Title: A STIM dependent dopamine-insulin axis maintains the larval drive to feed and grow in Drosophila

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Abstract:

Appropriate nutritional intake is essential for organismal survival. In holometabolous insects such as *Drosophila melanogaster*, the quality and quantity of food ingested as larvae determines adult size and fecundity. Here we have identified a subset of dopaminergic neurons (THD') that maintain the larval motivation to feed. Dopamine release from these neurons requires the ER Ca^{2+} sensor STIM. Larvae with loss of STIM stop feeding and growing, whereas expression of STIM in THD' neurons rescues feeding, growth and viability of STIM null mutants. Moreover STIM is essential for maintaining excitability and release of dopamine from THD' neurons. Optogenetic stimulation of THD' neurons activated neuropeptidergic cells, including median neuro secretory cells that secrete insulin-like peptides. Loss of STIM in THD' cells alters the developmental profile of specific insulin-like peptides including ilp3. Loss of ilp3 partially rescues STIM null mutants and inappropriate expression of ilp3 in larvae affects development and growth. In summary we have identified a novel

STIM-dependent function of dopamine neurons that modulates developmental changes in larval feeding behaviour and growth.

Author summary

The ability to feed appropriately when hungry is an essential feature for organismal survival and is under complex neuronal control. An array of neurotransmitters and neuropeptides integrate external and internal signalling cues to initiate, maintain and terminate feeding. In adult vertebrates and invertebrates dopamine serves as a reward cue for motor actions, including feeding. Larvae of holometabolous insects, including *Drosophila melanogaster*, feed and grow constantly followed by gradual cessation of feeding, once sufficient growth is achieved for transition to the next stages of development. Here we identified a subset of larval dopaminergic neurons in *Drosophila melanogaster*, activity in which maintains continuous feeding in larvae. By analysis of a null mutant we show that these neurons require the Stromal Interaction Molecule (STIM) an ER Ca^{2+} sensor, to maintain excitability. In turn they modulate activity of certain neuropeptidergic cells. Among these are the median neurosecretory cells (MNSc) that synthesize and secrete insulin-like peptides including ilp3. The identified dopaminergic neurons dysregulate the normal pattern of larval ilp3 expression leading to premature cessation of feeding and growth. Overall, our study identified a simple dopamine modulated mechanism for feeding and growth whose manipulation could be useful for model organism studies related to feeding disorders, obesity and diabetes.

Introduction:

Animal growth occurs primarily during the juvenile stage of development. In holometabolous insects, including *Drosophila*, larval development is considered equivalent to the juvenile stage(1,2). Steady and appropriate nutritional intake in larvae, is essential for growth and development, and ultimately determines both survival and fecundity of the animals. In *Drosophila*, the feeding rate increases in the second instar larval stage and larvae continue to feed voraciously till the wandering stage of third instar larvae(3). Increase in feeding rate is accompanied by acceleration of cell division and cell growth(4).

Feeding behaviour and its modulation in *Drosophila* larvae has been studied primarily in the third instar larval stage, where it is regulated by multiple neurotransmitters and neuropeptides that respond to both external cues and the internal metabolic state (5). Neuropeptide F (NPF; a human NPY homolog) serves as a motivational signal for foraging in larvae in response to appetitive odours. The activity of NPF neurons appears dependent on inputs from two pairs of central dopaminergic neurons that receive tertiary olfactory inputs (6,7). Mutants for the short neuropeptide F (sNPF), encoded by an independent gene from NPF, affect body size by regulating food intake in larvae (8). When food is restricted octopaminergic circuits regulate feeding independent of NPF signalling in 3rd instar larvae (9). Neurons that secrete the Hugin neuropeptide respond to averse gustatory signals and their activation suppresses larval feeding (10). Where essential amino acids are imbalanced a subset of dopaminergic neurons are required for rejection of food by larvae (11). In addition serotonergic neurons from the brain project to the gut where they potentially regulate feeding-related muscle movements (12). Insulin like peptides (ilps), are

secreted by the medial neurosecretory cells (MNSc) that access the internal metabolic state and release ilps into neurohaemal sites for circulation. In adults ilps terminate feeding based on the energy state of the organism (13). Analysis of the recently concluded larval connectome demonstrates that MNSc receive both direct and indirect inputs from the enteric nervous system found in the larval gut (14). The larval MNSc also receive synaptic inputs from central neurons that release the Hugin neuropeptide (10). Ilps released through neurohaemal sites from the MNSc circulate through the body and regulate energy metabolism (15), synthesis and release of the steroid hormone 20-OH ecdysone from the prothoracic gland which drives larval instar progression (16,17).

A key difference between larval and adult feeding behaviour is that adult *Drosophila* feed sporadically, driven by hunger and satiety signals (18) whereas *Drosophila* larvae accelerate feeding as second instar larvae and feed continuously till the wandering stage of third instar larvae to optimise growth. They stop feeding for a few hours prior to pupariation (19,20). Despite studies identifying several neurotransmitters and neuropeptides in feeding regulation and their cognate neurons as part of the feeding connectome in larvae (5) mechanisms that initiate and maintain persistent feeding in early larval stages are not fully understood. In this study, whilst characterizing the cellular and molecular phenotypes of null mutants for the ER-Calcium sensor protein STIM (**St**romal **I**nteraction **M**olecule) (21) we identified a novel dopaminergic-neuropeptide connection that drives persistent feeding and growth in early larvae. Growth deficits and lethality in STIM mutants appeared to have a focus in dopaminergic cells (22). However, the cellular basis of STIM function and systemic phenotypes arising from loss of STIM in dopaminergic neurons remained to be understood. Here, we show that STIM function is required in a subset of

central dopaminergic neurons for their excitability and dopamine release. These dopaminergic neurons impact larval growth by providing the motivation for persistent larval feeding and modulating neuropeptidergic cells, including the MNSc, to regulate levels of insulin-like peptides.

Results

Reduced food intake and growth deficits in STIM mutant larvae

STIM^{KO} larvae appear normal after hatching but their transition from first to second instar stages is slower than wild-type animals (**S1A and S1B Fig**) (22) and as second instars they die gradually between 86h to 326h after egg laying (**AEL; S1B Fig**). To identify the precise time window when *STIM*^{KO} larvae become sickly they were observed over 6h time intervals from 36h to 90h AEL. Whereas, wild type (*Canton-S* or CS) larvae transition from 1st to 2nd instar between 42-54h AEL, the same transition in *STIM*^{KO} larvae occurs between 60-72h AEL, indicating a delay of 18h (**Fig 1A and 1B**). The delay is followed by an inability to transition to 3rd instar (**Fig 1C**). *STIM*^{KO} larvae also exhibit retarded growth. At 72h they appear similar to CS larvae of 60h (**Fig 1D)**. After 72h however, there is a complete cessation of growth in *STIM*^{KO} larvae (**Fig 1D and 1E**), followed by gradual loss of viability after 80-86h (**S1B Fig**). From these results, it became evident that cessation of growth precedes loss of viability in *STIM*^{KO} larvae.

The momentum of larval growth is maintained primarily by cell growth in the endoreplication tissues (23,24). In a few organs like the brain and the imaginal discs growth is accompanied by constant cell division. Normally, at the end of embryonic development, mitotic cells such as a majority of neuroblasts (NBs) and imaginal disc cells enter a quiescent state (4,25,26). Postembryonic larval development is initiated in the late

first instar and early second instar stages by cell growth and renewed cell proliferation in the brain and imaginal discs, where it is nutrient dependent (26). Cessation of growth in *STIM*^{KO} larvae (**Fig 1D and 1E**), suggested a deficit in cell growth and/or cell division. To investigate the status of cell proliferation in *STIM*^{KO} larvae we chose the well-characterized system of thoracic neuroblasts (27). Upon comparison of thoracic segments of WT and *STIM*^{KO} larvae at 70-74h (**Fig 1F,** first two columns) it was evident that NBs exited from quiescence and entered the proliferative state in both genotypes. Both the NB marker Deadpan (red) and the post-mitotic cell marker Prospero (blue) appeared normal in *STIM*^{KO} larvae of 70-74h AEL. Subsequently, at 82-86h, the number of postmitotic cells (Prospero positive) decreased significantly in *STIM*^{KO} animals as compared to the controls but the number of thoracic neuroblasts remained unchanged (**Fig 1F,** compare third and fourth columns). Upon quantification, the ratio of dividing neuroblasts (Deadpan surrounded by Prospero positive cells) to non-dividing neuroblasts (Deadpan with either no or few Prospero positive cells) changed significantly in 86h aged *STIM*^{KO} larvae (**Fig 1G and 1H**). To identify the cause underlying the reduced number of postmitotic cells we analyzed different phases of the cell cycle of thoracic neuroblasts in *STIM*^{KO} larval brains. For this, we used the genetically encoded FUCCI system (28). Here, the G1, S, and G2 phases of interphase are marked by green, red and green+red (yellow) fluorescent tags respectively. At 72-76h, both control and *STIM*^{KO} showed an asynchronous pattern of division. But at 82-86h, control larvae persisted with the asynchronous pattern, whereas a majority of thoracic neuroblasts in *STIM*^{KO} animals remained in the G2/M state (**S1C Fig**).

Among other factors, the exit of quiescence and maintenance of neuroblast proliferation at the early second instar stage depends on

nutritional intake (26,29,30). The slow growth and delayed exit from quiescence suggested that *STIM*^{KO} larvae may lack adequate nutritional inputs. As a first step staged larvae were placed on yeast, mixed with a blue dye and tested for ingestion of food. Even as early as 40-44h AEL there was a significant reduction of food intake in *STIM*^{KO} larvae (**Fig 1I,** quantification in **Fig 1J**). By 80-84h AEL two classes of *STIM*^{KO} larvae were evident. One with reduced food intake and others with no food intake (**Fig 1I and S1D Fig**). The proportion of *STIM*^{KO} larvae with no food intake reached ~70% by 82-86h AEL (**S1E Fig**). The ability to feed was further quantified in *STIM*^{KO} animals by measuring mouth hook contractions through larval development (31). Control larvae (*CS*) exhibit a steady increase in mouth hook contractions with age, except prior to and during larval molts, indicating greater nutrient intake with age. In contrast, increase in mouth hook movements of *STIM*^{KO} larvae follows a slower developmental trajectory, with minimal increase as they progress from first to second instar larvae and a cessation at 74h AEL, that is further retarded at 86h (**Fig 1K; videos 1-4)**. Thus, the acceleration of mouth hook movements observed in *CS* larvae from first to third instar is retarded in *STIM*^{KO} larvae before the appearance of growth deficits (**Fig 1I-K**). suggesting that the consequent nutritional deficits prevent normal growth.

STIM function is required in a subset of larval dopaminergic neurons

To understand how the loss of STIM might affect larval feeding we identified specific cells that require STIM function for larval growth and viability. From previous work, we know that knock out of *STIM* in dopaminergic neurons marked by *THGAL4* (32) leads to larval lethality (22). Over-expression of a wildtype *UASSTIM*$^{+}$ transgene, henceforth referred to as *STIM*$^{+}$, in dopaminergic neurons marked by *THGAL4* rescued

larval lethality of *STIM*^{KO} animals to a significant extent (**S2A Fig**). Further to identify specific dopaminergic neurons that require STIM function for growth and viability we tested rescue by overexpression of STIM^{+} in two non-overlapping subsets of dopaminergic neurons marked by *THC'GAL4* and *THD'GAL4* (33) and henceforth referred to as *THD'* and *THC'.* Rescue of *STIM*^{KO} larvae from 2nd to 3rd instar (~90%) was evident upon over-expression of *STIM*$^{+}$ in THD' marked neurons (**Fig 2A and 2B**). The rescue by THD' was equivalent to the rescue by *THGAL4* (20$\pm$2 and 18$\pm$1.5 viable adults eclosed respectively from batches of 25 larvae; **S2A Fig;** dark and light green arrows). In contrast rescue by expression of *STIM*$^{+}$ in *THC'GAL4* marked neurons was considerably less (out of batches of 25 animals 5$\pm$1 adults eclosed**; S2A Fig;** blue arrow), indicating a greater requirement for STIM function in THD' marked dopaminergic neurons.

Next, we analysed *THD'* driven expression of mCD8GFP and identified two classes of GFP positive cells in the larval brain. All GFP expressing cells in the central brain (three cells of DL1 and two cells of DL2 clusters (11,34,35)), appear positive for Tyrosine Hydroxylase (TH) but a pair of THD' cells in the ventral ganglion (VG) appear TH negative (TH^{-ve}) (**Fig 2C**). To understand the relative contribution of the VG-localised TH^{-ve} cells to *THD'>STIM*$^{+}$ rescue of *STIM*^{KO} animals, we restricted *THD'GAL4* expression to VG localised THD' neurons using *THGAL80* (36) (**Fig 2D**). Expression of *STIM*$^{+}$ in the VG localised THD' neurons (*THD'GAL4, THGAL80*) reduced the rescue of *STIM*^{KO} larvae significantly (**S2A Fig and Fig 2E**) and was absent in adults (**Fig 2F**). Thus, the rescue of viability in *STIM*^{KO} animals derives to a significant extent from brain-specific THD' dopaminergic neurons.

Ingestion of food and frequency of mouth hook contractions is also rescued by *THD'>STIM*$^{+}$ expression in *STIM*^{KO} larvae (**Fig 2G – 2I**). To

further confirm the relevance of STIM function in THD' marked dopaminergic neurons we knocked down STIM using a previously characterized STIM RNAi (*dsSTIM*) (37). *THD'>dsSTIM* animals exhibit delayed larval growth (**Fig 2J and S2B Fig)** and reduced feeding (**Fig 2K and 2L**) but no larval lethality. Adults however exhibit reduced body weight (**S2C Fig**). Taken together these data identified a CNS-specific subgroup of dopaminergic neurons that require STIM function for persistent feeding during the early stages of larval growth.

Neuronal excitability and dopamine release requires STIM

The status of THD' marked central brain dopaminergic cell clusters, DL1 and DL2 was investigated next in $STIM^{KO}$ larval brains at 80-84h, when a few viable organisms are still present despite cessation of growth and feeding (**Fig 2G**). THD' cells were marked with GFP in controls, $STIM^{KO}$ and $STIM^{KO}$ animals with $STIM^{+}$ rescue and the brains were stained with anti-TH sera. *THD'>mCD8GFP* cells appeared no different in $STIM^{KO}$ as well as $STIM^{+}$ rescued $STIM^{KO}$ animals at 80-84h AEL when compared to controls at either 58-62h or 80-84h AEL (**S3A Fig**). Moreover, the numbers of THD' GFP cells and TH positive cells in the CNS also appeared identical (**S3B Fig**). Therefore, the loss of STIM does not lead to the loss of dopaminergic neurons in the larval brain.

In order to test if reduced feeding in $STIM^{KO}$ larvae is indeed due to a loss in dopamine signalling we measured larval feeding with knockdown of a key dopamine synthesising enzyme Tyrosine Hydroxylase (TH), in THD' neurons (*THD'>dsTH*). Knockdown of *TH* led to significantly fewer mouth hook contractions in larvae at 80-86h AEL (**Fig 3A, Video 5-7**), indicating reduced feeding, This was accompanied by slower progression through larval moults and some mortality at each larval stage. Finally out of

a total of 25 just 20±1.2 3rd instar larvae pupated, of which 15 adults emerged (**S3C Fig)**. In agreement with lower nutrient intake during larval stages, third instar larvae were smaller in size **(Fig 3B-C**), and gave rise to adults with significantly reduced body weight (**Fig 3D**).

To understand how loss of STIM in THD' marked neurons might affect their neuronal function, we investigated properties of excitation. For this purpose, Potassium Chloride (KCl, 70mM) evoked cytosolic calcium transients were measured from THD' neurons using the genetically encoded Ca^{2+} sensor GCaMP6m in *ex vivo* preparations of similarly staged control (58-62h AEL) and $STIM^{KO}$ (70-74h AEL) larvae. We chose these time points because at 72h $STIM^{KO}$ larvae appear healthy and developmentally similar to control larvae at 60h (**Fig 1D**). THD' cells responded with similar changes in GCaMP intensity, in control and $STIM^{KO}$ larvae at these time points (**Fig 3E-3G**). However, the ability to evoke and maintain cytosolic Ca^{2+} transients upon KCl depolarisation was lost in THD' neurons from the DL1 and DL2 clusters of $STIM^{KO}$ larvae at 76-80h AEL (**Fig 3E-3G**). Overexpression of $STIM^{+}$ in THD' cells of $STIM^{KO}$ larvae rescued the KCl evoked Ca^{2+} response in larvae as late as 80-84h AEL (**Fig 3E-3G**).

STIM requirement for maintaining excitability of THD' neurons was tested further by KCl stimulation of THD' neurons with STIM knockdown (*THD'>dsSTIM)* from 2nd instar larvae aged 58-62h. Two classes of responses to depolarisation by KCl were observed. Most cells (70%) responded with normal or greater changes in intensity as compared to control cells, whereas in 30% of cells KCl did not evoke a Ca^{2+} transient (**S3D-S3F Fig**). We attribute this heterogeneous response to differential STIM knockdown by the RNAi in individual THD' cells and a potential effect of STIM knock-down on ER-Ca^{2+} homeostasis (see below).

These data suggest that loss of STIM affects membrane excitability properties and the ability of THD' neurons to respond to stimuli. This idea was tested directly by the expression of transgenes that alter membrane potential. Over-expression of an inward rectifier K^+ channel, Kir2.1 in THD' neurons, that is known to hyperpolarise the plasma membrane (38), resulted in developmental delays followed by the lethality of second and third instar larvae (**S4A Fig**). Further, overexpression of a bacterial Na^+ channel NaChBac (39) in THD' neurons of *STIM*^{KO} larvae evinced a weak rescue of developmental deficits, including the transition to third instar larvae (4.4±0.4) and adult viability (2.4±0.6 from of batches of 15 animals**; Fig. 3H-3K**). Though weak, NaChBac's rescue was consistent. We attribute the variability to a stochastic effect of NaChBac in THD' cells. This is also evident from the variable extent of rescue in growth observed in *STIM*^{KO}; *THD'>NaChBac* larvae (**Fig 3I and 3J**). Alternatively, in addition to neuronal excitability, STIM might affect other cellular functions such as ER stress, that are not alleviated by expression of NaChBac, resulting in the weak rescue. Control animals with overexpression of *NaChBac* in THD' neurons exhibit delayed pupariation (**S4B Fig**).

Neuronal excitability is required for neurotransmitter release at presynaptic terminals. We hypothesized that dopamine release from THD' neurons might be affected in *STIM*^{KO} larvae. To test this idea, we identified the pre-synaptic (green) and post-synaptic (red) terminal regions of THD' neurons by marking them with *SyteGFP* and *Denmark* respectively (**Fig 4A)** (40)]**.** Analysis of pre-synaptic regions (SyteGFP expression) identified three distinct areas in the CNS. One at the centre of the CNS corresponding to the mushroom body (**Fig 4A;** asterisk), the second as a branched form in the basomedial protocerebrum of the CNS (**Fig 4A;** arrowhead) and the third one consisting of punctae spread across the

oesophageal regions where brain-gut interactions take place (**Fig 4A;** hash). Based on the projection patterns observed we speculate that cells marked by THD' correspond to DL1-2, DL1-5, DL1-6 from the DL1 cluster and DL2-2 and DL2-3 from the DL2 cluster (35).

Next, dopamine release was measured in the most prominent presynaptic areas of THD' neurons, corresponding to the MB and the basomedial protocerebrum, by change in fluorescence of the genetically encoded fluorescent GPCR-activation-based-DopAmine sensor ($GRAB_{DA}$**)** (41)**.** Dopamine release in THD' neurons of *STIM*^{KO} larvae at 76-80h is significantly attenuated as compared with controls (**Fig 4B and 4C**). Importantly, overexpression of $STIM^{+}$ in THD' neurons rescued dopamine release, though with altered dynamics from control animals (**Fig 4B and 4C;** see discussion). We chose to measure dopamine release in 76-80h *STIM*^{KO} larvae because THD' neurons in their brains no longer responded to KCl evoked depolarization (**Fig 3B**) even though the larvae appear normal (**Fig 1D**). Dopamine release was stimulated by Carbachol (CCh), an agonist for the muscarinic acetylcholine receptor (mAChR), that links to Ca^{2+} release from ER-stores through the ER-localised IP_3 receptor (42) and is expressed on THD' neurons (43). CCh-induced changes in ER-Ca^{2+} were tested directly by introducing an ER-Ca^{2+} sensor (44) in THD' neurons (**Fig 4D and E**). Though ER-Ca^{2+} release, in response to CCh could be measured in just 7 out of 23 cells, the subsequent step of ER-store refilling, presumably after Store-operated Ca^{2+} entry into the cytosol through the STIM/Orai pathway, could be observed in all control THD' cells (58-62h AEL), whereas it was absent in THD' neurons from *STIM*^{KO} brains (76-80h AEL**; Fig 4E**). The ER-Ca^{2+} response was rescued by over-expressing $STIM^{+}$ in THD' neurons (**Fig 4D, and E**). Taken together our data establish an important role for STIM-dependent ER-Ca^{2+} homeostasis in maintaining

optimal dopamine release from THD' neurons in turn required for the larval drive to feed constantly.

Because late third instar larvae stop feeding (3,19,20,45) we hypothesized that CCh-stimulated Ca^{2+} responses in THD' cells might change in wandering stage third instar larvae. To test this idea we monitored carbachol-stimulated GCaMP activity in THD' neurons from wild-type larvae at 96h AEL (mid 3rd instar), 120h AEL (early wandering stage) and 124h AEL (late wandering stage). A robust GCaMP response was observed at 96h, whereas at the beginning of the wandering stage (118-122h AEL), the peak response was both reduced and delayed. A further delay in the peak response time was observed in late wandering stage larvae (122-126h AEL) (**S4E and S4F Fig**). Thus with gradual cessation of feeding in late third instar larvae, the dynamics of CCh-stimulated Ca^{2+} responses in THD' neurons also undergo changes (see discussion).

THD' dopaminergic neurons both activate and inhibit specific neuropeptidergic cells

A role for neuropeptides in modulating larval feeding has been identified previously (5–7) Based on these findings we postulated that dopamine release from THD' cells might modulate neuropeptidergic cells in the larval brain. This idea was tested by optogenetic stimulation of red-shifted Channelrhodopsin (*CsChrimson*) (46) expressing in THD' (*THD'>UAS-Chrimson*) cells followed by Ca^{2+} imaging from GCaMP6f expressing neuropeptide cells *(C929LexA>LexAopGCaMP6f)* (**Fig 5A**). Upon optogenetic activation of THD' neurons, a change in cellular Ca^{2+} signals was observed in a total of 64 peptidergic neurons from 9 brains, including some lateral neurosecretory cells (LNCs), median neurosecretory cells (MNCs), and regions of the suboesophageal zone (SEZ) (**Fig 5B and**

5C). Elevated Ca^{2+} signals were observed in a subset of neuropeptidergic cells (**Fig 5B - 5D,** yellow asterisks, n=24), whereas in some cells Ca^{2+} signals were reduced (**Fig 5B, 5C and 5E,** red asterisks, n=40**)**. There was no change in cellular Ca^{2+} upon optogenetic stimulation in the remaining cells with visible GCaMP6f fluorescence (n=35). Upon cessation of optogenetic activation of THD' neurons Ca^{2+} levels returned to baseline (**Fig 5D and 5E**). Individual cells exhibit either activatory or inhibitory responses upon repeated optogenetic stimulation with pulses of red light **(Fig 5F (i) and (ii))**, indicating that THD' cells evoke specific responses of either stimulation or inhibition based on the class of neuropeptide cells. Specificity of the *THD'>Chrimson* evoked response was further confirmed by testing brains from larvae that were reared without all *trans*-Retinal (ATR, a cofactor for the function of Channelrhodopsin) (47) and by imaging in the absence of light (**Fig 5G**).

STIM expression in THD' neurons regulates the expression of insulin-like peptides

Though optogenetic stimulation of THD' cells shows dopaminergic modulation of neuropeptidergic neurons (**Fig 5A and 5B**) it does not allow identification of specific neuropeptides that function downstream of the THD' neurons. Optogenetic activation of THD' and Ca^{2+} responses in neuropeptidergic cells (marked by *C929LexA>LexAopmCherry*) helped identify three clusters of neuropeptidergic cells that are downstream of THD' neurons and include the well-characterised ilp expressing MNC cluster (48), as a putative target of THD' neurons (**Fig 5B**). Analysis of an RNAseq experiment, conducted independently to identify changes in gene expression in brains from second instar $STIM^{KO}$ larvae (72-76h AEL) with developmentally comparable CS brains (58-62h;

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE202098; to be published elsewhere), found significant changes in expression of *ilp2*, *ilp3* and *ilp5*. Whereas *ilp2* and *ilp5* were significantly downregulated, *ilp3* was upregulated more than 5 fold (**Fig 6A**). The differential regulation of *ilp2, -3* and *-5* expression in $STIM^{KO}$ brains was further validated by qPCRs (**Fig 6B**). Importantly, expression of *ilp3* and *ilp5* were restored back to normal in brains from $STIM^{KO}$ larvae, rescued by overexpression of $STIM^{+}$ in THD' neurons (**Fig 6B**). During normal larval development *ilp3* transcript levels are low in actively feeding larvae (L2 and L3, 12h) and are gradually upregulated in later stages of L3 as larvae stop feeding (**Fig 6C**; DGET (49)). Based on the up-regulation of *ilp3* in $STIM^{KO}$ larvae (**Fig 6A and 6B**), we hypothesised that knock down of *ilp3* in MNSc might rescue $STIM^{KO}$ larvae. Indeed, partial rescue of larval lethality in 2nd instar larvae followed by their transition to 3rd instar larvae (5$\pm$0.5) was observed (**Fig 6D - G**). A few of the rescued 3rd instar larvae grew to full size, and pupariated (**Fig 6E**, L3b type larvae) and some even eclosed as adults (out of 3 batches of 25 animals 2.3$\pm$0.3; **Fig 6D and 6E**). The partial rescue observed by *ilp3* knockdown may be due to roles of additional dopamine-modulated neuropeptides plus the lower expression of ilp2 and ilp5 in $STIM^{KO}$ animals (**Fig 6A and 6B**) because both ilps are growth signals in 2nd and 3rd instar larvae. A small proportion of $STIM^{KO}$ larvae rescued by *ilp3* knockdown appear significantly larger than control larvae (**Fig 6E and 6F,** L3b), indicating that loss of ilp3 can rescue growth in some animals. This idea is further supported by knock-down of *ilp3* in MNSc from wildtype animals that resulted in slower transition to pupariation and larger pupae, and over-weight adults (**Fig 6H-J; S5A-B Fig**). Conversely, over-expression of *ilp3* in MNSc resulted in delayed larval transition from L2 to L3 and smaller sized larvae (**Fig 6K-M)** similar to delayed larval development in ilp5 knockdown

animals (**S5A Fig**). However, overexpression of ilp3 had no effect on feeding as indicated by measurement of larval mouth hook movements (**Fig 6N**) even though adults were of reduced body weight (**S5B Fig**). Taken together, these data show that dopamine signals from THD' cells are required to maintain normal expression of growth promoting ILPs (*ilp2* and *ilp5*) and repress expression of *ilp3* that appears to function as an anti-growth signal.

A direct synaptic connection between central dopaminergic neurons and the MNSc has not been reported (5,50) and is likely not supported by data that mapped synaptic connections in the larval brain(35,50–52). However, a neuromodulatory role for dopamine is documented where it can effect a larger subset of neurons, beyond direct synaptic partners, by means of diffusion aided volumetric transmission (53–56). To test this possibility we began by measuring larval developmental transitions in animals with knockdown of three dopamine receptors, *Dop1R1*, *Dop2R2* and *DopEcR* in the MNSc. Among these, knockdown of *Dop1R1* delayed development, reduced larval and adult viability (**Fig 7A**) and negatively impacted growth **(Fig 7B and 7C**). A weaker phenotype of delayed development and loss of viability was observed with knockdown of DopEcR, whereas larvae with knockdown of Dop2R2 appeared normal (**Fig 7A**). The response of MNSc to dopamine was tested next. Brains expressing a Ca^{2+} sensor in the MNSc (*MNSc>GCaMP6*m) were stimulated with dopamine in the presence of a Na_v blocker Tetrodotoxin (57), to prevent extraneous neuronal inputs. Of the seven targeted MNSc in one hemisphere, we observed consistent activation of one cell whereas dopamine addition inhibited the Ca^{2+} response in three to four cells (**Fig 7D**; quantified in **Fig 7E**). No changes in the Ca^{2+} responses of MNSc were observed in the absence of dopamine (**Fig 7D and 7E**).

Discussion

In *Drosophila*, as in other holometabolous insects, growth is restricted to the larval stages. In early stages of larval development cells exit mitotic quiescence and re-enter mitosis resulting in organismal growth (1,25,58).This change is accompanied by an increase in the feeding rate of the organism so as to provide sufficient nutrition for the accompanying growth in organismal size. In *STIM*^{KO} larvae we observed a loss of this ability to feed persistently starting from early second instar larvae. The focus of this feeding deficit lies in a subset of central dopaminergic neurons that require STIM function to maintain excitability. Importantly, these dopaminergic neurons communicate with multiple neuropeptidergic cells in the brain including the MNSc (**Fig 5D**) to regulate appropriate changes in larval feeding behaviour and growth through development.

STIM and excitability of dopaminergic neurons

We identified the THD' cells as critical for larval feeding from their inability to function in the absence of the SOCE regulator STIM. Loss of excitability and the absence of dopamine release from THD' cells in *STIM*^{KO} larvae (**Fig. 3A-3C and Fig 4B-4D**) suggests that voltage-dependent receptor activity is required to maintain growth in early 2nd instar larvae. Changes in expression of ion channels and presynaptic components have been observed earlier upon knockdown of STIM in *Drosophila* and mammalian neurons (59,60). Moreover, loss of STIM-dependent SOCE in *Drosophila* neurons effects their synaptic release properties (61). Partial rescue of viability in *STIM*^{KO} organisms by over-expression of a bacterial sodium channel NaChBac (**Fig 3D-3G**) and restoration of dopamine

release upon rescue by STIM^{+} (**Fig. 4B and 4C**) supports the idea that STIM-dependant SOCE maybe required for appropriate function and/or expression of ion channels and synaptic components in THD' neurons. Changes in ER-Ca^{2+} (**Fig 4D and 5E**) suggest that STIM is also required to maintain neuronal Ca^{2+} homeostasis.

Dopaminergic control of larval food seeking

Whilst mechanisms that regulate developmental progression of *Drosophila* larvae have been extensively studied, neural control of essential changes in feeding behaviour that need to accompany each larval developmental stage have not been identified previously. Artificial manipulation of activity in the central dopaminergic neuron subset studied here (THD'), either by expression of an inward rectifying potassium channel (Kir2.1) (**S4A Fig**) or the bacterial sodium channel NaChBac (**S4B Fig**), suggests an important role for THD' neurons during larval development. This idea is supported by the altered dynamics of muscarinic acetylcholine receptor (mAChR) stimulated Ca^{2+} release observed in THD' neurons between early, mid and late third instar larvae when larval feeding slows down and ultimately stops (**S4C and S4D Fig**) and re-iterates that signaling in and from these neurons drives larval feeding whereas lower carbachol-induced Ca^{2+} responses signal cessation of feeding. A weaker rescue of *STIM*^{KO} larvae is also obtained from *STIM*$^{+}$ expression in the THC' neuron subset. Taken together these observations suggest a neuromodulatory role for dopamine, where DA release from THD' neurons has a greater influence on feeding than the DA release from THC' neurons, possibly due to the DL1 and DL2 cluster (among THD' marked neurons) receiving more feeding and metabolic inputs (7,35,58). A role for cells other than THD', in maintaining kinetics of dopamine release required for feeding behaviour are also indicated because expression of STIM^{+} in THD'

neurons did not revert kinetics of dopamine release to wild type levels (**Fig 4C**). The prolonged dopamine release observed in wild-type THD' neurons may arise from synaptic/modulatory inputs to THD' neurons from other neurons that require STIM function.

Though the cells that provide cholinergic inputs to THD' cells have not been identified it is possible that such neurons sense the nutritional state. In this context, two pairs of cells in the THD' subset also motivate the search for food in hungry adult *Drosophila* (62). Starved flies with knock down of the mAChR on THD' neurons exhibit a decrease in food seeking behaviour (43). Cholinergic inputs to THD' neurons for sensing nutritional state/hunger may thus be preserved between larval and adult stages.

Interestingly, dopamine is also required for reward-based feeding, initiation, and reinforcement of feeding behaviour in mice (63). These findings parallel past studies where prenatal mice genetically deficient for dopamine ($DA^{-/-}$), were unable to feed and died from starvation. Feeding could however be initiated upon supplementation with L-DOPA (64) allowing them to survive. More recent findings show that dopaminergic neurons in the ventral tegmental area (VTA), and not the substantia nigra, drive motivational behaviour and facilitate action initiation for feeding in mice (65).

Dopaminergic control of neuropeptide signaling

Both activation and inhibition of specific classes of neuropeptidergic cells by optogenetic activation of THD' cells suggests a dual role for dopamine possibly due to the presence of different classes of DA receptors. The *Drosophila* genome encodes four DA receptors referred to as Dop1R1, Dop1R2, DD2R and a non-canonical DopEcR (66). Dop1R1, Dop1R2 and DopEcR activate adenylate cyclase and stimulate cAMP

signaling whereas DD2R is inhibitory (66). Cell specific differences among dopamine receptors have been observed in adults. Down regulation of *Dop1R1* on AstA and NPF cells shifted preference towards sweet food whereas down regulation of *DopEcR* in DH44 cells shifted preference towards bitter food (67). In third instar larvae a dopaminergic-NPF circuit, arising from central dopaminergic DL2 neurons, two cells of which are marked by *THD'GAL4* (**Fig 2E**), motivates feeding in presence of appetitive odours (6,45). The dopamine-neuropeptide axis identified here demonstrates a broader role for dopamine in regulating neuropeptide release and/or synthesis, in the context of larval feeding behaviour, perhaps similar to the mammalian circuit described above.

Of specific interest is the untimely upregulation of *ilp3* transcripts in *STIM*^{KO} larvae. Rescue of lethality in *STIM*^{KO} larvae either by bringing back activity to THD' neurons or by reducing ilp3 levels suggests an interdependence of Dopamine-Insulin signaling that is likely conserved across organisms (68–73). Our data suggest that ilp3 expression is suppressed during the feeding and growth stages of larvae (**Fig 6H-6M**), and once enough nutrition accumulates expression of ilp3 is up-regulated, concurrent with a reduction in carbachol-induced Ca^{2+} signals in THD' neurons, possibly followed by upregulation and release of ilp3. The idea of ilp3 as a metabolic signal whose expression is antagonistic to larval growth is supported by the observation that knock-down of ilp3 in the MNSc leads to larger pupae in wild type animals and larger larvae in *STIM*^{KO} (**Fig 6F and G**). To our knowledge this is the first report of ilp3 as a larval signal that is antagonistic to growth. Given that *Drosophila* encode a single Insulin receptor for ilp2, ilp3 and ilp5 (74) the cellular mechanism of ilp3 action remains to be elucidated. Possibly, ilps with different affinity for the insulin receptor stimulate different cellular subsets and/or different intracellular

signaling mechanisms, including ecdysone signaling that is essential for larval transition to pupae (74). Interestingly, in *STIM*^{KO} larval brains there is a significant increase in expression of the Insulin Receptor (**S1 Table**).

Expression of other neuropeptides did not show significant changes in *STIM*^{KO} larval brains (**S1 Table**), suggesting that for neuropeptidergic cells in the LNC and SOG dopamine signals alter release properties rather than synthesis. However, we were unable to identify specific neuropeptides for cells in the LNC and the SOG that responded upon activation of THD'.

The importance of dopamine for multiple aspects of feeding behaviour is well documented in mice (63,64). Of interest are more recent findings linking dysregulation of dopamine-insulin signaling with the regulation of energy metabolism (75) and the induction of binge eating (76,77). The identification of a simple neuronal circuit where dopamine-insulin signaling regulates feeding and growth could serve as a useful model for investigating new therapeutic strategies targeted towards the treatment of psychological disorders for obesity and metabolic syndrome (75,76).

Material and methods

Fly rearing and Stocks:

Drosophila strains were reared on standard cornflour agar media consisting of 80 g corn flour, 20 g glucose, 40 g sugar, 15 g yeast extract, 4 ml propionic acid, 5 ml *p*-hydroxybenzoic acid methyl ester in ethanol, 5 ml ortho butyric acid in 1l at 25°C, unless otherwise specified, under a 12:12 hr light : dark cycle. In all studies the *Canton S* (*CS*) strain was used as a wild-type control and CRISPR-Cas9 generated deficiency for *STIM* referred

581 to as *STIM*^{KO} served as a null mutant for the *Drosophila STIM* gene (22).
582 Details of other fly lines used are provided in Table 1 below.
583
584 **Staging:**
585 Synchronized larvae of the appropriate ages as described below
586 were collected and transferred to agar less media containing yeast (4gm),
587 sucrose (8gm), cornflour (16gm), Propionic acid (1ml) and 0.05gm of
588 Benzoic acid in 1ml of absolute alcohol. The number of viable organisms
589 and the developmental stage were scored at specific time points as
590 mentioned below and in the figures and figure legends.
591 Larval staging experiments were performed to obtain lethality and
592 developmental profiles of the indicated genotypes as described previously
593 (78). Depending on the experiment, timed and synchronized egg-laying was
594 done either for 6h to allow development profiling at 60-66h, 80-86h, 128-
595 136h, 176-182h and 320-326h after egg laying or for 2h at 35-37h(36h), 41-
596 43h(42h), 47-49h(48h), 53-55h(54h), 59-61h(60h), 65-67h(66h), 71-
597 73h(72h), 83-85h(84h), and 89-91h(90h) after egg laying for identifying a
598 lethality window between 36-90h. Larvae were collected at either 60–66h or
599 35-37h after egg laying (AEL) in batches of 25 (for developmental profile) or
600 10 (for lethality window). They were screened and staged subsequently.
601 Heterozygous larvae were identified using dominant markers (*FM7iGFP*,
602 *TM6Tb,* and *CyOGFP*) and removed. Each batch of larvae was placed in a
603 separate vial and minimally three vials containing agar-less media were
604 tested for every genotype at each time point. The larvae were screened at
605 the indicated time points for the number of survivors and stage of
606 development, determined by the morphology of the anterior spiracles and
607 mouth hooks (79). Experiments to determine the viability of experimental
608 genotypes and their corresponding genetic controls were performed

simultaneously in all cases. Larval images were taken on the MVX10 Olympus stereo microscope using an Olympus DP71 camera.

Feeding assay:

Feeding assay was performed at specific developmental time points in larvae (40-44h, 58-62h, 80-84h AEL) of the specified genotypes. Larvae were placed in a 35mm punched dish with coverslip at the base thus creating a small depression in the centre of the coverslip. In this depression a coin sized cotton swab was placed containing 4.5.% of yeast solution with 0.25% eriogluasin dye (blue dye). For scoring the number of larvae that fed, 30 larvae per plate were taken and incubated in the feeding plate for 4hrs at 25°C. Larvae were removed from the paste, washed, collected and scored for presence of blue dye (Dye^{+ve}) and absence of blue dye within the gut (Dye^{-ve}).

For quantification of ingested blue dye, 12-15 larvae were incubated for 2h in yeast paste with the blue dye. Larvae were removed from the yeast paste and 10 larvae with blue dye in the gut were washed, and homogenized in 50µl of cold 1xPBS. The homogenate was spun at 5k for 2 minutes in a table top Eppendorf centrifuge. The supernatant (2µl) was taken for quantification of protein using the Thermo scientific Pierce Protein assay kit, Cat#23227. Optical density (OD) at 625nm as a measure of ingested blue dye was measured from 30µl of the homogenate. Due to variation in larval sizes between control and experimental samples, the OD was normalized to whole larval protein concentration (µg/µl). OD was obtained using the SkanIt Software 6.1.1 RE for Microplate Readers RE, ver. 6.1.1.7. Larval imaging and processing was performed on the Olympus MVX10 stereo microscope using FIJI software.

Larval imaging and measurement:

Staged larvae from specified genotypes were collected at specific development time points, anesthetized on ice for 1h and mounted with ice cold HL3 buffer. The mounted larvae were imaged immediately using an Olympus MVX10 stereo microscope. For measurement of larval length from mouth hook to tip of the posterior spiracle FIJI software was used. A minimum of 10-15 larvae were taken per genotype for length analysis.

Quantification of larval mouth hook contractions:

Mouth hook contractions were measured by placing 1-3 appropriately staged larvae in a drop of 2% yeast solution in a petridish. Videos were taken for 30 seconds on an Olympus MVX10 stereo microscope. For each genotype a minimum of 10 animals were imaged. Mouth hook contractions were counted manually from the visualised videos.

Pupal volume measurement:

Pupal volume was measured by obtaining the width and height of each pupal image from the Olympus MVX10 stereo microscope. A formula for obtaining the volume of a cylinder, ($\pi r^2 h$) was applied to calculate the volume (80).

Adult fly weight measurement:

For weight measurement of adult flies, 10 flies (5 females and 5 males) of the appropriate genotype were taken 6 – 10 hr post-eclosion and weighed after placing them in a small Eppendorf tube. Thereafter, the weight of the same empty tube was measured. Fly weights were

calculated by subtracting the weight of the tube from the total weight of flies + tube. A minimum of five such measurements were performed for each genotype.

Immunohistochemistry:

Larval brains were dissected in ice-cold 1xPBS and fixed with 4% paraformaldehyde in 1xPBS on the shaker for 20mins at room temperature. Fixed brains were washed with PBTx (0.3% TritonX-100 in 1XPBS) 3-4 times at 10minutes intervals, blocked with 5% normal goat serum (NGS) in PBTx for 2hrs at room temperature, and incubated with primary antibodies diluted in 5%NGS+PBTx at the appropriate concentration as mentioned below, overnight at 4°C. Antibody solution was removed and re-used upto three times. Brains were washed with PBTx 3 times at 10 minute intervals followed by incubation with secondary antibodies at the dilutions described below, for 2hrs at room temperature and three washes in PBTx of 10minute intervals each. Brains were mounted in 70% glycerol diluted in 1xPBS. Confocal images were acquired by using FV3000 LSM and the Fluoview imaging software.

Following primary antibodies were used: Chick anti-GFP (1:8000, Abcam Cat#13970 RRID: AB_300798), mouse anti-Prospero (1:100, DSHB Cat# Prospero (MR1A), RRID:AB_528440), Rabbit anti-Deadpan (1:400, Abcam– ab195172), Mouse anti-TH (1:5, ImmunoStar Cat#22941; RRID: AB_572268), Mouse anti-bruchpilot (NC82) (dilution1:50, monoclonal, Gift from Eric Buchner, University of Wuerzburg, Germany).

Following secondary antibodies were used :Goat anti-Chicken IgY (H+L), Alexa Fluor 488 (Thermo Fischer Scientific, Cat#A-11039; RRID: AB_2534096), Goat anti-Rabbit IgG (H+L), Alexa Fluor 568 (Thermo Fischer Scientific Cat# A-11011; RRID: AB_143157), Goat anti-Mouse IgG

692 (H+L) Alexa Fluor 633 (Thermo Fischer Scientific Cat#A-21052; RRID:
693 AB_2535719), Goat anti-Rabbit IgG (H+L) Alexa Fluor 594 (Thermo Fischer
694 Scientific Cat#A-11037; RRID: AB_2534095).
695
696 **Ex-vivo imaging of the larval brain:**
697 GCaMP signals were obtained from appropriately aged larval brains
698 dissected from the specified genotypes and dissected in hemolymph like
699 saline (HL3) (70mM NaCl, 5mM KCl, 20mM MgCl2, 10mM NaHCO3, 5mM
700 trehalose, 115mM sucrose, 5 mM HEPES, 1.5mM Ca2+, pH 7.2).
701 Dissected brains were transferred to a 35mm punched dish with a cover
702 slip adhered to the bottom. Brains were embedded in ~5µl of 0.8% low
703 melt agarose (Invitrogen, Cat#16520-100) and bathed in 86µl of HL3.
704 Images were acquired as a time series on an XY plane at an interval of
705 2sec using a 20X-oil objective on an Olympus FV3000 inverted confocal
706 microscope (Olympus Corp., Japan). For KCl stimulation, at the 40th frame,
707 7µl of HL3 was added and at the 80th frame 7µl of 1M KCl was added. The
708 final concentration of KCl in the solution surrounding the brain was 70mM.
709 For stimulation with Carbachol (Sigma Aldrich Cat# C4382), 10µl of HL3
710 was added at the 40th frame followed by 10µl of 100mM Carbachol at the
711 80th frame. Final carbachol concentration was maintained at 0.5mM Ca^{2+}
712 responses were imaged till the 300th frame (600sec).
713 Changes in ER-Ca^{2+} were measured using an ER-GCaMP-210
714 strain (44). The brain sample was prepared as above. Images were
715 acquired as a time series on an XY plane at an interval of 1 sec using a 20X
716 oil objective on an Olympus FV3000 inverted confocal microscope
717 (Olympus Corp., Japan). For Carbachol stimulation, 10µl of HL3 was added
718 at the 50th frame and 10µl of 100mM of Carbachol was added at the 100th

frame. Final carbachol concentration was maintained at 0.5mM. Images were obtained for 600 frames (600 secs).

Larval brain expressing *MNSc>GCaMP* is used for Dopamine (DA) (Sigma, Cat#H8502) stimulation. Dissected brains were transferred to a 35mm punched dish with a cover slip adhered to the bottom. Brains were embedded in ~5µl of 0.8% low melt agarose (Invitrogen, Cat#16520-100) and bathed in 80µl of HL3 having 1uM of TTX. Images were acquired as a time series on an XY plane at an interval of 1.5sec using a 20X-oil objective on an Olympus FV3000 inverted confocal microscope (Olympus Corp., Japan). At the 30th frame, 10µl of HL3 was added and at the 60th frame 10µl of 1mM DA was added. Ca^{2+} responses were imaged till the 250th frame (450sec).

Analysis of Optogenetic signals:

Larvae from the specified genotypes were reared in fly media containing 0.2mM ATR (All-trans retinal Sigma-Aldrich Cat#R2500). Brain samples were prepared as mentioned above. Images were acquired as a time series on an XY plane at an interval of 2 sec/frame using a 20X oil objective on an Olympus FV3000 inverted confocal microscope (Olympus Corp., Japan). For optogenetic stimulation of *CsChrimson*, a 633nm LED (from Thor labs) was used and GCaMP6f fluorescent images were obtained simultaneously using a 488nm laser line so as to measure changes in cytosolic Ca^{2+} upon CsChrimson activation. Image acquired till 200th frames (400 secs).

A minimum of 6 independent brain preparations were used for all live imaging experiments and the exact number of cells imaged are indicated in the figures. Raw fluorescence data were extracted from the marked ROIs

using a time series analyser plugin in Fiji. ΔF/F was calculated using the following formula for each time point (t): $\Delta F/F = (F_t/F_0)/F_0$, where F_0 is the average basal fluorescence obtained from the first 40 frames.

RNA isolation and quantitative PCR

Larval brains from appropriate genotypes and their respective age were dissected in ice cold 1XPBS. Larval brains (10-15) were transferred to a microtube containing 500µl TRIzol (Life Technologies), and vortexed immediately for 10-15secs. After vortexing the samples were either processed within 30 min or stored at −80 °C for processing for up to 2 weeks. RNA was isolated following manufacturer's protocol for TRIzol. Concentration and integrity of RNA was determined on a bioanalyzer (Agilent 2100). RNA (1µg) was taken for cDNA synthesis. DNAse treatment and first strand synthesis was performed as previously described (81). Reverse Transcription followed by PCR (RT-PCR) was performed in a reaction mixture of 25 µl with 1 µl of the cDNA. Quantitative real time PCRs (qPCRs) were performed in a total volume of 10µl with Kapa SYBR Fast qPCR kit (KAPA Biosystems) on a QuantStudio 3 Real-Time PCR system. Duplicates were performed for each qPCR reaction. Minimum of three biological replicates were taken for each qPCR reaction. *rp49* was used as the internal control. The fold change of gene expression in any experimental condition relative to wild-type was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct\ (\text{target gene}) - Ct\ (rp49))_{Expt.} - (Ct\ (\text{target gene}) - Ct\ (rp49))_{Control.}$

Sequences of PCR primers used are as follows:

rp49

F_5'CGGATCGATATGCTAAGCTGT3'

R_5'GCGCTTGTTCGATCCGTA3'

dilp2

F_5'CCATGAGCAAGCCTTTGTCC3'

R_5'TTCACTGCAGAGCGTTCCTTG3'

dilp3

F_5'ACTCGACGTCTTCGGGATG3'

R_5'CGAGGTTTACGTTCTCGGCT3'

dilp5

F_5'ACTCACTGTCGAGCATTCGG3'

R_5'GAGTCGCAGTATGCCCTCAA3'

Quantification and Statistical Analysis

All bar graphs and line plots show the means and standard error of means. In boxplots, horizontal lines in the box indicate median, box limits are from 25th-75th percentiles, and individual data points are represented by closed circles (unless otherwise specified in the figure legends). Unpaired student t-Test (for two genotypes) and one way ANOVA followed by post-hoc Tukey's significance test (for data with multiple genotypes) was performed to calculate P values, given for all figures in **S2 Table.** All graphs were plotted using Origin 8.0 software. Origin 7.5 MicroCal, Origin Lab, Northampton, MA, USA N/A, Fiji Open access (RRID:SCR_002285). Diagrammatic representative images are made with help of Biorender website (https://app.biorender.com).

Table 1: Fly strains

Fly line	Description	Source
Canton S	Wild type	
STIM^{KO}	Null mutant for STIM gene generated with help of	Generated in the lab

	CRISPR-Cas9 gene editing technique	
THGAL4	Dopaminergic GAL4 driver marks TH (Tyrosine Hydroxylase) positive cells in brain and hypoderma	Serge Birman CNRS, ESPCI Paris Tech, France
THGAL80	Inhibits Gal4 expression in Dopaminergic cells	Toshihiro_Kitamoto, University of Iowa, Carver College of Medicine.
THD'GAL4	Marks subgroup of Dopaminergic cells in larval brain	Mark N Wu, Johns Hopkins University, Baltimore
THC'GAL4	Marks dopaminergic cells of larval brain	
C929GAL4	Marks neuropeptidergic cells	P. H. Taghert, Washington University
c929LexA (DimmLexA)	Marks neuropeptidergic cells	Michael Texada, Janelia Farms, USA
transTango	Marks Post synaptic neurons with RFP	Michael Rosbash, Brandeis University, USA
UASSTIM+	*Stim* wildtype cDNA under UAS control	Generated in the lab (82)
UASGRAB$_{DA}$	A Genetically Encoded Fluorescent Sensor for	Yulong Li, Peking University School of

	Dopamine	Life Sciences, Beijing, China
LexAopmCherry	Expresses mCherry under LexAop control	Claude Desplan, New York University, USA
UASmCD8GFP	Expresses membrane tagged GFP under UAS control	RRID: BDSC_5130
UASKir2.1	Prevents membrane depolarization	RRID: BDSC_6595
UASGCaMP6m	Ca^{2+} Sensor with intermediate kinetics expresses under UAS	RRID: BDSC_42748 from Douglas Kim
LexAopGCaMP6f	Ca^{2+} Sensor with faster kinetics expresses under LexAop	RRID: BDSC_44277
UASSyteGFP,Denmark	Syt-GFP marks presynaptic terminal and Denmark (RFP) marks post synaptic part of the neuron.	RRID: BDSC_33065
UASCsChrimson	Optogenetically encoded cation channel that is activated at 625nm	RRID: BDSC_55136
InscGAL4	Marks neuroblasts of larval brain with GAL4	RRID: BDSC_8751
UASFUCCI	Marks different phases of cell cycle with fluorescent markers	RRID: BDSC_55100
UASNaChBac	Increases sodium conductance a activates the neuron	RRID: BDSC_9468

UASERGCaMP-210	ER specific Ca^{2+} sensor	Cahir O'Kane, Cambridge University, UK
dsSTIM (III)	UAS-RNAi against Stim gene	VDRC_47073
dsTH (III)	UAS-RNAi against *Tyrosine hydroxylase* (TH) gene	RRID: BDSC_25796
UASDicer2(X)	Enhancer for RNAi	RRID: BDSC_24648
MNScGAL4 (Dilp2GAL4)	Marks ilp producing MNSc cells of larval brain	RRID: BDSC_37516
UASilp3^{+}	Over expression of wildtype ilp3 gene	Ernst Hafen's lab
UASdsilp3	RNAi for ilp3 gene	RRID: BDSC_33681
UASDop1R1RNAi	RNAi for Dop1R1 receptor	RRID: BDSC_62193
UASDopEcR RNAi	RNAi for DopEcR receptor	RRID: BDSC_31981
UASDop2R2RNAi	RNAi for Dop2R receptor	RRID: BDSC_36824
THD'GAL4, THGAL80/CyO	Recombinant line made for this study	
THD'GAL4, UASGCaMP6m/CyO		
c929LexA, LexAopGCaMP6f, LexAopmCherry/TM6Tb		
THD'GAL4, UASCsChrimson/CyO		
STIM^{KO}*; +; THGAL4/TM3SerG*	Strains made for this study	
STIM^{KO}*;*		

THD'GAL4/CyOG; +	
STIM^{KO}; *THC'GAL4/CyOG; +*	
STIM^{KO}; *UASSTIM/CyOG; +*	
STIM^{KO}; *UASGCaMP6m/CyOG; +*	
STIM^{KO}; *UASmCD8GFP/CyOG; +*	
STIM^{KO}; *UASdsilp3/CyOG; +*	
STIM^{KO}; *UASMNScGAL4/CyOG; +*	
UAS-STIM+/CyOG; *UASGRAB$_{DA}$/Tb*	
UAS-STIM+/CyOG; *UASERGCaMP-210/Tb*	

Acknowledgements

This study was supported by core funds from NCBS (TIFR), a SERB distinguished fellowship to GH (SB/S9/YSCP/SERB-DF/2018-1), a DBT grant (BT/PR28450/MED/122/166/2018) to GH and a DST-WOS-A grant (SR/WOS-A/LS-703/2016) to NK. We thank Fly base for access to various databases, Bloomington and VDRC fly stock centres, Fly Facility at NCBS for fly lines and NCBS Central Imaging and Flow Facility (CIFF) for confocal imaging and NCBS lab support for timely help.

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Figure legends:

Fig 1. *STIM^{KO}* larvae exhibit reduced feeding followed by systemic growth arrest.

(A-C) Number of 1st instar (L1), 2nd instar (L2) and 3rd instar (L3) larvae from *CS* (grey) and *STIM^{KO}* (magenta) measured at 6h intervals after egg laying (AEL) at the specified time points (mean ± SEM). Number of sets (N) = 3, number of larvae per replicate (n) = 10. *P < 0.05, Student's *t*-test with unequal variances. P values are given in **S2 Table.**

(D) Representative images of larvae from *CS* and *STIM^{KO}* at the indicated time. Scale bar = 1 mm

(E) Measurement of larval length (mean ± SEM) from *CS* (grey) and *STIM^{KO}* (magenta) larvae at the specified time points. Number of larvae per genotype per time point is (n) ≥ 12. **P* < 0.05 for all time points, Student's *t*-test with unequal variances. P values are given in **S2 Table.**

(F) Representative images of thoracic neuroblasts marked with *Insc>mCD8GFP* (green), a neuroblast marker (anti-Deadpan, red) and a marker for post-mitotic cells (anti-Prospero, blue) from control

(*Insc>mCD8GFP*) and *STIM^{KO}; Insc>mCD8GFP* animals at the indicated ages. Similar images were obtained from four or more animals. Scale bar = 20µm.

(G) Diagrammatic summary of neuroblast proliferation in control (*Insc>mCD8GFP*) and *STIM^{KO}; Insc>mCD8GFP* animals.

(H) Stack bar graph showing number of dividing neuroblasts to non-dividing neuroblasts. *$P < 0.05$, Student's *t*-test with unequal variances, n = 4 animals from each genotype. P values are given in **S2 Table.**

(I) Representative images of dye-fed larvae from *CS* and *STIM^{KO}* at the indicated times AEL, scale bar = 200µm except for *CS* (80-84h) where scale = 1mm.

(J) Quantification (mean ± SEM) of ingested blue dye in *CS* and *STIM^{KO}* larvae at the indicated ages by normalizing optical density (OD) of the dye at 655nm to concentration of protein. Number of feeding plates per time point (N) = 6, number of larvae per plate (n) =10. *$P < 0.05$, Student's *t*-test with unequal variances. P values are given in **S2 Table.**

(K) Line graph with quantification of larval mouth hook contractions per 30 seconds (mean ± SEM) from *CS* and *STIM^{KO}* at indicated developmental time points. Number of larvae per genotype per time point is (n) ≥ 10. *$P < 0.05$ at all time points, Student's *t*-test with unequal variances. All P values are given in **S2 Table.**

Fig 2. Feeding and growth deficits of *STIM^{KO}* larvae arise from central dopaminergic neurons

(A) Number (mean ± SEM) of 3rd instar larvae (L3) are restored close to wildtype (CS) levels by expression of *STIM^{+}* in *THD'* cells of *STIM^{KO}* larvae (rescue). Larvae were monitored at 6h intervals from 66h to 96h AEL.

Number of sets (N) = 3, number of larvae per set (n) = 10. Letters represent statistically similar groups for the 90h and 96h time point. Also see **S2A Fig** for a complete developmental profile of CS, *STIM*^{KO} and rescue *(STIM*^{KO}; *THD'>STIM*$^{+}$) animals with appropriate genetic controls.

(B) Stack bars with the number of adults (mean ± SEM) that eclosed at 320 to 326h AEL from the indicated genotypes. The genotype of rescue larvae is *STIM*^{KO}*; THD'>STIM*$^{+}$*.* Number of sets (N) = 3, number of organisms per set (n) = 25.

(C) Representative confocal images of the larval brain from animals of the genotype *THD'>mCD8GFP*. Anti-GFP (green) indicates the expression of *THD'GAL4* and anti-TH (magenta) marks all dopaminergic cells. Asterisks mark TH^{+ve} cells in CNS whereas arrowheads mark non-TH positive cells in ventral ganglia of larval brain (i and ii). DL1 and DL2 clusters in the central brain of three and two dopaminergic cells respectively are marked (iii). Scale bars = 20µm.

(D) Representative confocal images of the larval brain from animals of the genotype *THGAL80,THD'>mCD8GFP*. *THD'GAL4* driven GFP expression (green) is suppressed in DL1 and DL2 clusters in the CNS (asterisk) by *THGAL80* but not in the ventral ganglia (arrowheads). Scale bar = 20µm.

(E) Line graph shows the number (mean ± SEM) of 3rd instar larvae from *CS, STIM*^{KO}, *STIM*^{KO}*;THD'>STIM*$^{+}$ *and STIM*^{KO}*;THGAL80,THD'>STIM*$^{+}$ at 6h intervals between 60 to 96h AEL. Number of sets (N) = 3, number of larvae per set (n) = 10. Different alphabet represent statistically significant groups for 84h, 90h and 96h. Also see **S2A Fig** for complete larval staging profile and additional genetic controls.

(F) Stack bar graph showing the number of adults eclosed (mean ± SEM) at 320 to 326h AEL from *CS (*wildtype*), STIM*^{KO} (mutant), *STIM*^{KO}*;THD'>STIM*$^{+}$ (rescue) and *STIM*^{KO}*;THGAL80,THD'>STIM*$^{+}$ (THGAL80 - restrictive rescue)

genotypes. Different alphabet represent statistically significant groups. Number of sets (N) = 3, number of organisms per set (n) = 25.

(G) Representative images of dye-fed larvae of *CS, STIM*^{KO} and rescue (*STIM*^{KO}*; THD'>STIM*$^{+}$) genotypes at 80-84h AEL Scale bar = 1mm.

(H) Bar graph with quantification of ingested food containing a blue dye in larvae of the indicated genotypes (*CS, STIM*^{KO} and *STIM*^{KO}*; THD'>STIM*$^{+}$ rescue) at the indicated developmental times. Mean (mean ± SEM) optical density (655nM) of blue dye in larval lysates after normalizing to larval protein concentration (OD/Protein concentration X 10^{-2}) was obtained from 6 feeding plates (N) each containing 10 larvae (n). Different alphabet represent statistically significant groups.

(I) Expression of *STIM*$^{+}$ in THD' cells rescues the feeding behaviour deficit of *STIM*^{KO} larvae. Box graph with quantification of larval mouth hook contractions of the indicated genotypes (*CS, STIM*^{KO} and *STIM*^{KO}*; THD'>STIM*$^{+}$ rescue). Circles represent single larvae in the box graph of 25th and 75th percentiles with the median (bar), and mean (square). Number of larvae per genotype per time point is (n) ≥10. Different alphabet represent statistically significant groups.

(J) Number of 3r^{d} instar larvae (mean ± SEM) from RNAi knockdown of STIM^{+} in THD' neurons (*THD'>dsSTIM*) along with control genotypes *THD'/+* and *dsSTIM/+* at 6h intervals between 66h to 96h AEL (After Egg laying). Number of sets (N) = 3, number of larvae per set (n) = 10. Also see **S2B** and **S2C Figs** for complete larval staging profile and adult weights. Different alphabet represent statistically significant groups.

(K) Representative images of dye-fed larvae of the indicated genotypes at 58-62h and 82-86h AEL. Scale bar = 200µm.

(L) Quantification (mean ± SEM) of blue dye containing ingested food (OD/Protein concentration X 10^{-2}; similar to panel D above) in larvae of the indicated genotypes at 58-62h and 82-86h AEL. No. of plates for each time point, (N) = 3, number of larvae per plate (n) = 10.

In all graphs and box plots, different alphabets represent distinct statistical groups as calculated by one way ANOVA followed by post-hoc Tukey's test. P values for individual panels are given in **S2 Table.**

Fig 3. **Dopamine synthesis and excitability in THD' neurons is important for larval growth.**

(A) Larval mouth hook movements that correlate with feeding are reduced in larvae with reduced dopamine synthesis in THD' neurons. Box graph with quantification of mouth hook contractions in larvae with knockdown of Tyrosine Hydroxylase (*dsTH*) in THD' neurons and appropriate control genotypes. Number of larvae per genotype per time point is (n) ≥10.

(B) Representative images of larvae with knockdown of Tyrosine Hydroxylase (*THD'>dsTH*) and controls (*THD'/+, dsTH/+*) at 82-86h. Scale bar = 1mm.

(C) Quantification of larval length from the indicated genotypes. n≥15.

(D) Quantification of weight of 10 flies from the indicated genotypes.. Each circle represents one set of adult flies consisting of 5 females and 5 males, 6-8h post-eclosion. A minimum of 5 sets were measured for each genotype.

(E) Representative images of the central brain (left panels) indicating the region of focus (boxed), followed by images of DL1 and DL2 clusters of THD' cells from two lobes of same brain with Ca^{2+} transients at the indicated time points before and after addition of a depolarizing agent (KCl – 70mM). Ca^{2+} transients were measured by changes in the intensity of

GCaMP6m fluorescence from *THD'>GCaMP6m* (control), *STIM^{KO}; THD'>GCaMP6m*, *STIM^{KO};THD'>GCaMP6m, STIM^{+}* (rescue). Scale bar 20µm

(F) Changes in GCaMP6m fluorescence (mean ± SEM of ΔF/F) from THD' neurons of the indicated genotypes. Number of brains, (N) ≥ 5, number of cells, (n) ≥ 15.

(G) Peak intensities of GCaMP6m fluorescence (ΔF) in THD' cells from the indicated genotypes. Box plots show 25th and 75th percentiles, the median (bar), and mean (square) of ΔF of each cell (small circles).

(H) Number of 3rd instar larvae of *CS, STIM^{KO}* and *STIM^{KO};THD'>NaChBac* genotypes at the indicated times AEL (mean ± SEM). Number of sets (N) = 3, number of larvae per set (n) = 15.

(I) Representative images of larvae from *CS, STIM^{KO}* and *STIM^{KO}; THD'>NaChBac* at 94-98h AEL, scale bar = 1mm.

(J) Quantification of larval length from the indicated genotypes. n=10.

(K) Number of adults eclosed at 320h AEL (mean ±_SEM) from wildtype (*CS),* mutant (*STIM^{KO}*) and NaChBac rescue (*STIM^{KO}; THD'>NaChBac*) animals. Numbers were obtained from three experiments (N = 3), number of organisms per experiment, n = 15.

In all box plots, circles represent single larvae or flies. The box plots span 25th and 75th percentiles with the median (bar), and mean (square). Alphabets represent distinct statistical groups as calculated by one way ANOVA followed by post-hoc Tukey's test. P values are given in **S2 Table.**

Fig 4. STIM is necessary for dopamine release and ER-Ca^{2+} homeostasis

(A) Axonal and dendritic projections of THD' neurons visualized by expression of SyteGFP (green) and Denmark (anti-RFP, magenta) respectively in a representative brain immunostained for anti-Brp (blue). Panels marked as (d) contain magnified images from (c) of presynaptic terminals (green) located at the base of the mushroom body in the CNS (asterisk), as branches extruding into basomedial protocerebrum of the CNS (arrowhead) and as punctae near the oesophageal region (hash).

(B) Representative images of dopamine release before and after addition of Carbachol (CCh) as measured by changes in intensity of $GRAB_{DA}$ at the presynaptic terminals of THD' neurons of control (*THD'>GRAB*$_{DA}$), *STIM*^{KO} (*STIM*^{KO}*;THD'>GRAB*$_{DA}$) and rescue (*STIM*^{KO}*;THD'>GRAB*$_{DA}$*, STIM*$^{+}$*)* genotypes. Scale bar = 20µm

(C) Normalized changes in fluorescence (ΔF/F) of $GRAB_{DA}$ measuring dopamine release from THD' neurons of control (*THD'>GRAB*$_{DA}$), *STIM*^{KO} (*STIM*^{KO}*;THD'>GRAB*$_{DA}$) and rescue (*STIM*^{KO}*;THD'>GRAB*$_{DA}$*, STIM*$^{+}$*)* genotypes. Traces show the average change in $GRAB_{DA}$ florescence (mean ± SEM of (ΔF/F)) measured from individual presynaptic regions of interest ($\geq$10) taken from N $\geq$ 6 brains of each genotype.

(D) Representative time series images of ER calcium transients as measured by changes in intensity of ER-GCaMP in THD' neurons of control (*THD'>ER-GCaMP-210*), *STIM*^{KO} (*STIM*^{KO}*;THD'> ER-GCaMP-210)* and rescue (*STIM*^{KO}*;THD'> ER-GCaMP-210, STIM*$^{+}$) genotypes.

(E) i) Traces of normalized ER-GCaMP (ΔF/F) responses (mean ± SEM) from THD' neurons of the indicated genotypes. Control (*THD'>ER-GCaMP-210*) Type 1, indicates cells that exhibit ER-Ca^{2+} release upon CCh addition followed by refilling of ER-stores. Control Type 2 indicates cells where ER-

Ca^{2+} release was not evident. Rescue indicates *STIM*^{KO}; *THD'>ERGCaMP-210,STIM*$^{+}$. For each genotype, number of cells (n) $\geq$ 15 and N $\geq$ 5 brains. (ii) Quantification of control cells that exhibit ER Ca^{2+} release (Type 1) and cells where ER Ca^{2+} release was not observed (Type 2). (iii) Boxed region from (i) enlarged to show ER Ca^{2+} response of Type 1 and Type 2 control cells.

Fig 5. Differential regulation of neuropeptidergic cell activity by THD' neurons

(A) Pictorial representation of an experimental setup for testing functional connectivity of dopaminergic (THD') and peptidergic (C929) cells in organisms of the genotype *THD'>Chrimson, C929LexA>LexAopGCaMP6m.* The lower panel shows the peptidergic neurons containing regions 1-3 in which change in Ca^{2+} transients were observed upon activation of THD' cells.

(B) Ca^{2+} transients in peptidergic Median Neurosecretory cells (MNSc, top), lateral neurosecretory cells (LNSc, middle) and suboesophageal region (SEZ, bottom) as measured by the intensity of GCaMP6m before Chrimson activation (100s) and after Chrimson activation (300s and 400s) of THD' cells. Activated cells (red asterisks) and inhibited cells (yellow asterisks) are marked. Scale bar = 20um.

(C) Quantification of total C929 marked cells from 9 brains that were either optogenetically activated or inhibited.

(D) Average GCaMP6m responses (mean ± SEM of $\Delta F/F$) across time in C929 marked cells that were activated upon Chrimson activation of THD' neurons at 633nm. The box plot shows 25th and 75th percentiles of peak responses measured by change in GCaMP6m of individual activated

peptidergic cells (circles), with the median (bar), and mean (square). n = 24 cells from (N) = 9 brains

(E) Average GCaMP6m responses (mean ± SEM of ∆F/F) across time in C929 marked cells that were inhibited upon Chrimson activation of THD' neurons at 633nm. The box plot shows 25th and 75th percentiles of peak responses measured by change in GCaMP6m of individual inhibited peptidergic cells (circles), with the median (bar), and mean (square). n = 40 cells from (N) = 9

(F) (i) Pulsed activation of THD' neurons produce corresponding pulses of activation in certain peptidergic neurons as evident from their GCaMP6m responses (mean ± SEM). Red panels indicate the time intervals (50s each) of *THD'>Chrimson* activation. n = 21 cells from N = 11 brains.

(ii) Pulsed activation of THD' neurons produce corresponding pulses of inhibition in certain peptidergic neurons as evident from their GCaMP6m responses (mean ± SEM). Red panels indicate the time intervals (50s each) of *THD'>Chrimson* activation. n = 19 cells from N = 9 brains.

(G) GCaMP6m responses (mean ± SEM) are absent in C929 cells in the absence of red light and in larvae that were not fed with All-trans Retinol (ATR). n = 50 cells from N=9 brains.

Fig 6. Developmental expression of insulin-like peptide 3 (ilp3) is regulated by STIM in THD' neurons

(A) FPKM values obtained for transcripts encoding the indicated insulin like peptides (*ilp*) obtained from an RNAseq experiment performed from *CS* brains (58-62h, mid 2nd instar stage) and *STIM^{KO}* brains (72-76h, mid 2nd instar stage).

(B) Quantification of *ilp2, ilp3* and *ilp5* transcripts in *CS*, *STIM^{KO}* and *STIM^{KO}; THD'>STIM^{+}* (rescue) brains. Expression changes of *ilp3* and *ilp5*

transcripts in *STIM*^{KO} brains were rescued upon over-expression of *STIM*$^{+}$ in THD' neurons. Transcripts were measured relative to controls. Data show (mean ± SEM) of fold changes of gene expression (N=3).

(C) Heatmap of *ilp3* expression from larval to pupal stages. FPKM values were obtained from the DGET database (https://www.flyrnai.org/tools/dget/web/).

(D) Knockdown of *ilp3* partially rescues viability in *STIM*^{KO} larvae. Stack bar graph showing the number of viable organisms and their developmental stage at the specified hours after egg laying for *CS, STIM*^{KO}*; MNScGAL4/+, STIM*^{KO}*; dsilp3/+* (control) genotypes followed by *STIM*^{KO}*;MNScGAL4>dsilp3*. Data are from three experiments, each with 25 organisms per genotype.

(E) Representative images of larvae from *CS* and *STIM*^{KO}*; MNSc>dsilp3* at 118-122h AEL, scale bar = 1mm.

(F) Quantification of larval length from *CS* and *STIM*^{KO}*; MNSc>dsilp3* (rescue, L3b) genotypes. Box plots show the 25th and 75th percentiles with median (bar), mean (square) and sizes of individual larvae (circles), n=10. *P<0.05 (unpaired t-test).

(G) Representative image of an adult *STIM*^{KO} mutant fly rescued by knockdown of *ilp3* (*STIM*^{KO}*; MNScGAL4>dsilp3*). Scale bar = 1mm.

(H) Number of 3rd instar larvae at the indicated times AEL (mean ± SEM) from the indicated genotypes with knockdown of *ilp3* in the MNSc (*MNSc>dsilp3*) and of controls (*MNSc/+ and dsilp3/+*). Alphabets indicate statistically different groups at 132h and 144h. Number of sets (N) = 3 and number of larvae per set (n) = 10.

(I) Representative images of pupae with knock-down of *ilp3* (*MNSc> dsilp3*) at 144-156h AEL and appropriate controls (*MNSc/+* and *dsilp3/+*). Scale bar = 1mm.

(J) Quantification of pupal volume from the indicated genotypes. Box plots show the 25th and 75th percentiles with median (bar), mean (square) and sizes of individual pupa (circles), n=30

(K) Number of 3rd instar larvae obtained by over-expression of *ilp3* (*MNSc>ilp3*$^{+}$*)* and in appropriate control genotypes (*MNSc/+, ilp3*$^{+}$/+) at the indicated time AEL (mean $\pm$ SEM). (N) = 3 and (n) = 10. Alphabets indicate statistically different groups.

(L) Representative images of larvae with over-expression of *ilp3* (*MNSc>ilp3*$^{+}$) and appropriate controls (*MNSc/+* and *ilp3*$^{+}$/+) at 94-98h AEL, scale bar = 1mm.

(M) Overexpression of ilp3 results in smaller sized larvae. Quantification of larval length from the indicated genotypes. Box plots show the 25th and 75th percentiles with median (bar), mean (square) and sizes of individual larvae (circles), n $\geq$24.

(N) Overexpression of ilp3 does not affect feeding. Box graph showing 25th and 75th percentiles, the median (bar), and mean (square) of number of larval mouth hook contraction per 30 seconds, where each circle represents a single larva of the indicated genotype. Number of larvae per genotype is (n)≥10.

For all graphs and box plots, alphabets represent distinct statistical groups as calculated by one way ANOVA followed by post-hoc Tukey's test, n.s. indicates non-significant groups. P values are given in **S2 Table.**

Fig 7. MNSc respond differentially to Dopamine stimulation

(A) Development profiles of larvae with RNAi mediated knockdown of dopamine receptors in the MNSc. Bar graphs represent number of viable organisms and their developmental stages at the specified hours after egg

laying for the indicated genotypes (*CS, MNScGAL4/+, dsDop1R1/+, dsDopEcR/+, dsDop2R1/+* (Controls), *MNSc>dsDop1R1, MNSc>dsDopEcR*, and *MNSc>dsDop2R2*). N=3 batches of n=25 larvae for every genotype. P values of all genotypes compared with appropriate controls were calculated by one way ANOVA followed by post-hoc Tukey's test and are given in **S2 Table.**

(B) Representative images of larvae from specified genotypes at 128-134hr. Scale bar = 1mm

(C) Quantification of weight of 10 flies from the indicated genotypes. Box plot shows the 25th and 75th percentiles with median (bar) and mean (square). Each circle represents one set of adult flies consisting of 5 females and 5 males, 6-8h post-eclosion. A minimum of 5 sets were measured for each genotype. Alphabets represent distinct statistical groups as calculated by one way ANOVA followed by post-hoc Tukey's test. P values are given in **S2 Table.**

(D) Representative images of larval brains expressing the calcium indicator GCaMP6 in MNSc. Brains were stimulated with either dopamine (DA) in presence of tetrodotoxin (top panel) or a control solution (HL3; lower panel) and imaged over time. Scale bar = 20µm

(E) Changes in GCaMP6m fluorescence (mean ± SEM of $\Delta F/F$) from MNSc cells with addition of either dopamine (DA) and Tetrodotoxin (TTX) (7 brains and 12-14 cells) or HL3 (No DA+ No TTX) (5 brains and 14 cells).

Supplementary figure legends:

Supplementary fig 1.

(A-B) Number of viable organisms (mean ± SEM) at the indicated developmental stage of *CS* and *STIM*^{KO} after egg laying. Number of sets (N) = 3, number of organisms per set (n) = 25.

(C) Confocal images of larval brains at the indicated time points expressing the FUCCI marker. Control genotype is *Insc>FUCCI* (top row) and the mutant genotype is *STIM*^{KO}; *Insc>FUCCI* (bottom row). Here late mitosis/G1 phase, S-phase and G2/early mitosis are marked by green, red, and yellow fluorescent indicators respectively. Scale bar = 20mm; n = 5 larval brains.

(D) Representative image of dye-fed larvae from *CS* and *STIM*^{KO} at 80-84h AEL. Scale bar: 2mm

(E) Bar graph showing the average number of Dye^{+ve} (presence of blue dye in the gut) and Dye^{-ve} (absence of blue dye in the gut) *CS* and *STIM*^{KO} larvae at the indicated ages (mean ± SEM). Number of feeding plates per time point (N) = 3, number of larvae per plate (n) = 30. *$P < 0.05$, Student's *t*-test with unequal variances. P values are given in **S2 Table**.

Supplementary fig 2.

(A) Stack bar graph showing the number of viable organisms (mean ± SEM) and their developmental stage at the specified hours after egg laying for the indicated genotypes. Coloured arrows mark bars that exhibit rescue of *STIM*^{KO} upon expression of STIM^{+} driven by *THGAL4* (dark green), *THC'GAL4* (blue), *THD'GAL4* (green) and restricted rescue in presence *of THGAL80* (red) with all appropriate genetic controls as indicated. Number of sets (N) = 3, number of organisms per set (n) = 25.

(B) Stack bar graph showing the number of viable organisms (mean ± SEM) and their developmental stage at specified hours after egg laying for the indicated genotypes; *THD'/+, dicer;+;dsSTIM/+* (controls) and

THD'>dicer,dsSTIM. Number of sets (N)=3, number of organisms per set (n)=25.

(C) Quantification of weight of 10 flies from indicated genotypes. Box plots show the 25th and 75th percentiles with median (bar), mean (square) and each circle represents one set. Each set consists 10 flies of which 5 are females and 5 are male adult flies collected at 6-8h after eclosion. Number of set (N) ≥5. Alphabets indicate different statistical groups.

In all panels significant changes between relevant genotypes at the indicated stages were calculated by one way ANOVA followed by post-hoc Tukey's test. P values are given in **S2 Table.**

Supplementary fig 3.

(A) Representative confocal images of larval brains showing THD' neurons marked with anti-GFP (green) and anti-TH (red) from control (*THD'>mCD8GFP*), *STIM^{KO} (STIM^{KO}; THD'>mCD8GFP)* and rescue (*STIM^{KO};THD'>mCD8GFP, STIM^{+}*) animals at the indicated developmental time points. Scale bars = 20µm.

(B) Numbers of TH positive cells (magenta) and THD' cells (green) in the larval CNS of the indicated genotypes. Cells were quantified from *THD'>mCD8GFP* (control) at 58-62h and 80-84h and from *STIM^{KO};THD'>mCD8GFP* (*STIM^{KO}*) and *STIM^{KO};THD'>mCD8GFP, STIM^{+}* (rescue) at 80-84h. The numbers of TH^{+ve} and GFP^{+ve} cells were counted manually and were no different among four hemi-lobes from four brains of a single genotype and among all brain hemi-lobes of all genotypes. Hence the absence of error bars.

(C) Stack bar graph showing the number of viable organisms (mean ± SEM) and their developmental stage at specified hours after egg laying for the

indicated genotypes *THD'/+, dsTH/+* (controls), and *THD'>dsTH*. Number of sets (N)=3, number of organisms per set (n)=25. Significant changes were calculated by one way ANOVA followed by post-hoc Tukey's test. P values are given in **S2 Table.**

(D) Representative images of the central brain (left panels) indicating the region of focus (boxed), followed by images of THD' cells with Ca^{2+} transients at the indicated time points after addition of a depolarizing agent (KCl, 70mM). Ca^{2+} transients were measured in the indicated genotypes: *THD'>GCaMP6m* and *THD'>dicer;dsSTIM,GCaMP6m* by measuring changes in the intensity of GCaMP6m fluorescence. Scale bar = 20µm

(E) Quantified changes in GCaMP6m fluorescence (mean ± SEM of $\Delta F/F$) from THD' neurons of the indicated genotypes. Number of brains, (N) $\geq$ 6, number of cells, (n) $\geq$11.

(F) Peak intensities of GCaMP6m fluorescence (ΔF) in THD' cells from the indicated genotypes. Box plots show 25th and 75th percentiles, the median (bar), and mean (square) of ΔF of each cell (small circles).

Supplementary fig 4.

(A-B) Altered excitability in THD' neurons by expression of either a mammalian inward rectifying potassium channel (Kir2.1) or a bacterial sodium channel (NachBac) affects larval developmental progression and viability. Stack bar graphs show the number of viable organisms (mean $\pm$ SEM) and their developmental stage at the indicated hours after egg laying (top) for the indicated genotypes. Number of sets (N) = 3, number of organisms per set (n) = 25. P values calculated after one way ANOVA followed by post-hoc Tukey's test are given for relevant stages and genotypes in **S2 Table.**

(C) Representative images of the central brain (left panels) indicating the region of focus (boxed), followed by images of THD' cells with Ca^{2+} transients at the indicated time points after addition of mAChR agonist (CCh 100µM). Ca^{2+} transients were measured in the indicated genotype by measuring the changes in the intensity of GCaMP6m fluorescence. Scale bar = 20µm

(D) Quantified changes in GCaMP6m fluorescence (mean ± SEM of $\Delta F/F$) from THD' neurons of the indicated genotypes. Number of brains, (N) $\geq$ 4, number of cells, (n) $\geq$12.

Supplementary fig 5.

(A) Larval developmental profile with overexpression of ilp3 in MNSc (red arrow) appears similar to knockdown of ilp5 (green arrow). Stack bar graph showing the number of viable organisms and their developmental stage at the specified hours after egg laying for the indicated genotypes. N=3 sets with 25 larvae in each set.

(B) Knockdown and overexpression of ilp3 in the MNSc affects adult weights in opposing directions. Quantification of weight of 10 flies from the indicated genotypes. Box plot shows the 25th and 75th percentiles with median (bar), mean (square). Each circle represents one set consisting of 10 flies in each of which 5 females and 5 males were collected at 6-8h post- eclosion. A minimum of 5 sets were quantified for each genotype. Alphabets indicate statistically different groups based on P values calculated after one way ANOVA followed by post-hoc Tukey's test, given for relevant stages and genotypes in **S2 Table.**

Supplementary video 1: Mouth hook contractions in *CS* larvae (70-74h).

Supplementary video 2: Mouth hook contractions in *STIM*^{KO} larvae (70-74h).

Supplementary video 3: Mouth hook contractions in *CS* larvae (82-84h).

Supplementary video 4: Mouth hook contractions in *STIM*^{KO} larvae (82-84h).

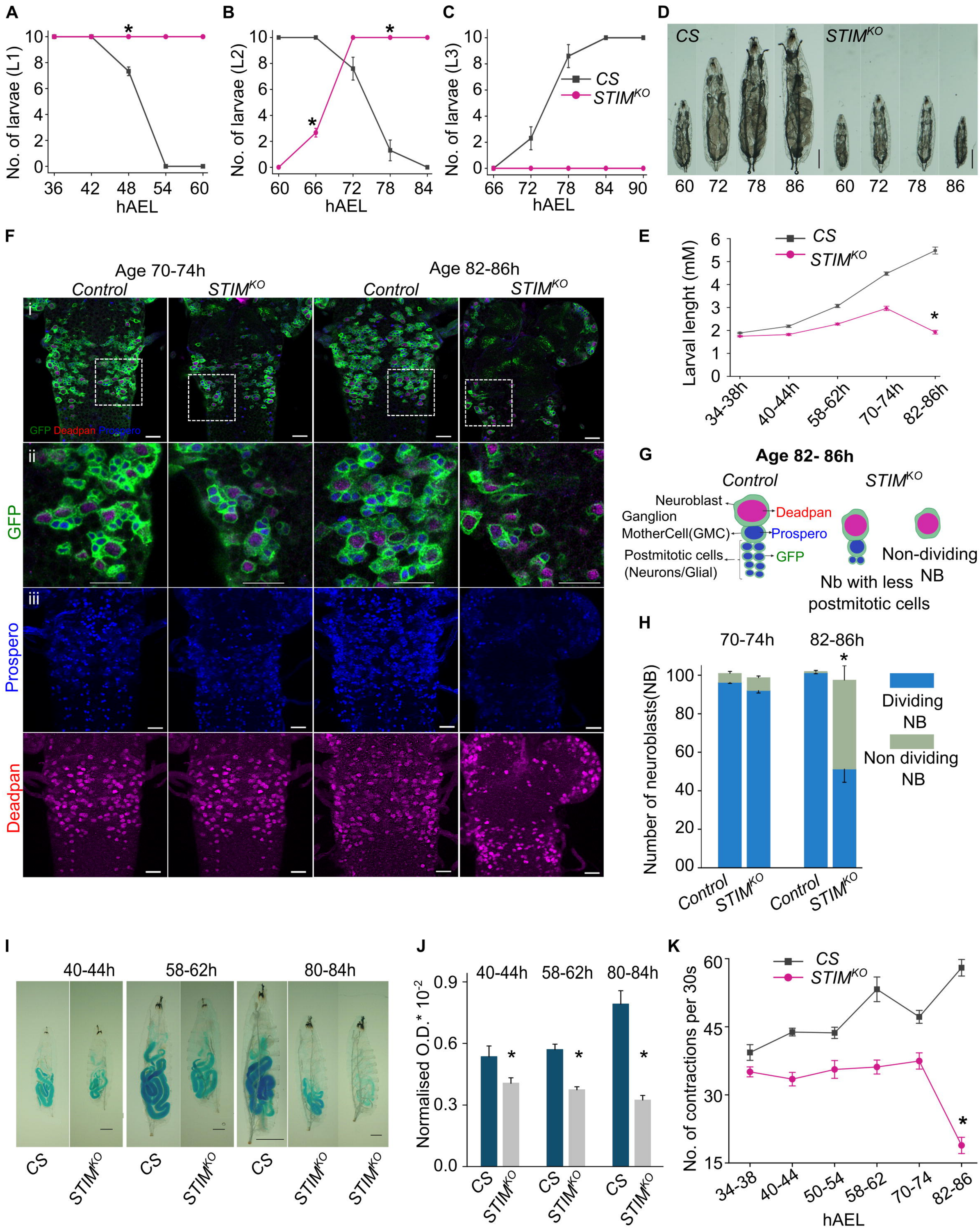
Supplementary video 5: Mouth hook contractions in *THD'>dsTH* larvae (82-86h).

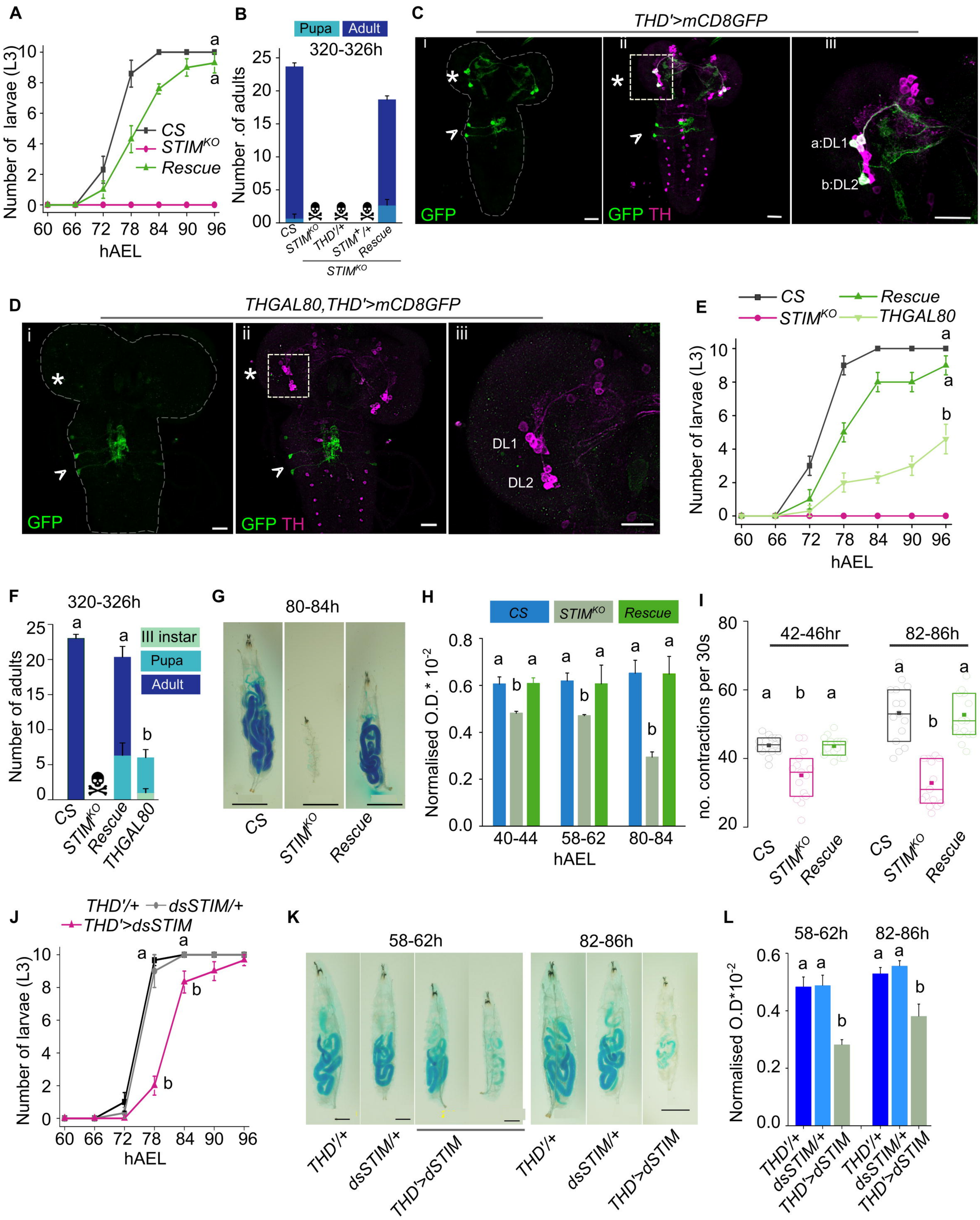
Supplementary video 6: Mouth hook contractions in *THD'/+* (control) larvae (82-86h).

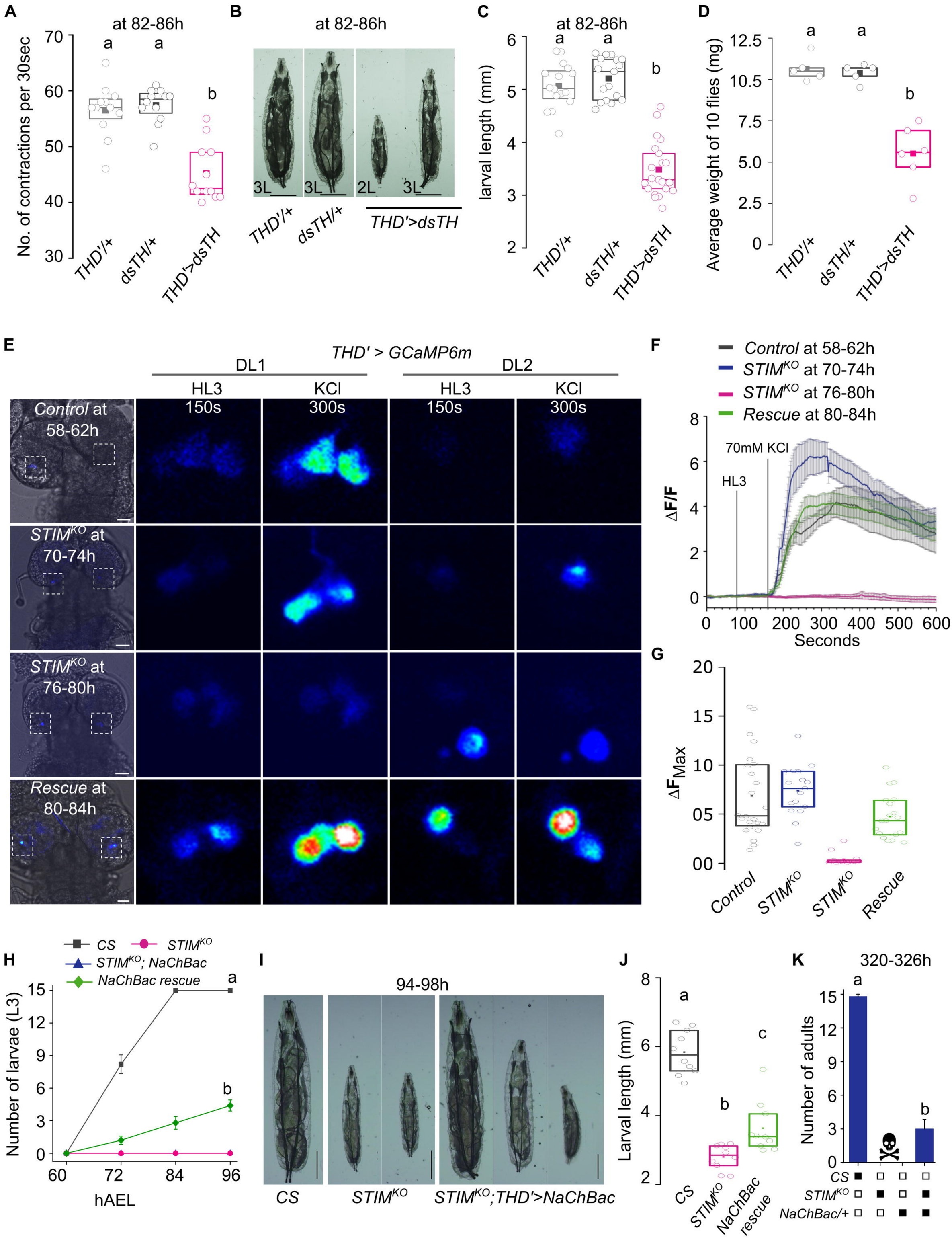
Supplementary video 7: Mouth hook contractions in *dsTH/+* (control) larvae (82-86h).

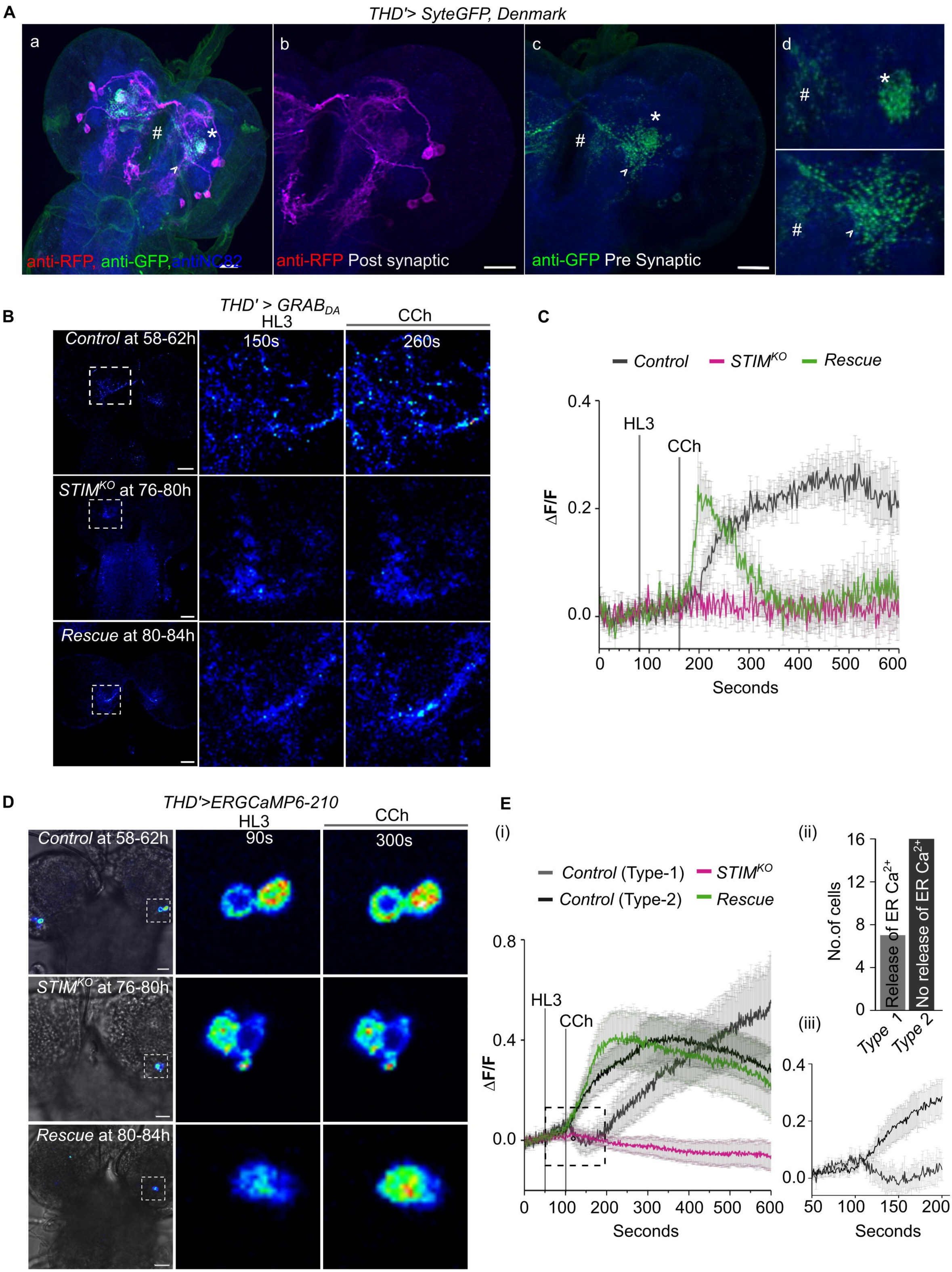
Supplementary Table 1: Expression status of genes encoding neuropeptides in *STIM*^{KO} brains.

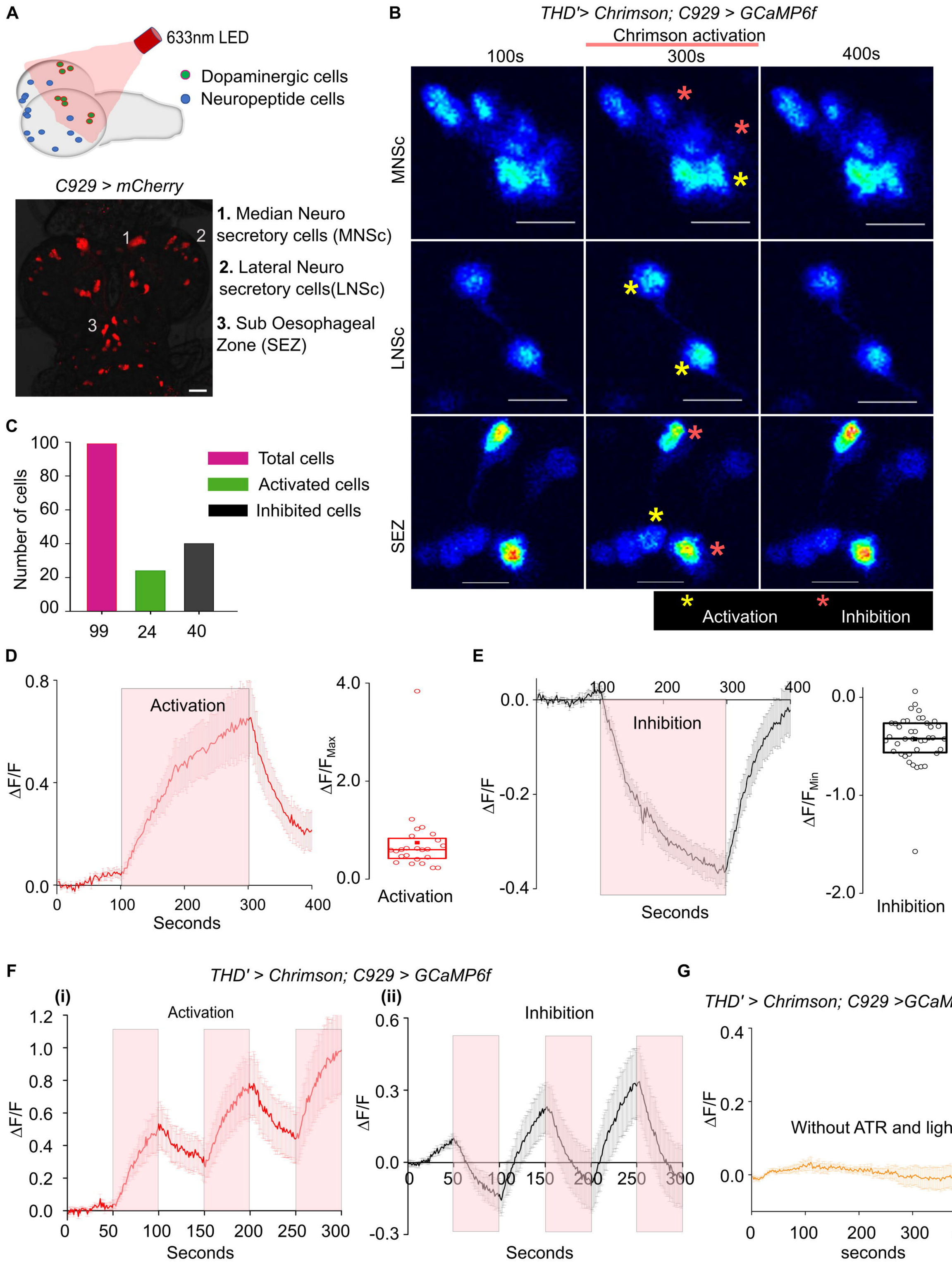
Supplementary Table 2: P values for main and supplementary figures.

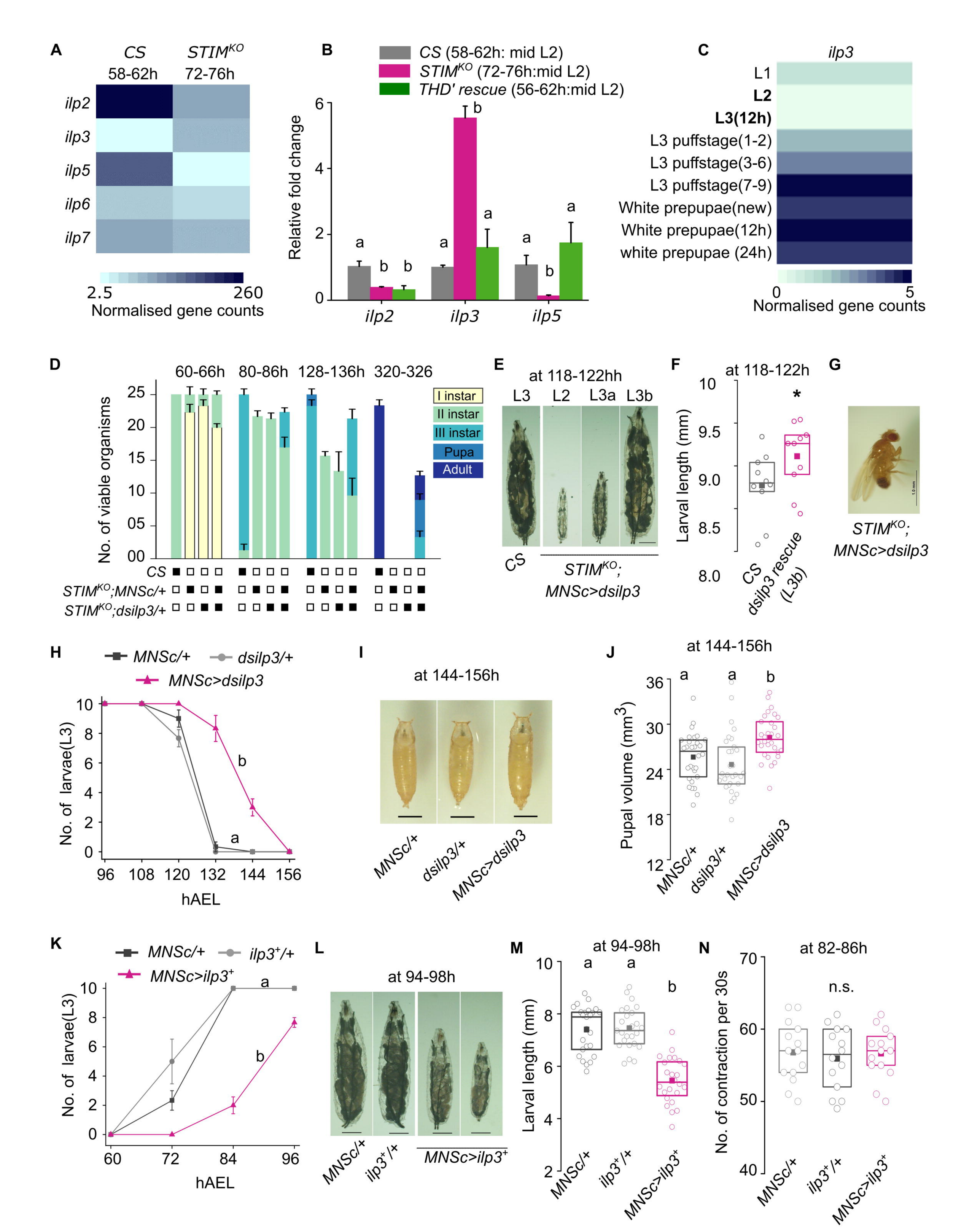

A
No. of larvae (L1)
hAEL
B
No. of larvae (L2)
hAEL
C
No. of larvae (L3)
hAEL
CS
STIMKO
D
CS
STIMKO
60 72 78 86 60 72 78 86
E
Larval lenght (mM)
CS
STIMKO
34-38h
40-44h
58-62h
70-74h
82-86h
F
Age 70-74h
Control
STIMKO
Age 82-86h
Control
STIMKO
GFP Deadpan Prospero
GFP
Prospero
Deadpan
G
Age 82- 86h
Control
STIMKO
Neuroblast
Ganglion MotherCell(GMC)
Postmitotic cells (Neurons/Glial)
Deadpan
Prospero
GFP
Non-dividing NB
Nb with less postmitotic cells
H
70-74h
82-86h
Number of neuroblasts(NB)
Dividing NB
Non dividing NB
Control
STIMKO
I
40-44h
58-62h
80-84h
CS
STIMKO
J
Normalised O.D.* 10-2
40-44h
58-62h
80-84h
CS
STIMKO
K
No. of contractions per 30s
CS
STIMKO
34-38
40-44
50-54
58-62
70-74
82-86
hAEL

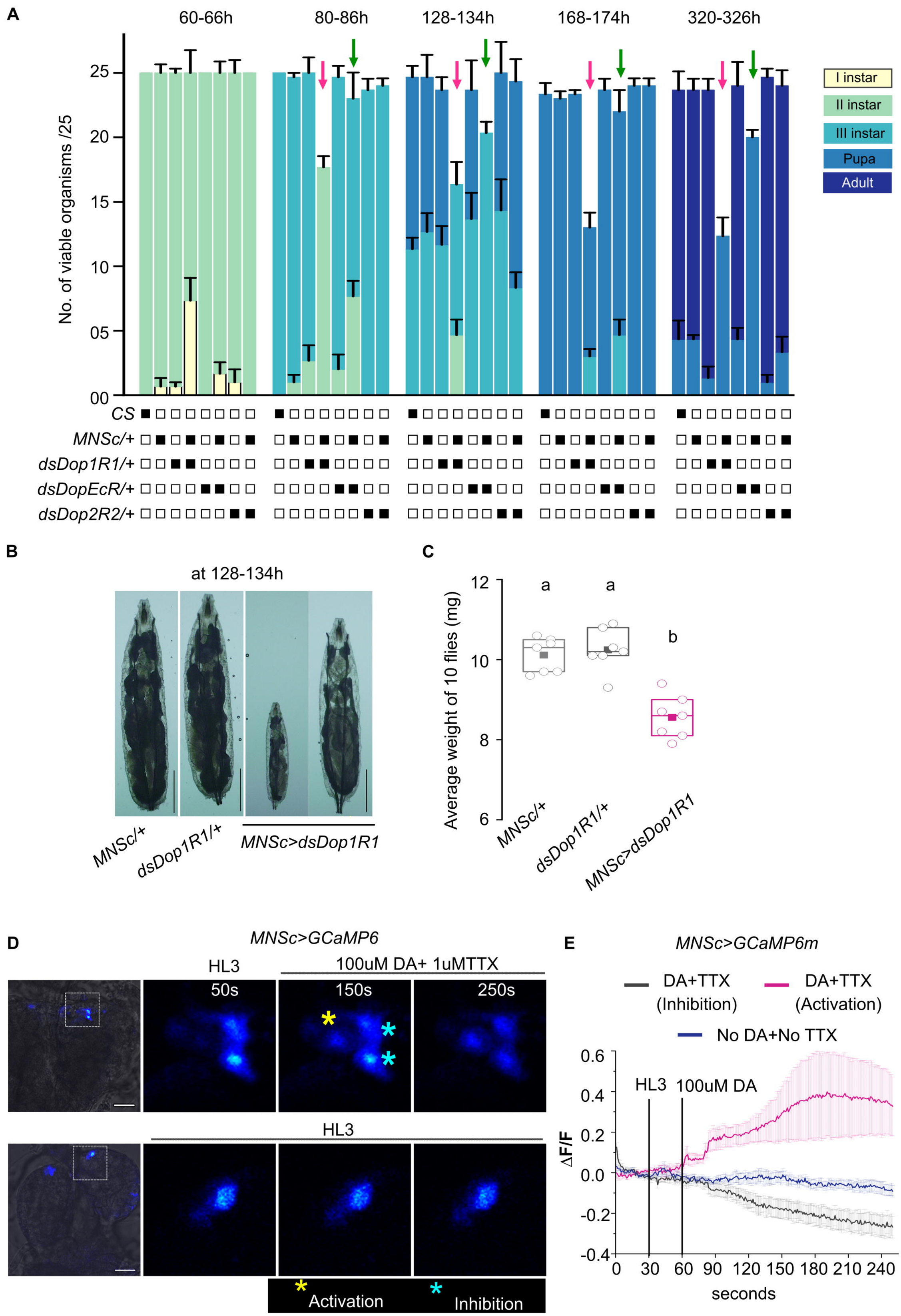

A
Number of larvae (L3)
hAEL
CS
STIMKO
Rescue
B
Pupa
Adult
320-326h
Number .of adults
CS
STIMKO
THD'/+
STIM+/+
Rescue
STIMKO
C
THD'>mCD8GFP
GFP
GFP TH
a:DL1
b:DL2
D
THGAL80,THD'>mCD8GFP
GFP
GFP TH
DL1
DL2
E
CS
STIMKO
Rescue
THGAL80
Number of larvae (L3)
hAEL
F
320-326h
Number of adults
III instar
Pupa
Adult
CS
STIMKO
Rescue
THGAL80
G
80-84h
CS
STIMKO
Rescue
H
CS
STIMKO
Rescue
Normalised O.D.* 10-2
40-44
58-62
80-84
hAEL
I
42-46hr
82-86h
no. contractions per 30s
CS
STIMKO
Rescue
J
THD'/+
dsSTIM/+
THD'>dsSTIM
Number of larvae (L3)
hAEL
K
58-62h
82-86h
THD'/+
dsSTIM/+
THD'>dsSTIM
L
58-62h
82-86h
Normalised O.D*10-2
THD'/+
dsSTIM/+
THD'>dsSTIM


A
at 82-86h
No. of contractions per 30sec
70
60
50
40
30
a
a
b
THD'/+
dsTH/+
THD'>dsTH
B
at 82-86h
3L
3L
2L
3L
THD'/+
dsTH/+
THD'>dsTH
C
at 82-86h
larval length (mm)
6
5
4
3
2
a
a
b
THD'/+
dsTH/+
THD'>dsTH
D
Average weight of 10 flies (mg)
12.5
10.5
7.5
5.0
2.5
0
a
a
b
THD'/+
dsTH/+
THD'>dsTH
E
THD' > GCaMP6m
DL1
DL2
HL3
KCl
HL3
KCl
150s
300s
150s
300s
Control at 58-62h
STIMKO at 70-74h
STIMKO at 76-80h
Rescue at 80-84h
F
Control at 58-62h
STIMKO at 70-74h
STIMKO at 76-80h
Rescue at 80-84h
70mM KCl
HL3
ΔF/F
8
6
4
2
0
0
100
200
300
400
500
600
Seconds
G
ΔFMax
20
15
10
05
00
Control
STIMKO
STIMKO
Rescue
H
CS
STIMKO
STIMKO; NaChBac
NaChBac rescue
Number of larvae (L3)
15
12
9
6
3
0
a
b
60
72
84
96
hAEL
I
94-98h
CS
STIMKO
STIMKO;THD'>NaChBac
J
Larval length (mm)
8
6
4
2
a
b
c
CS
STIMKO
NaChBac rescue
K
320-326h
Number of adults
15
12
9
6
3
0
a
b
CS
STIMKO
NaChBac/+


A
THD'> SyteGFP, Denmark
a
b
c
d
#
*
anti-RFP, anti-GFP,antiNC82
anti-RFP Post synaptic
anti-GFP Pre Synaptic
B
THD' > GRAB$_{DA}$
HL3
CCh
Control at 58-62h
150s
260s
STIM^{KO} at 76-80h
Rescue at 80-84h
C
Control
STIM^{KO}
Rescue
HL3
CCh
ΔF/F
0.4
0.2
0.0
0
100
200
300
400
500
600
Seconds
D
THD'>ERGCaMP6-210
HL3
CCh
Control at 58-62h
90s
300s
STIM^{KO} at 76-80h
Rescue at 80-84h
E
(i)
Control (Type-1)
STIM^{KO}
Control (Type-2)
Rescue
HL3
CCh
ΔF/F
0.8
0.4
0.0
0
100
200
300
400
500
600
Seconds
(ii)
No.of cells
16
12
8
4
0
Release of ER Ca^{2+}
No release of ER Ca^{2+}
Type 1
Type 2
(iii)
0.4
0.2
0.0
50
100
150
200
Seconds


A
633nm LED
Dopaminergic cells
Neuropeptide cells
C929 > mCherry
1
2
3
1. Median Neuro secretory cells (MNSc)
2. Lateral Neuro secretory cells(LNSc)
3. Sub Oesophageal Zone (SEZ)
B
THD'> Chrimson; C929 > GCaMP6f
Chrimson activation
100s
300s
400s
MNSc
LNSc
SEZ
Activation
Inhibition
C
Number of cells
Total cells
Activated cells
Inhibited cells
99
24
40
D
Activation
ΔF/F
Seconds
ΔF/F Max
Activation
E
Inhibition
ΔF/F
Seconds
ΔF/F Min
Inhibition
F
THD' > Chrimson; C929 > GCaMP6f
(i)
Activation
ΔF/F
Seconds
(ii)
Inhibition
ΔF/F
Seconds
G
THD' > Chrimson; C929 >GCaMP6f
Without ATR and light
ΔF/F
seconds


A
CS
STIMKO
58-62h
72-76h
ilp2
ilp3
ilp5
ilp6
ilp7
2.5
260
Normalised gene counts
B
CS (58-62h: mid L2)
STIMKO (72-76h:mid L2)
THD' rescue (56-62h:mid L2)
Relative fold change
ilp2
ilp3
ilp5
C
ilp3
L1
L2
L3(12h)
L3 puffstage(1-2)
L3 puffstage(3-6)
L3 puffstage(7-9)
White prepupae(new)
White prepupae(12h)
white prepupae (24h)
0
5
Normalised gene counts
D
60-66h
80-86h
128-136h
320-326
No. of viable organisms
I instar
II instar
III instar
Pupa
Adult
CS
STIMKO;MNSc/+
STIMKO;dsilp3/+
E
at 118-122hh
L3
L2
L3a
L3b
CS
STIMKO;
MNSc>dsilp3
F
at 118-122h
Larval length (mm)
CS
dsilp3 rescue
(L3b)
G
STIMKO;
MNSc>dsilp3
H
MNSc/+
dsilp3/+
MNSc>dsilp3
No. of larvae(L3)
hAEL
I
at 144-156h
MNSc/+
dsilp3/+
MNSc>dsilp3
J
at 144-156h
Pupal volume (mm3)
MNSc/+
dsilp3/+
MNSc>dsilp3
K
MNSc/+
ilp3+/+
MNSc>ilp3+
No. of larvae(L3)
hAEL
L
at 94-98h
MNSc/+
ilp3+/+
MNSc>ilp3+
M
at 94-98h
Larval length (mm)
MNSc/+
ilp3+/+
MNSc>ilp3+
N
at 82-86h
n.s.
No. of contraction per 30s
MNSc/+
ilp3+/+
MNSc>ilp3+


A
60-66h
80-86h
128-134h
168-174h
320-326h
No. of viable organisms /25
25
20
15
10
05
00
I instar
II instar
III instar
Pupa
Adult
CS
MNSc/+
dsDop1R1/+
dsDopEcR/+
dsDop2R2/+
B
at 128-134h
MNSc/+
dsDop1R1/+
MNSc>dsDop1R1
C
Average weight of 10 flies (mg)
12
10
8
6
a
a
b
MNSc/+
dsDop1R1/+
MNSc>dsDop1R1
D
MNSc>GCaMP6
HL3
100uM DA+ 1uMTTX
50s
150s
250s
HL3
Activation
Inhibition
E
MNSc>GCaMP6m
DA+TTX (Inhibition)
DA+TTX (Activation)
No DA+No TTX
ΔF/F
0.6
0.4
0.2
0.0
-0.2
-0.4
HL3
100uM DA
0
30
60
90
120
150
180
210
240
seconds