Direct Regulation of Gonadotropin Release by Neurokinin B in Tilapia (*Oreochromis niloticus*)

J. Biran, M. Golan, N. Mizrahi, S. Ogawa, I. S. Parhar, and B. Levavi-Sivan

Department of Animal Sciences (J.B., M.G., N.M., B.L.-S.), The Robert H. Smith Faculty of Agriculture, Food, and Environment, Hebrew University of Jerusalem, Rehovot 76100, Israel; and Brain Research Institute (S.O., I.S.P.), Monash University Sunway Campus, Jalan Lagoon Selatan, Bandar Sunway, Selangor 46150, Malaysia

Neurokinin B (NKB) was recently identified as a key regulator of reproduction in mammals and fish. Fish were found to possess a specific novel neuropeptide termed NKF. To study the role of NKB/NKF in the regulation of fish reproduction and to investigate the role of NKB/NKF and their receptors in the piscine pituitary, we have identified the NKB/tac3r system in tilapia. Bioinformatics and phylogenetic analyses have demonstrated that the tilapia holds 1 putative tac3 gene and 2 NKB receptor genes (tac3ra and tac3rb) that clustered with other piscine Tac3 and NKB receptor lineages. Furthermore, we found that in African cichlids, NKB peptides differ from other vertebrate NKBs in their C-terminal sequence, possessing isoleucine instead of valine as the X in the NKB FXGLM-NH2-terminal consensus sequence. Signal transduction analysis demonstrated that tilapia NKB (tiNKB), tiNKF, and human NKB activated both CRE-luc and SRE-luc transcriptional activity of both tilapia and human NKB receptors. Two hours after ip injection of tiNKB, the plasma levels of both FSH and LH were increased, whereas tiNKF was more effective in increasing LH levels. However, tiNKB was more effective than tiNKF in increasing both FSH and LH from tilapia pituitary dispersed cells. Using in situ hybridization and fluorescent immunohistochemistry, we have shown that LH cells possess tac3, tac3ra, and tac3rb mRNAs, whereas FSH cells possess mainly tac3rb and tac3 and tac3ra to a much lesser extent. These results suggest that the members of the NKB/tac3r system may serve as paracrine/autocrine regulators of gonadotropin release in fish pituitary. (Endocrinology 155: 0000–0000, 2014)

Reproductive function is tightly regulated by a complex network of central and peripheral factors, where the most important is GnRH. Recently, the neuropeptides kisspeptin (encoded by *Kiss1*) and neurokinin B (NKB, encoded by *Tac3*) have been placed as crucial at different stages of reproduction (1). Studies in humans have revealed that loss-of-function mutations in the genes encoding NKB or neurokinin 3 receptor (NK3R) lead to hypogonadotropic hypogonadism and infertility (2–4). The presence of specific neurons in the mammalian arcuate nucleus, often called Kiss1/NKB/dynorphin neurons because they coexpress kisspeptin, NKB, and dynorphin A, was recently established in mammals (5–7).

Fish possess a large diversity of reproduction strategies, can be found in different environmental niches and use different timing regimes of sexual maturation. When compared with other vertebrates, fish have several unique characteristics. There is no functional hypothalamo-pituitary portal system in fish. In contrast, the different cells of the pars distalis receive a direct innervation from the hypothalamus, through nerve terminals that secrete different neuropeptides (8). However, it is still unknown whether NKB or NKF neurons project to the pituitary in fish. In tilapia, cells of a given cell type, in the pituitary, are grouped together in a given region of the gland (9, 10). Unlike mammals, fish have 2 kisspeptin types (kiss1 and kiss2) and usually more than 1 kisspeptin receptor (Kiss1R and Kiss2R) (11, 12). Tilapia possess 3 GnRH ligand variants compared with mammals that have 1 or 2. A species-
specific variant (GnRH1) is mainly located in the preoptic area and plays a major role in the regulation of reproduction. A variant that is conserved in all vertebrates (GnRH2) in the midbrain may act as a neurotransmitter and/or neuromodulator. The third form, salmon GnRH (sGnRH [GnRH3]), has mainly been identified in the terminal nerve ganglion and olfactory bulbs and is indicated to have neuromodulatory effects (13). The C terminus exists in tilapia GnRH receptors, and there is no GnRH desensitization (14, 15).

To date, a large number of tachykinins have been identified in a wide range of species from invertebrates to mammals. Tac1 encodes both substance P (SP) and NKA through alternative splicing. Tac2/Tac3 produces the peptide NKB, and Tac4 encodes hemokinin-1. Three classes of mammalian tachykinin receptors (NK1, NK2, and NK3) have been identified, and these have preferential binding affinities for SP, NKA, and NKB, respectively (16). The mammalian TAC1 and TAC4 give rise to 2 active neuropeptides, whereas the TAC3 is the only TAC that give rise to only 1 neuropeptide, namely NKB (17). We have recently identified genes encoding NKB (tac3a/b) and tac3r from many fish species, and cloned 2 tac3 cDNA forms from zebrafish (18, 19). Phylogenetic analyses showed that piscine and mammalian Tac3 genes arise from 1 lineage (18). The piscine orthologous Tac3 gene encodes for 2 putative tachykinin peptides, namely NKB and a second fish putative peptide, referred to as NKF (12, 18, 20).

Zebrafish tac3a mRNA levels gradually increased during the first few weeks of life and peaked at pubescence. In the brain of zebrafish, tac3a and tac3b mRNA was observed in specific brain areas that are related to reproduction (12). Furthermore, a single ip injection of NKBa or NKF significantly increased LH levels in mature female zebrafish, and the tac3a and both tac3r genes were up-regulated by estrogen (18). These results suggest that the role of the NKB system in the neuroendocrine control of reproduction is evolutionarily conserved in vertebrates.

Tilapia have become one of the most commercially important cultured freshwater fish, due to their high growth potential, short generation time, ease of spawning, and disease resistance (21). Tilapia recently became the first aquaculture species to have its genome sequenced (22), and hence, in addition to the zebrafish, medaka, and goldfish, tilapia is now widely used as an animal model to investigate reproductive physiology and endocrinology. Using specific methods for the measurement of GTH previously established in our laboratory (23–25), the objective of the current study was to characterize the NKB system, with respect to reproduction, in tilapia. We cloned the tilapia tac3 and their receptors, tested their effects on FSH and LH release in vivo and in vitro, identified their signal transduction pathways, and localized the tac3 and its receptors in specific cells in the pituitary.

Materials and Methods

Animals

Sexually mature Nile tilapia (Oreochromis niloticus) were kept and bred in the fish facility unit at the Hebrew University in 500-L tanks at 26°C and with a 14-hour light, 10-hour dark photoperiod. Fish were fed daily ad libitum with commercial fish pellets (Raanan fish feed). All experimental procedures were in compliance with the Animal Care and Use guidelines of the Hebrew University and were approved by the local Administrative Panel on Laboratory Animal Care.

Isolation of tilapia Tac3 ligands and receptors and phylogeny analyses

The putative tac3 gene sequence was isolated from tilapia using tblastn against the scaffolds and contigs of the genome sequence, O.niloticus_wgs_v1, with zebrafish Tac3a protein (NP_001243318.1) as input. Significant similarity was found on Scaffold63, and exons were predicted. Because we could not detect homologous expressed sequence tags for the predicted gene, tilapia tac3 was cloned, and its sequence was confirmed at both the genomic and mRNA level. The tilapia tac3 open reading frame sequence was submitted to GenBank (accession number KF471673). Similarly, Tac3 sequences were also identified in the genomes of other African cichlids: zebra mbuna (Mylandia zebra; AGTA02027823.1 nucleotides [nt] 10 397–10 498 and 10 738–10 848; AGTA02027824.1 nt 267–317 and 402–470), Rhampbochondrom esox (ABPN01048277.1 nt 48–158; ABPN01048278.1 nt 225–275 and 360–425), Haplochombromis nyerei (Pundamilia nyerei; AFNX01017040.1 nt 520–419 and 176–66; AFNX01017039.1 nt 8760–8709 nt 8625–8557). Syntenity was observed using the University of California at Santa Cruz genome browser and the tilapia genome build (Broad oreNil1.1/oreNil2).

Tilapia tac3ra and tac3rb sequences were predicted from genomic sequence (Contig033115 and Scaffold113, respectively), and their mRNA sequences were cloned and were submitted to GenBank (accession numbers KF471674 and KF471675, respectively). Our tac3rb sequence differs from the GenBank predicted sequence (which was not available when we did the gene prediction) and has an alternative C-terminal exon upstream to the predicted exon. Cloning proved our version of the gene to be correct.

Phylogenetic analysis was generally performed according to Ref. 18. Both neighbor joining and maximum likelihood were used on the basis of alignments performed both by ClustalW (26) and MUSCLE (27). The topologies were the same in all combinations of multiple alignment and tree construction models with the exception of the zebrafish Tac3 peptide-containing clades that were orderly inverted between the neighbor-joining and maximum-likelihood trees. Bootstrapping of 500 was performed on both neighbor-joining and maximum-likelihood trees. Analysis was performed using Mega6 software (28).
Tissue distribution

Tissue distributions of tilapia tac3a, tac3ra, and tac3rb mRNAs were determined by real-time PCR as previously described (12). Tissue samples were collected from sexually mature post-vitellogenic female and milt-producing male tilapia (body weight [BW] 128.4 ± 10.8 g and GSI 0.29% ± 0.09%; BW 92.7 ± 7.06 g and GSI 2.63% ± 1.20%, respectively; n = 3). Total RNA was extracted from brain, pituitary, liver, intestine, fat, muscle, gill, retina, ovary, testis, heart, kidney, pancreas, and stomach. Total RNA and cDNA were prepared as previously described (29). Real-time PCR of tilapia tac3, tac3ra, and tac3rb were performed generally according to Ref. 29. To assess the relative abundance of tilapia ligand and receptor mRNAs, the genes were normalized against an endogenous reference (18). Serial dilutions were prepared from a cDNA sample, and the efficiencies of the specific gene amplifications were compared by plotting Ct vs log (template concentration). Gene-specific primers were designed with the aid of Primer Express version 2.0 software (Supplemental Table 1). The primer sequences, R^2 values, and slopes of the real-time PCR analyses, calculated by linear regression, are presented in Supplemental Table 1.

Amplification was carried out in a Stratagenе Mx3000p cycler according to the manufacturer’s protocol. The cDNAs were amplified simultaneously in separate tubes in duplicate, and the results were analyzed with the MxPRO QPCR Software (Stratagenе). A dissociation-curve analysis was run after each real-time experiment to confirm the presence of only 1 product. To control for false-positives, a reverse-transcriptase negative control was run for each template and primer pair. To verify amplification of the correct sequences, the real-time PCR products amplified with each gene primer were confirmed by sequencing.

Immunofluorescence and in situ hybridization on pituitary cryosections

Tilapia pituitaries were harvested from sexually mature fish and fixed in buffered 4% paraformaldehyde in PBS. After fixation, samples were cryoprotected in 20% sucrose and 30% Tissue-Tek OCT compound (Sakura), embedded in OCT, sagitally sectioned, and analyzed for mRNA expression by in situ hybridization (ISH). The hybridization was generally performed as described previously (18) with slight modifications. Tilapia pituitaries were sagitally sectioned, and development of color signal was made using Fast-Red tablets and light and fluorescent microscopy. After the confirmation of hybridization signals, immunofluorescence (IF) labeling was performed. Sections were blocked in 5% normal goat serum with 0.3% Triton X-100 for 1 hour at room temperature and incubated with specific antisera raised in rabbit against recombinant tilapia βLH (25) or recombinant tilapia βFSH (23) diluted 1:500 in antibody dilution buffer (1% BSA, 0.3% Triton X-100 in PBS) overnight at 4°C. Samples were stained using secondary antirabbit antibodies conjugated to Alexa fluorescent dyes (Invitrogen) diluted 1:300 and incubated for 2 hours at room temperature. After staining, slides were washed and mounted using antifade solution (2% propyl-gallate, 75% glycerol in PBS).

Peptide synthesis

Tilapia NKB (pyroglutamated [p]-EMDDIFGLM-NH2) and tilapia NKF (YNDLDYDSFVGLM-NH2) were synthesized by GL BioChem. Peptides were synthesized by the automated solid-phase method by applying Fmoc active-ester chemistry, purified by HPLC to >95% purity and the carbox terminus of each peptide was amidated. The peptides were dissolved to the desired concentration in fish saline (0.9% NaCl in DDW) for in vivo experiments and in culture media for in vitro experiments.

Receptor transactivation assay

To study the signaling pathways of the novel tilapia NKBs, the entire coding regions of tilapia tac3ra (GenBank accession no. KF471674) and tilapia tac3rb (GenBank accession no. KF471675) were inserted into pcDNA3.1 (Invitrogen). To differentiate between the protein kinase C (PKC) and PKA signal transduction pathways, we used a sensitive luciferase (LUC) reporter gene assay. We previously demonstrated that cAMP response element (CRE-LUC; Invitrogen) and serum response element (SRE-LUC; Invitrogen) reporter systems are useful tools for discriminating cAMP/PKA and Ca^2+/PKC signaling pathways, respectively (12). The cDNA clone for human TAC3R was obtained from the Missouri S&T cDNA Resource Center (www.cdna.org). Three micrograms of each construct together with 3 μg of a luciferase reporter plasmid were transiently transfected into COS-7 cells (American Type Culture Collection). Forty-eight hours after transfection, cells were treated with tilapia NKB (tiNKB), tiNKF, or huNKB at increasing doses. The hormone treatment and the subsequent measurement of luciferase activities were carried out as previously described (12, 18). The EC_{50} values were calculated from concentration response curves by means of computerized nonlinear curve fitting with Prism version 6 software (GraphPad).

Primary pituitary cell culture

Primary cultures of pituitary cells were prepared as described previously (30). Briefly, pituitary glands harvested from sexually mature tilapia males (BW 96.5 ± 9.5 g; GSI 0.39% ± 0.12%) were placed in culture media (M199, penicillin [100 IU/mL], streptomycin [0.1 mg/mL], nystatin [1.25 IU/mL], and 0.3% BSA). Pituitaries were diced into fragments and trypsinized using Trypsin-EDTA solution (Biological Industries). Cells were plated on a 48-well plate (250,000 cells/well/0.5 mL). The cells were allowed to recover for 4 days at 28°C under an atmosphere of 5% CO_{2} and then challenged for 6 hours with tiNKB, tiNKF, or sGnRH (Bachem, Inc) at concentrations ranging from 1nM to 1μM. Each experiment was performed in triplicate and repeated 3 times (separate cell preparations).

In vivo effect of tilapia NKB and NKF

Adult male tilapia (BW 89.29 ± 32.93 g, GSI 0.24% ± 0.40%) were injected ip with saline, sGnRH analog (10 μg/kg BW; [d-Ala^6,Pro^9-Net]-mammalian GnRH; sGnRHa; Bachem), or tiNKB or tiNKF each at 10 or 100 μg/kg BW (n = 8 fish per group). The fish were bled from the caudal blood vessels into heparinized syringes 2, 4, and 24 hours after injection. This time course is according to standard protocols used previously (23, 31) to test the effect of GnRH on circulating levels of LH and FSH in tilapia. Blood samples were collected from the caudal vascu-
ELISA for the measurement of tilapia FSH and LH

Levels of gonadotropins in the plasma and primary culture media were measured by a competitive specific ELISA developed for tilapia (24), based on recombinant gonadotropins, primary antibodies against recombinant tilapia LHβ or FSHβ, respectively, and recombinant tilapia LHβa (25) or FSHβa (23) for the standard curves. For culture media, the sensitivity was 2.43 and 1.52 ng/mL for LH and FSH, respectively. Interassay covariance was 14.8% and 12.5%, and intra-assay variation was 7.2% and 8% for LH and FSH, respectively.

Statistical analysis

The results are presented as the mean ± SEM. Two-way ANOVA was used to compare mean values of the in vivo experiment and one-way ANOVA for the in vitro cell-culture experiment. This was followed by an a posteriori Tukey-Kramer test (all pairs) only when ANOVA revealed the presence of statistical significant differences between groups, using JMP version 7 software.

Results

Identification, cloning, phylogenetic analyses, and synteny of tilapia tac3 and tac3 receptors

Because there are different names for the gene encoding NKB in different species (TAC3 in human or Tac2 in mouse), in this article we will refer to mRNA products of this gene as tac3 mRNA and to the peptides as NKB or NKF. The receptor that binds NKB, which is termed NKB or NKF in humans, will be termed tac3r at the mRNA level and Tac3r at the protein level.

We report here the identification of the full-length tac3 cDNA from tilapia brain using PCR with specific primers (Supplemental Table 1). The tilapia tac3 preprohormone cDNA contains a coding region of 357 bp, coding a 118-amino-acid peptide (Supplemental Figure 1). This polypeptide contained the tridecapeptide NKF sequence (YNDLDYDFVGLM-NH2) and the pyroglutamated decapetide NKB sequence (pEMDDIFIGLM-NH2). The tachykinin signature motif (FXGLM-NH2) was present in the amino acid translation of both tilapia tac3 cDNA sequences, where in tiNKB, X represents isoleucine, and in tiNKF, X represents valine. The signature motif in tilapia was different from that of all other fish examined (Figure 1). The C-terminal motif of NKBs is important to the binding of NKBs to their cognate receptors (32). We further searched for NKB of other cichlids. Surprisingly, we found that in a similar way to the Nile tilapia, NKB peptides of other cichlids, like M. zebra, R. esox, and H. nyererei all share the same signature motif of FVGLM, whereas all other fish examined share the canonical signature of FVGLM (Figure 1). Both NKB and NKF were flanked by potential dibasic cleavage sites and an adjacent glycine at the C terminus for amidation (33). At the protein level, the resulting Tac3 preprohormone precursor displayed 38% to 41% identity with human or mouse TAC3, 77.2% with medaka and 69.7% with salmon Tac3a, and only 51.5% or 46.3% with salmon and zebrafish Tac3b (Supplemental Table 2).

A phylogenetic tree of vertebrate Tac3 prepropeptides was generated by using the neighbor-joining method (Figure 2A). This analysis showed that the tilapia Tac3 clusters with other previously identified piscine peptides. The tilapia Tac3 was grouped with the Atlantic salmon, Grass rockfish, medaka, and puffer Tac3a, whereas both zebrafish Tac3a and Tac3b were grouped together (Figure 2A).

Chromosome syntenic analysis revealed that the locus of tac3 is highly conserved between teleosts (18). Tilapia tac3 is located on chromosome 20, whereas zebrafish tac3a is located on chromosome 23, and the only tac3 found in medaka (similar to tac3a) is located on chromosome 7 (Supplemental Figure 3 and Ref. 18). For both tilapia tac3 and zebrafish tac3a genes, the nearest neighboring gene (c1galt1a) is non-syntenic, whereas the next nearest ones (b4galt1a, slc6a1, and arh-
gef25) were found in inverse order relative to humans (Supplemental Figure 3 and Ref. 18).

The deduced amino-acid sequences for the tilapia Tac3ra and Tac3rb receptors translated from the cloned mRNAs are shown in Supplemental Figure 2. Tilapia tac3ra was highly homologous to tac3ra of other fish (medaka, fugu, and pufferfish 75%–93% similarity), whereas tilapia tac3rb sequences possess only 73%–85% similarity to other piscine tac3rb sequences (Figure 2B). Both receptors had around 65% similarity with the mammalian receptors (Supplemental Table 3). The tilapia tac3 receptors shared 71.8% identity with each other. Sequence analysis of the 2 types of tilapia receptors identified distinct potential sites for N-glycosylation, phosphorylation by PKC, PKA, casein kinase II, tyrosine kinase, and N-myristoylation (Supplemental Figure 3). The N and C termini are, as in other G protein-coupled receptors, the most divergent regions.

Phylogenetic analysis of the Tac3rs showed that the vertebrate Tac3r proteins fall into several distinct lineage groups; one lineage includes the mammalian, chicken, and frog Tac3r, whereas all other piscine Tac3rs, with the exception of the zebrafish, have 2 different branches, one for the type A receptor (Tac3ra) and the other for the type B receptor (Tac3rb; Figure 2B).

**Tissue distribution of tac3 and tac3rs**
Real-time quantitative PCR was performed to detect the tac3a, tac3ra, and tc3rb expression levels in various tissues of tilapia (Supplemental Figure 4). The tilapia brain was dissected into 3 parts, of which the anterior part contains the telencephalon and olfactory bulb; the midbrain...
contains the optic tectum and diencephalon; and the posterior brain contains the medulla oblongata and cerebellum. The tac3 mRNA was detected mostly in all brain parts, intestine, and retina. The expression level of tac3rb was high both in the hindbrain and in the periphery, including the pituitary, ovary, and testis, whereas tac3ra was highest in brain, retina, and intestine (Supplemental Figure 4).

**Activation and signaling of tilapia Tac3rs**

The EC50 values of NKBs for each receptor are summarized (Supplemental Table 4). Both tilapia and human receptors were activated by the human and tilapia NKBs dose-dependently in both signal transduction systems. For human TAC3R, huNKB was the most efficient peptide in the PKC signal transduction (EC50 = 0.008nM, 0.15nM, or 2.46nM for huNKB, tiNKB, and tiNKF, respectively), whereas tiNKF was the most efficient peptide at the CRE-luc pathway (EC50 = 1.56nM, 8.76nM, or 37.27nM, for tiNKF, huNKB, tiNKB, respectively; Figure 3, C and F). For all NKB peptides tested, tiTac3rb exhibited lower stimulation than tiTac3ra, in both signal transduction pathways. The tiNKF was more effective than tiNKB or huNKB in inducing tilapia Tac3ra activity (Figure 3, A and D). The human receptor gave a higher response than the fish receptors, maybe due to the mammalian origin of the COS-7 cells.

**In vivo and in vitro effects of tilapia NKB and BKF on GTH release**

To understand the physiological effects of tiNKB or tiNKF on gonadotropin release, we first examined the effect of ip injection (10 or 100 μg/kg BW) on plasma FSH and LH levels. Administration of tiNKB at 10 μg/kg significantly increased plasma levels of FSH and LH at 2 hours after the injection (Figure 4). The administration of tiNKF had no significant effect on FSH but increased LH levels after 2 hours at both concentrations (Figure 4B).

In vivo actions of neuropeptides potentially involve a combination of direct and indirect effects on the pituitary. To gain insight into the direct effects of NKB and NKF on gonadotropin release at the level of the pituitary, we tested the effect of the neuropeptides in static incubation of primary tilapia pituitary cell culture. tiNKB and tiNKF significantly increased the release of FSH from cultured pituitary cells, although the release in response to sGnRH was higher (Figure 5A). The release of LH from the pituitary cells was significant only at 1nM, when at this concentration NKB and sGnRH were equally efficient (Figure 5B).

**Localization of tac3, tac3ra, and tac3rb in the pituitary**

Because we found significantly higher release of both FSH and LH from dispersed pituitary cells, in response to...
tiNKb and tiNFk, we further aimed to examine the specific cellular localization of the tilapia tac3 and tac3r mRNAs in the tilapia pituitary via ISH and IF histochemistry methodologies. ISH for tac3 or tac3r transcript was followed by IF histochemistry using homologous specific antisera raised against tilapia LH/H9252 or FSH/H9252. Hybridizing pituitaries with the 3 corresponding sense probes gave no signal (data not shown). ISH demonstrated the localization of tac3 mRNA-containing cells in the median region (PPD), known to contain the gonadotrope cells (Figure 6). However, although tilapia tac3 was shown to colocalize with LH cells, a very low degree of colocalization was evident in FSH cells (Figure 6). A similar pattern of distribution and colocalization was observed when the tac3ra mRNA-containing cells were tested. Tilapia tac3ra mRNA was expressed in LH cells, whereas only few FSH cells contained the receptor (Figure 7). Dual ISH-IF showed that tilapia tac3rb mRNA was expressed in both LH and FSH cells at the PPD. Approximately 30% of FSH cells are double-labeled for tac3rb (228/736), whereas all LH cells express tac3rb mRNA (Figure 8).

Discussion

Tac3/Tac3r cloning in the tilapia

The tachykinin peptide family is one of the most prevalent peptide families described in animals. Over the past 40 years, more than 40 tachykinins have been identified from invertebrates and vertebrates (34). They perform multiple physiological functions such as smooth muscle contraction, vasodilation, inflammation, nerve signal processing, neuroprotection, and neurodegeneration (34). There is a growing body of evidence for the function of tachykinins and their receptors in the regulation of the reproductive system. However, most of these data relate to their effects at the brain level. We show here the identification of the Tac3 system as direct regulators of reproduction at the pituitary level in tilapia.

We have recently shown that fish tachykinin peptides contains the FVGLM-amide motif (18), which matches the tachykinin signature sequence FXGLM-amide, where X represents a hydrophobic amino acid (34). However, in the current study, we identified in silico Tac3 of several cichlids from the lakes of Africa and found that their NKBs contain a different hydrophobic amino acid (Ile). In addition, the sequences downstream of this motif, both on the tilapia NKB and NKF, contain all of the information necessary for the maturation of a tachykinin peptide. The basic sequence KR/RR provides a target for endoproteolytic cleavage by prohormone convertases (35) and subsequent trimming by carboxypeptidases (36). The last residue (Gly) at the carboxy terminus of the tachykinin motif provides the NH2 group from C-terminal amidation by peptidylglycine α-amidating mono-oxygenase (37).

We found 2 forms of tac3 genes in zebrafish and salmon, when both these species went through gene duplication during their evolution (38, 39); however, more
advanced fish, with genomes that did not go through this event (tilapia, medaka, puffer, and others), contained only 1 tac3 ortholog. Conserved syntenic blocks in different taxa of vertebrates indicate that evolutionary pressure has acted to preserve the NKB genes, suggesting that NKB plays important roles from fish to mammals.

The situation in Tac3r is different; all the tested fish species exhibit 2 forms of Tac3rs, except the zebrafish, which possesses 3 different variants (18, 20). Moreover, as suggested by the phylogenetic analysis of the Tac3 receptors, the 3 ortholog genes of the zebrafish receptors have high homology to the piscine type B of the Tac3r, whereas other fishes, including the tilapia, medaka, pufferfish, and fugu have 2 phylogenetically different receptors. In the present study, we cloned genes of tac3ra and tac3rb from the tilapia and revealed that the deduced amino acid sequences for both receptors were, as expected, highly homologous to those of the human and of other piscine NKB receptors.

**Signaling activity of Tac3r types**

After NKB is bound to its receptor, Tac3r activation increases intracellular Ca$^{2+}$ concentration through inositol phospholipid hydrolysis. Alternatively, Tac3r activation can increase intracellular cAMP levels through adenylate cyclase activation (40). The pharmacological profiles of tiTac3ra and tiTac3rb confirmed that both receptors have functional activities in the cAMP/PKA and Ca$^{2+}$/PKC pathways, as was shown before also for zebrafish (18). The order of affinity for cAMP/PKA pathway in tilapia Tac3ra was tiNKF > tiNKB > huNKB and for tiTac3rb was tiNKB > tiNKF = huNKB. Interestingly, tiNKF was more potent in activating human TAC3R than its cognate peptide, as was shown for the zebrafish (18).

**Figure 5.** In vitro effect of tiNKB, tiNKF, or sGnRH on FSH (A) and LH (B) release from primary cultures of tilapia pituitary cells. Cells were treated with graded concentrations of tiNKB, tiNKF, or sGnRH peptides for 6 hours. Gonadotropin levels released to the media were determined using specific ELISAs for tilapia gonadotropin. Gonadotropin concentrations are expressed as nanograms gonadotropin per well. Columns marked by asterisks significantly differ from basal: *, $P < .05$; **, $P < .01$; ***, $P < .001$.

**Figure 6.** Tilapia tac3 is expressed in LH and to a lesser extent in FSH cells. Double-labeled ISH-IF of tac3 mRNA (red) and βFSH (green, A–C) or βLH (green, D–F) in the tilapia pituitary. The drawing on the right marks the location of the imaged area within the pituitary. Scale bar, 100 μm.
affinity for Ca\(^{2+}\)/PKC pathway in tilapia Tac3ra was \(\text{tiNK} > \text{tiNKB} = \text{huNKB}\), whereas for tiTac3rb, very low activity was detected. The tiTac3rb was less effective than the other forms in eliciting luciferase activity by both signal transduction pathways, unlike the case in the zebrafish, where zebrafish Tac3ra and Tac3rb showed more similar patterns (18, 20), although both the zebrafish Tac3rs grouped phylogenetically with the tiTac3rb. The maximal response in the case of zebrafish NKBs was higher than in the case of tiNKB, and more similar to that of the human, probably because the zebrafish signature motif is more similar to that of the human.

All fish NKF\(\alpha\)s, including tilapia, contain 3 calcium-binding aspartic acid residues, which form the DxDxD sequence motif that is flanked by an \(\alpha\)-helix (Figure 1). Many calcium-binding proteins contain the same Ca\(^{2+}\)-binding helix-loop-helix structure, referred to as the EF-hand. In the canonical EF-hand, the loop contains 3 calcium-binding aspartic acid residues, which form the DxDxDG sequence motif, and is flanked by 2 \(\alpha\)-helices (41, 42).

**Tilapia NKB and NKF increase FSH and LH release**

There is a very limited number of studies on the in vivo effect of NKB/NKF on gonadotropin release in fish. Hence...
we used concentrations at a range that are similar to that used for GnRH (10 and 100 µg/kg BW). Administration of NKB or NKF peptides to primary cell culture of tilapia pituitaries resulted in a significant increase of FSH and LH release to the media, suggesting a direct effect of NKB peptides on gonadotropin release in tilapia. In mammals, the data on the direct effect of NKB on the secretion of pituitary hormones are very limited. NKB induced secretion of prolactin from rat anterior pituitary cells (43). Pituitary hormones are very limited. NKB induced secretion of prolactin from rat anterior pituitary cells (43). However, NKB failed to modulate prolactin secretion from fish to mammals and its ability to stimulate gonadotropin secretion in fish pituitary.

We thank Dr Shifra Ben-Dor from The Weizmann Institute for her help in the bioinformatics analyses.

Address all correspondence and requests for reprints to: Department of Animal Sciences, The Robert H. Smith Faculty of Agriculture, Food, and Environment, Hebrew University of Jerusalem, Rehovot 76100, Israel. E-mail: berta.sivan@mail.huji.ac.il.

This research was funded by the Binational Agricultural Research and Development Fund (BARD IS-4499-12) and by the Israel Science Foundation (237/12).

The nucleotide sequences reported in this paper have been deposited in the GenBank database under accession nos. KF471673, KF471674, and KF471675 for tilapia tac3, tac3ra, and tac3rb, respectively.

Disclosure Summary: The authors have nothing to disclose.

Localisation of NKB and tac3 receptors in the tilapia pituitary

In mammals, tachykinins are synthesized in the anterior pituitary gland as well as in the hypothalamus (48), and many experimental findings point to a possible role of tachykinins as paracrine modulators of pituitary hormone secretion (44, 49). Although all of the tachykinin receptors were found in the mammalian anterior pituitary gland, NKB receptors were found exclusively in lactotrophs and gonadotrophs (44). However, NKB failed to modulate gonadotropin gene expression in LβT2 cells, although TAC3R exists in these cells (44). Because GnRH neurons express the Kiss1R but apparently not TAC3R in sheep and mice and kisspeptin (KNDY) neurons express TAC3R, it was shown in humans that NKB secreted from KNDY neurons acts in an autocrine or paracrine manner to enhance kisspeptin secretion and that kisspeptin alone is sufficient to elicit GnRH pulsatility (50). It should be noted that in zebrafish brain, there is no coexpression of NKB and kisspeptin (19); hence, the localization of the tac3rs should be studied in brain neurons.

Taken together, our current data may support a paracrine role for Tac3 in the pituitary because both tac3 and its receptors are expressed in the tilapia pituitary. Moreover, both tac3 and its receptors were localized in tilapia gonadotrophs, suggesting that the Tac3 system may serve not only as paracrine but also as autocrine regulators of gonadotrophs in fish.

In summary, we have identified the Tac3 system in tilapia. We have demonstrated that in African cichlids, NKB peptides possess a unique FIGLM signature peptide rather than the FVGLM consensus sequence, which is common to all other known NKBs. We have further identified 2 tilapia tac3rs and showed them to differ in their phylogeny and signal transduction activity and in their tissue distribution. We have shown that both NKF and the unique NKB peptides were biologically active and demonstrated their ability to elicit FSH as well as LH release in vivo and in vitro. Finally, we localized the expression of tac3 and its receptors in LH and FSH cells, suggesting they may serve as paracrine/autocrine regulators of GTH release in fish pituitary.

Acknowledgments

We thank Dr Shifra Ben-Dor from The Weizmann Institute for her help in the bioinformatics analyses.

References


44. Mijiddorj T, Kanasaki H, Purwana IN, Oride A, Sukhabaatar U, Miyazaki K. Role of neurokinin B and dynorphin A in pituitary


