Supporting Information

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SI Materials and Methods

Digoxigenin-in Situ Hybridization of c-fos. The brains of treated fish ($n = 3$ each) were fixed in buffered 4% paraformaldehyde for 6 h, cryoprotected in 20% sucrose, and frozen in Tissue Tek OCT compound. Coronal sections (10 μm) were cut using a cryostat and thaw-mounted onto 3-aminopropylsilane-coated glass slides. The sense and antisense riboprobes for *c-fos* (898 nt) mRNA were labeled with digoxigenin (DIG), as described previously (1). To enhance the detection of DIG, the signals were identified with a peroxidase-conjugated anti-DIG antibody (Roche Diagnostics GmbH), which was amplified using the TSA Biotin System (Perkin-Elmer) and detected with 3,3′-diaminobenzidine, using an avidin-biotin peroxidase complex kit (Vector Laboratories).

Real-time PCR for kiss1 and Serotonin-Related Genes. The brain cDNA samples of the fish obtained from the novel tank diving test, alarm substance exposure, and kisspeptin injection studies were subjected to real-time PCR. Gene expression levels of *kiss1* and serotonin-related genes: plasmacytoma expressed transcript 1 (*pet1*), tryptophan hydroxylase 2 (*tph2*), and solute carrier family 6, member 4 (*slc6a4a*), which is a gene for serotonin transporter ([DDBJ/EMBL/GenBank accession no. AB245404 (*kiss1*), EF370169 (*pet1*), NM_214795 (*tph2*), and NM_001039972 (*slc6a4a*) GenBank release no. 192.0] were examined by real-time PCR with specific primers, as described previously (1). *pet1* is a transcription factor, expressed only in the central 5-HT system, which not only establishes and maintains the serotonergic phenotype, it also interacts with the regulatory regions of 5-HT related genes including *slc6a4a* and *tph2* (2). Expression of *pet1*, *tph2*, and *slc6a4a* were analyzed in the raphe, whereas *kiss1* mRNA levels were analyzed in the rest of the brain region containing the habenula. The preparation of the total RNA and cDNA and real-time PCR were performed as previously reported (1).

Luciferase Assay for Binding Affinity of Kiss1-SAP. The assay system of dual-luciferase reporter (Promega) was used to assay the luciferase activity, according to the manufacturer's protocol. HEK 293T cells were maintained in DMEM in the presence of 10% (vol/vol) FBS at 37 °C with a humidified atmosphere of at 5% CO_2. For luciferase assays, cells were transiently cotransfected with receptor plasmids (zebrafish Kiss-R1 or Kiss-R2, GenBank accession numbers EU047918 and EU047917), and a construct containing reporter gene (the nuclear factor of activated T-cells response element; pNFAT-RE, Promega), using Lipofectamine (Invitrogen). A construct containing Renilla luciferase gene under the herpes simplex virus thymidine kinase promoter (pRL-TK, Promega) was used as the internal control in each transfection, and mock vectors were used as controls. Forty-eight hours after transfection, cells were exposed to DMEM in the presence of 0.5% FBS and incubated in the absence or presence of Blank-SAP, Kiss1-SAP, and kisspeptin1-15 (10^{-5}M for each) for another 6 h. Luciferase activity assays were performed according to the protocols (Promega). Each experiment was performed in fourfold, and each assay was repeated at least three times. Each ratio representing the value of the firefly luciferase versus that of Renilla luciferase activities, and all data were analyzed by either statistics methods (independent *t* test and or one-way ANOVA followed by post hoc Fisher's PLSD test). The means of the data from four individual transfected wells are used for data presentation.

1. Ogawa S, Ng KW, Ramadasan PN, Nathan FM, Parhar IS (2012) Habenular Kiss1 neurons modulate the serotonergic system in the brain of zebrafish. *Endocrinology* 153(5):2398–2407.

2. Rand CM, Berry-Kravis EM, Zhou L, Fan W, Weese-Mayer DE (2007) Sudden infant death syndrome: Rare mutation in the serotonin system FEV gene. *Pediatr Res* 62(2):180–182.

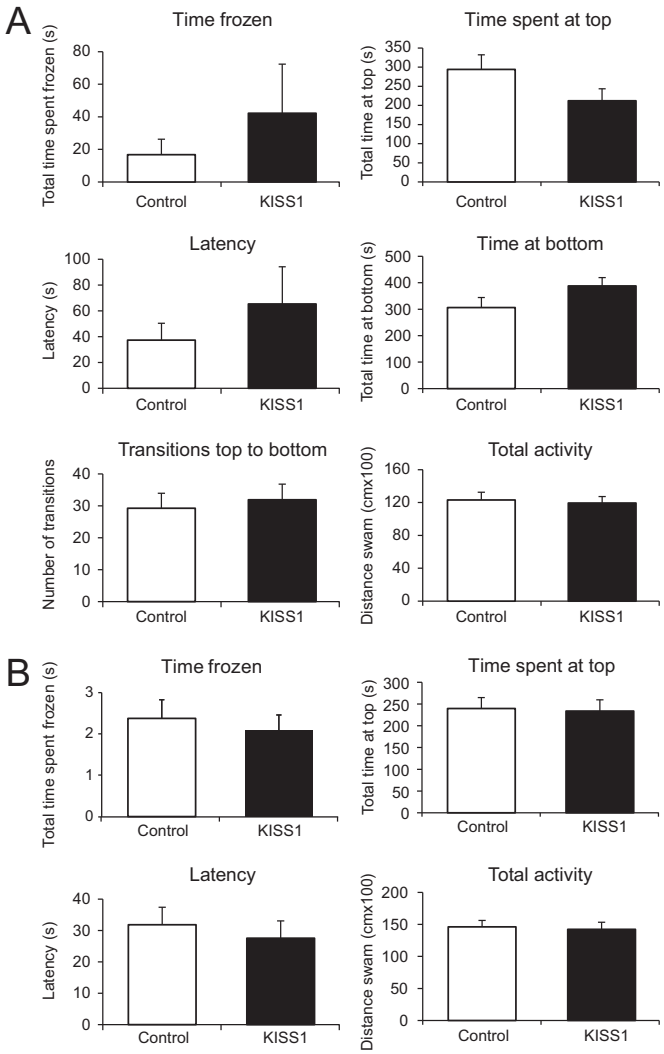

Fig. S1. Effect of administration of Kiss1 on anxiety. Graphs of the novel tank diving test during the 13-min observation in the fish injected with either distilled water (open bars) or Kiss1 (closed bars) after 1 h (*A*) and 4 h (*B*) postinjection. Data are presented as mean ± SEM. Independent *t*-test comparisons between control and Kiss1-injected fish.

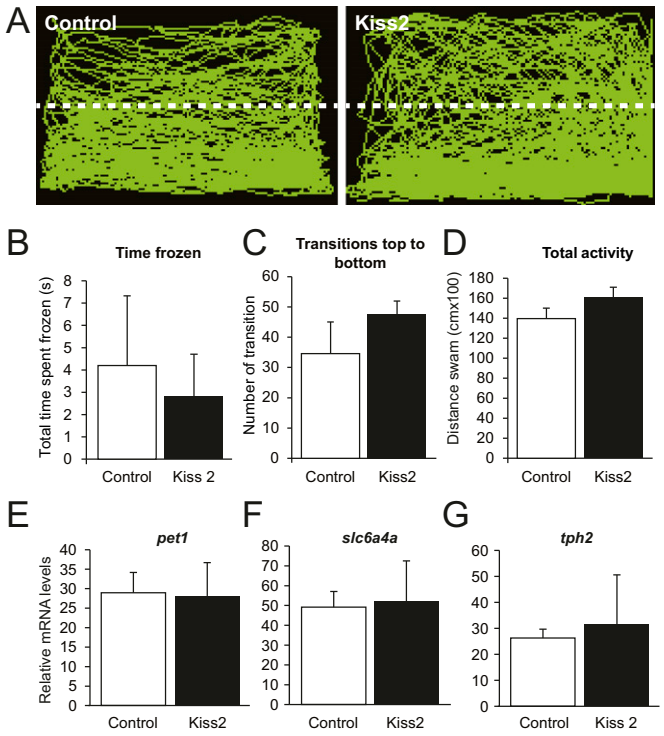

Fig. S2. Effect of Kiss2 administration on anxiety-like behavior and serotonergic genes. (*A*) Side view of behavioral tracking of an adult male zebrafish subjected to either distilled water (control) or Kiss2 (10^{-11} mol per fish, intracranial injection) for an 8-min recording 4 h after administration. (*B–D*) Graphs of the duration of freezing (*B*), the number of top to bottom transitions (*C*), and the total distance traveled (*D*) in the adult zebrafish injected with either distilled water (control, open bars) or Kiss2 (closed bars). (*E–G*) Graphs of relative mRNA levels of *pet1* (*E*), *slc6a4a* (*F*), and *tph2* (*G*) in the brain of adult zebrafish injected with either distilled water or Kiss1 at 6 h after administration. Data are presented as mean ± SEM. Independent *t*-test comparisons between control and Kiss2-injected zebrafish.

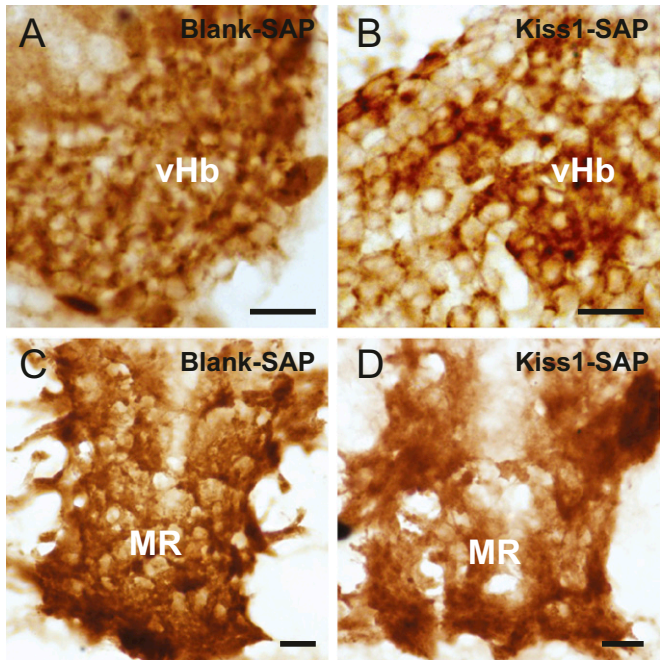

Fig. S3. Effect of Kiss1-SAP on Kiss1 immunoreactivity 3-d after injection. (*A–D*) Transverse sections of Kiss1 immunoreactivity in the ventral habenula (vHb; *A* and *B*) and the median raphe (MR; *C* and *D*) in the brain of zebrafish injected with Blank-SAP (*A* and *C*) or Kiss1-SAP (*B* and *D*) at 3 d after injection (10^{-3} mol per fish, intracranial administration; $n = 3$ per group). (Scale bars, 10 μm.) Note that there was no effect of Kiss1-SAP on Kiss1-immunoreactivity compared with in Blank-SAP treated fish at 3-d after injection, which could be due to a time course effect. Therefore, we decided to observe the effect of Kiss1-SAP at 12-d after injection (Fig. 6).

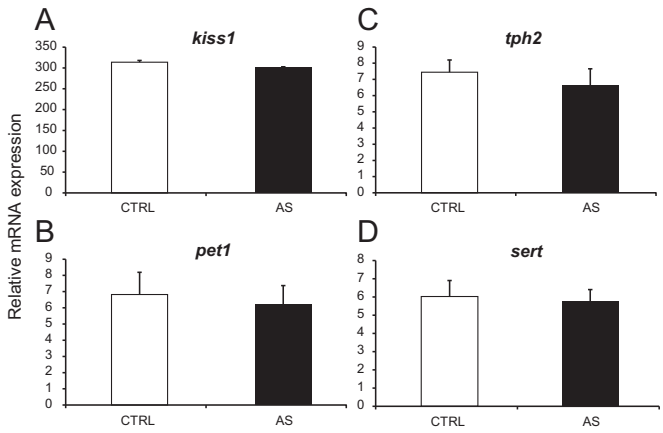

Fig. S4. Effect of a single alarm substance exposure on *kiss1* and 5-HT-related genes. (*A–D*) Graphs showing the effect of a single exposure of AS (8 min) on mRNA levels of *kiss1* (*A*), *pet1* (*B*), *tph2* (C), and *slc6a4a* (*D*) ($n = 5$ per group). Data are presented as mean ± SEM. Independent *t*-test comparisons between control and AS-exposed fish.

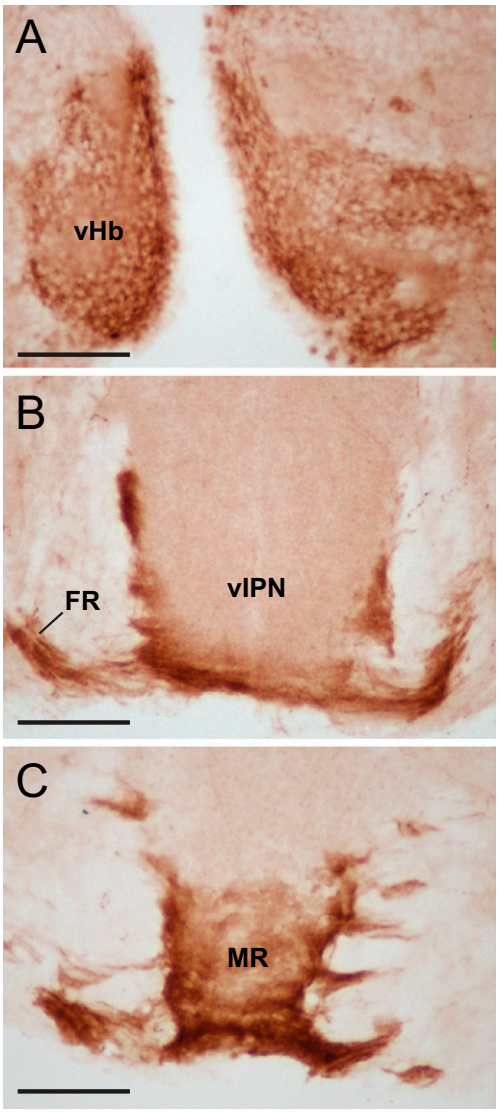

Fig. S5. Zebrafish Kiss1 immunoreactivity in the habenula and the median raphe. Immunohistochemistry using a newly generated antiserum to prepro-zebrafish Kiss1 showed Kiss1 immunoreactive cells in the ventral habenula (vHb; *A*) and immunoreactive fibers in the ventral interpeduncular nucleus (vIPN; *B*) and the median raphe (MR; *C*) through the fasciculus retroflexus (FR). No further immunoreactive fibers were seen after the MR, suggesting that the habenular Kiss1 neurons are terminated at the MR, but not in the vIPN. (Scale bars, 50 μm.)