Supporting information for:

Targeted mutation of secretogranin-2 disrupts sexual behavior and reproduction in zebrafish

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SI reference citations

Supporting Information (SI) Text

SI Materials and Methods

Gonadosomatic index. Adult male and female fish (4 months of age) were randomly selected from each tank, anaesthetized in ice water, weighed, and decapitated. Gonads of *scg2a*-/-, *scg2b*-/- and *scg2a*-/-; *scg2b*-/- and WT fish were dissected and weighed to calculate the gonadosomatic index (GSI; gonad weight/body weight x 100).

Sex steroid analysis. Sex steroids were extracted using the Folch method (1). Whole zebrafish were individually frozen with liquid nitrogen, ground in a mortar and pestle, and poured into a 50 mL plastic centrifuge tube. Chloroform:methanol (2:1) (15 mL) was added to each sample. Samples were homogenized for 30 sec with a Polytron (Luzern, Switzerland) homogenizer, then incubated for 15 min at room temperature. Five mL 2 M KCl containing 5 mM ethylenediaminetetraacetic acid (EDTA) were added to each sample and the tubes were vortexed for 1 min. The samples were left at room temperature for 20 min to allow layer separation and then the lower layer containing steroids, was removed and placed into a 10 mL glass test tube. The samples were evaporated under a gentle stream of nitrogen in a fume hood. The lipid extract was reconstituted in 0.3 mL ethylene glycol monomethyl ether (EGME), transferred to a 1.5 mL conical centrifuge tube, and stored in a -20°C freezer until analyzed. Whole body extraction efficiencies were determined by spiking homogenates with known amounts of their respective radioactive isotope (^{14}C or ^{3}H). Extraction efficiencies were relatively high (85-89%), therefore results were not corrected. Testosterone (T) (TECO Diagnostics, Anaheim, CA, TEST-96), estradiol (E2) (TECO Diagnostics, Anaheim, CA, ESTRA-96) and 11-keto-testosterone (11-KT) (Cayman Chemical, Ann Arbor, MI, 582751; 2.9% cross-reactivity with 11-ketoandrostenedione and T <0.01%) levels were assessed using enzyme immunoassay test kits according to the manufacturer's protocols.

In vitro germinal vesicle breakdown. Assay details have been reported in detail previously (2). Full growth stage follicles were isolated manually from mature zebrafish , then incubated with 100 IU/mL human chorionic gonadotropin (Ningbo Second Hormone Factory, Ningbo, China) in medium 199 (HyClone Laboratories Inc., Logan, UT) at 28°C. Oocytes were examined microscopically at 3 and 6 h for ooplasmic clearing, which occurs due to proteolytic cleavage of vitellogenin and is indicative of oocyte maturation.

Synthesis and purification of secretoneurin peptides. Zebrafish SNa (TNENAEEQYTPQKLATLQSVFEELSGIASSKTNT) and SNb (ATEDLDEQYTPQSLANMRSIFEELGKLSAAQ) peptides were prepared using an Intavis Multipep Peptide Synthesizer (Intavis Bioanalytical Instruments AG), using an Fmoc-tBu strategy. N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU) and N-Methylmorpholine (NMM) were used as the coupling reagent system. All Fmoc protected amino acids were purchased from Chempep Inc. Wellington, FL). All other chemicals were purchased from Sigma Aldrich (St. Louis, MI). Peptides were purified by semi-preparation HPLC (Varian ProStar) using Phenomenex Luna C-18 column (3 um 10X250 mm). The purity of each peptide was analyzed using high performance liquid chromatography (HPLC) with

Jupitor analytical C-18 column and diode array detector. The purity of each peptide used in experiment is over 95% based on the absorbance spectrum. Peptide sequences were confirmed using HPLC-ESI-MS/MS. The system consisted of an Agilent 1100 micro-HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled with an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA) equipped with a nano-electrospray interface operated in positive ion mode. All peptides were lyophilized and stored as trifluoroacetic salts at -80°C.

Table S1. PCR primer sets and reaction conditions.

Gene name	Gene abbreviation	Direction	Primer Sequence (5'-3')	Amplicon size	Annealing temperature (°C)
Luteinizing hormone beta	*lhb*	Forward Reverse	AATGCCTGGTGTTTCAGACC AACAGTCGGGCAGGTTAATG	144	59
Follicle stimulating hormone beta	*fshb*	Forward Reverse	TGTGGAGAGCGAAGAATGTG AGACCTTCTGGGTGTGCTGT	116	57
Glycoprotein alpha	*cga*	Forward Reverse	CTGCTGCTTTTCGAGAGCTT AGTGGCAGTCTGTGTGGTTG	155	59
Gonadotropin-releasing hormone 3	*gnrh3*	Forward Reverse	ATGGAGGCAACATTCAGGATGT CCTTTCAGAGGCAAACCTTCA	131	56
Gonadotropin-releasing hormone receptor 2	*ghrnr2*	Forward Reverse	TCCTCAACCCTCTGTCCATC TGCTTTGGGGAATCAATCTC	123	56
Isotocin	*oxt*	Forward Reverse	GATCTGCTGCTGAAGCTCCT TACAAAAGTGGGTGGCGAGT	134	59
Vasotocin	*avp*	Forward Reverse	AGGTCTGCATGGAAGAGGAG CTGCCTTCAGGACAGTCTGG	146	58
Secretogranin-IIa	*scg2a*	Forward Reverse	CAGGACGTACGGGTTATGCT GCGTTGGTCTTTGGTTTTGT	138	57
Secretogranin-IIb	*scg2b*	Forward Reverse	AAACAAAGCTCCGAGCAAAA AACTGGTGTCGGGATACTCG	116	57
Elongation factor alpha	*ef1α*	Forward Reverse	CAAGGAAGTCAGCGCATACA ACCGCTAGCATTACCCTCCT	150	59

Supporting results and figures

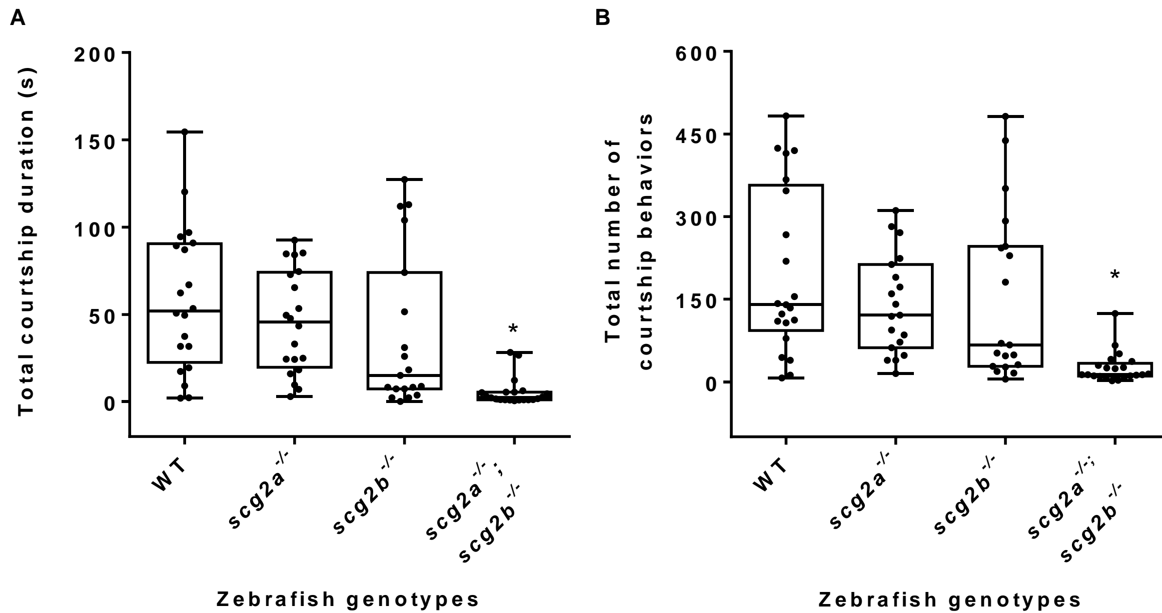


Figure S1. Total courtship behavior in WT and *scg2-/-* fish. (A) Total duration (s) and (B) total number of courtship behaviors assessed for 10 minutes in pairwise within-line mating crosses. Horizontal lines represent median values; boxes represent interquartile ranges and whiskers represent minimum-maximum values. Each dot represents one fish. Since the data was not normally distributed, significant differences were determined using a Kruskal-Wallis test on ranks followed by a Dunn's multiple comparison test (n=20-22 per genotype). Asterisks denote a significant difference from the wild-type pairs; *P≤0.05.

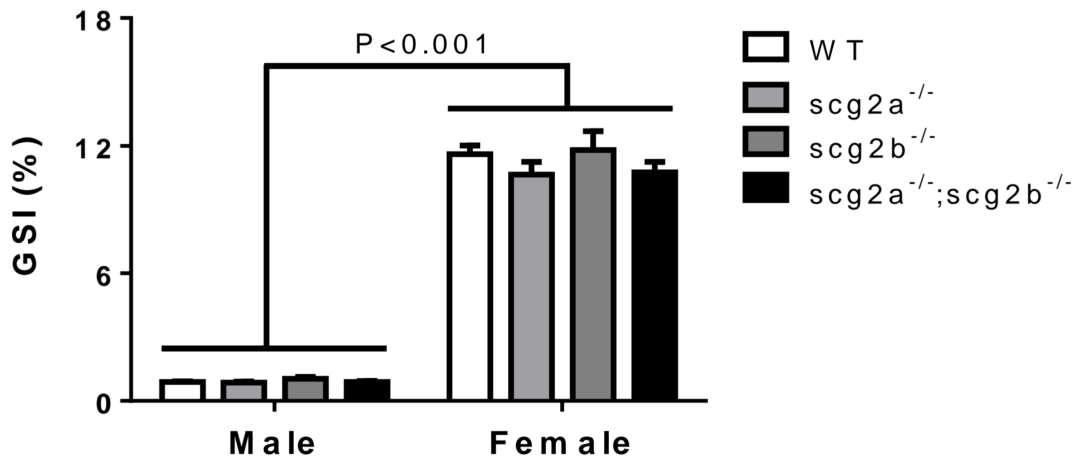


Figure S2. Gonadosomatic index (GSI) for WT and *scg2-/-* males and females. Means + SEM are presented (n = 20-90 fish per group). Lines connecting different bars represent the comparison made between the two (P<0.001).

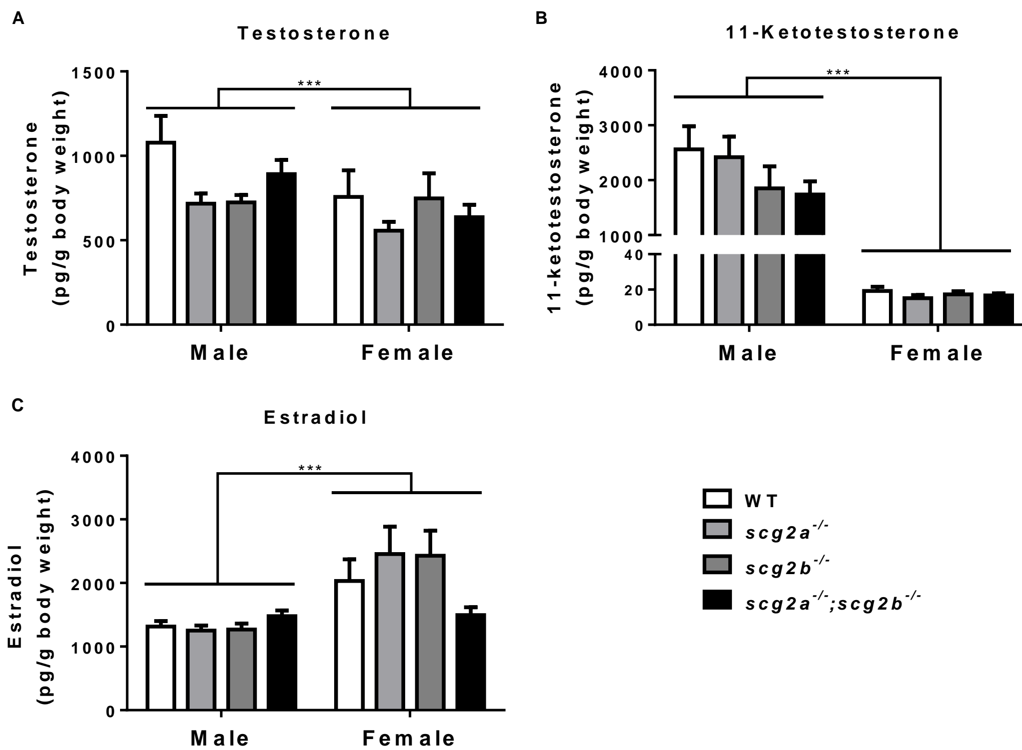
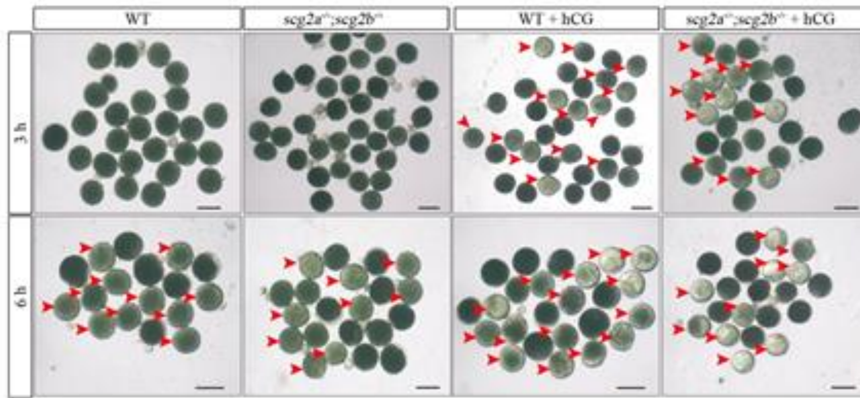


Figure S3. Whole body steroid levels in WT and *scg2*$^{-/-}$ fish. (A) Testosterone, (B) 11-ketotestosterone, (C) estradiol levels in male and female *scg2*$^{-/-}$ and WT fish. Means + SEM are presented (n=8-10). Lines above the graph indicate significant differences compared between sexes (*** P ≤ 0.001).

Whole-body sex steroid analysis in WT and *scg2*$^{-/-}$ fish. Given that sex steroids are essential for sexual differentiation, sexual maturation and reproduction, whole body sex steroid levels were analyzed to determine possible mechanism behind the fertility defects seen in *scg2*$^{-/-}$ fish. There was no main effect of genotype [F(3) = 1.716, P = 0.172] on the levels of testosterone; however, there was a significant main effect of sex [F(1) = 15.729, P < 0.001] in that males had higher levels than females (Fig. S3A). No genotype X sex interactions were observed [F(3) = 0.773, P = 0.513]. Similarly, for 11-ketotestosterone, there was no main effect of genotype [F(3) = 1.238, P = 0.302] but there was a significant main effect of sex [F(1) = 2065.543, P < 0.001]; males had higher levels than females (Fig S3B). No genotype X sex interactions were observed [F(3) = 1.337, P = 0.270]. For the levels of estradiol, there were no main effects of genotype [F(3) = 0.163, P = 0.921], a significant main effect of sex [F(1) = 15.425, P < 0.001] (Fig. S3C); females had higher levels than males. No genotype X sex interactions were observed [F(3) = 2.579, P = 0.060].

A



B

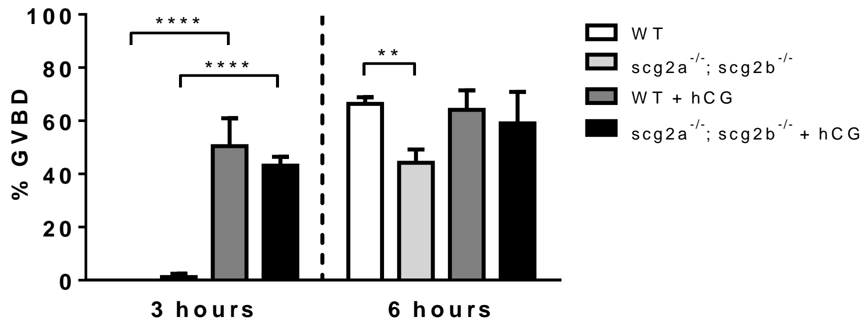


Figure S4. (**A**) Morphology of full grown follicles dissected from WT and *scg2a-/- ;scg2b-/-* ovaries after 3 hours and 6 hours incubation with or without hCG (100 IU/mL). Follicles undergoing germinal vesicle breakdown (GVBD) are marked by red arrows. Scale bars=400μm. (B) The percentage of follicles undergoing GVBD. Each value represents the mean + SEM of five independent experiments. Statistical significance of the number of immature versus mature oocytes undergoing GVBD alone and with hCG treatment was assessed Fisher's Exact test within each time period. ** P=0.005, *** P=0.0001.

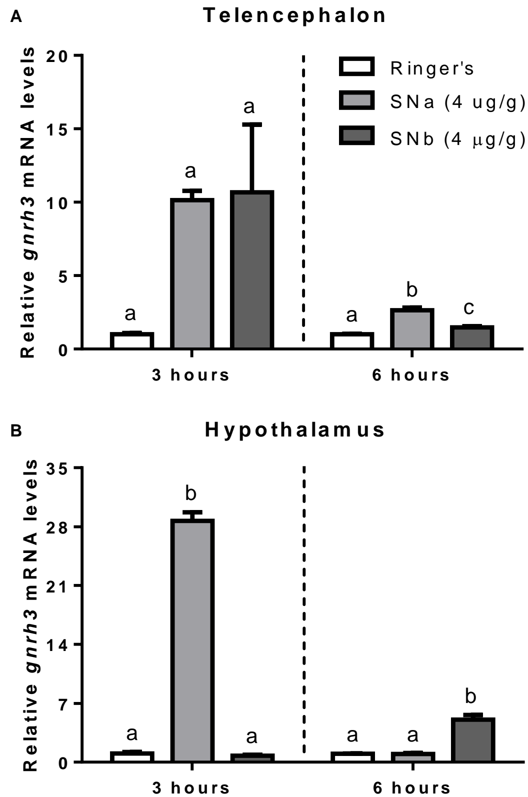
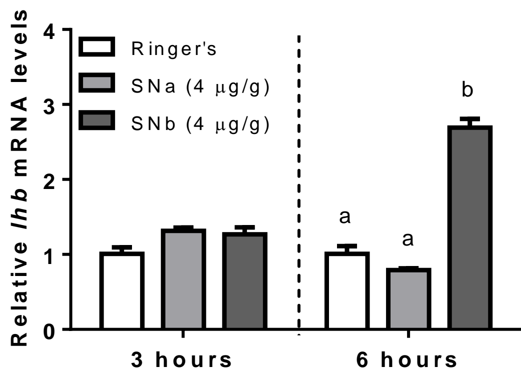
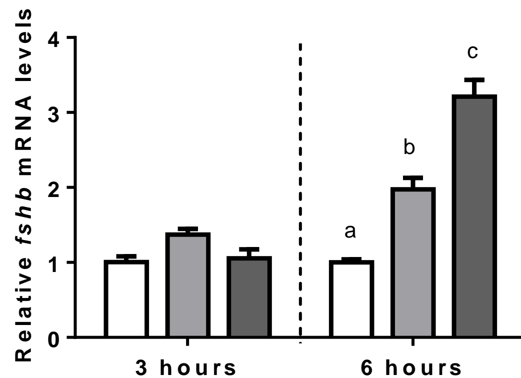
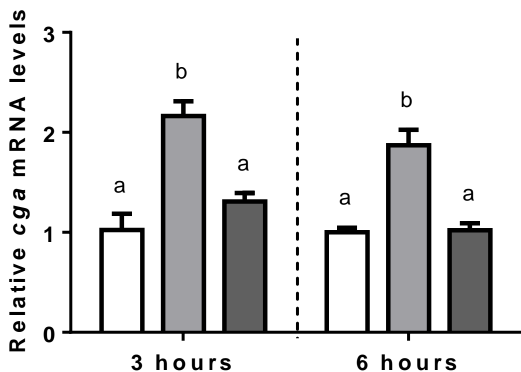
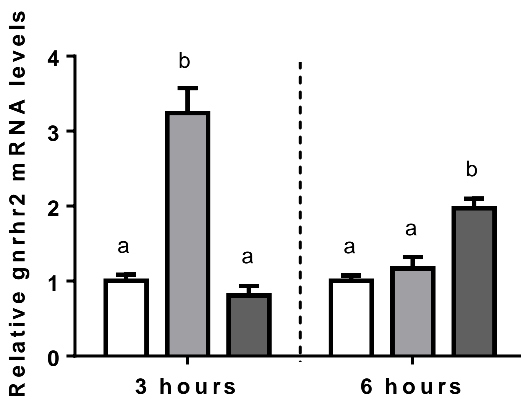


Figure S5. Effects of SN injection on relative mRNA levels of *gnrh3* in the telencephalon and hypothalamus of WT adult zebrafish pituitaries. Adult females (4 months old) were injected IP with Ringer's solution (control), SNa or SNb (4μg/g). Fish were anaesthetized, and pituitaries dissected and collected for RNA extraction and real time qPCR following standard methods (2, 3). We sampled at 3 or 6 hrs, following the time-course of action of SNa and SNb on brain gene expression reported previously (4). Primers are presented in Table S1, and data are expressed relative to *ef1α*. The mean +SEM (n=3 independent pools of 4-6 tissue samples per tube) are shown. All data passed the Shapiro-Wilk normality test. All but the hypothalamus (3 hour) data passed the equal variance test, so data were log transformed and passed both tests. Significant differences between groups at each time point were determined using a one-way ANOVA followed by the Holm-Sidak post-hoc test ($P \leqslant 0.05$). Different letters denote significant differences between groups within each time point.


A
Relative lhb mRNA levels
0
1
2
3
4
Ringer's
SNa (4 µg/g)
SNb (4 µg/g)
a
a
b
3 hours
6 hours


B
Relative fshb mRNA levels
0
1
2
3
4
a
b
c
3 hours
6 hours


C
Relative cga mRNA levels
0
1
2
3
a
b
a
a
b
a
3 hours
6 hours


D
Relative gnrhr2 mRNA levels
0
1
2
3
4
a
b
a
a
a
b
3 hours
6 hours

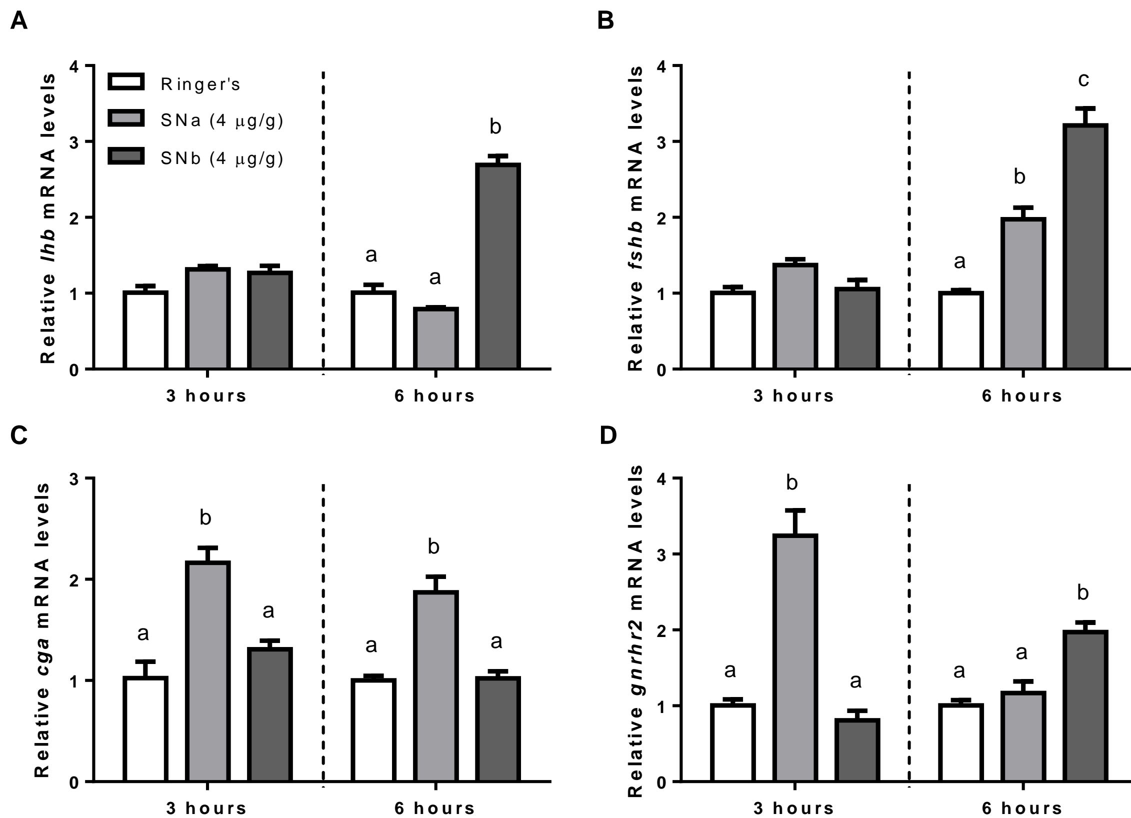


Figure S6. Effects of SN injection on relative mRNA levels of *lhb*, *fshb*, *cga* and *gnrhr2* in WT adult zebrafish pituitaries. Adult females (4 months old) were injected IP with Ringer's solution (control), SNa or SNb. Fish were anaesthetized, and pituitaries dissected and collected for RNA extraction and real time qPCR following standard methods (2, 3). We sampled at 3 or 6 hrs, following the time-course of action of SNa and SNb on pituitary gene expression reported previously (4). Primers are presented in Table S1, and data are expressed relative to *ef1α.* The mean +SEM (n=3 independent pools of 4-6 pituitaries per tube) are shown. Data passed the Shapiro-Wilk normality test and equal variance test. Significant differences between groups at each time point were determined using a one-way ANOVA followed by the Holm-Sidak post-hoc test ($P \leq 0.05$). Different letters denote significant differences between groups within each time point.

Genetic compensation does not take place in *scg2* mutant zebrafish. In the telencephalon, there was a significant main effect of genotype on the levels of *scg2a* [$F(3) = 19.087$, $P < 0.001$] (Fig. S6A). However, there was no significant effect of sex [$F(1) = 2.730$, $P = 0.106$] or interactions between genotype and sex [$F(3) = 0.885$, $P = 0.456$]. Since there were no statistically significant differences in females versus males in the levels of *scg2a*, the results were pooled across sex. There was a significant decrease in the *scg2a* levels in *scg2a-/-*and *scg2a-/-*; *scg2b-/-* ($P < 0.05$) and no difference in *scg2b-/-* compared to WTs. The levels of *scg2a* in the telencephalon were decreased by 55% and 73% in *scg2a-/-*and *scg2a-/-*; *scg2b-/-* fish, respectively, compared to WTs. The levels of *scg2a* were also higher in *scg2b-/-* fish compared to *scg2a-/-*and *scg2a-/-*;

scg2b$^{-/-}$ fish (P < 0.05) (Fig. S6A). In the telencephalon, there was a significant main effect of genotype on the levels of *scg2b* [F(3) = 40.231, P < 0.001] (Fig. S7B). No significant effects of sex [F(1) = 0.0068, P = 0.934] or genotype X sex [F(3) = 1.488, P = 0.231] interactions were evident. Since there were no statistically significant differences in females versus males in the levels of *scg2b*, the results were pooled across sex. There was a significant decrease in *scg2b* in all scg2 mutant lines compared to WTs (P < 0.05). The levels of *scg2b* in the telencephalon were decreased by 32%, 56% and 88% in *scg2a*$^{-/-}$and *scg2a*$^{-/-}$; *scg2b*$^{-/-}$ fish, respectively.

For the levels of *scg2a* in the hypothalamus, there was a significant main effect of genotype [F(3) = 9.612, P < 0.001], no main effect of sex [F(1) = 0.0197, P = 0.889], but a significant genotype X sex [F(3) = 3.330, P = 0.028] interaction was evident (Fig. S6B). Holm-Sidak post-hoc tests revealed that *scg2a*$^{-/-}$and *scg2a*$^{-/-}$; *scg2b*$^{-/-}$ males had significantly decreased levels of hypothalamic *scg2a* compared to WTs and *scg2b*$^{-/-}$ (P < 0.05). The *scg2a* levels in the hypothalamus were reduced by 64% and 76 % in *scg2a*$^{-/-}$and *scg2a*$^{-/-}$; *scg2b*$^{-/-}$ males, respectively. In females, no significant differences were observed in the levels of *scg2a* between genotypes. Furthermore, *scg2a*$^{-/-}$; *scg2b*$^{-/-}$ females had significantly higher levels of *scg2a* compared to *scg2a*$^{-/-}$; *scg2b*$^{-/-}$ males (P < 0.05) (Fig. S6B). For *scg2b* in the hypothalamus, there was a significant main effect of genotype [F(3) = 29.200, P < 0.001], no main effect of sex [F(1) = 0.0088, P = 0.926], but a significant genotype X sex [F(3) = 3.212, P = 0.032] interaction was evident (Fig. S7E). Post-hoc analyses showed a significant decrease in the levels of *scg2b* in *scg2b*$^{-/-}$ and *scg2a*$^{-/-}$; *scg2b*$^{-/-}$ males (P < 0.05). The *scg2a*$^{-/-}$; *scg2b*$^{-/-}$ males also had significantly lower hypothalamic levels of *scg2b* compared to *scg2b*$^{-/-}$ males (P < 0.05). The levels of *scg2b* were decreased by 74% and 83% in the hypothalamus of *scg2b*$^{-/-}$ and *scg2a*$^{-/-}$; *scg2b*$^{-/-}$ males, respectively. Hypothalamic levels of *scg2b* in all scg2 mutant lines were significantly lower compared to WT females (P < 0.05). The *scg2a*$^{-/-}$; *scg2b*$^{-/-}$ females also had significantly lower levels of hypothalamic *scg2b* compared to both the *scg2a*$^{-/-}$and *scg2b*$^{-/-}$ lines (P < 0.05). The levels of *scg2b* in the hypothalamus were reduced by 72%, 70% and 88% in *scg2a*$^{-/-}$ and *scg2a*$^{-/-}$; *scg2b*$^{-/-}$ females, respectively. Hypothalamic levels of *scg2b* were significantly lower in *scg2a*$^{-/-}$ females compared to *scg2a*$^{-/-}$ males (P < 0.05).

In the pituitary, significant main effects of genotype [F(3) = 19.238, P < 0.001] and sex [F(1) = 4.193, P = 0.047] were observed for *scg2a* but no significant interactions were present [F(3) = 1.477, P = 0.235] (Fig. S6C). Post-hoc analyses revealed that the levels of *scg2a* were significantly lower in *scg2a*$^{-/-}$ and *scg2b*$^{-/-}$ males, and *scg2a*$^{-/-}$, *scg2b*$^{-/-}$ and *scg2a*$^{-/-}$; *scg2b*$^{-/-}$ females compared to their respective within-sex WT controls (P < 0.05). The levels of *scg2a* in the pituitary decreased by 51% and 61% in *scg2a*$^{-/-}$ and *scg2b*$^{-/-}$ males, respectively. The levels of *scg2a* in the pituitary decreased by 55%, 54% and 29% in *scg2a*$^{-/-}$, *scg2b*$^{-/-}$ and *scg2a*$^{-/-}$; *scg2b*$^{-/-}$ females, respectively. Significant main effects of genotype [F(3) = 11.441, P < 0.001) were also observed for the levels of *scg2b* in the pituitary (Fig S7F). However, no significant effects of sex [F(1) = 0.0268, P = 0.871] nor interactions [F(3) = 0.0894, P = 0.965] were observed. Since there were no statistically significant differences in females versus males in the levels of *scg2b*, the results were pooled across sex. Post-hoc analyses showed that transcript levels of *scg2b* were decreased similarly in all mutant lines compared to WTs (P < 0.05). The levels of *scg2b* were decreased by 54%, 74% and 68% in *scg2a*$^{-/-}$; *scg2b*$^{-/-}$ and *scg2a*$^{-/-}$; *scg2b*$^{-/-}$ fish, respectively.

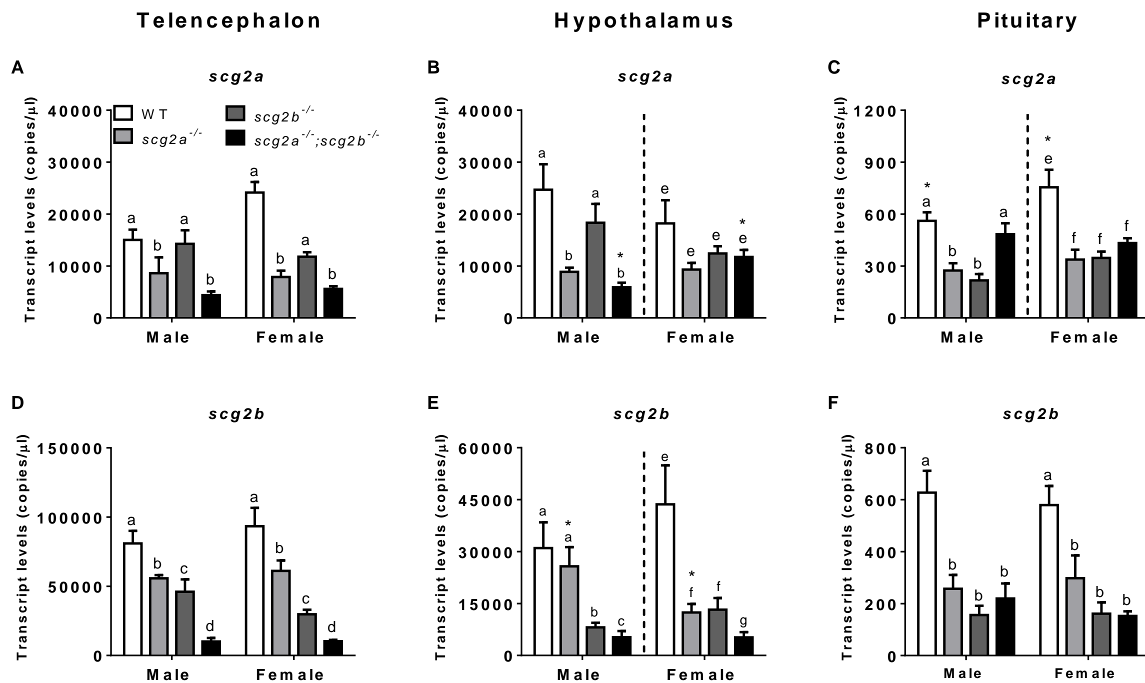


Figure S7. Levels of *scg2a* and *scg2b* in the adult zebrafish telencephalon, hypothalamus and pituitary. The relative abundance of the mRNAs (copies/µl) was normalized according to NORMA-gene. Results are presented as means + SEM (n = 5-7). Vertical lines between males and females indicate a main statistical effect of sex or interaction between sex and genotype. In these cases (B, C and E), means with different letters denote significant differences between genotypes within a sex (a-d for males; e-g for females) ($p \leq 0.05$). An asterisk (*) above a group indicates statistical differences between males and females for that genotype ($p \leq 0.05$). Graphs lacking a vertical line between male and female (A, D and F) indicate no statistical effect of sex; therefore, means with different letters (a-d) denote significant differences between genotypes, regardless of sex ($p<0.05$).

Supporting video files

Supporting Video 1. Two pairs of WT zebrafish in breeding tanks. Shown are the first 2 minutes of the 10 minute recordings. See main text for experimental details and Figure S1 for the summary data.

Supporting Video 2. Two pairs of *scg2a-/-* zebrafish in breeding tanks. Shown are the first 2 minutes of the 10 minute recordings. See main text for experimental details and Figure S1 for the summary data.

Supporting Video 3. Two pairs of *scg2b-/-* zebrafish in breeding tanks. Shown are the first 2 minutes of the 10 minute recordings. See main text for experimental details and Figure S1 for the summary data.

Supporting Video 4. Two pairs of *scg2a-/-/scg2b-/-* zebrafish in breeding tanks. Shown are the first 2 minutes of the 10 minute recordings. See main text for experimental details and Figure S1 for the summary data.

Supporting References

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2. H. Xia *et al.*, Mettl3 Mutation Disrupts Gamete Maturation and Reduces Fertility in Zebrafish. *Genetics* **208**, 729-743 (2018).
3. B. Tao *et al.*, Secretogranin-II plays a critical role in zebrafish neurovascular modeling. *J Mol Cell Biol* **10**, 388-401 (2018).
4. H. Shu *et al.*, Identification and functional characterization of two Secretogranin II genes in orange-spotted grouper (*Epinephelus coioides*). *General and Comparative Endocrinology* **261**, 115-126 (2018).