Running Title:
“Neuropeptides required for Drosophila development under nutritional stress are regulated by the ER-Ca$^{2+}$ sensor STIM”
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Significance
Elevation of cytosolic Ca$^{2+}$ is required for the release of neuropeptides from specialised secretory neurons called neuroendocrine cells. Release of Ca$^{2+}$ stored in the ER, which further triggers Ca$^{2+}$ entry from the extracellular milieu is called Store-operated Ca$^{2+}$ entry (SOCE). STIM, an ER protein, is a key regulator of SOCE and its contribution to neuroendocrine cell functioning is not well studied. Using Drosophila larval development under nutrient restriction as a paradigm, we identified two SOCE regulated neuropeptides, Corazonin and short Neuropeptide F. Reducing STIM alters the level of these neuropeptides in the fed as well as nutrient restricted condition. This study suggests that STIM-triggered SOCE may regulate the release of neuropeptides whose activity ultimately regulates adaptation to nutritional stress.

Key words: STIM, neuropeptides, exocytosis, nutrition, development
Abstract

Cytosolic Ca\textsuperscript{2+} levels are tightly regulated by the sequestration of Ca\textsuperscript{2+} within the endoplasmic reticulum (ER). Loss of ER Ca\textsuperscript{2+} is sensed by STromal Interacting Molecule (STIM), whose translocation to the plasma membrane triggers Store Operated Ca\textsuperscript{2+} Entry (SOCE), and a subsequent rise in cytosolic Ca\textsuperscript{2+}. Relatively little is known about SOCE’s contribution to neuroendocrine cells; a neuronal sub-type that specializes in the secretion of neuropeptides (NPs) which ultimately regulate animal physiology and behavior. To investigate how SOCE regulates NPs, Drosophila development under nutrient restriction (NR) was used as the biological context. Genetic experiments identified the requirement of two SOCE-regulated NPs -corazonin (Crz) and short neuropeptide F (sNPF) - for the development of NR larvae to pupae and finally, adulthood. Overexpression of SOCE regulators was sufficient to rescue the development of NR larvae with reduced sNPF or Crz levels. To facilitate cellular investigations, a restricted set of neurons that produce sNPF and Crz, and are activated by NR, were identified. Immunohistochemistry on these neurons and mass spectrometric measurements of their projections showed that dSTIM regulates Crz and sNPF levels at steady state and in response to NR, likely by modulating their release. Genetically increasing neuronal output, in the background of reduced dSTIM, robustly rescued development of NR larvae. Because NP action fundamentally underpins how animals adapt to stimuli, regulation of NPs by STIM triggered SOCE is likely to be important. In situations where SOCE is altered, such as neurodegenerative diseases, this regulation may contribute to disease manifestation and progression.
Introduction

Ca$^{2+}$ is one of the primary signals utilized by cells for potentiating signal transduction pathways. Depending on cell type and context, elevation of cytosolic Ca$^{2+}$ can trigger a diverse array of cellular processes. To keep basal cytosolic Ca$^{2+}$ levels low, Ca$^{2+}$ is sequestered into various organelles, the largest being the ER. ER-store Ca$^{2+}$ is released by ligand activated Ca$^{2+}$ channels such as the Ryanodine receptor or inositol 1,4,5-trisphosphate receptor (IP$_3$R). Loss of ER-Ca$^{2+}$ is sensed by STromal Interacting Molecule (STIM), an ER-resident transmembrane protein, which undergoes structural rearrangement to bind Orai, the store-operated Ca$^{2+}$ channel on the plasma membrane. Activation of Orai causes an influx of Ca$^{2+}$ from the extracellular milieu which may have up to ~1000 fold higher levels of Ca$^{2+}$ as compared to the cytosol. This type of capacitative Ca$^{2+}$ entry is termed Store-operated Ca$^{2+}$ entry (SOCE) (1). The proteins that regulate SOCE – IP$_3$R, STIM and Orai – are ubiquitously expressed in the animal kingdom, underscoring the importance of this pathway to cellular functioning.

Elevation of cytosolic Ca$^{2+}$ is central to neuronal function. To achieve context dependent outcomes, neurons possess many different strategies to modulate the frequency and amplitude of Ca$^{2+}$ signals (2). Of these, the properties and expression of a large repertoire of activity-dependent voltage gated Ca$^{2+}$ channels (VGCCs) and receptor-activated Ca$^{2+}$ channels, that bring in external Ca$^{2+}$, is perhaps the most well studied. The contributions of internal ER-Ca$^{2+}$ stores to neuronal Ca$^{2+}$ dynamics are also well recognized. However, the study of how STIM and subsequently SOCE may influence neuronal functioning is as yet a nascent field. In mice, STIM2 mediates SOCE in cortical (3) and hippocampal neurons (4), while STIM1-mediated SOCE has been reported for cerebellar granule neurons (5) and isolated Purkinje neurons (6). Although both STIM1 and STIM2 are expressed in the hypothalamus (Human Protein Atlas), the major
neuroendocrine center in vertebrates, a role for STIM or SOCE is poorly investigated in this tissue.

In flies (Drosophila melanogaster; Dmel), dSTIM regulates functioning of neurons (7–12), fat body cells (13) and intestinal stem cells (14). dSTIM-mediated SOCE has been demonstrated in Dmel neurons (7), with functional consequences in certain neuronal subtypes: regulation of flight in dopaminergic neurons (8, 15) and development in protein-deprived media in glutamatergic neurons (10). Further, dSTIM over-expression in insulin-producing neuropeptidergic neurons could restore Ca\textsuperscript{2+} homeostasis in a non-autonomous manner in other neurons of an IP\textsubscript{3}R mutant (16), indicating an important role for dSTIM in NE cell output as well as compensatory interplay between SOCE regulators IP\textsubscript{3}R and dSTIM. At least in Dmel, all three proteins - IP\textsubscript{3}R, dSTIM and Orai - interact to regulate SOCE in neurons (7, 12).

NE cells are secretory neurons that produce NPs, the most diverse group of neuronal signaling agents. Inside the brain, NPs typically modulate neuronal activity and wiring; when released systemically, they act as hormones. Dmel is typical in having a vast repertoire of NPs that together play a role in almost every aspect of its behavior and physiology (17, 18). Consequently, NP synthesis and release are highly regulated processes. As elevation in cytosolic Ca\textsuperscript{2+} is required for NP release, a contribution of STIM-mediated SOCE to NE function was hypothesized.

Dmel development under nutritional stress was chosen as the biological context to identify SOCE-regulated NPs, for four reasons. First, we previously reported that reducing IP\textsubscript{3}R or dSTIM or expressing a dominant-negative Orai (Orai\textsuperscript{E180A}), in a large number of Dmel NE cells, reduced larval development under NR conditions (11). Because these manipulations are also known to reduce SOCE in Dmel neurons (7, 8), it was likely that SOCE regulates NPs secreted by NE cells that are involved in the adaptation to NR conditions. Second, adaptation to poor nutritional conditions requires a number of
coordinated behavioral and physiological responses, each likely needing a distinct NP or a set of NPs, thus using this assay improved the likelihood of uncovering SOCE-regulated NPs. Third, most NPs in the nutritional and feeding context are studied in adult flies, where regulatory mechanisms involved in adaptation to dietary imbalances and starvation, may be different from the larval stage. Adults require nutrition for reproduction and survival, whereas larvae require them predominantly for growth and development. Thus far, a role only for three of eight *Dmel* insulin-like peptides (dILPs) which are neuropeptides, has been well-established in relation to nutrition and development (19, 20). Several NPs directly or indirectly regulate the dILPs, with consequences for maintaining metabolic homeostasis and regulating feeding behaviors, but of note, a majority of these studies are in adults (21). Therefore, the assay used in this study was expected to yield insights on NPs required during development, an uncommon area of study. Finally, a developmental readout allowed for the exploitation of the *Dmel* genetic tool kit to scale from cellular perturbations to systemic behavior.

**Results**

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

The ability to pupariate on nutrient-restricted (NR) media (100mM sucrose) as opposed to a complex mixture of yeast, sugar and corn flour (“normal” food), when transferred in the last stage of larval development (~88 hours post egg laying), was used as a measure of development. Collectively, more than 20 different NPs are made by the NE cells in which reducing SOCE components resulted in poor pupariation upon NR (11). From these NPs, those with established roles in feeding and metabolism were selected and, a curated GAL4-UAS genetic screen undertaken. Neuropeptide GAL4s were used to drive the knockdown of *IP\_3R (IP\_3R\textsuperscript{R})*, and pupariation of the resulting larvae was scored in normal vs NR food conditions (Fig. S1A). On normal food, a significant reduction of pupariation
was seen only with sNPFGAL4. Upon NR, the largest effect was seen with sNPFGAL4, followed by small but significant pupariation defect with AstA-GAL4, Crz-GAL4 and DSK-GAL4. Neurons that secrete NPs may also secrete neurotransmitters, therefore, a role specifically for sNPF was tested next. Directly reducing the level of sNPF (sNPFR) or reducing an enzyme required for neuropeptide processing (amontillado; amonIR) in sNPF-producing cells, as well as a hypomorphic sNPF mutation (sNPFG0448) resulted in impairment of larval development upon NR (Fig. S1B). These indicate that sNPF is required for pupariating under NR conditions.

sNPFGAL4 has a diverse expression pattern in the larval CNS (22) and also expresses in the larval midgut and epidermis. To identify a restricted set of neurons that contribute to the pupariation phenotype observed on NR media, we focused on a Crz-GAL4 strain, as sNPF and Crz are co-expressed in a set of three bilateral neurons in the larval brain lobes ((22); Fig. S1C). Reducing either sNPF or Crz (Fig. 1A) using the Crz-GAL4 driver resulted in larvae with a pupariation defect on NR food. Furthermore, reduction of Crz using sNPFGAL4 (Fig. S1D) presented a defect upon NR to the same extent as that was observed with Crz-GAL4 (Fig. 1A). Together, these suggest a role for neurons that express both sNPF and Crz in development under nutritional stress.

As reduction of IP3R resulted in a weak phenotype (Fig. S1A), the focus was shifted to dSTIM, as both proteins are known to positively regulate SOCE in Drosophila neurons (7, 12). Knockdown of dSTIM (dSTIMR) with either sNPFGAL4 or Crz-GAL4, resulted in reduced pupariation under NR to the same extent, with no effect on development on normal food (Fig. 1B). Over-expression of a dominant-negative version of Orai (OraiE180A), known to reduce SOCE in neurons (8), also reduced purariation rates in NR larvae when expressed in the Crz+ neurons (Fig S1E). Thus, SOCE is required in Crz+ neurons for development under nutritional stress.
Loss of either IP$_3$R or sNPF has previously been shown to affect larval feeding (23, 24). Hence, to eliminate the possibility that developmental defects on sucrose arise from compromised feeding, the intake of dye-colored food was measured. Age-synchronized larvae with knockdown of either dSTIM, IP$_3$R, Crz or sNPF in Crz-GAL4 expressing neurons exhibited no difference in feeding over a 2-hour period (Fig. S1G), suggesting that developmental defects in the NR assay are not arising from a fundamental feeding problem.

Reduction in either NPs - sNPF or Crz - or SOCE regulators - IP$_3$R and dSTIM – in Crz$^+$ neurons (Fig. 1A,B, S1A) resulted in reduced pupariation upon NR. To understand if these NPs and SOCE regulators interact genetically, compensation experiments were undertaken. In the background of reduced NPs, SOCE-regulators were over-expressed in Crz$^+$ neurons. NR larvae with this genetic make-up not only showed significant improvement in pupariation (Fig. 1C), but also in their ability to eclose as adults (Fig. 1D). Enhancing the expression of SOCE regulators leads to increased SOCE in Drosophila neurons (12), suggesting that reduced neuronal output of NPs can be overcome by enhancing intracellular Ca$^{2+}$ signaling.
Fig. 1 ER-Ca\(^{2+}\) signaling is required in Crz and sNPF producing neurons for development under NR conditions. 88h AEL larvae are transferred to either normal food (See Material and Methods) or nutrient restricted (NR) media. The number of pupae and adults is scored for a batch of 25 larvae transferred per vial. N = 6. (A) % pupae upon reduction of either Crz or sNPF in Crz\(^{+}\) neurons (B) % Pupae upon reduction of dSTIM (dSTIM\(^{IR}\)) in either sNPF\(^{+}\) or Crz\(^{+}\) neurons (C) % Pupae when in the background of either Crz or sNPF reduction (Crz\(^{R}\), sNPF\(^{R}\)), dSTIM or IP\(_{3}\)R are over-expressed. Results are not statistically significant. (D) % Adults recovered for genotypes in (C). Bars with the same
alphabet represent statistically indistinguishable groups. Two-way ANOVA with Sidak multi comparison test p<0.05 for (A) and (B), one-way ANOVA with a post hoc Tukey’s test p<0.05 for (C) and (D). Data represents mean ± SEM. KD: Knockdown. All experiments were carried out in presence of Dicer2 to enhance the efficiency of the interfering RNAi (IR).
Activation of Crz+ and sNPF+ DLP neurons is required to pupariate on NR diet

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) (25). Except the medial neurons, the *Crz-GAL4* used in this study recapitulates the known expression pattern of Crz+ neurons (Fig. S2A and C; Cartoon: Fig. 2A). Low ectopic expression of *Crz-GAL4* was observed in 3-4 neurons adjacent to DLPs (Fig. S2B). Crz+ DLPs also express sNPF (22). The Crz+ and sNPF+ DLPs have two major branches: the anterior branch culminates in a dense nest of projections at the ring gland (RG) which is considered the neurohaemal site for systemic release, while the posterior branch terminates in the gnathal ganglion (GNG). The VG neurons form a network amongst themselves with two parallel branches emerging that terminate in the brain lobes, close to the anterior branch of the DLPs, suggesting possible neuromodulation by the VG neurons. Atrial neuropeptide (ANF), a rat neuropeptide coupled to GFP (26) was used as a proxy to visualize the distribution of neuropeptides in the Crz+ neurons (Fig S2D). Firstly, ANF intensity was higher in the cell bodies of the DLPs than VG neurons. Secondly, within the DLPs, ANF::GPF was either in the cell bodies or RG projections, but not in the projections terminating at the GNG, suggesting that the RG is the major site of neuropeptide release for the DLPs (Fig. S2D).

Functional requirement of Crz+ neurons to survive NR was tested genetically in different ways: 1) by ablation of the neurons (using *UAS-hid::UAS-rpr*); 2) by reducing vesicle recycling using temperature-sensitive dynamin mutant (*shibrels*); 3) by impairing vesicle release using tetanus toxin (TeTxLc). All three perturbations resulted in reduced pupariation on NR media (Fig. 2C).

To probe the activation state of Crz+ neurons, the UV light-activated genetically encoded calcium sensor, CaMPARI (27), was utilised. The sensor fluorecesces in the GFP range ($F_{\text{green}}$) and is converted irreversibly to fluorecesce in the RFP range ($F_{\text{red}}$), when
exposed to UV light and in the presence of Ca\textsuperscript{2+}. The level of conversion positively titrates with Ca\textsuperscript{2+} concentrations. Larva expressing CaMPaRI in Crz\textsuperscript{+} neurons were placed in either normal or NR food for 24 hours (NR). Whole larvae were then exposed to UV light for 2mins. Control larva (Fig. 2E,F; no UV) were subject to the same treatment but without being exposed to UV light. Increased F\textsubscript{red}/F\textsubscript{green} ratios were observed in the DLPs after 24 hours on NR media, suggesting increased cytosolic Ca\textsuperscript{2+} levels and therefore, enhanced neuronal activity (Fig. 2D,E). F\textsubscript{red}/F\textsubscript{green} ratios did not appear to change in the VG neurons suggesting either that either they do not participate in nutritional stress response (Fig. 2F, S2E) or there is insufficient penetration of UV light into the VG. Therefore, expression of dSTIM was restricted to the DLPs by using tshGAL80 (28) that prevented expression of Crz-GAL4 in the VG (Fig. 2G). The level of pupariation under NR conditions observed with restricted expression was similar to that seen with full expression (Fig. 1B). Similar levels of reduced pupariation of NR larvae is also seen upon DLP restricted knockdown of Crz by sNPF-GAL4 (Fig. S1D). Together, these results suggest that a major role in the nutrient stress response is played by the Crz\textsuperscript{+} and sNPF\textsuperscript{+} DLP neurons.
Fig. 2 Crz\(^+\) and sNPF\(^+\) DLPs are functionally required for development under NR conditions (A) Cartoon of Crz\(^+\) neurons in the larval CNS marked by Crz-GAL4 used in
this study. Based on Figs S2A,C,D DLP: dorso lateral peptidergic neurons; VG: Ventral Ganglion; GNG gnatho ganglion (B) Distribution of Crz NP in Crz+ neurons visualized by genomically integrated mcherry-tagged Crz (29) (C) % Pupae when Crz+ neurons are either ablated using apoptotic genes hid and rpr (hid::rpr), or when vesicle release is perturbed by introducing a dynamin mutant activated at 30°C (Shibret\textsuperscript{R}) or over-expression of tetanus toxin light chain (TeTxLc). TeTxLC-IMP: inactivated TeTxLc. N=6 (D) Representative image. Expression of the UV-activated Ca\textsuperscript{2+} indicator, CaMPARI in Crz+ and sNPF+ DLPs. F\textsubscript{561} reflects Ca\textsuperscript{2+} levels, while F\textsubscript{488} reflects levels of the indicator. F\textsubscript{561/488} ratio in the presence and absence of UV-stimulation, in normal vs NR food for 24h in (E) DLPs (F) VG neurons (for representative image see Fig. S2E). N>7 larvae for UV-stimulated; N=3 for No UV stimulation. (G) Representative image. Expression of tsh-Gal80 transgene turns off the expression of Crz-GAL4 only in the VG (H) % Pupae when dSTIM\textsuperscript{R} is selectively active only in Crz+ and sNPF+ DLPs, by using the tsh-Gal80 transgene and in the presence of dicer2. N=6. Bars with the same alphabet represent statistically indistinguishable groups. one-way ANOVA with a post hoc Tukey’s test p<0.05 for (C). Mann-Whitney Test for (E) and (F). Two-way ANOVA with Sidak multi comparison test p<0.05 for (H). Data represents mean ± SEM for (C), (H).
**dSTIM affects Crz and sNPF peptide levels**

The identification of a restricted set of neurons, along with specific neuropeptides, provided a system to interrogate how dSTIM might regulate NPs at the cellular level. As both neuronal activation and dSTIM-regulated SOCE can regulate transcription (30, 31), relative levels of *Crz* mRNA in larval brains were measured by qRT-PCR. Larvae (~90hAEL) were fed either normal or NR food for 24hrs followed by measurement. Interestingly, elevated levels of *Crz* mRNA were found in the dSTIM knockdown condition as compared to controls in both normal and NR conditions (Fig. 3A). Notably, NR did not alter transcript levels of *Crz* in control brains (Fig. 3A). Systemic feedback from reduced Crz signaling has been reported to increase *Crz* transcript levels (32). To understand if the increased Crz mRNA levels in fed state of dSTIM knockdown might be explained by such feedback, we measured levels of Corazonin receptor (*CrzR*), which in larvae are predominantly expressed in the CNS (33). Reducing *Crz* results in increased levels of *CrzR* in larval brains on normal food, and a similar increase is seen when dSTIM is reduced in *Crz*+ neurons (Fig. S3A). This indicates that loss of *dSTIM* in *Crz*+ neurons results in reduced systemic Crz signaling; with likely feedback resulting in increased Crz mRNA levels.

Under conditions similar to that used for measuring transcript changes, we next measured relative Crz peptide levels by immunohistochemistry (IHC) on Crz+ DLPs. Two levels of interrogation were undertaken: in the cell body and on the RG. While NR caused no observable change in *Crz* mRNA levels, it induced a major increase of Crz peptide in the cell body as well as in projections (Fig. 3B,C, S3 C,D; See Control: Normal vs NR). Thus, increased activation of these neurons by NR (Fig. 2E), appears to result in Crz peptide mobilization. In comparison, Crz+ DLPs with reduced dSTIM displayed increased Crz peptide levels on normal food itself (Fig. 3B,C, S3C,D). Also, unlike in the control, NR
did not appear to significantly alter peptide levels in Crz+ DLPs with reduced dSTIM (Fig. 3B,C, S3 C,D; See dSTIM KD Normal vs NR).

Because sNPF is expressed broadly in several neurons of the larval CNS (Fig. S1C), and the phenotype under observation derives from a small set of neurons, the strategy used to assess Crz levels could not be used for sNPF. 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 neurohemal release sites (34). As Crz peptide levels measured by IHC were consistent between the cell bodies and projections, and Crz+ DLPs are major contributors to sNPF on the RG, this technique allowed us to infer sNPF peptide levels from a restricted set of neurons. RGs were collected from larvae with the same background and conditions as used for RT-PCR and IHC experiments. In the control, although starvation increased the average level of sNPF peptide in the RGs, the increase was not statistically significant; perhaps a resolution limit of the technique (Fig. 3D). However, when dSTIM was reduced in the Crz+ DLPs, RG preparations showed a sharp increase in sNPF levels on normal food and remained unaltered with NR (Fig. 3D). While Crz peptide was detectable in the RG preparations, it was of much lower intensity; thus, despite increases in average levels of Crz peptide as seen for sNPF, statistically higher levels of Crz were observed only under NR, dSTIM KD condition (Fig. S3E).

Cumulatively, these results show that reducing dSTIM affects both sNPF and Crz peptide levels similarly, suggesting that dSTIM alters function of specific NE cells, at a fundamental level. NR activates Crz+ and sNPF+ DLPs (Fig. 2E), causing a mobilization of Crz and sNPF peptide (Fig. 3C,D, S3C,D). This mobilization is likely required for the sustained release of Crz and sNPF from the neurohemal endings of DLPs on the RG, and promotes development in NR larvae. Loss of dSTIM in Crz+ DLPs increases peptide levels and this increase remains unchanged even under starvation. One interpretation of greater
retention is impaired release, which is consistent with the reduced systemic Crz signaling seen upon loss of \textit{dSTIM} (Fig. S3A).
Fig 3. dSTIM regulates Crz and sNPF levels. Here, Control: crz>dicer2; dSTIM KD: crz>stimIR, dicer2. Measurements on 3rd instar larvae subject to 24 hours of either normal or NR food. (A) crz mRNA levels from larval brains. N ≥ 6. (B) Representative images. Crz+ and sNPF+ DLPs stained with Anti-Crz antibody. (C) Relative Crz peptide levels (CTCF) in DLP cell bodies. N>12 brains. Cell numbers indicated atop bars. (D) Relative total sNPF peptide levels measured on dissected ring glands (N atop bars) and quantified using MALDI-MS. Externally added heavy standard (Hug-PK*) was used to normalise peptide levels between samples. Data represents mean ± SEM. Two-way ANOVA with Sidak multi comparison test p<0.05 for (A). Kruskal-Wallis Test with Dunn’s multicomparison correction p<0.05 for (C) and (D). Bars with the same alphabet represent statistically indistinguishable groups.
Development of NR larvae with reduced dSTIM in Crz⁺ neurons can be rescued by increasing neuronal output

Cellular studies suggested that reducing dSTIM decreases the release of Crz and sNPF in normal and NR food conditions. Enhancing neuronal activation leads to increased vesicle exocytosis and therefore serves as a means to increase NP release. This formed the basis of employing genetically encoded, externally triggered channels to enhance neuronal activity of Crz⁺ neurons. Two types of channels were over-expressed in Crz⁺ neurons: a) TrpA1, a temperature sensitive Ca²⁺ channel and b) Channelrhodopsin (ChR2-XXL), a light-sensitive cation channel. In the dSTIMIR background, over-expression of dTrpA1 and its activation by raising the temperature to 30°C acutely for 24 hours at the time point of transfer to NR media, resulted in a rescue of the pupariation as well adult eclosion defect of NR larvae (Fig. 4A). Over-expression of ChR2-XXL and exposure to white light post-transfer to NR media, resulted in modest rescue of pupariation of dSTIMIR NR larvae (Fig. S4A). Poorer rescue with ChR-XXL could be because sustained activation of this channel depress synaptic transmission (35). Similar genetic manipulation but in a hypomorphic IP₃R mutant (iptku) resulted in a small but significant rescue of pupariation on NR media (Fig. S4B,C). Together, these results strongly suggested that defects in intracellular Ca²⁺ signaling molecules may be overcome by increasing neuronal activity, which likely increases peptide release. Another strategy to increase NP release relied on the over-expression of the small GTPase Ral (crz-GAL4>RalWT) which functions as part of the exocyst complex and positively regulates NP release from dense core vesicles (15). NR Larvae with reduced dSTIM but over-expressing Ral displayed higher pupariation than controls (Fig. S4D).

Besides neuronal activation, neuronal output of NPs may be enhanced by increasing NP production. The simplest way to accomplish this is by NP overexpression via the GAL4-UAS system. However, in the peculiar case of NPs, for enhanced
biosynthesis of active peptide to translate to enhanced release, proteins involved in NP processing as well as regulated secretory system would need to be up-regulated. Furthermore, regulatory feedback from peptides to their transcription may complicate over-expression. To get around these issues, an indirect strategy to enhance overall protein synthesis was adopted. Over-expression of Insulin receptor (InR) increases the cell size and peptidergic capacity of DIMM\(^+\) NE cells with functional consequences (36). As the Crz\(^+\) DLPs are DIMM\(^+\) (37), we reasoned that Crz\(^+\) neurons may be sensitive to InR levels. Supporting this was the observation that reducing InR (InR\(^{ir}\)) significantly decreased pupariation of NR larvae (Fig. S4E). Also, over-expressing InR not only increased cell size (Fig. S4F), but also increased Crz peptide levels (Fig. 4B,C). Finally, in the dSTIM knockdown background, over-expression of InR resulted in increased pupariation as well as adult eclosion of NR larvae (Fig. 4D).

Thus, increasing either neuronal activity or protein synthesis, could override the loss of dSTIM in Crz\(^+\) neurons and result in a functional rescue of development of NR larvae. Both strategies would ultimately increase neuronal output or release of NPs, suggesting that dSTIM regulates NP release in Crz\(^+\) neurons. Similar decreases in pupariation of NR larvae upon loss of either NPs (sNPF, Crz) (Fig 1A), or SOCE regulators (IP\(_3\)R, STIM, Orai) (Fig S1A, 1B, S1E) in Crz\(^+\) neurons, further supports a role for dSTIM in regulating sNPF and Crz release. Together, these data provide evidence that dSTIM regulates Crz\(^+\) NE cells and this regulation has a major functional consequence for development under nutritional stress.
**Figure 4**

**Rescue: Increased neuronal activity**

(A) % Pupae upon NR

(B) Representative image. Over-expression of Insulin Receptor (InR) in Crz+ neurons visualised by genetically encoded nuclear-GFP (GFPnls). See also (Fig S4F)

(C) Relative Crz peptide levels detected by Crz antibody normalized to over-expressed GFP. N = 5 brains.

(D) % Pupae upon NR

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**Fig 4. Loss of dSTIM can be compensated by increasing neuronal output, to survive development under NR.** (A) % survivors upon over-expression of TrpA1 and raising the temperature to 30°C to increase its activation in Crz+ neurons, with or without reduced dSTIM. N=6 (B) Representative image. Over-expression of Insulin Receptor (InR) in Crz+ neurons visualised by genetically encoded nuclear-GFP (GFPnls). See also (Fig S4F) (C) Relative Crz peptide levels detected by Crz antibody normalized to over-expressed GFP. N = 5 brains. (D) % survivors upon over-expression of InR in Crz+ neurons, in the background of reduced dSTIM. N=6. Data represents mean ± SEM. One-way ANOVA with a post hoc Tukey’s test p<0.05 for (A) and (D). Student’s t test for (C). ****p<0.0001.
Discussion

This study employed an in vivo approach coupled to functional outcome to broaden our understanding of dSTIM’s regulation of NP producing NE cells. NPs are products of multi-step synthesis and post-translational modification. Notably, NPs are not exclusively secreted at synaptic junctions. Furthermore, NPs have diverse functions. Crz has a role in adult metabolism and stress responses (32, 38–40), and in behaviors such as sperm transfer and copulation (41), and regulation of ethanol sedation (42, 43). sNPF has roles in insulin regulation (39, 44), circardian behavior (45), sleeping (46, 47) and feeding (23). Such different functional outcomes are likely regulated by controlling NP release amount and timing, from fast quantile release to slow secretion (48). Because Ca^{2+} is required for NP vesicle exocytosis, various combinations of Ca^{2+} influx mechanisms can be used to fine tune the amount and timing of Ca^{2+} elevation and therefore, NP release. For example, in Dmel neuromuscular junction, octopamine elicits NP release by a combination of cAMP signaling and ER-store Ca^{2+}, and the release is independent of activity-dependent Ca^{2+} influx (49). 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 (50). In primary neuronal cultures from Dmel, loss of dSTIM not only reduces SOCE (7) but also KCl-stimulated vesicle exocytosis (15). Thus, it is conceivable that elevation of cytosolic Ca^{2+} resulting from dSTIM mediated SOCE is a strategy used by NE cells to fine tune peptide release in vivo across phyla.

Crz^{+} DLPs with reduced dSTIM displayed increased peptide accumulation in basal (fed) conditions as well as under chronic stimulation (24h NR) (Fig. 3C,D and S3C,D). Because Ca^{2+} levels monitored by CaMPARI (Fig. 2 D,E) show that Crz^{+} DLPs have basal activity in the fed state too, together, these results suggest that dSTIM regulates Ca^{2+} dynamics and therefore, NE cell 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 (51). However, in our case, this regulation appears to have functional significance only in NR conditions as pupariation of larvae with reduced levels of \textit{dSTIM} in Crz\textsuperscript{+} neurons is not affected on normal food (Fig 1B).

In the context of nutritional stress, this study provides evidence for a hypothesis first proposed by Jan Veenstra that Crz\textsuperscript{+} neurons play a central role in conveying nutritional status information (52). Crz\textsuperscript{+} DLPs are known to play a role in sugar sensing (53, 54) and express the fructose receptor Gr43a (53). Additionally, they express receptors for neuropeptides DH31 (55), DH44 (55) and AstA (52) produced in the gut. Together these observations and our study are strongly indicative of a role for Crz\textsuperscript{+} DLPs in directly or indirectly sensing nutrients, with a functional role in larval survival and development in nutrient restricted conditions.

In a broader context, STIM is a critical regulator of cellular Ca\textsuperscript{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. Certain NPs also exhibit functional conservation between flies and humans (dILPs/insulin; AKH/ glucagon). The intriguing similarity between Crz/GnRH (gonadotrophin-releasing hormone) and sNPF/PrRP (Prolactin-releasing peptide), at the structural (56) and developmental (57) level, along with the structural conservation of their receptors, leads to the speculation that GnRH and PrRP might play a role in mammalian development during nutrient restriction. Of note, like sNPF, PrRP has roles in stress response and appetite regulation (58). Most often, studies on mammalian NPs are performed in the postnatal phase. We hope this study provides an impetus to look at NPs at an early mammalian developmental time point, like the fetus.

Finally, there is growing evidence that SOCE is dysregulated in neurodegenerative diseases (59). In neurons derived from mouse models of familial Alzheimer’s disease (60)
and early onset Parkinson’s (51), reduced SOCE has been reported. How genetic mutations responsible for these diseases manifest in NE cells is unclear. If they were to also reduce SOCE in peptidergic neurons, it could be speculated that physiological and behavioural symptoms associated with these diseases, may in part stem from compromised SOCE in NE cells.

Material and Methods

Fly Husbandry

Flies were grown at 25°C in 12h:12h L:D cycle. Recipe for normal food (11). 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 used in this study are tabulated in supplementary information.

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 on the larvae through a 10X objective on Olympus BX60, for 2 minutes. Larvae were them 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. 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.

Immunohistochemistry

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 subject to permeabilisation (0.3% Triton X-100 + PBS; PBSTx) for 15 mins, 4 hr blocking in 5% normal goat serum in PBSTx at 4oC, 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 (61). 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 50 shots per sample. Data were analyzed with Data Explorer 4.10. Spectra were baseline corrected and de-isotopoded. The sum of the resulting relative intensities of the de-isotopded 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 thermogenic experiments

For thermogenic (dTrpA1, Shibre<sup>fs</sup>) experiments, larvae were matured to 88AEL 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 (Shibre<sup>fs</sup>). 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 lights on 24 hours till the end of observation time.

Author contributions

M, C.W and G.H designed research; M performed research, except MALDI-MS which was performed by C.W.; M, C.W and G.H. analysed data; M wrote the paper with inputs from C.W. and G.H.

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References

1. Prakriya M, Lewis RS (2015) Store-Operated Calcium Channels. *Physiol Rev* 95(4):1383–1436.

2. Brini M, Cali T, Ottolini D, Carafoli E (2014) Neuronal calcium signaling: function and dysfunction. *Cell Mol Life Sci* 71(15):2787–2814.
3. Berna-Erro A, et al. (2009) STIM2 Regulates Capacitive Ca^{2+}; Entry in Neurons and Plays a Key Role in Hypoxic Neuronal Cell Death. *Sci Signal* 2(93):ra67 LP-ra67.

4. Sun S, et al. (2014) Reduced synaptic STIM2 expression and impaired store-operated calcium entry cause destabilization of mature spines in mutant presenilin mice. *Neuron* 82(1):79–93.

5. Lalonde J, Saia G, Gill G (2014) Store-operated calcium entry promotes the degradation of the transcription factor Sp4 in resting neurons. *Sci Signal* 7(328):ra51.

6. Hartmann J, et al. (2014) STIM1 controls neuronal Ca(2)(+) signaling, mGluR1-dependent synaptic transmission, and cerebellar motor behavior. *Neuron* 82(3):635–644.

7. Venkiteswaran G, 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(25):10326–10331.

8. Pathak T, Agrawal T, Richhariya S, Sadaf S, Hasan G (2015) Store-Operated Calcium Entry through Orai Is Required for Transcriptional Maturation of the Flight Circuit in Drosophila. *J Neurosci* 35(40):13784–13799.

9. Agrawal N, Padmanabhan N, Hasan G (2009) Inositol 1,4,5- trisphosphate receptor function in Drosophila insulin producing cells. *PLoS One* 4(8):e6652.

10. Jayakumar S, et al. (2016) Drosophila larval to pupal switch under nutrient stress requires IP3R/Ca2+ signalling in glutamatergic interneurons. *Elife* 2016;5:e17. doi:10.7554/eLife.17495.

11. Megha, Hasan G (2017) IP3R-mediated Ca2+ release regulates protein metabolism in Drosophila neuroendocrine cells: implications for development under nutrient stress. *Development* 144(8):1484–1489.

12. Chakraborty S, et al. (2016) Mutant IP3 receptors attenuate store-operated Ca2+...
entry by destabilizing STIM-Orai interactions in Drosophila neurons. *J Cell Sci.*
doi:10.1242/jcs.191585.

13. Baumbach J, et al. (2014) A Drosophila in vivo screen identifies store-operated
calcium entry as a key regulator of adiposity. *Cell Metab* 19(2):331–343.

14. Deng H, Gerencser AA, Jasper H (2015) Signal integration by Ca(2+) regulates
intestinal stem-cell activity. *Nature* 528(7581):212–217.

15. Richhariya S, Jayakumar S, Kumar Sukumar S, Hasan G (2018) dSTIM and
Ral/Exocyst Mediated Synaptic Release from Pupal Dopaminergic Neurons Sustains
Drosophila Flight. *eneuro* 5(3).

16. Agrawal N, et al. (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(4):1301–1313.

17. Næssel DR, Winther AM (2010) Drosophila neuropeptides in regulation of physiology
and behavior. *Prog Neurobiol* 92(1):42–104.

18. Taghert PH, Nitabach MN (2012) Peptide neuromodulation in invertebrate model
systems. *Neuron* 76(1):82–97.

19. Zhang H, et al. (2009) Deletion of Drosophila insulin-like peptides causes growth
defects and metabolic abnormalities. *Proc Natl Acad Sci* 106(46):19617 LP-19622.

20. Næssel DR (2012) Insulin-producing cells and their regulation in physiology and
behaviour. *Can Journl Zool* 90:476–488.

21. Næssel DR, Broeck J Vanden (2016) Insulin/IGF signaling in Drosophila and other
insects: factors that regulate production, release and post-release action of the
insulin-like peptides. *Cell Mol Life Sci* 73(2):271–290.

22. Næssel DR, Enell LE, Santos JG, Wegener C, Johard HA (2008) A large population
of diverse neurons in the Drosophila central nervous system expresses short
neuropeptide F, suggesting multiple distributed peptide functions. *BMC Neurosci*
23. Lee KS, You KH, Choo JK, Han YM, Yu K (2004) Drosophila short neuropeptide F regulates food intake and body size. *J Biol Chem* 279(49):50781–50789.

24. Subramanian M, et al. (2013) Altered lipid homeostasis in Drosophila InsP3 receptor mutants leads to obesity and hyperphagia. *Dis Model Mech* 6(3):734–44.

25. J. CY, Gyunghee L, C. HJ, H. PJ (2005) Comparative analysis of Corazonin-encoding genes (Crz's) in Drosophila species and functional insights into Crz-expressing neurons. *J Comp Neurol* 482(4):372–385.

26. Rao S, Lang C, Levitan ES, Deitcher DL (2001) Visualization of neuropeptide expression, transport, and exocytosis in Drosophila melanogaster. *J Neurobiol* 49(3):159–172.

27. Fosque BF, et al. (2015) Neural circuits. Labeling of active neural circuits in vivo with designed calcium integrators. *Science* 347(6223):755–760.

28. Calleja M, Moreno E, Pelaz S, Morata G (1996) Visualization of gene expression in living adult Drosophila. *Science* 274(5285):252–255.

29. Varga K, et al. (2016) Loss of Atg16 delays the alcohol-induced sedation response via regulation of Corazonin neuropeptide production in Drosophila. *Sci Rep* 6:34641.

30. West AE, Griffith EC, Greenberg ME (2002) Regulation of transcription factors by neuronal activity. *Nat Rev Neurosci* 3:921.

31. Richhariya S, Jayakumar S, Abruzzi K, Rosbash M, Hasan G (2017) A pupal transcriptomic screen identifies Ral as a target of store-operated calcium entry in Drosophila neurons. *Sci Rep* 7:42586.

32. Kubrak OI, Lushchak O V, Zandawala M, Nässel DR (2016) Systemic corazonin signalling modulates stress responses and metabolism in Drosophila. *Open Biol* 6(11):160152.

33. Chintapalli VR, Wang J, Dow JAT (2007) Using FlyAtlas to identify better Drosophila
melanogaster models of human disease. *Nat Genet* 39(6):715–720.

34. Wegener C, Herbert H, Kahnt J, Bender M, Rhea JM (2011) Deficiency of prohormone convertase dPC2 (AMONTILLADO) results in impaired production of bioactive neuropeptide hormones in Drosophila. *J Neurochem* 118(4):581–595.

35. Dawydow A, et al. (2014) Channelrhodopsin-2–XXL, a powerful optogenetic tool for low-light applications. *Proc Natl Acad Sci* 111(38):13972.

36. Luo J, Liu Y, Nassel DR, Nässel DR (2013) Insulin/IGF-regulated size scaling of neuroendocrine cells expressing the bHLH transcription factor Dimmed in Drosophila. *PLoS Genet* 9(12):e1004052.

37. Park D, Veenstra JA, Park JH, Taghert PH (2008) Mapping peptidergic cells in Drosophila: where DIMM fits in. *PLoS One* 3(3):e1896.

38. Lee G, et al. (2008) Developmental regulation and functions of the expression of the neuropeptide coronasin in Drosophila melanogaster. *Cell Tissue Res* 331(3):659–673.

39. Kapan N, Lushchak O V, Luo J, Nässel DR (2012) Identified peptidergic neurons in the Drosophila brain regulate insulin-producing cells, stress responses and metabolism by coexpressed short neuropeptide F and coronasin. *Cell Mol Life Sci* 69(23):4051–4066.

40. Johnson EC, et al. (2010) Altered Metabolism and Persistent Starvation Behaviors Caused by Reduced AMPK Function in Drosophila. *PLoS One* 5(9):e12799.

41. Tayler TD, Pacheco DA, Hergarden AC, Murthy M, Anderson DJ (2012) A neuropeptide circuit that coordinates sperm transfer and copulation duration in Drosophila. *Proc Natl Acad Sci U S A* 109(50):20697–20702.

42. McClure KD, Heberlein U (2013) A Small Group of Neurosecretory Cells Expressing the Transcriptional Regulator apontic and the Neuropeptide coronasin Mediate Ethanol Sedation in Drosophila. *J Neurosci* 33(9):4044–4054.
43. Sha K, et al. (2014) Regulation of Ethanol-Related Behavior and Ethanol Metabolism by the Corazonin Neurons and Corazonin Receptor in Drosophila melanogaster. *PLoS One* 9(1):e87062.

44. Lee KS, et al. (2008) Drosophila short neuropeptide F signalling regulates growth by ERK-mediated insulin signalling. *Nat Cell Biol* 10(4):468–475.

45. Selcho M, et al. (2017) Central and peripheral clocks are coupled by a neuropeptide pathway in Drosophila. *Nat Commun* 8:15563.

46. Chen W, et al. (2013) Regulation of sleep by the short neuropeptide F (sNPF) in Drosophila melanogaster. *Insect Biochem Mol Biol* 43(9):809–819.

47. Shang Y, et al. (2013) Short neuropeptide F is a sleep-promoting inhibitory modulator. *Neuron* 80(1):171–183.

48. van den Pol AN (2012) Neuropeptide transmission in brain circuits. *Neuron* 76(1):98–115.

49. Shakiryanova D, Zettel GM, Gu T, Hewes RS, Levitan ES (2011) Synaptic neuropeptide release induced by octopamine without Ca2+ entry into the nerve terminal. *Proc Natl Acad Sci U S A* 108(11):4477–4481.

50. Wang Y, et al. (2017) Ligand- and voltage-gated Ca2+ channels differentially regulate the mode of vesicular neuropeptide release in mammalian sensory neurons. *Sci Signal* 10(484):eaal1683.

51. Zhou Q, et al. (2016) Impairment of PARK14-dependent Ca(2+) signalling is a novel determinant of Parkinson’s disease. *Nat Commun* 7:10332.

52. Veenstra JA (2009) Does corazonin signal nutritional stress in insects? *Insect Biochem Mol Biol* 39(11):755–762.

53. Miyamoto T, Amrein H (2014) Diverse roles for the Drosophila fructose sensor Gr43a. *Fly (Austin)* 8(1):19–25.

54. Mishra D, et al. (2013) The Molecular Basis of Sugar Sensing in *Drosophila* Larvae.
55. Johnson EC, et al. (2005) A novel diuretic hormone receptor in Drosophila: evidence for conservation of CGRP signaling. *J Exp Biol* 208(Pt 7):1239–1246.

56. Jékely G (2013) Global view of the evolution and diversity of metazoan neuropeptide signaling. *Proc Natl Acad Sci* 110(21):8702 LP-8707.

57. Hartenstein V (2006) The neuroendocrine system of invertebrates: a developmental and evolutionary perspective. *J Endocrinol* 190(3):555–570.

58. Onaka T, Takayanagi Y, Leng G (2010) Metabolic and stress-related roles of prolactin-releasing peptide. *Trends Endocrinol Metab* 21(5):287–293.

59. Secondo A, Bagetta G, Amantea D (2018) On the Role of Store-Operated Calcium Entry in Acute and Chronic Neurodegenerative Diseases. *Front Mol Neurosci* 11:87.

60. Popugaeva E, Bezprozvanny I (2013) Role of endoplasmic reticulum Ca2+ signaling in the pathogenesis of Alzheimer disease. *Front Mol Neurosci* 6:29.

61. Schachtner J, Wegener C, Neupert S, Predel R (2010) Direct peptide profiling of brain tissue by MALDI-TOF mass spectrometry. *Methods Mol Biol* 615:129–135.