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Identification of neuronal populations controlling Dilps secretion and body growth according to nutrition in *Drosophila melanogaster*

Eleonora Meschi

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THÈSE DE DOCTORAT

Identification de populations neuronales
contrôlant la sécrétion des insulines et
la croissance en fonction de la nutrition
chez *Drosophila melanogaster*

Eleonora MESCHI

Institut de Biologie Valrose iBV

**Présentée en vue de l'obtention
du grade de docteur en SVS
d'Université Côte d'Azur**
Dirigée par : Rénaud Delanoue / Pierre
Léopold
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Devant le jury, composé de :
Andrea Brand, Professeure, The Gurdon
institute
François Leulier, Docteur, IGFL
Irene Miguel-Aliaga, Professeure, Imperial
College
Marylène Poirié, Professeure, INRA

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contrôlant la sécrétion des insulines et la
croissance en fonction de la nutrition chez
Drosophila melanogaster

Jury :

Président du jury

Marylène Poirié, Professeure, INRA Sophia Antipolis

Rapporteurs

François Leulier, Docteur, IGFL Lyon

Irene Miguel-Aliaga, Professeure, Imperial College Londres

Examinatrice

Andrea Brand, Professeure, The Gurdon Institute Cambridge

THESIS

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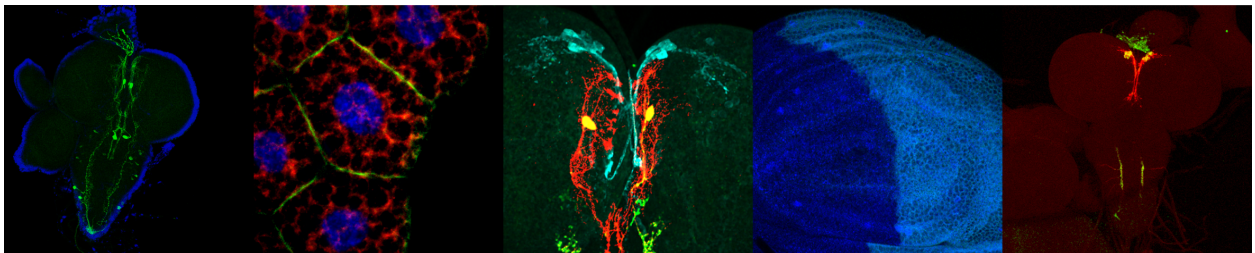
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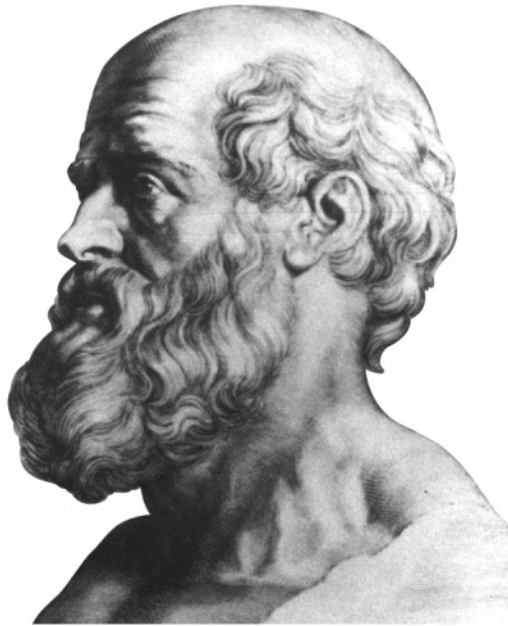
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Thesis defence: the 14th of November 2018



Per i miei nonni, i miei due angeli custodi...



“Let food be thy medicine and medicine be thy food.”

- Attributed to Hippocrates

“Who said anything about medicine? Let’s eat!”

- Attributed to one of Hippocrates forgotten (and skeptical) student

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Résumé

La taille finale des organismes dépend de la vitesse et de la durée de croissance. Ces paramètres sont contrôlés par différentes hormones. La production d'hormone stéroïdienne détermine la fin de la période de croissance en déclenchant la maturité sexuelle, alors que la vitesse de croissance est régulée par la voie de signalisation de l'insuline/IGF (IIS). La vitesse de croissance des organismes est influencée par la nutrition. En effet, des défauts de croissance sont observés chez les individus souffrant de carence protéique chronique. La nutrition contrôle la croissance grâce à la voie de signalisation de l'insuline/IGF. Cependant, le mécanisme par lequel la nutrition contrôle la voie IIS est complexe et reste à élucider. Afin d'explorer cette régulation, le laboratoire utilise *Drosophila melanogaster* comme modèle d'étude. Chez la drosophile, il existe 8 insulin-like peptides (Dilps). Parmi eux, Dilp2 est la principale insuline promouvant la croissance systémique. Elle est produite par des neurones spécialisés appelés les Insulin Producing Cells (IPC), homologues des cellules bêta du pancréas. La sécrétion de Dilp2 dans l'hémolymphe, équivalent du sang chez les vertébrés, est précisément ajustée en fonction de la nutrition. Cette régulation implique une communication inter-organe avec le corps gras, homologue du foie et du tissu adipeux blanc. Selon les conditions nutritionnelles, plusieurs signaux dérivés du corps gras (FDS) sont sécrétés et contrôlent la sécrétion de Dilp2. Ces FDS agissent directement ou indirectement sur les IPCs, *via* des relais neuronaux. Mon projet de thèse avait pour but de découvrir et d'étudier de nouvelles cibles neuronales contrôlant l'activité sécrétrice des IPCs, et par conséquent la croissance systémique, en fonction de la nutrition.

J'ai identifié une paire de neurones inhibiteurs des IPCs, que l'on a nommé IPC-Connecting Neurons (ICN). Actifs en carence en acides aminés, ils inhibent la sécrétion des Dilps. J'ai montré que la signalisation EGFR réprime l'activité de ces neurones en condition nourrie, ce qui augmente la sécrétion des Dilps et par conséquent la taille des individus. Cette activation est due à un nouveau ligand d'EGFR: Growth Blocking Peptide (GBP). J'ai montré que ce ligand de type EGF possède des propriétés particulières puisqu'il agit de façon endocrinienne. En effet, en condition nourrie, GBP est sécrété par le corps gras dans l'hémolymphe, et atteint les ICN afin d'activer la signalisation EGFR.

En conclusion, nous proposons que GBP produit par le corps gras en condition nourrie active la voie EGFR dans les neurones ICN, lève l'inhibition exercée sur les IPCs et stimule la sécrétion des Dilps.

Cependant, les mécanismes moléculaires par lequel le couple GBP/EGFR inhibe l'activité neuronale des ICNs reste à élucider.

Ce travail a permis d'identifier un nouveau mode de régulation de la sécrétion des insulines et de la croissance des organismes en fonction de la disponibilité et de la qualité nutritionnelle.

Mots clés : croissance systémique, communication inter-organe, nutrition, insuline/IGF, IPCs, GBP, EGFR.

Summary

Body growth is tightly regulated by nutrient availability. Upon nutritional shortage, animals harmoniously reduce their body size by modulating the activity of the insulin/IGF signaling pathway (IIS). To understand how nutrition controls the IIS, we used *Drosophila melanogaster* as a model. *Drosophila* has a conserved IIS with 8 insulin-like peptides (Dilps), a unique insulin receptor and a conserved downstream signaling cascade. Among the Dilps, Dilp2 is the main growth-promoting factor. Dilp2 is produced by specialized neurons located in the brain, the Insulin-Producing-Cells (IPCs), functionally related to vertebrate beta cells. Dilp2 secretion is precisely adjusted in response to nutrition: it is released in the hemolymph under normal nutrient condition, but not upon dietary amino acid scarcity. This regulation requires several inter-organ cross-talks between the producing neurons and the fat body, which is the equivalent of the vertebrate white adipose tissue and liver. Depending on diet composition, several fat-derived signals (FDS) are secreted into the hemolymph and control Dilp2 secretion from the IPCs. These FDS act either directly or indirectly through a neuronal relay, to control the IPCs secretory activity. The aim of my PhD project was to better understand these regulations and to discover new neuronal relay controlling the IPCs secretory activity and body growth, according to nutrition.

I identified a pair of neurons harboring synaptic connections with the IPCs (IPC-connecting neurons, ICNs). I determined that the ICNs activity is maximal upon amino acids shortage and is required to exert a blockage of the neighbouring IPCs. Moreover, in rich nutrient conditions, EGFR signaling prevents activation of the ICNs, allowing Dilp2 release from the IPCs. GBP1 and 2 are EGF-like peptides produced by the fat body in response to amino acids, and they can modify insulin release. However, the neural circuitries at play are unknown. I demonstrated that GBPs are atypical ligands for the EGF receptor (EGFR), with endocrine function. Using *ex-vivo* brain culture, I showed that the presence of the fat body-derived GBP1 in the hemolymph activates EGFR signaling in the ICNs and alleviates their inhibitory input on the IPCs, allowing Dilp2 release and therefore body growth.

In conclusion, I identified a novel neural circuitry responding to fat-derived EGF-like GBPs, coupling dietary amino acids to the release of insulin-like peptides and systemic growth.

Key words: systemic growth, inter-organ communication, nutrition, Dilp2, IPCs, GBP, EGFR.

Abbreviations

A

ACh : acetylcholine
AChR : acetylcholine receptor
adipoR: Adiponectin receptor
AKH: Adipokinetic hormone
AKT/PKB: protein kinase B
Ala: alanine
Alk: anaplastic lymphoma kinase
ALS: acid-label subunit
AMPC: cyclic adenosine monophosphate
AMPk: AMP-activated protein kinase
ARN: Adiponectin responsive neuron
Asp: aspartate
AstaA: Allatostatin A
AstaR1/2: AstA receptor 1/2
AREG: Amphiregulin
ATP: adenosine triphosphate

B

Babo: Baboon

C

CA: corpora allata
Ca²⁺: calcium
CamKII: Calmodulin Kinase II
CC: Corpora cardiaca
CCHa2: CCHamide2
CCHa2-R: CCHamide2 receptor
cGMP: cyclic guanosine monophosphate
ChAT: choline acetyltransferase
CRISPR: Clustered, Regularly Interspaced,
Short Palindromic Repeat
Crz: corazonin
CrzR: corazonin receptor

D

DAG: diacylglycerol

DAR-1/2: Drosophila Allatostatin Receptor 1/2
Daw: Dawdle
DH44: Diuretic hormone 44
DH44R1/2: DH44 receptor 1 and 2
DHR4: Drosophila Hormone Receptor 4
Dilp: Drosophila insulin like peptide
DLPs: dorsal lateral peptidergic neurons
DN1: dorsal neurons 1
Dome: Domeless
Dsor: Downstream of raf
dsRNA: double strand RNA
DTK: Drosophila Tachykinin
DTKR: Drosophila Tachykinin receptor

E

EcR: Ecdysone receptor
EGFR: Epidermal Growth Factor receptor
Egr: Eiger
EH: Eclosion Hormone
elf4E: eukaryotic initiation factor 4E
ERK: Extracellular signal-regulated kinases
ETH: Ecdysis triggering hormone
20-E: 20-hydroxyecdysone
4EBP: eukaryotic initiation factor 4E binding
protein

F

FBS: fat body-derived signals
FoxO: Forkhead Box protein O
Fru: Fruit-less
F₁F₀-ATPase: F₁-F₀ adenosine triphosphatase
synthase

G

GABA: gamma-aminobutyric acid
GABA(B)R1: GABA(B) Receptor 1
GBPs: Growth Blocking Peptides

GCL neurons: Growth coordinating Lgr3 neurons
GCN2: General control nonderepressible 2
GDH: glutamate dehydrogenase
GDP/GTP: Guanosine diphosphate/Guanosine triphosphate
GH: Growth hormone
GHR: Growth hormone receptor
Glu: glutamate
GPCR: G protein coupled receptor
GRASP: Golgi reassembly stacking protein
GRASP: GFP reconstitution across synaptic partners
Grnd: Grindelwald
Gr43a: Gustatory receptor 43a
Gr64a: Gustatory receptor 64a
G-6-P: glucose-6-phosphate

H

Hex-C: Hexokinase C
Hh: Hedgehog
Hugin-PC: protocerebrum Hugin
5-HT_{1A}: serotonin receptor

I

ICN: IPCs-Connecting Neurons
IGF: Insulin Growth Factors
IGFBPs: IGF Binding Proteins
IGF1R: Insulin Growth Factor 1 Receptor
IIS: insulin/IGF signaling pathway
Imp-L2: Imaginal morphogenesis protein-Late 2
Indels: insertions/deletions
InR: insulin receptor
ins1/ins2: insulin 1/insulin 2
IP3: inositol 1,4,5-triphosphate
IPC: Insulin-Producing Cells
IRS: Insulin receptor substrate
IUGR: intrauterine growth retardation

J

JAK/STAT: Janus kinases/signal transducers and activators of transcription
Jeb: Jelly belly
JH: Juvenile hormone
JNK: c-Jun N-terminal kinase

K

K_{ATP}: ATP-sensitive potassium channel
Kir: inward rectifying potassium channel

L

LAT1: L-type amino acid transporter 1
Lgr3: leucine-rich repeat-containing G protein-coupled receptor 3
LPD: low protein diet
Lst: Limostatin
LTP: Lipid Transfer Protein

M

MAPK: Mitogen-activated protein kinases
MAP4K3: Mitogen-activated protein kinase kinase kinase 3
MEK: MAPK/ERK kinase
MIP: MyoInhibitory Peptide
MND: Minidisc
mRNA: messenger RNA
Mth: Methuselah
Mth10: Methuselah like 10

N

NAT1: Nutrient amino acid transporter 1
NLaz: Neural Lazarillo
NS3: nucleostemin 3

O

OAMB: octopamine mushroom body receptor

P

PAT1: proton-assisted SLC36 amino acid transporters 1
PDK1: phosphoinositide dependent kinase 1
PEPCK1: phosphoenolpyruvate carboxykinase 1
PEPT1: oligopeptide transporter 1
PER: proboscis extension response
PG: prothoracic gland
PGC1: PPAR γ coactivator-1

PI: Pars Intercerebralis
PIP2: phosphatidylinositol (4,5)-biphosphate
PIP3: phosphatidylinositol (3,4,5)-triphosphate
PI3K: phosphatidyl-inositol 3'kinase
PKA: protein kinase A
PKC: Protein kinase C
PKG: Protein kinase G
PLC- γ : Phospholipase C γ
PRAS40: proline-rich Akt substrate 40
PTTH: prothoracicotropic hormone

Q

QF/QUAS :Q transcription factor/Q upstream activation sequence
QS : Q suppressor

R

RFP: Red Fluorescent Protein
Rheb: Ras-homolog enriched in brain
RNAi: Ribonucleic acid interference
rRNA: ribosomal RNA

S

SDR: Secreted decoy of InR
Shal: Shaker like
SH3BP4: SH3 domain-binding protein 4
Slif: Slimfast
sNPF: short Neuropeptide F

sNPF receptor 1: short Neuropeptide F receptor 1
Sun: Stunted
Sur: Sulphonylurea receptor
S6K: ribosomal protein S6 kinase

T

TACE: TNF- α converting enzyme
TetX: Tetanus toxin
TGF β : Transforming growth factor β
TH: tyrosine hydroxylase
TNF- α : Tumor necrosis factor α
TOR: Target of Rapamycin
TORC1/TORC2: TOR complex 1/2
TSC1/2: tuberous sclerosis complex 1/2

U

UAS: upstream activation sequence
Upd2: Unpaired 2
USP: ultraspiracle

V

VDCC: voltage-dependent calcium channels
VDRC: Vienna Drosophila Resource Center
VM: Ventromedial
VNC: ventral nerve cord

Table of figures

Figure 1: Regulation of body size.....	24
Figure 2: <i>Drosophila</i> life cycle.....	27
Figure 3: The minimal viable weight and the critical weight are two larval checkpoints.....	28
Figure 4: Ecdysteroidogenesis is promoted by several signaling pathways in the prothoracic gland.....	32
Figure 5: Peaks of ecdysone regulate developmental transitions..	33
Figure 6: Ecdysone pulses are mediated by feedback controls.	34
Figure 7: The insulin/IGF1 signaling pathway	38
Figure 8: Ligand receptor interactions.	40
Figure 9 : Striking conservation of the insulin/IGF signaling pathway between mammals and <i>Drosophila melanogaster</i>	41
Figure 10 : Ubiquitous overexpression of <i>dilp2</i> dramatically increases body size of flies..	42
Figure 11 : Modulation of the ILPs signal after release from the IPCs.	45
Figure 12 : Average height of Norwegian and Finnish boys aged 7-13 years from 1930 to 1960.....	47
Figure 13 : Dietary yeast concentration is positively correlated with final body size in <i>Drosophila melanogaster</i>	48
Figure 14 : The TOR signaling pathway in <i>Drosophila melanogaster</i>	50
Figure 15 : Crosstalks between IIS and TOR signaling pathways.....	51
Figure 16 : Specific knockdown of <i>Slif</i> in the fat body drastically reduces body size.	53
Figure 17 : The larval fat body is a sensor organ of amino acids levels.	54
Figure 18 : Kinetics of <i>Dilp2</i> accumulation within the IPCs upon refeeding.....	55
Figure 19 : Remote control of <i>Dilp2</i> release by the fat body.....	56
Figure 20 : Conventional microbiota and selected <i>Lactobacillus plantarum</i> both promotes body growth upon nutrient shortage in <i>Drosophila</i> and mice.....	58
Figure 21 : Model of central and peripheral sugar sensor by AKH and the cytokine unpaired 2.....	62
Figure 22 : <i>CCHA2</i> relay the sugar information from peripheral tissues to the larval brain and regulates <i>dilps</i> transcription and secretion from the IPCs.	63
Figure 23 : The fat derived <i>Egr</i> remotely controls <i>dilps</i> transcription in response to amino acids deprivation.	66
Figure 24 : Fat-derived GBPs respond to dietary amino acids and promote body growth through remote control of <i>Dilp2</i> secretion.....	67
Figure 25 : Model of direct leucine sensing in the IPCs.	69
Figure 26 : The kinase <i>GCN2</i> is an amino acid imbalance sensor in dopaminergic neurons.....	70
Figure 27 : Central amino acids sensing mediated by the kinase <i>GCN2</i> in <i>DH44</i> expressing neurons promotes food intake.	72
Figure 28 : <i>AKH</i> secretion is induced by hypoglycemia..	73
Figure 29 : <i>AKH</i> is secreted in a sugar-dependent manner.	74
Figure 30 : <i>Limostatin</i> is a decretin and suppress insulin secretion during fasting in adult flies.	75
Figure 31 : Internal fructose levels fluctuate in response to nutritious sugars and are sensed by the fructose receptor <i>Gr43a</i> in the <i>Drosophila</i> brain.....	76
Figure 32 : Sugar sensing by <i>DH44</i> positive neurons in the <i>Drosophila</i> adult brain.....	78
Figure 33 : GABAergic neurons are in close proximity with the IPCs and control their neuronal activity. shown in adults that GABA neurons functionally interact with the IPCs..	80
Figure 34 : Serotonergic neurons project on the IPCs both in <i>Drosophila melanogaster</i> larvae and adult.....	82
Figure 35 : Octopaminergic neurons project on the IPCs and control their neuronal activity.	83
Figure 36 : <i>DLPs</i> neurons projects towards IPCs processes and promote insulin/IGF signaling pathway in peripheral tissues..	84

Figure 37 : Tachykinin positive neurons project towards the IPCs processes and tachykinin signaling in the IPCs inhibits the insulin/IGF signaling pathway..	85
Figure 38 : AstA positive neurons regulate the IPCs activity in both <i>Drosophila</i> larva and adult.....	86
Figure 39 : Hugin neurons located in the protocerebrum make synapses with the IPCs and control their neuronal activity..	87
Figure 40 : Cholinergic neurons regulate the IPCs transcriptional activity. y.	88
Figure 41 : The central circadian clock circuit controls the IPCs electrical activity <i>via</i> their physical interaction with the DN1.....	89
Figure 42 : The ICNs ablation causes bigger animals independently of their gender.....	127
Figure 43 : The ICNs induce lipid mobilization from the fat body.	128
Figure 44 : GBP1 is internalized by EGFR expressing cells.	130
Figure 45 : GBP1 secretion from the fat body is TOR dependent..	131
Figure 46 : Working model explaining how GBPs promote systemic growth depending on dietary amino acids	133
Figure 47 : The fat-derived Sun modulates insulin levels through brain Mth in the IPCs according to nutrients.....	135
Figure 48 : EH neurons neuronal structure.....	137
Figure 49 : EH neurons induce Dilp2 secretion and promote systemic growth.	138
Figure 50 : Possible mechanisms by which EH neurons control the IPCs secretory activity.....	140
Figure 51 : EGF modes of signaling through its receptor EGFR.....	143
Figure 52 : Possible mechanism of Kv4.2 inactivation by MEK, leading to membrane hyperpolarization....	145
Figure 53 : Origins of mitochondrial and endocrine Sun peptide.....	148
Figure 54 : Multiplicity of adipose factors controlling Dilps activity and their modes of action.	150
Figure 55 : Secretory mechanisms of fat-derived signals promoting Dilps activity.	151
Annex 1: The neuronal identity of the ICNs.	159
Annex 2: How the ICNs and the IPCs communicate?	160
Table 1 : Summary of <i>dilps</i> expression during <i>Drosophila</i> development. Temporal and spatial expression pattern of different <i>dilps</i> were analyzed in normal conditions. IPCs: Insulin-Producing-Cells.	43
Table 2 : Anthropometric data of well-nourished versus malnourished children. Insufficient caloric intake or protein consumption both decrease the growth speed and lead to smaller children.	46

Table of contents

Acknowledgments	6
Résumé	11
Summary.....	13
Abbreviations.....	14
Table of figures.....	17
INTRODUCTION	22
Chapter I: How is body growth determined?	23
I- Growth parameters	23
II- <i>Drosophila melanogaster</i> as a model to study body growth.....	25
III- <i>Drosophila</i> life cycle: How do flies grow up?.....	26
Chapter II: Endocrine control of body growth.....	30
I- Ecdysone signaling times the developmental transition.....	30
a. Ecdysone biosynthesis and timing of ecdysone release	30
b. Tissue-specific response to ecdysone.....	33
c. Feedback regulation of ecdysone synthesis to shape ecdysone pulses	34
II- Insulin/IGF signaling promotes the growth rate	35
a. The insulin signaling controls metabolic homeostasis in vertebrates.....	35
b. The IGF signaling promotes systemic growth in vertebrates.....	36
c. Overlaps between insulin and IGF signaling pathways in vertebrates	38
d. Evolutionary conservation of the insulin/IGF pathway between vertebrates and <i>D.</i> <i>melanogaster</i>	40
e. The <i>Drosophila</i> insulin like peptides (Dilps)	41
f. Modulation of the ILPs signal after release	44
Chapter III: Nutritional control of body growth	46
I- Parallel between <i>drosophila</i> and vertebrates	46
II- Cellular nutrient sensing: the TOR signaling pathway	48
III- Crosstalk between the TOR and IIS pathways	51
IV- The fat body is the main nutrient sensing organ.....	52
V- Remote control of Dilps secretion	54

VI- Microbiota-dependent growth acceleration	56
Chapter IV: Central integration of nutrients information.....	59
I- Peripheral nutrient sensing which modulates Dilps secretion	60
a. Peripheral sensing of sugars and/or fat.....	60
1. Unpaired 2	60
2. CCHamide2	62
3. Dawdle.....	63
4. Adiponectin.....	64
b. Peripheral sensing of amino acids.....	65
1. Eiger.....	65
2. Growth-Blocking-Peptides	66
3. Stunted	67
II- Central sensing of nutrients	68
a. Amino acids sensing	68
1. LAT1 transporters: Minidisc.....	68
2. The kinase GCN2.....	69
3. TOR signaling and serotonin	71
4. DH44 positive neurons: CG13248.....	71
b. Carbohydrates sensing	72
1. Corpora Cardiac (CC) cells: AKH and Limostatin	72
2. The Insulin Producing Cells (IPCs) in adult flies	75
3. Gr43a positive neurons	75
4. DH44 positive neurons in adult flies.....	77
Chapter V: Neuronal circuitries at play to control the IPCs secretory activity	79
I- GABAergic circuitry	79
II- Serotonergic neurons	80
III- Octopaminergic circuitry	82
IV- DLP neurons: Short Neuropeptide F (sNPF) and Corazonin	83
V- Tachykinin positive neurons.....	84
VI- Allatostatin A expressing neurons.....	85
VII- Hugin positive neurons	86
VIII- Glia and Cholinergic neurons	87
IX- Dorsal Neurons (DN1)	89
Chapter VI: PhD project	90
RESULTS.....	91

Chapter I: An EGF-responsive neural circuit couples insulin secretion with nutrition in <i>Drosophila</i> .	92
I- Introduction	92
II- Manuscript	93
III- Unpublished results	127
a. The R22H11 construct is a <i>fruit-less</i> GAL4 line - no sex-specific phenotypes.....	127
b. The ICNs promote lipid mobilization	128
c. GBP1 is a <i>bona fide</i> ligand of EGFR	129
d. GBP1 secretion from the fat body is TOR-dependent	130
IV- Conclusion.....	132
Chapter II: <i>Drosophila</i> insulin release is triggered by adipose Stunted ligand to brain Methuselah..	134
I- Introduction	134
II- Publication	134
III- Conclusion.....	135
Chapter III: Eclosion hormone neurons control the IPCs secretory activity – Preliminary data.....	136
I- Processes of the eclosion hormone (EH) expressing neurons project towards the IPCs	136
II- EH neurons promote Dilps release and body growth.....	138
III- Discussion and Conclusion.....	139
DISCUSSION.....	141
I- Fat-derived GBPs are long-ranged EGF ligands	142
II- EGFR function in controlling the ICNs neuronal activity	144
III- ICNs and IPCs neuronal communication.....	146
IV- A conserved mechanism for insulin release	146
V- Sun: a mitochondrial protein with an endocrine function.....	147
VI- Multiplicity of adipose factors responding to nutrients	148
VII- Secretory mechanisms of insulinotropic adipose factors	151
VIII- Diversity of neuronal populations controlling the IPCs secretory activity	152
CONCLUSION	154
MATERIALS AND METHODS.....	156
Fly strains	157
Lipids staining	157
Fat body – Nile red	157
Oenocytes – OilredO	157
ANNEX	158
References	161

INTRODUCTION

Chapter I: How is body growth determined?

I- Growth parameters

How is the size of an organism determined? This is one of the most fundamental aspects of developmental biology that still remains mysterious. One of the most noticeable features among animals are the differences in size. Yet, how can species have different body size and what are the molecular mechanisms involved? Obviously, genes play the predominant part in determining body size but environmental cues such as nutrition also play a role. However, mechanisms underlying systemic size control remain still poorly understood.

Body size is an important feature that conditions many characteristics of adult life such as mate selection, fecundity, predation, tolerance to temperature or starvation (Edgar, 2006). To ensure the emergence of adult with correct body size and proportions, body growth has to be tightly regulated. The size of an organism depends mainly on the number and the size of each cells. This requires different processes. The cell growth defines cell size while the balance between the cell proliferation and cell death determines the cell number (Conlon and Raff, 1999).

These processes are controlled by both local and systemic programs that allows growth coordination between organs to maintain good proportions and plastic adaptation to external cues. Indeed, Donald Metcalf showed that multiple transplanted fetal thymus gland in an isologous mouse, are able to grow to their normal adult size. This indicates that organs have an intrinsic program to control their size (Metcalf, 1963). In contrast, transplanted fetal spleens do not grow to their normal adult size. It is rather the total mass of the transplanted spleens that reach the mass of one adult spleen, demonstrating that the spleen growth is regulated by external signals (Metcalf, 1964). These two experiments clearly show that animal organs reach their adult size under autonomous control which is then modulated by systemic factors like hormones. The stimulating growth effect of the mammalian growth hormone GH is one of the

Introduction

most striking example. Children lacking GH display short stature while children producing more GH present abnormal high stature.

Even though some species continue to grow throughout life, most of animals like mammals, stop growing at some point during development. This defines the entrance in the adulthood and indicates that adult size is fully determined by the growth occurring during juvenile stages. The transition between juvenile and adult stage, called puberty in humans or metamorphosis in invertebrates, allows individual to become sexually matured. Puberty is generally followed by a growth spurt. On the other hand, holometabolous invertebrates do not grow anymore during metamorphosis. However, in both cases, final body size is restricted to the growth period duration. Indeed, children affected by precocious puberty will be smaller adult because of the short period of growth.

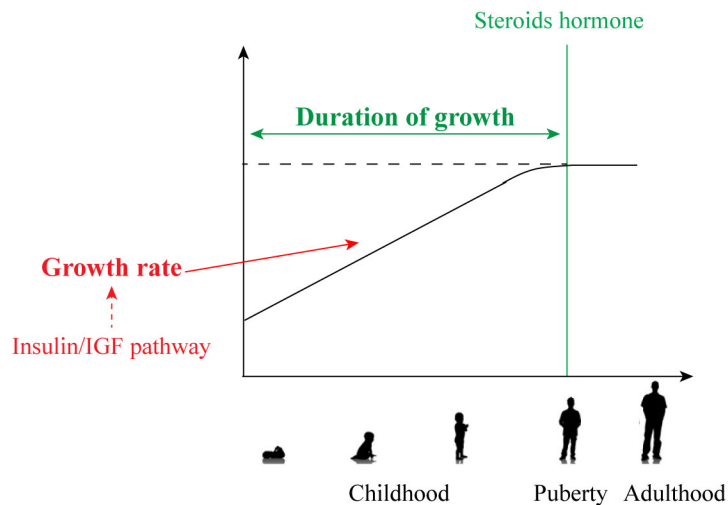


Figure 1: Regulation of body size. Body size is determined by the growth rate and the time of growth controlled by the insulin/IGF pathway and steroids hormone respectively.

Body size is determined by two main features: the speed of growth or growth rate and the duration of growth limited by the onset of maturation and adulthood. Both parameters are controlled by different signaling pathways. The growth rate is regulated by the insulin/IGF signaling pathway, while developmental transition and sexual maturation are determined by steroid hormones (Figure 1).

II- *Drosophila melanogaster* as a model to study body growth

Thomas Hunt Morgan was one of the first geneticist interested by the fruit fly *Drosophila melanogaster*. He identified the white eye-pigment mutation and created the first genetic map based on recombination frequencies. In 1933, he obtained the Nobel prize for his chromosomal theory of heredity. Later in the 20th century, the pioneering work of Ed Lewis, along with the large-scale genetics screens by Christiane Nüsslein-Volhard and Eric Wieschaus, and coupled with the incredible progress in molecular biology, made the drosophila a well-established model that became the main model organism used to study genetics and developmental biology (Arias, 2008; Bier, 2005).

Why is *Drosophila melanogaster* such an attractive model?

Even though drosophila and humans seem to be physically very different, they share the same fundamental biological processes and signaling pathways are highly conserved. Furthermore, the availability of the genome sequencing has shown that even genetically, flies and humans are not so different. Therefore, 75% of genes related to human diseases have their homologues in *Drosophila* (Adams et al., 2000; Banfi et al., 1996; Giot et al., 2003; Pickeral et al., 2000; Reiter et al., 2001). Besides, a broad spectrum of human diseases is recapitulated by disrupting a gene in *D. melanogaster* like neurological disorders, cardiovascular diseases, cancers, developmental disorders, metabolic dysfunctions, immune system disorders...

The fruit fly *Drosophila melanogaster* is one of the most widely used model system in biological research thanks to the development of powerful genetic tools. To assess gene function, loss-of-function mutant animals can be quickly made. In addition, gene relationships are also elucidated by epistasis experiments.

Gene expression in a specific tissue or group of cells can be altered by techniques like binary expression system such as the UAS/Gal4 system (Brand and Perrimon, 1993), LexA/LexAop (Lai and Lee, 2006) and QF/QUAS (Potter et al., 2010), or generation of mosaics within a wild type tissue (Xu and Rubin, 1993). Many signaling pathways and new genetic loci were discovered thanks to these techniques.

Furthermore, the combination between different binary expression systems (UAS/Gal4 and LexA/LexAop) allows to manipulate gene expression simultaneously in two different tissues *in vivo* and can be useful for the study of interorgan communication (Del Valle Rodríguez et al., 2011).

New mutant animals can now be quickly generated using the CRISPR-Cas9 (Clustered, Regularly Interspaced, Short Palindromic Repeat) technique. RNA-guided DNA endonuclease Cas9 has dramatically opened opportunities for creating double-strand breaks in the genome of organisms, including flies. In brief, the CRISPR/Cas9 can be used to induce mutagenesis but also to tag endogenous

Introduction

proteins (Korona et al., 2017), thus permitting to simplify protein imaging or biochemistry at the cellular or subcellular level.

Importantly, a large number of transgenic fly lines containing endogenous mutations, P element insertions, RNAi, inversions, duplications, are available (Bloomington Drosophila Stock Center, VDRC, Kyoto) and listed (flybase). Moreover, the genome of *Drosophila melanogaster* and its twelve closest related species have been entirely sequenced and annotated in databases reporting gene expression pattern as well as protein-protein interaction (St. Pierre et al., 2014).

Overall, *Drosophila melanogaster* has emerged as an excellent model to elucidate the basic regulatory mechanisms by which development ensures that organisms reach appropriate body size with correct proportions (Edgar, 2006; Mirth and Riddiford, 2007).

III- *Drosophila* life cycle: How do flies grow up?

Drosophila melanogaster has a very short life cycle that lasts around two months. The fruit fly development is very similar to the mammalian development and goes through 4 different developmental steps (Figure 2) (Tennessen and Thummel, 2011):

- the embryonic development,
- a juvenile growth phase, which corresponds to the larval stage in *Drosophila* or childhood for humans,
- the sexual maturation, called either metamorphosis/pupal stage in insects or puberty in humans, triggered by an increase in circulating steroid hormone,
- the adulthood which is the reproductive stage.

After fertilization, the embryo develops and body axes are formed, followed by cellularisation, gastrulation, segmentation and elaborated morphogenetic events that end up with eclosion of a larva (Rivera-Pomar and Jäckle, 1996).

Introduction

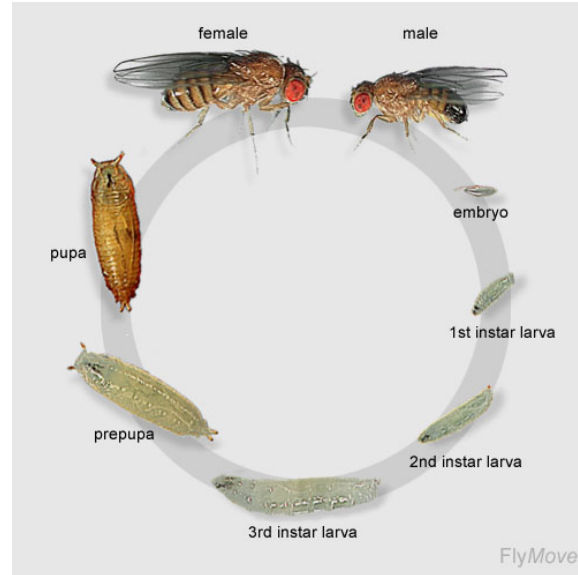


Figure 2: Drosophila life cycle. After fertilization, the embryo develops and the larva hatches. During the three larval stages, the larva grows, then undergoes metamorphosis and pupate. Pupa experiences morphogenetic events and finally give rises into an adult fly.

Adapted from <http://www.compostadores.com/descubre-el-compostaje/biodiversidad-en-mi-compostador/164-las-mosquitas-de-la-fruta.html>

The larval phase is fractioned in 3 different stages, from L1 to L3, all separated by molts. During molts, the old cuticle is shed and replaced by a larger one, accommodating the increase in animal body size. Two different types of tissues are present in larvae: the larval tissues and the imaginal tissues. The cell size of the larval tissue increases by endoreplication cycles, a process of chromosomal replications without any cellular division. This increase in DNA content allows cells to become severely larger in volume and is responsible for most of the remarkable 200-fold increase body mass (Church and Robertson, 1966). This body mass increase due to the growth of endoreplicative larval tissues, is mainly controlled by the TOR and insulin signaling pathways. Conversely, imaginal tissues are proliferative tissues. They are larval epithelial precursor organs that will give rise to foremost adult body parts and appendages. During the larval stage, even though their development fate in the adult is already established, the cells of the growing imaginal disc seem undifferentiated (Beira and Paro, 2016). By mid to late first instar, mitosis is restarted in imaginal disc and cells will divide exponentially during the L2 and L3 stages. This mechanism allows to end up with 50 000 cells per disc before pupariation.

Two size checkpoints, discovered in *Manduca sexta*, occur during larval development: the minimal viable weight and the critical weight. The minimal viable weight reflects the amount of nutrient storage necessary to survive to metamorphosis while the critical weight is the minimal size required to undergo complete metamorphosis (Mirth and Riddiford, 2007; Nijhout, 1975; Nijhout and Williams, 1974) (Figure 3).

Introduction

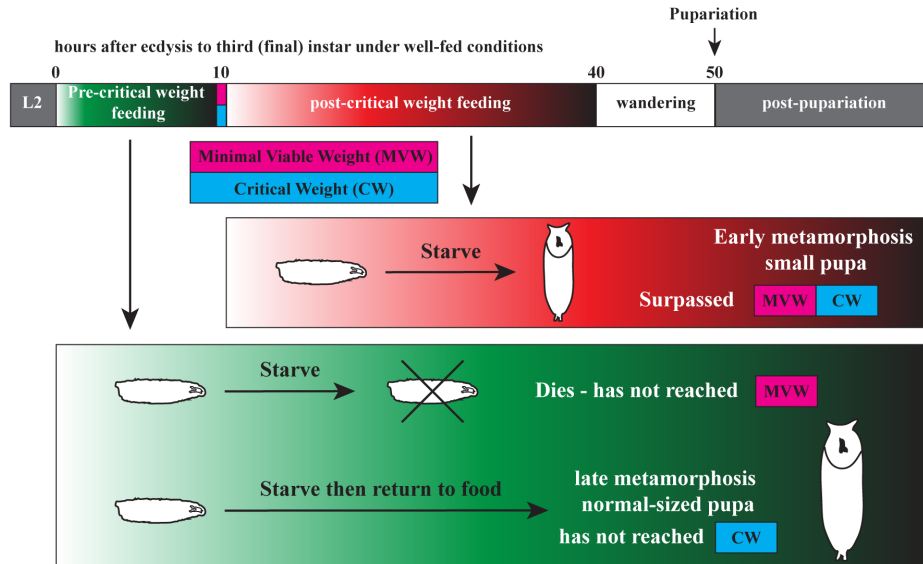


Figure 3: The minimal viable weight and the critical weight are two larval checkpoints. Upon starvation, larvae that did not reach the minimal viable weight die, while the ones that has not reach the critical weight stall their larval development until growing conditions improve. Conversely, post MVW and CW larvae undergo pupariation upon starvation.

Adapted from Mirth C.K. and Riddiford L.M. (2007) – Size assessment and growth control: how adult size is determined in insects.

Indeed, developmental progression and larval growth are coordinated with nutrient availability, uptake and utilization by genetic mechanisms. For instance, starved larvae prior to the critical weight, will stall their larval development until growth conditions improve without affecting adult size. Conversely, post-critical weight larvae that experience starvation will stop growing and will undergo metamorphosis, resulting in smaller fertile adults (Edgar, 2006; Mirth and Riddiford, 2007).

By the end of larval development, environmental cues (nutrition, temperature, oxygen) and hormonal factors (ecdysone) relieve the inhibition at play during larval stages, to trigger metamorphosis into pupa.

During metamorphosis, a major morphogenetic event takes place in mature imaginal discs that will evert through their stalk in an ecdysone dependent manner (Fristrom D. & Fristrom J.W., 1993). Most of the larval tissues (midgut, salivary glands, some muscles) undergo intense remodeling and histolysis by autophagy in response to ecdysone. Nevertheless, this cell removal requires upregulation of pro apoptotic genes and caspases (Cakouros et al., 2004; Lee et al., 2002; Martin and Baehrecke, 2004; Waldhuber et al., 2005). The fat body, functional homolog of the vertebrate fat and liver, experience intense remodeling since it is dissociated into individual fat cells that will be removed by cell death during early adulthood (Nelliot et al., 2006).

Introduction

Overall, during metamorphosis intense remodeling and changes occur in the pupal body structure and will result in adult eclosion with reproductive capabilities.

Holometabolous insects, like *Drosophila melanogaster*, do not grow as adults. This means that body size in insects is roughly due to the speed of growth during the juvenile/larval stage and the duration of this growth phase (Edgar, 2006). To adjust body size depending on external cues and intrinsic program, different signaling pathways are at play. The steroid hormone ecdysone controls the growth period while the insulin/IGF signaling pathway regulates the growth rate.

Chapter II: Endocrine control of body growth

I- Ecdysone signaling times the developmental transition

Three different hormones are key regulators of developmental timing: the molting hormone ecdysone, the prothoracicotropic hormone (PTTH) and the juvenile hormone (JH).

The central regulator of developmental transitions (molts and metamorphosis) in insects is the steroid hormone ecdysone. Temperature-sensitive ecdysone null mutants *ecd^l*, induced at early third-instar stage, fail to pupariate and persist as L3 instar larvae for three weeks. Providing ecdysone in the food restore pupariation (Garen et al., 1977). The actions of the ecdysone are modulated at different levels: ecdysone biosynthesis, timing of ecdysone release, tissue-specific response to ecdysone and feedback regulation of ecdysone signaling. Since I am interested in the growth period, I will mainly focus on the molecular events required for larval to pupal transition such as PTTH and ecdysone release.

a. Ecdysone biosynthesis and timing of ecdysone release

After reaching the critical weight, the molting hormone ecdysone is produced by the prothoracic gland (PG) (primary source of ecdysone), and therefore induces metamorphosis. The primary tropic factor for the ecdysteroidogenic activity of the PG is the prothoracicotropic hormone (PTTH), a neuropeptide produced by two pairs of bilateral neurosecretory cells in the brain (Smith and Rybczynski, 2012; Yamanaka et al., 2013).

PTTH is the major but not the only, developmental signal that triggers the onset of ecdysteroidogenesis. Indeed, PTTH neuronal ablation results in delayed pupariation by 4 to 6 additional days and gives rise to bigger flies while *pth^{-/-}* null mutant flies have only one day delay in pupariation,

Introduction

suggesting that additional ecdysteroidogenic signal(s) are produced by the PTTH neurons (Ghosh et al., 2010; McBrayer et al., 2007; Shimell et al., 2018). Furthermore, Garen et al. (1977) demonstrated that ecdysone is required and sufficient to trigger metamorphosis. However, even though it is delayed, metamorphosis still occurs in *ptth*^{-/-} null mutant and PTTH neuronal ablated flies, suggesting that PTTH and PTTH neurons are important to time the onset of metamorphosis but not essential for the metamorphosis to happen.

PTTH is produced as a prohormone, then processed into an active mature form (Kawakami et al., 1990). PTTH release occurs at particular developmental stages and depends on endocrine control but also environmental cues like photoperiod (Di Cara and King-Jones, 2013; McBrayer et al., 2007; Steel and Vafopoulou, 2006). However, several studies showed that multiple factors act on the PG to coordinate the ecdysone synthesis and release, suggesting that the PG itself is more likely to orchestrate developmental transition. The insulin/IGF signaling pathway in the PG promotes its growth (Colombani et al., 2005; Mirth et al., 2005) leading to the hypothesis that PG growth acts as a sensor for the metabolic status of the organism (Mirth and Riddiford, 2007). However, part of the ecdysteroidogenic effect of the insulin/IGF signaling could be due to its potential crosstalk with the MAPK signaling (Kim et al., 2004). Indeed, like insulin signaling, upon MAPK signaling activation, the PG cell growth is augmented while its downregulation results in developmental delay and body overgrowth similar to what is obtained in PTTH or Torso deficient larvae (Caldwell et al., 2005; Rewitz et al., 2009a). The TOR signaling pathway in the PG is also important to link nutritional inputs to ecdysone production after the critical weight (Layalle et al., 2008). Two others regulatory factors act on the PG to ensure proper steroidogenesis: the TGFβ /Activin signaling and the nitric oxide (Bialecki et al., 2002; Cáceres et al., 2011; Gibbens et al., 2011; Parvy et al., 2005) (Figure 4).

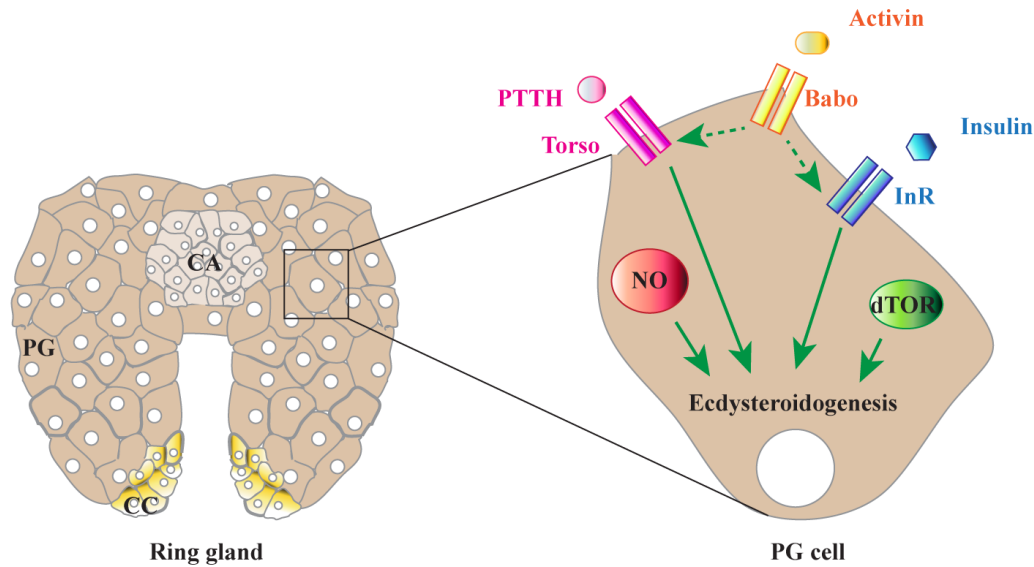


Figure 4: Ecdysteroidogenesis is promoted by several signaling pathways in the prothoracic gland. The PTTH, Activin, Insulin, TOR and NO signaling act in concert to ensure proper ecdysone production. Taken from Yamanaka et al., (2013) – Ecdysone control of developmental transitions: Lessons from *Drosophila* research.

To initiate a transition between two developmental stages, secreted PTTH will dimerize through cysteines bounds and activate its receptor TORSO located on the PG (Rewitz et al., 2009a). The MAPK signaling cascade is induced and controls acute and long-term regulation of ecdysteroidogenesis through translation and post-translational modifications as well as transcription of some ecdysteroidogenic enzymes in PG cells (Gibbens et al., 2011; McBrayer et al., 2007; Ou et al., 2011; Rewitz et al., 2009b).

In lepidopterans, whether the larvae undergo simple molt or metamorphosis depends also on the presence or absence of the juvenile hormone. When JH levels are high, larval to larval molt occurs while larval to pupal transition requires low JH levels (Doane, 1973; Mizoguchi, 2001; Riddiford, 1970a, 1970b; Truman and Riddiford, 1974). Nevertheless, whether JH titer drop induces PTTH secretion from the PG cells in *Drosophila* remains unclear (Riddiford, 2011; Riddiford et al., 2010).

Small peaks of secreted ecdysone by the PG in response to PTTH induce larval molts (L1 to L2 and L2 to L3 transition). During the third instar, ecdysone titers gradually increase to reach several plateaux: a first increase at the time of critical weight (8-10 hours after L2-L3 transition), a second rise at 18-20h followed by a steady rise to a peak at wandering stage (Shimell et al., 2018). The small peaks prior to wandering stage provoke behavioral and developmental changes (Warren et al., 2006) such as feeding cessation, wandering behavior, onset of *glue* gene expression in the salivary glands that allows the puparium to adhere to its substrate (Andres et al., 1993) and the fat body autophagy (Rusten et al.,

2004). At the end of the third instar, an elevated peak of ecdysone triggers the larval-pupal transition, cessation of growth and metamorphosis (Gokhale and Shingleton, 2015) (Figure 5).

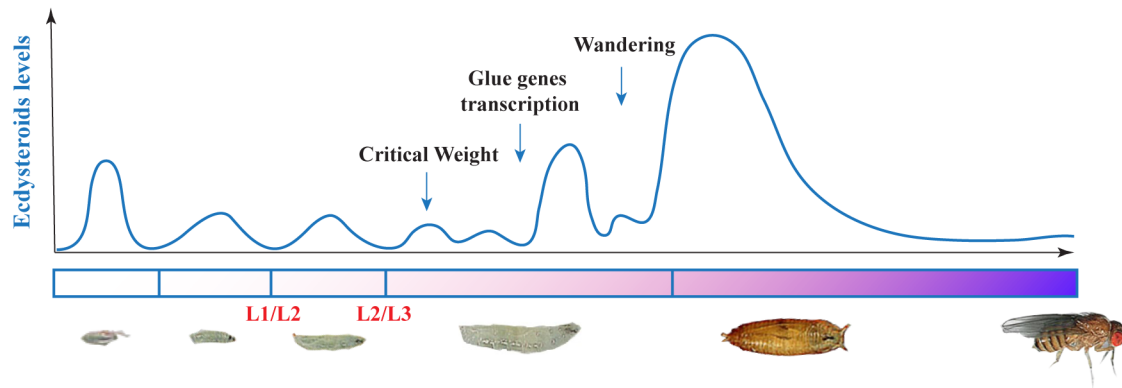


Figure 5: Peaks of ecdysone regulate developmental transitions. These peaks control larval molts (L1 to L2 and L2 to L3), critical weight attainment, glue genes transcription, feeding cessation and pupariation. Adapted from Gokhale and Shingleton (2015) – Size control: the developmental physiology of body and organ size regulation.

b. Tissue-specific response to ecdysone

After its secretion into the hemolymph, several peripheral tissues (fat body, gut, Malpighian tubules) uptake the ecdysone and the P450 enzyme converts it into the biological active hormone 20-hydroxyecdysone (20-E) (Petryk et al., 2003). The released 20-E will then act on a heterodimer of primary ecdysone-inducible nuclear receptors: ecdysone receptor (EcR) and ultraspiracle (USP) (Koelle et al., 1991; Oro et al., 1990). The 20-E-EcR-USP complex induces a primary response gene independent of protein synthesis and later a secondary response gene.

The 20-E is involved in different biological processes depending on the tissue target and the developmental stage such as morphogenetic, apoptotic, physiological, reproductive and behavioral responses. How does systemic 20-E achieve this wide range effect? Differential sensitivities of ecdysone-inducible genes to ecdysone concentration, differential EcR isoforms expression as well as primary ecdysone-inducible transcription factors could explain this diversity (Yamanaka et al., 2013).

c. Feedback regulation of ecdysone synthesis to shape ecdysone pulses

In order to obtain pulses of ecdysone temporally regulated, feedforward and feedback loops modulate ecdysone synthesis by the PG, allowing differential synthesis of ecdysone depending on the developmental stage. Indeed, *EcR* is expressed in the PG at the beginning of pupariation, implying that the PG directly sense circulating levels of ecdysone (Talbot et al., 1993).

Depending on ecdysone levels, differential regulations on ecdysteroidogenesis occur in the PG. Upon low ecdysone titer, ecdysteroidogenesis is promoted through increased *torso* expression, leading to augmented PTTH sensitivity (Young et al., 2012). Ecdysone signal is also amplified by the EcR autoregulatory loop, through which EcR stimulates its own expression (Koelle et al., 1991).

By contrast, upon high ecdysone titer, the PG sensitivity to PTTH is reduced (Gilbert et al., 1997; Song and Gilbert, 1998), probably because of *torso* and/or PTTH signaling components downregulation. Accordingly, PTTH has been shown to lower *torso* expression (Puig et al., 2003). Additionally, other studies demonstrated that ecdysone-inducible genes inhibit ecysteroidogenesis in the PG (King-Jones et al., 2005; Ou et al., 2011; Rewitz and O'Connor, 2011).

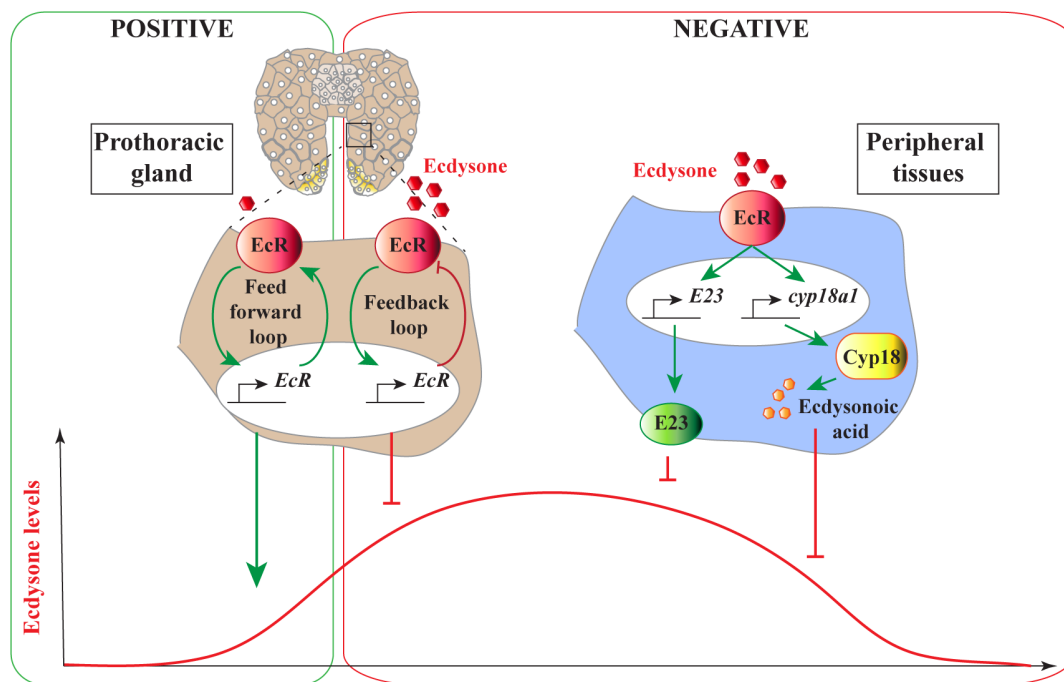


Figure 6: Ecdysone pulses are mediated by feedback controls. Feedforward and feedback loops on the PG controls ecdysone production whereas peripheral tissues adjust ecdysone clearance in order to generate ecdysone pulses.

Adapted from Rewitz K. et al. (2013) – Chapter One – Developmental checkpoints and feedback circuits time insect maturation.

Introduction

To generate ecdysone pulses, hemolymph has to be cleared. The oscillation of circulating ecdysone concentration is due to ecdysone synthesis regulation but also to regulated degradation by peripheral tissues. The ecdysone inducible gene *Cyp18a1* convert 20-E in the inactive form Ecdysoneic acid (Guittard et al., 2011; Rewitz et al., 2010) while *E23* which encodes an ABC transporter, is believed to pump 20-E out of the cell (Hock et al., 2000) (Figure 6).

Overall, these central and peripheral feedback mechanisms ensure proper ecdysone pulses to trigger metamorphosis at the right moment, allowing physiological growth period and consequently emergence of adult with correct size.

II- Insulin/IGF signaling promotes the growth rate

Key traits of life are regulated by the insulin/IGF molecules and their receptors like growth, metabolism and reproduction depending on the developmental stage. In vertebrates, insulin controls metabolic functions while Insulin Growth Factors (IGF) determine systemic growth (Nakae et al., 2001). In contrast, the insulin/IGF signaling pathway in invertebrates has a dual function: maintain the metabolic homeostasis and ensure body growth. First, I will briefly introduce the physiological function of insulin, IGFs, and their signaling pathways in vertebrates. Second, I will examine similarities and differences of these mechanisms in invertebrates, and finally, I will focus on the *Drosophila* insulin like peptides and their mode of action.

a. The insulin signaling controls metabolic homeostasis in vertebrates

Animals need energy to live. This energy comes from nutrition that has to be stored and used upon needs. In order to maintain this metabolic homeostasis, organisms developed different endocrine control involving anabolic hormones that promote nutrients absorption and storage, with catabolic hormones that induce breakdown of large molecules into smaller units used to produce energy. Insulin is the main anabolic hormone of the body. It is involved in carbohydrates, fats and proteins metabolisms by stimulating glucose absorption from peripheral tissues.

Introduction

The insulin is produced by the β cells of the pancreatic islets. Right after a meal, in response to increased circulating glucose levels, two waves of insulin are secreted by β cells: a rapid first phase release and a second sustained phase release.

Once insulin is secreted in the bloodstream, it activates the tyrosine kinase insulin receptor (InR) present on target tissues such as muscles, adipose tissue and liver. The InR is then autophosphorylated on tyrosines, and triggers a phosphorylation cascade that induces glycogenesis, lipogenesis, and stimulates protein synthesis while it inhibits glycolysis, glycogenolysis, lipolysis, proteolysis but also gluconeogenesis from the liver (Dimitriadis et al., 2011).

Some insulin resistant patients display mutations on the InR. The same mutations in mice recapitulate equivalent metabolic phenotype (Accili et al., 2001). Moreover, despite elevated insulinemia, mice deficient for InR fail to restore glycemia upon feeding (Nakae et al., 2001). Then, animals develop impaired insulin secretion, become diabetics and prematurely die. Apart from hyperglycemia, InR null mutant mice present other metabolic defects such as high levels of triglycerides and free fatty acids that lead to hepatic steatosis, reduced hepatic glycogen content and decreased amount of white and brown adipose tissues due to reduced cell fat content (Cinti et al., 1998).

InR deficiency phenotype mimics mice lacking both non-allelic insulin genes *ins1* and *ins2* (Duvill   et al., 1997). Similarly, mice lacking downstream components of the insulin signaling pathway also show such metabolic defects. For instance, null mutant mice for either Insulin receptor substrate 1 (IRS1) or 2 (IRS2) both develop insulin resistance (Araki et al., 1994; Kadowaki, 2000; Kubota et al., 2000; Tamemoto et al., 1994; Terauchi et al., 1997; Withers et al., 1998). Conversely, IRS3 null mutant mice do not display such features, probably because of IRS1 and IRS2 compensation (Liu et al., 1999).

Taken together, these data clearly demonstrate that the insulin and its downstream signaling pathway are mainly involved in glucose homeostasis in vertebrates.

b. The IGF signaling promotes systemic growth in vertebrates

Different genes encode insulin super family related peptides in vertebrates: the two non-allelic insulin genes (in rodents), the two *igf*, the insulin like peptides and relaxin molecules (Bathgate et al., 2013; Nakae et al., 2001). There are two Insulin Growth Factors named IGF1 and IGF2 in vertebrates which share high sequence similarities with pancreatic insulin. During development, they promote growth

Introduction

of somatic tissues such as skeletal muscles and bones (Maki, 2010) but they also participate in axon regrowth and central nervous system myelination (Beck et al., 1995).

Conversely to *Igf1*, most of the tissues do not express *Igf2* after birth (DeChiara et al., 1990). Indeed, IGF2 is only important for pre-natal growth while IGF1 is required for both pre-and post-natal growth (Liu et al., 1993; Lupu et al., 2001). Postnatally, IGF1 is known to act as a general growth promoter by stimulating cell division (Efstratiadis, 1998). Similar to murine models, human patients with homozygous *Igf1* deficiencies display severe intrauterine growth retardation and post-natal growth failure (Woods et al., 1996) as well as decreased bone mineral density (Woods et al., 2000), microcephaly and mental retardation (Hwa et al., 2013). Despite its ubiquitous expression, IGF1 is mainly produced by the liver, suggesting that IGF1 promotes tissue growth in an endocrine but also in an autocrine/paracrine way (LeRoith et al., 1995). In addition, tissue specific *Igf1* null mutant support the idea that circulating IGF1 exclusively comes from the liver (Sjögren et al., 1999; Yakar et al., 1999).

It is broadly recognized that IGF1 production by hepatic cells is stimulated by the Growth Hormone (GH) through its receptor GHR, to promote post-natal peripheral tissue growth (Daughaday and Rotwein, 1989). *Ghr* null mutant mice display a tremendous drop in hepatic IGF1 synthesis, undetectable IGF1 circulating levels and consequently, post-natal growth retardation. However, the GH and IGF1 mode of actions are not so linear. Indeed, *Igf1* and *Ghr* double null mutant mice are more growth retarded than single knockout mice, suggesting a synergic effect of GH and IGF1 (Lupu et al., 2001).

All these data strongly suggest that different type of growth exist: GH-dependent, IGF1-dependent, both GH and IGF1 dependent and last, GH and IGF1 independent growth (Lupu et al., 2001).

Equally to *Igf1* deficiency, null mutant mice for *Insulin Growth Factor 1 Receptor (Igf1r)* present intrauterine growth retardation, muscular hypoplasia, delayed ossification and thin epidermis (Liu et al., 1993). Despite their rarity, human patients with *Igf1r* deficiency present pre- and postnatal growth failure as well as mental retardation (Roback et al., 1991; Tamura et al., 1993).

Altogether, these studies undoubtedly assign the growth promoting role to the IGFs and their downstream signaling pathway in vertebrates.

c. Overlaps between insulin and IGF signaling pathways in vertebrates

The IGFs and the insulin are close related molecules that act on three different receptors: the IGF1R, the IGF2R and the InR respectively. The IGF1R and InR belong to the family of ligand-activated receptor kinases. The distinctive feature of these two receptors is their ability to exist at the cell surface as homodimers or as heterodimers (Ward et al., 2007). Upon ligand binding, they become autophosphorylated (Wei et al., 1995) which enables them to phosphorylate different substrate proteins in order to ensure growth or metabolic responses (Schlessinger, 2000). Phosphorylated receptors recruit the adaptor proteins IRS1, IRS2 and ShC. The interaction of IRS1 and IRS2 with InR/IGF1R induces the activation of the phosphatidylinositol 3'kinase (PI3K) which in turn converts PIP2 (phosphatidylinositol (4,5)-biphosphate) in PIP3 (phosphatidylinositol (3,4,5)-triphosphate). PIP3 recruits Akt /PKB (protein kinase B) and PDK1 (phosphoinositide dependent kinase 1) at the plasma membrane, which enables threonine phosphorylation of Akt by PDK1. To become fully active, Akt need to be phosphorylated in a serine residue by the TORC2 complex. Activated Akt will then phosphorylates downstream signaling molecules including the transcription factor Forkhead Box protein O (FoxO). Phosphorylation of FoxO triggers its translocation from the nucleus to the cytoplasm therefore promoting cell proliferation. In addition to FoxO phosphorylation, Akt also phosphorylates cytosolic and nuclear proteins involved in cell survival and metabolism (Manning and Cantley, 2007) (Figure 7).

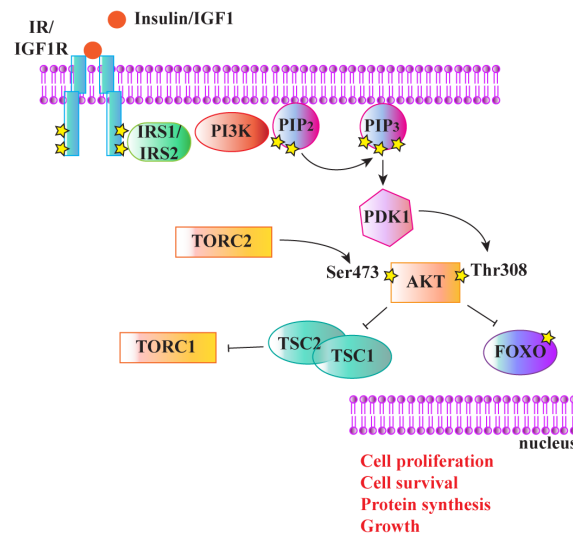


Figure 7: The insulin/IGF1 signaling pathway. Insulin/IGF1 ligand interact with the IR/IGF1R and leads to the receptor autophosphorylation and recruitment of IRS1 and IRS2. The PI3K is activated and converts PIP2 in PIP3. Then, AKT is recruited at the cell membrane and phosphorylated by PDK1 and TORC2 in order to become fully activated. Activated AKT phosphorylates several targets such as FoxO and promotes cell proliferation, survival, protein synthesis and growth.

Adapted from Jin Jung H. and Yousin Suh, (2014) - Regulation of IGF-1 signaling by microRNAs

Introduction

The distinction between insulin and IGFs functions in vertebrates is broadly recognized. For sake of simplicity, scientists have defined the following paradigm: insulin is devoted to metabolism while IGFs are involved in systemic growth. Despite these distinct developmental roles for insulin/IGFs receptors and signaling pathways, some overlaps have been discovered by targeted gene mutations.

Strikingly, severe pre- and postnatal growth retardation are observed in human lacking the InR, with decreased trophic actions on adipose tissue (Jospe et al., 1996; Krook et al., 1993; Wertheimer et al., 1993). Conversely, even though *ins1* and *ins2* null mutant mice display severe growth impairment, mice lacking *InR* are just slightly smaller in size at birth (-10%). This phenotype can be explained by partial IGF1R compensation (Louvi et al., 1997).

Additionally, *Igf1r* deficient mice also develop metabolic defects such as hyperglycemia and decreased β cell mass (Withers et al., 1999). Furthermore, according to its effector role of InR and IGF1R, deficiency in *Irs1* give rises to mice with pre- and postnatal growth retardation and metabolic defects (Tamemoto et al., 1994). These data strongly support the idea that both insulin and IGFs ensure developmental functions, control metabolism and systemic growth.

Mice with combined gene ablations helped the scientific community to decipher interactions among ligands and receptors of the Insulin/IGF family.

As *Igf1 Igf1r* double null mutant mice have the same phenotype than *Igf1r* deficient mice, it implies that IGF1 signals through IGF1R exclusively (Liu et al., 1993). However, deficient mice for *Igf1* show infertility while *Igf1r Igf2r* double null mutants are fertile, suggesting that IGF1 signaling through InR is sufficient to restore reproductive function (Ludwig et al., 1996). When mice lack both *Igf1r* and *Igf2r*, they do not have any abnormal phenotype, indicating that IGFs are able to promote tissue growth through InR (Ludwig et al., 1996). Since murine IGF2 have been shown to be involved in embryonic growth exclusively (DeChiara et al., 1990) it is most likely IGF2 binding on InR that promote tissue growth in the *Igf1r* and *Igf2r* deficient mice.

Surprisingly, new born *Igfr2* deficient mice showed increased circulating and tissue levels of IGF2, with 40% increase in size due to general organomegaly, polydactyly and edema (Lau et al., 1994). These data suggest that IGF2R is important for IGF2 clearance. Besides, IGF2 clearance defects result in fetal organ overgrowth suggesting that IGF2 signals through IGF1R.

Unexpectedly, combined gene ablations revealed distinct but certainly overlapping functions of insulin, IGFs and their receptors on metabolism, systemic growth and reproduction of vertebrates (Figure 8).

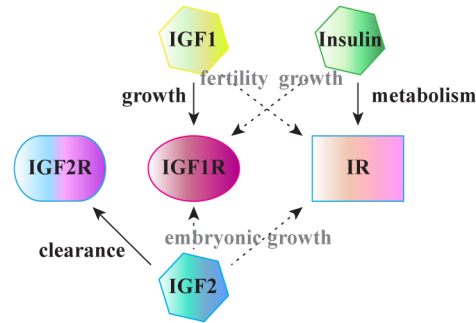


Figure 8: Ligand receptor interactions. Single and combined knockout mice reveal the possible interaction between insulin, IGF1, IGF2 and their receptors InR, IGF1R and IGF2R. Even though each ligand has a better affinity for its own receptor, it can bind to others in order to fulfill different physiological functions.

Adapted from Nakae J. et al. (2001) – Distinct and overlapping functions of insulin and IGF-I receptors.

d. Evolutionary conservation of the insulin/IGF pathway between vertebrates and *D. melanogaster*

The insulin/IGF signaling pathway (IIS) is highly conserved in *Drosophila*. In vertebrates, IGF1R, IGF2R and two InR isoforms, InR-A and -B exist. Conversely, only one InR exists in *Drosophila*. The *Drosophila* InR and the mammalian ones are highly homologous: they are tetramers, both composed of two α and two β subunits containing the ligand binding domain, and the transmembrane and cytoplasmic domains respectively (Seecof and Dewhurst, 1974). Additionally, the mammalian insulin, but not IGF1, binds the *Drosophila* InR and triggers the activation of the downstream components (Marin-Hincapie and Garofalo, 1995; Petruzzelli et al., 1985). Interestingly, the *Drosophila* InR, Human InR and IGF1R share a comparable level of amino acids identity (37% identity).

Intriguingly, InR loss of function mutations or knockdown in *Drosophila* demonstrate that the IIS promotes systemic growth, longevity and fertility (Partridge and Gems, 2002). Similarly, loss of Chico, which is the only *Drosophila* homolog of IRS1-4, induces developmental delay, reduced body size, increased fat and sterility (Böhni et al., 1999) while overexpression of InR, PI3K or Akt result in body and tissue overgrowth (Leevers et al., 1996). Mutations of the IIS lead to change in cell size but also cell number (Böhni et al., 1999; Brogiolo et al., 2001).

Furthermore, ligand binding to *Drosophila* InR triggers the recruitment of the Insulin Receptor Substrate Chico, leading to activation of the PI3K/PDK1/Akt signaling similar to what happens in vertebrates. Likewise, activated Akt will then phosphorylates downstream signaling molecules including

Introduction

the transcription factor FOXO, sole *Drosophila* homolog of FOXO 1,3,4 and 6, involved in cell proliferation and metabolism (Figure 9).

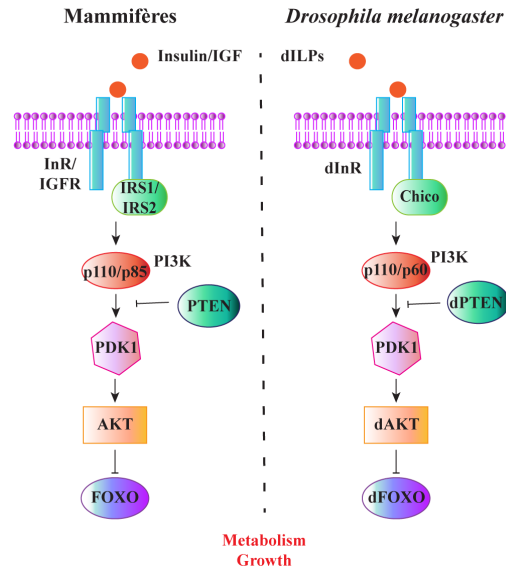


Figure 9 : Striking conservation of the insulin/IGF signaling pathway between mammals and *Drosophila melanogaster*.

Adapted from Garofalo R. S. (2002) – Genetic analysis of insulin signaling in *Drosophila*.

Overall, these data show a striking conservation of the IIS pathway and functions during evolution.

e. The *Drosophila* insulin like peptides (Dilps)

Drosophila possesses 8 *Drosophila* insulin like peptides (Dilps), named from Dilp1 to Dilp8. Dilp1-Dilp5, Dilp7 are structurally comparable to preproinsulin while Dilp6 is more similar to IGF (Brogiolo et al., 2001). Dilp8 is a relaxin peptide (Colombani et al., 2012; Garelli et al., 2012).

Except from Dilp8, the other seven Dilps bind InR, activate the canonical IIS promoting systemic growth and anabolism. Ubiquitous overexpression of each of these seven *dilps*, using the weak *armadillo GAL4* driver, result in increased adult body weight. The strongest overgrowth phenotype was obtained with *dilp2* (Ikeya et al., 2002), suggesting that Dilp2 is the most closely related Dilp to mature insulin (Brogiolo et al., 2001) (Figure 10).

Introduction

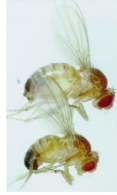


Figure 10 : Ubiquitous overexpression of *dilp2* dramatically increases body size of flies. *Drosophila* overexpressing *dilp2* (top) is bigger than control fly (bottom). Taken from Brogiolo W. et al. (2001) – An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control.

Conversely, individual or combined knockout mutations of the seven *dilp* genes lead to smaller animals with metabolic defects, confirming their implication on body growth control and metabolic homeostasis (Grönke et al., 2010). Additionally, these experiments reveal the compensatory regulation among *dilp* genes and demonstrate that they are partially redundant. Indeed, Gronke S. et al. (2010) showed that in *dilp2* and *dilp2-3* mutants, *dilp5* transcripts are upregulated while elevated transcripts levels of *dilp3* have been found in *dilp2* and *dilp5* mutants. Likewise, *dilp6* transcripts are increased in *dilp2-3-5* mutants.

Interestingly, studies have shown that *dilp* genes expression is spatially and temporally different (Table 1).

Gene	Expression throughout development
<i>dilp1</i>	High expression in larval IPCs and during non-feeding stages (pupa, early adulthood) (Liu et al., 2016; Rulifson et al., 2002; Slaidina et al., 2009)
<i>dilp2</i>	Ubiquitous low signal in imaginal discs (Brogiolo et al., 2001), high signal in the IPCs (Brogiolo et al., 2001; Broughton et al., 2005; Ikeya et al., 2002; Liu et al., 2016; Rulifson et al., 2002), in salivary glands (Brogiolo et al., 2001) and in a subset of glial cells (Chell and Brand, 2010). In the embryo, high signal in the midgut, low signal in mesoderm stage 12-16 (Brogiolo et al., 2001).
<i>dilp3</i>	High signal in the IPCs (Brogiolo et al., 2001; Ikeya et al., 2002; Rulifson et al., 2002), expressed in some glial and neural cells (Sousa-Nunes et al., 2011), highly expressed during early pupal stage (Okamoto et al., 2009). In the adult, <i>dilp3</i> is expressed in the midgut circular muscles (Veenstra et al., 2008) and the IPCs (Broughton et al., 2005; Liu et al., 2016).
<i>dilp4</i>	High expression in larval midgut. In the embryo, high signal in mesoderm stage 2-6, anterior midgut rudiment (Brogiolo et al., 2001).
<i>dilp5</i>	High signal in the IPCs (Brogiolo et al., 2001; Ikeya et al., 2002; Rulifson et al., 2002), moderate signal in gut (Brogiolo et al., 2001), signal in the ovary (Broughton et al., 2005), renal tubules (Söderberg et al., 2011) and IPCs of adults (Broughton et al., 2005; Liu et al., 2016)

Introduction

<i>dilp6</i>	Low signal in gut, High signal in larval, pupal and adult fat body (Bai et al., 2012; Okamoto et al., 2009; Slaidina et al., 2009) and in a subset of glial cells (Chell and Brand, 2010; Sousa-Nunes et al., 2011)
<i>dilp7</i>	High signal in ten cells of ventral nerve cord, the visceral dMP2 neurons (Miguel-Aliaga et al., 2008). In the adult, it is expressed in a subset of subesophageal ganglion neurons, in the thoracico-abdominal ganglion and in the female reproductive system (Yang et al., 2008). In the embryo, ubiquitous (except yolk) low signal, moderate signal in midgut (Brogiolo et al., 2001).

Table 1 : Summary of *dilps* expression during *Drosophila* development. Temporal and spatial expression pattern of different *dilps* were analyzed in normal conditions. IPCs: Insulin-Producing-Cells. (Liu Y et al 2016) – *Drosophila* insulin-like peptide 1 (*Dilp1*) is transiently expressed during non-feeding stages and reproductive dormancy

The space and time pattern for each *dilp* genes being different, although they can functionally interchange each other, imply that they all have different physiological functions *in vivo*.

In line with this, different physiological and environmental cues regulate *dilp* genes expression. In fact, *dilp6* transcription in the fat body occurs during developmentally and experimentally induced non-feeding state in a FOXO-dependent manner (Delanoue et al., 2010). Conversely, upon nutrient shortage, *dilp3* and *dilp5* transcript levels decrease in the IPCs while *dilp2* remains unchanged (Colombani et al., 2003; Ikeya et al., 2002). Glial expression of *dilp2* and *dilp6* depend on amino acids content (Chell and Brand, 2010; Sousa-Nunes et al., 2011).

Unlike *Dilp1* to *Dilp7*, *Dilp8* is related to the relaxin molecules, involved in coordinating growth between larval tissues and coupling organ growth with developmental timing to ensure emergence of adults with proper body proportions (Colombani et al., 2012; Garelli et al., 2012). Secreted by damaged or abnormal growing imaginal discs, *Dilp8* activates its orphan receptor leucine-rich repeat-containing G protein-coupled receptor 3 (*Lgr3*) present on the Growth coordinating *Lgr3* (GCL) neurons and postpones maturation in order to repair the damaged tissue by suppressing ecdysone production. Interestingly, axonal arborisations of GCL neurons surround the dendritic part of the PTH neurons, suggesting that the developmental delay induced by *Dilp8* could be due to its control on ecdysone biosynthesis through PTH neurons (Colombani et al., 2015). According to their role in coordinating growth between tissues, *dilp8* mutants as well as *lgr3* mutants or *Lgr3* knockdown in the GCL neurons, display bilateral asymmetry (Colombani et al., 2015; Garelli et al., 2012).

During my PhD, I was interested in understanding systemic growth control. Ikeya et al demonstrated that systemic growth is controlled by *Dilp1*-*Dilp7*, with *Dilp2* having the main effect.

Therefore, I will focus the rest of my thesis on Dilps growth promoting function and more precisely on Dilp2.

f. Modulation of the ILPs signal after release

Circulating IGF1 can be found in three different forms: free, binary or ternary complexes. To better modulate IGFs function, vertebrates possess six high affinity IGF Binding Proteins (IGFBP) named IGFBP-1 to IGFBP-6. The IGFBPs bind preferentially IGF1 or IGF2, in order to regulate their bioavailability by protecting them from degradation, limiting their binding to IGF receptors and modulating their actions (Ruan and Lai, 2010). Binary complexes result in one IGF and one IGFBP molecules and represent 10% of the total IGF1 serum level. Most of the remaining plasma IGFs is present as ternary complexes including one molecule each of IGF, IGFBP-3 or IGFBP-5 and the glycoprotein acid-label subunit (ALS), leaving only 1% of free IGFs in the plasma (Boisclair et al., 2001).

Surprisingly, some IGFBPs are also able to bind insulin and to impede the interaction with InR, therefore preventing insulin signaling activation (Yamanaka et al., 1997). Interestingly, patients with type 2 diabetes display high plasmatic level of the IGFBPs known to have enhanced affinity for insulin (Bang et al., 1994; López-Bermejo et al., 2006).

Similar to vertebrates, insulin/IGF binding proteins have been discovered in *Drosophila*. The secreted Imaginal morphogenesis protein-Late 2 (Imp-L2) is homolog to IGFBP-7, since they share similar sequence homology. Like IGFBP-7, *Drosophila* Imp-L2 binds to human insulin, IGF1, IGF2, proinsulin and Dilp2 *in vitro* (Andersen et al., 2000; Honegger et al., 2008). Overexpression of Imp-L2 in different tissues such as the fat body leads to smaller adults, while *Imp-L2^{-/-}* null mutants are bigger, suggesting that Imp-L2 inhibits IIS. Accordingly, *dilp2* and *ImpL2* co-overexpression in the fat body leads to viable flies of wild-type size, indicating that Imp-L2 antagonizes the growth-promoting function of Dilp2. Moreover, heterozygous *Imp-L2^{+/-}* mutant flies which overexpress *dilp2* ubiquitously, display enhanced overgrowth compared to *dilp2* overexpressing flies. This is probably due to increased circulating levels of free Dilp2.

Overall, these data strongly indicate that by direct binding, Imp-L2 is a potent antagonistic peptide of Dilp2 and therefore decrease insulin sensitivity in peripheral tissues (Honegger et al., 2008).

The *Drosophila* homolog of ALS has also been discovered. The dALS is expressed in the IPCs and in the fat body. Upon acute or genetic starvation, the mRNA levels of dALS are downregulated in

Introduction

both tissues (Colombani et al., 2003). Interestingly, dALS can directly bind to Imp-L2 and to the Imp-L2/Dilp2 complex but not to Dilp2. Overexpression or silencing of dALS in the larval fat body leads to reduced and increased final body size respectively. Moreover, overexpression of dALS in the fat body significantly neutralizes overgrowth and metabolic changes due to Dilp2 overexpression (low circulating levels of trehalose and increase total fat content). Overall, these data strongly support that dALS form ternary complex with Imp-L2 and Dilp2, in order to counteract Dilps growth and metabolic functions (Arquier et al., 2008).

Another Dilps binding protein is the glial secreted decoy of InR (SDR). SDR is continually secreted into the hemolymph and have been shown to directly interact with several Dilps because of its resemblance with the extracellular domain of InR. By binding to Dilps, SDR prevents and adjusts insulin signaling under adverse dietary conditions to fine-tune systemic growth against variations of circulating insulin levels (Okamoto et al., 2013).

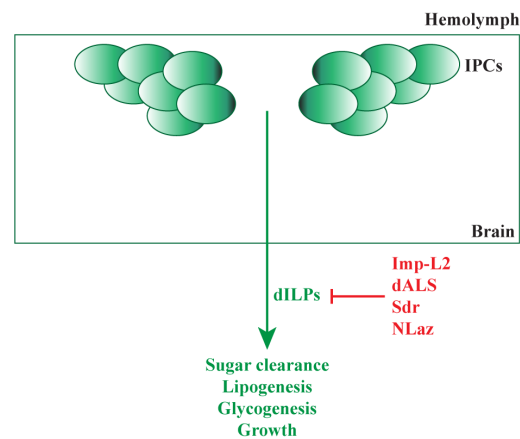


Figure 11 : Modulation of the ILPs signal after release from the IPCs. Dilps promoting effects are shown in green while binding molecules involved in inhibition of Dilps functions are depicted in red. Imp-L2: Imaginal morphogenesis protein–Late 2, dALS: drosophila Acid label subunit, SDR: secreted decoy, NLaz: Neural Lazarillo. Adapted from Mattila J. and Hietakangas (2017) – Regulation of carbohydrate energy metabolism in *Drosophila melanogaster*.

As well as insulin/IGF binding proteins, other mechanisms are at play to modulate IIS activity in peripheral tissues. High sugar diet causes insulin resistance through a lipocalin-like protein called Neural Lazarillo (NLaz). NLaz expression is activated in larval fat cells in a JNK-dependent manner and diminishes the IIS cascade sensitivity (Pasco and Léopold, 2012) (Figure 11).

Chapter III: Nutritional control of body growth

I- Parallel between drosophila and vertebrates

The interaction between genetic potential and environmental cues such as net nutrition determines the adult height during the growth period, most crucially in early childhood. The balance between food intake and food losses because of activities or diseases, defines the net nutrition. Among populations, differences in average height are mainly due to the environment. Indeed, well-nourished children from Europe, European descent, Africa, African descent, India or the Middle East, share the same growth profile and have similar stature (Steckel, 1995). However, the restriction of height by malnutrition still takes place in poor countries where average nutritional intake is low. For instance, children affected by marasmus or kwashiorkor, two forms of malnutrition caused by insufficient caloric intake and insufficient protein consumption respectively, display significantly lower body weight and height than healthy individuals (Kilic et al., 2004) (Table 2).

Measurements	Control	Marasmus	Kwashiorkor
Age (months)	10,33	8,95	9,87
Weight (kg)	10,35	4,77	6,10
Height (cm)	76,2	65,07	65,5

Table 2 : Anthropometric data of well-nourished versus malnourished children. Insufficient caloric intake or protein consumption both decrease the growth speed and lead to smaller children. Taken from Mehmet Kilic et al. (2004) – The evaluation of serum leptin level and other hormonal parameters in children with severe malnutrition.

In developed countries, children presenting eating disorders are shorter than healthy subjects (Favaro et al., 2007). Likewise, war periods are often associated with food restriction. During the world war II, children from Norway and Finland had 20% and 17% reduction in energy intake respectively, which is correlated to a decrease in the average height (Angell-Andersen et al., 2004) (Figure 12).

Introduction

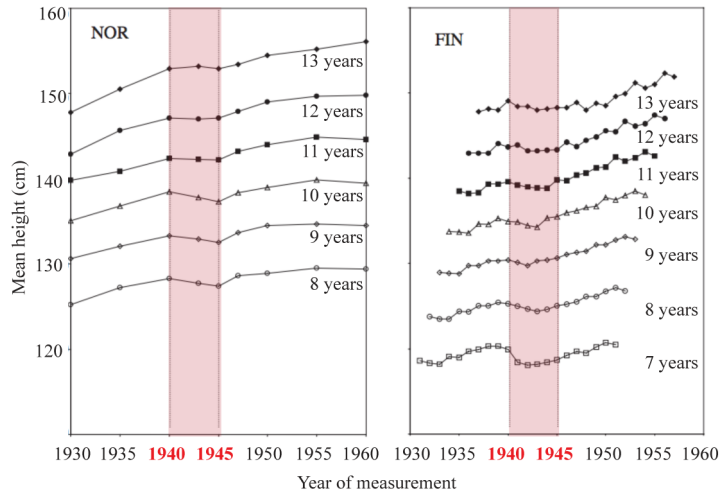


Figure 12 : Average height of Norwegian and Finnish boys aged 7-13 years from 1930 to 1960. During the World War II, the speed of growth dropped. This is concomitant with a food restriction period. Adapted from Angell-Andersen E. et al. (2004) – The association between nutritional conditions during World War II and childhood anthropometric variables in the Nordic countries.

Negative energy balance induces metabolic and hormonal changes, explaining the decrease in height. Plasmatic levels of several hormones and growth factors are reduced upon food restriction, like insulin, IGF1 and IGFBP-1 (Gat-Yablonski and Phillip, 2015).

Overall, these correlations strongly indicate that malnutrition inhibit systemic growth by reducing the IIS activity in peripheral tissues.

Invertebrates can also experience food restriction due to overcrowd environment in natural populations (Bubli et al., 1998) (Bubli et al., 1998). Indeed, multiple eggs are deposited in a rich nutritive environment. Nevertheless, due to environmental restriction until adult emergence, larvae continuously feed, and progressively drain this limited nutrient amount. Similar to humans, this food deprivation during juvenile stages causes size deficiency in invertebrates. Indeed, post-critical weight larvae that experience starvation will stop growing and will undergo metamorphosis, resulting in smaller fertile adults (Mirth and Riddiford, 2007). Similarly, flies raised on a low protein diet are smaller than flies raised on a normal protein diet (Figure 13).

Altogether, these observations support that nutrition plays a crucial role in determining appropriate final body size in multicellular organisms.

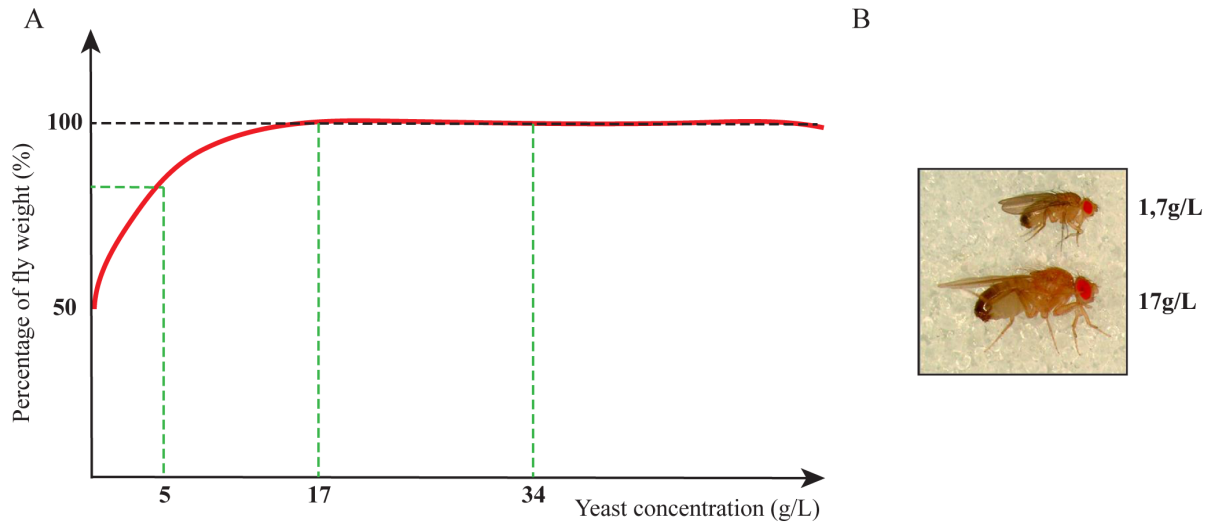


Figure 13 : Dietary yeast concentration is positively correlated with final body size in *Drosophila melanogaster*. A. Fly weight increases depending on yeast concentration in the diet until reaching a plateau. However, increasing over 17g/L the dietary yeast concentration, does not further induce bigger body size. B. Picture of flies raised either on poor protein diet (top) or on normal diet (bottom). Taken from Layalle S. and Géminard C. (unpublished data).

II- Cellular nutrient sensing: the TOR signaling pathway

Before trying to understand how systemic growth is coordinated with nutritional inputs, it is essential to decipher nutritional control on individual cells.

Cellular growth is defined by increased cellular mass and depends on dietary proteins. Upon starvation, cells must inhibit anabolic programs like protein synthesis and activate protein recycling through autophagy and proteosomal degradation. Cellular activation of these survival programs strongly suggests the existence of active nutrient sensing mechanisms.

The kinase Target of Rapamycin (TOR) is one of the main cellular nutrient sensing. TOR is highly conserved among unicellular and multicellular organisms and necessary for normal cell growth and proliferation, in part through regulation of translational effectors in response to amino acids (Oldham et al., 2000; Zhang et al., 2000). TOR exists in two different complexes called TORC1 and TORC2. While TORC2 is involved in cell survival and proliferation, TORC1 controls cell growth and size and couples growth cues to cellular metabolism (Saxton and Sabatini, 2017). The TORC1 complex includes TOR, Raptor (regulatory protein associated with TOR) and LST8 (Lethal with Sec13 protein 8). Raptor facilitates substrates recruitment to TORC1 while LST8 associates and stabilizes the TORC1 catalytic domain. TOR signaling pathway is dependent on growth factors, cellular energy levels through AMP-

Introduction

activated protein kinase (AMPK) (Inoki et al., 2003; Mihaylova and Shaw, 2011), but also on nutrition. Importantly, both cytosolic and lysosomal amino acids are important to stimulate TOR signaling.

In response to amino acids inside lysosomes, the GTPases RagA/C, tethered to the lysosomal membrane through interaction with Ragulator complex, are converted from their GDP to GTP form. This switch is promoted by the lysosomal v-ATPase, a Rag/Ragulator complex interactor, and mediates translocation of TORC1 to the lysosomal surface through Raptor binding to p62 (Duran et al., 2011; Kim et al., 2008; Zoncu et al., 2011). This p62-Raptor interaction is required for TOR activation (Hara et al., 2002; Lee and Chung, 2007). Besides, MAP4K3 is another kinase interacting with the Rag GTPases complex and required for amino acids-dependent TOR activation (Bryk et al., 2010).

Cytosolic amino acids activate TOR signaling through the GATOR1 and GATOR2 complexes (GAP activity towards Rags). In vertebrates, GATOR1 is tethered at the lysosomal membrane through KICSTOR interaction. However, *Drosophila melanogaster* lacks the KICSTOR components (Wolfson et al., 2017). GATOR1, mediates the GTP to GDP switch of Rag proteins, therefore inhibiting TORC1. Conversely, GATOR2 interacts with GATOR1 and indirectly stimulates TORC1 signaling. Interestingly, upon leucine withdrawal, the protein Sestrin2 binds to GATOR2, impede GATOR2-GATOR1 interaction resulting in TORC1 inhibition by GATOR1. Similarly, in vertebrates, cytosolic arginine directly binds to the arginine sensor CASTOR1 (Cellular Arginine Sensor for TORC1), blocking its inhibitory effect on GATOR2 (Saxton and Sabatini, 2017). However, no CASTOR homologs have been found in *Drosophila melanogaster*, suggesting the presence of another arginine sensing mechanism (Chantranupong et al., 2016).

After TORC1 lysosomal translocation in response to amino acids, the Ras-homolog enriched in brain (Rheb) protein, present at the lysosome membrane, directly binds and activates TOR.

The activated kinase TOR phosphorylates two main substrates: ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor 4E binding protein (4EBP), both involved in protein synthesis and cell growth. Phosphorylation of 4EBP prevents its binding to eukaryotic initiation factor 4E (eIF4E, a 7-methyl-guanosine mRNA cap binding protein), therefore allowing formation of the active translational complex and resulting in upregulated translation (Sonenberg, 1996). Likewise, S6K phosphorylation will phosphorylates and activates several substrates, including eIF4B and promote mRNA translation (Teleman, 2009).

Introduction

The major negative regulator of TOR, tuberous sclerosis complex 1 and 2 (TSC1/2), convert Rheb from its GTP active form to its GDP inactive form, leading to inhibition of TORC1 activity. In addition, under amino acids withdrawal, the formation of active Rag GTPases complex is also inhibited by the negative regulator SH3BP4 (Kim et al., 2012) (Figure 14).

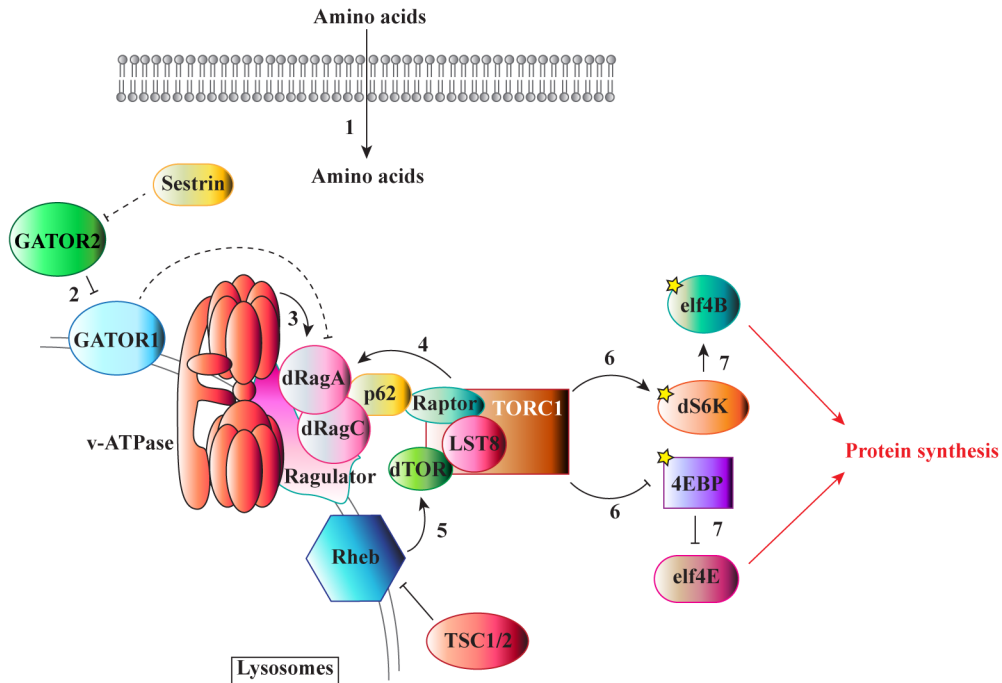


Figure 14 : The TOR signaling pathway in *Drosophila melanogaster*. Amino acids enter in cells (1). GATOR2 inhibits GATOR1 (2). v-ATPase promotes the GDP to GTP conversion of Rag proteins (3). This leads to TORC1 complex translocation on the lysosomal membrane by GTP-dRagA/C (4) through indirect interaction with p62. Then Rheb activates dTOR (5) which in turn phosphorylates both dS6K and 4EBP (6). Phosphorylation of 4EBP remove the basal inhibition on elf4E, while S6K phosphorylates elf4B (7). Therefore protein synthesis is promoted.

Consistent with the autonomous growth promoting function of TOR, mutant flies display severe growth defects due to slower growth rate and finally die. Importantly, these mutants phenocopy larvae deprived of amino acids. Moreover, mutant cell clones in cuticular structures are half the size of the wild type cells (Zhang et al., 2000). Likewise, flies lacking Rheb have reduced cell size and number resulting in smaller organisms while Rheb gain of function promotes systemic growth through S6K phosphorylation, even upon amino acids starvation (Saucedo et al., 2003; Stocker et al., 2003). In line with the inhibitory effect on TOR signaling, null mutant cell clones for TSC1/2 in *Drosophila* show overgrowth compared to wild type cells (Potter et al., 2001; Tapon et al., 2001). Additionally, decreased cell size is observed in flies with gain of function allele for 4EBP (Miron et al., 2001). Similarly, null mutant flies for *S6K*^{-/-} are smaller because of decreased cell number (Montagne et al., 1999).

Introduction

To conclude, the TOR signaling pathway integrates and links nutritional and energy status with the rate of protein synthesis in order to promote cell growth.

III- Crosstalk between the TOR and IIS pathways

The TOR pathway also integrates growth hormones signal. Actually, insulin and TOR signaling pathway share some common key regulators like Akt and FoxO. In absence of insulin, the nuclear FoxO increases 4EBP levels. In addition, activated Akt directly inhibits by phosphorylation both FoxO and TSC2, therefore indirectly activating the TORC1 complex (Hay, 2011; Lin and Smaghe, 2018) (Figure 15).

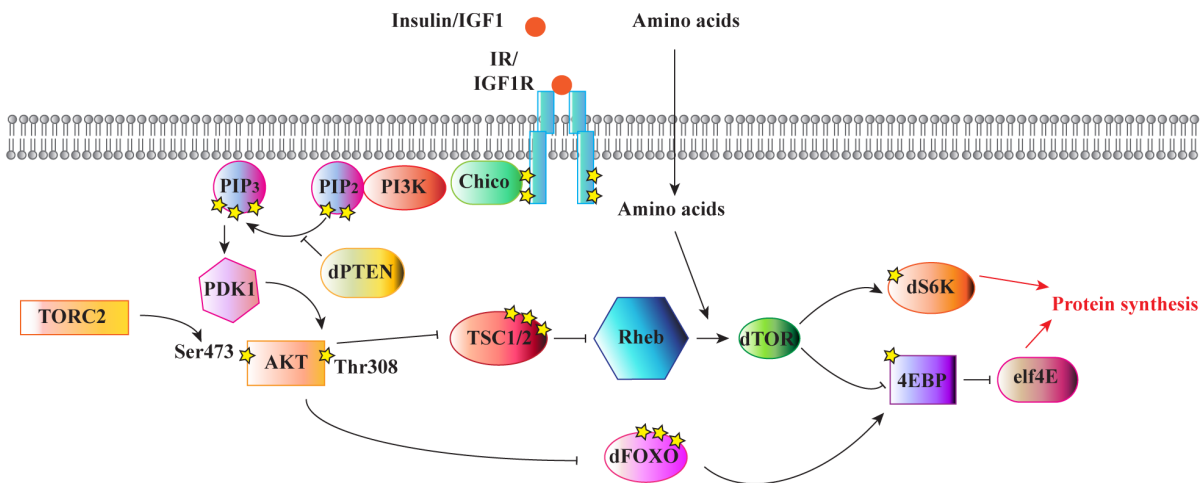


Figure 15 : Crosstalks between IIS and TOR signaling pathways. TOR activity is nutrient and hormonal dependent since it is activated directly by amino acids but also indirectly by AKT, a downstream component of the insulin/IGF signaling pathway. Interactions between TOR and IIS pathways allow coordination of cell growth among an organ.

Adapted from Nissim Hay (2011) – Interplay between FoxO, TOR and AKT.

Consistent with TORC1 activation, *Drosophila* cells treated with insulin display increased levels of phosphorylated TSC2 as well as larvae overexpressing Akt (Potter et al., 2002). However, removal of all TSC1/TSC2 phosphorylation site by Akt do not affect body size neither growth rate, indicating that phosphorylation of TSC1/TSC2 by Akt is not necessary for Akt to activate TORC1 and promote tissue growth (Dong and Pan, 2004; Schleich and Teleman, 2009). The relationship between TOR and insulin signaling could be done also by another substrate of Akt called proline-rich Akt substrate 40 (PRAS40). In mammals, it has been proposed that PRAS40 binds to TORC1. Upon insulin signaling activation, Akt phosphorylates PRAS40 and inhibits its binding to TORC1, hence allowing TORC1 to interact with its

Introduction

substrates (Haar et al., 2007; Sancak et al., 2007). Similarly, insulin stimulates PRAS40 phosphorylation in S2 cells. Furthermore, tissue specific overexpression of PRAS40 in the posterior part of the fly wing induces a reduction in size of this compartment, which can be partially counteracted by Rheb overexpression. Likewise, ubiquitous overexpression of PRAS40 leads to smaller animal and pupal lethality, equivalent to TOR deficiency phenotype. Surprisingly, *PRAS^{-/-}* mutant flies are viable, have normal size and display normal TORC1 activity in larvae. Nevertheless, TORC1 activity is increased in ovaries of *PRAS40^{-/-}* adults and restores fertility in IIS loss of function flies (Pallares-Cartes et al., 2012). These results indicate that PRAS40 regulate TORC1 activity specifically in non-somatic tissues.

Even though the molecular mechanism by which IIS and TOR signaling pathways are linked within a cell is not established yet, they are both nutrition-sensitive pathways that regulate the growth rate and interact, in order to coordinate systemic growth.

IV- The fat body is the main nutrient sensing organ

Nutrition has to be tightly detected in order to modulate different physiological features such as feeding behavior, metabolism, longevity and growth. After their absorption in the hemolymph, nutrients distribution throughout the body takes place to provide energy, essential cellular components and to promote growth. These nutrients are sensed by specific cells/organs in order to maintain nutritional homeostasis. Nutritional information is mostly perceived by peripheral organ such as digestive tract and adipose tissues, and is subsequently conveyed to other peripheral organs or the brain. In turn, the brain integrates the incoming signals and orchestrates physiological and behavioral responses, like body growth.

To coordinate body growth of multicellular organisms with nutritional inputs, humoral responses appeared during evolution and in particular the humoral Dilps and the IIS pathway (Partridge and Gems, 2002). Indeed, inhibition of the PI3K signaling ubiquitously or in endoreplicative tissues phenocopy starvation or inhibition of protein synthesis and arrest cell growth (Britton and Edgar, 1998; Britton et al., 2002; Galloni and Edgar, 1999). Furthermore, PI3K activity in larval tissues depends on proteins availability in the diet (Britton et al., 2002). These data suggest that IIS promotes peripheral tissue growth according to nutrition. However, amino acids deprivation in culture cells do not prevent insulin to activate InR autophosphorylation, IRS, PI3K nor Akt phosphorylation, suggesting that the IIS do not directly

Introduction

respond to nutrition (Hara et al., 1998). Consequently, IIS activity in peripheral tissues must be linked to nutrient availability through an intermediate sensor mechanism.

Apart from its storage function and endocrine activity, the fat body has been proposed as the main nutrient sensing organ, which coordinates systemic growth through a humoral mechanism. (Colombani et al., 2003). In order to identify growth-related gene, a genetic screen was performed. The gene *slimfast* (*slif*) which encodes a conserved cationic amino acids transporter mediating arginine and leucine uptake, has been discovered. The hypomorphic mutant *slif^d* shows a body size reduction due to decreased cell size and number, and larval lethality suggesting that it probably suffers amino acids deprivation. Similarly, the ubiquitous knockdown of *slif* (*slif^{Anti}*) induces growth deficiency, larval lethality, strong decrease of S6K activity and increase of PEPCCK1 transcripts levels. All of these parameters reflect and mimic amino acids deprivation.

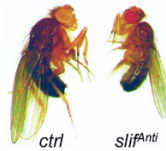


Figure 16 : Specific knockdown of Slif in the fat body drastically reduces body size.

Importantly, specific knockdown of *slif* in the fat body entirely recapitulates the *slif^d* mutant phenotypes: delayed larval development, reduced growth rate, decreased growth and reduced PI3K activity in endoreplicative tissues and pupal lethality. At 18°C, emerged adults are 54% smaller compared to controls (Figure 16). These results are similar to TOR activity inhibition in the fat body and strongly show that amino acids deficiency in the fat body is sufficient to inhibit systemic growth. Therefore, these data designate the fat body as the main amino acids sensing organ.

Different studies have established that TOR signaling pathway is cell autonomously required for growth according to nutrients (Oldham et al., 2000; Zhang et al., 2000). Coexpression of the S6K in the fat body partially rescues the growth defect and pupal lethality caused by *slif^{Anti}*. This data demonstrates the amino acids transporter Slimfast in the fat body is upstream the TOR signaling pathway and triggers the amino acid sensor.

Overall this study established the fat body as the main amino acids sensing organ. This sensor mechanism depends on TOR signaling and induces a remote control of InR/PI3K activity in endoreplicative tissues (Colombani et al., 2003) (Figure 17).

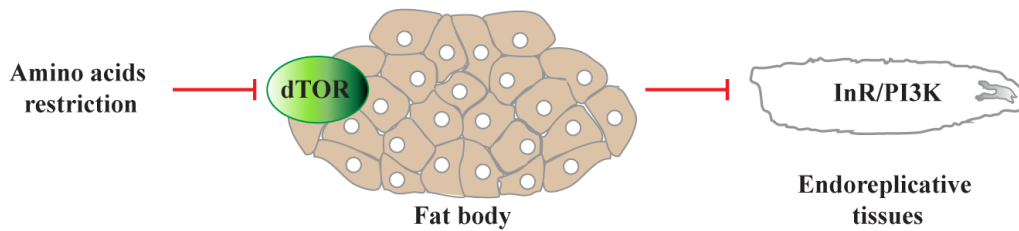


Figure 17 : The larval fat body is a sensor organ of amino acids levels. The fat body senses amino acids deprivation in a TOR dependent manner and remotely suppresses the InR/PI3K activity in endoreplicative tissues. Adapted from Colombani J. et al. (2003) – A nutrient sensor mechanism controls *Drosophila* growth.

V- Remote control of Dilps secretion

Upon genetic impairment of amino acid uptake in the fat body, the InR/PI3K signaling is reduced in endoreplicative tissues, leading to decreased body size. It has been shown that reduced IIS in these tissues is not due to changes in Dilps transcription levels in the IPCs, main production site of Dilp2 (Brogiolo et al., 2001; Colombani et al., 2003). Similarly, upon nutritional shortage, *dilp3* and *dilp5* transcription are decreased in the IPCs, while *dilp2* expression, which contributes to 80% of *dilps* gene transcription in the IPCs, remains unchanged (Buch et al., 2008; Ikeya et al., 2002). Besides, IPCs neuronal ablation induces a growth retardation fully rescued by Dilp2 transgene indicating that brain IPCs are the main source of circulating Dilp2 in the hemolymph involved in systemic growth control (Rulifson et al., 2002). This strongly suggests that nutritional cues probably affect circulating levels of Dilps through altered *dilp2* translation or Dilp2 secretion, therefore controlling the IIS in peripheral tissues.

To evaluate whether secretion of Dilp2 by the IPCs is dependent on nutrients, the Dilp2 antibody have been used. It has been a key tool to uncover by which mechanism the fat body induces a remote control of InR/PI3K activity in endoreplicative tissues according to nutrients (Géminard et al., 2009). Indeed, endogenous Dilp2 strongly accumulates within the insulin containing granules in the IPCs of starved larvae. Equally, starved larvae overexpressing a tagged form of Dilp2 in the IPCs also display accumulation in the IPCs, and 70% reduction in circulating levels of Flag-Dilp2. These results indicate that upon nutrition deprivation, the increased labeling of Dilp2 in the IPCs results in retention and lack of secretion into the hemolymph. Furthermore, Dilp2 accumulation in the IPCs is completely reverted after 2 hours of amino acids re-feeding (Figure 18). Forcing the IPCs depolarization under low protein diet, abolishes Dilp2 accumulation in the IPCs, leading to 50% pupal lethality while their hyperpolarization

Introduction

induces Dilp2 retention in the IPCs upon normal diet and results in extremely smaller hyperglycemic adults.

Taken together, these experiments prove that the IPCs couple neurosecretion and importantly, Dilp2 secretion with nutritional input.

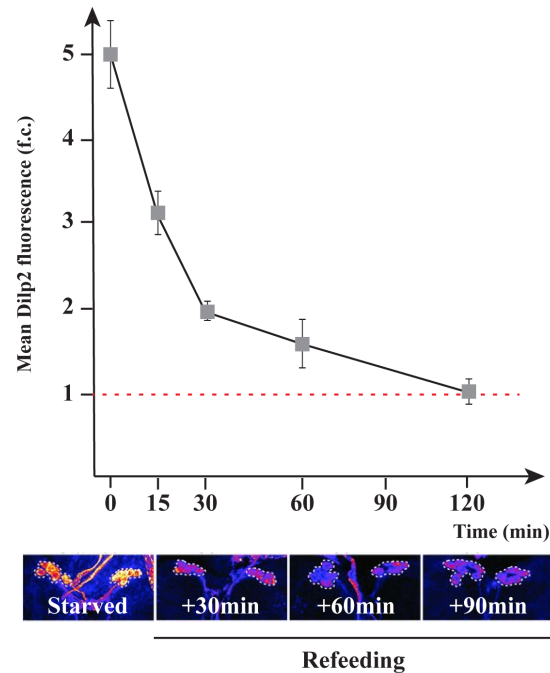


Figure 18 : Kinetics of Dilp2 accumulation within the IPCs upon refeeding. Third instar larvae were starved on PBS/1% sucrose for 24 hours and transferred in rich diet for indicated times.

Adapted from Géminard C. et al. (2009) - Remote control of insulin secretion by fat cells in *Drosophila* and from Delanoue R. et al. (2016) – *Drosophila* insulin release is triggered by adipose Stunted ligand to brain Methuselah receptor.

It has been shown that IPCs do not directly respond to nutrients and that the fat body is a nutrient sensing organ (Colombani et al., 2003; Kim and Rulifson, 2004). Géminard et al. (2009) showed that both specific knockdown of *slif* or reduced TOR activity in the fat body induce a strong accumulation of Dilp2 in the IPCs. Conversely, activation of the TOR pathway in the fat body of underfed larvae prevents Dilp2 retention. This indicates that the fat body acts as an amino acids sensor organ through TOR signaling pathway and remotely control Dilp2 secretion in the hemolymph. Likewise, *ex vivo* cocultures of brains coming from starved larvae with fat bodies or hemolymph collected from fed larvae show decreased retention of Dilp2 in the IPCs, providing evidence of a humoral signal emitted by the fat body that remotely control Dilp2 release (Géminard et al., 2009).

Introduction

Overall, results from Colombani et al. (2003) and Géminard et al. (2009) established a model where amino acids are sensed by the fat body, activate the TOR signaling pathway and produce a humoral signal subsequently conveyed to the brain. This signal stimulates Dilp2 secretion from the IPCs into the hemolymph and activates the IIS pathway in larval tissues, therefore promoting systemic growth (Figure 19).

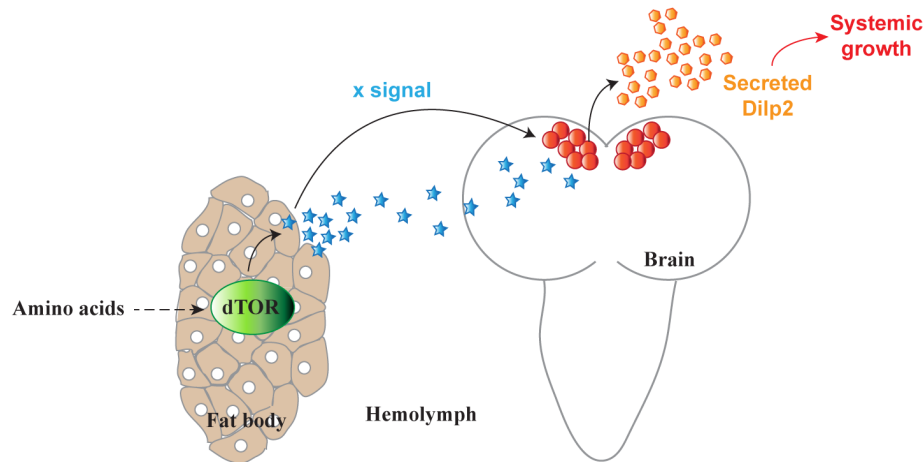


Figure 19 : Remote control of Dilp2 release by the fat body. Upon dietary amino acids, TOR is activated in fat cells and generates a positive signal released in the hemolymph. This signal reaches the brain IPCs, induces Dilp2 secretion and therefore body growth. Adapted from Géminard C. et al. (2009) – Remote control of insulin secretion by fat cells in *Drosophila*.

VI- Microbiota-dependent growth acceleration

It has been shown that intestinal microbiota is important to modulate physiological features of the host. The term microbiota defines numerous species of microbes such as symbionts and commensals which colonize a specific host-environment like the gastrointestinal tract, in order to influence the host metabolism, immune system and other activities (Hooper and Gordon, 2001). For instance, some enzymatic activities are provided by intestinal bacteria, allowing degradation and digestion of dietary carbohydrates (Hooper et al., 2002). Furthermore, strong relationships have been established between the microbiota composition and some metabolic diseases such as diabetes (Burcelin et al., 2009). In farm animals, antibiotics but also probiotics, which both modulate microbiota composition, are considered like growth promoters since their use increase animal's body weight (Simon, 2005).

More recently, using *Drosophila melanogaster* as a model, it appears that one commensal bacteria of the gut microbiota, *Lactobacillus plantarum*, promotes body growth upon nutrients restriction

Introduction

(Storelli et al., 2011). Under proteins scarcity, flies raised on germ free condition pupate 2,9 days later than both conventionally reared flies and flies associated with *L. plantarum* only. Storelli et al demonstrated that by promoting proteins absorption, *L. plantarum* indirectly increases the TOR signaling pathway in the fat body and the PG. Consequently, both IIS and ecdysone signaling are activated in peripheral tissues. Augmented IIS activity in peripheral tissues accelerates the growth rate of all developmental stages, while increase the ecdysone production through TOR activation in the PG (Layalle et al., 2008), leads to advanced pupariation. The increased growth rate with a reduction of the growth period length explain why flies raised on germ free conditions and flies associated with *L. plantarum* display the same final body size. This study strongly supports that the gut microbiota, and more precisely *L. plantarum*, promotes optimal larval development by modulating hormonal growth signaling upon nutrient scarcity.

Comparable studies have been conducted in vertebrate models. Germ free mice are 14,5% lighter and 4% shorter compared to conventional mice. The reduced size is due to lower circulating levels of IGF and IGFBP-3, despite identical GH levels. Consistent with the promoting-bone growth function of IGF1, germ free mice display reduced bone growth parameters such as femur length, cortical thickness, cortical bone fraction and the trabecular fraction of the femur (Schwarzer et al., 2016). These data support that conventional mice have a higher sensitivity to GH than germ free mice. Yan et al. also confirmed that the gut microbiota is required for an ideal somatic and bone growth through IGF1. Indeed, eight months after conventional gut microbiota colonization, mice displayed increased circulating levels of IGF1 correlated with a longer femur, larger L5 vertebra and greater periosteal and endosteal area than germ free mice, suggesting that gut microbiota stimulates radial and longitudinal bone growth (Yan et al., 2016). However, whether the gut microbiota sustains the GH/IGF1 axis directly or through an optimization of nutrient uptake from enterocytes remains to clarify.

Similar results were found in mice raised upon low protein and fat diet. Moreover, as in *Drosophila*, colonized mice with *L. plantarum* recapitulate the bone growth benefits seen in mice colonized with conventional microbiota (Poinsot et al., 2018).

Introduction

Even though the relationship between gut microbiota, GH/IGF1 axis and bone growth is not completely understood yet, all these studies strongly demonstrated that gut microbiota influences systemic growth and that nutrition seems to play an important role (Figure 20). Accordingly, undernourished children displaying GH resistance are smaller than well-nourished siblings and present immature microbiota with fewer bacterial species and lower relative abundance (Subramanian et al., 2014).

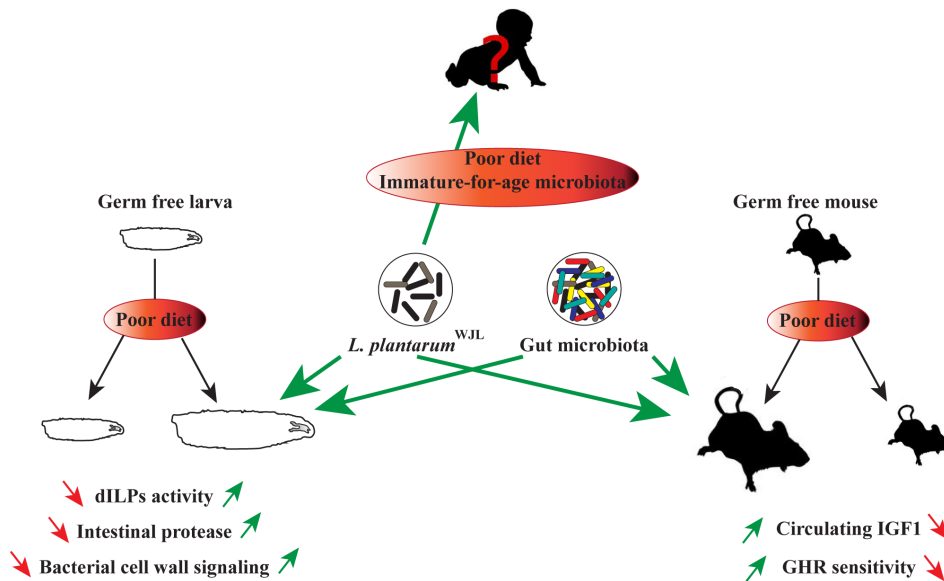


Figure 20 : Conventional microbiota and selected *Lactobacillus plantarum* both promotes body growth upon nutrient shortage in *Drosophila* and mice.

Adapted from Poinso P. et al. (2018) – 40 years of IGF1: The emerging connections between IGF1, the intestinal microbiome, *Lactobacillus* strains and bone growth.

Overall, by using different animal models, these studies open a new and robust field of investigations to evaluate the potential benefit of selected microbiota on systemic growth of undernourished children by counteracting the GH resistance. In addition, they also strengthen the link between nutrition and body growth by modulating hormonal signaling.

Chapter IV: Central integration of nutrients information

Nutrition has to be tightly detected in order to modulate different physiological features such as feeding behavior, metabolism, longevity and growth. Nutrient sensors respond to a specific nutrient component such as sugar, amino acids, fat, water, salt and micronutrients, and induce a cellular response, eventually leading to physiological changes like feeding behavior. The nutrient sensing occurs at different levels: external, in the intestine and post-ingestive (Miyamoto et al., 2013).

The external nutrient sensing is composed by taste receptors for sugar, amino acids and salts in sensory neurons. The main goal of this first sensing is the perception of these nutrients as pleasant and with high nutritional value, in order to choose the most suitable nutrient.

Within the intestine, before the nutrients breakdown and absorption, nutrient sensing still happens through different receptors, transporters or transceptors and the nutrient value is reevaluated. Despite a good molecular evolutionary conservation of proteins, the same major intestinal cell types, and the technical advantages of the model, only few studies highlight the functions of the intestinal transporters and taste receptors in *Drosophila melanogaster* (Miguel-Aliaga, 2012). Two sugar taste receptors are expressed in *Drosophila* enterocytes: Gr64a and Gr43a, however their function in the midgut remains to be clarified. Dietary proteins are cut into single, di- or tri-peptides by peptidases enzymes and several amino acids transporters have been identified in the *Drosophila* intestine such as PAT1, NAT1, MND and the PEPT1 gene homolog: *opt1* (Miguel-Aliaga, 2012).

Several post-ingestive nutrient sensing occurs in the digestive tract and adipose tissue. Indeed, the fat body is the main nutrient sensing organ devoted to growth control (Colombani et al., 2003). The nutritional information is then conveyed to other peripheral organs or the brain, in order to orchestrate physiological and behavioral responses. Nevertheless, different studies point out the relevance of communication between peripheral organs to control metabolism.

Introduction

For example, in response to sugar, the transforming growth factor β (TGF- β) ligand, Dawdle, is secreted by the fat body and activates the TGF- β /activin signaling in the midgut. This results in the repression of digestive enzymes highly expressed during starvation and hence regulation of sugar metabolism (Chng et al., 2014). Moreover, upon chronic high sugar diet, the Activin β (Act β) has been shown to be secreted by enteroendocrine cells and to enhance AKH signaling in the fat body through its receptor Baboon, resulting in hyperglycemia (Song et al., 2017). Additionally, upon nutrient shortage, systemic Hedgehog (Hh) is produced by the gut and directly targets the fat body and the PG in order to slow down larval growth and delay pupariation. Besides, circulating Hh stimulates survival upon starvation by promoting lipid mobilization in the fat body. Taken together, these experiments reveal a new hormonal function for Hh which coordinates growth and maturation with nutrient availability (Rodenfels et al., 2014).

During my PhD, my aim was to understand how nutritional information is transmitted to the IPCs in order to precisely adjust body growth. Therefore, I will mainly focus on post-ingestive nutrient sensing which relays the nutritional information to the brain.

I- Peripheral nutrient sensing which modulates Dilps secretion

Peripheral nutrient sensing in *Drosophila* occurs mainly in the adipocytes of the fat body and maybe even gut endocrine cells (Géminard et al., 2009; Ren et al., 2015). After sensing the systemic nutrient status, adipocytes secrete adipokines in order to communicate this information systemically, including to the brain. These fat body-derived signals have been shown to modify Dilps secretion in the IPCs.

a. Peripheral sensing of sugars and/or fat

1. Unpaired 2

Unpaired 2 (Upd2) is the first fat body-derived signal that has been identified and is a Leptinlike Janus kinase-signal transducers and activators of transcription (JAK-STAT) ligand (Rajan and Perrimon,

Introduction

2012). *Upd2* transcription in the fat body responds to high fat and high sugar diet. Its knockdown in the fat body as well as *upd2*^{-/+} hemizygous mutants induce smaller larvae, strong Dilps retention in the IPCs and hyperglycemic flies with reduced stored fat. *Upd2* overexpression in the fat body rescues the mutant phenotype. The oenocytes are hepatocyte-like cells that accumulate lipid droplets exclusively upon starvation. Oenocytes of well-fed *upd2*^{-/-} larvae display abnormal lipid accumulation.

These results indicate that fat-derived *Upd2* is required to sense nutritional state downstream of fats and sugars. Importantly, inhibiting the JAK/STAT signaling pathway in fat cells, does not recapitulate *upd2* specific knockdown or mutant phenotype. This shows that fat-derived *Upd2* plays a nonautonomous role to regulate fat storage and systemic growth and rather functions as a hormone to remotely control Dilps secretion.

Rajan and Perrimon (2012) further demonstrate that *Upd2* activate the JAK/STAT signaling in GABAergic neurons juxtaposed to the adult IPCs. Indeed, reducing the activity of the JAK/STAT signaling in these neurons impaired systemic growth, Dilps secretion and metabolic homeostasis.

To conclude, dietary fats and sugars stimulate *Upd2* release from the fat body. This Leptinlike ligand activates the JAK/STAT signaling pathway in GABAergic neurons and blocks the release of the inhibitory neurotransmitter GABA. Hence, IPCs are no longer inhibited. This double inhibition promotes Dilps secretion allowing appropriate body growth and metabolism homeostasis (Figure 21).

Interestingly, the authors further investigate the *Upd2* secretory mechanism. Upon starvation, AKH signaling in the fat body increases cytosolic Ca²⁺ levels and Calmodulin Kinase II (CamKII) activity. Activated CamKII inhibits the non-conventional protein secretion machinery called Golgi reassembly stacking protein (GRASP), blocking *Upd2* secretion (Rajan et al., 2017). Upon sugars deprivation, the CC secretes AKH which inhibits release of the fat-derived *Upd2* and leads to Dilps retention (Figure 21).

This suggests that the fat-derived *Upd2* is not the only primary fat and sugar sensor.

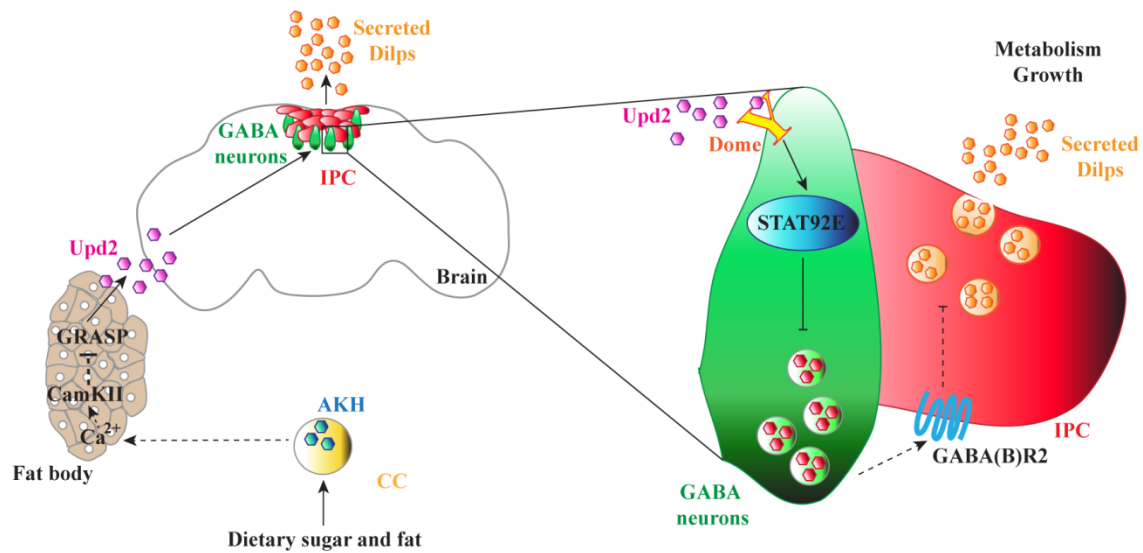


Figure 21 : Model of central and peripheral sugar sensor by AKH and the cytokine unpaired 2. Sugar and fat promote production and secretion of the fat-derived Upd2. Upd2 binds to its receptor Domeless in GABAergic neurons, activates STAT signaling and inhibits GABA release therefore allowing Dilps secretion. Conversely, upon starvation, AKH is secreted by the CC cells, increases calcium levels in the fat body and activates CamKII which in turn, phosphorylates and inhibits GRASP. This leads to Upd2 retention within fat cells and consequently, induces GABA release from GABAergic neurons. Through GABA(B)R2 in the IPCs, GABA abolishes Dilps secretion and therefore prevents systemic growth. CamKII: calmodulin kinase II; GRASP: Golgi reassembly stacking protein; Upd2: unpaired 2; Dome: Domeless.

Adapted from Rajan A. et al. (2012)- *Drosophila* cytokine Unpaired 2 regulates physiological homeostasis by remotely controlling insulin secretion and Rajan a. et al. (2017) – A mechanism coupling systemic energy sensing to adipokine secretion.

2. CCHamide2

The fat body and gut endocrine cells express the CCHamide2 (CCHa2) peptide in a nutrient-dependent manner. Transcription of *ccha2* decreases upon starvation and increases after sugars and proteins refeeding. Its receptor (CCHa2-R) is expressed in several neuroendocrine cells in the brain including the IPCs.

Interestingly, both *CCHa2^{-/-}* and *CCHa2-R^{-/-}* null mutants display increased Dilp2 staining within the IPCs and a strong reduction in *dilp5* expression. *CCHa2* overexpression in the fat body restores the *CCHa2^{-/-}* mutant phenotypes. Similarly, larvae expressing either *CCHa2 RNAi* in the fat body/gut or *CCHa2-R RNAi* in the IPCs, phenocopy *CCHa2^{-/-}* and *CCHa2-R^{-/-}* null mutants, respectively. This demonstrates that both fat-derived CCHa2 and CCHaR in the IPCs promote Dilps transcription and secretion.

Introduction

Besides, CCHA2 peptide addition on *ex vivo* brain culture provokes a strong calcium increase in the IPCs of control but not *CCHA2-R^{-/-}* mutants brain, indicating that CCHA2 directly activates the IPCs through its receptor CCHA2-R.

Upon sugar diet, the fat body/gut produces and secretes CCHA2. CCHA2 activates CCHA2-R on the IPCs and enhances both *dilp5* transcription and Dilp2 secretion to promote body size (Sano et al., 2015) (Figure 22). However, mechanisms by which sugars activate *CCHA2* transcription and secretion from the fat body is still unknown.

Taken together, these experiments show that CCHA2/CCHA2-R form a nutrient sensing that coordinates systemic growth with nutrients availability.

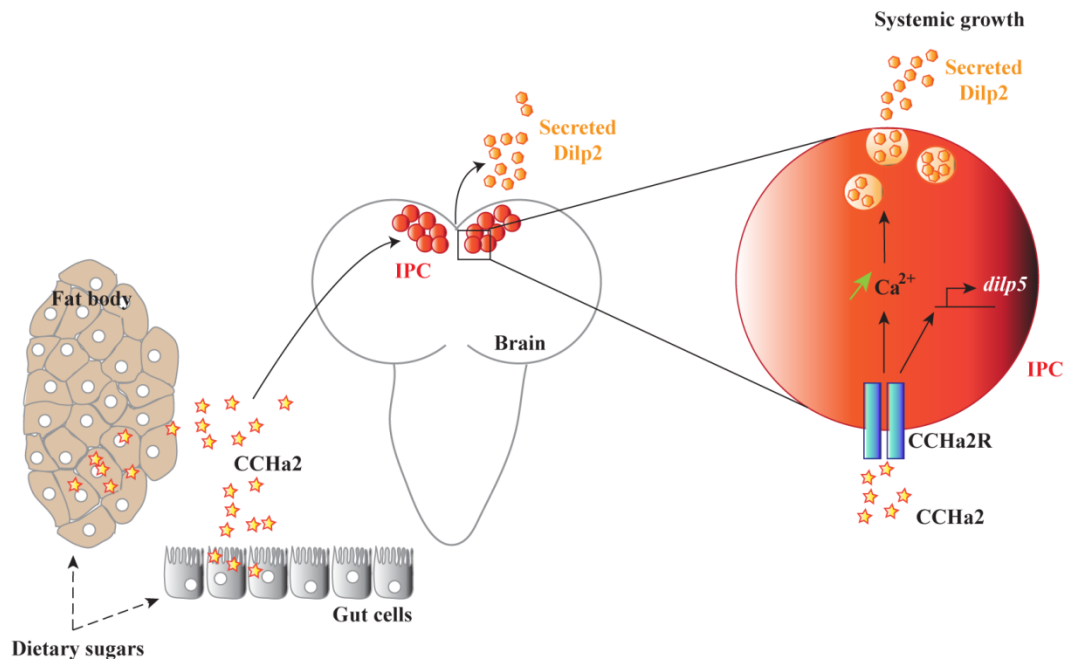


Figure 22 : CCHA2 relay the sugar information from peripheral tissues to the larval brain and regulates *dilps* transcription and secretion from the IPCs. Upon dietary sugars, CCHA2 is produced by the gut and the fat body and is probably secreted into the hemolymph. Through CCHA2R on the IPCs, CCHA2 promotes *dilp5* transcription and activates the IPCs neuronal activity leading to Dilp2 secretion.

Adapted from Sano H. et al. (2015) – The nutrient-responsive hormone CCHamide-2 controls growth by regulating insulin-like peptides in the brain of *Drosophila melanogaster*.

3. Dawdle

The Activin-like ligand Dawdle (Daw) is expressed in several organs even though it is predominant in the fat body and its secretion is sugar-dependent (Chng et al., 2014). Daw is essential for

Introduction

sugar tolerance in larvae (Ghosh and O'Connor, 2014). Its effects are mediated through the receptor Babo and the Smad signaling in peripheral tissues. Even though Daw controls pH balance and mitochondrial metabolism in an insulin independent manner, it also controls carbohydrates homeostasis by positively regulating Dilps secretion. Indeed, *Daw*^{-/-} null mutants display decreased insulin signaling and increased retention of Dilps in the IPCs. This suggest that Daw acts as a hormone to control Dilps secretion (Ghosh and O'Connor, 2014). However, no direct evidence proves an interorgan communication between the fat derived Daw and the brain IPCs.

4. Adiponectin

In mammals, adiponectin is a hormone secreted by the white adipose tissue, involved in insulin sensitivity (Ruan and Dong, 2016). Serum concentration of adiponectin is inversely correlated with insulin sensitivity. For instance, patients with type 2 diabetes mellitus or insulin resistance display low circulating adiponectin levels in the blood (Hotta et al., 2000; Pellmé et al., 2003).

The *Drosophila* homolog of adiponectin is still unknown. However, the adiponectin receptor (adipoR) is expressed in two clusters of seven neurons located in the larval optic lobes, that innervate the ring gland, including the corpora allata (CA) (Arquier et al., in preparation). These neurons are called the adiponectin responsive neurons (ARN). As in mammals, reducing AdipoR in these neurons increases circulating Dilps levels and decreases IIS activity in peripheral tissues. Both are characteristics of peripheral insulin resistance. In line with this, larvae with reduced AdipoR in ARN are affected by the metabolic syndrome. Conversely, activating adiponectin signaling in ARNs reduces circulating Dilps levels. Furthermore, Arquier et al. shows that ARNs neuronal activity responds to dietary sugars. Upon high sugar diet, ARN display a strong neuronal activation which is abolished after incubation with human adiponectin.

However, even though the adiponectin ligand in drosophila is still unknown, its expression in the fat body should be repressed by high sugar diet, raising the possibility of adiponectin as a potential nutrient sensor.

b. Peripheral sensing of amino acids

1. Eiger

Another fat body-derived signal has been identified: Eiger (Egr) (Agrawal et al., 2016). Under low protein diet (LPD), silencing Egr in the fat body partially rescue the body size reduction. Conversely, overexpression of a soluble form of Egr in the fat body leads to decreased body size in both LPD and normal conditions. These results suggest that Egr is required to reduce body size upon chronic amino acid deprivation.

Surprisingly, Egr expression in the fat does not respond to dietary amino acids restriction nor to TOR inhibition in the fat body. Egr is cleaved by the TNF- α converting enzyme (TACE) to release the soluble active form. Upon LPD or TOR inhibition in the fat body, TACE expression in fat cells is upregulated. Moreover, like Egr, the fat body knockdown of TACE partially rescue the body size reduction in LPD. This clearly indicates that upon chronic amino acids deprivation, TOR signaling is inhibited, leading to TACE transcription in the fat body. TACE cleaves Egr which is released in the hemolymph. Accordingly, Egr is no longer detected in the hemolymph of well-fed larvae or when TACE is silenced in fat cells.

Egr activates the JNK signaling pathway through its receptor Grindelwald (Grnd) (Andersen et al., 2015). Both silencing of Grnd or reducing JNK activity in the IPCs result in partial body size rescue with increased *dilp2* and *dilp5* transcription upon LPD.

Overall, these experiments strongly demonstrate a role of Egr as a metabolic hormone coupling body growth with nutrient availability through regulation of *dilps* transcription in *Drosophila* (Agrawal et al., 2016). Upon low protein diet, TACE is produced by the fat body and cleaves Egr. The soluble Egr secreted into the hemolymph, activates Grnd and the JNK signaling pathway in the brain IPCs. This pathway inhibits *dilps* transcription and therefore reduces body growth (Figure 23).

Remarkably, TNF- α signaling inhibits *insulin* expression in culture cells of mammals (Agrawal et al., 2016; Miyazaki et al., 1990). Additionally, TNF- α and JNK pathway are involved in insulin resistance and metabolic disorders in both mammals and invertebrates (Agrawal et al., 2016; Hirosumi et al., 2002). These studies unravel a conserved mechanism by which TNF- α mediates direct physiological and metabolic responses to nutrient deprivation.

Introduction

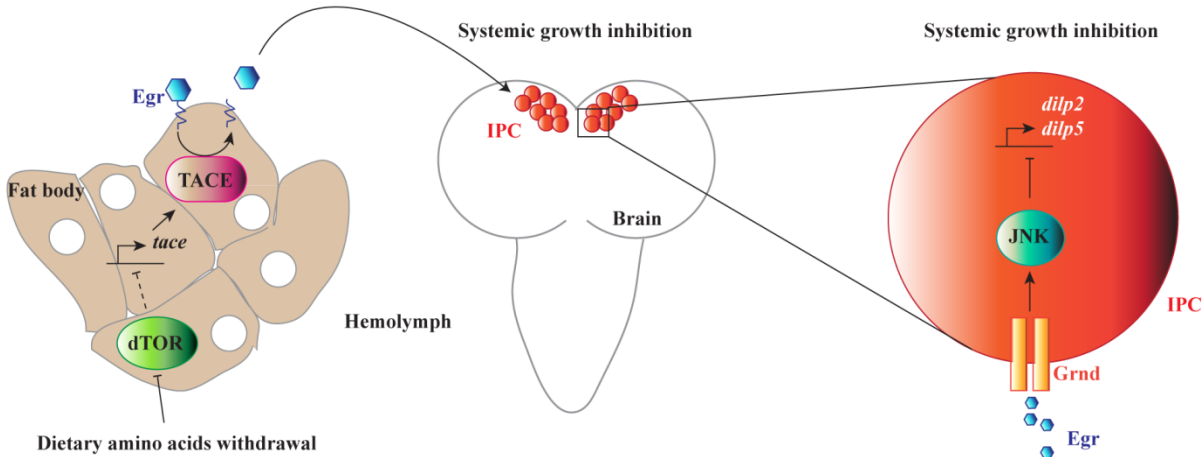


Figure 23 : The fat derived Egr remotely controls *dilps* transcription in response to amino acids deprivation. Upon chronic amino acids restriction, TACE is produced by fat cells and cleaves the full Egr. Thus, soluble Egr is released into the hemolymph and acts through its TNF receptor Grnd on brain IPCs. Consequently, the JNK signaling pathway is activated in the IPCs and limits *dilps* transcription. This results in an inhibition of systemic growth. TACE: TNF- α converting enzyme; Egr: Eiger; Grnd: Grindelwald. Adapted from Agrawal N. et al. (2016) – The *Drosophila* TNF Eiger is an adipokine that acts on Insulin-Producing Cells to mediate nutrient response.

2. Growth-Blocking-Peptides

The Growth Blocking Peptides (GBPs) have been described as fat body-derived signals responding to dietary amino acids (Koyama and Mirth, 2016).

Three GBPs exist, from GBP1 to GBP3 but only GBP1 and GBP2 strongly affect body growth. Indeed, silencing GBP1 or GBP2 or both GBP1/GBP2 specifically in the fat body, induce smaller adults because of reduced growth rate. Additionally, *gbp1* and *gbp2* expressions in the larval fat body strongly decreased upon acute starvation and are totally rescued after protein refeeding. Similarly, reducing TOR activity in the fat body diminishes both *gbp1* and *gbp2* mRNA levels. These results indicate that *gbps* expression in the larval fat body is sensitive to TOR signaling and to dietary amino acids.

Importantly, null mutant for GBP1 and GBP2, *ex67^{-/-}*, and larvae with fat specific knockdown of both *gbp1/gbp2*, display strong Dilp2 and Dilp5 retention in the IPCs. This retention is similar to what is obtained upon acute starvation or reduced TOR activity in fat cells (Géminard et al., 2009; Koyama and Mirth, 2016) and is consistent with the decreased body size. As expected, Dilp2 is not detected in the hemolymph of *ex67^{-/-}* mutant larvae, confirming that GBP1 and GBP2 from the fat body regulate Dilp2 secretion.

Notably, overexpression of both *gbp1* and *gbp2* in the fat body rescue the *ex67^{-/-}* mutant phenotypes: increased body size, increased growth rate, less Dilps retention and higher peripheral IIS

Introduction

activity. Besides, *gbp1* and *gbp2* overexpression in the fat body also partially relieved the body size reduction due to TOR inhibition, therefore suggesting that GBP1 and GBP2 act downstream of TOR signaling in fat cells.

Finally, Koyama and Mirth (2016) also demonstrate by *ex vivo* brain culture that fat-derived GBPs act on the brain to induce Dilps secretion in a dose-dependent manner. Nevertheless, whether fat-derived GBPs directly act on the IPCs or through another neuronal population remains to be clarified.

To conclude, dietary amino acids stimulate *gbps* transcription in the fat body in a TOR-dependent manner. Then, GBPs are most probably secreted in the hemolymph, act on the brain to induce Dilps secretion from the IPCs, and therefore allow systemic growth (Figure 24).

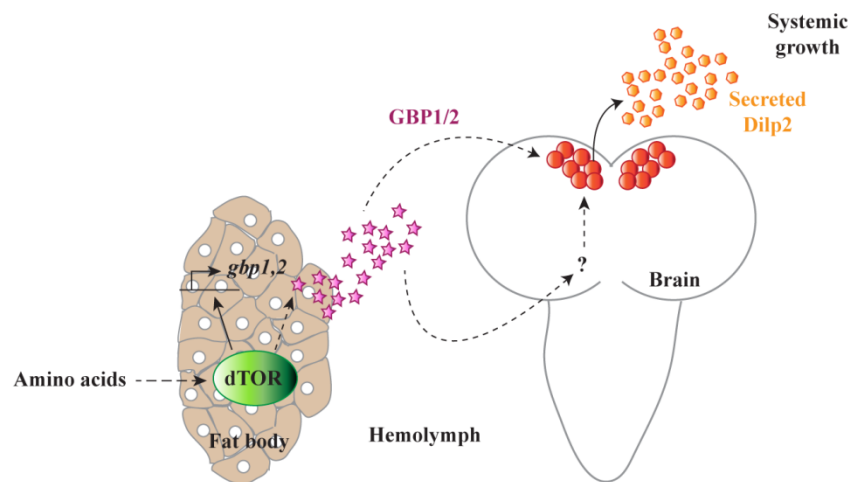


Figure 24 : Fat-derived GBPs respond to dietary amino acids and promote body growth through remote control of Dilp2 secretion. Activation of the TOR signaling pathway by dietary amino acids in the fat body leads to production and most probably secretion of GBPs into the hemolymph. Secreted GBPs act on the brain to induce Dilp2 secretion and promote body growth. Nevertheless, the neuronal target of GBPs remains unknown. Adapted from Koyama T. et al. (2016) – Growth-Blocking Peptides as nutrition-sensitive signals for insulin secretion and body size regulation.

3. Stunted

The mitochondrial protein Stunted (Sun) is the latest discovered fat body derived signal responding to dietary amino acids (Delanoue et al., 2016). Since I participated to this study, I will present it in more details in the Results section and the Discussion.

II- Central sensing of nutrients

a. Amino acids sensing

Among tissues that act as nutrient sensor, the brain plays also a role. Indeed, specific neuronal populations express nutrient transporters and respond to particular macronutrients such as amino acids.

1. LAT1 transporters: Minidisc

One striking example is the IPCs which directly sense the essential amino acid L-leucine. Dilp release from the IPCs depends on specific dietary amino acids like leucine (Géminard et al., 2009). This regulation was thought to be only indirect through the fat body derived signals. However, brains from starved larvae incubated with leucine, display an increased neuronal activity in the IPCs which is abolished upon specific knockdown of the leucine transporter minidisc (MND) (Manière et al., 2016). MND is one of the two large neutral amino acids LAT1-like transporters existing in *Drosophila*. MND is expressed in several tissues including the larval IPCs. As expected, brains from starved larvae present strong accumulation of Dilp2 in the IPCs. This accumulation is reverted upon leucine incubation in control brains but not after *Mnd* knockdown in the IPCs, demonstrating that leucine directly activates the IPCs neuronal activity and Dilp2 secretion through MND in cultured brains. Furthermore, Manière et al. showed that leucine transport through MND has an insulintropic effect mediated by glutamate dehydrogenase (GDH) signaling.

Dietary leucine supplementation induces overgrowth with reduced glycemia in control flies, consistent with an increase in Dilp2 secretion by the IPCs. These phenotypes are abolished upon specific *Mnd* silencing in the IPCs. Nevertheless, neither body size nor glycemia change in larvae deficient for *Mnd* specifically in the IPCs compared to control flies when raised on poor protein diet without leucine addition. This suggests that MND in the IPCs is a leucine sensor only upon specific dietary conditions.

Introduction

Taken together, these data establish that dietary leucine is directly transported within the IPCs through MND and activate the GDH signaling pathway in order to stimulate Dilp2 secretion into the hemolymph, therefore allowing the promoting-body growth effect of high dietary leucine concentration (Figure 25). This suggests that MND is a primary leucine sensor in the IPCs. Interestingly, in mammals, both leucine and isoleucine increase the free cytosolic Ca^{2+} in β cells and therefore stimulate insulin secretion, probably through GDH signaling pathway (Bolea et al., 1997; Göhring and Mulder, 2012; Newsholme et al., 2005).

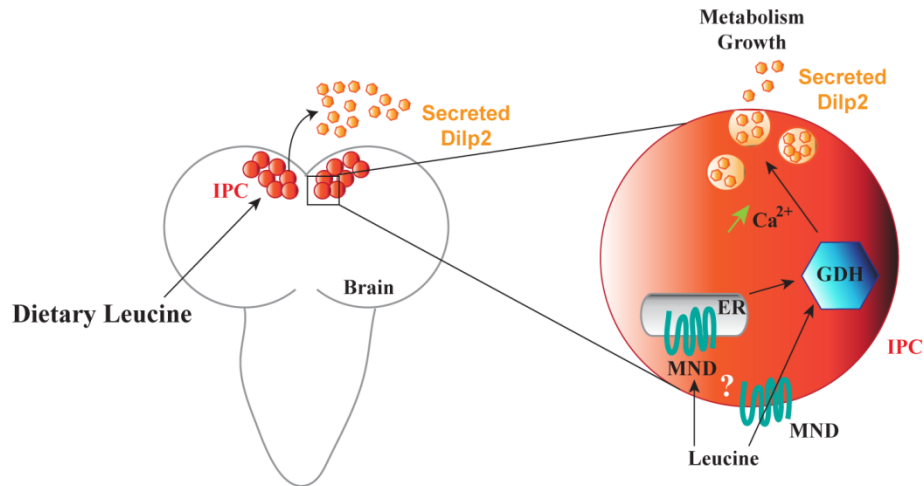


Figure 25 : Model of direct leucine sensing in the IPCs. Leucine is transported in the IPCs by the amino acids transporter MND and subsequently stimulates the IPCs activity through the GDH signaling. This results in Dilp2 and Dilp5 secretion. Nevertheless, whether MND is present at the cell surface or just at the ER membrane is still unclear.

Adapted from Manière G. et al. (2016) – Direct sensing of nutrients via a LAT1-like transporter in *Drosophila* insulin-producing cells.

2. The kinase GCN2

The kinase GCN2 is another central amino acids sensor (Bjordal et al., 2014). Larvae raised on an imbalanced diet lacking the essential amino acids tryptophane and lysine, have a decreased food intake and increased roaming behavior. Genetic depletion of amino acids or neuronal activation specifically in dopaminergic neurons strongly inhibits feeding, suggesting that a dopaminergic circuitry detects dietary amino acids imbalance and therefore negatively regulates feeding behavior. Bjordal et al. nicely proved that GCN2 kinase activity is involved in sensing the amino acids imbalance in dopaminergic neurons. Indeed, overexpression of a constitutively activated form of GCN2 during the mid L3 stage, leads to feeding inhibition and roaming. Conversely, GCN2 knockdown or reduction of dopamine signaling in

Introduction

dopaminergic neurons decrease the feeding inhibition and the roaming behavior induced by imbalanced diet. Furthermore, the addition of an amino acids imbalanced mix on *ex vivo* cultured brains triggers a strong neuronal activation of a subset of 3 dopaminergic neurons which is completely suppressed after integration of the missing amino acids. Besides, inhibition of the GABA signaling by silencing GABA(B) Receptor 1 promotes food avoidance in well fed larvae, suggesting that GABA signaling is probably involved in the regulation of food intake by dopaminergic neurons. Genetic interaction experiments nicely reveal that GCN2 signaling acts upstream of GABA(B) Receptor1.

Overall, this study proves that exposure to imbalanced amino acids diet leads to activation of the kinase GCN2 which in turn inhibits the GABA signaling in a cluster of three dopaminergic neurons. This inhibition induces dopamine release in order to arrest the food intake (Figure 26).

Remarkably, such amino acids imbalanced sensor has also been identified in rodent brain (Hao et al., 2005) and mice displaying a mutation in the gene encoding GCN2 do not avoid imbalanced diet (Maurin et al., 2005). Additionally, GCN2 signaling interacts with GABA(B) receptor 1 in mammals (Nehring et al., 2000; Ritter et al., 2004; Vernon et al., 2001; White et al., 2000). All these studies, strongly propose a clear conserved molecular mechanism between vertebrates and invertebrates to adjust feeding behavior according to amino acids composition in the diet.

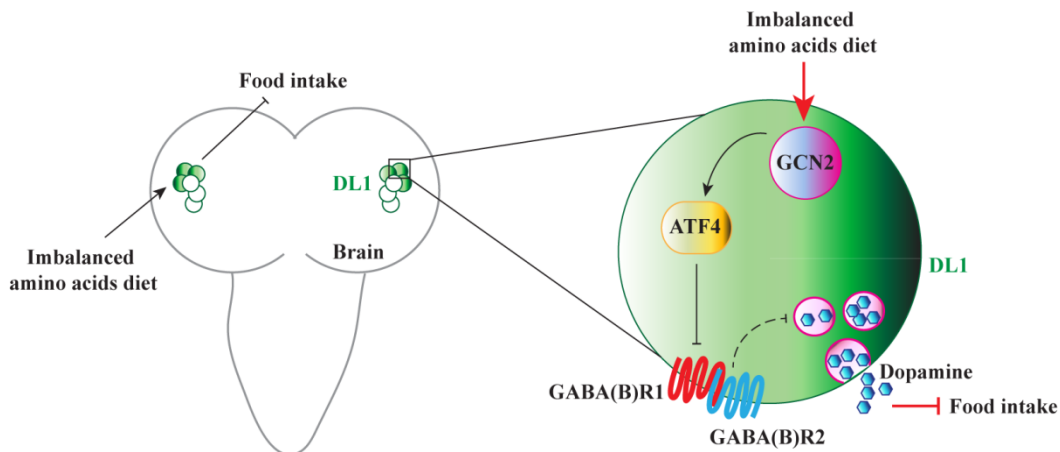


Figure 26 : The kinase GCN2 is an amino acid imbalance sensor in dopaminergic neurons. Three dopaminergic neurons of the DL1 cluster sense amino acids imbalance through activation of the GCN2 kinase. GCN2 activates its target ATF4 which abolishes GABA signaling, suppressing the basal dopamine release inhibition. Consequently, dopamine is secreted and induces feeding cessation.

Adapted from Bjordal M. et al. (2014) – Sensing of amino acids in a dopaminergic circuitry promotes rejection of an incomplete diet in *Drosophila*.

3. TOR signaling and serotonin

The TOR signaling pathway is considered as a cellular nutrient sensor, especially in the fat body (Colombani et al., 2003). However, two studies reveal that the TOR signaling component S6K couple to the neurotransmitter serotonin, are involved in nutrient sensing and feeding preference. This regulation takes place in the fly brain (Ribeiro and Dickson, 2010; Vargas et al., 2010). Nevertheless, the neuronal population and the exact mechanism of such amino acids sensor are still unidentified.

4. DH44 positive neurons: CG13248

Very recently, a preliminary work demonstrates the existence of another neuronal population involved in central amino acids sensing (Yang et al., 2017). They demonstrated that three amino acids, glutamate, alanine and aspartate, promote feeding consumption through activation of six Diuretic hormone 44 (DH44) positive neurons in the fly adult brain. This activation requires the cationic amino acids transporter encoded by the CG13248 gene (Park et al., 2011). These results suggest that Glu, Ala and Asp may enter into DH44 positive neurons through the amino acids transporter CG13248 to activate their neuronal activity and promote food consumption. They also claimed that these three amino acids would decrease the activity of the well-known kinase GCN2. However, no genetic evidence is shown to support this hypothesis (Figure 27).

Introduction

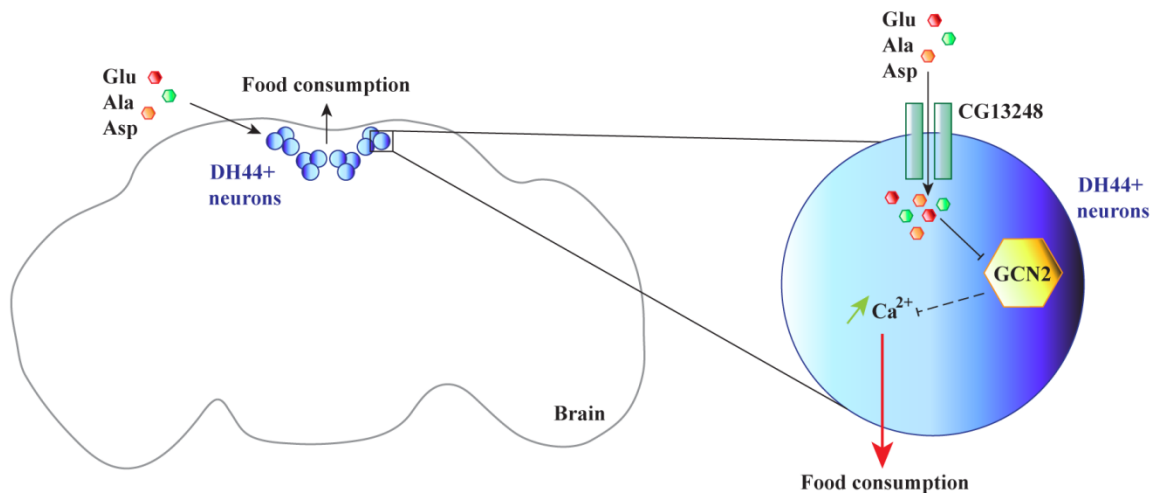


Figure 27 : Central amino acids sensing mediated by the kinase GCN2 in DH44 expressing neurons promotes food intake. In *Drosophila* adult brain, the Glutamate, Alanine and Aspartate enter in DH44 positive neurons through the cationic amino acid transporter encoded by the *CG13248* gene. These amino acids presumably inhibit the kinase GCN2 and allow a calcium levels increase. The neuronal activation of the DH44 positive neurons promotes food consumption.

Adapted from Yang Z. et al. (2017 – BioRxiv) – An internal sensor detects dietary amino acids and promotes food consumption in *Drosophila*.

b. Carbohydrates sensing

Amino acids are not the only macronutrients that can be detected centrally. Indeed, specific neuronal populations express nutrient transporters and respond to carbohydrates.

1. Corpora Cardiaca (CC) cells: AKH and Limostatin

Dietary carbohydrates determine glycemia. Circulating levels of glucose in *Drosophila* are composed of monomeric free glucose and trehalose, which is a disaccharide of glucose. The IPCs are known to partially regulate glycemia (Ikeya et al., 2002; Rulifson et al., 2002), however larval IPCs do not express the Sulphonylurea receptor Sur and the inward rectifying potassium channel Kir. These two proteins form an ATP-sensitive potassium channel (K_{ATP}) involved in hormone secretion by glucose-sensing cells (Aguilar-Bryan et al., 1995; Seino and Miki, 2003). Instead, Sur1 and Kir are expressed in the corpora cardiaca (CC) cells (Kim and Rulifson, 2004). The CC cells produce the Adipokinetic hormone (AKH), similar to the mammalian glucagon (Van der Horst, 2003). Interestingly, CC cell ablation in larvae induces hypoglycemia which is partially restored upon *akh* ubiquitous overexpression, suggesting that AKH is a master regulator of glycemia in *Drosophila*. Furthermore, starvation enhances

Introduction

the hypoglycemic phenotype of CC cell ablation, confirming that AKH participates to the compensatory mechanism leading to circulating glucose homeostasis during food withdrawal. Both CC cells ablation and hyperpolarization prevent the hyperglycemic effect of the tolbutamide, a drug which induces K_{ATP} channel closure leading to cellular depolarization and hormone secretion in mammalian cells. This demonstrates that AKH secretion from CC cells is controlled by the K_{ATP} channel activity to adjust glycemia in *Drosophila*.

Kim and Rulifson revealed that CC cells directly respond to glucose/trehalose. In fact, low extracellular concentration of glucose or trehalose provokes an increase in calcium concentration in *ex vivo* CC cells. This increase in intracellular calcium levels has been previously correlated with AKH secretion in *Locusta migratoria* (Pannabecker and Orchard, 1987).

This study strongly demonstrates that larval CC cells directly sense glucose/trehalose circulating levels through expression of the cellular sensor K_{ATP} channels. Hypoglycemia increased intracellular levels of calcium in the CC cells, membrane depolarization and subsequently AKH secretion into the hemolymph in order to restore normoglycemia (Kim and Rulifson, 2004) (Figure 28). Remarkably, this mechanism is very similar to what is observed in mammalian pancreatic α cells.

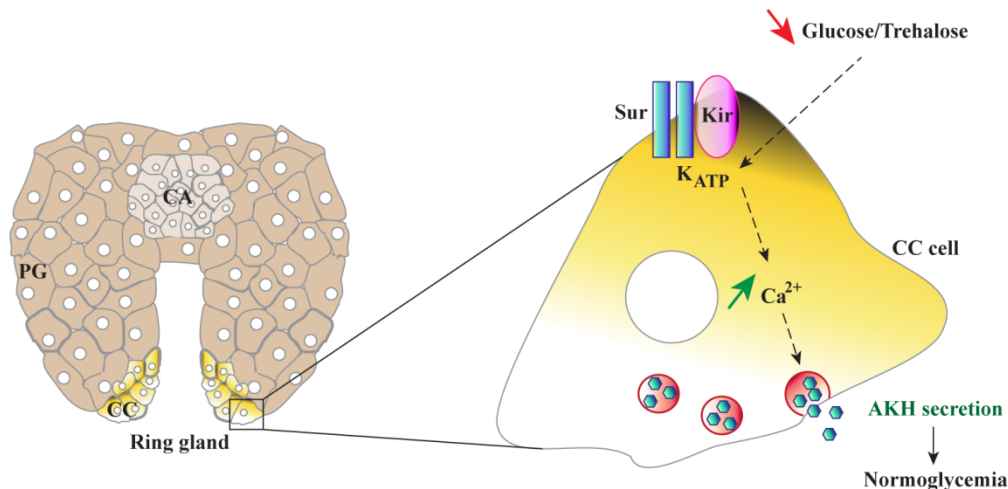


Figure 28 : AKH secretion is induced by hypoglycemia. The glucose/trehalose cellular sensor K_{ATP} is expressed in corpora cardiaca (CC) cells. Upon hypoglycemia, intracellular calcium levels increase in the CC cells, inducing depolarization and adipokinetic hormone (AKH) secretion in order to restore normoglycemia.

CA: corpora allata; PG: prothoracic gland; CC: corpora cardiac; Sur: Sulphonylurea; Kir: inward rectifying potassium channel; K_{ATP} : ATP sensitive potassium channel; AKH: Adipokinetic hormone.

Unexpectedly, Dilp3 secretion from the IPCs is stimulated by glucose and trehalose in an AKH signaling-dependent manner (Kim and Neufeld, 2015). Indeed, they showed that trehalose induces AKH secretion from the CC leading to AKH signaling activation in the IPCs. This induces Dilp3 release. Consequently, Dilp3 activates the TOR signaling pathway in the fat body and prevents autophagy.

Introduction

To conclude, in rapidly growing *Drosophila* larvae, dietary sugars promote both Dilps and AKH secretion. IIS will promote growth and energy storage. Concomitantly, the sugar-dependent secretion of AKH will counteract the excessive storage of trehalose by stimulating energy use (Figure 29).

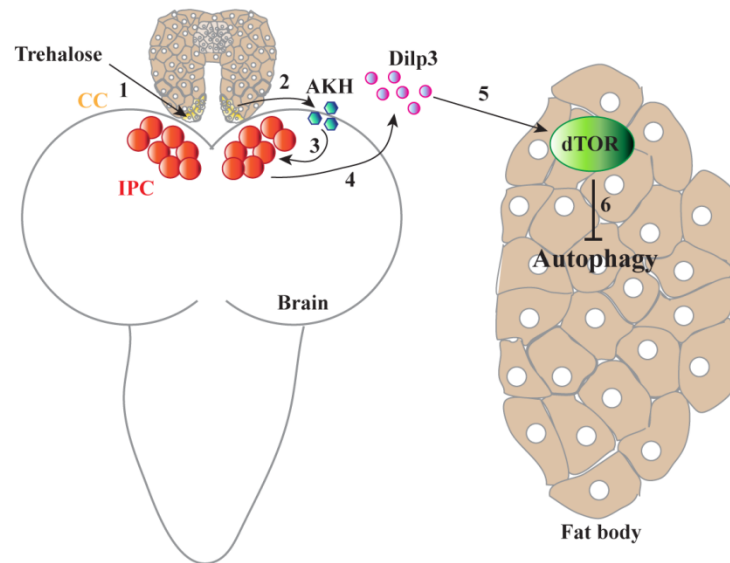


Figure 29 : AKH is secreted in a sugar-dependent manner. Upon high circulating levels of trehalose (1), AKH is secreted from the CC (2) and activates the AKH signaling pathway in the IPCs (3). This triggers Dilp3 secretion (4) in the hemolymph in order to activate TOR signaling pathway (5) in the fat body and therefore inhibit autophagy (6).

The gut associated-CC cells express also the secreted hormone Limostatin (Lst) and colocalize with AKH (Alfa et al., 2015). Specific knockdown of *lst* in CC cells provokes hypoglycemia, elevated circulating Dilps and obesity in adult flies. Upon starvation, *lst* expression increases and is rescued after carbohydrates refeeding. This is concomitant with the post-prandial increase of circulating Dilps. This indicates that Lst functions as a decterin because its expression is regulated by dietary carbohydrate and is required to suppress insulin during fasting periods. Consistent with its decterin role, the small peptide Lst-15 corresponding to the highly conserved Lst region, decreases the IPCs neuronal activity *in vivo* and decreases Dilps secretion in *ex vivo* head culture. Furthermore, the authors identify *CG9918*, that encodes a GPCR in the IPCs, as a strong candidate for Lst receptor. Overall, these findings demonstrate that upon dietary carbohydrates withdrawal, CC cells secrete Lst. The decterin hormone Lst acts on its receptor *CG9918* present on the IPCs, in order to inhibit the IPCs neuronal activity and therefore block Dilps secretion (Figure 30).

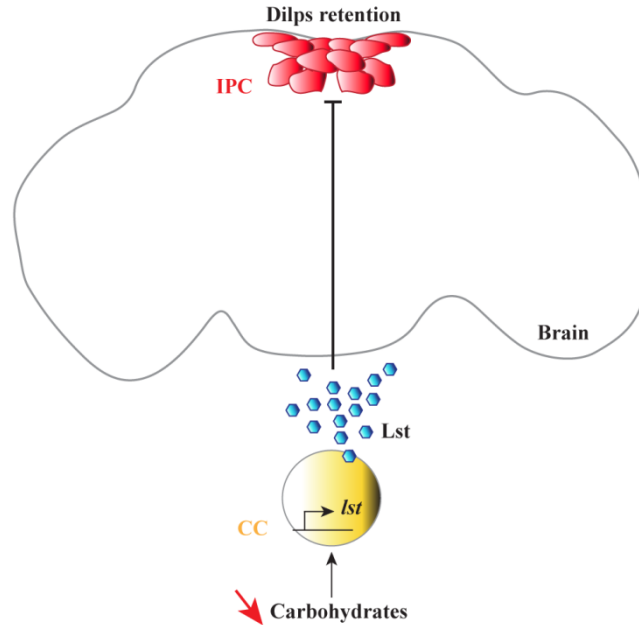


Figure 30 : Limostatin is a dectetin and suppress insulin secretion during fasting in adult flies. Upon dietary carbohydrates withdrawal, the CC produces and secretes Limostatin (Lst). Lst binds to its receptor and inhibit the IPCs neuronal activity, therefore impeding Dilps secretion. Adapted from Alfa R. W. et al. (2015) – Suppression of insulin production and secretion by a dectetin hormone.

2. The Insulin Producing Cells (IPCs) in adult flies

Unlike larval IPCs, IPCs from adult flies are able to directly sense glucose. Indeed, the subunit Sur of the K_{ATP} channel is expressed in adult IPCs (Haselton et al., 2010). Electrophysiological records measured a membrane depolarization in dissociated IPCs cultured with glucose or glibenclamide, a K_{ATP} channel inhibitor. Likewise, intracellular Ca^{2+} concentration increases in both conditions. These results strongly support the idea that the adult IPCs directly sense and respond to glucose through inhibition of functional K_{ATP} channels in order to stimulate a firing response (Haselton et al., 2010; Kréneisz et al., 2010). This mechanism is very similar to what is observed in pancreatic β cells.

3. Gr43a positive neurons

A fructose sensor also exists in *Drosophila* brain. The gustatory receptor 43a (Gr43a) is a fructose receptor present in taste neurons but also in the adult brain, where it functions as a sensor of circulating fructose levels (Miyamoto et al., 2012). Indeed, in *ex vivo* brain culture, Gr43a positive central neurons display high intracellular calcium levels upon elevated extracellular fructose concentration. This neuronal

Introduction

activation by fructose is abolished in *Gr43a*^{-/-} mutant flies and can be restored by specifically expressing *Gr43a* in the Gr43a positive neurons. After a sugar meal, while glucose and trehalose levels in the hemolymph stay stable, fructose concentration abruptly arises and serves as an indicator for sugar consumption, suggesting that the fructose sensor Gr43a probably assigns nutrient valence to carbohydrates. Accordingly, null mutant flies for *Gr43a*^{-/-} consume small quantity, do not evaluate nutritional value of the tasteless sugar sorbitol, become increasingly hungry and die of starvation. A phenotype restored by *Gr43a* expression specifically in the Gr43a positive central neurons. These data suggest that brain Gr43a has a role upon non-satiating conditions. Interestingly, Miyamoto et al. (2012) showed that well-fed *Gr43a*^{-/-} flies display an overconsumption phenotype of highly desirable sugars but not of fructose and trehalose, which is rescued by the *UAS-Gr43a* transgene. This suggests that brain Gr43a suppresses carbohydrates feeding upon satiety. Indeed, *Gr43a*^{-/-} flies equally consume palatable and non-nutritive sugars which are not metabolized into fructose.

Overall, these results indicate that after a sugar meal, fructose circulating levels rise and activate the Gr43a in central neurons. Depending on the satiety/hunger state, activation of the Gr43a positive central neurons differently affects feeding behavior. In hungry flies, activation of Gr43a positive neurons promotes carbohydrates feeding while upon satiety, these neurons inhibits food intake. This means that Gr43a positive central neurons integrate the satiety information (Figure 31). However, the molecular mechanism by which Gr43a regulates food intake remains still poorly understood.

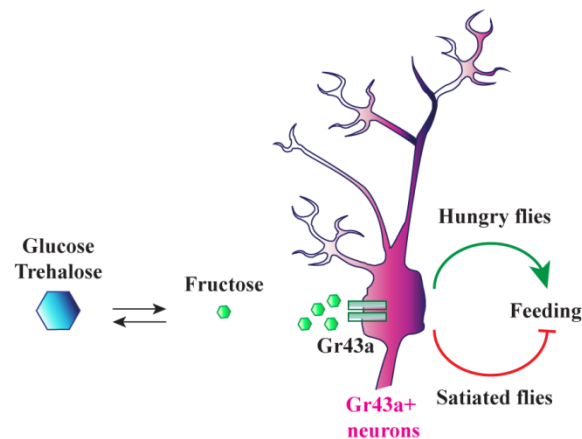


Figure 31 : Internal fructose levels fluctuate in response to nutritious sugars and are sensed by the fructose receptor Gr43a in the *Drosophila* brain. In response to fructose levels, Gr43a positive neurons are activated and either promote or abolish feeding behavior depending on the satiety/hunger state.

Adapted from Miyamoto T. et al. (2012) – A fructose receptor functions as a nutrient sensor in the *Drosophila* brain.

Similar to adults, *Drosophila* larvae also express Gr43a in taste neurons, proventricular neurons and sensory neurons in the brain. Yet, Gr43a in brain neurons of larvae serves as sensor for all main dietary sugar in order to generate a slow and late sugar preference (Mishra et al., 2013). However, neither

the exact identity of brain neurons nor the molecular mechanism involved in carbohydrates sensing have been discovered.

4. DH44 positive neurons in adult flies

In the adult fly brain, six DH44 positive neurons that sense three amino acids (Yang et al., 2017), also sense nutritive sugars independently of taste input (Dus et al., 2015). Indeed, upon starvation, hyperpolarization of DH44 positive neurons enhances consumption of the non-nutritive sugars while their depolarization induces equal consumption of nutritive and non-nutritive sugars. These results suggest that DH44 neurons control food choice behavior and mediate the selection of nutritive sugars.

DH44 neuronal activity is activated by hemolymph and nutritive sugars, leading to DH44 secretion. Glucose entry and its conversion to glucose-6-phosphate by hexokinase C (Hex-C) are required to stimulate DH44 secretion. The authors demonstrated that nutritive sugars selection rely on DH44-DH44 receptor 1 and 2 axis, to promote proboscis extension response (PER) and excretion respectively.

To conclude, upon nutritive sugar ingestion, glucose enters in the DH44 positive neurons and is converted in glucose-6-phosphate. DH44 neurons are activated and release DH44 neuropeptide. Consecutively, DH44 binds DH44 R1 and DH44 R2 in target tissues and promotes PER response, gut motility and excretion through a positive feedback loop therefore stimulating nutritive sugar consumption (Figure 32). To conclude, the six central DH44 neurons are post-ingestive sugar sensor.

Remarkably, in the mammalian hypothalamus and hindbrain, glucose-sensing neurons have also been identified even though their biological role in feeding behavior is still mysterious (Anand et al., 1964; Levin, 2007; Oomura et al., 1964).

Introduction

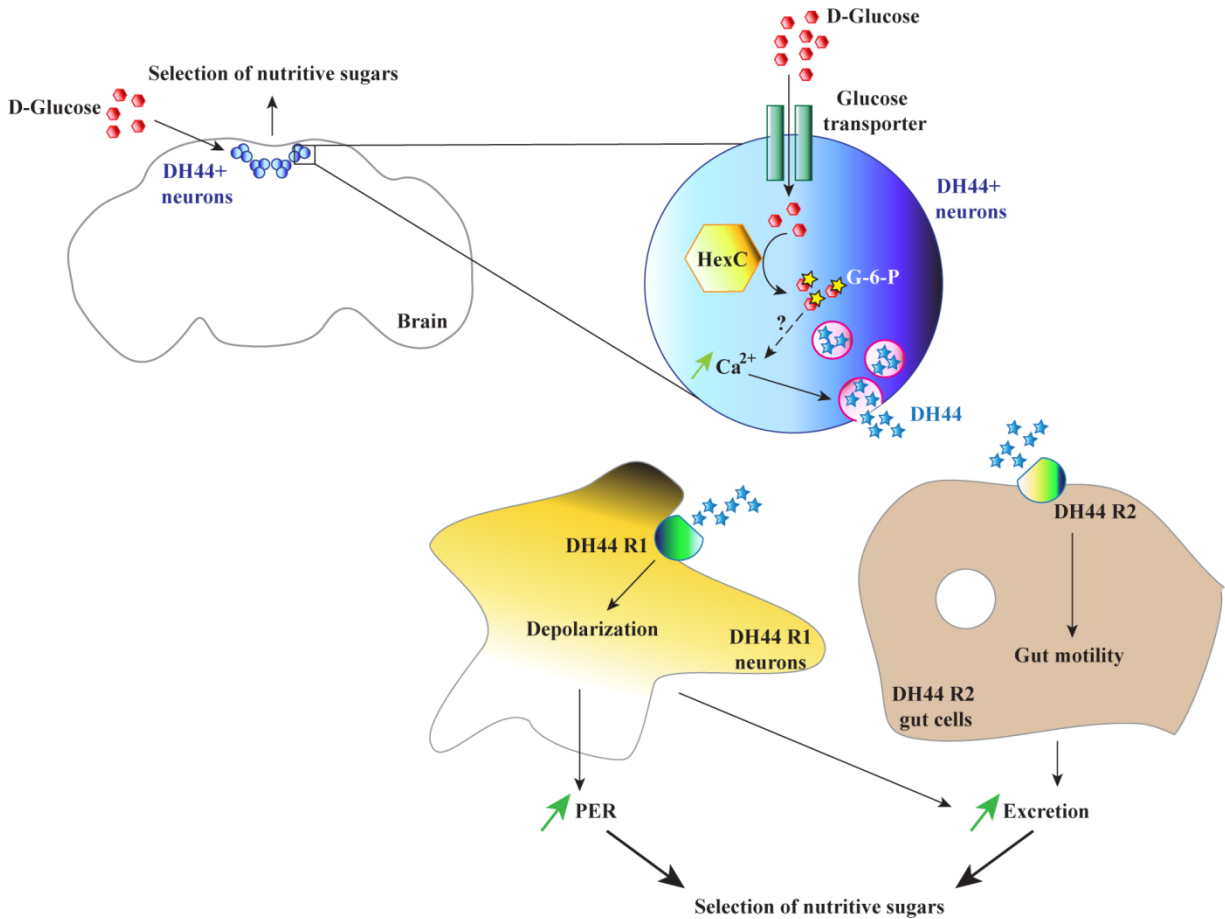


Figure 32 : Sugar sensing by DH44 positive neurons in the *Drosophila* adult brain. Nutritive sugar ingestion activates DHR44 positive neurons and leads to DH44 release. Then, DH44 binds to DH44 R1 in neurons and DH44 R2 in enteroendocrine cells. DH44 signaling promotes PER and excretion, therefore increasing nutritive sugar consumption. This results in a positive feedback loop.

Adapted from Dus M. et al. (2015) – Nutrient sensor in the brain directs the action of the brain-gut axis in *Drosophila*.

Chapter V: Neuronal circuitries at play to control the IPCs secretory activity

In the previous chapter, we have seen how nutritional input is sensed by the body and integrated by the brain in order to control feeding behavior, metabolism and body growth. Most of these nutrients and/or signals act on the IPCs either directly or through a neuronal relay. Several studies further show that the IPCs are not only involved in growth control depending on nutrient availability. In this chapter, I will briefly introduce the neuronal circuitries at play that regulate several physiological functions by controlling the IPCs neuronal activity.

I- GABAergic circuitry

The inhibitory neurotransmitter GABA acts through ionotropic and metabotropic receptors, GABA_A and GABA_B respectively. However, only the metabotropic GABA_B receptor is expressed along neurites of the IPCs (Enell et al., 2010). In addition, a large number of neurons produce GABA in both larvae and adults, including some cell bodies adjacent to the IPCs. Interestingly, GABAergic branches are found around the IPCs dendrites, suggesting that GABA_B receptors on IPCs are postsynaptic (Figure 33). Specific knockdown of GABA_B receptor in the IPCs induces strong retention of Dilps, reduces lifespan, decreases resistance to desiccation and starvation, and altered lipid and carbohydrates metabolism (Enell et al., 2010). These phenotypes imply that GABA signaling inhibits the IPCs activity.

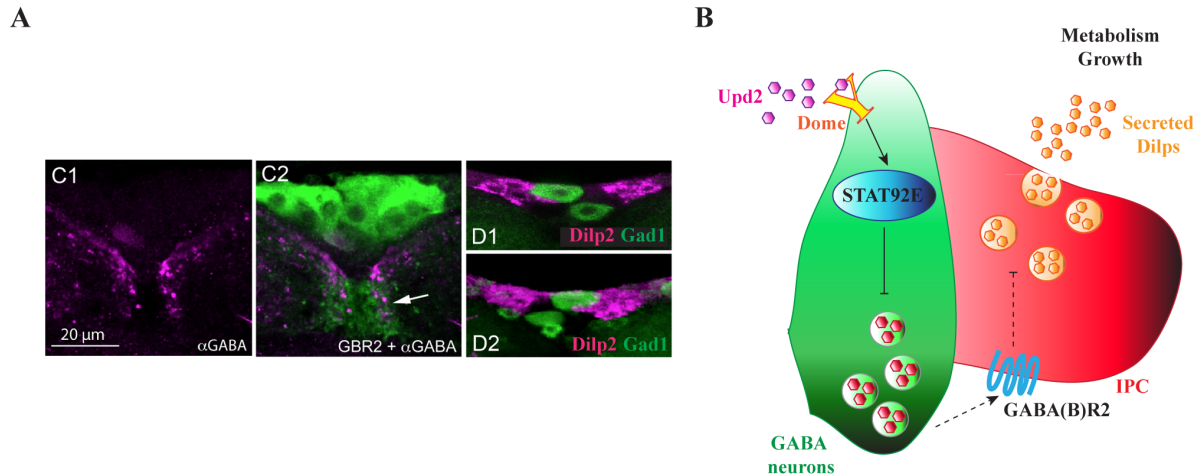


Figure 33 : GABAergic neurons are in close proximity with the IPCs and control their neuronal activity. A. Immunostaining of GABAergic neurons (magenta) and adult IPCs (green). In C1 and C2, GABA antibody labels neuronal processes that superimpose (arrows) the IPCs (GBR2-GAL4-GFP). In D1 and D2, several neurons adjacent to the IPCs (magenta – Dilp2 antiserum) express the biosynthetic enzyme Gad1 and therefore are GABAergic neurons (green – Gad1-GAL4-GFP). B. Furthermore, it has been shown in adults that GABA neurons functionally interact with the IPCs. Indeed, secreted GABA acts on GABA(B)R2, which is present on the IPCs, and inhibits the IPCs secretory activity.

Taken from Enell L. E. et al. (2010) – Insulin signaling, lifespan and stress resistance are modulated by metabotropic GABA receptors on Insulin Producing Cells in the brain of *Drosophila* and adapted from Rajan A. et al. (2012)- *Drosophila* cytokine Unpaired 2 regulates physiological homeostasis by remotely controlling insulin secretion.

Upd2 is the fat-derived ligand which acts on the GABAergic neurons adjacent to IPCs, through its receptor Dome and the JAK/STAT signaling (Rajan and Perrimon, 2012). The current model proposes that upon dietary fat and sugars, the leptin like Upd2 is released from the fat body, cross the blood brain barrier and activate the JAK/STAT signaling through the receptor Dome in GABAergic neurons. JAK/STAT signaling activation suppresses the tonic inhibition of the IPCs and allows Dilps release.

Taken together, these two study show that GABAergic circuitry control the IPCs neuronal activity in adult flies in a nutrient-dependent manner.

II- Serotonergic neurons

Serotonergic neurons have been found to control the IPCs secretory activity (Kaplan et al., 2008). Interestingly, they express the nucleostemin (NS) 3, which encodes a GTPase regulating cell proliferation and cell fate.

Introduction

In *Drosophila*, NS3 is involved in systemic growth control through modulation of Dilps signaling (Kaplan et al., 2008). Indeed, embryos injected with dsRNA for *ns3* display a strong growth impairment (-40%), delayed pupariation, reduced viability, strong Dilp2 accumulation in the IPCs and high serotonin levels. Serotonin levels being inversely correlated with growth, it suggests that serotonin has a growth inhibitory effect (Kaplan et al., 2008; Valles and White, 1986).

ns3 is ubiquitously expressed but its specific overexpression in serotonergic neurons rescued the developmental delay, Dilp2 retention and growth defects of *ns3* mutants. Remarkably, serotonergic neurons processes project all around the cell body and major tract of the larval IPCs. Importantly, overexpression of a constitutively active Akt in different peripheral tissues of *ns3* mutants larvae, rescue the organ size, suggesting that NS3 in serotonergic neurons acts upstream to insulin signaling. However, the signal inducing *ns3* expression in serotonergic neurons is still unknown.

Adult IPCs express the serotonin receptor 5-HT_{1A} (Luo et al., 2012). Serotonergic neurons also send processes towards the IPCs of adult flies. Unexpectedly, both 5-HT_{1A}^{-/+} mutants or specific knockdown of 5-HT_{1A} in the IPCs induces Dilp2 accumulation in the IPCs, a decreased resistance to starvation, to heat and longer recover from cold coma. These results suggest that 5-HT_{1A} stimulates the insulin signaling since IPC ablation also results in decreased tolerance to heat and cold treatment (Broughton et al., 2005). This is contradictory with the predicted inhibitory effect of serotonin (Kaplan et al., 2008; Valles and White, 1986).

Furthermore, no growth defects were observed in 5-HT_{1A} mutant larvae nor upon 5-HT_{1A} downregulation in the IPCs. This could be explained by the absence of 5-HT_{1A} in larval IPCs, suggesting that in larvae, serotonin effect is probably mediated by another receptor.

Altogether, these studies show that serotonergic circuitry can control the IPCs activity. Whether this circuitry is inhibitory or excitatory requires further investigations in both *Drosophila* larvae and adults (Figure 34).

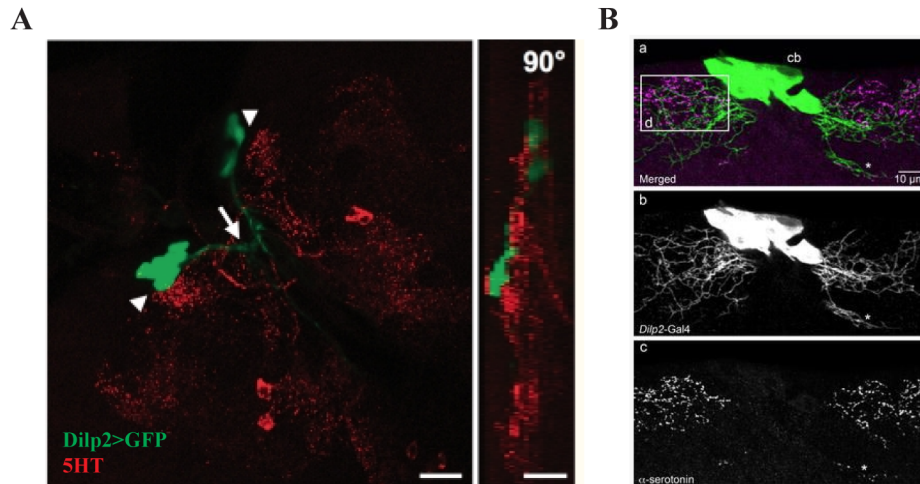


Figure 34 : Serotonergic neurons project on the IPCs both in *Drosophila melanogaster* larvae and adult. A. Larval brain Z-stack from a larva expressing GFP in the IPCs (green) and immunostained for 5-HT (red). Arrowheads denote regions where serotonergic processes are in close proximity to the cell body of the IPCs while the arrow show apposition between the IPCs and serotonergic processes. B. Adult brain expressing GFP in the IPCs (b - green) and stained for serotonin (c - magenta). The merge (a) demonstrate that IPC branches superimpose serotonergic branches, suggesting a possible connection between serotonergic neurons and the IPCs. Taken from Kaplan D. D. et al. (2008) – A nucleostemin family GTPase, NS3, acts in serotonergic neurons to regulate insulin signaling and control body size and Luo J. et al. (2011) – Insulin-producing cells in the brain of adult *Drosophila* are regulated by the serotonin 5-HT_{1A} receptor.

III- Octopaminergic circuitry

The octopaminergic circuitry is involved in wake/sleep behavior of adult flies (Crocker et al., 2010). The neurotransmitter octopamine is the equivalent of the mammalian norepinephrine and both promotes wakefulness (Crocker and Sehgal, 2008; Sara, 2009).

Interestingly, octopaminergic processes project on the IPCs and the IPCs express the octopamine mushroom body receptor (OAMB) (Figure 35). Similar to the decreased wakefulness obtained by electrical silencing of octopaminergic neurons, the IPCs depolarization decreases the sleep while their hyperpolarization increases it, independently of the day/night cycle.

Activation of one isoform of OAMB increases cAMP signaling and Ca²⁺ signaling (Lee et al., 2009). Importantly, the effect of octopamine on sleep/wake behavior is mediated by PKA and cAMP (Crocker and Sehgal, 2008; Crocker et al., 2010). Decreased PKA activity in the IPCs suppresses the wake-promoting effect of octopamine, confirming that PKA acts downstream of octopamine and its receptor in the IPCs, in order to modulate wake/sleep behavior.

Introduction

Overall, Crocker et al proved that octopaminergic neurons release octopamine and activate the OAMB present on the adult IPCs. This leads to increased cAMP signaling and reduced potassium current, resulting in IPCs depolarization and therefore promoting wakefulness (Figure 36).

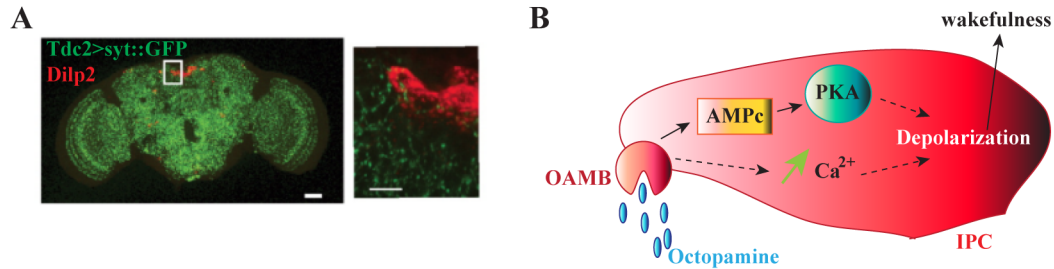


Figure 35: Octopaminergic neurons project on the IPCs and control their neuronal activity. A. The synaptically targeted GFP is expressed in octopaminergic neurons (*Tdc2>syt::GFP*) (green) and the IPCs are labeled by the Dilp2 antibody (red). B. Working model of how octopaminergic neurons control the IPCs neuronal activity in order to promote wakefulness.

Taken and adapted from Crocker A. et al. (2010) – Identification of a neural circuit that underlies the effects of octopamine on sleep:wake behavior.

Unexpectedly, Dilps and InR have been shown to promote sleep in adult flies. Nevertheless, the clock neurons LNvs display positive Dilp2 signal and express *oamb* raising the possibility that octopamine through LNvs neurons could promote sleep (Abruzzi et al., 2017; Cong et al., 2015).

These studies reveal a differential function of IPCs and Dilps on wake/sleep behavior.

IV- DLP neurons: Short Neuropeptide F (sNPF) and Corazonin

The short Neuropeptide F, an orthologue of mammalian neuropeptide Y, promotes feeding behavior and body growth by inducing Dilps expression in the IPCs in an ERK-dependent manner (Lee et al., 2008b, 2004).

This peptide is produced in a bilateral set of neurons called dorsal lateral peptidergic neurons (DLPs). DLP neurons are located in the pars intercerebralis and projects on the IPCs processes both in larvae and adult fly (Kapan et al., 2012) (Figure 36). These neurons also express another neuropeptide: the corazonin. Interestingly, both the sNPF receptor 1 (sNPFR1) and the corazonin receptor (CrzR) are expressed in the adult IPCs (Kapan et al., 2012; Nässel and Broeck, 2016). Silencing either sNPFR1 or CrzR in the IPCs results in decreased IIS in peripheral tissues. Accordingly, specific knockdown of sNPF in DLP neurons reduces *dilp2* and *dilp5* transcription, but not corazonin silencing, suggesting that corazonin and sNPF differently regulate the IPCs activity (Kapan et al., 2012).

Introduction

Surprisingly, some DLPs neurons express the fructose receptor Gr43a and the DH44 receptor, suggesting that DLP neurons potentially directly sense nutrients and in turn modulate the IPCs functions (Miyamoto et al., 2012).

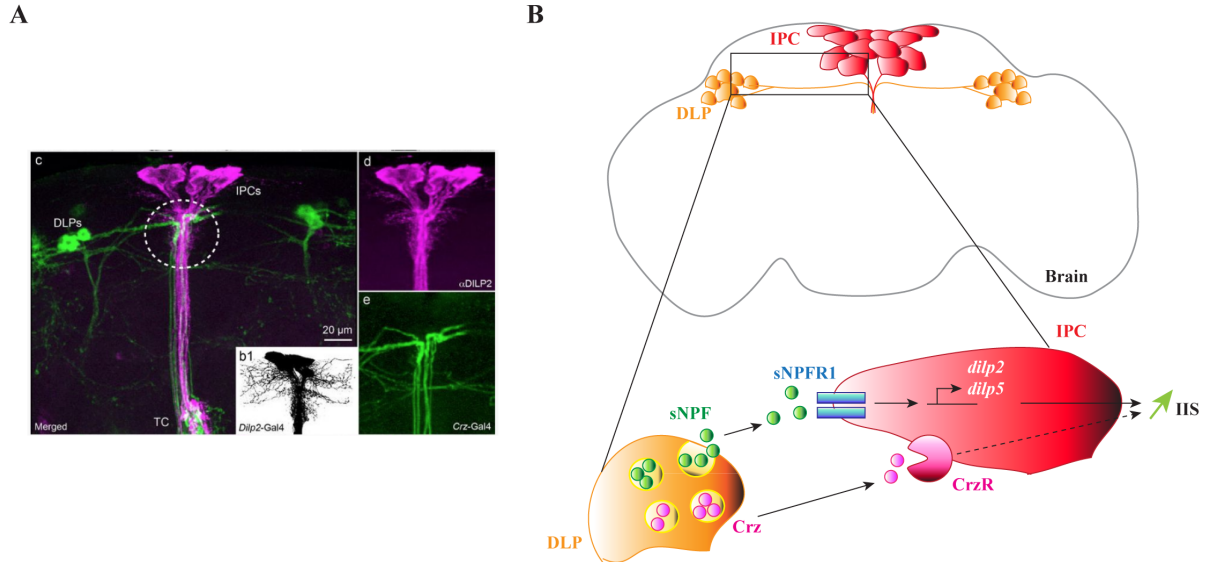


Figure 36 : DLPs neurons project towards IPCs processes and promote insulin/IGF signaling pathway in peripheral tissues. A. Adult brain expressing GFP in corazonin/sNPF neurons (DLPs neurons - green) and stained for *dilp2* antibody (IPCs - magenta). B. Working model summarizing how DLPs neurons differently controls the IPCs activity with both sNPF and Corazonin signaling in order to increase the insulin/IGF signaling pathway in peripheral tissues. Taken and adapted from Kapan N. et al. (2012) – Identified peptidergic neurons in the *Drosophila* brain regulate insulin-producing cells, stress responses and metabolism by coexpressed short neuropeptide F and corazonin.

V- Tachykinin positive neurons

Five different tachykinin peptides are encoded by the same gene, from DTK-1 to DTK-5. They are expressed in several tachykinin positive neurons but also in the enteroendocrine cells of the midgut and potentially regulate the IPCs (Siviter et al., 2000). Indeed, the IPCs express the Tachykinin receptor (DTKR) and neuronal processes of tachykinin positive neurons converge toward the IPCs (Birse et al., 2011) (Figure 37).

DTKR silencing in the IPCs increases *dilp2* and *dilp3* transcription and Dilp2 staining in the IPCs. Furthermore, upon starvation DTKR downregulation in the IPCs causes decreased lifespan and a faster decrease of trehalose levels, both features of increased IIS (Belgacem and Martin, 2007; Partridge et al., 2011). Taken together, these data suggest that DTKR controls the brain IPCs and inhibits the IIS.

Introduction

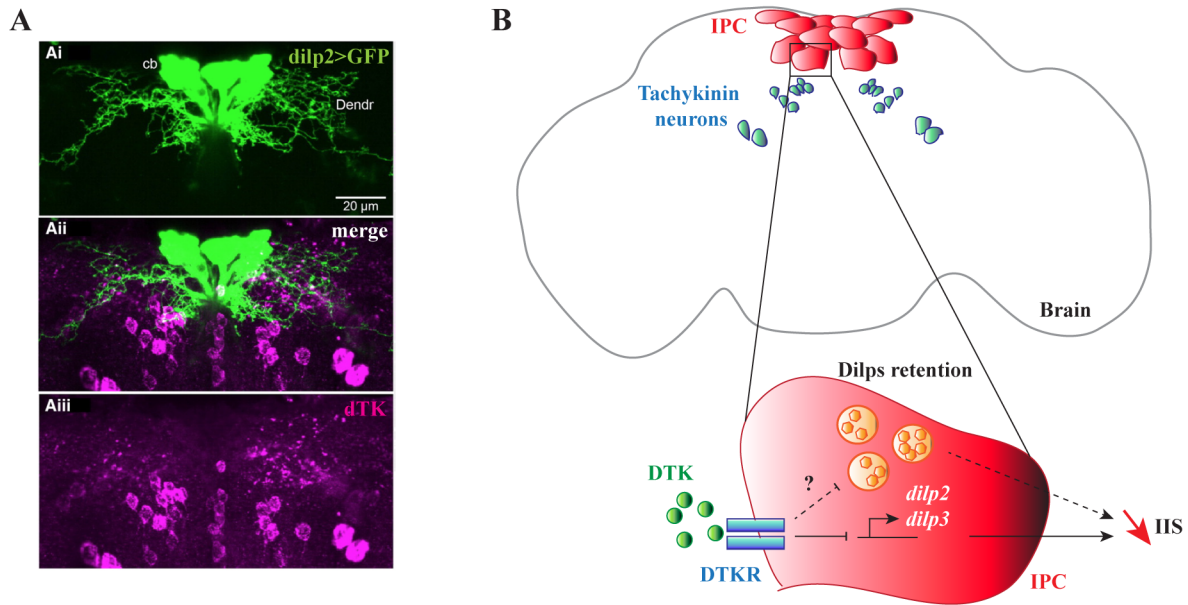


Figure 37 : Tachykinin positive neurons project towards the IPCs processes and tachykinin signaling in the IPCs inhibits the insulin/IGF signaling pathway. A. Adult brain expressing the GFP in the IPCs (green) and labeled with DTK antibody (magenta). Processes of tachykinin neurons impinge on the IPCs. B. Tachykinin signaling in the IPCs inhibits *dilp2* and *dilp3* transcription and probably block Dilps secretion, therefore leading to decreased IIS in peripheral tissues.

Taken and adapted from Birse T. et al. (2011) – Regulation of insulin-producing cells in the adult *Drosophila* brain via the tachykinin peptide receptor DTKR.

VI- Allatostatin A expressing neurons

Four allatostatin A (Asta) peptides have been identified and are expressed both in the intestinal endocrine cells and the brain (Lenz et al., 2000). They signal through two GPCR receptors called *Drosophila* Allatostatin Receptor DAR-1 and DAR-2, both homologs of the mammalian galanin receptor (Birgöl et al., 1999; Larsen et al., 2001; Lenz et al., 2001). AstA is involved in feeding and foraging behavior (Hergarden et al., 2012; Wang et al., 2012).

Interestingly, DAR-2 is expressed in the IPCs of adult brains, and GFP reconstitution across synaptic partners reveals several synaptic contacts between the IPCs and the AstA expressing neurons. Moreover, silencing DAR-2 in the IPCs leads to reduced peripheral IIS, suggesting that AstA is a positive regulator of the IPCs and stimulates Dilps signaling (Hentze et al., 2015) (Figure 38).

In the lab, it has been shown that AstaR1 is also expressed in the larval IPCs. Positive GRASP signal confirmed that IPCs and AstA positive neurons make potential synaptic contacts. Additionally, specific knockdown of AstaR1 in the IPCs results in decreased body size due to reduced growth rate. This

Introduction

is correlated with increased Dilp2 accumulation in the IPCs and less Dilp2 circulating levels in the hemolymph (Deveci D. et al., in preparation) (Figure 39).

These two studies strongly suggest that Allatostatin A positive neurons promotes the insulin signaling through AstA receptors in the IPCs of both *Drosophila* adults and larvae.

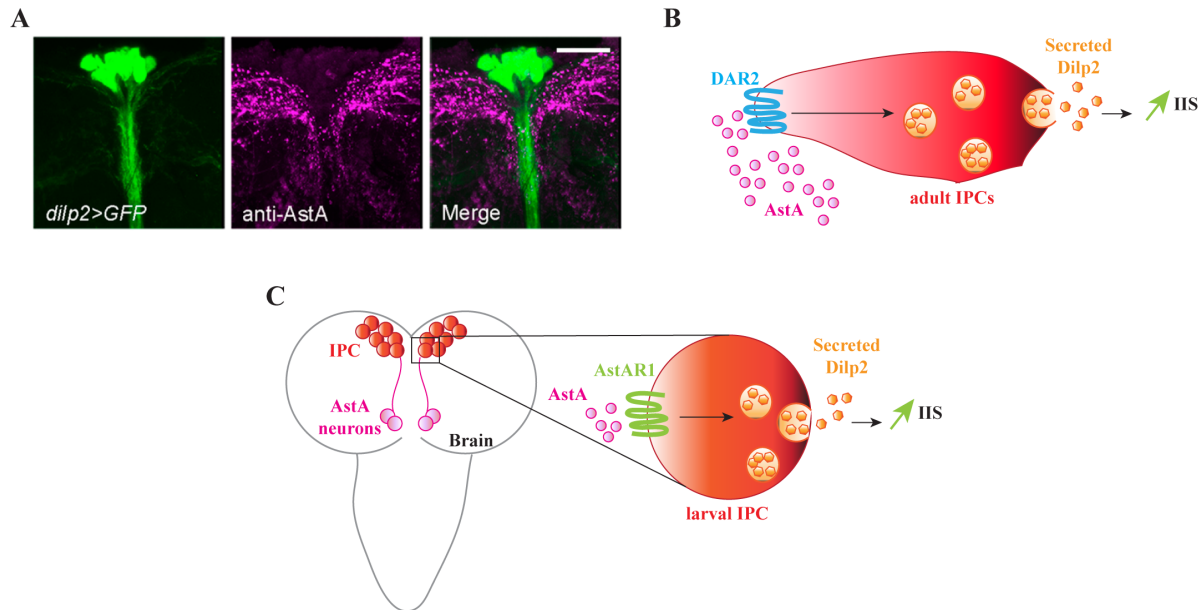


Figure 38 : AstA positive neurons regulate the IPCs activity in both *Drosophila* larva and adult. A. Adult brain expressing the GFP in the IPCs (green) and labeled with the AstA antibody (magenta). AstA peptide is localized all around the IPCs cell bodies and processes. B-C. AstA positively regulates Dilps secretion and increases peripheral IIS through two receptors: AstAR1 in larval IPCs and DAR2 in adult IPCs.

Taken and adapted from Hentze J. et al. (2015) – The neuropeptide Allatostatin A regulates metabolism and feeding decisions in *Drosophila* and from Deveci D. et al. – In preparation.

VII- Hugin positive neurons

Hugin is a neuropeptide expressed in 20 neurons in the larval brain. Interestingly, there are 8 protocerebrum Hugin (Hugin-PC) neurons, which make extensive synaptic contacts with the IPCs (Figure 39). Furthermore, the Hugin-PC neurons express the Hugin receptor encoded by the CG8784, and Hugin treatment in *ex vivo* brain culture, induces high calcium activity in the IPCs (Schlegel et al., 2016).

Interestingly, both insulin signaling and PC neurons strongly inhibits the feeding behavior (Hückesfeld et al., 2016; Wu et al., 2005a, 2005b), further suggesting that Hugin-PC neurons controls the IPCs activity.

Additionally, Hugin-PC neurons are also acetylcholinergic neurons and the IPCs express the muscarinic acetylcholine (ACh) receptor (Cao et al., 2014).

Introduction

Because 98% of synapses have dense core vesicles, the authors suggest that the neurotransmitter ACh and the neuropeptide Hugin are potentially co-released, in order to tightly modulate neuronal transmission (Schlegel et al., 2016).

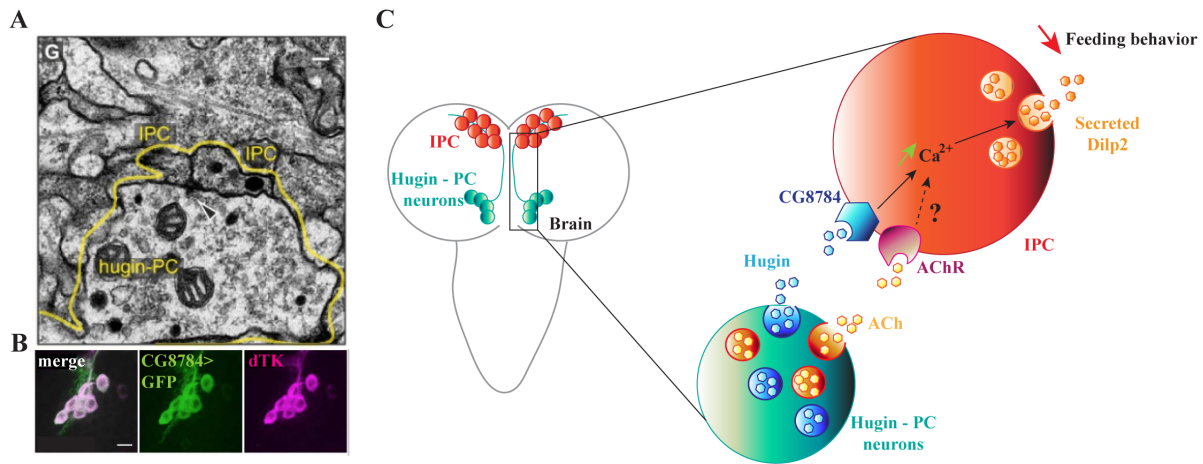


Figure 39 : Hugin neurons located in the protocerebrum make synapses with the IPCs and control their neuronal activity. A. Electron microscopy picture showing synaptic connection between Hugin-PC neurons and the IPCs. B. The Hugin receptor encoded by CG8784 gene (magenta) is localized in the IPCs (green). C. Hugin and ACh are co-released by Hugin-PC neurons and act through their receptors on the IPCs. Hugin signaling increases calcium levels and most probably induces Dilps secretion in order to inhibit feeding behavior. Even though the link between ACh and calcium increase is unclear, both Hugin and ACh are necessary to regulate feeding. Taken and adapted from Schlegel P. et al. (2016) – Synaptic transmission parallels neuromodulation in a central food-intake circuit.

VIII- Glia and Cholinergic neurons

A neuronal circuitry involving both glial cells and cholinergic neurons, have been shown to remotely control *dilp5* transcription in the IPCs of *Drosophila* larvae (Okamoto and Nishimura, 2015).

Dilp5 expression strongly decreases upon starvation. Unlike fed condition, upon nutrient restriction FoxO is localized in the IPCs nucleus. This indicates that *dilp5* transcription and FoxO cellular localization in the IPCs are nutrient-dependent and inversely correlated, suggesting that FoxO acts as a negative regulator of *dilp5* transcription. In line with this, ectopic expression of nuclear FoxO in the IPCs increases *dilp5* transcription upon starvation.

The authors demonstrated that the PI3K signaling in the IPCs is activated under fed condition through the anaplastic lymphoma kinase (Alk). Alk is the receptor for Jelly belly (Jeb), which is mainly expressed in central neurons. Importantly, knockdown of *jeb* in cholinergic neurons reduces *dilp5* transcription and induces nuclear localization of FoxO in the IPCs.

Introduction

In addition, Okamoto N. and Nishimura T. proved that the effect of Jeb is dependent of InR and IIS in cholinergic neurons. Furthermore, they showed that the surface glia function as a nutrient-sensing cells in order to regulate *dilp5* transcription. Interestingly, upon nutrients availability, the surface glia secretes Dilp6 in the brain (Chell and Brand, 2010; Spéder and Brand, 2014). Consistently, *dilp6* specific silencing in the glia induces decreased *dilp5* transcription and partial nuclear localization of FoxO in the IPCs. While *dilp6* overexpression rescues *dilp5* expression and cytoplasmic localization of FoxO in the IPCs upon starvation. Besides, *dilp6* transcription in the glia is IPCs-derived Dilps-, TOR- and nutrient-dependent.

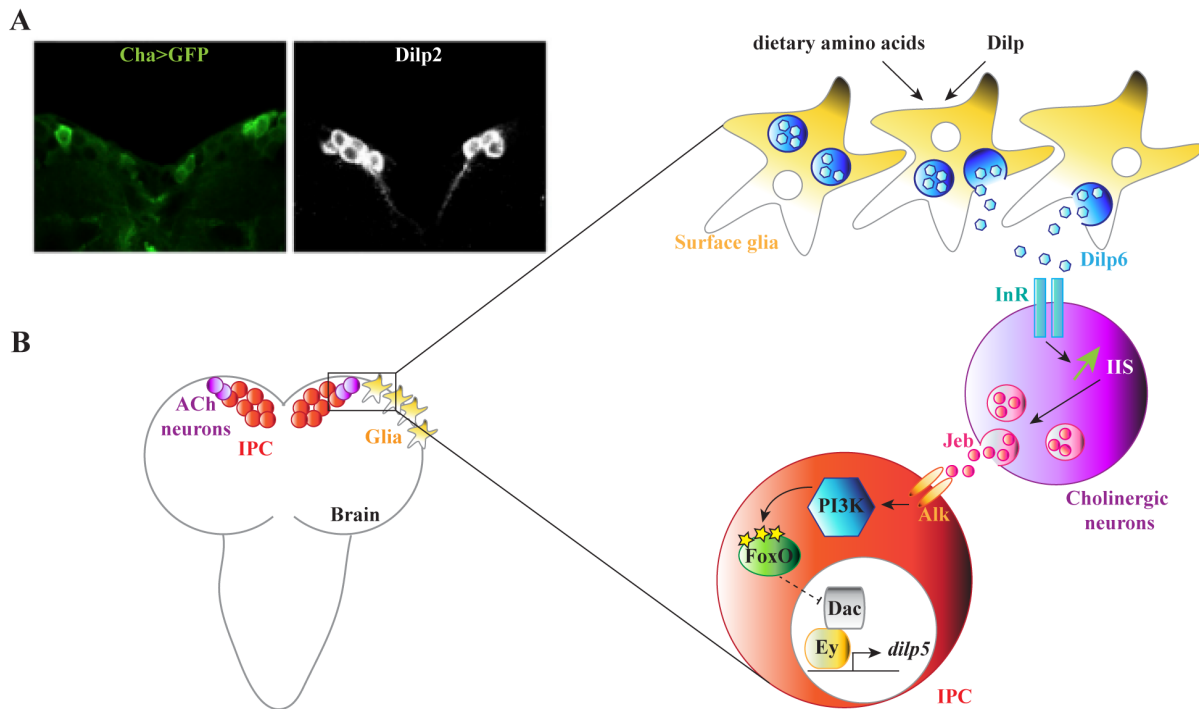


Figure 40 : Cholinergic neurons regulate the IPCs transcriptional activity. Upon normal diet, amino acids and circulating Dilps are sensed by glial cells at the brain surface. Consequently, Dilp6 is secreted and activates IIS in cholinergic neurons resulting in Jeb secretion. Jeb increase PI3K activity through its receptor Alk, restricting FoxO in the cytoplasm. Thus, Dac and Ey promote *dilp5* transcription. Dac: Dachshund; Ey: Eyeless; Alk: Anaplastic lymphoma kinase; Jeb: Jelly belly.

Adapted from Okamoto N. and Nishimura T. (2015) – Signaling from glia and cholinergic neurons controls nutrient-dependent production of an insulin peptide for *Drosophila* body growth.

Taken together, these data demonstrate that both amino acids and IPCs-derived Dilps induce Dilp6 production by surface glia. In response to Dilp6, IIS pathway is activated and Jeb secreted by cholinergic neurons. Jeb then remotely induces *dilp5* transcription in the IPCs through Alk and PI3K activity (Okamoto and Nishimura, 2015) (Figure 40). Even though no direct evidence shows direct

contact between cholinergic neurons and the IPCs, this study reveals a neuronal circuit controlling Dilp5 production by the IPCs.

IX- Dorsal Neurons (DN1)

Recently, in addition to their role in wake/sleep behavior, the adult IPCs have been shown to coordinate metabolic rhythms in the fat body (Barber et al., 2016). Interestingly, IPCs have daily rhythm of electrical activity controlled by the circadian clock. This is due to physical and functional interaction between the IPCs and the dorsal neuron 1 (DN1) of the clock network (Figure 41). Nevertheless, functional experiment showed that activation of DN1 cells do not stimulate all 14 IPCs, suggesting that some IPCs do not receive direct input from DN1 neurons.

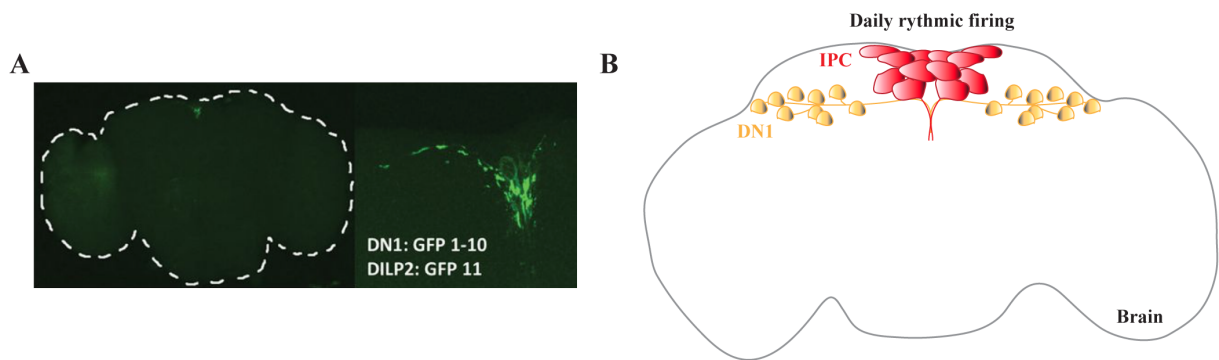


Figure 41 : The central circadian clock circuit controls the IPCs electrical activity *via* their physical interaction with the DN1. A. Positive GRASP signal is observed between the IPCs and DN1. B. Schematic representation of the physical connections between IPCs and DN1. GRASP: GFP reconstitution across synaptic partners; DN1: Dorsal Neuron 1.

Taken and adapted from Barber A. F. et al. (2016) – Circadian and feeding cues integrate to drive rhythms of physiology in *Drosophila* insulin-producing cells.

Chapter VI: PhD project

In the introduction, I explained how body size results from the integration between hormonal inputs and environmental cues, such as nutrition. Nutrition is essential for different physiological features, notably the metabolism homeostasis, body growth and fecundity. That is why nutrient sensing is so complex and diversified. During my PhD, I was interested in further elucidating how nutritional information is sensed, conveyed and integrated in order to fine-tuned hormonal growth control. Indeed, the nutritional control of the growth rate is mediated by the IIS. Even though *dilps* transcription is important, Dilps secretion from the IPCs remains the most efficient way to rapidly increase insulin signaling in peripheral tissues and therefore promote body growth. Moreover, Dilps secretion from the IPCs is precisely adjusted in response to nutrition (Géminard et al., 2009). Most of the nutrient sensors controlling *Drosophila* larvae growth, involve the IPCs. However how the secretory activity of the IPCs is controlled by dietary nutrients remains imprecise.

The IPCs are in the center of an elaborated network which undergo different regulations thanks to different signals. In response or in absence of nutrients, some of these signals act directly on the IPCs, such as the fat-derived Egr and CCHa2, while others require a neuronal relay like Upd2 (Agrawal et al., 2016; Rajan and Perrimon, 2012; Sano et al., 2015). Besides, the IPCs are located in an optimum position, allowing them to receive inputs from several neuronal populations in order to accurately control their neuronal activity, both in *Drosophila* larvae and adults.

My PhD project consisted in understanding how the IPCs secretory activity is regulated by identifying neuronal populations which affect Dilps secretion and control body size according to nutrition.

RESULTS

Chapter I: An EGF-responsive neural circuit couples insulin secretion with nutrition in Drosophila

I- Introduction

In order to maintain energy homeostasis and adapt body growth to environmental cues, organisms have to assess their nutritional status. Thus, insulin-like peptides release is tightly regulated by nutrient availability. In *Drosophila*, 8 different Dilps (Dilp1-Dilp8) exist. Apart from Dilp8, the other Dilps exert metabolic and growth-promoting functions through a conserved IIS (Colombani et al., 2012; Garofalo, 2002; Ikeya et al., 2002). At least four of them are produced by two clusters of seven neurosecretory cells located in the brain: the IPCs. These peptidergic neurons are functionally equivalent to pancreatic β cells and release Dilps into the hemolymph to control systemic growth (Rulifson et al., 2002). The IPCs indirectly evaluate the nutritional status through an interorgan communication with the fat body. This tissue is homologous to the vertebrate liver and white adipose tissue and has been presented as the main nutrient sensing organ (Colombani et al., 2003). Through production of different fat body-derived signals (FBS), the fat body conveyed the nutritional information to brain IPCs and remotely controls Dilps secretion (Géminard et al., 2009). Several FBS have been identified and act on the IPCs by different mechanisms: either directly or indirectly through neuronal relay (Agrawal et al., 2016; Rajan and Perrimon, 2012; Sano et al., 2015). Others FBS such as the GBPs have been discovered but the neuronal circuitry at play is unknown (Koyama and Mirth, 2016). Furthermore, the position and neuronal structure of the IPCs suggest that they probably receive several inputs from other neurons to fine-tune their secretory activity.

Results

During my PhD, I explored the possibility that other neuronal populations could control the IPCs secretory activity in order to modulate Dilps secretion and ultimately body growth, according to nutrients availability. Interestingly, I unraveled a novel neural circuit controlling the IPCs secretory activity in response to dietary amino acids. I discovered one pair of inhibitory neurons located in the Pars Intercerebralis which display synaptic connections with the IPCs. These IPCs-Connecting Neurons (ICNs) are active upon acute amino acid withdrawal and block Dilp2 secretion. Surprisingly, I demonstrated that ICNs respond to the fat hormone GBPs through Epidermal Growth Factor receptor (EGFR). Upon dietary amino acids, GBPs released from fat cells activate the ICNs EGFR signaling, suppressing their inhibitory effect on the IPCs and leading to Dilp2 secretion. Unexpectedly, this study elucidates the mode of action by which GBPs promotes growth and adds a complexity level to the elaborated network involving the IPCs.

II- Manuscript

An EGF-responsive neural circuit couples insulin secretion with nutrition in *Drosophila*

Eleonora Meschi¹, Pierre Léopold^{1*} and Renald Delanoue¹

¹Université Côte d'Azur, CNRS, Inserm, Institute of Biology Valrose, Parc Valrose, 06108 Nice, France.

* corresponding author and lead contact: leopold@unice.fr

Running title: EGFR-driven neural circuitry controls insulin release in *Drosophila*

Summary (150 words max.)

Developing organisms use fine-tuning mechanisms to adjust body growth to ever-changing nutritional conditions. In *Drosophila*, the secretory activity of insulin-Producing Cells (IPCs) is central to couple systemic growth with amino acids availability. Here, we identify a subpopulation of inhibitory neurons contacting the IPCs (IPCs-connecting neurons, or ICNs) that play key role in this coupling. We show that ICNs respond to Growth Blocking Peptides (GBPs), a family of fat body-derived signals produced upon availability of dietary amino acids. We demonstrate that GBPs are atypical ligands for the fly EGF receptor (EGFR). Upon activation of EGFR by adipose GBPs, ICN-mediated inhibition of IPC function is relieved, allowing insulin secretion. Our study reveals an unexpected role for EGF-like metabolic hormones and EGFR signaling as critical modulators of neural activity, coupling insulin secretion to the nutritional status.

Introduction

Developing organisms must evaluate their nutritional status, adapt growth and maintain energy homeostasis. In vertebrates, variations in the circulating levels of insulin and insulin-like growth factor 1 (IGF-I) trigger nutrient storage and release, and control systemic growth. Therefore, the release of insulin-like peptides by their producing cells is tightly regulated by nutrients. Alterations in this coupling is associated with major metabolic diseases and growth defect (Donadon et al., 2009).

Insulin-like peptides act on an evolutionary conserved pathway and serve the same physiological role in coupling nutrition and growth in all developing multicellular organisms. In *Drosophila*, eight Insulin-like peptides (Dilp1-8) have been identified. With the exception of Dilp8, which has distinct functions (Colombani et al., 2012), all the other Dilps carry out both the metabolic functions of the vertebrate insulin and the growth-promoting functions of IGFs, through a unique receptor (InR) and a conserved intracellular insulin/IGF signaling pathway (IIS) (Garofalo, 2002; Ikeya et al., 2002). In flies, the insulin-producing cells (IPCs), which are functionally related to the pancreatic beta cells, are found in the brain (Rulifson et al., 2002). The IPCs are peptidergic neurons, located in the median neurosecretory cluster (mNSC), producing at least four of the Dilps (Brogiolo et al., 2001; Ikeya et al., 2002; Liu et al., 2016). IPCs processes present at the surface of the heart tube release Insulin-like peptides into the hemolymph, inducing organ growth and controlling carbohydrate homeostasis (Rulifson et al., 2002).

During the growth period, IPC secretory activity is indirectly coupled to the nutrient status through an inter-organ communication involving the fat body (FB), a functional equivalent of vertebrate liver and white adipose tissue that acts as a nutrient sensor. This function relies on the amino acid sensor Target Of Rapamycin Complex 1 (TORC1) in the FB, controlling the production of fat body-derived signals (FBS) that remotely adjust Dilps secretion from the IPCs (Colombani et al., 2003; Géminard et al., 2009). Both growth-promoting and –inhibiting FBSs, produced in response to various nutritional cues, participate in

this tight control. The cytokine Unpaired 2 (Upd2) and the small peptide CCHamide-2 (CCHa2) stimulate IPCs activity after sugar and/or lipid intakes (Rajan and Perrimon, 2012; Sano et al., 2015). In response to dietary amino acids, adipose cells release the Stunted (Sun) peptide and the Growth Blocking Peptides 1 and 2 (GBP1, 2), which remotely activate brain Dilp secretion (Delanoue et al., 2016; Koyama and Mirth, 2016). Conversely, chronic exposure to a low protein diet induces the release of Tumor Necrosis Factor (TNF)- α from fat cells, which in turn represses brain *dilp2* expression (Agrawal et al., 2016). These FBSs do not share any common mode of action on the IPC. They act directly through their receptors on the IPCs, such as the CCHa2 receptor, the Sun receptor Methuselah or the TNF receptor Grindelwald (Agrawal et al., 2016; Delanoue et al., 2016; Sano, 2015). Alternatively, like in the case of Upd2, a neuronal relay is used (Rajan and Perrimon, 2012). In the case of the GBPs, the mechanism of action remains unknown (Koyama and Mirth, 2016).

Here, we report the identification of a short neural circuit required to adjust Dilp secretion in response to dietary amino acids, consisting in one pair of bilateral neurons making direct synaptic connections with the IPCs. Under low protein diet, these IPC-connecting neurons (ICNs) inhibit Dilp2 secretion. In fed larvae, ICNs activity is blocked, allowing Dilp release and animal growth. We further show that ICNs activity is controlled by fat GBPs, which act as long range hormonal agonists of EGFR signaling. In fed conditions, GBPs activate EGFR signaling in the ICNs, relieving their inhibitory effect on the IPCs, resulting in Dilp secretion. Altogether, our study reveals an unexpected role for EGF-like metabolic hormones and EGFR signaling as critical modulators of neural activity, coupling insulin secretion to the nutritional status.

Results

Identification of a pair of neurons contacting the IPCs

The IPCs are at the center of an elaborated network integrating various nutritional cues delivered in part by peripheral sensor tissues. In order to better characterize this intricate network, we first sought to image the neuronal architecture of the IPCs. Using the IPC driver *dilp2-Gal4*, we expressed DenMark to visualize post-synaptic vesicles (Nicolai et al., 2010) and a Synaptotagmine::GFP (SyteGFP) fusion as a presynaptic marker (Zhang et al., 2002). As previously described, we observed IPCs axons projecting towards the *corpora cardiaca* (CC). More surprisingly, we could also observe a dense dendritic compartment surrounding the soma with branching running caudally along the midline through the subesophageal ganglion (Figure 1A). This suggested that the IPCs receive inputs from afferent neurons connecting through their dendritic arborisations.

We then screened the *Janelia* neuronal GAL4 collection to identify driver lines expressing in neurons projecting towards the IPCs dendrites. We focused our attention on the *R22H11* line marking one pair of neurons located in the Pars Intercerebralis (PI) of the optic lobes, and one pair of CAPA-positive neurons in the posterior, ventral part of the medial subesophageal ganglion (named CC-MS 2, Corpora Cardiaca innervating neurosecretory neuron of the medial subesophageal ganglion 2) (Siegmund and Korge, 2001) (Suppl. Figure 1). Interestingly, the *R22H11* PI neurons project their neurites towards the IPC dendrites (Figure 1B). By using the *dvGlut::GFP* fusion that marks presynaptic compartments (Riemensperger et al., 2013), we found that presynaptic vesicles of the *R22H11* PI neurons co-localize with the IPCs (Figure 1C). This suggests that *R22H11* neurons project axons towards the IPCs. Using the *GFP reconstitution across synaptic partners* (GRASP) (Feinberg et al., 2008) we could confirm direct contact between the two neuronal populations (Figure 1D). These findings therefore suggest that the

R22H11 PI neurons could directly contact the IPCs and thereafter these neurons were named IPCs Connecting Neurons (ICNs).

The ICNs are inhibitory afferent neurons controlling IPC function.

To test whether the ICNs could functionally interfere with IPC secretory activity, we ablated the ICNs by expressing the pro-apoptotic gene *hid* with the *R22H11* driver (hereafter named *ICN-Gal4*). ICNs ablation induces pupal overgrowth (+15,6%) (Figure 2A and S2A) associated with a decrease in Dilp2 staining in the IPCs (Figure 2B). We confirmed by Dilp2 ELISA (Park et al., 2014) that reduced Dilp2 staining in the IPCs was the consequence of increased Dilp2 release in the hemolymph (Figure 2C). In line with this, pupal overgrowth was due to augmented growth rate (Figure 2D) with no effect on developmental timing (Figure S2C) or food intake (Figure S2D). Both hyperpolarization of the ICNs by expression of the potassic channel Kir2.1, or impaired secretion by tetanus toxin (TetX) led to pupal overgrowth, associated with reduced accumulation of Dilp2 in the IPCs and increased circulating Dilp2 levels (Figure 2A-C). Converse experiment using the bacterial sodium channel NaChBac to induce a chronic depolarization of the ICNs led to a strong reduction of circulating Dilp2 associated with accumulation of Dilp2 in the IPCs (Figure 2B and C). However, in our experimental conditions, this was not sufficient to modify pupal volume (Figure 2A).

Importantly, no phenotype was observed using a CAPA-GAL4 line expressed in the CAPA-positive subset of the *R22H11*-positive neurons (Figure S2B). This demonstrates that the ICNs located in the PI are sole responsible for the observed growth regulations. Since mRNA levels of *dilp2* remain unchanged after manipulating ICNs activity (Figure S2E), we conclude that the ICNs are part of an inhibitory neuronal relay specifically acting on Dilp2 secretion by the IPCs.

ICNs and IPCs present opposite responses to nutrition.

Since the IPCs modulate Dilps secretion according to nutritional cues, we then assessed whether ICNs respond to nutrition. The CaLexA reporter allows cumulative tracing of neuronal activity (Masuyama et al., 2012). Using this reporter, IPCs show a robust neuronal activity in fed conditions, which is abolished under amino acids starvation (Figure 3A). By contrast, ICNs exhibit basal activity in fed conditions, which dramatically increases after acute amino acids starvation (Figure 3B). In addition, IPCs showed decreased activity at late wandering stage (a physiological state of cessation of feeding) compared to younger feeding larvae (Figure 3C), while ICNs activity strongly increases during this stage (Figure 3D). Therefore, IPCs and ICNs show opposite responses to nutrition, in line with the inhibitory action of ICNs on IPCs.

Ectopic expression of GBPs in the ICNs promotes body growth

We next tested whether ICNs could constitute a neuronal relay between the fat body and the IPCs in the nutritional response. To evaluate this possibility, we expressed various fat body-derived factors (FBS) in the ICNs and tested their possible autocrine action on ICNs function and animal growth. While Stunted or Unpaired 2 expression in the ICNs (*icn>sun*, *icn>upd2*) had no effect on pupal volume (Figure 4A, S3A-B), expression of GBP1 and GBP2 (*icn>gbp1*, *icn>gbp2*) led to pupal overgrowth similar to what obtained after GBP overexpression in the fat body (*lpp>gbp1*, *lpp>gbp2*) (Figure S3C). Like in the case of ICNs activity manipulation, this pupal overgrowth was coupled with increased Dilp2 secretion from the IPCs (Figure 4B). This autocrine response is specific for the ICNs since ectopic expression of GBPs in a neighboring neuronal population (*eh>gbp1*, *eh>gbp2*), or in the CAPA neurons targeted by the *R22H11* driver do not induce body growth increase (Figure S3D, E). These results suggest that ICNs respond to nutrients through variation in GBPs levels.

GBPs activate the EGFR signaling pathway

GBPs share 3-dimensional structure in their core region with EGF ligands (Aizawa et al., 2002) and bind to EGF receptor (EGFR) in human keratinocytes (Ohnishi et al., 2001). To better understand the mode of action of GBPs on ICNs, we first tested whether GBPs activate *Drosophila* EGFR signaling in insect cells. After activation by its ligands, EGFR dimerizes and undergoes auto-phosphorylation in trans on tyrosine. We therefore tested whether GBPs induce EGFR phosphorylation in co-cultured S2R+ cells expressing EGFR::V5 and GBP1::HA as previously described (Lahusen et al., 2007) Anti-phosphotyrosine immunoprecipitation followed by anti-V5 western blotting indicates an increase of Tyr-phosphorylated EGFR::V5 in the presence of GBP1-HA (Figure 4C). This is accompanied by increased ERK phosphorylation (Figure 4D), as previously described (Tsuzuki et al., 2014). In addition, ectopic expression of GBP1 in the wing pouch (*nub>gbp1*) increases the level of phosphorylated ERK (Figure 4E). This is abolished upon EGFR silencing (*nub>egfrRi, gbp1*) showing that GBP1 requires EGFR to activate ERK signaling *in vivo*. EGFR signaling controls wing vein patterning and, when in excess, leads to formation of ectopic veins in the adult wing (Butchar et al., 2012). Forced expression of both GBP1 and GBP2 in the wing pouch induces an extra vein phenotype (Figure 4F), confirming that GBPs are potent *in vivo* activators of EGFR signaling.

EGFR signaling in the ICNs induces Dilps secretion and systemic growth

We next wondered whether GBPs action in the ICNs requires EGFR. For this, we first modified EGFR signaling in the ICNs and observed systemic growth effects. Inhibition of EGFR signaling in the ICNs (*icn>egfr-ri, icn>dsor-ri*) reduces pupal size (-8%) (Figure 5A and S4A), and Dilp2 release from the IPCs (Figure 5B). Conversely, increased EGFR signaling (*icn>egfr^{A887T}*) induces overgrowth (Figure 5A and S4A) and Dilp2 release (Figure 5B), while *dilp2* gene expression is unchanged (Figure S4B). Notably, no growth phenotype is observed when the CAPA-GAL4 line is used to drive expression of the modified forms of

EGFR (Figure S4C). Remarkably, overexpression of an activated form of Spitz (*icn>sspitz*) or its membrane tethered version (*icn>nrt::sspitz*) in the ICNs phenocopy GBP-induced growth (Figure S4D), but not a non-processed form of Spitz (*icn>mspitz*, Figure S4D). This overall indicates that activation of EGFR in the ICNs is sufficient to promote systemic growth *via* Dilp release. Although EGFR signaling could potentially impact cell proliferation, differentiation and migration (Shilo, 2003), we did not observe alteration in the number or the macroscopic organization of the ICNs in any of these conditions. In addition, ICNs where EGFR signaling is experimentally modified, still produce Tackykine (dTK) (Figure S4E) and Myoinhibitory Peptide (MIP) (Figure S4E'), suggesting that their neuronal identity is not changed.

We further established that the growth inhibition produced by reducing EGFR signaling in the ICNs (*icn>egfr-ri* or *icn>dsor-ri*) was epistatic to GBP1 overexpression (*icn>egfr ri; gbp1* or *icn>dsor ri; gbp1*) (Figure 5C,D, Figure S4F). This indicates that the GBPs exert their effect on the ICNs through activation of EGFR signaling. Altogether, these results indicate that GBPs produced by fat body cells in response to nutrients act remotely on the ICNs *via* EGFR signaling to promote systemic growth.

GBPs produced by fat cells repress ICNs activity through EGFR signaling.

We next tested whether GBP1 release from fat cells relies on dietary amino acids. For this, we generated a functional HA-tagged GBP1 allele. Its overexpression either in the fat body (*lpp>gbp1HA*) (Figure S5A) or in the ICNs (*icn>gbp1HA*) (Figure S5B) induces an overgrowth similar to that observed with unmodified GBP1. Upon acute amino acids starvation, we did not detect changes in GBP1::HA protein levels in adipose cells (*lpp>gbp1HA*), while hemolymph levels severely dropped (Figure 6A). Noticeably, in these conditions, fat body GBP1::HA-containing vesicles were enlarged compared to fed controls (Figure S5C). Importantly, using anti-HA antibody, we could detect fat-derived GBP1::HA decorating the ICNs of fed larvae (Figure 6F and S5D). These results indicate that the secretion of GBPs

from fat cells is tightly controlled by nutrition, and that GBP1 molecules secreted by fat cells travel to the brain ICNs.

Since fat body derived-GBP1 has insulinotropic properties (Koyama and Mirth, 2016), we next assessed whether this effect is achieved *via* a relay using EGFR signaling in the ICNs. For this, hemolymph from control larvae (*lpp>w*) or larvae overexpressing *gbp1* in the fat body (*lpp>gbp1*) was collected to perform *ex vivo* brain culture experiments. When incubated with dissected control brains (*icn>w*), hemolymph from *lpp>gbp1* larvae efficiently triggered Dilp2 secretion, confirming the insulinotropic function of GBPs. This effect was abolished upon EGFR silencing in the ICNs (*icn>egfr-ri*) brains (Figure 6B), indicating that fat body derived-GBP1 requires EGFR in the ICNs to stimulate Dilp2 secretion.

To better understand the function of GBP/EGFR signaling in ICNs, *icn>calexa* brains of wandering larvae were incubated with hemolymph collected from control larvae (*lpp>w*), larvae overexpressing *gbp1* in the fat body (*lpp>gbp1*) or larvae bearing a deletion of both *gbp1* and *gbp2* genes (*ex67^{-/-}*). While hemolymph from both control and *gbp1*-overexpressing larvae induced a decrease in ICNs neuronal activity, hemolymph from *ex67^{-/-}* mutant larvae had no effect (Figure 6C), demonstrating that circulating GBP1 represses ICNs neuronal activity. Similarly, overexpressing a constitutively activated form of EGFR in the ICNs (*calexa; icn>egfr^{A887T}*) efficiently repressed ICNs in wandering larvae (Figure 6D), while silencing EGFR (*calexa; icn>egfr-ri*) activated them in L3 larvae (Figure 6E). Hence, we conclude from these data that activation of EGFR signaling by circulating GBPs inhibits ICNs neuronal activity, therefore releasing ICNs-mediated inhibition of the IPCs.

Discussion

EGFR signaling exerts a central control on cell growth and differentiation, and as such is essential in multiple developmental processes. Our study proposes a new paradigm for EGFR signaling by

establishing that EGF receptor and its atypical GBP ligands play a pivotal role in controlling neuronal activity and, as a consequence, adapt the rate of tissue growth according to nutritional cues.

GBPs act as long distance EGFR ligands.

RMN analysis suggests that the core region of GBPs has structural similarity with the C-terminal domain of EGF (Aizawa et al., 1999, 2002). Several experimental approaches provided conclusive evidence of direct binding of GBPs to EGFR in keratinocytes, other results suggested that unidentified receptor and co-receptor or adaptor protein might exist in insect cells (Aizawa et al., 2002; Oda et al., 2010; Ohnishi et al., 2001). More recently, GBPs were proposed to interact with the GPCR Mthl10, but there is no direct evidence that this interaction has functional significance for the control of IPCs function (Sung et al., 2017).

Our study establishes that *gpb1* overexpression can efficiently stimulate EGFR-dependent signaling, both in cultured cells and in developing organs. We also provide genetic evidence that the function of fat body derived-GBPs in controlling Dilp secretion entirely relies on the presence of EGFR in the ICNs.

In both mammals and flies, several ligands activate EGFR signaling to fulfill a variety of biological responses (Ceresa and Peterson, 2014). The main ligand for EGFR, Spitz, is palmitoylated, which reduces its secretion and its range of action (Miura et al., 2006). By contrast, we show that GBPs produced by the fat body can travel over long distances and bind to the brain ICNs, therefore providing a signal for inter-organ communication (see Figure 6F and S5D). We do not know how GBPs are transported in the hemolymph, but recent data suggest that mammalian EGFR ligands with endocrine function could be packed into signaling competent exosomes (Singh et al., 2016).

The mechanisms of GBP released into the hemolymph is uncharacterized. However, we showed that GBP secretion is highly dependent on the nutritional status, since HA-tagged GBP1, although highly

produced in fat cells under an ectopic Gal4 system, is undetectable in the hemolymph when larvae are subjected to amino acids starvation. TORC1 signaling controls *gbp* gene transcription (Koyama and Mirth, 2016). It will be interesting to know whether GBP secretion is also controlled by TORC1 activity or by alternative pathways.

EGFR function in the ICNs

In the CNS, EGFR and its various ligands promote differentiation, maturation and survival of a variety of neurons (Wong and Guillaud, 2004). In the present work, we observed that EGFR signaling in the ICNs is not needed for their architecture or their neuronal identity. Surprisingly, activation of EGFR reduces intracellular calcium and represses ICNs activity. Other pathways than MAPK signaling could be activated downstream of EGFR, such as PI3K/AKT or phospholipase-C γ (PLC- γ), which could promote intracellular calcium release. These branches of EGFR signaling control cell migration and invasion (Wee and Wang, 2017). However, in the context of ICNs, activation of EGFR leads to calcium decrease, and we found that dSOR, one downstream component of the MAPK pathway, is involved in this process. Interestingly, some MAPK components are present in distal neural structures such as dendrites and synapses, suggesting that their function is not limited to the regulation of gene expression and could control local synaptic activity in a more acute response. Indeed, several synaptic proteins have been identified as ERK/MAPK substrates such as scaffolding PSD proteins, Kv4.2 potassium channels or group I metabotropic glutamate receptors. Phosphorylation of these components by ERK/MAPK modifies the trafficking and synaptic delivery of these proteins and thus determines the strength and the efficacy of excitatory synapses (Mao and Wang, 2016). These would be interesting targets for a role of EGFR in ICNs to evaluate in future studies.

A general mechanism for Insulin-like peptides secretion.

Our work identifies a central relay that is instrumental in regulating Insulin-like peptides secretion in response to dietary amino acids. In mammals, deficiencies in dietary proteins are associated with impaired insulin secretion and amino acids or dietary proteins have insulinotropic effects (Newsholme et al., 2007; Zhang and Li, 2013). One possible mechanism for this regulation could involve a central relay through direct innervation of the pancreatic islets (Horiuchi et al., 2017; Rosario et al., 2016). In addition, it is known that EGF ligands can stimulate insulin release from pancreatic islets (Lee et al., 2008). However, it is not clear what tissue could be producing the EGF signal in such regulation. Therefore, the present work gives a prospect for further studies identifying key circuitries required for insulin/IGF secretion.

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Materials and Methods

Fly strains and media

The following fly strains were used: white¹¹¹⁸ (*w*), *dilp2-GAL4* (Rulifson et al., 2002), *lpp-GAL4* (gift from S. Eaton), *lexAop-CD8-GFP-2A-CD8-GFP*; *UAS-mLexA-VP16-NFAT*, *LexAop-rCD2-GFP* (gift from Jing W. Wang), *UAS-dvglut::GFP* (gift from S. Birman), *UAS-sun* (gift from W Ja), *UAS-upd2* (gift from N. Perrimon). K. Mirth and T. Koyama kindly provided the following lines: *UAS-gbp1*, *UAS-gbp2*, *ex67^{-/-}*. The *UAS-mspi*, *UAS-sspi* and *UAS-nrt::sspi* strains were a kind gift by J. Treisman.

R22H11-GAL4 (RRID:BDSC 48043) (Pfeiffer et al., 2010) and *R22H11-LexA* (RRID:BDSC_54109) lines come from Janelia Farm (BDSC).

RNAi lines come from the Vienna Drosophila RNAi Stock Center: *UAS-egfrRi* (107130KK), *UAS-dsorRi* (107276KK).

nubbin-GAL4; *UAS-hid*; *UAS-tetx*; *UAS-kir2.1*; *UAS-NaChBac*; *UAS-egfr^{A887T}*; *EH-GAL4* (C21); *UAS-mCD8-GFP*, *CAPA-GAL4*; *UAS-DenMark*, *UAS-syteGFP* and *lexAop-rCD2-RFP*; *UAS-CD4-spGFP1-10*, *lexAop-CD4-spGFP11* were provided by the Bloomington Drosophila Stock Center.

Animals were reared at 25°C on fly food containing per liter, 10g of agar, 83g corn flour, 60g white sugar, 34g dry yeast and 3,75g Moldex (diluted in ethanol).

Plasmids and generation of transgenic lines

gbp1 and *gbp2* coding sequences were PCR amplified from DGRC cDNA clones IP05665 and GH14572 respectively. They were cloned into the pENTR/D-TOPO vector using the following gene-specific primers: sense primer CAC CGA ATT CAG GAT GTT GAT ACG TAT TAA TCC ATT GGT G and antisense primer CCG GAA TTC CGC CGG CTT TCT GCA TCG TTT TCC for *gbp1* and sense primer CAC CGG TAC CAG GAT GTT TTT GCA ACT CTT ATC TAT A and antisense primer CCG GGT ACC GGC TTC CTT CCT GCA ACG TCC TGC for *gbp2*. To generate the *UAS-gbp1::HA* and *UAS-gbp2::HA* lines, coding sequences were cloned into the Gateway Destination vector (Drosophila Gateway Vector Collection) by using of the pTWH. *UAS-gbp1::HA* construct was then introduced into the germ line by injections in *w¹¹¹⁸* fly line (BestGene).

pBDP2 gdilp2-BHA-FLAGA (gift from S. K. Kim) was injected in the Bloomington stock #24484 to generate a new *y¹ w¹¹¹⁸*; *gd2HF(attP2)*. J. Treisman kindly provided the *mt-egfr::V5* and *UAS-cSpi::GFP* plasmids. *UAS-hh::HA* was kindly provided by K. Basler.

Amino acids starvation

72h AEL larvae are transferred for 16h on PBS 1x, 2% agar plates and supplemented with 1% sugar, but no protein source.

Feeding behavior

Blue food dye (Erioglaucine Disodium Salt, Sigma-Aldrich) was added at 1,5% in the food. Larvae were left in blue food for 3 hours at 25°C, washed, put in microtubes (8 larvae per tube), and frozen. Samples were homogenized in water and spun down for 5 min at 13000 rpm, and the amount of blue dye in the supernatant was measured by spectrophotometry (OD₆₂₉ Nanovue). For a given experiment all values are normalized to one single condition.

Pupal volume analysis

Larvae were synchronized at 24h AED and reared under controlled conditions (30 larvae/vial). Pupal volume was measured using ImageJ and calculated by using the formula

$$\frac{4}{3} * \pi * \left(\frac{length}{2}\right) * \left(\frac{width}{2}\right)^2$$

. For a given experiment, all values are normalized to one control condition to give the “Δ pupal volume ratio”.

Ex vivo Organ culture

We performed ex vivo organ culture as described in (Géminard et al., 2009). Brains of staged larvae were dissected in Schneider (Sigma) using sterile tools. Brains were transferred into tube containing 30µl of Schneider medium supplemented with 2,5% FBS and 1% streptomycin. 20µl of hemolymph was collected from larvae and carefully added to dissected brains. Co-cultures were incubated overnight at 18°C.

Immunofluorescence on larval tissues

Larval tissues were dissected in 1x PBS, fixed in 4% formaldehyde (Polyscience) in PBS for 30 min at room temperature and then washed several times in PBS containing 0,3% Triton X-100 (PBT). They were then blocked into PBT+10% FBS. Primary antibodies were incubated overnight at 4°C. After several washes, secondary antibodies were incubated 2h at room temperature. Dissected tissues were mounted into Vectashield (Vector). Fluorescence images were acquired using a Leica SP5 DS confocal microscope.

The following antibodies were used: rabbit anti Dsred (1/200) (632496 Clontech), chicken anti GFP (1/10000) (ab13970 abcam), rat anti Dilp2 (1/400), rabbit anti pERK (1/200) (4370S Cell Signalling), rat anti HA (1/200) (3F10 Sigma). The rabbit anti ITK (*Aedes locusta* tachykinin used to visualize dTK) (1/500), rabbit anti CAPA precursor (1/500), rabbit anti MIP (myoinhibitory peptide) (1/500) were kindly gifted by J. Veenstra.

Fluorescence quantification

After acquisition of confocal z stacks of dissected tissue with a 0,5 μ m step and identical laser power and scan settings, images were analyzed by ImageJ. We performed the sum-intensity 3D projections to measure total fluorescent intensity across the object of interest (IPCs or ICNs) and subtracted the background fluorescent intensity. For a given experiment, all values are normalized to one control condition to give the “ Δ fluorescent intensity ratio” (Dilp2 or GFP accumulation).

For ex-vivo organ culture presented in Fig 6E, GFP positive cells were counted instead of total fluorescent intensity. For a given experiment, all values are normalized to one control condition to give the “ Δ GFP positive cell ratio”.

Hemolymph collection

Hemolymph was collected as described in Rodenfels et al. (2014). It was obtained by bleeding larvae in ice cold PBS 1x. To remove hemocytes, a first centrifugation at 500g for 30min is performed and then followed by a second one at 16000g for 20min at 4°C to remove cellular fragments. The supernatant is then directly used for western blotting.

Protein extraction

After dissection, 5 to 10 fat bodies were transferred in an eppendorf with 60 μ l of RIPA buffer containing protease (Complete – Roche) and phosphatase inhibitor cocktails (Thermo Scientific). Tissues were then manually disrupted. Samples were then rotated for 30min at 4°C and then spun down at full speed, 4°C for 10min. Proteins were precipitated from the supernatant with chloroform-methanol mix (except for S2 cells extract). Pellets were dried 5 min and then dissolved into 1,5mM Tris, 6M Urea and inhibitor cocktails. Proteins measurements were done with the Bio-Rad Protein Assay (Bradford) (BIO-RAD).

Blue 4x (NuPage, Novex) and reducing agent 10x (NuPage, Novex) were added before heating at 100°C 5min.

Cell culture and phospho-MAPK assay

S2R+ cells were maintained in Schneider medium supplemented with 10% FBS and 1% streptomycin. All UAS plasmids were co-transfected with *actin-GAL4* using lipofectamine (ThermoFisher Scientific).

For the phospho-MAPK assay, on day 3 after transfection with *mt-egfr::V5*, cells were treated with 100 μ M of CuSO₄ to induce EGFR::V5 expression. On day 4, cells transfected with *GAL4-UAS* plasmids were resuspended in Schneider medium and cocultured with EGFR::V5 expressing cells for 3 hours at 22°C. Cells were harvested and proteins were extracted.

Western Blotting

Proteins were resolved by SDS-PAGE 12% gels (NuPage, Novex gel, Invitrogen) using the MOPS running buffer and transferred electrophoretically to Nitrocellulose membranes (Biorad). Membranes were incubated 30 minutes in blocking buffer PBS-T (1X PBS, 0,1% Tween-20, 5% milk), and then incubated in the same buffer with primary antibodies, overnight at 4°C. After several washes, membranes were incubated 2h with PBS-T containing the secondary antibody and then washed again. Chemiluminescence was observed using the ECL-Plus Western Blotting detection system (Biorad). Images were taken with the Fujifilm Multi Gauge software and analyzed using imageJ.

The following antibodies were used: rabbit anti ERK (1/2000) (4695 Cell Signalling), rabbit anti pERK (1/2000) (4370S Cell Signalling), rat anti HA (1/500) (3F10 Sigma), guinea pig anti Cvd (1/2000) (gift from S. Eaton), mouse anti tubulin (1/2000) (T9026 Sigma).

Immunoprecipitation

A protein A or G-sepharose beads with CL6B beads mix was incubated with the primary antibody 3h at 4°C, after several washes in IP buffer (50mM Tris pH8, 150mM NaCl, 1mM EGTA, 10%NAF, protease (Complete – Roche) and phosphatase inhibitor cocktails (Thermo Scientific). After proteins extraction, 800 μ g of proteins were added to the mix and rotated at 4°C overnight. After several washes, blue 4x (NuPage, Novex) and reducing agent 10x (NuPage, Novex) were added and samples were heated at 100°C 5min.

The following antibodies were used: rabbit anti phosphor-tyrosine (1/1000) (8654S Cell Signalling) and mouse anti V5 (1/2000) (R960-25 Invitrogen).

ELISA Test

Experimental procedure was adapted from Park et al. (2014) on 1 μ l or 2 μ l of hemolymph collected from larvae, transferred in 55 μ l of PBS, centrifuged at 1000g for 1min.

Quantitative RT-PCR

qRT-PCR experimental procedure is described in Delanoue et al. (2010).

Wing analysis

Adult flies were collected, stored in ethanol and wings were mounted in Euparal solution. Pictures were acquired using a Leica Fluoresce StereomicroScope M205 FA with a Leica digital camera MC 190 HD.

Statistical Analysis

P values are the result of ANOVA or Student's test provided by Graphpad Prism (* $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$, **** $p < 0,0001$).

Figure Legends

Figure 1: *R22H11* neurons present axonal projections making physical contacts with the IPCs

(A) *UAS-DenMark*; *UAS-SyteGFP* are driven by the IPCs driver, *dilp2-GAL4*. The dendritic marker DenMark is used to visualize the IPCs dendrites (red), and SyteGFP reveals their axonal architecture (green).

(B) The *R22H11-GAL4* line drives *mCD8GFP* (green) in one neuron in each brain hemisphere, sending projections towards the IPCs (anti-Dilp2, red) (hereafter referred as ICNs, see text).

(C) Expression of *UAS-DVglut::GFP* (a vesicular transporter of glutamate) driven by *R22H11-GAL4* (hereafter referred to as *icn-Gal4*) labels presynaptic vesicles (green). Analysis of z sections reveals that ICNs axons (green) co-localize with the IPCs (anti-Dilp2, red) along their neuronal tracts and cell bodies.

(D) GFP reconstitution across synaptic partners (GRASP) shows physical connections between IPCs and ICNs. *icn-LexA* and *dilp2-GAL4* are used to express membrane-tethered split-GFP *LexAop-CD4::spGFP11* and *UAS-CD4::spGFP1-10*, respectively. IPCs are stained by the anti-Dilp2 (cyan) and ICNs are labeled with a membrane associated RFP (DsRed, red). The GRASP signal is visualized in green.

Figure 2: ICNs inhibit body growth by blocking Dilps secretion

(A) Pupal volume measurement after ICNs ablation (*icn>hid*), hyperpolarization (*icn>kir2.1*), blockade of secretory activity (*icn>tetx*) or chronic depolarization (*icn>nachbac*). (n≥238)

(B) Dilp2 accumulation levels in the IPCs upon ICNs ablation (*icn>hid*), hyperpolarization (*icn>kir2.1*) secretion blockade (*icn>tetx*) or depolarization (*icn>nachbac*). (n≥62) Fluorescence intensity is measured as delta ratio compared to control (*icn>w*).

(C) ELISA test of Dilp2HF (*gd2HF*) circulating levels in the hemolymph upon ICNs ablation (*icn>hid*), hyperpolarization (*icn>-kir2.1*), secretion blockade (*icn>tetx*) or depolarization (*icn>nachbac*). (n≥4 for 3 independent experiments)

(D) Larval growth curve after ICNs ablation (*icn>hid*), compared to controls (*icn>w* and *w hid*). (n≥57).

In all graphs, means are shown and errors bars represent ±SEM, ****p<0,0001, **p<0,01, *p<0,05.

Figure 3: IPCs and ICNs show antagonistic regulation by nutrition and developmental stage

A. Analysis of IPCs neuronal activity in fed and starved (1% sucrose) conditions using the calcium reporter *UAS-CaLexA* crossed with *dilp2-Gal4*. (n≥68)

B. Analysis of ICNs neuronal activity in fed and starved (1% sucrose) conditions using the calcium reporter *UAS-CaLexA* crossed with *icn-Gal4*. (n≥56)

C. Analysis of IPCs neuronal activity at 80h AEL and 120h AEL (wandering stage) using the calcium reporter *UAS-CaLexA* crossed with *dilp2-Gal4*. (n≥48)

D. Analysis of ICNs neuronal activity at 80h AEL and 120h AEL (wandering stage) using the calcium reporter *UAS-CaLexA* crossed with *icn-Gal4*. (n≥69)

In graphs, means are shown and errors bars represent ±SEM, ****p<0,0001.

Figure 4: Growth-Blocking-Peptides (GBPs) are putative EGF ligands inducing systemic body growth when expressed in the ICNs

A. Ectopic expression of *gbp1* or *gbp2* in the ICNs (*icn>gbp1* and *icn>gbp2*) leads to increased pupal volume. (n≥408)

B. Dilp2 signal in IPCs decreases upon *gbp1* and *gbp2* ectopic expression in ICNs. (n≥34)

C. GBP1 induces tyrosine auto-phosphorylation of EGFR in S2 cells (co-culture of GBP1::HA expressing cells with EGFR::V5 expressing cells, immunoprecipitation with anti-phospho-Tyr antibodies, western blotting using anti-V5 antibody).

D. Co-culture of EGFR::V5 expressing cells with either GBP1- or GBP2-expressing cells tested for ERK phosphorylation (pERK). The EGFR ligand Spitz (cSpi::GFP) and Hedgehog-HA (Hh::HA) are used as positive and negative controls, respectively.

E. ERK phosphorylation (pERK) observed upon ectopic *gbp1* expression in the wing pouch (*nub>gbp1*) requires EGFR (*nub>egfrRi, gbp1*).

F. Ectopic *gbp1* and *gbp2* expression in the wing pouch using *nub-GAL4* driver (*nub>gbp1* and *nub>gbp2*) induce extra veins (L2).

In graphs, means are shown and errors bars represent \pm SEM, ****p<0,0001.

Figure 5: GBP1 in the ICNs promotes body growth through EGFR signaling pathway

A. Pupal volumes after silencing *egfr* (*icn>egfrRi*) or *dsor*, (*icn>dsorRi*) or activating EGFR signaling (*icn>egfr^{A887T}*) in ICNs. (n \geq 83)

B. IPCs-Dilp2 retention after silencing *egfr* (*icn>egfrRi*) or *dsor*, (*icn>dsorRi*) or activating EGFR signaling (*icn>egfr^{A887T}*) in ICNs. (n \geq 58)

C. Pupal volumes after ectopic expression of *gbp1* alone (*icn>gbp1*), or upon co-silencing EGFR (*icn>egfrRi, gbp1*) in the ICNs. (n \geq 77)

D. IPCs-Dilp2 accumulation after ectopic expression of *gbp1* alone (*icn>gbp1*), or upon co-silencing EGFR (*icn>egfrRi, gbp1*) in the ICNs. (n \geq 57)

In graphs, means are shown and errors bars represent \pm SEM, ****p<0,0001.

Figure 6: Fat body-derived GBP1 is secreted in the hemolymph and acts on ICNs neuronal activity through EGFR

A. Western blotting experiment (anti-HA) on hemolymph extracts and dissected fat bodies from *lpp>gbp1::HA* larvae in either fed or acute amino acids starvation state.

B. Brain culture with hemolymph collected from control larvae or larvae overexpressing *gbp1* in the fat body (*lpp>gbp1*) tested for IPC-Dilp2 retention. Brains were extracted from either *icn>w* control or *icn>egfrRi* animals. (n \geq 48)

C. Brain culture with hemolymph collected from control larvae, larvae overexpressing *gbp1* in the fat body (*lpp>gbp1*) or larvae deficient for *gbp1* and *gbp2* (*ex67^{/-}*) tested for ICNs neuronal activity using the CaLexA calcium marker (*icn>calexa*). (n \geq 47)

D. The CaLexA reporter is used to test ICNs activity upon constitutive active EGFR expression (*icn>calexA, egfr^{A887T}*) at late wandering stage (123h. AEL). (n \geq 44)

E. The CaLexA reporter is used to test ICNs activity upon silencing EGFR expression (*icn>calexA, egfrRi*) during L3 feeding stages (96h. AEL). (n≥33)

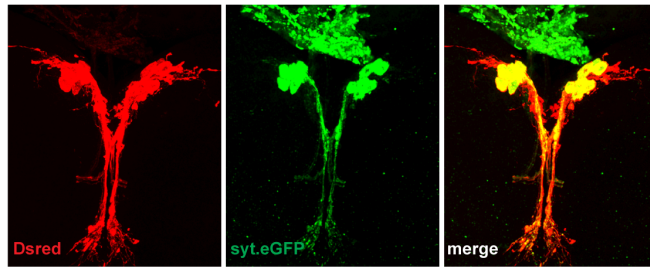
F. GBP1::HA (green) specifically expressed in the fat body (*lpp>gbp1::HA*) is detected in the ICNs, labelled with an anti-Tachykinine (dTK, red) antibody.

In graphs, means are shown and errors bars represent \pm SEM, ****p<0,0001, **p<0,01.

Meschi - Figure 1

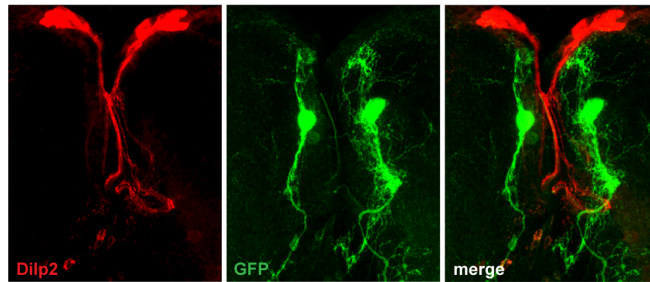
A

dilp2>UASDenMark; syteGFP



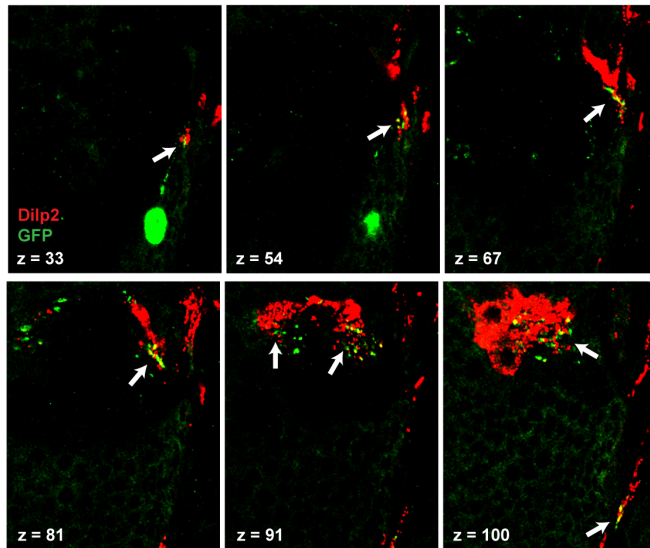
B

R22H11>UASmCD8GFP



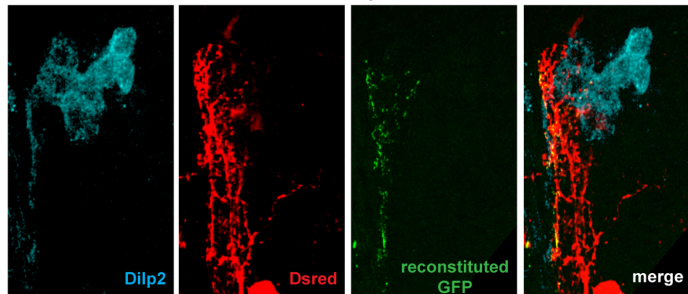
C

R22H11>UAS-dvGlut::GFP



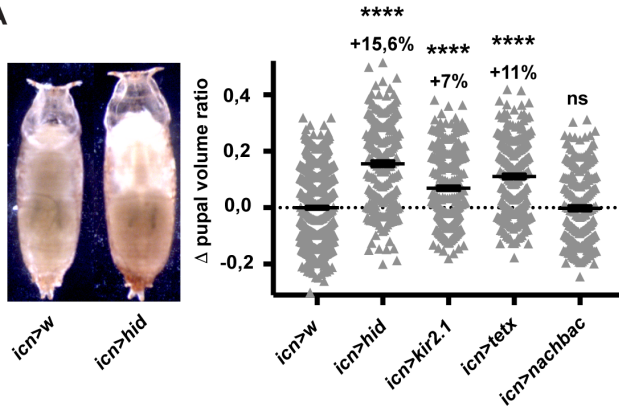
D

R22H11LexA; dilp2>GRASP

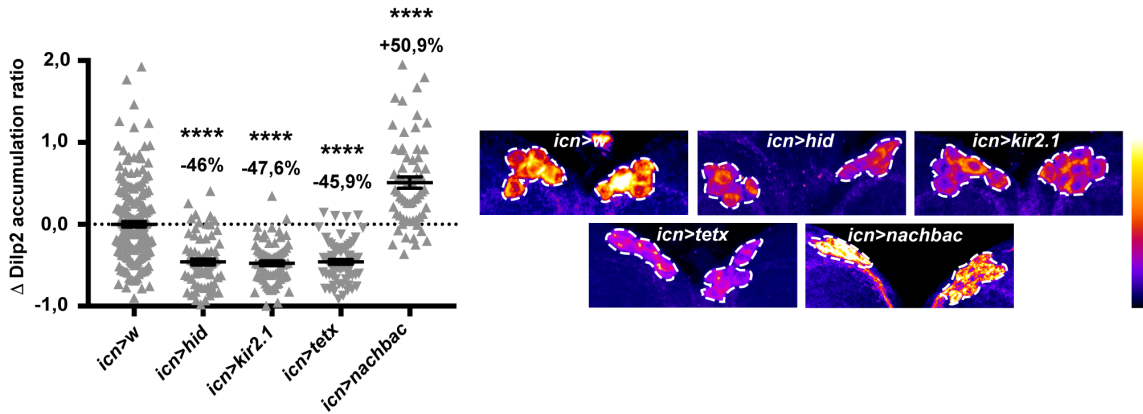


Meschi - Figure 2

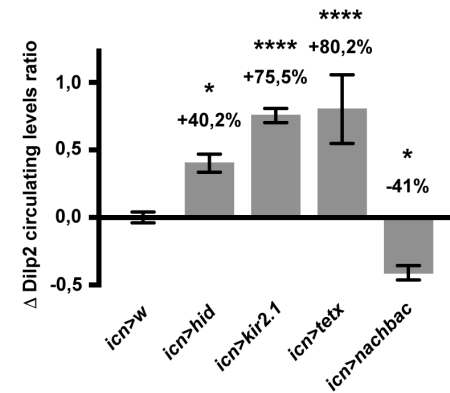
A



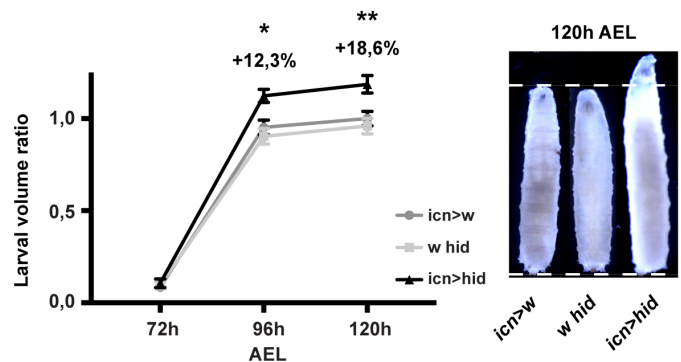
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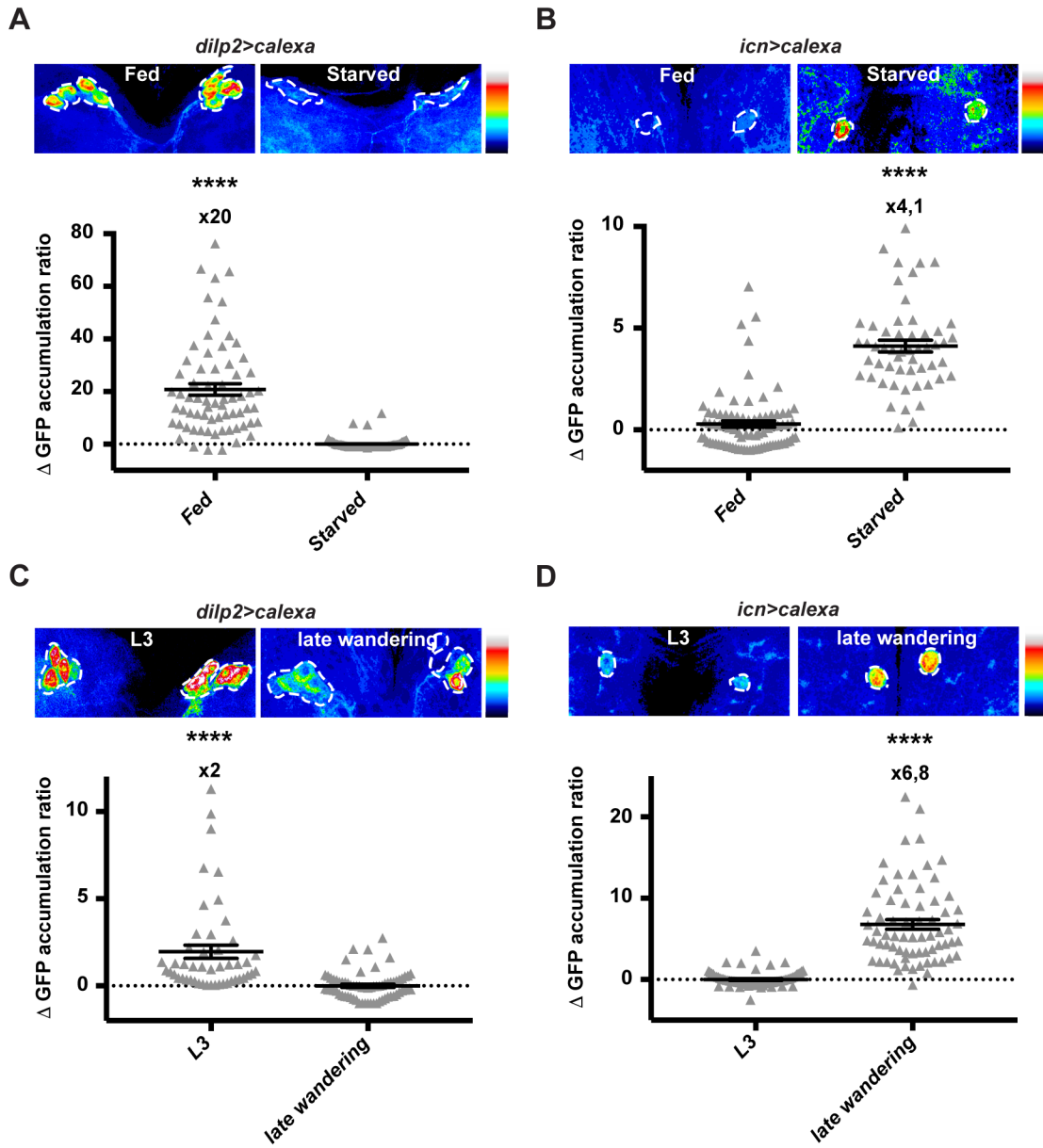
C



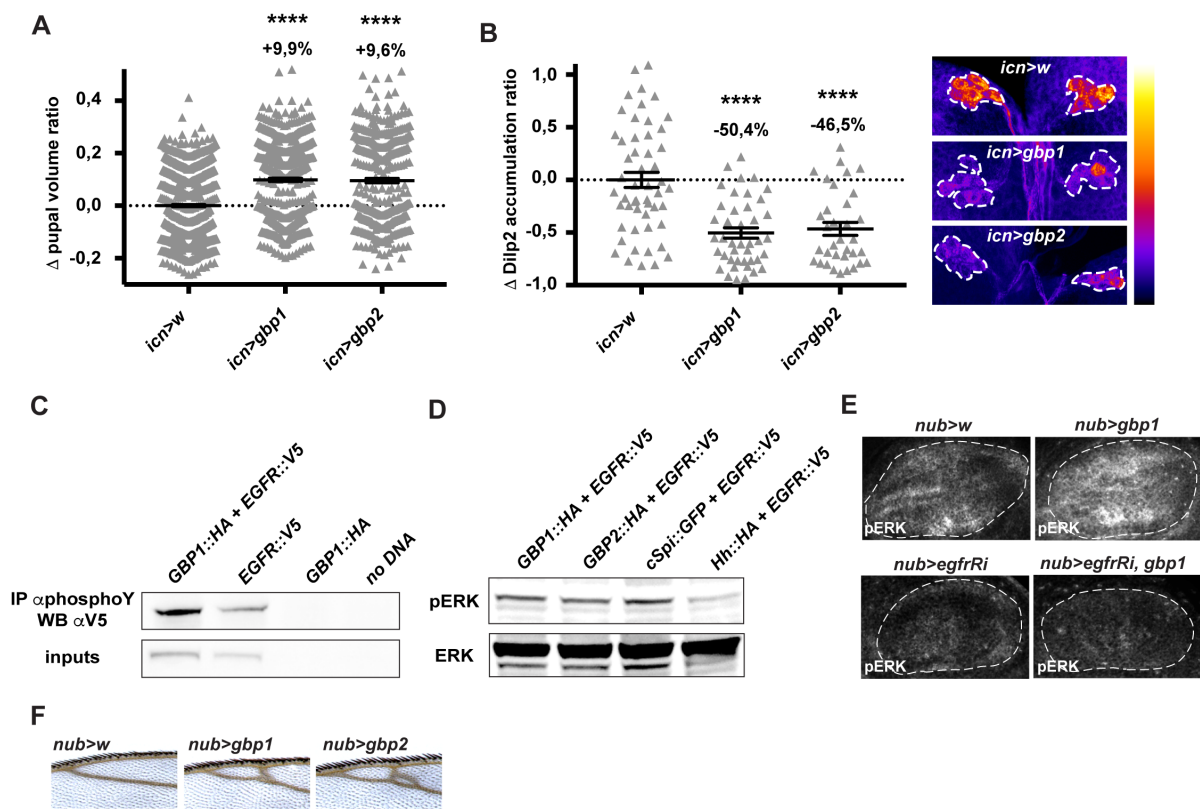
D



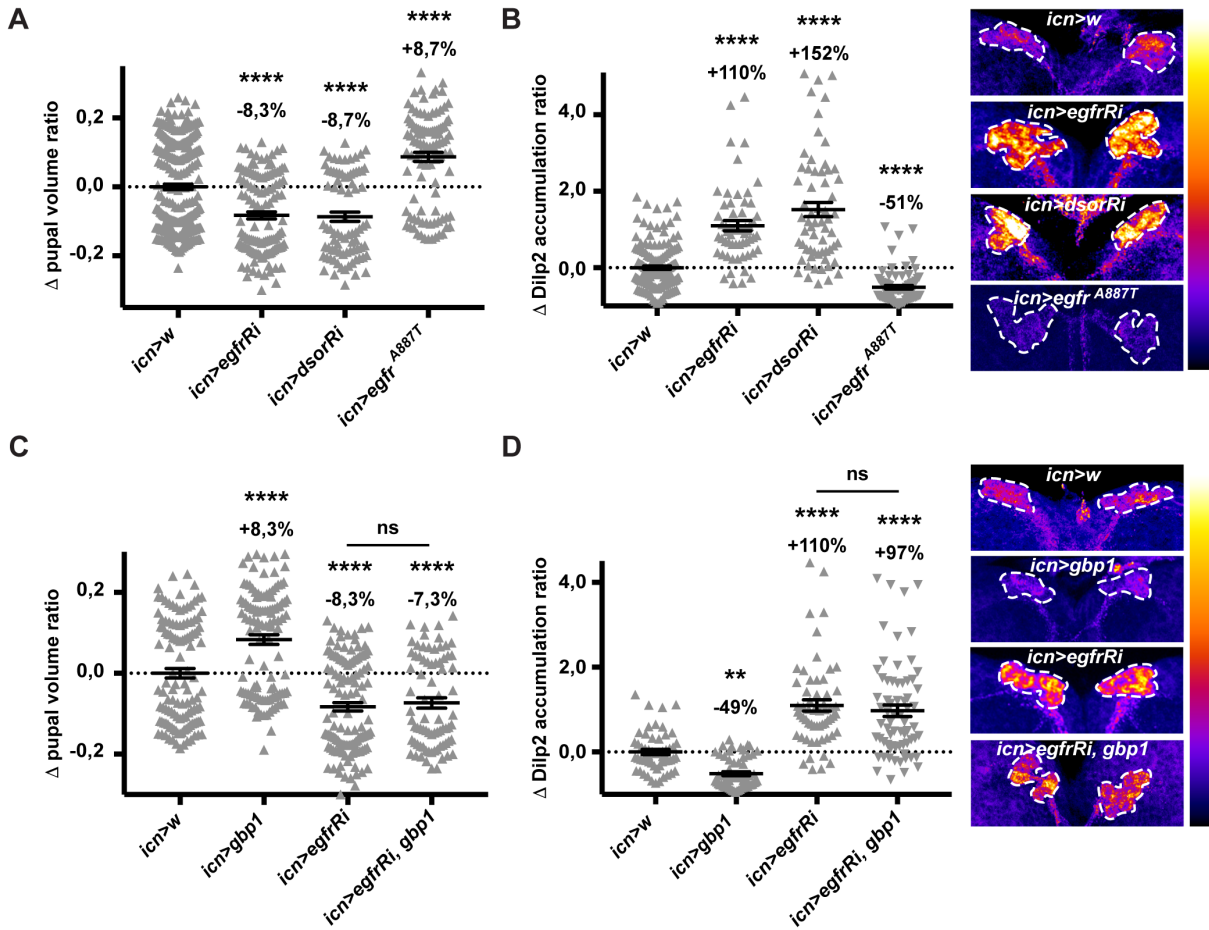
Meschi - Figure 3



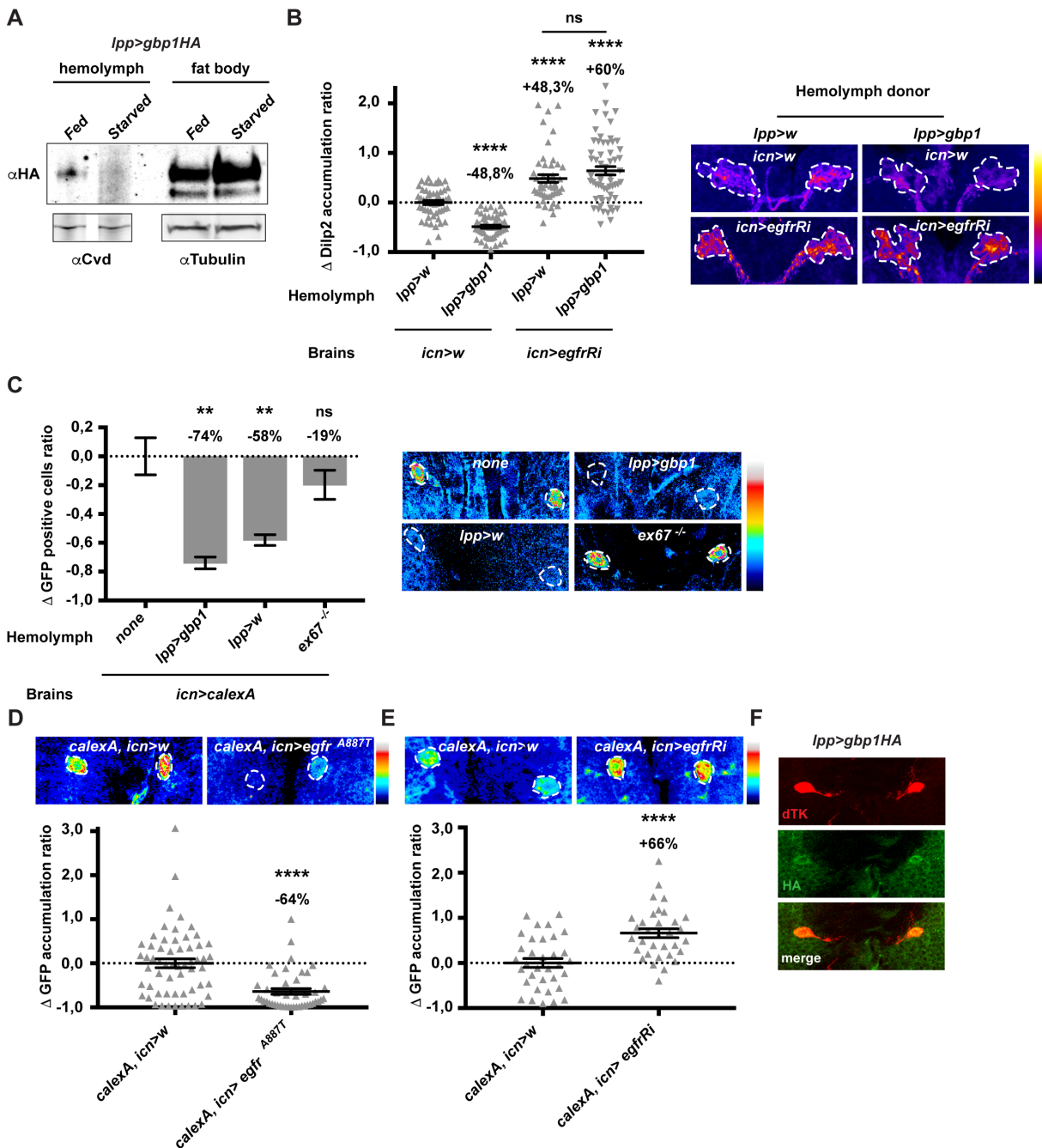
Meschi - Figure 4



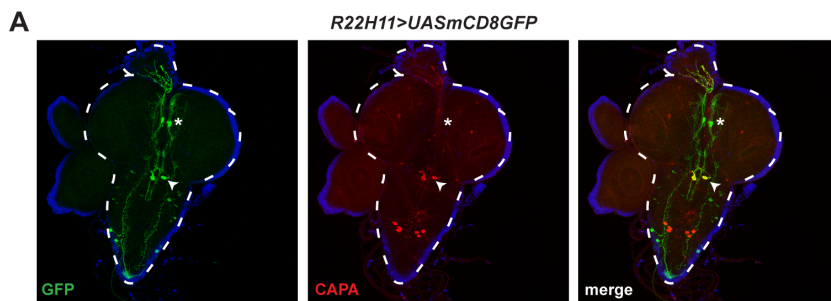
Meschi - Figure 5



Meschi - Figure 6



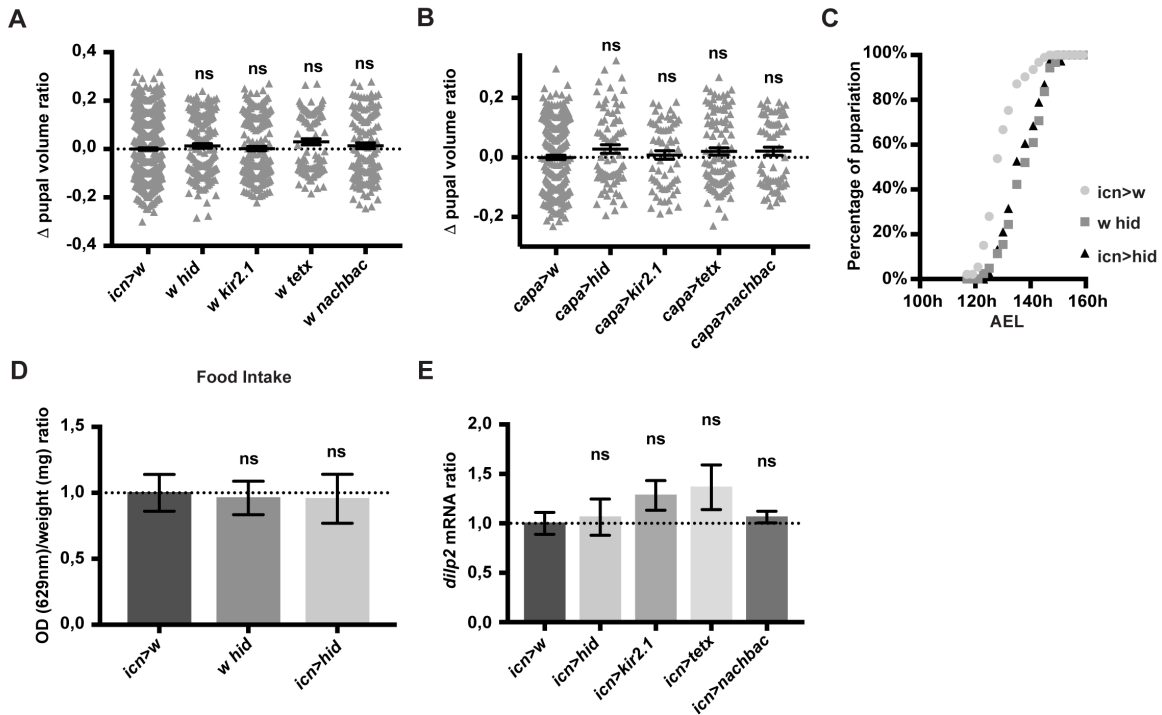
Meschi - Suppl Fig 1



Supplementary Figure 1: Complete expression pattern of the R22H11 driver

A. The *R22H11-GAL4* line drives *mCD8GFP* (green) in one neuron in each brain hemisphere, (hereafter referred as ICNs, see text) indicated by an asterisk*, and in one pair of CAPA positive neurons (red), shown with a white arrowhead.

Meschi - Suppl Fig 2



Supplementary Figure 2: Overgrowth phenotypes are exclusively due to the ICNs effect on Dilps secretion, with no effect on developmental timing, feeding behavior nor dilp2 expression

A. Pupal volume measurements in all control conditions (*icn>w*, *w UAS*-). (n>94)

B. Pupal volume measurements after CAPA neurons ablation (*capa>hid*), hyperpolarization (*capa>kir2.1*), blockade of secretory activity (*capa>tetx*) or chronic depolarization (*capa>nachbac*). (n>60)

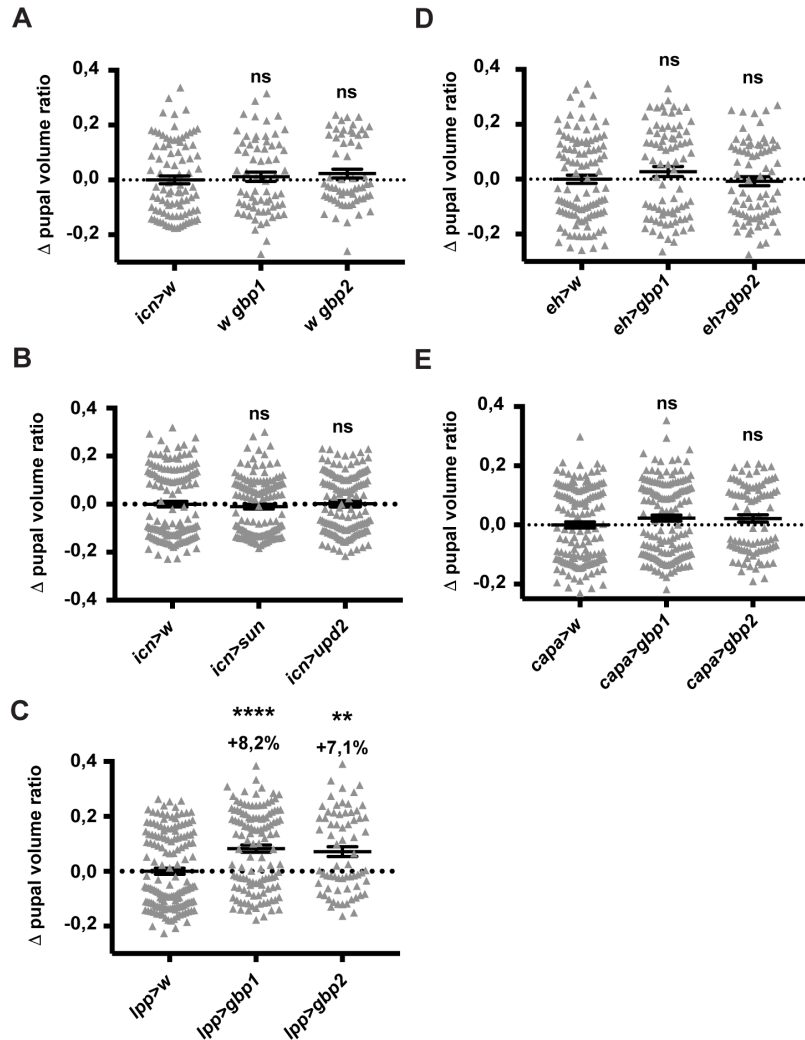
C. Developmental timing measurements after ICNs ablation (*icn>hid*). (n>40)

D. Feeding behavior was tested after ICNs ablation (*icn>hid*). (n=9)

E. *dilp2* mRNA levels in dissected brains were quantified upon ICNs ablation (*icn>hid*), hyperpolarization (*icn>kir2.1*), secretion blockade (*icn>tetx*) or depolarization (*icn>nachbac*). (n=3)

In graphs, means are shown and errors bars represent \pm SEM.

Meschi - Suppl Fig 3



Supplementary Figure 3: GBPs stimulate body growth specifically through ICNs

A. Pupal volume measurements in all control conditions (*icn>w*, *w UAS-*). (n>60)

B. Pupal volume measurements upon ectopic expressions of the fat body signals *sun* (*icn>sun*) and *upd2* (*icn>upd2*) in ICNs. (n>144)

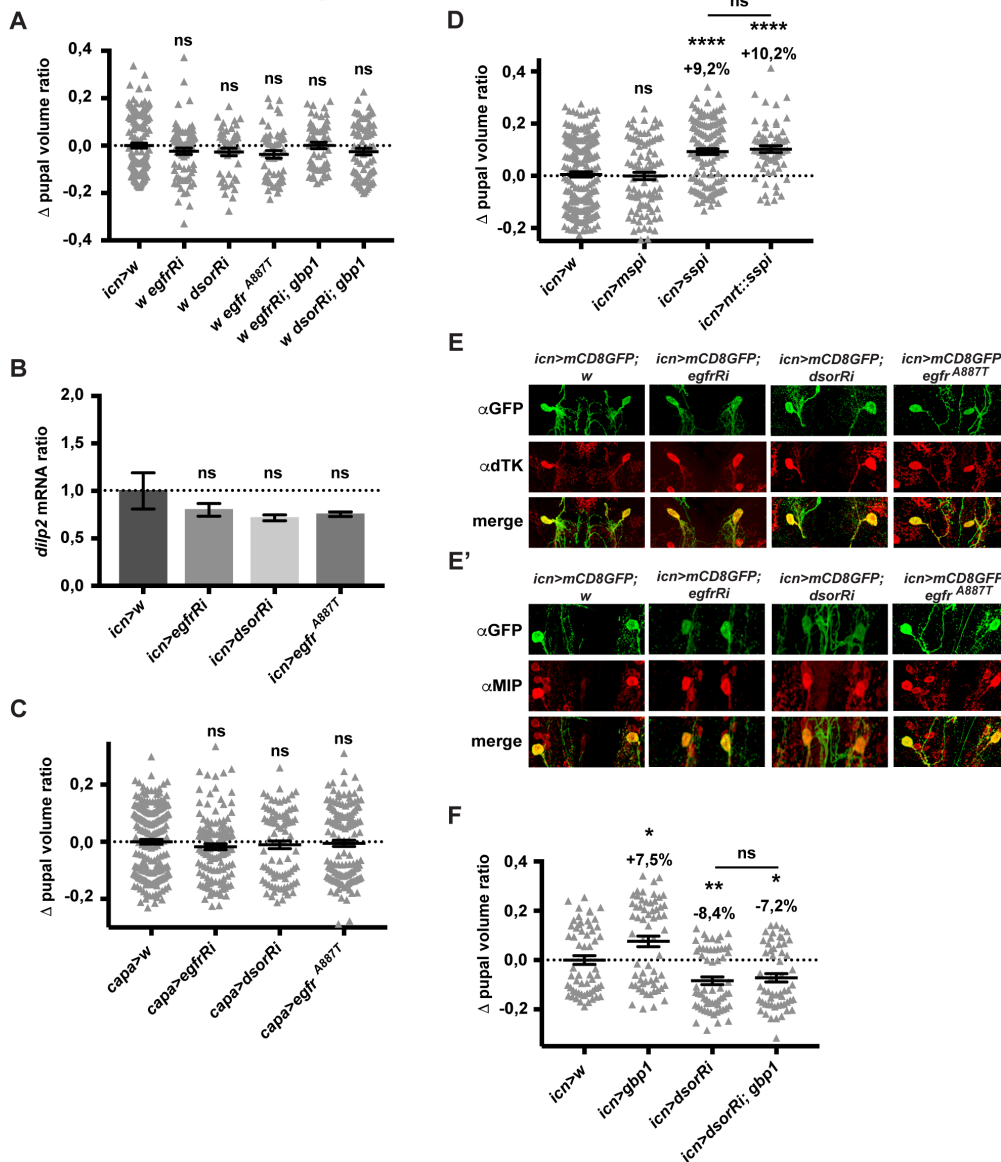
C. Pupal volume measurements upon *gbp1* (*lpp>gbp1*) or *gbp2* (*lpp>gbp2*) overexpression in the fat body. (n>67)

D. Pupal volume measurements upon ectopic expressions of *gbp1* and *gbp2* in the EH (eclosion hormone) neurons (*eh>gbp1* and *eh>gbp2*). (n>75)

E. Pupal volume measurements upon ectopic expressions of *gbp1* and *gbp2* in the CAPA neurons. (n>88)

In graphs, means are shown and errors bars represent \pm SEM, ****p<0,0001, **p<0,01.

Meschi - Suppl Fig 4



Supplementary Figure 4: Activation of EGFR signaling in ICNs promotes body growth via its effect on Dilps secretion without altering the ICNs identity nor dilips expression

A. Pupal volume measurements in all control conditions (*icn>w*, *w UAS-*). (n>44)

B. Quantification of *dilp2* mRNA levels in dissected brains upon EGFR signaling pathway inhibition (*icn>egfrRi* and *icn>dsorRi*) or activation (*icn>egfr^{A887T}*) in the ICNs. (n=3)

C. Pupal volume measurements upon EGFR signaling pathway inhibition (*capa>egfrRi*, *capa>dsorRi*) or activation (*capa>egfr^{A887T}*) in the CAPA neurons. (n>83)

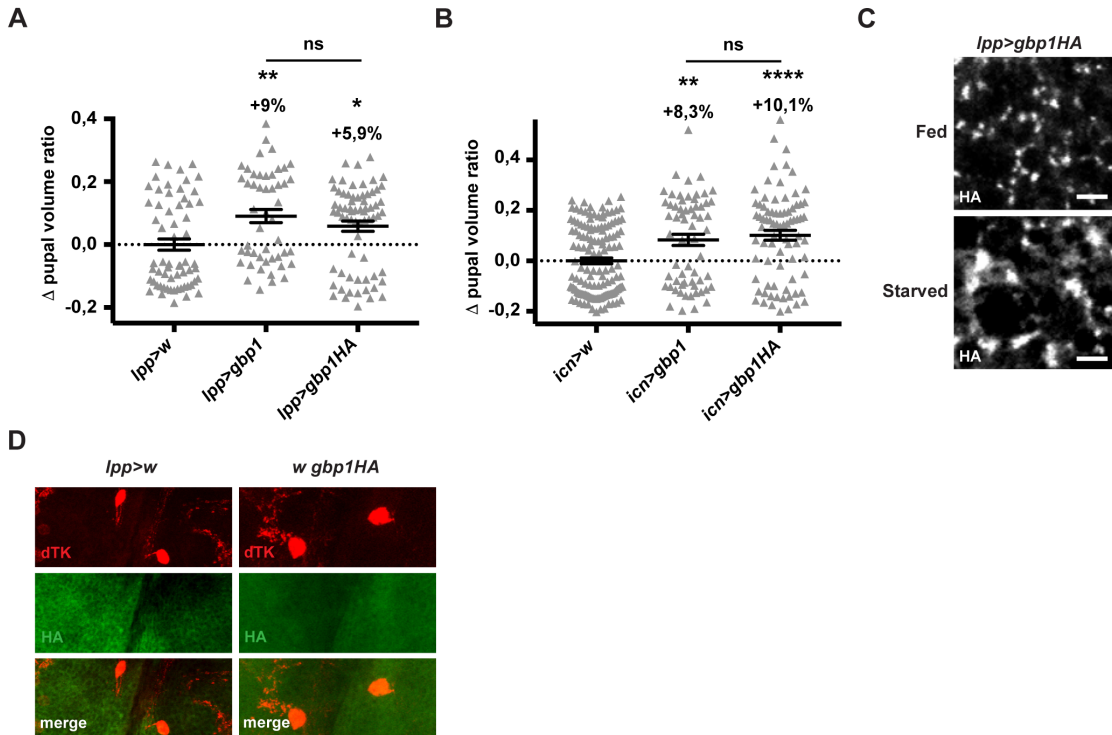
D. Pupal volume measurements upon ectopic expressions in the ICNs of a secreted activated form of Spitz (*icn>sspi*), its membrane-tethered form (*icn>UAS::sspi*) or the unprocessed form of Spitz (*icn>UASmspi*). (n>62)

E-E': ICNs labelled by GFP (green) and either dTK (tachykinine) (E, red) or MIP (Myoinhibitory peptide) (E', red) antibodies upon EGFR signaling inhibition (*icn>mCD8GFP*, *egfrRi* and *-dsorRi*) or activation (*icn>mCD8GFP*, *egfr^{A887T}*).

F. Pupal volumes after ectopic expression of *gbp1* alone (*icn>gbp1*), or upon co-silencing *dsor* (*icn>dsorRi*, *gbp1*) in the ICNs. (n>55)

In graphs, means are shown and errors bars represent \pm SEM, ****p<0,0001, **p<0,01, *p<0,05.

Meschi - Suppl Fig 5



Supplementary Figure 5: The fusion protein GBP1::HA is functional and can be used to study secretion and localization of GBP1.

A. Pupal volumes after overexpression of the fusion protein GBP1::HA in the fat body (*lpp>gbp1::HA*). (n>48)

B. Pupal volumes after overexpression of the fusion protein GBP1::HA in the ICNs (*icn>gbp1::HA*). (n>62)

C. GBP1::HA containing vesicles stained in the fat body upon fed condition and acute amino acids starvation (*lpp>gbp1::HA*). Scale bar represents 2,5 μ m.

D. GBP1::HA (green) stainings in the ICNs, labelled with dTK (red) antibody in control genotypes (*lpp>w* and *w gbp1::HA*).

In graphs, means are shown and errors bars represent \pm SEM, ****p<0,0001, **p<0,01, *p<0,05.

III- Unpublished results

a. The R22H11 construct is a *fruit-less* GAL4 line - no sex-specific phenotypes

The construct R22H11 is a fragment of the *fruit-less* promoter. *fruit-less* transcript undergoes sex-specific splicing and codes for transcription factors. These molecular differences between male and female underlie some sexually dimorphic features of neural circuitry and behavior in *Drosophila melanogaster*, such as the courtship behavior (Siwicki and Kravitz, 2009). Thus, we wondered whether the overgrowth phenotype obtained by ablating the ICNs was sex-specific. To facilitate males and females sorting, we used the wing area as a read out for animal size instead of pupal volume (Cavicchi et al., 1981; Robertson, 1962; Sokoloff, 1966). Interestingly, ICNs ablation induces increased wing area (+7%) in both males and females (Figure 42). This result strongly suggests that *R22H11-GAL4* is expressed in both male and female and that the ICNs are growth inhibitory neurons independently of the animal gender.

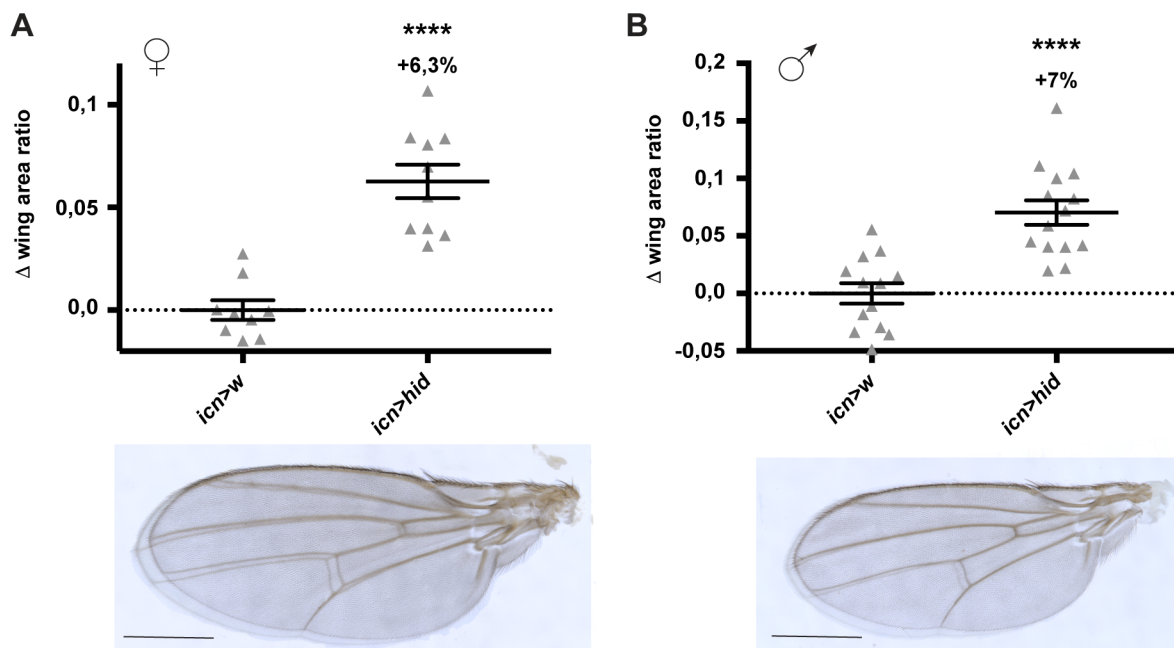


Figure 42 : The ICNs ablation causes bigger animals independently of their gender. A. Wing area measurements in female with ablated ICNs (n>9). B. Wing area measurements in male with ablated ICNs (n>13). Scale bars represent 500 μ m.

b. The ICNs promote lipid mobilization

Altering the IIS activity in peripheral tissues induces changes in fat content and lipid metabolism. Indeed, adults with ablated IPCs display higher levels of trehalose, glycogen and total lipids, suggesting that stored energy is increased (Broughton et al., 2005). In order to measure lipids content in our conditions, we stained fat bodies with Nile Red. This dye allows to stain neutral lipids and is used to assess size and shape of lipid droplets (Tennessee et al., 2014).

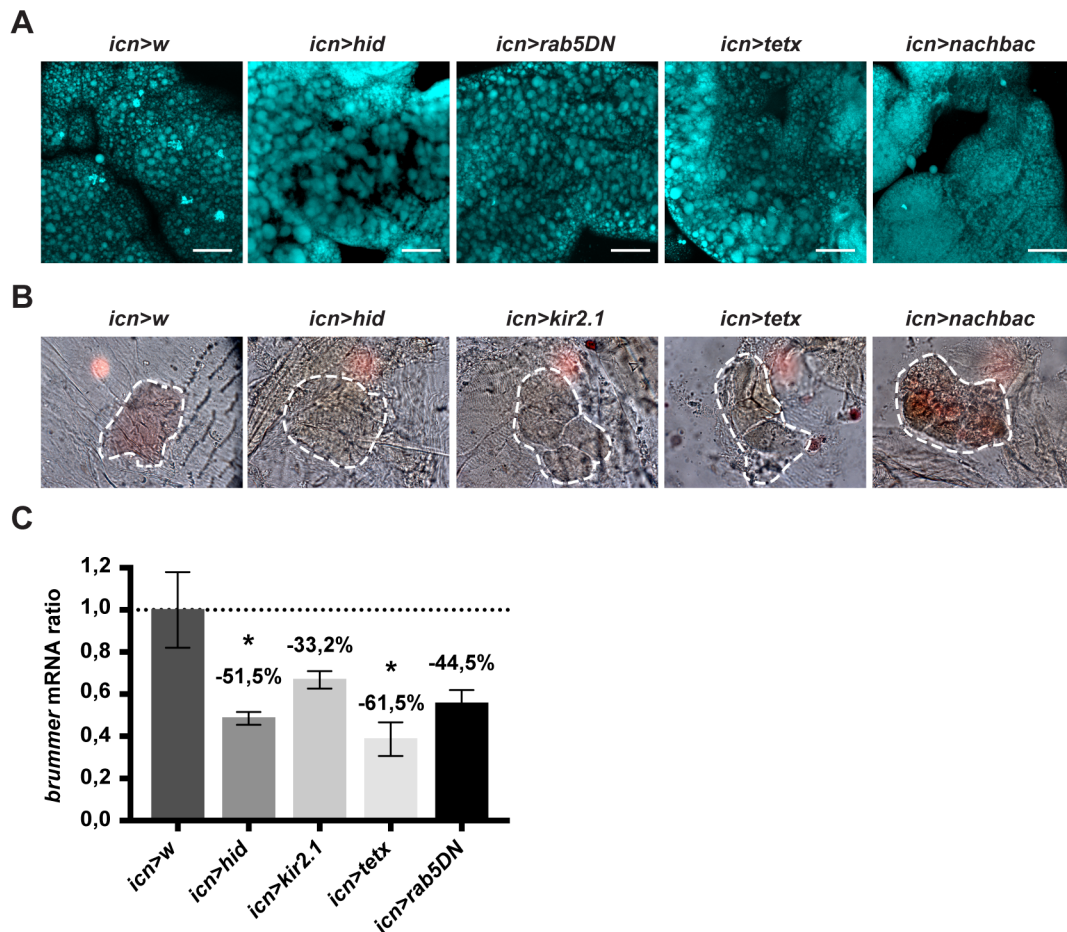


Figure 43 : The ICNs induce lipid mobilization from the fat body. A. Nile red staining in the fat body after ICNs ablation (*icn>hid*), blockade of secretory activity (*icn>tetx*) (*icn>rab5DN*) or chronic depolarization (*icn>nachbac*) to visualize lipid droplets. Scale bars represent 50 μ m. B. Oil Red O staining in the oenocytes after ICNs ablation (*icn>hid*), hyperpolarization (*icn>kir2.1*), blockade of secretory activity (*icn>tetx*) or chronic depolarization (*icn>nachbac*). C. mRNA levels of the lipase *brummer* in the fat body by qRT-PCR upon ICNs ablation (*icn>hid*), hyperpolarization (*icn>kir2.1*) and blockade of secretory activity (*icn>tetx*) (*icn>rab5DN*).

Results

ICNs ablation by expressing the pro apoptotic gene *hid*, as well as impaired secretion due to the tetanus toxin (TetX) or Rab5 dominant negative (Rab5DN) expression, induce bigger lipid droplets. Conversely, larvae expressing the sodium channel NaChBac in the ICNs present smaller lipid droplets in the fat body (Figure 43A). This change in lipid droplets size suggest that the ICNs probably through modification of peripheral IIS activity, alter the lipid metabolism.

To further investigate how the ICNs modify the lipid metabolism, we stained the oenocytes with the Oil Red O dye to visualize neutral lipids (Tennessee et al., 2014). Upon nutrient deprivation, lipids accumulation in the oenocytes is observed and is used as an indicator of lipid mobilization from fat cells (Arquier et al., 2008; Gutierrez et al., 2007). Consistent with altered lipid metabolism, the ICNs ablation (*icn>hid*), hyperpolarization (*icn>kir2.1*) or with impaired secretion (*icn>tetx*) decrease the lipids staining while their depolarization (*icn>nachbac*) promotes lipid accumulation in the oenocytes (Figure 43B). These results strongly indicate that ICNs promote lipid mobilization from fat cells, as starvation, by inhibiting Dilp2 secretion and therefore decreasing IIS in peripheral tissues (see Figure 2 of the manuscript).

According to the literature, some fru-GAL4 positive neurons promote lipid mobilization in *Drosophila* adult (Al-Anzi and Zinn, 2011). Indeed, silencing these neurons induce fat stores accumulation due to impaired lipolysis. Decreased expression of the lipase *brummer* is partially responsible for this phenotype. The lipase Brummer stimulates fat mobilization in flies (Grönke et al., 2005). Its expression is inhibited upon feeding because of increased peripheral IIS activity due to Dilps (Choi et al., 2015). Hence, we asked whether *brummer* expression in the fat body could be modified in our conditions. In line with both the literature and our previous results, inhibition of the ICNs leads to decreased *brummer* expression in the fat body (Figure 43C).

Altogether, these data demonstrate that by inhibiting Dilp2 secretion, the ICNs promote lipid mobilization from the fat body by increasing expression of the lipase *brummer* in *Drosophila* larvae.

c. GBP1 is a *bona fide* ligand of EGFR

In order to test whether GBP1 could be an EGFR ligand, we cocultured S2 cells expressing either a tagged-form of EGFR or tagged-forms of GBP1, Spitz and Hedgehog (Hh). Spitz and Hedgehog were used as a positive and negative control respectively. While none of the EGFR expressing cells showed Hh staining, they were positive for Spitz, suggesting that EGFR positive cells have the ability to uptake Spitz

Results

but not Hh. Interestingly, EGFR expressing cells also display GBP1 staining, and therefore indicate that GBP1 can be internalized by EGFR expressing cells (Figure 44). These results are consistent with data presented in Figure 4 and 5 of the paper and further demonstrate that GBP1 is a new *bona fide* EGFR ligand.

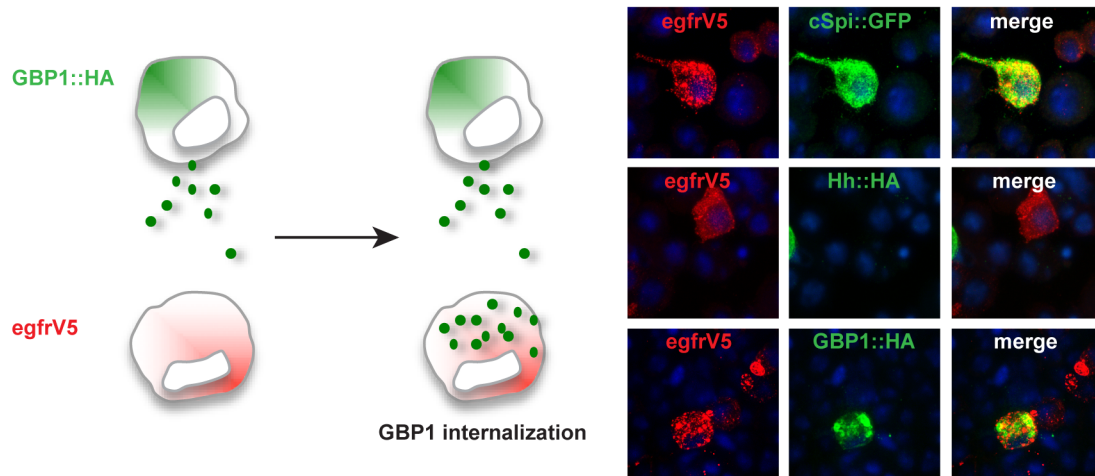


Figure 44 : GBP1 is internalized by EGFR expressing cells. EGFR expressing cells are cocultured with GBP1, Spitz or Hh expressing cells. GBP1 and the EGFR known ligand Spitz, but not Hh, are internalized in S2 cells expressing EGFR.

d. GBP1 secretion from the fat body is TOR-dependent

We previously showed that GBP1 release from fat cells relies on dietary amino acids (Figure 6A paper). Thanks to a functional HA-tagged GBP1 allele expressed in the fat body, we observed that GBP1::HA levels severely dropped in the hemolymph upon acute amino acids starvation, while they do not change in the fat body.

Interestingly, a secreted version of RFP (RFPsec) partially colocalizes with GBP1::HA in secretory vesicles of the fat body (Figure 45A). Thus, we wanted to assess whether all the fat body secretory machinery was blocked upon acute amino acids starvation. We overexpressed the secreted form of RFP specifically in the fat body, and performed western blot on hemolymph samples. Unexpectedly, we could detect RFPsec in hemolymph of both fed and starved larvae, suggesting that a specific secretory route for GBPs exists in fat cells, and further, that this specific secretory machinery is highly sensitive to nutrition (Figure 45B).

As previously mentioned the TOR pathway is the core of the amino acid sensing mechanism in fat cells, and the overexpression of TSC1/TSC2 can efficiently block this activity (Colombani et al., 2003). We examined the possibility that fat-derived GBP1 secretion responds to TOR activity. We generated a fly line expressing both TSC1/2 and GBP1::HA in the fat body (*lpp>gbp1::HA, tsc1/2*).

Results

Remarkably, these larvae are delayed with reduced growth rate compared to larvae overexpressing only GBP1::HA (Figure 45C). Eventually, some of them can pupate but die before adult emergence. This phenotype is consistent with TSC1/2 as organ size inhibitors (Potter et al., 2001). Importantly, while it is expressed in the fat body, GBP1::HA levels dramatically dropped in the hemolymph of larvae where TSC1/2 is co-overexpressed (*lpp>gbp1::HA, tsc1/2*) (Figure 45D) compared to figure 6A of the manuscript. This data undoubtedly reveals that GBP1 secretion from the fat body is dependent on the TOR signaling pathway.

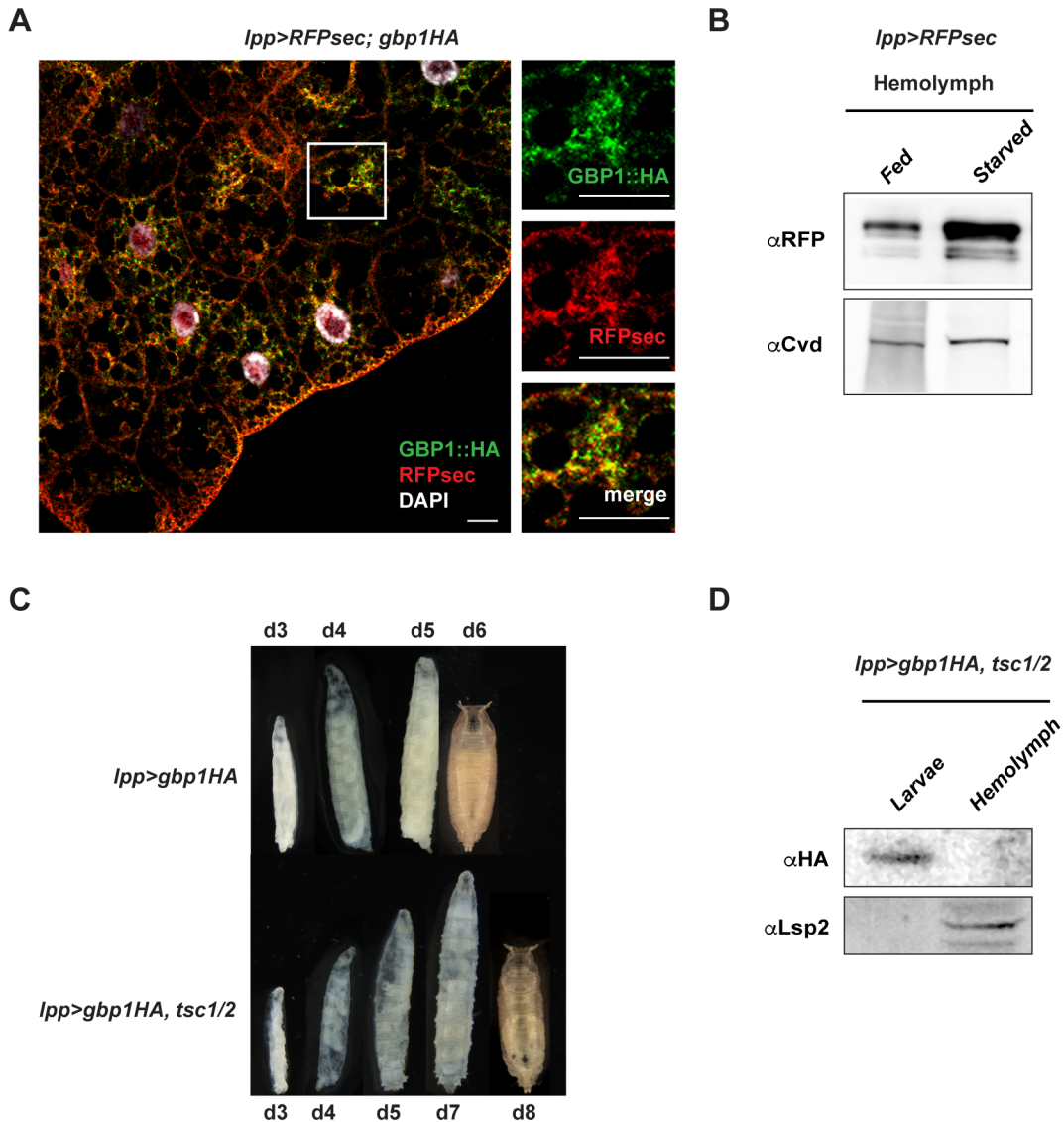


Figure 45 : GBP1 secretion from the fat body is TOR dependent. A. Immunostaining showing colocalization between GBP1::HA and secreted RFP positive vesicles (*lpp>RFPsec; gbp1HA*). Scale bars represent 20 μ m. B. Western blot on hemolymph coming from larvae that overexpress RFPsec in the fat body under fed and starved conditions (*lpp>RFPsec*). C. Larval development after overexpression of either *gbp1HA* (*lpp>gbp1HA*) or *gbp1HA* and TSC1/2 in the fat body (*lpp>gbp1HA, tsc1/2*). D. Western blot on all larvae or hemolymph collected from larvae that overexpress *gbp1HA* and TSC1/2 in fat cells (*lpp>gbp1HA, tsc1/2*).

IV- Conclusion

Nutritional status has to be sensed in order to maintain energy homeostasis and adapt body growth to environmental changes. The lab previously demonstrated that the fat body is the main nutrient sensing organ that remotely control Dilps secretion from the IPCs to adjust systemic growth (Colombani et al., 2003; Géminard et al., 2009). Different fat body-derived signals have been established to convey nutritional information to brain IPCs. Among them, fat-derived GBPs ligands have been shown to respond to dietary amino acids in order to stimulate Dilps secretion and promote body growth. However, both their receptor and neuronal target were unknown (Koyama and Mirth, 2016).

Here, I revealed a short neuronal circuit controlled by GBPs ligand coupling insulin secretion to the nutritional status. Surprisingly, the insulintropic effect of GBPs is EGFR-dependent, supporting the idea that GBPs is a new long-distance ligand for EGFR. I propose that GBPs metabolic hormones and their EGF receptor play a fundamental function in inhibiting the ICNs neuronal activity. This removes the basal inhibition exerted on the IPCs and as a consequence, allows adaptation of the tissue growth rate according to nutritional cues (Figure 46).

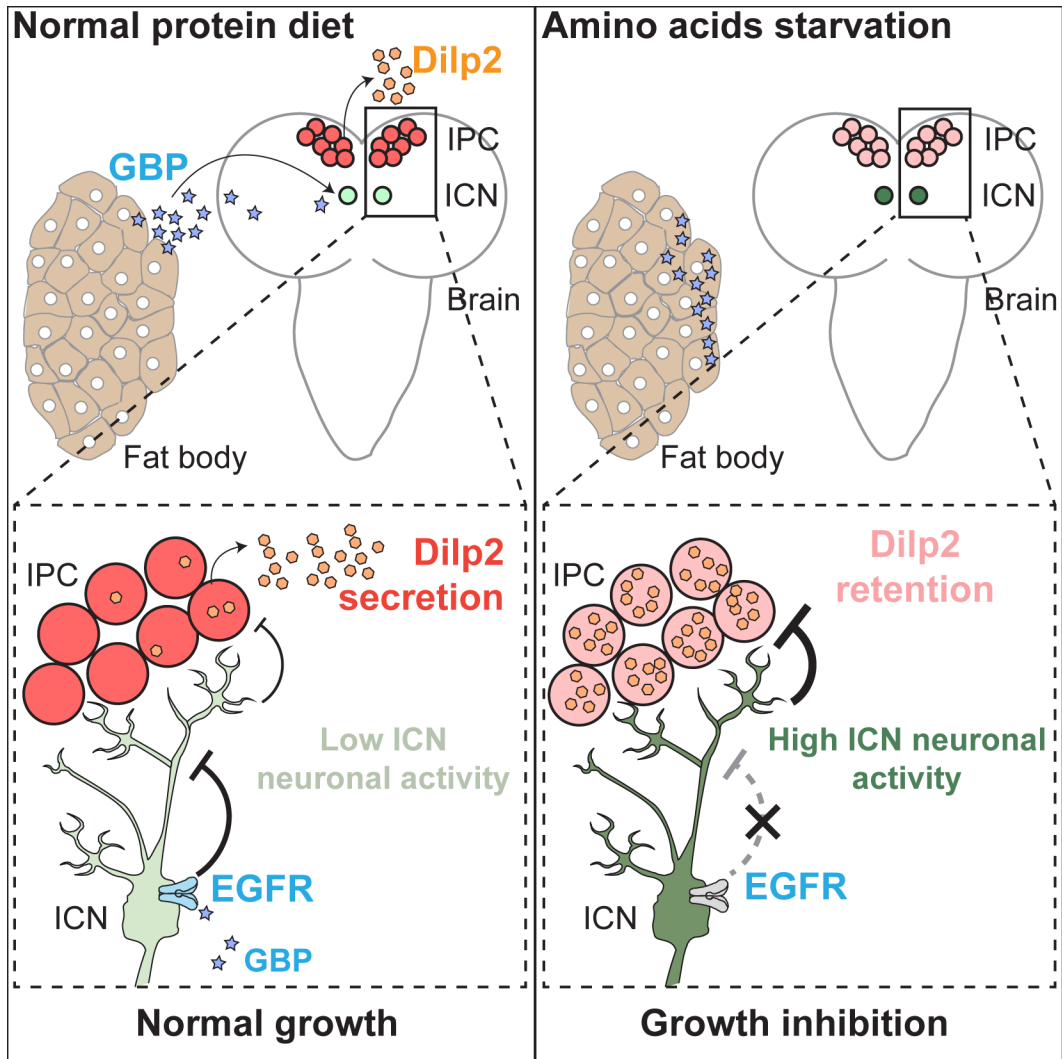


Figure 46 : Working model explaining how GBPs promote systemic growth depending on dietary amino acids

Chapter II: Drosophila insulin release is triggered by adipose Stunted ligand to brain Methuselah

I- Introduction

Environmental cues such as nutrient availability, influence several traits of animal physiology like growth, longevity, feeding, fecundity and metabolism (Andersen et al., 2013). Nutritional information is converted by the fat body into several growth-controlling signals which remotely govern Dilps secretion (Colombani et al., 2003; Géminard et al., 2009). In response to fat and/or sugar, the leptin-like ligand Upd2 and the CCHa2 peptide are produced by fat cells while two others fat-derived ligands, GBPs, respond to dietary amino acids (Koyama and Mirth, 2016; Rajan and Perrimon, 2012; Sano et al., 2015). These adipose factors have insulinotropic effect and promote systemic growth. Another adipose factor, Egr, has been shown to inhibit *dilps* transcription to adapt body growth to long-term protein scarcity (Agrawal et al., 2016). Even though several FBS have been discovered, during my PhD, I also contributed to the identification of a new adipose signal, Stunted (Sun), which is secreted in response to dietary amino acids in a TOR-dependent manner. Fat-derived Sun directly acts on brain IPCs through its receptor Methuselah (Mth) to stimulate Dilps secretion and systemic growth. The multiplicity and possible partial redundancy of existing fat-derived signals indicate how nutrient sensing is essential for an organism to adapt to dietary composition.

II- Publication

SIGNALING

Drosophila insulin release is triggered by adipose Stunted ligand to brain Methuselah receptor

Renald Delanoue,^{1‡} Eleonora Meschi,^{1†} Neha Agrawal,^{1,*†} Alessandra Mauri,¹ Yonit Tsatskis,² Helen McNeill,² Pierre Léopold^{1‡}

Animals adapt their growth rate and body size to available nutrients by a general modulation of insulin–insulin-like growth factor signaling. In *Drosophila*, dietary amino acids promote the release in the hemolymph of brain insulin-like peptides (Dilps), which in turn activate systemic organ growth. Dilp secretion by insulin-producing cells involves a relay through unknown cytokines produced by fat cells. Here, we identify Methuselah (Mth) as a secretin-incretin receptor subfamily member required in the insulin-producing cells for proper nutrient coupling. We further show, using genetic and ex vivo organ culture experiments, that the Mth ligand Stunted (Sun) is a circulating insulinotropic peptide produced by fat cells. Therefore, Sun and Mth define a new cross-organ circuitry that modulates physiological insulin levels in response to nutrients.

Environmental cues, such as dietary products, alter animal physiology by acting on developmental and metabolic parameters like growth, longevity, feeding, and energy storage or expenditure (1). The systemic action of this control suggests that intermediate sensor tissues evaluate dietary nutrients and trigger hormonal responses. Previous work in *Drosophila melanogaster* established that a specific organ called the fat body translates nutritional information into systemic growth-promoting signals (2–4). The leptinlike Janus kinase–signal transducers and activators of transcription (JAK-STAT) ligand unpaired 2 and the CCHamid2 peptide are produced by fat cells in response to both sugar and fat and trigger a metabolic response (5, 6). Dietary amino acids activate TORC1 signaling in fat cells and induce the production of relay signals that promote the release of insulin-like peptides (Dilps) by brain insulin-producing cells (IPCs) (3, 7). Two fat-derived peptides (GBP1 and GBP2) activate insulin secretion in response to a protein diet, although their receptor and neural targets remain uncharacterized (8). To identify critical components of this organ crosstalk, we conducted a genetic screen in *Drosophila* larvae (fig. S1A). The gene *methuselah* (*mth*), which encodes a heterotrimeric GTP-binding protein (G protein)-coupled receptor belonging to the subfamily of the secretin-incretin receptor subfamily (9–12) came out as a strong hit. Impair-

ing *mth* function in the IPCs reduces larval body growth (Fig. 1A), whereas silencing *mth* in a distinct set of neurons or in the larval fat body had no impact on pupal volume (fig. S1B). Larvae in which expression of the *mth* gene is reduced by RNA interference (RNAi), specifically in the IPCs (hereafter, *dilp2>mth-Ri*), present an accumulation of Dilp2 (Fig. 1B) and Dilp5 (fig. S1G) in the IPCs, whereas *dilp2* gene expression remains unchanged (fig. S1H), a phenotype previously described as impaired Dilp secretion (13). Indeed, forced depolarization of the IPCs rescues pupal volume and Dilp2 accumulation upon IPC-specific *mth* depletion (knockdown) (fig. S1, J and K). Therefore, Mth is required for Dilps secretion and larval body growth.

Two peptides encoded by the *stunted* (*sun*) gene, SunA and SunB, serve as bona fide ligands for Mth and activate a Mth-dependent intracellular calcium response (14, 15) (see fig. S3E for peptide map). Silencing *sun* in fat cells, but no other larval tissue, of well-fed larvae mimics the *mth* loss-of-function phenotype (Fig. 1, C and D, and fig. S1I) with no effect on the developmental timing (fig. S1L). Conversely, overexpression of *sun* in the larval fat body (*lpp>sun*) partially rescues the systemic growth inhibition observed upon feeding larvae a diet low in amino acids (Fig. 1E and fig. S1M) or upon “genetic starvation” [silencing of the *slimfast* (*slif*) gene in fat cells (3)] (fig. S1N). This growth rescue is abolished in *mth*¹ homozygous mutants (Fig. 1F). This shows that Sun requires Mth to control growth. However, *sun* overexpression has no effect in animals fed a normal diet (Fig. 1E). A modification of *sun* expression does not prevent fat body cells from responding to amino acid deprivation as seen by the level of TORC1 signaling, general morphology, and lipid droplet accumulation (fig. S2, A and B) but affects the ability of larvae to resist to starvation (fig. S2C).

Dilp2-containing secretion granules accumulate in the IPCs following starvation and are rapidly released upon refeeding (7) (fig. S3A). Mth is required in the IPCs to promote Dilp secretion after refeeding (Fig. 2A and fig. S3B), and forced membrane depolarization of IPCs using a bacterial sodium channel (*dilp2>NaChBac*) is dominant over the blockade of Dilp2 secretion in *dilp2>mth-Ri* animals (Fig. 2A). This dominance indicates that Mth acts upstream of the secretion machinery. In addition, Dilp2 secretion after refeeding is abrogated in *lpp>sun-Ri* animals (Fig. 2B), and overexpression of *sun* in fat cells prevents Dilp2 accumulation upon starvation (Fig. 2B). Altogether, these findings indicate that Mth and its ligand Sun are two components of the systemic nutrient response controlling Dilp secretion.

Hemolymph from fed animals triggers Dilp2 secretion when applied to brains dissected from starved larvae (7) (Fig. 3A). This insulinotropic activity requires the function of Mth in the IPCs (Fig. 3A and fig. S3C) and the production of Sun by fat body cells (Fig. 3B). Conversely, overexpressing *sun* in the fat body (*lpp>sun*) is sufficient to restore insulinotropic activity to the hemolymph of starved larvae (fig. S3D). A 2-hour incubation with a synthetic peptide corresponding to the Sun isoform A (Sun-A) is also sufficient to induce Dilp secretion from starved brains (Fig. 3C). A similar effect is observed with an N-terminal fragment of Sun (N-SUN) that contains the Mth-binding domain (14, 15) but not with a C-terminal fragment (C-SUN) that does not bind Mth (fig. S3, E and F). The insulinotropic effect of N-SUN is no longer observed in brains from larvae of the *mth* allele, *mth*¹ (fig. S3F). This absence of effect indicates that N-SUN action requires Mth in the brain. In addition, preincubation of control hemolymph with antiserum containing Sun antibodies specifically suppresses its insulinotropic function (Fig. 3D). These results indicate that Sun is both sufficient and necessary for insulinotropic activity in the hemolymph of protein-fed animals.

To directly quantify the amount of circulating Sun protein, we performed Western blot experiments on hemolymph using antibodies against Sun. A 6-kD band was detected in hemolymph collected from fed larvae (Fig. 4A), and size was confirmed using Schneider 2 (S2) cell extracts (Fig. 4C). The band intensity was reduced upon *sun* knockdown in fat body cells but not in gut cells (Fig. 4C). Therefore, circulating Sun peptide appears to be mostly contributed by fat cells, as suggested by functional experiments (see Fig. 1C). The levels of circulating Sun are strongly reduced upon starvation (Fig. 4A). In line with this, *sun* transcripts are drastically reduced after 4 hours of protein starvation and start increasing after 1 hour of refeeding (Fig. 4B), whereas expression of the *sun* homolog *CG31477* is not modified (fig. S4A). *sun* transcription is not affected by blocking TORC1, the main sensor for amino acids in fat body cells (3) (*lpp>TSC1/2* in fig. S4B). However, adipose-specific TORC1 inhibition induces a dramatic reduction of circulating Sun (Fig. 4C), indicating that TORC1 signaling controls

¹Université Côte d’Azur, CNRS, INSERM, Institute of Biology Valrose (IBV), 06100 Nice, France. ²Lunenfeld Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada

*Present address: The Gurdon Institute and Department of Physiology, Development and Neuroscience, University of Cambridge, Tennis Court Road, Cambridge CB2 1QN, UK.

†These authors contributed equally to this work.

‡Corresponding author. Email: delanoue@unice.fr (R.D.); leopold@unice.fr (P.L.)

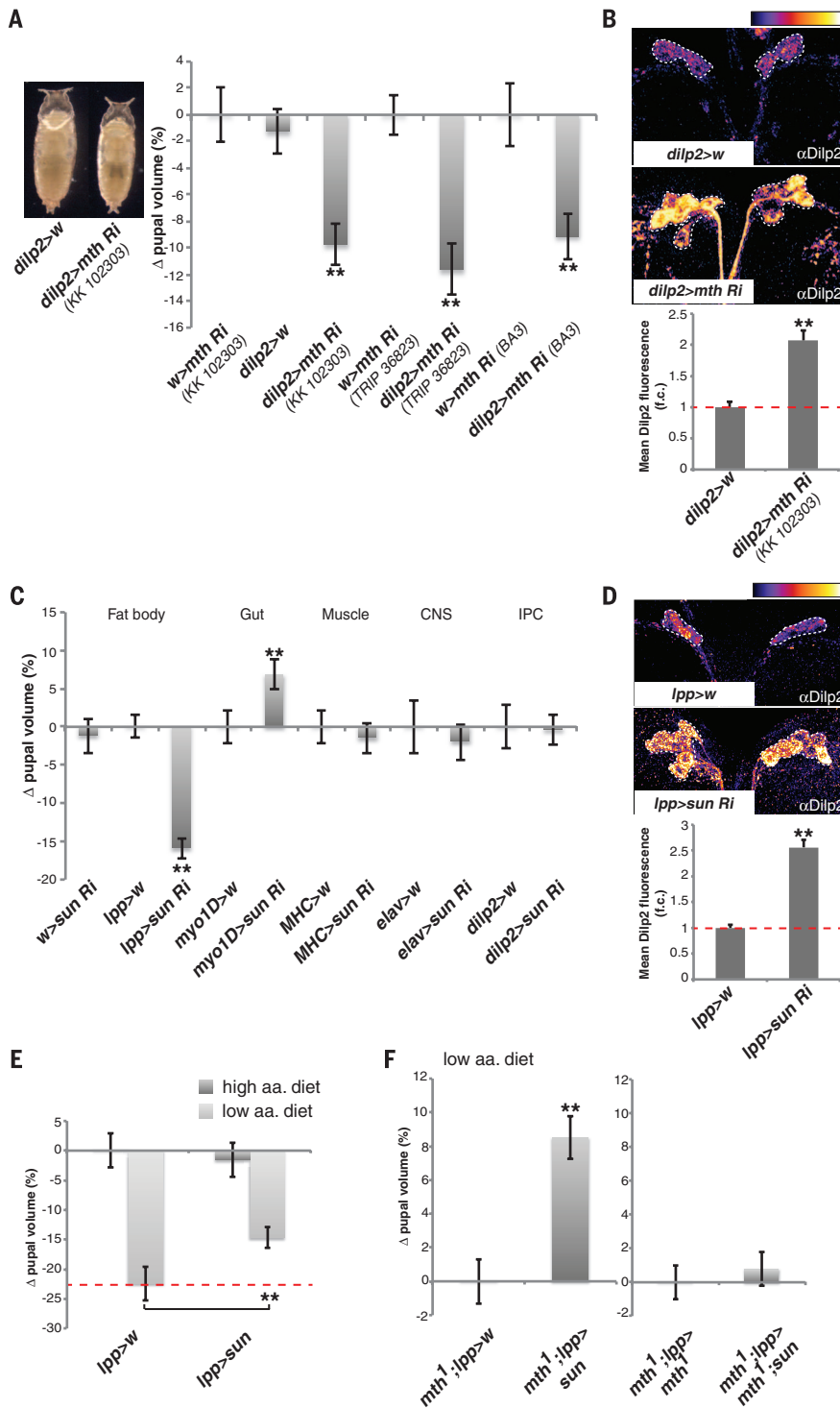


Fig. 1. Mth and Sun are required for systemic growth. (A) Three different *mth* RNAi constructs (KK 102303, TRIP 36823, and BA3) decrease pupal size when driven in the IPC (*dilp2>*) ($n > 20$). (B) Silencing *mth* in the IPC induces accumulation of Dilp2 ($n > 20$). Fluorescence intensity measured as fold change (f.c.). (C) Silencing *sun* in the fat body (*lpp-Gal4>sun-Ri*) reduces pupal volume. No defect is observed when silencing in other tissues (*myo1d-Gal4*, gut; *MHC-Gal4*, muscle; *elav-Gal4*, CNS; or *dilp2-Gal4*, IPC). (D) *sun* silencing in the fat body causes Dilp2 accumulation in the IPC ($n > 60$). (E) *sun* overexpression in the fat body partially rescues the pupal size reduction observed in larvae fed a low-protein diet compared with those fed a high-protein diet ($n > 20$). Amino acid, aa. Red dashed line here and below shows the level of controls. (F) Overgrowth observed upon forced fat body expression of *sun* in larvae fed a low-amino acid diet is observed in *mth*^{+/+} but not in *mth*^{1/1} homozygous flies ($n > 40$). In graphs, means are shown, and error bars represent \pm SEM; ** $P < 0.01$.

Sun peptide translation or secretion from fat cells. PGC1-Spargel is a transcription activator, the expression of which relies on nutritional input (fig. S4D) (16). We find that *PGC1* is required for *sun* transcription (Fig. 4D) and that fat body silencing of *PGC1* and *sun* induce identical larval phenotypes (Fig. 4E and fig. S4C). Although *PGC1* expression is strongly suppressed upon starvation, blocking TORC1 activity in fat cells does not reduce *PGC1* expression (fig. S4E). Conversely, knocking down PGC1 does not inhibit TORC1 activity (fig. S4F). This finding suggests that PGC1 and TORC1 act in parallel. Therefore, Sun production by fat cells in response to nutrition is controlled at two distinct levels by PGC1 and TORC1.

The Sun peptide is identical to the ϵ subunit of the mitochondrial F_1F_0 -adenosine triphosphatase (F_1F_0 -ATPase) synthase (complex V) (14, 17). Indeed, both endogenous Sun and Sun labeled with a hemagglutinin tag (Sun-HA) (fig. S5A) colocalize with mitochondrial markers in fat cells (fig. S5B), and the Sun peptide cofractionates with mitochondrial complex V in blue native polyacrylamide gel electrophoresis (fig. S5C). In addition, silencing *sun* in fat cells decreases mitochondrial Sun staining (fig. S5B) and the amounts of adenosine triphosphate (ATP) (fig. S5D). However, recent evidence indicates that an ectopic (ecto) form of the F_1F_0 -ATP synthase is found associated with the plasma membrane in mammalian and insect cells (18–21). In addition, coupling factor 6, a subunit of complex V, is found in the plasma (22). Therefore, Stunted could participate in two separate functions carried by distinct molecular pools. To address this possibility, we used a modified form of Stunted carrying a green fluorescent protein (GFP) tag at its N terminus (GFP-Sun), next to the mitochondria-targeting signal (MTS) (fig. S5A). When expressed in fat cells, GFP-Sun does not localize to the mitochondria (fig. S6A), contrarily to a Sun peptide tagged at its C-terminal end (Sun-GFP) (fig. S6C). This suggests that addition of the N-terminal tag interferes with the MTS and prevents mitochondrial transport of Sun. However, both GFP-Sun and Sun-GFP are found in the hemolymph (fig. S6B) and rescue pupal size and Dilp2 accumulation in larvae fed a low-amino acid diet as efficiently as wild-type Sun (*wt-Sun*) (Figs. 4F and 1E and fig. S6E) and do so in a *mth*-dependent manner (fig. S6D). This indicates that the growth-promoting function of Sun requires its secretion but not its mitochondrial localization and suggests the existence of one pool of Sun peptide located in the mitochondria devoted to F_1F_0 -ATP synthase activity and ATP production and another pool released in the hemolymph for coupling nutrient and growth control. In this line, although fat body levels of Sun are decreased upon starvation (fig. S6F), its mitochondrial localization is not reduced (fig. S6G). This finding indicates that starvation affects a nonmitochondrial pool of Sun. In support of this, starved fat bodies contain normal levels of ATP and lactate (fig. S6, H and I), indicating that mitochondrial oxidative

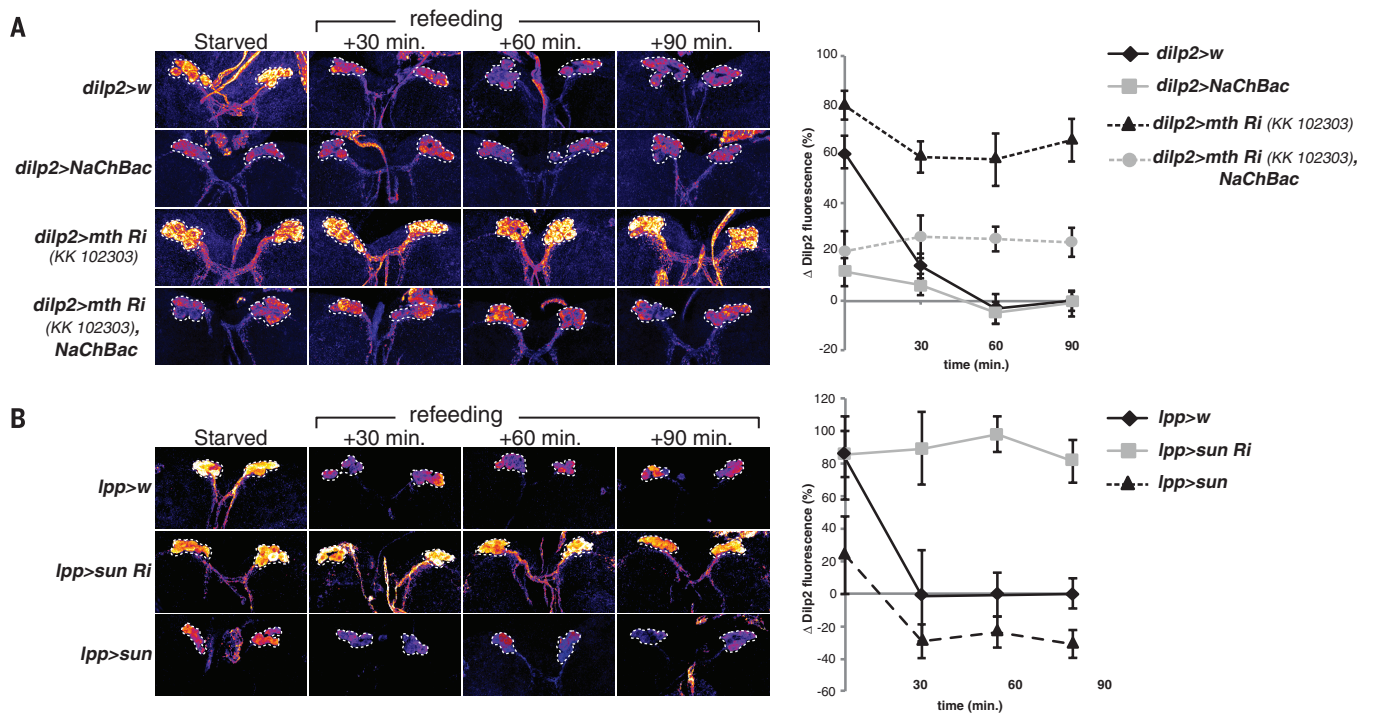


Fig. 2. Mth and Sun control Dilp2 secretion from brain IPCs. (A) Representative pictures of Dilp2 staining in IPCs (dashed outline) showing the kinetics of Dilp2 accumulation upon refeeding after prolonged starvation. Genotypes are as indicated. (B) *sun* silencing (*lpp>sun-Ri*) in the fat body prevents Dilp2 secretion upon refeeding, whereas *sun* overexpression (*lpp>sun OE*) reduces Dilp2 accumulation upon starvation. Graphs represent quantifications of Δ Dilp2 fluorescence relative to *lpp>w* control after 90 min of feeding (means \pm SEM; $n > 20$).

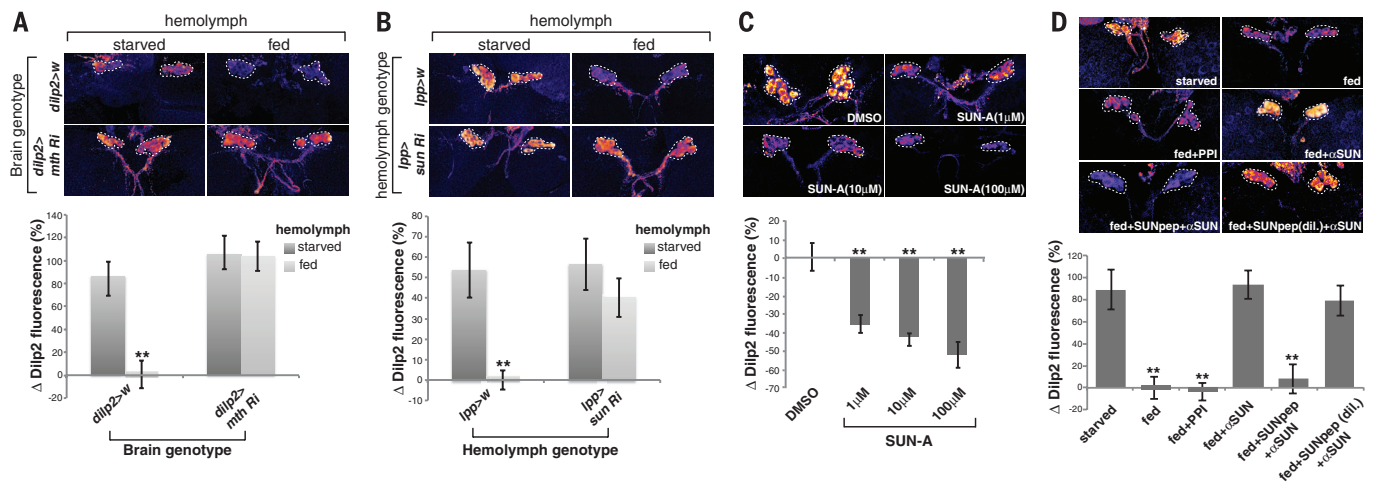


Fig. 3. Sun is a fat body–derived insulinotropic signal. (A) Hemolymph collected from fed, but not starved, larvae activates Dilp2 secretion when incubated on dissected brains from starved control (*dilp2>w*), but not *mth*-deficient (*dilp2>mth-Ri*), larvae. (B) Hemolymph from fed larvae deficient for adipose *sun* (*lpp>sun-Ri*) does not induce Dilp2 secretion. (C) Incubation of brains from starved larvae with various concentrations of SUN-A stimulates Dilp2 secretion. DMSO, dimethyl sulfoxide. (D) Hemolymph collected from fed

larvae preincubated with preimmune serum (fed+PPI), but not from larvae treated with antibodies against SUN (α SUN), induces Dilp2 secretion. Titration of α SUN with blocking peptides allows reactivation of Dilp2 secretion. Diluted (dil.) blocking peptides do not block α Sun action [fed+SUNpep(dil.) + α SUN]. Graphs represent quantifications of Δ Dilp2 fluorescence relative to control brains (brains from starved larvae incubated with hemolymph, from fed larvae, or with DMSO) (means \pm SEM; $n > 20$); $^{**}P < 0.01$.

phosphorylation is preserved in fat cells in poor nutrient conditions. Last, other subunits from complex V (ATP5a) or complex I (Ndufs3)

were not detected in circulating hemolymph (fig. S6J). Therefore, the release of Sun in the hemolymph relies on a specific mechanism.

In conclusion, we provide evidence for a molecular cross-talk between fat cells and brain IPCs involving the ligand Stunted and its receptor

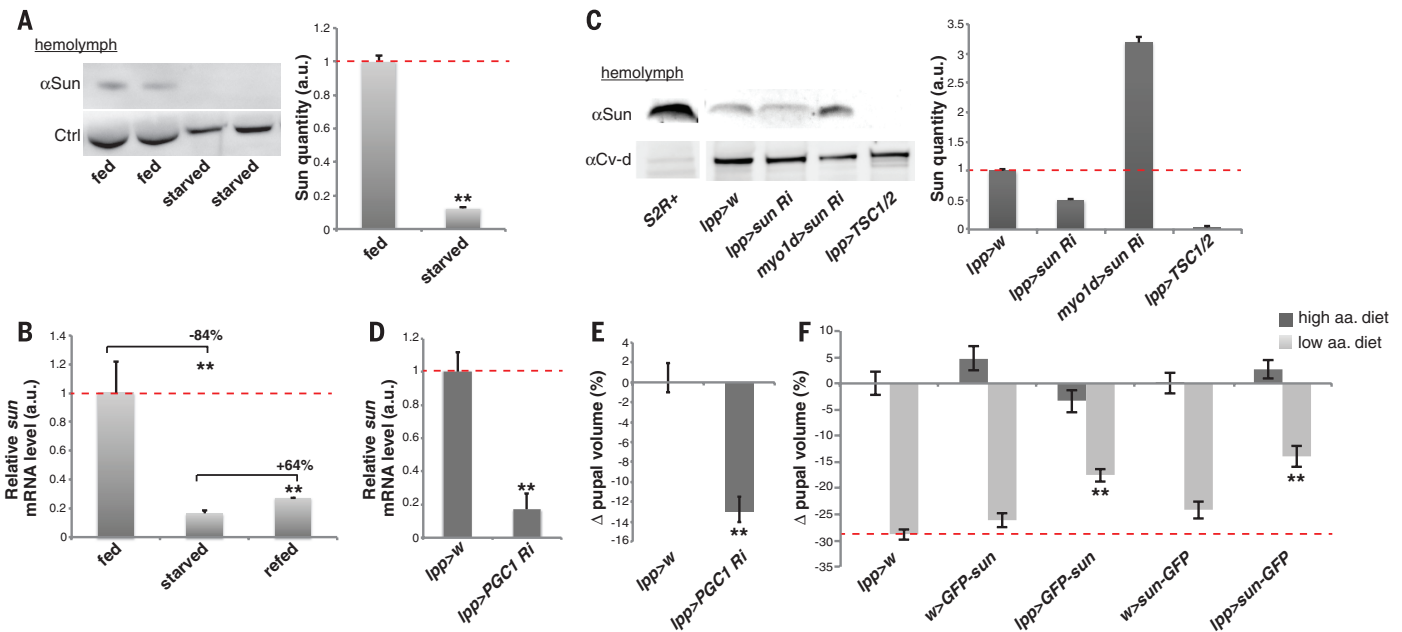


Fig. 4. The level of circulating Sun relies on TOR and PGC1 in fed animals. (A) Circulating Sun peptide is detected by Western blotting of hemolymph from fed larvae (lanes 1 and 2) but not starved larvae (lanes 3 and 4). Antibodies against Crossveinless d lipoprotein (α Cv-d) are used as a loading control. Quantification in arbitrary units (a.u.) of the normalized circulating Sun detected in the hemolymph according to nutritional conditions (means \pm SEM; $n = 4$); $**P < 0.01$. (B) The *sun* transcript levels in the fat body decrease upon starvation and increase upon refeeding (measured by quantitative reverse transcription polymerase chain reaction) (means \pm SEM; $n = 4$);

$**P < 0.01$. (C) Circulating Sun levels decrease upon *sun* silencing in fat body cells (*lpp>sun-Ri*) but not in gut cells (*myo1d>sun-Ri*). Blocking TORC1 in fat cells (*lpp>TSC1/2*) strongly decreases circulating Sun. Quantification of normalized circulating Sun. (D) *sun* expression is severely reduced when *PGC1* is silenced in fat body cells (means \pm SEM; $n = 3$); $**P < 0.01$. (E) Silencing *PGC1* in the fat body (*lpp>PGC1-Ri*) decreases pupal size (means \pm SEM; $n > 40$); $**P < 0.01$. (F) Forced fat body expression of GFP-Sun (*lpp>GFP-Sun*) or Sun-GFP (*lpp>Sun-GFP*) rescues pupal size reduction observed from larvae fed the low-amino acid diet (means \pm SEM; $n > 40$); $**P < 0.01$.

Methuselah. Stunted is a moonlighting peptide present both in the mitochondria as part of the F_1F_0 -ATP synthase complex and as an insulinotropic ligand circulating in the hemolymph. The mechanism of Stunted release remains to be clarified. The beta subunit of the ectopic form of F_1F_0 -ATP synthase is a receptor for lipoproteins (18–21), which serve as cargos for proteins and peptides. In addition, *Drosophila* lipid transfer particle-containing lipoproteins were shown to act on the larval brain to control systemic insulin signaling in response to nutrition (23). This suggests that Sun could be loaded on lipoproteins for its transport. Given the role of insulin-like growth factor (IGF) signaling in aging, our findings could help in understanding the role of Sun/Mth in aging adult flies (9–11, 13, 14).

The same genetic screen previously identified the fly tumor necrosis factor α Eiger (Egr) as an adipokine necessary for long-term adaptation to protein starvation (24), and recent work pointed to other adipose factors (5, 6, 8), illustrating the key role of the larval fat body in orchestrating nutrient response. The multiplicity of adipose factors and their possible redundancy could explain the relatively mild starvation-like phenotype obtained after removal of only one of them. Overall, these findings suggest a model whereby partially redundant fat-derived signals account for differential response to positive and negative

valence of various diet components, as well as acute versus long-term adaptive responses.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/353/6307/1553/suppl/DC1
Materials and Methods
Fig. S1 to S6
References (25–30)

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Supplementary Materials for

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Renald Delanoue,* Eleonora Meschi, Neha Agrawal, Alessandra Mauri, Yonit Tsatskis,
Helen McNeill, Pierre Léopold*

*Corresponding author. Email: delanoue@unice.fr (R.D.); leopold@unice.fr (P.L.)

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This PDF file includes

Materials and Methods
Figs. S1 to S6
References

Supplementary Materials

Materials and methods

Fly strains

The following fly strains were used: *white1118* (*w*), *lpp-Gal4* (gift from S. Eaton), *dilp2-Gal4* (25), *kurs6-Gal4* (26), *UAS-TSC1/2* (27), *mth¹*, *mthΔ*, *UAS-mth RNAi* (BA3) and *UAS-sun* (from W. Ja). RNAi lines come from the Vienna Drosophila RNAi Stock Center: *UAS slif Ri* (KK 110725), *UAS-sun RNAi* (GD 23685), *UAS-mth RNAi* (KK 102303). From Bloomington Stock Center were obtained a third independent *UAS-mth RNAi* line (TRIP 36823), *UAS-PGC1 RNAi* (TRIP 33914), *nSyb-Gal4*, *phm-Gal4*, *esg-Gal4*, *elav-Gal4*, *UAS-NaChBac*, *UAS-mitoGFP*. Efficiency and specificity of the RNAi lines were assessed by qPCR (Fig. S1, C-F). Animals were reared at 25°C on fly food containing, per liter, 10 g agar, 83 g corn flour, 60 g white sugar, 34 g dry yeast and 3.75 g Moldex (in ethanol), supplemented with yeast paste. For low aa. diet, larvae were raised on a similar medium where only yeast quantity was reduced to 8.5g/l.

Amino acid starvation

72h. AEL larvae are transferred for 16 h. on PBS 1x, 1.5% agar plates and supplemented with 1% sugar, but no protein source.

Plasmids and transgenic flies

sun coding sequences were PCR amplified from BDGP EST cDNA clones RE19513 and cloned into the pENTR/D-TOPO vector using the following gene-specific primers: sense primer CAC CAT GAC TGC CTG GAG AGC TGC and antisense primers CTA GGA TTC CGA TTG GGT TT or GGA TTC CGA TTG GGT TTG AC (with or without stop codon). To generate *UAS-SUN-GFP* and *UAS-GFP-SUN* transgenic lines, coding sequences were cloned into the Gateway Destination vectors (Drosophila Gateway Vector Collection) by using of the pTWG plasmid for generation of *UAS-SUN-GFP* and pTGW for *UAS-GFP-SUN* (Fig. S5A). Constructs were introduced into the germ line by injections in the presence of the integrase (BestGene).

Genetic screen

We performed a biased genetic screen to identify molecules that regulate Dilp2 secretion in the IPCs. We used the *dilp2-Gal4, UAS-nlsGFP* line to screen a set of *phiC31* (KK) RNAi lines from the Vienna *Drosophila* RNAi Center (VDRC) for selected GO terms: membrane targeted proteins, intracellular trafficking and vesicular mediated transport. 100 lines were tested each week. 20 virgins containing the *dilp2-GAL4* driver were crossed with 10 males of each RNAi lines. Three replicates were collected from each cross with collections of 4 h. 168 candidates significantly affect pupal volume. In a secondary screen, positive hits were tested for Dilp2 immunostainings of larval brains. 88 Candidates were showing both decrease in pupal volume and changes in Dilp2 secretion.

Pupal volume analysis

Larvae from different genotypes were synchronized at 24 h. AED and cultured under controlled conditions (30 larvae/vial). Pupal volume was measured using ImageJ and calculated by using the formula $(4/3)\pi(L/2)(l/2)^2$ (L, length; l, diameter). For a given experiment, all values are normalized to one control condition to give the “ Δ pupal volume”.

Ex Vivo Organ culture

Ex vivo organ culture was performed using a method based on (7). 72 h. AEL larvae are starved for 16 h on plates made with PBS 1x, agar 1.5% and 1% sugar. Prior dissection, larvae were surface-sterilized in a solution of 70% ethanol for 30 sec, rinsed in sterile PBS and dissected in Schneider (Sigma) using sterile tools. Brains were transferred to a microfuge tube containing 40 μ l of Schneider medium with 2.5% FBS. Co-culture experiments were performed by carefully adding 20 μ l the hemolymph collected from either fed or starved 3rd instar larvae. Cultures were incubated at room temperature for 2 h. In some experiments, hemolymph was pretreated using the anti-Sun (1 μ g) either pure or mix with 2 μ g of the peptides used for immunization (see below) (SUNpep) or 0.01 μ g (SUNpep dil.).

Synthetic full Sun isoform A (SUN-A), the 30 first residues (N-SUN) or the last 30 residues (C-SUN) of SUN-A were used in this study. Synthetic peptides are re-suspended in DMSO and added directly at the indicated concentrations to the cultures brains for 2 h.

Immunofluorescence on larval tissues

Tissues were dissected in 1x PBS, fixed in 3.7% formaldehyde (Polyscience) in PBS for 30 min at room temperature and extensively washed in PBS containing 0.1% Triton X-100 (PBT). Tissues were then blocked for 1 h in PBT containing 10% FCS. Primary antibodies were incubated overnight at 4°C. Secondary antibodies were incubated 2-3 hours at room temperature. Membranes were stained with FluorProbe 647-phalloidin (Interchim) at 1/200. After washing, tissues were mounted in Vectashield with DAPI for staining DNA (Vector). Fluorescence images were acquired using a Leica SP5 DS confocal laser scanning microscope (40x objectives). The following primary antibodies were used in this study: anti-Sun was generated in Guinea pigs using two peptides containing amino acids 19-33 (CARILRESLKTGLRAD) and amino acids 27-41 (KTGLRADAAKRDASH) (Eurogentec, Belgium) and diluted at 1:200; rat anti-Dilp2 (7) at 1/400; mouse anti-ATP5A (Abcam) at 1/500, chicken anti-GFP (Abcam) at 1/10000; rat anti-HA (clone 3F10; Roche) at 1/1000.

Fluorescence Quantification

Mean Dilp2 fluorescence was measured by acquiring confocal Z series of the IPCs with a 0.5 μm step and identical laser power and scan settings. ImageJ software was used to generate sum-intensity 3D projections and to measure total fluorescent intensity across the IPCs.

Hemolymph preparation

Hemolymph was obtained by bleeding larvae in ice cold PBS. Hemocytes were removed by centrifugation for 30 min at 500 g at 4°C; and cellular fragments were removed by a subsequent centrifugation at 16000 g for 20 min at 4°C. The supernatant from the second centrifugation step was directly used for Western blotting as in (28).

Western blotting.

Proteins were resolved by SDS-PAGE using 12% gels (NuPAGE Novex gel, Invitrogen) using the MES running buffer and transferred electrophoretically to Nitrocellulose membranes (Biorad). The

membranes were incubated for 20 min. in blocking buffer (PBS, 5% milk) and incubated overnight at 4°C in the same buffer containing primary antibodies. Membranes were washed three times in PBS-T and probed with secondary antibodies in blocking buffer for 2 h at room temperature. After three washes in PBS-T (PBS + 0.1% Tween20), chemiluminescence was observed using the ECL-Plus Western blotting detection system (Biorad). Images were generated using the Fujifilm Multi Gauge software and quantified using ImageJ software. The following primary antibodies were used in this study: anti-Sun was used at 1/50, mouse anti-ATP5A at 1/200, mouse anti-Ndufs3 (Abcam) at 1/1000, rabbit anti-Lsp2 at 1/4000 and guinea pig anti-CvD at 1/1000 (gifts from S. Eaton).

ATP measurement

An ATP bioluminescence assay kit CLS II (Roche) was used to measure ATP concentration on dissected larval fat bodies. In brief, tissues were homogenized in the boiling squishing buffer (100 mM Tris, 4 mM EDTA, pH 7.75) and incubated for 2 min at 95°C. After spinning at 3500 rpm for 20 min, supernatant was transferred and centrifuged again for 10 min at 5500 rpm. The extract was diluted 96-well flat microplate and luciferase activity was measured using a luminometer (Centro LB 960 Berthold). Samples were normalized with protein concentration measured by Bradford assays.

Lactate measurement

Dissected larval fat bodies were homogenized in 100 µl of PBS. Samples were heat shocked at 60°C for 15 min and centrifuged at 12 500 rpm for 5 min; 5–10 µl of the supernatant was used for lactate determination using a Lactate Assay Kit (BioVision). Lactate levels are normalized to protein content.

Blue Native gels

Mitochondria were purified from 3rd instar larvae by differential centrifugation using mitochondrial isolation medium (MIM; 250 mM sucrose, 10 mM Tris/HCl [pH 7.4], 0.15 mM MgCl₂, with protease inhibitor). Mitochondria were resuspended in 1× NativePAGE Sample Buffer (Invitrogen) with 1% digitonin and protease inhibitors, and incubated for 15 min on ice. Samples were centrifuged at 16,200 × *g* for 30 min at 4°C, and supernatant was resuspended with G250 sample additive and NativePAGE Sample Buffer. For immunodetection, mitochondria were run on the Novex NativePAGE

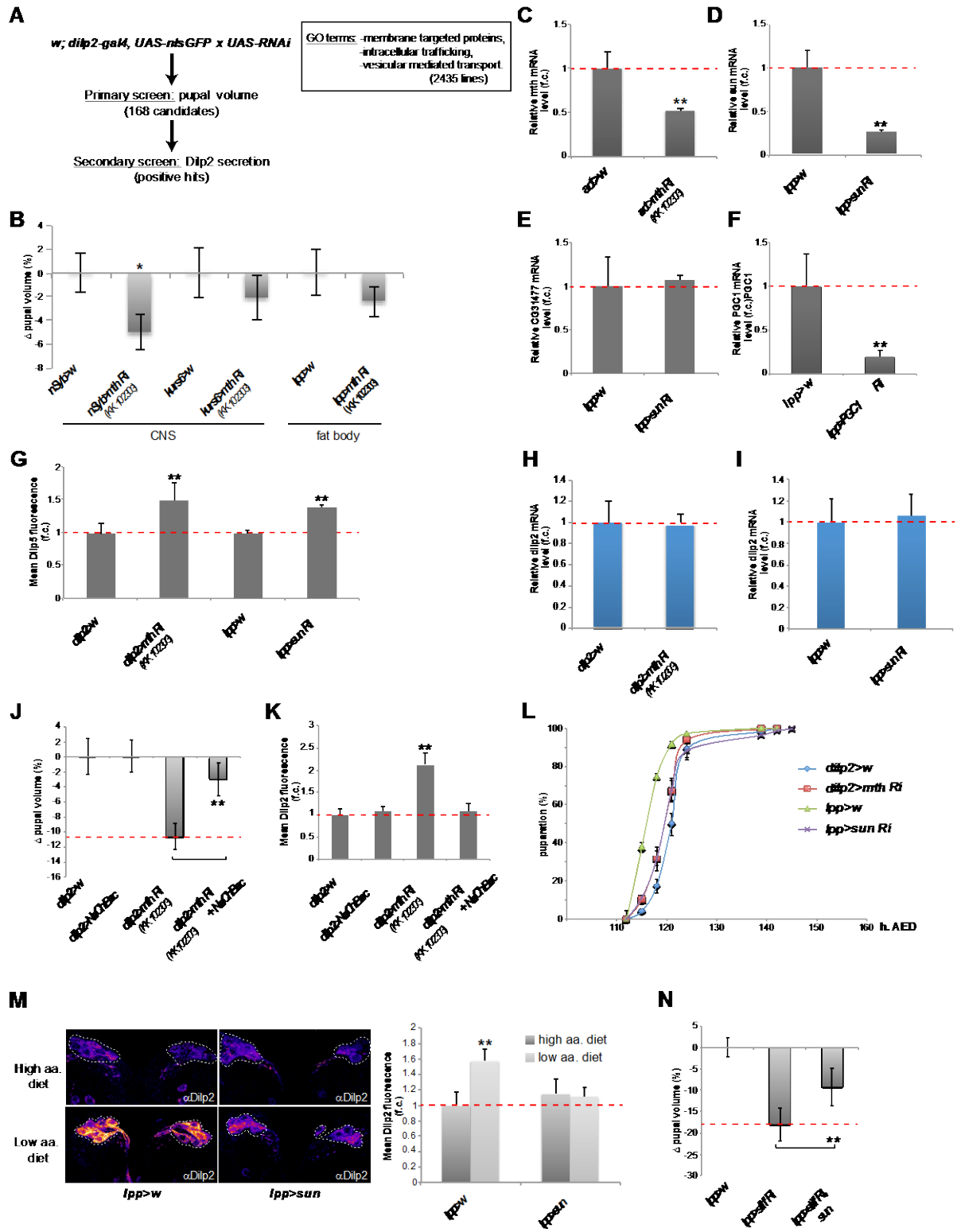
Bis Tris Gel System (Invitrogen) using 3%–12% Bis Tris Native PAGEs as previously described by (29), and transferred to PVDF membranes with transfer buffer containing 20% methanol and no SDS. Membranes were blocked in 5% skim milk powder in Tris-buffered saline for 1hr and incubated with primary and secondary antibodies. Chemiluminescence detection was performed on a Versadoc imager (Bio-Rad).

Quantitative RT-PCR

Larval tissues were dissected in PBS 1x and frozen in liquid nitrogen. Total RNA was extracted using Qiagen RNeasy lipid tissue minikit according to the manufacturer protocol. RNA samples (3 µg per reaction) were treated with DNase and reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen) and the generated cDNA used for real time RT-PCR (StepOne Plus, Applied Biosystem) using PowerSYBRGreen PCR mastermix (Applied Biosystem), using 8 ng of cDNA template and a primer concentration of 300 nM. Samples were normalised with RP49. Three separate samples were collected for each experiment and triplicate measurements were conducted. For each gene, two independent sets of primers were used. Primers were designed using the PrimerExpress software (Applied Biosystem) and sequences are available on request.

Supplementary Figures

Figure S1



(A) Schematic representation of the primary and secondary screens that lead to the identification of *mth*.

(B) Silencing of *mth* in neuronal cells (*nSyb-Gal4*) reduces animal size. *Mth* loss of function in non-IPC neurons (*kurs6-Gal4*) or in the fat body (*lpp-Gal4*) have no effect on final body volume. n>60.

(C) *mth* expression is efficiently reduced in *act>mth Ri* larvae. Graph represents means \pm SEM; n=3.

(D) *sun* expression is efficiently downregulated in *lpp>sun Ri* fat cells. n=3.

(E) But expression of CG31477, gene homologous to *sun*, is unchanged showing the specificity of the *UAS-sun RNAi* line.

(F) *PGC1* expression is efficiently reduced in *lpp>PGC1 Ri* fat bodies. n=3.

(G) Silencing *mth* in the IPC and *sun* in the fat body results in Dilp5 peptide accumulation. n>10.

(H) *dilp2* expression is unchanged upon *mth* silencing in the IPC. n=3.

(I) *dilp2* expression remains unaffected by *sun* silencing in the fat body. n=3.

(J-K) Ectopic expression of a depolarizing channel (NaChBac) in the IPC partially rescues the growth defect (J) and Dilp2 accumulation (K) of *mth* animals. n>20.

(L) Silencing *mth* in the IPC (*dilp2>mth Ri*) or *sun* in the fat body (*lpp>sun Ri*) has limited effects on developmental timing. n>60.

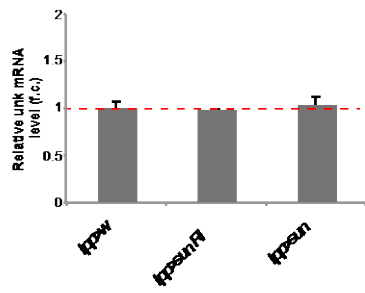
(M) Forced *sun* expression in the fat body rescues Dilp2 accumulation in the IPC observed in low aa. diet. n>20

(N) *Sun* overexpression rescues body size reduction due to *slif* loss of function in the fat body. n>60

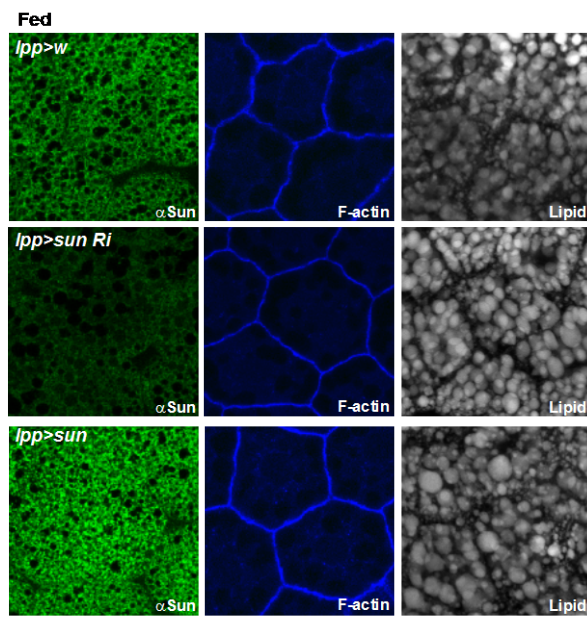
Graph represents mean \pm SEM; ** p<0.01.

Figure S2.

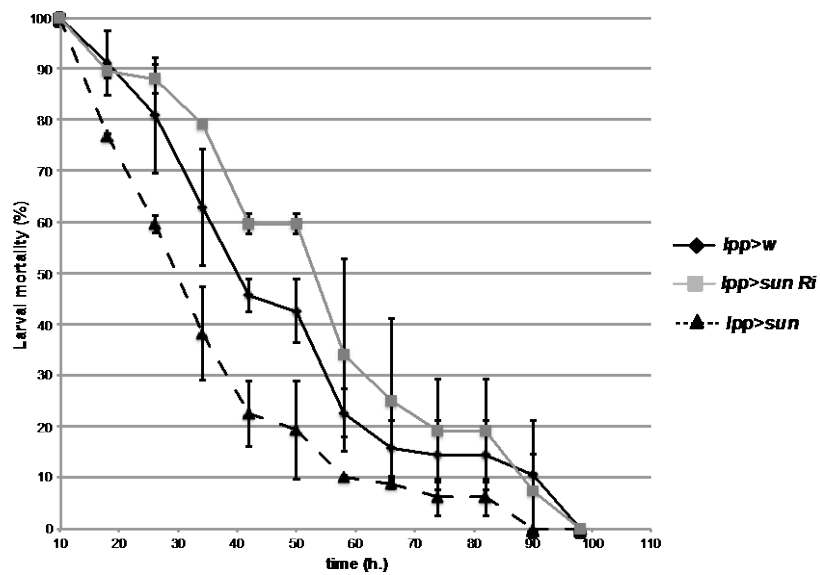
A



B



C

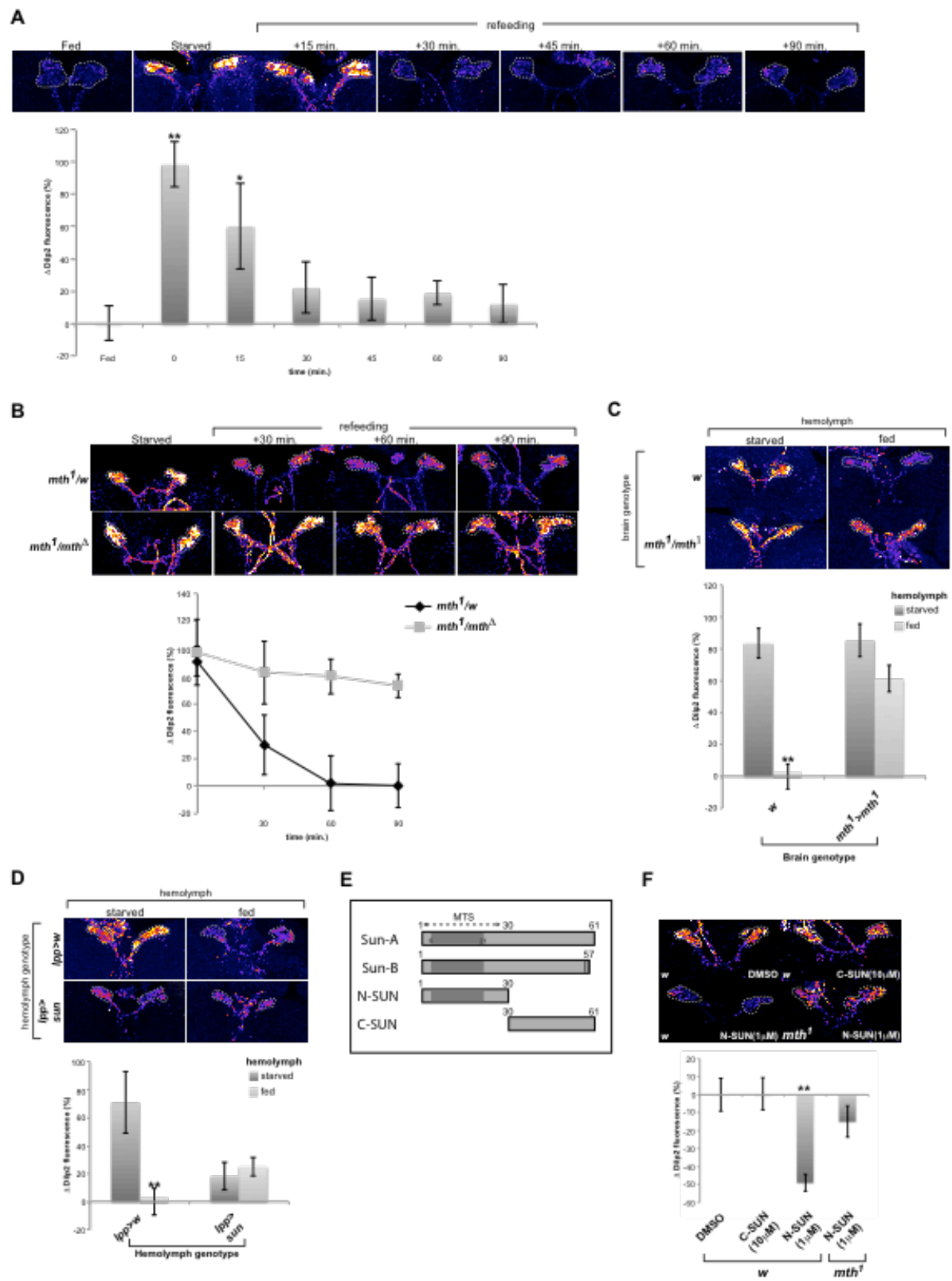


(A) Modifying *sun* expression using *lpp-Gal4* has no effect on *unk* expression in the fat body, suggesting that TOR activity remains unchanged in these conditions. Graph represents mean \pm SEM; n=3.

(B) *sun* LOF or GOF in the fat body, *lpp>sun Ri* and *lpp>sun* respectively, labeled with anti-Sun antibody (green), does not have any significant impact on fat cells morphology and physiology, such as actin organization (phalloidin in blue) or to lipid storage (lipid droplets stained with Nile red).

(C) *lpp>sun* animals are starvation intolerant. Overexpressing *sun* in the fat body strongly decreases larval viability when *lpp>sun* larvae are exposed to acute aa. starvation (PBS 1x, agar 1.5% + 1% sugar). On the contrary, silencing *sun* in fat cells, *lpp>sun Ri* gives larvae that are more resistant to aa. starvation. Graph shows percentage of larval mortality according to time after exposure to aa. deprived medium. n>40.

Figure S3.



(A) Representative pictures of Dilp2 staining in IPCs (dotted outline) showing the kinetic of Dilp2 accumulation upon refeeding. 72h AEL wild type larvae were starved for 16h and re-fed with yeast paste. Brains were dissected and stained at the given time points. Quantification of Dilp2 fluorescence on IPCs shows that yeast refeeding swiftly triggers Dilp2 secretion, and fluorescence levels are nearly similar to fed state within 30 min. n>20.

(B) Representative pictures of Dilp2 staining in IPCs (dotted outline) showing the kinetic of Dilp2 accumulation upon refeeding after prolonged starvation. *mth* loss of function (*mth¹/mthΔ*) precludes IPC response to refeeding. n>20.

Graphs **(A and B)** represent quantifications of ΔDilp2 fluorescence relative to control brains (from normally fed wt or *mth¹/w* larvae) (mean ± SEM)

(C) Hemolymph collected from normally fed larvae activates Dilp2 secretion when incubated on dissected brains from starved control larvae (*w*). Brains dissected from larvae lacking *mth* function (*mth¹/mth¹*) (bottom row) do not respond to hemolymph extracted from normally fed larvae. n>20

(D) Hemolymph from starved larvae overexpressing *sun* in the fat body (*lpp>sun*) activates Dilp2 secretion. n>20.

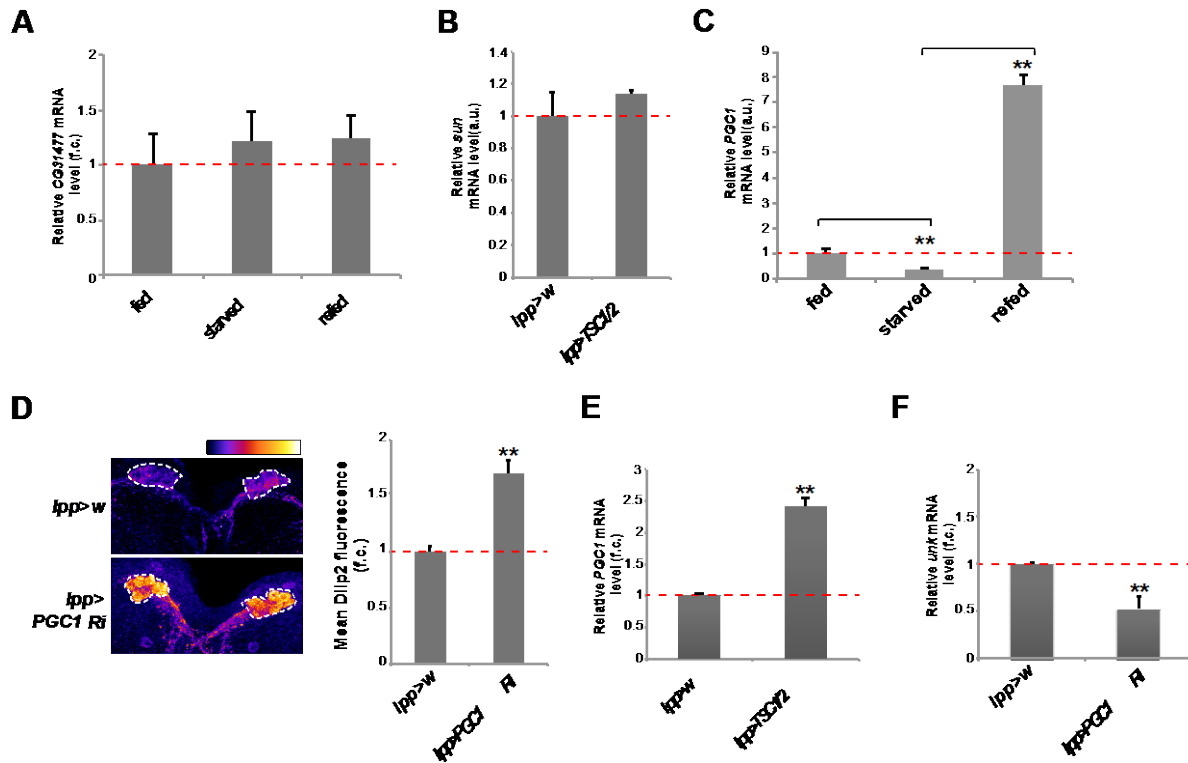
(E) Schematic representation of the Sun derived constructs and peptides used in this study. Sun-A and Sun-B isoforms only in their Cter ends. The first 30 aa. of Sun have all the features of a mitochondrial targeting signal (MLS). However, Sun homolog in yeast, ATPsynε, belongs to a group of nuclear encoded mitochondrial proteins which have no processed NH₂- terminal sequence (30).

Dark grey box represents the Mth-interacting domain characterized by (14) and (15).

(F) Incubation of brains from starved larvae with various synthetic forms of SUN. The C-terminal part (C-SUN) has no effect on Dilp2 secretion. The N-terminal part (N-SUN, 1μM) stimulates Dilp2 secretion by *wt*, but not *mth¹* brains. n>20.

Graphs **(C, D and F)** represents quantifications of ΔDilp2 fluorescence relative to control brains (brains from starved larvae incubated with hemolymph of normally fed larvae or DMSO) (mean ± SEM); ** p<0.01.

Figure S4.



(A) Expression of CG31477, *sun* homolog, is unchanged upon starvation and refeeding treatment.

Graph represents means \pm SEM; n=4.

(B) *sun* expression remains unaffected by blocking TORC1 but is severely reduced when *PGC1* is silenced in fat body cells. Graphs represent mean \pm SEM; n=3; ** p<0.01.

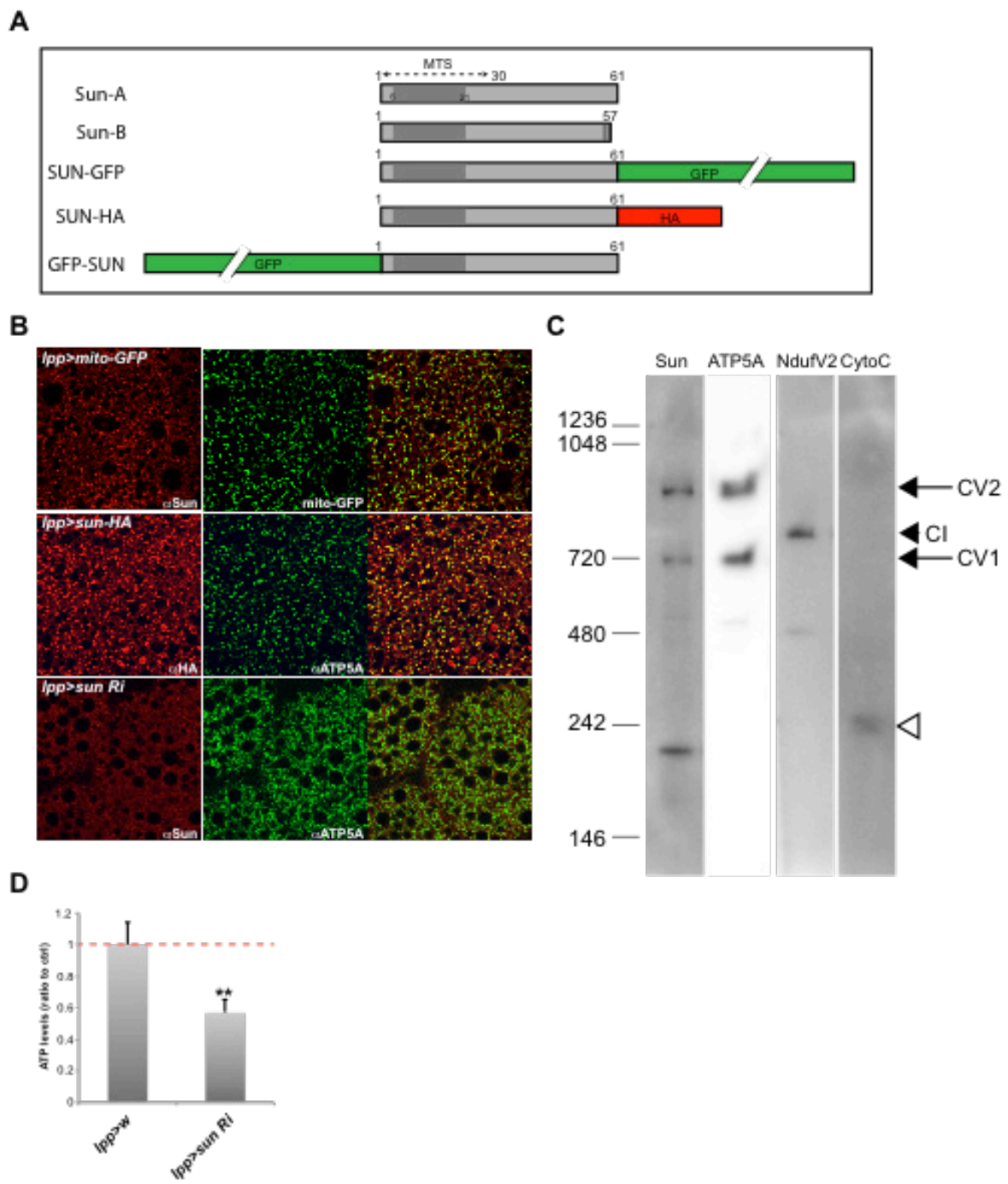
(C) Levels of *pgc1* transcripts in the fat body (qRT-PCR) strongly decrease in starvation, and promptly increase when larvae are normally refed. Graph represents means \pm SEM; n=4; ** p<0.01.

(D) Silencing *PGC1* in the fat body (*lpp>PGC1 Ri*) induces Dilp2 retention. Graph represents mean \pm SEM; n>40 ; ** p<0.01.

(E) Altering TOR activity by overexpressing the TOR inhibitors, TSC1 and TSC2 (*lpp>TSC1/2*), leads to an increase of *PGC1* expression in the fat body. Graph represents means \pm SEM; n=3; ** p<0.01.

(F) Silencing *PGC1* expression significantly decreases *unk* expression in the fat cells. Graph represents means \pm SEM; n=3; ** p<0.01.

Figure S5.



(A) Schematic representation of the Sun derived constructs and peptides used in this study. Sun-A and Sun-B isoforms only in their Cter ends. The first 30 aa. of Sun have all the features of a mitochondrial targeting signal (MLS). However, Sun homolog in yeast, ATPsyn ϵ , belongs to a group of nuclear encoded mitochondrial proteins which have no processed NH₂- terminal sequence (30).

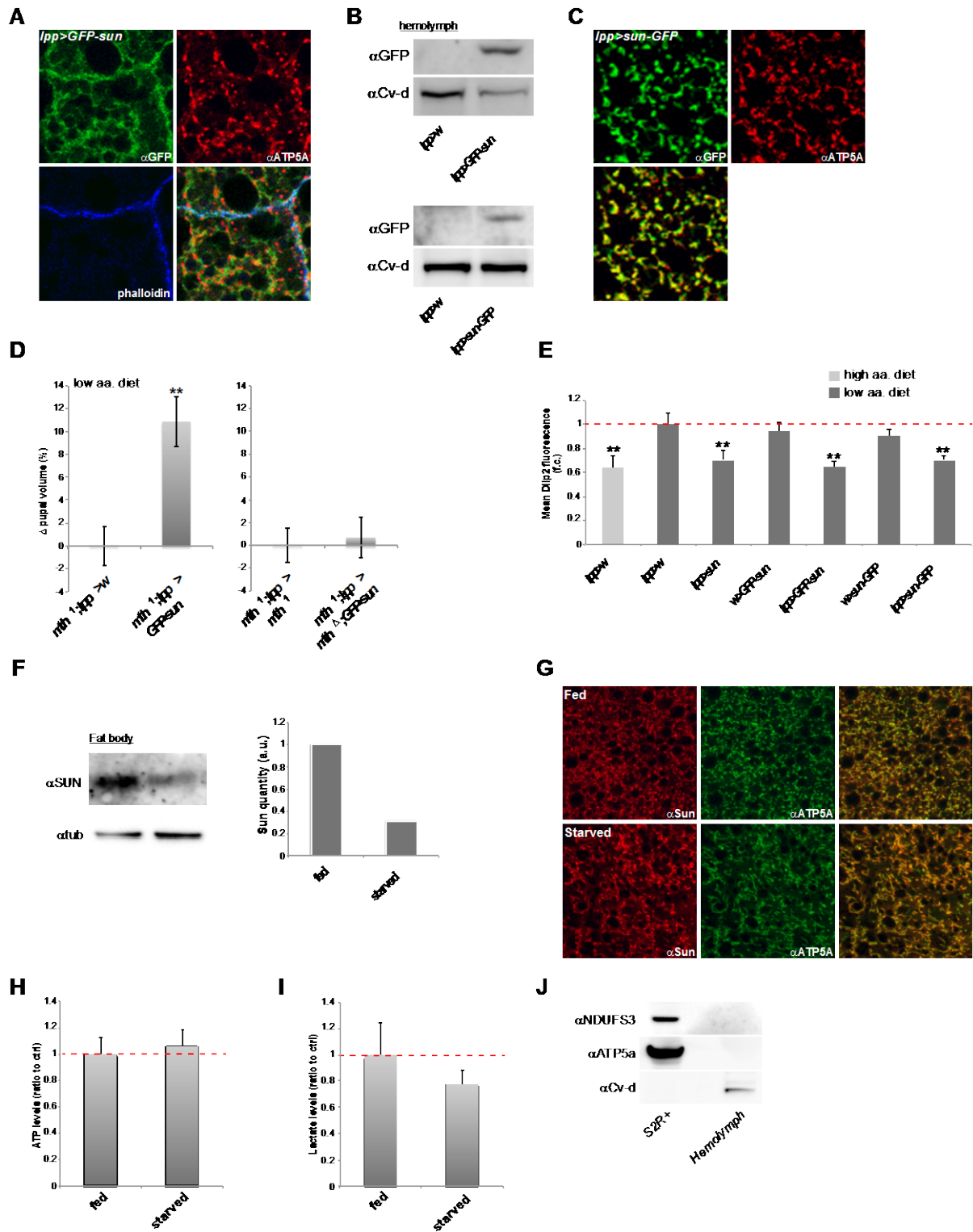
Dark grey box represents the Mth-interacting domain characterized by (14) and (15). GFP is represented by a green box, while HA tag by a red box.

(B) Immunostaining of fat body cells expressing a mitochondrial marker (*lpp>mito-GFP*) labeled with anti-GFP (green) and anti-Sun (red) (top row). Overexpression of HA-tagged version of Sun labeled with anti-HA (red) shows strong co-localization with a mitochondrial marker (ATP5A, green) (middle row). Sun staining (red) is abolished upon sun silencing in the fat body, *lpp>sun Ri*, but ATP5A mitochondrial labeling is conserved (green) (bottom row).

(C) Immunoblot of BN-PAGE confirms the presence of Sun protein on complex V dimer (CV2) and CV monomer (CV1). Complex I (CI) is detectable with antibody against Ndufv2 (black arrowhead), and CV with antibodies against ATP5A (arrowhead). The Cytochrome complex (CytoC) is marked (white arrowhead).

(D) ATP levels are reduced in fat bodies dissected from *lpp>sun Ri* larvae, relative to *lpp>w* animals. Graph represents mean \pm SEM, n>6; ** p<0.01.

Figure S6.



(A) High magnification reveals that adding a GFP tag at the Nter end of Sun completely disrupts mitochondrial localization (ATP5A, red) when expressed in fat body cells of *lpp>GFP-sun* larvae. Mis-localized GFP-sun (green) is detected in the entire cell (phalloidin, blue).

(B) Western blot shows that this GFP-sun and Sun-GFP proteins can be readily detected in the hemolymph of *lpp>GFP-sun* and *lpp>sun-GFP* larvae, respectively.

(C) Sun tagged with GFP (green) in Cter extremity localizes into the mitochondria (red, ATP5A) at high magnification.

(D) Overgrowth observed upon forced fat body expression of *UAS-GFP-sun* in low aa diet is observed in *mth^{1/+}*, but not in *mth¹/mth Δ* trans-heterozygote flies. Graph represents mean \pm SEM; n>40 ; ** p<0.01. Graph represents mean \pm SEM; n>40 ; ** p<0.01.

(E) Ectopic expression in the fat body of *sun*, *GFP-sun* or *sun-GFP* (*lpp>w*, *lpp>GFP-sun* and *lpp>sun-GFP* respectively) partially rescues Dilp2 accumulation in the IPC observed in controls (*lpp>w*, *w>GFP-sun* and *w>sun-GFP*) in low aa. Diet. The levels are comparable to Dilp2 staining observed in normally fed larvae. Graph represents means \pm SEM; n>10; ** p<0.01.

(F) Representative western blot showing that the quantity of Sun protein decreases in aa.-starved fat body compared to fed conditions. Graph represents normalized Sun quantity.

(G) Immunostaining reveals that upon aa. starvation, mitochondria become elongated as revealed by mitochondrial marker ATP5A (green), but Sun labeling (red) appears to be mostly unchanged in the mitochondria.

(H) ATP levels are unchanged in fat bodies dissected from fed or starved *wt* larvae. Graph represents mean \pm SEM, n=6.

(I) Lactate levels are unchanged in fat bodies dissected from fed or starved *wt* larvae. Graph represents mean \pm SEM, n=3.

(J) NDUFS3 and ATP5A can be readily detected in extract from S2r+ cells, but not in hemolymph collected from a normally fed larvae.

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III- Conclusion

During my PhD, I had the opportunity to participate to the identification of a new adipose factor, Sun, involved in Dilps secretion according to dietary amino acids. Surprisingly, Sun is a component of the F_1F_0 -ATP synthase complex in the mitochondria. Therefore, I mainly contributed to prove that Sun is also an insulinotropic peptide present in the hemolymph. As expected, I showed that fat-derived Sun is produced and released in the hemolymph in a nutrient- and TOR- dependent manner. Furthermore, I proved that most of the circulating Sun is coming from the fat body. Indeed, specific downregulation of *sun* in the fat body, but not in the gut, reduces circulating Sun levels in the hemolymph (-50%).

This study provides evidence of a new organ crosstalk between the fat ligand Sun and the brain Mth receptor in the IPCs controlling insulin levels in response to nutrition (Figure 47).

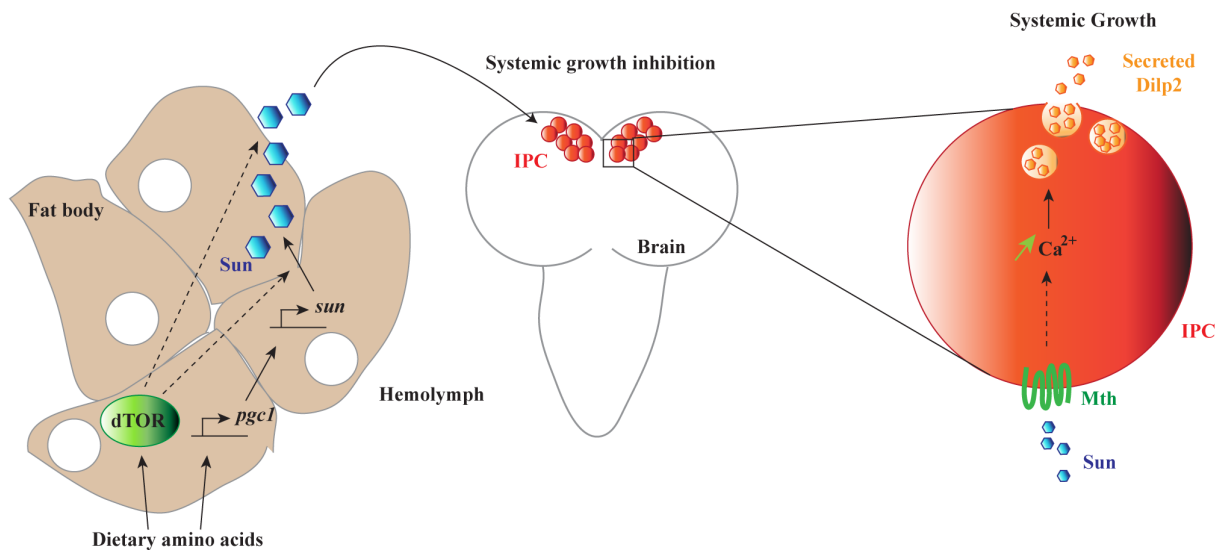


Figure 47 : The fat-derived Sun modulates insulin levels through brain Mth in the IPCs according to nutrients. Upon dietary amino acids, *pgc1* transcripts increases in the fat body and promotes *sun* transcription. In parallel, TOR activation leads to Sun translation and/or secretion in the hemolymph. Secreted Sun acts on its receptor Mth, located in the brain IPCs and most probably increases calcium levels therefore triggering Dilp2 secretion.

Chapter III: Eclosion hormone neurons control the IPCs secretory activity – Preliminary data

My PhD project consisted in understanding how the IPCs secretory activity is regulated by identifying neuronal populations which affect Dilps secretion and control body size according to nutrition. Apart from the ICNs, we also identified another neuronal population, the eclosion hormone (EH) expressing neurons which seem to control Dilps secretion through direct synaptic contacts with the IPCs.

I- Processes of the eclosion hormone (EH) expressing neurons project towards the IPCs

We identified another driver line expressed in the EH neurons (*C21>*) which project towards the IPCs dendrites (McNabb et al., 1997). The EH neurons are constituted of one pair of neurons located in the Pars Intercerebralis (PI) of the optic lobes. Interestingly, the EH neurons project their neurites towards the IPC dendrites and the IPCs axons in the ring gland (Figure 48A). By using the *DenMark* which is a dendritic marker (Nicolai et al., 2010) and the *syn.e::GFP* fusion that marks presynaptic compartments (Zhang et al., 2002), we found that both post- and presynaptic parts of the EH neurons co-localize with the IPCs (Figure 48B). This suggests that EH neurons project both dendrites and axons towards the IPCs. Using the GFP reconstitution across synaptic partners (*GRASP*) (Feinberg et al., 2008) we could confirm direct contact between the two neuronal populations (Figure 48C) both in the PI and the ring gland (Figure 48C' and 48C''). Both dsRed staining and GRASP signal are also observed in the PG. Conversely to what it has been proposed, this suggests that the IPCs and EH neurons project on the PG and not only on the CC (Siegmond and Korge, 2001). These findings therefore suggest that the EH neurons could directly contact the IPCs.

Results

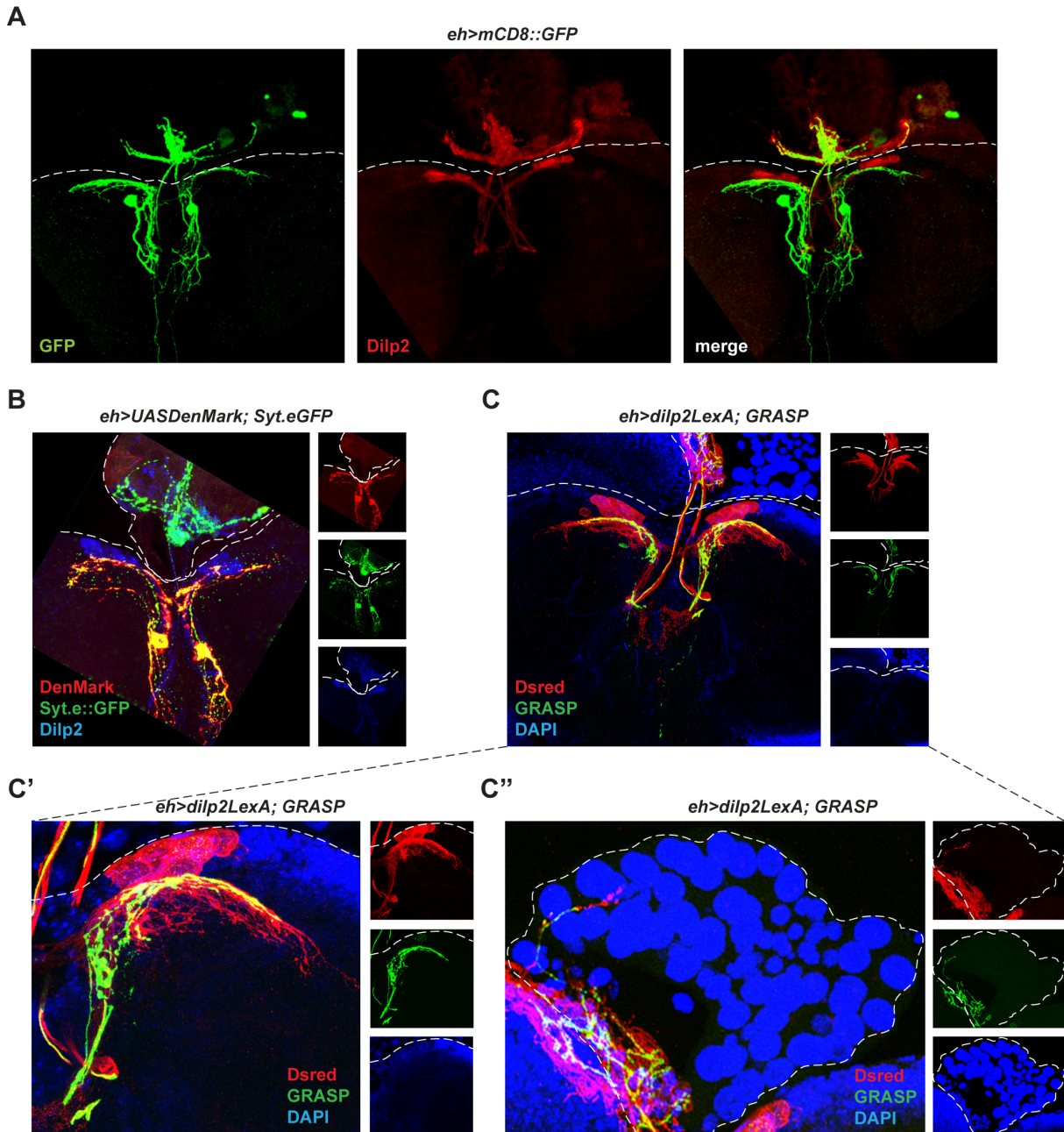


Figure 48 : EH neurons neuronal structure. A. The *eh-GAL4* line drives *mCD8GFP* (green) in one neuron in each brain hemisphere, sending projections towards the IPCs (anti-Dilp2, red) and the ring gland. B. *UAS-DenMark; UAS-SyteGFP* are driven by the EH neuronal driver, *eh-GAL4*. The dendritic marker DenMark is used to visualize the EH neurons dendrites (red), and SyteGFP reveals their axonal architecture (green). C. GFP reconstitution across synaptic partners (GRASP) shows physical connections between IPCs and EH neurons. *dilp2-LexA* and *eh-GAL4* are used to express membrane-tethered split-GFP *LexAop-CD4::spGFP11* and *UAS-CD4::spGFP1-10*, respectively. IPCs are labeled with a membrane associated RFP (DsRed, red). The GRASP signal is visualized in green. C'. Zoom of the GRASP signal around the IPCs arborisations. C''. Zoom of the GRASP signal on the ring gland. C, C' and C'' acquisitions were done by Delanoue R.

II- EH neurons promote Dilps release and body growth

To test whether the EH neurons could functionally interfere with the IPC secretory activity, we impaired their neuronal activity. Both hyperpolarization of the ICNs by expression of the potassium channel Kir2.1, or impaired secretion by tetanus toxin (TetX) led to smaller pupae (Figure 49A), associated with increased accumulation of Dilp2 in the IPCs (Figure 49B). Converse experiment using the bacterial sodium channel NaChBac to induce a chronic depolarization of the ICNs gave rise to bigger animals (Figure 49A). Nevertheless, Dilp2 accumulation in the IPCs remains to be tested in this condition.

Even though brain *dilp2* mRNA levels have to be assessed after EH neuronal activity manipulation, we propose that the EH neurons are part of an excitatory neuronal relay specifically acting on Dilp2 secretion by the IPCs.

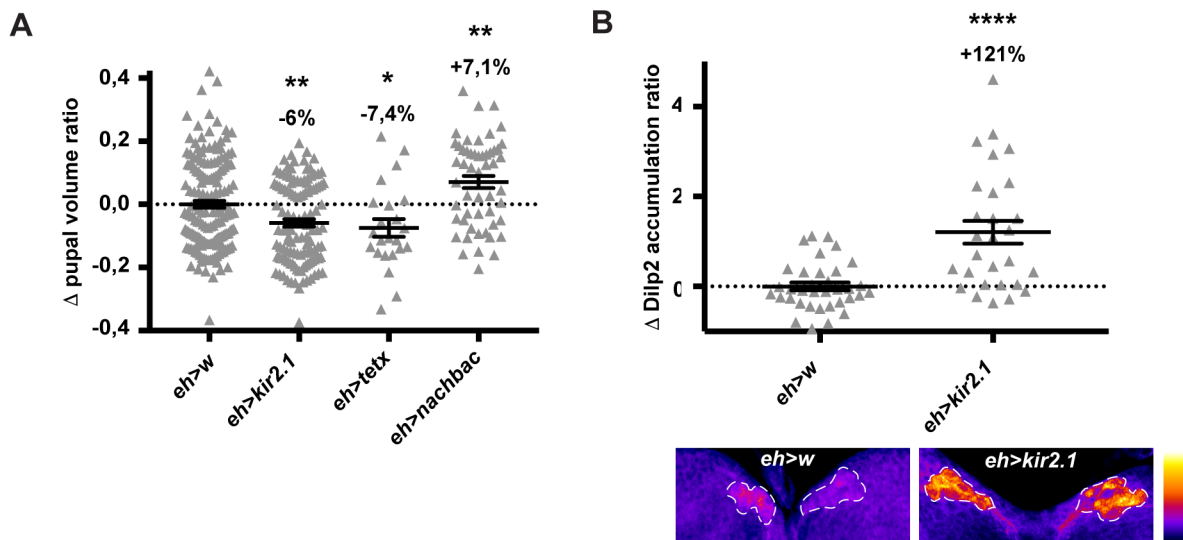


Figure 49 : EH neurons induce Dilp2 secretion and promote systemic growth. A. Pupal volume measurement after EH neurons hyperpolarization (*eh>kir2.1*), blockade of secretory activity (*eh>tetx*) or chronic depolarization (*eh>nachbac*). (n≥23) B. Dilp2 accumulation levels in the IPCs upon EH neurons hyperpolarization (*eh>kir2.1*). (n≥28) Pupal volume and fluorescence intensity are measured as delta ratio compared to control (*eh>w*).

III- Discussion and Conclusion

EH neurons or ventromedial (VM) neurons consist in two peptidergic neurons which produce the eclosion hormone (EH) (McNabb et al., 1997; Siegmund and Korge, 2001). The eclosion hormone acts on both peripheral tissues and the brain in order to control ecdysis (Clark et al., 2004; Kim et al., 2006; Krüger et al., 2015). Accordingly, *eh* null mutant larvae invariably die around the ecdysis timing (Krüger et al., 2015).

Surprisingly, EH receptor has not been identified yet in *Drosophila melanogaster*. Nevertheless, the receptor guanylyl cyclase BdmGC-1 has been proposed as an EH receptor in the fruit fly *Bactrocera dorsalis* (Chang et al., 2009). Furthermore, EH has been shown to activate second messenger cascades leading to cGMP and Ca²⁺ accumulation in order to promote ecdysis triggering hormone (ETH) secretion (Kingan et al., 2001).

Even though in the literature, no scientific evidence suggests a possible control of EH neurons on insulin signaling, we found that electrical manipulation of EH neurons affect insulin secretion and ultimately systemic growth. However, to confirm that only secretion of insulin is affected, we should check if *dilp2* mRNA levels in the brain are unchanged. Additionally, we provided evidences of physical contacts between the IPCs and the EH neurons. Interestingly, it has been shown that insulin secretion is sustained and modulated by several factors including cGMP and protein kinase G (PKG) in mammalian pancreatic β cells (Lazo-de-la-Vega-Monroy and Vilches, 2014). This raises the possibility that EH neurons could control the IPCs secretory activity through EH release, activation of its receptor (EH Receptor) and subsequently intracellular cGMP and Ca²⁺ increase. Another option is that EH neurons produce other neuropeptides or neurotransmitters that could act on the IPCs to modulate Dilps secretion.

Altogether, these preliminary data suggest a new function for the EH neurons in controlling the IPCs secretory activity (Figure 50).

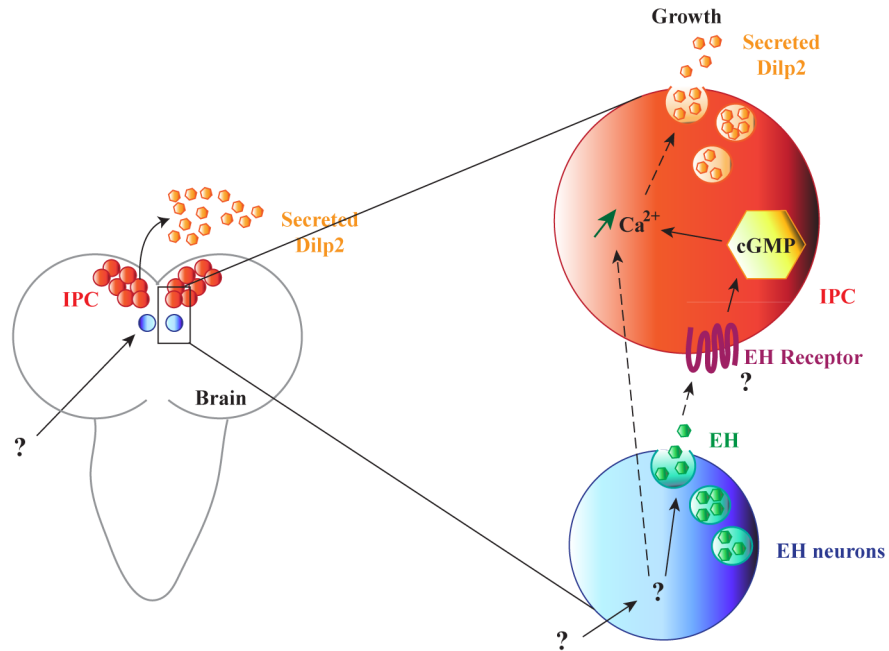


Figure 50 : Possible mechanisms by which EH neurons control the IPCs secretory activity. EH neurons could stimulate insulin secretion through EH release and consequently cGMP signaling activation in the IPCs or through an unknown mechanism. In both cases, the IPCs intracellular calcium levels have to increase in order to promote Dilps secretion. However, the upstream signal inducing EH neuronal activity remains unidentified.

However, further investigations are necessary to confirm these results. To which physiological or environmental cue the EH neurons respond? Dilps secretion as well as the IPCs neuronal activity are precisely adjusted in response to nutrition (Géminard et al., 2009) (Meschi E. et al., in preparation). Therefore, EH neuronal activity could also be partially controlled by dietary nutrients. The CaLexA reporter allows cumulative tracing of neuronal activity (Masuyama et al., 2012) and could be used to investigate whether the EH neurons are sensitive to nutrition.

It would be interesting to study how the EH neurons and the IPCs communicate. Since both neuronal populations are peptidergic neurons, either EH is a new insulinotropic hormone, either EH neurons produce other insulinotropic neuropeptides and/or neurotransmitters. Specific EH silencing in the EH neurons would determine whether EH neurons and the IPCs communicate through EH.

Additionally, EH has been shown to increase intracellular levels of cGMP and Ca²⁺ (Kingan et al., 2001). *Ex vivo* calcium and cGMP reporter imaging could be performed on IPCs prior and after incubation with EH. This would strengthen that EH neurons and the IPCs communicate through EH and would also suggest the presence of EH Receptor on the IPCs. Besides, since cGMP sustains insulin secretion from pancreatic β cells, this study would reveal a conserved signaling among mammals and invertebrates that promotes insulin secretion.

DISCUSSION

Discussion

Systemic growth of an organism is precisely coupled with nutrients availability. This coupling requires specific nutrient sensing and central integration of the nutritional information in order to fine-tune hormonal secretion involved in growth control. The Dilps and IIS control the growth rate. Dilp2 is mainly produced by the IPCs and its secretion is precisely adjusted in response to nutrition through an interorgan communication with the fat body (Colombani et al., 2003; Géminard et al., 2009). Several fat-derived signals have been identified such as Upd2, CCHA2, Egr, GBPs and Sun (Agrawal et al., 2016; Delanoue et al., 2016; Koyama and Mirth, 2016; Rajan and Perrimon, 2012; Sano et al., 2015). These FBS act either directly on the IPCs or through a neuronal relay in order to control the IPCs secretory activity. My PhD project aimed to better understand how the IPCs secretory activity is regulated by both fat-derived signals and neuronal populations, affecting Dilps secretion and body size according to nutrition.

During my PhD, I deciphered the neuronal circuitry by which GBPs control the IPCs secretory activity and discovered a new neuronal population inhibiting the IPCs secretory activity: called the ICNs. Importantly, GBPs insulintropic effect is EGFR and Dsor dependent. I also participated to the identification of a new fat-derived signal, Sun, involved in Dilps secretion according to dietary amino acids (Delanoue et al., 2016) and I collected some preliminary data on another neuronal circuitry that potentially controls the IPCs secretory activity: the EH neurons.

In this section, I will discuss three major points:

- GBPs/EGFR non-canonical axis has an insulintropic effect, conserved in vertebrates.
- Multiplicity of insulintropic fat-derived signals and their secretory mechanisms
- Elaborated network which regulates the IPCs secretory activity

I- Fat-derived GBPs are long-ranged EGF ligands

By RMN analysis, GBPs have been classified as EGF like ligand because of their three-dimensional similarities with the C-terminal region of EGF (Aizawa et al., 1999, 2002). Accordingly, direct binding of GBPs to EGFR has been revealed in human keratinocytes (Ohnishi et al., 2001). Nevertheless, this study also suggests the existence of an unidentified 58kDa GBP receptor (GBPR) (Ohnishi et al., 2001).

Recently, GBPs has been found to bind to the G protein coupled receptor (GPCR) Methuselah-like 10 (Mthl10) and to induce ERK phosphorylation in insect cells culture (Sung et al., 2017). Although manipulating GBP and Mthl10 give similar phenotype on Dilps secretion, there is no direct evidence that

Discussion

GBPs/Mth10 interaction has relevant significance for the control of the IPCs function. Indeed, even though GBPs overexpression decreases lifespan while Mth10 knockdown increases it, the genetic interaction experiment reveals that the effect of GBPs on lifespan is independent of Mth10.

GBPs is known to mediate the humoral aspect of innate immune response during infectious and non-infectious stress (Tsuzuki et al., 2012). GBPs trigger JNK signaling activation and stimulate the expression of antimicrobial peptides. While Mth10 can also stimulate antimicrobial peptide, no evidence suggests a potential role of EGFR on innate immune response. This suggests that GBPs could act on different receptors to promote different physiological responses.

In line with the EGFR phosphorylation induced by GBPs in human keratinocytes (Ohnishi et al., 2001), our study establishes that *gbp1* overexpression can efficiently stimulate EGFR-dependent signaling, both in cultured cells and in developing organs. We also provide genetic evidence that the function of fat body derived-GBPs in controlling Dilp2 secretion entirely relies on the presence of EGFR in the ICNs. This clearly shows that GBPs metabolic hormones act as long-range distance EGFR ligand.

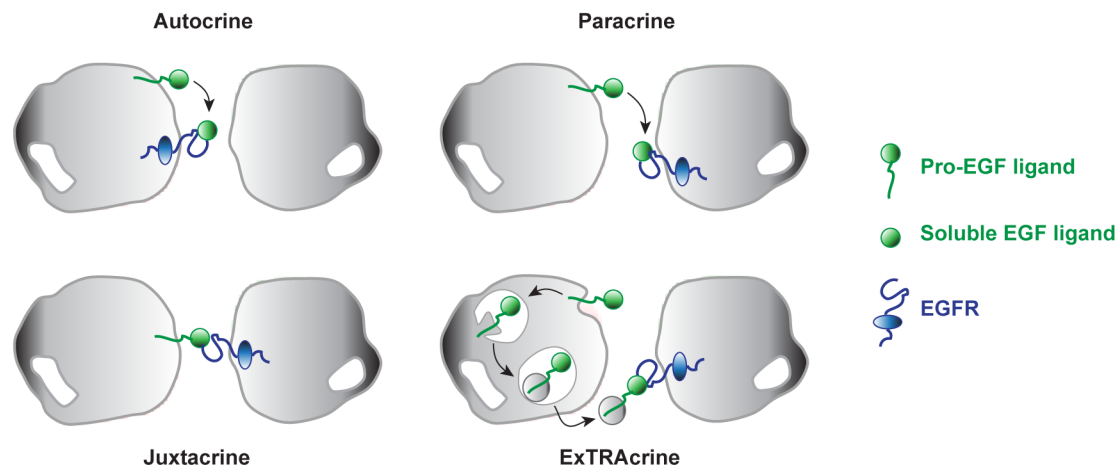


Figure 51 : EGF modes of signaling through its receptor EGFR. Autocrine signaling: EGF is produced, released and binds to EGFR on the same cell. Paracrine signaling: Soluble EGF activate EGFR on a nearby cell. Juxtacrine: The pro-EGF binds to EGFR on an adjacent cell. ExTRAcrine signaling: EGF is packed in exosomes, and activate EGFR probably on distant cells.

Adapted from Singh B. et al. (2016) – EGF receptor ligands: recent advances

In mammals, seven EGFR ligands exist (Ceresa and Peterson, 2014). These ligands activate EGFR through different mode of signaling: autocrine, paracrine, juxtacrine and extracrine (Figure 51) (Singh et al., 2016). Likewise, in *Drosophila* four ligands and one antagonist interact with EGFR through different molecular mechanisms: Spitz, Vein, Keren, Gurken and Argos (Lusk et al., 2017). Despite their redundancy, the expression pattern of each ligand and the strength of EGFR activation are responsible for different EGF signaling responses. Additionally, while Gurken is considered as a long-ranged EGFR

Discussion

ligand acting over more than 10 cell diameters, Spitz is known as a short-ranged ligand acting over 3-4 cell diameters (Goentoro and Shvartsman, 2006). Nevertheless, at the scale of an organism, both are short-ranged EGF ligands.

By contrast, we show that GBPs produced by the fat body can be secreted in the hemolymph in a nutrient-dependent manner and cover long distances in order to bind EGFR in the brain ICNs. In the same line, plasmatic exosomes containing EGFR and the EGFR ligand Amphiregulin (AREG) are detected in humans, which would be compatible with a possible endocrine function of EGF (Higginbotham et al., 2016).

Therefore, GBPs are long-ranged EGF ligands allowing inter-organ communication. However, the secretory mechanism by which GBPs are released from fat cells upon dietary amino acids remains to be elucidated.

II- EGFR function in controlling the ICNs neuronal activity

EGF is a mitogenic factor which promotes cellular proliferation and tissue differentiation during development. Besides, in mammalian adult brain, EGF is expressed in several structures such as the pituitary gland while EGFR is localized in cortical neurons and the hippocampus; the last being a neuronal structure known to continuously undergo neurogenesis (Tucker et al., 1993; Wong and Guillaud, 2004). These specific localizations and some *in vitro* data strongly suggest a role of EGF/EGFR axis in neurogenesis and neuronal differentiation in adults. Unexpectedly, neither structural nor identity changes were detected in the ICNs upon EGFR signaling alterations, demonstrating that the EGFR signaling pathway is not important for the ICNs neurogenesis and differentiation.

In our conditions, EGFR alterations modify the calcium signaling. Since neuronal activity relies on calcium signaling, we determined that only the ICNs neuronal activity is changed upon EGFR signaling alterations. We observed that EGFR activation in the ICNs reduces intracellular calcium levels and therefore inhibits the ICNs neuronal activity. By contrast, in the literature, EGFR signaling activation is usually correlated with an intracellular calcium increase. This increase is due to activation of downstream components such as phospholipase-C γ (PLC- γ) which catalyzes phosphatidylinositol-4,5-bisphosphate hydrolysis into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). Subsequently, IP3 induces calcium release from intracellular stores (Wong and Guillaud, 2004), while DAG activates protein kinase C (PKC) which in turn, phosphorylates and potentiates voltage-dependent calcium

Discussion

channels (VDCC) (Strong et al., 1987). Furthermore, several EGFR interacting kinases such as Src, have been shown to modulate different ion channels, like Slowpoke (Jonas and Kaczmarek, 1996; Ling et al., 2000). Our results show that body growth induction by fat-derived GBPs requires dSOR, a MAP kinase downstream of EGFR pathway which is the equivalent of MEK. This suggests that GBPs inhibit the ICNs activity through both EGFR and at least one downstream kinase component.

Remarkably, several MAPKs downstream of EGFR pathway, modulate the synaptic plasticity by phosphorylation of different synaptic components such as scaffolding proteins (PSD-95 and PSD-93), cadherin-associated protein, potassium channels including Kv4.2 and group I metabotropic glutamate receptors (Mao and Wang, 2016; Schrader et al., 2006). Phosphorylation of these components by ERK/MAPK modifies the trafficking and synaptic delivery of these proteins and thus determines the strength and the efficacy of excitatory synapses. Importantly, pharmacological MEK inhibition induces faster Kv4.2 inactivation (Yuan et al., 2006). During inactivation, the potassium channel Kv4.2 cannot open even though the transmembrane voltage is favorable. This demonstrates that MEK inhibits Kv4.2 inactivation. Consequently, Kv4.2 remains open longer, more K^+ passively go out of the cell and cellular hyperpolarization is triggered (Figure 52). Though, whether MEK exert its inhibitory effect on Kv4.2 channels in a kinase-dependent manner or through direct action on gating mechanism is still unclear.

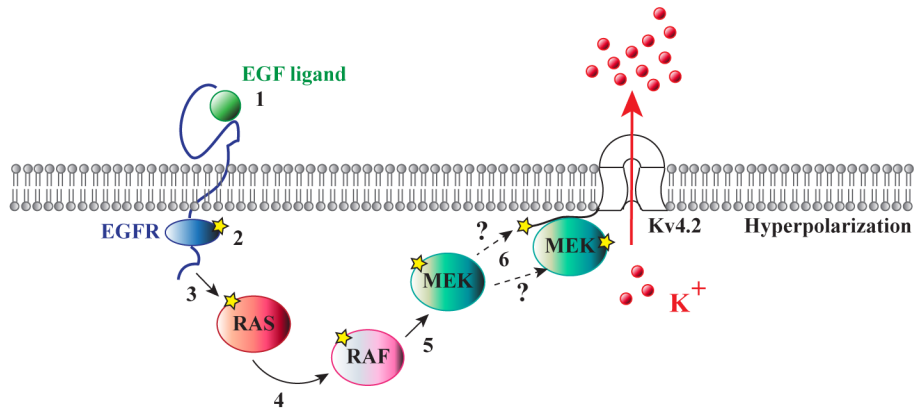


Figure 52 : Possible mechanism of Kv4.2 inactivation by MEK, leading to membrane hyperpolarization. After EGF binding (1), EGFR autophosphorylates (2) and triggers a phosphorylation cascades on RAS/RAF/MEK (3-5). Subsequently, MEK inhibit Kv4.2 inactivation either by direct phosphorylation or through gating mechanism (6). This leads to longer opening of Kv4.2, K^+ ions exit and therefore cell hyperpolarization. Adapted from Yuan L-L. et al. (2006) – Acceleration of K^+ channel inactivation by MEK inhibitor U0126.

The *Drosophila* homolog of Kv4.2 is a member of the Shaker-like family (Shal-type) and dSOR is the equivalent of MEK. It would be very interesting to test whether ICNs activity inhibition by EGFR and dSOR is mediated through Kv4.2 modulation.

III- ICNs and IPCs neuronal communication

Our results suggest that the ICNs are inhibitory on the IPCs since ICNs ablation, hyperpolarization or impaired secretion lead to increased circulating Dilp2 levels in the hemolymph. Thus, what is the inhibitory signal released by the ICNs?

The ICNs are not GABAergic, nor aminergic, since they do not present positive staining for GABA and tyrosine hydroxylase (TH) (Annexe 1A-1B). Intriguingly, even though the ICNs colocalize with the ChAT-GAL4, suggesting that they could be cholinergic, they are not labeled with the choline acetyltransferase (ChAT) antibody (Annexe 1C-1D). In addition, downregulation of ChAT in the ICNs does not lead to changes in pupal volume, suggesting that the ICNs do not use acetylcholine to inhibit the IPCs (Annexe 2A).

ICNs are also peptidergic neurons (Annexe 1E). They express at least two different neuropeptides: Tachykinin (dTK) and MyoInhibitory Peptide (MIP). Silencing MIP in the ICNs does not change the pupal volume (Annexe 2B). Interestingly, the IPCs express the tachykinin receptor. In the adults IPCs, published data suggest that DTKR inhibits the IIS by preventing *dilps* transcription and probably Dilps secretion (Birse et al., 2011). This is in line with our hypothesis. If the ICNs inhibit the IPCs through the tachykinin signaling, both silencing dTK in the ICNs and dTKR in the IPCs should lead to bigger pupae. Unfortunately, we could not detect any change in pupal size upon specific dTKR knockdown in the IPCs or dTK in the ICNs (Annexe 2C-2D).

Neuropeptide hormones are synthesized as prohormone and are processed by Amontillado, the *Drosophila* prohormone convertase 2. Loss of Amontillado is associated with a loss of neuropeptide hormone signals (Wegener et al., 2010). Importantly, ICNs specific knockdown of Amontillado does not alter the pupal volume (Annexe 2E), strongly suggesting that neuropeptides are not required to exert the inhibitory activity of the ICNs on the IPCs.

How the ICNs inhibit the IPCs remains still unclear and will need further studies to be elucidated. It will be noteworthy to test other neurotransmitters such as serotonin or octopamine, since their receptors are expressed in the adults IPCs (Crocker et al., 2010; Luo et al., 2012).

IV- A conserved mechanism for insulin release

Insulin-like peptides secretion is precisely adjusted according to dietary amino acids (Géminard et al., 2009). Likewise, insulin secretion is also controlled by dietary amino acids in mammals. Indeed,

Discussion

dietary proteins scarcity is associated with impaired insulin secretion whereas amino acids or dietary proteins have insulintropic effects (Newsholme et al., 2007; Zhang and Li, 2013). During my PhD, I showed that a central relay contributes to adjust insulin secretion.

Similarly, pancreatic islets are directly innervated by central neurons. Remarkably, dietary amino acids have been shown to control insulin secretion partially through this central relay (Horiuchi et al., 2017).

Furthermore, it has been demonstrated that EGF ligands promote insulin secretion from pancreatic islets (Lee et al., 2008a). However, the producing-source of this metabolic EGF remains undetermined. EGF is produced by the submaxillary gland, small intestines, kidney, pancreas, pituitary gland and the brain. EGF is detected in different body fluids such as saliva, cerebrospinal fluid, urine, amniotic fluid, prostatic fluid, pancreatic juice, breast milk and blood (Wong and Guillaud, 2004). This would suggest that EGF ligands can be secreted and act as a hormone in mammals. Furthermore, the existence of plasmatic exosomes containing EGFR and the EGFR ligand Amphiregulin (AREG) in humans would be coherent with such properties (Higginbotham et al., 2016).

Overall, these studies emphasize a possible conserved endocrine function for EGF ligands in regulating insulin like peptide secretion.

V- Sun: a mitochondrial protein with an endocrine function

The protein Sun has been identified as another fat-derived signal responding to dietary amino acids (Delanoue et al., 2016). Intriguingly, Sun is a mitochondrial protein included in the mitochondrial complex V of the F_1F_0 adenosine triphosphatase (F_1F_0 -ATPase) synthase.

How a mitochondrial protein can be secreted into the hemolymph and act as a hormone? Interestingly, an ectopic form of the F_0F_1 -ATP synthase also localizes to the plasma membrane and another complex V subunit, coupling factor 6, was identified in the plasma (Martinez et al., 2003; Osanai et al., 2001; Zalewska et al., 2009). These studies suggest that mitochondrial protein can be secreted. Nevertheless, is Sun mitochondrial localization required to its endocrine role?

To unravel this possibility, either a N- or C-terminal tagged form of Sun were overexpressed in fat cells. Both constructs are found in the hemolymph and partially rescue pupal volume and Dilp2 secretion upon dietary protein scarcity. Surprisingly, while the tagged version in C-terminal is detected in mitochondria, the N-terminal tagged Sun is not. This reveals that secretion of Sun and not its mitochondrial localization is required for Sun endocrine role on systemic growth. According to these data,

Discussion

we propose the presence of two distinct Sun pools: one dedicated to the mitochondrial function and another devoted to couple nutrition and systemic growth (Figure 53). In line with this hypothesis, mitochondrial functions are preserved in larvae exposed to amino acids starvation even though Sun protein levels in the fat body decrease. This indicates that only the endocrine Sun pool is dependent on dietary amino acids and PGC1 (PPAR γ coactivator-1).

However, the secretory mechanism by which Sun is secreted from fat cells upon dietary amino acids remains to be elucidated.

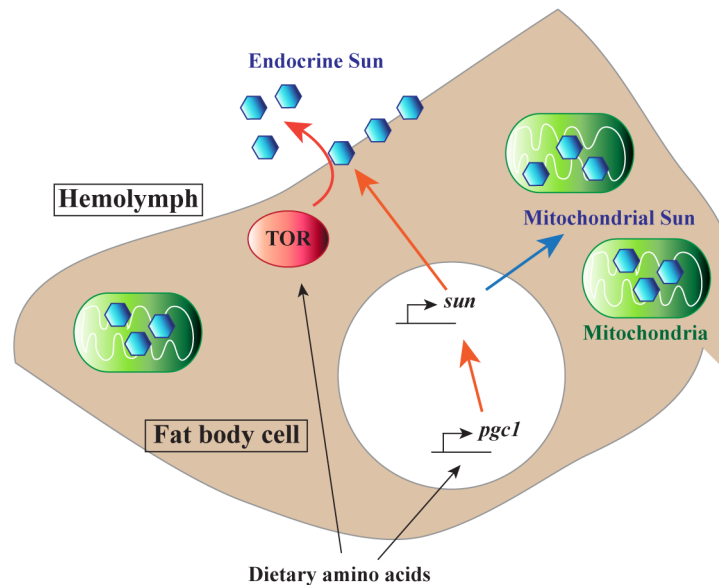


Figure 53 : Origins of mitochondrial and endocrine Sun peptide. Dietary amino acids induce *pgc1* transcription which in turn promotes *sun* transcription. In parallel, dietary amino acids also activate TOR signaling pathway and stimulates secretion of endocrine Sun into the hemolymph. Orange arrows indicate the endocrine Sun origin while the blue arrow shows Sun devoted to the mitochondrial function. Interestingly, dietary amino acids only control the endocrine Sun production/secretion.

Adapted from Delanoue R. et al. (2016) – Drosophila insulin release is triggered by adipose Stunted ligand to brain Methuselah receptor.

VI- Multiplicity of adipose factors responding to nutrients

Different adipose factors have been identified: the Upd2, CCHa2 peptide, Egr, GBPs and Sun (Agrawal et al., 2016; Delanoue et al., 2016; Koyama and Mirth, 2016; Rajan and Perrimon, 2012; Sano et al., 2015). Each of them display different peculiarities but they all link insulin activity to nutrition. Why are so many adipose factors needed in order to control insulin activity?

Discussion

Insulin signaling is required for different essential traits of life such as growth, metabolism, lifespan and reproduction (Edgar, 2006). This explains why insulin signaling has to be tightly regulated and therefore, the accurate detection of different nutrients by various FDS.

While fat-derived Upd2 and CCHa2 respond to dietary fat and/or sugars, GBPs and Sun promotes growth in an amino acids-dependent manner. Conversely, Egr is a negative regulator of insulin signaling used to decrease the growth rate and adapt larvae to protein scarcity.

Moreover, these factors differently control insulin activity. In fact, while Upd2, Sun and GBPs promote Dilps secretion from the IPCs, the ligands CCHa2 and Egr act on *dilps* transcription. According to these mode of action, we can emphasize on the rapidity of insulin activity modulation. Upd2, Sun and GBPs immediately promote IIS in response to nutrition and allow the body to quickly adapt to nutritional changes. By contrast, CCHa2 and Egr exert a long-term control on IIS and induce adaptation to sugar and/or protein scarcity.

Besides, these FDS do not share a unique neuronal target. Sun, Egr and CCHa2 act directly on the IPCs through their receptors Mth, Grnd and CCHa2R respectively. Conversely, Upd2 and GBPs need a neuronal relay to convey the nutritional information to the IPCs: Upd2 signals to its receptor Dome on GABAergic neurons while GBPs signal to EGFR on the ICNs.

For most of them, the discovery of these FDS involved in systemic growth control, has been done in larvae (Agrawal et al., 2016; Delanoue et al., 2016; Koyama and Mirth, 2016; Sano et al., 2015). Intriguingly, the interorgan communication mediated by Upd2 has been mainly studied in adults (Rajan and Perrimon, 2012). Nevertheless, some of their results were also obtained in larvae, therefore suggesting a conserved mechanism across development.

Taken together, all the FDS are essential to temporally and accurately adjust IIS activity and systemic growth according to diet composition. Indeed, the removal of only one fat-derived factors leads to body size changes. Nevertheless, the mild-starvation like phenotype due to their removal could be explained by their possible redundancy (Figure 54).

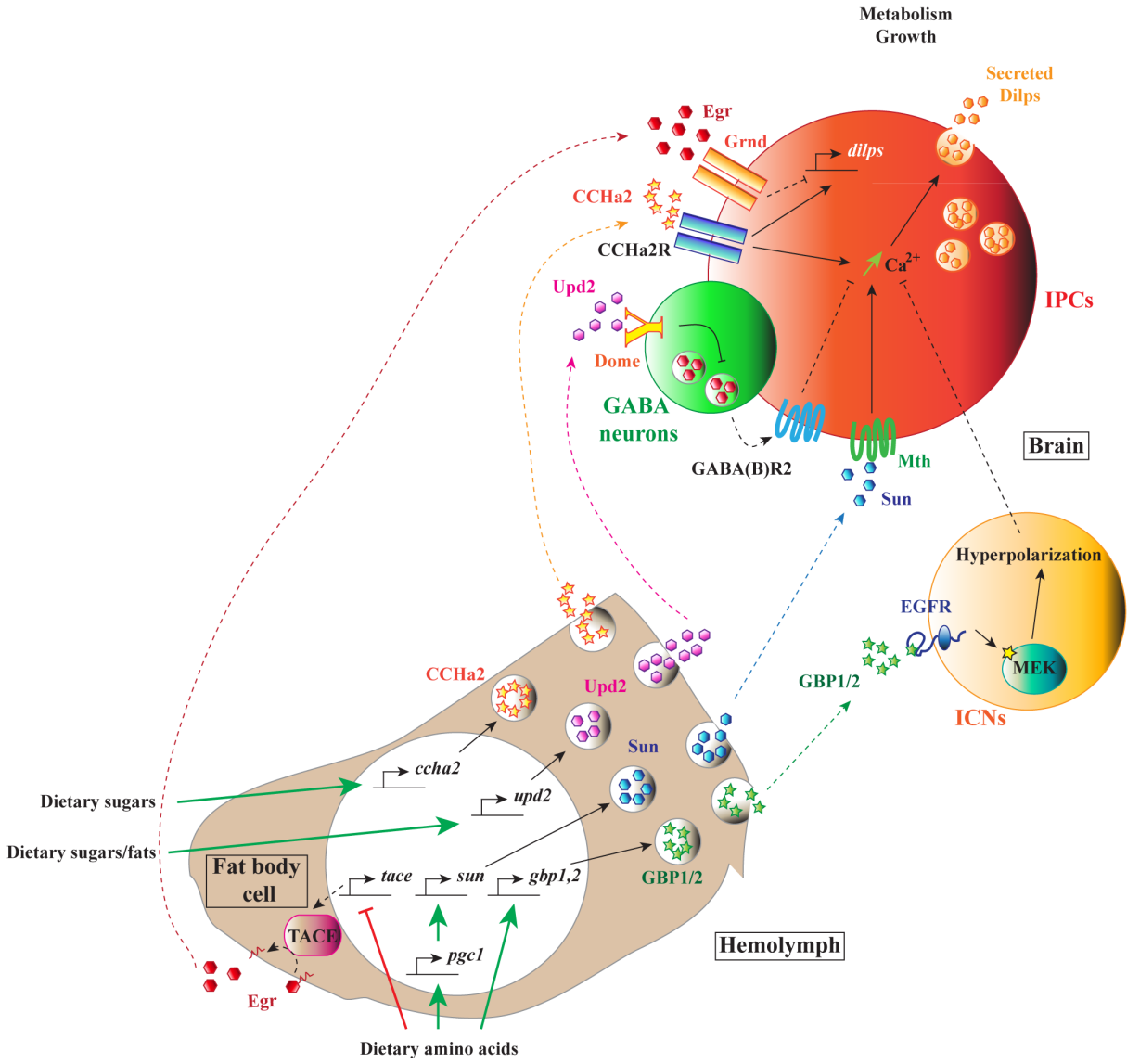


Figure 54 : Multiplicity of adipose factors controlling Dilps activity and their modes of action. Dashed lines represent actions that did not happen upon dietary nutrients. Green arrows indicate positive regulators of Dilps activity while red arrows show negative regulators of Dilps activity.

VII- Secretory mechanisms of insulinotropic adipose factors

Among the FDS, four of them are insulinotropic: Upd2, CCHa2, GBPs and Sun. Even though they all respond to dietary nutrients, they probably do not share a common secretory mechanism. Indeed, a secreted version of RFP expressed in the fat body is detected in hemolymph of both fed and starved larvae, showing that starvation does not block a general secretory machinery of fat cells but rather specific ones. Accordingly, secretion of these FDS depends on different signaling pathways (Figure 55).

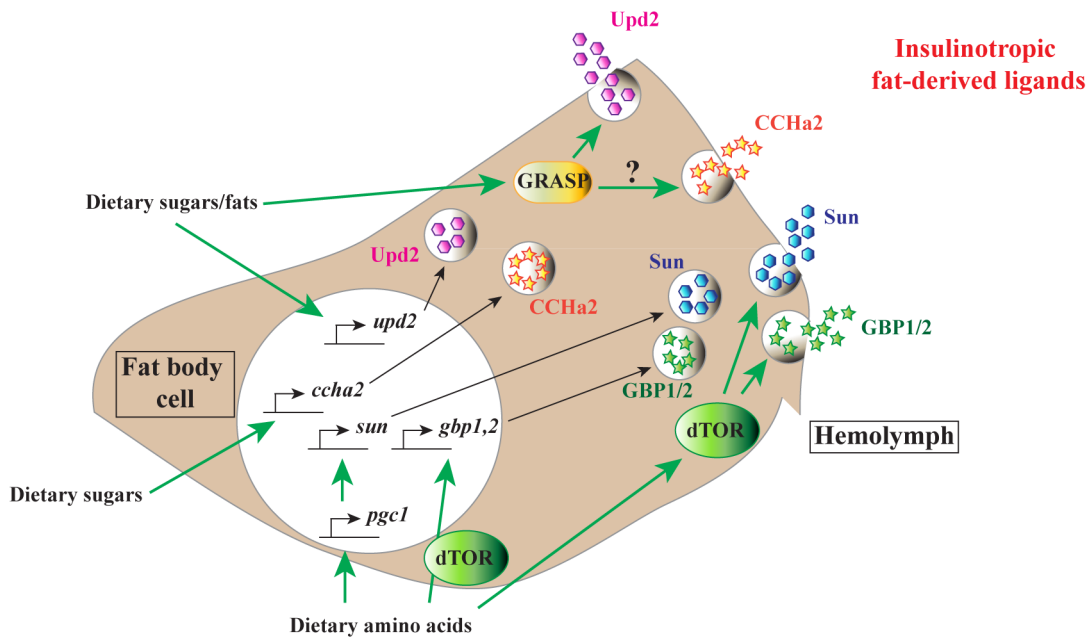


Figure 55 : Secretory mechanisms of fat-derived signals promoting Dilps activity. Green arrows represent positive regulators of Dilps activity.

upd2 and *ccha2* transcripts levels in fat cells have been shown to respond to dietary sugars and/or fat. Likewise, their secretion from fat cells is also dependent on nutrition. Upd2 is released into the hemolymph through a non-conventional protein secretion machinery: the GRASP (Rajan et al., 2017). Interestingly, the absence of dietary sugars will induce two distinct responses. In fat cells, *upd2* is no longer transcribed (Rajan and Perrimon, 2012). Concomitantly, AKH is secreted by CC cells and inhibit the GRASP machinery, therefore blocking Upd2 secretion from the fat body (Rajan et al., 2017).

Likewise, the transcriptional regulation of *ccha2* in fat cells has been described (Sano et al., 2015), but no direct experimental evidence shows that CCHa2 is secreted according to dietary sugars. Nevertheless, indirect experiments demonstrate that fat-derived CCHa2 act on brain IPCs, suggesting that CCHa2 must be secreted to ensure its endocrine function. The exact secretory mechanism by which CCHa2 is secreted by the fat body upon dietary sugars remains to be elucidated. It would be very

Discussion

interesting to test whether the non-conventional protein secretion machinery, GRASP, is specific to sugar-induced FDS and is involved in this process.

GBPs and Sun are insulinotropic fat-derived signals that respond to dietary proteins (Delanoue et al., 2016; Koyama and Mirth, 2016) (Meschi E. et al., in preparation). Both transcripts levels in fat cells are dependent on amino acids. However, while the TOR signaling pathway controls *gbp1* and *gbp2* transcription (Koyama and Mirth, 2016), *sun* transcription requires the nutrient-responsive coactivator PGC1 (Delanoue et al., 2016). Interestingly, secretion of both Sun and GBP1 requires TOR signaling pathway (Delanoue et al., 2016) (Unpublished results – Figure 45D). Yet, how GBP1 and Sun are secreted into the hemolymph remains unclear.

TOR can mediate endocytosis and requires the key regulator Hsc4, a clathrin-uncoating ATPase (Hennig et al., 2006). Besides, *Drosophila* Hsc4, is also involved in exocytosis of neurotransmitters *in vivo* (Bronk et al., 2001). It would be interesting to determine whether TOR promotes exocytosis of FDS through Hsc4.

The ectopic β chain of the F_0F_1 -ATP synthase is a lipophorin binding protein involved in lipids, lipoproteins and proteins transport (Fruttero et al., 2014; Martinez et al., 2003; Zalewska et al., 2009). Moreover, the delivery of the lipoprotein Lipid Transfer Protein (LTP) from the fat body to specific brain neurons has been shown to control insulin signaling according to dietary lipids composition (Brankatschk et al., 2014). These studies highlight the possibility that lipophorin binding proteins, such as the ectopic β chain of the F_0F_1 -ATP synthase, bind to Sun and/or GBPs and transport them to the brain.

VIII- Diversity of neuronal populations controlling the IPCs secretory activity

Several physiological functions like growth, metabolism, reproduction, lifespan, feeding behavior, sleep/wake behavior and coordination of the fat body metabolism rhythms are regulated by the IPCs neuronal activity (see Introduction-Chapter V). Indeed, across development, different neuronal populations directly connect to the IPCs and stimulate or inhibit their neuronal activity in order to achieve these physiological functions (Nässel et al., 2013). The IPCs have to couple physiological conditions with environmental cues to better modulate insulin activity. To do so, the IPCs integrate different information conveyed by several neuronal populations or peripheral-derived factors. This explains why a better knowledge of conditions and signals controlling the IPCs secretory activity need to be elucidated.

Discussion

Here, we identified two other peptidergic neuronal populations in *Drosophila* larvae that directly modulate the IPCs secretory activity: the ICNs and the EH neurons. While the ICNs inhibit the IPCs secretory activity, the EH neurons stimulate it in order to control systemic growth.

Taken together, these data strengthen the physiological relevance of the IPCs in controlling different life features. Nonetheless, due to their central localization in the brain, the IPCs might connect to unknown neuronal populations. To address this possibility, the IPCs connectome could be performed and would provide a better understanding on their function.

CONCLUSION

- GBPs secretion from the fat body into the hemolymph depends on dietary amino acids and requires TOR signaling activity
- GBPs is a long-ranged EGF ligand and act as a hormone
- ICNs are one pair of peptidergic neurons located in the PI which inhibit the IPCs secretory activity and therefore reduce systemic growth
- ICNs transduce the fat-derived GBPs signal in an EGFR- and Dsor-dependent manner
- GBPs insulinotropic effect is EGFR and Dsor dependent in the ICNs
- EGFR signaling activation reduces the calcium signaling and therefore hyperpolarize the ICNs
- This double inhibition allows to couple dietary amino acids with insulin secretion and systemic growth
- GBPs metabolic hormones and their EGF receptor in the ICNs play a fundamental function and adapt the tissue growth rate to nutritional cues

*MATERIALS AND
METHODS*

Materials and Methods is described in the manuscript. Here is an additional method related to the unpublished results.

Fly strains

The following fly strains were used: *UAS-rab5DN* (Bloomington 42704), *Chat*> (Bloomington 60317), *386y*> (Bloomington 25410), *Chat 25856 TRIP*, *mip 106076KK*, *dtk 103662KK*, *dtk 25800TRIP*, *dtkR99D 43329GD*, *amon 110788KK*, *amon 29010 TRIP* and *UAS-tsc1/2* (Tapon et al., 2001). Susan Eaton kindly provided the *UAS-RFPsec* line. The *Dilp2-LexA* strain is a kind gift of Zhefeng Gong.

Lipids staining

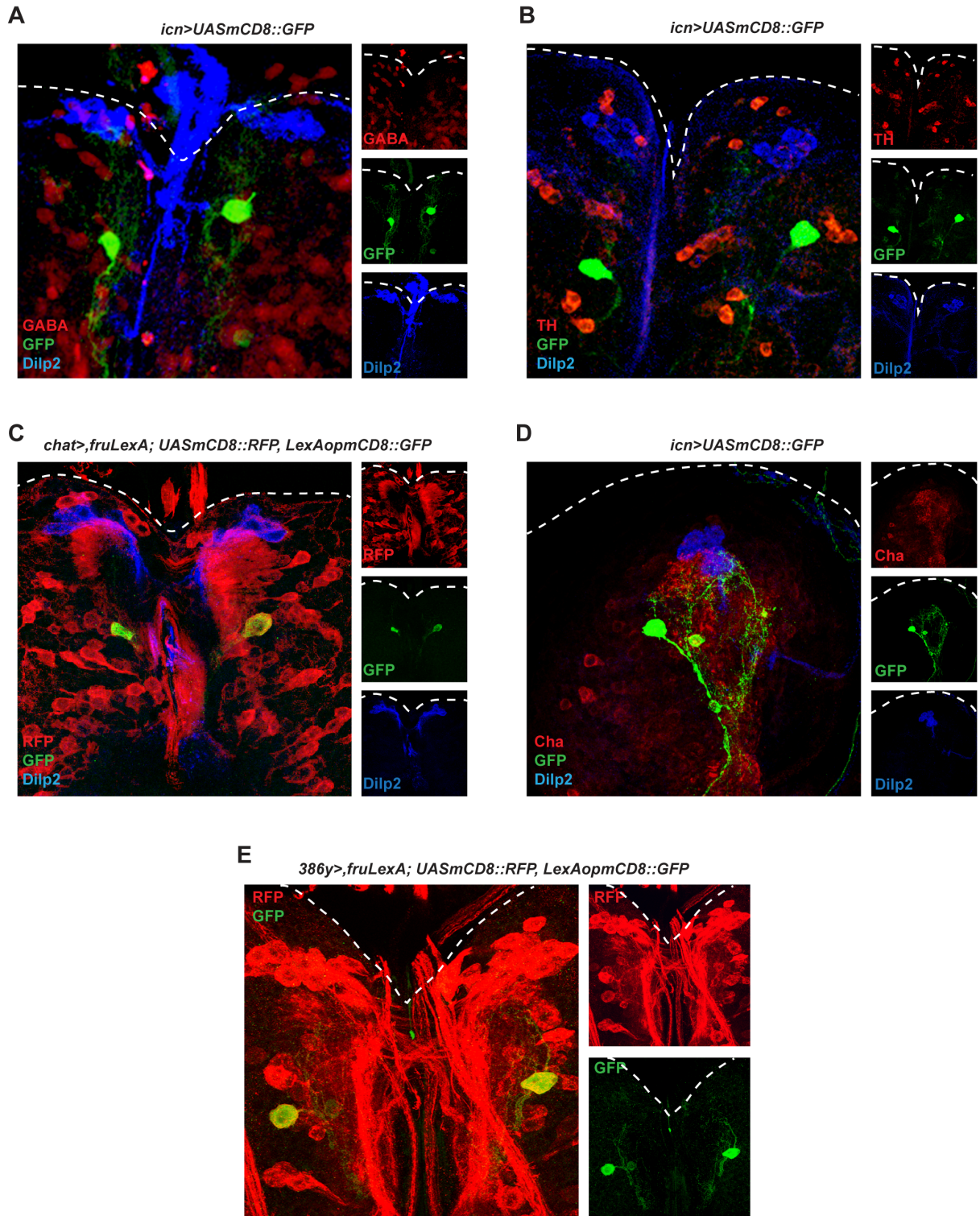
Fat body – Nile red

Fat bodies were dissected in a 0,00002% Nile red solution supplemented with 75% glycerol. Fluorescence images were immediately acquired using a Leica SP5 DS confocal microscope.

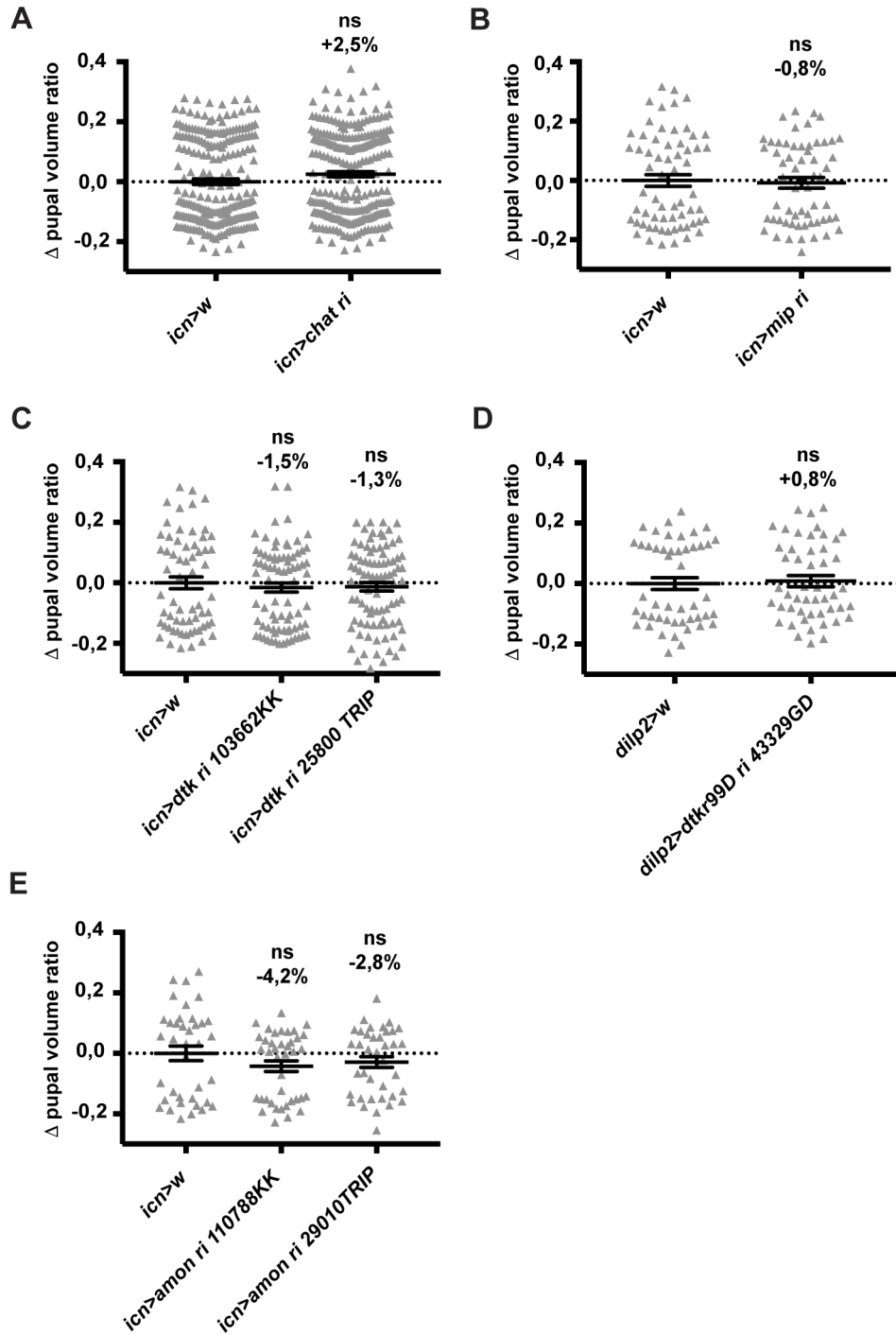
Oenocytes – OilredO

Larvae were fixed 15 minutes in paraformaldehyde 4%, rinse with water and incubated with 0,5% Oil Red O solution for 20 minutes. After several washes with water, cuticles were mounted in a PBS-Glycerol 80% solution. Pictures were acquired using a Leica Fluoresce StereomicroScope M205 FA with a Leica digital camera MC 190 HD.

ANNEX



Annex 1 : The neuronal identity of the ICNs. A. GABAergic neurons are labeled in red (GABA), the ICNs in green (GFP) and the IPCs are visualized in blue (α Dilp2). B. Aminergic neurons are labeled with the tyrosine hydroxylase (TH) antibody (red), the ICNs with the GFP (green) and the IPCs are visualized with the α Dilp2 (blue). C. *ChaT*> is expressed in cholinergic neurons (red), the ICNs are labeled with the GFP (green) and the IPCs with α Dilp2 (blue). D. Cholinergic neurons are labeled with an α Cha (red), the ICNs with the GFP (green) and the IPCs with α Dilp2 (blue). E. *386y*> is expressed in peptidergic neurons (red) and the ICNs are visualized with the GFP (green).



Annex 2: How the ICNs and the IPCs communicate? A. Pupal volume measurement after acetylcholine transferase silencing in the ICNs (*icn>chat ri*). (n>237) B. Pupal volume measurement after *mip* downregulation in the ICNs (*icn>mip ri*). (n>58) C. Pupal volume measurement after tachykinin knockdown in the ICNs (*icn>dtk ri*). (n>62) D. Pupal volume measurement after tachykinin receptor silencing in the IPCs (*dilp2>dtkr99D ri*). (n>48) E. Pupal volume measurement after amontillado knockdown in the ICNs (*icn>amon ri*). (n>38)

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