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A multi-component neuronal response encodes the larval decision to pupariate upon amino acid starvation

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Title: A multi-component neuronal response encodes the larval decision to pupariate upon amino acid starvation

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Author Contributions

S.J., S.R., and G.H. conceived the study, S.J. and S.R. performed all experiments except for Figure 7f which was performed by B.K.D., S.J. and S.R. analysed data and wrote the manuscript with critical revisions from G.H.

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Abstract

Organisms need to co-ordinate growth with development, particularly in the context of nutrient availability. Thus, multiple ways have evolved to survive extrinsic nutrient deprivation during development. In *Drosophila*, growth occurs during larval development. Larvae are thus critically dependant on nutritional inputs but post critical weight they pupariate even when starved. How nutrient availability is coupled to the internal metabolic state for the decision to pupariate needs better understanding. We had earlier identified glutamatergic interneurons in the ventral ganglion that regulate pupariation on a protein-deficient diet. Here we report that *Drosophila* third instar larvae (either sex) sense arginine to evaluate their nutrient environment using an amino-acid transporter Slimfast. The glutamatergic interneurons integrate external protein availability with internal metabolic state through neuropeptide signals. IP_3 mediated calcium release and store-operated calcium entry are essential in these glutamatergic neurons for such integration and alter neuronal function by reducing the expression of multiple ion channels.

Significance Statement

Co-ordinating growth with development, in the context of nutrient availability is a challenge for all organisms in nature. Post attainment of “critical weight” insect larvae can pupariate even in the absence of nutrition. Mechanism(s) that stimulate appropriate cellular responses and allow normal development on a nutritionally deficient diet remain to be understood. Here, we demonstrate that nutritional deprivation, in post-critical weight larvae, is sensed by special sensory neurons

through an amino acid transporter that detects loss of environmental arginine. This information is integrated by glutamatergic interneurons with the internal metabolic state through neuropeptide signals. These glutamatergic interneurons require calcium-signalling regulated expression of a host of neuronal channels to generate complex calcium signals essential for pupariation on a protein-deficient diet.

Introduction

The coordination of organismal growth with development requires monitoring of nutrient availability and its integration with the internal metabolic state (Boulan et al., 2015). How nutrients inform developmental decisions however remains poorly understood. An example of a nutrient dependent developmental decision is pupation in insects (Nijhout and Williams, 1974). In *Drosophila*, third instar larvae feed voraciously and undergo an important developmental checkpoint based on their nutritional status which is the achievement of critical weight, when larvae can pupariate even upon subsequent starvation (Rewitz et al., 2013). Changes in neuro-endocrine signals that might encode the decision to pupariate post critical-weight need elucidation (Nijhout and Williams, 1974; Mirth et al., 2005; Rewitz et al., 2013).

Amino acid sensors like GCN2 (General Control Non-derepressible 2) and mTOR have been thought to encode responses to nutrient deprivation (Hao et al., 2005; Bjordal et al., 2014; Goberdhan et al., 2016) though recently a GCN2 independent mechanism was described (Leib and Knight, 2015). Interestingly, specific but different amino acids elicit differing responses upon encountering a nutrient poor diet in *Drosophila* (Bjordal et al., 2014; Corrales-Carvajal et al., 2016; Croset et al., 2016). Moreover, loss of amino acid alters the internal metabolic state and affects systemic behaviour (Ribeiro and Dickson, 2010; Fontana and Partridge, 2015; Corrales-Carvajal et al., 2016). In a recent study, we demonstrated that the

ability to pupariate post-critical weight in the absence of essential amino acids, requires cholinergic stimulation of the muscarinic acetylcholine receptor (mAChR) present on a set of glutamatergic interneurons in the ventral ganglion (Jayakumar et al., 2016). These glutamatergic neurons lie at the heart of a neural circuit required for the decision to pupariate under protein starvation and depend on intracellular calcium signalling. The nature of the sensory information received and its integration with stage-specific internal metabolic state(s) warranted further investigation.

The mechanism(s) by which intracellular calcium signalling in neurons modulates cell function is only beginning to be understood (Hartmann et al., 2014; de Juan-Sanz et al., 2017; Richhariya et al., 2017). Similar to what is known from immune cells (Feske, 2007), intracellular calcium signalling through the IP_3R and Store-operated calcium entry (SOCE) has recently been implicated in regulating gene expression in excitable cells (Somasundaram et al., 2014; Richhariya et al., 2017).

We report here that loss of environmental amino acids, especially arginine, elicits complex calcium transients in a population of glutamatergic neurons. These transients are shaped both by sensory inputs and neuro-peptidergic modulation. Gene expression analysis of the glutamatergic interneurons supports SOCE-regulated gene expression as a driver of neuronal plasticity required for handling nutrient stress during development.

Materials and Methods

Fly stocks and rearing

Drosophila strains were grown on cornmeal medium supplemented with yeast (Normal Diet, ND) as described in (Subramanian et al., 2013) at 25°C unless

otherwise noted. The protein-deprived diet (PDD) contained 100 mM sucrose with 1% agar. For the single amino acid rescues, corresponding amino acids at 1X concentration of the commercially available EAA Mixture (Gibco) were added to PDD. Single amino acids were obtained from Sigma-Aldrich and used at following concentrations: L-Arginine-2.995 mM, L-Cystine 0.5 mM, L-Histidine 1 mM, L-Isoleucine 2 mM, L-Leucine 2 mM, L-Lysine 1.98 mM, L-Methionine 0.505 mM, L-Phenylalanine 1 mM, L-Threonine 2 mM, L-Tryptophan 0.25 mM, L-Tyrosine 0.99 mM and L-Valine 2 mM. For optogenetic experiments, egg laying was carried out in ND supplemented with 200 µM all-trans-retinal (ATR), and larvae were transferred at 84 ± 4 h onto the ND with 400 µM ATR. A table of all stocks used is appended as Table 3. The *itpr IR* was used with *UAS-dicer2* in all experiments.

Pupariation assay

Larvae at 84 ± 4 h after egg laying (AEL) of either sex were transferred to desired media in batches of 25 and were scored for pupariation after ten days. At least six independent batches were performed for each genotype on each media. These are reported as percentage pupariation. For rate of pupariation, genotypes were monitored every 12 h after transfer of larvae.

Live imaging from larval central nervous systems

Larval CNS were dissected in hemolymph-like saline (HL3) (70 mM NaCl, 5 mM KCl, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose, 5 mM HEPES, 1.5 mM Ca^{2+}, pH 7.2), embedded in 0.2% low-melt agarose (Invitrogen), and bathed in HL3. GCaMP6m was used as the genetically encoded calcium sensor. ANF::GFP was expressed genetically to quantify vesicular release. For creating the effect of an acute loss of amino acid levels, we incubated the *ex vivo* preparations in

144 either 0.5x EAA, obtained from a 50x EAA mixture lacking glutamine (Thermo Fisher
145 Scientific) or 0.3 mM arginine (Sigma), dissolved in HL3. At the point of withdrawal,
146 the amino acid levels were diluted 10-fold using more HL3 thus creating the effect of
147 amino acids withdrawal. Mock withdrawals were performed by increasing the volume
148 without changing the amino acid concentrations. For recording from class IV
149 multidendritic neurons on the cuticle, semi-intact preparations were used where the
150 CNS along with the anterior half of the larva was embedded in 0.2% low-melt
151 agarose.

152 Images were taken as a time series on an XY plane across 6 Z-planes at an
153 interval of 4 s using a 10x objective with an NA of 0.4 and an optical zoom of 4 on a
154 Leica SP5 inverted confocal microscope with a resonant scanner at 8KHz. A Z-
155 project across time was then obtained as a time series. This time series across
156 depth was then used for further analysis. For optogenetic inhibition experiments, a
157 green 561-nm laser line was driven simultaneously with image acquisition scans
158 using the 488-nm laser line. Conversely, for activation experiments a 488-nm laser
159 was driven simultaneously while acquiring image scans with the 561-nm laser. All
160 live imaging experiments were performed with at least five independent CNS
161 preparations and the exact number of cells for each experiment is indicated in the
162 figures.

163 Raw images were extracted using Image J1.48 and regions of interest (ROI)
164 selected using the Time Series Analyser plugin (Balaji,
165 https://imagej.nih.gov/ij/plugins/time-series.html). $\Delta F/F$ was calculated using the
166 formula $\Delta F/F = (F_t-F_0)/F_0$, where F_t is the fluorescence at time t and F_0 is baseline
167 fluorescence corresponding to the average fluorescence over the first ten frames.
168 Any cell in which the GCaMP signal rose above an arbitrarily value of $\Delta F/F = 1.5$ at

any point post withdrawal or stimulation was classified as a responder. Percent responders were calculated as the (number of responder cells / total number of cells) x 100. ROI heatmaps, were mapped on to segments T3-A5 of the CNS and then percentage responders in each segment were plotted using Matrix2png (Pavlidis and Stafford Noble, 2003). Area under the curve was calculated from the point of stimulation till 600 s using Microsoft Excel (Microsoft) and plotted using BoxPlotR (Spitzer et al., 2014).

For measuring peptide release decrease in intracellular fluorescence was measured and quantified as $\Delta F/F$ Release calculated by $(F_0-F_t)/F_0$ where F_t is the fluorescence at time t and F_0 is baseline fluorescence corresponding to the average fluorescence over the first 10 frames. Area under the curve was calculated from point of withdrawal to 600s using Microsoft Excel (Microsoft), and box plots were plotted using BoxPlotR (Spitzer et al., 2014). Peptide sequences used were FMRFa-DPKQDFMRFa (NeoBioLab), CCHamide-2 - GCQAYGHVCYGGH-NH2 and Allatostatin - SRPYSFGL-NH2 (LifeTein, LLC).

Immunohistochemistry

To visualize CaLexA-GFP, larvae were either exposed to normal diet or protein deprived diet for 12 hours. The CNS were then dissected in cold PBS, fixed with 4% paraformaldehyde, washed with 0.2% PTX, blocked and incubated overnight in primary rabbit anti-GFP antibody (1:10,000; A6455, Life Technologies, RRID:AB_221570). They were then washed and incubated with an anti-rabbit Alexa Fluor 488 (#A1108, Life Technologies, RRID:AB_143165) and mounted. Confocal images were obtained on the Olympus Confocal FV1000 microscope (Olympus) with

192 a 20x, 0.7 NA objective. Images were visualized using either the FV10-ASW 4.0
193 viewer (Olympus) or Fiji (RRID:SCR_002285) (Schindelin et al., 2012).

194 **RNA-seq from larval central nervous systems**

195 RNA was isolated from approximately 15 larval CNS of 84(±1) h AEL larvae of
196 both control *(UAS-itpr IR/+; UAS-dicer2/+)* and *itpr* KD *(VGN6341GAL4>UAS-itpr IR;*
197 *UAS-dicer2)* genotypes using Trizol (Thermo Fisher Scientific) following
198 manufacturer's protocol. Libraries with approximately 500ng total RNA per sample
199 were prepared as described in (Richhariya et al., 2017). Libraries were run on a
200 Hiseq2500 platform at AgriGenome labs Pvt. Ltd. Biological triplicates were
201 performed for the control and duplicates were performed for *itpr* KD.

202 Per sample, 45-73 million reads were obtained after sequencing. Sequencing
203 reads were aligned to the dm6 release of the *Drosophila* genome using TopHat
204 (Trapnell et al., 2009) and >97% mapping was obtained for all samples. PCR
205 duplicates were identified and removed using Samtools (Li et al., 2009)
206 (http://samtools.sourceforge.net). 18-23% reads were unique and correspond to 10-
207 14 million reads. These unique reads were used for further analysis. Differential
208 expression analysis was performed using three independent methods - CuffDiff2
209 (Trapnell et al., 2013), DESeq (Anders and Huber, 2010) and edgeR (Robinson et
210 al., 2010). Alignment files obtained from TopHat were used for differential analysis
211 with CuffDiff2. Read counts were also obtained by GenomicRanges (Lawrence et al.,
212 2013) which were then used to estimate differential expression by DESeq and
213 edgeR. A fold change cut-off of a minimum 25% change was used. Genes with non-
214 zero values in both conditions were considered. Significance cut-off was set at q-
215 value<0.05 for CuffDiff2 and FDR-corrected p-value<0.05 for DESeq and edgeR.
216 Heat maps were generated using Matrix2png (Pavlidis and Stafford Noble, 2003).

Comparison of gene lists and generation of Venn Diagrams was carried out using Whitehead BaRC public tools (http://jura.wi.mit.edu/bioc/tools/). Gene ontology analysis for molecular function was carried out using two platforms - GOrilla (Eden et al., 2009) and Panther GO-slim (Mi et al., 2017). In both methods, 287 down-regulated genes were used as the target set and all genes in *Drosophila* were used as background.

Cell Sorting

Fluorescence-activated cell sorting (FACS) was used to sort cells of interest from larval CNS, where neurons of interest were genetically labelled by green fluorescent protein using the GAL4/UAS system. Control (*VGN6341-GAL4>UAS-eGFP*) and *dStim* KD (*VGN6341-GAL4>eGFP; UAS-dStim IR; UAS-dcr2*) CNS were dissected in Schneider's medium (Thermo Fisher Scientific). Approximately 20 CNS were pooled per sample. These CNS were treated with an enzyme solution (0.75 µg/µl collagenase and 0.4 µg/µl dispase in Schneider's medium) at room temperature for 30 min. They were then washed and re-suspended in cold Schneider's medium and gently triturated several times using a pipette tip to obtain a single-cell suspension. This suspension was then passed through a 40µm mesh filter to remove clumps and kept on ice until sorting (less than an hour). Flow cytometry was performed on a FACS Aria cell sorter (BD Biosciences). The threshold for GFP positive cells was set using dissociated neurons from a non-GFP expressing wild-type strain, *Canton S*. The same gating parameters were used to sort other genotypes in the experiment. GFP-positive cells were collected directly in Trizol and then frozen immediately in dry ice until further processing.

RNA isolation and qRT-PCR from sorted cells

Approximately 1200 GFP cells were collected per sample in 500ul Trizol (Thermo Fisher Scientific) and frozen in dry ice until processing. RNA was isolated following the manufacturer's protocol using glycogen as a carrier (total 5 µg per sample). Isolated RNA was re-constituted in 8ul nuclease free water and all of it was used for making cDNA. cDNA synthesis was carried out using the SMART-Seq v4 Ultra Low Input RNA Kit (Clontech) following manufacturer's instructions. 10 cycles of PCR were performed for amplification. Three independent sets of sorted cells were used for each genotype. Quantitative real time PCRs (qPCRs) were performed in a total volume of 10 µl with Kapa SYBR Fast qPCR kit (KAPA Biosystems) on an ABI 7500 fast machine (Applied Biosystems). Technical duplicates were performed for each qPCR reaction. A melt analysis was performed at the end of the reaction to ensure the specificity of the product. The fold change of gene expression in any experimental condition relative to control was calculated as $2^{-\triangle\triangle Ct}$ where $\triangle\triangle Ct$ = (Ct (target gene) –Ct (housekeeping gene))$_{Expt.}$ - (Ct (target gene) – Ct (housekeeping gene))$_{Control}$. *Act5c* (Fig. 7), *rp49* and *tubulin* (Table 2) were used as housekeeping gene controls and all three yielded similar results. All primer sequences are listed in Table 4.

RNA isolation and qRT-PCR for *slif*

Body wall preparations from the anterior half of the larvae were obtained by dissecting the skin in phosphate buffer saline prepared in double distilled water treated with diethyl pyrocarbonate (Sigma) and washing to remove any remnant fat body. Body walls from five larvae were pooled for one sample and these were homogenized in 500 µl TRIzol (Thermo Fisher Scientific) by vortexing immediately after dissection. At least three biological replicate samples were performed for each genotype. After homogenization, the sample was kept on ice and processed within

30 min or stored at −80°C until processing for up to 4 weeks. RNA was isolated following manufacturer's protocol. Purity of the isolated RNA was estimated by NanoDrop spectrophotometer (Thermo Scientific) and integrity was determined by running it on a 1% Tris-EDTA agarose gel.

Approximately 500 ng of total RNA was used per sample for cDNA synthesis. DNAse treatment and first strand synthesis were performed as described previously (Richhariya et al., 2017). Quantitative real-time PCRs (qPCRs) were performed in a total volume of 10 µl with Kapa SYBR Fast qPCR kit (KAPA Biosystems) on an ABI QS3 fast machine (Applied Biosystems). Technical duplicates were performed for each qPCR reaction. A melt analysis was performed at the end of the reaction to ensure the specificity of the product. The fold change of gene expression in any experimental condition relative to the RNAi control was calculated as $2^{-\triangle\triangle Ct}$. *rp49* was used as the housekeeping gene control.

Preference assay

To record larval preference, Arginine was obtained from Sigma-Aldrich. 1% agarose solution was prepared and cooled to <50°C before adding the desired quantity of arginine to reach the final concentration of 20 mM. The plate was divided into four agarose quadrants, two with and two without arginine. The quadrants were diametrically opposite to one another. To visually differentiate the quadrants, we used Bromophenol blue on the agarose only quadrants. To ensure that preference was not affected by colour, we also performed experiments where the colour was mixed in the arginine quadrants and did not observe any difference in preference. Plates were prepared fresh roughly two hours before the behavioural assays were performed. Third instar larvae were collected, rinsed with double distilled water and

starved for at 1.5-2 hours on 1% agarose. Groups of 20 animals were placed at the centre of each Petri dish under diffuse light. The plates were not covered and any larvae that came out were ignored for final analyses. We scored for preference at 10 minutes post addition of the larvae. For optogenetic inhibition experiments, we additionally illuminated the arena with a green LED (Thorlabs) with a central wavelength of 525 nm. Groups of at least 15 animals were placed and larvae were scored for assessing preference index at 8 minutes post the green LED being turned on as well as 8 minutes post the green LED being turned off.

The arena was custom designed from styrofoam and the videos were acquired using a PiCamera coupled to a Raspberry Pi 3.

Data Representation and Statistics

All bar graphs and line plots indicate means and error bars, standard error of means. In the box plots, horizontal lines in the box represent medians, crosses indicate the means, box limits indicate the 25th and 75th percentiles, whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, individual data points are represented as open circles and the numbers below represent the n number for each box. All statistical tests are mentioned in the figure legends and were performed using Origin 8.0. Figure1-1 has all statistical tests performed for each figure and their exact p-values.

Results

Ca^{2+} transients in glutamatergic neurons encode environmental arginine levels

Glutamatergic neurons located in segments T3-A5 of the ventral ganglion (VG) marked by the *vglut*^{VGN6341GAL4} (*VGN6341-GAL4* henceforth) are integral for the decision to pupariate on a protein-deficient diet (PDD) (Jayakumar et al., 2016). To

314 test if *VGN6341-GAL4* marked glutamatergic interneurons in the central nervous
315 system (CNS) of third instar larvae respond to the loss of dietary protein, we
316 expressed in them an activity dependent reporter, CaLexA (Masuyama et al., 2012).
317 CNS from larvae placed in a protein-deprived diet (PDD) showed a subset of
318 CaLexA positive neurons whereas larvae on a normal diet did not, indicating that
319 loss of dietary protein activates a set of glutamatergic neurons in the ventral ganglion
320 (Figure 1a).

321 To test the real-time effect, if any, of amino acid withdrawal on *VGN6341-*
322 *GAL4* marked glutamatergic interneurons calcium transients were measured from
323 semi-intact *ex vivo* preparations. Here, an artificial environment of nutrient
324 withdrawal was created by dilution of amino acids (see Methods, Figure 1b and 1c).
325 Withdrawal of essential amino acids (EAA) from the environment of third instar larval
326 CNS yielded robust calcium transients in about 50% of marked neurons as reported
327 by the calcium sensor GCaMP6m (Figure 1d, 1e and Figure 2c). To understand if
328 there existed a specific subset of glutamatergic interneurons in segments T3-A5 that
329 respond consistently to amino-acid withdrawal, we quantified the responsive cells in
330 each hemi-segment. Interestingly, cells that responded within the T3-A5 region did
331 not explicitly follow an anatomical bias for any segment or hemi-segment across
332 CNS from multiple larvae (Figure 1f).

333 The observed calcium transients were specific to mid-third instar larvae, as
334 they were not elicited from glutamatergic neurons in the VG upon EAA withdrawal
335 from the CNS of second instar larvae (Figure 1e, Figure 2c and d). These data
336 indicate that the responses for sensing nutrient withdrawal are stage-specific and are
337 possibly required for the decision to pupariate. In agreement with this idea,
338 optogenetic activation of *VGN6341-GAL4* marked neurons of mid-third instar larvae,

339 on a normal diet, resulted in premature pupariation (Figure 1g, 1h, and 1i), where the
340 puparia appeared visually similar to controls (Figure 1g). However, they were not
341 viable (Figure 1i) possibly because they lacked other physiological parameters for
342 normal pupariation.

343 We hypothesized that the observed calcium transients in glutamatergic
344 neurons encode a neural decision to continue development and pupariate under
345 nutrient stress. To understand the molecular basis of the observed neuronal calcium
346 transients, we first tested the nature of calcium signalling required for generating
347 these transients. We previously identified intracellular calcium signalling in this
348 subset of glutamatergic neurons as essential for pupariation on a protein deficient
349 diet (PDD) (Jayakumar et al., 2016). Indeed, calcium transients observed upon EAA
350 withdrawal in glutamatergic neurons in the VG were significantly attenuated upon
351 knockdown of components of intracellular calcium signalling and store-operated
352 calcium entry (SOCE), such as the IP_3R, dSTIM and dOrai (Figure 2a, 2c and 2d).
353 The loss of transients also correlated with loss of pupariation on PDD in these
354 genotypes (Figure 2b).

355 Glutamatergic neurons in the *Drosophila* ventral ganglion include both motor
356 neurons and interneurons. To test if *VGN6341-GAL4* marked neurons were indeed
357 interneurons and did not include many motor neurons an optogenetic activation
358 experiment was performed. Disruption of motor neuron activity followed by freezing
359 behaviour has been demonstrated earlier upon optogenetic activation of motor
360 neurons (Dawydow et al., 2014). Similar freezing behaviour was evident upon
361 optogenetic activation of larval motor neurons marked by *OK6-GAL4* (Figure 2e and
362 2f). However, normal larval movement was evident upon optogenetic activation of

363 the *VGN6341-GAL4* neurons (Figure 2e and 2f) indicating that a majority are likely to
364 be interneurons and are not involved in larval locomotion.

365 Next, we tested if specific amino acids were required in the diet for pupariation
366 of larvae with IP_3R knockdown in neurons of the ventral ganglia. We tested
367 supplementation of PDD with all essential amino acids (except glutamate). Strikingly,
368 supplementation of arginine alone was sufficient for a complete rescue of the
369 pupariation deficit observed upon reduced intracellular calcium signalling in larvae at
370 84 h after egg-laying (AEL) (Figure 3a and 3b). Withdrawal of arginine from the CNS
371 of 84 h AEL larvae resulted in robust calcium transients in about 42% cells compared
372 to 0% in cells with no change in environmental arginine levels (Figure 3d-3f).
373 Moreover, withdrawal of a mixture of EAAs except arginine elicited a less
374 pronounced response from just 27% cells and withdrawal of a mixture of non-
375 essential amino acids (NEAA) elicited a further attenuated response (Figure 3d-3f).
376 Cells that responded to arginine withdrawal did not exhibit an obvious anatomical
377 focus within T3-A5 segments of the ventral ganglion (Figure 3c) similar to what was
378 observed upon EAA withdrawal. These data indicate that although withdrawal of all
379 amino acids together yields a strong response, withdrawal of arginine alone is
380 sufficient to elicit calcium transients in glutamatergic interneurons.

381 Glutamatergic interneurons regulate peptide release from medial
382 neurosecretory cells (mNSC) required for pupariation on PDD (Jayakumar et al.,
383 2016). We thus tested the physiological relevance of transients observed upon loss
384 of arginine. Peptide release was monitored with ANF::GFP from all cells marked by
385 *dimm-LexA* that specifically drives expression in neuro-peptidergic cells (Rao et al.,
386 2001; Jayakumar et al., 2016). Withdrawal of arginine resulted in peptide release
387 from numerous peptidergic cells in the larval CNS (Figure 4a). Peptide release from

all the medial neurosecretory cells (mNSCs) elicited by withdrawal of arginine was attenuated by optogenetic inhibition of activity in glutamatergic neurons marked by *VGN6341-GAL4* (Figure 4a and 4b). Similar inhibition of peptide release was not observed from other peptidergic cells in the CNS (Figure 4c). Thus, though amino acid withdrawal elicits peptide release from various peptidergic cells, dependence of peptide release on glutamatergic neuron activity appears limited to the mNSCs (Figure 4b). Earlier, we observed that release of *Dilp2* from the mNSCs is diet dependant and stimulated by activation of *VGN6341-GAL4* neurons (Jayakumar et al., 2016). In agreement with this loss of pupariation was observed in larvae on PDD upon blocking peptide release from *Dilp2* producing cells of the mNSCs. On a normal diet however pupariation does not require peptide release from the mNSCs (Figure 4d). These results demonstrate that loss of dietary arginine stimulates calcium transients in glutamatergic interneurons and these transients stimulate peptide release from the mNSCs.

The mNSCs are known to send projections to the ring gland (Cao and Brown, 2001), and possibly stimulate synthesis of the moulting hormone ecdysone (Jayakumar et al., 2016). However, on a normal diet it is well established that the Prothoracicotropic hormone (PTTH), produced by a pair of bilateral neurons in the central brain, has an essential role in ecdysone production (McBrayer et al., 2007). Previous work has demonstrated that stimulation of glutamatergic interneurons marked by *VGN6341-GAL4* elicits peptide release from mNSCs, as monitored by ANF::GFP (Jayakumar et al., 2016). However, in a similar experiment, peptide release from the PTTH neurons was significantly lower than from the mNSCs (Figure 4e and 4f). Taken together these data suggest that peptide release from PTTH neurons is not stimulated directly by *VGN6341-GAL4* marked glutamatergic

interneurons. However, they do not rule out either an independent role of PTTH neurons or indirect stimulation of PTTH neurons by mNSC secreted peptides, in pupariation on PDD.

Peptidergic modulation of Ca^{2+} transients upon amino acid withdrawal

Reduction in environmental arginine levels elicited peptide release from various neuro-peptidergic cells in the brain (Figure 4a and 4b). A screen for receptors on glutamatergic neurons in the ventral ganglion, that modulate pupariation on PDD, identified several neuropeptide receptors as well as the mAChR, all of which couple with intracellular Ca^{2+}-signalling (Jayakumar et al., 2016). Calcium transients upon EAA withdrawal were lost upon knockdown of mAChR (Figure 2a, 2c and 2d). To test whether neuropeptide modulation upon amino acid withdrawal is additionally responsible for the observed calcium transients, we knocked down expression of three neuropeptide receptors viz. FMRFaR, CCHa2-R and AstA-R2 identified in the screen for pupariation deficits on PDD (Jayakumar et al., 2016). The number of cells that responded to withdrawal of amino acids fell to less than 10 percent when either FMRFaR or CCHa2-R were knocked down in glutamatergic neurons (Figure 5a-5c). Response amplitudes of the responding cells were however comparable to control cells (Figure 5a). On the other hand, knockdown of AstA-R2 resulted in both an increase in the percentage of responding cells and a significant increase in response amplitude (Figure 5a and 5b). Thus, EAA withdrawal mediated calcium transients in glutamatergic neurons are modulated by multiple neuropeptide receptors in addition to cholinergic inputs through the mAChR.

The ability of neuropeptides to stimulate calcium responses in the glutamatergic neurons of the VG was tested next. Calcium transients in

437 glutamatergic interneurons were significantly attenuated upon EAA withdrawal when
438 peptide release was blocked from neuropeptide secreting neurons (Figure 5d, 5e
439 and 5f). Interestingly, blocking of peptide release did not affect the response of
440 glutamatergic interneurons immediate to the event of amino acid deprivation, but
441 there was a significant effect on the maintenance of calcium transients (Figure 5e
442 and 5f). Direct addition of either FMRFa or CCHa2 yielded calcium transients from
443 34 and 43 percent of *VGN6341-GAL4* marked glutamatergic neurons respectively in
444 the VG (Figure 5i, 5j, 5l and 5m). Consistent with AstAR knockdown data (Figure
445 5a), addition of AstA peptide led to a significant decay in the calcium response
446 (Figure 5k and 5n). Glutamatergic neurons in the ventral ganglia thus receive
447 neuropeptide signals through FMRFa, AstA, CCHa2 and possibly other
448 neuropeptides in response to amino acid withdrawal.

449 The role of CCHa2 and FMRFa for pupariation on PDD was tested next.
450 Knockdown of CCHa2 specifically in the fat body led to pupariation deficits (Figure
451 5g). Similarly, blocking vesicle recycling and therefore peptide release from
452 established Tv FMRFa neurons in the larval CNS (Santos et al., 2007) resulted in
453 pupariation deficits upon protein deprivation (Figure 5h). These data support the idea
454 that glutamatergic neurons in the VG integrate information regarding protein
455 deprivation by neurohormonal peptide release from multiple internal tissues besides
456 the external environment.

457 **_ppk_ neurons express _slimfast_ for sensing arginine as a proxy for diet quality**

458 Next, we tested if glutamatergic neurons in the VG sense loss of arginine
459 directly. For this, we bath applied tetrodotoxin (TTX) to inhibit polysynaptic inputs.
460 Upon withdrawal of EAA in the presence of 2 μM TTX, calcium transients in the

461 glutamatergic neurons were abolished (Figure 6a) indicating that these glutamatergic
462 neurons respond to loss of arginine indirectly. A potential class of input neurons,
463 implicated from our earlier study (Jayakumar et al., 2016), were the class IV
464 multidendritic neurons labelled by *ppk-GAL4* which are cholinergic (Imlach et al.,
465 2012). Optogenetic inhibition of these multidendritic neurons with a light activated
466 chloride pump (halorhodopsin; eNpHR3) concurrent with withdrawal of amino acids
467 abolished the transients (Figure 6b) whereas inhibition after the onset of transients
468 failed to affect the maintenance of transients (Figure 6c). In agreement with previous
469 data (Jayakumar et al., 2016), optogenetic activation of ppk neurons activated
470 glutamatergic neurons marked by *VGN6341-GAL4* in third instar larval CNS.
471 Interestingly, optogenetic activation of *ppk* neurons activation of glutamatergic
472 neurons marked by *VGN6341* was absent in second instar larvae (Figure 6d). These
473 data indicate that cholinergic inputs from class IV multidendritic neurons initiate the
474 calcium transients upon loss of arginine in third instar larvae. The multidendritic
475 neurons marked by *ppk-GAL4* are nociceptive (Hwang et al., 2007; Zhong et al.,
476 2010) and their ability to sense arginine has not been reported previously.

477 A possible candidate as a sensor for arginine is the amino acid transporter
478 Slimfast (encoded by the gene *slif*) with a known preference for arginine (Boudko et
479 al., 2015; Croset et al., 2016), but its expression has been reported only in the larval
480 fat-body (Colombani et al., 2003). To test for *slif* expression in class IV multidendritic
481 neurons, *slif* levels were measured from the body wall of control larvae and larvae
482 expressing *slif* RNAi driven by *ppk-GAL4. slif* expression in the body wall was
483 significantly reduced upon knock down by *ppk-GAL4* in class IV multidendritic
484 neurons (Figure 6e). Larvae with *slif* knockdown in *ppk* neurons also exhibit
485 significant pupariation deficits on PDD (Figure 6f). The pupariation deficit could be

rescued to a significant extent by supplementing PDD with just arginine (Figure 6f). To test whether *ppk* neurons sense arginine directly, we performed withdrawal of either EAA or arginine alone and observed calcium transients under both conditions from *ppk* labelled neurons in semi-intact preparations (Figure 6g). Importantly, these transients were abrogated by *slif* knockdown in *ppk* neurons (Figure 6h). Taken together these data confirm that *slif* is required in class IV multidendritic neurons for the physiological response to loss of arginine in the diet.

Drosophila larvae exhibit an innate preference for arginine (Croset et al., 2016). Interestingly, knockdown of *slif* by *ppk-GAL4* attenuated this innate preference (Figure 6i and 6j). Requirement of class IV multidendritic neurons in sensing and mediating the response to arginine in real-time was confirmed by inhibiting *ppk* neurons with halorhodopsin. When activity in *ppk* neurons was inhibited, larvae lost their preference for arginine whereas upon removal of the inhibition, their innate preference for a diet with arginine was restored (Figure 6k). Thus, *slif* on ppk neurons appears to sense arginine in the environment, in addition to perhaps arginine levels in the hemolymph. Together these results suggest that arginine functions as a proxy for assessing diet quality in *Drosophila* larvae. This information encodes a real-time behavioural change, as well as a physiological response that controls the developmental decision to pupariate in the absence of arginine.

IP_3R mediated Ca^{2+}-release and SOCE regulate neuronal function through gene expression

Next, we addressed why loss of mAChR and neuropeptide receptor stimulated intracellular calcium signalling through the IP_3R and dSTIM abrogated the

polysynaptic Ca^{2+} transients in VG glutamatergic neurons observed upon withdrawal of essential amino acids (Figure 2b). IP_3-mediated Ca^{2+}-release is followed by dSTIM/dOrai mediated Store operated Ca^{2+}-entry (SOCE) in *Drosophila* neurons (Venkiteswaran and Hasan, 2009; Chakraborty et al., 2016). Both IP_3-mediated Ca^{2+} release and SOCE alter cytosolic Ca^{2+} levels and affect function in mammalian neurons (Futatsugi et al., 1999; Fujii, 2000; Hartmann and Konnerth, 2005) though the precise cellular mechanisms remain to be elucidated. STIM/Orai mediated SOCE also regulates gene expression in non-excitable and excitable cells (Feske, 2007; Richhariya et al., 2017). As the calcium transients are stage-specific (Figure 1e) we hypothesized that gene expression changes may underlie the ability of glutamatergic neurons to respond to nutrient stress and loss of Ca^{2+} transients in genotypes affecting intracellular Ca^{2+} signalling (Figure 2b) may arise in part from altered gene expression. To test this, we profiled the transcriptome of the third instar larval CNS with and without knockdown of the *IP_3R* by RNA-seq. Analysis by three independent methods (See Materials and Methods), identified expression of 20 genes as up-regulated and 287 genes as down-regulated in *IP_3R* knockdown larval CNS (Figure 7a and 7b, Figure7-1), indicating positive regulation of gene expression by IP_3-mediated Ca^{2+} signalling. *IP_3R* knockdown was confirmed by RNA-seq (Figure 7c). Importantly, gene ontology analysis of the 287 down-regulated genes revealed significant enrichment in categories of ion transport, membrane depolarization and synaptic signalling (Figure 7d, Table 1). Indeed, the expression of genes encoding various ion channels was down-regulated upon IP_3R knockdown (Figure 7e). Taken together, these data suggest that attenuation of calcium signalling through the IP_3R in the larval CNS of third instar larvae alters neuronal function by change in the expression of ion channel genes. To test whether such expression of ion channels is

the reason why glutamatergic interneurons in 2nd instar larval CNS remain unresponsive to EAA withdrawal, we compared expression of selected calcium signalling and ion channel genes between second and third instar larval brains (Figure 7f). The genes tested do not appear to be differentially expressed between the CNS of second and third instar larvae, indicating that the absence of response to EAA withdrawal in the ventral ganglia of 2nd instar larvae might not be a consequence of IP_3R/SOCE regulated gene expression.

To test if changes in ion-channel expression also occur in the subset of glutamatergic neurons where Ca^{2+}-transients are observed upon withdrawal of EAA we isolated these neurons by Fluorescence-activated cell sorting (FACS) post genetic labelling with GFP (Figure 7g, h). Because knockdown of *dStim* in glutamatergic neurons resulted in abrogation of calcium transients (Figure 2b) and a pupariation defect on PDD (Figure 2a), we tested the effect of *dStim* knockdown on the expression of specific ion channel genes identified from the RNA-seq. About 0.5 percent of total cells in the CNS were identified as GFP positive in the control sample where *UAS-eGFP* was driven with the *VGN6341-GAL4.* Knockdown of *dStim* had no effect on the number of GFP positive cells (Figure 7i). The sorted glutamatergic cells expressed *vGlut* (a marker for glutamatergic neurons) (Mahr and Aberle, 2006) at levels almost 3 fold higher than the housekeeping gene *rp49* (Figure 7j). Significant reduction in *dStim* levels was observed from control normalised to the housekeeping gene *Act5c* upon knockdown of *dStim* (Figure 7k) whereas levels of another housekeeping gene, *β-tubulin* did not change between the two conditions (Table 2). These data indicate that sorting was specific to the *VGN6341-GAL4* marked cells in the control and knockdown conditions. Importantly, mRNA levels of genes encoding mAChR (*mAChR-A;* Figure 7k*),* and several voltage-gated ion channels specific for

sodium (*NaCP60Ek)*, potassium (*Hk, eag*) and calcium (*cac, Ca-alpha1D*) were reduced upon knockdown of *dStim* (Figure 7K). This observation was similar to gene expression changes observed in the whole larval CNS (Figure 7e). Similar results were obtained when fold changes were calculated upon normalization to two other housekeeping genes, *rp49* and *tubulin* (Table 2). Moreover, knockdown of intracellular calcium signalling components in glutamatergic neurons, also attenuated the calcium response to a depolarizing stimulus (Figure 8a, b). These data support a role for SOCE in the maintenance of excitability in *VGN6341-GAL4* marked glutamatergic neurons by regulating expression of ion channels.

To test if reduction in ion channel gene expression is relevant for the calcium transients observed upon amino acid withdrawal, toxins specific to either sodium, potassium or calcium channels (Wu et al., 2008) were expressed with *VGN6341-GAL4*. Interestingly, such chronic inhibition of either Na^+, K^+ or Ca^{2+} ion channels caused only a modest reduction in the percentage of responding cells and did not alter the nature of the transients in the initial phase post-amino acid withdrawal (Figure 8c, d, e). However, expression of every toxin altered maintenance of calcium transients in the latter phase from 300 to 600 seconds (Figure 8c, f). Expression of these toxins also resulted in severe pupariation deficits on PDD (Figure 8g). Taken together, these data identify regulation of ion channel gene expression changes as a mechanism by which the IP_3R and resultant SOCE regulate neuronal function and plasticity in the larval CNS.

Discussion

In this study, we have explored the neural mechanisms that control pupariation on a protein deficient diet. We find that presence of arginine in the

environment serves as an important proxy for nutrition in *Drosophila* larvae. Loss of arginine is sensed by the amino-acid transporter *slimfast* in peripheral sensory neurons that are cholinergic. This information is conveyed to glutamatergic interneurons in the ventral ganglion, where it initiates calcium transients. Whereas arginine appears to be an important signal for sensing nutrient deprivation, other essential amino acids play smaller, and possibly additive roles for evoking calcium transients in glutamatergic interneurons and in pupariation. Neuro-peptidergic modulation is equally necessary for the calcium transients seen in response to loss of amino acids. The calcium transients in turn stimulate neuropeptide secretion from the mNSCs, required for pupariation when the diet of mid-third instar larvae lacks protein. Both cholinergic and peptidergic inputs to the glutamatergic interneurons are dependent on receptors that stimulate intracellular calcium signalling through IP_3R/SOCE. We have identified regulation of ion channel gene expression by IP_3R/SOCE as a long-term mechanism for modulating neuronal function and plasticity of the glutamatergic interneurons (Figure 8h).

Sensing of arginine by cholinergic neurons through *slimfast*

Nutrient sensing mechanisms have evolved in response to epochs of nutrient deprivation (Efeyan et al., 2015). Dietary arginine levels have also been shown to regulate insulin release (Liang et al., 2017). In newts, because of the cannibalistic nature of adult newts, arginine serves as a threat assessment for larvae (Ferrer and Zimmer, 2007a). Similar to newt larvae (Ferrer and Zimmer, 2007b) our findings suggest that *Drosophila* third instar larvae sense environmental levels of arginine. In *Drosophila* larvae, arginine presumably serves to estimate diet quality and availability. Biochemically, arginine is converted to ornithine and polyamines required

608 for cell proliferation (Auvinen et al., 1992; Lange et al., 2014). This property of
609 arginine may be significant for tissue remodelling during pupariation.

610 The ability of class IV multidendritic sensory neurons in nociception and
611 response to parasitoid wasp attacks is well established (Hwang et al., 2007; Zhong
612 et al., 2010; Ohyama et al., 2015). However, their function in the context of nutrient
613 deprivation is a recent finding (Jayakumar et al., 2016). In this study, we identify
614 them as direct sensors of arginine. Recent reports have described how levels of
615 dietary amino acids affecting their dendritic branching (Watanabe et al., 2017) and
616 subsequently that nutrient-dependant changes in dendritic branching are distinct
617 from their nociceptive function (Brown et al., 2017), thus strengthening the role of
618 multidendritic sensory neurons in nutrient sensing. We have explored the response
619 to dietary deprivation of amino acids using a semi-intact preparation. Though, this
620 has been informative, systemic humoral responses would not have been measured
621 and these could well be relevant for pupariation on a protein-deficient diet. With the
622 existing improvements in imaging methods, it would be of interest to investigate the
623 effects of nutrient-deficiency on the decision to pupariate in freely behaving larvae as
624 well.

625 Our data support expression of *slif* in multidendritic sensory neurons and
626 functionally demonstrate that pupariation on PDD requires *slif* in *ppk-GAL4*
627 expressing neurons. In the fat-body *slif* is required for arginine transport (Colombani
628 et al., 2003). A recent study on homologs of *slif* however revealed that it could
629 function as a “transceptor”, a hybrid of a receptor as well as a transporter (Boudko et
630 al., 2015). A transceptor role for Slimfast in sensory neurons is supported by our
631 observation of loss of calcium transients in *ppk* neurons upon withdrawal of arginine
632 in the absence of Slimfast (Figure 6e). Interestingly we also observed a significant

633 decrease in the preference for arginine upon knockdown of *slif* in neurons marked by
634 *ppk-GAL4*, suggesting that these larvae may be repelled by arginine. However, the
635 regulation of acute diet preference through Slimfast needs to be investigated further.

636 **Neuronal regulation of pupariation**

637 Response to starvation in *Drosophila* is thought to be primarily regulated by
638 the fat-body (Owusu-Ansah and Perrimon, 2014). Both the *Drosophila* brain and the
639 fat-body are tissues that exert metabolic control (Colombani et al., 2003;
640 Hietakangas and Cohen, 2009; Bjordal et al., 2014). However, premature pupariation
641 observed by optogenetic activation of VG glutamatergic interneurons (Figure 1g-i)
642 supports the idea that neuronal regulation described here might override other
643 metabolic signals.

644 Calcium responses encoded by glutamatergic interneurons regulate
645 ecdysteroid-synthesizing gene expression by stimulating peptide-release from the
646 mNSCs and this release is essential for pupariation on PDD (Jayakumar et al.,
647 2016). Therefore, we hypothesize that neuropeptide release from the mNSCs, which
648 is regulated by the glutamatergic interneurons regulates the expression of
649 ecdysteroid-synthesizing genes. These cells produce various peptides, including
650 Dilp2 whose release is affected by activity in glutamatergic interneurons of the VG
651 (Jayakumar et al., 2016). The role for other neuropeptides such as Dilp3, Dilp5,
652 DSK, DH44 and SIFa (Brogiolo et al., 2001; Cabrero et al., 2002; Rulifson et al.,
653 2002; Terhzaz et al., 2007; Park et al., 2008) produced by the mNSCs needs to be
654 tested in pupariation during nutrient deprivation. Even though our data support the
655 role of mNSC peptides, specifically Dilp2, in ecdysteroid synthesis and pupariation,
656 they do not exclude the possibility that such regulation might also occur indirectly

657 through modulation of the PTTH neurons (Nijhout and Williams, 1974; Mirth et al.,
658 2005).

659 **Integration of metabolic state by glutamatergic interneurons**

660 Interestingly, our data suggest that calcium transients from a fraction of
661 neurons (amidst glutamatergic interneurons of segments T3-A5 in the VG and
662 marked by *VGN6341-GAL4)* encode the loss of dietary amino acids. The systemic
663 response to loss of amino acids thus requires that the underlying cellular response
664 reach a signalling threshold in a certain number of glutamatergic neurons from the
665 potential responsive pool and not necessarily all the cells. Such a population code,
666 for the integration of sensory information is not unprecedented and has been shown
667 in different contexts (Erickson, 2000; Carleton et al., 2010; Cohn et al., 2015;
668 Tantirigama et al., 2017).

669 In addition to peptide release regulated by the glutamatergic interneurons,
670 reduction in the environmental levels of amino acids elicits peptide release from
671 other neuro-peptidergic cells. Furthermore, the calcium transients in the
672 glutamatergic neurons that encode loss of arginine are initiated by sensory inputs,
673 but also require inputs through multiple neuropeptide receptors. The knockdown of
674 neuropeptide receptors altered the number of responding cells and it is therefore
675 likely that neuro-peptidergic signals help propagate the calcium response initiated by
676 cholinergic inputs. Whereas FMRFa and CCHa2 positively regulate transient
677 propagation, AstA is a negative regulator of the response. Peptidergic modulation of
678 neural circuits has been well studied in *Drosophila* and other invertebrates (Taghert
679 and Nitabach, 2012).

CCHa2 is expressed predominantly in the fat-body (Sano et al., 2015) as well as the brain and the midgut (Ren et al., 2015) of *Drosophila* larvae and conveys nutritional information about environmental proteins and carbohydrate levels in *Drosophila* larvae (Sano et al., 2015). The role of FMRFa in nutritional sensing is not understood though FMRFa regulates glucose metabolism in snails (Roszer et al., 2014). AstA regulates feeding of *Drosophila* larvae depending on the available nutrient content in the environment (Hentze et al., 2015; Chen et al., 2016) and is known to negatively regulate feeding in adult flies (Hergarden et al., 2012).

The ability of different neuropeptides secreted from various internal tissues to drive antagonistic neuronal responses suggests that nutrient sensing glutamatergic neurons are regulated differentially based on their expression of neuropeptide receptors (Figure 8h). Knockdown of the putative hugin receptor, PK-2 on *VGN6341-GAL4* marked neurons, also leads to pupariation deficits upon nutrient deprivation (Jayakumar et al., 2016). Peptidergic modulation of glutamatergic neurons likely allows for integration of external sensory cues (starvation in this case), with metabolic changes in the fat-body, possibly the gut and the central brain. Thus, the glutamatergic interneurons are modulated by various neuropeptides in a state-dependent manner (starved vs non-starved), further supporting complex neuromodulation for pupariation, similar to neuro-peptidergic modulation of circuits that regulate ecdysis (Diao et al., 2017). Together this integrated response at the appropriate developmental stage enables larvae to make the decision to continue development and pupariate in the absence of external nutrients. This finding supports the well-established concept of third instar larvae reaching “critical weight” when 50% of *Drosophila* larvae continue towards pupariation even under nutrient deprived conditions (De Moed et al., 1999; Mirth et al., 2005).

Modulation of neuronal response by IP_3R through gene expression

The dependence of neuronal calcium transients on intracellular calcium signalling components as well as ion-channels, indicates that the two mechanisms work synergistically to generate this complex cellular response in a network of multiple neurons. This is a likely explanation for why the neuronal response is not sustained upon expression of ion channel toxins. IP_3R and SOCE have been implicated in activity-dependent plasticity of mammalian neurons (Emptage et al., 2001; Rose and Konnerth, 2001), in the context of motor coordination (Fujii, 2000; Ichise et al., 2000; Hartmann et al., 2014) as well as for enabling long-term potentiation in hippocampal neurons (Fujii, 2000), synaptic plasticity in pyramidal neurons (Narayanan et al., 2010) and long-term depression in Purkinje neurons (Futatsugi et al., 1999; Hartmann et al., 2014). However, the mechanism(s) by which IP_3R/SOCE alter neuronal function and plasticity are open to debate (Hartmann et al., 2011; Majewski and Kuznicki, 2015). It is possible that attenuation of IP_3R/SOCE signalling reduces neuronal activity, that in turn affects the expression of ion-channels encoding genes. Store-calcium release is known to increase the density of cation-nonspecific h channels in rodent hippocampus (Narayanan et al., 2010). Also synaptic scaling is dependent on homeostatic plasticity modulated by changes in calcium levels (Ibata et al., 2008; Goold and Nicoll, 2010). Similar to our findings, IP_3-mediated Ca^{2+} release and SOCE might affect ion-channel gene expression in mammalian neurons and thus underlie aspects of neuronal plasticity in addition to other acute mechanisms by which IP_3 mediated Ca^{2+} release and SOCE regulate ion channel function. Plasticity in the neural circuit(s) that respond to nutrient stress may be advantageous for adapting to the stress in adults as well and needs further investigation.

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1039 **Figure Legends**

1040 **Figure 1: Glutamatergic neurons in larval ventral ganglion respond to acute**
1041 **amino acid deprivation** (**a**) Confocal images from the whole CNS of third instar
1042 larval brains where *UAS-CaLexA* has been driven using *VGN6341-GAL4* on a
1043 protein deprived diet (PDD) for 12 hours. The scale bar indicates 50μm. (**b**)
1044 Schematic of the preparation used to observe calcium transients in *VGN6341-GAL4*
1045 marked glutamatergic neurons upon amino acid withdrawal. For details please refer
1046 to methods. (**c**) Schematic depicting how cells from T3-A5 are imaged with a sample
1047 response of cells classified as responders and non-responders. (**d**) Images of a
1048 sample ventral ganglion across different times post withdrawal of amino acids
1049 (Arrowheads indicate responder cells). (**e**) Line plots of calcium transients observed
1050 in glutamatergic neurons marked by *VGN6341-GAL4* when essential amino acids
1051 (EAA) were withdrawn from CNS of corresponding age. Red dot indicates the point
1052 of withdrawal of amino acids. The transients are from cells that responded and
1053 crossed an arbitrary threshold of a minimum change (ΔF/F) of 1.5 post withdrawal as
1054 described in methods. The same control data has been used in 1b and 1c. (**f**)
1055 Heatmaps showing distribution of percentage responders in segments T3-A5 from
1056 control CNS preps in response to withdrawal of EAA. The diagram on the right
1057 indicates average responders (±SEM) from each hemi-segment. (**g**) Representative
1058 pictures of puparia when either glutamatergic neurons marked by *VGN6341-GAL4* or
1059 a control genotype were optogenetically activated on ND. (**h** and **i**) Rate of
1060 pupariation and percentage adults respectively on ND, when glutamatergic neurons

marked by *VGN6341-GAL4* were optogenetically activated for two days post 84±2 h AEL. Pupariation at Day 1 was significantly different with $p = 1.14*10^{-07}$ and at Day 2, $p = 5.98*10^{-05}$ using Two-tailed Student's t-test. In (**i**) percentage adults was significantly lower with $p = 6.68*10^{-20}$, using Two-tailed Student's t-test. Bars with the same alphabet represent statistically indistinguishable groups. Exact p-values are provided in Figure 1-1.

Figure 2: SOCE in larval glutamatergic neurons is important for pupariation under amino acid deprivation (**a**) Box plots represent percentage pupariation on PDD of the indicated genotypes where intracellular calcium signalling was perturbed in glutamatergic neurons. One-way ANOVA: $F(5,30) = 652.2215$, $p < 0.05$; with post hoc Tukey's Multiple Comparison Test (MCT). (**b**) Line plots of calcium transients observed in glutamatergic neurons marked by *VGN6341-GAL4* when essential amino acids (EAA) were withdrawn from CNS of indicated genotypes. Red dot indicates the point of withdrawal of EAA. The transients are from cells that responded above an arbitrary threshold of $\Delta F/F \geq 1.5$ post withdrawal, as described in methods. The control genotype trace is from the same experiments as shown in Figure 1e. (**c** and **d**) Percent responders, one-way ANOVA: $F(6,31) = 16.1339$, $p < 0.05$; with post hoc Tukey's MCT and (**d**) area under the curve from the line plots shown in Figure 1e and 2b, one-way ANOVA: $F(6,255) = 9.25345$, $p < 0.05$; with post hoc Tukey's MCT. (**e**) Images showing the location of larvae of the indicated genotypes at the indicated time points in either absence or presence of white light. (**f**) Bars represent percentage "freezing behaviour" upon exposure to light from the indicated genotypes. Exact p-values are provided in Figure 1-1.

Figure 3: Arginine is critical amino acid for pupariation (**a** and **b**) Percentage pupariation of the indicated genotypes on PDD upon supplementation with indicated

specific amino acids, numbers indicate batches of 25 larvae each. One-way ANOVA: $F(11,60) = 78.45015$, $p < 0.05$; with post hoc Tukey's MCT for (**a**) and one-way ANOVA: $F(11,58) = 0.73875$, $p < 0.05$; with post hoc Tukey's MCT for (**b**). (**c**) Heat maps showing the distribution of percentage of cells that responded to withdrawal of Arginine in segments T3-A5 from five control CNS preparations. The diagram on the right indicates the mean and SEM of number of responding cells in each indicated hemi-segment as calculated from the five CNS preparations. (**d**) Line plots of calcium transients in responding CNS cells observed upon withdrawal of either arginine, a mixture of essential amino acids lacking arginine, a mixture of non-essential amino acids or no withdrawal (mock-withdrawal). The mock-withdrawal trace is from all cells as none of them crossed the threshold. (**e** and **f**) Percent responders and area under the curve quantified from the line plots shown in Figure 3d. One-way ANOVA: $F(3,14) = 16.0049$, $p < 0.05$; with post hoc Tukey's MCT for (**e**) and one-way ANOVA: $F(3,146) = 14.00764$, $p < 0.05$; with post hoc Tukey's MCT for (**f**). Bars and boxes with the same alphabet represent statistically indistinguishable groups. Exact p-values are provided in Figure 1-1.

Figure 4: Withdrawal of amino acid in the media can induce glutamatergic neuron dependent peptide release (**a**) Line plots of peptide release from medial neurosecretory cells (mNSCs) marked by *dimm-LexA*. Peptide release was measured by decrease in fluorescence of the Atrial Natriuretic Factor linked to GFP (ANF::GFP) following withdrawal of arginine from CNS with and without optical inhibition of glutamatergic neurons with eNpHR2. Blue dot indicates the point of withdrawal of arginine and the green line indicates the duration of inhibition by eNpHR2. **(b and c)** Box plots represent area under the curves quantified from the peptide release response of mNSCs(**b**) and non-mNSC peptidergic cells(**c**). The

release was found to be significantly lower during inhibition from the mNSC in (**b**) with p = 0.00525, Two-tailed Student's t-test, whereas the release from the non-mNSC (**c**) cells were not, p = 0.13651, Two-tailed Student's t-test. All data are from a minimum of four CNS of the individual genotypes. Boxes and bars with the same alphabet represent statistically indistinguishable groups. (**d**) Percentage pupariation of the indicated genotypes on indicated diets concurrent with a temperature shift from permissive (18°C) to restrictive temperature (29°C) thereby blocking peptide release on PDD. Two-way ANOVA: $F(3,21) = 41.09651$, $p < 0.05$; with post hoc Tukey's MCT. (**e**) Line plot showing peptide release from *PTTH-GAL4* marked cells assayed by expression of the ANF::GFP construct and measured upon stimulation with 50μM carbachol. (**f**) Box plots represent release as estimated by area under the curves for either mNSC cells or from PTTH cells, p = 0.02858, Two-tailed Student's t-test. The mNSC data is the same as in Figure 6a from (Jayakumar et al., 2016). Bars and boxes with the same alphabet represent statistically indistinguishable groups. Exact p-values are provided in Figure 1-1.

Figure 5: Neuropeptides modulate calcium transients and pupariation during protein deprivation (**a**) Line plots of calcium transients from larval CNS observed in *VGN6341-GAL4* marked glutamatergic neurons upon withdrawal of EAA in animals with the indicated neuropeptide receptor knockdowns. Red dot indicates the point of withdrawal of EAA. The transients are from cells that responded above an arbitrary threshold of $\Delta F/F \geq 1.5$ post withdrawal, as described in methods. The control genotype trace is from the same experiments as shown in Figure 2b. (**b**) Bars represent percentage of cells that responded to EAA withdrawal above the threshold from the indicated genotypes as shown in Figure 5a. One-way ANOVA: $F(3,19) = 212.26$, $p < 0.05$; with post hoc Tukey's MCT. (**c**) Box plots represent area under the

curve from the graph in Figure 5a. One-way ANOVA: $F(3,225) = 9.32574$, $p < 0.05$; with post hoc Tukey's MCT. (**d**) Line plots of calcium transients from larval CNS observed in *VGN6341-GAL4* marked glutamatergic neurons upon withdrawal of EAA in animals upon blocking peptide release either at restrictive (31°C) or permissive (22°C) temperatures. Red dot indicates the point of withdrawal of EAA. The transients are from cells that responded above an arbitrary threshold of $\Delta F/F \geq 1.5$ post withdrawal, as described in methods. (**e** and **f**) Box plots represent area under the curve from the graph in Figure 5d, for the initial phase (60 -300s) were not significant $p = 0.15686$, Two-tailed Student's t-test; but were significant in the later phase (300-600s), $p = 4.30*10^{-06}$, Two-tailed Student's t-test. (**g**) Bars represent percentage pupariation observed when CCHa2 knockdown was performed in the fat-body, $p = 3.69*10^{-05}$, Two-tailed Student's t-test. (**h**) Bars represent percentage pupariation observed when FMRFa release is either permitted ($p = 0.35576$, Two-tailed Student's t-test) or inhibited from the CNS under conditions of nutrient deprivation, $p = 3.75*10^{-08}$, Two-tailed Student's t-test. (**i**, **j** and **k**) Line plots of calcium transients observed upon addition of 5 μM of the corresponding neuropeptide. Open red circles indicate the point of addition of neuropeptide. Percent cells that responded are indicated on top of the graphs. For AstA all cells were below the threshold. The mock trace was obtained from all cells and the same trace is shown in the three graphs. All data are from a minimum of five CNS of the individual genotypes. (**l**, **m** and **n**) Box plots represent area under the curve from graphs in Figure 5i, 5j and 5k respectively, with $p = 3.41228*10^{-05}$ (**l**), $p = 0.000527221$ (**m**) and $p = 0.000535367$ (**n**), Two-tailed Student's t-test. Boxes with the same alphabet represent statistically indistinguishable groups. Exact p-values are provided in Figure 1-1.

Figure 6: *slif* in ppk neurons senses arginine as a proxy for nutritional quality

(**a**) Line plots show calcium transients observed from *VGN6341-GAL4* marked glutamatergic neurons upon withdrawal of EAA from CNS with and without application of 2 μM TTX. Responses from all cells upon TTX application have been plotted. The control data is the same as used in Figure 2b. (**b** and **c**) Line plots of calcium transients from glutamatergic neurons observed upon withdrawal of EAA when inputs from *ppk-GAL4* marked sensory neurons were inhibited either concurrent with withdrawal (**b**) or after withdrawal (**c**). Red dot indicates the point of withdrawal of EAA. Green line on top of the graph indicates duration of inhibition. The transients are from all cells in (**b**) and from the responding cells (~55%) in (**c**). Responding cells were classified by an arbitrary threshold of $\Delta F/F \geq 1.5$ post withdrawal, as described in methods. All data are from a minimum of five CNS of the individual genotypes and 57 cells. (**d**) Line plots of calcium transients from glutamatergic neurons observed upon activating *ppk* neurons in either second (68 ± 2 h AEL) or third instar larvae (84 ± 2 h AEL). Blue line on top of the graph indicates duration of activation. (**e**) Bars represent fold change in mRNA levels of *slif* normalized to *rp49* in the indicated genotypes, $p = 0.021$, Two-tailed Student's t-test. (**f**) Bars represent percentage pupariation in the indicated genotypes, one-way ANOVA: $F(2,19) = 390.6136$, $p < 0.05$; with post hoc Tukey's MCT. (**g**) Line plots show calcium transients observed in *ppk* neurons upon withdrawal of EAA from semi intact preparations. Red dot indicates the point of withdrawal of EAA. The transients shown are from all cells. (**h**) Line plots show calcium transients observed upon withdrawal of arginine from semi-intact preparations in control as well as knockdown of *slif* using *ppk-GAL4.* Blue dot indicates the point of withdrawal of arginine. The transients shown are from all cells. (**i**) Representative images from the preference

assay at the indicated times in control animals (*slif IR/+*) and animals with *slif* knockdown in *ppk-GAL4* expressing sensory neurons. (**j** and **k**) Box plots of the preference index calculated either at the end of 10 minutes in animals of the indicated genotypes, p = 0.000105775, Two-tailed Student's t-test (**j**) or during and after real-time optical inhibition of *ppk-GAL4* neurons expressing eNpHR3, p = $1.6009*10^{-05}$, Two-tailed Student's t-test. (**k**), where the numbers indicate the number of batches of 20 larvae that were tested. Bars and boxes with the same alphabet represent statistically indistinguishable groups. Exact p-values are provided in Figure 1-1.

Figure 7: Intracellular calcium signalling through IP3R/SOCE in glutamatergic neurons regulates expression of genes encoding ion channels (a) Venn diagrams representing the number of genes identified as differentially expressed by three independent, indicated methods. *IP_3R* knockdown majorly leads to down-regulation of a set of genes. For list of genes, refer to Figure 7-1. **(b)** Bars indicate the number of genes up- or down-regulated upon knockdown of the *IP_3R* in the CNS as measured by RNA-seq. **(c)** Bars indicate the expression levels of *IP_3R* in the indicated conditions, q-value refers to the corrected p-value obtained from CuffDiff. **(d)** Bars represent the fold enrichment in the number of genes of the indicated GO molecular function categories in the set of genes down-regulated upon *IP_3R* knockdown, as compared to all genes in *Drosophila*. Numbers on top of the bars indicate FDR corrected p-values. This analysis was performed using the Panther GO Slim Molecular Function option. **(e)** Heatmap indicates the fragments per kilobase per million (FPKM) values as a proxy for expression level of the indicated cation channel genes in control (UAS-*IP_3R IR/+; UAS-dicer2/+)* and *IP_3R* knockdown (*$elav^{C155}$-GAL4>UAS-IP_3R IR; UAS-dicer2*) conditions. Red labels indicate genes

whose expression is significantly altered by *IP_3R* knockdown identified by all three methods (CuffDiff, DESeq and edgeR), pink labels indicate differential gene expression significant by any two methods and grey labels indicate differential expression of genes that are not significant. **(f)** Bars indicate the fold change in expression levels of the indicated genes normalized to *rp49* measured by qRT-PCR from central nervous systems of second instar larvae to that of third instar, p = 0.019984 for *cac,* Two-tailed Student's t-test. **(g)** Cartoon representation of the procedure used to sort glutamatergic neurons of interest. **(h)** Representative dot plots of flow cytometric analysis of cell suspensions made from the indicated genotypes. X-axis denotes the extent of fluorescence and the Y-axis is a measure of granularity based on the side-scatter. Threshold was set using the non-fluorescent wild type and pink dots were collected as GFP positive cells. **(i)** Bars represent the percentage of GFP-positive glutamatergic cells obtained by FACS from the indicated genotypes. One-way ANOVA: $F(2,9) = 24.3$, $p < 0.05$; with post hoc Tukey's MCT. Bars with the same alphabet represent statistically indistinguishable groups. **(j)** Comparison of the levels of *VGlut* in whole CNS vs. sorted glutamatergic neurons as compared to the housekeeping gene, *rp49*. *VGlut* expression is enriched in the sorted glutamatergic neurons. **(k)** Bars indicate the fold change in expression levels of the indicated genes normalized to *Act5c* measured by qRT-PCR from sorted glutamatergic cells of the control (*VGN6341-GAL4>UAS-eGFP*) and *dStim* KD (*VGN6341-GAL4>UAS-eGFP; UAS-dStim IR; dcr2*) genotypes; p = 0.012 (*dStim*), p = 0.003 (mAChR), p = 0.023 (*NaCP60E*), p = 0.040 (*Hk*), p = 0.040 (*eag*), p = 0.077 (*cac*), p = 0.008 (Ca-alpha1D). RNA was isolated from ~1200 sorted neurons and amplified using the SMART-seq method before performing qRT-PCR. *p<0.1, **p<0.05; ***p<0.01; two tailed t-test. Exact p-values are provided in Figure 1-1.

Figure 8: IP_3R/SOCE in glutamatergic neurons regulates neuronal excitability **(a)** Line plots show calcium transients observed upon depolarization by KCl in *VGN6341-GAL4* marked glutamatergic neurons from CNS of the indicated genotypes. Grey box indicates the window of addition of KCl. Responses from all cells have been plotted. (**b**) Box plots indicate the peak change in fluorescence from traces in a. One-way ANOVA: $F(3,80) = 13.44943$, $p < 0.05$; with post hoc Tukey's MCT. **(c)** Line plots indicate calcium transients observed upon withdrawal of essential amino acids in control CNS and in CNS with expression of the indicated toxins in *VGN6341-GAL4* marked glutamatergic neurons. Red dot indicates the point of withdrawal of EAA. The transients are from cells that responded above an arbitrary threshold of $\Delta F/F \geq 1.5$ post withdrawal, as described in methods. Data are from a minimum of five CNS of the individual genotypes. Control trace is the same as in Figure 2b. (**d**) Percent responders from the trace in Figure c, one-way ANOVA: $F(3,23) = 30.58001$, $p < 0.05$; with post hoc Tukey's MCT. (**e** and **f**) Area under the curve quantified from the trace in Figure 2m for the initial phase from 60-300 seconds, one-way ANOVA: $F(3,243) = 0.28392$, $p < 0.05$; with post hoc Tukey's MCT (**e**) and the later phase from 300 to 600 seconds, one-way ANOVA: $F(3,243) = 5.04391$, $p < 0.05$; with post hoc Tukey's MCT (**f**). (**g**) Bar graphs indicate percentage pupariation of the indicated genotypes on an amino acid deficient diet, one-way ANOVA: $F(3,12) = 120.0924$, $p < 0.05$; with post hoc Tukey's MCT. Boxes and bars with the same alphabet represent statistically indistinguishable groups. **(h)** Schematic summarizing cholinergic activation (mAChR) and peptidergic modulation (FMRFaR, CCHa2R and AstAR) of glutamatergic neurons required for pupariation on a protein deficient diet. GPCRs stimulate calcium release through the IP_3R followed by SOCE in glutamatergic neurons. The intracellular calcium signalling

regulates expression of genes encoding several ion channels as well as the mAChR. Activation of intracellular calcium signalling mechanisms and ion channels stimulates a complex calcium response across glutamatergic neurons upon amino acid withdrawal. The neuronal response is necessary for pupariation on the protein deficient diet. Exact p-values are provided in Figure 1-1.

Table Legends

Table 1: GO classification of down-regulated genes

GO classification of the Molecular Function of the genes down-regulated upon *IP_3R* knockdown was performed using Gene Ontology enRIchment anaLysis and visuaLizAtion tool (GORILLA). Enriched categories with associated fold enrichment, p-value and FDR corrected p-value are indicated. Various categories related to voltage gated ion channels were identified and are highlighted in red.

Table 2: Fold changes in mRNA upon *dStim* knockdown in VGN6341 neurons. Fold changes normalized to two housekeeping genes, *rp49* and *tubulin*. *p-values* obtained from a two-tailed t-test. FC - Fold Change, KD- knockdown, SEM- Standard error of mean. n=3.

Table 3: List of Fly Stocks

Table 4: List of primer sequences

Extended Data Legends

Figure 1-1: File contains exact p-values for all statistical tests performed in this paper.

Figure 7-1: File contains lists of genes that were identified as differentially regulated by all three methods used.

Table 1

GO term	Description	P-value	FDR q-value	Enrichment
GO:0008010	structural constituent of chitin-based larval cuticle	8.27E-18	2.02E-14	15.26
GO:0042302	structural constituent of cuticle	2.15E-17	2.62E-14	13.37
GO:0005214	structural constituent of chitin-based cuticle	1.19E-16	9.7E-14	13.53
GO:0005261	cation channel activity	3.34E-10	2.03E-7	7.25
GO:0022843	voltage-gated cation channel activity	7.15E-10	3.48E-7	13.78
GO:0005216	ion channel activity	9.33E-10	3.79E-7	5.89
GO:0022838	substrate-specific channel activity	1.8E-9	6.26E-7	5.67
GO:0005198	structural molecule activity	3.1E-9	9.46E-7	3.23
GO:0022836	gated channel activity	3.64E-9	9.84E-7	7.35
GO:0046873	metal ion transmembrane transporter activity	7.05E-9	1.72E-6	5.22
GO:0022803	passive transmembrane transporter activity	1.08E-8	2.39E-6	5.09
GO:0015267	channel activity	1.08E-8	2.19E-6	5.09
GO:0022832	voltage-gated channel activity	1.34E-8	2.51E-6	10.67
GO:0005244	voltage-gated ion channel activity	1.34E-8	2.33E-6	10.67
GO:0008324	cation transmembrane transporter activity	2.06E-7	3.35E-5	3.55
GO:0015075	ion transmembrane transporter activity	2.72E-7	4.15E-5	3.00
GO:0022891	substrate-specific transmembrane transporter activity	6.25E-7	8.95E-5	2.81
GO:0022890	inorganic cation transmembrane transporter activity	9.3E-7	1.26E-4	3.65
GO:0022892	substrate-specific transporter activity	2.26E-6	2.9E-4	2.52
GO:0015085	calcium ion transmembrane transporter activity	7.21E-6	8.78E-4	7.56
GO:0022857	transmembrane transporter activity	7.67E-6	8.9E-4	2.42

Table 2

Gene	Normalized to *rp49*					Normalized to *tubulin*				
	Control		*dStim KD*			*Control*		*dStim KD*		
	Mean FC	SEM	Mean FC	SEM	p-value	Mean FC	SEM	Mean FC	SEM	p-value
Act5c	1.01	0.07	1.01	0.05	0.93	1.01	0.08	0.92	0.13	0.58
itpr	1.01	0.07	0.79	0.07	0.11	1.00	0.03	0.83	0.19	0.43
Stim	1.00	0.07	0.58	0.02	0.00	1.02	0.15	0.54	0.10	0.05
mAChR	1.04	0.20	0.41	0.03	0.04	1.02	0.16	0.42	0.09	0.03
NaCP60E	1.04	0.23	0.52	0.06	0.09	1.03	0.18	0.55	0.14	0.10
Hk	1.08	0.29	0.44	0.02	0.10	1.06	0.26	0.46	0.10	0.09
eag	1.05	0.21	0.49	0.04	0.06	1.06	0.26	0.46	0.10	0.09
cac	1.00	0.06	0.63	0.15	0.08	1.01	0.13	0.61	0.23	0.20
ca-alpha1D	1.00	0.06	0.49	0.04	0.00	1.02	0.14	0.45	0.10	0.03
VGlut	1.01	0.12	0.58	0.07	0.03	1.01	0.10	0.58	0.07	0.02

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Fly line	Description	Source
VGN6341-GAL4	Subset Glutamatergic driver	(Syed et al., 2015)
ppk-GAL4	Class IV multidendritic driver (very weak expression in class III)	BL32078 (Zhong et al., 2010)
OK6-GAL4	Motoneuron driver	(Aberle et al., 2002)
Dilp2-GAL4	Expresses GAL4 in insulin producing cells from the mNSCs (Dilp2 driver)	(Rulifson et al., 2002)
PTTH-GAL4	PTTH driver	(McBrayer et al., 2007)
FMRFa-GAL4	FMRFa driver	(Park et al., 2008) Gift from Paul Taghert
Cg-GAL4	Fat-body driver	BL7011
UAS-ANF::GFP	Expresses ANF::GFP	BL 7001 (Rao et al., 2001)
UAS-Shi^{ts}	Inhibits vesicle recycling	(Kitamoto, 2001)
UAS-ChR2.XXL	Channelrhodopsin variant	(Dawydow et al., 2014) Gift from Christian Wegener
UAS-itpr IR; UAS-dicer	RNAi line for *itpr* combined with dicer	1063-R2 from NIG
UAS-dStim IR; UAS-dicer	RNAi line for *dStim* combined with dicer	V47073/GD from VDRC
UAS-dOrai IR; UAS-dicer	RNAi line for *dOrai* combined with dicer	12221/GD from VDRC
UAS-mAChR60C IR	RNAi line for mAChR	VDRC 101407 CG4356 (Agrawal et al., 2013)
UAS-Kir2.1	Inhibitor of neuronal activity (Inward rectifying potassium channel)	(Baines et al., 2001)

UAS-eGFP	Cytosolic GFP	Gift from Michael Rosbash
UAS-FMRFaR IR	RNAi for FMRFaR	CG2114-R1 from NIG
UAS-CCHa2R IR	RNAi for CCHa2R	CG14593, 1658 from VDRC
UAS-AstAR IR	RNAi for AstA R	CG10001, 108648 from VDRC
UAS-slif IR	RNAi for slif	CG11128 v45589 from VDRC
UAS-CCHa2 IR	RNAi for CCHa2	BL57183
UAS-GCaMP6m	Genetically encoded Calcium indicator	BL42748
UAS-jRCaMP1b	Red shifted Calcium indicator	BL63793 (Dana et al., 2016)
UAS-PLTXII	Toxin against presynaptic insect Ca^{2+} channels	(Wu et al., 2008)
UAS-κ-ACTX-Hv1c	Toxin against K^+ channels	(Wu et al., 2008)
UAS-δ-ACTX-Hv1a	Toxin against Na^+ channels	(Wu et al., 2008)
UAS-CsChrimson	Red shifted optogenetic activator	BL 55135 (Klapoetke et al., 2014)
UAS-eNpHR2	Optogenetic inhibitor (Chloride channel)	Gift from Leslie Griffith (Berni et al., 2012)
dimm-LexA(C929)	LexA under *dimm* promoter	(Jayakumar et al., 2016)
LexAop-ANF::GFP	Expresses ANF::GFP under LexAop	(Jayakumar et al., 2016)
LexAop-Shi^{ts}	Shi^{ts} under LexAop	BL44276 (Chen et al., 2013)
ppk-QF	Class IV multidendritic driver (very weak expression in class III)	BL36348 (Petersen and Stowers, 2011)
QUAS-eNpHR3	Optogenetic inhibitor (Chloride channel) under QUAS	BL 36355 (Petersen and Stowers, 2011)

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Gene	Forward (5'>3')	Reverse (5'>3')
Act5c	GTCCACCTTCCAGCAGATG	CCTCCTCCAGCAGAATCAAG
Ca-alpha1D	GCGAATGCCATTAACTATGACAAC	ACTCGGAGTCGCAGTATTTACC
cac	TGTTCGATTGCGTCGTGAAC	TGGCACTCTGCGGAAGTATG
eag	AGGATGTTTCCCTGCTCGTG	TGGGTCACTAACAACCTCGC
Hk	CTGTTCGACATCTCGGAGGC	GGTGCTCCAGTAGACCTTCG
itpr	CCAGGGTTTGCGAAATGGC	CAGGTCGTCTTCAGAATGGC
mAChR	ATGACACCTGGCGACGTCC	CGCAATGCACCACTCCTTG
NaCP60E	GACTTTTCCGAGCTACGGGG	TTAAGACAGCCGTCCTCACG
rp49	CGGATCGATATGCTAAGCTGT	GCGCTTGTTCGATCCGTA
slif	GCTGGACCTGAACACATGGA	TGCGGCTCCTACTTATCTGC
Stim	GTACGCTAGATCATGGCCCG	CGTTGTGAGGCAACATTGGG
VGlut	CTGTGTTCATTTGGTTGGCTGC	GATCCGTGTTGGTAATGGCAC
β-Tubulin	CCAAGGGTCATTACACAGAGG	ATCAGCAGGGTTCCCATACC

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