ER-Ca\(^{2+}\) sensor STIM regulates neuropeptides required for development under nutrient restriction in \textit{Drosophila}

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Abstract

Neuroendocrine cells communicate via neuropeptides to regulate behaviour and physiology. This study examines how STIM (Stromal Interacting Molecule), an ER-Ca\(^{2+}\) sensor required for Store-operated Ca\(^{2+}\) entry, regulates neuropeptides required for \textit{Drosophila} development under nutrient restriction (NR). We find two STIM-regulated peptides, Corazonin and short Neuropeptide F, to be required for NR larvae to complete development. Further, a set of secretory DLP (Dorso lateral peptidergic) neurons which co-express both peptides was identified. Partial loss of \textit{dSTIM} caused peptide accumulation in the DLPs, and reduced systemic Corazonin signalling. Upon NR, larval development correlated with increased peptide levels in the DLPs, which failed to occur when \textit{dSTIM} was reduced. Comparison of systemic and cellular phenotypes associated with reduced \textit{dSTIM}, with other cellular perturbations, along with genetic rescue experiments, suggested that \textit{dSTIM} primarily compromises neuroendocrine function by interfering with neuropeptide release. Under chronic stimulation, \textit{dSTIM} also appears to regulate neuropeptide synthesis.

Introduction

Metazoan cells commonly use ionic Ca\(^{2+}\) as a second messenger in signal transduction pathways. To do so, levels of cytosolic Ca\(^{2+}\) are dynamically managed. In the resting state, cytosolic Ca\(^{2+}\) concentration is kept low and maintained thus by the active sequestration of Ca\(^{2+}\) into various organelles, the largest of which is the ER. Upon activation, ligand-activated Ca\(^{2+}\) channels on the ER, such as the ryanodine receptor or inositol 1,4,5-trisphosphate receptor (IP\(_3\)R), release ER-store Ca\(^{2+}\) into the cytosol. Loss of ER-Ca\(^{2+}\) causes STromal Interacting Molecule (STIM), an ER-resident transmembrane protein, to dimerize and undergo structural rearrangements. This facilitates the binding of STIM to Orai, a Ca\(^{2+}\) channel on the plasma membrane, whose pore now opens to allow Ca\(^{2+}\) from the extracellular milieu to flow into the cytosol. This type of capacitative Ca\(^{2+}\) entry is called Store-operated Ca\(^{2+}\) entry (SOCE) [1]. Of note, key components of SOCE include the IP\(_3\)R, STIM and Orai, that are ubiquitously...
expressed in the animal kingdom, underscoring the importance of SOCE to cellular functioning. Depending on cell type and context, SOCE can regulate an array of cellular processes [2].

Neuronal function in particular is fundamentally reliant on the elevation of cytosolic Ca\(^{2+}\). By tuning the frequency and amplitude of cytosolic Ca\(^{2+}\) signals that are generated, distinct stimuli can make the same neuron produce outcomes of different strengths [3]. The source of the Ca\(^{2+}\) influx itself contributes to such modulation as it can either be from internal ER-stores or from the external milieu, through various activity-dependent voltage gated Ca\(^{2+}\) channels (VGCCs) and receptor-activated Ca\(^{2+}\) channels or a combination of the two. Although the contributions of internal ER-Ca\(^{2+}\) stores to neuronal Ca\(^{2+}\) dynamics are well recognized, the study of how STIM and subsequently, SOCE mediated by it, influences neuronal functioning, is as yet a nascent field.

Mammals have two isoforms of STIM, STIM1 and STIM2, both which are widely expressed in the brain. As mammalian neurons also express multiple isoforms of Orai and IP\(_3\)R, it follows that STIM-mediated SOCE might occur in them. Support for this comes from studies in mice, where STIM1-mediated SOCE has been reported for cerebellar granule neurons [4] and isolated Purkinje neurons [5], while STIM2-mediated SOCE has been shown in cortical [6] and hippocampal neurons [7]. STIM can also have SOCE-independent roles in excitable cells, that are in contrast to its role via SOCE. In rat cortical neurons [8] and vascular smooth muscle cells [9], Ca\(^{2+}\) release from ER-stores prompts the translocation of STIM1 to ER-plasma membrane junctions, and binding to the L-type VGCC, Ca\(_V\)1.2. Here STIM1 inhibits Ca\(_V\)1.2 directly and causes it to be internalized, reducing the long-term excitability of these cells. In cardiomyocyte-derived HL1 cells, STIM1 binds to a T-type VGCC, Ca\(_V\)1.3, to manage Ca\(^{2+}\) oscillations during contractions [10]. These studies indicate that STIM regulates cytosolic Ca\(^{2+}\) dynamics in excitable cells, including neurons and that an array of other proteins determines if STIM regulation results in activation or inhibition of neurons. Despite knowledge of the expression of STIM1 and STIM2 in the hypothalamus (Human Protein Atlas), the major neuroendocrine centre in vertebrates, studies on STIM in neuroendocrine cells are scarce. We therefore used Drosophila melanogaster (Drosophila), the vinegar fly, to address this gap.

Neuroendocrine cells possess elaborate machinery for the production, processing and secretion of neuropeptides (NPs), which perhaps form the largest group of evolutionarily conserved signalling agents [11,12]. Inside the brain, NPs typically modulate neuronal activity and consequently, circuits; when released systemically, they act as hormones. Drosophila is typical in having a vast repertoire of NPs that together play a role in almost every aspect of its behaviour and physiology [13,14]. Consequently, NP synthesis and release are highly regulated processes. As elevation in cytosolic Ca\(^{2+}\) is required for NP release, a contribution for STIM-mediated SOCE to NE function was hypothesized.

Drosophila possess a single gene for STIM, IP\(_3\)R and Orai, and all three interact to regulate SOCE in Drosophila neurons [15,16]. In dopaminergic neurons, dSTIM is important for flight circuit maturation [15–17], with dSTIM-mediated SOCE regulating expression of a number of genes, includingRal, which controls neuronal vesicle exocytosis [17]. In glutamatergic neurons, dSTIM is required for development under nutritional stress and its’ loss results in down-regulation of several ion channel genes which ultimately control neuronal excitability [18]. Further, dSTIM over-expression in insulin-producing NE neurons could restore Ca\(^{2+}\) homeostasis in a non-autonomous manner in other neurons of an IP\(_3\)R mutant [19], indicating an important role for dSTIM in NE cell output, as well as compensatory interplay between IP\(_3\)R and dSTIM. At a cellular level, partial loss of dSTIM impairs SOCE in Drosophila neurons [15,17,20] as well as mammalian neural precursor cells [21]. Additionally, reducing dSTIM in Drosophila dopaminergic neurons attenuates KCl-evoked depolarisation and as well as vesicle release [17]. Because loss of dSTIM specifically in dimm\(^+\) NE cells results in a pupariation
defect on nutrient restricted (NR) media [22], we used the NR paradigm as a physiologically relevant context in which to investigate STIM’s role in NE cells from the cellular as well as systemic perspective.

Material and methods

Fly husbandry

Flies were grown at 25˚C in 12h:12h L:D cycle. Normal 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) For nutritional stress assay, flies were allowed to lay eggs for 6 hours on normal food. After 88 hours, larvae were collected and transferred to either normal or NR (100mM Sucrose) food. Pupae and adults were scored after 10 days of observation.

Fly strains

Canton S was used as the wild type (+) control.

The following strains were obtained from Bloomington Drosophila Stock Centre: AKH-GAL4 (25684), Crz-GAL4 (51976), DSK-GAL4 (51981), sNPF-GAL4 (51981), UAS-CrzR525999 (25999), UAS-dicer2 (24651), UAS-TeTxLC (28837), UAS-TeTxLC-IMP (28838), UAS-CaMPAR1 (58761), UAS-GFP (4776), UAS-mRFP (32218), UAS-TrpA1 (26263), UAS-InsR (8248), UAS-amonIR (28583), UAS-RalDN (32094).

The following strains were obtained from Drosophila Genetic Resource Center, Kyoto: sNPF-GAL4 (113901).

The following were obtained from Vienna Drosophila Research Centre stock collection: UAS-IP2IR (106982), UAS-STIMIR (47073), UAS-CrzR (30670), UAS-InsRIR (999).

The following was from Exelixis at Harvard Medical School: sNPF (c00448).

The following were kind gifts: AstA1-GAL4 (David Anderson), dILP2-GAL4 (Eric Rulifson), hug-GAL4 (Michael Pankratz), NPF-GAL4 (Ping Shen), UAS-sNPRIR (Kweon Yu), Crz::mCherry (Gábor Juhasz), UAS-hid::UAS-rpr (Tina Mukherjee), UAS-Shibire (Toshihiro Kaitamoto), ts-h-GAL80 (Julie Simpson), UAS-prepANF::GFP (Edwin Levitan), UAS-ChR2XXL (Robert Kittel and Georg Nagel).

The following were previously generated in our laboratory: itprka1091, itprug3, UAS-OraiE180A, UAS-iptp, UAS-Stim, UAS-RalWT.

Larval feeding

3rd instar larvae (10 total) were collected and placed on cotton wool soaked with solution of 4.5% dissolved yeast granules and 0.5% Erioglaucine (Sigma, 861146). Controls contained no dye. Feeding was allowed for 2 hours at 25˚C. 5 larvae per tube were crushed in 100μL of double distilled water. Solution was spun at 14000 rpm for 15 minutes and 50μL was withdrawn for absorbance measurement at 625nm in a 96-well plate. 5μL was used to measure protein content using the Pierce BCA Protein Assay kit (#23227).

qRT-PCR

RNA was isolated from 12–15 larval brains at the specified time points using Trizol. cDNA synthesis was carried out as described [23]. All mRNA levels are reported as fold change normalized to rp49. Primer sequences:

- rp49, F:CGGATCCTGATATCGCTAGCTG, R:GCCGTTGTTGATCCGTA.
- Crz, F:CCCTTTAAGCCGCGCATCTCC, R:CTTTGGACCGTCCTAGACA.
- CrzR, F:CTGTGCACTCCTGGGCGAC, R:GGCCTTGTATCAGCCTCT.
Measuring neuronal activation using CaMPARI

Early third instar larvae were transferred to either normal or NR food. After 24 hours, larvae were recovered and immobilized on double sided tape. UV light from a Hg-arc lamp was focused using the UV filter, on the larvae through a 10X objective on Olympus BX60, for 2 minutes. Larvae were then immediately dissected in ice-cold PBS, mounted in PBS and imaged using Olympus FV-3000 Confocal microscope using a 40X objective and high-sensitivity detectors. Microscope settings for laser intensity, PMT settings and magnification were kept identical for all measurements. Each experiment always had a no UV control, in which larvae were subject to immobilisation but not UV light. Fluorescence intensity was calculated for each cell body using Image J.

Immunofluorescent staining

For expression patterns, 3rd instar larval brains with RGs attached were dissected in ice-cold PBS and fixed in 3.7% formaldehyde at 4°C for 20mins. The samples were washed 4 times in PBS and mounted in 60% glycerol. Endogenous fluorescence was acquired on Olympus FV-3000 using a 20X, 40X or 60X objective, and processed used ImageJ. For samples requiring antibody staining brains were similarly processed and then subjected to permeabilisation (0.3% Triton X-100 + PBS; PBSTx) for 15 mins, 4 hr blocking in 5% normal goat serum in PBSTx at 4°C, followed by overnight incubation in primary antibody (1:1000 Chicken-GFP, Abcam: ab13970) and secondary with Alexa 488 or Alexa 594 (1:400; Abcam). For corazonin (1:1000; raised in Rabbit; Jan Veenstra, University of Bordeaux), all the above steps remained the step, except that dissected brains were fixed for 1hr at RT in 4% PFA and the secondary was anti-rabbit Alexa 405 (1:300, Abcam). Cell bodies were outlined manually and integrated density was used to calculate CTCF (Corrected Total Cell Fluorescence). For all samples, a similar area was measured for background fluorescence.

Direct peptide-profiling by MALDI-TOF MS

Ring glands were dissected in cold HL3.1 and transferred to a MALDI plate as previously described [24]. 0.2 μl of matrix (saturated solution of recrystallized α-cyano-4-hydroxycinnamic acid in MeOH/EtOH/water 30/30/40% v/v/v) was added, containing 10 nM of stable isotope-labeled HUG-pyrokinin (HUG-PK⁺ (Ser–Val[d8]–Pro–Phe–Lys–Pro–Arg–Leu–amide, Mw = 950.1 Da; Biosyntan, Berlin, Germany)) and 10 nM labeled myosuppressin (MS⁺ (Thr–Asp–Val[d8]–Asp–His–Val–Phe–Leu–Arg–Phe–amide, Mw = 1255.4 Da; Biosyntan) MALDI-TOF mass spectra were acquired in positive ion mode on a 4800 Plus MALDI TOF/TOF analyzer (MDS Sciex, Framingham, MA, USA) in a mass range of 900–4000 Da and fixed laser intensity with 20 subspectra and 1000 shots per sample. Data were analyzed with Data Explorer 4.10. Spectra were baseline corrected and de-isotoped. The sum of the resulting relative intensities of the de-isotoped peaks was calculated for the different ion adducts (H⁺, Na⁺, K⁺) of each peptide as well as the labeled peptides⁺. Then, the ratios sNPF/HUG-PK⁺ and corazonin/MS⁺ were calculated, using the labeled peptide with the most similar molecular weight. For sNPF, all isoforms (1/2-short, 1-long, -3 and -4) variants were totaled.

Optogenetic and thermogenetic experiments

For thermogenetic (dTrpA1, Shibire⁺) experiments, larvae were matured to 88 hours AEL at 25°C. After transfer to either NR or normal food, vials were placed at 22°C, 25°C or 30°C for either 24 hours (dTrpA1) or till the end of observation time (Shibire⁺). For optogenetic experiments (Chr2-XXL), larvae were matured to 88AEL in the dark. After transfer to either NR or
normal food, one set was placed in the dark while another was placed in an incubator with regular white lights that were on continuously till the end of observation time.

Results and discussion

SOCE is required in sNPF and Crz producing cells for development under nutritional stress

Collectively, more than 20 different NPs are known to be made by the neuroendocrine cells in which reducing SOCE components resulted in poor pupariation upon NR [22]. To shortlist specific NPs important for this paradigm, we undertook a curated GAL4-UAS screen. NP-GAL4s were used to drive the knockdown of IP$_3$R (IP$_3$R$^{IR}$) [25], and pupariation of the resulting larvae were scored on normal vs NR media (S1A Fig). On normal food, a significant reduction of pupariation was seen only with sNPF-GAL4 (S1A Fig), whose expression strongly correlates with neurons producing sNPF [26]. Upon NR, the largest effect was seen with sNPF-GAL4, followed by small but significant pupariation defect with AstA-GAL4 and DSK-GAL4 (S1A Fig). Neurons that secrete NPs may also secrete neurotransmitters, therefore, a role specifically for sNPF was tested. Reducing the level of sNPF (sNPF$^{IR}$) or reducing an enzyme required for neuropeptide processing (amontillado; amon$^{IR}$) [27] in sNPF-GAL4 expressing cells, as well as a hypomorphic sNPF mutation (sNPF$^{00448}$) resulted in impairment of larval development upon NR (S1B Fig). These data indicate that sNPF is required for pupariating under NR conditions.

sNPF-GAL4 expresses in large number of neurons (>300) in the larval brain [26] (S1C Fig), and also expresses in the larval midgut and epidermis. To further refine sNPF$^+$ neurons on which we can perform cellular investigations, we tested a Crz-GAL4 driver. This driver expresses in fewer neurons (~22), all of which express the neuropeptide Corazonin (Crz). Importantly, a small subset of these, three bilateral neurons in the brain lobe, make Crz and sNPF. [26] (Fig 1A). Reducing SOCE in Crz neurons, by reducing either IP$_3$R or STIM (dSTIM$^{IR}$) [15,16] or over-expressing a dominant-negative version of Orai (Orai$^{E180A}$) [23], resulted in reduced pupariation on NR (Fig 1B). The absence of a developmental defect on normal food suggests that SOCE in these neurons is primarily required to survive NR.

To test if both sNPF and Crz were required, they were specifically reduced (sNPF$^{IR}$; Crz$^{IR}$) in Crz neurons. Knockdown of either NP resulted in larvae with a pupariation defect on NR media but not, on normal food (Fig 1C; S1D Fig). In Drosophila neurons, enhancing the expression of SOCE regulators leads to increased SOCE [16]. To test the positive effect of SOCE on Crz and sNPF, a genetic compensation experiment was carried out. The SOCE-regulators, IP$_3$R or dSTIM were over-expressed in Crz neurons which also expressed reduced levels of either sNPF or Crz. NR larvae with this genetic make-up showed a significant improvement in pupariation on NR media, as compared to NR larvae with only reduced NPs (Fig 1C). Interestingly, the compensation was sufficient to also increase the number of adults that emerged (Fig 1D). Notably, over-expression of either of the two SOCE molecules, dSTIM and IP$_3$R on their own, did not affect pupariation on either normal or NR media (Fig 1C), but unlike on normal food (S1E Fig), did reduce development to adulthood on NR media (Fig 1D). These data underscore the sensitivity of Crz neurons to ER-Ca$^{2+}$ homeostasis during NR.

Loss of IP$_3$R and sNPF has previously been shown to affect larval feeding [28–30]. Hence, larval intake of dye-colored food in a 2-hour span was measured. Age-synchronized larvae with knockdown of either dSTIM, IP$_3$R, Crz or sNPF in Crz neurons exhibited no difference in the amount of dye ingested (S1F Fig), suggesting that developmental defects in the NR assay do not arise from a fundamental feeding problem.
STIM regulates neuropeptides during Drosophila development

A

\[ crz>mRFP, GFP^{nis} \]

mRFP  GFP\(^{nis} \)  Merge

B

\[ \begin{array}{ccc}
\text{Normal} & \text{NR} \\
\begin{array}{ccc}
\text{a} & \text{a} & \text{a} \\
\text{a} & \text{b} & \text{c} \\
\text{a} & \text{b} & \text{c} \\
\end{array}
\end{array} \]

\[ \begin{array}{c}
\% \text{Pupae} \\
\begin{array}{c}
1x \\
\text{IP3R}^{R} \\
\text{dSTIM}^{O/E} \\
\text{Orb} \text{E19A} \\
\end{array}
\end{array} \]

C

\[ \begin{array}{ccc}
\text{Normal} & \text{NR} & \text{dSTIM}^{O/E} \\
\text{a} & \text{a} & \text{a} \\
\text{b} & \text{b} & \text{b} \\
\end{array} \]

\[ \begin{array}{ccc}
\text{IP3R}^{O/E} \\
\text{NR} & \text{NR} & \text{NR} \\
\text{a} & \text{a} & \text{a} \\
\text{a} & \text{a} & \text{a} \\
\end{array} \]

D

\[ \begin{array}{ccc}
\text{a} & \text{a} & \text{a} \\
\text{a} & \text{b} & \text{b} \\
\text{c} & \text{c} & \text{c} \\
\text{ac} & \text{c} & \text{c} \\
\end{array} \]

\[ \begin{array}{c}
\% \text{Adults} \\
\begin{array}{c}
\text{crz}^{R} \\
\text{SNP}^{R} \\
\text{SNP}^{R} \\
\text{crz}^{R} \\
\end{array}
\end{array} \]

\[ \begin{array}{c}
\begin{array}{c}
\text{crz}^{R} \\
\text{SNP}^{R} \\
\text{SNP}^{R} \\
\text{crz}^{R} \\
\end{array}
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\[ \begin{array}{c}
\begin{array}{c}
\text{crz}^{R} \\
\text{SNP}^{R} \\
\text{SNP}^{R} \\
\text{crz}^{R} \\
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\[ \begin{array}{c}
\begin{array}{c}
\text{crz}^{R} \\
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\text{SNP}^{R} \\
\text{crz}^{R} \\
\end{array}
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Altogether, these genetic experiments helped identify a set of NP expressing neurons, the Crz neurons, a subset of which also express sNPF, in which SOCE plays an important role during development on NR.

Crz$^+$ and sNPF$^+$ DLP neurons majorly contribute to development on NR, and are activated by NR

In the larval CNS, Crz is expressed in 3 pairs of DLPs (Dorso Lateral Peptidergic neurons) in the pars lateralis region of the brain lobes, 1 pair of neurons in dorso-medial region and 8 pairs of interneurons in the VG (ventral ganglion) [31]. Other than the dorso-medial neurons, the Crz-GAL4 used in this study recapitulates the known expression pattern for Crz. (S2A and S2B Fig; Cartoon: Fig 2A). Additionally, adjacent to DLPs, low expression of Crz-GAL4 was observed in 3–4 neurons that do not express Crz (S2A and S2C Fig). As mentioned previously, Crz$^+$ DLPs co-express sNPF [26]. In terms of neuronal architecture, the DLP neurons have two major branches: the anterior branch culminates in a dense nest of neurites at the ring gland (RG), while the posterior branch terminates in the subesophageal zone (SEZ). The VG neurons form a network amongst themselves to ultimately give rise to two parallel bundles that travel anteriorly, and end in the brain lobes. To visualize the overall distribution of NPs in the Crz neurons, we ectopically expressed a rat neuropeptide coupled to GFP (ANF::GFP), a popular tool used to track NP transport and release in Drosophila [32] (S2D Fig). Firstly, within the DLPs, like Crz::mcherry (S2A Fig), ANF::GFP was either in the cell bodies or RG projections, but not in the projections terminating at the subesophageal zone (S2D Fig), suggesting selective NP transport to the RG, which is a major neurohaemal site for systemic release of neuropeptides. Secondly, ANF::GFP intensity was higher in the cell bodies of the DLPs than VG neurons (S2D Fig).

The close proximity of the terminal projections of the Crz$^+$ VG neurons and the anterior branch of the Crz$^+$ DLP neurons in the brain lobe suggested possible neuromodulation between the two sets of neurons. Therefore, we undertook experiments to distinguish the contribution of DLPs vs VG localized Crz neurons, to the development in NR media. First, we utilized tshGAL80 to restrict Crz-GAL4 expression to the DLPs (S2E Fig). The level of pupariation under NR conditions observed with restricted expression of dSTIM$^{IR}$ (Fig 2B; Mean: 51% ±4.2) was similar to that seen with full expression (Fig 1B; Mean: 40.7%±13.3), suggesting a major contribution of the DLP neurons to the NR phenotype. Furthermore, sNPF$^{IR}$ larvae have levels of pupariation of NR larvae (S2F Fig; Mean: 30.9%±7.8) similar to Crz$^{IR}$ NR larvae (Fig 1C; Mean: 33.8%±5.9). Because sNPF-GAL4 marks only the Crz$^+$ DLP neurons and not the Crz$^+$ VG neurons (S1C Fig), this too suggests a major role for the Crz$^+$ DLP neurons.

Requirement of SOCE in Crz neurons for pupariation on NR (Fig 1C) suggested that these neurons experience elevated cytosolic Ca$^{2+}$ in NR conditions and are therefore, stimulated by
chronic starvation. To test this, the UV light-activated genetically encoded calcium sensor, CaMPARI [33], was utilised. The sensor fluoresces in the GFP range (F$_{488}$) and is converted irreversibly to fluoresce in the RFP range (F$_{561}$), when exposed to UV light and in the presence of Ca$^{2+}$. The level of conversion positively titrates with Ca$^{2+}$ concentrations. Larva expressing CaMPARI in Crz$^+$ neurons were placed in either normal or NR media for 24 hours (24h NR). Whole larvae were immobilized, and exposed to UV light for 2mins. Control larva were subject to the same treatment but, without being exposed to UV light (Fig 2C and 2D; no UV). Detection of F$_{561}$ in the fed state suggests that these neurons are active even under normal food conditions (Fig 2C and 2D). Notably, after 24 hours on NR media, chronic starvation caused a ~2-fold increase in average levels F$_{561}$ and therefore, of neuronal activation (Fig 2D). F$_{561}$/F$_{488}$ ratios did not appear to change in the VG neurons (S2G and S2H Fig). While there is a possibility that VG neurons do not exhibit higher F$_{561}$ because of insufficient penetration of UV light, the CaMPARI results together with the genetic experiments (Fig 2B, S2F Fig), formed the basis for selecting the DLP neurons for further analysis on how dSTIM affects Crz and sNPF.

dSTIM regulates NP synthesis and release in Crz neurons

Crz peptide levels were measured in DLP neurons by staining larval brains with an antiserum raised against the mature Crz peptide sequence [34]. Two locations on the DLP neurons were chosen for measurement: neuronal cell body/soma and neurite projections on the RG. In control DLP neurons, 24 hrs of NR caused average levels of Crz levels to increase, in both locations (Fig 3A, S3A and S3B Fig). In comparison, DLP neurons expressing dSTIM$^{IR}$ displayed increased Crz peptide levels on normal food itself, and this remained unaltered upon NR, for both locations (Fig 3A, S3B and S3C Fig). sNPF levels could not be similarly measured by immunofluorescence because sNPF is expressed in many neurons close to the DLPs (S1C Fig), making measurements specifically from the DLP soma difficult to quantify. Instead, semi-quantitative, direct, mass spectrometric profiling of dissected RGs was employed. This technique can measure peptide levels relative to stable isotopic standards at single neurohaemal release sites [35]. As Crz levels between the cell bodies and projections correlated, and Crz$^+$ DLPs are the sole contributors of sNPF on the RG [26], this technique allowed us to infer sNPF levels in DLPs. In controls, 24hrs of NR, increased the average level of sNPF ~5-fold on the RG (Fig 3C). In comparison, RG preparations from larvae where DLPs express dSTIM$^{IR}$, displayed increased sNPF levels on normal food itself, and this remained unaltered upon NR (Fig 3C). Although Crz was detected in the RG preparations, it was of much lower intensity. Average Crz levels increased with NR in the control, and in dSTIM$^{IR}$ condition, but statistically higher levels of Crz were seen only in the NR, dSTIM$^{IR}$ condition (S3D Fig). Nonetheless, broad agreement in trends, between Crz using immunofluorescence and sNPF using MALDI-MS, suggest that the two peptides are similarly regulated by NR and dSTIM. This is consistent with genetic experiments which showed that over-expression of dSTIM can rescue loss of both, sNPF as well as Crz (Fig 1C and 1D).

Thus, increased activation of DLP neurons by NR (Fig 2D), appears to result in peptide accumulation. Loss of dSTIM increases peptide levels on normal food, and prevents an increase in peptide levels upon NR.

As an ER-Ca$^{2+}$ sensor, dSTIM may potentially regulate several cellular processes that would affect NPs such as their synthesis, processing, trafficking and/or release. As STIM$^{IR}$ increased peptide levels in the cell body as well as neurite projections on the RG, a major trafficking defect was unlikely (Fig 3A vs S3B and S3C Fig). This does not rule out a role for dSTIM in dense-core vesicle trafficking, but merely indicates that trafficking of Crz is not observably
Fig 2. Crz⁺ and sNPF⁺ DLPs are required for development on NR media and activated by NR. (A) Cartoon of Crz⁺ and sNPF⁺ neurons in the larval CNS marked by Crz-GAL4. DLP: dorso lateral peptidergic; VG: Ventral Ganglion; SEZ: Subesophageal zone (B) % Pupae when dSTIM is selectively down-regulated only in Crz⁺ and sNPF⁺ DLPs, by using the tsh-Gal80 transgene and in the presence of dicer2. Control: tshGal80/+;dSTIM⁺/+; Data represents mean ± SEM (C) Representative image. Expression of the UV-activated Ca²⁺ indicator, CaMPARI in Crz⁺ and sNPF⁺ DLPs, in larvae on 24 hours of normal (Fed) or NR media (24h NR). Fluorescence at 561nm (F₅₆₁) reflects Ca²⁺ levels, while at 488nm (F₄₈₈) reflects levels of the indicator CaMPARI. (D) Quantification of Ca²⁺ levels as reported by F₅₆₁/F₄₈₈ ratio in DLPs in larvae on 24 hours of normal or NR media, in the presence and absence of UV-stimulation. N=7 larvae for UV-stimulated; N=3 for No UV stimulation. Bars with the same alphabet represent statistically indistinguishable groups. Two-way ANOVA with Sidak multi comparison test p<0.05 for (B). Mann-Whitney Test for (D). See also S2 Fig and for source data, S2 Table.

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Fig 3. dSTIM regulates Crz and sNPF levels. Larvae were subjected to 24 hours of normal (fed) or nutrient restricted (NR) media. Crz levels were measured in DLP neurons by immunofluorescence on larval brains. All manipulations were performed using the Crz-GAL4 driver (A) Relative levels of Crz peptide in DLP neuron cell bodies. Control = crz>dicer2, dSTIMIR = crz>dSTIMIR,dicer2. Number of cells measured shown atop bars. N=12 brains (B) Representative images for cell bodies measured in (A). (C) Relative levels of total sNPF peptides measured on dissected ring glands (N atop bars) and quantified using MALDI-MS. Externally added heavy standard (Hug-PK) was used to normalize peptide levels between samples. (D) % Pupae on normal or NR media, upon reduced peptide processing (amonIR,dicer2) and quantified using MALDI-MS. Externally added heavy standard (Hug-PK) in Crz’ neurons. Data represents mean ± SEM (E) Relative levels of Crz upon expression of amonIR and dicer2. N=10 brains. (F) Relative levels of Crz upon indicated cellular perturbation of Crz’ neurons. N≥6 brains. control: Crz-GAL4/+; (G) Crz mRNA levels from larval brains when Crz is reduced by two different RNAi lines. N ≥ 5. (H) Corazonin receptor (CrzR) mRNA levels from larval brains with reduced Crz. N ≥ 4. (I) CrzR mRNA levels from larval brains expressing indicated cellular perturbations in Crz neurons. N≥6 (I) Crz mRNA levels from larval brains. Control = crz>dicer2, dSTIMIR = crz>dSTIMIR,dicer2 N ≥ 6. Bars with the same alphabet represent statistically indistinguishable groups. Kruskal-Wallis Test with Dunn’s multicomparison correction p<0.05 for (A), (C), (F), Mann-Whitney Test for (E). Two-way ANOVA with Sidak’s multi comparison test p<0.05 for (D), (J), One-way ANOVA with Tukey multi comparison test p<0.05 for (G), (H), (I). See also S3 Fig and for source data, S3 Table.

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We previously found that InRIR causes Crz levels to decrease upon NR. InDrosophila pacemaker neurons, Ral has been shown to bias the sensitivity of a neuropeptide receptor, the Pigment Dispensing Factor Receptor [41]. Perhaps, functions distinct from Ral’s contribution to vesicle exocytosis contribute to this observation. Nonetheless, the lack of increase in Crz levels in DLP neurons upon NR, when InRIR and RalDN are expressed, assumes significance in the...
context of pupariation of NR larvae. Control larvae subject to 24hrs of NR, display increased 
Crz (Fig 3A) and sNPF (Fig 3C) levels, and then proceed to successfully complete development 
to pupae (Fig 1C). Whereas, in dSTIMIR, InRIR and RaIDN conditions, on NR, neither do DLPs 
display increased Crz levels (Fig 3A and 3F), nor do all larvae pupariate (Figs 1B, 1C and 3D). 
Thus, an increase in peptide levels on NR correlates with larval ability to pupariate on NR. 

In context with increased neuronal activation during NR (Fig 2D), and evidence that 
functional vesicle exocytosis (Fig 3D, S3F Fig) as well as adequate peptide production (Fig 3D) 
is required for survival on NR, these data suggest that increased production and release of Crz 
and sNPF during NR, is required for NR larvae to successfully complete development. 

To prove that Crz and sNPF are released during NR, the ideal experiment would be to mea-
sure levels of secreted NPs. But small size (8–10 amino acids), low hemolymph titres and high 
complexity of hemolymph, make peptide measurements by biochemical means highly chal-
lenging in Drosophila. Moreover, NPs exhibit endocrine as well as paracrine signalling [42]; 
and the latter will not be reflected in hemolymph measurements. Fortunately, in the Crz sig-
nalling system there is feedback compensation between secreted Crz and Corazonin receptor 
(CrzR) mRNA levels, providing a means to gauge secreted Crz levels indirectly. In adults, 
expression of CrzIR in Crz neurons, increased levels of CrzR on the fat body [43]. We thus 
tested if CrzR, which in larvae appears to be expressed in the salivary glands and CNS [44], 
was subject to similar feedback. In larval brains, reducing Crz, using two different CrzIR 
strains, not only caused a reduction in Crz mRNA levels (Fig 3G), but also a concomitant increase in 
CrzR mRNA levels (Fig 3H). Conversely, reducing CrzR levels (CrzRIR) in the larval CNS, 
results in up-regulation of Crz mRNA (S3G Fig). This confirmed the existence of feedback in 
the Crz signalling system, and the use of neuronal CrzR transcript levels as a measure of 
secreted Crz levels. In line with this inference, we observed an up-regulation of CrzR mRNA in 
larval brains where Crz neurons are expressing either InRIR or amonIR or RaIDN (Fig 3I). 
Therefore, the observation that in the STIMIR condition CrzR mRNA levels are high (Fig 3I), 
supports the idea that dSTIM function is necessary for the secretion of optimal levels of Crz. 

Because dSTIM-mediated SOCE is known to induce changes in gene expression [38], we 
probed if Crz expression is sensitive to NR and dSTIM. In the control, NR did not change Crz 
mRNA levels (Fig 3I), suggesting that a post-transcriptional mechanism is responsible for 
increasing Crz peptide levels upon NR (Fig 3A, S3B and S3C Fig). In the STIMIR condition, 
Crz transcript levels were up-regulated on normal food conditions (Fed) and no further 
increase was observed upon NR (Fig 3I). The straightforward explanation for high Crz peptide 
levels in STIMIR condition could therefore be attributed to higher gene expression of Crz. 

However, data from other perturbations in Crz neurons suggested that a linear interpretation 
between Crz mRNA and peptide levels cannot not be made. When CrzIR is expressed, Crz 
mRNA is reduced (Fig 3G), but peptide levels are elevated (S3I Fig); whereas, when amonIR is 
expressed, Crz mRNA is increased (S3H Fig), but peptide levels are decreased (Fig 3E). Mean-
while, in three conditions, Crz mRNA as well as peptide levels are higher than controls: CrzRIR 
(S3G vs S3I Fig), InRIR (S3H vs S3F Fig) and RaIDN (S3H vs S3F Fig). Note also that both higher 
(dSTIMIR, InRIR, RaIDN) or lower (amonIR) Crz peptide levels in DLP cell bodies, result in 
reduced systemic Crz signalling (CrzR mRNA levels; Fig 3I). These data indicate that Crz 
transcription, translation and release are independently regulated. A simple explanation for ele-
vated levels of Crz transcript as well as peptide levels in STIMIR is therefore, feedback 
compensation. Moreover, there is no change in Crz mRNA upon 24hrs of NR, when STIMIR is 
expressed (Fig 3I). Together, this argues against a direct role for dSTIM in regulating Crz gene 
expression. 

In summation, these data have been inferred as follows: on normal food, partial loss of 
dSTIM reduces systemic Crz signalling, indicating a requirement for dSTIM in Crz secretion.
On NR media, Crz+ DLPs are stimulated to increase peptide synthesis and release, in order for NR larvae to complete development. Peptide up-regulation upon NR is abrogated when dSTIM is reduced. These add up to suggest that dSTIM compromises NE cell function in a manner that affects peptide synthesis and release, with functional consequences for survival on NR.

Systemic and cellular phenotypes observed with reduced dSTIM in Crz neurons can be rescued by increasing synthesis and release of peptides

To validate a role for dSTIM in peptide synthesis and release, we tested genetic perturbations that can compensate for this deficiency, to rescue developmental and cellular phenotypes associated with dSTIMIR expression in Crz neurons. In the case of NPs, genetic over-expression may not translate to enhanced release, as proteins involved in NP processing as well as the regulated secretory pathway would need to be up-regulated. Furthermore, regulatory feedback from peptides to their transcription may complicate over-expression, as seen for the Crz signalling system (S3I Fig). To get around these issues, and because InRIR phenocopied STIMIR (Fig 3A, 3D, 3F and 3I and S3H Fig), we opted to increase protein synthesis by over-expression of the Insulin receptor (InR). Cell size (S4A Fig) as well as Crz levels (S4B and S3C Figs) in DLP neurons scaled with InR over-expression, supporting the effectiveness of InR. To increase release, we over-expressed RalWT as Ral over-expression can compensate for vesicle release in dopaminergic neurons expressing STIMIR [17]. In Crz neurons with reduced dSTIM, over-expression of either InR or RalWT rescued pupariation on NR media (Fig 4A); restored peptide up-regulation upon NR (Fig 4A and 4B) and decreased CrzR mRNA back to control levels (Fig 4D). Of note, over-expressing RalWT or InR by itself, in Crz neurons, did not alter CrzR mRNA levels (Fig 4D), suggesting that neuronal activation, which happens on NR media (Fig 2D) potentiates their activity.

To increase neuronal activity, we utilised the temperature and voltage-gated cation channel, TrpA1 [45]. Over-expression of dTrpA1 and its activation by raising the temperature to 30˚C for 24 hours, in the dSTIMIR background, rescued pupariation of NR larvae (Fig 4E). It also restored the ability of DLP neurons to increase Crz levels upon NR (Fig 4F) and decreased levels of CrzR mRNA (Fig 4G). In line with the feedback between CrzR mRNA levels and systemic Crz signaling, over-expression of dTrpA1 alone in Crz neurons resulted in a decrease in CrzR mRNA levels (Fig 4G), supporting the role for neuronal activation in secreting Crz. Interestingly, development to adulthood on NR, for dSTIMIR larvae, was also significantly increased upon over-expression of InR (S4E Fig) and TrpA1 (S4F Fig), but not RalWT (S4E Fig).

An optogenetic approach, utilizing the over-expression of Channelrhodopsin (ChR2-XXL), a light-sensitive cation channel, also rescued pupariation but not to the same extent (S4D Fig). Poorer rescue with ChR2-XXL could be because sustained activation of this channel depresses synaptic transmission and the channel is less conductive for Ca\textsuperscript{2+} compared to TrpA1 [46]. Similar genetic manipulation, with TrpA1 and Chr2-XXL, in a hypomorphic IP\textsubscript{3}R mutant (itpr\textsuperscript{hu}) resulted in a small but significant rescue of itpr\textsuperscript{hu} pupariation on NR media (S4G and S4H Fig).

Together, these results strongly suggested that defects arising from dysregulated intracellular Ca\textsuperscript{2+} signalling, may be overcome by increasing vesicle exocytosis (RalWT, TrpA1, ChR2-XXL rescue) or protein synthesis (InR rescue). Importantly, the rescues observed with InR, RalWT and dTrpA1 are effective at the molecular (CrzR levels), cellular (Crz peptide levels upon NR) as well as systemic (NR larvae) level.

Conclusions

This study employed an in vivo approach coupled to a functional outcome, in order to broaden our understanding of how STIM regulates neuropeptides. A role for dSTIM-mediated SOCE
STIM regulates neuropeptides during *Drosophila* development

**A**  
% Pupae on NR  
dSTIM<sup>f3</sup>  
InR rescue  
Ra<sup>f1</sup> rescue  
InR control  
Ra<sup>f1</sup> control

**B**  
Relative Crz Intensity  
Fed  
24h NR  
Fed  
24h NR  
Ra<sup>f1</sup> Rescue  
InR Rescue

**C**  
Relative Crz Intensity  
Fed  
24h NR  
Fed  
24h NR  
Ra<sup>f1</sup> Rescue  
InR Rescue

**D**  
Fold Change CrzR / rp49  
1<sup>st</sup> dicer  
dSTIM<sup>f3</sup>  
InR rescue  
Ra<sup>f1</sup> Rescue  
InR  
Ra<sup>f1</sup>

**E**  
% Pupae upon NR  
30°C (24h)  
22°C  
Fed  
24h NR  
Fed  
24h NR  
TrpA1 control  
dSTIM<sup>f3</sup> rescue  
TrpA1 rescue

**F**  
Relative Crz Intensity (30°C)  
Fed  
24h NR  
Fed  
24h NR

**G**  
Fold Change (30°C)  
CrzR / rp49  
dicer  
dSTIM<sup>f3</sup>  
TrpA1  
TrpA1 rescue
in *Drosophila* neuroendocrine cells for survival on NR was previously established [22]. The previous study offered the opportunity to identify SOCE-regulated peptides, produced in these neuroendocrine cells, that could be investigated in a physiologically relevant context.

In *Drosophila*, both Crz and sNPF have previously been attributed roles in many different behaviours. Crz has roles in adult metabolism and stress responses [43,47–49], sperm transfer and copulation [50], and regulation of ethanol sedation [51,52]. While, sNPF has been implicated in various processes including insulin regulation [28,48] circadian behaviour [53], sleep- ing [54,55] and feeding [28]. Thus, the identification of Crz and sNPF in coping with nutritional stress is perhaps not surprising, but a role for them in coordinating the larval to pupal transition under NR is novel.

A role for Crz in conveying nutritional status information was originally proposed by Jan Veenstra [56], which this study now supports. In larvae, Crz+ DLPs are known to play a role in sugar sensing [57] and in adults, they express the fructose receptor Gr43a [58]. Additionally, they express receptors for neuropeptides DH31 [59], DH44 [59] and AstA [56], which are made in the gut as well as larval CNS. Together, these observations and our study are strongly indicative of a role for Crz+ DLPs in directly or indirectly sensing nutrients, with a functional role in larval survival and development in nutrient restricted conditions.

Several neuropeptides and their associated signalling systems are evolutionarily conserved [11,12]. The similarities between Crz and GnRH (gonadotrophin-releasing hormone), and sNPF and PrRP (Prolactin-releasing peptide), at the structural [11], developmental [60] and receptor level therefore, is intriguing. Structural similarity of course does not imply functional conservation, but notably, like sNPF, PrRP has roles in stress response and appetite regulation [61]. This leads to the conjecture that GnRH and PrRP might play a role in mammalian development during nutrient restriction.

dSTIM regulates Crz and sNPF at the levels of peptide release and likely, peptide synthesis upon NR. We speculate that neuroendocrine cells can use these functions of STIM, to fine tune the amount and timing of peptide release, especially under chronic stimulation (such as 24hrs NR), which requires peptide release over a longer timeframe. Temporal regulation of peptide release by dSTIM may also be important in neuroendocrine cells that co-express peptides with multifunctional roles, as is the case for Crz and sNPF. It is conceivable that such different functional outcomes may require distinct bouts of NP release, varying from fast quantile release to slow secretion [62]. As elevation in cytosolic Ca**2+** drives NP vesicle release, neurons utilise various combinations of Ca**2+** influx mechanisms to tune NP release. For example, in *Drosophila* neuromuscular junction, octopamine elicits NP release by a combination of cAMP signalling and ER-store Ca**2+**, and the release is independent of activity-dependent Ca**2+** influx [63]. In the mammalian dorsal root ganglion, VGCC activation causes a fast and complete
release of NP vesicles, while activation of TRPV1 causes a pulsed and prolonged release [64]. dSTIM-mediated SOCE adds to the repertoire of mechanisms that can regulate cytosolic Ca^{2+} levels and therefore, vesicle release. This has already been shown for Drosophila dopaminergic neurons [17] and this study extends the scope of release to peptides. Notably, dSTIM regulates exocytosis via Ral in neuroendocrine cells, like in dopaminergic neurons.

In Drosophila larval Crz^{+} DLPS, dSTIM appears to have a role in both fed, as well as NR conditions. On normal food, not only do Crz^{+} DLPS exhibit small but significant levels of neuronal activity (Fig 2D) but also, loss of dSTIM in these neurons reduced Crz signalling (Fig 3I). Thus, dSTIM regulates Ca^{2+} dynamics and therefore, neuroendocrine activity, under basal as well as stimulated conditions. This is consistent with observations that basal SOCE contributes to spinogenesis, ER-Ca^{2+} dynamics as well as transcription [65]. However, in our case, this regulation appears to have functional significance only in NR conditions as pupariation of larvae, with reduced levels of dSTIM in Crz^{+} neurons, is not affected on normal food (Fig 1B). In a broader context, STIM is a critical regulator of cellular Ca^{2+} homeostasis as well as SOCE, and a role for it in the hypothalamus has been poorly explored. Because STIM is highly conserved across the metazoan phyla, our study predicts a role for STIM and STIM-mediated SOCE in peptidergic neurons of the hypothalamus. There is growing evidence that SOCE is dysregulated in neurodegenerative diseases [66]. In neurons derived from mouse models of familial Alzheimer’s disease [67] and early onset Parkinson’s [65], reduced SOCE has been reported. How genetic mutations responsible for these diseases manifest in neuroendocrine cells is unclear. If they were to also reduce SOCE in peptidergic neurons, it’s possible that physiological and behavioural symptoms associated with these diseases, may in part stem from compromised SOCE-mediated NP synthesis and release.

Supporting information

S1 Fig. Identification of SOCE-regulated neuropeptides to survive NR. A. Genetic Screen. GAL4s for various peptides were used to drive IP_{RIR} and the corresponding larvae were tested for their ability to pupariate on normal or NR media. AstA: Allatostatin A; AKH: Adipokinetic Hormone; DSK: Drosulphakinin; Hug: Hugin; NPF: Neuropeptide F; sNPF: short Neuropeptide F. B. % Pupae in NR media when sNPF is reduced by RNAi (sNPR_{IR}) or by reducing an enzyme required for neuropeptide processing (amon_{IR}) in sNPF-GAL4 expressing cells, or in a hypomorphic sNPF mutant (sNPF_{00448}). C. 3rd instar larval brain expressing GFP in sNPF-GAL4 producing neurons and a mcherry-tagged corazonin (Crz::mcherry). Note the co-localisation of sNPF-GAL4 with Crz::mcherry, only in the brain lobes (arrow). D. % Pupae on normal and NR media when Crz is reduced in Crz^{+} neurons, using a second RNAi line (crz_{IR}25999). E. % Pupae and adults that developed on normal food from larvae where Crz^{+} neurons over-expressed either dSTIM or IP_{IR}. Differences are not statistically significant. F. Food intake as measured by absorbance of coloured food (A_{625}) fed to larvae for the indicated genotypes. N = 8 sets of 5 larvae each. Differences are not statistically significant. one-way ANOVA with a post hoc Tukey’s test p<0.05 for (A), (B), (F). Ordinary two-way ANOVA with Sidak’s multi-comparison test p<0.05 for (D). Bars with the same alphabet represent statistically indistinguishable groups. Data represents mean ± SEM. (TIFF)

S2 Fig. Characterization of Crz^{+} neurons. A. and B. Expression pattern of Crz-GAL4 (observed via GFP_{plts}) closely matched the expression of Crz (observed by a genomically integrated, mCherry-tagged corazonin: Crz::mCherry). C. Crz-GAL4 also expresses in ~4–5 additional neurons in the DLP region, that are not marked by Crz::mCherry (See A). The level of expression also varies with the marker. GFP_{plts} vs histone-tagged to RFP (H2B::RFP). D. Overall
distribution of NPs in Crz neurons followed by the expression of GFP-tagged rat ANF (ANF::GFP) and mCD8-tagged membrane bound RFP (UAS-mRFP). Note the exclusion of ANF from projections that end in the SEZ. E. Representative image. Expression of tsh-GAL80 in crz> GFP expressing brains causes the loss of GFP expression in the VG. F. % Pupae when Corazonin is reduced (CrzIR) in sNPF-GAL4 expressing neurons. This allows restricted expression of CrzIR only in DLPs (S1C Fig). Ordinary two-way ANOVA with a post hoc Sidak’s multi-comparison test p<0.05. Data represents mean ± SEM. G. Representative image. Expression of the UV-activated Ca2+ indicator, CaMPARI in Crz VG neurons. F56i reflects Ca2+ levels, while F488 reflects levels of the indicator. H. Quantification of F56i/F488 ratio in the presence and absence of UV-stimulation, after 24hrs in either normal or NR food, in Crz VG neurons. N>7 larvae for UV-stimulated; N = 3 for No UV stimulation. Kruskal-Wallis Test with Dunn’s multi-comparison correction p<0.05. Bars with the same alphabet represent statistically indistinguishable groups. (TIFF)

S3 Fig. dSTIM regulates Crz peptide and mRNa levels similar to InR and Ral. A. Representative image. How “Box” and “projection” areas were delineated for measuring levels of Crz by immunofluorescence, at the ring gland (RG). Box is a 50X50 px square. Lines are a 50X15px rectangle. These measurements were made in a subset of DLPs for which Crz intensity were measured in cell bodies, plotted in Fig 3A. B. and C. Quantification of Crz levels at the RG. D. Relative Crz peptide levels measured on dissected RGs (N atop bars) and quantified using MALDI-MS. Externally added heavy standard (MS) was used to normalise peptide levels between samples. Kruskal-Wallis Test with Dunn’s multi-comparison correction p<0.05. E. Relative levels of the Rat Neuropeptide ANF::GFP measured by GFP fluorescence, in Crz+ DLPs in either control (crz>dicer2) or dSTIMIR condition (crz>dstimIR,dicer2). Two-tailed t-test. Total number of cell bodies counted mentioned atop bars. N>15 brains. F. % Pupae when vesicle release is perturbed either by expressing a dynamin mutant activated at 30°C (Shibire3i), or tetanus toxin light chain (TeTxLc). TeTxLC-IMP: inactivated TeTxLc. Ordinary one-way ANOVA with a post hoc Tukey’s test p<0.05. Data represents mean ± SEM. G. Crz mRNA levels in larval brain with pan-neuronal reduction in CrzR (crzRIR). Two-tailed t-test. N = 4. H. Crz mRNA levels in larval brains where protein synthesis (InRIR) or peptide processing (amnIR) or vesicle exocytosis (RalDN) are perturbed in Crz+ neurons. Ordinary one-way ANOVA with a post hoc Tukey’s test p<0.05. N>4. I. Relative levels of Crz measured on cell bodies of Crz+ DLP neurons when either Crz (CrzIR) is reduced in Crz+ neurons or its receptor CrzR (crzRIR) is reduced pan-neuronally. Kruskal-Wallis Test with Dunn’s multicomparison correction p<0.05. N>10 brains. Bars with the same alphabet represent statistically indistinguishable groups. (TIFF)

S4 Fig. Increasing neuronal activity or protein synthesis can overcome phenotypes observed with loss of dSTIM in Crz neurons. A. Cell size of DLP neurons, upon over-expression of Insulin receptor (InR) in Crz+ neurons. N = 5 brains. Student’s t-test. Representative images in B. C. Relative Crz peptide levels in cell bodies of Crz+ DLP neurons expressing InR. N = 5. Student’s t-test. p<0.001 D. % Pupae upon NR, when in Crz+ neurons, dSTIM was reduced, and ChR2-XXL, the light activated channel was ectopically expressed. Larvae were reared in the dark and post-transfer to NR, either continued to be kept in the “Dark” or moved to an incubator with 24 lights (white light) ON (Light) till the end of the pupariation assay (~10days). two-way ANOVA with a post hoc Sidak’s multi-comparison test p<0.05. E. % Adults recovered upon over-expression of Insulin receptor (InR) or Ral (RalWT) in Crz+ neurons expressing dSTIMIR. InR rescue: crz>InR,dSTIMIR,dicer2. RalWT rescue: crz> RalWT,
$dSTIM^{IR}$, $dicer2$. InR control: $dicer2; InR/+$; $dSTIM^{IR} /+$. $Ral^{WT}$ control: $Ral^{WT} /+$; $dSTIM^{IR} /+$. Ordinary one-way ANOVA with a post hoc Tukey’s test $p < 0.05$. F. % Adults recovered upon over-expression of $TrpA1$ in $Crz^+$ neurons expressing $dSTIM^{IR}$. $TrpA1$ control: $dicer2; TrpA1/+$; $dSTIM^{IR} /+$. $TrpA1$ rescue: $crz > TrpA1$, $dSTIM^{IR} ; dicer2$ Ordinary one-way ANOVA with a post hoc Tukey’s test $p < 0.05$. N = 6 Ordinary one-way ANOVA with a post hoc Tukey’s test $p < 0.05$. N = 6. G and H. % Pupae upon NR, in an hypomorphic IP$_3$R mutant ($itpr^{ka1091/ug3}$) with Crz$^+$ neurons over-expressing either $dTrpA1$, and post-transfer incubating at 30˚C for 24hours, or $ChR2-XXL$ and reared in light till the end of the assay. Ordinary one-way ANOVA with a post hoc Tukey’s test $p < 0.05$. for (G). Student’s t-test for (H). Bars with the same alphabet represent statistically indistinguishable groups. Data represents mean ± SEM.

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