The Molecular Mechanism of Aggression and Feeding Behaviour in *Drosophila melanogaster*

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Abstract

Obesity is a complex disorder which has become a growing health concern. Twin studies have demonstrated a strong genetic component to the development of obesity and genome wide association studies have identified several genetic loci associated with it. However, most of these loci are still poorly understood in a functional context. Interestingly, many of the hormones and neurobiological messengers responsible for regulating feeding behaviour and metabolism are also linked to controlling aggression, but it is still not understood how they interact to maintain metabolic homeostasis. In this thesis, the model organism Drosophila melanogaster was employed to dissect the molecular mechanisms of the genetic cascades regulating aggressive behaviour and metabolic homeostasis.

In paper I and II, the role of transcription factor AP-2 (TfAP-2) and Tiwaz Twz, Drosophila homologues of two human obesity-linked genes were investigated in aggression and feeding behaviour. Paper I demonstrated that TfAP-2 and Twz genetically interact in octopaminergic neurons to modulate male aggression by controlling the expression of genes necessary for octopamine (fly analogue of noradrenaline) production and secretion. Moreover, it was revealed that octopamine in turn regulates aggression through the Drosophila cholecystokinin (CCK) satiation hormone homologue Drosulfakinin (Dsk). Paper II revealed that TfAP-2 and Twz also initiate feeding through regulation of octopamine production and secretion. Octopamine then induces Dsk expression leading to inhibition of feeding.

Paper III established that the activity of the small GTPase Ras-related C3 botulinum toxin substrate 2 (Rac2) is required in Drosophila for the proper regulation of metabolic homeostasis, as well as overt behaviours. Rac2 mutants were starvation susceptible, had less lipids and exhibited disrupted feeding behaviour. Moreover, they displayed aberrant aggression and courtship behaviour towards conspecifics.

Paper IV studied Protein kinase D (PKD), the homologue of a third obesity-linked gene PRKD1, and another kinase Stretchin-Mlck (Strn-Mlck). Reducing PKD transcript levels in the insulin producing cells led to flies with increased starvation susceptibility, decreased levels of lipids and diminished insulin signalling compared to controls. Reduced Strn-Mlck expression resulted in a starvation phenotype and slight reduction in insulin signalling and lipid content. These findings imply a function for PKD and Strn-Mlck in insulin release.

Keywords: Drosophila, aggression, obesity, homeostasis
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


IV **Goergen P**, Khan Z, Akber M, Schiöth HB, Williams MJ. Two serine/threonine kinases Protein kinase D and Stretchin-Mlck are necessary for the secretion of brain derived insulin-like peptides in *Drosophila*. Manuscript

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Additional Publications


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Abbreviations

BMI   body mass index
CAFE  capillary feeding
CCK   cholecystokinin
CNS   central nervous system
DAG   diacylglycerol
Dsk   drosulfakinin
FTO   fat mass and obesity associated
GABA  $\gamma$-aminobutyric acid
GPCRs G protein coupled receptors
Gr32a gustatory receptor 32a
GWAS genome wide association studies
HIF   high intensity fighting
HPA   hypothalamic-pituitary-adrenal
ILP   insulin-like peptide
IPC   insulin producing cell
KCTD  potassium channel tetramerization domain
LIF   low intensity fighting
MARCM mosaic analysis with a repressible cell marker
MLCK  myosin light chain kinase
PH    plekstrin homology
PI    pars intercerebralis
PKD   protein kinase D
PLA   proximity ligation assay
Rac2  ras-related C3 botulinum toxin substrate 2
SOG   subesophageal ganglion
Strn-Mlck stretchin-mlck
TbH   tyramine $\beta$ hydroxylase
TfAP-2 transcription factor AP-2
Twz   tiwaz
Vmat  vesicular monoamine transporter
WHO   world health organization
Introduction

Obesity is a chronic metabolic disease and has become a growing health concern in the 21st century. The world health organization (WHO) estimated in 2008 that over 1.4 billion adults are overweight and that one third of these men and women are considered clinically obese. These proportions have become so high over the last years that obesity is now classified as a global epidemic. Individuals with a high body mass have an increased risk to develop cardiovascular diseases, cancer and type 2 diabetes inter alia. The increase in obesity can certainly be partly attributed to an increased intake in energy dense food and a general reduction in activity, common to a western lifestyle. However, twin studies and family association studies showed that the genetic blueprint of an individual also plays an important role in determining if an individual is likely to get obese. It is even suggested that genetic factors explain about 65% of the variance in body mass index (BMI). The BMI is derived from the height and weight of an individual and is the most commonly used measure to estimate body size. It is easily calculated and although it does not differ between fat and muscle it is a good proxy-marker for obesity. Obesity is commonly not caused by a mutation in a single gene but triggered through variations in multiple genes. However, rare cases of monogenic obesity have been reported. One example is leptin, a hormone secreted by the adipose tissue, where deficiencies of it lead to severe obesity.

Starting in the late 90s more and more human genetic studies were published that tried to identify genes associated with obesity. These early studies had low resolution compared to modern methods and also, due to their low replicate numbers, were rarely replicable. Over the years, the technology has developed: modern microarrays can now genotype several million genetic variants and genome-wide association studies (GWAS) became very important to the discovery of possible novel genes involved in obesity. GWA studies, however, only identify genomic regions associated with a disease, not individual genes, and the possibility exists that the linked genes do not have a causative effect on the BMI. Of the 32 loci identified by Speliotes et al. only 15 contain genes that could be biologically linked to obesity. The first gene to come out of a GWAS for BMI was a gene termed the fat mass and obesity associated gene or short FTO gene. FTO has been the focus of intense research but its molecular mechanism is still not fully understood. A further 36 possible obesity linked genes have been identified since.
Studies trying to decipher the role of these genes commonly used vertebrates such as rodents, frogs and fish as animal models and these models have greatly increased our knowledge of human biology and physiology. However, they are all, both genetically and metabolically, complex, making it difficult to study the effects of a single gene and to provide understanding of the fundamental processes. Interestingly, a majority of the obesity-linked genes have orthologues in invertebrate models such as *Drosophila melanogaster*. Flies are genetically simpler and have less redundancy than vertebrates. There are nevertheless remarkable similarities between the metabolic pathways and organs regulating energy balance in *Drosophila* and vertebrates (Table 1.). For example, flies have a hypothalamus-like structure known as the *Pars intercerebralis* (PI). Similar to the hypothalamus, it has a very heterogeneous configuration that is composed of many different cell types, including hormone producing neuroendocrine cells. The PI also produces insulin-like peptides (ILPs) and hence fulfils the role of the mammalian β-pancreatic cells. These neurosecretory cells from the PI innervate two other glands, the corpora cardiaca and the corpus allatum, and together they may form the fly version of the mammalian hypothalamic-pituitary-adrenal axis, linking the nervous system to the endocrine system. The corpora cardiaca is both the *Drosophila* equivalent of the pituitary gland and α-pancreatic cells, producing adipokinetic hormone (*Drosophila* glucagon analogue). The midgut fulfils the function of the stomach and intestine to digest and absorb food. The metabolic and storage function of the mammalian liver is shared between two organs in *Drosophila*, the fat body and the oenocytes. Carbohydrate and lipid storage, including glycogen and triglycerides, is performed mainly by the fat body. Similar to mammalian adipocytes, the fat body cells contain lipid droplets and even use many of the same enzymes to regulate glycogen synthesis and breakdown as mammals. Oenocytes fulfil the function of lipometabolism and, finally, the malphighian tubules function as the counterpart of the vertebrate kidneys.

All these organs assimilate information obtained from the environment, as well as monitor the internal status, to coordinate proper physiological activities to maintain energy homeostasis. Thus, not only are many metabolic pathways conserved, the regulation of energy homeostasis requires interplay between metabolically active tissues, reminiscent of the metabolic regulation in mammals, making *Drosophila* an ideal model organism to study the molecular mechanisms regulating homeostasis and unravel the function of the obesity-linked genes.
Table 1. **Comparison between tissues regulating energy balance in humans and Drosophila.**

<table>
<thead>
<tr>
<th>Function</th>
<th>Human</th>
<th>Drosophila</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin/insulin-like peptide production</td>
<td>β pancreatic cells</td>
<td>Pars intercerebralis</td>
</tr>
<tr>
<td>Glucagon/adipokinetic hormone production</td>
<td>α pancreatic cells</td>
<td>Corpora cardiaca</td>
</tr>
<tr>
<td>Hormone secretion</td>
<td>Pituitary gland</td>
<td>Corpora cardiaca</td>
</tr>
<tr>
<td>Excretory and osmoregulatory system</td>
<td>Kidney</td>
<td>Malphigian tubules</td>
</tr>
<tr>
<td>Digestion and absorption of food</td>
<td>Stomach &amp; intestine</td>
<td>Midgut</td>
</tr>
<tr>
<td>Carbohydrate and lipid storage</td>
<td>Liver</td>
<td>Fat body</td>
</tr>
<tr>
<td>Lipometabolism</td>
<td>Liver</td>
<td>Oenocytes</td>
</tr>
<tr>
<td>Corticoids &amp; androgens/juvenile hormone produc</td>
<td>Andrenal gland/gonad</td>
<td>Corpus allatum</td>
</tr>
</tbody>
</table>

*Drosophila* not only shares important basic functions with vertebrates, but also has further advantages. Firstly, compared to mammalian model systems, it has a short generation time and a low maintenance cost. The *Drosophila* generation time is about two weeks with a life span of about two months. This allows large-scale crosses to be followed through several generations in a matter of months and at a fraction of the cost compared to the vertebrate models. Secondly, *Drosophila* has been a model organism over a century now and mutant flies with defects in any of several thousand genes are readily available. Thirdly, it is possible to phenotypically analyse with ease the effects of altered gene expression, either loss of expression or misexpression, and thus deduce the functions of different gene products26. Finally, compared to vertebrate model organisms, *Drosophila* has far fewer ethical concerns associated with *in vivo* studies.

Considering the similarities to the mammalian system and the advantages over other model organisms, research on fly metabolism has the possibility to provide insights with high clinical relevance for humans.

**Aggression and Metabolism**

Aggression is a complex behaviour affected by genetic, physiological and environmental factors. It is also widely conserved throughout the animal kingdom as it is an important behavioural trait offering the winner food resources, mating opportunities, and territory. However, aggression is a costly behaviour, requiring more energy, increasing the likelihood of injury and increasing the chances of predation. Somehow these costs need to be balanced with the benefits for an animal to function properly and maintain en-
ergetic homeostasis. This can be accomplished by both adjusting outward behaviours, as well as regulating internal metabolics. Failure to correctly regulate homeostasis can lead to diseases and ultimately death.

The decision to be aggressive is at least partially determined by systems known to control metabolism, such as the monoamine system\textsuperscript{27-30}. Actually, these pathways may have evolved in order to maintain homeostasis during energy-limiting conditions. As evidence in support of this theory, the hydra (\textit{Hydra japonica} and \textit{Hydra vulagaris}), with its simple neural net, is able to react to the monoamines dopamine and noradrenaline to regulate feeding behaviour\textsuperscript{31,32}.

During evolution these same pathways could have assumed more complicated roles, such as regulating overt behaviours, while still maintaining their original function of regulating metabolic homeostasis. In humans, continued disruption of the homeostatic system can lead to metabolic syndrome, including obesity and type 2 diabetes. A current hypothesis about the evolutionary basis for metabolic syndrome postulates that modern societies have switched from aggressive to more non-aggressive behaviours, leading to the accompanying metabolic changes\textsuperscript{33}. They argue that the molecular machinery responsible for the control of aggression, for example, induces insulin resistance. Acquiring insulin resistance allows the organism to shift the allocation of energy from the muscles to the brain and enables a physically submissive but socially smart lifestyle. Furthermore, the investment into wound healing is reduced since the occurrence of injury should be less likely in a non-aggressive individual. In other words, this theory predicts that the choice to be aggressive or passive will affect an animal’s physiology. In vertebrates, less aggressive individuals typically have low testosterone, high plasma cholesterol and elevated serotonin signalling, and more aggressive animals have higher levels of testosterone, low brain serotonin and lower plasma cholesterol\textsuperscript{33-35}. In fact, chronically elevated serotonin signalling in the hypothalamus has been shown to induce peripheral insulin resistance\textsuperscript{36,37}. Interestingly, preliminary trials of behavioural intervention indicate that games and exercises involving physical aggression can reduce systemic inflammation and improve glycemic control\textsuperscript{33}.

The transition in allocation of energy and immune response that is typical of the so-called soldier (aggressive) to diplomat (non-aggressive) transition did therefore evolve as a natural adaptive response. The modern human lifestyle, however, is characterized by extremely low aggression and reduced injury-proneness. This extremism may have pushed this natural body response, soldier-to-diplomat transition, to the utmost and turned it pathological. Therefore, it is of great interest to study the relationship between aggression and energetics.
Aggression in *Drosophila*

Aggressive behaviour in *Drosophila melanogaster* was first documented by Alfred H. Sturtevant nearly a hundred years ago\(^3\), but only in the last two decades have the phenotypic behaviours associated with aggressive behaviour\(^3\), as well as various hormones, pheromones and neurotransmitters that modulate it\(^4,41\), been described.

Aggression between *Drosophila* males is composed of behavioural modules, which were described by the research team of Edward Kravitz\(^3\), and aggressive interactions can be divided into high intensity fighting (HIF) and low intensity fighting (LIF). Most of the aggression encounters are LIF, while the HIF are rare events. LIF is characterized by shoving (side-by-side pushing with a leg) and wind flicks (quick wing flicking). Lunging (one fly throws himself on the opponent), fencing (boxing face-to-face with the two front legs), wing threat (holding the wings at a 30-45° angle) and chasing (pursuing one another) make up the HIF repertoire. The modules can follow as long interaction with increasing intensity or each can appear on their own as an individual event. Although most research focuses on male aggression, aggressive interaction between female flies exists as well, but they differ both in the behavioural patterns displayed and the motivational drive. For example, females head-butt their opponents, a behaviour not seen in males\(^42\).

The role of octopamine in Aggression

Octopamine was first discovered over sixty years ago in the salivary glands of the Octopus (*Octopus vulgaris*)\(^43\). Octopamine is structurally related to Noradrenaline and acts as a neurohormone, a neurotransmitter and a neuromodulator\(^44\). Together with tyramine, octopamine composes the invertebrate counterpart of the vertebrate adrenergic transmitters Noradrenaline and Adrenaline. Noradrenaline is best known to be a key modulator in mammalian aggression and comparably octopamine affects aggression in invertebrates\(^44\). Tyramine and octopamine have been the most extensively studied in the context of invertebrate aggressive behaviour. The effects of octopamine on the aggressiveness of invertebrates are species specific\(^45\). In crustaceans, for example, injection of octopamine caused crabs to take a submissive-looking posture\(^46,47\) and Pedetta et al. (2010)\(^45\) showed that octopamine injection decreased aggressiveness in the crab *Chasmagnathus*. On the other hand, increasing octopamine levels in honeybees and crickets lead to a correlating increase in aggressive behaviour. As for *Drosophila*, using automated video analysis, it was shown that flies lacking octopamine performed significantly fewer lunges than controls\(^28\). Further studies by Baier et al. (2002)\(^30\) and Zhou et al. (2008)\(^30\), who also worked with octopamine mutant flies, reported a decrease in aggressiveness, reinforcing the role of octopamine as
a key modulator in insect aggression. In an adult *Drosophila*, the brain has approximately 100 octopaminergic neurons, which are involved in the initiation and maintenance of complex behaviours and general internal states. However, it was shown that only a distinct subset of octopaminergic neurons in the subesophageal ganglion (SOG) is necessary for a proper aggressive response. Although, octopamine has been extensively studied in the context of aggression in *Drosophila*, to this day only one downstream target of it have been determined.

**Transcription factor AP-2 and Tiwaz**

Multiple GWA studies have identified loci near the transcription factor activator enhancer binding protein family member TFAP2B to be associated with BMI, central obesity and fat distribution. Members of this family are sequence specific DNA binding proteins that play a role in the regulation of transcription, either as transcriptional activators or repressors. The AP-2 members can either form homodimers or act as a heterodimer with other family members. AP-2 proteins are composed of a highly conserved helix-span-helix dimerization motif at the C-terminal, a central basic region and a less conserved region at the N-terminal. In humans and mice, five members of the transcription factor AP-2 family exist (TFAP2A-E in humans, Tcfap2a-e, in mice respectively). The related proteins are all translated from different genes and they are key regulators of various developmental processes. Interestingly, TFAP2 family members seem to be important in the regulation of monoamines. AP2α (encoded by TFAP2A) and AP2β (TFAP2B) have been positively correlated to several monoamines in rats, notably AP2β had a high correlation with dopamine and serotonin turnover in the rat forebrain. Furthermore, AP2α coexpresses with tyrosine hydroxylase in both noradrenergic and adrenergic neurons in the mouse brain and AP2 binding sites in the upstream regions of both tyrosine hydroxylase and dopamine β hydroxylase have been identified. Null mice for the gene Tcfap2b have reduced levels of noradrenaline due to improper development of the noradrenergic neurons in the peripheral and central nervous system (CNS).

In *Drosophila*, the TFAP2 family is represented by the homologue TfAP-2. During embryogenesis it is expressed in the maxillary segment and neural structures. During larval development, it is expressed in CNS as well as leg, antennal and labial imaginal disks and highly expressed in the adult *Drosophila* CNS. Mutation of TfAP-2 causes defects in proboscis development and leg joint formation.

How TFAP2 family members are regulated post-transcriptionally is just beginning to be understood and is the focus of intense research. One candidate for regulating TFAP2B function is KCTD15 (Tiwaz in *Drosophila*), a
potassium channel tetramerization domain-containing protein. Similar to TFAP2B, KCTD15 emerged as a possible obesity-linked gene in multiple GWAS\textsuperscript{51-53}. Common for all KCTD members is a Broad complex, Tramtrack and Bric-a-brac domain, which is a versatile protein-protein interaction motif enabling homo- and heterodimerization. The human KCTD family consists of 26 members which have a range of functional roles. For example, roles in GABA signalling\textsuperscript{23,74} or in the developmentally important FGF pathway\textsuperscript{75} have been attributed to its members. Studies in zebrafish and frog embryos determined that Kctd15 inhibits neural crest induction by acting on the Wnt pathway\textsuperscript{76}. Interestingly, hedgehog, a Wnt responsive gene, is a factor in Drosophila adipogenesis and plays an important role in determining the fate of brown and white adipose cells in mice\textsuperscript{77}. In zebrafish, Kctd15 inhibits the activity of the AP-2β parologue AP-2α\textsuperscript{76,78}. Furthermore, KCTD1, a vertebrate KCTD15 parologue, was also shown to be necessary for AP-2α sumoylation, pointing to a possible role for the interaction between Kctd15 and Ap-2α in zebrafish\textsuperscript{89}. Moreover, preliminary evidence from a yeast two-hybrid screen revealed that in Drosophila, TfAP-2 associates with Tiwaz (Twz) and, similar to TfAP-2, Twz is highly expressed in the CNS\textsuperscript{80}. Thus, both genes may interact in Drosophila to regulate octopamine signalling and metabolic homeostasis.

Crocker \textit{et al.} (2010)\textsuperscript{81} used the mosaic analysis with a repressible cell marker (MARCM) method to determine that octopaminergic neurons innervate the insulin producing cells (IPCs). The IPCs are situated in the PI, the fly’s endocrinological equivalent of the mammalian hypothalamus. As the name suggests, the IPCs produce ILPs that are involved in energy metabolism\textsuperscript{20}, which is in it-self interesting as the human homologues of TfAP-2 and Twz have been identified as risk loci for obesity in several GWAS\textsuperscript{80,82-84}. IPCs also produce Drosulfakinin (Dsk), Dsk was shown to be necessary for control of locomotor behaviour\textsuperscript{85}, same as octopamine\textsuperscript{86}. Dsk is the Drosophila homologue of the mammalian satiation hormone cholecystokinin (CCK)\textsuperscript{87}. CCK was postulated to act as a neurotransmitter and neuromodulator in the CNS\textsuperscript{88}. Interestingly, levels of CCK are correlated with aggression\textsuperscript{89}. However, CCK is not only produced in the CNS but also in the gut where it acts a gastrointestinal hormone to suppress hunger. CCK is secreted by the gut when food enters the lumen. After being released CCK binds to its receptor, the cholecystokinin A receptor, which is located on vagal sensory nerve terminals. Signals delivered to the nucleus of the solitary tract situated in the brain stem lead to the inhibition of feeding\textsuperscript{90,91}. The insect sulfakinins are functionally and structurally related to the vertebrate CCK family and seem to also function as satiety signals. It was recently shown that Dsk is necessary to prevent overfeeding under starved conditions\textsuperscript{87}. This strengthens the role of the IPCs as an important site for energy metabolism regulation. The same study also reported that Dsk is necessary in larvae and adult
Drosophila to determine food palatability. Interestingly, Zhang et al. (2013) showed that octopamine has an important role in the regulation of the acquisition of palatable food.

**Ras-related C3 botulinum toxin substrate 2**

Monoamines such as serotonin, dopamine and noradrenaline are not the lone central regulators of the homeostatic system and aggressive behaviour in Drosophila; a large repertoire of G protein coupled receptors (GPCRs) have also been identified to play a role in the regulation aggression and metabolism. For example, receptors for the previously mentioned monoamines are all GPCRs.

A common regulator for GPCR signal transduction is the binding protein Arrestin2. When, for example, NinaE, a Rhodopsin-like GPCR required for the visualization of visible light, is activated, Arrestin2 will bind to the receptor and desensitise it. Arrestin2 then acts as a binding site for proteins involved in GPCR recycling and consequently leads to the salvaging of the receptor from the plasma membrane. Without this turnover, all the receptors would quickly be bound by ligands and the system would become saturated; no new signals could be transmitted, leading to a system shutdown.

Recently, it was discovered in Drosophila that Ras-related C3 botulinum toxin substrate 2 (Rac2) was necessary for the proper localization of Arrestin2 after NinaE activation. Rac2 null mutants failed to shuttle the Arrestin2 to the rhabdomeres in the photoreceptor cells after a light stimulation, severely impairing the termination of the photoresponse. Rac2 is a small GTPase involved in many cellular processes, such as cytoskeletal organization and the regulation of cellular adhesion. It even plays an important role in the cellular immune response, as Rac2 mutants were unable to mount a proper immune response against invading parasitoids. Interestingly, a large scale RNAi screen for obesity genes found that flies with reduced Rac2 expression had significantly less stored lipids than controls.

**Protein kinase D and Stretchin-Mlck**

A major regulator of energy homeostasis, in vertebrates and invertebrates alike, is insulin and its signalling pathway is highly conserved throughout the animal kingdom. Together with glucagon, insulin secretion is fine-tuned to match the metabolic needs of an individual.

In mammals, food ingestion leads to increased levels of blood glucose and amino acids which stimulate secretion of insulin by the pancreatic β-cells. Insulin then binds to specific receptors on fat and muscle cells and stimulates these cells to increase glucose uptake. Furthermore, insulin inhib-
its glucagon secretion from the pancreatic α-cells, thus stopping gluconeogenesis and glycogenolysis\textsuperscript{98}. Finally, insulin signals to the liver to promote glycogen synthesis\textsuperscript{99}. Insulin is secreted primarily in response to glucose, which is a principal food component and can accumulate immediately after food intake. However, other factors such as amino acids\textsuperscript{100,101}, fatty acids\textsuperscript{102} and hormones\textsuperscript{103-105} can also induce insulin secretion.

The secretion of insulin is finely regulated from synthesis, through granule formation, to release. Problems of insulin production and trafficking manifest early in the development of diseases such as diabetes, and with the current global obesity epidemic, there is good incentive to gain a better understanding of this pathway at the molecular level.

\textit{Drosophila} produces eight ILPs\textsuperscript{106}. ILP production in the brain is specific to two clusters of seven cells, known as the insulin producing cells (IPCs), which are located in the PI and produce three of the eight ILPs (ILP2, 3 and 5)\textsuperscript{20}. All three ILPs display a similar structure and are also structurally related to human insulin\textsuperscript{107}. Disruption of the insulin signalling pathway can cause a range of abnormalities. These depend on the stage of development of the organism, where along the signalling pathway it occurs and on the type of disruption. In first instar larvae IPC ablation causes delayed development and retarded growth, whereas ablation in third instar larvae leads to adults with increased lipid content and an extended lifespan\textsuperscript{108}.

An important factor for the trafficking of ILPs in \textit{Drosophila} could be the serine/threonine kinase \textit{Protein kinase D (PKD)}. It is an effector of a diacetyl-glycerol (DAG)-regulated signalling pathway\textsuperscript{109}. It consists of a kinase domain, required for the phosphorylation of target proteins. It is predicted to contain two cysteine rich domains (C1, C2) important for membrane localization and DAG binding. And finally it has a plekstrin homology (PH) domain, which possesses multiple functions\textsuperscript{110}. In comparison to human PKD, the \textit{Drosophila} orthologue lacks the alanine and proline rich sequences\textsuperscript{110}. Not much is known of PKD in \textit{Drosophila}. Maier et al. (2007)\textsuperscript{111} investigated its role in development and found that manipulation of PKD activity will lead to incomplete wing development, retina degeneration and tissue loss underlining PKD as a kinase with multiple roles. In mammals, three isoforms are known (PRKD1-3) and they are involved in various important biological processes, such as cell growth and angiogenesis, secretory transport from the trans-Golgi network and cytoskeleton regulation\textsuperscript{112-114}. \textit{PRKD1}, similar to \textit{TFAP2B} and \textit{KCTD15}, was identified as a possible obesity-linked gene in multiple GWAS\textsuperscript{80,82-84}

Evidence for a role of PKD in insulin secretion comes firstly from cell studies. Blocking Prkd1 in INS-1 cells (rat insulinoma-derived pancreatic β cell line) lead to an inhibition of insulin secretion\textsuperscript{109}. Upon addition of glucose, Prkd1 was activated and led to membrane fission at the trans-Golgi network\textsuperscript{109}. Secondly, it was found, that \textit{Caenorhabditis elegans} deprived of the gene \textit{dfk-2} (PKD homologue) had an increased lifespan by as much as
40%\textsuperscript{115}. This is reminiscent of a study in \textit{Drosophila}, where ablation of the IPCs also improved longevity\textsuperscript{108}. Furthermore, loss of \textit{DAF-16} function (\textit{FOXO} orthologue) rescues the extended lifespan in \textit{djk-2} deficient nematodes. As it is known in mammals that insulin signalling inhibits the translocation of FOXO to the nucleus, these results from \textit{C. elegans} provide further evidence that PKD could be involved in the regulation of insulin signalling.

A second \textit{Drosophila} serine/threonine kinase of interest is Stretchin-Mlck (Strn-Mlck), which is a member of the Titin/Myosin Light Chain Kinase (MLCK) family\textsuperscript{116}. Members of this family play an essential role in the organization of the actin/myosin cytoskeleton. Again, evidence for an involvement of the gene in insulin secretion comes from cell studies. RNAi screens on \textit{Drosophila} S2 cells showed that diminishing Strn-Mlck transcript levels lead to a reduction of \textit{dFOXO}\textsuperscript{117}, a transcription factor involved in the regulation of ILPs\textsuperscript{118}. Insulin release that was either induced in mouse and rat pancreatic \(\beta\) cells by glucose or \(\text{Ca}^{2+}\) could be inhibited by an MLCK inhibitor\textsuperscript{119}. Moreover, applying the MLCK inhibitor to MIN6 cells resulted in a reduction of granule movement\textsuperscript{120}. All these studies indicate that Strn-Mlck might be required for the translocation of secretory granules to the plasma membrane.
AIMS

The overall aim of this thesis was to better understand the molecular mechanisms that underlie aggression and feeding behaviour. Although aggression and energetics have been extensively studied, what is still not understood is how they interact to maintain metabolic homeostasis. For this purpose, the model organism *Drosophila melanogaster* was employed. Although it has a simpler neuroendocrinological pathway than mammals, it shares the important basic functions. Furthermore, it shows stereotypic aggressive behaviour allowing the dissection of the molecular mechanisms of the genetic cascades regulating aggressive behaviour and metabolic homeostasis. The specific aims for each study were:

**Paper I**

In this study, the aim was twofold. Firstly, it was determined whether *TfAP-2* and *Tiwaz* interact and if they play an important role in proper octopamine signalling. It was hypothesized that manipulation of *TfAP-2* and/or *Tiwaz* would lead to a misregulation of octopamine, which would result in abnormal aggression behaviour.

Secondly, it was inquired if the satiation hormone Dsk is a downstream target of octopamine. It was hypothesised that octopamine modulates Dsk to regulate aggression and manipulation of one of these should lead to changes in the other one and ultimately to changes in aggressive behaviour.

**Paper II**

Here, the aim was to examine the involvement of *TfAP-2* and *Tiwaz* in regulating *Drosophila* adult feeding behaviour and it was hypothesized that misregulation of *TfAP-2* and *Tiwaz* would have abnormal feeding behaviour as a consequence. Furthermore, the role of Dsk and octopamine were studied and possible interaction between the two to regulate feeding in adult flies was examined.
Paper III
Herein we sought to establish if the small GTPase Rac2 is involved in regulating *Drosophila* metabolism. It was hypothesised that with reduced or no Rac2 expression, flies would have severe metabolic phenotypes.

Paper IV
In Paper IV, the aim was to investigate the *in vivo* role of the serine/threonine kinases PKD and Strn-Mlick in the IPCs. It was hypothesized that changes of either of these genes would lead to aberrant production or secretion of insulin-like peptides.
Materials and Methods

The most commonly used methods are discussed below. More detailed descriptions of the experimental procedures used in the thesis are provided in the individual papers.

Fly husbandry & common behavioural set-up

All flies were maintained on an enriched Jazz mix standard fly food, and maintained at 25°C, 50-60% humidity, on a 12:12 light:dark cycle. To inhibit the GAL4 driver, flies involved in an experiment using the GAL4/UAS system were kept at 18°C. Once the progeny eclosed, they were shifted to 29°C to achieve best working of the bipartite system. Male flies, aged 5-7 days, were used for all experiments unless otherwise indicated. Behavioural tests were carried out at room temperature with 60% humidity and the behavioural arena consisted of a cylindrical chamber (2 cm x 2.5 cm; height x diameter), filled with 1% agarose. All flies, prior to starting a behavioural assay, were anesthetized using an ice-water bath, transferred to the behavioural chamber and left to recover for at least 3 minutes before the recording period started. A High Definition (HD) camera was used to film the behaviour.

Behavioural studies

Aggression Assay

Two male flies were raised in isolation prior to the assay. They were transferred to the behavioural chamber, where a camera, positioned above the chamber, was used to record activity for 20 minutes. Distinct stereotypic aggressive interactions were scored as described by Chen et al.\textsuperscript{39} and Nilsen et al.\textsuperscript{121} Aggressive interactions were further scored as either low or high intensity engagements. LIF was scored as side-by-side pushing with a leg (shoving), or quick wing flicking (wing flick); HIF was graded as frontal lunging (lunging) or boxing face-to-face with the two front legs (fencing), holding the wings at a 30-45° angle (wing threat), as well as chasing one another (chasing). Courtship behaviour was marked as one-wing extended at
a 90° angle (singing), circling to the posterior (circling), tapping the abdomen (tapping), licking the genitalia (licking), or bending the abdomen towards the other fly (abdomen bending).

Mating Assay

Individual males, raised in isolation, and 3-4 day old virgin wild type females were transferred to the behavioural chamber. A camera positioned above the chamber, was used to record activity and the behavioural interactions between the male and female were scored for 20 minutes or until copulation occurred. Scoring of the courtship behaviours was performed as described by Becnel et al. Latency, courtship index as well as the frequency of mating behaviours were measured. Latency was calculated by counting the time it took a male to initiate mating and courtship index is calculated as the percentage of time a male spends actively courting a female over a 20 minute period.

CAFE Assay

The feeding assay was adapted from Ja et al. In brief, five flies were kept in a vial containing 1% agarose. The vial was covered with paraffilm and a 5μl capillary, filled with a liquid food solution consisting of 5% (wt/vol) sucrose, 5% (wt/vol) inactive dry yeast, and 0.5% food-colouring dye, was pierced through the top. The initial food level in the capillary was marked and the capillary was filmed with a HD camera for 24 hours. After the recording period the final food level was marked and total food intake per day could be determined. The number of feeding bouts per fly was counted from the recording, and the average meal size of the fly was calculated by dividing the total food intake by the number of feeding bouts (meal size = Total food intake fly⁻¹ 24 hours⁻¹ / Total number of feeding bouts fly⁻¹ 24 hours⁻¹).

Metabolic studies

Lipid extraction

Lipid content was determined according to the method of Service (1987). Groups of five flies (males or females) were transferred into a 60°C incubator for 24 hours. Their dry weight was determined and the flies were then placed in 10 mL of diethyl ether for 24 hours at room temperature to extract all lipids. The diethyl ether was filtered out and the flies were placed back in the incubator at 60°C for 24 hours to evaporate any residual diethyl ether. Flies were weighed again to give the lipid-free weight; the difference between the two measurements was considered the lipid content of the flies.
Starvation Assay
Twenty male or female flies were transferred to vials containing 1% agar, and were placed in an incubator at 25°C in 12:12 hour light:dark conditions. The number of dead flies was recorded at twelve hour intervals allowing the calculation of the median time of death and the survival rate.

Gene expression analysis
Quantitative real-time PCR
The phenol-chloroform method was used for RNA extraction from tissue samples. Fifty fly heads were homogenized with TRIzol and chloroform to extract RNA. Isopropanol was added for RNA precipitation followed by washing of the pellets with 75% ethanol. Next, the RNA pellets were air dried and dissolved in RNase free water. DNase was added to degrade any DNA contamination. Total RNA concentration was determined using a Nanodrop. RNA samples were diluted in MilliQ water. To synthesise cDNA, 20mM dNTP and random Hexamers were added and the samples were incubated. Then, 5xFS buffers, 0.1 M DTT and murine leukemia virus transcriptase were added followed by another incubation period. Lastly samples were subjected to PCR followed by gel electrophoresis to confirm cDNA synthesis. The qRT-PCR mastermix consisted of MgCl₂ free 10x Buffer, 50mM MgCl₂, 20mM dNTP, MilliQ water, both forward and reverse primer, dimethyl sulfoxide, Sybr Green, and Taq polymerase. Samples were run in duplicates and negative controls were included in each plate. In paper I and II, EF-1, Rpl11, and Rp49 were run as housekeeping genes, Paper III and IV used Rp49 as a housekeeping gene. Amplification was performed as follows: denaturation at 95°C for 3 min, 50 cycles of denaturing at 95°C for 15 sec, annealing at an appropriate temperature established for the primers for 15 sec, and extension at 72°C for 30 sec. Reactions were run on iCycler temperature cyclers and fluorescence was measured using MyiQ single colour real time PCR detection system. Data were analysed using iQ5 software. All samples were analysed in duplicates, and the measured concentration of mRNA was normalized relative to control values of the housekeeping genes. The relative levels of a given mRNA were quantified from the normalized data according to the ΔΔCT analysis.
Results and Discussion

Paper I

In Paper I, the role of the Drosophila obesity-linked homologues TfAP-2 and Twz in regulating aggressive behaviour through octopamine signalling was investigated. It was determined that TfAP-2 protein is expressed in octopaminergic neurons by staining adult fly male brains with a human anti-AP-2γ. To identify the octopaminergic neurons, they were labelled using GFP. TfAP-2 expression overlapped with green fluorescent staining in the subesophageal ganglion (SOG), an area known to control aggression in flies. Having established that TfAP-2 is expressed in neurons known to regulate aggression, flies were tested for any behavioural changes when the transcript levels of TfAP-2 or Twz were manipulated. Male flies with diminished TfAP-2 or Twz expression in octopaminergic neurons showed reduced high intensity fighting behaviour and higher levels of courtship behaviour towards males than controls. Overexpressing TfAP-2 induced aggressive behaviour. When pairing the males with virgin females and scoring courtship behaviour, TfAP-2 and Twz knockdowns courted virgin wild-type females more enthusiastically than controls, whereas flies with increased TfAP-2 expression showed little or no interest in mating. Interestingly, knocking down Twz in flies also overexpressing TfAP-2 could rescue the phenotypes. These results indicate that TfAP-2 and Twz are necessary in males for a normal behavioural response towards conspecifics. These observed phenotypes reflect very closely the behaviours of flies where octopamine levels were manipulated.

To confirm that TfAP-2 and Twz were actually regulating octopamine signalling, it was firstly shown that the transcript levels of Tyramine β hydroxylase (TbH) and Vesicular monoamine transporter (Vmat), two genes involved in octopamine synthesis and secretion, are affected by changes in the expression levels of TfAP-2 and Twz. Secondly, feeding hyperactive TfAP-2 overexpressing flies two different octopamine antagonists reduced the phenotype in a dose dependent manner.

Having established that TfAP-2 and Twz are necessary for proper octopamine control, the question of how this control of octopamine production may regulate aggressive behaviour was studied. In rodents, levels of the satiation hormone CCK are correlated with aggression. In flies, the CCK homologue, Dsk is produced in the IPCs and it is known that octopaminergic neu-
rons innervate the IPCs\textsuperscript{81}. Therefore, the effects of overexpressing *Dsk* in the IPCs was examined, and aggression and activity assays were performed. The flies showed increased HIF and hyperactivity, similar to mice with increased CCK signalling\textsuperscript{125,126}, as well as to flies overexpressing *TfAP-2*.

To determine whether *Dsk* is working downstream of octopamine, the CCK antagonist, SR27897 was fed to *TfAP-2* overexpressing flies. The antagonist reduced the hyperactivity of the *TfAP-2* overexpressing flies to control levels. Similar results were seen for the aggression phenotype. SR27897 successfully rescued the high intensity fighting phenotype of the *TfAP-2* overexpressing flies. Furthermore, it was shown that *Dsk* expression was highly influenced by octopamine, *TfAP-2* and *Twz*. Feeding the octopamine agonist Chludimeform to wild type flies increased *Dsk* transcript levels. Moreover, *Dsk* levels were reduced in *TfAP-2* and *Twz* knockdowns and increased in *TfAP-2* overexpressing flies. Feeding phentolamine, an octopamine antagonist, to *TfAP-2* overexpressing flies blocked *Dsk* induction. These findings strongly consolidated the hypothesis that octopamine modulates *Dsk* expression to regulate male behaviour and the following model of regulation of aggressive behaviour in *Drosophila* was proposed:

*TfAP-2* and *Twz* interact in octopaminergic neurons and are involved in regulating the activity of octopamine by inducing expression of *Tbh* and *Vmat*. *Tbh* and *Vmat* are necessary for proper synthesis and release of octopamine. Octopamine signals to the IPC to stimulate *Dsk* activity. *Dsk* promotes male aggressive behaviour, while reducing mating behaviour in males (Figure 1).

**Figure 1.** Proposed pathway for the regulation of aggression in *Drosophila*. Transcription factor AP-2 (*TfAP-2*) and Tiwaz (*Twz*) are found in the octopaminergic neurons and are inducing the transcription of genes coding for Tyramine β hydroxylase (*Tbh*) and Vesicual monoamine transporter (*Vmat*). *Tbh* is necessary for the synthesis of octopamine and *Vmat* in the release of it. Octopamine producing neurons are innervating the insulin producing cells (IPC) where they stimulate the production of Drosulfakinin (*Dsk*). Increased levels of *Dsk* lead to increased aggression and decreased mating behaviour in male *Drosophila*. 
Paper II

It was shown in Paper I that in adult males TfAP-2 and Twz genetically interact to control aggressive behaviour by regulating octopamine production and secretion which in turn regulates the expression of Dsk. In mammals Dsk homologue CCK is known to inhibit feeding90,91. In Drosophila, Dsk is necessary to prevent overeating after starvation87. Furthermore, Dsk is necessary to determine food palatability in which octopamine also plays an important role87,92. Therefore, the focus of Paper II was to investigate the role of TfAP-2 and Twz in the regulation of feeding.

It was examined how starvation and diet affects TfAP-2, Twz and their downstream targets, that were discovered in Paper I. It was found that TfAP-2 transcript levels are up-regulated under conditions of dietary restriction and down-regulated when flies are fed a high calorie diet, while Twz transcription is only influenced by severe starvation. Transcriptional levels of Tbhl and Vmat, two genes necessary for octopamine production and secretion, were also measured under these conditions. It was discovered that starvation conditions regulate both Tbhl and Vmat, but only Tbhl was regulated by macronutrient content. Next, a re-feeding assay was performed to examine the consummatory behaviour after starvation of flies with abnormal TfAP-2 or Twz expression levels. Similar to loss of Dsk in the insulin producing cells, knocking down TfAP-2 or Twz in the octopaminergic neurons caused the flies to overeat after starvation. This indicates that both TfAP-2 and Twz may be upstream of Dsk regulation of consummatory behaviour. Interestingly, overexpression of TfAP-2 in octopaminergic neurons resulted in the strongest overeating phenotype observed. Having established a role of TfAP-2 and Twz under starved condition, a CAFE assay was conducted to determine their function under normal circumstances. It was revealed that down-regulation of TfAP-2 and Twz induced the flies to consume more over a 24 hour period and overall eat larger meals, although, Twz knockdowns did not reach significance. Flies, which overexpressed TfAP-2, ate not only more food than controls but also had threefold as many feeding bouts as controls. These results indicate that TfAP-2 and Twz are involved in Drosophila consummatory behaviour.

Next, it was determine if the feeding behaviour regulation of TfAP-2 and Twz is regulated via octopamine signalling. Rats, getting chronic noradrenaline infusions, gained considerable weight and showed hyperphagia and an irregular feeding pattern127. Flies overexpressing TfAP-2 were firstly fed an octopamine antagonist. The octopamine antagonist successfully reduced total food intake, as well as the number of feeding bouts in the TfAP-2 overexpressing flies but did not show any significant effect on the controls. Secondly, the voltage-activated bacterial sodium channel NaChBac was expressed in the octopaminergic neurons in order to activate them specifically and a CAFE assay was performed. Similar to TfAP-2 expressing flies, these
flies had a significant increase in total food intake and feeding bouts when compared to controls, indicating that increased octopamine signalling is sufficient to induce elevated food intake. Furthermore, activating octopaminergic neurons is enough to increase Dsk expression as flies, where NaChBac was overexpressed in the octopaminergic neurons, had higher Dsk transcript levels than controls. Overexpressing Dsk in the IPC, however, did not significantly change food intake or the number of feeding bouts. Dietary condition affected Dsk similar to Vmat, with increased transcript levels in flies starved for 24 hours and little variations across the different diets.

From the above data the following model is presented: TfAP-2 and Twz regulate the production and secretion of octopamine, which in turn initiates feeding, while at the same time, in a negative feedback loop, octopamine induces the expression of Dsk to inhibit feeding frequency (Figure 2).

![Figure 2. Proposed pathway for the regulation of feeding in Drosophila.](image)

The focus of the study was then shifted towards the mouse model and firstly immunohistochemistry was performed to investigate the expression pattern of the mammalian Tfap2b and Kctd15 genes in the brain. AP-2β and Kctd15 overlapped in neurons in the arcuate hypothalamic nucleus, the ventromedial hypothalamic nucleus and in the core of the accumbens nucleus in the ventral striatum. All these areas are known to be involved in the regulation of food intake. Secondly transcript levels of Kctd15 and Tfap2b were measured in the hypothalamus of mice which were assigned to the different food restrictions. Mice were fed either normal chow, normal chow but were food deprived for 24 hours before being sacrificed or fed a high fat diet to induce obesity. Tfap2b was effects both by fasting and the high fat diet and in both cases its transcript levels were upregulated. Kctd15 expression was little affected by dietary status and showed only a slight, yet nor significant increase under starved conditions. These results are reminiscent for what was observed for TfAP-2 and Twz in flies. Thirdly, a proximity ligation assay
(PLA) using mHypoE-N25/2 was conducted to establish that AP-2β and Kctd15 interact in vivo. PLA allows detecting and localizing proteins with single molecule resolution, hence permits to determine directly if proteins interact. Lastly, the PL assay was conducted to resolve if AP-2β and Kctd15 interact with the sumoylation enzyme Ube2i. In mice, Ube2i interacts with AP-2β. In Drosophila Twz interacts with Lesswright (Ube2i homologue) and AP-2γ is sumoylated. Performing a Western blot on mouse brain using an anti-AP-2β antibody, two bands were observed. One band corresponding to the predicted size of AP-2β, the second band has the predicted size of sumoylated AP-2β. It is therefore possible that Twz/KCTD15 acts like scaffold where TFAP-2/AP-2β is either sumoylated, similar to Kctd1 and AP-2α or ubiquitinated. Overall, the data suggest that in flies and mammals the initiation and cessation of consummatory behaviour is controlled by a conserved signalling system.

Paper III

It was recently discovered in a high throughput screen that flies with reduced expression of the small GTPase Rac2 have reduced stored lipids and, by visual inspection, Rac2 mutants appeared thinner than controls. A low level of stored lipids has been correlated with an increase in starvation susceptibility. It was established that male and female Rac2 mutants were indeed more susceptible to starvation than controls and possible factors such as lipid storage, feeding behaviour and activity were inspected. Rac2 mutants, both male and female, had lower lipid content than controls, they did not consume more food than control but ate fewer and larger meals and finally single Rac2 mutants where not more active than controls. However, when they were paired with wild type males or virgin females, they were significantly more active than controls, indicating that Rac2 mutant males are hyperactive in the presence of another fly. To begin to gain a better understanding of the underlying cause of this increase in activity, an aggression assay was performed. When two Rac2 mutant males were paired with each other they performed more low intensity fighting and displayed all courtship behaviours males would normally present towards virgin females. Male Rac2 mutants seem to be unable to make the distinction between males and females, as they vigorously court each other over a prolonged period of time and even show copulation attempts. To further elucidate this, Rac2 mutant males were individually marked by painting a coloured dot on their abdomen and the behaviour of each fly was scored. It was discovered that the courting or chasing, male never initiated aggressive behaviour; all aggressive behaviour was started by the male being courted and the flies would even switch roles within a recording period. Pairing a Rac2 male with a wild type fly lowered his aggression behaviour significantly and he spent most of its time courting.
the wild type male. Rac2 mutants also showed aberrant courting behaviour towards wild type females. When confronted with a virgin female, Rac2 flies behaved similar to control flies. No difference in the time until they started courting the female or in their courtship enthusiasm was observed. However, when mated females were added, control males avoided them as expected, whereas Rac2 males on the other hand courted the females vigorously. All these findings supported strongly the idea that Rac2 males are capable of executing aggressive behaviour but fail to distinguish between sexes and cannot differentiate between different reproductive stages of females.

Put forth as a possible explanation was that Rac2 is necessary for the proper working of the GPCR Gustatory receptor 32a (Gr32a). Gr32a, found in neurons in the forelegs of flies, is needed to recognize non-volatile aversive cues on males and to subsequently suppress conspecific male-male courtship. Gr32a neurons project to the SOG, an area, as discussed in Paper I, responsible to regulate aggression in flies. Similar to the way Rac2 regulates the translocation of Arrestin2 after the GPCR NinaE is activated, Rac2 could be necessary for the proper signalling of Gr32a. Rac2 mutants no longer recycle Gr32a, leading to its shutdown. No information from these neurons is delivered to the brain and the fly’s default program is to mate with the other fly.

It was shown that Rac2 mutant males were hyperactive when paired with other flies. This constant hyperactivity could be a possible explanation for the observed feeding and metabolism phenotypes. Rac2 mutant males have a strong need to mate and are constantly chasing and harassing other flies, this higher number of interactions increases the energy demand and leads to the observed reduced lipid storage. Depending on how strong the mating desire is, it might even override hunger signals which could explain the fewer but larger meals. Only when the hunger signal is strong enough are Rac2 mutants going to eat. However, many receptors involved in modulating feeding behaviour are GPCRs, including Drosophila cholecystokinin-like and Leucokinin receptors, both targets of the meal termination signals, Dsk and Leucokinin, respectively. It could be that disruption of Rac2 in the Rac2 mutants is causing non-proper signalling of these receptors leading to the misregulation of satiation signals. Interestingly, these GPCRs have been shown to interact with Kurtz, a Drosophila non-visual Arrestin. However, this is only conjecture and further experiments are needed to find out what is/are the true reason(s) of the feeding and metabolism phenotypes observed.

Paper IV

Similar to TFAP2B and KCTD15 studied in Papers I & II, protein kinase D (PRKDI) was identified as an obesity-linked gene in multiple GWA studies. Cell studies linked PKD family members to insulin release.
Together with a second serine/threonine kinase Stretchin-Mlck (Strn-Mlck), the roles of these genes in the insulin secretion in Drosophila was investigated in Paper IV.

In Drosophila, an increase in circulating insulin leads to larger amount of stored lipids in the fat body\textsuperscript{140}, which is positively correlated with a higher starvation resistance\textsuperscript{134}. Therefore, the survival of flies with reduced PKD and Strn-Mlck expression under starved conditions and their lipid content were measured at different time points during starvation. Flies with lowered transcript levels of PKD and Strn-Mlck in the CNS were less starvation resistant than controls and had a lower lipid content than controls. Interestingly, knocking down PKD or Strn-Mlck specifically in the IPCs in the fly brain was enough to recapitulate the starvation phenotype. The total lipid content, however, was only reduced in flies with diminished PKD transcript levels in the IPCs. Whether these flies were starved for 6 hours, 12 hours or fed \textit{ad lib}, a significant reduction in lipid content was observed compared to controls. These findings are in accordance with the phenotypes reported in mice and humans that are insulin-deficient\textsuperscript{141}. However, they are in contrast with previously published results in Drosophila, where it was shown that IPC ablation leads to increased starvation survival and higher lipid content\textsuperscript{20}. It is important to note that these studies performed a complete ablation of the IPCs and therefore affected all genes produced in the IPCs of which some are involved in lipid metabolism and feeding regulation\textsuperscript{142}.

Expression levels of the brain derived \textit{dilps} were also measured in flies with diminished PKD expression. Not \textit{dilp2}, \textit{dilp3} nor \textit{dilp5} were affected pointing to a post-translational role of PKD. For that reason, an insulin signalling activity indicator, GFP-PH domain fusion protein (tGPH), was employed and the Inr/PI3K signalling activity in the fat boy was monitored. Control larvae showed proper response to glucose stimulation after starvation and insulin activation of the Inr/PI3K pathway was observed. Larvae with reduced PKD expression in the IPCs failed to respond to glucose stimulation. This results indicate that PKD is necessary for the proper release of brain derived ILPs.

The second kinase studied, Strn-Mlck, although starvation susceptible, did not have a strong lipid storage phenotype. This is in agreement with a previous directed RNAi study, which did also not observe a reduction in lipid storage in cells with reduced Strn-Mlck\textsuperscript{143}. Overall, Strn-Mlck showed no significant variation under the measured environmental conditions, but did show a reduction in glucose induced Inr/PI3K signalling in the fat body, which were however not as strong as in flies with reduced PKD expression. Studies in rat and mouse pancreatic β cell lines have revealed that the Strn-Mlck homologue MLCK is necessary for the movement of insulin granules. However, this transport is probably not exclusive because Strn-Mlck is expressed throughout the fly body\textsuperscript{76}, pointing to a more ubiquitous role in granule movement. Similar to the ablation of the IPCs, the global role of
Strn-Mlck could lead to confounding results and more work is needed to unravel the precise role of Strn-Mlck in the regulation of insulin signalling.
Conclusions and perspectives

The aim of this thesis was to gain a better understanding of how overt behaviour, such as aggression and metabolic modulators interact to maintain homeostatic control.

It was determined for three of the investigated genes that they play a role in metabolic homeostasis as well as being involved in regulating overt behaviours. In Paper I and II, TfAP-2 and Twz were found to be important genes necessary for the production and release of octopamine and elevated octopamine signalling induced aggression and consummatory behaviour in flies. The small GTPase Rac2 emerged, in paper III, as a third gene linked to behaviour and metabolism. Rac2 mutants showed modified male aggressive, mating and feeding behaviour. These findings support the idea that regulatory pathways are conserved and may have initially evolved to maintain energy homeostasis, and then over time assumed other roles such as the regulation of aggression. It is therefore possible that the discovered role of TfAP-2 and Twz on octopaminergic control in Drosophila is a conserved pathway, modulating aggression and feeding in higher organisms. Studies in model organisms such as zebrafish and rodents could offer further insight for this hypothesis. Tfap2b knock-out mice die perinatally making behavioural studies impossible but one could, for example, breed hyperaggressive and non-aggressive mice and compare transcript levels of Tfap2b and Kctd15. Furthermore, zebrafish show stereotypic aggression behaviour and similar to fruit flies, the GAL4-UAS bipartite system could be used to knock down transcript levels of the zebrafish homologues of TfAP-2 and Twz and the effects on behaviour could be studied.

In a similar way one could argue with the findings concerning Rac2. A mechanism was proposed suggesting that Rac2 is necessary for the desensitization of the GPCR Gr32a. Gr32a is a taste receptor with a dual function. Firstly it is required in feeding behaviour, where it is needed for bitter tasting. Secondly, the Gr32a receptor is involved in social behaviour as studies have shown that flies lacking GR32a present improper conspecific recognition. Interestingly, it was recently reported that the Gr32a receptor mediates the overt behaviours via octopamine signalling. The next step would now be to find out if this proposed mechanism is correct and if Rac2 regulation of Arrestin-like protein translocation is not limited to the Drosophila eye, but is a general mechanism of many GPCRs. Determining this is important as it could provide another piece to the molecular mechanism.
that is regulating aggression in *Drosophila*. Furthermore, it appears that a majority of the *Drosophila* and vertebrate neuropeptide GPCR signalling pathways share common evolutionary origins\textsuperscript{136}, thus opening the possibility that findings made in this model organism also hold true for vertebrates.

Paper I and II also demonstrated that Dsk acts downstream of octopamine, altering aggression and inhibiting food intake. Dsk is primarily considered a hormonal satiety signal that is involved in insect feeding behaviour\textsuperscript{87} and has not been linked to aggression before. Understanding Dsk’s involvement in aggression (Paper I) and feeding (Paper II) in *Drosophila* is again of particular interest for human studies of eating disorders, as many eating disorders are commonly accompanied by changes in impulsive and aggressive behaviour indicating a possible similar control\textsuperscript{149,150}.

In Paper IV, a homologue of a third human obesity-linked gene was studied. The serine/threonine kinase PKD was established to be important for the secretion of insulin. These findings together with the results from Paper I and II demonstrate the strength of *Drosophila* as a model organism to study the function of disease genes, such as the obesity-linked genes in a whole animal setting. Findings in flies can offer a good starting point and can then lead to more targeted studies in higher animals.

Regarding PKD, the next steps would be to characterize the social behaviour of flies with reduced PKD expression. Immunohistochemistry could be performed on *Drosophila* brains using an anti-PKD antibody to map expression patterns of PKD in the fly brain. Lastly, the approach to measure could be refined. In Paper IV, insulin release was assessed visually and not via a quantitative method. It would therefore be of interest to also measure circulating ILPs in the hemolymph.

With the conservation of metabolic functions and molecular pathways between flies and humans, combined with the immense number of genetic tools available, this thesis has demonstrated that *Drosophila* is a valuable model organism to better understand how genes regulate both metabolism and overt behaviours.
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