Supplementary Methods

<u>Fly stocks</u>

The following stocks used in this study have been described previously. <u>*Drivers:*</u> *mex1-Gal4*[51] (*mex*), *mex1-Gal4 Tubulin-Gal80*^{TS} (*mex*^{TS}), *mex1-Gal4 UAS-2x-GFP (mex > GFP), esg-sfGFP* (*esg-GFP*, generated by David Doupé), *ChAT*^{MI04508}*-Gal80* (*ChAT-Gal80*, BDSC:60321)[52], *Tsh-Gal80* (gift from Todd R. Laverty), *R49E06-Gal4* (*ARCENs*, BDSC:38689)[53], *Tubulin-Gal80*^{TS}; *R49E06-Gal4* (*R49E06*^{TS} and *ARCENs*^{TS}), *VT004958-p65AD* (BDSC: 71993)[54], *VT047163-Gal4DBD* (BDSC: 75312)[54], *VT004958-p65AD;VT047163-Gal4DBD* (spECs)[54], *hml-Gal4Δ UAS-GFP (hml,* BDSC: 30142)*, Tubulin-Gal80*^{TS} (BDSC:7018,7019)[55]. Perrimon lab stocks: *Myo31DF*^{NP0001}*-Gal4* (*myo1A-Gal4*)[56], *myo1A-Gal4 UAS-GFP* (*myo1A > GFP*), *Myo1A-Gal4 Tubulin-Gal80*^{TS}(*myo1A*^{TS}), *esg-Gal4 Tubulin-Gal80*^{TS} *(esg*^{TS}), *Tubulin-Gal80*^{TS}*; esg-Gal4 (esg*^{TS}*), esg-Gal4*, *Su(H)GBE-Gal4 UAS-CD8-GFP; Tubulin-Gal80*^{TS} (*Su(H)GBE*^{TS}), *Tubulin-Gal80*^{TS}*; pros-Gal4* (*pros*^{TS}), *elav-Gal4; Tubulin-Gal80*^{TS} (*elav*^{TS}), *hml-Gal4ΔUAS-GFP; Tubulin-Gal80*^{TS} (*hml*^{TS}), *Tubulin-Gal80*^{TS} *; how*^{24B}*-Gal4* (*how*^{TS}), *upd3-Gal4 UAS-GFP* (*upd3>GFP*). <u>*Reporters:*</u> *20XUAS-IVS-jGCaMP7c* (*UAS-GCAMP7c,* BDSC: 79030)[57], TOE.GS01624 (*gRNA-ACE*, BDSC: 79471)[58], *UAS-3XFLAG-dCas9-VPR* (*UAS-dCas9VPR*, BDSC: 66562)[58], *UAS-nAChRβ3*^{RNAi}(JF01947, BDSC: 25927)[59], *UAS-2x-GFP* (*2xGFP*, BDSC: 6874), *LexAop-CD8-GFP-2A-CD8GFP;UAS-mLexA-VP16-NFAT LexAop-rCD2-GFP* (*NFAT-CaLexA*, BDSC:66542)[60], *UAS-Orai*[61], *UAS-ChAT*^{RNAi} (JF01877, BDSC:25856)[59], *UAS-sc*^{RNAi} (JF02104, BDSC: 26206), *20XUAS-6XGFP* (*6xGFP*, BDSC: 52262), *10XUAS-mCD8::GFP* (*mCD8:GFP*, BDSC:32184, 32186), *UAS-mCD8::GFP QUAS-mtdTomato-3xHA* (*UAS-GFP QUAS-Tomato-HA*, BDSC:30118), *QUAS-mtdTomato-3xHA* (*QUAS-Tomato-HA*, BDSC: 30005), *20xUAS-6xGFP* (*6xGFP*, BDSC: 52262), *20xUAS-6xmCherry-HA* (*6xmCherry*, BDSC: 52268), *5xUAS-IVS-Syt1::smGdP-HA* (*Syt1-HA*, BDSC:62142)[62], *10xUAS-IVS-myr::GFP* (*myrGFP*, BDSC: 32198), *13xLexAop2-6xGFP* (*LexAop6xGFP*, BDSC: 52265)[63], *UAS-shibire*^{TS} (BDSC: 44222)[64], *UAS-CsChrimson* (*20XUAS-IVS-CsChrimson.mCherry*, BDSC: 82181)[65], *QUAS-CsChrimson* (gift from Chris Potter)[66], *13xLexAop-sfGFP* (*LexAopGFP*, generated by Pedro Saavedra), *UAS-TrpA1*[67], *UAS-wgn*^{RNAi} (HMC03962, BDSC: 55275), *UAS-wgn*$^{RNAi-2}$ (GLC01716, BDSC: 50594), *UAS-grnd*^{RNAi} (GD12580/v43454), *UAS-Inx2*^{RNAi} (JF02446, BDSC: 29306), *UAS-Inx7*^{RNAi} (JF02066, BDSC:26297), *UAS-eiger*^{IR} (*egr*^{RNAi}*,* BDSC: 58993), *UAS-DenMarK* (BDSC:33061)[68], *lexAop-UAS-morphotrap.ext.mCh* (*UAS-morphotrap,* BDSC:68171)[69]*,* 13xLexAop-*IVS-jGCaMP7c* (*LexAopGCaMP7c*, BDSC:80916), 4x*UAS-PV-Myc* (*UAS-PV*, BDSC:25030)[70], *UAS-emptyVK37*

(gift from Hugo Bellen), *UAS- Luciferase*^{RNAi} (JF01355, BDSC: 31603), *UAS-Luciferase* (BDSC: 35789). Protein trap: *Egr-GFP* (BDSC: 66381)[71]. Enhancer traps: *Diap1-LacZ* (BDSC:12093), *Vn-LacZ* (BDSC:11749), *Ex-LacZ* (BDSC: 44248).

The following stocks were generated in this study. Reporters: 1) To generate the *UAS-nAChRβ3*$^{RNAi-2}$ line, we used the Valium20 vector and followed a previous protocol[72]. Guide strand: CGGCGAGAAGATCATGATCAA, Passenger strand: TTGATCATGATCTTCTCGCCG. 2) To generate the *UAS-nAChRβ3* and *LexAop-nAChRβ3* line, we recombined *nAChRβ3* cDNA from DmCD00481061 (from FlyBi Consortium) into pValium10-roe and LexAop-GW, respectively, using Gateway™ LR Clonase™ II Enzyme Mix (Invitrogen). *nAChRβ3*$^{RNAi-2}$*,* *nAChRβ3* and *LexAop-nAChRβ3* were integrated into the attp40 site following injection[72]. *LexAop-nAChRβ3* was also integrated into the VK27 site by BestGene Inc. Drivers: 1) To generate *mex1-LexA::GAD* (*mex-LexA*)*,* we linearized the pDPPattB-LHG[63] vector with Acc65I and BssHII enzymes and cloned gBlock1 and gBlock2 (see at the end of Methods, generated by IDT) in the vector using Gibson Assembly (NEB). *Mex1-LexA::GAD* was integrated into attp40 and attp2 sites[72] as well as VK27 site (BestGene). 2) To generate *R49E06-QF* (*ARCENs-QF*), we amplified the R49E06 promoter with primers R49E06QF-F and R49E06QF-R from *Ore R* gDNA. R49E06QF-primers were based on the primers used to generate *R49E06-Gal4*[53]. We next linearized pattB-DSCP-QF#7-hsp70 (Plasmid #46133)[73] using BamHI and NsiI and with Gibson Assembly (NEB) cloned *R49E06* promoter in the plasmid.
R49E06QF-F: ACATCCAGTGTTTGTTCCTTGTGTAGACTGACATTCCGTTGCCAAGAAGCGC
R49E06QF-R: TCGATCCCCGGGCGAGCTCGGATCAGCGTGTCCTGTAGTACCAGCATAC
R49E06-QF was integrated into attp40 and attp2 sites[72].
nAChRβ3-flag: We generated the nAChRβ3-flag genomic construct using Scarless gene editing as described previously[74]. In brief, to generate ~ 1kb Left and Right homology arms that flank *nAChRβ3* cleavage site (2L: 546,835-546,836), we designed primers Left-F, Left-R, Right-F and Right-R (see below). Right-R primer included a silent mutation to disrupt the PAM sequence. We used gDNA from Cas9 flies to amplify the homology arms which we cloned to pScarlessHD-3xFLAG-DsRed plasmid (gift from Kate O'Connor-Giles, Addgene plasmid # 80820) with Gibson Assembly (NEB).
Left-F: AATTGTAATACGACTCACTATAGGGGCGTGTGGCAACCGT,
Left-R: ACCTCCAGATCCACCACCTCGGTGTGGTTGATGCCCA
Right-F: TTCTGGTGGTTCAGGAGGTTCCGCCGTGTGCCAAGG
Right-R: AATTAACCCTCACTAAAGGGATTCCACGGTCTGATGGCCG.

nAChRβ3 gRNA guides were cloned into the pCFD4 vector (Addgene #49411)[75] as described at http://www.crisprflydesign.org/wp-content/uploads/2014/06/Cloning-with-pCFD4.pdf
Guide strand: ATCAACCACACCGAGGTGCC, Passenger strand: GGCACCTCGGTGTGGTTGATC. After injection, once CRISPR alleles were identified with the DsRed marker, we used pBac transposase to generate scarless *nAChRβ3-flag* genomic flies as described at https://flycrispr.org/scarless-gene-editing/ .

Inducing injury and recovery

We used DSS to damage the epithelium because it stimulates regeneration slower than bacterial infections (DSS: few days, infection: few hours)[76,77], thus giving more time to experiment on pathways activated during and after injury. For DSS-injury, flies were fed daily with ~2% DSS (Dextran Sulfate Sodium Salt; MP Biomedicals) mixed in standard fly food for 4 days at either 23°C or 29°C as specified. To induce recovery following DSS-injury, flies were transferred to standard fly food at 29°C as specified. For immunocytochemistry experiments, fly guts were cleared for 2hrs with 5% sucrose (using Kimwipe, KimTech) when testing for homeostasis and recovery, and with 5% sucrose together with ~2% DSS when testing for injury.

For Bleomycin-injury which causes a distinct type of injury from DSS[78], flies were fed with 25μg/ml Bleomycin in 5% sucrose for 2 days at 29°C. For recovery they were transferred in standard food for 2 days (29°C) after Bleomycin-injury. To test for homeostasis, flies were processed as the experimental conditions without DSS- or Bleomycin- feeding, respectively. For bacterial infection flies were starved for 2hrs and then fed with *Ecc15* at OD100 in 5% sucrose while uninfected flies were treated with 5% sucrose alone.

Immunocytochemistry

Vibratome-sectioned flies: Whole flies were fixed in 4% PFA and 0.4mg/ml Pierce™ DSP (Thermo Scientific) in PBS while rotating for 4-5 hrs at 4°C. Fixed flies were then placed in warm 4% agarose solution inside a 15mmx15mmx5 mold/adaptor (VWR) and left on ice for 30min. Agarose coated flies were sectioned in 150μm slices with a Leica VT1000 vibratome in cold PBS using MX35 Ultra microtome blades (Thermo Scientific). Sectioned flies were removed from agarose and fixed again for 15min at RT. Samples were washed with 0.25% Triton-X in PBS 3 times for 10min at RT and then transferred to Blocking solution (5% Normal Donkey Serum, 0.25% Triton X-100 in PBS) overnight at 4°C. Primary antibodies were added with blocking solution for 48hrs at 4°C. Sectioned flies were then washed with 0.25% Triton-X in PBS, 5 times for 30min at 4°C. Secondary antibodies, Phalloidin and DAPI were added in

blocking solution at 4°C overnight in the dark. Samples were then washed with 0.25% Triton-X in PBS, 5 times for 30min at 4°C in the dark and mounted with Vectashield medium using bridge and covered with No.1 thickness cover glass (ThermoScientific).

Dissected guts: Guts were dissected in cold PBS, fixed in 4% PFA in PBS for 25min at RT, washed 3 times for 10min in PBS and incubated for 1hr in Blocking solution (5% Normal Donkey Serum, 0.1% Triton X-100 in PBS). They were then stained with primary antibodies in Blocking Solution overnight at 4°C. Afterwards, guts were washed 3 times for 10min in 0.1% Triton X-100 in PBS and stained with secondary antibodies and DAPI in Blocking Solution at 4°C overnight in the dark. Guts were washed with 0.1% Triton X-100 in PBS 3 times for 10min and mounted in Vectashield medium.

Dissected VNC and Brain: VNC and brains were dissected in cold PBS and fixed in 4% PFA in PBS for 30min at RT. They were washed for 10min with 1% Triton X-100 in PBS then 2 times for 10 min in 0.5 % Triton X-100 in PBS, incubated for 1hr in Blocking solution (5% Normal Donkey Serum, 0.25% Triton X-100 in PBS) and then stained with primary antibodies in Blocking Solution overnight at 4°C. Afterwards they were washed 3 times for 10min in 0.25% Triton X-100 in PBS. Next, they were stained with secondary antibodies and DAPI in Blocking Solution at 4°C overnight in the dark and washed with 0.25% Triton X-100 in PBS 3 times for 10min before mounted in Vectashield medium.

The following antibodies were used: rabbit anti-Flag (Sigma, F7425; 1:100), mouse anti-β-galactosidase (Promega Z378A; 1:500), rabbit anti-pH3 (Millipore #06-570; 1:3000), mouse anti-GFP (Invitrogen A11120; 1:500), mouse anti-ChAT (DSHB; 1:500) rabbit anti-GFP (Invitrogen A6455; 1:3000), rabbit anti-DsRED (Clontech #632496; 1:200), chicken anti-GFP (AVES; 1:2000), rat anti-HA (Sigma 3F10; 1:500), mouse anti-Pros (DSHB MR1A; 1:50), rabbit anti-Syt1 (gift from Hugo Bellen; 1:1500), rabbit cleaved anti-Dcp1(Cell Signaling Asp216; 1:100), rabbit anti-pdm1 (gift from Xiaohang Yang; 1:500), mouse anti-pdm1 (DSHB 2D4; 1:20), guinea pig anti-Inx2 (gift from Guy Tanentzapf; 1:1000), Phalloidin Alexa-647 (Invitrogen A2284; 1:50), Phalloidin Alexa-633 (Invitrogen A2287; 1:50), Phalloidin Alexa-405 (Invitrogen A30104; 1:50), DAPI (1:3000), Alexa Fluor-conjugated donkey-anti-mouse, donkey-anti-rabbit, goat-anti-chicken, goat-anti-guinea pig and donkey-anti-rat secondary antibody (ThermoFisher; 1:1000).

Ion dye assays

Flies were fed DSS-food for 4 days at 23°C and then transferred to 29°C with standard food for 1 day and then changed for 18hrs (overnight) to 5% sucrose together with SodiumGreen or MQAE dye. Specifically, flies were fed 2μM Sodium Green Tetraacetate

Indicator (Invitrogen, S6900) or 2.5mM MQAE ((N-(Ethoxycarbonylmethyl)-6-Methoxyquinolinium Bromide) (Thermo Fisher, E3101) diluted in 5% sucrose (as previously described[79]). Next guts were dissected and fixed in 4% PFA in PBS for 30min in the dark. After one rinse and one wash (10min) with PBS, DAPI or PI (1:3000) was added with PBS for 10min. Guts were washed one last time with PBS, mounted in Vectashield and taken immediately for confocal imaging.

Optogenetic activation

CsChrimson activation in ARCENs: Flies were raised in the dark at 19°C. To induce recovery flies were first fed with 2%DSS-food for 4 days at 23°C in the dark before transferring to standard food for ~6hrs (recovery d1). During recovery flies were at 29°C without light, except for pulsed red light (~630nm) using 1-meter RGB LED Strip (SMD5050, eTopxizu).

CsChrimson activation in spECs: Flies were raised in the dark at 25°C. To induced recovery flies were fed 2% DSS-food for 2 days at 29°C in the dark before transferring to standard food for ~7hrs. For homeostasis flies were kept in standard food at 29°C for 2 days. During 7hr CsChrimson activation flies were at 29°C in the dark along with pulsed red light (~630nm) using 1-meter RGB LED Strip (SMD5050, eTopxizu). Flies that did not undergo CsChrimson activation were in similar conditions but covered with aluminum foil.

Confocal imaging and quantification

Confocal imaging was conducted with Zeiss LSM710, LSM780 and LSM980 confocal microscope using 25x, 40x and 63x oil objective lenses with identical acquisition conditions for all samples of a given experiment. For whole fly imaging, tile scans were stitched while imaging with the Zeiss Zen Blue acquisition software. Fiji (https://imageJ.net/Fiji) was used to assemble all images and measure mean fluorescence. Brightness was adjusted equally across comparable images and background signal was subtracted (Despeckle) equally across comparable images for clarity. The number of pH3+ cells were counted with an epi-fluorescence microscope.

To quantify the dynamics of Syt1+ boutons in GFP+ ARCEN enteric innervations we used Imaris 10 provided by the IAC facility of Harvard Medical School. Specifically, we used the filaments module in Imaris 10 to segment GFP+ innervations. Then we used the spots module to identify and segment Syt1+ boutons with the shortest distance to filaments. *Bouton volume*: the median volume (μm^3) of Syt1+ spots with the shortest distance to filaments per image.

Bouton density: the number of Syt1+ spots with the shortest distance to filaments per image divided by the maximum filament length (μm) per image.

GCaMP7c live imaging

Guts were dissected in fresh HL3 buffer (1.5mM Ca^{2+}, 20mM $MgCl_2$, 5mM KCl, 70mM NaCl,10mM $NaHCO_3$, 5mM HEPES, 115mM Sucrose, 5mM Trehalose) and placed in eight-well clear bottom cell culture chamber slides with HL3. Guts were stabilized with a Nylon mesh (Warner instruments, 64-0198) and paper clips cut in small identical pieces.

ACh and Nicotine sensitivity assay: ACh and Nicotine sensitivity assay depicted in Fig.1-2 and Ext. Data Fig.5 was done using LSM710 and LSM780 microscopes with 40x water objective lens. Each frame (~5 sec/frame) is the maximum projection of 5-6 z-stacks (2.96μm/z) and was acquired with 488nm excitation for GFP. 5mM Acetylcholine (Acetylcholine Chloride, Sigma A2661) or 1mM Nicotine (Sigma, N3876) were added at the 10th frame (~50 sec). Nicotine sensitivity assay shown in Fig.5 was conducted with LSM980 microscope using 40x oil objective lens. 0.5mM Nicotine was added at the 10th frame (~30 sec, ~3 sec/frame) and each frame is the maximum projection of 4 z-stacks (2.96μm/z). All images were taken from similar areas in the posterior midgut between R4-R5. Fiji was used for assembly and calculation of fluorescence per frame. $\Delta F/F_0 = F_{fr}-F_0/ F_0$. F_{fr} is the fluorescence per frame and F_0 (baseline fluorescence) is the average fluorescence intensity of the first 9 frames (fr_1-fr_9).

CsChrimson-induced Ca^{2+} currents in the gut: Each gut was dissected in HL3 without any light from the dissecting scope and immediately as described above placed in clear bottom chamber slide in HL3 and covered in aluminum foil. Immediately, each gut was taken for imaging in LSM980 microscope using 40x oil objective lens. Imaging was conducted in a dark chamber and minimum light was used to locate R4c region of each gut. Each frame (~3 sec) is the maximum projection of 4 z-stacks (2.96μm/z) and was acquired with 488nm excitation for GFP. After the 10th frame (~30 sec), 590nm light-emitting laser was added. 300μM 1-Heptanol (Sigma, H2805) was added right before imaging started. Fiji was used for assembly and calculation of fluorescence per frame. $\Delta F/F_0 = F_{fr}-F_0/ F_0$, F_0 is the average fluorescence intensity of the first 10 frames. Identical acquisition conditions were used for all samples and genotypes per experiment.

Quantitative Real-Time PCR

RNA was extracted from 15 adult fly guts per biological independent sample using the Ambion PureLink RNA Mini Kit, including PureLink DNase. 500ng of RNA was amplified and

converted to cDNA using the iScript cDNA Synthesis kit (Bio-Rad). cDNAs were analyzed using the SYBR Green kit (Bio-Rad) and Bio-Rad CFX Manager software. *Rpl32* or *α-tubulin* were used as internal controls. Each RT-qPCR was performed with at least three biological replicates. Expression levels were normalized to control genotypes or Homeostasis or control during Homeostasis.

ACE: F- AGGTGCATGTCTACACGGG, R- ACGTTGGTGTTGGGGTTCC
upd3: F- ATCCCCTGAAGCACCTACAGA, R- CAGTCCAGATGCGTACTGCTG
egr: F- AGCTGATCCCCCTGGTTTTG, R- GCCAGATCGTTAGTGCGAGA
nAChRβ3: F- ATGACGACGACTCCCAAGATA, R- AAGAAGCATCCCCATTAGCATTT
pdm1: F- AGCTGTCCTAACGAGTTCCG, R- ACATCGCGCATATTTGTGTCAA
esg: F- ATGAGCCGCAGGATTTGTG, R- CCTCCTCGATGTGTTCATCATCT
prospero: F- CTGCCCCAGAGTTTGGACAA, R- CCTGATGCGAGTGACTGGA
upd: F- CAGCGCACGTGAAATAGCAT, R- CGAGTCCTGAGGTAAGGGGA
upd2: F- CGGAACATCACGATGAGCGAAT, R- TCGGCAGGAACTTGTACTCG
vn: F- TCACACATTTAGTGGTGGAAG, R- TTGTGATGCTTGAATTGGTAA
spi: F- CGCCCAAGAATGAAAGAGAG, R- AGGTATGCTGCTGGTGGAAC
krn: F- CGTGTTTGGCAACAACAAGT, R- TGTGGCAATGCAGTTTAAGG
dpp: F- GCCAACACAGTGCGAAGTT, R- ACCACCTGTTGACTGAGTGC
gbb: F- CGCTGGAACTCTCGAAATAAA, R- CCACTTGCGATAGCTTCAGA
wg: F- GATTATTCCGCAGTCTGGTC, R- CTATTATGCTTGCGTCCCTG
hh: F- GGATTCGATTGGGTCTCCTAC, R- GGGAACTGATCGACGAATCT
Inx7: F-CCTACAGGCCGGGAAGTGA, R-CAAAGGGCACCCACTGGTA
α-tubulin: F- CAACCAGATGGTCAAGTGCG, R- ACGTCCTTGGGCACAACATC
Rpl32: F- GCTAAGCTGTCGCACAAATG, R- GTTCGATCCGTAACCGATGT

Expansion Microscopy

Expansion was conducted as previously described[80]. In brief, guts were fixed, stained with primary and secondary antibodies (as described above), and after the final wash were placed briefly in 1x PBS. Then PBS was exchanged with 0.1mg/ml AcX in PBS and guts were left overnight without shaking. The next day, gelation chambers were made consisting of a stack of 2 #1.5 coverslips in each side of a slide. Stock X, 4HT, TEMED and APS (47:1:1:1) were mixed at 4°C to generate the gelling solution. Guts were transferred in the solution and incubated for 30min in the dark at 4°C. Within 5 min after incubation, guts were placed in the gelation chamber (2 guts per chamber) with 20μl of gelling solution and covered with a lid

without causing air pockets. The gelation chambers were then placed at 37°C for 2hrs in the dark and without moving. Next, using a razor the lid was removed and excess gel was trimmed off. A wet brush with digestion buffer (with ProK 1:100) was used to remove the gels (with the guts) which were then placed in a 6 well Glass Bottom Plate with Lid (Cellvis, 1-2 guts per well) and immersed in ~2-3ml of digestion buffer overnight, at RT and in the dark. The next day, the digestion buffer was exchanged with PBS and stored at 4°C until imaging. Prior to imaging, gels were trimmed to the smallest possible size, then washed with UltraPure™ Distilled water (Invitrogen) 3 times for 20min. During imaging most of the water was removed without drying the guts.

Statistics and Reproducibility

Prism (https://www.graphpad.com/scientific-software/prism/) was used to create all graphs as well as to perform statistical analysis. Shapiro-Wilk normality tests were conducted. Normally distributed data were analysed with unpaired two-tailed Student's t-tests (two groups) or with one-way Anova (Tukey's multiple comparison test or Dunnett's multiple comparison test) or two-way Anova (Sidak's multiple or Tukey's comparison test). Non-normally distributed data were analysed with two-tailed Mann-Whitney tests (two groups) or using Kruskal-Wallis with Dunn's tests for multiple comparisons. Statistical tests are indicated in figure legends. No statistical analysis was conducted to determine sample sizes. Randomization was not performed. Blinding experiments were conducted during mitotic division counts. No data were excluded. Confocal images shown in figures are representative of two or three independent experiments with similar results. Data found in charts and boxplots derive from two or more independent experiments. Each RT-qPCR was performed with at least three biologically independent samples per genotype or condition. Charts show mean values $\pm$ SEM. Violin plot shows median and 1st and 3rd quartile. Boxplots show median, 1st and 3rd quartile and whiskers show minimum and maximum values.

Single nuclei gut profiling

Sample preparation: For homeostasis 3-5 days old *Ore R* female flies were fed standard lab food at 29°C for 6 days. For recovery d2 3-5 days old *Ore R* female flies were fed 2%DSS for 4 days at 29°C and then transferred to standard food at 29°C for 2 days.

Single-Nucleus suspension and FACS: Single-nucleus suspension was conducted as previously described[81]. Briefly, 70 guts per condition were dissected in cold Schneider's medium, flash-frozen and stored at -80°C. Prior to FACs sorting, samples were spined down

and Schneider's medium was exchanged with homogenization buffer [250mM Sucrose, 10mM Tris pH8, 25mM KCl, 5mM MgCl, 0.1% Triton-X, 0.5% RNasin Plus (Promega, N2615), 50x protease inhibitor (Promega G6521), 0.1mM DTT]. Using 1ml dounce (Wheaton 357538), nuclei were released by 20 loose pestle strokes and 40 tight pestle strokes while keeping samples on ice and avoiding foam. Next, nuclei were filtered through 5 ml cell strainer (40 µm), and using 40 µm Flowmi (BelArt, H13680-0040). Nuclei were centrifuged, resuspended in PBS/0.5%BSA with 0.5% RNase inhibitor, filtered again with 40 µm Flowmi and stained with DRAQ7™ Dye (Invitrogen, D15106). Single nuclei were sorted with Sony SH800Z Cell Sorter at PCMM Flow Cytometry Facility at Harvard Medical School and 100k nuclei per sample were collected in PBS/BSA buffer. Gating strategy is shown in SI Fig.1.

10x genomics and sequencing: Single nuclei RNA-seq libraries were prepared using the Chromium Next GEM Single Cell 3' Library and Gel Bead Kit v3.1 according to the 10xGenomics protocol. Approximately 16,500 nuclei were loaded on Chip G with an initial concentration of 700 cells/µl based on the 'Cell Suspension Volume Calculator Table'. Sequencing was conducted with Illumina NovaSeq 6000 at Harvard Medical School Biopolymers Facility.

10x data processing: We used cellranger count pipeline 6.1.1 to process Chromium single-cell data and generated the feature-barcode matrices. The reads were aligned using the include-introns option with *Drosophila melanogaster* BDGP6.32 reference. All the matrices in different batches were aggregated into a single feature-barcode matrix by cellranger aggr pipeline and normalized by equalizing the read depth among libraries. Graph-based clustering in cellranger was applied to identify cell clusters (Ext. Data Fig.1c). Heatmap (Ext. Data Fig.1f) and Dot plot (Ext. Data Fig.1e) were generated using the Seurat DoHeatmap and DotPlot functions, respectively. P-values were computed using a two-tailed negative binomial exact test, following the sSeq method as employed in the Cell Ranger pipeline. To account for multiple testing, these p-values were then adjusted using the Benjamini-Hochberg procedure. The number of nuclei and gene expression per cluster were extracted from the single-cell gene expression matrix and visualized by box plot and bar plot. Visualizing and querying the data from the study can be done at:
https://www.flyrnai.org/tools/rna_seq_base/web/showProject/39/plot_coord=1/sample_id=all [82]. In this viewer site the back end was written using PHP with the Symfony framework. The front end HTML pages take advantage of the Twig template engine. The JQuery library is used for handling AJAX calls and datatables is used for displaying table views. The Vega-Lite javascript libraries is used for the graphing (http://idl.cs.washington.edu/papers/vega-lite/)[83]. The website

and databases are hosted on the O2 high-performance computing cluster made available by the RC at Harvard Medical School. Raw RNA-seq reads have been deposited and are publicly available in the NCBI Gene Expression Omnibus (GEO) database under accession code: GSE218641.

Clustering: The seven EC clusters are the anterior (aEC 1-3), middle (mEC) and posterior ECs (pEC 1-3) all of which highly express EC markers like *pdm1*, *myo1A* and *mex1* (Ext. Data Fig. 1e) and were further identified as anterior and posterior 1-3 clusters based on previous single-cell profiling[84]. The two PC clusters (ISC/EBs, proECs) are enriched for mesenchymal markers like *esg* and *Notch* (Ext. Data Fig. 1e). The ISC/EB cluster contains ISCs and EBs as determined by markers from previous single-cell profiling[84] and the proECs cluster (progenitors of ECs) consist of EBs dedicated to EC differentiation based on markers like the elevated *Sox21a* expression[85] (Ext. Data Fig. 1e). The EE cluster expresses EE markers *pros*, *piezo*, *AstA* (Ext. Data Fig. 1e). Cardia and LFC,Cu,Fe clusters were identified based on previous single-cell profiling[84]. Two clusters that resembled previous unidentified clusters[84] were assigned as unknown.

gBlock1:

AACGAGGATTATCATCAAAAGAGCGCCGGAGTATAAGTAGAGGCGCTTCGTCTACG
GAGCGACAATTCAATTCAAACAAGCAAAGTGAACACGTCGCTAAGCGAAAGCTAAGCAAAT
AAACAAGCGCAGCTGAACAAGCTAAACAATCTGCAGTAAAGTGCAAGTTAAAGTGAATCAA
TTAAAAGTAACCAGCAACCAAGTAAATCAACTGCAACTACTGAAATCTGCCAAGAAGTAATT
ATTGAATACAAGAAGAGAACTCTGAATACTTTCAACAAGTTACCGAGAAAGAAGAACTCACA
CACAGCGGCCAATTCGGTACCGCGGCCGCTAAGCAA

gBlock2:

ATATTTTTTATATACATACTTTTCAAATCGCGCGCCCTCTTCATAATTCACCTCCACCACACC
ACGTTTCGTAGTTGCTCTTTCGCTGTCTCCCACCCGCTCTCCGCAACACATTCACCTTTTGT
TCGACGACCTTGGAGCGACTGTCGTTAGTTCCGCGCGATTCGGTTCGCTCAAATGGTTCC
GAGTGGTTCATTTCGTCTCAATAGAAATTAGTAATAAATATTTGTATGTACAATTTATTTGCT
CCAATATATTTGTATATATTTCCCTCACAGCTATATTTATTCTAATTTAATATTATGACTTTTTA
AGGTAATTTTTTGTGACCTGTTCGGAGTGATTAGCGTTACAATTTGAACTGAAAGTGACATC
CAGTGTTTGTTCCTTGTGTAGATGCATCTCAAAAAAATGGTGGGCATAATAGTGTTGTTTAT
ATATATCAAAAATAACAACTATAATAATAAGAATACATTTAATTTAGAAAATGCTTGGATTTCA
CTGGAACTGCTACACCGATCACTTAGGACCCATAATGCATCATTGGAGGTGAAGAAAATCT

GCTGAATGTTCTTCGATGTGATATATATGATTTCGTTGCCCTCGGCTGTGATGTTGGCTAGT
TTGGCACATCGTACGACAGCTCCGAATTTGGGGATAACATAGTACAATGTTCCGAAATTGT
CGATGATCATGCTGCGACTGCTGCCCAGAAGACTGCCCAAATGTGTGAGTTTAATGCTATT
GTTAATTGGTTTGCTTGCTAATTCGCTTTTGCCTTCAATTTCCAGTCTGTCCACCGTACTGT
ATAGATCACCATCCTGGTCGGAGAGTATGAGTTCCCCTTGAATTCCGAAGATGAAATCCAC
TGGCTTGATGGGAAACGATTGATTCATATTCTCGTACTTATTGCTCTCCAAGGATAGGCGG
TGCCACGTCTGCTCCAGGATGTCGTAGGCTAGAATTTCTGGTACCTTTCCAAGGATGAAGT
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GGGTGTCATTAGCACCTATATGGTGTCCACAATCGATTCTCAATAGCTCCGCGTTGTTACG
CATCAGATCGAAGACAAACAAACGGGGTGAGCACGTGCTGCCGGGAAATCCGATGTCCAT
GACCCACAGTCGACTCAGTGCATCCACCTGGGACCAGTGTGCCTGTTGTACCAGAGAGCA
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CTTTCAGTTTTTAGTACATTCGTATTTTTTTAAGTATTAAAATTATTTAATCACACGAATAAAC
ATTAAGAAATATTTTATTCTGGAAACTTTCTTCGTTATAGAGCATTTATCGTTGTAAAAAAAAT
AATTTCATTAGTTTACATTACATTTTCGTTTATATGTTTAGATTTTTTCTAATTAAATGATATTG
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GAAAAATGTGGTAAGCTTTATTTTCTAACACCTCTCTTCTGATTTTATTGATTATAACTGGAA
TCTTTATCGGTTTCTCTCTTTTTCCATCAGTAGGTTGAGAGCTTTTCATACTTTTCACGCCGA
AATGGGAAAAATGGCACTGCGCGTAGAGTCAGTTATCCATTTGAGTTGGCCAAGATCAGAT
CGAAATACGCTAAAATCATTTTCGGGAGCACAATCGAATACTCATACAACACACACTTCAAA
CGAGGATTATCATCAAAAGAGCGCCGG

Full Genotypes

Fig. 1:

Ore R corresponds to *Oregon R*

Myo1A^{TS}> dCas9VPR corresponds to +; Myo1A-Gal4 Tubulin-Gal80^{TS}/+; UAS-3XFLAG-dCas9-VPR/+

Myo1A^{TS}> dCas9VPR, gRNA-ACE corresponds to +; Myo1A-Gal4 Tubulin-Gal80^{TS}/gRNA-ACE; UAS-3XFLAG-dCas9VPR/+

Mex^{TS}>GCAMP7c corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/+; 20XUAS-IVS-jGCaMP7c/+

Mex^{TS}>GCAMP7c + dCas9VPR, gRNA-ACE corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/gRNA-ACE; 20XUAS-IVS-jGCaMP7c/UAS-3XFLAG-dCas9-VPR

Fig. 2:

Myo1A^{TS} > + corresponds to +; Myo1A-Gal4 Tubulin-Gal80^{TS}/+; UAS- Luciferase^{RNAi}/+

Myo1A^{TS} > nAChRβ3^{RNAi} corresponds to +; Myo1A-Gal4 Tubulin-Gal80^{TS}/+; UAS-nAChRβ3^{RNAi}/+

Mex^{TS} > + corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/UAS- emptyVK37; +

Mex^{TS} > nAChRβ3^{RNAi} corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/+; UAS-nAChRβ3^{RNAi}/+

Mex^{TS} > + / esg-GFP corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/esg-GFP; +

Mex^{TS} > nAChRβ3^{RNAi}/ esg-GFP corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/esg-GFP; UAS-nAChRβ3^{RNAi}/+

Mex > GFP / nAChRβ3-flag corresponds to +; Mex1-Gal4 UAS-2x-GFP/nAChRβ3-flag; +

Myo1A > GFP / nAChRβ3-flag corresponds to +; Myo1A-Gal4 UAS-GFP/nAChRβ3-flag; +

Mex^{TS}>GCAMP7c corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/+; 20XUAS-IVS-jGCaMP7c/+

Mex^{TS}>GCAMP7c + nAChRβ3^{RNAi} corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/+; 20XUAS-IVS-jGCaMP7c/UAS-nAChRβ3^{RNAi}

Fig. 3:

Myo1A^{TS} > + corresponds to +; Myo1A-Gal4 Tubulin-Gal80^{TS}; UAS-Luciferase^{RNAi} and +; Myo1-Gal4 Tubulin-Gal80^{TS}/UAS-2x-GFP; +

Myo1A^{TS} > nAChRβ3^{RNAi} corresponds to +; Myo1A-Gal4 Tubulin-Gal80^{TS}/+; UAS-nAChRβ3^{RNAi}/+

Mex^{TS} > + corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/UAS- emptyVK37; +

Mex^{TS} > nAChRβ3^{RNAi} corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/+; UAS-nAChRβ3^{RNAi}/+

Myo1A^{TS} *> NFAT-CaLexA* corresponds to +*; Myo1A-Gal4 Tubulin-Gal80*^{TS}*/LexAop-CD8-GFP-2A-CD8GFP; UAS-mLexA-VP16-NFAT LexAop-rCD2-GFP/+*

Mex^{TS} *> NFAT-CaLexA* corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*/LexAop-CD8-GFP-2A-CD8GFP; UAS-mLexA-VP16-NFAT LexAop-rCD2-GFP/ +*

Mex^{TS} *> NFAT-CaLexA + nAChR*β*3*^{RNAi} corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*/LexAop-CD8-GFP-2A-CD8GFP; UAS-mLexA-VP16-NFAT LexAop-rCD2-GFP/UAS-nAChR*β*3*^{RNAi}

Myo1A^{TS} *>* + corresponds to +*; Myo1A-Gal4 Tubulin-Gal80*^{TS}*/UAS-emptyVK37; +/+*

Myo1A^{TS} *> nAChR*β*3*$^{RNAi-2}$ corresponds to +*; Myo1A-Gal4 Tubulin-Gal80*^{TS}*/UAS-nAChR*β*3*$^{RNAi-2}$*; UAS-Luciferase/+*

Myo1A^{TS} *> Orai* corresponds to +*; Myo1A-Gal4 Tubulin-Gal80*^{TS}*/UAS-emptyVK37; UAS-Orai / +*

Myo1A^{TS} *> nAChR*β*3*$^{RNAi-2}$ + Orai corresponds to +*; Myo1A-Gal4 Tubulin-Gal80*^{TS}*/UAS-nAChR*β*3*$^{RNAi-2}$*; UAS-Orai /+*

Mex^{TS} *> nAChR*β*3* corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*/UAS-nAChR*β*3; +*

Myo1A^{TS} *> nAChR*β*3* corresponds to +*; Myo1A-Gal4 Tubulin-Gal80*^{TS}*/UAS-nAChR*β*3; +*

<u>Fig. 4:</u>

R49E06^{TS}*> myrGFP+syt1HA* corresponds to +; *Tubulin-Gal80*^{TS}*/10xUAS-IVS-myr::GFP; R49E06-Gal4/5xUAS-IVS-Syt1::smGdP-HA*

R49E06^{TS}*> syt1HA + mexLexA > LexAop6xGFP* corresponds to +; *Tubulin-Gal80*^{TS}*/ 13xLexAop2-6xGFP; R49E06-Gal4 mex1-LexA::GAD/ 5xUAS-IVS-Syt1::smGdP-HA*

R49E06^{TS}*>* + corresponds to +; *Tubulin-Gal80*^{TS}*/+; R49E06-Gal4; UAS-Luciferase*^{RNAi}

R49E06^{TS}*> ChAT*^{RNAi} corresponds to +*; Tubulin-Gal80*^{TS}*/+; R49E06-Gal4; UAS-ChAT*^{RNAi}

ARCENs + mexLexA^{TS} > + corresponds to +*; Tubulin-Gal80*^{TS}*/ UAS-emptyVK37; R49E06-Gal4 mex1-LexA::GAD / +*

ARCENs> shibire^{TS} corresponds to +*; +; R49E06-Gal4/UAS-shibire*^{TS}

mexLexA^{TS} *> LexAopnAChR*β*3* corresponds to +*; Tubulin-Gal80*^{TS}*/LexAop-nAChR*β*3*; *mex1-LexA::GAD/+*

ARCENs > shibire^{TS} + *mexLexA*^{TS}*> LexAopnAChR*β*3* corresponds to +*; Tubulin-Gal80*^{TS}*/ LexAop-nAChR*β*3*; *R49E06-Gal4 mex1-LexA::GAD / UAS-shibire*^{TS}

QUASCsChrimson + Mex^{TS} *> NFAT-CaLexA* corresponds to *QUASCsChrimson/+; Mex1-Gal4 Tubulin-Gal80*^{TS}*/LexAop-CD8-GFP-2A-CD8GFP; UAS-mLexA-VP16-NFAT LexAop-rCD2-GFP/+*

ARCENsQF > QUASCsChrimson + Mex^{TS} *> NFAT-CaLexA* corresponds to *QUASCsChrimson/+; Mex1-Gal4* $Tubulin\text{-}Gal80^{TS}$*/LexAop-CD8-GFP-2A-CD8GFP; UAS-mLexA-VP16-NFAT LexAop-rCD2-GFP/R49E06-QF*

$ARCENs^{TS}$*>* wgn^{RNAi} corresponds to +*;* $Tubulin\text{-}Gal80^{TS}$*/* $UAS\text{-}wgn^{RNAi}$*; R49E06-Gal4;+*

$ARCENs^{TS}$*>* $grnd^{RNAi}$ corresponds to +*;* $Tubulin\text{-}Gal80^{TS}$*/*$UAS\text{-}grnd^{RNAi}$*; R49E06-Gal4;+*

ARCENs> mCD8GFP corresponds to +*; 10xUAS-mCD8::GFP/+; R49E06-Gal4/+*

ARCENs> mCD8GFP+ wgn^{RNAi} corresponds to +*; 10xUAS-mCD8::GFP/* $UAS\text{-}wgn^{RNAi}$*; R49E06-Gal4/+*

Fig. 5:

spECs > CsChrimson + mexLexA > LexAopGCAMP7c corresponds to +*; VT004958-p65AD/ UAS-CsChrimson ;VT047163-Gal4DBD/ Mex1-LexA::GAD 13xLexAop-IVS-jGCaMP7c*

spECs > CsChrimson corresponds to +*; VT004958-p65AD/ UAS-CsChrimson ;VT047163-Gal4DBD/+*

Mex^{TS} > + corresponds to +*; Mex-Gal4* $Tubulin\text{-}Gal80^{TS}$*/UAS- emptyVK37;* + and +*; Mex1-Gal4* $Tubulin\text{-}Gal80^{TS}$*/ +;* $UAS\text{-}Luciferase^{RNAi}$*/+*

Mex^{TS} > $Inx2^{RNAi}$ corresponds to +*; Mex1-Gal4* $Tubulin\text{-}Gal80^{TS}$*;* $UAS\text{-}Inx2^{RNAi}$/+ and +*; Mex1-Gal4* $Tubulin\text{-}Gal80^{TS}$*/UAS-emptyVK37;* $UAS\text{-}Inx2^{RNAi}$*/+*

Mex^{TS} > $Inx7^{RNAi}$ corresponds to +*; Mex1-Gal4* $Tubulin\text{-}Gal80^{TS}$*;* $UAS\text{-}Inx7^{RNAi}$*/+*

Mex^{TS} > *NFAT-CaLexA* corresponds to +*; Mex1-Gal4* $Tubulin\text{-}Gal80^{TS}$*/ LexAop-CD8-GFP-2A-CD8GFP; UAS-mLexA-VP16-NFAT LexAop-rCD2-GFP/ +*

Mex^{TS} > *NFAT-CaLexA +* $Inx2^{RNAi}$ corresponds to +*; Mex1-Gal4* $Tubulin\text{-}Gal80^{TS}$*/ LexAop-CD8-GFP-2A-CD8GFP; UAS-mLexA-VP16-NFAT LexAop-rCD2-GFP/*$UAS\text{-}Inx2^{RNAi}$

Mex^{TS} > *NFAT-CaLexA +* $Inx7^{RNAi}$ corresponds to +*; Mex1-Gal4* $Tubulin\text{-}Gal80^{TS}$*/ LexAop-CD8-GFP-2A-CD8GFP; UAS-mLexA-VP16-NFAT LexAop-rCD2-GFP/*$UAS\text{-}Inx7^{RNAi}$

Mex^{TS} > $Inx2^{RNAi}$ + $nAChR\beta3$ corresponds to +*; Mex1-Gal4* $Tubulin\text{-}Gal80^{TS}$*/* $UAS\text{-}nAChR\beta3$*;* $UAS\text{-}Inx2^{RNAi}$*/+*

Mex^{TS} > $nAChR\beta3$ corresponds to +*; Mex1-Gal4* $Tubulin\text{-}Gal80^{TS}$*/* UAS-nAChR$\beta$3*;*$UAS\text{-}Luciferase^{RNAi}$*/+*

Mex^{TS}*>GCAMP7c* corresponds to +*; Mex1-Gal4* $Tubulin\text{-}Gal80^{TS}$*/UAS-empty; 20XUAS-IVS-jGCaMP7c/+*

Mex^{TS}*>GCAMP7c +* $Inx2^{RNAi}$ corresponds to +*; Mex1-Gal4* $Tubulin\text{-}Gal80^{TS}$*/+; 20XUAS-IVS-jGCaMP7c/*$UAS\text{-}Inx2^{RNAi}$

Ext. Data Fig. 1:

Ore R corresponds to *Oregon R*

Ext. Data Fig. 2:

Ore R corresponds to *Oregon R*

Myo1A^{TS}> dCas9-VPR corresponds to +*; Myo1A-Gal4 Tubulin-Gal80^{TS} /+; UAS-3XFLAG-dCas9-VPR/+*

Myo1A^{TS}> dCas9-VPR, gRNA-ACE corresponds to +*; Myo1A-Gal4 Tubulin-Gal80^{TS} / gRNA-ACE; UAS-3XFLAG-dCas9-VPR/+*

Mex^{TS}> dCas9-VPR corresponds to +*; Mex1-Gal4 Tubulin-Gal80^{TS}/+ ; UAS-3XFLAG-dCas9-VPR/+*

Mex^{TS}> dCas9-VPR, gRNA-ACE corresponds to +*; Mex1-Gal4 Tubulin-Gal80^{TS} / gRNA-ACE; UAS-3XFLAG-dCas9-VPR/+*

How^{TS}> dCas9-VPR corresponds to +*; Tubulin-Gal80^{TS} /+; how^{24B}-Gal4/ UAS-3XFLAG-dCas9-VPR*

How^{TS}> dCas9-VPR, gRNA-ACE corresponds to +*; Tubulin-Gal80^{TS} / gRNA-ACE; how^{24B}-Gal4/ UAS-3XFLAG-dCas9-VPR*

Hml^{TS} > dCas9-VPR corresponds to +*; hml-Gal4Δ UAS-GFP/+; Tubulin-Gal80^{TS}/ UAS-3XFLAG-dCas9-VPR*

Hml^{TS} > dCas9-VPR, gRNA-ACE corresponds to +*; hml-Gal4Δ UAS-GFP/ gRNA-ACE; Tubulin-Gal80^{TS}/ UAS-3XFLAG-dCas9-VPR*

mex^{TS} > GCAMP7c + corresponds to +*; Mex1-Gal4 Tubulin-Gal80^{TS}/+; 20XUAS-IVS-jGCaMP7c/+*

Mex^{TS}>GCAMP7c + dCas9VPR, gRNA-ACE corresponds to +*; Mex1-Gal4 Tubulin-Gal80^{TS}/gRNA-ACE; 20XUAS-IVS-jGCaMP7c/UAS-3XFLAG-dCas9-VPR*

Ext. Data Fig. 3:

Myo1A^{TS} > + corresponds to +*; Myo1A-Gal4 Tubulin-Gal80^{TS}/+;* +

Myo1A^{TS} > nAChRβ3^{RNAi} corresponds to +*; Myo1A-Gal4 Tubulin-Gal80^{TS}/+; UAS-nAChRβ3^{RNAi}/+*

Myo1A^{TS} > nAChRβ3^{RNAi-2} corresponds to +*; Myo1A-Gal4 Tubulin-Gal80^{TS}/ UAS-nAChRβ3^{RNAi-2};+*

Mex^{TS}> GCAMP7c corresponds to +*; Mex1-Gal4 Tubulin-Gal80^{TS}/+; 20XUAS-IVS-jGCaMP7c/+*

Mex^{TS}> GCAMP7c / nAChRβ3^{RNAi} corresponds to +*; Mex1-Gal4 Tubulin-Gal80^{TS}/+; 20XUAS-IVS-jGCaMP7c/ UAS-nAChRβ3^{RNAi}*

Esg^{TS} > + corresponds to +*; esg-Gal4 Tubulin-Gal80*^{TS} */ +; UAS- Luciferase*^{RNAi}*/+*

Esg^{TS} *> nAChR*β*3*^{RNAi} corresponds to +*; esg-Gal4 Tubulin-Gal80*^{TS}*; UAS-nAChR*β*3*^{RNAi} */+*

Su(H)GBE^{TS} > + corresponds to +*; Su(H)Gbe-Gal4 UAS-CD8-GFP/+; Tubulin-Gal80*^{TS}*/UAS-Luciferase*^{RNAi}

Su(H)GBE^{TS} *> nAChR*β*3*^{RNAi} corresponds to +*; Su(H)Gbe-Gal4 UAS-CD8-GFP/+; Tubulin-Gal80*^{TS}*/ UAS-nAChR*β*3*^{RNAi}

Pros^{TS} > + corresponds to +*; Tubulin-Gal80*^{TS} */ +; prospero-Gal4/ UAS-Luciferase*^{RNAi}

Pros^{TS} *> nAChR*β*3*^{RNAi} corresponds to +*; Tubulin-Gal80*^{TS} */ +; prospero-Gal4/ UAS-nAChR*β*3*^{RNAi}

How^{TS} > + corresponds to +; *Tubulin-Gal80*^{TS} */ +; how*^{24B}*-Gal4/ UAS-Luciferase* ^{RNAi}

How^{TS} *> nAChR*β*3*^{RNAi} corresponds to +; *Tubulin-Gal80*^{TS} */ +; how*^{24B}*-Gal4/ UAS-nAChR*β*3*^{RNAi}

Hml^{TS} > + corresponds to +*; hml-Gal4*Δ *UAS-GFP/+; Tubulin-Gal80*^{TS}*/ UAS-Luciferase*^{RNAi}

Hml^{TS} *> nAChR*β*3*^{RNAi} corresponds to +*; hml-Gal4*Δ *UAS-GFP/+; Tubulin-Gal80*^{TS}*/ UAS-nAChR*β*3*^{RNAi}

Ore R corresponds to *Oregon R*

Mex^{TS} *> + / nAChR*β*3-flag* corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS} */ nAChR*β*3-flag; +*

Mex^{TS} *> UAS-nAChR*β*3*^{RNAi} */ nAChR*β*3-flag* corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS} */ nAChR*β*3-flag; UAS-nAChR*β*3*^{RNAi} */+*

Mex^{TS}*>GCAMP7c* corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*/+; 20XUAS-IVS-jGCaMP7c/+*

Mex^{TS}*>GCAMP7c + nAChR*β*3*^{RNAi} corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*/+; 20XUAS-IVS-jGCaMP7c/UAS-nAhcR*β*3*^{RNAi}

<u>Ext. Data Fig. 4:</u>

Mex^{TS} > *+ / Egr-GFP* corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*/ Egr-GFP;+*

Mex^{TS} > *nAChR*β*3*^{RNAi}*/ Egr-GFP* corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*/Egr-GFP; UAS-nAChR*β*3*^{RNAi}*/+*

Mex^{TS} > *+ / Vn-LacZ* corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*/ +; Vn-LacZ/+*

Mex^{TS} > *nAChR*β*3*^{RNAi}*/ Vn-LacZ* corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*/+; UAS-nAChR*β*3*^{RNAi}*/Vn-LacZ*

Myo1A^{TS} > + corresponds to +*; Myo1A-Gal4 Tubulin-Gal80*^{TS}*/UAS-emptyVK37; +/+* and +*; Myo1-Gal4 Tubulin-Gal80*^{TS}*/UAS-2x-GFP; +*

Myo1A^{TS} > *nAChR*β*3*^{RNAi} corresponds to +*; Myo1A-Gal4 Tubulin-Gal80*^{TS}*/+; UAS-nAChR*β*3*^{RNAi}*/+*

Myo1A^{TS} > *+ / Diap1-LacZ* corresponds to +*; Myo1A-Gal4 Tubulin-Gal80*^{TS}*/+; Diap1-LacZ / +*

Myo1A^{TS} > nAChRβ3^{RNAi} / Diap1-LacZ corresponds to +; Myo1A-Gal4 Tubulin-Gal80^{TS}/+; Diap1-LacZ /UAS-nAChRβ3^{RNAi}

Myo1A^{TS} > + / Ex-LacZ corresponds to +; Myo1A-Gal4 Tubulin-Gal80^{TS}/ Ex-LacZ ; +

Myo1A^{TS} > nAChRβ3^{RNAi} / Ex-LacZ corresponds to +; Myo1A-Gal4 Tubulin-Gal80^{TS}/ Ex-LacZ; UAS-nAChRβ3^{RNAi}/+

Mex^{TS} > + corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/UAS- emptyVK37; +

Mex^{TS} > nAChRβ3^{RNAi} corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/+; UAS-nAChRβ3^{RNAi}/+

Esg^{TS} > NFAT-CaLexA corresponds to Tubulin-Gal80^{TS}/+; esg-Gal4 / LexAop-CD8-GFP-2A-CD8GFP; UAS-mLexA-VP16-NFAT LexAop-rCD2-GFP/ +

Ext. Data Fig. 5:

Mex^{TS} > + corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/UAS- emptyVK37; +

Mex^{TS} > nAChRβ3^{RNAi-2} corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/UAS-nAChRβ3^{RNAi-2}; +

Mex^{TS}> Orai corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/+ ; UAS-Orai/+

Mex^{TS}> nAChRβ3^{RNAi-2} + Orai corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/UAS-nAChRβ3^{RNAi-2}; UAS-Orai /+

Myo1A^{TS} > + corresponds to +; Myo1A-Gal4 Tubulin-Gal80^{TS}/UAS-emptyVK37

Myo1A^{TS} > nAChRβ3^{RNAi-2} corresponds to +; Myo1A-Gal4 Tubulin-Gal80^{TS}/UAS-nAChRβ3^{RNAi-2}; UAS-Luciferase/+

Myo1A^{TS}> Orai corresponds to +; Myo1A-Gal4 Tubulin-Gal80^{TS}/UAS-emptyVK37; UAS-Orai/+

Myo1A^{TS}> nAChRβ3^{RNAi-2} + Orai corresponds to +; Myo1A-Gal4 Tubulin-Gal80^{TS}/UAS-nAChRβ3^{RNAi-2}; UAS-Orai /+

Mex^{TS} > PV corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/2xUAS-PV-Myc; 2xUAS-PV-Myc/ +

Myo1A^{TS}> nAChRβ3 corresponds to +; Myo1A-Gal4 Tubulin-Gal80^{TS}/ UAS-nAChRβ3; +

MexLexA> LexAopGFP corresponds to +; 13xLexAop2-sfGFP/+; mex1-LexA::GAD/ +

MexLexA^{TS}> + corresponds to +; Tubulin-Gal80^{TS}/ +; mex1-LexA::GAD / +

MexLexA^{TS}> LexAopnAChRβ3 corresponds to +; Tubulin-Gal80^{TS}/ LexAop-nAChRβ3; mex1-LexA::GAD / +

Mex^{TS}> GCAMP7c corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/+; 20XUAS-IVS-jGCaMP7c/+

Mex^{TS}> GCAMP7c + nAChRβ3 corresponds to +; Mex1-Gal4 Tubulin-Gal80^{TS}/ nAChRβ3; 20XUAS-IVS-jGCaMP7c/ +

MexLexA^{TS}> +/ Upd3>GFP corresponds to +; Tubulin-Gal80^{TS}/ upd3-Gal4 UAS-GFP ; mex1-LexA::GAD / +

MexLexA^{TS}*> LexAopnAChR*β*3 / Upd3>GFP* corresponds to +*; Tubulin-Gal80*^{TS}*/ upd3-Gal4 UAS-GFP* ; *mex1-LexA::GAD / LexAop-nAChR*β*3*

MexLexA^{TS}*> +/ Egr-GFP* corresponds to +*; Tubulin-Gal80*^{TS}*/ Egr-GFP* ; *mex1-LexA::GAD / LexAop-nAChR*β*3*

MexLexA^{TS}*> LexAopnAChR*β*3 / Egr-GFP* corresponds to +*; Tubulin-Gal80*^{TS}*/ Egr-GFP* ; *mex1-LexA::GAD / LexAop-nAChR*β*3*

Mex^{TS} *> nAChR*β*3* corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*/UAS-nAChR*β*3; +*

<u>Ext. Data Fig. 6:</u>

Pros^{TS} > + corresponds to +; *Tubulin-Gal80*^{TS} */ +; prospero-Gal4/ UAS-Luciferase*^{RNAi}

Pros^{TS} > *ChAT*^{RNAi} corresponds to +; *Tubulin-Gal80*^{TS} */ +; prospero-Gal4/ UAS-ChAT*^{RNAi}

Esg^{TS} > + corresponds to *Tubulin-Gal80*^{TS}*/+; esg-Gal4 / +; UAS- Luciferase* ^{RNAi}*/+*

Esg^{TS} > *ChAT*^{RNAi} corresponds to *Tubulin-Gal80*^{TS}*/+; esg-Gal4 / +; UAS- ChAT*^{RNAi} */+*

Myo1A^{TS} > + corresponds to +; *myo1A-Gal4 Tubulin-Gal80*^{TS} */+ ; UAS-Luciferase* ^{RNAi}*/+*

Myo1A^{TS} > *ChAT*^{RNAi} corresponds to +; *myo1A-Gal4 Tubulin-Gal80*^{TS} */+; UAS-ChAT*^{RNAi}*/+*

Hml^{TS} > + corresponds to +*; hml-Gal4*Δ *UAS-GFP/+; Tubulin-Gal80*^{TS}*/ UAS-Luciferase*^{RNAi}

Hml^{TS} > *ChAT*^{RNAi} corresponds to +*; hml-Gal4*Δ *UAS-GFP/+; Tubulin-Gal80*^{TS}*/ UAS-ChAT*^{RNAi}

How^{TS} > + corresponds to +; *Tubulin-Gal80*^{TS} */ +; how*^{24B}*-Gal4/ UAS-Luciferase* ^{RNAi}

How^{TS} > *ChAT*^{RNAi} corresponds to +; *Tubulin-Gal80*^{TS} */ +; how*^{24B}*-Gal4/ UAS-ChAT*^{RNAi}

Esg > + corresponds to +; *esg-Gal4 / UAS-emptyVK37; +*

Esg > *sc*^{RNAi} corresponds to +; *esg-Gal4/+; sc*^{RNAi} */+*

R49E06> mCD8GFP corresponds to +*; 10xUAS-mCD8::GFP/+; R49E06-Gal4/+*

R49E06> 2xGFP corresponds to +*; UAS-2x-GFP/+; R49E06-Gal4/+*

R49E06> mCD8GFP + ChAT-Gal80 corresponds to +*; 10xUAS-mCD8::GFP/+; R49E06-Gal4/ ChAT-Gal80*

R49E06 > 6xmCherry corresponds to +; +; *R49E06-Gal4/20xUAS-6xmCherry-HA*

R49E06^{TS} *> syt1HA* corresponds to +*; Tubulin-Gal80*^{TS}*/* +; *R49E06-Gal4/5xUAS-IVS-Syt1::smGdP-HA*

R49E06^{TS} *> DenMarK* corresponds to +*; Tubulin-Gal80*^{TS}*/* +; *R49E06-Gal4/UAS-DenMarK*

R49E06^{TS}*> syt1HA + mexLexA > LexAopGFP* corresponds to +;*Tubulin-Gal80*^{TS}*/13xLexAop2-6xGFP;R49E06-Gal4 mex1-LexA::GAD/5xUAS-IVS-Syt1::smGdP-HA* and +;*Tubulin-Gal80*^{TS}*/13xLexAop2-sfGFP; R49E06-Gal4 mex1-LexA::GAD/ 5xUAS-IVS-Syt1::smGdP-HA*

Ext. Data Fig. 7:

R49E06^{TS}> + corresponds to +; Tubulin-Gal80^{TS}/+; R49E06-Gal4; UAS-Luciferase^{RNAi}

R49E06^{TS}> ChAT^{RNAi} corresponds to +; Tubulin-Gal80^{TS}/+; R49E06-Gal4; UAS-ChAT^{RNAi}

R49E06^{TS}> ChAT^{RNAi} / Tsh-Gal80 corresponds to +; Tubulin-Gal80^{TS}/ Tsh-Gal80; R49E06-Gal4; UAS-ChAT^{RNAi}

ARCENs>+ corresponds to +; UAS-2xGFP/+; R49E06-Gal4/+

ARCENs> TrpA1 corresponds to +; UAS-TrpA1/+; R49E06-Gal4/+

ARCENs> TrpA1/ChAT-Gal80 corresponds to +; UAS-TrpA1/+; R49E06-Gal4/ChAT-Gal80

R49E06-QF> QUAS-TomatoHA corresponds to +; +; R49E06-QF/ QUAS-mtdTomato-3xHA

ARCENs^{TS}> + corresponds to +; Tubulin-Gal80^{TS}/+; R49E06-Gal4; UAS-Luciferase^{RNAi}

ARCENs^{TS}> wgn^{RNAi-2} corresponds to +; Tubulin-Gal80^{TS}/+; R49E06-Gal4/ UAS-wgn^{RNAi-2}

ARCENs^{TS}> wgn^{RNAi-2} / Tsh-Gal80 corresponds to +; Tubulin-Gal80^{TS}/ Tsh-Gal80; R49E06-Gal4/ UAS-wgn^{RNAi-2}

Esg^{TS} > + corresponds to +; esg-Gal4 Tubulin-Gal80^{TS} /+; / +; UAS- Luciferase^{RNAi}/+

Esg^{TS} > egr^{RNAi} corresponds to +; esg-Gal4 Tubulin-Gal80^{TS} /+; UAS-eiger^{IR} /+

Mex^{TS} > + corresponds to +; mex1-Gal4 Tubulin-Gal80^{TS} /+ ; UAS-Luciferase^{RNAi}/+

Mex^{TS} > egr^{RNAi} corresponds to +; mex1-Gal4 Tubulin-Gal80^{TS} /+; UAS-eiger^{IR} /+

Hml^{TS} > + corresponds to +; hml-Gal4Δ UAS-GFP/+; Tubulin-Gal80^{TS}/ UAS-Luciferase^{RNAi}

Hml^{TS} > egr^{RNAi} corresponds to +; hml-Gal4Δ UAS-GFP/+; Tubulin-Gal80^{TS}/ UAS-eiger^{IR}

Esg + mex^{TS} > + corresponds to +;esg-Gal4 / mex1-Gal4 Tubulin-Gal80^{TS}; UAS-Luciferase^{RNAi}/+

Esg + mex^{TS} > egr^{RNAi} corresponds to +; esg-Gal4 Tubulin-Gal80^{TS} /mex1-Gal4; UAS-eiger^{IR} /+

Mex+ hml^{TS} > + corresponds to +; mex1-Gal4 Tubulin-Gal80^{TS} /+; UAS- Luciferase ^{RNAi}/ hml-Gal4Δ UAS-GFP

Mex+ hml^{TS} > egr^{RNAi} corresponds to +; mex1-Gal4 Tubulin-Gal80^{TS}/+ ; UAS-eiger^{IR} / hml-Gal4Δ UAS-GFP

Esg + hml^{TS} > + corresponds to +; esg-Gal4 Tubulin-Gal80^{TS} / +; hml-Gal4Δ UAS-GFP/+

Esg + hml^{TS} > egr^{RNAi} corresponds to +; esg-Gal4 Tubulin-Gal80^{TS} / +; hml-Gal4Δ UAS-GFP/ UAS-eiger^{IR}

Esg + hml + mex^{TS} > + corresponds to +; esg-Gal4 Tubulin-Gal80^{TS} / mex1-Gal4 ; hml-Gal4Δ UAS-GFP/+

Esg + hml + mex^{TS} > egr^{RNAi} corresponds to +; esg-Gal4 Tubulin-Gal80^{TS} / mex1-Gal4 ; hml-Gal4Δ UAS-GFP/UAS-eiger^{IR}

ARCENs^{TS}*>Morphotrap/Egr-GFP* corresponds to +*; Tubulin-Gal80*^{TS}*/Egr-GFP; R49E06-Gal4/ lexAop-UAS-morphotrap.ext.mCh*
ARCENs^{TS}*>Cherry /Egr-GFP* corresponds to +*; Tubulin-Gal80*^{TS}*/Egr-GFP; R49E06-Gal4/ 20xUAS-6xmCherry-HA*

Ext. Data Fig. 8:
spECs > 6xGFP corresponds to +*; VT004958-p65AD/ + ;VT047163-Gal4DBD/20xUAS-6xGFP*
spECs > CsChrimson + mexLexA >LexAopGCAMP7c corresponds to +*; VT004958-p65AD/ UAS-CsChrimson ;VT047163-Gal4DBD/ Mex1-LexA::GAD 13xLexAop-IVS-jGCaMP7c*
Ore R corresponds to *Oregon R*
Mex > GFP corresponds to +*; Mex1-Gal4/UAS-2xGFP; +*
Mex^{TS} > + corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*/UAS- emptyVK37;* + and +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*/ +; UAS-Luciferase*^{RNAi}*/+*
Mex^{TS} > *Inx2*^{RNAi} corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*; UAS-Inx2*^{RNAi}/+ and +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*/UAS-emptyVK37; UAS-Inx2*^{RNAi}*/+*
Mex^{TS} > *Inx7*^{RNAi} corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*; UAS-Inx7*^{RNAi}*/+*
spECs > CsChrimson corresponds to *VT004958-p65AD/ UAS-CsChrimson ;VT047163-Gal4DBD/+*
spECs > CsChrimson+Inx2^{RNAi} corresponds to *VT004958-p65AD/ UAS-CsChrimson ;VT047163-Gal4DBD/ UAS-Inx2*^{RNAi}
Mex^{TS} > *Inx2*^{RNAi} + *nAChR*β*3* corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*/ UAS-nAChR*β*3; UAS-Inx2*^{RNAi}*/+*
Mex^{TS} > *nAChR*β*3* corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*/* UAS-nAChRβ3*;UAS-Luciferase*^{RNAi}*/+*
Mex^{TS}*>GCAMP7c* corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*/+; 20XUAS-IVS-jGCaMP7c/+*
Mex^{TS}*>GCAMP7c + Inx2*^{RNAi} corresponds to +*; Mex1-Gal4 Tubulin-Gal80*^{TS}*/+; 20XUAS-IVS-jGCaMP7c/UAS-Inx2*^{RNAi}