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

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Edited by R. Michael Roberts, University of Missouri, Columbia, MO, and approved April 24, 2020 (received for review February 3, 2020)

The luteinizing hormone surge is essential for fertility as it triggers ovulation in females and sperm release in males. We previously reported that secretoneurin-a, a neuropeptide derived from the processing of secretogranin-2a (Scg2a), stimulates luteinizing hormone release, suggesting a role in reproduction. Here we provide evidence that mutation of the *scg2a* and *scg2b* genes using TALENs in zebrafish reduces sexual behavior, ovulation, oviposition, and fertility. Large-scale spawning within-line crossings (n = 82 to 101) were conducted. Wild-type (WT) males paired with WT females successfully spawned in 62% of the breeding trials. Spawning success was reduced to 37% (P = 0.006), 44% (P = 0.0169), and 6% (P < 0.0001) for *scg2a*$^{-/-}$, *scg2b*$^{-/-}$, and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ mutants, respectively. Comprehensive video analysis indicates that *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ mutation reduces all male courtship behaviors. Spawning success was 47% in saline-injected WT controls compared to 11% in saline-injected *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ double mutants. For these mutants, spawning success increased 3-fold following a single intraperitoneal (i.p.) injection of synthetic secretoneurin-a (P = 0.0403) and increased 3.5-fold with injection of human chorionic gonadotropin (hCG). Embryonic survival at 24 h remained on average lower in *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish compared to WT injected with secretoneurin-a (P < 0.001). Significant reductions in the expression of gonadotropin-releasing hormone 3 in the hypothalamus, and luteinizing hormone beta and glycoprotein alpha subunits in the pituitary provide evidence for disrupted hypothalamo-pituitary function in *scg2a* and *scg2b* mutant fish. Our results indicate that secretogranin-2 is required for optimal reproductive function and support the hypothesis that secretoneurin is a reproductive hormone.

secretogranin-2 | secretoneurin | reproduction | behavior | ovulation

The integrated neuroendocrine control of vertebrate sexual behavior, hormone production, and coordinated gamete release optimizes fertility. A central principle in mammalian reproductive physiology is that hypothalamic gonadotropin-releasing hormone 1 (GNRH1) neurons perform critical roles. Nerve terminals projecting to the median eminence release GNRH1 into the portal blood system, activating GNRH receptors stimulating the synthesis and secretion of the pituitary gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary. These gonadotropins in turn bind to their receptors in the gonads, stimulating steroidogenesis and gametogenesis. Sex steroids produced by the gonads feed back both positively and negatively on the brain and pituitary to finely control gonadotropin secretion and sexual behaviors. Since the discovery of the link between mutations in the human *GPR54*/*KISS1R* genes and isolated hypogonadotropic hypogonadism (1, 2), the kisspeptins have also become key to the central dogma of neuroendocrine regulation of mammalian reproduction. Research on several mammalian species has now established that specific kisspeptin neuronal populations directly control GNRH1 neurons to regulate LH release (3, 4). In teleosts, there are also highly complex and organized interactions between a multitude of stimulatory and inhibitory factors in the brain that regulate gonadotropin release from the pituitary (5, 6). Notable neuropeptides and neurotransmitters include the multiple coexisting Gnrh forms,[†] dopamine, gamma-aminobutyric acid (GABA), neuropeptide-Y, and recent research has focused on gonadotropin-inhibitory hormone (7), kisspeptins (8), and tachykinins (9), as in mammalian model systems.

Fish, while sharing numerous similarities with mammals, offer unique features that can provide alternative strategies for the discovery of new regulatory systems critical for reproduction and innovative ways in which to enhance fertility. To varying degrees, teleost species have lost the hypothalamo-hypophysial portal system typical of earlier diverging actinopterygian groups and in mammals. In teleost fishes, the neurohypophysis has interdigitated with the adenohypophysis, and hypophysiotropic neurons such as those producing Gnrh3 terminate on or near the highly regionalized anterior pituitary cells they control. In some cases, there are synapse-like direct contacts of hypophysiotropic neurons with gonadotrophs in the anterior pituitary; this direct innervation offers the possibility of independent regulation of the gonadotrophs by >20 neuropeptides and neurotransmitters (6, 10–12). In teleosts, Lh and Fsh are produced in separate gonadotroph subtypes, contrasting the situation in the mammalian pituitary. Additionally, genetic mutations in zebrafish leading to the disruption of well-known reproductive regulators, such as the Gnrhs and kisspeptins, surprisingly have little to no impact (8, 13, 14). This does not imply they are nonessential, but has revealed an incredible ability of fish to physiologically compensate for genetic

Significance

The secretory granule protein secretogranin-2 is processed to the bioactive neuropeptide secretoneurin. While these are expressed in hypothalamic neurons and anterior pituitary cells, a role in hormonal control of reproduction is unknown. We provide in vivo evidence that secretogranin-2 plays critical roles in fertility regulation. Mutation of the secretogranin-2a and -2b genes in zebrafish leads to disrupted sexual behaviors, reduced ovulation and egg laying, and suboptimal fertility and embryo survival. Injection of a synthetic secretoneurin peptide enhanced reproductive outcomes in the mutant fish. Our secretogranin-2 mutants offer an amenable model system in which to screen potential injectable stimulators of reproduction, be they for spawning in cultured fish species, or to help with the search for new human infertility treatments.

Author contributions: K.M., B.T., L.C., W.H., and V.L.T. designed research; K.M., C.L., B.T., L.C., and V.L.T. performed research; C.L., W.H., and V.L.T. contributed new reagents/analytic tools; K.M., W.S.Z., B.T., L.C., W.H., and V.L.T. analyzed data; and K.M., W.H., and V.L.T. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2002004117/-/DCSupplemental.

First published May 28, 2020.

[†]When referring to fish, we have adopted the zebrafish gene/protein nomenclature conventions; otherwise, we use human nomenclature according to https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Conventions.

losses (15). Moreover, there are other lesser known but potentially important neurohormones that must be investigated.

One such candidate is secretogranin-2 (SCG2) and is our focus here. This precursor protein is mainly distributed in dense-core vesicles of many neurons and endocrine cells and is overexpressed in some human neuroendocrine tumors (16, 17). While numerous potential peptides may arise from specific SCG2 processing by prohormone convertases in secretory granules, secretoneurin (SN) is the only derived neuropeptide thus far with known biological activities (16). Mammalian SN has various proposed roles including angiogenesis, neuroinflammation, and neurotransmitter release (17). The human *SCG2* gene is located on chromosome 2. Similarly, tetrapods, such as frogs and mice, have only one *SCG2* gene, resulting in the production of one SN peptide 33 amino acids in length. Only a discrete domain in the middle of the SCG2 precursor protein, representing the SN peptide, is relatively well-conserved across all vertebrate taxa, sharing the central YTPQ-X-L-X8-EL core we identified previously (18). Teleosts have two *scg2* paralogs, *scg2a* and *scg2b*, likely a consequence of the early whole genome duplication event that occurred in fishes (19). In zebrafish, *scg2a* and *scg2b* are respectively located on chromosomes 15 and 2. The two zebrafish Scg2 subtype precursor proteins generate SNa and SNb peptides, respectively, 34 and 31 amino acids long. Of the overlapping 31 amino acids, only 14 (~45%) are identical between the two SNs (18, 20).

There are important data suggesting that SCG2 and/or SN are involved in vertebrate reproduction. We originally uncovered the teleost secretogranin-2 transcript, now referred to as *scg2a*, using a differential display screen of pituitaries of goldfish injected with the GABA degradation inhibitor gamma-vinyl-GABA to enhance Lh release (21). Subsequently, we found that synthetic SNa could stimulate Lh release both in vivo and in vitro in the goldfish model (22–25). Anatomical and functional evidence indicates neuroendocrine, endocrine, and paracrine sources of SN that all stimulate Lh release in fish (18). Moreover, microarray analysis of dispersed goldfish pituitary cells incubated with SNa suggests other roles in cellular metabolism and hormone synthesis (18). In rat pituitary and mouse LβT2 gonadotroph cells, GnRH1 stimulates the release of SN-immunoreactive proteins concomitantly with LH secretion (26–28). That mouse SN also stimulates LH release from LβT2 cells through a cAMP and ERK-dependent signaling pathway, suggests an involvement of SN in autocrine/paracrine regulation of mammalian gonadotrophs (26). This growing body of evidence supports a fundamental and evolutionarily conserved role for SCG2/SN in gonadotropin regulation in the pituitary.

Most previous studies related to SN used exogenously applied peptides, often with in vitro cell systems. Nothing has been reported on the role of the endogenous SCG2 precursor protein or any of the SCG2-derived neuropeptides on the ability of animals to reproduce. Until our report (29), there were no mutant models for any species, and deleterious human SCG2 mutations have yet to be identified. Employing the recently described zebrafish *scg2a* and *scg2b* single and double mutant lines (29), we specifically tested the hypothesis that the Scg2 genes play critical stimulatory roles in reproduction. To accomplish this, we undertook comprehensive analysis of sexual behavior, reproductive outcomes, and expression of key genes in the brain and pituitary of wild-type (WT) and *scg2a* and *scg2b* mutant female and male zebrafish. We present in vivo data that normal functioning of the secretograninergic system is required for optimal reproductive outcome and propose that SN is a reproductive hormone.

Results

Mutation of *scg2* Decreases Courtship Behaviors and Spawning Success. Large-scale pairwise mating and behavioral tests were performed, recorded (Movies S1–S4), and analyzed. Male zebrafish exhibit typical courtship behaviors toward females that include chase, tail-nose, encircle, zig-zag, and quiver (30). The five male courtship behaviors we observed followed a similar sequence in both WT and *scg2* mutant spawning trials. Significant reductions in the duration of chase [$H(9) = 44.650$, $P < 0.001$] (Fig. 1*A*), tail-nose [$H(9) = 36.694$, $P < 0.001$] (Fig. 1*B*), encircle [$H(9) = 23.851$, $P = 0.005$] (Fig. 1*C*), and quiver [$H(9) = 27.230$, P 0.001] (Fig. 1*D*) behaviors, as well as a significant reduction in the frequency of chase [$H(9) = 45.811$, $P < 0.001$] (Fig. 2*A*), tail-nose [$H(9) = 43.664$, $P < 0.001$] (Fig. 2*B*), encircle [$H(9) = 27.829$, $P = 0.001$] (Fig. 2*C*), and quiver [$H(9) = 30.437$, $P < 0.001$] (Fig. 2*D*) behaviors were observed in mutant lines as compared to WT within-line crosses. Dunn's post hoc test revealed that $scg2a^{-/-};scg2b^{-/-}$ within-line crosses had reduced durations and frequencies of chase, tail-nose, encircle, and quiver ($P < 0.05$) (Figs. 1 *A–D* and 2 *A–D*). The number and duration of encircles were also reduced when $scg2a^{-/-};scg2b^{-/-}$ females were paired with WT males ($P <$ 0.05) (Figs. 1*C* and 2*C*). The sum of the durations [$H(9) = 43.405$, $P < 0.001$] and frequencies [$H(9) = 44.478$, $P < 0.001$] of all male courtship behaviors was significantly different comparing WT within-line crosses to *scg2* mutants. The total courtship duration of $scg2a^{-/-};scg2b^{-/-}$ within-line crosses and $scg2a^{-/-};scg2b^{-/-}$ females paired with WT males was reduced ($P < 0.05$) (*SI Appendix*, Fig. S1*A*). The frequency of courtships of $scg2a^{-/-};scg2b^{-/-}$ within-line crosses was also reduced compared to WT within-line crosses ($P <$ 0.05) (*SI Appendix*, Fig. S1*B*).

Within-line crossing revealed that spawning success was significantly reduced to 37% ($P = 0.0006$), 44% ($P = 0.0169$), and 6% ($P < 0.0001$) in $scg2a^{-/-}$, $scg2b^{-/-}$, and $scg2a^{-/-};scg2b^{-/-}$ fish, respectively. Relative to the 62% success rate in WT fish, this corresponds to a 40%, 29%, and 90% decrease in the $scg2a^{-/-}$, $scg2b^{-/-}$, and $scg2a^{-/-};scg2b^{-/-}$ crosses, respectively (Fig. 3*A*). The spawning success of $scg2a^{-/-}$, $scg2b^{-/-}$, and $scg2a^{-/-};scg2b^{-/-}$ males paired with WT females increased to 52% ($P = 0.1872$), 59% ($P = 0.6579$), and 49% ($P = 0.0748$) (Fig. 3*A*). Similarly, spawning success in both $scg2a^{-/-}$ and $scg2b^{-/-}$ females paired with WT males was 50% ($P = 0.1077$ and $P = 0.0996$, respectively) (*SI Appendix*, Fig. S12*A*). In marked contrast, the $scg2a^{-/-};scg2b^{-/-}$ females paired with WT males spawned in only 11% ($P < 0.0001$) of all trials (Fig. 3*A*). Fecundity (number of embryos/clutch) was significantly affected by genotype [$H(9) = 53.727$, $P \leq 0.001$]. Significantly higher fecundities were observed in reciprocal crosses of $scg2a^{-/-};scg2b^{-/-}$ males paired with WT females ($P < 0.001$) compared to WT within-line crosses (Fig. 3*B*). The survival of the embryos was also significantly affected by genotype [$H(9) = 44.960$, $P \leq$ 0.001]. Increased mortality was observed in reciprocal crosses of $scg2a^{-/-};scg2b^{-/-}$ males paired with WT females ($P < 0.001$) (Fig. 3*D*). A small but statistically significant difference was observed between genotypes in the percent natural fertilization [$H(9) =$ 97.908, $P \leq 0.001$] (Fig. 3*C*), but all were within the 85 to 100% range. The percent fertilization was 7% and 6% higher in $scg2a^{-/-}$ males paired with WT females ($P < 0.002$) and $scg2b^{-/-}$ males paired with WT females ($P < 0.003$), respectively. In contrast, in vitro fertilization rates for $scg2a^{-/-}$, $scg2b^{-/-}$, and $scg2a^{-/-};scg2b^{-/-}$ fish were 90%, 89%, and 89% ($n = 6$), respectively, not significantly different from the 88% in WT, and well within normal levels (31).

Mutation of *scg2* Does Not Affect Gonadosomatic Index, Gonad Morphology, or Sex Steriods but Reduces Ovulation. The gonads of $scg2a^{-/-}$, $scg2b^{-/-}$, and $scg2a^{-/-};scg2b^{-/-}$ fish were examined to potentially delineate the cause of the observed fertility defects. Gross gonadal morphology of mutant fish is similar to WT animals. No significant main effects of genotype [$F(3) = 0.989$, P 0.398] were observed on the gonadosomatic index (GSI; % gonad/body weight) but there was the expected main effect of sex; females have proportionally larger gonads than males [$F(1) =$ 1001.425, $P < 0.001$] (*SI Appendix*, Fig. S2). No genotype × sex interactions were evident for GSI [$F(3) = 0.773$, $P = 0.510$]. The ovaries and testes of all $scg2^{-/-}$ fish appeared normal by histology

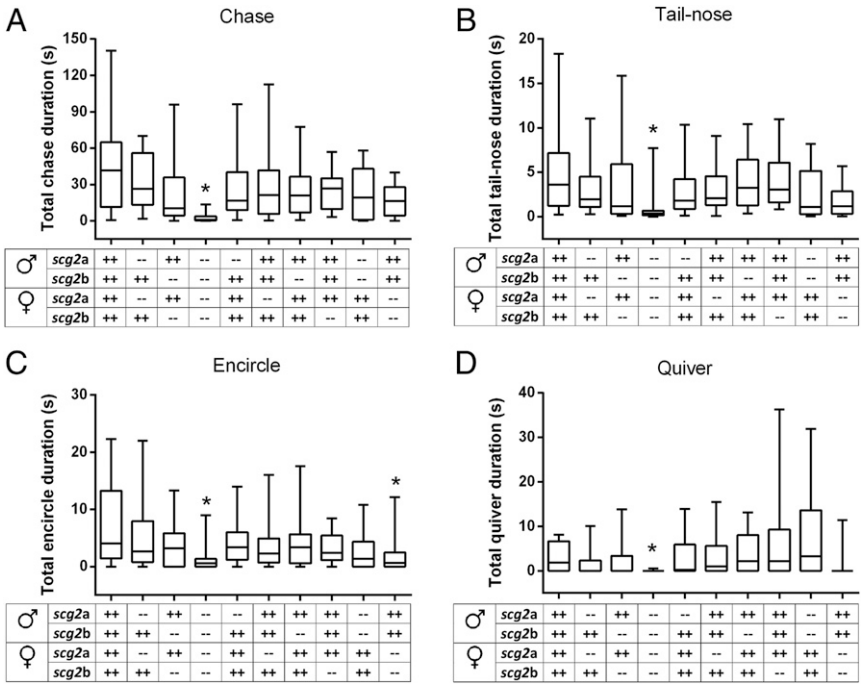

Fig. 1. Box plots of total chase (*A*), tail-nose (*B*), encircle (*C*), and quiver (*D*) behavior durations in seconds (s) assessed for 10 min in pairwise within-line and reciprocal mating crosses. Horizontal lines represent median values; boxes represent interquartile ranges and whiskers represent minimum–maximum values. Since the data were not normally distributed, significant differences were determined using a Kruskal–Wallis test on ranks followed by a Dunn's multiple comparison test (n = 20 to 22). Asterisks denote a significant difference from the wild-type pair; *$P \leq 0.05$.

(Fig. 4). In the ovaries, follicles of various stages including primary growth, previtellogenic, early-vitellogenic, midvitellogenic, and full-grown oocytes could be observed in all of the genotypes (Fig. 4 *A–D*), indicating that folliculogenesis was not affected (Fig. 4*I*). In the testes, all stages of sperm cells, including spermatogonia, spermatocytes, and spermatids, were observed in all genotypes (Fig. 4 *E–H*); however, *scg2a*$^{-/-}$ and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish had fewer spermatozoa compared to WT, suggesting that *scg2a* mutation had

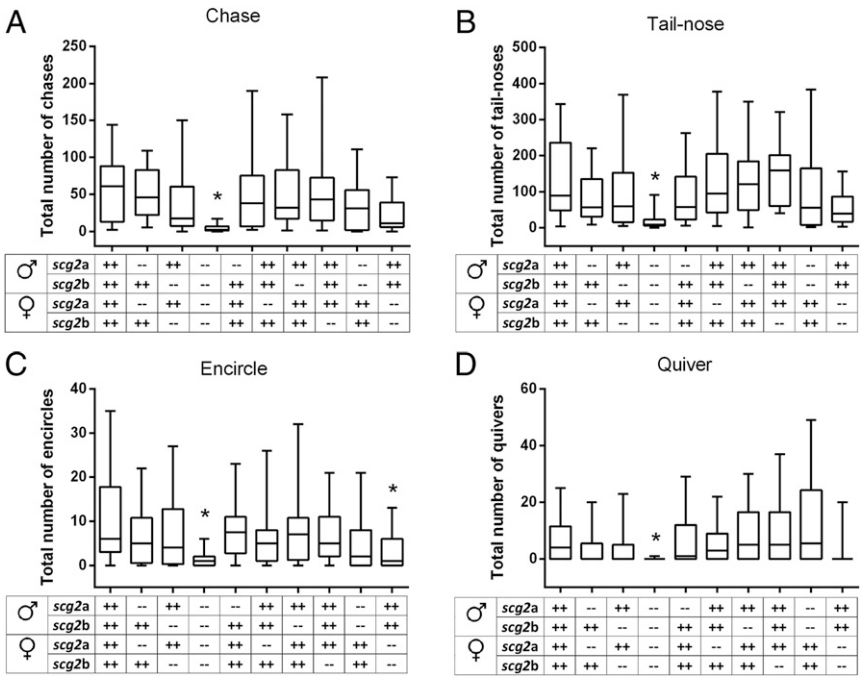

Fig. 2. Box plots of number of chase (*A*), tail-nose (*B*), encircle (*C*), and quiver (*D*) behaviors assessed for 10 min in pairwise within-line and reciprocal mating crosses. Horizontal lines represent median values; boxes represent interquartile ranges and whiskers represent minimum–maximum values. Since the data were not normally distributed, significant differences were determined using a Kruskal–Wallis test on ranks followed by a Dunn's multiple comparison test (n = 20 to 22). Asterisks denote a significant difference from the wild-type pair; *$P \leq 0.05$.

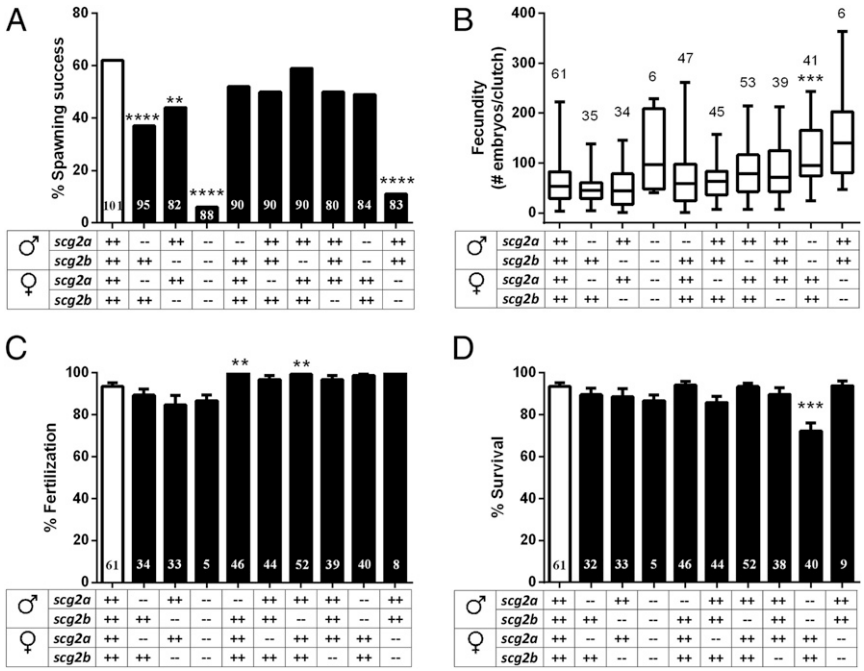

Fig. 3. Spawning success, fecundity, fertilization, and survival of embryos produced during zebrafish pairwise matings. (*A*) Percent of spawning success of within-line and reciprocal crosses. Significant differences were determined using Fisher's exact test. (*B*) Box plots of the number of embryos produced per couple. Horizontal lines represent median values; boxes represent interquartile ranges and whiskers represent minimum–maximum values. *P* values present the comparison of that group against the wild-type pair using Kruskal–Wallis and Dunn's test for post hoc comparisons. (*C*) Mean (+SEM) % fertilization and (*D*) % embryo survival of mating crosses. Since the data were not normally distributed, significant differences were determined using a Kruskal–Wallis test on ranks followed by a Dunn's multiple comparison test. Sample sizes (*n*) are indicated in or above each bar for that group. Asterisks denote a significant difference from the wild-type within line cross (white bar); ****$P \leq 0.0001$; ***$P \leq 0.001$; **$P \leq 0.01$.

a minor but significant ($P < 0.05$) effect on spermatid development (Fig. 4*J*). Total body contents of testosterone, estradiol, and 11-keto-testosterone were expectedly sexually dimorphic but were not impacted by genotype (*SI Appendix*, Fig. S4).

Considering the normal ovarian appearance in mutant females, we hypothesized that failed ovulation is a likely mechanism underlying reduced oviposition. Indeed, for WT, 11/15 (73%) females ovulated, while for *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish, only 1/10 (10%) females ovulated ($P = 0.0036$; Fisher's two-tailed exact test). These data on ovulation rates compare favorably with the respective oviposition/spawning rates in WT (62%) and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ (6%) pairs in the large-scale within-line crosses (Fig. 3*A*). Oocytes were then tested for their capacity to undergo in vitro maturation. They had a normal appearance (*SI Appendix*, Fig. S4*A*) and both WT and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ females exhibit similar rates of germinal vesicle breakdown in response to hCG (*SI Appendix*, Fig. S4*B*), additionally supporting that ovarian function in *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ females is relatively normal.

Single Injection of hCG or SN Improves Reproductive Outcomes. It was next important to determine if poor reproductive outcomes in the *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ mutants could be improved by hormonal treatments. Under the conditions of anesthesia, handling and intraperitoneal (i.p.) injection of Ringer's solution (control) immediately prior to the breeding trial, within 20 min of first light, WT pairs successfully spawned in 47% of trials (Fig. 5*A*). Expectedly, this acute stress somewhat reduced spawning success compared to unhandled, unanesthetized, and uninjected WT controls in optimal conditions. Spawning success was significantly increased to 83% in WT ($P = 0.0013$) injected with the LH agonist hCG (Fig. 5*A*). Zebrafish SNa and SNb (see synthesis and purification protocol in *SI Appendix*, *SI Materials and Methods*) increased spawning success equally in WT, 1.3-fold from 47 to 61%, an intermediate level of activity that was not statistically different from controls ($P = 0.2062$) (Fig. 5*A*). Spawning success in *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ pairs injected with Ringer's was 11%, which is not different from noninjected *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ at 6%, but significantly lower than WT controls ($P = 0.0006$) (Fig. 5*A*). Injection of hCG in *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish significantly increased spawning success to 38% compared to Ringer's-injected *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish ($P = 0.0063$) and was therefore not significantly different from WT controls ($P = 0.2662$) (Fig. 5*A*). Injection of SNa increased spawning success in *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ mutants almost 3-fold to 30% compared to *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish injected with Ringer's only ($P = 0.0403$). Spawning success in SNa-injected *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish were not different compared to WT fish ($P = 0.0973$) (Fig. 5*A*). The spawning success of SNb-injected *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish was only 8%, which was not different from Ringer's-injected *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish ($P = 0.5$), and this was significantly lower compared to WT controls ($P = 0.0002$) (Fig. 5*A*).

There were no significant main effects of genotype [$F(1) = 0.275$, $P = 0.073$] or hormone injection [$F(3) = 1.338$, $P = 0.266$] on fecundity. Furthermore, no genotype × injection interaction was evident [$F(3) = 0.964$, $P = 0.413$] (Fig. 5*B*). For fertilization rate, there were no significant main effects of genotype [$F(1) = 1.162$, $P = 0.284$], injection [$F(3) = 0.646$, $P = 0.587$], or genotype × injection interaction [$F(3) = 0.201$, $P = 0.896$] (Fig. 5*C*). There were significant effects of genotype [$F(1) = 6.244$, $P = 0.014$] on embryo survival. Generally, *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish had a lower survival than WT. No significant effects of hormone injection [$F(3) = 2.650$, $P = 0.053$] or genotype × injection interactions [$F(3) = 2.656$, $P = 0.52$] were evident for survival (Fig. 5*D*).

SN Injection Up-Regulates Brain and Pituitary Gene Expression in Adult Female WT Zebrafish. There was a variable increase in the relative mRNA levels of *gnrh3* in the telencephalon at 3-h post

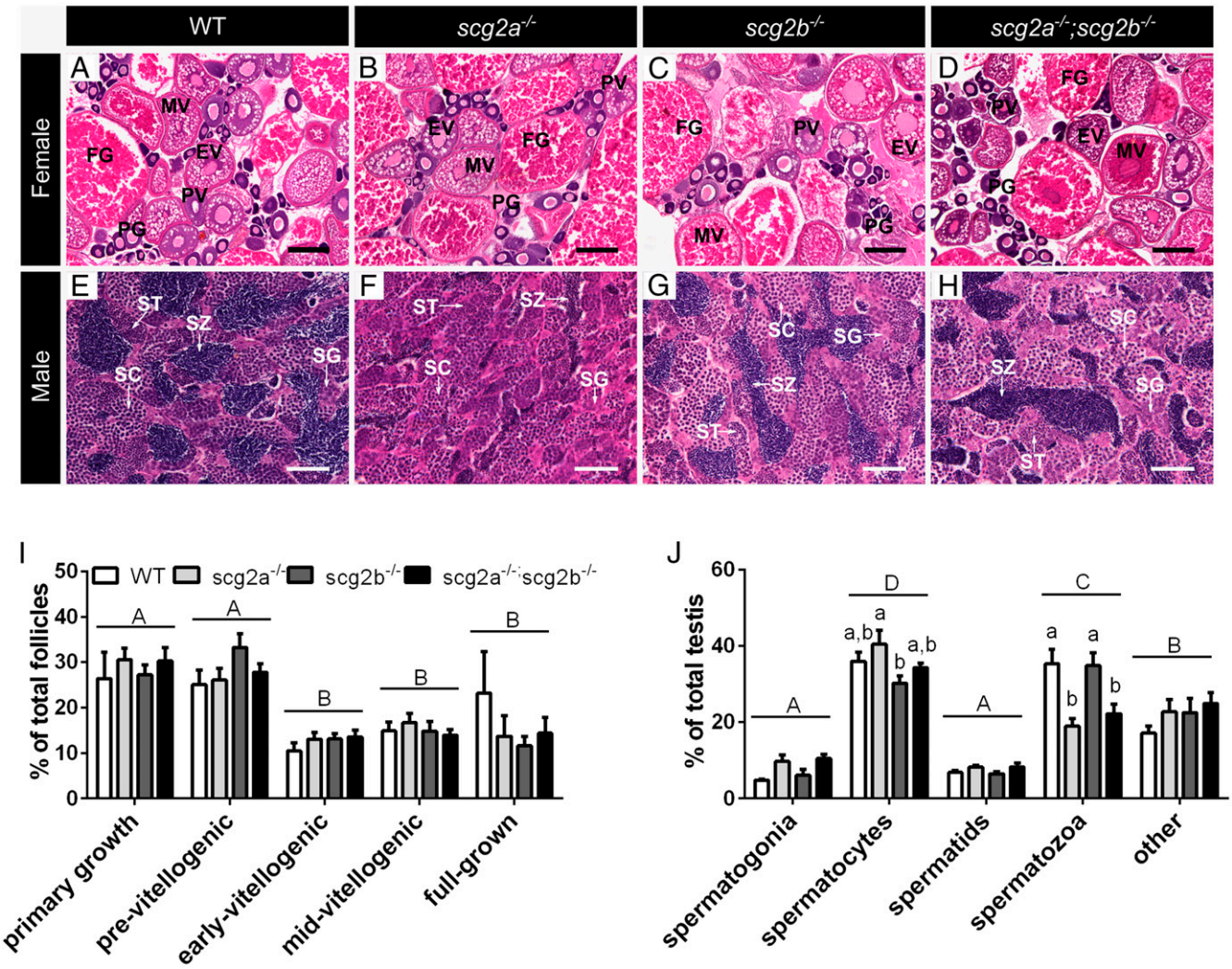

Fig. 4. Histological assessments of ovaries (*A–D* and *I*) and testes (*E–H* and *J*) of WT, scg2a$^{-/-}$, scg2b$^{-/-}$, and scg2a$^{-/-}$;scg2b$^{-/-}$ zebrafish at 4 mo of age. Ovarian follicular stages are labeled on top of their respective structure and abbreviated as follows: PG, primary growth follicle; PV, previtellogenic follicle; EV, early-vitellogenic follicle; MV, midvitellogenic follicle; FG, full-grown follicle. Spermatogonial cells in testes are indicated by arrows; SG, spermatogonia; SC, spermatocytes; ST, spermatids; SZ, spermatozoa. (Scale bars, 200 μm for females and 50 μm for males.) Gonads fixed in Bouin's solution underwent standard histological procedures and 5- to 8-μm-thick sections were stained with hematoxylin-eosin stain. Sections shown were selected to be representative of mean values shown in *I* (ovaries) and *J* (testes). (*I*) Percentages of primary growth, previtellogenic, early-vitellogenic, midvitellogenic, and full-grown oocytes in ovaries from females (mean + SEM, $n = 5$). (*J*) Percentage of area covered by spermatogonia, spermatocytes, spermatids, spermatozoa, and other tissues in testes from males (mean + SEM, $n = 5$). Uppercase letters (A, B, C, D) denote differences between gonad stages ($P < 0.05$), whereas lowercase letters (a, b, c, d) signify differences between genotypes within a stage ($P < 0.05$). Other details for histology are presented in *SI Appendix, SI Materials and Methods*.

SN peptide injection. For SNa, *gnrh3* increased 10.1-fold, and for SNb, the response was more variable but still represented on average a 10.7-fold increase. The overall response in the telencephalon at 3 h, however, did not reach statistical significance ($P = 0.076$) (*SI Appendix*, Fig. S5). At 6 h, SNa and SNb, respectively, increased *gnrh3* 2.6-fold and 1.5-fold ($P < 0.001$ for both) in the telencephalon (*SI Appendix*, Fig. S5). In the hypothalamus, SNa rapidly increased *gnrh3* 1.5-fold at 3 h ($P < 0.001$), whereas SNb increased *gnrh3* 5.1-fold at 6 h ($P < 0.001$) (*SI Appendix*, Fig. S5).

Injection of SNa increased relative mRNA levels of glycoprotein alpha (*cga*) ($P = 0.003$) and gonadotropin-releasing hormone receptor 2 (*gnrhr2*) ($P < 0.001$), by ∼2.2- and ∼3.2-fold, respectively, at 3 h. Additionally, SNa increased *cga* ($P = 0.003$) and follicle stimulating hormone beta (*fshb*) ($P = 0.005$) by ∼1.9- and ∼2-fold, respectively, at 6 h (*SI Appendix*, Fig. S6). Following SNb injection, no changes were noted at 3 h. In contrast, relative mRNA levels of *lhb* ($P < 0.001$), *fshb* ($P < 0.001$), and *gnrhr2* ($P = 0.004$) were respectively elevated following SNb injection by ∼2.7-, ∼3.2, and 2-fold at 6 h ($P \leq 0.05$) (*SI Appendix*, Fig. S6).

Mutation of *scg2* Modulates Expression of Key Neuropeptigergic Genes Regulating Reproduction. We used droplet digital PCR (ddPCR) to quantify mRNA levels for gonadotropin-releasing hormone (*gnrh3*), isotocin (*oxt*), and vasotocin (*avp*) in the telencephalon and hypothalamus. We first focused on *gnrh3* because it codes for the hypophysiotropic Gnrh peptide in zebrafish and many other cyprinid species. There were clear and significant decreases in the levels of *gnrh3* in both the telencephalon (Fig. 6*A*) and hypothalamus (Fig. 6*B*) of mutant fish. We found a significant main effect of genotype [$F(3) = 4.638$, $P = 0.007$], no effect of sex [$F(1) = 2.663$, $P < 0.110$], but there was significant genotype × sex interaction [$F(3) = 3.318$, $P = 0.028$] on the levels of *gnrh3* in the telencephalon. The *scg2b*$^{-/-}$ and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ double mutant females had significantly lower transcript levels of

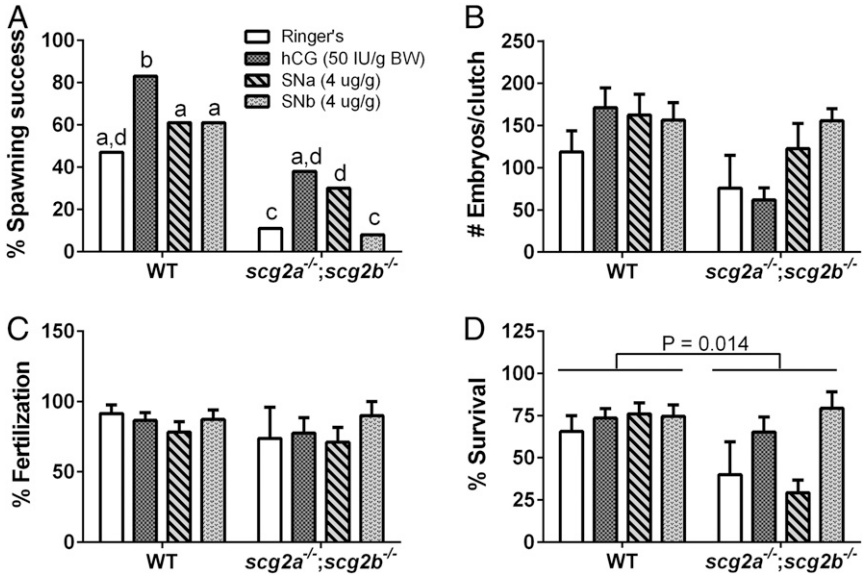

Fig. 5. Spawning success, fecundity (no. of embryos/clutch), fertilization, and survival of embryos produced during zebrafish pairwise spawning following i.p. injections. (*A*) Percent of spawning success of WT and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ matings. Means of 28 to 40 spawning trials are presented. Significant differences were determined using a one-tailed Fisher's exact test. Letters (a, b, c, d) above bars signify differences between groups and treatments ($P < 0.05$). (*B*) Fecundity or the number of embryos produced per spawning couple. Mean + SEM of 3 to 30 successful spawning events are presented. (*C*) Mean + SEM % fertilization and (*D*) % embryo survival of mating crosses ($n = 3$ to 30). Significant differences were determined using a two-way ANOVA followed by a Holm–Sidak post hoc test. Lines connecting different bars represent the comparison between WT and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish.

gnrh3 compared to WT females ($P < 0.05$), whereas *scg2a*$^{-/-}$ females were not statistically different from WT females. The levels of *gnrh3* were reduced by 61% and 63% in the telencephalon of *scg2b*$^{-/-}$ and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ females compared to WT females. The levels of *gnrh3* in the telencephalon were also reduced in all scg2 mutant males; however, none were statistically significant compared to the WT male controls ($P > 0.05$). The WT females had significantly higher telencephalic *gnrh3* levels compared to males ($P < 0.05$), while this sex difference was not observed in the mutants (Fig. 6*A*). A significant main effect of genotype [$F(3) = 36.421$, $P < 0.001$] on the levels of *gnrh3* was also observed in the hypothalamus; however, there was no significant effect of sex [$F(1) = 0.096$, $P = 0.758$] and there was no genotype × sex interaction evident [$F(3) = 2.273$, $P = 0.093$]. The hypothalamic levels of *gnrh3* were significantly decreased in all scg2 mutant fish ($P < 0.05$). In *scg2a*$^{-/-}$, *scg2b*$^{-/-}$, and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ lines, *gnrh3* was reduced by 71%, 62%, and 73% compared to WT, respectively. The *scg2a*$^{-/-}$ and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ mutants also had lower levels of *gnrh3* compared to the *scg2b*$^{-/-}$ line ($P < 0.05$) (Fig. 6*B*).

The levels of *oxt* in the telencephalon (Fig. 6*C*) were significantly affected by genotype [$F(3) = 4.241$, $P = 0.010$]. However, there were no significant effects of sex [$F(1) = 0.0185$, $P = 0.892$] or genotype × sex interaction [$F(3) = 0.837$, $P = 0.481$]. Levels of *oxt* were decreased similarly in *scg2a*$^{-/-}$ and *scg2b*$^{-/-}$ fish compared to WT ($P < 0.05$), while this difference was not observed in the double *scg2* mutants. Specifically, there was a 59% and 50% decrease in *oxt* in the telencephalon of *scg2a*$^{-/-}$ and *scg2b*$^{-/-}$ fish, respectively, compared to WT (Fig. 6*C*). In contrast, no main effect of genotype [$F(3) = 1.318$, $P = 0.281$] was observed on the levels of *oxt* in the hypothalamus. There was a main effect of sex [$F(1) = 15.669$, $P < 0.001$] but also no genotype × sex interaction [$F(3) = 1.087$, $P = 0.364$]. The hypothalamic levels of *oxt* were highly variable but *scg2a*$^{-/-}$ and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ females had lower levels of *oxt* compared to males ($P = 0.003$ and $P = 0.021$, respectively) (Fig. 6*D*).

The levels of *avp* in the telencephalon (Fig. 6*E*) were significantly affected by genotype [$F(3) = 7.306$, $P < 0.001$]. However, there were no significant effects of sex [$F(1) = 2.353$, $P = 0.132$] or genotype × sex interaction [$F(3) = 1.980$, $P = 0.130$]. Levels of *avp* were decreased by 66% in *scg2b*$^{-/-}$ fish compared to WT ($P < 0.05$). Levels of *avp* were also significantly higher in the telencephalon of *scg2a*$^{-/-}$ and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish than those in *scg2b*$^{-/-}$ fish (Fig. 6*E*). Similarly, in the hypothalamus (Fig. 6*F*), the levels of *avp* were significantly affected by genotype [$F(3) = 6.079$, $P < 0.001$] but no main effect from sex [$F(1) = 3.331$, $P = 0.074$]. There was however an interaction between genotype and sex [$F(3) = 6.693$, $P < 0.001$]. The hypothalamic levels of *avp* in *scg2a*$^{-/-}$ and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ males were higher than those of WT and *scg2b*$^{-/-}$ males ($P < 0.05$). Specifically, the levels of *avp* were increased by 57% and 50% in *scg2a*$^{-/-}$ and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ males, respectively, compared to WT males. The levels of *avp* in *scg2a*$^{-/-}$ females were lower than in *scg2a*$^{-/-}$ males ($P < 0.001$) (Fig. 6*F*).

Mutation of scg2 Modulates the Expression of Pituitary Genes That Positively Regulate Reproduction in Adult Males and Females. The levels of the *lhb* mRNA in the pituitary in response to *scg2* mutation in both male and female adult zebrafish followed a pattern resembling *gnrhr2* in the four different genotypes. There were main effects of genotype [$F(3) = 0.462$, $P < 0.001$] and sex [$F(1) = 34.312$, $P < 0.001$] on the levels of *lhb*. A significant genotype × sex interaction [$F(3) = 3.341$, $P = 0.028$] was also evident for *lhb*. Post hoc analyses revealed that the levels of *lhb* were significantly lower in both male and female mutant fish relative to WT controls ($P < 0.05$). In males, there was a 93%, 97%, and 66% decrease in *lhb* in *scg2a*$^{-/-}$, *scg2b*$^{-/-}$, and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish, respectively, compared to WT males. Similarly, *scg2a*$^{-/-}$, *scg2b*$^{-/-}$, and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ female levels of *lhb* were reduced by 91%, 92%, and 60%, respectively, compared to WT females. The levels of *lhb* were also significantly higher in *scg2a*$^{-/-}$ and *scg2b*$^{-/-}$ females compared to males ($P < 0.05$) but no clear sex differences were observed in WT or *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish (Fig. 7*A*).

The expression pattern observed for *fshb* in the pituitary was somewhat different from that of *lhb*. Main effects of both genotype [$F(3) = 123.053$, $P < 0.001$] and sex [$F(1) = 85.607$, $P < 0.001$] were evident and there was a significant genotype × sex

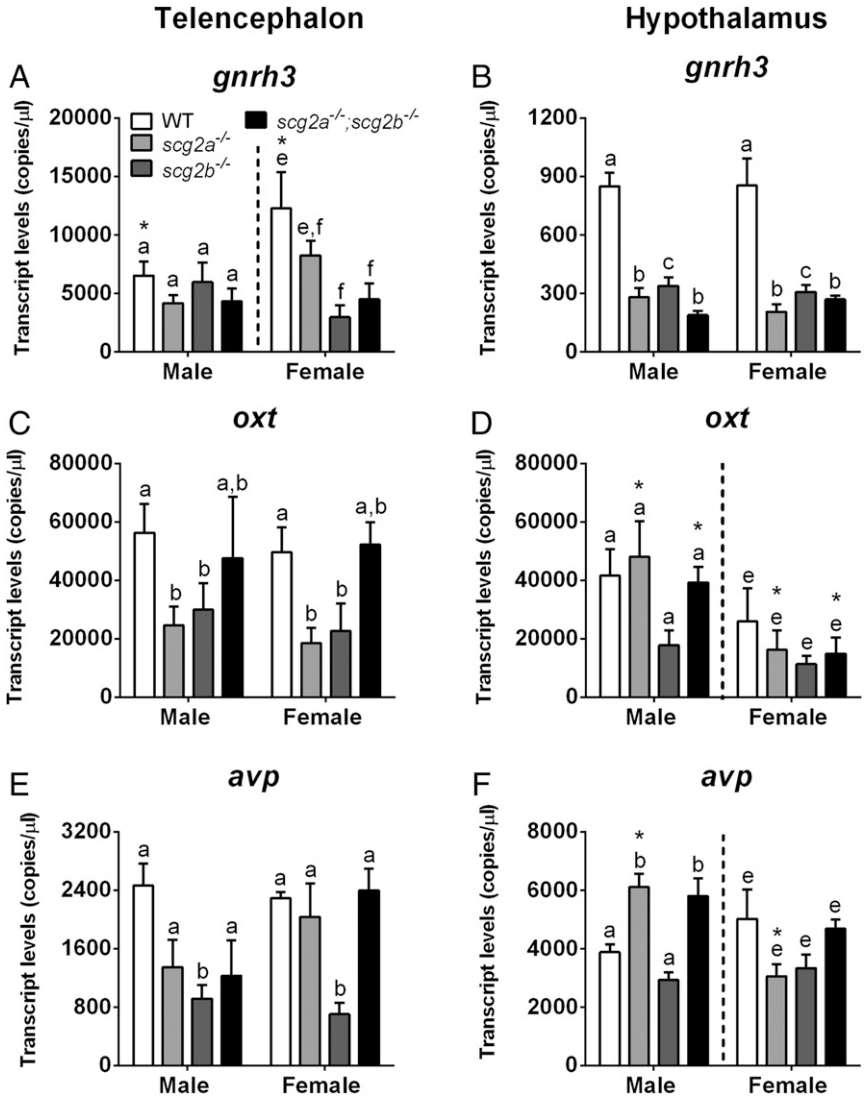

Fig. 6. The effect of scg2 mutation on the expression of neuropeptidergic genes that positively regulate reproduction in adult males and females. Gene expression of *gnrh3* (*A* and *B*), *oxt* (*C* and *D*), and *avp* (*E* and *F*) in the telencephalon and hypothalamus, respectively, of adult males and females. Results are presented as means + SEM (n = 5 to 7). Vertical lines between males and females indicate a main statistical effect of sex or interaction between sex and genotype. In these cases (*A*, *D*, and *F*), means with different letters denote significant differences between genotypes within a sex (a–d for males; e–h 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 (*B*, *C*, and *E*) indicate no statistical effect of sex; therefore, means with different letters (a–d) denote significant differences between genotypes, regardless of sex ($P < 0.05$).

interaction [$F(3) = 5.756$, $P = 0.002$]. Both male and female levels of *fshb* were lower in single *scg2* mutant lines compared to WT ($P < 0.05$). Specifically, there was a 76% and 87% decrease in *fshb* in *scg2a*$^{-/-}$ and *scg2b*$^{-/-}$ males, respectively, compared to WT males. The *scg2a*$^{-/-}$ and *scg2b*$^{-/-}$ females also had a reduction of 80% and 73% in *fshb* levels, respectively, compared to WT females. Interestingly, no difference was observed in *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ males or females when compared to their same sex WT controls. Females had significantly higher transcript levels of *fshb* compared to males ($P < 0.05$) (Fig. 7*B*).

The levels of *cga* exhibited the same general pattern as that of *lhb* in the pituitary. There were main effects of genotype [$F(3) = 26.472$, $P < 0.001$] and sex [$F(1) = 9.554$, $P = 0.004$], and there was a significant genotype × sex interaction [$F(3) = 3.181$, $P = 0.034$]. Levels of *cga* were significantly lower in all *scg2* mutant males and females compared to WT ($P < 0.05$). Male levels of *cga* were decreased by 90%, 96%, and 78% in *scg2a*$^{-/-}$, *scg2b*$^{-/-}$, and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish, respectively, compared to WT males. Female pituitary levels of *cga* were decreased by 94%, 95%, and 61% in *scg2a*$^{-/-}$, *scg2b*$^{-/-}$, and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$, respectively, compared to WT females. The WT females also had significantly higher levels of *cga* compared to WT males, whereas no sex differences were observed in any *scg2* mutants (Fig. 7*C*).

The levels of *gnrhr2* in the pituitary were significantly affected by genotype [$F(3) = 36.466$, $P < 0.001$] and sex [$F(1) = 77.391$, $P < 0.001$]. There was no significant genotype × sex interaction [$F(3) = 0.758$, $P = 0.524$], indicating that the overall pattern of expression of *gnrhr2* was similar between the sexes. The levels of *gnrhr2* were lower in *scg2a*$^{-/-}$ or *scg2b*$^{-/-}$ males and females compared to their same sex WT controls ($P < 0.05$). Male pituitary *scg2a*$^{-/-}$ and *scg2b*$^{-/-}$-*gnrhr2* levels were reduced by 86% and 82%. Female pituitary *scg2a*$^{-/-}$ and *scg2b*$^{-/-}$*gnrhr2* levels were reduced by 85% and 78%. In the pituitaries of *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish of both sexes, *gnrhr2* returned to levels similar to WT animals. Levels of *gnrhr2* were overall higher

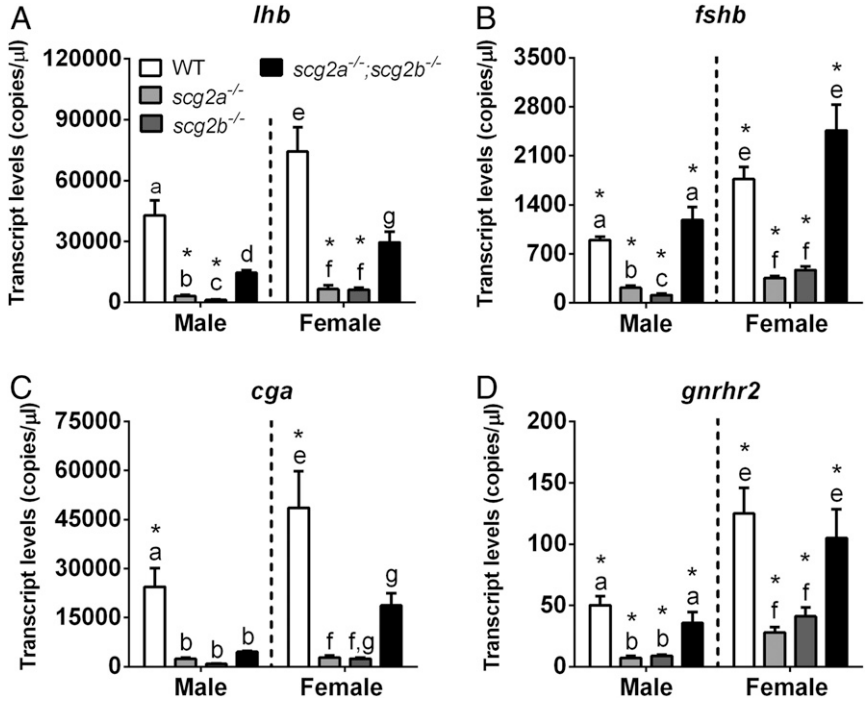

Fig. 7. The effect of scg2 mutation on the expression of pituitary genes that positively regulate reproduction in adult males and females. Gene expression of *lhb* (*A*), *fshb* (*B*), *cga* (*C*), and *gnrhr2* (*D*) in the pituitary of adult males and females. Results are presented as means + SEM ($n = 5$ to 7). Vertical lines between males and females indicate a main statistical effect of sex or interaction between sex and genotype. Means with different letters denote significant differences between genotypes within a sex (a–d for males; e–h for females) ($P \leq 0.05$), and an asterisk (*) above a group indicates statistical differences between males and females for that genotype ($P \leq 0.05$).

in females ($P < 0.01$) and, *scg2a*$^{-/-}$ and *scg2b*$^{-/-}$ females had higher transcript levels than their male counterparts ($P < 0.05$) (Fig. 7*D*).

We found little evidence for genetic compensation in response to mutations of the *scg2* paralogs. In the telencephalon, hypothalamus, and pituitary, we found that *scg2a* and *scg2b* were significantly decreased in *scg2a*$^{-/-}$, *scg2b*$^{-/-}$, and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ correspondingly (*SI Appendix*, Fig. S7). This supports our observations on decreased *scg2a* and *scg2b* in 2-d-old whole mutant embryos (29).

Discussion

We report that mutation of *scg2* leads to disruption of sexual behavior and spawning success in zebrafish. These data are significant for three main reasons: 1) we provide in vivo evidence for a role of *scg2* in reproduction; 2) we demonstrate that mutation of the *scg2* paralogs leads to changes in the expression of key neuroendocrine and pituitary genes well known for their importance in the hormonal control of reproduction (18, 32); and 3) our data on *scg2a* and *scg2b* contrast earlier work showing that mutant zebrafish lines lacking the two Gnrhs (14), or the two kisspeptins and their receptors (8), exhibit only minor defects and still reproduce reasonably well.

Spawning is the coordinated release of sperm from males and eggs from females for optimum fertilization. Spawning success is a composite measure of reproduction but is especially indicative of successful ovulation and subsequent egg laying by the female interacting with a male. Therefore, we performed large-scale analysis of reproductive output. Mutation of either *scg2a* or *scg2b* caused a significant reduction in the spawning success of within-line breeding pairs. This negative effect was further amplified in double mutant animals. In contrast, spawning success was similar to WT fish when the majority of the mutant lines were paired with WT. The clear exception was when *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ females were paired with WT males; spawning success was only 11% and similar to the very low levels seen with the double mutant within-lines crosses. This indicates the fundamental importance of *scg2* in zebrafish reproduction. Our results on spawning success somewhat resemble those observed in mutant zebrafish and medaka lacking *lhb*, the critical gonadotropin subunit for Lh synthesis. For example, *lhb*$^{-/-}$ females paired with WT males are unable to spawn (33–35), although, homozygous or heterozygous *lhb* receptor (*lhr*) knockout zebrafish can spawn with WT males but the number of embryos produced was decreased slightly (33). On the other hand, male *lhb*$^{-/-}$ or *lhr*$^{-/-}$ zebrafish and *lhb*$^{-/-}$ medaka are fertile (33–36). Similarly, *fshb*$^{-/-}$ zebrafish of both sexes are fertile (35), whereas only *fshb*$^{-/-}$ male medaka are fertile (34).

Morphological and histological analysis showed no noticeable impact of *scg2* mutation on ovarian development; all lines contained a full range of developing follicles, and whole-body testosterone, estradiol, and 11-keto-testosterone levels in females were similar across all genotypes. Yet, *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ females are very poor ovulators when paired with active, courting WT males. Our results are similar to those reported for *lhb*$^{-/-}$ zebrafish lines in which the mutation did not noticeably affect ovarian development, but it did cause infertility due to failed ovulation (34, 35). Similarly, *gnrh1*$^{-/-}$ and *lhb*$^{-/-}$ knockout medaka females had fully developed ovaries but none of the 10 tested pairs ovulated (34). Histological analysis of *scg2*$^{-/-}$ testes showed that all spermatogonial cell types were present but *scg2a*$^{-/-}$ and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish had a somewhat lower percentage of total testes area occupied by spermatozoa compared to WT zebrafish. This decrease appears to have little impact on male reproductive capacity, as shown by the reciprocal crosses of double mutant males. All stages of spermatogenesis were also present in *lhb*$^{-/-}$, *lhr*$^{-/-}$, and *fshb*$^{-/-}$ zebrafish testes, indicating that normal testicular development is possible even in the absence of Lh and Fsh (33, 35).

The hypothesis that disruption of sexual behavior is correlated to the suppressed reproductive success in *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ double

mutants was supported by our comprehensive quantitative analysis for all within-line and reciprocal crosses. Our study revealed important decreases in duration and frequency of chase, tail-nose, encircle, and quiver behaviors in *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ within-line crosses, as well as the encircle behavior in *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ females paired with WT males. In contrast, the *scg2a*$^{-/-}$ and *scg2b*$^{-/-}$ mutant lines exhibited a milder behavioral phenotype, and we did not detect major shifts in number and duration of the various spawning behaviors. To our knowledge, only two other gene mutation studies in zebrafish have examined courtship behavior similarly to our report. The first study examined androgen receptors (*ar*) which are responsible for mating behaviors (37). The *ar*$^{-/-}$ males ($n = 14$) were slower to initiate courtship and exhibited fewer chases, tail-nose interactions, zig-zags, and quivers; however, encircling was not significantly different between genotypes (38). The second study assessed the importance of female reproductive hormone prostaglandin F2α ($PGF_{2\alpha}$) which facilitates ovulation and spawning (39, 40) and is a sex pheromone that induces male reproductive behaviors in some zebrafish (41). Mutation of the olfactory $PGF_{2\alpha}$ receptor (*or114-1*$^{-/-}$) in zebrafish (41) resulted in males encircling and touching females less frequently and chasing less than WT males ($n = 4$ per group). These smaller scale studies and ours are consistent in showing that all mutant individuals exhibit typical courtship behaviors, but at a lower frequency and duration than WT counterparts. Double mutation of *scg2a* and *scg2b* therefore disrupts the ability of females to lay eggs regardless of the genotype of the courting male.

We next tested whether the severe phenotype in the double mutant line could be improved by hormone treatments. While our data indicate that hCG and SNa improved spawning success in the mutants, embryonic survival at 24 h remained on average lower in *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish compared to WT fish. Injection of hCG significantly elevated spawning success from 11 to 38% in *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ zebrafish, confirming that the gonads of *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ mutants are at least partially sensitive to Lh. Since the Lh agonist hCG induces oocyte maturation, ovulation, and spawning in several fish species (42, 43), our results indicate that hormonal induction of spawning is possible in some pairs of *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ mutant fish, but that many of these fish are unresponsive to hCG in vivo. Similarly, in lh$^{-/-}$ mutant zebrafish, hCG can only partially rescue ovulation (39). Some individuals cannot be fully rescued with hCG, so it will be important to determine other possible pathways that may be disrupted by *scg2* mutations.

Importantly, a single i.p. injection of SNa increased spawning and the release of fertilizable eggs in *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ mutants by about threefold compared to their saline-injected controls. As such we provide direct evidence that SNa alone stimulates successful reproduction. Previous reports were suggestive of a role for SN in reproduction but lacked direct evidence for impacts on reproductive outcomes. We previously demonstrated that goldfish SNa stimulated Lh release in vivo, when dopaminergic inhibition was blocked by the D2 receptor antagonist domperidone or when applied directly to dispersed goldfish pituitary cells in vitro (21, 23–25). Similarly, we demonstrated that mouse SN stimulated LH release from the LβT2 tumor-derived gonadotroph cell line (26). The only other animal where SN action on Lh has been studied is the orange-spotted grouper, a commercially important marine fish. It was shown that both SNa and SNb in vivo and in vitro increased hypothalamic *gnrh3* and pituitary *lhb* and *fshb* mRNA levels in grouper (32). Following SNa injection in female zebrafish, we found a rapid increase in *gnrh3* in the hypothalamus and *cga* and *gnrhr2* in the pituitary at 3 h that is on a similar time scale (e.g., ~3 h) as the actions of SNa in increasing spawning success in *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ mutant zebrafish. In contrast, SNb injection in WT female zebrafish did not affect hypothalamic *gnrh3* or *cga* at 3 h. There were several brain and pituitary transcripts that were elevated at 6 h postinjection, but such a time course of action appears not to be associated with increased spawning. We found that injection of SNb into *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ mutants was unable to rescue impaired reproductive function in our zebrafish. The Scg2b precursor protein and SNb peptide are not well studied. In our recent report, we demonstrated that *scg2b* but not *scg2a* mutation transiently disrupted formation of central arteries in 2-d-old zebrafish larvae, an effect rescued by injection of a synthetic SNb mRNA (29), but much remains unknown. The zebrafish SNa peptide is 34 amino acids long and represents the evolutionarily conserved central core of the larger 539 amino acid Scg2a precursor protein. Similarly, the SNb peptide is a 31 amino acid fragment from the 583 amino acid Scg2b precursor. Furthermore, SN may not be the only bioactive Scg2-derived peptide. A preliminary peptidomic analysis of whole male zebrafish brain revealed 11 and 22 peptides derived from the Scg2a and Scg2b precursors, respectively (44). A multitude of other peptides is theoretically possible, given the number of potential dibasic and monobasic cleavage sites in both the Scg2a and Scg2b precursor proteins. It will be important in the future to test the bioactivities of these other Scg2-derived peptides.

The clear reductions in sexual behavior, ovulation, and spawning in *scg2* mutant lines are indicative of disrupted hypothalamopituitary function, so to assess the underlying neuroendocrine mechanisms, we quantified expression of key genes known to positively regulate reproduction. Gonadotropin-releasing hormone is considered the main stimulator of LH and FSH (6, 45); it also has neuromodulatory and behavioral roles (46) in reproduction. In zebrafish, laser ablation of the hypophysiotropic Gnrh3 neurons early in development negatively impacts reproduction in adults (47). In medaka, mutation of the hypophysiotropic *gnrh1* gene revealed an important role in ovulation (34): female medaka had well-developed ovaries but failed to ovulate. In our study, we found that hypophysiotropic *gnrh3* mRNA was significantly decreased in *scg2* mutant fish in both the telencephalon and hypothalamus. This effect was most evident in the hypothalamus in which *gnrh3* transcripts were quantified by sensitive droplet digital PCR and were reduced by 71%, 62%, and 73% in *scg2a*$^{-/-}$, *scg2b*$^{-/-}$, and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish, respectively, compared to WT. Regulation of Gnrh3 neurons by Sgc2 or derived peptides is further supported by observations in females of increased hypothalamic *gnrh3* following injection of SNa and to a lesser extent SNb.

The expression of *oxt* and *avp* in the telencephalon and hypothalamus was determined for the *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ mutants. There were several reasons to do this. Firstly, immunohistological analyses indicate the SNa peptide is coexpressed with OXT and AVP in fish as in mammals (18, 48, 49). Equally important is that OXT and AVP are well-known regulators of sociosexual behaviors (50) and thus it is possible that mutation of *scg2a*$^{-/-}$ and/or *scg2b*$^{-/-}$ could have disrupted *oxt* and/or *avp* expression in our zebrafish. While we did observe some alterations in *oxt* and *avp* expression in the brain, they could not explain the severely reduced spawning behavior and oviposition in *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ mutant lines. For example, *oxt* expression in the telencephalon and hypothalamus was not different between WT and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ males and females. Importantly, using *oxt* and *oxtr1* mutations in medaka, Yokoi et al. (51) demonstrated that *oxt* function is required for mate choice based on familiarity recognition. They concluded that *oxt* is important for female recognition of familiar males. Moreover, mutation of *oxt* or *oxtr1* did not affect spawning success in these medaka, supporting the concept that the nonapeptides modulate sociosexual behaviors rather than ovulation itself. However, there are data in some species demonstrating actions of vasotocin on teleost ovaries (52). The changes in *oxt* or *avp* mRNAs we observed in the brains of some of the *scg2* mutant zebrafish, alone, cannot explain changes in sexual behavior and spawning in the *scg2* mutant zebrafish. The roles of *scg2* are distinct from coexpressed genes such as *oxt* and *avp* in magnocellular cells of the teleost preoptic area. The impact of *scg2* mutation on

spawning is also in marked contrast to that seen in all other neuropeptide mutations in teleosts reported to date that do not show any effects on spawning success (8, 13–15, 51, 53).

Disrupted anterior pituitary function is also a consequence of *scg2* mutation. The expression of the gonadotropin subunits (*lhb*, *fshb*, and *cga*) were significantly lowered in the pituitaries of *scg2* mutants except for *fshb* in *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish. Interestingly, the *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish had slightly higher *lhb* and *cga* transcript levels than single mutant lines, while concurrently having very low expression of *gnrh3* in the telencephalon and hypothalamus. This suggests that animals were attempting to somehow compensate physiologically for the deleterious effects of the *scg2a*$^{-/-}$ and *scg2b*$^{-/-}$ mutations, but this was clearly inadequate because they were still unable to reproduce effectively. We measured the pituitary expression of *gnrhr2*, because it is the most sensitive receptor to the Gnrh3 peptide (54). We found that the single *scg2a*$^{-/-}$ and *scg2b*$^{-/-}$ mutants had lower pituitary mRNA levels of *gnrhr2* than WT or *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ males and females; *scg2a*$^{-/-}$ and *scg2b*$^{-/-}$ females also had higher mRNA levels of *gnrhr2* than their male counterparts. This reduction in *gnrhr2* in the mutants could be part of the mechanism leading to decreased gonadotropin mRNA levels. It is also noteworthy that injection of SNa increased pituitary *gnrhr2*, further delineating a potential mechanism whereby SN could enhance gonadotropin production to regulate ovulation.

This presumed physiological compensation response in the *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ double mutants is clearly different from the situation observed in both the Gnrh and kisspeptin mutants, where compensatory changes in neuronal and pituitary gene expression are associated with maintenance of normal reproduction in both sexes (14, 55). Most remarkable are the data on *scg2a* and *scg2b* expression in fish with targeted mutations of *gnrh3* and *kiss1*/*kiss2* genes (*gnrh3*$^{-/-}$;*kiss1*$^{-/-}$;*kiss2*$^{-/-}$). These other zebrafish lines undergo normal gonadal maturation and exhibit normal fertility (55). Of the various neuropeptide mRNAs that were measured in that study, expression of *scg2a* was increased more than all others in whole brain extracts in the triple knockout fish; the respective fold increases were 1.4 and 2.7 for males and females (55). In the study of *gnrh2*$^{-/-}$;*gnrh3*$^{-/-}$ double mutants, Marvel et al. (14) observed a 1.6-fold increase in whole brain expression of *scg2b* in female zebrafish. In situ hybridization revealed increased expression of *scg2b* in lateral and lateral-dorsal hypothalamus, among other regions. These data reinforce the notion that neurons increasing their expression of *scg2a* and/or *scg2b* are important for maintaining reproduction in zebrafish carrying mutations in the classical reproductive regulators Gnrh and kisspeptin. Additionally, the expression patterns of *lhb* and *fshb* observed in our study are different for those reported in the kisspeptin mutant zebrafish. The transcript levels of *fshb* in both sexes and *lhb* in males in the *kiss1*$^{-/-}$;*kiss2*$^{-/-}$ mutant zebrafish lines were significantly downregulated (8). The reduction in *lhb* transcripts observed by Tang et al. (8) was far less than the dramatic 60 to 97% reductions of *lhb* in our study. In the adult pituitaries of *gnrh3*$^{-/-}$ zebrafish (13), *gnrh2*$^{-/-}$;*gnrh3*$^{-/-}$ zebrafish (14), *kiss1*$^{-/-}$;*kiss2*$^{-/-}$;*gnrh3*$^{-/-}$ zebrafish (55), and *gnrh1*$^{-/-}$ medaka (34), the gonadotropin subunit transcript levels were similar between the mutant and WT animals. Thus, we conclude that *scg2* is important for gonadotroph function.

We have provided in vivo evidence that *scg2* plays a critical role in reproductive processes, including sexual behavior, brain and pituitary gene expression, and spawning. We demonstrate that injection of the Scg2a-derived neuropeptide SNa enhances impaired reproduction in *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ mutants within a few hours, as does the LH analog hCG. Assessment of ovulation, oviposition, fertilization, and embryonic survival are all robust reproductive parameters and relatively easy to perform in zebrafish. Our *scg2* mutants, therefore, offer an amenable model system in which to assay potential stimulators of reproduction, be they for improved spawning in economically important cultured fish species, or to help with the search for new infertility treatments in humans.

Materials and Methods

Animals. Procedures conducted in this study were approved by the University of Ottawa Animal Care Protocol Review Committee and follow the guidelines of the Canadian Council on Animal Care for the use of animals in research. We follow conventional gene/protein nomenclature for zebrafish; see https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Conventions. WT (AB strain), *scg2a*$^{-/-}$, *scg2b*$^{-/-}$, and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ double mutant zebrafish (29) were bred in house and raised according to standard husbandry procedures. As previously established (29) the *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ homozygous mutant line (*scg2a*$^{-/-}$−7,+2 bp; *scg2b*$^{-/-}$ −5,+7 bp) was obtained by crossing the *scg2a*$^{-/-}$ homozygote (−7,+2 bp) with the *scg2b* $^{-/-}$ homozygote (−5,+7 bp). Fish were maintained in 10-L tanks of dechloraminated tap water at 28 °C on a 14:10 light/dark cycle in the University of Ottawa aquatics facility. Experiments (gene expression in WT zebrafish following i.p. injection of SNa and SNb and in vitro germinal vesicle breakdown (GVBD) were conducted under the same environmental conditions at the China Zebrafish Resource Center (Wuhan), following approval by the Institutional Animal Care and Use Committee of the Institute of Hydrobiology, Chinese Academy of Sciences.

Gene Expression Analysis in Mutant Zebrafish. Sexually mature adults (8 mo postfertilization [mpf]) were anesthetized in an ice bath, then their telencephalon, hypothalamus, and pituitary were dissected and collected (four fish pooled per tube). All samples were flash frozen in liquid nitrogen and stored at −80 °C until RNA isolation took place. Total RNA (RNA) was extracted from telencephalon, hypothalamus, and pituitary according to the manufacturer's protocols using TRIzol (Thermo Scientific, cat. no. 15596018) and Phasemaker tubes (Invitrogen, cat. no. A33248) with minor modifications. RNA was stored frozen at −80 °C. Total cDNA (cDNA) was synthesized using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, cat. no. K1642) according to the manufacturer's protocol and stored at −20 °C. A total of 1 μg of total RNA was used for cDNA synthesis in all samples except the pituitary in which 250 ng was used.

The ddPCR setup, droplet generation, and transfer of emulsified samples to PCR plates were performed according to the manufacturer's protocols. The PCRs were performed on a Bio-Rad c1000 Touch thermal cycler according to the following cycling conditions: 1× (95 °C for 5 min); 40× (95 °C for 30 s, annealing temperature for 1 min, 4 °C for 5 min) with a ramp rate of −2 °C/s; and 1× (90 °C for 5 min). Specific primers used for ddPCR are shown in *SI Appendix*, Table S1. The fluorescent intensity of the droplets was measured with the QX200 Droplet Reader (Bio-Rad). Data analysis was performed with QuantaSoft software (Bio-Rad). The absolute transcript levels of telencephalon, hypothalamus, and pituitary samples were normalized using the algorithm NORMA-Gene (56) and presented as means + SEM (n = 5 to 7).

Characterization of Reproductive Outputs. Reproduction was assessed via mating crosses between pairs of fish that were within the same mutant lines and from reciprocal crosses of mutants with WT zebrafish. Males and females (6 to 9 mpf) were separated 2 wk before an experiment. They were kept in 3-L tanks at a density of four fish/L. The night before each experiment, fish were then transferred to 1-L breeding tanks. Each tank contained one male and one female, separated overnight with a divider. Breeding tanks were covered overnight with opaque boxes to prevent visual disturbance. On the morning of an experiment, covers were removed, water was changed, and tanks were placed in the recording arena. The dividers were removed, and the fish were filmed for 10 min with a digital camcorder. The pairs were filmed consecutively in random order, all within the first hour of light (9 to 10 AM). The fish were left undisturbed for 1.5 h at which point oviposition was noted. Clutches were placed in 1× E3 medium (1× E3 medium: 3 mL methylene blue, 16 mL 60× embryo medium [34.8 g NaCl, 1.6 g KCl, 9.78 g $MgCl_2{\cdot}6H_2O$, 5.8 g $CaCl_2{\cdot}2H_2O$ to 2 L with ddH_2O], to 1 L with ddH_2O), to assess fertilization rate, then kept in an incubator (28 °C) until 24 h postfertilization (hpf) to assess survival rate. Ovulation was assessed in pairs of adult female and male WT or *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ fish (7 mpf). A male and female were placed in breeding traps for 17 h. At lights on (9:00 AM), females were anesthetized, whole ovary dissected and checked for ovulation (57). Gonad histology was examined in adult WT and *scg2*$^{-/-}$ males and females. Fish were maintained in 10-L tanks of water at 28 °C on a 14:10 light/dark cycle until 4 mo of age. Five female and five male fish from each line were randomly selected at lights on (9:00 AM), anesthetized in ice water, weighed, and dissected. The gonads were fixed in Bouin's solution overnight at 4 °C. The fixed samples were

washed twice in PBS, embedded in 1 to 1.5% agarose, trimmed, and prepared for standard histological processing and analysis. Samples were dehydrated in increasing ethanol (EtOH) concentrations, cleared with xylene, infiltrated with paraffin, and embedded in individual paraffin blocks. Serial sections of 5- to 8-μm thickness were prepared on a microtome (Kedee rotary microtome) and suspended on a water bath at 45 °C. Once the sections expanded to normal size, they were mounted on glass slides (Citoglas, Citotest). Sections were deparaffinized in xylene, rehydrated in EtOH, stained with hematoxylin and eosin-Y, and covered with a coverslip.

Assessment of Gonadal Morphology. After standard dissection and fixation protocols, a CRI Nuance multispectral imaging camera in conjunction with an Olympus BX53 microscope was used to capture digital images of the sections (Perkin-Elmer). Pictures were assigned a random number to perform unbiased, blind analyses. Follicles were divided into five groups: primary growth, previtellogenic, early vitellogenic, midvitellogenic, and full grown (58). Follicle stages are presented as a percentage of the total follicles examined from each female. In males, four major stages were examined: spermatogonia, spermatocytes, spermatids, and spermatozoa (59). All other space or interstitial tissue is referred to as "other." Stages are presented as a percentage of the total area of each picture.

Analysis of Courtship Behaviors. Zebrafish exhibit an elaborate premating ritual consisting of a series of behaviors to attract mates (30). The following courtship behaviors were assessed in male zebrafish: chase (following or swimming alongside female), tail-nose (touching the female's side or tail with nose or head), zig-zag (tail sweep and circle along female's body), encircle (circling around or in front of the female), and quiver (rapid tail oscillation against the female's side) (30). The only female courtship behavior that was assessed was egg laying (oviposition). Courtship behaviors were assessed by several observers that were blind to the genotypes of interacting pairs. Videos were viewed using VideoPad Editor (http://www.nchsoftware.com/videopad/) at 40 to 50% speed. The start time, end time, and behavior type were manually recorded. Twenty-five percent of all videos ($n = 20$ to 22 per group) were analyzed at random and the ratio of nonspawning and spawning pairs analyzed in each subset reflect the total dataset. The data presented here were derived from the analysis of ∼37 h of videos footage, corresponding to nearly 8 million frames. Samples (2 min) of the video recordings from all four genotypes (WT, *scg2a*$^{-/-}$, *scg2b*$^{-/-}$, and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$) are provided (Movies S1–S4).

Characterizing Reproductive Output Following Injection with hCG, SNa, or SNb. The reproductive capabilities of *scg2a*$^{-/-}$;*scg2b*$^{-/-}$ zebrafish following i.p. injection of synthetic zebrafish SNa and SNb and hCG were assessed via within-line mating crosses. Two weeks prior to the spawning experiment, fish were separated by sex into 3-L tanks at a density of four fish/L and maintained at 28 °C on a 14:10 light/dark. The night before each experiment, fish were transferred to 1-L breeding tanks. Each tank contained one male and one female, separated overnight with a divider. At lights on (9:00 AM), both fish were anesthetized with tricaine (3-amino benzoic acid ethyl ester) according to standard zebrafish husbandry procedures (31), then weighed using an analytical balance. Fish were injected using a 32-gauge Hamilton syringe with either Ringer's solution (pH 7) as control, hCG (50 IU/g body weight) (Millipore, cat. no. 230734-1MG) zebrafish SNa peptide (4 μg/g body weight), or zebrafish SNb peptide (4 μg/g body weight). The doses of SNa and SNb were chosen after preliminary range-finding tests (0.5 to 4 μg/g) for spawning induction. Fish were immediately returned to their breeding tanks to recover for 20 to 30 min. Dividers separating male and female were removed, and fish were left to breed for 6 h at which point the absence or presence of a clutch was noted. Clutches were placed in E3 medium to assess fertilization rate, then kept in an incubator (28 °C) until 24 hpf to assess survival rate.

Data Analysis. Online freeware was used for the statistical analysis of spawning success data. The percent spawning success of mating pairs were compared to the WT within line cross with a Fisher's exact test (http://graphpad.com/quickcalcs/contingency1/). All other statistical analyses were performed using SigmaPlot 11.0 software (Systat Software, Inc.). Normality and homoscedasticity were tested using a Shapiro–Wilk test and Levene's test, respectively. Two-way analyses of variance (ANOVA), with sex and genotype as main factors, followed by a Holm–Sidak post hoc test were used to analyze the tissue-specific mRNA levels, GSI, and sex steroid concentrations. Two-way ANOVA, with sex and gonadal stage as main factors, followed by a Holm–Sidak post hoc test were used to analyze oocyte percentages and total area occupied by sperm cells of different stages. A Kruskal–Wallis test on ranks followed by a Dunn's multiple comparison test was used to analyze the percent fertilization, percent survival, and all behavioral data. Information about the presentation of each two-way ANOVA and its associated statistics are described in each figure caption.

Data Availability. All data, methods, and results of statistical analyses are reported in this paper and associated *SI Appendix*. We welcome any specific inquiries.

ACKNOWLEDGMENTS. This research was funded by the Ontario Graduate Scholarship program (to K.M.), the Natural Sciences and Engineering Research Council of Canada Discovery Grant (RGPIN-2016-04182 to V.L.T.), the University of Ottawa Research Chair program (to V.L.T.), the University of Ottawa International Research Acceleration Program (to V.L.T. and W.H.), the Chinese Academy of Sciences (Grants 152342KYSB20180019 and 2019FBZ05 to W.H.) and the National Natural Science Foundation of China (31721005 to W.H.). We acknowledge with appreciation the help of W. Fletcher, C. Archer, and Ming Li for care of animals and Peng Di for ovulation tests.

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