Figures and figure supplements

Drosophila larval to pupal switch under nutrient stress requires IP$_3$/Ca$^{2+}$ signalling in glutamatergic interneurons

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Figure 1. Pupariation in a protein-deprived environment requires intracellular calcium signaling in neurons. (A) Representative images of larvae and pupae of indicated genotypes subjected to either protein-deprived diet (PDD) or normal diet (ND). Percentages refer to pupariation. B and D Percentage pupariation of indicated genotypes represented as mean ± SEM over hours after transfer to the indicated media at 80–88 hr after egg laying (AEL). Dotted lines indicate 50% viability. C and E Bars represent mean percentage pupariation at 120 hr (± SEM). Larvae were transferred to the indicated diet at 80–88 hr AEL. All pupariation experiments were performed with N ≥ 6 batches, with 25 larvae in each batch. Bars with the same alphabet represent statistically indistinguishable groups (one-way ANOVA with a post hoc Tukey’s test p < 0.05).

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Figure 1—figure supplement 1. Pupariation under protein-deprivation requires neuronal IP$_3$R. (A) Schematic representation of the amino acid starvation paradigm. (B) High-magnification images of the anterior region of 80–88 hr AEL larvae of indicated genotypes. Bars show indicated genotypes subjected to lipid-depleted food (C) and 100 mM sucrose (PDD) supplemented with 5x RPMI (growth supplement) (D). N ≥ 6 batches with 25 larvae each. (E) Bars represent normalized food intake as measured by amount of red dye fed mixed with food. No significant interaction was observed between genotype and diet (p = 0.98). N ≥ 6 batches with 25 larvae each. (F) Bars represent weight of indicated genotypes at 80–88 hr AEL. Bars with the same alphabet represent statistically indistinguishable groups (one-way ANOVA with a post hoc Tukey's test p<0.05). For two-way ANOVA, numbers represent the variable genotype and alphabets represent diets (p<0.05).

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**Figure 2.** Knockdown of the IP$_3$R in glutamatergic neurons prevents pupariation upon PDD. (A) Expression patterns of GAL4 drivers used in (B) determined using UAS-eGFP and co-stained with anti-nc82. (B and C) Bars show mean percentage pupariation (± SEM) of the indicated genotypes on PDD. N ≥ 6 batches with 25 larvae each. (D) Images of selected substacks of the ventral ganglion of VGN6341-GAL4, with and without tsh-GAL80, expressing UAS-eGFP, double labelled with anti-nc82. (E) Selected substacks showing overlap of all dvGlut-positive cells and GFP-positive cells marked by VGN6341-GAL4 in the ventral ganglion. Scale bars indicate 50 µm. Bars with the same alphabet represent statistically indistinguishable groups (one-way ANOVA with a post hoc Tukey’s test p<0.05).

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Figure 2—figure supplement 1. Knockdown of the IP$_3$R in glutamatergic neurons prevents pupariation upon protein-deprivation. (A and B) Bars indicate mean percentage pupariation (± SEM) upon itpr knockdown with different neuronal drivers on PDD. N ≥ 6 batches with 25 larvae each. (C) VGN6341-GAL4 driven GFP-positive cells overlap with dvGlut-positive cells in the larval ventral ganglion. Scale bar indicates 50μm. Bars with the same alphabet represent statistically indistinguishable groups (one-way ANOVA with a post hoc Tukey’s test p<0.05). DOI: 10.7554/eLife.17495.006
Figure 3. Cholinergic inputs convey nutrient-stress signals to glutamatergic neurons of the ventral ganglion. (A) Bars indicate mean percentage pupariation (± SEM) of indicated genotypes subjected to PDD. N ≥ 6 batches with 25 larvae each. (B) Schematic illustrating the setup used to image
neurons of interest from the larval ventral ganglion. (C) Representative images showing calcium activity measured by GCaMP6m in the mVG neurons of indicated genotypes at indicated time points from a time series. (D and E) Traces represent time series of the mean normalized GCaMP6m responses (± SEM) from the mVG neurons of the indicated genotypes upon stimulation with carbamylcholine (CCh). (F) and (G) Box plots represent Area under the Curve (F) and Peak change in fluorescence (G) quantified from (E). In box plots, center lines show the medians; box limits indicate the 25th and 75th percentiles, whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, open circles represent each data point and numbers below represent total number of cells measured. (H) Bars indicate mean percentage pupariation (± SEM) of the indicated genotypes on PDD. N ≥ 6 batches with 25 larvae each. (I) Image showing GRASP between pickpocket-GAL4 and VGN6341-LexA. J and K Traces represent time series of mean normalized GCaMP6m responses (± SEM) from mVG glutamatergic cells upon optogenetic activation of either cholinergic (J) or aminergic (K) domains. Grey box indicates duration of optogenetic activation. Bars with the same alphabet represent statistically indistinguishable groups (one-way ANOVA with a post hoc Tukey’s test p<0.05).

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Figure 3—figure supplement 1. Identification of GPCRs that stimulate IP$_3$-mediated calcium signaling in response to PDD. (A) Schematic represents the total number of genes tested in the forward genetic screen with RNAi lines for GPCRs and the modifier screen with AcGq and dSTIM yielding the validated hits. The hits are summarized in Table 1 N ≥ 3 batches with 25 larvae each.
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Figure 3—figure supplement 2. Cholinergic neurons are important for responding to nutrient stress. A and B Traces represent time series of mean normalized GCaMP6m responses (± SEM) from the glutamatergic cells in the mVG. (A) Stimulation with either saline or carbachol after incubation with a mAChR specific antagonist, atropine. (B) GCaMP6m and RFP channels upon CCh stimulation. (C) Area under the curve of indicated genotypes on ND calculated from time series curves in Figure 3D. D and E Area under the curve for indicated genotypes at either 2 hr or 18 hr on ND (D) and PDD (E). The response in the mutant was reduced irrespective of media at 2 hr. On ND, the response was unchanged over time in both, control and itpr mutant. However, the response declined over time on PDD in both. Correspondingly, there was a significant interaction between the mutation and time on PDD (p<0.001) but not on ND (p = 0.91) by two-way ANOVA; same numbers represent the variable genotype, and same alphabets represent the variable time as statistically indistinguishable (p<0.05).

F G and H Bars represent mean percentage pupariation (± SEM) of the indicated genotypes when synaptic activity is blocked using UAS Shi′. Animals were subjected to restrictive (29°C) or permissive (22°C) temperature for 48 hr from 80 to 88 hr AEL on indicated media. N >5 batches of 25 larvae each. I Bars represent mean percentage pupariation (± SEM) of the indicated genotypes on Figure 3—figure supplement 2 continued on next page
Figure 3—figure supplement 2 continued

PDD. N > 4 batches of 25 larvae each. Bars with the same alphabet represent statistically indistinguishable groups (one-way ANOVA with a post hoc Tukey’s test p<0.05).

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Figure 3—figure supplement 3. ppk class IV multidendritic neurons activate VGN6341 marked glutamatergic interneurons. A and B Complete confocal stacks (z-project) of GFP and nc82 patterns obtained when the two GRASP constructs (UAS-CD4GFP1-10; LexAop-CD4GFP11) are driven by either ppk-GAL4 (A) or VGN6341-LexA (B). (C) Expression pattern of ppk-QF in the larval brain. (D) Traces represent time series of mean normalized jRCaMP1b responses (± SEM) from mVG glutamatergic cells upon optogenetic activation of the ppk domain. Blue box indicates duration of optogenetic activation.

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Figure 4. Glutamatergic neurons in the larval ventral ganglion project to peptidergic neurons in the mNSC. A and B Selected confocal stacks showing the neurites marked by VGN6341-GAL4 driven UAS-eGFP (green) and their

Figure 4 continued on next page
merged patterns with dimm-LexA-driven expression of LexAop-mCherry (red). The boxed area in A is shown in B as a high-magnification image. Arrow heads indicate VGN6341-GAL4 expressing neurites projecting toward the mNSCs. Asterisks mark dimm-LexA labelled projections. (C) Neurites marked by VGN6341-GAL4-driven eGFP (arrow heads) overlap with projections of the mNSCs marked by Dilp2mCherry (asterisks). (D) Selected high-magnification confocal images of VGN6341-GAL4 driven UAS-eGFP with an anterior projecting neurite from a midline mVG neuron. The white arrow head marks the same co-ordinates in all three images. The yellow arrow head shows the ascending projections. Scale bars represent 50 μm in A and B and 10 μm in C and D.

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Figure 5. Glutamatergic neurons in the larval ventral ganglion convey signals to peptidergic neurons of the mNSC. (A) High-magnification images of the mNSC area in a GRASP experiment between the peptidergic and the glutamatergic domains stained for GFP and Dilp2. (B) Bars represent mean percentage pupariation. 

dimm-LexA > LexAop-GCaMP6m; UAS-Chrimson 
- No GAL4  ● VGN6341  ● VGN6341-GAL4; ilpr Mutant

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percentage pupariation (± SEM) of larvae subjected to mGluR₆ knockdown using indicated GAL4 drivers on PDD. N ≥ 6 batches with 25 larvae each.

(C) A z-project of selected substacks at higher magnification showing the mNSC region from (A). Scale bar indicates 10 μm. Arrow heads point to weakly stained cells. (D) Confocal images showing the mNSC region of the Dilp2-GAL4 simultaneously driving an axonal and dendritic marker (Dilp2>UAS-DenMark, UAS-SyteGFP). E and F Traces represent time series of mean normalized GCaMP responses (± SEM) from peptidergic cells in the mNSC of the indicated genotypes on ND (E) or PDD (F). G and H Quantification of area under the curve from (E) and (F). Box plots and symbols are as described for Figure 3F. (I) Bars represent mean percentage pupariation (± SEM) of indicated genotypes subjected to PDD. N ≥ 6 batches with 25 larvae each. Scale bars indicate 50 μm unless specified otherwise Bar s with the same alphabet represent statistically indistinguishable groups (one-way ANOVA with a post hoc Tukey’s test p<0.05).

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Figure 5—figure supplement 1. IP$_3$R signaling in glutamatergic neurons is required for activation of medial neurosecretory cells. (A) Confocal image showing GRASP between the peptidergic (dimm-GAL4) and the glutamatergic (VGN6341-LexA) domains stained for GFP and Dilp2. (B) Bars represent mean percentage pupariation (± SEM) of indicated genotypes on PDD. N ≥ 6 batches with 25 larvae each. (C) and (D) Representative images of the mNSC showing GCaMP6m response upon thermogenic activation by VGN6341-GAL4. (E) Traces represent time series of mean normalized GCaMP6m responses (± SEM) from mNSCs of the indicated genotypes upon thermogenic activation using VGN6341-GAL4. The grey box indicates duration of thermogenic activation. (F) Traces represent time series of mean normalized GCaMP6m responses (± SEM) from the VGN6341 neurons upon self-optogenetic activation with CsChrimson. The red box indicates duration of optogenetic activation. (G) Area under the curves of GCaMP6m responses from the VGN6341 neurons upon self-optogenetic activation. (H) Traces represent time series of mean GCaMP6m responses (± SEM) from the mNSC in itpr mutants upon activation of glutamatergic cells either acutely or chronically. (I) Traces represent time series traces of mean GCaMP6m responses (± SEM) of oscillating cells in the mNSC (from E) observed upon thermogenic activation of VGN6341 neurons in control larvae (4/33 on ND and 17/36 on PDD). Bars with the same alphabet represent statistically indistinguishable groups (one-way ANOVA with a post hoc Tukey’s test p<0.05).

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Figure 5—figure supplement 2. Neurons of the mVG activate peptidergic neurons in the mNSCs. A and B A z-project image of GFP and the nc82 patterns obtained when either dimm-GAL4 (A) or VGN6341-LexA (B) drive the complete GRASP constructs (UAS-CD4GFP\textsuperscript{1-10}, LexAop-CD4GFP\textsuperscript{11}). The VGN-6341-LexA image is the same as that shown in Figure 3—figure supplement 2. (C) Traces represent time series of mean normalized GCaMP6m responses (± SEM) from the peptidergic mNSCs upon optogenetic activation. Red boxes indicate duration of optogenetic activation. The VGN6341-GAL4 activation trace is the same as that shown in Figure 5F.

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Figure 6. mACHR stimulation in glutamatergic neurons modulates enhanced peptide release from the mNSCs upon protein-deprivation. (A–C) Traces represent a time series of mean normalized peptide release (ANF::GFP; ± SEM) from mNSCs of the indicated genotypes upon Carbachol (CCh) stimulation. D and E Traces represent a time series of mean normalized peptide release (ANF::GFP; ± SEM) on PDD from the mNSCs upon optogenetic activation of VGN6341-GAL4 (E) and using CCh under acute inhibition from the VGN6341-GAL4 (F). Red and green boxes indicate duration of activation and inhibition, respectively. (F–I) Box plots of CCh stimulated peptide release (ANF::GFP) quantified by area under the curve from the mNSCs of the indicated genotypes on PDD. Box plots and symbols are as described for Figure 3F. Bars with the same alphabet represent statistically indistinguishable groups (one-way ANOVA with a post hoc Tukey’s test p<0.05). Figure 6 continued on next page
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Figure 7. mAChR stimulation of glutamatergic neurons modulates peptide release from varicosities at the ring gland upon protein-deprivation. (A) Time series of ANF::GFP release from varicosities in the ring gland of the indicated genotypes at the indicated time intervals, after stimulation by Carbachol (CCh). (B–D) Traces represent a time series of mean normalized peptide (± SEM) release from varicosities at the ring glands of the indicated genotypes after Carbachol (CCh) stimulation. (E) Box plots representing CCh-stimulated peptide release with ANF::GFP quantified by area under the curve of the indicated genotypes on PDD. (F–G) Traces represent a time series of mean normalized peptide release (ANF::GFP; ± SEM) from varicosities at the ring glands of indicated genotypes upon Carbachol (CCh) stimulation. (H) Box plots representing CCh-stimulated peptide release with ANF::GFP quantified by area under the curve of the indicated genotypes on PDD. (B–D) and (F–G). Box plots and symbols are as described for Figure 3F. Bars with the same alphabet represent statistically indistinguishable groups (one-way ANOVA with a post hoc Tukey’s test p<0.05).

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Figure 8. Glutamatergic neurons regulate Dilp2 release upon protein-starvation. (A) Dilp2 staining in larval brains from the indicated genotypes before and after stimulation with 50μM CCh for 30 min. (B) and (C) Box plots representing percentage release of Dilp2 from the respective genotypes subjected to ND or PDD. Box plots and symbols are as described for Figure 2F. A significant interaction was observed between genotype and diet (p<0.001). (D) Bars represent percentage pupariation as mean ± SEM of indicated genotypes on PDD. N ≥ 6 batches with 25 larvae each. Bars with the same alphabet represent statistically indistinguishable groups (one-way ANOVA with a post hoc Tukey’s test p<0.05). For two-way ANOVA, numbers represent the variable genotype and alphabets represent diets (p<0.05). DOI: 10.7554/eLife.17495.025
Figure 8—figure supplement 1. *dilp2* mRNA levels are not altered in the *itpr* mutant. (A) Bar graph of the mean fold change (± SEM) of *dilp2* mRNA levels in the larval CNS. All larvae were fed normal food until 82–86 hr AEL and then transferred to the respective media for 18 hr before dissection at 100–104 hr AEL. Two way ANOVA revealed a significant effect of diet (p = 0.00018) but not of the *itpr* mutation (p = 0.804) on *dilp2* mRNA levels. No significant interaction between diet, and the mutation was observed (p = 0.706). Same numbers represent the variable genotype, and same alphabets represent the variable time as statistically indistinguishable (p<0.05).

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Figure 9. IP₃R signaling in glutamatergic neurons regulates the expression of ecdysone biosynthetic genes during protein-deprivation. (A) Normalized fold changes in the mRNA levels of the indicated genes represented as means ± SEM at indicated time points after 83–85 hr AEL on PDD from wild-type ring glands (n ≥ 3). (B) Bars represent mean fold changes (± SEM) of expression levels of respective ecdysteroid-synthesizing genes as shown in (A) from the ring glands of indicated genotypes at indicated time points (n ≥ 5). Bars with the same alphabet represent statistically indistinguishable groups (one-way ANOVA with a post hoc Tukey’s test p<0.05). (C) Schematics of the neuronal circuit required for pupariation under protein-deprivation in early third instar larvae (80–88 hr AEL). Upon amino acid deprivation, glutamatergic neurons of the mVG are activated by ppk inputs. These glutamatergic neurons activate peptidergic cells in the mNSC to release peptides to further modulate ecdysteroid gene expression. In itpr mutants upon amino acid deprivation, glutamatergic inputs from the mVG to the mNSCs remain silent. (D) Schematic model of the signaling mechanisms observed in the circuit for pupariation under protein-deprived conditions.

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