The Medio-Basal Hypothalamus as a Dynamic and Plastic Reproduction-Related Kisspeptin-gnrh-Pituitary Center in Fish

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Kisspeptin regulates reproductive events, including puberty and ovulation, primarily via GnRH neurons. Prolonged treatment of prepubertal striped bass females with kisspeptin (Kiss) 1 or Kiss2 peptides failed to enhance puberty but suggested a gnrh-independent pituitary control pathway. Kiss2 inhibited, but Kiss1 stimulated, FSHβ expression and gonadal development, although hypophysiotropic gnrh1 and gnrh receptor expression remained unchanged. In situ hybridization and immunohistochemistry on brains and pituitaries revealed a differential plasticity between the 2 kisspeptin neurons. The differences were most pronounced at the prespawning phase in 2 regions along the path of gnrh1 axons: the nucleus lateralis tuberis (NLT) and the neurohypophysis. Kiss1 neurons appeared in the NLT and innervated the neurohypophysis of prespawning males and females, reaching Lh gonadotropes in the proximal pars distalis. Males, at all reproductive stages, had Kiss2 innervations in the NLT and the neurohypophysis, forming large axonal bundles in the former and intermingling with gnrh1 axons. Unlike in males, only preovulatory females had massive NLT-neurohypophysis staining of kiss2. Kiss2 neurons showed a distinct appearance in the NLT pars ventralis-equivalent region only in spawning zebrafish, indicating that this phenomenon is widespread. These results underscore the NLT as important nuclei for kisspeptin action in 2 facets: 1) kisspeptin-gnrh interaction, both kisspeptins are involved in the regulation of gnrh release, in a stage- and sex-dependent manner, especially at the prespawning phase; and 2) gnrh-independent effect of Kiss peptides on the pituitary, which together with the plastic nature of their neuronal projections to the pituitary implies that a direct gonadotropic regulation is plausible. (Endocrinology 155: 1874–1886, 2014)

The involvement of kisspeptin in the control of reproduction has been demonstrated in most vertebrates, including in numerous fish species (1–7). It has become common knowledge that kisspeptin mainly acts via the control of GnRH neurons. Unlike most mammals that possess 1 kisspeptin form (kiss1), most teleosts possess 2 kisspeptin systems: kiss1 and kiss2 genes (and Kiss1 and Kiss2 peptides); and 2 cognate receptors, kiss1r and kiss2r (8). The kisspeptin system in fish follows suit and has a similar role as found in mammals, but the exact function of the 2 kisspeptin forms largely varies among species. The task of determining the roles of Kiss1 and Kiss2 peptides has proven challenging, because variations are observed not only between species but also within the same species. For example, in Morone species, Kiss1 and Kiss2 peptides alternate from stimulation to inhibition, depending on the reproductive stage (9). In the medaka and the European sea bass, kiss1 neurons express estrogen receptor (10) and
are sexually dimorphic (11, 12), whereas in the zebrafish, kiss2 neurons respond to estrogen (13). Results also depend upon different administration modes, acute vs chronic, different doses, and different forms (the core kiss decapeptides, the dodeca-kiss2, the pentadeca-kiss1, or the pyro-Glu pentadeca-Kiss1) (2, 6, 9, 14).

There are indications that kisspeptin can also directly act at the pituitary level to stimulate Lh release (15–20). However, despite the fact that kisspeptin is found in the median eminence-hypophysial portal blood (21), and that kisspeptin and its receptor are expressed in the pituitary (19, 22, 23), this pathway is considered negligible in mammals (21, 24). The presence of kisspeptin receptor in fish pituitary is also reported (10) as well as in vitro stimulatory effect on Lh and Gh secretion in the goldfish (18) or an inhibitory effect on Lh, as in the case of the European eel (23).

The kisspeptin system in mammals is known to be sexually dimorphic and highly plastic. The changes are evident by the numbers of kiss1 neurons (25, 26) and by their stage-dependent responsiveness to sex steroids and their mode of action (27). The same seems to occur in fish species, but it is difficult to discern a common pattern because of the presence of 2 kisspeptin forms and the diverse reproductive strategies among teleosts.

The initial aim of the study was to determine whether chronic exposure of kisspeptin can promote puberty in striped bass females undergoing the unique stage of “dummy run.” This phase occurs in the female’s third year of life, a year before their actual puberty, in which the first reproductive cycle deviates from normal cycle, resulting in ovarian regression (28–30). The results obtained from this part of the study pointed to a gnrh-independent action of kisspeptin on the pituitary, which further revealed differences in the unique appearance of kisspeptin neurons in the hypothalamic nucleus lateralis tuberis (NLT) at the prespawning stage. The location of the NLT in close contact with the pituitary indicates that these kisspeptin neurons may be involved in both the regulation of gnrh1 release and gonadotropes in the pituitary.

Materials and Methods

Animals

For the kisspeptin chronic administration experiment, striped bass (Morone saxatilis) were obtained as juveniles from Maryland Department of Natural Resources and maintained at ambient conditions in a 20-m³ tank, supplied with constant exchange of artificial 8–10 parts per trillion sea-water, until 3 years of age. Animal maintenance and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. Brain histological samples were obtained from males and females at different reproductive stages, either from the Chesapeake Bay Department of Natural Resources or from the Aquaculture Research Center at Institute of Marine and Environmental Technology.

Implant preparation and experimental procedure

Sixty milligrams of either synthetic kiss1 (15 amino acids, (pGLU)DVSYYNLNSFGLRY-NH₂) or kiss2 (12 amino acids, SKFNFPNFLARF-NH₂) peptides (Genescipt) were incorporated into an inert matrix of polyethylene-vinyl acetate (EVAc) (Dupont, Inc) implants (31, 32). The release kinetics of this type of implants has been characterized both in vivo and in vitro using GnrHa-loaded EVAc implants, where GnrHa plasma levels peaked at 3 days after implantation, gradually decreased to an approximately 10% of maximal level at day 10, and remained above control levels until day 21 (32).

The experiment lasted 10 weeks from early November to mid-January in 3 year old females. Initial stages of ovarian development take place during this time range in dummy-run and mature (≥4 y old) females. In January, the difference in the ovarian development between dummy-run and mature females is noticeable: mature females will complete the cycle and spawn in April-May, whereas dummy-run ovaries regress (33). The doses of 25 and 75 μg/kg body weight (BW) were selected based on our single injection treatments that showed a dose-dependent plasma Lh stimulation in the range of 50–100 and 3–5 ng/kg BW for Kiss1 and Kiss2, respectively (9). Five groups of 8–12 females at previtellogenic stages (gonado-somatic index [GSI], 0.51 ± 0.10%; average oocyte diameter, 170 μm) were treated with implants containing no peptide as a control group, 25 or 75 μg/kg BW of Kiss1 or Kiss2 (referred to hereafter as Kiss1-25, Kiss1-75, Kiss2-25, and Kiss2-75, respectively). Fish were reimplemented every 3–4 weeks, during which blood was collected at each treatment point. Ten weeks after first implantation, the fish were bled, killed, GSI was recorded, and brain, pituitary, and gonads were snap frozen and stored at −80°C. Gonadal tissues were sampled from the midportion of the gonads and were immediately placed in 4% formaldehyde, 1% glutaraldehyde fixative for histological examination. The tissues were dehydrated through a 75%–90% ethanol series, embedded in glycol methacrylate plastic (JB-4 Plus; Sigma), and sectioned to 6-μm sections. The sections were stained with Harris hematoxylin solution (Sigma). Oocyte diameter was determined microscopically in each fresh ovarian sample (34).

Hormones and gene transcript measurements

Lh levels in the plasma were measured using Lh ELISA as previously described (35). Circulating levels of FSh were measured using a specific ELISA developed for tilapia, Oreochromis niloticus (36), and validated for use in Morone saxatilis. Competitive ELISAs were performed using specific primary antibodies against tilapia FShβ and recombinant tilapia FShβa (37) for the standard curves. The wells were coated with recombinant FShβ, and the antibodies were diluted 1:50 000. The intra- and interassay coefficients of variation were 8.0% and 12.5%, respectively. The sensitivities of the assays were 0.55 ng/mL⁻¹. Serial dilutions of striped bass plasma curve was parallel to that of the tilapia FSh standards, approving the use of the tilapia FSh ELISA to measure striped bass FSh (36).

For the measurement of gnrh1, gnrh2, gnrh3, Lh, and FSh transcript levels, brains and pituitary total RNA (1 μg) extracted...
using TRIzol reagent (Invitrogen) was reverse transcribed by Quantitect RT kit (QIAGEN). Real-time PCR was performed on 50-ng cDNA using SYBR Green PCR mix (Applied Biosystems) in duplicate, with 0.1 μM gene-specific primers (primers appear with the preface TAQ in Table 1), as previously described (9, 38), in an ABI Prism 7500 Detection System (Applied Biosystems). C_{T} values of each sample were normalized against the levels of 18S RNA amplified from 0.2-ng cDNA (39) and then converted to the fold change of the mean threshold cycle (C_{T}) value. Amplification reactions were carried out at 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Melting points for all primers ranged between 59°C and 61°C. Proper and specific amplification was verified using gel electrophoresis and by the dissociation curve of the primer sets. In each run, 2 negative water controls and a reference control, added to each plate, were included in addition to the standard curve.

### In situ hybridization (ISH) and immunohistochemistry (IHC) of brain and pituitary

#### Generation of antibodies against Kiss1 and Kiss2

The cDNAs encoding the precursors of each kiss1 and kiss2 (from start to stop codon) were amplified using primers expkiss1F and Rev and expkiss2F and Rev, respectively (Table 1), cloned into a pET-15b vector, and expressed in the presence of 1 mM isopropyl β-D-1-thiogalactopyranoside in Rosetta-DI th laboratories. C_{T} values of each sample were normalized against the levels of 18S RNA amplified from 0.2-ng cDNA (39) and then converted to the fold change of the mean threshold cycle (C_{T}) value. Amplification reactions were carried out at 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Melting points for all primers ranged between 59°C and 61°C. Proper and specific amplification was verified using gel electrophoresis and by the dissociation curve of the primer sets. In each run, 2 negative water controls and a reference control, added to each plate, were included in addition to the standard curve.

### Brain histology

#### Striped bass

Males and females at different reproductive stages: 1) 1-year-old juveniles; 2) recrudescent, at the middle of gonadal development, sampled in January, having GSI values of 2%–3%; 3) “prespawning” stage fish sampled in mid-April, when males are spermatizing and females carry “green” ovaries (9–15 h before ovulation) with GSI values of 13%–14% for females and approximately 10% for males; and 4) postovulatory (spent) females within a few days postspawning (n = 2–3 in each group).

#### Zebrafish

Adult zebrafish pairs were conditioned to spawn overnight in spawning containers with dividers as described (41). The fish were sampled by decapitation at either 18 hours of prespawning, during the afternoon before the scheduled morning spawning (n = 3 pairs), or the next morning at the initiation of spawning after removal of the divider (n = 7 pairs).

Brains were removed immediately after decapitation, fixed in buffered 4% paraformaldehyde overnight at 4°C, cryoprotected in 15% (30% for zebrafish brains) sucrose overnight at 4°C, and embedded in Tissue Tek OCT (Electron Microscopy Sciences). Coronal sections of 12 μm were mounted onto Plus glass slides and stored at −80°C. ISH, which followed the protocol described earlier (9), IHC, or combined ISH/IHC, all used Tyramide Signal Amplification kit (TSA, PerkinElmer), according to the manufacturer’s protocol. Anti-digoxigenin-horseradish peroxidase (Roche) was used to detect Dig-labeled probe (kiss1, kiss2 of both striped bass and zebrafish, and striped bass FSH riboprobes encompassing the entire coding region of each gene). Fluorescence was obtained via Cy3 (red) or fluorescein (green) from the kit. Anti-Kiss1, anti-Kiss2, anti-sea bass-GnRH1 Gnrh associated peptide (9, 42, 43), and anti-stripped bass βLh primary sera, as well as the secondary antibody goat antirabbit con-

### Table 1. Degenerate and Gene-Specific Primers Used for RT-PCR and Real-Time Quantitative PCR

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Abbreviations: F, forward; R, reverse.
jugated to horseradish peroxidase (Lanza), were all diluted 1:1000. Negative control IHC slides were similarly treated with the corresponding recombinant-precursors preabsorbed sera of Kiss1 and Kiss2.

**Statistical analysis**

Statistical analyses were performed by one-way ANOVA and Tukey’s post hoc test for multiple comparisons using Instat3 (GraphPad). For LH and FSH plasma level analyses, statistical significance was determined using a two-way ANOVA followed by a Bonferroni-Dunn post hoc test (Aabel 3.0.6; Gigawiz). Statistical difference was accepted when $P \leq .05$.

**Results**

The effect of kisspeptin on mRNAs levels of reproduction-related genes in the brain and pituitary

mRNA levels of gnrh1, gnrh2, and gnrh3 were measured in brains sampled at the conclusion of the experiment using quantitative RT-PCR as described earlier (9). Kiss1-25 had no effect on any of the gnrh and gnrh receptor expression, whereas the higher Kiss1 dose and both doses of Kiss2 resulted in a decrease in their transcript levels (Figure 1a). Treatment with Kiss2-25 and Kiss2-75 decreased gnrh1 mRNA levels compared with control to $48 \pm 8\%$ and $36 \pm 7.5\%$, respectively (Figure 1a). gnrh2 mRNA levels were dose dependently reduced to $29 \pm 10\%$, $24.3 \pm 5\%$, and $13.3 \pm 4.4\%$ of control with Kiss1-75, Kiss2-25, and Kiss2-75, respectively (Figure 1b). gnrh3 mRNA levels were affected only by the higher dose of Kiss2-75 and were decreased to $29.4 \pm 6\%$ of control (Figure 1c).

In the pituitary, gnrh receptor mRNA levels decreased to $50 \pm 9\%$, $30 \pm 8\%$, and $47 \pm 13\%$ of control in response to Kiss1-75, Kiss2-25, and Kiss2-75 treatments, respectively, but not with Kiss1-25 (Figure 1d). No significant effect was observed on the expression of the Lhβ, but a 50% increase in FShβ expression was obtained in the Kiss1-25 treatment.

![Figure 1](image1.png)

**Figure 1.** The effect of chronic treatment of Kiss1 and Kiss2 peptides on brain and pituitary mRNA levels of major reproductive axis genes (a) gnrh1, (b) gnrh2, (c) gnrh3, (d) gnrh1r, (e) FShβ, and (f) Lhβ. mRNA levels were measured in dummy-run female striped bass treated for 10 weeks with EVAc implants containing, respectively, (A) no peptide (Con) (n = 8); (B) pyroGluKiss1-15, 75 μg/kg BW (n = 11); (C) pyroGluKiss1-15, 25 μg/kg BW (n = 11); (D) pyroGluKiss1-15, 75 μg/kg BW (n = 12); (E) Kiss2-12, 25 μg/kg BW (n = 12); and (F) Kiss2-12, 75 μg/kg BW (n = 11). Results are presented as mean ± SEM. Statistical significance was determined using a two-way ANOVA followed by a Bonferroni-Dunn test to compare treatments (stars) and for each treatment as a factor of time. Significant difference between the treatments was obtained only at the 6-week time point. C, Oocyte diameter. Measurements were taken at the beginning (open bars) and at the conclusion of the experiment (closed black bars). The largest oocytes in the sample and their abundance were recorded. Inset 1 is a caption of a representative histological sample of Kiss1-25 μg/kg BW and inset 2 is of Kiss2-25 μg/kg BW taken at the end of the experiment. PG, primary growth oocyte; SGI, secondary growth oocyte stage I; SGII, secondary growth oocyte stage II. Scale bar, 50 μm. In all experiments, statistical difference was accepted when *, $P \leq .05$; **, $P \leq .01$; ****, $P \leq .005$.

The effect of kisspeptin treatment on plasma FSH and LH levels and ovarian development

LH and FSH levels in the blood were measured using ELISAs in samples collected at 0, 3, 6, and 10 weeks im-

![Figure 2](image2.png)

**Figure 2.** The effect of chronic treatment of Kiss1 and Kiss2 on plasma levels and gonadal development. A, LH; B, FSH. Gonadotropin levels were measured via specific ELISAs at 0, 3, 6, and 10 weeks during the course of the experiment, in which females were treated with EVAc implants containing 1) no peptide (Con) (n = 8); 2) pyroGluKiss1-15, 25 μg/kg BW (n = 11); 3) pyroGluKiss1-15, 75 μg/kg BW (n = 12); 4) Kiss2-12, 25 μg/kg BW (n = 12); and 5) Kiss2-12, 75 μg/kg BW (n = 11). Results are presented as mean ± SEM. Statistical significance was determined using a two-way ANOVA followed by a Bonferroni-Dunn test to compare treatments (stars) and for each treatment (letters) as a factor of time. Significant difference between the treatments was obtained only at the 6-week time point. C, Oocyte diameter. Measurements were taken at the beginning (open bars) and at the conclusion of the experiment (closed black bars). The largest oocytes in the sample and their abundance were recorded. Inset 1 is a caption of a representative histological sample of Kiss1-25 μg/kg BW and inset 2 is of Kiss2-25 μg/kg BW taken at the end of the experiment. PG, primary growth oocyte; SGI, secondary growth oocyte stage I; SGII, secondary growth oocyte stage II. Scale bar, 50 μm. In all experiments, statistical difference was accepted when *, $P \leq .05$; **, $P \leq .01$;***, $P \leq .005$.
mediately before implantation. FSh plasma levels in the control and in the 2 Kiss1-treated groups displayed a specific pattern of a marked 3-fold increase only at the 6-week point (sampled in December). This specific increase was reduced to basal level in the Kiss2 treatment groups, resulting in significantly lower FSh levels at this time point (Figure 2A). In general, plasma Lh levels have not changed substantially spermiating (Figure 4Ac), and juvenile (Figure 4Ad), suggesting that bundles of axons reach this region. Mature spermiating males display kiss2 somas also in the NLTm (IHC, Figure 3, A and B, and ISH, Figure 3, C and D). These neurons were not observed in any fish at other stages, such as recrudescence male (Figure 3Ab), recrudescence female (Figure 3Bb), or spent female (Figure 3Bc).

Using ISH, kiss1 neuronal somas are detected in the NLTm of all prespawning males and females (IHC, Figure 3, A and B, and ISH, Figure 3, C and D). Hence, Kiss1 and Kiss2 preparations were tested for the presence of the recombinant proteins on a 16.5% Tris-

**Histology**

Using ISH and IHC, we detected the appearance of neurons expressing kiss1 in the NLT pars medialis (NLTm) and NLT pars lateralis (NLTl) of all prespawning males and females (IHC, Figure 3, A and B, and ISH, Figure 3, C and D). Hence, Kiss1 neuronal somas are detected in the NLTm of all prespawning males and females (Figure 3, Ca and Da) and also in the NLTl of females (Figure 3Db). Kiss2 staining is detected only by IHC in the NLTl region of all males at all stages: prespawning (Figure 4Aa), precociously spermiating (Figure 4Ac), and juvenile (Figure 4Ad), suggesting that bundles of axons reach this region. Mature spermiating males display kiss2 somas also in the NLTm (IHC, Figure 4Aa and ISH, Figure 4Ca). In females, however, kiss2 neurons are observed by both methods in the NLTm (Figure 4, Ba and Da) and in the NLTl (IHC, Figure 4Bb and ISH, Figure 4Db), only at the prespawning stage, and are lacking the structures detected by IHC only in males. Kiss2 cell bodies are abundantly expressed in the nucleus recessus lateralis (NRL) region of both sexes at all stages and are detected by the anti-Kiss2 serum. These neurons send axonal projections down the NLT pars ventralis (NLTV) along the third ventricle towards the NLTm (Figure 4Ae).

**Stage- and sex-related Kiss1 and Kiss2 presence in the NLT**

**Striped bass**

**Antibodies**

The rec-Kiss1 and Kiss2 preparations were tested for the presence of the recombinant proteins on a 16.5% Tris-

Tricine SDS-PAGE. Kiss1 precursor resolved as an approximately 13-kDa band and Kiss2 precursor as a 12-kDa band. The specificity of each antiserum was determined via a combination of Western blot analysis and ISH/IHC labeling and found to be highly specific with no cross-binding (Supplemental Figures 1–4, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). Sections displaying NLTs of prespawning males and females were treated with sera preabsorbed with the recombinant Kiss1 or Kiss2 and subsequently lost the staining observed in the neighboring sections treated with antiserum (Figures 3Bd and 4Ab and Supplemental Figures 2–4).

**Zebras**

kiss1 and kiss2 neurons were detected by ISH in brains of 18-hour prespawning and spawning zebrafish males

**Figure 3.** The distribution and plasticity of kiss1 neurons in the hypothalamic NLT of male and female striped bass at different reproductive stages. IHC using the specific anti-Kiss1 serum of (A) male or (B) female, and ISH using kiss1 antisense riboprobe of (C) male and (D) female. Aa, prespawning male; Ab, recrudescence male; Ba, preovulatory female; Bb, recrudescence vitellogenic female; Bc, postovulatory (spent) female; Bd, preovulatory female treated with Kiss1 preabsorbed serum; Ca, prespawning male; Da, preovulatory female, NLTm region Db, preovulatory female, NLTl region. Kiss1 positive neurons are labeled in red. Pit, pituitary. Scale bars, 100 μm.
and females. Kiss1-expressing neurons were detected only in the extrahypothalamic habenula (data not shown). Kiss2 neuronal bodies were detected, as expected, in the dorsal zone of periventricular hypothalamus in all fish (Figure 5). However, only males and females sampled at the time of spawning had a strong expression of kiss2 along the ventral zone of periventricular hypothalamus (Hv), close to the pituitary (Figure 5, C and F). Brain sections of 18-hour prespawning fish lacked kiss2 in the Hv (Figure 5, A–D). Comparison of the sections of the striped bass and zebrafish depicting the NLT and Hv, respectively, clearly show that these are 2 equivalent regions, which are referred to by different names in the 2 corresponding atlases used for our neuroanatomical studies (44, 45).

Stage- and sex-related Kiss1 and Kiss2 presence in the neurohypophysis

Using IHC, we show that Kiss1 axonal projections penetrate the neurohypophysis (NH) of prespawning females (Figure 6, A and G) and are absent in juvenile and adult recrudescent males (Figure 5, H and I, respectively) or juvenile, recrudescent and spent females (Figure 6, B–D). Kiss1 axon terminals reach the proximal pars distalis (PPD) and are dispersed between Lh gonadotropes (Figure 6G).

Kiss2 staining is found throughout the entire NH in proximity to the gonadotropes (Figure 7, A, B, and G) in males and females at the prespawning stage. Kiss2 axon terminals are clearly spread in the PPD in between Fsh gonadotropes of a preovulatory female (Figure 7G). The same is seen in males at all stages: juveniles (Figure 7F) and recrudescent (Figure 7D). These projections reside side by side with the hypophysiotropic gnrh1 projections (Figure 7, H and I).

Discussion

The aim of the present study was 2-fold. First, to test whether chronic treatment of kisspeptin, known as the gatekeeper of puberty in mammalian species, can advance pubertal processes in female striped bass. The second aim, which emerged from the first, was to examine kisspeptin-pituitary interactions. Several studies in fish have already shown that kisspeptin functions as a regulator of reproduction (1, 2, 9). However, with the exception of some indirect evidence, the control of puberty by kisspeptin in fish remains largely unclear. These studies reported an increase in kisspeptin receptor gene expression at the onset of puberty (46, 47), increased sensitivity of prepubertal fish to acute doses of kisspeptin peptides (Kiss) (2, 9), or advanced gonadal development in juvenile chronically treated with Kiss-10 deca-peptide, as demonstrated in Morone spp. males (6) and Seriola lalandi (14).

Kisspeptin chronic treatment did not advance puberty in dummy-run females

In order to test the direct involvement of Kiss in puberty, we exploited females at the dummy-run phase, in which females initiate ovarian development without completion of the process (33).

Treatment with either Kiss1-15 or Kiss2-12 via sustained release implants ensured a constant presence of the
Kisspeptin effects on gnrhs and gnrh receptor

Kiss2 had a general down-regulating effect on the expression of the 3 brain gnrh forms and the pituitary gnrh receptor form (49). Unlike Kiss2, Kiss1 did not cause a decrease in gnrh2 and gnrh3 transcript levels, and gnrh1 was down-regulated only by the higher Kiss1 dose. In support, a dose-dependent negative effect of acute Kiss treatment on gnrh1 was already reported in recrudescent mature striped bass, which was noted only with Kiss2 and not with Kiss1 (9). Indeed, chronic treatment of kisspeptin in mammalian species, as opposed to acute administration, often causes desensitization and negatively affects reproduction (50, 51). However, our results show that in the striped bass, this is not always the case, particularly with low doses of Kiss1.

Kisspeptin effect on gonadotropins

Kiss2 treatment did not change Lhβ and FShβ transcript levels in the pituitary, but at 6 weeks after treatment, an inhibitory effect was observed for FSh plasma levels, which are higher in the control and Kiss1-treated fish. The higher levels are probably the result of a natural increase correlating the stage of gonadal development at this time of the year (mid-December), associated with an increase in FShβ mRNA levels at this time point (52). Moreover, FSh mRNA increased in response to Kiss1-25 treatment, coinciding with the advancement in oocyte development. When Kiss1 was injected to pubertal hybrid striped bass, FSh plasma levels increased dose dependently 4 hours after injection in the 8.8 and 44 μg/kg BW and were attenuated in the 88 μg/kg BW (Supplemental Figure 5). This finding strongly supports the suggestion that Kiss1 is capable of inducing the release of FSh from the pituitary when given at low levels at the right reproductive stage. A stimulatory effect of Kiss1 on FShβ and Lhβ mRNA levels was reported in prepubertal male S. lalandi during the reproductive season and gonadal development, again not correlating with gnrh1 mRNA levels in the brain. This may suggest a gnrh1-independent relationship between FSh and hypothalamic Kiss1 at the level of the pituitary. The fact that the kiss1r expression was detected in the PPD of the European sea bass (22) strongly supports this option. Another support comes from a recent study, in which kisspeptin directly induced Lhβ- and FShβ-subunits early gene expres-

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**Figure 5.** Kiss2 neuronal distribution in male and female zebrafish before and at spawning. ISH using kiss2 antisense riboprobe on females (A–C) and males (D and E) medio-basal hypothalamic sections. A, Female brain 18 hours before spawning. B, Higher magnification of A focusing on the Hv region. C, Female brain at spawning. D, Male brain 18 hours before spawning. E, Higher magnification of D focusing on the Hv region. F, Male brain at spawning. G, Upper panel illustrates the relevant brain cross-section demonstrating the organization of the regions, black dots represent kiss2-expressing neuronal cell bodies exist at all times, and gray dots represent those observed only at spawning; lower panel illustrates sagittal whole brain demonstrating the anatomical location of the presented cross-section (illustrations were adopted from Ref. 45). Hv, ventral zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; ATN, anterior tuberal nucleus. Scale bars, 100 μm.
sion in LβT2 cells and murine gonadotropes in vitro (53). Although steroids do not directly affect fshβ expression in the pituitary of recrudescence females in vitro (54, 55), we cannot rule out the effect of the administered Kiss1 on the gonads, which may, in turn, feedback directly the pituitary to induce Fsh. Further investigations are required to determine this issue. Because it is known that the gonadal feedback can be exerted via various pathways (56), a direct Kiss1-pituitary action may be one such pathway. The fact that kisspeptins are members of the arginine-phenylalanin (RF)-amide peptide family, which contains several peptides, including gnh, raises the possibility of cross-receptor activation at pharmacological levels, to exert their negative effect. It remains to be determined whether kisspeptin strictly acts via its cognate receptor in experimental administrations of this type.

The NLT as a central hub for kisspeptin neurons

To investigate the different pathways that kisspeptin may use to interact with the pituitary, we examined the neuro-anatomical dynamic of the 2 kisspeptin neurons and their projections into the pituitary at different reproductive stages. The focus in the NLT region was based on previous reports of the appearance of kiss1-expressing neurons in the NLT or the nucleus ventralis tuberis (NVT) in the medio-basal hypothalamus in the European sea bass and medaka, respectively (10–12). Our results in the striped bass confirmed a similar appearance of kiss1 neurons at the prespawning stage of both males and females. Consistent with the European sea bass (22), no apparent differences in cell number were observed between the 2 sexes, as was reported in the medaka (11, 12). The location of the NLT in close contact with the pituitary and on the gnrh1 axonal path offers 2 optional modes for the kiss neurons: acting on gnrh projections en route to the pituitary and/or a direct action on the pituitary.

Most mammalian hypothalamic neurohormones, including GnRH and kisspeptin, reach the pituitary via the neurohemal median eminence, whereas the teleost neurohypophysis serves as a path for all neurons penetrating the pituitary (57, 58). This allows a unique opportunity for

Figure 6. IHC analysis of Kiss1 axonal distribution in the pituitary/neurohypophysis of males and females at different reproductive stages. Panels depict stage-specific IHC of (A) preovulatory female, upper NH region; (B) recrudescent vitellogenic female; (C) postovulatory (spent) female; (D) juvenile female (FSH detected using ISH); (E) preovulatory female, bottom NH inside the PPD region; (F) preovulatory female treated with preimmune serums, bottom NH inside the PPD region; (G) Kiss1 axon terminals penetrating the PPD and are near Lh gonadotrophs (arrows) in the pituitary of a preovulatory female; (H) juvenile male, and (I) recrudescent male. NH, neurohypophysis; Kiss1, red; LH/FSH, green. Scale bars, 100 μm.
tracking axon terminals to their destination in the adenohypophysis. The specificity of the anti-Kiss1 and anti-Kiss2 serums, which pose a major concern when dealing with the RF-amide peptide family, was high without cross-reactivity, thus providing, for the first time in teleosts, an opportunity to track also kiss1 neurons, which in perciforms (unlike the habenular expression in zebrafish) have a hypothalamic expression and reproductive relevance.

**Kiss1 neuronal distribution and projections**

The combined ISH and IHC clearly show that kiss1 neurons appear in the NLTm and NLTl at the prespawning phase and project to the pituitary reaching the PPD. The fact that this kiss1 neuronal population expresses estrogen receptors α and β (11, 22) and kiss1 expression is enhanced by gonadal steroids (11, 12) indicates its involvement in the gonadal steroid feedback of spermiation and preovulatory Lh surge.

**Kiss2 neuronal distribution and projections**

Using IHC, we detected an intensive presence of Kiss2 in the NLTl of both males and females, but although males exhibited this pattern at all stages, it was observed only in prespawning females. Furthermore, ISH detected kiss2 neuronal bodies in the NLT only at the prespawning phase, suggesting that 1) the constant innervations of the NLT by kiss2 neurons at all stages happen only in males, similar to the European sea bass (10); and 2) neuronal cell bodies expressing kiss2 appear in the NLTm/l in the prespawning stage. Taken together, this suggests that both kiss1 and kiss2 NLT populations are involved in the mediation of spermiation and the Lh surge. Based on the strong axonal projections towards the NLTv, and the presence of dense population of kiss1r- and kiss2r-expressing neurons in this region (9), it is logical to conclude that the axonal bundles in the NLTl originate from the NRL, where most Kiss2 neurons reside (9). The NRL kiss2 neurons also express low levels of kiss1 in the striped bass.
(9), which were undetectable by IHC in the present study and presumably under the IHC detection limit of this technique. Indeed, kiss1 mRNA was not detected in the NRL of the European sea bass (10). Several possible reasons may cause the differences, including the sample preparation technique, eg, paraffin-embedded sea bass brains vs frozen brains combined with signal amplification in the case of the striped bass. The NRL neurons do not exhibit sex or reproductive stage variability nor do they express estrogen receptor or display gonadal steroid sensitivity (11, 12). However, the lack/low prevalence of the projections in the NLT of females at the nonprespawning stages is surprising and requires further investigation.

The massive Kiss2 innervations pattern of the NLT of males is also distributed throughout the NH, whereas this pattern is seen only in preovulatory females. Interestingly, Kiss2 is also detected in the NH of a spent female, but this may represent only remnants of the preovulatory phase. Taken together, these data indicate that the innervations of Kiss2 in the NLT of males, which probably arrive from the NRL, with their unchanged axonal presence, suggest that they may not relay gonadal feedback. These neurons probably chaperon gnrh1 axons as they make their way into the pituitary. Thus, if correct, the NRL kiss2 neurons not only regulate the hypophysiotropic gnrh neurons at their origin in the preoptic area, as was shown in zebrafish (13), but also as they reach the pituitary. In fact, the arcuate nucleus Kiss1 neurons in mammals is known to serve also as the pacemakers of the GnRH release, partly by directly abutting their nerve endings (59), specifically in the median eminence (60).

Kiss1 and Kiss2 neurons temporal appearance in the NLT at the prespawning stage

Despite evidence for Kiss1 neuro-plasticity via synaptic outputs and hypopolarization (61, 62), the appearance of an additional population of kiss1 neurons has never been documented in mammals. Nonetheless, Kiss1 is crucial for the preovulatory Lh surge (63, 64), and the appearance of both kiss1 and kiss2 at exactly this time suggests that this is the case also in teleosts. Interestingly, little is known to date about the importance of kiss1 in male gonadal development, particularly in spermiation. Thus, our observation provides the first evidence that the kiss system dynamically regulates the hypothalamus-pituitary-gonadal axis also in males. In order to substantiate our hypothesis that this is a widespread phenomenon among teleosts, we determined the appearance of kiss1- and kiss2-expressing neurons in this region in zebrafish. The zebrafish is a nearly daily spawner under laboratory conditions (constant 16-h light, 28°C), and our results show that an episodic kisspeptin expression occurs around the time of spawning. This phenomenon was observed only with kiss2, which is the reproductively relevant kisspeptin form in the zebrafish (3, 13). Interestingly, it is kiss1 that is expressed in the NVT in breeding medaka (under long-day regime) (3), which like zebrafish is a near-daily spawner. On one hand, these observations support the already suggested diverse reproductive roles of kiss1 and kiss2 among teleosts. On the other hand, they also highlight the importance of the time of sampling and indicate that the duration of the gonadal cycle must be considered as a basis for comparative studies.

The possibility that the NLT kiss neurons project to the POA cannot be ruled out, and in fact, kiss1 NVT neurons in the medaka do innervate this region (65). However, the finding that NLT kiss2 and Kiss1 coincide with NH projections in prespawning females and males suggests that these neurons also innervate the pituitary. The dual presence of the 2 kisspeptins in the NH may enable the kisspeptins not only to interact with gnrh projections but also bind gonadotropes and somatotropes (16, 18) and, in turn, perhaps enhance the gnrh effect. Moreover, it is likely that at least in preovulatory females, the effect of kiss1 and kiss2 neurons is positive, because, as alluded to earlier, kisspeptin is essential for this process and the Lh surge that precedes it. On the other hand, it is possible that...
the kiss2 axonal bundles in the NLT exert a negative regulatory role, as is also suggested by the inhibitory effect of Kiss2 on gnrh1 in striped bass demonstrated in both the current study and in a previous study (9).

**Summary**

In conclusion, this study aimed at testing the ability of the kisspeptins to induce puberty in prepubertal striped bass females. Although the results do not support this action, a possible gnrh-independent direct interaction of Kiss1 with FSH gonadotrophs has emerged. Kiss-pituitary interactions were pursued neuro-anatomically by tracing the neuronal distribution and projections of kiss1 and kiss2 in the brain and pituitary. We have revealed the hypothalamic NLT as a major region, where a high level of plasticity occurs, correlating to reproductive stages. By showing that kiss1 and kiss2 neurons innervate the gonadotropes, we demonstrated that, like gnrh neurons, these neurons have the potency to affect all cells in the PPD. Furthermore, both kiss1- and kiss2-expressing neurons seem to appear in the NLT of males and females before spawning and help to execute the hormonally regulated events of spermiation and ovulation. In addition, we observed sexually dimorphic kiss2 neuronal innervations in the NLT and the neurohypophysis that probably reach the NLT and the NH from the NRL and are present predominantly in males. A schematic illustration of the dynamics of the kisspeptin neurons before and immediately before spawning is presented in Figure 8. Altogether, Kiss2 that originates from the NRL probably acts on gnrh1 innervations in the NLT and NH, whereas Kiss1 and Kiss2 NLT populations probably act on both the gnrh axons and the gonadotropes at the prespawning stage. Although many questions remain to be answered, this study shows that both kiss1 and kiss2 are integrally involved in the regulation of reproductive processes in the striped bass, probably via plastic and diverse mode of actions.

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