAS-TyrR I-RNAi	yw*; UAS- TyrR I ^{RNAi}	VDRC 26876

2.1.2 Fly media

2.1.2.1 Standard medium

The ingredient of the food comprised of 6.25% autolysed yeast, 6.25 cornmeal, 1% agar powder, 2% glucose and 3% molasses and syrup. It was dissolved in sterile water

and boiled for 15 minutes. After cooling, 3% nipagin and 1% propionic acid were added as preservatives.

2.1.2.2 Concentrated medium (CM)

The concentrated medium was prepared in a volume of 200 ml included 10 g yeast extract, 10 g sucrose, 17.2 g cornmeal and 2 g agar.

2.1.2.3 High fat medium (HF)

The high fat fly food was based on a recipe of CM added 15% w/v of pure coconut fat. After loading the preservative, the diet was dispensed after cooling down to 45°C.

2.1.3 Solutions

Phosphate Buffered Saline Tween-20 (0.3% PBST): dissolve the following in 800 ml of distilled water, 8 g NaCl, 0.2 g KCl, 1.44 g Na₂PO₄, 0.24 g KH₂PO₄, 3 ml Tween-20. Subsequently, the pH was adjusted to 7.2 and additional distilled water was added up to 1 liter. The solution was autoclaved.

Paraformaldehyde in PBS (4% PFA): 2 g paraformaldehyde powder was dissolved in 50 ml PBS solution under continuous stirring at 45°C. The fixing solution was aliquoted and stored at 4°C after it became clear.

Hemolymph-like saline (HL3): 70 mM NaCl, 5 mM KCl, 4 mM MgCl₂·6H₂O, 1.5 mM CaCl₂·2H₂O, 10 mM NaHCO₃, 115 mM sucrose, 5 mM trehalose, 5 mM HEPES were dissolved in 800 µl deionized water, adjusted pH to 7.2 and brought to the final volume of 1 liter. The buffer was sterile filtered and stored at 4°C.

20× Saline Sodium Citrate (SSC): 3 M NaCl, 0.3 M Sodium citrate trisodium were dissolved in 1 liter distilled water, adjust pH to 7.0 with 1 M HCl, sterilized by autoclaving.

2.1.4 Antibodies

Rat monoclonal anti-GFP	Santa Cruz Biotechnology, Inc.
Rabbit monoclonal anti-Dilp2	Gift from Manfred Schmid, ETH
Rabbit monoclonal anti-DH44	Gift from Jan A. Veenstra, France
Rabbit monoclonal anti-DH31	Gift from Jan A. Veenstra, France
Donkey anti-Rat DyLight™ 488	Jackson ImmunoResearch Europe,

	Hamburg, Germany
Goat anti-Rabbit DyLight™ 549	Jackson ImmunoResearch Europe, Hamburg, Germany
Rat anti-mouse CD8a Biotin	eBioscience

2.1.5 Oligonucleotides

Table 2. Oligonucleotides used for PCRs.

primers	sequence (5' to 3')
ATPsyn-Cf6-F	CACATCACTAACACCAACTGC
ATPsyn-Cf6-R	GGGATTGCGACAGCATCTTA
bsk-F	CGTAAATGGCCGATATGCT
bsk-R	TACCGACTGTGGATGGTGAA
CF-Sp6-PCR	CAGCGGCCGAGATTTAGGTGACACTATAG
CF-Sp6-rG	CAGCGGCCGAGATTTAGGTGACACTATAGArGrGrG
CG1172-F	CCACGAGACTATCAGGTGGC
CG1172-R	GTAGCGCATCCCAGATCTCC
CG16837-F	CGGTTATTCGAGTCGATCCT
CG16837-R	AACACGAAGGTGCGTCTCTG
CG5656-F	GCCGTCTATGCTCATGGTCC
CG5656-R	TCCTAAGCAAGAGGCGAAGC
CG7236-F	CTGGCGACGTGCAATGTTTC
CG7236-R	GTGGTCTGTA CTGCTGGTC
Ddc-F	AAGTGGGATTTGCCAGTGAC
Ddc-R	TGCTGGTGAAC TTTGACTGC
DH44-F	TGGAACACGGA ACTCACAGG
DH44-R	GATTCAGTTCCGACCTGGCGG
Dhc93AB-F	GCCCAAGGAAGTGACCGTAA
Dhc93AB-R	CATCTCCTTGACGGCCTTGT
Dilp2-F	CTGAGTATGGTGTGCGAGGA
Dilp2-R	ACAACTGCAGGGGATTGAG
Dilp3-F	GGCCGCAA ACTGCCGAAAC
Dilp3-R	GGGAACGGTCTTCGAAGCCATCG
Dilp5-F	ATGCTGAGGGTTGCCTGTCCC
Dilp5-R	TCCAAATCCGCAAGTGGTCCTC
fit-F	ATCCGCACTCTGTCAACTGG
fit-R	GGCGTAGTTCTCGATCCGTT
OAMB-F	CGGTTAACGCCAGCAAGTG
OAMB-R	AAGCTGCACGAAATAGCTGC
OA2-F	GGCAACGAGTAACGGTTTGG
OA2-R	TCATGGTAATGGTCACGGGC
OAMB-AS-F	CTGCCGTGAGAACGACGAG
OAMB-AS-R	ATGTATGCGCAATGTGAGGC

OAMB-K3-F	CTGCCGTGAGAACGACGAG
OAMB-K3-R	GCGCAATATGAGCTGGGACT
Octb2R-F	TCCTGTGGTACACACTCTCCA
Octb2R-R	CCACCAATTGCAGAACAGGC
Octb3R-F	TGTGGTCAACAAGGCCTACG
Octb3R-R	GTGTTCCGGCGCTGTTAAGGA
Oligo-dT7-I	GAGAGAGGATCCAAGTACTAATACGACTCACTATAGGGAGA(T) ₂₅
Oligo-dT7-II	GAGAGAGGATCCAAGTACTAATACGACTCACTATAGG
rdgA-F	TGTAAGCCACCTATCGGGA
rdgA-R	GGAAAGAAGCTTGCCGCACTG
Rpl32-F	CCGCTTCAAGGGACAGTATC
Rpl32-R	GACAATCTCCTTGCGCTTCT
TyrR I-F	AGACGAGGTGCAAGGTGTTG
TyrR I-R	TTCCCCGACTTCTTTGACTGC
TyrR II-F	TGCAGTCTTTGCCACCTTCA
TyrR II-R	GTTGCCACGAGCCTATGAGA
TyrR III-F	GAAGTGGCCATCACCGACT
TyrR-III-R	GTGACGGCGAGATACCTGTC
yip2-F	TGAAGGGCATCAACCAGACC
yip2-R	GAGGAGGACGCGATCACATT

F: forward, R: reverse

2.1.6 Enzymes

Superscript III TM Reverse Transcriptase	Invitrogen, Darmstadt, Germany
Pwo DNA Polymerase	Peqlab, Erlangen, Germany
RNase inhibitor (40U/μl)	Invitrogen, Darmstadt, Germany
Taq DNA Polymerase (5 U/μl)	Invitrogen, Darmstadt, Germany
Porcine kidney trehalase	Sigma-Aldrich, Steinheim, Germany

2.1.7 Chemicals and kits

Agarose	Biozym, Oldendorf, Germany
Alexa Fluor(555/647)	Invitrogen, Darmstadt, Germany
BODIPY(493/503)	Molecular Probes life technologies, Darmstadt, Germany
Chloroform	Carl Roth, Karlsruhe, Germany
Coconut Fat	Peter Kölln KGaA, Elmshorn, Germany
DAPI	Invitrogen, Darmstadt, Germany

DIG Easy Hyb	Roche Diagnostics GmbH, Mannheim
DMSO	Carl Roth, Karlsruhe, Germany
dNTPs	Promega, Mannheim, Germany
FD&C Blue No.1	Ruth, Bochum, Germany
FocusClear	CelExplorer, TaiWan, China
GeneRuler™ 1kb DNA Marker	Fermentas, St.Leon-Roth, Germany
Glucose (HK) Assay Kit	Sigma-Aldrich, Steinheim, Germany
Goat serum	Sigma-Aldrich, Steinheim, Germany
Isopropanol	Carl Roth, Karlsruhe, Germany
Microarray 14K	Canadian <i>Drosophila</i> Microarray Center
Mifepristone	Sigma-Aldrich, Steinheim, Germany
MEGAscript® T7 Kit	Ambion Inc, USA
Nipagin	Carl Roth, Karlsruhe, Germany
NucleoSpin® RNAII Kit	Macherey-Nagel, Düren, Germany
Octopamine	Sigma-Aldrich, Steinheim, Germany
Paraformaldehyde	Sigma-Aldrich, Steinheim, Germany
Paraquat	Sigma-Aldrich, Steinheim, Germany
Propionic acid	Carl Roth, Karlsruhe, Germany
RNAmagic	Bio-Budget, Kredeld, Germany
SureClean	Bioline, Luckenwalde, Germany
SYBR® Green	Invitrogen, Darmstadt, Germany
Triglyceride	Invitrogen, Darmstadt, Germany
Triolein	Sigma-Aldrich, Steinheim, Germany
TritonX-100	Sigma-Aldrich, Steinheim, Germany
Tyramine	Sigma-Aldrich, Steinheim, Germany

2.1.8 Equipments

Agarose Gel electrophoresis unit	Biometra GmbH, Göttingen, Germany
Centrifuge 5415D	Eppendorf, Hamburg, Germany
<i>Drosophila</i> Activity Monitor DAM2	Trikenetics, Waltham, USA
GenePix™ 4000B scanner	Molecular Devices GmbH, München,

	Germany
Imager.Z1, Zeiss	Zeiss, Oberkochen, Germany
Kontes glass tissue grinder	Kimble Chase, Meiningen, Germany
Leica TCS SP1 confocal Microsystems	Leica, Heidelberg, Germany
MagnaRack	Invitrogen, Darmstadt, Germany
NanoDrop (ND-1000UV)	Peqlab, Erlangen, Germany
Olympus SZX12 microscope	Olympus GmbH, Hamburg, Germany
Omni Bead Ruptor 24	Omni International Inc, Kennesaw, USA
PCR amplification	Bio-RadLaboratories GmbH, München
Spectrophotometer	Multiscan MCC/340P version 2.32
StepOnePlus™ System	Applied Biosystems, Life Technologies
Thermocycler (Labcycler)	SensQuest GmbH, Göttingen, Germany

2.2 Methods

2.2.1 *GAL4/UAS crossing*

Without otherwise noted, the crossings were raised at 25°C, and the F1-generation was collected for the analysis.

2.2.2 *Lifespan measurement*

Adult male and female flies were separated within 8 h after eclosion and placed in vials supplied with standard medium. No more than 30 flies of each line were kept in a vial to avoid too high density. For the cross of *TβH-GAL4>UAS-dTrpA1*, the parental lines crossed to *w¹¹¹⁸* were used as controls, the F1 generation of these crossings was shifted to 30°C. For each genotype, four or five replicate cages were combined to analyze the different survival between the experimental and control groups. The flies were transferred to fresh medium every other day, and the number of dead flies was recorded daily.

2.2.3 *Starvation resistance*

During starvation experiments, all flies were kept at 25°C for 3 days, and then I

transferred them onto starvation medium containing 1% agar only. The progeny of crossings involving dTrpA1 were assayed at 30°C. The procedure ensured all the flies were at the same age and under identical treatment environment. The assays were performed employing more than 100 flies per genotype. Dead flies were counted every 2 h.

2.2.4 Cold coma recovery assay

To induce cold chill stress, flies without CO₂ anaesthesia were placed individually on a cell of 96-wells plate while being put on ice inside a fridge for 16 hours. The recovery period was recorded at room temperature until the fly regained consciousness to stand up. The experiments were performed by using at least 100 flies of each genotype.

2.2.5 Oxidative and osmotic stress

Adult flies were gathered in vials with standard food, males and females housed separately. After 3-5 day-old, flies were transferred to various media for stress challenges. Oxidative stress medium contained a final concentration of 20 mM paraquat, and food containing 0.6 M NaCl was used to investigate longevity of animal response to osmotic stress. Percent survival was recorded every 12 h.

2.2.6 Body fat quantification

Total triacylglycerol (TAG) in flies was measured using the Coupled Colorimetric Assay (CCA) method as described previously (Hildebrandt et al., 2011). Glyceryl trioleate was used as TAG standard for standard curve establishment, which was diluted in a series of 2-fold dilution with starting the quantity of 100 µg. For experiments, 5 females or 8 males per group as one biological replicate were weighted. Then the flies were transferred to a 2 ml screw-cap tube containing 1 ml 0.05% Tween-20 and homogenized with the help of 4 ceramic beads at a speed of 3.25 m/s for 2 min in a Bead Ruptor 24. Homogenates were heat-inactivated at 70°C for 5 min followed centrifugation for 3 min at 3000 rpm. Sample supernatant of 50 µl was pipetted to a

96 well microtiter plate. The various concentrations of TAG standard were treated in an identical way. The absorbance of samples was measured at 562 nm using a microplate reader. 200 μ l of pre-warmed triglyceride reagent was added into each well and incubated 30 min at 37°C with mild shaking. Then the absorbance was read at 562 nm again and the standard curve was set up by subtraction of blank values. The final quantity of samples was calculated per mg of fly wet weight base on the standard curve. Each genotype was measured in triplicate.

2.2.7 Bodipy staining

The neutral lipids in the whole body were stained with the amine-reactive 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY[®] 493/503) dye, which could be used to create green-fluorescent bioconjugates. The dye was dissolved in dimethyl sulfoxide (DMSO, 1mg/ml), and diluted 1:1000 (v/v) in PBS for working solution.

For each genotype, 5 flies were put in the tube without any liquid and frozen them in liquid nitrogen. After washing twice with 500 μ l cold 1 \times PBS, the PBS buffer was removed completely and 500 μ l 4% (w/v) paraformaldehyde (PFA) was added for fixation for 15 min at RT. Subsequently, the flies were repeatedly frozen in liquid nitrogen and thawed on ice for three times so as to disrupt the cells. Next, 200 μ l of BODIPY dye was given over the flies, incubated for 1 h in the darkness at RT. It was followed by two more washes in PBS and the preparations were observed under fluorescent microscope.

2.2.8 Glucose and trehalose measurement

To ensure accurate and reproducible glucose and trehalose measurement, a good quality of hemolymph droplets from fruit fly is the most important step, only the pure hemolymph derived from peripheral blood could accurately reflect the state of circulatory carbohydrates. The novel technique of hemolymph collection was performed in my study as described previously (Haselton and Fridell, 2011), which has advantages over traditional ways of isolation involving decapitation, such as the droplet samples would not be contaminated by intestinal contents, and it minimizes

any fat body tissue exude to the hemolymph sample.

In summary, newly hatched flies were maintained for 7 days. Before hemolymph isolation, all flies were starved for 3 h. Each fly was fixed with both wings pasted on double-sided adhesive to the surface of ice. They were arranged in even rows with head aligned and ensured that the flies are stabilization during the whole procedure. A sharp needle was used to gently puncture the central head capsule above the ptilinal suture. Once all the flies in one group have been punctured, 1 μ l microcapillary was used to collect the hemolymph droplets via capillary action along with pressing the fly's abdomen (Fig. 6).

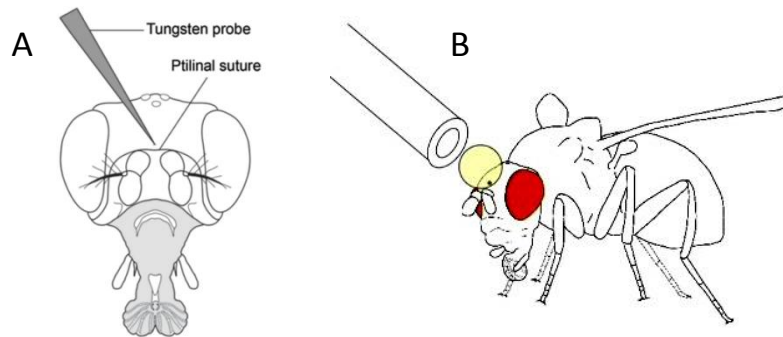


Figure 6. The positions of needle puncture in the head and hemolymph collection. (A) Frontal view of *Drosophila* head showing puncture location in the center of the head capsule just above the ptilinal suture. (B) Micracapillary collects hemolymph coming from the location of needle puncture by pressing thorax or abdomen. Modified from (Haselton and Fridell, 2011).

Usually, 0.3-0.5 μ l of hemolymph sample was pooled from 15-20 adult flies. The sample was diluted 1:10 in deionized water. The concentration of glucose was determined depending on standard curve established by known glucose concentrations. Glucose standard solution was diluted in a 2-fold series dilution starting from 1 mg/ml to 0.06 mg/ml with deionized water, 3 μ l of each diluted solution was loaded in 50 μ l glucose assay reagent (which was supplied in the glucose assay kit). The same volume of water added in 50 μ l glucose assay reagent was set as a blank. Mixed tubes and incubated them for 15 min at RT (25°C). Subsequently, the absorbance was measured at 340 nm with a Nanodrop 1000.

For trehalose determination, 0.25 μ l porcine kidney trehalose (1:200) was added in the remaining to convert trehalose into glucose, then the mixture was incubated at

37°C over night and measured absorbance again at 340 nm. The amount of trehalose was calculated as the second absorbance value subtracted from the first value.

2.2.9 Food intake assay

For the feeding assay, the processes were conducted as described (Wong et al., 2009) with some modifications. Flies were starved for 24 h and then located onto fresh food containing 0.5% (w/v) blue food dye (FD & C Blue Dye no. 1). Groups of 5 flies were homogenized in 1 ml of water with 4 ceramic beads, the suspension were flowed through a 0.3 µm filter to skim the lipid and debris. The absorbance of 800 µl of the suspension was measured at 629 nm with a spectrophotometer. The flies were kept on non-dyed medium and used as a baseline for calibration. The amount of food flies intake was calculated underlying a standard curve, which was previously established by serial dilutions of blue food in water.

2.2.10 CAFE assay

The Capillary Feeder (CAFE) assay was performed as a modified version as previously described in (Ja et al., 2007). Briefly, a hole was drilled into the container with cap (84×30 mm) where a yellow pipette tip was inserted, and some distilled water was added at the bottom to supply adequate internal humidity. A medium-sized tube (84×16 mm) was installed in the container. The tube had several fitted holes to ensure humidity coming in instead of flies crawling out. For each container, a 5 µl glass capillary was applied with liquid food which consisted of 5% (w:v) sucrose and 5% (w:v) autolyzed yeast. To minimize evaporation, about 0.5 µl mineral oil was sucked in by capillary action, the capillary filled with liquid was inserted through the tip that placed 5 mm below the cap, so the flies could approach the food easily (Fig. 7). For each assay, 2 seven-day-old flies were loaded in the internal tube and allowed to recover for one day prior to the measurement. The experiment was carried out at 25°C under constant conditions. The descent of the top meniscus of liquid was monitored for 24 h, and then one photo was taken and the length of food consumption was measured by the stereo microscope and cellA program, two blank tubes without flies were set up as evaporation control, the final consumption was

calculated as the test consumed volume subtract the evaporated volume.

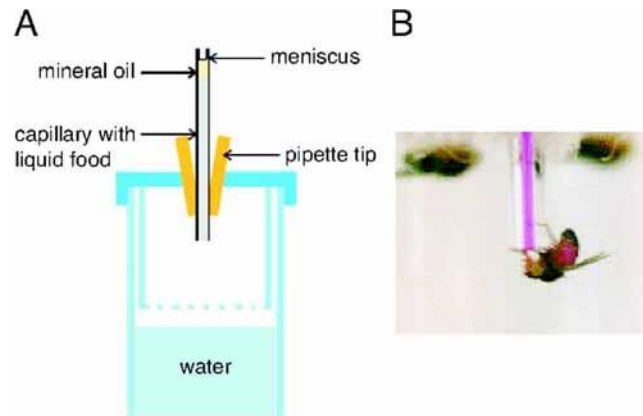


Figure 7. Scheme of the CAFE assay. (A) A 5 μ l glass capillary with liquid food was held in place by a pipette tip. Mineral oil was introduced to minimize evaporation. Flies were housed in a pierced inner chamber. Water in an outer chamber provides humidity. (B) Fly feeding could be visualized with a red dye added to the liquid food. Source is from Ja et al., 2007.

2.2.11 Measurement of metabolic rate

This protocol was described in (Yatsenko et al., 2014) and used with some modifications. A scheme showing the construction of the respirometer for CO_2 measurement is shown (Fig. 8). In detail, a long capillary micropipette was settled and sealed in the head of a 1 ml pipette tip. Soda lime, which was used as a CO_2 absorbent, was placed in the tip and insulated by two pieces of foam in front and back. 3 adult flies of the genotype of interest were placed in the pipette tip. Using plasticine the respirometer was sealed completely at the top. Two respirometers without flies were used as the atmospheric control. At the bottom of the chamber, a blue dye solution was added to make the liquid visible. The respirometer was placed in straight with the micropipette tips submerged into the colored solution. The chamber was covered with glass to prevent it from temperature and pressure fluctuations. The assay was performed for 2 h; subsequently a photograph of the chamber was taken. The amount of CO_2 production ($\mu\text{l/hr/fly}$) was calculated with the formula:

$$\frac{(\pi \times R^2) \times (\Delta d - \Delta c) \times 1000}{n \times h}$$

R: radius of capillary micropipette (cm).

Δd : height of the liquid in experimental samples far from the colored solution (cm).

Δc : height of the liquid in negative controls far from the colored solution (cm).

n: number of flies used.

h: hours of assay performed.

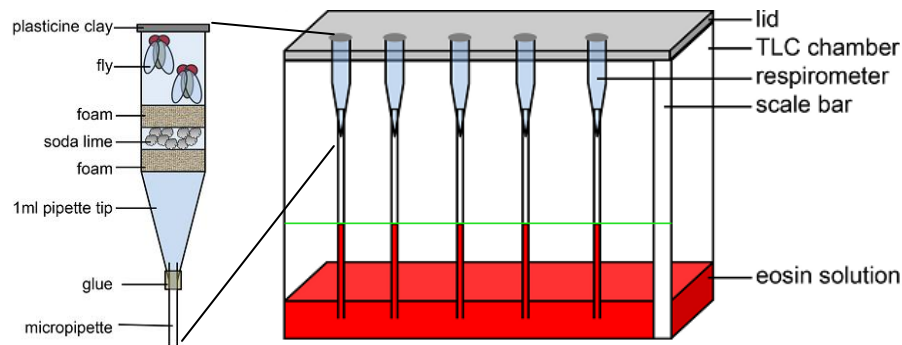


Figure 8. Scheme of setups for measurement of CO₂ production. The construction of the individual respirometer and the chamber show details. Eosin solution was alternatively replaced by blue dye (FD&C Blue No.1). Source is adopted from Yatsenko et al., 2014.

2.2.12 Immunohistochemistry and imaging

Tissues of 7-day-old adult flies were dissected in Hemolymph-like saline (HL-3) buffer under a microscope, fixed it in 4% PFA for 30 min at room temperature, and rinsed three times for 5 min each in PBST (0.3% Triton X-100), blocked in blocking-buffer (1×PBS + 1% Triton X-100 + 10% goat serum) for 30 min at RT. Subsequently, the primary antibody was added to incubate overnight at 4°C, after three times 5 min washing, the diluted secondary antibody was added and incubated for 3 h at RT in a constant darkness. The material was washed three times for 10 min each, finally the tissue was mounted on slide by immersing in 10 μl FocusClear medium. Each image was photographed by Carl Zeiss fluorescent microscope and Zeiss AxioVision 4.8 software.

The following primary antibodies and dilutions were used in this study: rat anti-GFP (1:200), rabbit anti-dILP2 (1:200), rabbit anti-DH31 (1:400) and rabbit anti-DH44 (1:400). Secondary antibodies used were: Dylight 488 conjugated donkey anti-Rat (1:500) and Dylight 549 conjugated goat anti-Rabbit (1:500).

For fluorescent intensity measurement, Zeiss Axio Imager Z1 with Apotome was applied for optical tissue sections. To facilitate comparison of the difference, several

fixed 2 μm -thickness of focal plane images were gathered under identical exposure time and then overlapped, the quantification of cell fluorescent intensity was measured using Image J and calculate the corrected total cell fluorescence (CTCF). $\text{CTCF} = \text{Integrated Density} - \text{Area of selected cell} \times \text{Mean fluorescence of background readings}$ (Gavet and Pines, 2010).

2.2.13 Fly behavioral assays

Fly behavioral activities were monitored using the *Drosophila* Activity Monitoring System (DAMS) (Fig. 9). To assemble the monitor, 10 adult flies for the big ones and one individual fly each for the small ones were located into glass tubes which contained roughly one-sixth volume of fly food in one end. The activity monitors carrying the glass tubes containing fly and food were housed in thermostatic incubator at 25°C and entrained for 5 d under a 12 h light/dark (LD) cycle condition. When flies crossed an infrared beam, the DAMS would record the sum of movement automatically every 2 min during the testing period.

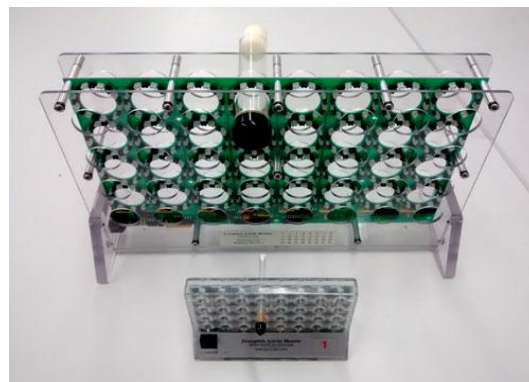


Figure 9. *Drosophila* activity monitors. The big monitor (upper) holds 32 tubes (2.5 cm \times 12.5 cm) for majority flies, the small one (lower) holds 32 tubes (0.5 cm \times 8 cm) for individual fly.

2.2.13.1 Circadian rhythm

Before recording, male and female flies were raised at standard condition for 5 d. 10 flies were placed in a glass vials for free running and entrained to LD cycles for 4 d at 25°C, followed by constant darkness (DD) for 4 d, then transferred to LD cycles again for another 4 d. The data were recorded every 2 min in the whole period. For the

point line graphs, the mean of every one hour was taken as a single data point. The data were shown in periodogram analyses.

2.2.13.2 Locomotor activity

Locomotor activity was quantified as the sum of total movement records during a phase period. Individual periods for days 2–4 were taken and determined using histogram analysis.

2.2.13.3 Sleep analyses

For sleep analyses, only the small monitor could be used. The protocol was following a previous description (Crocker and Sehgal, 2008). All flies were kept at 25°C on a LD cycle for 5 d. Individual flies were placed in small glass tube containing the appropriate food source. Flies were entrained for about 36 h before sleep recording. Locomotor activities were collected in LD with DAMS monitors as described above. Sleep was defined as bouts of 5 min of inactivity, in other words, at least 1 movement detected within 5 min was considered as wakefulness. The average of minute counts per hour of sleep was calculated as a single time point for each genotype.

Latency to sleep was confirmed as minutes of duration that from the moment lights were turned off to the first bout of sleep. Sleep scores were generated based on the amount of fragmentation seen in sleep, as measured by brief wakefulness and the length of sleep bouts.

2.2.14 Negative geotaxis

In the negative geotaxis assay, 20 flies at 5-days old were transferred without anesthesia into a tall clean cylinder, which has a scale ruler on it. After a 15 min acclimation period, the cylinder was tapped down three times and climbing was photographed once using a camera per 5 s post startle. This procedure was performed 10 times for each group at a 10 min interval. Four to five replicates for each genotype were tested in identical condition. Finally, the distance of flies have climbed was calculated base on the scale ruler. The difference between experimental and control group was analyzed using a one way ANOVA for multiple groups or

two-tailed Student's t-test for two groups in GraphPad Prism package.

2.2.15 Copulation test

The test was performed in the morning at a temperature of 25°C. Single virgin females, 1 to 5 days old, was placed together with two wild type w^{1118} males in a mating chamber (2 cm in height, 1.5 cm in diameter) supplied with a wetted piece of filter paper. The flies were allowed to recover for 10 min. Copulation was scored every 5 min and the number of copulations within 1 h was recorded. At least 20 virgin females were assayed for each genotype. Statistical analysis was performed using two-tailed Student's t-test with the Prism 6.0 program package.

2.2.16 Egg laying assay

Mated females collected from larger copulation setups were shifted to fresh vials. Five females in one vial were fed with yeast paste at 25°C and the number of laid eggs was counted every 24 h. At least four vials per genotype were analyzed. Statistical analyses were performed using ANOVA and the unpaired two-tailed Student's t-test. All data are presented as mean values \pm S.E.M. For the RNAi experiments, the number of laid eggs per day was counted.

2.2.17 Quantification of ovary sizes

Ovaries of mated females were prepared under the stereomicroscope. Ovaries of 1 day old females and those of 15 day old females were analyzed. Pictures of ovaries were taken and the area of individual ovary was measured using the Cell sense program package. Statistical analyses were performed using GraphPad Prism Software 6.0 with ANOVA and the unpaired two-tailed Student's t-test. All data are presented as mean values \pm S.E.M.

2.2.18 DAPI staining

Eggs were collected from the surface of the medium within 2 h after oviposition, washed with 1 \times PBS and fixed in 4% PFA for 5 min. After washing three times using

PBST (0.3% Triton X-100 in PBS), 5 min for each, all eggs were mounted on slides and stained with DAPI for 10 min before observation with epifluorescence microscopy.

2.2.19 Molecular biological methods for microarray

2.2.19.1 Total RNA extraction

Tissues of interest were dissected in HL-3 buffer on ice. After that, the dissected tissues were homogenized in 1 ml RNAmagic for 1 min at 3.25 m/s speed, with the help of ceramic beads using Bead Ruptor 24 implement. Following addition of 200 μ l of chloroform with shaking by hands 15 times, the mixture was centrifuged for 5 min at 15000 g. The upper phase was transferred carefully into a clean tube, mixed with an equal volume of isopropanol. The mixture was centrifuged for 30 min at maximum speed at 4°C after incubated at -20°C overnight. The RNA pellet was washed twice with 500 μ l cold 70% ethanol. Finally, all aqueous phase materials were discarded and air dried, the RNA resolved in RNase-free water and stored at -80°C.

2.2.19.2 First-Strand cDNA synthesis

The procedure was designated to convert mRNA into first-strand complementary DNA. The following components were used: 500 ng of total RNA, 0.5 μ l Oligo-dT7 I (10 pmol), 0.5 μ l CapFinder SP6rG1 (10 pmol), 1 μ l dNTPs mix (10 mM), add water up to 6.75 μ l, the mixture was incubated at 65 °C for 5 min, then placed on ice for at least 1 min. The following components were mixed in the indicated order: 2 μ l 5 × FSB buffer, 0.25 μ l RNase Inhibitor (10 U), 0.5 DTT (0.1 M) and 0.5 μ l Superscript III Reverse Transcriptase (100 U). Gently mixed and incubated at 50 °C for 1 h, and the reaction was terminated at 70°C for 15 min to inactivate the transcriptase. Then it was chilled on ice for immediate usage or stored at -20°C.

2.2.19.3 Second-Strand cDNA synthesis

The second-strand cDNA synthesis was performed by PCR. The following reagents were added: 5 μ l 10 × LA buffer, 8 μ l dNTPs mix (10 mM), 1 μ l cDNA template, 1 μ l AdaptorSp6-PCR (10 pmol), 1 μ l OdT T7 II (10 pmol), 0.5 μ l LA-Taq and 33.5 μ l ddH₂O was added up to total volume of 50 μ l. Vortex the mixture and centrifuged in a short

time.

The reaction was carried out in a thermocycler with the following program:

95 °C	1 min	
95 °C	20 sec	} 30 cycles
58 °C	20 sec	
72 °C	2 min 30 sec + 5 sec/cycle	
72 °C	5 min	
4 °C	∞	

A small volume of PCR products was estimated by gel electrophoresis on 1% w/v agarose. The samples were loaded in gel wells with staining by loading buffer. The voltage at 100 V was applied for 30 min, and 1 kb DNA ladder served as a reference. DNA fragments were visualized underlying Molecular Imager Gel Documentation camera.

2.2.19.4 Purification of PCR Products

For purification of PCR products, the reagent SureClean™ was used according to the manufacturer's instruction. An equal volume of PCR products and SureClean solution was mixed thoroughly, incubated at RT for 10 min, and then centrifuged at maximum speed (21000 g) for 10 min and the pellet retained. The pellet was washed twice in cold 70% ethanol at 2 × original volume, and resuspended it in 15 µl water. The final concentration and purity of cDNA was measured using a NanoDrop 1000.

2.2.19.5 Amino-allyl-cRNA synthesis

The synthesis of aa-cRNA *in vitro* was performed using T7-MEGAscript® Kit according to the manufacturer's manuals with some modifications in the following components. To avoid the precipitate of buffer, all materials were mixed at room temperature: 2 µl ATP (75 mM) + 2 µl GTP (75 mM) + 2 µl CTP (75 mM) + 1 µl UTP (75 mM) + 1,5 µl aaUTP (50 mM) + 2 µl 10 × reaction buffer + 3 µl template cDNA + 2 µl T7 enzyme and add DEPC-H₂O up to total volume 20 µl per sample. The mixture was incubated for 16 h at 37°C.

2.2.19.6 Purification of aa-cRNA

The aa-cRNA was cleaned with NucleoSpin RNA II Kit.

- Filled up above RNA to 100 μ l with RNase free water.
- Added 350 μ l of RA1 lysis buffer and mixed by vortex.
- Added 350 μ l of 70% ethanol and mixed again.
- Two times of loading 400 μ l of the mixture to the RNA-binding column and centrifuged at 11,000 g, 4 °C for 30 s. The rest of the mixture was flowed down with the same way.
- The column was washed twice with 600 μ l RA3 buffer and centrifuged as above.
- For the last washing, 250 μ l RA3 was added and centrifuged for 2 min at 11,000 g, 4°C.
- The column was placed into a new tube and the RNA was eluted with 50 μ l RNase-free water, centrifuged the tube at 11,000 g for 1 min after incubation 1 min at 65 °C. The collected flow was reloaded to the column and centrifuged again.
- Precipitated the RNA by adding 10 μ l of 3 M Sodium Acetate and 250 μ l of 96% ethanol. The mixture was incubated at -20°C for 30 min and centrifuged 30 min at 17,000 g, 4 °C.
- The pellet was retained and washed twice by 250 μ l 70% ethanol each and centrifuged. The pellet was air-dried and resolved in 8 μ l RNase-free water. The amount and purity of the aa-cRNA were measured.

2.2.19.7 Labeling of aa-cRNA

The Alexa Fluor dye 555 and 647 were used to label the equal amounts of aa-cRNA from control and treated sample respectively. Both dyes were restored to RT, and dissolved in 40 μ l DMSO. Basically, one reaction consisted of one volume sample around 20 μ g for each template, two volumes NaHCO₃ buffer (0.3 M, pH 9) and 3 volumes Alexa Fluor 555 or 647. Gently mixed it by pipetting up and down several times and protected at RT for 2 h from light. Then the sample was cleaned with the protocol as depicted above.

2.2.19.8 Hybridization of the microarray

The colored aa-cRNA was hybridized to one microarray 14 K chip and for further analysis of gene expression. In preparation of hybridization buffer, the total volume of 800 pmol of each dye mixed totally by pipetting up and down, and denatured with that of 345 μ l DIG Easy hybridization buffer, 16.6 μ l salmon testis and 16.6 μ l yeast tRNA at 65 °C for 10 min. During the period, a coverslip was cleaned and covered on the chip. Slowly avoiding any bubbles, the whole mixture was transferred onto the array along the brim of the coverslip. The array was then placed on a closed hybridization chamber and incubated at 42 °C for 16 h.

2.2.19.9 Washing the microarray

During the washing process, the array was always protected from light in a falcon tube that was wrapped with aluminum foil. Purification was carried out in the following washing steps with gentle rotation:

- 60 min in 1 x SSC at RT.
- 2× 30 min each in 1 × SSC + 0.1% Triton X-100 at 60 °C.
- 2× 30 min each in 0.1 x SSC + 0.1% Triton X-100 at 37 °C.
- 30 s in 0.1% Triton-X-100 at RT.
- 10 s in ddH₂O.
- Rinse briefly in ddH₂O.
- Immediately dry with nitrogen.

2.2.19.10 Image scanning and analyzing

The scanning was performed using the GenePixTM 4000B scanner. The quantification of fluorescent intensity and ratio of each spot were imaged with the corresponding software GenePix Pro 6.0. The further analysis of the data normalization, quality control and screening were applied by the Acuity 4.1 software. After filtering of the data generated from 3 independent biological replicates, those genes ratio change >2 fold for up- or down-regulated were selected for further study, and the representation of these clusters was created according to the description (Eisen et al.,

1998). Gene ontology terms relevant to regulated gene expression enrichment was analyzed in DAVID Bioinformatics Resources (Jiao et al., 2012) and the FlyBase that a basic gene database for *Drosophila* (<http://flybase.org/>).

2.2.20 Reverse transcriptase PCR analysis

The reverse transcriptase polymerase chain reaction (RT-PCR) was used to test the expression of different genes in various tissues of larval or adult wild type flies. The protocol of total RNA extraction was described in (2.2.18.1). The isolated RNA was free from contaminating DNA. In brief, cDNA-synthesis was performed at 50°C for 60 min using a conventional oligo (dT) primer and SuperScript III reverse transcriptase. PCR amplification was carried out using Taq DNA polymerase in a total volume of 25 µl and the gene specific primers were used for PCR. The program was processed according to table 3. Subsequently, the PCR products were checked by 1 % agarose gel electrophoresis.

Table 3. RT-PCR program.

window	Denaturation 95°C	Primer-Annealing 55°C	Elongation 72°C	Cycles
1	1 min	---	---	1
2	30 s	30 s	1 min	30
3	---	---	5 min	1
4	Cool down 4°C			∞

2.2.21 Relative quantification of qRT-PCR

For relative quantitative PCR, the reaction was performed using StepOnePlus™ Real-Time PCR System. The total volume of 10 µl each reaction was loaded into 48 wells plate which consists of 5 µl SYBR Green MasterMix, 0.25 µl ROX, 0.5 µl forward primer (5 µM), 0.5 µl reverse primer (5 µM), 1 µl cDNA (20 ng) and 2.75 µl water. The amplification was run as below:

95 °C	10 min	} 40 cycles
95 °C	15 sec	
60 °C	20 sec	
72 °C	35 sec	

95 °C 5 min
 60 °C + 0.3 °C until 95 °C
 95 °C 15 sec

} Melting curve

In a preliminary test, the primer efficiencies for both gene of interest and reference were analyzed basing on the standard curve and the slope, which was derived from serial dilutions of cDNA from 50 ng to 3.12 ng. The primer efficiencies were calculated according to $E=10^{-1/\text{slope}}$ formula (Pfaffl, 2001). Thus, the relative expression ratio (fold change) of a target gene was determined based on E and C_T values in comparison to a reference gene. In triplicates, the fold changes for each group were represented as mean \pm S.E.M for statistical analysis.

In this case, *RPL32* was served as a reference gene for all of assays.

$$\text{Ratio} = \frac{(E_{\text{target}})^{-\Delta CT_{\text{target}}(\text{sample-control})}}{(E_{\text{ref}})^{-\Delta CT_{\text{ref}}(\text{sample-control})}}$$

2.2.22 Statistical analysis

In all experiments, GraphPad Prism version 6.00 was mainly used for statistical analyses. The comparison between two groups, the unpaired two-tailed student's *t*-test was used, if multiple comparisons were done, one-way ANOVA with Tukey Post-hoc test were performed. All histograms were represented as mean \pm S.E.M. The survival curves of lifespan, starvation resistant, cold chill, oxidative and osmotic stress were analyzed with Log-rank (Mantel-Cox) test. Cell fluorescent intensity was analyzed using Image J software package. Venn diagram was created with the online tool Venn diagram generator of the pangloss laboratory. Statistical analysis of significant difference were defined as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$.

3 Results

3.1 Octopamine regulates insulin-like peptide secretion in *Drosophila melanogaster*

3.1.1 Octopamine signaling is essential for lifespan extension

Lifespan of many organisms can be extended by both genetic factors and environmental conditions, such as inactivation of insulin/IGF or Jun-N-terminal Kinase (JNK) signaling, reducing high calorie food intake and increasing stress-protective genes expression. Additionally, previous studies showed that the lifespan was also affected by hormonal signaling pathways (Jeong et al., 2012). To examine whether OA acts on lifespan in *Drosophila*, I performed a longevity test of transgenic flies with altered OA level.

Flies deficient in OA synthesis had a decreased natural lifespan compared to the genetic-matched controls for both, males and females. In males, the median survival of $T\beta H^{nM18}$ mutants has significantly decreased by 27% (Fig. 10A, Log-rank test: $p < 0.001$), and shortened median survival by 37% (Fig. 10B, Log-rank test: $p < 0.001$) to the control in female. It was confirmed that the median survival of control females (44 days) lived longer than males (37 days), but no change in median survival was observed in both sexes (27 days) of OA deficient flies.

To further investigate the role of OA in lifespan regulation, we genetically manipulated expression of T β H using the GAL4/UAS system. The $T\beta H-GAL4$ was used to drive a heat-sensitive channel dTrpA1 under transcriptional control of an UAS promoter. The dTrpA1 is an ion channel that can be switched by a temperature shift, thus depolarizing the cell and inducing release of its hormones/transmitters (Hamada et al., 2008; Chadha and Cook, 2012). Thus, the progeny of $T\beta H-GAL4 > UAS-dTrpA1$, that flies release more OA at a temperature of 29-30 °C. To minimize the genetic background aberration, the parental control lines $T\beta H-GAL4$ and $UAS-dTrpA1$ were crossed to w^{1118} respectively and the lifespan measurements were done at the same temperature conditions. Flies releasing more OA had a

significantly increased lifespan. The median survival increased in males by 7.1-11.1 % (Fig. 11A, Log-rank test: $p < 0.001$) and in females by 10-13.8 % (Fig. 11B, Log-rank test: $p < 0.001$).

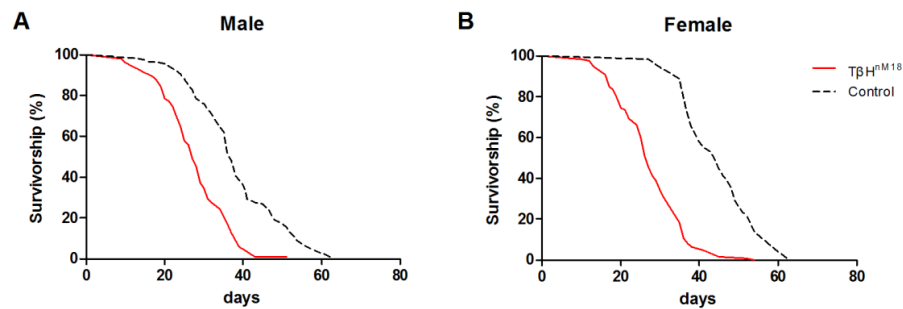


Figure 10. OA deficient flies show decreased lifespan in males and females. Lifespan was assayed using normally feeding flies. OA deficient males (A) and females (B) lived 27% and 38.6% shorter than control males and females respectively, in males the median survival time are 27 days for $T\beta H^{nM18}$ ($n=102$), 37 days for controls ($n=116$) and in females the median survival time are 27 days for $T\beta H^{nM18}$ ($n=121$), 44 days for controls ($n=123$). Log-rank test for survival analysis showed that $p < 0.001$ in both sexes.

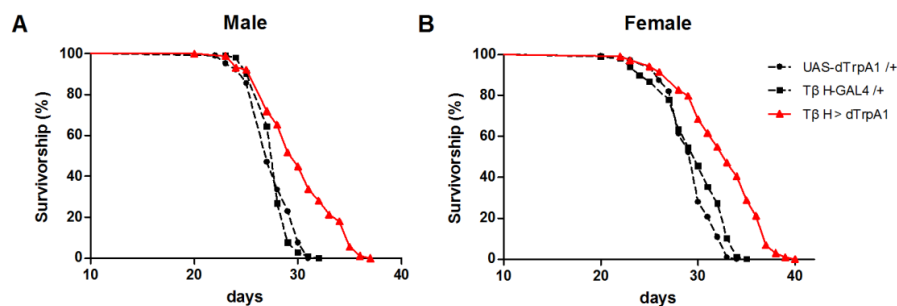


Figure 11. Flies releasing more OA show an extended lifespan in males and females. The experiments were carried out at 29°C. Triggered OA release in males (A) and females (B) induced longer lifespans than in either of both controls in males and well as in females; in males the median survival is 30 days for $T\beta H > dTrpA1$ ($n=91$), 27 days for $w^{1118} > dTrpA1$ ($n=105$) and 28 days for $T\beta H > w^{1118}$ ($n=105$), and in females the median survival time is 33 days for $T\beta H > dTrpA1$ ($n=104$), 30 days for $w^{1118} > dTrpA1$ ($n=112$) and 30 days for $T\beta H > w^{1118}$ ($n=100$). Log-rank test for survival analysis showed that $p < 0.001$ in both sexes.

3.1.2 Manipulation of octopamine signaling affects tolerance against starvation and cold coma but not oxidative stress

Long lifespan is associated with enhanced stress resistance (Kenyon, 2005). Thus, I tested whether altered OA level affects survival during starvation. Experimental and control flies were exposed to food deprivation by only feeding water. However, flies deficient in OA synthesis displayed a significant higher survival with respect to the controls. The median survival of $T\beta H^{nM18}$ mutant increased in males by 37.5 % (Fig. 12A, log-rank test: $p < 0.001$), and in females by 40% (Fig. 12B, log-rank test: $p < 0.001$). Although OA deficient flies show a large decrease in lifespan, they are more tolerated to starvation.

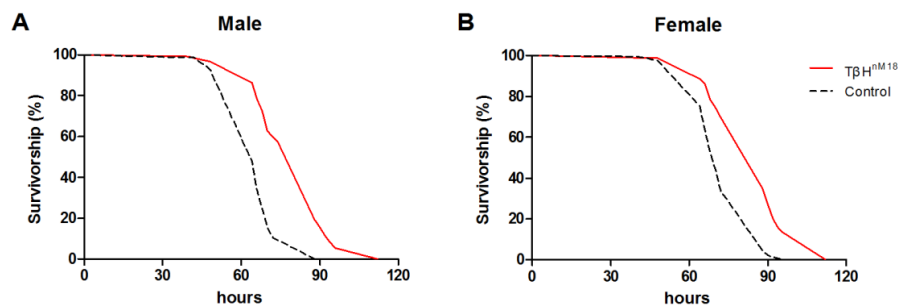


Figure 12. $T\beta H$ mutant flies display starvation resistance both in male and female. The survival of $T\beta H^{nM18}$ shown 37.5% longer in male (A) and 25.7% longer in female (B) than controls upon starvation, the median survivals are 88 h for $T\beta H^{nM18}$ males ($n=124$), 64 h for controls ($n=227$) and 88 h for $T\beta H^{nM18}$ females ($n=159$), 70 h for control females ($n=244$). Log-rank test for survival analysis showed that $p < 0.001$ in both sexes.

As noted previously, two monoamines of OA and TA are products of tyrosine metabolism. OA is converted from TA by the $T\beta H$ enzyme, TA is converted from tyrosine by the Tdc enzyme. Therefore, synthesis of both amines requires Tdc2, the disruption of neuronal Tdc2 results in flies devoid of both, TA and OA (Cole et al., 2005). However, $T\beta H^{nM18}$ mutant flies carrying a lesion in the $T\beta H$ locus were devoid of OA but retained 10 fold higher TA levels (Monastirioti et al., 1996). To further dissect the requirement for OA and TA in starvation stress, the survival time of $Tdc2^{RO54}$ mutants and their matched control flies were assayed under constant starvated conditions. Similar with $T\beta H^{nM18}$ mutant, flies devoid of OA and TA revealed a significant increased survival as compared with the controls. The median survival of $Tdc2^{RO54}$ mutant increased 17.8% in both sexes (Fig. 13, log-rank test: $p < 0.001$).

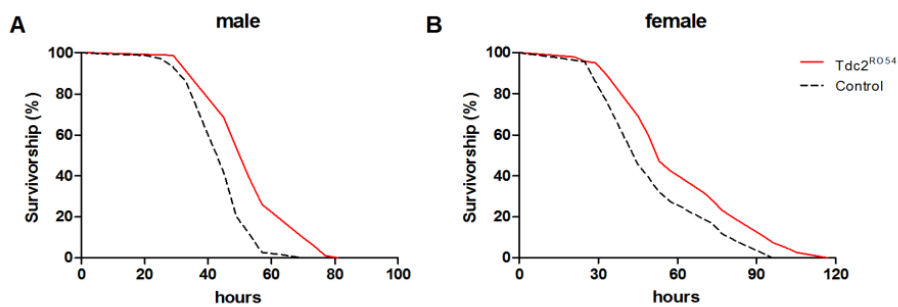


Figure 13. *Tdc2* mutant flies display starvation resistance both in male and female. The survival of *Tdc2*^{R054} shown 17.8% longer in males (A) and 17.8% longer in females (B) than controls upon starvation, the median survivals are 53 h for *Tdc2*^{R054} males (n=92) and females (n=199), 45 h for control males (n=144) and females (n=112), respectively. Log-rank test for survival analysis showed that $p < 0.001$ in both sexes.

In contrast to what was observed of OA deficient flies in response to starvation, the *TβH>dTrpA1* flies with induced release of OA were sensitive to starvation at 29 °C, with 12.1 % and 9.1 % decrease when compared with their parental control, respectively (Fig. 14, log-rank test: $p < 0.001$), observed in their median survival (30 h for *TβH>dTrpA1* flies, 34 h for *TβH-GAL4 /+* flies and 33 h for *UAS-dTrpA1 /+* flies). Taken together, these experiments indicated a fundamental role of OA in the regulation of starvation resistance in *Drosophila*.

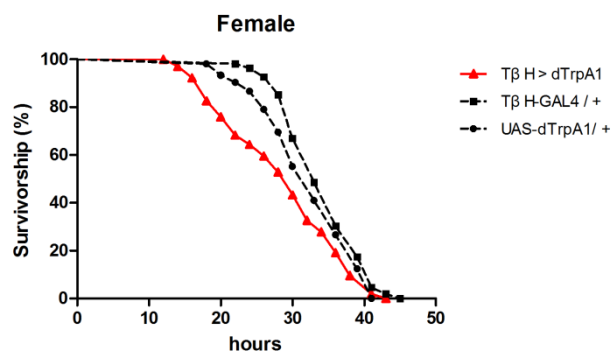


Figure 14. Flies releasing more OA are sensitive to starvation stress. All the flies were maintained at 29°C. *TβH* overexpression flies lived shorter than its parental controls, the median survival is 30 hours for *TβH>dTrpA1* (n=105), 33 hours for *w¹¹¹⁸>dTrpA1* (n=105) and 33 hours for *TβH>w¹¹¹⁸* (n=109). Log-rank test for survival analysis showed significant difference, $p < 0.001$.

To further demonstrate the reaction of OA signaling to stressors, I performed tests of cold coma recovery and oxidative stress resistance. *Drosophila* is unable to modulate

its body temperature; the most advantageous temperature for its development ranges from 18°C to 29°C. Therefore, to induce chill coma, flies were placed on ice for 16 hours and then returned to room temperature, the duration of recovery was monitored when the flies were able to stand. Base on the method described previously (Colinet et al., 2010), we compared the ‘short-term recovery’ between the different genotypes.

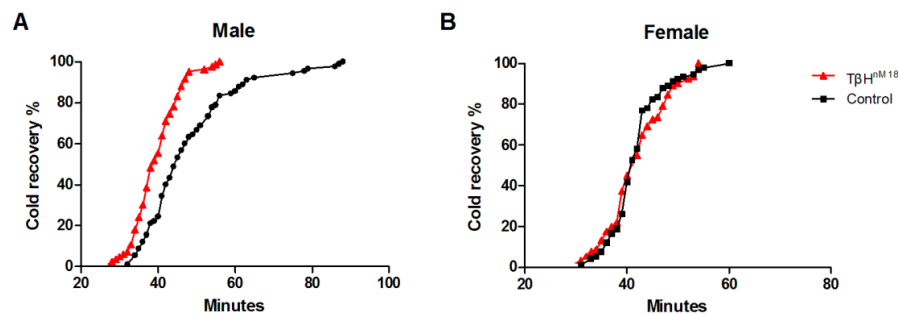


Figure 15. $T\beta H$ mutant males display different recovery times from chill coma. Flies deficient in OA showed short recovery time from chill coma only in male (A) but not in female (B) when compared with that of controls. The median recovery times are 39 min for $T\beta H^{NM18}$ males (n=83), 45 min for control males (n=90) and 42 min for $T\beta H^{NM18}$ females (n=91), 41 min for control females (n=91). Log-rank test for cold recovery analysis showed that $p < 0.001$ only in males, no significant difference in females.

The $T\beta H^{NM18}$ mutant male flies showed 15.4 % shorter time in recovery from chill coma than the male controls (Fig. 15A, log-rank test: $p < 0.001$). Nevertheless, no difference was observed between female flies (Fig. 15B, log-rank test: $p > 0.05$), indicating that cold stress-related phenotypes are association with fecundity.

Oxidative stress is another factor that affects lifespan. I induced this by feeding 20 mM paraquat added in normal medium. It was noted that, $T\beta H^{NM18}$ mutant flies did not alter their longevity when challenged to oxidative induction in both male and female flies (Fig. 16A and 16B, log-rank test: $p > 0.05$), although they were resistant to starvation and cold coma stress in this study. These data reveal a more complex mechanism that octopamine coordinates in the regulation of longevity and stress resistance.

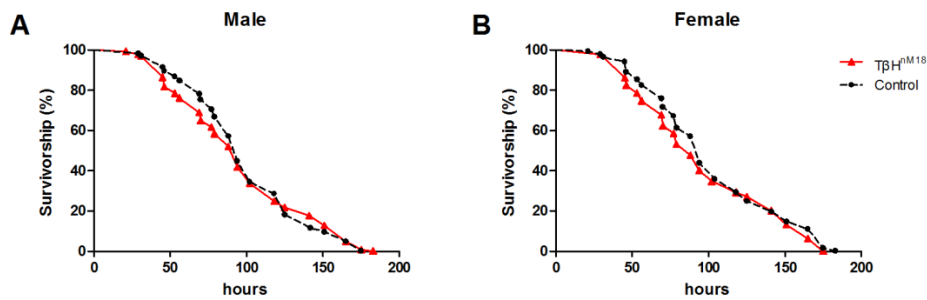


Figure 16. OA deficient flies are not more resistant to oxidative stress. OA deficient males (A) and females (B) showed the same survivorship if compared with controls under oxidative stress induced using 20 mM paraquat. The number of flies used for assay are $T\beta H^{nM18}$ males (n=125) and females (n=130); for the control males (n=105) and females (n=137). Log-rank test for survival analysis showed that $p > 0.05$ in both sexes.

3.1.3 Manipulation of octopamine levels change metabolic phenotypes

In *Drosophila*, energy accumulation is strongly associated with intervention that regulates lifespan and stress resistance (Djawdan et al., 1998). To test whether OA plays a role in modulating energy metabolism, I measured the levels of triglycerides (also called triacylglycerol, TAG), the major form of lipid droplet storage in insect. Whole body TAG levels were measured in adult flies raised on standard food. Compared to the controls, a significant increase of lipid content was observed in $T\beta H^{nM18}$ mutant flies, with 32.8 % increased levels in females (Fig. 17A left, $p < 0.01$) and 42.8 % higher ones in males (Fig. 17A right, $p < 0.01$). $T\beta H^{nM18}$ females can mate normally and produce fully developed ovaries, but their abdomens become swollen due to egg retention. To test whether increased lipid contents in females were caused by retained eggs in the ovaries, I measured triglyceride levels in ovary-enucleated females. Similarly, $T\beta H^{nM18}$ females without ovaries also showed the obesity phenotype with 18.1% higher TAG level than controls (Fig. 17B, $p < 0.05$). In contrast to OA deficient flies, $T\beta H > dTrpA1$ with triggered release of OA showed significantly reduced TAG levels, with 46.3% decreases at 29°C (permissive temperature) compared to that of at 19°C (restrictive temperature; Fig. 18, $p < 0.001$). Notably, decreased triglyceride levels in $T\beta H > dTrpA1$ flies are unlikely to be caused by temperature induction because no significant changes at 19°C were observed in their control flies. These data indicated that the regulatory role of octopamine is

involved in lipid metabolism.

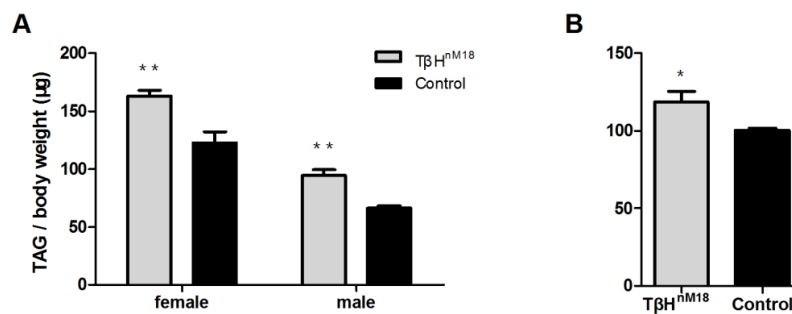


Figure 17. TβH mutants store more total TAG. Total TAG levels were measured in $T\beta H^{nM18}$ and control flies under normal feeding condition. The $T\beta H^{nM18}$ flies have significantly higher TAG levels, both in females and males (A), with 32.8% higher in female flies ($T\beta H^{nM18}$, n=8; control, n=8) and 42.8% higher in male flies ($T\beta H^{nM18}$, n=6; control, n=6). (B) TAG µg per body weight in females with removed ovaries. $T\beta H^{nM18}$ (n=8) showed 18.1% higher than control (n=8) in TAG level. Data are presented as mean ± SEM; two-ways ANOVA and the unpaired two-tailed Student's t-test were used for analysis, *p<0.05, **p<0.01.

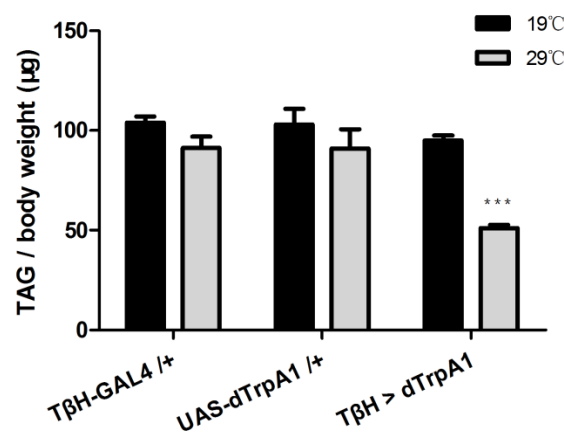


Figure 18. Flies releasing more OA had reduced TAG levels. $T\beta H > dTrpA1$ induced release of OA significantly reduced TAG levels, with 46.3% decreases at 29°C (n=14) compared at 19°C (n=8). For the parental controls, there were no different TAG levels between at 29°C and at 19°C ($T\beta H-GAL4/+$, n=12; $UAS-dTrpA1/+$, n=12). Data are presented as mean ± SEM; two-ways ANOVA was used for analysis, ***p<0.0001.

Next, I evaluated if the body fat deposition is changed in animal deficient of OA and TA. Compared to the controls, no significant differences of fat content were observed in $Tdc2^{RO54}$ mutant male and female flies (Fig. 19, p>0.05). Flies that lack of only OA are obese, while flies lack of both OA and TA have normal fat deposits. In other

words, animals decrease their body fat results from TA-scarcity, suggesting that OA and TA play contrary effects in fat deposition.

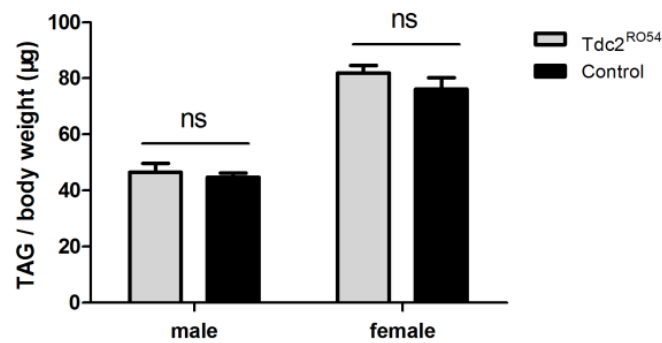


Figure 19. TAG levels are not changed in *Tdc2* mutant flies. Total TAG levels were measured in *Tdc2*^{RO54} mutant and control flies under normal feeding condition. The *Tdc2*^{RO54} flies have no significantly different TAG levels both in males (*Tdc2*^{RO54}, n=26; control, n=26) and females (*Tdc2*^{RO54}, n=26; control, n=26) related to controls, respectively. Data are presented as mean ± SEM; two-ways ANOVA and the unpaired two-tailed Student's t-test were used for analysis: ns, no significant difference.

Next, I tested whether different nutritional conditions can alter triglyceride levels in *TβH*^{nM18} flies. The *TβH*^{nM18} and control flies were shifted to concentrated medium (CM) or high fat medium (15 % fat oil added to CM, HF) for 5 days and the changes of TAG levels were examined. Consistently, *TβH*^{nM18} flies showed a significant increase in whole body TAG levels relative to controls, either the flies were maintained on CM (Fig. 20A, 35.6 % and 41 % increase in female and male, p<0.001) or HF (Fig. 20B, 30.4 % and 46.3 % increase in female and male, p<0.05). Moreover, both *TβH*^{nM18} and control flies fed on HF medium significantly increased their lipid storage relative to that seen in flies fed on CM.

In addition to lipids, the carbohydrates content is another form of energy storage in insects. In the hemolymph, circulating glucose and trehalose (a disaccharide used in insects) are two types of sugar which function as a source of energy and as a source of starting material for most types of biosynthesis, and their levels are strictly controlled by insulin (Ikeya et al., 2002). In the experiments, I tested the circulating levels of glucose and trehalose. Hemolymph was extracted from the *TβH*^{nM18} flies that were maintained on normal condition. Compared with the controls, a significant

decrease of the sugar levels was observed in $T\beta H^{nM18}$ flies, with 43.2 % decrease in males and 34.5 % decrease in females, respectively (Fig. 21A, $p < 0.05$). In order to confirm the effect of OA regulating carbohydrate metabolism, I measured the circulating glucose and trehalose levels in the $T\beta H > dTrpA1$ flies. The $T\beta H > dTrpA1$ flies with increased OA release (29°C) showed 25.2 % higher levels of these two sugars than those flies held at 19°C (Fig. 21B, $p < 0.05$).

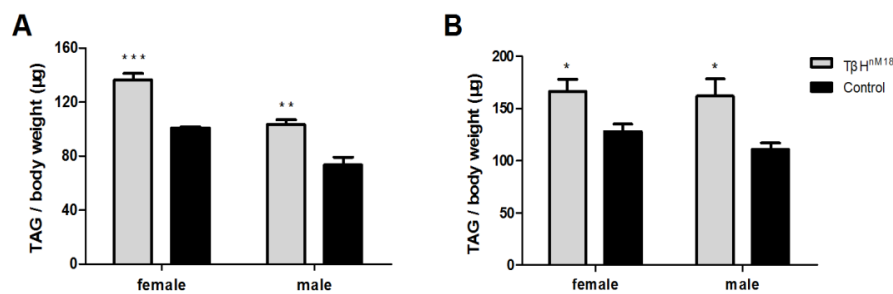


Figure 20. $T\beta H$ mutants show increased TAG storage in CM and HF medium. Total TAG levels were measured in $T\beta H^{nM18}$ and control flies under different nutritional conditions. (A) The $T\beta H^{nM18}$ flies have significantly high TAG levels both in female and male fed on CM, with 35.6% higher in female flies ($T\beta H^{nM18}$, $n=8$; control, $n=8$) and 41% higher in male flies ($T\beta H^{nM18}$, $n=8$; control, $n=8$). (B) The $T\beta H^{nM18}$ flies fed on HF also have significantly high TAG levels both in female and male, with 30.4% higher in female flies ($T\beta H^{nM18}$, $n=8$; control, $n=8$) and 46.3% higher in male flies ($T\beta H^{nM18}$, $n=8$; control, $n=8$). Data are presented as mean \pm SEM; two-ways ANOVA was used for analysis, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

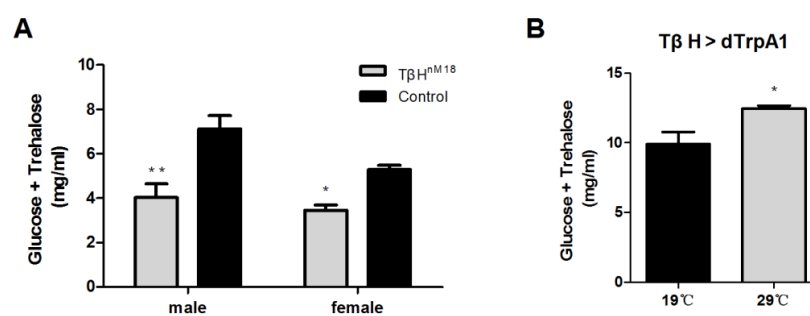


Figure 21. Manipulation of OA levels alters glucose and trehalose levels in the hemolymph. The hemolymph was extracted from flies, which were already pre-starved for 3 h (see details in *Material and Methods*). (A) The $T\beta H^{nM18}$ flies have significantly lower glucose and trehalose levels both in male and female, with 43.2% lower in male flies ($T\beta H^{nM18}$, $n=8$; control, $n=8$) and 34.5% lower in female flies ($T\beta H^{nM18}$, $n=7$; control, $n=7$). (B) Flies releasing more OA have high glucose and trehalose levels. The $T\beta H > dTrpA1$ flies with increased release of OA held at 29°C ($n=6$) showed 25.2 % higher levels of these two sugars than that of determination in flies fed at 19°C ($n=5$). Data are presented as mean \pm SEM; two-ways ANOVA and the unpaired two-tailed

Student's t-test were used for analysis, * $p < 0.05$, ** $p < 0.01$.

However, the circulating carbohydrate level displays unsynchronized aspects in *Tdc2^{RO54}* mutant males and females. Compared to the wild type controls, *Tdc2^{RO54}* males were significantly decreased by 49.1% in glucose and trehalose levels (Fig. 22, left, $p < 0.0001$), which is consistent with the observation in *TβH^{nM18}* flies. In opposite, a significant increase of these sugar levels was detected in *Tdc2^{RO54}* females, with 98.2 % increase (Fig. 22, right, $p < 0.0001$).

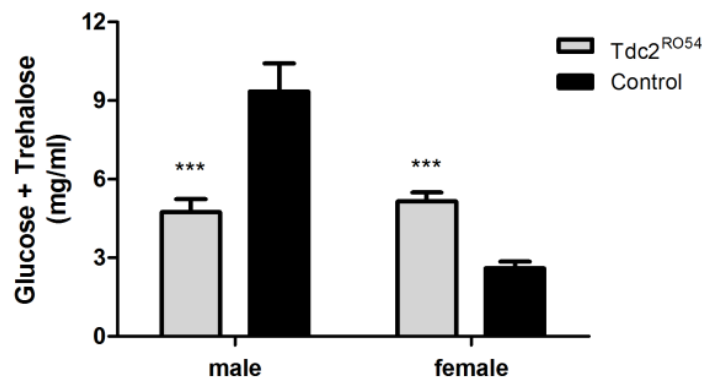


Figure 22. *Tdc2^{RO54}* mutants show different carbohydrate levels in male and female flies. *Tdc2^{RO54}* males were significantly decreased by 49.1% in glucose and trehalose levels (*Tdc2^{RO54}*, $n=22$; control, $n=18$); whereas *Tdc2^{RO54}* females were significantly increased by 98.2% in glucose and trehalose levels (*Tdc2^{RO54}*, $n=26$; control, $n=28$). Data are presented as mean \pm SEM; two-ways ANOVA was used for analysis, *** $p < 0.0001$.

3.1.4 Food intake is reduced in OA-deficient flies

It is known that an increase of energy storage could result from elevated food consumption. To test whether octopamine functions in metabolism by affecting food intake, I performed food intake assays using a standard system of blue dye food (Xu et al., 2008) and capillary feeder assay (Ja et al., 2007). When the flies were fed blue dye food, it showed that *TβH^{nM18}* female flies show a slight reduction in the amount of blue dye food relative to the control (Fig. 23A, 23.5 % decrease in female flies, $p < 0.05$). However, no change in food content was seen between *TβH^{nM18}* male flies and control male flies. In the CAFÉ assay, the volume of liquid food consumption from a glass microcapillary was measured over a 24 h period. Flies deficient in OA

synthesis displayed a significant decrease in food intake compared to the controls. The mean food consumption of $T\beta H^{nM18}$ flies decreased in males by 28.2 % (Fig. 23B, left, $p < 0.01$), and in females by 41.2 % (Fig. 23B, right, $p < 0.01$).

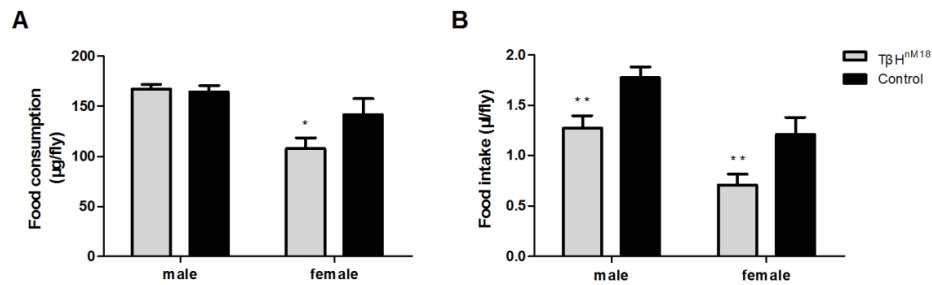


Figure 23. $T\beta H$ mutants decrease food intake. (A) Food consumption was measured by an experiment using food contained a non-absorbed blue dye. The $T\beta H^{nM18}$ female flies decreased 23.5% when compared to female control ($T\beta H^{nM18}$, $n=6$; control, $n=6$), whereas $T\beta H^{nM18}$ male flies showed no difference to the control ($T\beta H^{nM18}$, $n=11$; control, $n=11$). (B) CAFÉ assay. The $T\beta H^{nM18}$ flies had significantly reduced the amount of food intake both in female and male, with 28.2% lower in male flies ($T\beta H^{nM18}$, $n=10$; control, $n=10$) and 41.2% lower in female flies ($T\beta H^{nM18}$, $n=10$; control, $n=10$). Data are presented as mean \pm SEM; two-ways ANOVA was used for analysis, * $p < 0.05$, ** $p < 0.01$.

The CAFÉ assay showed that flies without OA and TA displayed a significantly decreased food intake only in females. The mean food consumption was decreased by 44.1% in $Tdc2^{RO54}$ female flies (Fig. 24, right, $p < 0.0001$), moreover, there was no difference detectable in male flies compared with the control male flies (Fig. 24, left, $p > 0.05$).

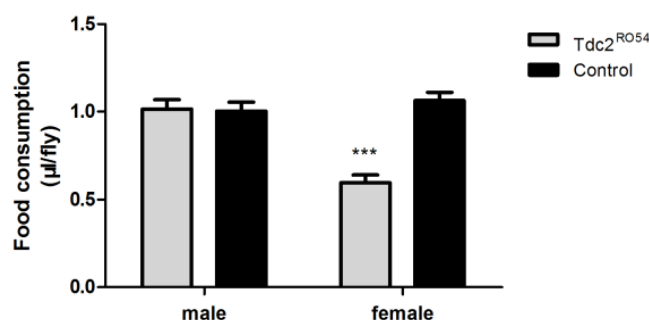


Figure 24. CAFÉ assay for $Tdc2$ mutant flies. The $Tdc2^{RO54}$ flies have significantly reduced the amount of food intake, with 44.1% lower in female flies ($Tdc2^{RO54}$, $n=16$; control, $n=20$) but no difference was seen in male flies ($Tdc2^{RO54}$, $n=20$; control, $n=20$). Data are presented as mean \pm SEM; two-ways ANOVA was used for analysis, *** $p < 0.0001$.

3.1.5 Metabolic rate has decreased in flies deficient in OA

To further confirm the roles of OA on regulation of metabolism, I performed a modified respirometry approach to measure CO₂ output from flies, which is a good indicator to identify changes in metabolic rates. It was found that, when compared to the control flies, $T\beta H^{nM18}$ flies have a significant decrease of CO₂ production per hour × fly, with 59.1% decrease in females and 60.9% decrease in males, respectively (Fig. 25A, $p < 0.01$).

To distinguish the effects for OA and its precursor TA in metabolism regulation, measurement of CO₂ production in $Tdc2^{RO54}$ flies was performed as well. Interestingly, results showed that $Tdc2^{RO54}$ flies had slightly but not significantly increased CO₂ production corresponding to the controls in both sexes (Fig. 25B, $p > 0.05$).

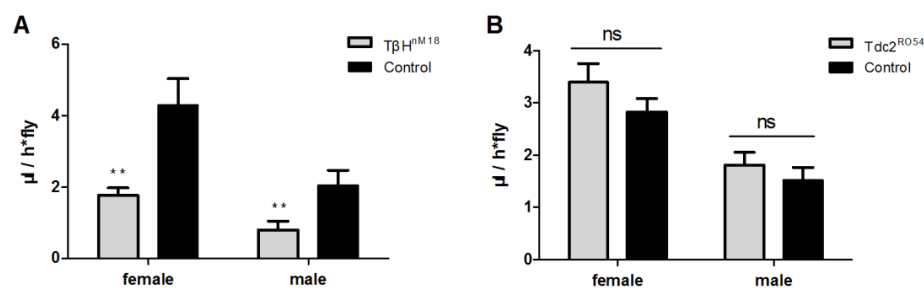


Figure 25. CO₂ production is different in flies deficient in OA and/or TA. (A) CO₂ production was measured in $T\beta H^{nM18}$ flies using respirometry. The $T\beta H^{nM18}$ flies showed significantly decreased CO₂ production both in males and females, with 59.1% decrease in female flies ($T\beta H^{nM18}$, $n=10$; control, $n=10$) and 60.9% decrease in male flies ($T\beta H^{nM18}$, $n=9$; control, $n=9$). (B) Measurement of CO₂ production in $Tdc2^{RO54}$ flies. The $Tdc2^{RO54}$ flies and control flies did not show significant differences in CO₂ production in both sexes. Data are presented as mean ± SEM; two-ways ANOVA was used for analysis, * $p < 0.05$, ** $p < 0.01$.

3.1.6 Octopamine signaling does not affect growth

Next, I tested whether flies devoid of OA have a defect in growth. The weight and head-size of adult flies (3–5 days old) were measured. There was no significant difference in body weight between $T\beta H^{nM18}$ flies and controls (Fig. 26A, $p > 0.05$). Moreover, head sizes of adult male and female flies were also similar (Fig. 26B, $p > 0.05$). Thus, these experiments suggest that diminishing octopamine did not affect growth at least in normally fed adults.

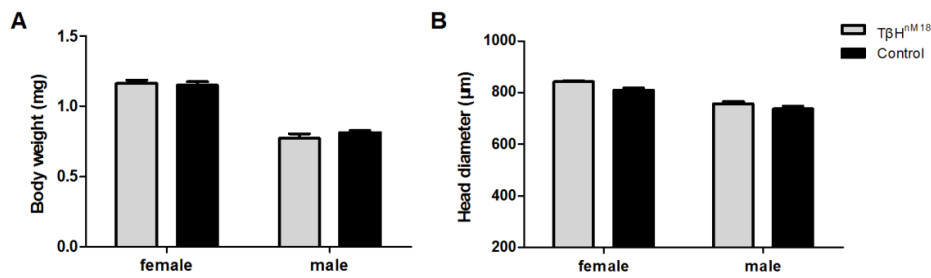


Figure 26. Growth is not affected in TβH mutant flies. (A) 3-5 day-old adult flies were weighed. There was no significant difference in body weight between TβH^{nM18} flies and controls (TβH^{nM18} female, n=17, male, n=17; control females, n=19, male, n=18). (B) Head diameter of adult flies was evaluated. No significant difference was seen between the genotypes (TβH^{nM18} female, n=26, male, n=22; control females, n=20, male, n=24). All data are presented as mean ± SEM.

In contrast, the body weight and body length of *Tdc2*^{RO54} flies are different from that of in TβH^{nM18} flies. Surprisingly, *Tdc2*^{RO54} flies display a significant decrease in body weight both of males and females when compared to the control flies, with 12.3% decrease in males and 13.9% decrease in females, respectively (Fig. 27A, p<0.05). However, there was a slightly increased body length of *Tdc2*^{RO54} flies detected in both sexes (Fig. 27B, p>0.05). Thus, in our experiments it appears that TA and OA might exert different effects in control of organismal growth as determined by body weight.

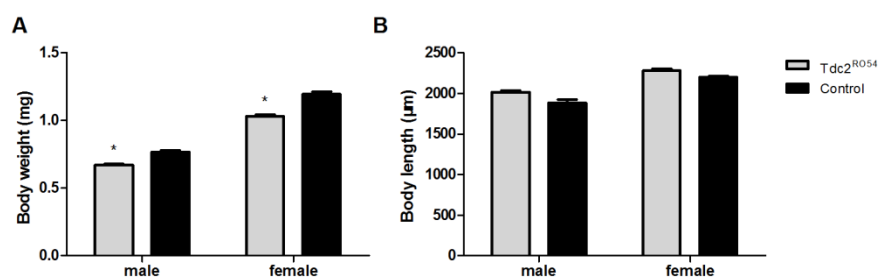


Figure 27. Tdc2 mutant flies show differences in body weight and length. (A) 3-5 day-old adult flies were weighted. The *Tdc2*^{RO54} flies have significantly lower body weight both in males and females, with 12.3% lower in male flies (*Tdc2*^{RO54}, n=26; control, n=30) and 13.9% lower in female flies (*Tdc2*^{RO54}, n=26; control, n=30). (B) Body length of adult flies was evaluated. A slight but not significant increase was seen between *Tdc2*^{RO54} mutant control flies (*Tdc2*^{RO54} male, n=23, female, n=20; control male, n=30, female, n=30). Data are presented as mean ± SEM; two-ways ANOVA and the unpaired two-tailed Student's t-test were used for analysis, *p<0.05.

3.1.7 Octopamine signaling is relevant for Insulin secretion in IPCs

Previous studies showed that genetic manipulation of dILPs signaling affected lifespan, stress resistance, lipid and carbohydrate metabolism and also the pattern of animal activity. In my study, flies deficient in OA synthesis showed phenotypical peculiarities that were consistent with animals showing increased insulin signaling levels. To test whether metabolic phenotypes in OA-deficient flies are due to increased insulin mRNA transcription. I performed qRT-PCR to check the level of *dilp2*, *dilp3* and *dilp5* transcripts in IPCs. Although the three dILPs have been considered to be expressed in the same cells, they are independently regulated by distinct combination of transcription factors (Ikeya et al., 2002). It is noted that the level of *dilp2* transcript in OA deficient flies was indistinguishable from that of control flies. Nevertheless, *dilp3* transcript was slightly but significantly decreased in OA-deficient flies, and *dilp5* transcription was increased (Fig. 28).

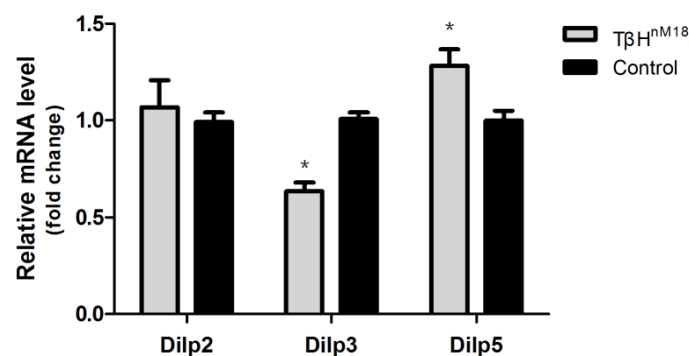


Figure 28. Relative expression levels of dILPs transcripts in TβH mutant flies. *Dilp2* transcript level showed no difference in OA deficient flies and controls. The value for control was set to 1. *Dilp3* transcript was significantly decreased in OA-deficient flies. Nevertheless, *dilp5* transcription was increased (n=3 replicates for each genotype), *p<0.05.

Interestingly, compensated *dilp3* and *dilp5* transcription levels do not have adequately mirror the phenotypical peculiarities that I observed in OA deficient flies without altered *dilp2* transcript. Thus, I performed a quantification of those insulin-like peptides remaining in the IPCs in normally fed flies and those starved for 24 h. The fluorescence intensity of dILPs was quantified in means of dILP2-specific immunostaining. Stained brains were analyzed under identical conditions, meaning

with identical laser power and stacks settings. The immunostaining of IPCs reflects the levels of three dILPs due to dILP2 antibody likely intercross with all three peptides (Cao and Brown, 2001). It was found that dILP2 signals were lower in IPCs of flies deficient OA if compared with controls, with 26.8 % decreases in males and 26.1 % decreases in females (Fig. 29A, B and E), reflecting increased release of insulin from IPCs. After 24 h starvation, the starved control flies showed higher accumulation of dILP2 than the fed flies (Fig. 29C and D), consistent with previous studies (Luo et al., 2012). However, the accumulation of dILP2 in OA deficient flies seems not to be increased as much as in control flies because the differences between them are up to 46.2 % in males and 27.9 % in females under starved condition (Fig. 29F, $p < 0.05$). But anyhow, these data indicated that OA has a role in secretion of dILP2.

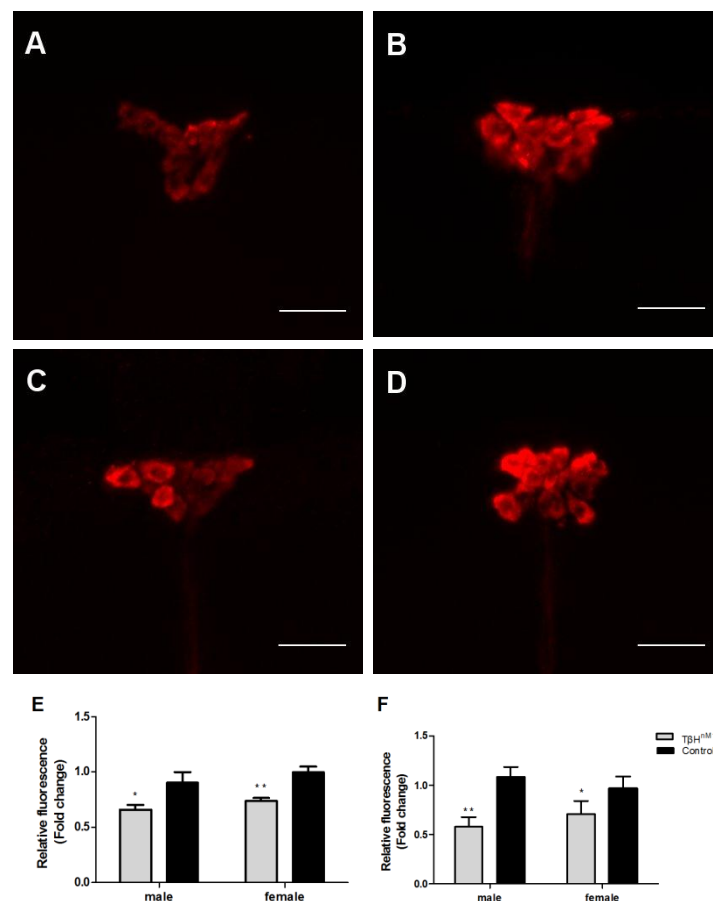


Figure 29. DILP levels are affected in $T\beta H$ mutant fed and starved flies. Relative dILP immunofluorescence in IPCs in $T\beta H$ mutant fed and starved flies (A and C) compared to control flies under identical conditions (B and D). (E) The $T\beta H^{M18}$ flies have significantly decreased dILP2 levels in IPCs both in males and females, with 26.8% decrease in male flies ($T\beta H^{M18}$, $n=5$; control, $n=5$) and 26.1% decrease in female flies ($T\beta H^{M18}$, $n=17$; control, $n=19$). (F) After starvation, the

TβH^{nM18} flies significantly decreased 46.2% dILP2 level in male (*TβH^{nM18}*, n=13; control, n=12) and 27.9 % in female (*TβH^{nM18}*, n=10; control, n=10). Data are presented as mean ± SEM; two-ways ANOVA was used for analysis, *p<0.05, **p<0.01.

Since OA appears to act as an inhibitory signal for dILP2 secretion, it is informative that increasing OA synthesis may improve the accumulation of dILP2 in IPCs. To address this, I applied two inducible cation-channel systems to activate octopaminergic/tyraminerpic neurons. The heat-induced activation of dTrpA1 channel has already been described above. Another valuable tool, channelrhodopsin-2 (ChR2), is a blue light-induced cation-selective membrane channel from the green algae *Chlamydomonas reinhardtii* (Nagel et al., 2003). The utility of transgenic flies expressing ChR2 under the control of UAS in the presence of all-trans retinal was already demonstrated (Schroll et al., 2006). Here, the ectopic expression of ChR2 was targeted to defined neurons by crossing the *UAS-ChR2* line to *TβH-GAL4* or *Tdc2-GAL4* lines with induction of blue light for a period of 4 h. Activation of *TβH*- and *Tdc2*-positive neurons by means of dTrpA1 expression resulted in an increase in relative dILP2 signals compared to the control flies, which increased 28.7 % in progeny of *TβH>dTrpA1* and was 1.5 fold higher in progeny of *Tdc2>dTrpA1* (Fig. 30A, p<0.01). Similar to the heat-induced activation, blue light-induced activation of octopaminergic/tyraminerpic neurons also resulted in an increase in dILP2 immunofluorescent intensity (Fig. 30B, p<0.05). Taken together, these data indicated that OA could inhibit dILP release from IPCs.

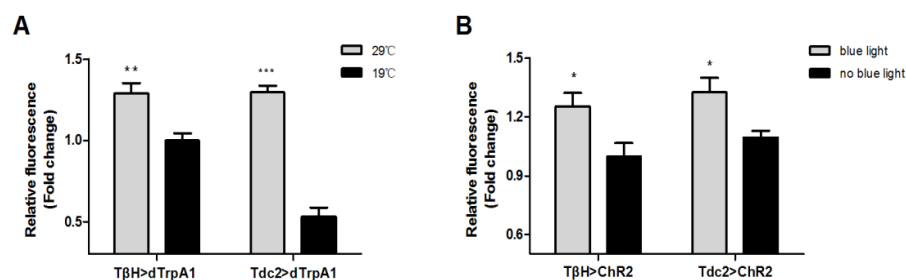


Figure 30. Flies produced more OA resulted in increased dILP2 level in IPCs. (A) dTrpA1 mediated release of OA significantly increased dILP2 levels, with 28.7% increases in *TβH>dTrpA1* (29°C, n=9; 19°C, n=8) and 1.5 fold higher in *Tdc2>dTrpA1* (29°C, n=12; 19°C, n=9). (B) ChR2 mediated release of OA also significantly increased dILP2 levels in IPCs, with 25.4% increase in *TβH>dTrpA1* (blue light, n=12; no blue light, n=10) and 21.1% increase in *Tdc2>dTrpA1* (blue light, n=12; no blue light, n=9). Data are presented as mean ± SEM; two-ways ANOVA was used for

analysis, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

To distinguish the requirement for OA and TA in the process of dILP2 release from IPCs, I analyzed the dILP2 level in the brain of *Tdc2^{RO54}* mutant flies. It was found that dILP2 immunostaining showed lower intensity in the IPCs of flies without OA and TA than that of in the controls, which was decreased 20.7% in males (Fig. 31, left, $p < 0.001$), and a 12% decrease, although not significant, was observed in females (Fig. 31, right, $p > 0.05$). Because both mutants are devoid of octopamine, the difference is that *Tdc2^{RO54}* mutants in addition lack TA, thus, the similar phenotypes in dILPs secretion are most likely attributable to the scarcity of OA.

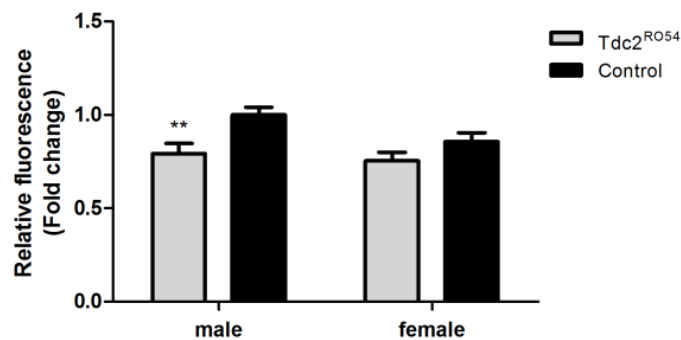


Figure 31. *Tdc2* mutant flies display decreased dILPs level in IPCs. Relative dILP immunostaining in IPCs in *Tdc2^{RO54}* mutants were compared to control flies held under identical conditions. The *Tdc2^{RO54}* flies have significantly decreased dILP2 level in IPCs in males, with 20.7% decrease in male flies (*Tdc2^{RO54}*, $n=13$; control, $n=14$) and 12% decrease in female flies (*Tdc2^{RO54}*, $n=13$; control, $n=14$). Data are presented as mean \pm SEM; two-ways ANOVA was used for analysis, ** $p < 0.01$.

3.1.8 Oral feeding of OA or TA alter dILP2 levels in IPCs

To dissociate the role for OA and its precursor TA in insulin secretion, *T β H^{nM18}* and its matching control flies were fed 10 mg/ml OA in standard food for 3 d, *Tdc2^{RO54}* and its matching control flies were fed 7.5 mg/ml TA for 2 d respectively. The pharmacological approach facilitated me isolating the individual roles of OA and TA as well as their united roles. In this study, it was found that 10 mg/ml TA was toxic for flies, oral intake OA have effectively rescued the egg-laying phenotype in mutant flies, which is consistent with previous studies that animals feeding 10 mg/ml OA have

elevated levels of this neurotransmitter in the brain (Monastirioti et al., 1996; Barron et al., 2007). $T\beta H^{nM18}$ mutant flies fed 10 mg/ml OA had strongly increased dILP2 accumulation in the IPCs, which was 48.5 % increased when compared to control flies fed on standard food alone (Fig. 32A, $p < 0.0001$). Unsurprisingly, $Tdc2^{RO54}$ flies fed 10 mg/ml OA also had 24.6 % higher dILP2 level than control flies (Fig. 32B, $p < 0.05$). However, $Tdc2^{RO54}$ flies fed 7.5 mg/ml TA slightly increased dILP2 levels, which may indicate that ingested TA was converted to OA due to T β H activity.

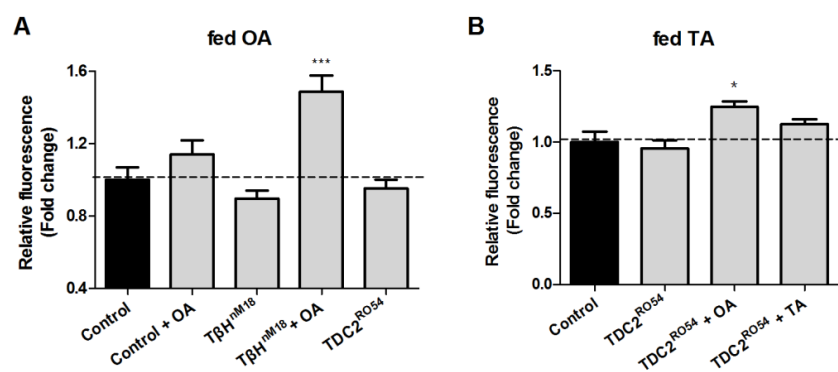


Figure 32. Oral feeding of OA increases dILP2 level in IPCs. (A) Female flies of $T\beta H^{nM18}$ and controls were treated with 10 mg/ml of OA. There was a significant increase dILP2 accumulation in the IPCs in $T\beta H^{nM18}$ flies fed on 10 mg/ml OA compared to control flies fed on standard food alone ($T\beta H^{nM18} + OA$, $n=11$; control, $n=11$). (B) Female flies of $Tdc2^{RO54}$ and controls were treated with 7.5 mg/ml of TA. Relative dILP2 level was increased in $Tdc2^{RO54}$ flies fed on TA compared to control flies fed on standard food ($Tdc2^{RO54} + TA$, $n=11$; control, $n=9$). Data are presented as mean \pm SEM; two-ways ANOVA was used for analysis, * $p < 0.05$, *** $p < 0.0001$.

3.2 Analyses of Octopamine and Tyramine receptors functioning in regulation of dILP2 secretion

As most nonpeptide transmitters/hormones in invertebrates, octopamine and tyramine share similarities not only regarding their structure, but also their structural and pharmacological characteristics as well as their corresponding sets of G-protein coupled receptors (Roeder, 2005). Recent studies revealed that the two monoamines transmit various effects independently from each other, suggesting that OA exerts its effects through specific OA receptors (OAMB, OA2/Oct β 1R, Oct β 2R, Oct β 3R) and TA through a different class of specific TA receptors (TyrR I, TyrR II, TyrR III). Based on comprehensive pharmacological studies of utilizing different insect models, OA has gained a substantial interest as it modulates multiple physiological responses through specific receptors. However, except for TyrR I, the gene expression patterns and signaling properties of TA receptors were characterized only recently. In this part, I aim to identify the role of these receptors in regulating dILP2 secretion.

3.2.1 All OA and TA receptors gene transcription profiles in larval and adult tissues

To analyze all OA and TA receptor genes expression in major tissues in larvae and adult flies, RNA was isolated from larval brain, intestine, trachea, fat body, salivary gland, malpighian tubules and from adult brain, leg muscle, intestine, fat body, trachea, malpighian tubules and male or female reproductive system. RT-PCR analysis was performed and is shown in (Fig. 33 for larvae and Fig. 34 for adult).

It is noted that all seven receptor genes are strongly expressed in larvae and adult brains. OAMB is only found in brain at larval stage, in addition to the adult brain, it is also present in adult muscle, trachea and both male and female reproductive system. Similar to OAMB, OA2 is expressed in the same tissues except in male and female reproductive systems. Strikingly, Oct β 2R is expressed in almost all larval and adult tissues, indicating its comprehensive roles in regulating peripheral organs. Oct β 3R is present in brain and malpighian tubules in both larval and adult flies. Additionally, it is weakly present in adult muscle and trachea.

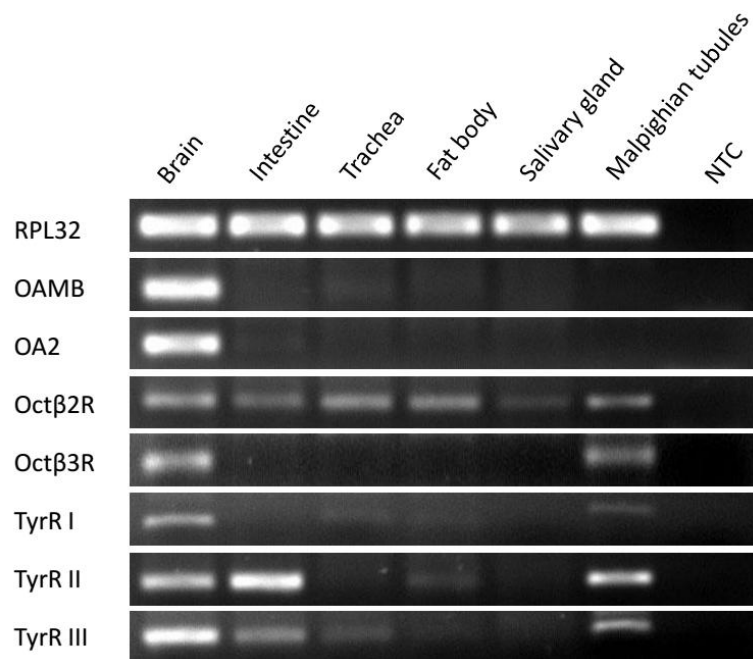


Figure 33. OA and TA receptors gene expression in larval tissues. RT-PCR analysis with RNA isolated from brain, intestine, trachea, fat body, salivary gland and malpighian tubules of wild type larvae. RPL32 serves as a positive control, NTC is no template control.

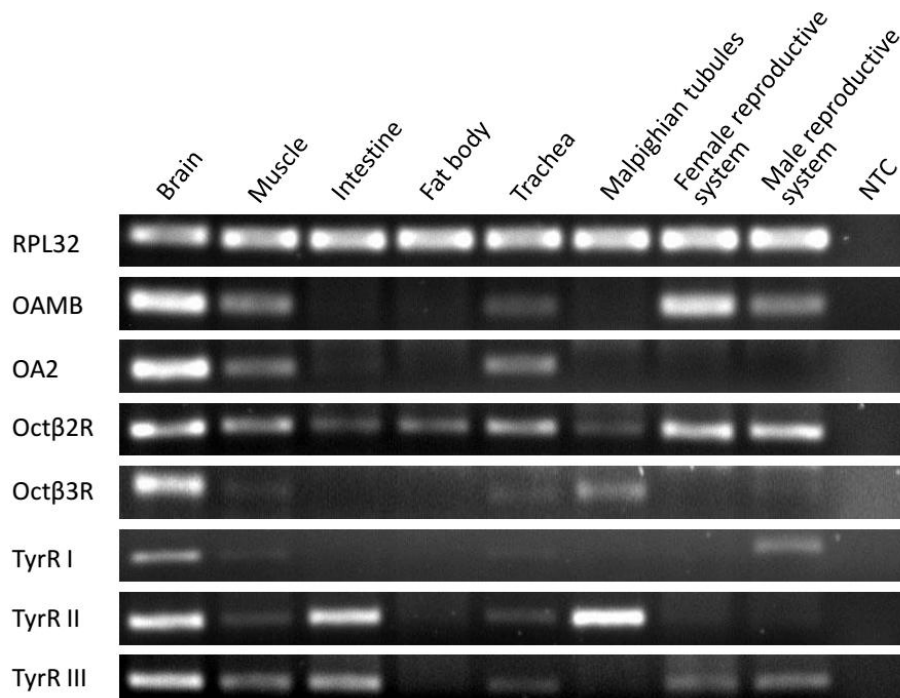


Figure 34. OA and TA receptors gene expression in adult tissues. RT-PCR analysis with RNA isolated from brain, muscle, intestine, fat body, trachea, malpighian tubules and male or female reproductive system of wild type adult flies. RPL32 serves as a positive control, NTC is the no template control.

For TA receptors, expression of TyrR I is limited in larval brain, malpighian tubules and ambiguously in trachea and fat body, as well as in adult brain, weakly in adult muscle and trachea and uniquely in the male reproductive system. TyrR II is not detected in larval trachea, salivary gland, adult fat body and reproductive system but in all other tissues. TyrR III is expressed in larval brain, intestine, trachea and malpighian tubules, as well as in all adult tissues except fat body and malpighian tubules (Fig. 33 and Fig. 34).

3.2.2 Expression analyses of distinct receptors in adult CNS with restriction in neurons of the *Pars intercerebralis*

As mentioned above, we found that all seven receptors were positively expressed in brains. Thus, we would ask whether there are receptors showing expression in IPCs that OA might act through to promote dILP2 secretion. To check the gene expression in PI, we performed RT-PCR analyses for all seven receptor genes. The region of PI was cut down from the central brain of adult flies which expressed $20\times UAS-mCD8::GFP$ driven by *dILP2-GAL4*. RNA isolation was performed using the magnetic beads cell sorting method as described in (Iyer et al., 2009).

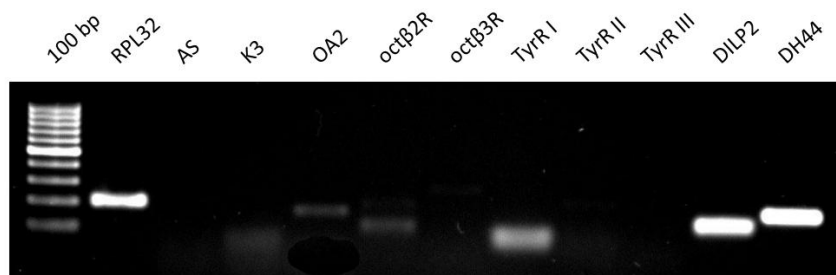


Figure 35. OA and TA receptors gene expression in the PI of the adult brain. RT-PCR analysis of gene expression of OAMB-AS, OAMB-K3, OA2, Oct β 2R, Oct β 3R, TyrR I, TyrR II and TyrR III restricted to PI region. RPL32 served as a positive control. Dilp2 and DH44 were used to evaluate the purity of RNA sample isolated using magnetic beads cell sorting method.

As shown, with OAMB-K3, OA2, Oct β 2R and TyrR I, four out of seven receptors were present in the PI neurons (Fig. 35). Of these, only one OAMB isoform, K3 but not AS, was found in the PI. Indeed, K3 couples to both cAMP and calcium (Crocker et al.,

2010). It should be noticed that, both genes of *dilp2* and *DH44* were simultaneously transcribed in the sample derived from PI. Although we expected to capture pure *dILP2* cells using that way, subsequent RT-PCR analysis demonstrated that, at least in some cases, multiple PI cells attached to each other and contributed to a single sample.

To further detect the expression of receptors in PI, especially in IPCs, we used double antibody immunostaining analysis. The GAL4/UAS system was employed to express a visible marker (GFP) under transcriptional control of receptor gene-specific promoters. The resulting GAL4 lines were crossed to *UAS-GFP* lines to visualize the almost complete expression pattern and their progenies were used for analysis. Colocalization in the brain was analyzed following anti-GFP and anti-*dILP2* or anti-*DH44* immunohistochemistry. Within the CNS of adults, Oct β 2R signal is strongly shown in mushroom body calyces. Two large clusters of varicose ramifications connect a pair of somata in the ipsilateral inferior lateral protocerebrum and the ventrolateral protocerebrum, and another send axon along the midline in each protocerebral hemisphere. Notably, although a large soma appears in the PI, it does not show colocalization with *dILP2* (Fig. 36A-C). A striking region showing expression of Oct β 3R gene is β lobe and γ lobe of mushroom bodies, which the former one sends projections to antennal nerves. However, the PI region did not show any signal overlap with *dILP2* (Fig. 36D-F). A very remarkable gene expression of TyrR I could be seen in a cluster cells, located in PI, which are six large *DH44* cells (Fig. 36G-I), the corticotropin-releasing factor (CRF) homolog in *Drosophila*. This part will be described in detail in the section 3.4. The adult CNS shows a comprehensive TyrR III expression, whereas no signal detected in the IPCs (Fig. 36J-L). According to the result of RT-PCR analyses, we expected to check the expression pattern of two other OA receptors, *OAMB* and *OA2*, using immunohistochemical analyses. Unfortunately, the promoter lines we created failed to drive GFP expression due to the experimental condition.

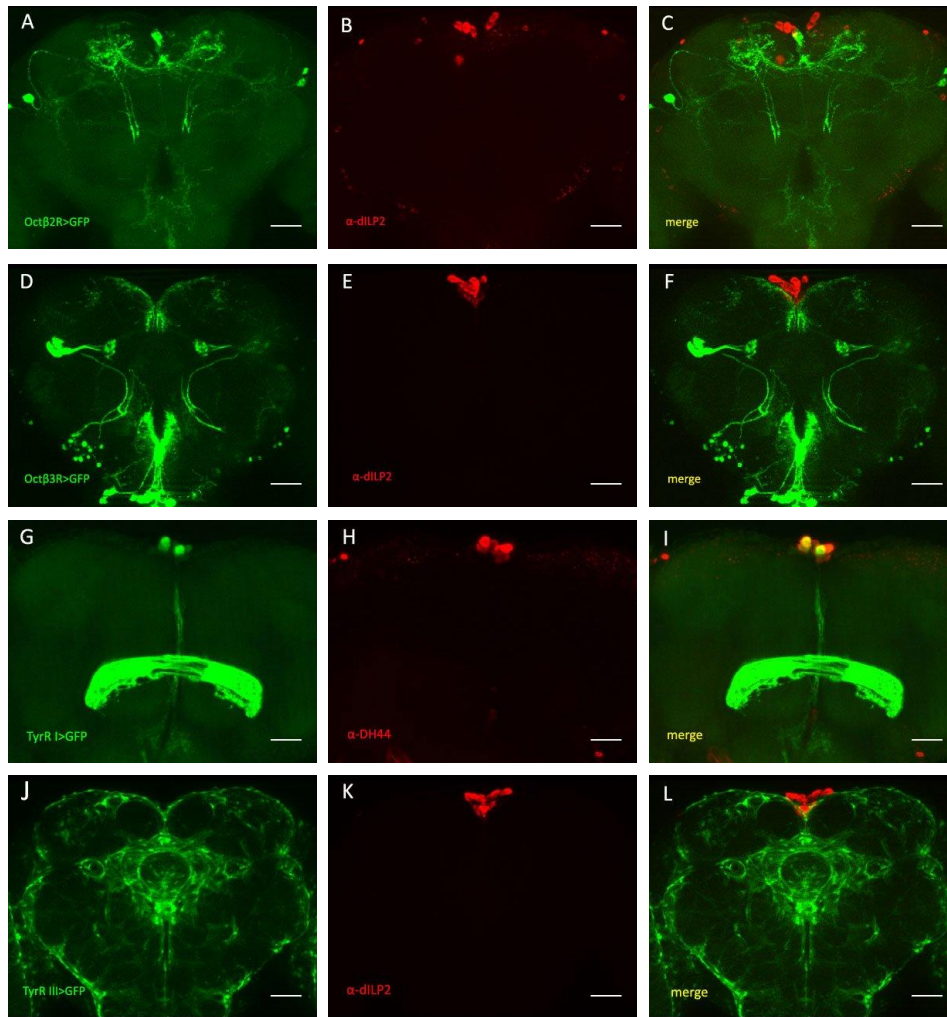


Figure 36. Visible expression pattern of OA and TA receptors in the adult brain. Distinct receptors drove GFP expression was visualized in adult brains using an immunohistochemical staining with anti-GFP antibody (green). Co-staining with anti-dILP2 antibody or anti-DH44 antibody (red) was used to display morphology of IPCs. (A-C) GFP signal shows that although one of Octβ2R positive neurons is present in the PI, it doesn't show colocalization with IPCs (*octβ2R-gal4 > uas-gfp*). (D-F) There is no Octβ3R expression in the dILP2-immunolabeled IPCs (*octβ3R-gal4 > uas-gfp*). (G-I) The TyrR I receptor gene is expressed in a set of median neurosecretory cells, most of which also express DH44-immunolabeling (*TyrR I-gal4 > uas-gfp*). (J-L) *TyrR III-GAL4* shows widespread expression pattern, but no one coexpresses dILP2 (*TyrR III-gal4 > uas-gfp*). Scale bar is 50 μm.

3.2.3 *Dilp2* secretion is differently mediated in all receptor mutants

To provide insights into the roles played by OA or TA receptors in dILP2 secretion, we then employed seven receptor mutants (include those for the three tyramine receptors) for subsequent investigation.

The intensity of dILP2 immunofluorescence was measured in IPCs body obtained from seven mutants according to the way mentioned in section 3.1.6, the genetic-

matched background, w^{1118} served as a control line. Strikingly, dILP2 accumulation was significantly higher in brains of OA2 and Oct β 2R mutant flies (Fig. 37, 28.5% increase in OA2 mutant; 23.3% increase in Oct β 2R mutant). Consistent with T β H mutant fly, OAMB mutant and Oct β 3R mutant flies showed reduction of dILP2 accumulation in brains (Fig. 37, 14.2% decrease in OAMB mutant; 8.6% decrease in Oct β 3R mutant). These data suggested that OAMB and Oct β 3R act as the dominant receptors mediated the dILP2 secretion effect of octopamine, OA2 and Oct β 2R receptors exerted reverse action. It is known that two isoforms of OAMB, K3 and AS, functioned in different second message signaling pathways (Lee et al., 2009). Thus, the activation of specific isoform of the OAMB receptor mediated above OA effects need to be further confirmed.

In addition, TyrR II mutants also showed higher dILP2 level in brains than it in control line (Fig. 37, 23.2% higher dILP2), suggesting the possibility that tyramine plays a role on dILP2 secretion and that this effect is mediated by the TyrR II receptor.

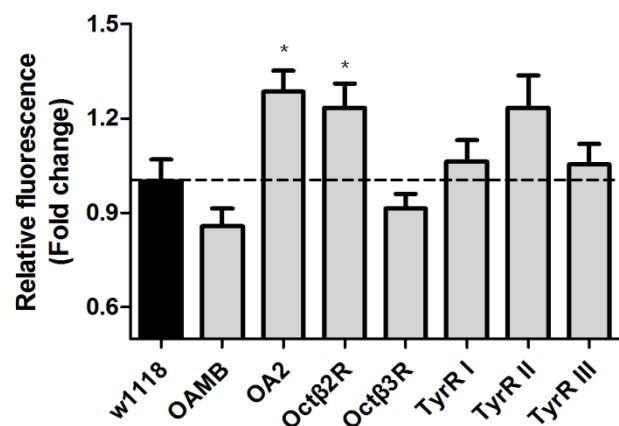


Figure 37. Relative dILP2 levels in all receptor mutant flies. Wild type w^{1118} , the background for all the lines, was used as a control line. Relative to the control, OA2, Oct β 2R and TyrR II mutant flies have significantly increased dILP2 level in IPCs, with 28.5% increase in OA2 mutant flies (n=10), 23.3% increase in Oct β 2R mutant flies (n=9) and 23.3% increase in TyrR II mutant flies (n=10). However, OAMB and Oct β 3R mutant flies showed slight reductions of dILP2, with 14.2% decrease in OAMB mutant (n=6); 8.6% decrease in Oct β 3R mutant (n=9). Data are presented as mean \pm SEM; the unpaired two-tailed Student's t-test was used for analysis, *p<0.05.

3.2.4 OA- or TA-receptor deficiency affects lipid storage

Since OA and TA receptor mutant flies showed altered dILP2 levels in the brain, it was

interesting to test their fat content in all kind of flies because insulin-like peptide is crucial in regulation of triglyceride storage in flies (Belgacem and Martin, 2006; Baker and Thummel, 2007; DiAngelo and Birnbaum, 2009; Zhang et al., 2009).

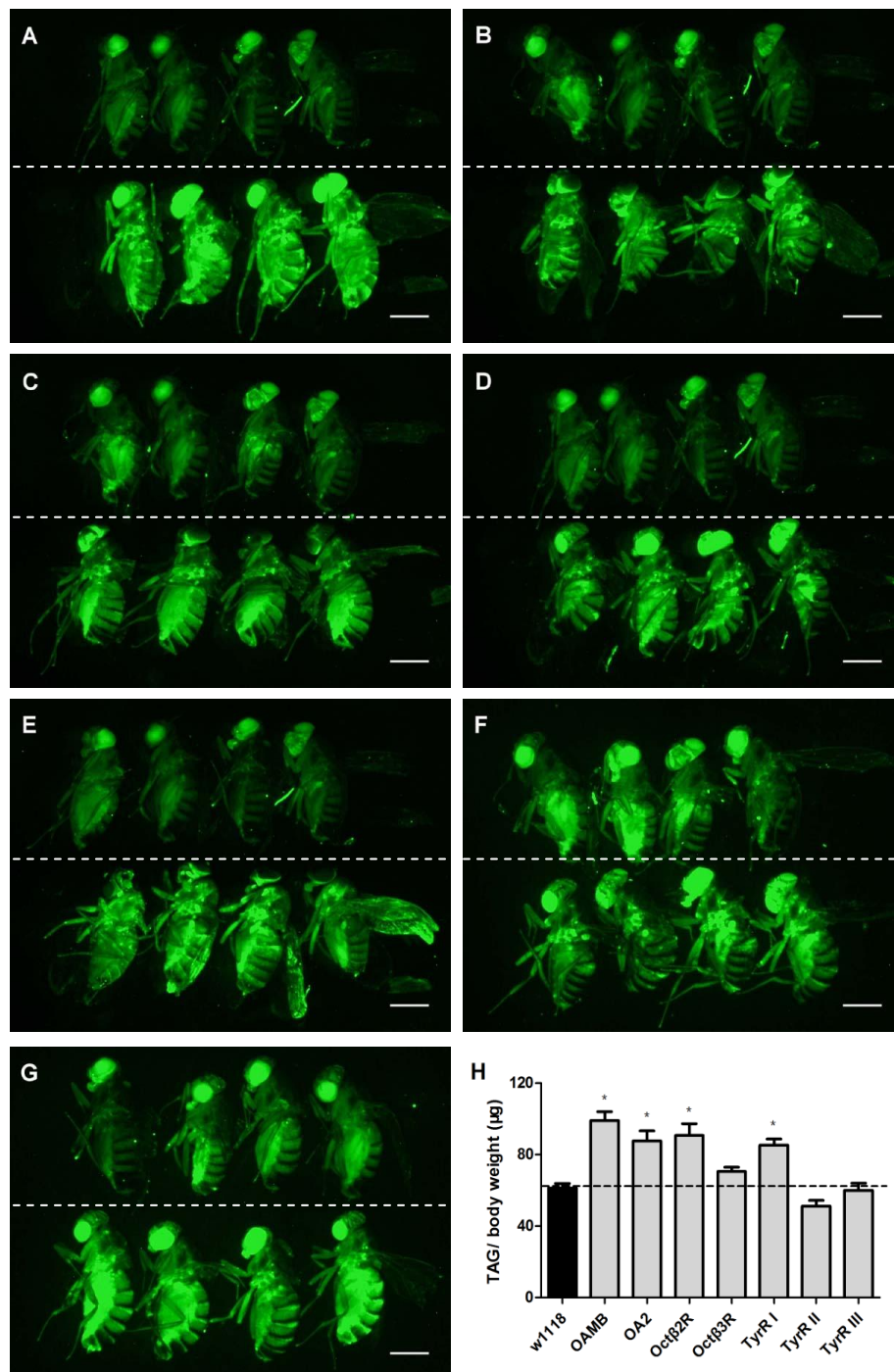


Figure 38. Fat content was altered in receptor mutant flies. Bodipy staining of the whole body shows high fat content in mutant flies of OAMB (A), OA2(B), Oct β 2R (C), Oct β 3R (D), TyrR I (E), TyrR II (F) and TyrR III (G), the upper line in every photo shows control flies. (H) Quantification of TAG level reveals that OAMB-, OA2-, Oct β 2R- and TyrR I mutant flies are significantly increased fat

storage profile, with that of 61.6% increase for OAMB mutant (n=31), 43.1% increase for OA2 mutant (n=25), 47.9% increase for Oct β 2R mutant (n=33) and 39.2% increase for TyrR I mutant (n=32), respectively. Data are presented as mean \pm SEM; the unpaired two-tailed Student's t-test was used for analysis, *p<0.05.

Fluorescent bodipy staining of the whole animals showed intuitional evidence that four OA-receptor mutants and three TA-receptor mutants show much higher total intensity in lipid staining than the wild type *w¹¹¹⁸*, the background for all the lines (Fig. 38A-G, the upper line: *w¹¹¹⁸*; the lower line: mutants). Quantification of TAG / body weight showed that OAMB-, OA2-, Oct β 2R- and TyrR I deficient flies were significantly increased triglyceride storage profile, with that of 61.6% increase for OAMB mutant, 43.1% increase for OA2 mutant, 47.9% increase for Oct β 2R mutant and 39.2% increase for TyrR I mutant, respectively (Fig. 38H, p<0.05). Compared to controls, no significant difference in fat content was detectable in the three other receptor-mutant strains (Fig. 38H).

3.2.5 OA- or TA-receptor deficiency affects response to starvation

As not only OA deficient flies but also the corresponding receptor deficient flies showed altered energy metabolisms, I investigated the influence of these receptors on the response to starvation. With comparison to controls, OAMB, OA2, Oct β 2R and TyrR I mutant flies obviously extended the survival under starved condition, observed in their median survival with 63 h for OAMB mutant (34 % increase), 57 h for OA2 mutant (21.3 % increase), 59 h for Oct β 2R mutant (25.5 % increase) and 64 h for TyrR I mutant (Fig. 39A-C and E, p<0.0001). However, there was 4.2 % decrease of longevity for TyrR II mutant flies (45 h in the median survival) suffering starvation (Fig. 39F). No significant changes in median survival for Oct β 3R mutant and TyrR III mutant flies were seen (Fig. 39D and G).

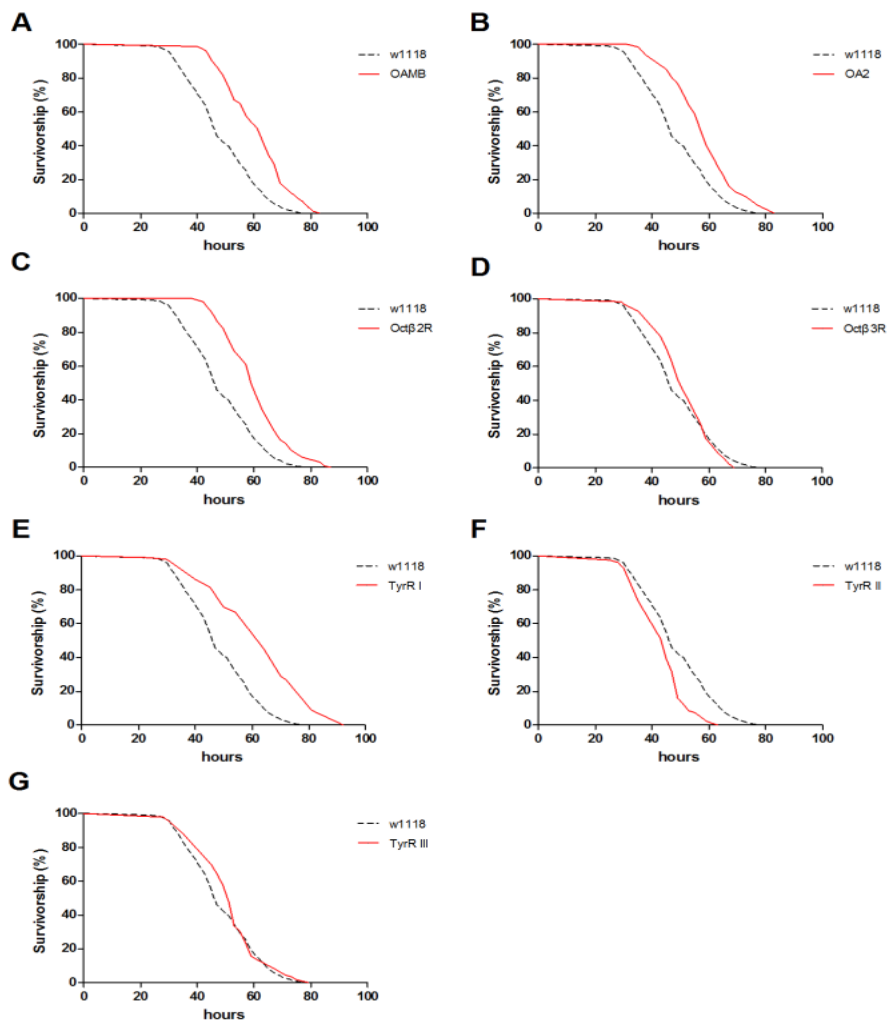


Figure 39. OA or TA receptor deficiency affects response to starvation. Under starved conditions, OAMB, OA2, Oct β 2R and TyrR I mutant flies significantly extended their longevity compared to control flies (47 h for the median survival, n=118). The median survivals of each genotype are 63 h for OAMB mutant (A, n=113), 57 h for OA2 mutant (B, n=122), 59 h for Oct β 2R mutant (C, n=97) and 64 h for TyrR I mutant (E, n=136). However, TyrR II mutant flies have a slight decrease in survival in response to starvation (F, n=82). No significant changes were seen in Oct β 3R mutant (D, n=126) and TyrR III mutant flies (G, n=109). Log-rank test for survival analysis showed significant difference, $p < 0.001$.

3.2.6 Functional analysis of diminished OA or TA receptor in specific tissue

To address the role of OA and its precursor in the relevant tissue I was interested in, a RNAi strategy to knockdown the endogenous receptor mRNA was employed. Transgenic flies carrying either the *UAS-ds-Oct β 2R*, *UAS-ds-OA2*, *UAS-ds-TyrR I* or *UAS-ds-TyrR II* were crossed to flies carrying GAL4 promoter which drives gene

expression in tissue-specific patterns. The F1 generation was used for testing and referred hereafter as *Octβ2R-RNAi*, *OA2-RNAi*, *TyrR I-RNAi* and *TyrR II-RNAi*, respectively. When *nsyb-GAL4* or *oenocyte-GAL4* were used to drive double-stranded RNAi expression, all four genotypes showed no significant and discordant difference in their fat content in both male and female flies (Fig. 40A-D). Nevertheless, we next used *ppl-GAL4* to drive expression of four RNAi constructs and found that the fat level in all lines were obviously higher than their parental controls (Fig. 40E and F), suggesting that OA played a FB-specific role in regulating lipid storage.

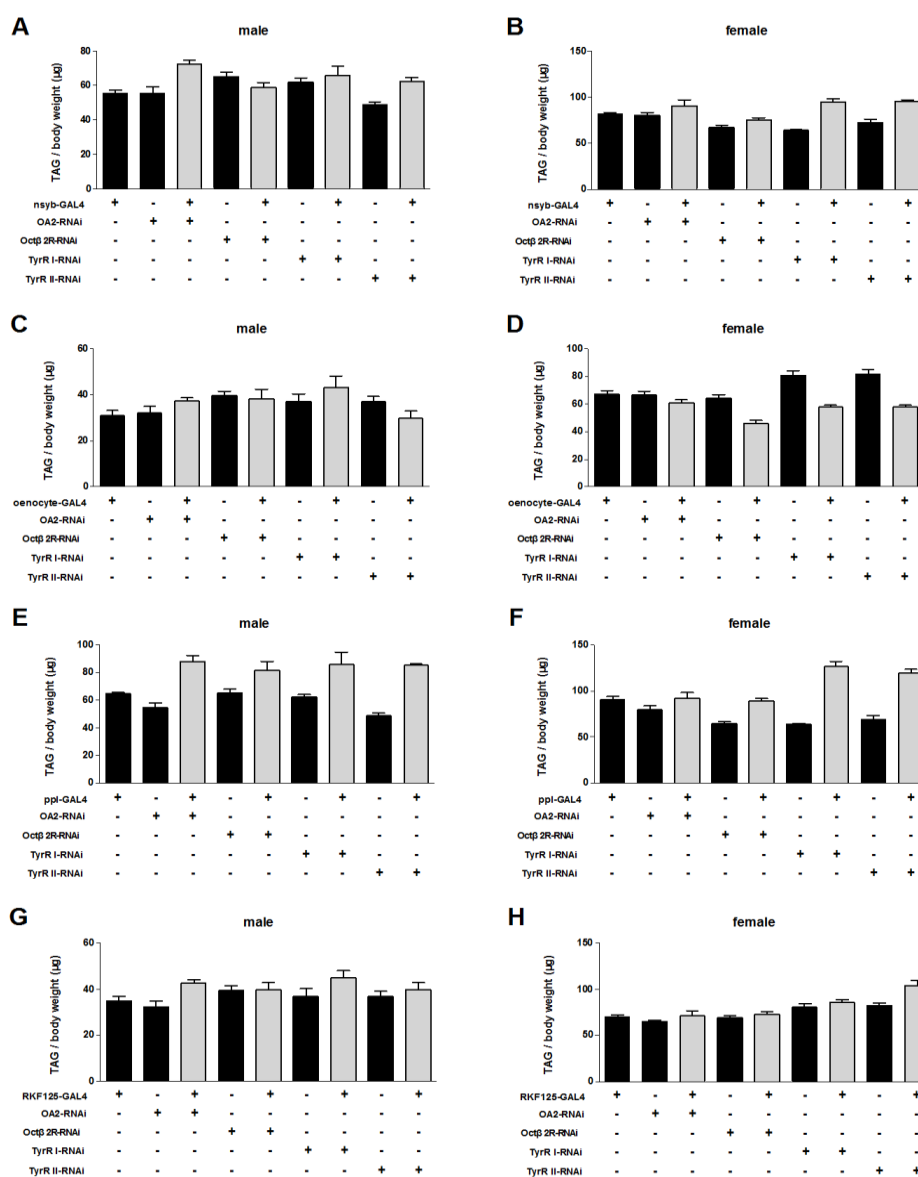


Figure 40. Fat content of flies with distinct receptor knockdown in specific tissues. RNAi mediated knockdown of Octβ2R, OA2, TyrR I and TyrR II with *nsyb-GAL4* (A and B) and

oenocyte-GAL4 (C and D) show uncoordinated changes in TAG level in male and female flies. (E-F) When we used *ppl-GAL4* or *RKF 125-GAL4* to drive knockdown of four receptors specificity in fat body, it shows significantly higher TAG level than controls. Data are presented as mean \pm SEM; the unpaired two-tailed Student's t-test was used for analysis, * $p < 0.05$.

To further confirm the role of OA in the fat body, *RKF 125-GAL4*, another FB-specific driver was applied to drive RNAi expression. However, the fat level changes were not as robust as seen in flies which expressed RNAi driven by *ppl-GAL4* (Fig. 40G and H).

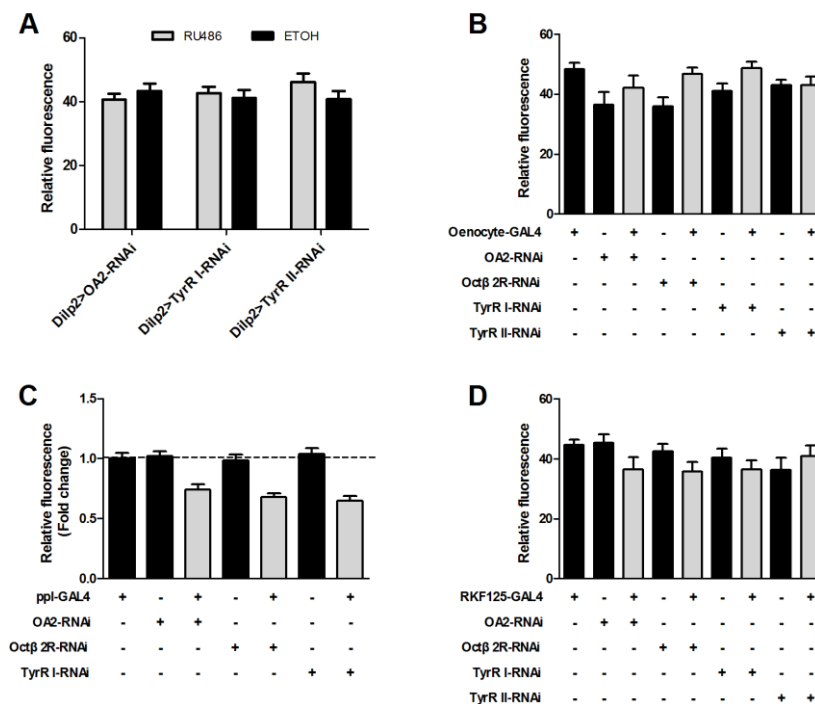


Figure 41. Dilp2 level in brain of flies with distinct receptor knockdown in specific tissues. RNAi mediated knockdown of OA2, Octβ2R, TyrR I and TyrR II in IPCs (A) and oenocyte (B) show no significant difference in dILP2 level. (C-D) FB-specific promoter of *ppl-GAL4* and *RKF 125-GAL4* drives receptors-RNAi showed significantly reduced dilp2 level corresponding to their controls. Data are presented as mean \pm SEM; the unpaired two-tailed Student's t-test was used for analysis, * $p < 0.05$.

As well, the remaining of dILP2 level in the brain of each receptor knockdown was determined. Comparing the mifepristone induced animals and the uninduced controls, RNAi-mediated expression of each in the IPCs did not alter dILP2 level in *OA2-RNAi*, *TyrR I-RNAi* and *TyrR II-RNAi* flies, the same results could be seen in the flies, in which the expression of RNAi constructs was targeted to oenocytes (Fig. 41A and B). As expected, the flies of which RNAi-mediated knockdown of OA2, Octβ2R or

TyrR I was achieved with *ppl-GAL4* showed a decrease in dILP2 level, which of 25.9 % decrease for *OA2-RNAi*, 31.9 % decrease for *Oct β 2R-RNAi* and 35.3 % decrease for *TyrR I-RNAi* (Fig. 41C, $p < 0.05$). Similar to *ppl-GAL4*-driven expression, *RKF 125-GAL4* driven RNAi expression also reduced dILP2 level in flies except for *TyrR II-RNAi* (Fig. 41D). These experiments provided strong evidence that OA acts through multiple receptors including a FB-specific role in the regulation of insulin-like peptide secretion.

3.3 Characterization of starvation-induced gene expression in the brain of *Drosophila* deficient in octopamine

It is known that starvation resistance is dependent on insulin-like peptide signaling and flies display increased resistance to starvation due to decreased dILP signaling (Rulifson et al., 2002; Broughton et al., 2005; Giannakou and Partridge, 2007). However, our finding as described in section 3.1.2 above was contradictory with previous studies, of that increased release of DILP signaling into hemolymph leads to increased resistance to starvation. Thus, the discrepancy has drawn my attention to the global changes in the neuronal transcriptomes in response to starvation.

3.3.1 Comparison of gene expression profiles between *TβH* mutants and controls

To elucidate the modulatory effect of OA on transcriptional events responding to starvation in *Drosophila*, cDNA microarray analyses were applied to compare genes differentially expressed in animals from 24 h fasting-treated and untreated in both *TβH* mutant and control strains. I chose a single time point of 24 h after starvation as this is the time just before the survival starts to decline. In order to minimize the experimental variation, three independent sets of RNAs were hybridized to *Drosophila* GeneChip consisting of 16128 probes representing 13885 genes. Genes different expression were recognized by setting up the mean of ratio changes at 2-fold (>2 as up-regulated or <-2 as down-regulated). The majority of genes showing only small changes were not identified statistically significant ($P > 0.05$).

According to the screening strategy described above, there are in total 81 genes whose expression changed in the brain of *TβH* mutant flies responding to starvation. On the contrary, 124 genes induced by starvation are differently expressed in control flies. In comparison, 65% of these transcripts are uniquely induced in *TβH* mutant flies, whereas 77% of transcripts are uniquely induced in control flies. Surprisingly, we noticed that only 11 upregulated genes and 17 downregulated genes overlapped in both strains respectively (Fig. 42A and B).

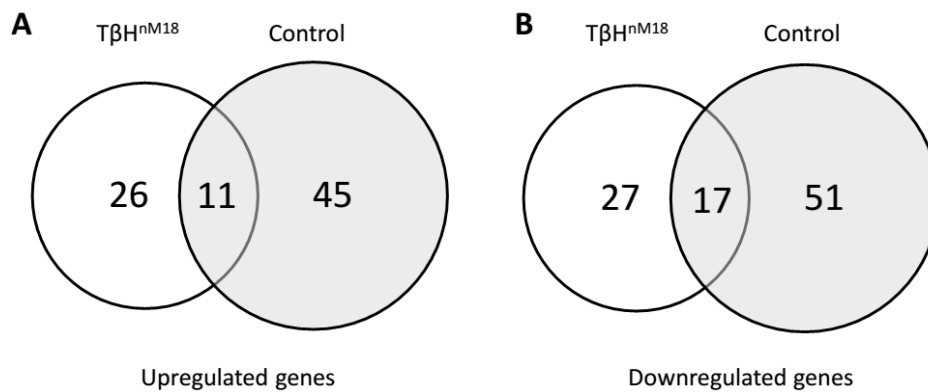


Figure 42. Venn diagram analysis for regulated genes classification. Venn diagrams compare the differentially expressed genes with at least two-fold changes in the two strains in response to starvation. Numbers in each unique circle display the total number of genes distribute to the specified strain. 11 genes overlap in upregulated group (A) and 17 genes overlap in downregulated group (B), respectively.

To elucidate the biological annotations from independent lists of up- and downregulated genes, I uploaded all data to Flybase (<http://flybase.org/>) and the database of annotation, visualisation and integrated discovery (DAVID, <http://david.abcc.ncifcrf.gov/>) for identification of putative functions. As the analysis, the functional classification of genes showed different transcription between TβH mutants and controls. Furthermore, gene ontology analyses (GOTERM_BP_FAT) were performed for biological process categories. In control sample, 8 main biological processes were significantly enriched: protein folding (GO:0006457), purine nucleotide metabolic process (GO:0006163), microtubule-based process (GO:0007017), intracellular transport (GO:0046907), nitrogen compound biosynthetic process (GO:0044271), gland morphogenesis (GO:0022612), macromolecular complex subunit organization (GO:0043933) and autophagic cell death (GO:0048102), see Table 4A. In contrast, 6 different biological processes were significantly enriched in TβH mutant sample: vesicle-mediated transport (GO:0016192), nitrogen compound biosynthetic process (GO:0044271), ribonucleotide metabolic process (GO:0009259), skeletal muscle tissue development (GO:0007519), membrane invagination (GO:0010324) and endocytosis (GO:0006897), see Table 4B. Taken together, these data demonstrate that different genes involved in cellular processes are evoked due to OA deficiency in response to starvation.

Table 4. Biological processes enrichment analysis.

ID	Biological process	Genes	%	P-Value
A				
GO:0006457	Protein folding	8	6.5	3.80E-04
GO:0006163	Purine nucleotide metabolic process	8	6.5	7.90E-04
GO:0007017	Microtubule-based process	14	11.3	8.40E-04
GO:0046907	Intracellular transport	12	9.7	1.00E-03
GO:0044271	Nitrogen compound biosynthetic process	10	8.1	1.60E-03
GO:0022612	Gland morphogenesis	7	5.6	2.10E-03
GO:0043933	Macromolecular complex subunit organization	10	8.1	5.50E-03
GO:0048102	Autophagic cell death	5	4	8.10E-03
B				
GO:0016192	Vesicle-mediated transport	12	14.8	1.20E-04
GO:0044271	Nitrogen compound biosynthetic process	9	11.1	3.00E-04
GO:0009259	Ribonucleotide metabolic process	6	7.4	1.40E-03
GO:0007519	Skeletal muscle tissue development	4	4.9	2.20E-03
GO:0010324	Membrane invagination	8	9.9	2.80E-03
GO:0006897	Endocytosis	8	9.9	2.80E-03

3.3.2 Categorization of starvation-induced genes associated with energy metabolism

The finding that flies deficient in OA have a longer survivorship under starved condition draw our attention on the genes associate with energy metabolism. I categorized genes that were regulated either in T β H mutant or control strain according to their molecular functions upon starvation, the genes were classified into three categories: (a) genes that were regulated in control, but not in T β H mutant strain; (b) genes that were regulated in the T β H mutant strain, but not in control; (c) genes were regulated in both strains but in opposite directions. In the first category, genes shown different expression were considered to be only affected upon starvation. In the list, 34 (27%) genes consist of 16 upregulated genes and 18 downregulated genes met category (a) are listed (Table 5). Many of the upregulated genes are related to function in carbohydrate catabolism, including *ATPsyn-Cf6* (hydrogen-exporting ATPase), *CG4988* (UDP-glucose 4-epimerase) and *P5cr* (pyrroline-5-carboxylate reductase). Two genes function in fat breakdown respond to nutrient deprivation, including *CG31091* (triglyceride lipase) and *Pde11* (3',5'-cyclic-AMP phosphodiesterase). There are also many genes downregulated in

the list involve in amino acid metabolism, such as *Ddc* (aromatic-L-amino-acid decarboxylase) and *CG1673* (branched-chain-amino-acid transaminase).

Table 5. Gene induced only in the control strain in response to starvation.

Symbol	Gene ID	Molecular function	Fold change
CR17024	32437	unknown	5.16
ATPsyn-Cf6	16119	hydrogen-exporting ATPase activity, phosphorylative mechanism	3.39
Dhc93AB	13812	ATPase activity, coupled	2.80
gek	23081	protein kinase	2.60
CG4988	32372	UDP-glucose 4-epimerase	2.60
Pde11	85370	3',5'-cyclic-AMP phosphodiesterase	2.58
hola	1174	unknown	2.54
PPO2	33367	L-DOPA monooxygenase	2.42
CG31091	51091	triglyceride lipase	2.41
Madm	27497	protein serine/threonine kinase	2.40
Dcp-1	10501	cysteine-type endopeptidase	2.36
spn-E	3483	ATP-dependent RNA helicase	2.36
P5cr	15781	pyrroline-5-carboxylate reductase	2.35
gammaTry	10359	serine-type endopeptidase	2.28
RanGAP	3346	Ran GTPase activator	2.25
Pkn	20621	protein serine/threonine kinase	2.18
Hsc70-1	1216	ATPase	-2.17
Ada	37661	adenosine deaminase	-2.25
CG6432	39184	acetate-CoA ligase	-2.27
Cchl	38925	holocytochrome-c synthase	-2.43
Pka-C2	274	cAMP-dependent protein kinase	-2.46
CG8611	27602	ATP-dependent RNA helicase	-2.47
Vha14-1	262512	proton-transporting ATPase activity, rotational mechanism	-2.52
Hsp60B	11244	ATPase activity, coupled	-2.56
aux	37218	protein kinase	-2.60
Torsin	25615	ATP binding	-2.62
CG1969	39690	glucosamine 6-phosphate N-acetyltransferase	-2.67
Arp53D	11743	structural constituent of cytoskeleton	-2.83
Eph	25936	transmembrane receptor protein tyrosine kinase	-2.93
Ddc	422	aromatic-L-amino-acid decarboxylase	-3.03
CG33298	32120	phospholipid-translocating ATPase	-3.69
CG1673	30482	branched-chain-amino-acid transaminase	-7.72
Ac3	23416	adenylate cyclase	-15.26
CG16837	35009	ATPase activity, coupled	-15.40

There were 22 (27%) genes (13 upregulated and 9 downregulated) regulated in response to starvation only in T β H deficient animals as shown (Table 6). Similar to the category I, many genes encoding metabolic enzymes are found in the

upregulated group, including *CG5656* (alkaline phosphatase), *CG17486* (asparagine synthase), *CG6726* (aminoacylase) and *slgA* (proline dehydrogenase). As well, there are three of these genes, *bur* (pyrophosphatase), *rdgA* (diacylglycerol kinase) and *CG7236* (protein serine/threonine kinase) in the downregulated group.

Table 6. Gene expressed only in TβH mutant strain.

Symbol	Gene ID	Molecular function	Fold change
Hsp70Ba	13277	unknown	8.55
CG5656	37083	alkaline phosphatase	8.34
Arr1	120	opsin binding	4.85
CG17486	32997	asparagine synthase (glutamine-hydrolyzing)	3.98
CG1234	37489	unknown	3.49
hang	26575	zinc ion binding	3.42
blow	4133	unknown	3.13
CG32000	52000	cation-transporting ATPase	3.09
Atpalpha	2921	sodium:potassium-exchanging ATPase	3.04
Yp2	5391	catalytic activity	2.70
Yp1	4045	catalytic activity	2.63
CG6726	39049	aminoacylase	2.52
slgA	3423	proline dehydrogenase	2.36
mats	38965	protein binding	-2.20
bur	239	pyrophosphatase	-2.27
MKK4	24326	MAP kinase	-2.32
Rab7	15795	GTPase	-2.48
shg	3391	protein homodimerization	-2.51
nrv3	32946	sodium:potassium-exchanging ATPase	-2.88
rdgA	261549	diacylglycerol kinase	-4.38
mei-S332	2715	unknown	-5.21
CG7236	31730	protein serine/threonine kinase activity	-8.27

In the third category, I list those genes whose expression are highly induced upon starvation in control strain, meanwhile, where its effect is also changed in TβH mutant strain in a same or opposite direction (Table 7). For example, a particular transcript upregulated in both strains in this category is the female-specific independent of transformer (*fit*), which was identified to be a female-biased gene and highest regulated in the adult head after starvation. Interestingly, *bsk* (JUN kinase) is increased in control strain, but decreased in the TβH mutant strain upon starvation. *Bsk* is central to the JNK signaling pathway, which is known to be involved in metabolic homeostasis control and sensitivity to starvation.

Table 7. Genes with changed expression in both strains following starvation.

Symbol	Gene ID	Molecular function	Fold change	
			<i>TβH^{nM18}</i>	Control
fit	38914	unknown	31.94	11.49
CG1172	264712	unknown	8.18	-4.67
Mlp60A	259209	zinc ion binding	5.11	-1.98
Mst57Db	11669	unknown	3.41	3.01
Or71a	36474	olfactory receptor	3.15	2.57
Tm2	4117	actin binding	2.57	5.68
Pkcdelta	259680	protein kinase C	1.89	2.59
Cbp20	22943	nucleotide binding	1.79	-2.33
Pph13	23489	protein homodimerization	-1.69	2.63
CG4301	30747	calcium-transporting ATPase UDP-glucose:glycoprotein	-1.91	3.74
Ugt	14075	glucosyltransferase	-2.06	2.46
Adk1	22709	adenylate kinase	-2.19	2.24
Ntmt	33457	histone methyltransferase	-2.24	2.11
yip2	40064	acetyl-CoA C-acyltransferase	-2.28	3.29
CG17746	35425	protein serine/threonine phosphatase	-2.34	2.95
CG12267	38057	DNA-directed RNA polymerase	-2.57	2.11
CycD	10315	protein kinase binding	-2.64	1.72
pad	38418	zinc ion binding	-2.77	1.94
t	86367	beta-alanyl-dopamine hydrolase	-3.12	1.67
bsk	229	JUN kinase	-3.24	3.06
l(1)G0334	28325	pyruvate dehydrogenase	-3.38	1.85
Uba1	23143	ubiquitin-protein ligase	-3.59	1.86
aph-1	31458	endopeptidase	-3.96	1.92
pes	31969	scavenger receptor	-6.62	1.56

3.3.3 Quantitative real-time PCR validation of differential gene expression

To verify and validate the microarray analysis data, the relative abundance of selected genes were reassessed using qRT-PCR with gene-specific primers for each target. Among 81 genes that show significant differences in transcript levels in three categories, 11 genes associated with energy metabolism were selected for further validation. The data calculated from three biological replicates were shown in (Fig. 43), the transcript levels of retinal degeneration A (*rdgA*), *CG5656* and *CG7236* were regulated only in starved *TβH* mutant strain but not in control, whereas ATPase coupling factor 6 (*ATPsyn-Cf6*) and *CG16837* were only changed in starved control strain. However, the expression of yippee interacting protein 2 (*yip2*), basket (*bsk*)

and *CG1172* were regulated in either of the two lines but they shown in opposite direction. The special transcript of female-specific independent of transformer (*fit*) was validated the highest expression level changes in both samples. In the comparison of two analyses, 9 out of 11 genes (82%) tested via real-time qPCR corresponded with my microarray results.

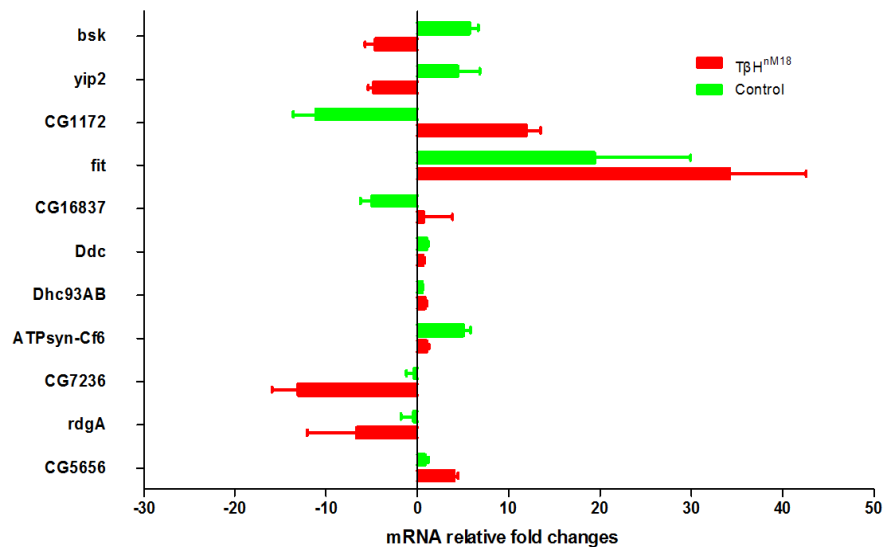


Figure 43. Validation of differential expression levels using relative qRT-PCR. The relative fold change of eleven selected individuals in three categories from the microarray analysis data was normalized by an endogenous reference gene of *RPL32*. The red bars and green bars indicate gene expression levels in CNS of TβH mutant and control after starvation respectively. All values represent as mean ± SEM. from three independent replicates.

3.4 Circadian behaviors are regulated by tyramine receptor I on DH44 cells in the brain of adult *Drosophila*

Most animals including insects exhibit an extensive range of daily behavioral rhythms in physiological processes under the control of an internal timing-clock system, which organizes these processes. Circadian rhythmicity is such a daily behavior controlled by clock neurons, which includes input pathway, circadian clock pacemaker and output pathway. In this part, I will study the roles of TA and OA on output pathway regulation, which transmits circadian signals to drive rhythmic behaviors.

3.4.1 TA but not OA is required for maintaining circadian rhythms

Through the genome-wide studies of circadian transcription analysis, *Tdc2* is expressed in clock neurons and required for normal circadian activity (Huang et al., 2013). Neuronal *Tdc2* encodes the tyrosine decarboxylase, which is essential for TA and OA synthesis (Cole et al., 2005). To testify the role of *Tdc2* in circadian rhythms, I performed studies on *Tdc2*^{RO54} mutants using a locomotor-based assay in which the animals were entrained to light-dark (LD), constant darkness (DD) and returning to LD conditions. Consistent with recent studies (Huang et al., 2013), I found that *Tdc2*^{RO54} flies exhibited severely aberrant circadian rhythms in LD as well as in DD conditions, and this arrhythmicity is especially present in suppressed evening peaks before light-off (Fig. 44A-C). In general, the activities of animals show a morning peak and an evening peak close to the light-on and light-off (see control flies), the phenotype of *Tdc2*^{RO54} flies in evening peak defect observed in LD during the light-off, was not different from that of observed in DD, indicating that there was an anticipation of input pathway (light).

Based on these data, I found that *Tdc2*^{RO54} flies show dramatically reduced locomotor activities (Fig. 46A, $p < 0.01$), which is consistent with previous studies (Hardie et al., 2007; Huang et al., 2013). To exclude the causality of locomotor defect result in arrhythmic behavior, LD-entrained *Tdc2*^{RO54} flies were placed in DD for 3 days followed by a return to LD. Similarly, the animals continue to show arrhythmicity in DD, suggesting that *Tdc2* is required for 24 h free-running rhythms and the arrhythmic

behavior is not a consequence of impaired locomotor activity.

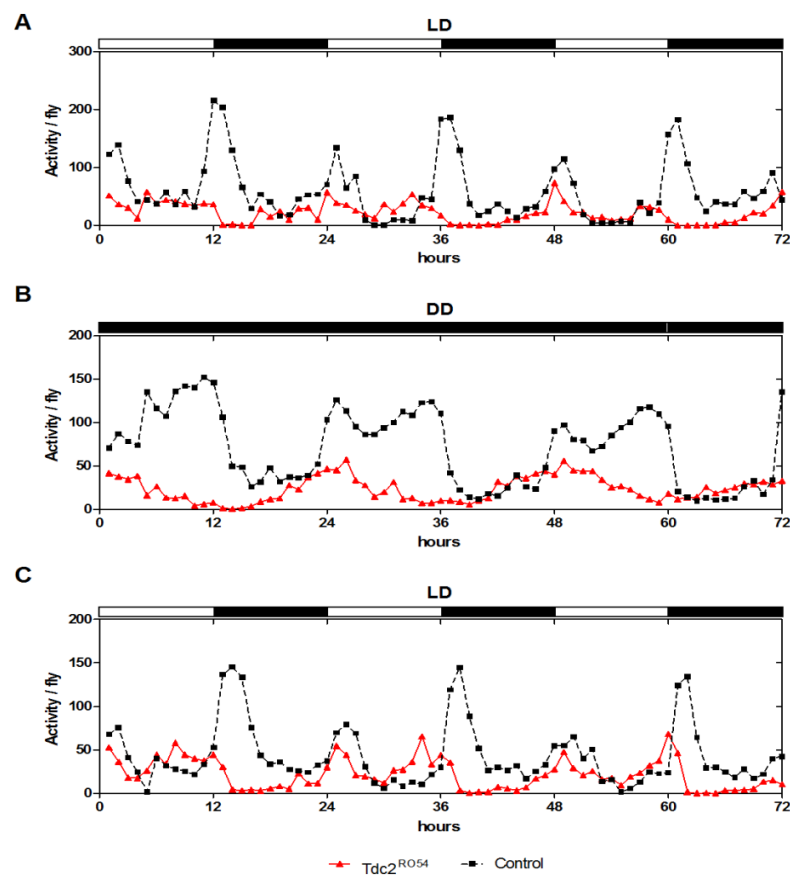


Figure 44. Circadian rhythm activity of *Tdc2^{RO54}* flies in different conditions. Both groups of male flies were firstly entrained to a LD cycle (A), followed by 3 d in DD (B), and then released to LD again (C). The *Tdc2^{RO54}* flies exhibited severely aberrant circadian rhythms in LD or DD conditions, and this arrhythmicity is especially present in suppressed evening peaks before light-off.

Previous evidence showed that *Tdc2* defective animals different from *Drosophila* have several behavioral abnormalities that are different from those observable in $T\beta H$ mutants (Alkema et al., 2005; Fussnecker et al., 2006). To distinguish the roles of TA and OA on circadian rhythmic formation in *Drosophila*, I tested the rhythmic behavior of $T\beta H^{nM18}$ flies, which are devoid of OA but contain 10 fold level of elevated TA (Monastirioti et al., 1996). Surprisingly, $T\beta H^{nM18}$ flies showed almost normal circadian rhythms not resembling those seen in *Tdc2^{RO54}* flies (Fig. 45A-C), although both mutants have a similar locomotor deficit (Fig. 46B, $p < 0.05$). Basically, $T\beta H^{nM18}$ flies have a normal circadian rhythmicity, except a slightly decreased evening

peak of activity. Because both mutants are deficient in OA, the difference in circadian rhythms is most likely attributable to difference in TA levels, indicating TA not OA signaling as a potential modulator is required for circadian rhythms.

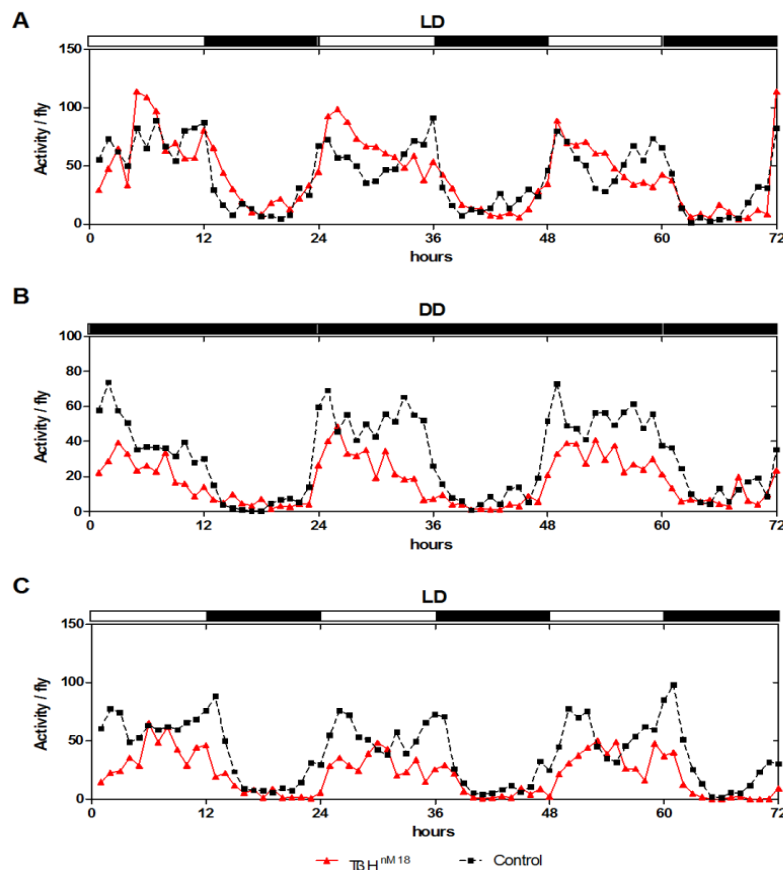


Figure 45. Circadian rhythm activity of $T\beta H^{nM18}$ flies in different conditions. Both groups of male flies were firstly entrained to a LD cycle (A), followed by 3 d in DD (B), and then released to LD again (C). Although the $T\beta H^{nM18}$ flies exhibited defect in locomotor activity, there was a normal circadian rhythm in LD or DD conditions, excepting a slight lower level in evening peak that resemble in $Tdc2^{RO54}$ flies.

3.4.2 TA- and OA-deficient flies show defects in negative geotaxis

The negative geotaxis is an innate escape response of flies to move against the gravitational vector, which directly relates to reflect the animals locomotor capacity. Understanding the hormonal basis of behavioral response to normal gravity will get insight into the mechanism underlying proprioception. Comparison of the distance climbed by control flies, both $Tdc2^{RO54}$ and $T\beta H^{nM18}$ flies showed significant declines

in the height climbed at 10 s post-startle (Fig. 47A and B).

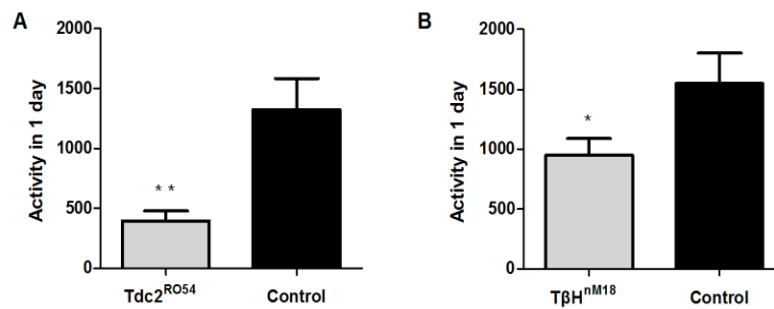


Figure 46. Locomotor activity of *Tdc2^{RO54}* and *TβH^{nM18}* flies. The histograms show the total activity counts of mutant male flies compared to their matched controls for 1 day in LD condition. There is a significant reduction of total activity in *Tdc2^{RO54}* flies (A) and in *TβH^{nM18}* flies (B), respectively. Data are presented as mean ± SEM; the unpaired two-tailed Student's t-test was used for analysis, * $p < 0.05$, ** $p < 0.01$.

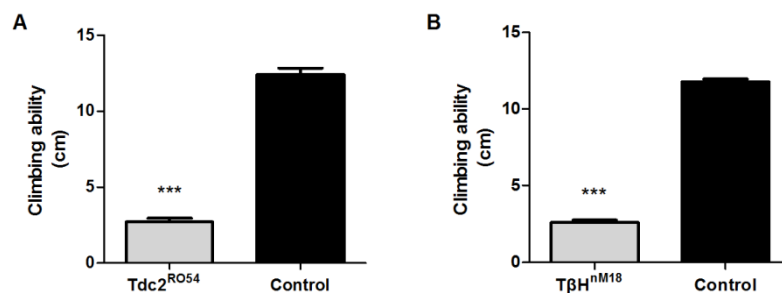


Figure 47. The *Tdc2^{RO54}* and *TβH^{nM18}* flies show defects in climbing ability. (A) The *Tdc2^{RO54}* male flies have severe defect in climbing ability compared with the control flies, with 78.3% decrease in male flies (*Tdc2^{RO54}*, $n=100$; control, $n=89$). (B) The *TβH^{nM18}* male flies also have significantly decreased their climbing distance, with 77.8% decrease in male flies (*TβH^{nM18}*, $n=396$; control, $n=391$). Data are presented as mean ± SEM; two-ways ANOVA was used for analysis, *** $p < 0.0001$.

3.4.3 Mutants with reduced OA have altered sleep

The sleep behavior is regulated by environmental and homeostatic processes, which is in contact with the circadian clock system. Recently published data showed a neural circuit that underlies the effects of OA on sleep:awake behavior (Crocker and Sehgal, 2008; Crocker et al., 2010). *Tdc2^{RO54}* and *TβH^{nM18}* flies with their corresponding controls were used for sleep analysis. Both mutants exhibited increases in sleep levels (Fig. 48A and B), and the total sleep times were significantly

increased compared to their matching controls (Fig. 48C, mean \pm SEM; $Tdc2^{RO54}$, 1170 \pm 47, control, 825 \pm 57, $p < 0.0001$; $T\beta H^{nM18}$, 1242 \pm 27, control, 947 \pm 58, $p < 0.0001$). Our findings are consistent with a recent publication (Crocker and Sehgal, 2008). The difference between these two mutants is that $Tdc2^{RO54}$ flies displayed sleep in left-shifted tendency, whereas $T\beta H^{nM18}$ flies displayed sleep in right-shifted tendency. The increased total sleep was accompanied by a decreased latency to sleep in the $Tdc2^{RO54}$ flies, which recognized the time from light-off until the animal's first bout of sleep. However, the $T\beta H^{nM18}$ flies have an increased in the latency to sleep, which is not significant (Fig. 48D). Interestingly, $Tdc2^{RO54}$ flies quickly went to sleep at the moment of light-off, which resulted in left-shifted tendency and decreased latency to sleep. On the contrary, $T\beta H^{nM18}$ flies quickly woke up when the light-off, and spend some time to sleep again, which resulted in right-shifted tendency and a slightly increased latency to sleep. Taken together, these data suggested that the loss of OA (common in both mutants) underlies the increase in sleep, whereas differences in TA levels (deficient in $Tdc2^{RO54}$ flies but elevated in $T\beta H^{nM18}$ flies) account for effects on light-triggered arrhythmic behavior.

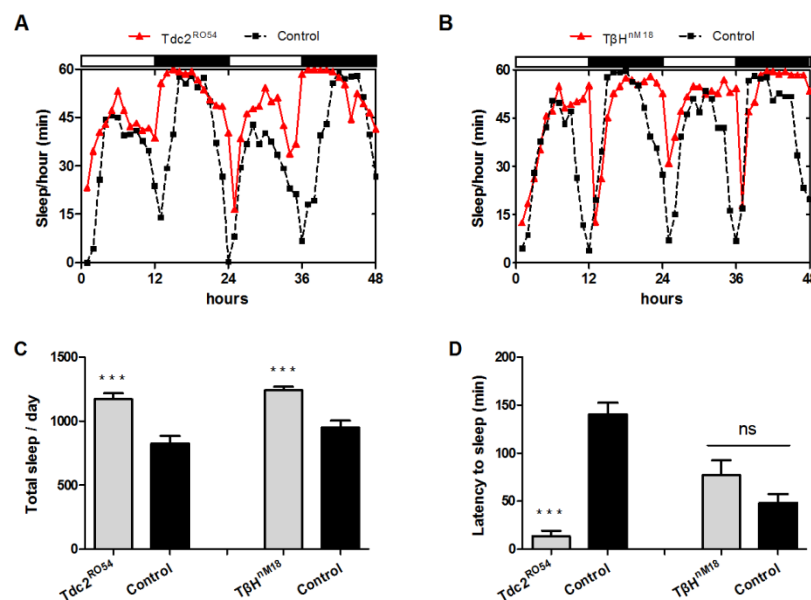


Figure 48. Mutants with reduced OA have increased sleep. (A) Two days of sleep time recording in $Tdc2^{RO54}$ male flies. The $Tdc2^{RO54}$ line (red, n=8) shows significantly more sleep than its control (black, n=8). Dark bars and white bars on top indicate nighttime and daytime, respectively. (B) Two days of sleep time recording in $T\beta H^{nM18}$ male flies. The $T\beta H^{nM18}$ line (red, n=8) shows

significantly more sleep than its controls (black, n=8). Dark bars and white bars on top indicate nighttime and daytime, respectively. (C) Total sleep is significantly increased in *Tdc2^{RO54}* and *TβH^{nM18}* male flies, with 41.7% increase in *Tdc2^{RO54}* flies (n=8) and 31.1% increase in *TβH^{nM18}* flies (n=8). (D) Latency to sleep is significantly lower in *Tdc2^{RO54}* male flies (with 90.2% decrease, n=24). However, latency to sleep is slightly but not significantly increased in *TβH^{nM18}* male flies (n=24). Data are presented as mean ± SEM; two-ways ANOVA was used for analysis, ***p<0.0001.

3.4.4 *TyrR I* mutants have aberrant circadian rhythms

Most of the cases demonstrate the actions of TA and OA have been mediated via the activation of G-protein-coupled receptors. To test which receptor mediates the role of TA in regulating circadian rhythms, we performed locomotor-based assay in which the receptor mutant displays aberrant circadian rhythms. We found that tyramine receptor I (also called octopamine/tyramine receptor), hereafter referred to as *TyrR I*, showed obviously arrhythmicity especially regarding the evening peak compared to the wild type controls (Fig. 49A-C). The *TyrR I* shows a preference for TA over OA in pharmacological binding studies and in the inhibition of adenylyl cyclase activity, thus presumably in our case, *TyrR I* is mediated the effects of TA on rhythmic behavior by stimulating cAMP signaling.

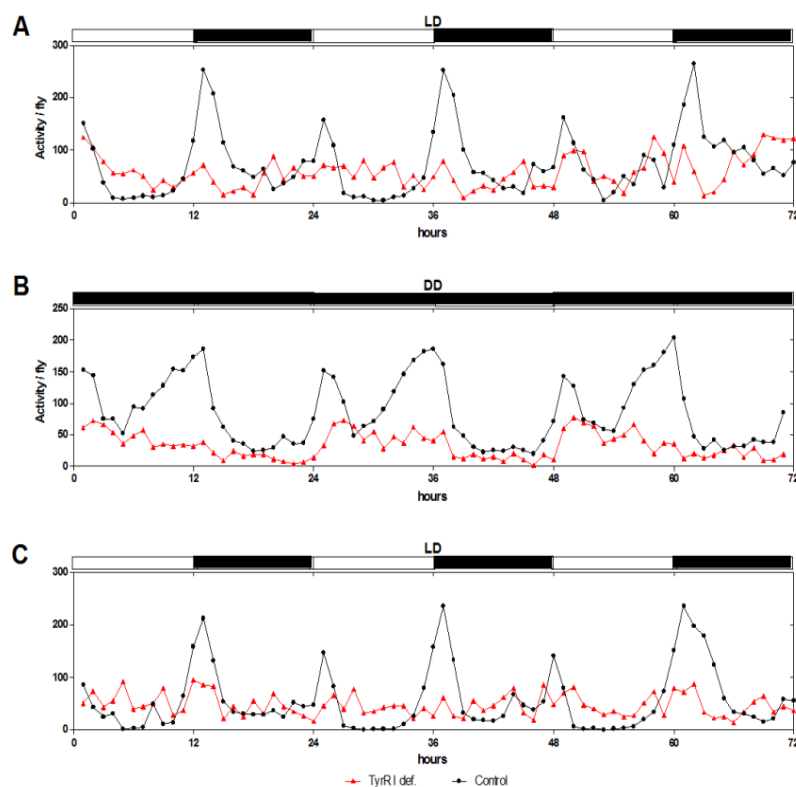


Figure 49. Circadian rhythm activity of TyrR I mutants in different conditions. Both groups of male flies were firstly entrained to a LD cycle (A), followed by 3 d in DD (B), and then released to LD again (C). The TyrR I flies exhibited severely aberrant circadian rhythms in LD or DD conditions.

The total locomotor activities and negative geotaxis were compared between TyrR I mutants and controls, respectively. As shown below, the TyrR I mutants have significantly decreased locomotor activities and climbing ability (Fig. 50A, $p < 0.05$ in total activity in 3 days; Fig. 50B, $p < 0.0001$ in climbing ability).

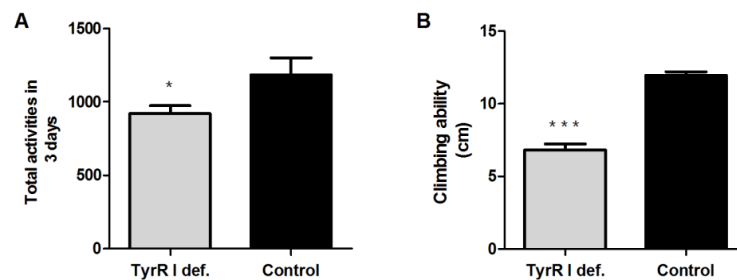


Figure 50. Locomotor activity and climbing ability of TyrR I mutant flies. (A) The histogram shows the total activity counts per TyrR I mutant male flies compared to its matched controls for 3 day in LD condition which is significantly decreased of total activity per TyrR I mutant flies (with 22.5% decrease). (B) The TyrR I mutant flies show defects in climbing ability. The TyrR I mutant males decreased 42.8% of climbing distance relative to control male flies (TyrR I mutant, $n=116$; control, $n=118$). Data are presented as mean \pm SEM; the unpaired two-tailed Student's t-test was used for analysis, * $p < 0.05$, *** $p < 0.01$.

3.4.5 Mutation of TyrR I affects body fat

Animal behaviors are closely associated with metabolism, because any generation and control of different type of behaviors need energy supply. Since TA and OA have different actions on fat accumulation I have identified in section 3.1.3, it is interesting whether TyrR I mediated the effect of TA or OA on fat accumulation. Consistent with the results obtained in section 3.2.4, both male and female TyrR I mutants displayed a significant increase in TAG level, with 110 % increase in males and 48.4 % increase in females (Fig. 51, $p < 0.001$). Interestingly, TAG increase in TyrR I mutants was uniform with that of in $T\beta H^{nM18}$ flies but not in $Tdc2^{RO54}$, these data combined with section 3.2.6 suggesting that TyrR I mediated fat body-specific role of OA to regulate fat storage.

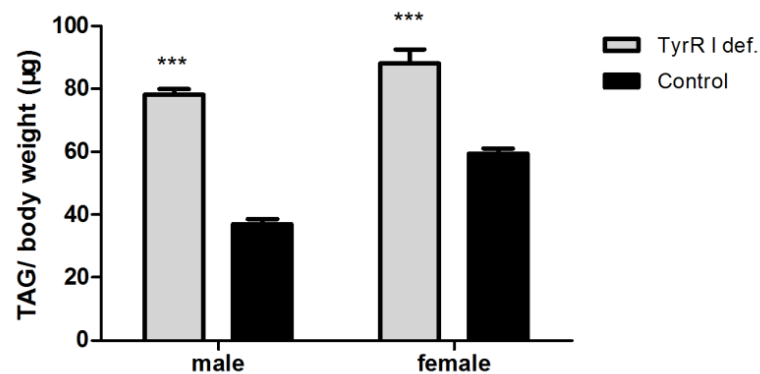


Figure 51. TyrR I mutants increased total TAG storage. Total TAG levels were measured in TyrR I mutants and control flies under normal feeding condition. The TyrR I mutants have significantly high TAG levels, both in females and males, with 110% higher in male flies (TyrR I, n=6; control, n=6) and 48.4% higher in female flies (TyrR I, n=8; control, n=8). Data are presented as mean \pm SEM; two-ways ANOVA was used for analysis, ***p<0.0001.

3.4.6 TyrR I is expressed in six DH44 neurons in brain

To investigate where TyrR I is expressed to mediate the action of TA on modulating of circadian rhythms, the classic GAL4/UAS system was employed to visualize the complete expression pattern of TyrR I. The *TyrR I-GAL4* was used to drive the Green fluorescent protein transgene (*UAS-GFP*). We found that the TyrR I is expressed in a cluster of neurons located in the *Pars intercerebralis* of larval and adult brains. Using the immunohistochemical double staining approach, anti-GFP and anti-dILP2 antisera were used to identify if the TyrR I expressing cells are IPCs (Fig. 52A-F). Furthermore, the TyrR I expressing cells are not DH31 producing cells either, because the two signals were not co-localized at the cellular level (Fig. 53A-F).

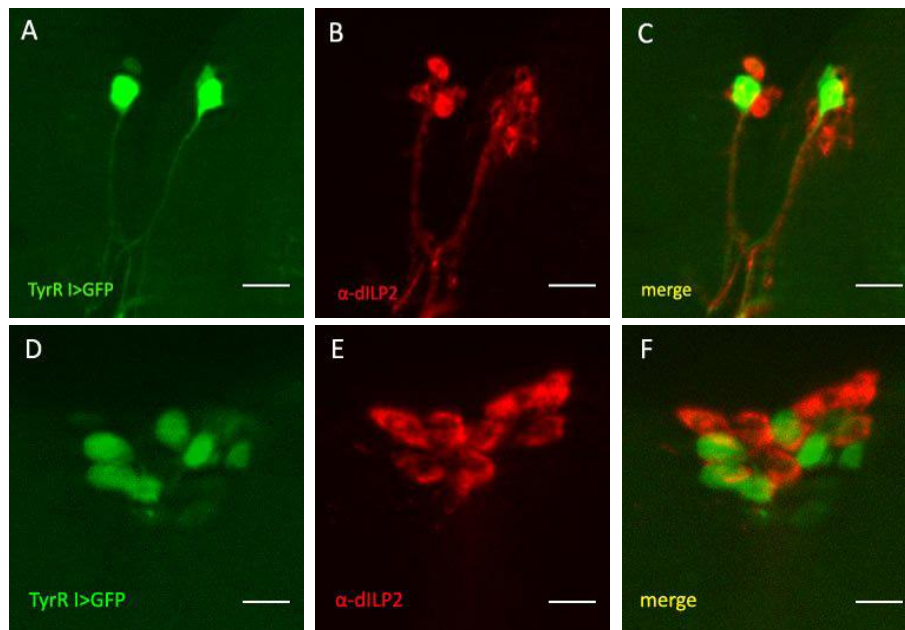


Figure 52. TyR I is not expressed on IPCs. (A-C) In larval brain, GFP driven by *TyrR I-Gal4* (*TyrR I-Gal4 > UAS-GFP*, green) displays a cluster of neurosecretory TyR I positive neurons that are located in the *Pars intercerebralis*, a couple of arborizations could be seen in the subesophageal ganglion and descending to the ventral nerve cord, while combined with dILP2 antibody immunolabeling (α -dILP2, red), it validates the TyR I neurons do not belong on IPCs. (D-F) In adult brain, the *TyrR I-Gal4* expressing cells are also not immunolabeled with dILP2 antiserum in PI region. Scale bar is 50 μ m.

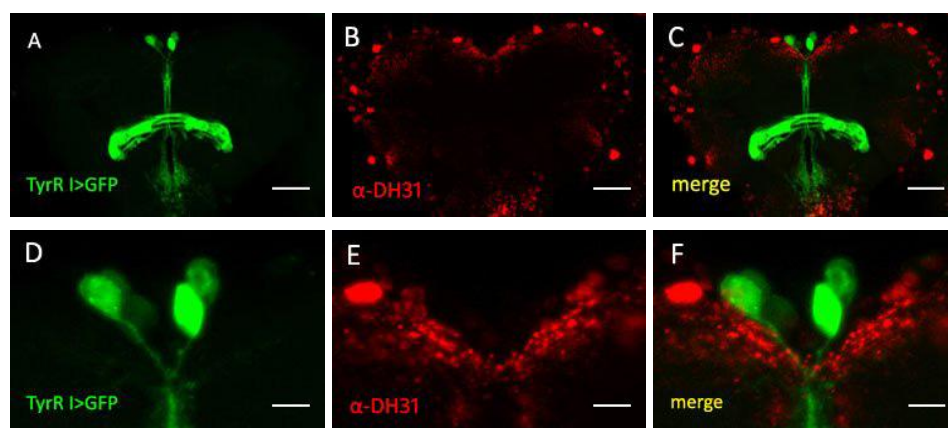


Figure 53. TyR I is not expressed on DH31 neurons. (A-C) show immunofluorescence by GFP staining and co-staining with anti DH31 in adult brain, the *TyrR I-Gal4* expressing cells are also not colocalized with DH31 neurons in PI region. (D-F) Enlargement of PI, the *TyrR I-Gal4* (*TyrR I-Gal4 > UAS-GFP*, green) and DH31 neurons (red) are not merged. Scale bar: A-C is 100 μ m; D-F is 20 μ m.

To identify which of the TyR I expressing neurons in the PI, an antiserum recognizing DH44 was applied. It was obvious that DH44 neurons displayed TyR I expression. In

agreement with previous studies (Cabrero et al., 2002), immunohistochemical analysis showed that DH44 was restricted to six PI neurons, which all expressed TyrR I (Fig. 54A-F).

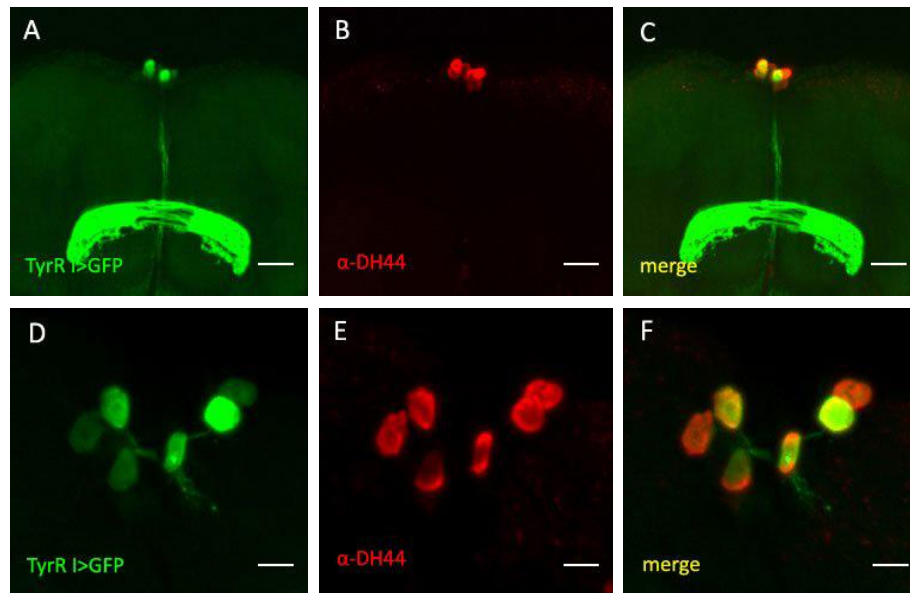


Figure 54. TyrR I is expressed on DH44 neurons. (A-C) The adult DH44 neurons co-localize GFP expression (*TyrR I-Gal4>UAS-GFP*, green) and anti DH44 immunostaining (α -DH44, red). All the DH44 immunoreactivity also displays GFP expression. Enlargement of PI showing six TyrR I neurons (D, green) and six DH44 neurons (E, red) are completely co-localized (F, yellow). Scale bar: A-C is 100 μ m; D-F is 20 μ m.

3.4.7 Knockdown of TyrR I in DH44 neurons elevates DH44 level

An important recently published study provided strong evidence that DH44 was responsible for rhythmic behaviors (Cavanaugh et al., 2014). To test whether TA contributions to circadian rhythms is directly mediated by TyrR I on DH44, I performed an RNAi strategy to knockdown TyrR I expression. First of all, I tested two GAL4 drivers specific to DH44, which will be referred to as *DH44^{FL}-GAL4* and *DH44^{VT}-GAL4*. Both lines were dominantly restricted to DH44 neurons, except that some additional expression outside the PI was detected in the *DH44^{FL}-GAL4* line (Fig. 55). These data were totally consistent with those recently published (Cavanaugh et al., 2014).

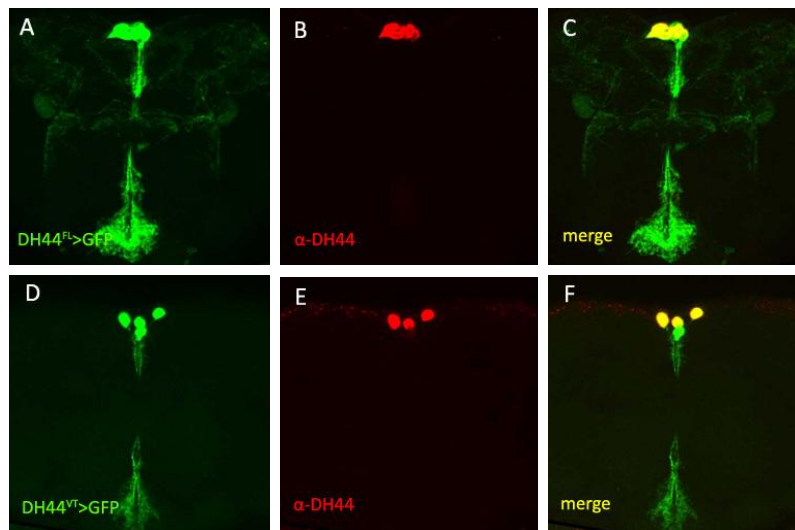


Figure 55. Identification of two DH44 driver lines. (A) $DH44^{FL}-GAL4>UAS-GFP$ brains show that GFP expression is dominantly restrict to PI neurons and additionally weak expression projecting to the fan-shaped body. (B and E) Anti DH44 immunolabeling (red). (D) $DH44^{VT}-GAL4>UAS-GFP$ brains only show GFP in PI neurons. (C and F) The merged images show overlapped signal in DH44 neurons.

To investigate the role of TyrR I on DH44, qPCR was therefore performed to measure the transcript level of the DH44 gene in adult brains with restricted to the PI, which is excluded the region of TyrR I additionally expressed on the superior slope of the oesophageal foramen, after knockdown TyrR I in DH44 by means of the cross $DH44^{VT}-GAL4>UAS-TyrR\ I-RNAi$. Two independent RNAi lines of TyrR I allele genes were used. Results showed that DH44 mRNA levels in both crossings were similar to those of the $DH44-Gal4$ control (Fig. 56A).

Next, I undertook a quantification of the DH44 immunofluorescence in DH44 neurons in response to TyrR I knockdown driven by either $nsyb-GAL4$ or $DH44-Gal4$. It was found that the DH44 immunostaining increased in the DH44 neurons with diminished TyrR I, both in globally brain and in DH44 neurons (Fig. 56B, $p<0.0001$ in both crosses), suggesting that diminished TyrR I has a inhibitory role on DH44 secretion over DH44 production.

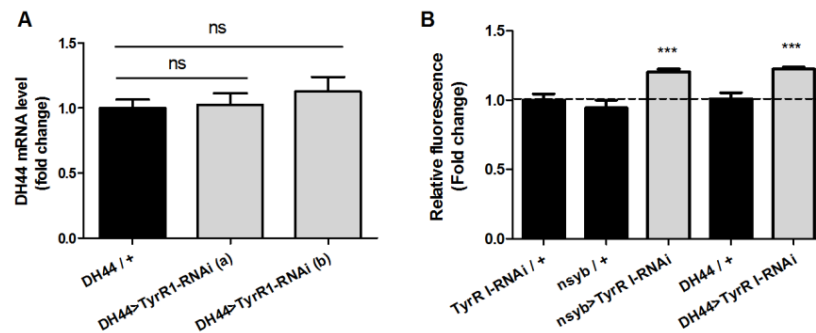


Figure 56. TyrR I knockdown in DH44 neurons affects DH44 secretion over production. (A) DH44 transcript level showed no difference between flies of two TyrR I gene alleles RNAi mediated knockdown on DH44 neurons and control flies, $P > 0.05$. The value for control was set to 1. (B) The F1 generation of *nsyb-GAL4>UAS-TyrR I-RNAi* shows significantly higher level of DH44 immunofluorescence than the control lines (*nsyb-GAL4>UAS-TyrR I-RNAi*, $n=12$; *nsyb-GAL4/+*, $n=10$; *UAS-TyrR I-RNAi/+*, $n=9$). As the same, *DH44-GAL4>UAS-TyrR I-RNAi* also shows significantly higher level of DH44 immunofluorescence than its control lines (*DH44-GAL4>UAS-TyrR I-RNAi*, $n=12$; *DH44-GAL4/+*, $n=10$; *UAS-TyrR I-RNAi/+*, $n=9$). Data are presented as mean \pm SEM; one-way ANOVA was used for analysis, *** $p < 0.01$.

3.4.8 Circadian rhythms were affected in TyrR I knockdown on DH44 neurons

It has been shown that DH44 cells as a neuronal population is part of the circadian output pathway, and knockdown of TyrR I in DH44 cells inhibited DH44 secretion, I ask whether diminished TyrR I in DH44 neurons is sufficient to degrade rhythmic behavior. To address that, I monitored the locomotor activity of their progeny from *DH44^{FL}-GAL4>UAS-TyrR I-RNAi*. Comparison with their parental controls, RNAi mediated knockdown of TyrR I in DH44 neurons resulted in circadian arrhythmicity, the degeneration of rhythmicity occurred primarily in morning and evening oscillators (Fig. 57A-C). These data are consistent with what I observed in *Tdc2^{RO54}* and TyrR I mutant flies, indicating that TyrR I mediated the role of TA on circadian rhythms via activation of DH44 output pathway.

Simultaneously, the total activities of flies in LD conditions were calculated. Nevertheless, there was no significant difference between *DH44-GAL4>UAS-TyrR I-RNAi* and its controls (Fig. 58). This phenotype could be explained by the increased arrhythmic activity of TyrR I diminished flies during the experimental period.

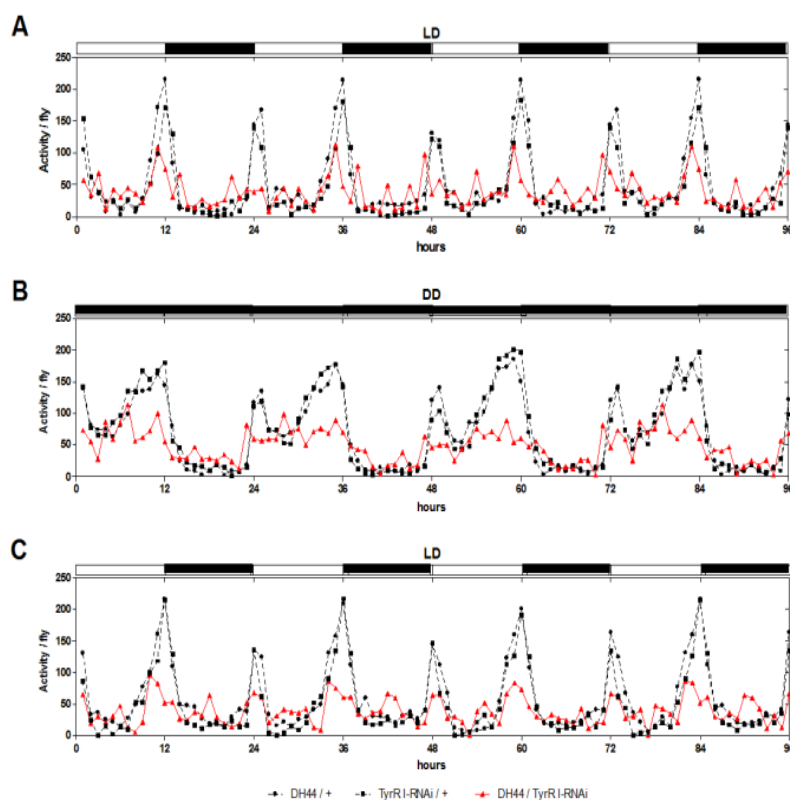


Figure 57. TyrR I knockdown on DH44 neurons show circadian arrhythmicity. Both groups of male flies were firstly entrained to a LD cycle for 4 days (A), followed by 4 days in DD (B), and then released to LD again (C). The flies with diminished TyrR I ($DH44^{FL-GAL4}>UAS-TyrR$ *I-RNAi*) exhibited severely aberrant circadian rhythms in different conditions, and this arrhythmicity occurred primarily in morning and evening oscillators.

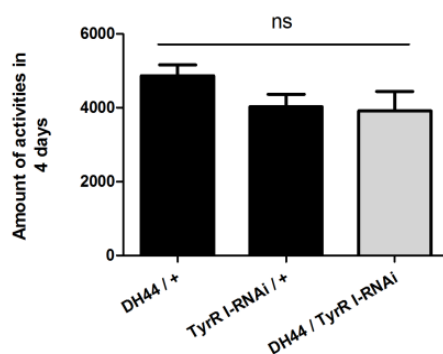


Figure 58. Locomotor activity of flies with diminished TyrR I on DH44 neurons. The histograms show the total activity counts of male flies with diminished TyrR I on DH44 neurons compared to its parental controls for 4 days in LD condition. It shows no significant difference of total activity among them ($n=8$ for each genotype). Data are presented as mean \pm SEM; one-way ANOVA was used for analysis, $p>0.05$.

3.4.9 Knockdown of *TyrR I* on *DH44* neurons alters fat storage

To assess whether RNAi-mediated knockdown *TyrR I* in *DH44* neurons affects energy homeostasis, whole TAG levels in the two RNAi lines crossed to either *nsyb-GAL4* or *DH44-GAL4* driver lines were measured. Compared to the controls, there were significantly increased TAG levels in *nsyb-GAL4* driven both *TyrR I-RNAi* (Fig. 59A). Although only one *TyrR I-RNAi* expression in *DH44* neurons showed TAG level increases in male flies (Fig. 59B), two *TyrR I-RNAi* lines crossed to *DH44-GAL4* showed both higher TAG levels than controls in female flies (seen Fig. A11 in appendix). Taken together, it indicates that altered *DH44* level impacts fat storage.

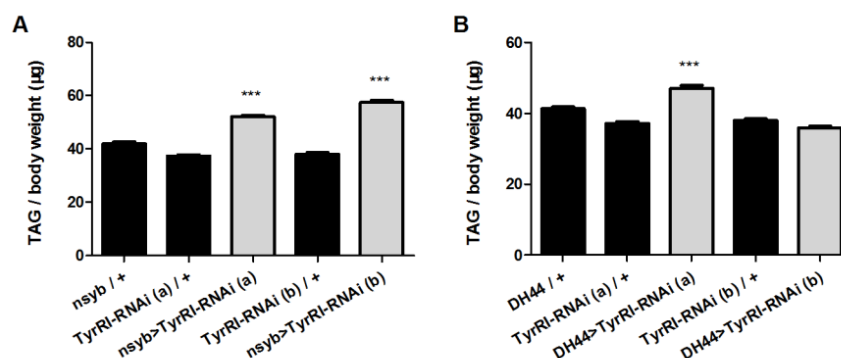


Figure 59. Knockdown *TyrR I* on *DH44* neurons alters fat storage. Total TAG levels were measured in male flies with diminished *TyrR I* and control flies under normal conditions. (A) The TAG levels are significant higher in male flies with RNAi mediated knockdown *TyrR I* on *DH44* neurons (*nsyb-GAL4 > UAS-TyrR I-RNAi*) than their parental controls (n=8 for each genotype). (B) The TAG levels are also significant higher in male flies with only one RNAi mediated knockdown *TyrR I* on *DH44* neurons (*DH44^{VT}-GAL4 > UAS-TyrR I-RNAi*) than their parental controls (n=8 for each genotype). Data are presented as mean \pm SEM; one-way ANOVA was used for analysis, ***p<0.0001.

To exclude the possibility that increased nutritional intake result in higher fat storage, I measured feeding using the CAFE assay. After performed six to eight replicated tests using age- and population-matched flies, it showed that knockdown of *TyrR I* neither in whole brain nor specific in *DH44* neurons flies induced changes in feeding behavior (Fig. 60A and B).

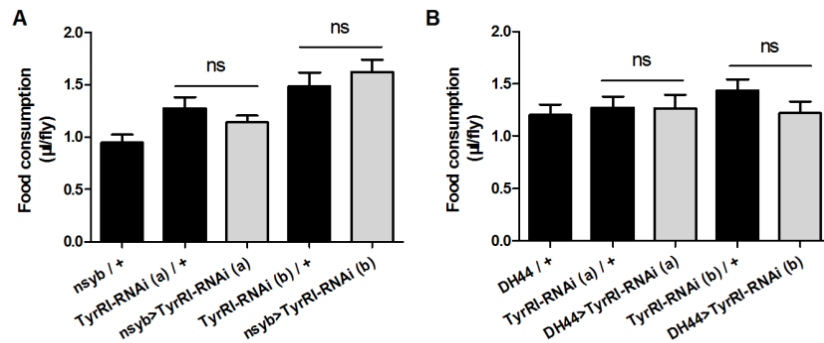


Figure 60. Food consumption is not changed in flies with diminished TyrR I expression. (A) CAFE assay was performed in male flies with RNAi mediated knockdown TyrR I and controls. There was no significant difference in liquid food consumption among flies with diminished TyrR I in brain (*nsyb-GAL4 > UAS-TyrR I-RNAi*, n=8 for each genotype). (B) The food consumption also doesn't show significant difference in male flies with diminished TyrR I on DH44 neurons (*DH44^{VT}-GAL4 > UAS-TyrR I-RNAi*, n=7 for each genotype). All data are presented as mean \pm SEM. one-way ANOVA was used for analysis, $p > 0.05$.

3.4.10 Flies with diminished TyrR I in DH44 neurons show stress resistance

As DH44 is related to mammalian corticotropin releasing factor (CRF), which is linked to central processing of stress responses, I reasoned that control of DH44 production or secretion in *Drosophila* leads to differences in survival upon different stressors. Thus, the sensitivity of flies with knockdown of TyrR I in the whole brain and in DH44 neurons to starvation and osmotic stress was measured. Under starvation conditions, survival curves of *nsyb-GAL4* and *DH44^{VT}-GAL4* driving both *TyrR I-RNAi* lines showed similar significant differences from those of control lines, and the median survival times between *nsyb>TyrR I-RNAi* and *DH44>TyrR I-RNAi* revealed no significant difference (Fig. 61), suggesting that knockdown of TyrR I in DH44 cells lead to starvation resistance.

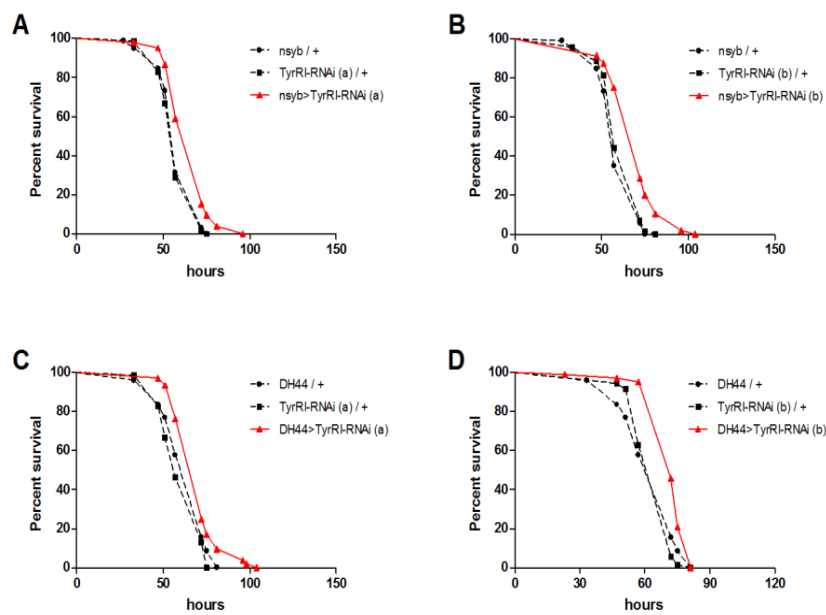


Figure 61. Flies with diminished TyrR I show starvation resistance. (A-B) Under starved conditions, male flies with knockdown of TyrR I in the brain show significantly extended lifespan compared to parental control flies. The median survivals of each genotype are 72 h for *nsyb>TyrR I-RNAi* (a) (n=105), 57 h for *nsyb-Gal4/+* (n=105), 57 h for *UAS-TyrR I-RNAi* (a) (n=96) and 72 h for *nsyb>TyrR I-RNAi* (b) (n=107), 57 h for *UAS-TyrR I-RNAi* (b) (n=112). (C-D) The male flies with knockdown of TyrR I in DH44 neurons also significantly extended their longevity compared to parental control flies. The median survivals of each genotype are 72 h for *DH44>TyrR I-RNAi* (a) (n=105), 62 h for *DH44-Gal4/+* (n=104) and 72 h for *DH44>TyrR I-RNAi* (b) (n=106). Log-rank test for survival analysis showed significant difference, $p < 0.001$.

Under osmotic conditions, survival times of *nsyb-GAL4* and *DH44-GAL4* driving either of both *TyrR I-RNAi* lines were significantly longer than those of their control lines. Furthermore, comparison of the estimates of median survival of these lines between *nsyb>TyrR I-RNAi* and *DH44>TyrR I-RNAi*, it did not show any difference (Fig. 62). Just like starvation stress, the flies with knockdown of TyrR I in DH44 cells also displayed osmotic resistance.

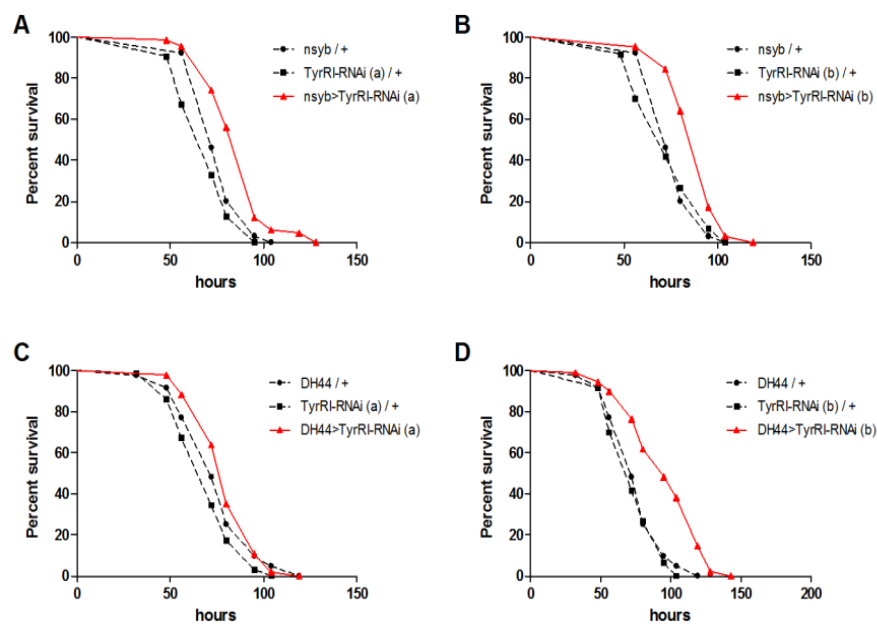


Figure 62. Flies with diminished TyrR I show increased osmotic resistance. (A-B) Under starved conditions, the male flies with knockdown of TyrR I in brain significantly extended their lifespan compared to parental control flies. The median survivals of each genotype are 95 h for *nsyb>TyrR I-RNAi* (a) (n=132), 72 h for *nsyb-Gal4/+* (n=130), 72 h for *UAS-TyrR I-RNAi* (a) (n=128) and 95 h for *nsyb>TyrR I-RNAi* (b) (n=128), 70 h for *UAS-TyrR I-RNAi* (b) (n=132). (C-D) The male flies with knockdown of TyrR I in DH44 neurons also significantly extended their longevity compared to parental control flies. The median survivals of each genotype are 80 h for *DH44>TyrR I-RNAi* (a) (n=94), 72 h for *DH44-Gal4/+* (n=83) and 72 h for *DH44>TyrR I-RNAi* (b) (n=89). Log-rank test for survival analysis showed significant difference, $p < 0.001$.

3.5 The octopamine receptor oct β 2R is essential for ovulation and fertilization in the fruit fly *Drosophila melanogaster*

It is known that OA has a key hormonal and neurochemical role in controlling the ovulation process in *Drosophila*. A set of octopaminergic cells that reside in the thoracic-abdominal ganglion innervate the ovaries and oviducts, the dysfunction of this sites lead to an egg retention phenotype (Lee et al., 2003; Monastirioti, 2003; Lee et al., 2009; Lim et al., 2014).

3.5.1 Oct β 2R mutant flies are defective in egg-laying

To characterize the contribution of different OA receptors to the phenotypes observed in $T\beta H^{nM18}$ mutant flies, we used a set of flies impaired in the expression in one of the four different OA receptors.

Analogous to OAMB mutant flies that were almost unable to lay eggs (Lee et al., 2003), Oct β 2R mutant animals showed a very similar phenotype, being unable to lay fertilized eggs. To quantify this effect, groups of five females each together with the corresponding males were set up and the number of eggs laid per day was counted. The w^{1118} strain served as the control (Fig. 63). Whereas w^{1118} flies showed a high level of egg-laying with a maximum at day 3, the Oct β 2R mutant flies showed only negligible quantities of laid eggs.

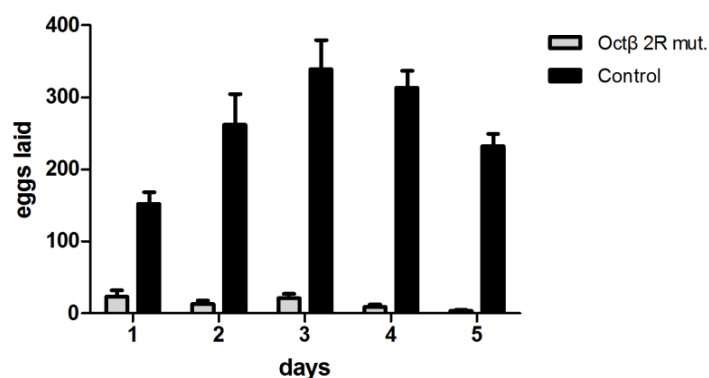


Figure 63. Oct β 2R mutant animals have a reduced ovulation rate. Flies deficient in the Oct β 2R (gray bars) show a reduced egg-laying rate when compared with that of the wild-type control (black bars). All values are statistically different between the Oct β 2R mutant animals and the

matching controls (N=10; $P < 0.0001$).

3.5.2 *Octβ2R is essential for female fertilization*

The very few eggs that were laid in the Octβ2R mutant animals always were final-stage (stage 14) oocytes or of the subsequent stage (“mature oocyte”). They failed to further develop because they were not fertilized. Eggs from control animals showed after DAPI-staining a large nucleus (Fig. 64A, arrow), which is typically seen in a completed meiosis (Ejima et al., 2004), whereas eggs of Octβ2R mutant animals showed a complex pattern of DAPI-negative signals as seen in unfertilized eggs (Fig. 64B), suggesting that Octβ2R is required at least for female fertilization.

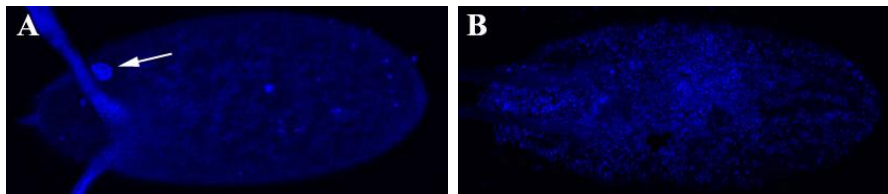


Figure 64. DAPI staining the eggs collected within 2 h after oviposition. Eggs from control animals showed after DAPI-staining a large nucleus (A), while eggs of Octβ2R mutant animals showed a complex pattern (B).

3.5.3 *The sterility phenotype is not due to defect in copulation*

Many physiological factors affect female fecundity, such as the age and health of the female, copulation with a male, sperm storage and mature eggs ovulating from ovarioles, which one of process is defect would lead to sterility. To investigate whether females or males are defective in fertility, Octβ2R homozygous mutant males or virgin females were placed with w^{1118} virgin females or males, respectively. All flies were fed on standard food with extra yeast paste. The sterile phenotype was female-specific as we observed very few eggs were laid in females of the Octβ2R mutant flies when crossed to w^{1118} males, while crossing of Octβ2R mutant males with w^{1118} females gave normal egg-laying rates.

Next I determined whether mating was impaired and used cohorts of males and females to quantify the number of copulations. The w^{1118} and TβH mutant flies

showed almost identical copulation rates, whereas the Oct β 2R mutant flies showed a strong delay in the copulation rates (Fig. 65).

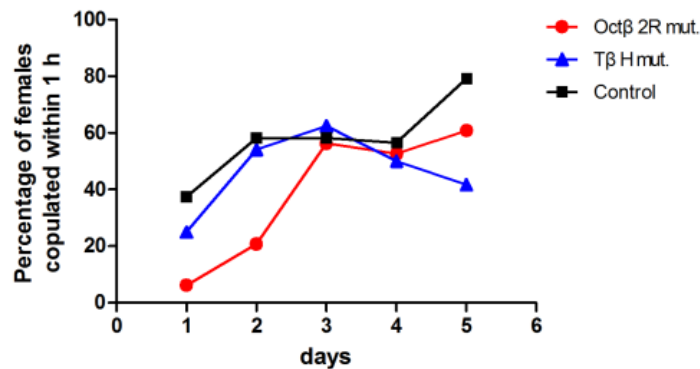


Figure 65. The Oct β 2R mutant females show a delayed copulation. Number of copulations was scored in Oct β 2R mutant animals (red circles), tyrosine β -hydroxylase mutant flies (green triangles) and control animals (black squares). Females of the corresponding genotype were confronted with wild-type males.

3.5.4 Abnormal egg retention in Oct β 2R mutant females

Females with the sterility phenotype are easily visible due to their notably enlarged abdomens. To get insights into the underlying reason for this phenotype, we analyzed the ovaries of control and Oct β 2R mutant flies. Ovaries of the latter ones are much larger than those of control animals. This holds true for very young (5d; Fig. 66A and B) as well as for older females (15d; Fig. 66D and E). Usually, oocytes are organized in one ovariole in rows of maximal two eggs (Fig. 66F). In the Oct β 2R mutant animals we observed up to five oocytes in a row (Fig. 66C). Moreover, all oocytes were staying at stage 14, while oocytes between stage 5 and 13 were essentially lost, indicating that eggs development proceed successfully and there were severe problems with the process of ovulation.

A quantitative evaluation of the different ovary sizes was performed using photographs taken from isolated ovaries followed by quantification of the corresponding areas. Both, for 5 day old females (Fig. 66G) as well as for 15 day old females (Fig. 66H), these differences are highly significant ($P < 0.0001$). Taken together, the above experiments elucidate that the sterility of Oct β 2R mutant females was due

to defective ovulation resulting in majority of mature eggs retained in ovarioles, instead of due to defective copulation.

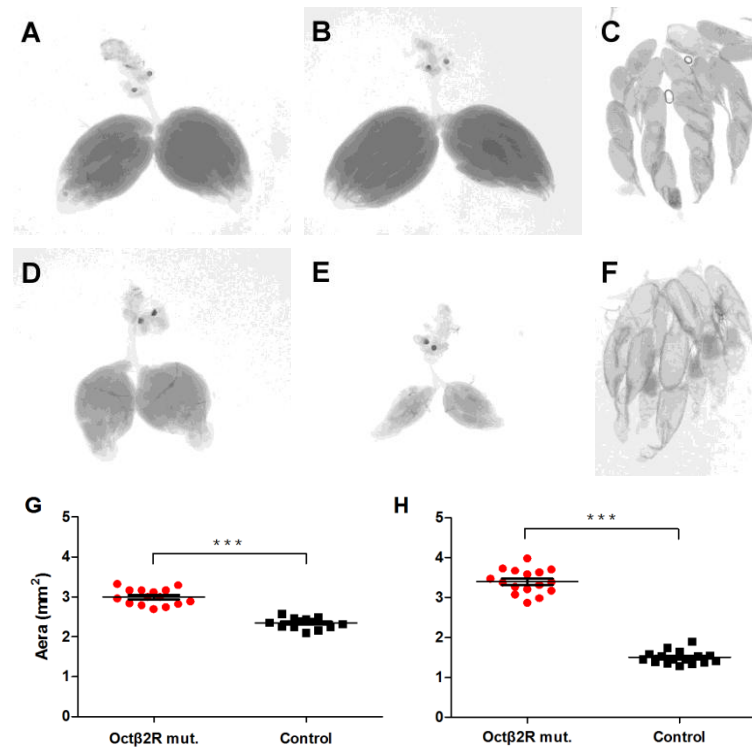


Figure 66. The ovaries of Octβ2R mutant flies are significantly larger than those of control females. Ovaries of Octβ2R mutant females at an age of 5 days (A) are larger than those of control females (D) of the same age. Ovaries of 15 day old Octβ2R mutant females (B) and ovaries from females of the same age are also shown (E). Photomicrograph of ovarioles containing late stage oocytes of Octβ2R mutant (C) and control animals are displayed (F). Quantification of the area of semi-ovaries dissected from 5 day old females (G) and 15 day old females (H) was measured (n>8, P<0.0001).

3.5.5 Octβ2R and other OA receptors expression in female reproductive system

In the next step, I aimed to evaluate the expression pattern of the Octβ2R within the female reproductive organs. For this purpose, Gal4-lines containing the presumptive Octβ2R-promoter had been prepared. Crossing this line with a *UAS-gfp* line utilizing the binary Gal4/UAS system allowed visualizing the expression sites of the receptor gene within the female reproductive organ (Fig. 67A). Expression is seen in the oviduct, but also in other structures including the spermatheca (Fig. 67B). To obtain further information about the expression in these different parts of the female reproductive organ, RT-PCR experiments were performed, with all four different OA

receptor genes (*OAMB*, *OA2*, *Oct β 2R*, *Oct β 3R*). Thus, the female reproductive system of young virgin females and of older mated females was manually isolated and different parts including the ovary, the oviduct, the spermatheca, and the uterus/seminal receptacle were dissected to isolate RNA usable for cDNA synthesis. As a loading control the *Rpl32* gene was employed (Fig. 67C top). All four different OA receptors were tested. Except the *OA2* receptor that is not present in the female reproductive organ, all others show a relatively broad expression in the different parts of this structure (Fig. 67C). While the *OAMB* and the *Oct β 2R* show very strong signals in the oviduct, expression levels of the *Oct β 3R* are slightly lower. Regarding the *Oct β 2R*, the broad presence in the different parts of the female reproductive system is obvious, with maximal expression in the oviduct and in the spermatheca (Fig. 67C).

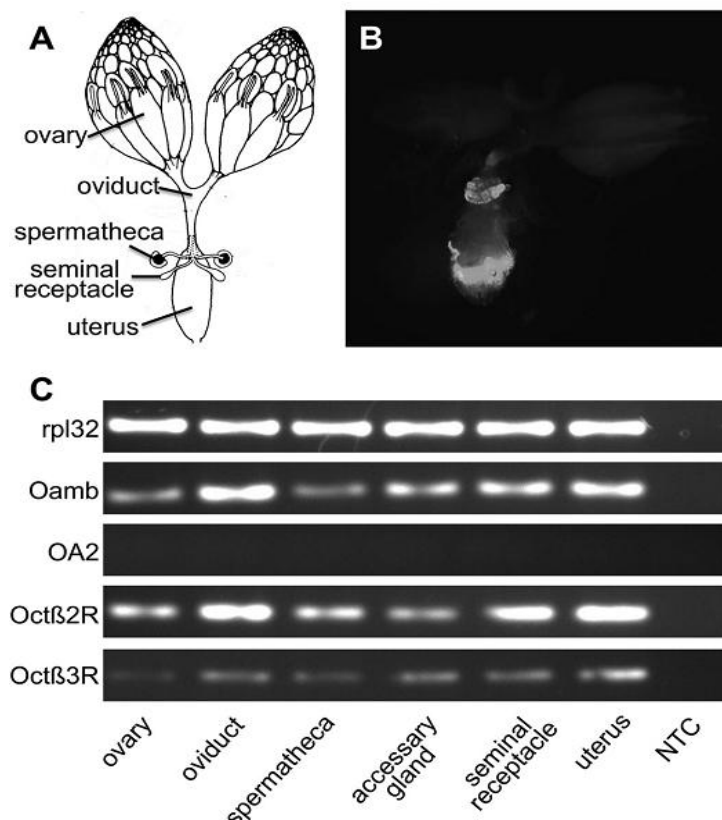


Figure 67. Expression analysis of OA receptors in the female reproductive system. The female reproductive organ consists of different regions including the ovary, the oviduct, the spermatheca, the seminal receptacle and the uterus (A, modified after (Middleton et al., 2006)). The *Oct β 2R*-promoter-Gal4 line (crossed to *UAS-gfp*) revealed expression in different parts of the

female reproductive organ (B). Reverse transcriptase PCR analysis of the different OA receptors are expressed in the different substructures of the female reproductive organ (C). *Rpl32* is used as a loading control. NTC means no template control.

3.5.6 *Octβ2R* is required in muscle tissue for ovulation

Because the thoracic CNS and female reproductive system are joined by abdominal nerves, the site of *Octβ2R* action could be the CNS neurons or in the reproductive system. To further dissect the role of the *Octβ2R* in the process of ovulation, I used RNAi targeted against the corresponding gene. Using three different Gal4-lines driving RNAi-mediated expression into different organs, the site of *Octβ2R* action for OA controlling of ovulation could be evaluated.

Crossing the tubulin-promoter Gal4 line with *UAS-mCD8::GFP* (membrane tagged GFP) show positive signals in most organs of the fly including females reproductive system (Fig. 68A). In *tubP-GAL4>UAS-Octβ2R-RNAi* line, we observed a downregulation of the ovulation rate by approximately 80% corresponding to their parental controls (Fig. 68C). On the other hand, targeting the RNAi-effect to the nervous system with the *nsyb-Gal4* driver had no effect on the ovulation rate. Moreover, when driven by a muscle-specific *mef2-GAL4*, *UAS-mCD8::GFP* was conspicuously visible in the oviduct muscle, shown in (Fig. 68B). Using the *mef2-Gal4* driver to decrease the expression of the *Octβ2R* resulted in a significant decrease of the ovulation rate indicating that expression of this receptor in the oviduct muscle is responsible for transmitting the effects on ovulation (Fig. 68C).

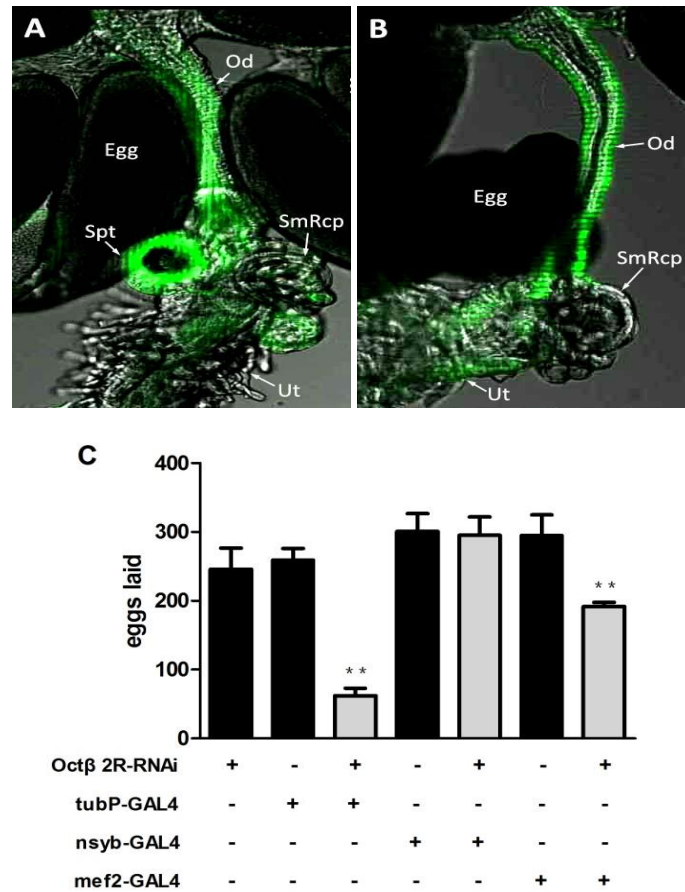


Figure 68. RNAi-mediated gene silencing of the Octβ2R revealed that visceral muscles as its site of action. The *tubP-GAL4>UAS-mCD8::GFP* show extensive expression in female reproductive system (A). The expression of *UAS-mCD8::GFP* driven by *mef2-GAL4* is conspicuously visible in the oviduct muscle (B). Egg-laying was quantified in different RNAi experiments (C). Controls (black bars) are parental lines crossed to the genetic background of all RNAi liners (*w¹¹¹⁸*). Egg-laying was quantified in groups of five females and listed as eggs laid per day ($P < 0.005$).

4 Discussion

The monoamines OA and TA, the counterparts of vertebrate norepinephrine and epinephrine, are major modulators of metabolism, behavior and fitness in invertebrates. In this project, I used the fruit fly *Drosophila melanogaster* as a simple model in order to study basic aspects of adrenergic signaling. Taking the advantage of the wealth of genetic tools available in *Drosophila* such as the GAL4/UAS system, RNAi and gene disruption technologies, I tried to dissect the roles of octopaminergic /tyraminerpic signaling in metabolism, ovulation and circadian rhythmicity. The fly shares a similar organ composition with mammals, for example, the fat body and oenocytes are functional analogues of the mammalian adipose tissue and liver, respectively (Gutierrez et al., 2007). In addition, the homologues of many mammalian genes could be found in the *Drosophila* genome, making it an adaptable model for behavior and metabolic research. Furthermore, to gain insight into the genetic mechanism of OA mediating metabolism underlying nutrient conditions, I performed microarray analysis to identify transcriptional profiles regulated by OA in the adult *Drosophila* brain under starvation conditions. Characterization of these target genes and their potential roles may be beneficial to understand the multiple mechanisms by which OA orchestrates the energy-demanding behaviors.

4.1 Octopamine regulates insulin-like peptide secretion

In this part, I focused on the role of OA in controlling production and release of dILPs from IPCs. Flies deficient in OA synthesis show phenotypical peculiarities that are consistent with animals having increased insulin secretion. This impacts lifespan, stress resistance, lipid and carbohydrate levels but also the animal's activity. Increasing the release of OA induces exactly the opposite effects indicating that the level of OA regulates various aspects of metabolism in the fly via insulin secretion. Furthermore, flies deficient in both OA and TA synthesis show most of metabolic phenotypes consistent with that in OA-deficient flies. As both mutant flies process different TA level, the similarity in metabolic phenotypes is not likely mediated by TA,

instead, I could show that these effects are attributable to the roles of OA-mediated regulation of dILP release, thus representing an interaction between stress signalling pathways and important metabolic control systems.

Both compounds (OA and TA) deploy different, in some cases even contrary effects. This striking difference is apparent especially for body fat deposition. While animals without TA and OA (*Tdc2*-deficient) are slightly leaner than their matching controls, those animals that lack only OA (*TβH*-deficient) are obese. These different phenotypes imply that TA counteracts the effects of OA on this metabolic process. The observation that TA and OA deficient flies are leaner is in accordance with data presented earlier, where ectopic induction of transmitter release from *Tdc2*-producing cells that contain both, OA and TA, increased body fat (Erion et al., 2012). Altogether, our results show that OA affects various facets of the fly's physiology and this in a well-coordinated way. Depleting or increasing OA levels in the fly induced effects that are almost congruent with those observable in mammals in comparable experimental situations. Flies devoid of OA (*TβH^{nM18}*) develop a typical couch potato syndrome, which is characterized by increased body fat, reduced physical activity, a reduced metabolic rate and consequently, reduced life span. Increasing the release of OA consistently induced exactly the opposite effects, resulting in animals that are lean, active and long living. In order to increase body fat, the balance between energy intake and energy expenditure has to be shifted into the direction of energy intake, either by increasing food intake or by reducing energy expenditure (Spiegelman and Flier, 2001). The relevant parameter in flies depleted of OA appears to be the reduction of energy expenditure. Energy expenditure has two major components, the basal metabolic rate and the activity induced expenditure. Both of them are apparently under the control of OA, meaning the reducing the OA level reduces the metabolic rate and the movement activity. Regarding the control of metabolic parameters, the system described has the almost identical architecture as the adrenergic system of vertebrates. While reduction of adrenergic signaling by ingestion of e.g. β-blockers induced weight in human patients, adrenergic agonist that enhance signaling via the corresponding receptors are known to act as fat burners. This induced reduction of body fat is believed to depend on an increase of

the metabolic rate.

The results of this study implied that reduced OA levels led to an increase in insulin release, which shifts the organisms' metabolic system into "uptake" mode, which may be equivalent with reduced metabolic rate. Thus, it is assumed that the metabolic effects of OA are mediated, at least in part, by regulation of insulin release. However, the insulin signaling release from IPCs is complex, dILP2, 3 and 5 are found to be produced in a same set of neurons in the brain. They are the most investigated peptides and their transcription seems to be regulated independently (Ikeya et al., 2002). Interestingly, the transcription of *dILP5* is upregulated and the transcription of *dILP3* is downregulated in flies devoid of OA, whereas *dILP2* transcript is not affected. Because there is no evidence to show the interaction and feedback among the dILPs, it could not be concluded if the different dILPs have different roles as well as different release kinetics from IPCs. It is known that *dILP5* transcript is affected by different nutritional conditions (Broughton et al., 2010) and *dILP3* may act in stimulatory regulation of production of dILP2 and dILP5 in the IPCs (Broughton et al., 2008; Gronke et al., 2010). Thus, we presume a compensatory regulation in transcriptional level occurs among these dILPs.

OA not only controls central aspects of metabolism and behavior, but also influences life span substantially. Flies without OA, which have increased body fat and a reduced metabolic rate have also a reduced life span. Insulin holds the potential to make the casual relationship between these different phenotypes (Hwangbo et al., 2004; Broughton et al., 2005). OA deficient flies are also characterized by increased insulin release, which is in turn known to be inversely related with life span. Thus, regulation of insulin secretion by OA and/or TA has a great impact on this very important parameter. In *Drosophila*, increasing OA signaling increases life span, presumably via decreasing insulin release, thus mimicking caloric restriction. In the last years, studies of dILP-ablated flies showed that extended lifespan are often accompanied with enhanced resistance to various stressors (Broughton et al., 2005), such as starvation and cold chill resistance. However, OA-deficient flies improved insulin release, but they displayed starvation resistance, suggesting that OA influences resistance to starvation not only by release of dILPs, but also in other ways. In fact, response to

starvation was found to be associated with the metabolic status, specifically, with the storage of carbohydrates and lipids. Moreover, OA-deficient flies have high lipid levels and low concentrations of glucose and trehalose in the hemolymph indicative for high carbohydrates storage. This association of increased lipid levels with resistance to starvation was obvious in T β H mutant flies and also in some OA and TA receptor mutant flies. In addition, sleep and locomotor activity during starvation are depended on metabolic demands. Upon food deprivation, animals exhibit starvation-induced hyperlocomotion and sleep reduction, which speeds-up the consumption of energy sources (Mattaliano et al., 2007). OA-deficient flies are impaired in locomotor activity and have increased sleep behavior, thus they accumulate more energy in response to starvation.

Early studies have demonstrated that ablation of IPCs or interference with DILPs diminished growth showing in smaller body size (Ikeya et al., 2002; Teleman, 2010). In our study, no obvious peculiarities on growth (both in body weight and length) were observable between OA-deficient flies and its controls, similar to the observation in studies focusing on GABA_B, 5-HT_{1A} or adiponectin receptor knockdowns in IPCs (Enell et al., 2010; Luo et al., 2012; Kwak et al., 2013). Whereas a reduction of body weight was observed for flies without OA and TA, the decreased body weight probably resulted from the decreased lipid amounts in the entire body. Although partial increase of insulin release by lack of OA alters lifespan, metabolic status and stress resistance, the amount of increase of insulin into the hemolymph may not be sufficient to increase growth accordingly.

Insulin functions throughout the animal kingdom as a master switch of metabolism. The action of OA on insulin release may be directly or indirectly mediated via receptors located on insulin-like peptide producing cells. Recently, it has been shown that all OA and TA receptors are expressed in the CNS, and the immunochemical figures showed that most of them are expressed in the *Pars intercerebralis*, the brain region that contains the IPCs (El-Kholy et al., 2015). In our study, we go further to show that OAMB (OAMB-K3 slice form), OA2, Oct β 2R and TyrR I are actually expressed in PI using conventional RT-PCR. Among them is the OAMB, the first OA receptor that was identified and has been characterized in greater detail. Regarding

many physiological roles of OA, OAMB has been shown to mediate these effects including OA's role on female ovulation, courtship, appetitive, learning and memory (Lee et al., 2009; Zhou et al., 2012; Kim et al., 2013). Recently, activation of OAMB in IPCs generates different effects on dILPs transcription, metabolism and social behavior (Luo et al., 2014). Additionally, effects of OA on sleep:wake behavior are mediated by the cAMP-dependent isoform of the OAMB receptor expressed in IPCs (Crocker et al., 2010). Pathways that control sleep cycles and metabolism are highly related to each other in mammals (Horne, 2009) and in *Drosophila*. In *Drosophila*, two conflicting studies were published: Erion and colleagues showed that sleep-inhibiting effects of OA are not mediated by insulin-like peptides (Erion et al., 2012), in contrast, Cong and colleagues showed that upregulation of dILPs and dInR in the nervous system led to increased sleep (Cong et al., 2015). Our study supports the latter one, because OA-deficient flies have increased sleep behavior and they release more insulin, thus, it is most probable that the effect of OA on sleep is mediated by regulation of insulin, and presumably through the OAMB receptor. Other receptors, such as Oct β 2R and TyrR I, had been shown to be expressed in PI but not in IPCs. Among them, TyrR I is confirmed to be part of DH44-producing cells repertoire; its physiological function is discussed in detail below. The Oct β 1R (OA2) receptor that is present in the PI region has not yet been studied and an in-depth analysis of its physiological significance may promise new functions of OA to various fields.

Alternatively, the effect of OA on insulin secretion may be indirectly controlled by some peripheral organs. To address this, we selectively knocked-down OA or TA receptors in the fat body, oenocytes as well as CNS using RNAi mediated gene-silencing. Strikingly, flies with RNAi mediated knockdown of OA or TA receptor in the fat body but not in oenocytes and CNS show phenotypes in lipid storage, starvation resistance and insulin release consistent with that of in OA deficient flies. These data indicate that OA may act in a FB-specific role in remotely controlling insulin secretion. If this is the case, what is the secreted factor of the FB exerting the function in communicating OA effects to the brain IPCs. Recently, a study found that a leptin-like protein, upd2, is produced in fat body and acts through JAK/STAT

pathway on GABAergic neurons linked to IPCs (Rajan and Perrimon, 2012). Unraveling the interaction between OA and upd2 or other factors may be an interesting task for the future research.

4.2 Starvation-induced gene expression analysis

In this study, I firstly reported that genetic ablation of OA in *D. melanogaster* resulted in increased starvation resistance. Various organic compounds and macromolecules including triglycerides, trehalose, glucose and other sugars, as well as soluble proteins can be used for energy usage to adapt to fasting conditions (Rion and Kawecki, 2007). To better understand the molecular mechanism of how *Drosophila* devoid of OA respond to starvation stress, the global brain mRNA profiles were analyzed using microarray analyses and they were validated using real-time RT-PCR, which is a powerful approach to get insight on gene expression in response to a special stimulus (Yuen et al., 2002; Dallas et al., 2005). In the experimental process, I screened a series of genes whose transcription levels were significantly altered in the CNS of T β H-deficient adults compared to control flies in response to starvation, by using a 2-fold change cut-off. Furthermore, I compared the changes of mRNA profiles involved in energy metabolism after 24 h of starvation. Transcription levels were validated by qRT-PCR for 9 out of 11 selected genes, which shows the high confidence of these data.

The microarray analysis shows that only relatively few genes are regulated in common between the T β H mutant and the control strain in response to starvation. Previous transcriptomic analysis showed that two of the lipase genes, *lip3* and *CG6113*, are upregulated in the whole larvae in response to starvation (Zinke et al., 2002), and two genes (*CG6277* and *CG6271*) encoding triacylglycerol lipases are upregulated in the CNS of starved larvae (Ryuda et al., 2008). However, transcription of these genes is not significantly changed in the experimental condition; this disagreement may be attributable to the difference in tissue samples and the different fly life-stages and also to the different criteria we applied to define upregulated and downregulated genes. Apart from this, there is another gene (*CG31091*), which also encodes a triglyceride lipase that was upregulated upon

starvation in this study, indicative for fat break-down process. The *Drosophila* genome contains over 60 lipase genes (Rubin et al., 2000), implying that fat break-down is a highly complex process.

Another study performed a comparable analysis of starvation responses in the head of Mel6 and TW1 strains and identified 65 upregulated and 48 downregulated genes upon starvation (Fujikawa et al., 2009). Among them, two important genes are also identified in this study. One is *yippee interacting protein 2 (yip2)*, encoding a homolog of mammalian *Ypel* gene family (Hosono et al., 2004). Yippee was identified in a yeast interaction screen that physically interacts with the *Hyalophora cecropia* *Hemolin*, which has a putative zinc-finger protein binding domain and functions as acetyl-CoA C-acyltransferase activity (Roxstrom-Lindquist and Faye, 2001). This gene is upregulated upon starvation in our control strain and in Mel6 and TW1 strains (Fujikawa et al., 2009), however, it is downregulated in the T β H defective strain, providing insights into the roles played by OA in improving the fatty acid beta-oxidation process. Another gene that is commonly found in these two studies is *female specific independent of transformer (fit)*, which shows the highest fold-changes in our study and validates a significant increase of mRNA transcription product in both strains by real time qRT-PCR. This gene belongs to one of four sex-specific genes and is expressed mainly in the fat cells attached to the brain (Fujii and Amrein, 2002). Whereas the previous study showed that *fit* was downregulated under starvation (Fujikawa et al., 2009), the current study showed the opposite, presumably, because the fat body attached to the brain was excluded in the current study. Moreover, another difference was the use of mated females in comparison to virgin females (Lawniczak and Begun, 2004; McGraw et al., 2004).

In responding to the nutrient deprivation, I found many differentially expressed genes involved in amino acid degradation, but clearly, the specific degradation pathway of these amino acid residues are inconsistent between the T β H deficient flies and the matching control strain. Two key enzymes, a cysteine- and a serine-type endopeptidase were upregulated in the control strain but not in T β H defective flies. Interestingly, one gene encoding an asparagine synthase was upregulated in the T β H defective strain. This may indicate that glycolysis and lipolysis are sufficient for

energy supply, whereas proteolysis is not required in the first 24 h of starvation in OA deficient flies.

In addition, expression of many genes encoding metabolic enzymes is changed in response to OA deficiency. A set of genes are implicated to function as protein serine/threonine kinases, which phosphorylate serine and threonine residues and regulate many cellular pathways involved in cell proliferation and apoptosis (Shi, 2009). Three genes, *Pkn*, *Madm* and *CG11746* were upregulated upon starvation. However, another gene encoding protein serine/threonine kinase, *CG7236*, as well as *CG11746*, was downregulated in OA deficient animals. One downregulated gene observed only in T β H defective flies, *rdgA*, encoding an eye-specific diacylglycerol kinase (DGK) (Masai et al., 1993), plays a key role for phototransduction in terminating the light response (Raghu et al., 2000). This is consistent with the observation that octopaminergic neurons project to the optic lobe mediating desensitization or arousal of the visual system (Stern et al., 1995).

By analyzing signaling pathway of interest among the differentially expressed genes in response to starvation in the T β H mutant compared to control strain, I found the Jun-N-terminal Kinase (JNK) pathway was activated in an opposite direction. Two of genes, *bsk* and *MKK4* (1.74-fold upregulated in control strain), were upregulated in control strain upon starvation but downregulated in OA deficient flies. The JNK signaling pathway as an important component of metabolic adaption can be induced by oxidative stress, which represses Insulin/IGF signaling activity and activates the transcription factor FoxO to extend lifespan in worms and mammals (Essers et al., 2004; Oh et al., 2005). Studies in *Drosophila* elucidated that JNK regulates growth and lifespan by inhibition of the expression of the insulin-like peptide 2 (Wang et al., 2005). Here increased JNK signaling activity was observed in the control strain under starved condition, indicating that JNK activation could be induced by starvation. However, this stress-responsive JNK activation is likely to be repressed in the CNS of flies deficient in OA.

4.3 Tyramine controls locomotor activity and circadian rhythms

Biogenic amines are important molecules in the CNS acting as behavioral regulators.

Here, a new role of TA and OA in modulation of the animal's locomotor rhythms was identified. It was demonstrated that decreased levels of TA in Tdc2 mutants (*Tdc2^{RO54}*) showed behavioral arrhythmicity, whereas increased levels in TβH mutants (*TβH^{nM18}*) showed normal rhythmicity, the differences in circadian rhythms are attributable to the lack of TA. Furthermore, we found that one of TA receptors, TyrR I, is expressed in DH44 producing neurons in the PI and affects DH44 secretion. Global knockout or restricted knockdown of TyrR I in the DH44 producing neurons results in behavioral arrhythmicity, increased lipid stores and stress resistance. Consistent with previous findings (Cavanaugh et al., 2014), DH44 is a circadian output molecule that influences circadian activity. The data implies that TA effects on the DH44 signaling pathway is mediated via the TyrR I receptor in order to modulate locomotor rhythms.

Early studies of *per*⁰ mutations showed abnormal circadian rhythms and a decrease in Tdc2 activity, suggesting a clock control of TA and/or OA synthesis (Livingstone and Tempel, 1983). In addition, flies defective for period, clock, cycle and doubletime, but not for timeless, showed defects in cocaine sensitization due to a lack of induction of Tdc2 activity, indicating that these clock genes function as regulators of Tdc2 (Andretic et al., 1999). Recently, a genome-wide screening approach identified a novel rhythmic factor, Tdc2, which is expressed in clock neurons and localized to the PDF populations (Huang et al., 2013). Thus, it will be of interest to evaluate the roles for TA and/or OA in regulating downstream neurons that drive locomotor rhythms. By comparing the line graphs for circadian activity observed from fly defective in TβH and Tdc2, it was possible to distinguish the roles of TA and OA in this context. *Tdc2^{RO54}* flies display reduced rhythmicity, accompanied by increased sleep, whereas *TβH^{nM18}* mutants also have an increase in sleep, but their locomotor rhythm is not altered. Thus, the results suggest that the deficit in behavioral rhythms is attributable to the loss of TA. In fact, these data are consistent with the previous study indicating that rhythmic release of transmitters from Tdc2 neurons is required for normal circadian behavior (Huang et al., 2013). However, it is noticed that *Tdc2^{RO54}* mutants display abnormal behavior rhythms while in particularly dismissed evening peak of activity in the LD condition. As mentioned earlier, circadian clocks are separated two connected clocks in the brain: morning (M) and evening (E) oscillators. The

PDF-positive neurons (s-LNVs) promote morning behavior, whereas the PDF-negative neurons (including LNds and DN1s) are required for the evening behavior in the presence of LD cycles (Grima et al., 2004; Stoleru et al., 2004). Beside these, the s-LNVs are thought to be the most important clock neurons, which functionally generate robust rhythms and are sufficient to mediate free running cycles under DD conditions (Yoshii et al., 2009; Collins et al., 2012). The findings of the current study show that these flies lack evening peak activity in the LD cycle and when transferred to DD condition, they show a uniform increase in rhythmic activity, suggesting that TA influences the E oscillator's activity instead of affecting the pacemaker s-LNV neurons or PDF release. Indeed, there is evidence derived from immunohistochemical studies showing that Tdc2 protein is present in two clusters of clock cells, including l-LNVs and LN_{ds}, as well as a few DN1 cells (Huang et al., 2013). Expression of Tdc2 in clock neurons is consistent with a role for TA in this process, but the mechanisms of how TA modulates locomotor rhythms through influencing the clock neurons is yet unknown.

In *Drosophila* larvae, OA and TA have opposite effects on larval locomotor behavior, in particular larvae have a severe locomotion phenotype when they have reduced OA levels ($T\beta H^{nM18}$ and $Tdc2^{RO54}$ mutants) (Saraswati et al., 2004). In contrast to larvae, adult flies have decreased locomotor activity owing to the reduced level of TA (Hardie et al., 2007). Results in this study show both $T\beta H^{nM18}$ and $Tdc2^{RO54}$ mutants have decreases in locomotor activity, whereas $Tdc2^{RO54}$ flies have more severe activity deficit than $T\beta H^{nM18}$ flies, indicating TA as well as OA play important roles in locomotion but TA is the dominant one, whereas the decrease in locomotor activity of $T\beta H^{nM18}$ mutants is probably due to increases in sleep time. In addition to locomotor activity and sleep, it was found that another behavioral parameter, negative geotaxis, is decreased in both mutants. Because the negative geotaxis assay is performed to analyze how quickly a fly is able to climb vertically, it is not associated with sleep-wake states. Thus, it may allow to elucidate the role that OA has in skeletal muscle control and activity.

TA acts through a set of specific receptors that belonging to the family of GPCR. Three different TA receptors (TyrR I, TyrR II, TyrR III) are present in *Drosophila*. Of

them, only TyrR I (CG7485) has been characterized in detail although various aspects of its physiological relevance are still not understood. Although early pharmacological studies suggested that this receptor has a dual function because OA couples it to increase Ca^{2+} signaling and TA preferentially couples it to inhibit cAMP signaling, recent studies clearly showed that TyrR I is a genuine TA receptor that, upon activation, leads to inhibition of adenylyl cyclase activity (Bayliss et al., 2013). Our recent studies revealed that expression of TyrR I is predominantly present in muscles and the tracheal system as well as in other peripheral organs, whereas in the CNS, strong expression of TyrR I is confined to six cells in the *Pars intercerebralis* (El-Kholy et al., 2015). Regarding the finding that TyrR I is highly expressed in the heart muscle, this receptor is proposed to be the most relevant one for transduction of TA effects on heart performance. Strikingly, I have identified that TyrR I receptor is expressed on all six DH44 neurosecretory cells in the PI and investigated its function by DH44-specific TyrR I inhibition in flies. However, those cells are close to but not the insulin-producing cells. I also confirmed that DH31 cells do not express this receptor. Importantly, recent studies have suggested that DH44, the homolog of corticotropin-releasing factor (CRF), is a circadian output molecule that required for normal locomotor rhythms, showing genetic knockdown of DH44 or selective activation of DH44 producing cells resulted in weakened locomotor rhythms (Cavanaugh et al., 2014). Based on our study, TyrR I mutants and flies with RNAi-mediated knockdown of TyrR I in DH44 neurons displayed weakened rhythmicity and diminished evening peak of activity. In addition, the TyrR I mutant flies also showed a decrease in locomotor behavior. Thus, it is likely that the physiological significance of TyrR I for locomotor rhythms generation in adult flies is mainly mediated by DH44 signaling.

Here it was shown that the DH44 immunostaining is increased in DH44 neurosecretory cell bodies after TyrR I knockdown. This would indicate a reduced DH44 release instead of modulated DH44 production, as the transcript level was not changed. However, in contrast to majority studies of dILPs release from IPCs (Enell et al., 2010; Luo et al., 2012; Rajan and Perrimon, 2012), indirect quantification of DH44 seems not to be as sensitive and efficient. Thus, directly monitoring DH44 levels in

small volumes of hemolymph samples would be necessary in the future to tackle this important question experimentally. Moreover, although DH44 producing cells are adjacent to IPCs, the knowledge where they have their axon terminals is largely unknown.

DH44 signaling is not well understood in *Drosophila*. The experiments showed that lipid levels were increased in TyrR I defective flies as well as in flies where TyrR I was knocked-down in DH44 neurons only. An alternative explanation for the increase in lipid accumulation in animals with altered DH44 levels may be that the reduced locomotor activity or weakened rhythmicity results in reduced energy expenditure. On the other hand, it can't be excluded that TyrR I has alternative effects on lipid storage, as TyrR I is also expressed in peripheral tissues such as the fat body (El-Kholy et al., 2015).

In mammals, glucocorticoids act as stress hormones and as resetting signals for the ubiquitous peripheral clocks, which is enabled by their production and secretion in diurnal cycles (Balsalobre et al., 2000). In *Drosophila*, DH44, as a candidate stress hormone, may be involved in the stress response of the fly. It is shown that its receptor is expressed in corazonin positive cells in the brain (Johnson et al., 2005). Genetic ablation and activation of corazonin neurons lead to altered locomotor behavior in response to stress induction (Zhao et al., 2010). The experiments provide evidence that starvation resistance and osmotic tolerance were improved in flies where TyrR I was diminished in DH44 cells. These phenotypes are most likely attributable to the TyrR I-inhibited DH44 release, which in turn leads to increased lipid accumulation. Furthermore, DH44 is a diuretic hormone, which stimulates fluid secretion rates in *Drosophila*. Notably, treatment with DH44 stimulates fluid secretion mediated via intracellular cAMP signaling in principal cells of the Malpighian tubules (Cabrero et al., 2002). Thus, the hypothesis that DH44 plays a significant physiological role in regulation of circadian rhythms through cAMP signaling awaits testing.

4.4 Oct β 2R regulates ovulation and fertilization

The experiments presented here extend the previous knowledge about the

neurohormonal control of ovulation and fertilization. To maximize successful reproduction, the activities of the ovary, the oviduct, and sperm storage organs have to be highly coordinated with the availability of sperm (Sun and Spradling, 2013). In insects, OA, the invertebrate equivalent of epinephrine and norepinephrine is central to this complex neurohormonal control system (Monastirioti et al., 1996). OA containing neurons in the thoracoabdominal ganglion innervate all major parts of the female reproductive organ, including the ovary, the oviduct and the spermatheca (Monastirioti, 2003; Lange and da Silva, 2007). Its essential role for successful ovulation has already been shown utilizing OA-deficient animals. Moreover, restoring the ability to produce OA in neurons only was sufficient to rescue the egg-laying phenotype. Locally, within the female reproductive organ, the OA receptor OAMB is essential for this process. It is present in the epithelial cells to produce secretions that are required for ovulation upon stimulation with OA (Lee et al., 2003; Lee et al., 2009). In the current project, I showed that the octopaminergic control of egg-laying is more complex than previously anticipated. A second OA receptor, the Oct β 2R is essential for transmitting another component in the well-orchestrated process of ovulation. In contrast to the OAMB receptor, whose activation induces a rise in cytosolic Ca²⁺-levels, Oct β 2R activation leads to an increase of cAMP in all cells expressing this receptor. Increasing the levels of cAMP is known to induce relaxation in vertebrate smooth muscles (Kotlikoff and Kamm, 1996; Tanaka et al., 2005). Activation of β 2-adrenergic receptors in smooth muscles controlling the lumen of arteries or bronchi induces exactly this relaxation. Mechanistically, the link between cAMP level increase and muscle relaxation should involve a Ca²⁺-downregulation leading to an inhibition of Ca²⁺/calmodulin mediated signaling and activity of the myosin light chain kinase or it involves regulation of K⁺-channels such as the MaxiK channel (Kotlikoff and Kamm, 1996; Tanaka et al., 2005). Following the model proposed by Lee et al (Lee et al., 2009), these seemingly contra-productive activities, increasing Ca²⁺-mediated signaling via the OAMB, while the Oct β 2R does the opposite, may nevertheless come together, in the case that both receptors are expressed in different cells within the oviduct system. OAMB is supposed to be expressed in the epithelial cells, where it induces fluid secretion, which is essential

for ovulation. The Oct β 2R on the other hand is expressed in the visceral oviduct muscle, where it induces relaxation via increase in cAMP levels. OA-induced relaxation of the oviduct muscle appears to be mediated via the Oct β 2R. Moreover, it might be operative in all insects, as locusts show a very similar type of OA action on contraction output of the oviduct muscles (Lange and Orchard, 1986; Nykamp and Lange, 2000). The role of Oct β 2R in the control of the female reproductive system is not confined to the oviduct muscles. We observed very high expression levels also in other parts of the female reproductive tract, most notably in the ovaries and, at even higher levels, in the spermatheca. Especially the expression in the spermatheca is of interest, as this might explain the lack of fertilization observed in the few eggs that are laid in the Oct β 2R-deficient animals. In locusts, the OA/TAergic control of the spermatheca is essential for successful fertilization, which may thus also be the case in the fly (Lange and da Silva, 2007).

Thus, these results imply that OAergic neurohormonal signaling orchestrates a very complex processor in the reproductive system that ensures that ovulation and fertilization are well-coordinated. For this, copulation is the primary signal that triggers release of OA from neurons located in the thoracoabdominal ganglion. As mentioned above, this induces contraction/relaxation of the ovary/oviduct muscles mediated via Oct β 2R as well as the release of secretions mediated by the OAMB. Moreover, an OA signal initiated in an identical temporal situation targets the spermatheca. This signal induces contraction of the spermatheca, which expels sperms into the uterus to ensure proper fertilization. The role of the third OA receptor in this organ, the Oct β 3R is completely unknown, although it is expressed in all major parts of the female reproductive system.

Although, OA is obviously of central importance for controlling ovulation and fertilization, other hormonal signals also influence this complex process. These other hormones act at different temporal scales, where they are involved in e.g. maturation of the female reproductive system (Simonet et al., 2004). The role of other hormones directly acting on the oviduct, especial that of the diverse set of peptide hormones is much less understood.

Early studies utilizing mammals revealed a surprisingly high degree of similarities

regarding the architecture of adrenergic control of ovulation. In rats, application of adrenergic agonists was able to increase ovulation in rats, while that of adrenergic antagonists had the opposite effect (Kannisto et al., 1985).

Taken together, the results imply that the Oct β 2R is an essential part of the OAergic control system required to temporarily connect ovulation and fertilization in female *Drosophila*. Moreover, taken the similar signaling properties as β -adrenergic receptors into account, this system might be very useful as a model for smooth muscle control by aminergic compounds in the future.

Summary

The monoamines octopamine (OA) and tyramine (TA), the biological counterparts of epinephrine and norepinephrine in invertebrate, are important neurotransmitters, neuromodulators and neurohormones that are centrally involved in a variety of physiological and behavioral tasks. Although the contributions of OA and TA have been studied for several decades, their promising new insights into various fields are just opening. In this study, I have elucidated the mechanisms that OA and/or TA play in the control of metabolism, circadian rhythms and female ovulation and fertilization in detail. Additionally, starvation-induced metabolic gene expression profiles related to OA regulation were characterized.

Insulin-like peptides (ILPs) act as key effectors regulating lifespan, metabolism, growth, development, reproduction, stress resistance and feeding behaviors in multicellular organisms. In this part, a focus was on the role of OA and TA in controlling production and release of dILPs from IPCs. Flies deficient in OA show phenotypical peculiarities including decreased hemolymph carbohydrate level and food intake, increased body fat and an increased stress resistance. Increasing the release of OA consistently induced exactly the opposite effects indicating that the level of OA regulates various aspects of metabolism in the fly. Compared with flies with altered TA levels, similar phenotypes except a reversed effect on body fat became apparent. Thus, most of the phenotypical peculiarities were caused by OA-mediated effects on release of dILPs.

Experiencing starvation requires to modify a series of cellular circuits to modulate behavior and energy homeostasis including, e.g. fat mobilization. Thus, I performed cDNA microarray analyses to identify gene expression signatures in response to starvation, both in normal as well as in OA-deficient flies. The results implied that starvation induced transcript changes in the brain that are primarily involved in metabolic processes. Including OA-deficient animals in this study revealed that OA plays a central role for orchestrating the starvation-induced alterations on gene expression in the brain.

The fly's locomotor activity assays revealed that TA but not OA is required for normal

rhythmic behavior. Notably, *Tdc2*-deficiency produced a phenotype of arrhythmicity, whereas *TβH*-deficiency had no obvious effect on rhythmic behavior. These effects of TA are mediated via a specific TA receptor, *TyrR I*, which is expressed in six DH44-positive neurons. Controlling the release of DH44 by *TyrR I* mediated signaling is responsible not only for the rhythmicity, but also for a variety of metabolic parameters. Thus, these findings provide a novel pathway how TA regulates rhythmic behavior in *Drosophila*.

OA is known to be essential for ovulation and fertilization in insects. Here I showed that the effects of OA on ovulation are mediated by at least two different OA receptors. In addition to the *OAMB* receptor that is present in the epithelium of the oviduct, the *Octβ2R* receptor is essential for ovulation and fertilization. *Octβ2R* is widely expressed in the female reproductive tract. Most prominent is expression in the oviduct muscle and the spermathecae. Animals deficient in expression of the receptor show a severe egg-laying defect. The corresponding females have a much larger ovary that is caused by egg retention in the ovary. Moreover, the very few laid eggs are not fertilized, indicating problems in the process of sperm delivery. It is assumed that *Octβ2R* acts in a similar way as β 2-adrenoreceptors in smooth muscles, where activation of this receptor induces an increase in cAMP levels that lead to relaxation of the muscle. Taken together, our findings show that octopaminergic control of ovulation and fertilization is more complex than anticipated and that various receptors located in different cells act together to enable a well orchestrated activity of the female reproductive system in response to copulation.

Zusammenfassung

Die Monoamine Octopamin (OA) und Tyramin (TA) fungieren als Neurotransmitter, Neurohormone und Neuromodulatoren und können als funktionelle Äquivalente von Adrenalin und Noradrenalin in Wirbellosen angesehen werden. Sowohl OA als auch TA sind an der Regulation unterschiedlichster Verhaltensweisen und physiologischer Prozesse beteiligt. Obwohl die Bedeutung beider Monoamine seit Jahrzehnten intensiv untersucht wird, sind viele Aspekte ihrer physiologischen Bedeutung bislang kaum bekannt. Im Rahmen dieser Arbeit habe ich die Kontrolle des Metabolismus, der zirkadianen Rhythmen, sowie der die Ovulation eingehend untersucht, die durch OA und TA in der Taufliege *Drosophila melanogaster* erfolgt. Außerdem habe ich mich der Untersuchung Hunger-induzierter Genexpression gewidmet, was speziell auf die Bedeutung des Octopamins für diesen Prozess ausgerichtet war.

Insulin und *Insulin-like peptides* (ILPs) sind von zentraler Bedeutung für die Regulation der Lebensspanne, des Stoffwechsels, des Wachstums und der Entwicklung, der Reproduktion, der Stressantwort sowie des Fressverhaltens in nahezu allen Tieren. Ich habe mich u.A. der Bedeutung von OA und TA für die Kontrolle der Produktion sowie der Freisetzung von *Insulin-like peptides* aus Insulin-produzierenden Zellen gewidmet. Fliegen, die kein OA produzieren können weisen einige Charakteristika auf, zu denen eine verringerte Zuckerkonzentration in der Hämolymphe, eine erhöhte Körperfetttrate sowie eine erhöhte Hungerresistenz gehören. Ektopische Induktion der Octopaminfreisetzung induzierte exakt die gegenläufigen Phänotypen. Fliegen ohne OA und TA zeigten vergleichbare phänotypische Besonderheiten, jedoch zeigten sie keine Veränderung des Körperfettanteils. Das lässt den Schluss zu, dass viele dieser phänotypischen Besonderheiten auf eine Regulation der ILP Freisetzung durch OA erfolgen.

Hunger induziert eine passende physiologische Antwort, die einerseits in einer Veränderung des Verhaltens, andererseits in eine Veränderung des Stoffwechsels mündet. Aus diesem Grund habe ich Transkriptom-Studien durchgeführt, in den Kontroll und OA-defiziente Tiere einer kurzen Hungerperiode ausgesetzt wurden. Die Auswertung der Ergebnisse lässt den Schluss zu, dass OA eine zentrale Rolle bei der

transkriptionellen Antwort des Gehirns spielt.

Die Analyse der Bewegungsaktivität der Fliege zeigte, dass TA, nicht aber OA für eine normale rhythmische Aktivität erforderlich ist. Diese Effekte des Tyramins werden über einen spezifischen Rezeptor, TyrRI vermittelt, der in sechs DH44-produzierenden Zellen des Gehirns exprimiert wird. Die durch TyrRI vermittelte Kontrolle der DH44 Freisetzung reguliert nicht nur die Rhythmik, sondern auch einige metabolische Parameter.

Es ist bekannt, dass OA essentiell für die Ovulation und die Fertilisierung in Insekten ist. Ich konnte im Rahmen dieser Arbeit zeigen, dass die Effekte des Octopamins durch zwei unterschiedliche Rezeptoren vermittelt werden. Zusätzlich zu OAMB, dessen Bedeutung schon bekannt war, ist der Rezeptor oct β 2R essentiell für Ovulation und Fertilisierung. Dieser Rezeptor ist in vielen Bereichen des weiblichen Geschlechtsapparats exprimiert, u. A. im Oviduktmuskel und der Spermatheca. Fliegen, die den Rezeptor nicht exprimieren zeigen *de facto* keine Eiablage, was auf einer Retention der Eier im Ovar beruht. Außerdem erfolgt keinerlei Befruchtung der Eier. Man kann annehmen, dass oct β 2R in gleicher Weise wie β 2-adrenerge Rezeptoren auf glatten Muskelzellen wirkt, wo eine Aktivierung dieser Rezeptoren die cAMP Konzentration erhöht, was zur Muskelrelaxation führt. Zusammengefasst kann gesagt werden, dass die octopaminerge Kontrolle der Eiablage sehr viel komplexer ist als angenommen wurde und dass OA die Aufgabe hat, die verschiedenen Prozesse im Geschlechtsapparat zu koordinieren, die für eine erfolgreiche Befruchtung und Eiablage erforderlich sind.

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Appendices

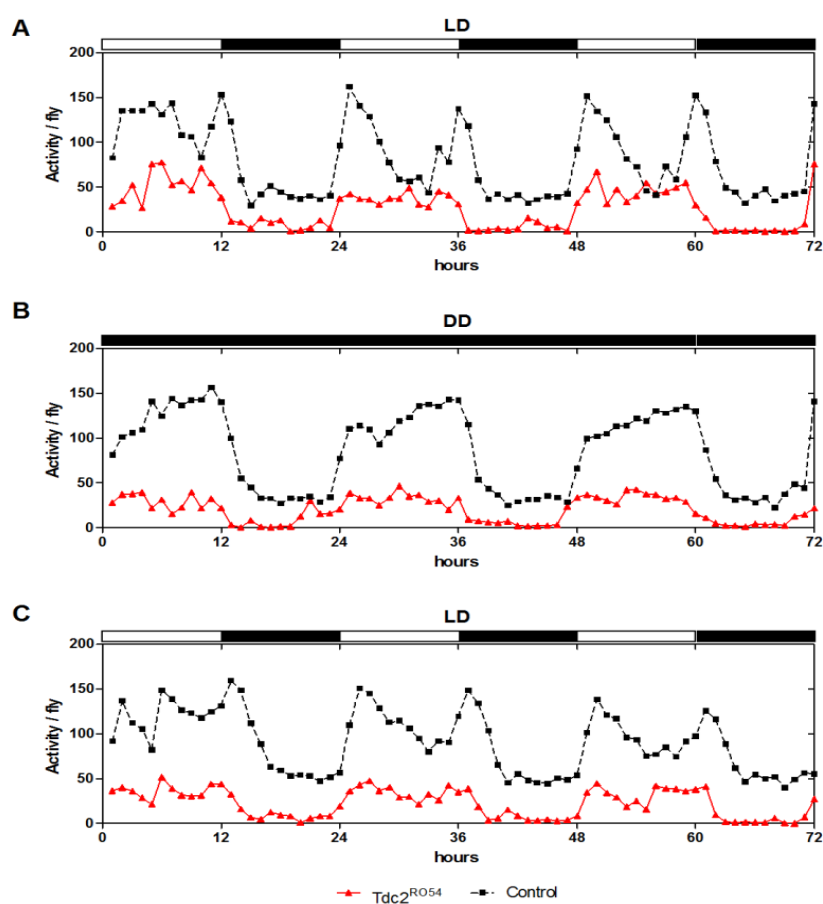


Figure A1. Circadian rhythm activity of *Tdc2*^{RO54} flies in different conditions. Both groups of female flies were firstly entrained to a LD cycle (A), followed by 3 d in DD (B), and then released to LD again (C). The *Tdc2*^{RO54} flies exhibited severely aberrant circadian rhythms in LD or DD conditions, and this arrhythmicity is especially present in suppressed evening peaks before light-off.

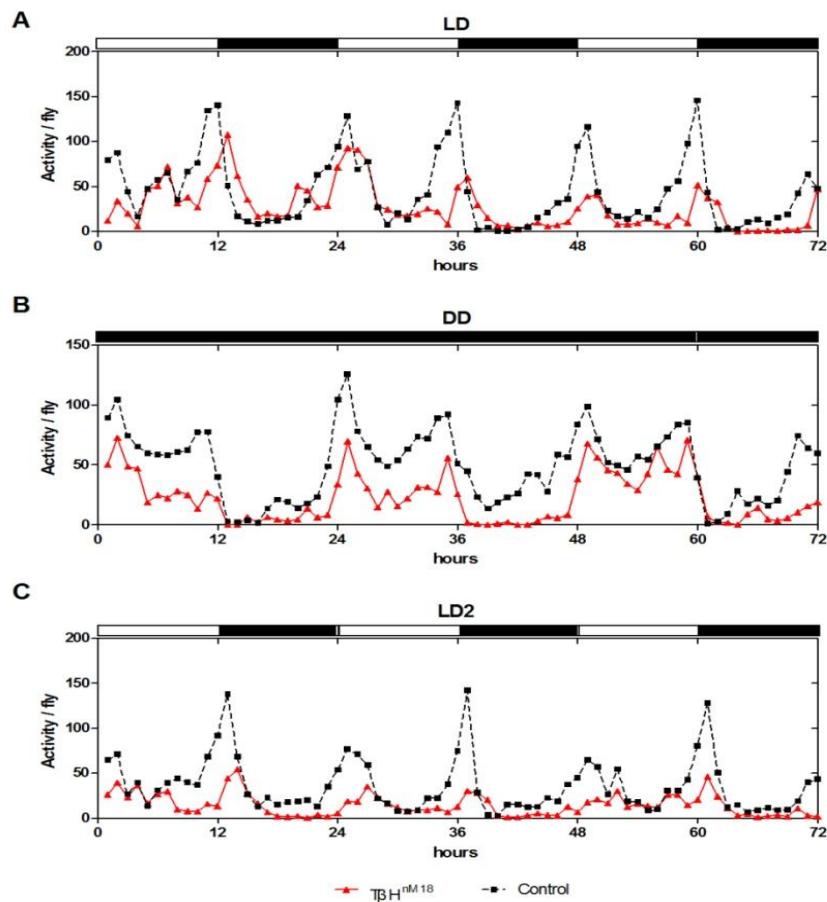


Figure A2. Circadian rhythm activity of $T\beta H^{nM18}$ flies in different conditions. Both groups of female flies were firstly entrained to a LD cycle (A), followed by 3 d in DD (B), and then released to LD again (C). Although the $T\beta H^{nM18}$ flies exhibited defect in locomotor activity, there was a normal circadian rhythm in LD or DD conditions, excepting a slight lower level in evening peak that resemble in $Tdc2^{RO54}$ flies.

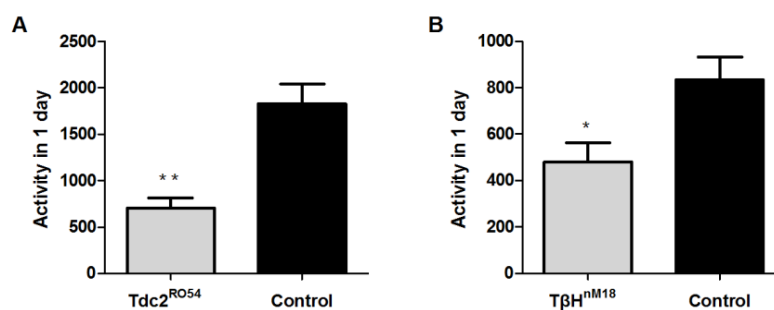


Figure A3. Locomotor activity of $Tdc2^{RO54}$ and $T\beta H^{nM18}$ flies. The histograms show the total activity counts of mutant female flies compared to their matched controls for 1 day in LD

condition. There is a significant reduction of total activity in *Tdc2^{RO54}* flies (A) and in *TβH^{nM18}* flies (B), respectively. Data are presented as mean ± SEM; the unpaired two-tailed Student's t-test was used for analysis, *p<0.05, **p<0.01.

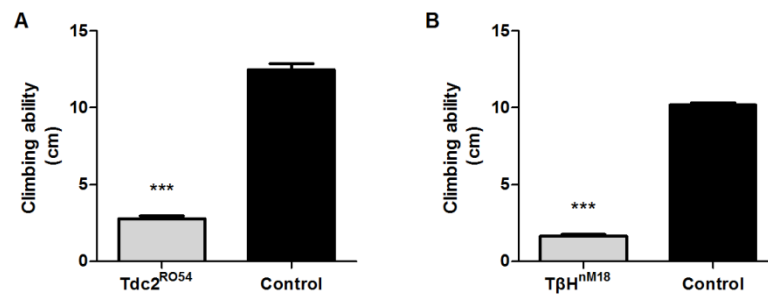


Figure A4. The *Tdc2^{RO54}* and *TβH^{nM18}* flies show defects in climbing ability. (A) The *Tdc2^{RO54}* female flies have severe defect in climbing ability compared with the control flies, with 77.8% decrease in female flies (*Tdc2^{RO54}*, n=100; control, n=89). (B) The *TβH^{nM18}* female flies also have significantly decreased their climbing distance, with 83.9% decrease in female flies (*TβH^{nM18}*, n=396; control, n=391). Data are presented as mean ± SEM; two-ways ANOVA was used for analysis, ***p<0.0001.

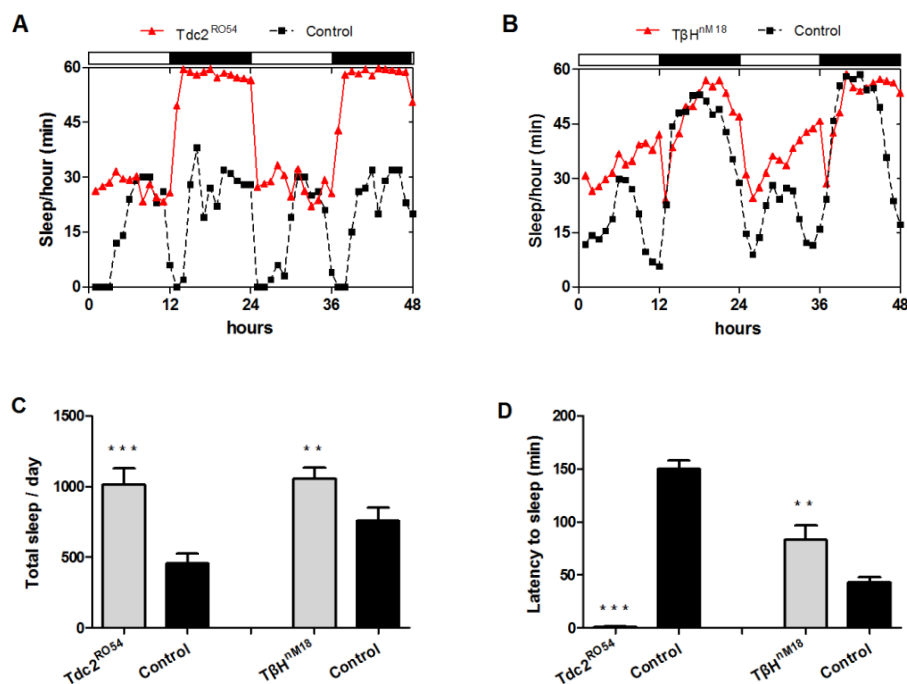


Figure A5. Mutants with reduced OA have increased sleep. (A) Two days of sleep time recording in *Tdc2^{RO54}* female flies. The *Tdc2^{RO54}* line (red, n=8) shows significantly more sleep than its controls (black, n=8). Dark bars and white bars on top indicate nighttime and daytime, respectively. (B) Two days of sleep time recording in *TβH^{nM18}* female flies. The *TβH^{nM18}* line (red, n=8) shows significantly more sleep than its controls (black, n=8). Dark bars and white bars on top indicate nighttime and daytime, respectively. (C) Total sleep is significantly increased in *Tdc2^{RO54}* and *TβH^{nM18}* female flies, with 123% increase in *Tdc2^{RO54}* flies (n=8) and 38.6% increase in *TβH^{nM18}* flies (n=8). (D) Latency to sleep is significantly lower in *Tdc2^{RO54}* female flies (with 98.6% decrease, n=24). However, latency to sleep is significantly increased in *TβH^{nM18}* female flies (with 93.8% increase, n=24). Data are presented as mean ± SEM; two-ways ANOVA was used for analysis, **p<0.001, ***p<0.0001.

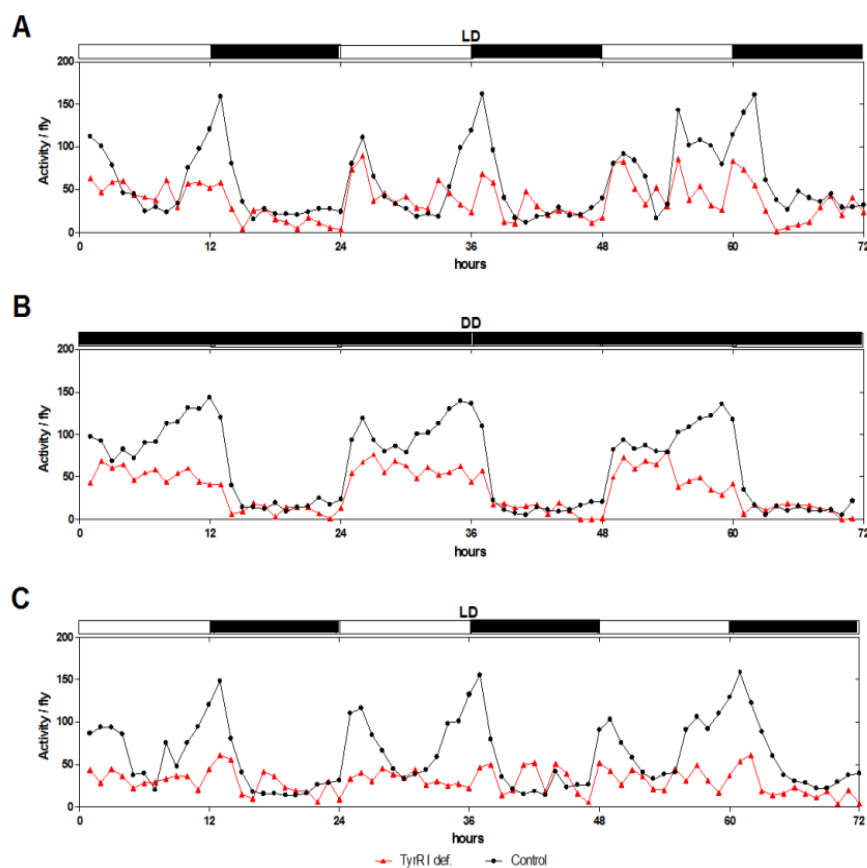


Figure A6. Circadian rhythm activity of TyrR I flies in different conditions. Both groups of female flies were firstly entrained to a LD cycle (A), followed by 3 d in DD (B), and then released to LD again (C). The TyrR I flies exhibited severely aberrant circadian rhythms in LD or DD conditions.

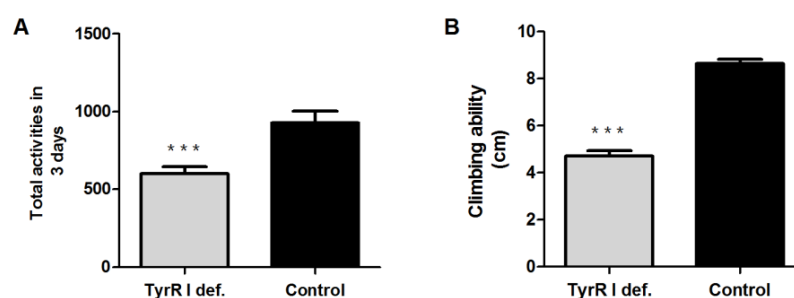


Figure A7. Locomotor activity and climbing ability of TyrR I mutant flies. (A) The histogram shows the total activity counts per TyrR I mutant female flies compared to its matched control for 3 day in LD condition which is significantly decreased of total activity per TyrR I mutant fly (with 35% decrease). (B) The TyrR I mutant flies show defects in climbing ability. The TyrR I mutant female decreased 45.5% of climbing distance relative to control female flies (TyrR I mutant, n=116; control, n=118). Data are presented as mean \pm SEM; the unpaired two-tailed Student's t-test was used for analysis, ***p<0.01.

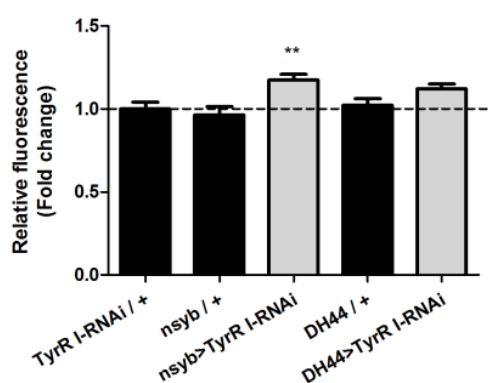


Figure A8. TyrR I knockdown on DH44 neurons affects DH44 secretion. The F1 generation of *nsyb-GAL4>UAS-TyrR I-RNAi* (a) shows significantly higher level of DH44 immunofluorescence than the control lines (*nsyb-GAL4>UAS-TyrR I-RNAi* (a), n=11; *nsyb-GAL4/+*, n=11; *UAS-TyrR I-RNAi/+* (a), n=9). As the same, *DH44-GAL4>UAS-TyrR I-RNAi* (a) also shows significantly higher level of DH44 immunofluorescence than its control lines (*DH44-GAL4>UAS-TyrR I-RNAi*, n=11; *DH44-GAL4/+*, n=11; *UAS-TyrR I-RNAi/+* (a), n=9). Data are presented as mean \pm SEM; one-way ANOVA was used for analysis, **p<0.01.

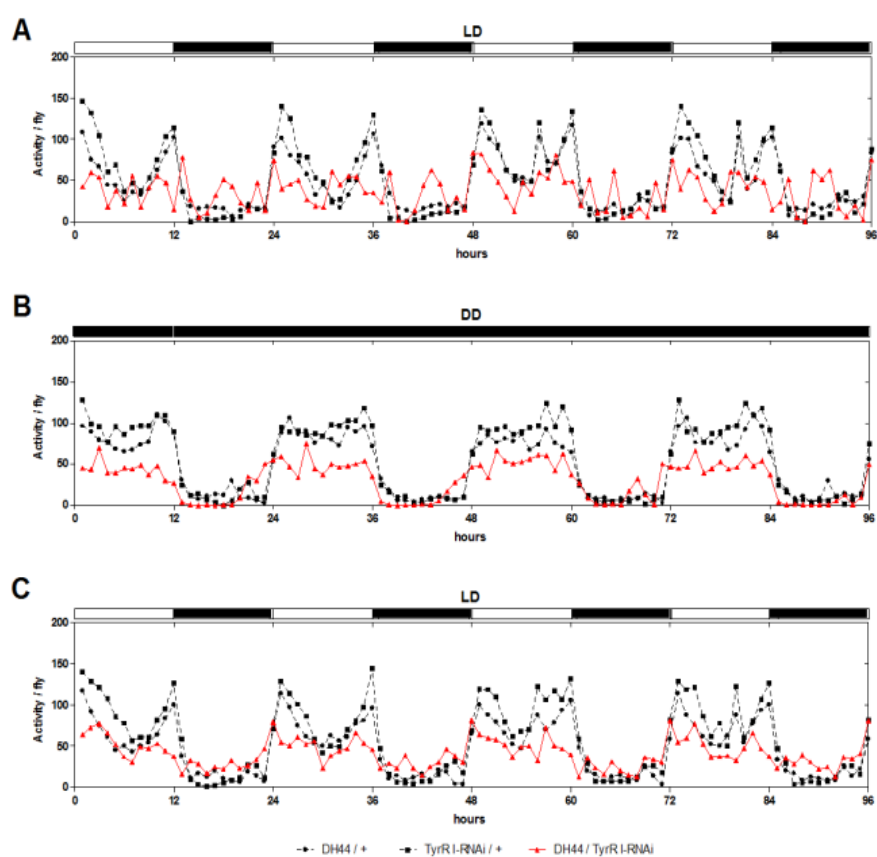


Figure A9. TyrR I knockdown on DH44 neurons show circadian arrhythmicity. Both groups of female flies were firstly entrained to a LD cycle for 4 days (A), followed by 4 days in DD (B), and then released to LD again (C). The flies with diminished TyrR I ($DH44^{FL}-GAL4>UAS-TyrR\ I-RNAi$) exhibited severely aberrant circadian rhythms in different conditions.

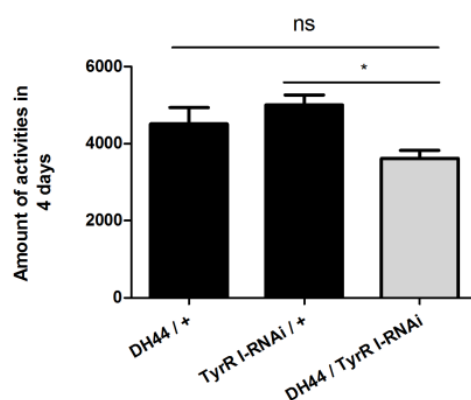


Figure A10. Locomotor activity of flies with diminished TyrR I on DH44 neurons. The histograms show the total activity counts of female flies with diminished TyrR I on DH44 neurons compared to its parental controls for 4 days in LD condition. It shows no significant difference of total activity among them (n=8 for each genotype). Data are presented as mean ± SEM; one-way ANOVA was used for analysis, $p > 0.05$.

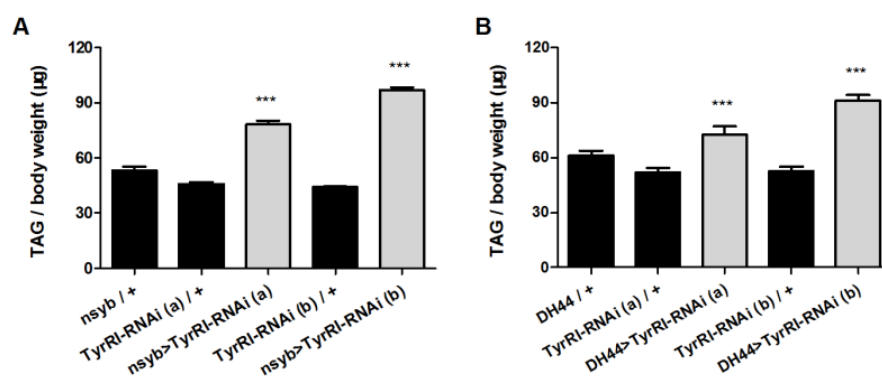


Figure A11. Knockdown TyrR I on DH44 neurons alters fat storage. Total TAG levels were measured in female flies with diminished TyrR I and control flies under normal conditions. (A) The TAG levels are significant higher in female flies with RNAi mediated knockdown TyrR I on DH44 neurons (*nsyb-GAL4 > UAS-TyrR I-RNAi*) than their parental controls (n=8 for each genotype). (B) The TAG levels are also significant higher in male flies with only one RNAi mediated knockdown TyrR I on DH44 neurons (*DH44^{VT}-GAL4 > UAS-TyrR I-RNAi*) than their parental controls (n=16 for each genotype). Data are presented as mean ± SEM; one-way ANOVA was used for analysis, *** $p < 0.0001$.

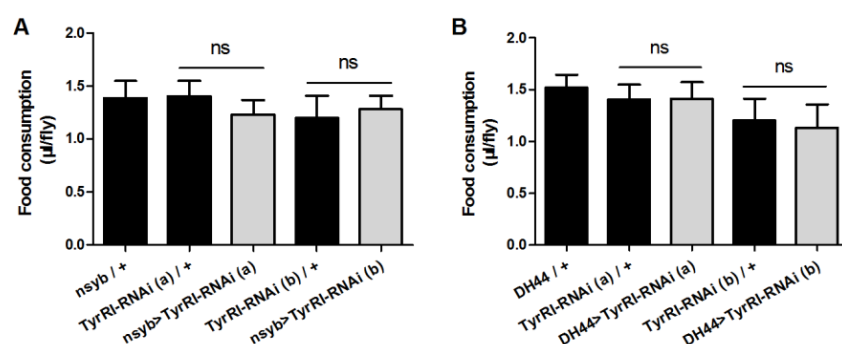


Figure A12. Food consumption is not changed in flies with diminished TyrR I expression. (A) CAFE assay was performed in female flies with RNAi mediated knockdown TyrR I and controls. There was no significant difference in liquid food consumption among flies with diminished TyrR I in brain (*nsyb-GAL4 > UAS-TyrR I-RNAi*, $n=8$ for each genotype). (B) The food consumption also doesn't show significant difference in female flies with diminished TyrR I on DH44 neurons (*DH44^{VT}-GAL4 > UAS-TyrR I-RNAi*, $n=7$ for each genotype). All data are presented as mean \pm SEM. one-way ANOVA was used for analysis, $p>0.05$.

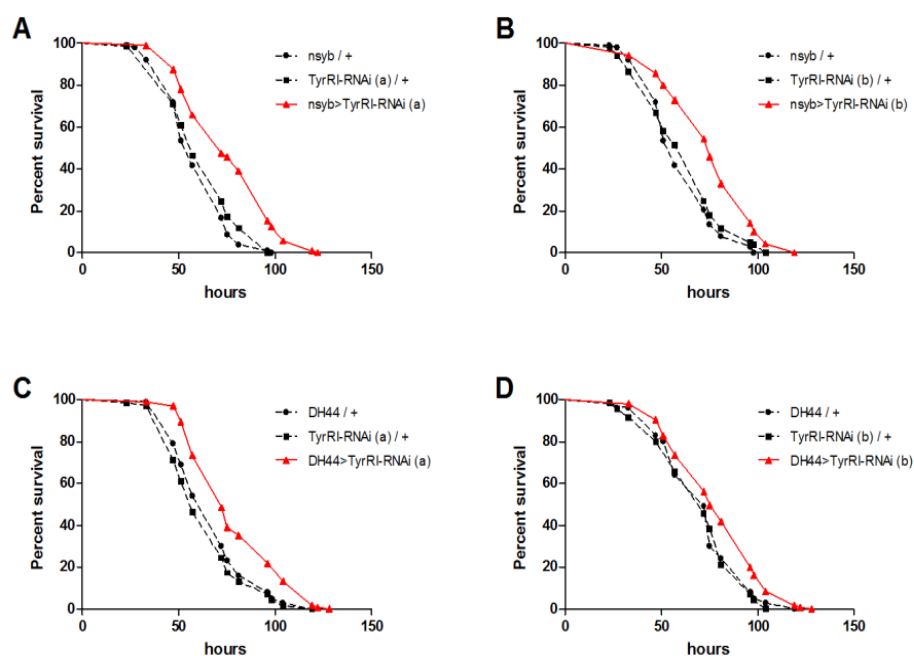


Figure A13. Flies with diminished TyrR I show starvation resistance. (A-B) Under starved conditions, female flies with knockdown of TyrR I in the brain show significantly extended their lifespan compared to parental control flies. The median survivals of each genotype are 72 h for

nsyb>TyrR I-RNAi (a) (n=105), 57 h for *nsyb-Gal4/+* (n=103), 72 h for *UAS-TyrR I-RNAi* (a) (n=138) and 72 h for *nsyb>TyrR I-RNAi* (b) (n=140), 57 h for *UAS-TyrR I-RNAi* (b) (n=105). (C-D) The female flies with knockdown of TyrR I in DH44 neurons also significantly extended their longevity compared to parental control flies. The median survivals of each genotype are 72 h for *DH44>TyrR I-RNAi* (a) (n=105), 62 h for *DH44-Gal4/+* (n=100) and 75 h for *DH44>TyrR I-RNAi* (b) (n=105). Log-rank test for survival analysis showed significant difference, $p < 0.001$.

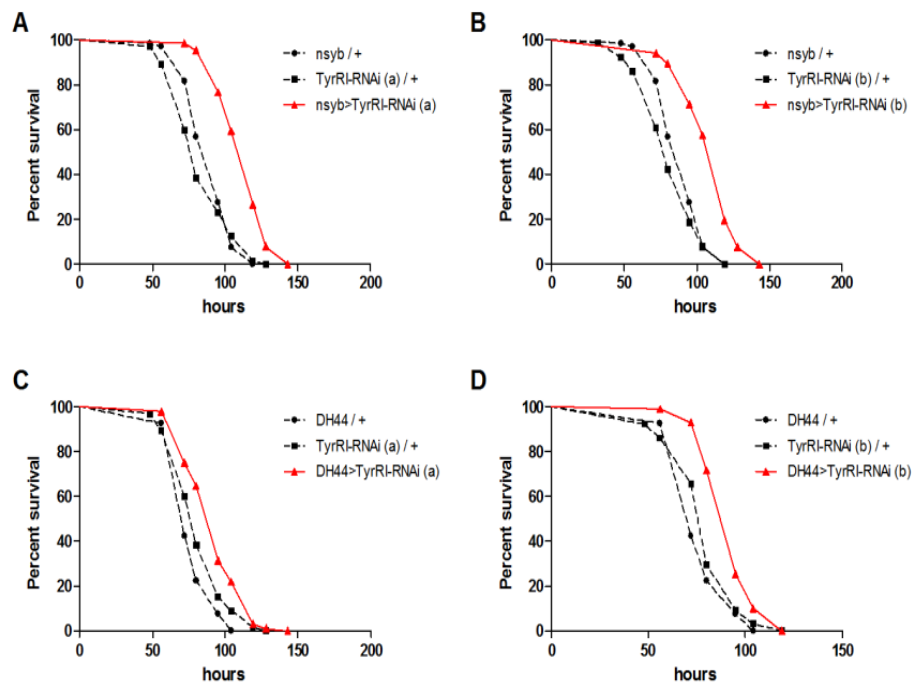


Figure A14. Flies with diminished TyrR I show increased osmotic resistance. (A-B) Under starved conditions, the female flies with knockdown of TyrR I in brain significantly extended their lifespan compared to parental control flies. The median survivals of each genotype are 119 h for *nsyb>TyrR I-RNAi* (a) (n=128), 95 h for *nsyb-Gal4/+* (n=130), 80 h for *UAS-TyrR I-RNAi* (a) (n=130) and 119 h for *nsyb>TyrR I-RNAi* (b) (n=132), 80 h for *UAS-TyrR I-RNAi* (b) (n=128). (C-D) The female flies with knockdown of TyrR I in DH44 neurons also significantly extended their longevity compared to parental control flies. The median survivals of each genotype are 95 h for *DH44>TyrR I-RNAi* (a) (n=96), 72 h for *DH44-Gal4/+* (n=80) and 95 h for *DH44>TyrR I-RNAi* (b) (n=99). Log-rank test for survival analysis showed significant difference, $p < 0.001$.

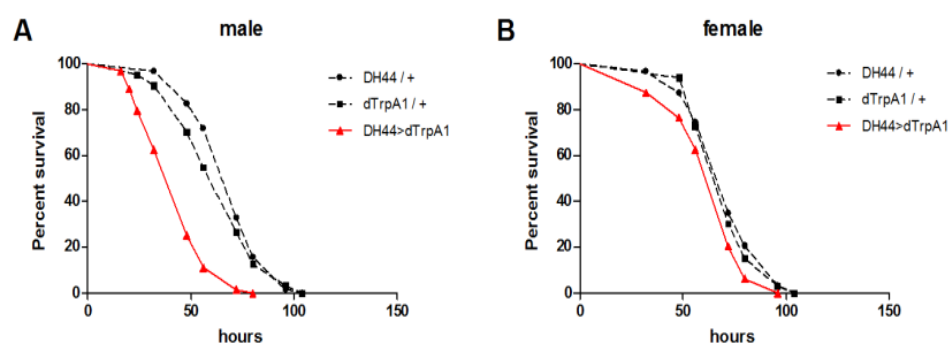


Figure A15. Activation of DH44 neurons shows sensitive to osmotic stress. The experiments were carried out at 29°C. *DH44>dTrpA1* males (A) and females (B) lived longer than both control males and females respectively, in males the median survival is 48 h for *DH44>dTrpA1* (n=64), 72 h for *DH44/+* (n=64) and 72 h for *UAS-dTrpA1/+* (n=64), and in females the median survival time is 62 for *DH44>dTrpA1* (n=64), 72 h for *DH44/+* (n=63) and 72 h for *UAS-dTrpA1/+* (n=66),. Log-rank test for survival analysis showed that $p < 0.001$ in both sexes.

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Publications

1. **Li Y**, Fink C, El-Kholy S, Roeder T. The octopamine receptor Oct β 2R is essential for ovulation and fertilization in the fruit fly *Drosophila melanogaster*. *Archives of insect biochemistry and physiology* 2015; 88(3): 168-178.
2. **Li Y**, Hoffmann J, Stephano F, Fink C, Roeder T. An ancient hormonal system regulates behavior and metabolism in invertebrates. (submitted)
3. Hoffmann J, Romey R, Fink C, **Li Y**, Roeder T. Overexpression of Sir2 in the adult fat body is sufficient to extend lifespan of male and female *Drosophila*. *Aging* 2013; 5(4): 315-327.
4. El-Kholy S, Stephano F, **Li Y**, Bhandari A, Fink C, Roeder T. Expression analysis of octopamine and tyramine receptors in *Drosophila*. *Cell Tissue Research* 2015 Mar, 7.

Declaration

I, Yong Li, here declare that my doctoral thesis entitled “Adrenergic signaling in the fruit fly *Drosophila melanogaster*: establishment and analysis of models for obesity and diabetes” and the work presented in it are my own work apart from supervisor’s guidance and design of the paper. This dissertation has not been submitted for the award of doctoral degree to another examining body and was prepared according to the Rules of Good Scientific Practice of the German Research Foundation.

Erklärung

Hiermit erkläre ich, Yong Li, dass diese Dissertation mit dem Titel:” Adrenergic signaling in the fruit fly *Drosophila melanogaster*: establishment and analysis of models for obesity and diabetes”, sowie die darin beschriebenen Arbeiten, abgesehen von der Beratung durch meinen akademischen Lehrer, nach Inhalt und Form meine eigene Arbeit ist. Diese Arbeit wurde weder im Ganzen noch zum Teil an anderer Stelle im Rahmen eines Promotionsvorhabens eingereicht. Sie wurde nach den Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft angefertigt.

Kiel,

Yong Li