IP$_3$R mediated Ca$^{2+}$ release regulates protein metabolism in *Drosophila* neuroendocrine cells: implications for development under nutrient stress

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**Summary statement:** This paper reports that intracellular Ca$^{2+}$ signaling regulates protein translation. This regulation can compensate for insulin signaling in specialized neuro-hormonal cells, and enables *Drosophila* larval to pupal development under acute starvation.
Abstract: Successful completion of animal development is fundamentally reliant on nutritional cues. Adaptations for surviving nutritional loss are coordinated in part by neural circuits. As neuropeptides secreted by neuroendocrine (NE) cells critically modulate neural circuits, we investigated NE cell function during development under nutrient stress. Starved *Drosophila* larvae exhibited reduced pupariation, if either insulin signaling or IP$_3$/Ca$^{2+}$ signaling, were down-regulated in NE cells. Moreover, an IP$_3$R (Inositol 1,4,5-trisphosphate receptor) loss-of-function mutant displayed reduced protein synthesis, which was rescued by over-expression of either InR (insulin receptor) or IP$_3$R in NE cells of the mutant, suggesting that the two signaling pathways may be functionally compensatory. Furthermore, cultured IP$_3$R mutant NE cells, but not neurons, exhibited reduced protein translation. Thus cell-specific regulation of protein synthesis by IP$_3$R in NE cells influences protein metabolism. We propose that this regulation helps developing animals survive poor nutritional conditions.
Introduction

Nutritional poverty during development has long-lasting effects on growth and behavior of an animal. Although under-nutrition causes overall body size to decrease, the brain grows to near-normal size, a process termed “Brain Sparing” (Dobbing and Sands, 1971). This suggests unique mechanisms in neuronal tissues to weather nutritional stress. *Drosophila* is an attractive model system to uncover these mechanisms because larvae subjected to nutrient restriction exhibit ‘Brain Sparing’ (Cheng et al., 2011) and nutritional effects on the larval to pupal development are easily monitored. Additionally, growth signaling pathways activated by dietary cues such as insulin receptor (InR) and TOR signaling, are conserved in *Drosophila* (Padmanabha and Baker, 2014).

When starved, larval neural stem cells (NSCs) continue to proliferate by using an InR orthologue, Alk (Anaplastic lymphoma kinase) (Cheng et al., 2011). This study focuses on neuroendocrine (NE) cells which unlike NSCs, are differentiated and non-dividing. Importantly, neuropeptides released by NE cells modulate neural circuits that regulate processes associated with animal physiology and behavior (Nassel and Winther, 2010; Taghert and Nitabach, 2012), eventually influencing how animals adapt to external or internal stimuli. Critically, NE cells produce peptide hormones that regulate feeding behaviour and metabolism (Nassel and Winther, 2010), processes required for larvae to complete development successfully.

The IP$\_3$R is an ER channel that releases store-Ca$^{2+}$, and is downstream of G-protein coupled receptor activation. The ER-resident protein STIM (STromal Interaction Molecule) conveys loss of store-Ca$^{2+}$ to Orai, a plasma membrane Ca$^{2+}$ channel thereby enabling store-operated Ca$^{2+}$ entry (SOCE) from the extracellular milieu. SOCE occurs in both mammals (Prakriya and Lewis, 2015) and flies (Agrawal et al., 2010; Venkiteswaran and Hasan, 2009). Thus, all three molecules - IP$\_3$R, STIM and Orai – function during stimulus-dependent elevation of cytosolic Ca$^{2+}$ that potentiates diverse signaling outcomes, depending on cellular context.

Loss of IP$\_3$R (Subramanian et al., 2013b) and dSTIM (Baumbach et al., 2014) leads to obesity in adult *Drosophila*. Importantly, adults of a hypomorphic IP$\_3$R mutant heteroallelic combination, *itpr$^{ka1091/ug3}$* (hereafter: *itpr* $^{ku}$) exhibit obesity, starvation resistance and hyperphagia, which are all rescued by over-expression of
IP$_3$R in NE cells (Subramanian et al., 2013a). This adult metabolic phenotype prompted an investigation on a role for IP$_3$R and InR in NE cells, during larval development.

**Results and Discussion**

**Down-regulation of InR or IP$_3$R mediated intracellular Ca$^{2+}$ signaling in NE cells reduces pupariation under starvation**

In *Drosophila*, a large subset of NE cells express the transcription factor *dimm* (Park et al., 2008). We down-regulated InR, TOR and intracellular Ca$^{2+}$ signaling pathways in *dimm* NE cells using the UAS-GAL4 system (Brand and Perrimon, 1993), and monitored pupariation of larvae on a sucrose-only diet from 88h AEL (After Egg Laying; Fig. 1A), a time-point used previously (Cheng et al., 2011).

Less than 25% of larvae pupariated on sucrose either after InR knockdown or over-expression of a negative regulator of InR signaling, PTEN (Fig. 1B). Manipulation of InR/TOR signaling components by over-expression of dominant-negative versions (TOR$^{TED}$, S6K$^{KQ}$) or RNAi (Akt1, Rheb) affected pupariation mildly or not at all (Fig. 1C). Unlike NSCs (Cheng et al., 2011), neither over-expression of dominant-negative Alk (Alk$^{DN}$) nor reduction via Alk RNAi, in NE cells, affected larval development, regardless of diet (Fig. 1B, C). Perhaps because NE cells are differentiated, they employ another mechanism to maintain insulin signaling during starvation. InR/TOR signaling affects NE cell size (Luo et al., 2013) and interestingly, pupariation rate on sucrose correlates with this study. For e.g., InR knock-down resulted in a NE cell size reduction of ~18% (Luo et al., 2013) and gave a strong phenotype in our assay, whereas reduction of Rheb or Alk, which does not change NE cell size, gave no phenotype in our assay (Fig. 1B). Robust pupariation on normal food for the above genetic manipulations (Fig. 1C) suggested that dietary nutrients compensate for reduced InR/TOR signaling in NE cells. Together, these observations underscore the importance of NE cell function to overcome nutrient stress.

Reducing intracellular Ca$^{2+}$ signaling in NE cells by knockdown of either IP$_3$R or dSTIM, or over-expression of a dominant negative form of Orai (Orai$^{E180A}$) (Pathak et al., 2015), reduced pupariation on the sucrose-only diet (Fig. 1D) but not on normal food (Fig. 1E). The similarities in outcome upon down-regulation of either
InR/TOR signaling or intracellular Ca$^{2+}$ signaling prompted testing of genetic interactions amongst components of the two pathways. Over-expressing IP$_3$R in NE cells with InR knockdown led to increased pupariation on sucrose, when compared to InR reduction alone (Fig. 1B), suggesting that under nutrient stress, IP$_3$R can compensate for InR. Next, we investigated the IP$_3$R mutant, itpr$^{ku}$.

**IP$_3$R mutant larvae are deficient in protein metabolism**

While itpr$^{ku}$ exhibited robust pupariation on normal food (Fig. S2A), its pupariation was sensitive to reduction in yeast, the major source of dietary protein in ‘normal food’ (Fig. 2A). Pupariation was also reduced on sucrose (Fig. S2A), and rescued by supplementation with amino acids (Jayakumar et al., 2016) or amino acids and vitamins, but not lipids or vitamins alone (Fig. S2A).

At 88h, equal proportion of 2$^{nd}$ (2L) and 3$^{rd}$ (3L) instar itpr$^{ku}$ larvae co-exist (Fig. S2B), suggesting pleiotropic development delay. Additionally, when pupariation rate of 88h 3Ls was monitored, itpr$^{ku}$ displayed a lag of ~24h (Fig. 2B). Surprisingly, longer development time did not result in greater pupal volume (Fig. 2B, S2C), typically seen when larvae spend more time feeding (McBrayer et al., 2007). While weight of 3L itpr$^{ku}$ at 88h, 112h and as wandering larvae were not different from control (Fig. S2D), protein and TAG levels were different (Fig. S2E, F). At 88h and 112h, itpr$^{ku}$ had higher TAG levels and lower protein levels. In wandering 3L these levels were near normal (Fig. S2E, F). When plotted as protein/TAG ratio (Fig. 2C) it appeared that itpr$^{ku}$ had a slower rate of protein assimilation. Increased developmental time on normal food by itpr$^{ku}$ may therefore be a strategy to accumulate sufficient protein, and also explain why it doesn’t result in increased body size.

Abnormal protein/TAG ratios suggested perturbed insulin signaling in itpr$^{ku}$. We therefore measured transcript levels of *Drosophila* insulin-like peptides (dILPs) 2, 3, 5 and 6 from larval brains on normal food (Fig. 2D, S3A). Except dILP5, which varied temporally to a significant degree (Fig. 2D), the trend for other dILPs (Fig. S2F) was similar to control. Although produced in the same set of NE cells (IPCs: insulin-producing cells), dILPs 2, 3 and 5 transcripts are independently regulated. dILP2 is considered to be a systemic response, whereas dILP3 is regulated by sugar (Kim and Neufeld, 2015), and dILP5 by protein concentration (Geminard et al., 2009;
Okamoto and Nishimura, 2015). Selective variation of dILP5 thus indicated dysfunctional protein sensing in itpr<sup>ku</sup>. Over-expression of dILP2 results in larger adults (Sato-Miyata et al., 2014); in contrast, size of itpr<sup>ku</sup> pupae (Fig. S2C) are similar to controls, suggesting that the small increase of dILP2 at 112h (Fig. S2F) may be a response to dILP5 up-regulation.

We next down-regulated IP<sub>3</sub>R in various cells/organs known to coordinate metabolism and development (Fig. S3B). Expectedly, IP<sub>3</sub>R knockdown in the prothoracic gland (PG) decreased pupariation on sucrose (Fig. S3B) because IP<sub>3</sub>R is required for ecdysone release from the PG (Venkatesh and Hasan, 1997; Yamanaka et al., 2015). However, unlike itpr<sup>ku</sup> or larvae with reduced IP<sub>3</sub>R in either NE cells or all neurons (Fig. S3B), supplementation with amino acids did not improve viability. This suggested that IP<sub>3</sub>R functions differently in PG cells and neurons. It is also likely that PG function in itpr<sup>ku</sup> is not as compromised as it is in PG-IP<sub>3</sub>R-knockdown condition, as Ca<sup>2+</sup> release in itpr<sup>ku</sup> neurons is reduced but not abolished (Joshi et al., 2004; Srikanth et al., 2004; Venkiteswaran and Hasan, 2009). Notably, reduction of IP<sub>3</sub>R in the fat body, or oenocytes (Fig. S3B), other sites of metabolic regulation in Drosophila, had no effect on larval development on sucrose.

IP<sub>3</sub>R reduction in NE cells also resulted in larvae with lowered protein/TAG ratio (Fig. 2E) and elevated dILP5 expression (Fig. 2F). These features were similar to, but reduced in magnitude, from itpr<sup>ku</sup> (Fig. 2C,D), suggesting the contribution of non-NE cells to itpr<sup>ku</sup> phenotype. Indeed, a set of glutamatergic neurons have been identified where over-expression of IP<sub>3</sub>R is sufficient to rescue lethality of itpr<sup>ku</sup> on sucrose (Jayakumar et al., 2016).

Because itpr<sup>ku</sup> displayed abnormal transcript levels of dILP5 and 2, loss of IP<sub>3</sub>R specifically in the IPCs was tested, and found not to affect pupariation on sucrose (Fig. S3B). This is consistent with previous observations where IP<sub>3</sub>R knockdown in IPCs does not phenocopy IP<sub>3</sub>R mutant phenotypes (Agrawal et al., 2009; Subramanian et al., 2013a). Together, these suggest that IP<sub>3</sub>R, in the IPCs, does not affect dILPs directly. Increases in dILP5 and dILP2 transcripts in itpr<sup>ku</sup> (Fig 2D, S3A) may instead be diet-dependent compensatory systemic responses. This is supported by the modest rescue of itpr<sup>ku</sup> pupariation on sucrose, with either over-expression of dILP2 in IPCs (Jayakumar et al., 2016) or dILP5 in NE cells (Fig. S3C). Over-expression of dILP5 in itpr<sup>ku</sup> IPCs caused embryonic lethality.
Increasing InR/TOR or intracellular Ca$^{2+}$ signaling in NE cells restores protein synthesis levels of the IP$_3$R mutant

Next, we tested the effect of up-regulation of InR and intracellular Ca$^{2+}$ signaling components in NE cells of itpr$^{ku}$. Over-expression of positive regulators of either InR (InR, PI3K$^{CaaX}$, Akt1) or TOR (S6K, Rheb) pathway rescued itpr$^{ku}$ development under nutritional stress (Fig. 2G, Fig. S3D). These manipulations increase growth by promoting ribosomal biogenesis (Grewal, 2009) and in NE cells, increase their size (Luo et al., 2013). Restoring intracellular Ca$^{2+}$ signaling by over-expression of wild-type IP$_3$R or dSTIM, as well as over-expression of CamKII, a kinase that propagates Ca$^{2+}$ signaling, in NE cells of itpr$^{ku}$, also rescued larval lethality on sucrose (Fig. 2G; Fig. S3D).

At the systemic level, over-expression of either InR or IP$_3$R in NE cells (dimm>lnr/ip3r, itpr$^{ku}$) was sufficient to increase protein/TAG ratios of itpr$^{ku}$ (Fig. 2H) at 112h, to levels similar to wandering stage itpr$^{ku}$ on normal food (Fig 2C) suggesting that both pathways ultimately affected systemic protein metabolism. Protein/TAG ratios are compared to dimm>lnr/ip3r as non-linear increases in weight, protein and TAG levels, were observed in these genotypes (Fig. S2D-F). Of note are TAG levels in dimmGAL4;itpr$^{ku}$ control and dimm>lnr/ip3r itpr$^{ku}$ rescues (Fig. S2E). In both rescue conditions, protein levels increase (Fig. S2F), whereas TAG levels remain high (like dimmGAL4;itpr$^{ku}$). Thus insufficient protein, and not higher TAGs correlates with the pupariation defect of itpr$^{ku}$ on sucrose.

As over-expression of dILP5 rescued itpr$^{ku}$ partially and itpr$^{ku}$ displayed up-regulated dILP5 at 112h on normal food, we asked if dimm>lnr/ip3r rescues involved dILP5. Nutrient withdrawal typically reduces dILP 2,3, and 5 transcript levels significantly (Ikeya et al., 2002) and 88h CS larvae tested for levels of these dILPs after 24 hours on sucrose showed expected reductions (Fig. S3E). Interestingly, itpr$^{ku}$ displayed greater reduction in dILP5 (Fig. 2I) but not dILP2 (Fig. S3F), when tested 24 hours after transfer to sucrose. This reduction in dILP5 likely affects itpr$^{ku}$ because at this time point (112h) on normal food, it requires a ~3 fold up-regulation of dILP5 (Fig. 2D). On sucrose, over-expression of InR or IP$_3$R in NE cells increases dILP5 levels in itpr$^{ku}$ (Fig. 2I) to control levels, without affecting dILP2 levels (Fig. S3F). This suggests dimm>lnr/ip3r rescues itpr$^{ku}$ in part by systemically up-regulating dILP5. Similar to dILP5, over-expression of either InR or Rheb in IPCs of
itpr
ku resulted in embryonic lethality, suggesting that insulin signaling in itpr
ku is complicated.

**IP₃R positively regulates protein translation in NE cells**

As systemic protein levels in itpr
ku were rescued by over-expression of IP₃R in NE cells, a cellular role for IP₃R in protein translation was investigated. In itpr
ku NE cells, obtained by culturing larval CNS, protein translation was reduced by ~50%; similar to NE cells treated with the protein synthesis inhibitor, cycloheximide (Fig. 3A, B). This was rescued by over-expression of IP₃R (Fig. 3A, B), strengthening the idea that IP₃R, like InR, has a positive effect on protein synthesis.

This observation is opposite to that reported for mammalian cell cultures (Brostrom and Brostrom, 2003), suggesting novel regulation of protein synthesis in neuropeptidergic cells. Reduced protein synthesis observed in mammalian cells treated with vasopression, angiotensin II and cholecystokinin (Brostrom et al., 1986; Kimball and Jefferson, 1990), agents that mobilise IP₃R mediated ER Ca²⁺ stores, can be rescued by addition of extracellular 2mM Ca²⁺ during stimulation (Brostrom et al., 1986; Kimball and Jefferson, 1990; Sans et al., 2002). This suggests that extracellular Ca²⁺ entry counteracts ER-store Ca²⁺ effects on protein synthesis. Interestingly, when IP₃R function is compromised in neurons, extracellular Ca²⁺ entry via SOCE is diminished (Venkiteswaran and Hasan, 2009). Thus, it is possible that a signaling cascade connects SOCE to protein translation, via IP₃R.

Unlike NE cells, the rate of protein translation in itpr
ku neurons was found to be no different from control neurons (Fig. S4A, B), suggesting IP₃R compensation of InR signaling to be cell-specific. Consistent with this, InR over-expression in cholinergic neurons of itpr
ku did not rescue its viability on sucrose (Fig. S4C).

Peptide release from a subset of NE cells is regulated by IP₃R-mediated Ca²⁺ transients from a subset of glutamatergic neurons (Jayakumar et al., 2016). Our results show that IP₃R mediated Ca²⁺ release also regulates protein translation in NE cells. Together these illustrate the plurality of cellular processes controlled by IP₃/Ca²⁺ signaling in the context of nutrient stress.

In summary, IP₃R mediated Ca²⁺ signaling helps maintain normal protein translation levels in NE cells, and this activity promotes systemic protein metabolism, during larval development. On a nutrient-rich diet, loss of IP₃R signaling is not
detrimental, because dietary cues maintain insulin/TOR signaling, and thereby keep protein levels normal for completing development. Under starvation, dietary cues are lost. IP₃/Ca²⁺ signaling possibly provides a nutrient-independent mechanism, to maintain protein synthesis in cells essential to survive nutrient stress, such as NE cells where increased levels of cell surface receptors or neuropeptides may be required for modulating relevant neural circuits. As yet, there are no receptors or neuropeptides reported to be up-regulated upon starvation in dimmt NE cells, but there is precedence to suggest their existence. For e.g., in starved Drosophila, the receptor for short Neuropeptide F is up-regulated in the antenna (Root et al., 2011) and in starved mammals, levels of Agouti-related peptide, that affects appetite and feeding, are increased (Henry et al., 2015). A recent screen identified IP₃/Ca²⁺-coupled neuropeptide receptors, on glutamatergic neurons, required for larval adaptation to nutrient stress (Jayakumar et al., 2016). Neuropeptides from NE cells that couple to such receptors may function during starvation in our model (Fig S5).

By focusing on animal development, this study integrates cellular observations to organismal phenotype. Therefore, it sets the framework to discover mechanistic details of how stimulus-coupled increases in cytosolic Ca²⁺ can regulate protein synthesis in a cell specific manner, and how that consequently regulates protein metabolism in the whole animal.
Materials and methods

Fly husbandry and stocks
Flies were reared on “normal” laboratory food (1L recipe: 80g corn flour, 20g Glucose, 40g Sugar, 15g Yeast Extract, 4mL propionic acid, p-hydroxybenzoic acid methyl ester in ethanol 5mL, 5mL ortho butyric acid) in 12h/12h L/D incubator at 25°C. Fly strains are listed in Supplemental Information.

Larval nutritional stress assay
Eggs were collected on normal laboratory food for 6-8 hours (depending on cross fecundity; ~100 eggs per bottle) and allowed to mature for 88h. For each genotype 6 batches of 25 3rd Instar larvae of similar size were transferred to a fresh vial of normal food or 100mM Sucrose in 1% agar. Pupae were scored 10 days after transfer. For development time, pupariation was scored every 24 hrs.

Pupal volume measurement
Pupal volume was approximated by measuring the width and height from pupal pictures, and applying volumetric formula for cylinders $\pi r^2 h$.

Weight, Protein and TAG measurements on whole larvae
30 larvae were weighed on normal laboratory food for 6-8 hours (depending on cross fecundity; ~100 eggs per bottle) and allowed to mature for 88h. For each genotype 6 batches of 25 3rd Instar larvae of similar size were transferred to a fresh vial of normal food or 100mM Sucrose in 1% agar. Pupae were scored 10 days after transfer. For development time, pupariation was scored every 24 hrs.

RT-PCR
RNA was isolated from 10-12 larval brains at specified time points. cDNA synthesis was carried out as described (Pathak et al., 2015). All mRNA levels are reported as fold change normalized to rp49. Primers are listed in Supplementary Information.

in vivo protein translation assay
Neuronal cultures from late 3rd instar larval brains were prepared as described (Deb et al., 2016). 16-18h old cultures were processed for in vivo protein synthesis labeling using manufacturer’s protocol provided with the Click-iT® Plus OPP Protein Synthesis Assay Kit (C10458). Confocal fluorescence images were collected on Olympus FV1000 at 60X with 0.5um z stacks. 10-15 cells were imaged per dish and
at least 3 independent dishes were cultured for each genotype. Identical confocal settings were used for all imaging. Total fluorescence in each channel for the entire stack was measured using ImageJ and background in each channel for each individual cell was subtracted for the measured ROI.
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Competing interests

No competing interests declared.

Author Contributions

M performed the experiments. M and GH designed experiments, analyzed results and wrote the manuscript.

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References

Agrawal, N., Padmanabhan, N. and Hasan, G. (2009). Inositol 1,4,5- trisphosphate receptor function in Drosophila insulin producing cells. PLoS One 4, e6652.

Agrawal, N., Venkiteswaran, G., Sadaf, S., Padmanabhan, N., Banerjee, S. and Hasan, G. (2010). Inositol 1,4,5-trisphosphate receptor and dSTIM function in Drosophila insulin-producing neurons regulates systemic intracellular calcium homeostasis and flight. J Neurosci 30, 1301–1313.

Baumbach, J., Hummel, P., Bickmeyer, I., Kowalczyk, K. M. M., Frank, M., Knorr, K., Hildebrandt, A., Riedel, D., Jäckle, H., Kühnlein, R. P., et al. (2014). A Drosophila in vivo screen identifies store-operated calcium entry as a key regulator of adiposity. Cell Metab 19, 331–343.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.

Brostrom, M. A. and Brostrom, C. O. (2003). Calcium dynamics and endoplasmic reticular function in the regulation of protein synthesis: implications for cell growth and adaptability. Cell Calcium 34, 345–363.

Brostrom, C. O., Bocckino, S. B., Brostrom, M. A. and Galuska, E. M. (1986). Regulation of protein synthesis in isolated hepatocytes by calcium-mobilizing hormones. Mol. Pharmacol. 29, 104–111.

Cheng, L. Y., Bailey, A. P., Leevers, S. J., Ragan, T. J., Driscoll, P. C. and Gould, A. P. (2011). Anaplastic lymphoma kinase spares organ growth during nutrient restriction in Drosophila. Cell 146, 435–447.

Deb, B. K., Pathak, T. and Hasan, G. (2016). Store-independent modulation of Ca2+ entry through Orai by Septin 7. Nat Commun 7,.

Dobbing, J. and Sands, J. (1971). Vulnerability of developing brain. IX. The effect of nutritional growth retardation on the timing of the brain growth-spurt. Biol. Neonate 19, 363–378.

Geminard, C., Rulifson, E. J. and Leopold, P. (2009). Remote control of insulin secretion by fat cells in Drosophila. Cell Metab 10, 199–207.
Grewal, S. S. (2009). Insulin/TOR signaling in growth and homeostasis: A view from the fly world. *Int. J. Biochem. Cell Biol.* **41**, 1006–1010.

Henry, F. E., Sugino, K., Tozer, A., Branco, T. and Sternson, S. M. (2015). Cell type-specific transcriptomics of hypothalamic energy-sensing neuron responses to weight-loss. *Elife* **4**, e09800.

Ikeya, T., Galic, M., Belawat, P., Nairz, K. and Hafen, E. (2002). Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in Drosophila. *Curr Biol* **12**, 1293–1300.

Jayakumar, S., Richhariya, S., Reddy, O. V., Texada, M. J. M., Hasan, G., Richhariya, S., Reddy, V. O., Texada, M. J. M. and Hasan, G. (2016). Drosophila larval to pupal switch under nutrient stress requires IP3R/Ca2+ signalling in glutamatergic interneurons. *Elife* 2016;5:e17.

Joshi, R., Venkatesh, K., Srinivas, R., Nair, S. and Hasan, G. (2004). Genetic dissection of itpr gene function reveals a vital requirement in aminergic cells of Drosophila larvae. *Genetics* **166**, 225–236.

Kim, J. and Neufeld, T. P. (2015). Dietary sugar promotes systemic TOR activation in Drosophila through AKH-dependent selective secretion of Dilp3. *Nat Commun* **6**, 6846.

Kimball, S. R. and Jefferson, L. S. (1990). Mechanism of the inhibition of protein synthesis by vasopressin in rat liver. *J. Biol. Chem.* **265**, 16794–16798.

Luo, J., Liu, Y., Nassel, D. R. and Nässel, D. R. (2013). Insulin/IGF-regulated size scaling of neuroendocrine cells expressing the bHLH transcription factor Dimmed in Drosophila. *PLoS Genet* **9**, e1004052.

McBrayer, Z., Ono, H., Shimell, M., Parvy, J. P., Beckstead, R. B., Warren, J. T., Thummel, C. S., Dauphin-Villemant, C., Gilbert, L. I. and O’Connor, M. B. (2007). Prothoracicotropic Hormone Regulates Developmental Timing and Body Size in Drosophila. *Dev. Cell* **13**, 857–871.

Nassel, D. R. and Winther, A. M. (2010). Drosophila neuropeptides in regulation of physiology and behavior. *Prog Neurobiol* **92**, 42–104.

Okamoto, N. and Nishimura, T. (2015). Signaling from Glia and Cholinergic Neurons Controls Nutrient-Dependent Production of an Insulin-like Peptide for Drosophila Body Growth. *Dev. Cell* **35**, 295–310.
Padmanabha, D. and Baker, K. D. (2014). Drosophila gains traction as a repurposed tool to investigate metabolism. *Trends Endocrinol. Metab.* **25**, 518–527.

Park, D., Veenstra, J. A., Park, J. H. and Taghert, P. H. (2008). Mapping peptidergic cells in Drosophila: where DIMM fits in. *PLoS One* **3**, e1896.

Pathak, T., Agrawal, T., Richhariya, S., Sadaf, S. and Hasan, G. (2015). Store-Operated Calcium Entry through Orai Is Required for Transcriptional Maturation of the Flight Circuit in Drosophila. *J. Neurosci.* **35**, 13784–13799.

Prakriya, M. and Lewis, R. S. (2015). Store-Operated Calcium Channels. *Physiol. Rev.* **95**, 1383–1436.

Root, C. M., Ko, K. I., Jafari, A. and Wang, J. W. (2011). Presynaptic Facilitation by Neuropeptide Signaling Mediates Odor-Driven Food Search. *Cell* **145**, 133–144.

Sans, M. D., Kimball, S. R. and Williams, J. A. (2002). Effect of CCK and intracellular calcium to regulate eIF2B and protein synthesis in rat pancreatic acinar cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **282**, G267-76.

Sato-Miyata, Y., Muramatsu, K., Funakoshi, M., Tsuda, M. and Aigaki, T. (2014). Overexpression of dilp2 causes nutrient-dependent semi-lethality in Drosophila. *Front. Physiol.* **5 APR**.

Srikanth, S., Wang, Z., Tu, H., Nair, S., Mathew, M. K., Hasan, G. and Bezprozvanny, I. (2004). Functional properties of the Drosophila melanogaster inositol 1,4,5-trisphosphate receptor mutants. *Biophys J* **86**, 3634–3646.

Subramanian, M., Jayakumar, S., Richhariya, S. and Hasan, G. (2013a). Loss of IP3 receptor function in neuropeptide secreting neurons leads to obesity in adult Drosophila. *BMC Neurosci* **14**, 157.

Subramanian, M., Metya, S. K., Sadaf, S., Kumar, S., Schwudke, D. and Hasan, G. (2013b). Altered lipid homeostasis in Drosophila InsP3 receptor mutants leads to obesity and hyperphagia. *Dis. Model. Mech.* **6**, 734–44.

Taghert, P. H. and Nitabach, M. N. (2012). Peptide neuromodulation in invertebrate model systems. *Neuron* **76**, 82–97.

Venkatesh, K. and Hasan, G. (1997). Disruption of the IP3 receptor gene of Drosophila affects larval metamorphosis and ecdysone release. *Curr Biol* **7**, 500–509.
Venkiteswaran, G. and Hasan, G. (2009). Intracellular Ca2+ signaling and store-operated Ca2+ entry are required in Drosophila neurons for flight. Proc Natl Acad Sci U S A 106, 10326–10331.

Yamanaka, N., Marques, G. and O'Connor, M. B. (2015). Vesicle-Mediated Steroid Hormone Secretion in Drosophila melanogaster. Cell 163, 907–919.
Fig. 1: Down-regulation of InR, TOR and intracellular Ca²⁺ signaling pathways in NE cells impairs larval development on sucrose. (A) Assay schematic (B,C) Pupariation upon reducing InR/ TOR signaling in *dimmGAL4* cells on sucrose (B) or normal diet (C). (D, E) % Pupariation with reduced intracellular Ca²⁺ signaling on sucrose (D) or normal diet (E). Bars with the same alphabet represent statistically indistinguishable groups (one-way ANOVA with a post hoc Tukey’s test p<0.05). n = 6 batches of 25 larvae each. Data represents mean ± SEM.
Fig. 2. Dysregulated protein metabolism in the IP₃R hypomorph, itprₖᵤ can be rescued by over-expression of either lnR or IP₃R in NE cells. (A) % pupariation of 65h larvae transferred into media with varying amounts of yeast. CS - Canton S. Ordinary 2 way ANOVA. **** p<0.0001 (B) pupariation time post-88h transfer on normal food. Inset: Representative pupa; relative pupal volume in Fig. S2C. (C,E) Temporal changes in Protein/ TAG ratio normalized to weight, for different
genotypes. n≥5. See also Fig. S2D-F. (D,F) dlLP5 transcript levels in larval CNS normalized to rp49. n=6. (G) % Pupariation of itpr1ku on sucrose diet upon over-expression of positive regulators of InR and TOR signaling (orange and green) or intracellular Ca\(^{2+}\) signaling (purple) in NE cells. See also Fig. S3D. (H) Protein/TAG ratios normalized to weight. See also Fig. S2D-F. n≥8. (I) dlLP5 transcript levels in larval CNS normalized to rp49. n=4. Statistics: (C), (D), and (F) Unpaired t test: * p <0.05; ** p <0.01; **** p<0.0001. (E), (G), (H), (I): Ordinary one-way ANOVA. Bars with the same alphabet represent statistically indistinguishable groups (one-way ANOVA with a post-hoc Tukey’s test p<0.05). Data represents mean ± SEM.
Fig. 3 NE cells from itpr<sup>ku</sup> display reduced protein synthesis. (A) Representative confocal images of NE cells (dimm<sup>+</sup>, GFP positive) in culture from the indicated genotypes and conditions. Newly synthesized peptides (F<sub>647</sub>) and nuclear volume (F<sub>405</sub>) were measured. Cells were treated with 10μM cycloheximide (CHX) for 30mins. Scale bar 2μm (B) Quantification of F<sub>647</sub> and F<sub>405</sub> from confocal images. n≥ 40. Ordinary one-way ANOVA. Post hoc Holms-Sidak. **p <0.01; *** p <0.001; **** p<0.0001 Data represents mean ± SEM.
Fig. S1A-D. Pupation rate for controls. UAS lines used in Fig 1 were crossed to CS (+) and larvae were scored for pupation on normal or sucrose-only media. n=6 batches of 25 each.
% pupation of itprku larvae on normal food or 100mM sucrose with 1mM Total Brain Lipids (+ Lipids), 1X Grace’s insect media (+ AAs & Vits) or 1X RPMI vitamin solution (+ Vits). n = 6 batches of 25 larvae, except + Vits where n = 3 batches of 25 larvae. Bars with the same alphabet represent statistically indistinguishable groups (2way ANOVA with Sidak’s multiple comparisons p<0.05).

% of 3rd instar larvae recovered at 88h AEL. Note that in itprku there were only two populations of larvae: 2nd or 3rd instar. n ≥ 4 batches of > 50 larvae each. Unpaired t-test **** p<0.0001.

Relative pupal volume. n=40. Unpaired t-test was ns.

Weight, TAGs and protein levels of larvae. These values were used to compute protein/TAG ratios for Fig. 2D, 2E and 2H. For CS vs itprku measurements were made across three time points (n = 4 for 88h and WL; n = 8 for 112h). Note that the time of wandering larvae for CS and itprku are not the same as itprku delays pupation (Fig. 2B). For the rest of the genotypes, measurements were performed at 112h AEL (n≥8). Bars with the same alphabet represent statistically indistinguishable groups (one-way ANOVA with post hoc Tukey’s test p<0.05).
**Fig. S3 (A)** Transcript levels of various *dilps* in larval brains normalized to *rp49*. Unpaired *t* test was ns for each time point except *ilp2* at 112AEL * p<0.05 (B) % pupae formed on normal, sucrose-only or AAs & Vits (1X Grace’s insect media) by larvae with RNAi mediated knockdown of IP₃R in various tissues and organs as indicated. Except for *dimm-GAL4*, all knockdowns were in the presence of *UAS-dicer*. IP₃R RNAi control: *UAS-IP₃R RNAi/+;UAS-dicer/+*. n=6 batches of 25 larvae, except for OK72-GAL4 where n=4 batches of 25 larvae (C) % pupae formed on normal or sucrose-only media, by larvae with over-expression of *dILP5* in NE cells of *itpr⁹⁸*. (D) % pupae formed on sucrose-only media by UAS controls of various over-expressed molecules, in the background of *itpr⁹⁸*. Note that *UAS-S6K* was leaky, as it rescued *itpr⁹⁸* in the absence of the driver *dimm-GAL4*. n=6 batches of 25 larvae (E) *dILP 2,3* and *5* transcript in 88h AEL CS larvae starved for 24hours on sucrose-only media. n=3. Unpaired t-test **p<0.01, ****p<0.0001** (F) Relative transcript levels of *dILP2* relative to *rp49*, in 88h AEL 3rd instar larvae starved for 24h on sucrose-only media. n=4. Fold change normalized to *rp49*. Bars with the same alphabet represent statistically indistinguishable groups (one-way ANOVA with post hoc Tukey’s test p<0.05).
Fig. S4 (A) Representative confocal z-projection stack of primary neuronal cells in culture, treated with *in vivo* protein synthesis detection kit. Scale bar 5 μm (B) Quantification of F<sub>647</sub> and F<sub>405</sub> from confocal images. n ≥ 80 Unpaired t test. (C) % pupation when 88h AEL *itpr<sup>ku</sup>* over-expressing InR in cholinergic neurons were transferred to normal or sucrose-only media.
Figure S5

Proposed Model in NE cells
Supplemental Experimental Procedure

Fly strains

Canton S (CS) was used as the wild type control. The following strains were obtained from Bloomington Stock Centre: UAS-InRRNAi (#31594), UAS-AktRNAi (#33615), UAS-TOR^{TED} (#7013), UAS-S6K^{KQ} (#6911), UAS-Alk RNAi (#27518), UAS-Rheb RNAi (#33966), UAS-InR (#8262), UAS-Rheb (#9689), UAS-Akt1(#8191), UAS-S6K (#6911), Elav^{155}-GAL4 (#458), Isp2-GAL4 (#27451), ok72GAL4 (#6486), UAS CAMKII (#29662), UAS dicer (#24648), The following were from Vienna Drosophila Research Centre stock collection: UAS-itpr RNAi (1063), UAS-STIM RNAi (47073)
The following were kind gifts: phm:gfp-GAL4 (Michael O’Connor), dimm^{929}-GAL4 (Paul H Taghert), UAS-dmycp110^{CaaX} (Ernst Hafen), UAS-eGFP (Michael Roshbash), UAS-Alk^{DN} (Manfred Frasch), UAS-PTEN (Bruce Edgar), Cha-GAL4 (Toshihiro Kitamoto)
The following were generated in our laboratory: itpr^{ka1091}, itpr^{ug3}, UAS-Oral^{E180A}, UAS-itpr^{+}, UAS-Stim

RT-PCR primers

dilp2
5’ CCATGAGCAAGCCTTTGTCC 3’
5’ TTCACTGCAGAGCGTTCCTTG3’
dilp3
5’ ACTCGACGTCTTCGGGATG3’
5’ CGAGGTTTACGTTCGGCT3’
dilp5
5’ ACTCACTGAGCATTCGG3’
5’ GAGTCGCAGTATGCCCTCAA3’
dilp6
5’ TGGTTCTCAAGTGGCGAC3’
5’ GAAATACATCGCCAAGGGC3’