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 pre-pubertal striped bass females with Kiss1 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 two kisspeptin neurons. The differences were most pronounced at the pre-spawning phase in two 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 pre-spawning 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 pre-ovulatory females had massive NLT-neurohypophysis staining of kiss2. Kiss2 neurons showed a distinct appearance in the NLTv-equivalent region only in spawning zebrafish, indicating that this phenomenon is widespread. These results underscore the NLT as important nuclei for kisspeptin action in two 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 pre-spawning 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.

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 one kisspeptin form (kiss1), most teleosts possess two kisspeptin systems: kiss1 and kiss2 and two 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 two kisspeptin forms largely varies among species. The task of determining the roles of Kiss1 and Kiss2 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

Abbreviations:

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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 two 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 prior to 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 (MD-DNR) and maintained at ambient conditions in a 20 m³ tank, supplied with constant exchange of artificial 8–10 ppt sea-water, until three 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 DNR (Oxford, MD) or from the Aquaculture Research Center at IMET.

**Implant preparation and experimental procedure**

Sixty mg of either synthetic Kiss1 (15 aa, (pGLU)DVSSYN-LNSFGLRY-NH₂), or Kiss2 (12aa, SKFNFNPFGLRF-NH₂) peptides (Genescript) were incorporated into an inert matrix of poly[ethylene-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 post implantation, gradually decreased to ~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 YO females. Initial stages of ovarian development take place during this time range in ‘dummy run’ and mature (≥4 year 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 while ‘dummy-run’ ovaries regress (33). The doses of 25 and 75 μg/Kg BW were selected based on our single injection treatments that showed a dose dependent plasma Lh stimulation in the range of 50–100 and 5–25 ng/Kg BW for Kiss1 and Kiss2, respectively (9). Five groups of 8–12 females at previtellogenic stages (GSI: 0.51 ± 0.10%, average oocyte diameter: 170 μm) were treated with implants containing no peptide as a control group, 25 μg or 75 μg / Kg body weight (BW) of Kiss1or Kiss2 (referred to hereafter as: Kiss1–25, Kiss1–75, Kiss2–25 and Kiss2–75, respectively). Fish were reimplanted every three-four weeks, during which blood was collected at each treatment point. Ten weeks after first implantation, the fish werebled, sacrificed, gonado-somatic index (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 FShα (37) for the standard curves. The wells were coated with recombinant FShβ, and the antibodies were diluted 1:50,000. The intra-assay 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, 2, 3, Lh and FSh transcript levels, brains and pituitary total RNA (1 μg) extracted using Trizol reagent (Invitrogen), was reverse-transcribed by Quantitect RT kit (Qagen). 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 TÅQ 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 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 59–61°C. Proper and specific amplification was verified using gel electrophoresis and by the dissociation curve of the primer sets. In each run, two negative water controls and a reference control, added to each plate, were included in addition to the standard curve.

In situ hybridization and immunohistochemistry 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 IPTG in E.coli Rosetta-gami B(DE3)pLysS and stored at –80°C. In situ hybridization (ISH), which followed the protocol described earlier (9), immunohistochemistry (IHC) or combined ISH/IHC, all of which used Tyramide Signal Amplification kit (TSA, Perkin Elmer), according to the manufacturer’s protocol. Anti-DIG HRP (Roche) was used to detect Dig-labeled probe (kiss1, kiss2 of both stripped fish 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 GAP (9, 42, 43) and antistriped bass G-15 desalting columns (Pharmacia) and then used for antiserum production in rabbits (ProteinTech Group, USA). The final bleed-antiserum was used in all immuno-staining.

**Brain histology**

Stripped 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 spermiating and females carry ‘green’ ovaries (9–15 hours prior to ovulation) with GSI values of 13%-14% for females and ~10% for males, 4) and postovulatory (spent) females within a few days postspawning (n = 3 pairs) or the next morning at the initiation of spawning following 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. In situ hybridization (ISH), which followed the protocol described earlier (9), immunohistochemistry (IHC) or combined ISH/IHC, all of which used Tyramide Signal Amplification kit (TSA, Perkin Elmer), according to the manufacturer’s protocol. Anti-DIG HRP (Roche) was used to detect Dig-labeled probe (kiss1, kiss2 of both stripped fish 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 GAP (9, 42, 43) and antistriped bass G-15 desalting columns (Pharmacia) and then used for antiserum production in rabbits (ProteinTech Group, USA). The final bleed-antiserum was used in all immuno-staining.

**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 compared the treatments and also each treatment as

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**Table 1.** Degenerate and gene-specific primers used for RT-PCR and Real Time Quantitative PCR

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<th>Sequence 5’ to 3’</th>
<th>Direction</th>
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a factor of time using 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 mRNA 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 QRT-PCR as described earlier (9). Kiss1–25 had no effect on any of the $gnrh$ and $gnrh3$ receptor expression while 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 to 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.

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 immediately before implantation. FSh plasma levels in the control and in the two Kiss1 treated groups displayed a specific pattern of a marked 3 fold increase only at the 6 weeks point (sampled in Dec). 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.

Figure 2. The effect of chronic treatment of Kiss1 and Kiss2 on plasma FSh 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 treated with EVAc implants containing: A) no peptide (n = 8), B) pyroGluKiss1–15, 25 μg/Kg BW (n = 11), C) pyroGluKiss1–15, 75 μg/Kg BW (n = 12), D) Kiss2–12, 25 μg/Kg BW (n = 12), and E) 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 timepoint (similar treatments are circled). 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 $* = P \leq .05$, **$= P \leq .01$, ***$= P \leq .005$. 

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during the experiment, except for a significant decrease to 37 ± 7% of the control in the Kiss2–75 treatment at the 10 weeks point (Figure 2B).

No significant difference was observed in the gonadosomatic index (GSI) values between the different groups at the end of the experiment (not shown) but oocyte diameter increased from 120 µm to 220 µm in the control group and to 290 µm in the Kiss1-25 treated group (Figure 2C). About 75% of the Kiss1–25 females had a more advanced stage of oocyte development including several larger oocytes (>230 mm) with numerous lipid droplets (Fig. 2Ca).

**Stage and sex-related Kiss1 and Kiss2 presence in the nucleus lateralis tuberis**

**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 a ~13 kDa 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 (Supplementary Figures 1–4). 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, 4Ab and S 2–4).

**Histology** - Using ISH and IHC, we detected the appearance of neurons expressing Kiss1 in the nucleus lateralis tuberis medialis (NLTm) and NLT lateralis (NLTl) of all prespawning males and females (IHC-Figures 3Aa, B, ISH- Figures 3C, D). These neurons were not observed in any fish at other stages such as recrudescent male (Fig. 3Ab), recrudescent female (Fig. 3Bb) or spent female (Fig. 3Bc). Using ISH, kiss1 neuronal somas are detected in the NLTm of all prespawning males and females (Fig 3Ca and 3 Da) and also in the NLTl of females (Fig. 3Db).

Kiss2 staining is detected only by IHC in the NLTl region of all males at all stages: prespawning (Fig. 4Aa), precociously spermiating (Fig. 4Ac) and juvenile (Fig. 4Ad), suggesting that bundles of axons reach this region. Mature prespawning males display kiss2 somas also in the NLTm (IHC-Fig. 4Aa, ISH-Fig. 4Ca). In females, however, kiss2 neurons are observed by both methods in the NLTm (Figs. 4Ba and 4Da) and in the NLTl (IHC-Fig. 4Ba, ISH-Fig. 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 NRL region of both sexes at all stages and are detected by the anti-Kiss2 serum. These neurons send axonal projections down the NLT ventrals along the third ventricle towards the NLTm (Fig. 4Ae).

**Zebrafish**

kiss1 and kiss2 neurons were detected by ISH in brains of 18 hours prespawning and spawning zebrafish males and females. Kiss1-expressing neurons were detected only in the extrahypothalamic habenula (not shown). Kiss2 neuronal bodies were detected, as expected, in the dorsal zone of periventricular hypothalamus (Hd) 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.
(Figures 5C and F). Brain sections of 18 hours prespawning fish lacked kiss2 in the Hv (Figures 5, A-B, C-D). Comparison of the sections of the striped bass and zebrafish depicting the NLT and Hv, respectively, clearly show that these are two equivalent regions, that are referred to by different names in the two corresponding atlases used for our neuronatomical studies (44, 45).

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

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

Kiss2 staining is found throughout the entire NH in proximity to the gonadotropes (Figures 7A, B, 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 7H, 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, 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 peptide in the body and eliminated the need for frequent injections. The pyroGlu-Kiss1–15 and Kiss2–12 peptides, more likely occur naturally as is deduced from the predicted cleavage sites (9), and were shown to be more potent in activating their cognate receptors than the...
commonly used Kiss-10 decapeptides (4).

Kisspeptin effect on the ovaries

In accordance with our previous results with single Kiss injections (9), Kiss1 and Kiss2 demonstrated different effects: only Kiss1 at a dose of 25 μg/Kg BW advanced oocyte growth by ~50% over the control group, characterized by secondary growth oocytes that are typically seen two months later in the reproductive season. Treatment with the higher dose of Kiss1 (75 μg/Kg BW) and both Kiss2 doses did not affect the gonads. Similarly, chronic Kiss1 treatment of immature males advanced gonadal development in Scomber japonicus and Seriola lalandi (7, 14, 48). Interestingly, opposite effects were re-

Figure 5. Kiss2 neuronal distribution in male and female zebrafish before and at spawning. In situ hybridization using kiss2 antisense riboprobe on females (A-C) and males (D-E) medio-basal hypothalamic sections. A) female brain 18 hours before spawning, B) higher magnification of Plate A focusing on the HV region, C) female brain at spawning, D) male brain 18 hours before spawning, D) higher magnification of Plate D focusing on the Hv region, F) male brain at spawning. G) Upper panel illustration of 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 – sagittal whole brain illustration demonstrating the anatomical location of the presented cross-section (illustrations were adopted from (45)). Hd – dorsal zone of periventricular hypothalamus, ATN – anterior tuberal nucleus, Hv- ventral zone of hypothalamic nucleus. Scale bars = 100 μm.

Figure 6. Immunohistochemistry analysis of Kiss1 axonal distribution in the pituitary/neurohypophysis of males and females at different reproductive stages. Plates 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) preimmune sera of preovulatory female- 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, PPD- proximal pars distalis. Kiss1 – red, Lh/Fsh- green. Scale bars = 100 μm.
ported in *Morone* spp. for chronic administration of Kiss2 and Kiss1 decapeptides (250 μg/Kg BW), which was associated with a reduction in gonad size with Kiss1–10 treatment (6). The differences may be attributed to the 10 times higher dose of Kiss1. The gonadal stimulatory effect of kiss1 corroborates other studies in immature fish and underscores the importance of kiss1 in puberty. Nevertheless, the GSI levels and oocyte size remained small with all treatments implying that none of the treatments triggered puberty.

**Kisspeptin effects on gnrh’s and gnrh receptor**

Kiss2 had a general down-regulating effect on the expression of the three 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 post-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 timepoint (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 4h post in-

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**Figure 7.** Immunohistochemistry analysis of Kiss2 axonal distribution in the pituitary/neurohypophysis of male and female at different reproductive stages. A-F, red- kiss2 and green- Lh. G, red-Kiss2, green FSh (detected by ISH), I-H, red- Kiss2, green-GnRH1. A. preovulatory female- upper and lower NH regions. B. postovulatory (spent) female, C. recrudescent-vitellogenic female, D. prespawning male, E. recrudescent male. F. juvenile male. G. Kiss2 axon terminals penetrating the PPD and are near FSh gonadotropes (arrows) in the pituitary of a preovulatory female, H. double labeling of Kiss2 axons (red) and GnRH1 (green) in the neurohypophysis of prespawning male demonstrating the massive presence of both types in the neurohypophysis, I. Higher magnification of the squared region in H clearly show the interactions between kiss2 and GnRH1 axons. NH- neurohypophysis, PPD- proximal pars distalis. Scale bars = 100 μm.
jection in the 8.8 and 44 μg/Kg BW and were attenuated in the 88 μg/Kg BW (Fig. S5). 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 Kiss-1 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 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 Lh- and Fsh- beta subunits early gene expression in Lβl2 cells and murine gonadotropes in vitro (53). Although steroids do not directly affect fshβ expression in the pituitary of recrudescent 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. Since 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 RF-amide 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 utilize to interact with the pituitary, we examined the neuro-anatomical dynamic of the two 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 two 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 two optional modes for the kiss1 neurons: acting on gnrh projections enroute 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 tracking axon terminals to their destination in the adenohypophysis. The specificity of the anti-Kiss1 and –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 ERα 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 while 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 happens 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 NLTl, 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 NLT originate from the nucleus recessus lateralis (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

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the projections in the NLT of females at the non prespawning 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 suggests 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 POA, as was shown in zebrafish (13), but also as they reach the pituitary. In fact, the arcuate nucleus Kiss1 neurons in mammals are known to serve also as the pace-makers 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 males gonadal development, particularly in spermiation. Thus, our observation provides the first evidence that the kiss system dynamically regulates the HPG axis also in males. In order to substantiate our hypothesis that this is a wide-spread 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 hours 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 suggest that these neurons also innervate the pituitary. The dual presence of the two 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 since, 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 prior to 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 predominately in males. A schematic illustration of the dynamics of the kisspeptin neurons before and immediately prior to spawning is presented in Figure 8. Altogether, Kiss2 that originates from the NRL probably acts on gnrh1 innervations in the NLT and NH, while 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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References


A-Male

B-Female

Figure 8. Schematic illustration summarizing the changes in the distribution of kiss1 and kiss2 neurons and their projections in the NLT and NH before and immediately before spawning. A. Male, B. Female. Gray circle and lines denote kiss1 neuronal soma and axons, respectively. The two hemispheres of the brain (the optic tectum above the hypothalamus is not shown) are divided by a gray dashed line in which the hemisphere on the left represents reproductive stages before the prespawning phase (Pre P-S) and the hemisphere on the right represents the prespawning stage (P-S). Black stars represent either neuronal soma or axon terminal bundles, black lines denote Kiss2 axonal projections. NLTm - nucleus lateralis tuberis, pars medialis, NLTl - nucleus lateralis tuberis pars lateralis, NLTv - nucleus laeralis tuberis, pars ventralis, P – pituitary.


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