Neurokinin B regulates reproduction via inhibition of kisspeptin in a teleost, the striped bass

Nilli Zmora¹, Ten-Tsao Wong¹, John Stubblefield¹, Berta Levavi-Sivan² and Yonathan Zohar¹

¹Department of Marine Biotechnology, Institute of Marine and Environmental Technology, University of Maryland Baltimore County, Baltimore, Maryland, USA
²Department of Animal Sciences, Faculty of Agriculture, Food and Environment, The Hebrew University, Rehobot, Israel

Abstract

Kisspeptin and neurokinin B (NKB) are neuropeptides co-expressed in the mammalian hypothalamus and coordinately control GnRH signaling. We have found that Nkb and kisspeptin neurons are distinct in the teleost, striped bass (STB) and capitalized on this phenomenon to study the mode of action of Nkb and its related neuropeptide-F (Nkf), both of which are encoded by the tac3 gene. In vitro brain slices and in vivo administration studies revealed that Nkb/f consistently downregulated kiss2, whereas antagonist (AntD) administration restored this effect. Overall, a minor effect was noted on gnrh1 expression, whereas Gnhr1 content in the pituitaries was reduced after Nkb/f treatment and increased with AntD. Concomitantly, immunostaining demonstrated that hypothalamic Nkb neurons border and densely innervate the largest kiss2 neuronal population in the hypothalamus, which also coexpresses Nkb receptor. No expression of Nkb receptor or Nkb neuronal projections was detected near/in Gnrh1 soma in the preoptic area. At the level of the pituitary, however, the picture was more complex: both Nkb/f and AntD upregulated lhb and fshb expression and Lh secretion in vivo. Together with the stimulatory effect of Nkb/f on Lh/Fsh secretion from pituitary cells, in vitro, this may indicate an additional independent action of Nkb/f within the pituitary, in which the hypothalamic pathway is more dominant. The current study demonstrates that Nkb/f utilizes multiple pathways to regulate reproduction in the STB and that in the brain, Nkb mainly acts as a negative modulator of kiss2 to regulate the release of Gnrh1.

Introduction

Reproduction is a fundamental process, tightly regulated by central and peripheral factors at the brain/hypothalamus, pituitary and gonad (HPG) levels. The principal components of the HPG axis have long been described in many vertebrate species, for which the hypothalamic gonadotropin-releasing-hormone (GnRH) is pivotal to the regulation of reproduction. During the past decade, numerous known and novel neuropeptides have been implicated in the upstream control of GnRH neurons and gonadotropes in the pituitary.

Neurokinin B (NKB) has recently emerged as an important component of the network controlling reproductive function. Similar to the KISS1/GPR54 system, the crucial reproductive role of NKB was discovered via a
null mutation in the gene encoding NKB (TAC3 in humans and Tac2 in rodents) or its receptor (TAC3r) encoded by Tac3r gene, in hypogonadotropic hypogonadal human patients and infertile mice (Topaloglu et al. 2009, Yang et al. 2012). In mammals, NKB is coexpressed with dynorphin-A in a subset of Kiss1 neurons in the arcuate nucleus (ARC), also termed KNDy neurons (Goodman et al. 2007, Navarro et al. 2009, Lehman et al. 2010, Wakabayashi et al. 2010). Together, these three neuropeptides are believed to generate and pace the GnRH pulses via a sex steroid-dependent mechanism (Navarro 2012). Although the stimulatory effect of KISS1 (Navarro et al. 2009, 2011a, 2012, Wakabayashi et al. 2010, Garcia-Galiano et al. 2012) and the inhibitory effect of dynorphin-A (Gallo 1990, Grachev et al. 2014) on the GnRH pulse are widely acknowledged, the effect of NKB remains controversial as studies have reported both NKB inhibition and stimulation of the reproductive axis (Sandoval-Guzman & Rance 2004, Navarro et al. 2011a, Garcia-Galiano et al. 2012, Grachev et al. 2012a, Kinsey-Jones et al. 2012, Ruiz-Pino et al. 2014). However, despite the contradictory findings in mammals (Krajewski et al. 2005, Todman et al. 2005, Burke et al. 2006a, Amsteldam et al. 2009, Navarro et al. 2011b), the emerging notion is that NKB does not act directly on GnRH neurons (Navarro 2012). This is supported by the observation that senktide (NKB agonist) treatment did not trigger action potentials in GFP-labeled GnRH neurons of mouse (Navarro et al. 2011b). A growing body of recent data from GRPR54-knockout mice (Garcia-Galiano et al. 2012), rats (Grachev et al. 2012b) and monkeys (Ramaswamy et al. 2011) suggests that NKB affects the reproductive axis via kisspeptin and that kisspeptin is essential for NKB signaling.

Even more confusing are the sparse reports on the involvement of NKB in regulating reproduction in teleosts. The first indication that Nkb is widely expressed in fish species and has a stimulatory reproductive effect was shown in zebrafish and goldfish (Ogawa et al. 2012, Qi et al. 2015). Teleost tac3 gene encodes two peptides, Nkb- and Nkb-related peptide, also termed neurokinin F (Nkf) (Biran et al. 2012). Subsequent studies have shown that Nkb acts at the level of the pituitary (Biran et al. 2014, Hu et al. 2014), but very little is known regarding the effect of Nkb at the level of the brain and its relationships with GnRH and kisspeptin neurons in the context of reproduction in teleosts.

In this study, we localized Tac3 and Tac3r neurons in male striped bass (STB), Morone saxatilis, reproductively relevant brain regions and showed that, unlike in mammals, Tac3 is not localized in kisspeptin neurons. This feature helped answer questions regarding the interactions between Nkb and kisspeptin neurons. The relationships of Tac3, kisspeptin and GnRH1 neurons were examined neuro-anatomically. The effect of Nkb and Nkf on GnRH1 and kisspeptin was studied both in vivo and in vitro. Our results establish the role of Nkb as a direct negative modulator of Kiss2, but not of GnRH1, in the hypothalamus.

**Materials and methods**

**Animals**

Animal maintenance and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine IACUC #0613018 and #0516021. STB were obtained as juveniles from the Maryland Department of Natural Resources and maintained at ambient conditions in a 2.5 m³ tank supplied with constant exchange of artificial 8–10 ppt seawater. Fish were anesthetized for ~10min in water containing 200mg/L tricaine methanesulfonate (MS-222) (Sigma) before killing or treatment. Treated fish were immediately transferred to clean water for recovery.

**Peptides**

The full cDNA of tac3 and tac3r genes of the STB were cloned from STB brain cDNA library (Supplementary material 1, see section on supplementary data given at the end of this article) and deposited in GenBank (accession #: KT361626 and KT361627). Only one variant for each gene was detected. The deduced STB Nkb ((pGLU) MHDIFIGLM-NH₂) and neurokinin-related peptide (Nkf: YSDLDYDSFVGLM-NH₂) and mammalian Nkb antagonists (AntD: (d-Pro2, d-Trp6,8, Nleu10)-neurokinin B or DPDTNLNE-NB: (Asp-d-Pro-His-Asp-Phe-d-Trp-Val-d-Trp-Leu-Nle-NH₂)) (Jacoby et al. 1986) peptides were synthesized at >95% purity (Genscript). The authenticity of the peptides was confirmed by mass spectrometry. Other screened antagonists: SB222200 was obtained from Santa Cruz Biotechnology and GR159897 from Tocris Biosciences. R486 (Asp-Ser-Phe-Trp-P-Ala-Leu-Met-NH2) (Almeida et al. 2004), neurokinin A (NKA) and substance P (SP) and NK1 antagonist, 3,5-bis(trifluoromethyl)benzyl N-acetyltryptophan were purchased from Sigma.
Receptor activation and antagonist selection using in vitro luciferase reporter gene assays

To generate the activation profiles of the neurokinin B receptor (Tac3r) by Nkb and Nkf, we employed a luciferase (LUC) reporter gene assay using LUC transcriptionally regulated by either a serum response element (SRE; Invitrogen) or a cyclic AMP (cAMP) response element (CRE; Invitrogen). STB tac3r was cloned into pcDNA3.1 expression vector (Zeo; Invitrogen) driven by the cytomegalovirus (CMV) promoter. Transient transfection, cell procedures and stimulation protocols followed the procedures previously described (Deoraj et al. 2000, Alok et al. 2001, Levavi-Sivan et al. 2005, 2010, Biran et al. 2008). COS7 cells were co-transfected with tac3r (30ng/well), a reporter plasmid (30ng/well), and pCMV-Renilla luciferase as transfection control (Promega). Nkb and Nkf peptides were used to generate an initial dose–response and to determine the signal transduction pathway in 10× serial dilutions from 1 fM to 10μM (in triplicate). EC50 values were calculated via analysis by a 3-parameter response curve formula using GraphPad Prism software from 3 individual trials.

Peptides and non-peptide molecules known to antagonize mammalian NK3R were tested for their ability to antagonize Tac3r of the STB, which was activated with a fixed dose of 1 nM Nkb or 0.1 nM Nkf combined with graded doses of the antagonists. The antagonists were added ~30min prior to the addition of Nkb/f peptides. IC50 values were extrapolated by Sigmoidal 4PL analysis using GraphPad Prism software.

Brain and pituitary incubation assays in vitro

Nkb, Nkf and AntD activities were further examined in STB brains and pituitaries using brain slices and primary pituitary culture assays, respectively (Klenke 2006). Brains and pituitaries of precociously spermiating mature males collected during the reproductive season (February through March) were used, mainly due to the relatively long period of this stage that allowed the conduction of multiple rounds of trials.

Brain slices Whole brains were sliced to 300um slices using a Mcllwain Tissue Slicer. Slices were placed in 74μm mesh inserts in a six-well plate, containing 4 mL of glucose DMEM at pH 7.44, followed by 3× 1-h washes, and then incubated with medium containing the tested peptides for six hours at 20°C with gentle agitation. Tissues and medium were then stored frozen until further analysis. RNA was extracted from the brains, and its quality and integrity were verified in roughly 25% of the samples via denaturing paraformaldehde agarose gel electrophoresis. cDNAs were prepared and followed by quantitative RT-PCR as described below.

Primary pituitary culture The culture procedure was carried out under aseptic conditions. Pituitaries were grossly chopped and cells were dispersed with 1 mL 0.25% trypsin–EDTA in DMEM (Gibco) for 5 min at room temperature, during which the medium was run through a 1 mL syringe equipped with 18- or 21-gauge needles. The reaction was stopped by the addition of 1 mL fetal calf serum. The cells were counted using a hemocytometer and populated at a density of 50,000 or 25,000 cells per well in 24 or 48-well plates, respectively. The cells were incubated for three days at 20°C and then incubated with serum-free medium (with 0.5% BSA) containing the tested peptides for 24h. The cells and media were collected separately and stored frozen until analysis.

Treatment of male STB with Nkb, Nkf and AntD in vivo

Nkb and Nkf injection study Precociously spermatizing males sampled in mid-December (n=7; average body weight±s.d.: 683±153g; gonad somatic index (GSI)=6.6) were treated with single intramuscular injection (between the gills cover and the peritoneum) of Nkb or Nkf peptides in saline at doses of 0, 10 or 100 µg/kg body weight (BW). Blood was sampled immediately before and at 2, 4 and 24 h after injection. Fish were killed by decapitation after 24 h, and brains, pituitaries and gonads were sampled for gene expression and ELISA assays of various components along the reproductive axis and for gonadal histology.

Nkb and Kiss antagonists treatment studies (1) Spermatizing males at the time of spawning in mid-April, (n=8; average BW±s.d.: =355 ± 110g; GSI=10.2) were injected intramuscularly with poly(D,L-lactide-co-glycolide) 50:50 (PLGA, Sigma) (Mylonas & Zohar 2001) slow-release microspheres containing AntD at doses of 0,
5 or 50 μg/kg BW. The experiment was concluded 10 days after implantation. (2) A similar 9-day experiment was conducted with microspheres containing the kisspeptin antagonists, pep 234 or pep 359, at doses of 0, 5 or 30 μg/kg BW (Zmora et al. 2015).

Blood was sampled immediately prior to implantation and at the trial termination for Lh and Fsh plasma levels measurements. Sperm from each fish was collected at the end of the experiment to determine milt volume and weight. Brains and pituitaries were collected and frozen.

Hormones and gene transcript measurements

Lh levels in the plasma were measured using ELISA as previously described (Mananos et al. 1997). Levels of Fsh were measured using a specific ELISA developed for tilapia Fsh (Aizen et al. 2007) and validated for use in

**Table 1** Gene-specific primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5′–3′</th>
<th>Direction</th>
<th>GeneBank accession #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tac3F</td>
<td>GATCCGAACGGATCT-CACTGCA</td>
<td>F</td>
<td>KT361626</td>
</tr>
<tr>
<td>Tac3R</td>
<td>CAGCTGGTAGATCT-TAGTCTGACG</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>Tac3recF1</td>
<td>TGTGGACAGGTACATGCG-CATCAT</td>
<td>F</td>
<td>KT361627</td>
</tr>
<tr>
<td>Tac3recF2</td>
<td>CCTGAAGCCTGCTTGCTGTC</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>Tac3recR</td>
<td>CACGGACAGGTAGCAGAGG</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>TAQgnrh1F</td>
<td>GGAACGGACGGCCCTCTCA</td>
<td>F</td>
<td>AF056314</td>
</tr>
<tr>
<td>TAQgnrh1R</td>
<td>GTGAGAAGGGCCCCCGACTA</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>TAQFSHbF</td>
<td>TGGGCTACAGGGGCTCAT</td>
<td>F</td>
<td>L35070</td>
</tr>
<tr>
<td>TAQFSHbR</td>
<td>CAGTCTCCGTGAGATATGAGGTTGCAT</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>TAQLHbF</td>
<td>CTTGGGACAGCCCTCCTCTCTGC</td>
<td>F</td>
<td>L35096</td>
</tr>
<tr>
<td>TAQLHbR</td>
<td>CTTGGGACACATCTGACAT</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>TAQTac3F</td>
<td>TGTGCTGATGACCAACGCTG-TAGATGC</td>
<td>F</td>
<td>KT361626</td>
</tr>
<tr>
<td>TAQTac3R</td>
<td>CAGGGACATTACAGGGCTGAGT</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>TAQKiss1F</td>
<td>CAGATGCCAGACGTAGTCTTGAG</td>
<td>F</td>
<td>GU351864</td>
</tr>
<tr>
<td>TAQKiss1R</td>
<td>CTATCTCCTTACTGGGGTTAGCTGAGTTT</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>TAQKiss2F</td>
<td>CGGAGCTGCTGCTGCA</td>
<td>F</td>
<td>GU351865</td>
</tr>
<tr>
<td>TAQKiss2R</td>
<td>GCCCTTCTGAAAAGGTAGC- GCTGTTT</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>TAQEF1xF</td>
<td>GAGTAGGAAGACGTGCTGAGCT</td>
<td>F</td>
<td>AJ866727</td>
</tr>
<tr>
<td>TAQEF1xR</td>
<td>GGGGGCCTGGCTGAAGGCTAAG</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>TAQ18SF</td>
<td>ACCCCCACAGAATATGAGAAA</td>
<td>R</td>
<td>AF147741</td>
</tr>
<tr>
<td>TAQ18SR</td>
<td>GCCCTGCGCCTTAAATTGACT</td>
<td>R</td>
<td></td>
</tr>
</tbody>
</table>

In situ hybridization and immunohistochemistry

Brains of precociously spermatizing males were removed and fixed in buffered 4% paraformaldehyde and cryo-protected in 15% sucrose overnight at 4°C and embedded in Tissue Tek OCT (Electron Microscopy Sciences). Sections of 12 μm were mounted onto Plus glass slides and stored at −80°C. In situ hybridization (ISH) and immunohistochemistry (IHC) were conducted using a Tyramide Signal Amplification Kit (TSA, Perkin Elmer) as described earlier (Zmora et al. 2012, 2014). Anti-Dig HRP (Roche) was used to detect Dig-labeled probe encompassing the entire coding region of each gene. HRP activity between the different stainings was quenched with 0.2 M HCl for 10 min. Anti-Kiss2, anti-seabass GnrH1-associated peptide (GAP) (Gonzalez-Martinez et al. 2002, Zmora et al. 2002, 2012) primary serums, as well as the goat anti-rabbit horseradish peroxidase secondary antibody (Lonzia, USA), were all diluted 1:1000. Anti-mammalian NKB (Phoenix Pharmaceuticals, Inc, cat# H-046-26) were diluted 1:100. Sense tac3 RNA and anti-Nkb was preadsorbed with STB Nkb, and Nkb produced no signal (Supplementary Fig. 2).
Statistical analysis

Statistical analyses were performed by one-way ANOVA and Tukey’s post hoc test for multiple comparisons, using Instat3 (GraphPad). Lh and Fsh plasma levels statistical analysis included one-way ANOVA, or two-way ANOVA for repeated measures, followed by Tukey HSD. Statistical difference was accepted when $P \leq 0.05$.

Results

Nkb and Nkf activation of Tac3r and inhibition by Nkb antagonist

As a first step, dose–response activation curves of Tac3r by Nkb and Nkf were generated via SRE or CRE, responsive elements that drive the PKC and PKA pathways, respectively, and ultimately transcribe the firefly luciferase gene. The PKA/CRE and PKC/SRE reporter systems both generated dose–response curves, albeit with different activation capacities for each ligand. Using the PKA pathway, Nkb was ~4.5 times more potent than Nkf, having an EC$\text{50}$ value of 3.5 pM vs an EC$\text{50}$ of 76 pM for NKF ($n=3$) (Fig. 1A). Using the PKC pathway, Nkb and Nkf showed similar EC$\text{50}$ values of 4.6 pM and 4.3 pM, respectively ($n=3$) (Fig. 1B). The maximal activation level mediated by PKA/CRE was 2 times higher than that of the PKC/SRE, suggesting that PKA is the dominant pathway for Nkb. Based on the EC$\text{50}$ values, Tac3r showed a 4–100 times higher preference to Nkb over NKA and SP ($n=2$) (Fig. 1C and D).

Six different neurokinin antagonists were tested by combining serial doses of each antagonist with 1 nM Nkb or Nkf (Supplementary Fig. 1 depicts the five antagonists that did not exhibit antagonistic activity). Of the six antagonists, only the peptide Asp-dPro-His-Asp-Phe-dTrp-Val-dTrp-Leu-Nle-NH2 (AntD) displayed a clear antagonistic activity. Starting at a dose of 10 pM, AntD reduced the activation of 1 nM Nkb by 75% and a starting dose of 1 nM significantly reduced that of 0.1 nM Nkf by 40% ($n=3$) (Fig. 1E and F).

![Figure 1](https://www.bioscientifica.com)
The effect of Nkb and Nkf on the expression of reproduction-related neuropeptides and gonadotropins in vivo

The effect of a single injection of Nkb and Nkf on reproduction-related factors along the HPG in precociously spermatizing males (mid-December) was examined. Although Nkb and Nkf peptides reduced kiss1 and kiss2 mRNA levels by 40–70% of control (Fig. 2C and D), the effect of Nkb at 100 µg/kg BW was not significant. gnrh1 mRNA levels did not change after the treatments (Fig. 2A), but Gnrh1 pituitary content was significantly reduced by 10 µg/kg BW Nkb and more profoundly (~75% reduction) by both doses of Nkf (Fig. 2B). Nkb at the two doses upregulated ldb by ~250%, whereas Nkf upregulated the levels of fshb by 350–400% of control (Fig. 2E). Plasma Lh levels in the control group at 2, 4 and 24 h after treatment were significantly lower than those of 0 time point (10:00 h, probably due to diurnal changes, as seen earlier (Zmora et al. 2012)) and Nkb/Nkf treatment prevented this decrease (Fig. 2F), whereas Fsh levels were unchanged (Supplementary Fig. 3).

The effect of Nkb and Nkf on the expression of reproduction-related neuropeptides in the brain, in vitro

Two sets of experiments were conducted on precociously spermatizing males. In the first set, whole brain slices were incubated with graded doses of Nkb or Nkf (from 10 pM to 10 nM), and in the second, 10 nM AntD was co-incubated with 10 nM Nkb or Nkf. The effect of Nkb, Nkf and AntD, alone or in combination, was determined via qPCR. gnrh1: Of the 4 tested doses, only the higher dose of 10 nM Nkb and Nkf upregulated the expression of gnrh1 by 300% and 180% of the control levels, respectively (Fig. 3A). In the second experiment, no such increase was obtained with 10 nM Nkb or Nkf, with or without AntD (Fig. 3B). kiss2 and kiss1: Nkb and Nkf downregulated kiss2 expression to 40–50% of control, starting at a dose as low as 10 pM (Fig. 3C). Nkb downregulated kiss1 mRNA levels starting at 100 pM. The effect of Nkf on kiss1 did not produce a typical dose-response (Fig. 3E). AntD restored the decreased mRNA levels of kiss2 when incubated with Nkb or Nkf (Fig. 3D). No change in kiss1 mRNA levels was noted in the second experiment (Fig. 3F). AntD alone had no effect on the expression of either kisspeptin form.

Figure 2
The effect of single injection of Nkb and Nkf on neuropeptides and gonadotropins in vivo. Nkb and Nkf peptides at doses of 0 (Cont), 10 or 100 µg/kg BW were injected into precociously spermatizing males intraperitoneally (n = 7 per group). Samples were taken 24 h after injection (A, B, C, D and E). Transcript levels in the brains were measured using qRT-PCR of (A) gnrh1, (C) kiss1 and (D) kiss2. (B) Gnrh1 decapeptide content in the pituitaries of treated males was measured using ELISA. (E) Transcript levels of lhh (black bars) and fsh (white bars) in the pituitaries. (F) Lh levels in the blood sampled immediately prior to injection and 2, 4 and 24 h after injection. Statistical analysis included one-way ANOVA (A, B, C, D and E) or two-way ANOVA for repeated measures (F) followed by Tukey HSD. Results are presented as mean ± s.e.m. of either absolute quantities or % of control calculated from the absolute copy number of mRNA or absolute numbers. Statistical difference was accepted when P ≤ 0.05 compared to control levels. *P ≤ 0.05, **P ≤ 0.01.
The effect of Nkb and Nkf on Lh and Fsh secretion from pituitary cells in vitro

Lh levels in the medium were augmented with 1 and 10 nM Nkb and 10 nM Nkf by 200% and 250% of control, respectively (Fig. 4A). Fsh levels in the medium increased in a dose-dependent manner starting at 0.1 nM by 2- and 5-fold, respectively (Fig. 4B). No change was recorded in lhb or fshb transcript levels (Fig. 4C and D).

The effect of AntD on the HPG axis in vivo

To test whether Nkb/Nkf is crucial to the spawning process, spermiating STB males, at the time of spawning (mid-April), were treated with microspheric implants at doses of 0, 5 or 50 µg/kg BW AntD for 10 days. AntD, at 50 µg/kg BW, upregulated kiss2 mRNA levels by ~3 times the control (Fig. 5A) and increased Gnrh1 peptide content in the pituitary by ~40% compared to that in control levels (Fig. 5D). However, AntD had no effect on the expression of gnrh1 and kiss1 (Fig. 5B and C). A notable increase in the expression of lhb and fshb was observed in both AntD treatments (Fig. 6A). Although the increase in lhb mRNA levels ranged between 2 and 4 times, fshb mRNA displayed a marked increase of 10 times the control levels (Fig. 6B). Lh and Fsh plasma levels were measured immediately before and 10 days after treatment. Although Lh levels significantly increased after 10 days in all treatments, levels in the AntD-treated fish did not differ from those of control fish (Fig. 6C). Fsh levels of the AntD-treated fish were significantly higher than those of control fish at the 10-day point (analyzed by one-way ANOVA) and also compared to the 0 time point (analyzed by paired t-test) (Fig. 6D).

No difference in milt weight was observed with any of the AntD treatments (Fig. 6E).

The effect of Kiss1, Kiss2 and Kiss antagonists on tac3 expression

The effect of kisspeptin on Tac3 was tested in two ways: (1) brain slices from precocious spermiating males were incubated with either Kiss1 or Kiss2 peptides at doses of
Nkb directly moderates Kiss2 in fish brain

0.5–10 nM, no effect on tac3 transcript levels was observed (Supplementary Fig. 4A); (2) when kisspeptin antagonists (Pep234 and Pep359) at doses of 5 and 30µg/kg BW were administered via slow-release implants to spermatizing males, no changes in tac3 mRNA levels were observed (Supplementary Fig. 4B) despite the dramatic decrease in sperm production (Zmora et al. 2015).

Brain distribution and co-localization of Tac3 and Tac3r neurons

Tac3 neuronal distribution in the brain was determined by ISH and IHC. Tac3 neurons are found in the telencephalon, in the dorsal habenula and preoptic area (Fig. 7A, B and C) and in the hypothalamic anterior tuberal nucleus (NAT) (Fig. 7D, E and F).

In the hypothalamus, Tac3 neurons in the NAT strongly innervated proximal Kiss2 neurons in the dorsal and ventral nucleus recessus lateralis (NRL) (Fig. 8E), which in turn express tac3r (Fig. 8A, B and C). Kiss2 neuronal projections can be found around Tac3 neurons in the NAT but are relatively sparse (Fig. 8F and G).

Neurons expressing tac3 mRNA in the nucleus preopticus magnocellularis and pars parvocellularis (PMpc) are not innervated by Gnrh1 fibers (Fig. 9A). However, a few Nkb-ir fibers are observed in the vicinity of Gnrh1 somas in the nucleus preopticus parvocellularis, pars anteroventralis (NPOav) (Fig. 9B). Gnrh1 somas

Figure 4
The effects of Nkb and Nkf on Lh and Fsh in pituitary cells in vitro. Primary pituitary cells of precociously spermatizing males were treated with 0.1, 1 or 10 nM Nkb (black bars) or Nkf (grey bars) for 24h. (A) Lh levels in medium, (B) Fsh levels in medium, (C) lhb mRNA levels, (D) fshb mRNA levels. Results are presented as mean ± s.e.m. of absolute ng/mL for protein levels in the medium. Statistical difference was accepted when P ≤ 0.05 compared to control levels. *P ≤ 0.05, **P ≤ 0.01.

Figure 5
The effect of antagonist D on gnrh1 and kiss expression in the brain and Gnrh1 pituitary content in vivo. Fully spermatizing male STB in the spawning season were treated with implants sustainably releasing AntD at doses of 0, 5 or 50 µg/kg BW (n=8 per group). The effect of the treatment on the expression of (A) kiss1, (B) kiss2 and (C) gnrh1 was measured using qRT-PCR after 10 days and compared to that of the control fish. (D) Gnrh1 content in the pituitaries of the same fish was measured using a Gnrh1 decapeptide ELISA. Results are presented as mean ± s.e.m. Statistical difference was accepted when P ≤ 0.05 compared to control treatment levels. *P ≤ 0.05, **P ≤ 0.01.
do not express tap3r, whose expression is observed in neurons in the nucleus anteriors periventricularis (NAPv) and in the pituitary (Fig. 9D and E). Nkb neuronal projections innervate the pituitary and are detected in the neurohypophysis as well as Nkb-positive cells in the proximal pars distalis (Fig. 9F).

Discussion

The present study aimed at better understanding how Nkb and Nkf regulate reproduction in the STB. Combined neuroanatomical and induction studies in vivo and in vitro with the Nkb/Nkf and Nkb antagonist, AntD, indicated that Nkb acts via kisspeptin to affect GnRh1 neurons.

A heterologous cell culture-expressing Tac3 receptor confirmed its identity and specificity as Nkb receptor, which was demonstrated by the clear preference for Nkb and Nkf over the other neurokinins, NKA and SP. When activated, Tac3r can utilize both PKA and PKC signal transduction pathways, as also reported for other species (Biran et al. 2012, 2014, Glidewell-Kenney et al. 2014). As for the antagonists, interestingly SB222200, the widely used non-peptide mammalian NK3R antagonist (Sarau et al. 2000, Malherbe et al. 2011), was non-potent on the STB Tac3r, suggesting that SB222200 is acting through a non-conserved region within the receptor. Of the three specific NK3R peptide antagonists, only AntD consistently and efficiently inhibited the activation of STB Tac3r (Jacoby et al. 1986).

As in other vertebrates, GnRh1 and kisspeptin (i.e., Kiss1 and Kiss2 in many teleosts) are major neuropeptides controlling reproduction in the STB. Kiss1 and Kiss2 act on GnRh1 neurons to modulate its expression and secretion in a stage-dependent manner (Zmora et al. 2012, 2014), and their neuronal subsets in the medio-basal hypothalamus display episodic appearances at the time of spawning (Zmora et al. 2014). This, and the fact that KISS1, NKB and dynorphin-A are co-expressed in the hypothalamic KNDy neurons in the arcuate-nucleus (ARC) to co-regulate GnRH1 in mammals, prompted us to examine the relationships between Nkb, kisspeptin and GnRh1 in the brain of the STB. Since the discovery of the KNDy neurons, scientists have wondered about the exact role of each neuropeptide and their interactions in the regulation of GnRH1 (Lehman et al. 2010, Navarro et al. 2011a, Grachev et al. 2012b, 2014, Navarro 2012, Angell & Steiner 2015). The finding that the Nkb and Kiss
neurons in the STB are distinct, as was found in zebrafish (Ogawa et al. 2012), suggested that this feature may be conserved in teleosts. Moreover, it provided insight into how these two neuropeptides interact and whether both regulate Gnrh1. tac3-expressing cells are widely distributed in the brain of the STB, in agreement with what is described in the goldfish and zebrafish (Ogawa et al. 2012, Qi et al. 2015). In the rostral hypothalamus, Nkb is found in the habenula and in the PMpc nucleus within the preoptic area. In the lateral hypothalamus, Nkb is found mainly in the NAT in the vicinity of the major Kiss2 neuronal population in the NRLd (that also co-expresses Kiss1 (Zmora et al. 2012)) and in the NRLv in the medio-basal hypothalamus. Although Gnrh1 neurons are sparsely innervated by POA Nkb fibers and do not express tac3 or tac3r, Kiss2 neurons in the NRLd are surrounded by Nkb fibers and do express tac3r. A similar pattern is found in mammals, where KNDy neurons do not express GPR54 but express NK3R and are densely surrounded by NKB fibers that loop back to the ARC (Burke et al. 2006a, Krajewski et al. 2010, Yeo & Herbison 2011). Indeed, the current study only detected projections of Nkb around the largest NRLd Kiss2 population; however, we cannot rule out weaker projections on other Kiss populations present in the brain of the STB, some of which may be temporal and stage specific.
First, we examined the possibility that kisspeptin and Nkb affect each other. A series of brain culture experiments with kisspeptin or Nkb/Nkf revealed that although kisspeptin does not modulate \(tac3\) transcript levels (Supplementary Fig. 3), Nkb and Nkf modulate the expression of \(kiss1\) and \(kiss2\). Unlike the effect on \(gnrh1\), that on \(kiss2\) was consistently inhibitory in nature. Congruently, treatment with AntD upregulated \(kiss2\) mRNA levels in the brain in vitro. The effect on \(kiss1\) mRNA in vitro was less consistent and more sporadic than the effect on \(kiss2\), probably due to differences in steroid levels that affect the potency of Nkb-containing fibers surrounds Kiss2 somas. Kiss2 neurons do not contain Nkb. (G) A different section displaying double-IHC of Kiss2 (green) and Nkb (red) and depicting the NRld nucleus, where neighboring kiss2 and Nkb neurons are clearly seen separated. NRld, nucleus recessus lateralis, pars dorsalis; NAT, nucleus anterior tuberis; NLTv, nucleus recessus nucleus lateralis tuberis, pars ventralis.

Figure 8
Nkb somas in the hypothalamus are localized in the vicinity of, and projected to, the major Kiss2 hypothalamic population that co-expresses Nkb receptor. (A and B) Double and combined in situ hybridization and immunocytochemistry demonstrating co-localization of (A) Nkb receptor (\(tac3r\), red) and (B) kiss2 somas (green) in the NRld. (C) Merged image of A and B. (D) A schematic drawing of the relevant coronal section in the hypothalamus demonstrating the close contact between the NAT and the NRld (adopted from Cerda-Reverter et al. 2001). (E) Combined ISH (\(tac3\), red) and IHC (Kiss2, green) used to localize \(tac3\) somas in the NAT where a single Kiss2 fiber is observed. The majority of Kiss2 projections are seen along the NLTv. \(tac3\)-expressing neurons do not contain Kiss2. (F) Double-IHC of Kiss2 (green) and Nkb (red) depicting the NRld nucleus where a dense network of Nkb-containing fibers surrounds Kiss2 somas. Kiss2 neurons do not contain Nkb. (G) A different section displaying double-IHC of Kiss2 (green) and Nkb (red) and depicting the NRld nucleus, where neighboring kiss2 and Nkb neurons are clearly seen separated. NRld, nucleus recessus lateralis, pars dorsalis; NAT, nucleus anterior tuberis; NLTv, nucleus recessus nucleus lateralis tuberis, pars ventralis.

Next, we examined the effects of Nkb and Nkf on Gnrh1. In mammals, two different modes of action were reported for NKB: an effect on GnRH1 soma in the POA and another on GnRH1 axon terminals that induces the release of GnRH1 to the median eminence (ME) in rats (Burke et al. 2006b, Krajewski et al. 2010) and mice (Todman et al. 2005, Gaskins et al. 2013). In the STB, Gnrh1 neurons do not co-express \(tac3\) at least not in spermatizing males, although there is an abundant presence of neurons expressing \(tac3\) and \(tac3r\) in the POA that includes a few fibers in the vicinity of Gnrh1 somas. These results, together with the brain incubation studies in vitro, led us to infer that Nkb most likely does not directly regulate Gnrh1 expression in the STB. In fact, in most in vivo and in vitro trials, except on one occasion, Nkb and Nkf had no effect on \(gnrh1\) transcript levels. In this single case, a dose of 10 nM Nkb/Nkf upregulated \(gnrh1\) expression in brain cultures in vitro. This confusion is also found in other teleosts such as the Nile tilapia, in which one study reported that Nkf stimulates Lh and Fsh (Biran et al. 2014) and another noted that Nkb downregulated \(gnrh1\), \(lhb\) and \(fshb\) (Jin et al. 2016).
In addition, in the goldfish, Nkb upregulated \textit{gnrh3}, \textit{lh} and \textit{fsh} (Qi \textit{et al.} 2015), results that may be attributed to variable factors, including the pharmacological dose of Nkb used in the goldfish (100 µg/g BW), which is 2000 times higher than the dose used in the current study. Another possibility is differences in reproductive stage-specific actions, as seen with Kiss2 (Zmora \textit{et al.} 2012) and gonadotropin-inhibiting hormone (Moussavi \textit{et al.} 2014). Altogether, it is safe to conclude that in the STB, any direct action(s) of Nkb on the POA Gnrh1 soma is probably minor.

To truly determine the direct effect of Nkb on Gnrh1 release, we took advantage of the fact that fish hypothalamic neurons directly innervate the pituitary; hence, Gnrh1 content in the pituitary may be indicative of its translational levels and/or release (Holland \textit{et al.} 2001). To our surprise, Nkb and Nkf systemic administration caused a decrease in Gnrh1 content in the pituitaries. This effect was not associated with the downregulation of \textit{gnrh1} mRNA in the brain, but rather with downregulation of \textit{kiss1} and \textit{kiss2}. Indeed, Kiss1 and Kiss2 were shown to play a critical role at the time of spawning via specific and timely neuronal appearances and via their elimination by antagonists (Zmora \textit{et al.} 2014, 2015). Similarly, this effect was associated with a minor decrease in \textit{gnrh1} mRNA levels and a significant decrease in Gnrh1 pituitary content (Zmora \textit{et al.} 2015), indicating that disruption of normal kiss levels has a pronounced effect on Gnrh1 peptide levels rather than on its expression. Alternatively, Nkb/Nkf acts on Gnrh1 nerve terminals to release Gnrh1 and, in turn, to induce the secretion of Lh, as reported in rats, mice and sheep (Navarro \textit{et al.} 2011b, Gaskins \textit{et al.} 2013, Li \textit{et al.} 2015). In this scenario, decreased \textit{kiss1/2} transcript levels do not affect \textit{gnrh1} mRNA levels and as a result, Gnrh1 peptide is not replenished, whereas simultaneously, Nkb induces the release of Gnrh1, altogether resulting in lower Gnrh1 pituitary content and higher Lh plasma levels.

This study in the STB and another in tilapia demonstrate a direct effect on the pituitary by Nkb and Nkf that elicits both Fsh and Lh secretion from primary pituitary cultures (Biran \textit{et al.} 2014). Likewise in rats, direct action of tachykinins on the pituitary stimulated...
or inhibited LH release in intact or castrated male rats, respectively (Kalra et al. 1992). Interestingly, only Lh and Fsh secretion is augmented, whereas expression is not. This is different from the in vivo results that noted an increase in mRNA levels of lhb and fshb. It is noteworthy that, in many cases, the results of in vivo systemic treatment do not directly match the in vitro effects, probably due to potential feedback from other neurons (e.g., lack of neuronal connections in the brain slices preparations) and from the periphery (mainly the gonads). Another possibility is deficient paracrine interactions between the dispersed pituitary cells, insufficient to upregulate lh and fsh mRNA.

As Nkb neuron immunoreactivity and transcript levels, as well as function, are affected by gonadal steroids (Kalra et al. 1992, Ruiz-Pino et al. 2012, Overgaard et al. 2014, Qi et al. 2015), the differences noted between the experiments may be due to gonadal steroid feedback. To test this possibility, we measured 11-ketotestosterone (11-KT, the major androgenic steroid in the striped bass) levels in the blood of the experimental fish, all of which (11-KT, the major androgenic steroid in the striped bass) levels in the blood of the experimental fish, all of which were similar in fish sampled in Dec, Feb and Apr–May. Other gonadal factors, however, including different steroids and peptides can still feedback to Nkb neurons. Overall, the effect of Nkb/Nkf on pituitary gonadotropins appears to be both direct and indirect. The direct effect is independent of kisspeptin and GnRH1 neurons and comprises direct innervations and paracrine/autocrine interactions between pituitary endogenous Nkb-expressing cells (Kalra et al. 1992, Biran et al. 2014) and supported by the expression of both tac3r and tac3 in pituitary cells, including in the proximal pars distalis. The indirect effect is exerted via kisspeptin neurons and GnRH1 that innervate the pituitary. Together, these actions explain the puzzling stimulatory effects of both Nkb and AntD on Lh/Fsh secretion in vivo, which in theory are expected to generate opposite responses. The net result of the stimulatory effect of AntD on kisspeptin and thereby GnRH1 overshadows its effect on the pituitary, suggesting that the hypothalamic action of Nkb is more dominant. With regard to the differential actions of Nkb and Nkf, Nkf has a more pronounced effect at the pituitary level, especially on Fsh, whereas Nkb is more potent at the brain level, inhibiting both kiss1 and kiss2. As is seen in the receptor-binding studies, AntD had little potency in antagonizing Nkf, which together with the dominance of Nkf in the pituitary may explain the increased secretion of gonadotropins in fish treated with AntD. Interestingly in the tilapia, two forms of tac3r are expressed in the pituitary: tac3rb is expressed only in Lh gonadotropes, whereas tac3ra is expressed in both Lh and Fsh cells and is more potently activated by Nkf than by Nkb (Biran et al. 2014). This may suggest that a yet unknown second form of Tac3r, with higher preference for Nkf and lower affinity to AntD, is also expressed in the pituitary of the STB and may be responsible for the differential effects of Nkb and Nkf.

Finally, although kisspeptin antagonist had a remarkable effect on sperm production (Zmora et al. 2015), similar treatment in the current study with AntD resulted in no change in sperm volume. This result suggests that Nkb is not as crucial to spawning as kisspeptin. However, because AntD did not affect Lh and stimulated Fsh circulation levels, it is not surprising that there was no detrimental effect on sperm production. On the other hand, as Nkb inhibits kisspeptin, it would be interesting to test whether chronic administration of Nkb will indeed hinder sperm production.

In summary, in this study, we characterized the mode of action by which Nkb and Nkf exert their effects on the reproductive axis, with specific attention to their relationships with GnRH1 and Kiss1/Kiss2 in the brain of male STB. Overall, Nkb/f has the capacity to utilize multiple pathways to influence reproduction: negatively affecting kisspeptin neurons in the nrl and positively affecting GnRH1 release and gonadotropin secretion. The net result of Nkb/f administration in an experimental setting is a reduction of GnRH1 peptide content in the pituitary and an induction of Lh and Fsh expression and secretion. However, because Nkb/f action in the pituitary is likely paracrine/autocrine, the Nkb-Kiss2 pathway may be more dominant under natural conditions. Although the current study briefly touched upon the effects of gonadal steroids on Nkb/f actions, this topic needs to be explored in greater detail.

**Supplementary data**

This is linked to the online version of the paper at [http://dx.doi.org/10.1530/JOE-16-0575](http://dx.doi.org/10.1530/JOE-16-0575).

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**

Research award (IS-4499-12CR) from the United States-Israel Binational Agricultural Research and Development (BARD) Fund.
References


Krajewski SJ, Burke MC, Anderson MJ, McMullen NT & Rance NE 2010 Forebrain projections of arcuate neurokinin B neurons


Ramassamy S, Seminara SB & Plant TM 2011 Evidence from agonal juvenile male rhesus monkey (Macaca mulatta) for the view that the action of neurokinin B to trigger gonadotropin releasing hormone release is upstream from the kisspeptin receptor. *Neuroendocrinology* 94 237–245. (doi:10.1159/000329045)


Received in final form 11 February 2017
Accepted 20 February 2017