Cell Reports, Volume *23*

Supplemental Information

Diet-Induced Growth Is Regulated via Acquired Leptin Resistance and Engages a Pomc-Somatostatin-Growth Hormone Circuit

Heiko Löhr, Simon Hess, Mafalda M.A. Pereira, Philip Reinoß, Sandra Leibold, Christel Schenkel, Claudia M. Wunderlich, Peter Kloppenburg, Jens C. Brüning, and Matthias Hammerschmidt

Supplemental Figures

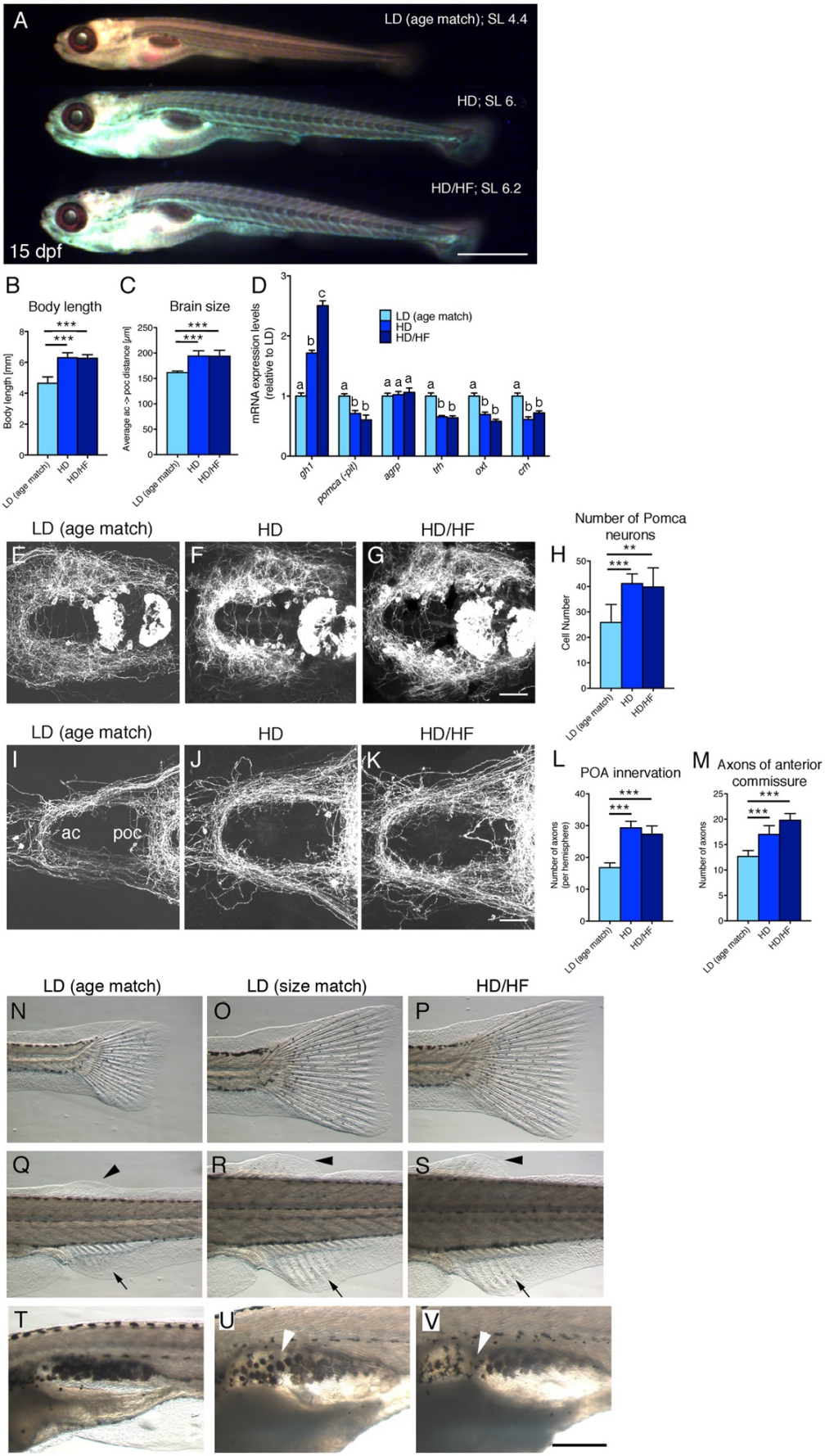

Figure S1: Strong impact of caloric input on somatic growth, neuronal circuit formation and developmental pace of zebrafish larvae – comparisons between age-matched larvae. Related to Figures 1 and 2.

Effects of caloric input on zebrafish larvae raised under LD, HD, or HD/HF feeding conditions from 5 -15 dpf. Analyses were performed at 15 dpf for all groups (age matched). (A) Lateral overview of larvae at 15 dpf stained with Nile red revealing strong differences in body length between LD (age match; standard body length (SL) = 4.4 mm) and HD (standard body length = 6.0 mm), HD/HF larvae (standard body length = 6.2 mm), respectively. Also note differences in fluorescent color profiles of Nile red likely reflecting differences in body fat composition. (B) Quantifications of body length (n=50) and (C) distance between anterior and postoptic commissures as a measure for brain size in *pomca:EGFPras* transgenic fish (n=5). (D) qRT-PCR analyses: mRNA levels for *gh1*, *pomca* (only neuronal fraction), *agrp*, *trh*, *oxt* and *crh*. Columns with different superscript letters (a,b,c) are significantly different from each other ($p<0.05$) according to ANOVA followed by a post hoc Tukey test. (E-M) Reduced number of Pomca hypothalamic neurons (E-H) and reduced innervation of preoptic area (POA) and anterior commissure (ac) in LD (age match) larvae compared to HD and HD/HF larvae assessed by anti-GFP IF on *pomca:EGFPras* transgenics (E-G; I-K) and subsequent quantifications of cell (H; n = 10) and axon numbers (L-M; n = 10). (N-V) Evaluation of developmental progress using anatomical criteria according to Parichy et al. (2009) revealing slower postembryonic development of LD (age match) larvae compared to HD and HD/HF larvae. (N-S) Development of caudal (N-P), dorsal (arrowhead in Q-S) and anal fins (arrow in Q-S) showing differences between LD (age match) versus HD and HD/HF larvae. (T-V) Budding of the anterior lobe of the swimbladder (arrowheads in T-V) has already taken place in HD and HD/HF but not in LD (age match) larvae. Scale bars: (A) 1 mm; (G) 50 µm for (E-G); (K) 50 µm for (I-K); (V) 250 µm for (N-V). p-values: (B, C, H, L, M) ** $p < 0.05$; *** $p < 0.01$ relative to LD (age match). Error bars in (B, C, D, H, L, M) show SD. (A, N-V) lateral views; (E-G; I-K) ventral views.

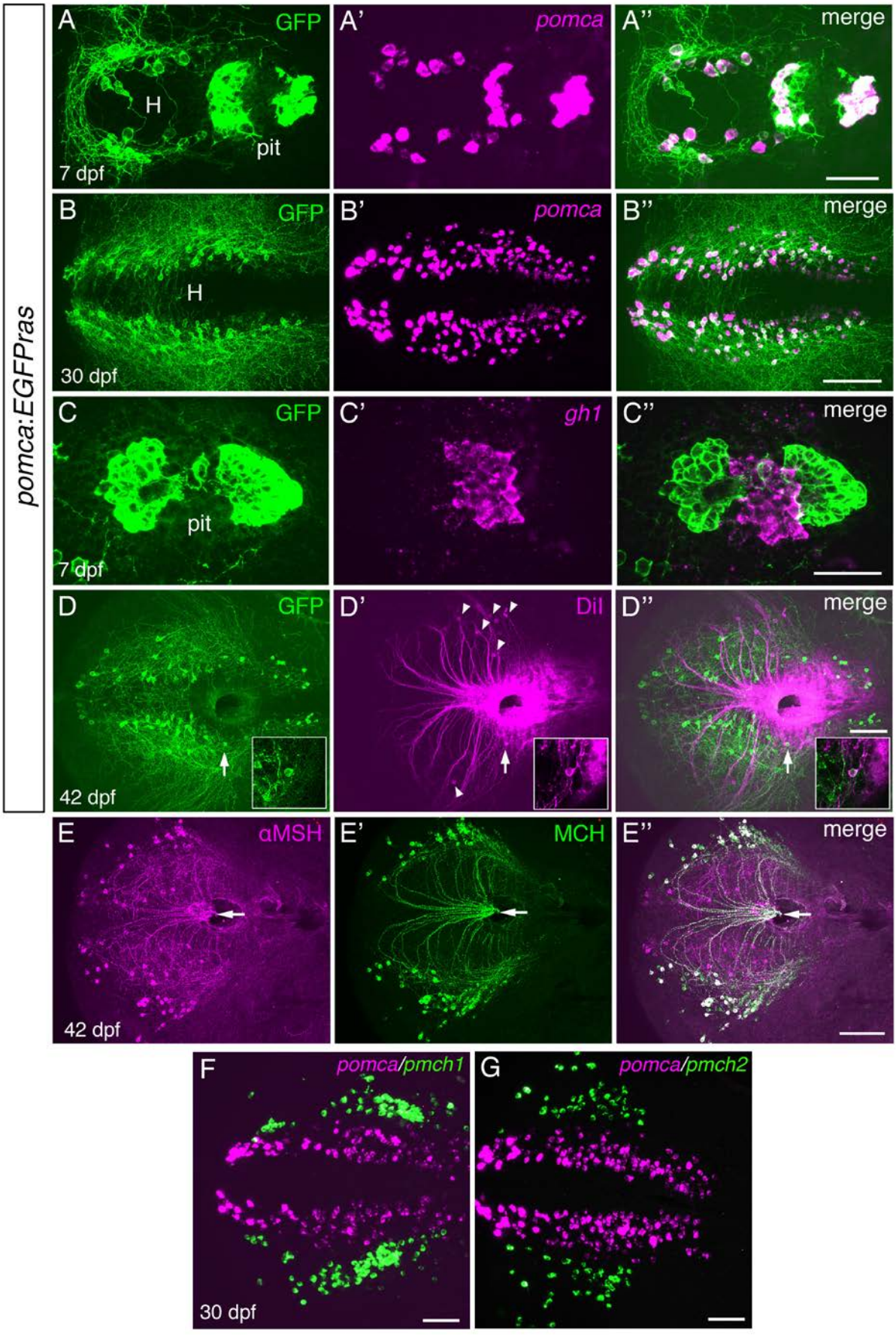
pomca:EGFPras
A
GFP
H
pit
7 dpf
A'
pomca
A''
merge
B
GFP
H
30 dpf
B'
pomca
B''
merge
C
GFP
pit
7 dpf
C'
gh1
C''
merge
D
GFP
42 dpf
D'
DiI
D''
merge
E
αMSH
42 dpf
E'
MCH
E''
merge
F
pomca/pmch1
30 dpf
G
pomca/pmch2

Figure S2. *pomca*:*EGFPras* transgenic zebrafish recapitulate endogenous *pomca* expression and reveal absence of adenohypophyseal innervation. Related to Figure 3.

(A-D'') *pomca*:*EGFPras* transgenic line. (A-B'') Fluorescent *in situ* hybridization (FISH) for *pomca* (magenta) followed by anti-GFP immunofluorescence (IF; green). All Pomca cells in hypothalamus (H) and pituitary (pit) are GFP+ at 7dpf. (B-B'') Co-localization of *pomca* transcripts and GFP in the hypothalamus at 30 dpf. (C-C'') FISH for *gh1* (magenta) and GFP IF (green) at 7 dpf revealing absence of Pomca cell-derived axons in regions of somatotropic cells of the pituitary. (D-D'') DiI injection into the pituitary at 42 dpf labels various hypothalamic neurons lateral to the Pomca cell domain (arrowheads). In three analyzed brains, only one single Pomca neuron (GFP+; arrow) was found to be co-labeled with DiI tracer (see D-D'' insets with magnified views). (E-E'') Co-IF for αMSH and MCH at 42 dpf reveals cross-reactivity of the anti-αMSH antibody with MCH peptide. All projections towards the pituitary (arrows) are double-positive and thus derived from MCH neurons. (F-G) Double FISH for *pmch1* (E, magneta) or *pmch2*, (F, magneta) and *pomca* (green) in wild-type brains at 30 dpf. Both *pmch1* and *pmch2* expressing cells are located lateral to the Pomca cell domain, most likely corresponding to the αMSH+ but GFP- cell bodies in (E). Scale bars: (A'') 50 µm, (B'') 100 µm, (C'') 50 µm, (D'', E'', F, G) 100 µm.

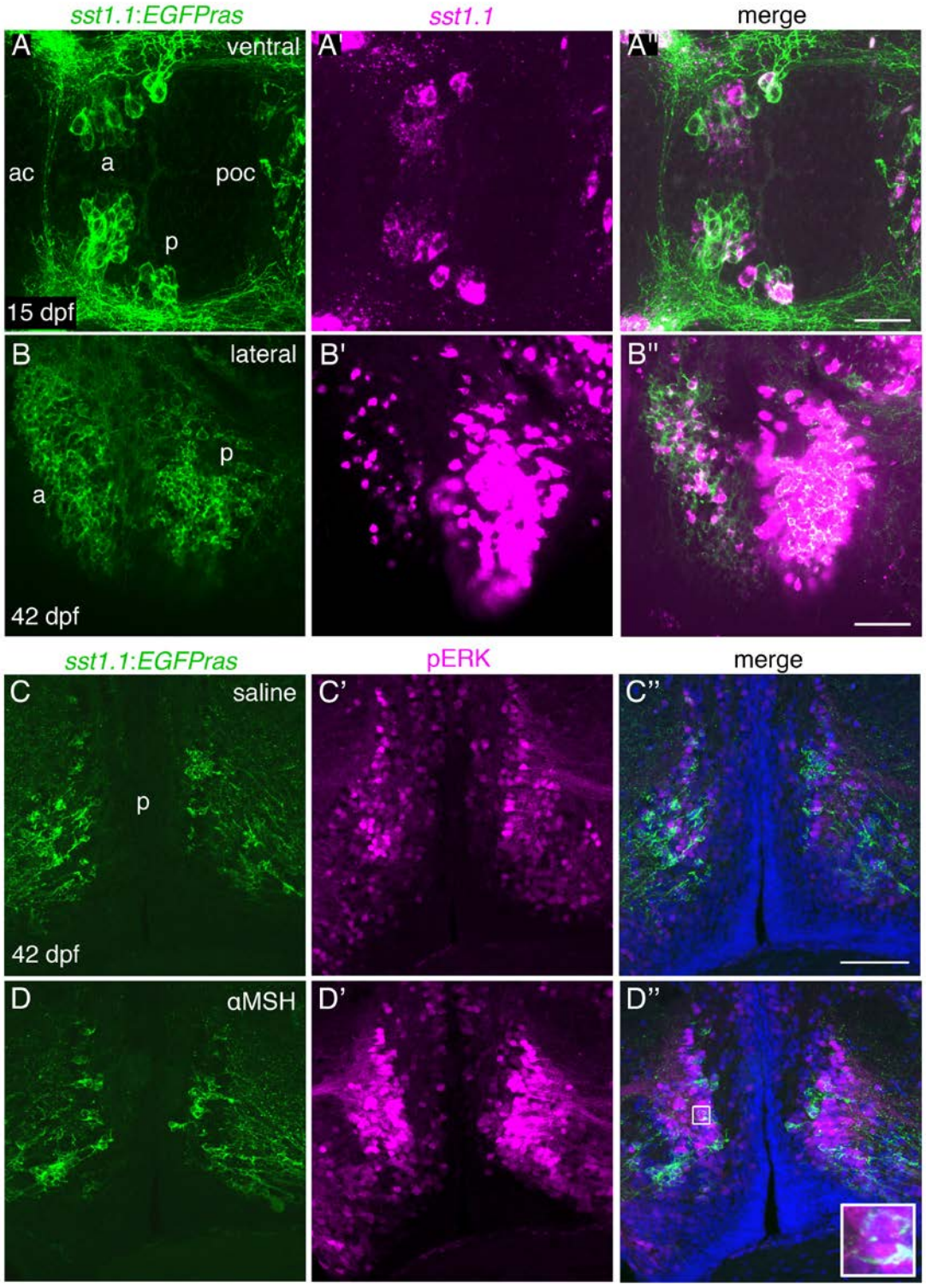

Figure S3. The *sst1.1:EGFPras* transgenic line recapitulates endogenous *sst1.1* expression and reveals activation of hypophysiotropic Sst1.1 neurons after αMSH ICV injection. Related to Figures 5 and 7.

(A-B'') *sst1.1* FISH (magenta) in combination with GFP IF (green) in *sst1.1:EGFPras* transgenic fish at 15 dpf (A-A'') and 42 dpf (B-B'') revealing co-localization of *sst1.1* transcripts and GFP both in the anterior (a) and posterior (p) domains of the preoptic

area (POA). (C-D'') Co-IF for GFP (green) and pERK (magenta) on cross-sections (12 µm) at the level of posterior Sst1.1 POA cell clusters of *tg(sst1.1:EGFPras)* fish (42 dpf), 30 min after cerebroventricular injection of saline (C-C'') or αMSH/saline (D-D''). pERK levels are strongly increased after αMSH application in a broad region of the POA including Sst1.1 neurons (see arrowheads and inset in D''). Scale bars: (A''): 50 µm, (B'', C'') 100 µm. ac: anterior commissure, poc, postoptic commissure.

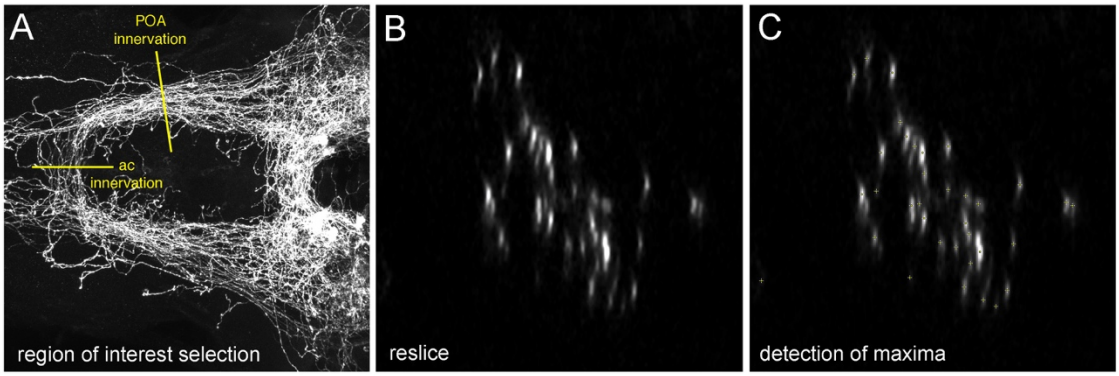

Figure S4. Automated quantification of axon numbers in larval zebrafish brains. Related to Experimental Procedures.

(A-C) Anti-GFP IF on a *Tg(pomca:EGFPras)*^{fr38Tg} larvae at 14 dpf. (A) Confocal image series (z-series) of the preoptic area (POA). Yellow lines depict regions of interest for quantification of axonal innervation of the POA or anterior commissure (ac), respectively. (B) Generation of optical cross sections using the „Reslice“ tool, from Fiji Software (Image J, NIH) allows for visualization of single axons in the respective area. (C) Automated quantification of axons using the „Find Maxima“ function of Fiji Software. Quantified spots are indicated by yellow crosses.

Supplemental Tables

Table S1. Primers used for BAC recombination. Related to Experimental Procedures.

Name	**Sequence (5'-3')**
pomca-hom-F-EGFP	ACAATATGAATTTAACATGCTTAAATGTGAATTGTATTGTGTTC TCAGAGaccatggtgagcaagggcgaggag
pomca-hom-F-mCherry	ACAATATGAATTTAACATGCTTAAATGTGAATTGTATTGTGTTC TCAGAGgccgccaccatggtgagcaagggcgaggaggac
pomca-hom-F-KalTA4	ACAATATGAATTTAACATGCTTAAATGTGAATTGTATTGTGTTC TCAGAGgccgccaccatgaaactgctctcatccatc
pomca-hom-R-KanR	TTTCCCAACACTGAGCTCTGACTTCAGATCCTCCTGCGCAGA GAACAGCCggactagtctattccagaagtagtgaggag
pTarbac2.1_iTol2-hom-F	GTCGACGGCCAGGCGGCCGCCAGGCCTACCCACTAGTCAAT TCGGGAGGAC*cctgctcgagccgggcccaagtg*
pTarbac2.1_iTol2-hom-R	GTTCATGTCTCCTTCTGTATGTACTGTTTTTTGCGATCTGCCG TTTCGA*attatgatcctctagatcagatct*
sst1.1-hom-F-EGFP	ACGCATCTCTCTTCTTTTACTCTGAGACCAAATAAACACTTTA ATAAAAGaccatggtgagcaagggcgaggag
sst1.1-hom-R-KanR	GCTGACGGCGAGCGCGAGGGACAGGAGCGCCAGTGCGCAC TGGATACGCGggactagtctattccagaagtagtgaggag
pIndigoBAC_iTol2-hom-F	GGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGC TCACTGCCC*cctgctcgagccgggcccaagtg*
pIndigoBAC_iTol2-hom-R	GGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACT GGAAAGCG*attatgatcctctagatcagatct*

(upper case letters: BAC homology arms; lower case letters: cassette specific sequences)

Table S2. Primers used for gateway cloning. Related to Experimental Procedures.

Name	**Sequence (5'-3')**
pomca(pit)5'_attB4	**GGGG ACA ACT TTG TAT AGA AAA GTT G**GA GCT CCT ATC AAA CCA TGT T
pomca(pit)5'_attB1r	**GGGG AC TGC TTT TTT GTA CAA ACT TG**C CTC TGA GAA CAC AAT ACA ATT CAC
CFP-nfsb -attB1	**GGGG ACA AGT TTG TAC AAA AAA GCA GGC T**CC GCC ACC ATGGTGAGCAAGGGCGAGGAGCTGT
CFP-nfsb-attB2	**GGGG AC CAC TTT GTA CAA GAA AGC TGG GT**A TTACACTTCGGTTAAGGTGATGTT

(bold letters: att sites; normal letters: cassette specific sequences)

Table S3. Primers used for generation of R26-fl-rxΔ-ZsGreen mice. Related to Experimental Procedures.

Name	Sequence (5'-3')
5Asc2a ZsGreen	GGCGCGCCACCATGGGTTTAAACCGCGCGGAAGGCCGCGGCAG CCTGCTGACCTGCGGCGATGTGGAAGAAAACCCAGGGCCGGGC AAGAAGAAGAGGAAGGTGGCCCAGTCC AAGCACGGCCTGACCA
3Sma ZsGreen	CCCGGGATTTAAATTCAGGGCAAGGCGGA
5Mlu roxwss	ACGCGTTAACTTTAAATAATTGGCATTATTTAAAGTTACTCGAG GGATCTTTGTGAAG
3Asc roxwss	GGCGCGCCTAACTTTAAATAATGCCAATTATTTAAAGTTAAAGC TTACTTACCATGTCAG

Table S4. SYBR-Green qPCR primer sequences. Related to Experimental Procedures.

Gene	Sequence F (5'-3')	Sequence R (5'-3')
pparg	TGCCGCATACACAAGAAGAG	ATGTGGTTCACGTCACTGGA
fabp11a	GGCAAACTTGTGCAGAAACA	GAACTGAGCCTGGCATCTTC
adipoqb	ACAAGAACGACAAGGCCATC	AAAACCGGAGAAGGTGGAGT
rps23	TGTGCTTGAGAAAGTTGGTGTTG	AGCTGGACTCTCACACACTTCCT
trh	CGCTCCATCCTCACACAGAT	CTGTCGCTTCTCCATCCACT
insa	CCACCACCATATCCACCATT	ACCAACAGGACCAACAGAGC
gh1	GGTGGTTAGTTTGCTGGTGAA	CGTCTCGATGGAGTCAGAGTT
oxt	AACGCTCTGTTCAGGACTGG	AGGGAGAAAATCCTCCTCCA
crh	GCGCAAAGTTCAAAAACCAT	GCTGCTCTCGATGGCTCTAC

Table S5. TaqMan gene expression assays. Related to Experimental Procedures.

Gene	Specifications
pomca	commercially available Assay ID: Dr03112624_m1
rps23	commercially available Assay ID: Dr03430371_m1
agrp	custom made F: ATCATCTGCCCTGCTGCAA R: GCCTTAAAGAAGCGGCAGTAG Probe: CCCCTGCGACACCTG
lepa	custom made F: CATCGTCAGAATCAGGGAACACA R: GTCCTGGATCCCCAATGATGAG Probe: TTGACGGGCAAAATT
lepb	custom made F: GAACCACCATCAGCCGAATTAAAA R: GCCGAAATCAATCTCTGGAGACAT Probe: CTGGAAGTGCTCATCTTT

Table S6. Primary antibodies. Related to Experimental Procedures.

Antibody	Source	Cat#
Chicken Anti-GFP Antibody	Thermo Fisher Scientific	A10262
Mouse Anti-GFP Antibody	Millipore	MAB3580
Mouse Anti-GFP Antibody (Living Colors A.v. Monoclonal Antibody JL08)	Clontech	632380
Rabbit Anti-RFP Antibody	MBL	PM005
Sheep Anti-Alpha-Melanocyte Stimulating Hormone Antibody	Chemicon	AB5087
Rabbit Melanin Concentrating Hormone Antibody	Phoenix Pharmaceuticals	H-070-47
Rabbit Anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody	Cell Signaling	4370
Goat Anti-mouse IgG Biotinylated Antibody	Vector Laboratories	BA-9200

Supplemental Experimental Procedures

BAC recombination, plasmid construction and generation of transgenic zebrafish lines

The BAC clones CH211-133P19, and DKEY-29L4 containing the *pomca* and *sst1.1* gene loci, respectively, were used for BAC recombination. PCR products of EGFPras-KanR and KaITA4-KanR cassettes flanked by homology arms for insertion at the start ATG of *pomca* or *sst1.1* were derived from pPCR-EGFP-KanRand pPCR-KaITA4-KanR plasmids, respectively (for primer sequences see Table S1). For generation of pPCR-EGFPras-KanR a Ras tag was fused to the EGFP coding sequence of pPCR-EGFP-KanR by a site directed mutagenesis PCR. For generation of pPCR-KaITA4-KanR, the KaITA4 cassette was removed from pCSKalTA4GI (Distel et al., 2009) by an *Eco*RI/*Hpa*I digest and inserted into pPCR-GFP-KanR from which the EGFP cassette was removed by an *Eco*RI/*Sna*BI digest. In addition, iTol2-Amp cassettes were placed in the vector backbones of BACs CH211-133P19 (pTARBAC2.1) and DKEY-29L4 (pIndigoBAC). iTol2-Amp cassettes flanked by homology arms for insertion into the respective BAC vector backbones were generated by amplification from pPCR8GW-itol2-Amp plasmid (Suster et al., 2009) using pTarbac2.1_iTol2-hom or pIndigoBAC_iTol2-hom primers (Table S1). BAC recombinations were performed using the pRed/ET technique according to manufacturer protocols (Quick & Easy BAC Modification Kit, Gene Bridges).

The plasmid for generation of the *Tg(pomca(pit):CFP-nfsb;cmlc2:GFP)*^{fr41Tg} line was cloned using the Gateway Tol2 kit (Kwan et al., 2007). For generation of p5E-pomca(pit), the pituitary specific *pomca* promoter (Liu et al., 2003) was amplified from genomic DNA, subcloned, re-amplified to add attB4-B1r attachment sites and inserted into pDONRP-P1R via a BP reaction. pME-CFP-nfsb was generated by addition of attB1-B2 attachment sites to a CFP-nfsb fusion construct via PCR followed by a BP reaction (for primers see Table S2). Finally, p5E-pomca(pit), pME-CFP-nfsb and p3E-pA (Kwan et al., 2007) were cloned into the destination vector pDestTol2CG2 (Kwan et al., 2007) via LR reaction. Stable transgenic lines were generated by standard injection and screening procedures.

Generation of R26-fl-rxΔ-ZsGreen mice

The SERCA ROSA26 targeting vector (Belgardt et al., 2008) was modified such that

the FRT-flanked IRES GFP cassette was replaced by a 2A-NLS-ZsGreen via *Asc*I/*Sma*I restriction and that a rox-flanked stop cassette was inserted into the *Asc*I site. Briefly, ZsGreen was amplified from pIRES2-ZsGreen1 vector (Clontech), using primers 5Asc2aZsGreen and 3SmaZsGreen (see Supplemental Table S3 for sequences), and verified by sequencing. Subsequently, a rox-flanked stop cassette (Anastassiadis et al., 2009) was amplified from SERCA using primers 5Mluroxwss 3Ascroxwss (see Supplemental Table S3 for sequences) and inserted 3' of the existing loxP-flanked neo stop cassette (Lasko et al., 1992) using *Asc*I and *Mlu*I/*Asc*I digestion. The resulting rosa26 targeting vector named B9-36 was linearized by *Asi*SI and 40 µg were transfected into C57BL/6-derived Bruce4 ES cells. Correctly targeted clones were identified by southern blotting on *Eco*RI-digested clonal DNA using the ROSA26 *Eco*/*Pac* and neo probes respectively. ES cells were injected into CB20 blastocysts to generate chimeric mice that were backcrossed to C57BL/6 animals to obtain germline transmission of the R26-fl-rx-ZsGreen allele. These mice were crossed with CAGGS Dre mice (Anastassiadis et al., 2009) to remove the rox-flanked stop cassette to obtain R26-fl-rxΔ-ZsGreen allele in which a loxP site-flanked neo stop cassette prevents expression of 2A-NLS-ZsGreen from the CAG promoter.

Sst-IRES-Cre; R26-fl-rxΔ-ZsGreen double transgenic mice were obtained by crossing homozygous R26-fl-rxΔ-ZsGreen mice to hemizygous Sst-IRES-Cre mice, yielding approximately 50% double transgenics with ZsGreen-labelled Sst cells. Genotyping was performed by PCR. The following primers were used for Sst-IRES-Cre: WT: 5’-CTGCAGTTCGATCACTGGAAC-3’ (forward), 5’-AAAGGCCTCTACAGTCTATAG-3’ (reverse), yielding 542 bp band; mutant: 5’-TCCAATTTACTGACCGTACA-3’ (forward), 5’-TCCTGGCAGCGATCGCTATT-3’ (mutant), yielding 450 bp band. The following primers were used for R26-fl-rxΔ-ZsGreen: 5’-AAAGTCGCTCTGAGTTGTTATC-3’ (shared forward), 5’-GATATGAAGTACTGGGCTCTT-3’ (reverse WT), 5’-TGTCGCAAATTAACTGTGAATC-3’ (reverse mutant). The WT allele gave a 570 bp band, the mutant allele a 380 bp band.

Electrophysiology

Perforated patch clamp experiments were performed on coronal brain slices (250-300 µm) from female and male $Sst^{ZsGreen}$ mice (6-9 weeks of age), which contained the PeVN. Experiments were carried out essentially as described previously (Hess et al., 2013; Könner et al. 2011). PeVN somatostatin neurons were identified according to their anatomical location and reporter expression. The brain slices were continuously superfused with carbogenated artificial cerebrospinal fluid (aCSF) (~31°C) at a flow rate of ~2 ml/min (recording chamber volume: ~2 ml). aCSF contained (in mM): 125 NaCl, 2.5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.2 NaH_2PO_4, 21 $NaHCO_3$, 10 HEPES, 5 glucose, adjusted to pH 7.2 with NaOH, resulting in an osmolarity of ~310 mOsm. Recordings were performed with pipette solution containing 1% biocytin (Sigma) and (in mM): 128 K-gluconate, 10 KCl, 10 HEPES, 2 $MgCl_2$, and adjusted to pH 7.3 with KOH. Initially, the patch pipette was tip filled with internal solution and back filled with 0.02% tetraethylrhodamine-dextran (D3308, Invitrogen, Eugene, OR, USA) and amphotericin B-containing internal solution (200 µg/ml; A4888, Sigma) to achieve perforated patch recordings (Horn and Marty, 1988; Akaike and Harata, 1994). Amphotericin B was dissolved in dimethyl sulfoxide (DMSO; final concentration 0.4–0.5%; D8418, Sigma) as described previsously (Rae et al., 1991) and was added to the modified pipette solution shortly before use. To block GABAergic and glutamatergic synaptic input the aCSF contained 10^{-4} M picrotoxin (P1675; Sigma-Aldrich), 5 x 10^{-5} M D-AP5; A5282; Sigma-Aldrich), and 10^{-5} M CNQX (C127; Sigma-Aldrich). αMSH (M4135; Sigma-Aldrich) was added to the aCSF at a concentration of 250 nM for durations of 5-7 min, as previously described (Ghamari-Langroudi et al., 2011). To analyze the αMSH responsiveness, the neuron's firing rate averaged from 10 sec intervals was taken as one data point. To determine the mean firing rate and standard deviation, 12 data points were averaged. On the single cell level a neuron was considered αMSH-responsive if the change in firing induced by αMSH was 3 times larger than the standard deviation (Dhillon et al., 2006; Kloppenburg et al., 2007).

In situ hybridization, immunolabelling and Nile Red staining of zebrafish larvae

Whole-mount fluorescent *in situ* hybridization, and whole-mount immunofluorescence staining (IF) of zebrafish were carried out as described (Filippi et al., 2007). To

increase signal intensity, *in situ* hybridization buffer was supplemented with 5-10% dextran sulfate (Sigma-Aldrich). DIG labeled probes for *pomca*, *gh1*, *oxt*, *trh*, *crh* and *sst1.1* were prepared as previously reported (see www.zfin.org). For generation of *mc4r* probes, partial coding sequences were cloned into pCRII (Invitrogen) and transcribed (sequence information available on request).

For information on antibodies used for zebrafish immunohistochemistry or immunofluorescence, see Table S6. Species-specific secondary antibodies coupled to Alexa Fluor 488 or 555 (Thermo Fisher Scientific) were used for IF experiments. Nuclear counterstaining was performed with DAPI (Thermo Fisher Scientific). For non-fluorescent immunostaining, samples were processed with standard protocols for DAB staining with the Vectastain Elite ABC-Peroxidase Kit (Vector Laboratories) using mouse anti-GFP primary (Millipore) and goat anti-mouse IgG biotinylated secondary antibodies (Vector Laboratories). For sections of DAB immunostained larvae or juvenile brains, samples were embedded in Durcupan (Sigma-Aldrich) and sectioned at a microtome (20 µm). For Durcupan embedding, larvae were dehydrated with increasing concentrations of Ethanol, then washed twice with acetone and incubated overnight in an acetone:Durcupan (1:1) mix. In turn, samples were transferred to sectioning molds containing pure Durcupan and incubated at 65°C until hardened. Cryo-sections (12 µm) of heads from juvenile fish and subsequent immunohistochemistry was performed as reported (Uribe and Gross, 2007).

RNAscope on mouse brain sections

Mice (n=5; 12 weeks of age) were perfused transcardially using a 0.9% saline solution at room temperature and fixed with 4% paraformaldehyde (pH 7.4) at 4°C. The brains were dissected and post-fixed for 18 h in 4% paraformaldehyde (pH 7.4) at RT. The solution was changed to 25% sucrose in 0.1 M PBS (pH 7.4) and incubated overnight at 4°C. The brains were cut at a freezing microtome. The brain sections were 14 µm thick and were collected in a 30% ethylene glycol and 20% glycerol in PBS solution. On the day before the assay, every 8th section throughout the PVN and PeVN was mounted on SuperFrost Plus Gold slides (Thermo Fisher Scientific) and incubated overnight at 60°C. One section from each animal was mounted to be used as negative control. Fluorescent *in situ* hybridization for Sst and Mc4r mRNA detection was performed using RNA scope technique. Reagents were

purchased from Advanced Cell Diagnostics (Hayward, CA), if not mentioned otherwise. In brief, sections were pre-treated for 10 min in hydrogen peroxide (Cat# 322381) at RT, followed by submersion in Target Retrieval (Cat# 322000) for 8 min at 98-99°C. The slides were rinsed twice in autoclaved Millipore water and quickly dehydrated in 100% ethanol. After air drying, a hydrophobic barrier was made around the sections using an ImmEdge hydrophobic barrier pen. The incubations were performed at 40°C, using the HybEz Hybridization System for Manual Assays. Sections were incubated for 40 min with protease III (Cat# 322381), followed by probe hybridization for 2 h. C1-probe for MC4R, no dilution, (Cat# 402741) and C3-probe for Sst (Cat# 404631-C3), diluted 1:50, were used. A 3-plex positive (Cat# 320881) and a 3-plex negative (Cat# 320871) control probes were processed in parallel with the target probes to assess the quality of the assay. Probe hybridization was followed by 2x2 min washes in Wash buffer (Cat# 310091). The manufacturer protocol for RNAscope® Multiplex Fluorescent v2 Assay (Cat# 323110) was followed for the remaining steps (amplification and detection). Briefly, AMP1 and AMP2 were incubated for 30 min, followed by AMP3 incubation for 15 min. Between each amplification step, 2x2 min washes were performed. Afterwards a TSA Plus amplification (Perkin Elmer, Cat# NEL760001KT) protocol was used. C1-probe tyramide fluorophore was Cy3 and C3-probe fluorophore was Fluorescein. DAPI was used for counterstaining and ProLong® Gold Antifade Mountant (Thermo Fisher Scientific, Cat# P36931) was used to coverslip the sections.

Imaging and quantitative evaluations of fluorescent images

Confocal z-stacks of zebrafish fluorescences were recorded using a Zeiss LSM 710 microscope. For confocal imaging, whole larvae were mounted in 80% glycerol containing 1.2 % low melting agarose/PBS. Juvenile brains or brain hemispheres were mounted in 80% glycerol in PBS only. Brains of DiI injected samples were imaged in PBS without optical clearing to avoid dye diffusion. Light microscopy was performed at a Zeiss M2 compound microscope for which samples were mounted in 80% glycerol/PBS. Cryo-sections were covered with Mowiol mounting medium. Imaging of whole larval brains at 7 dpf or older stages required careful removal of all ventral tissue underneath the skull in order to optimize optical quality. For image processing, ZEN (Zeiss), Photoshop CS2 (Adobe) and Fiji (Image J, NIH) softwares were used. Optical midline sections were generated with Imaris (Bitplane).

Imaging of mouse RNAscope labelings was performed at a Leica TCS SP-8-X confocal microscope. Tile scans and Z-stacks (optical section of 1.0 µm) of the PeVN were captured. Laser intensities for the two probe channels were kept constant throughout the entire material. Images were imported into FIJI software (NIH) where maximum intensity projections were generated.

For measurement of pERK levels in Pomca or Sst1.1 neurons of *Tg(pomca:EGFPras)*^{fr38Tg} or *Tg(sst1.1:EGFPras)*^{fr40Tg} transgenic zebrafish, respectively (Figures 2G,H and 7E), samples were subjected to immunostaining for GFP (Chicken Anti-GFP Antibody, Thermo Fisher Scientific; secondary antibody A488 coupled) and pERK (rabbit Anti-pERK Antibody, Cell Signaling; secondary antibody A555 coupled). Following staining, the samples of pharmacologically treated and control groups were imaged at a confocal microscope with identical acquisition settings. Z-axis distance of single focal planes was 1.5 µm. Scanned raw images were in turn processed using a MATLAB-based image segmentation algorithm (Fluo_traces_v2.m) according to published protocol (Wong et al., 2010). For pERK levels of *sst1.1:EGFPras* cells, an average of 73 ± 19 cells per brain hemisphere derived from seven brains were used for quantification. For pERK levels of *pomca:EGFPras* cells, 119 (ND saline), 54 (ND leptin), 57 (HD/HF saline) and 81 (HD/HF leptin) individual Pomca neurons derived from 4 brains per group were used for quantifications.

For quantification of axonal innervation of the preoptic area in zebrafish larvae (Figure 3K,L), confocal z-stacks (1 µm optical sections) of anti-GFP IF processed *Tg(pomca:EGFPras)*^{fr38Tg} larvae were generated (see Figure S4). Automatic quantification of axon numbers was performed according to the following protocol using Fiji software: Image sequences were loaded into Fiji. As a region of interest, a single line was chosen perpendicular to the axon tract crossing the preoptic area (middle distance between anterior and posterior commissures), or perpendicular to the tract of the anterior commissure (at the level of the midline). Using the „Reslice“ tool, optical cross sections were generated and single axons were quantified using the „Find Maxima“ function of Fiji. For n numbers see respective Figure Legends.

For quantification of DAPI-, Sst- and Mc4R-positive cells in the mouse PVN (Figure 6F), images were imported and fused into Halo software (Indica Labs). Cell identification was obtained based on DAPI staining, and the presence of 3-5 dots per

cell was used as threshold for probe recognition. Only cells with labelling above this threshold were considered positive. Absolute numbers of DAPI-, Sst- and Mc4R-positive cells were determined for equivalent, equally-sized and manually defined rectangular areas of the PeVN, and Sst/DAPI and Mc4R/DAPI ratios were calculated from these absolute numbers.

Supplemental References

Akaike, N., and Harata, N. (1994). Nystatin perforated patch recording and its applications to analyses of intracellular mechanisms. Jpn J Physiol *44*, 433-473.

Anastassiadis, K., Fu, J., Patsch, C., Hu, S., Weidlich, S., Duerschke, K., Buchholz, F., Edenhofer, F. and Stewart, A. F. (2009) Dre recombinase, like Cre, is a highly efficient site-specific recombinase in E. coli, mammalian cells and mice. Dis Model Mech. *2*, 508-515

Belgardt, B. F., Husch, A., Rother, E., Ernst, M. B., Wunderlich, F. T., Hampel, B., Klöckener, T., Alessi, D., Kloppenburg, P. and Brüning, J. C. (2008). PDK1 deficiency in POMC-expressing cells reveals FOXO1-dependent and -independent pathways in control of energy homeostasis and stress response. Cell Metab *7*, 291-301.

Distel, M., Wullimann, M.F., and Köster, R.W. (2009). Optimized Gal4 genetics for permanent gene expression mapping in zebrafish. Proc Natl Acad Sci U S A *106*, 13365-13370.

Dhillon, H., Zigman, J. M., Ye, C., Lee, C. E., McGovern, R. A. , Tang, V. , Kenny, C. D., Christiansen, L. M., White, R. D. , Edelstein, E. A. , Coppari, R., Balthasar, N., Cowley, M. A. , Chua, S. Jr, Elmquist, J. K. and Lowell, B. B. (2006). Leptin directly activates SF1 neurons in the VMH, and this action by leptin is required for normal body-weight homeostasis. Neuron *49*, 191-203.

Filippi, A., Durr, K., Ryu, S., Willaredt, M., Holzschuh, J., and Driever, W. (2007). Expression and function of nr4a2, lmx1b, and pitx3 in zebrafish dopaminergic and noradrenergic neuronal development. BMC Dev Biol *7*, 135.

Horn, R., and Marty, A. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. J Gen Physiol *92*, 145-159.

Kloppenburg, P., Zipfel, W. R., Webb, W. W. and Harris-Warrick, R.M. (2007). Heterogeneous effects of dopamine on highly localized, voltage-induced Ca2+ accumulation in identified motoneurons. J Neurophysiol *98*, 2910-2917.

Kwan, K.M., Fujimoto, E., Grabher, C., Mangum, B.D., Hardy, M.E., Campbell, D.S., Parant, J.M., Yost, H.J., Kanki, J.P., and Chien, C.B. (2007). The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. Dev Dyn *236*, 3088-3099.

Lakso, M., Sauer, B., Mosinger, B. Jr, Lee, E. J., Manning, R. W., Yu, S. H., Mulder, K. L. and Westphal, H. (1992). Targeted oncogene activation by site-specific recombination in transgenic mice. Proc Natl Acad Sci U S A. *8*9, 6232-6236.

Liu, N.A., Huang, H., Yang, Z., Herzog, W., Hammerschmidt, M., Lin, S., and Melmed, S. (2003). Pituitary corticotroph ontogeny and regulation in transgenic zebrafish. Mol Endocrinol *17*, 959-966.

Rae, J., Cooper, K., Gates, P. and Watsky, M. (1991). Low access resistance perforated patch recordings using amphotericin B. J Neurosci Meth *37*, 15–26.

Suster, M.L., Sumiyama, K., and Kawakami, K. (2009). Transposon-mediated BAC transgenesis in zebrafish and mice. BMC Genomics *10*, 477.

Wong, L.C., Lu, B., Tan, K.W., and Fivaz, M. (2010). Fully-automated image processing software to analyze calcium traces in populations of single cells. Cell Calcium *48*, 270-274.