Coevolution of the Spexin/Galanin/Kisspeptin Family: Spexin Activates Galanin Receptor Type II and III

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The novel neuropeptide spexin (SPX) was discovered using bioinformatics. The function of this peptide is currently under investigation. Here, we identified *SPX* along with a second *SPX* gene (*SPX2*) in vertebrate genomes. Syntenic analysis and relocating *SPX*s and their neighbor genes on reconstructed vertebrate ancestral chromosomes revealed that *SPX*s reside in the near vicinity of the *kisspeptin* (*KISS*) and *galanin* (*GAL*) family genes on the chromosomes. Alignment of mature peptide sequences showed some extent of sequence similarity among the 3 peptide groups. Gene structure analysis indicated that *SPX* is more closely related to *GAL* than *KISS*. These results suggest that the *SPX*, *GAL*, and *KISS* genes arose through local duplications before 2 rounds (2R) of whole-genome duplication. Receptors of KISS and GAL (GAL receptor [GALR]) are phylogenetically closest among rhodopsin-like G protein-coupled receptors, and synteny revealed the presence of 3 distinct receptor families KISS receptor, GALR1, and GALR2/3 before 2R. A ligand-receptor interaction study showed that SPXs activate human, *Xenopus*, and zebrafish GALR2/3 family receptors but not GALR1, suggesting that SPXs are natural ligands for GALR2/3. Particularly, SPXs exhibited much higher potency toward GALR3 than GAL. Together, these results identify the coevolution of *SPX*/*GAL*/*KISS* ligand genes with their receptor genes. This study demonstrates the advantage of evolutionary genomics to explore the evolutionary relationship of a peptide gene family that arose before 2R by local duplications. **(*Endocrinology* 155: 1864–1873, 2014)**

A novel secreted neuropeptide called spexin (SPX), which is encoded by the *C12ORF39* gene, was recently identified using bioinformatics tools (1, 2). The *C12ORF39* gene encodes a protein containing a signal peptide, followed by a precursor sequence with a predicted mature peptide (SPX) of 14 amino acids flanked by conserved dibasic cleavage sites (1–3). This mature peptide sequence is evolutionarily conserved across vertebrate species (4, 5). The mRNA and/or protein expression levels for *SPX* have been determined in brain regions and peripheral tissues of human, mouse, rat, and goldfish, including the hypothalamus, cerebral cortex, hippocampus, optic tectum, pons, retina, esophagus, stomach, kidney, ovary, and adrenal glands (1–8). These results suggest multiple physiological functions of SPX. Recent analyses using goldfish revealed the involvement of SPX in reproduction and appetite control (4, 5). Treatment of goldfish with SPX decreased the secretion of LH (4) and suppressed appetite (5). Recent work also demonstrates the central control of SPX in cardiovascular/renal function and nociception (9). Despite accumulating evidence for physiological roles of SPX, the detailed mechanism of action has not been clearly demonstrated because of a lack of information on the SPX receptor. The peptidergic nature of

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Abbreviations: GAL, galanin; *GALP*, *GAL-like peptide*; GALR, GAL receptor; GPCR, G protein-coupled receptor; KISS, kisspeptin; KISSR, KISS receptor; 2R, 2 rounds; SPX, spexin; SRE, serum-response element; SRE-luc, SRE-driven luciferase activity; VAC, vertebrate ancestral chromosome; WGD, whole-genome duplication.

SPX allows us to speculate that SPX may interact with an orphan G protein-coupled receptor (GPCR) or one of the peptide GPCRs.

Neuropeptide and receptor families expanded their family members through 2 rounds (2R) of whole-genome duplication (WGD) and local duplications before and after 2R (10–14). Phylogeny and synteny analyses are useful to understand the evolutionary history and relationship among paralogous genes. In addition, tracing the family genes on reconstructed pre-2R vertebrate ancestral chromosomes (*VAC*s) is a fast and relatively accurate tool to explore the relationships among members of a family that contains a large number of paralogous genes (14, 15). These analyses revealed that paralogous genes that arose through local duplications reside in the near vicinity on the same chromosomes or on reconstructed ancestral chromosomes (14). While searching for the evolutionary mechanism that generated the *SPX* gene, we detected the presence of a SPX paralog (*SPX2*) in vertebrate chromosomes. *SPX* (hereafter *SPX1*) and *SPX2* are closely located to *galanin* (*GAL*) and *kisspeptin* (*KISS*) family genes on the same reconstructed *VAC*. The sequence alignment of mature peptides for *SPX*, *GAL*, and *KISS* revealed sequence identity to some extent. We proposed a possible scenario that the 3 ancestral genes are likely to have emerged through local tandem duplications before 2R. The GAL receptor (GALR) and KISS receptor (KISSR) are phylogenetically closest to each other among other peptide GPCR families (16). Phylogeny and synteny analyses also revealed the presence of 3 ancestors for GALR1, GALR2/3, and KISSR before 2R, which raises the possibility that SPX may interact with one of these receptor subfamilies. We found that SPX exhibited substantial potency in activation of human GALR2 and GALR3, indicating that SPX is another ligand for the GALR2/3 subfamily.

Materials and Methods

Data acquisition and phylogenetic analysis of peptides and receptors

The amino acid sequences of peptides and receptors were retrieved from the genome database of human, anole lizard, chicken, *Xenopus tropicalis*, coelacanth, zebrafish, medaka, stickleback, *Tetraodon*, lamprey, amphioxus, *Ciona*, *Caenorhabditis elegans*, and *Drosophila* with the TBLASTN algorithm provided by the Ensembl Genome Browser (http://www.ensembl.org), the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), or the amphioxus genome database (http://genome.jgi-psf.org/Brafl1/Brafl1.home.html). Orthology or paralogy of the genes was investigated using synteny and search tools from the Ensembl Genome Browser. The exons and splicing junctions of novel genes were defined manually or using the HMMgene (v.1.1) program (http://www.cbs.dtu.dk/services/HMMgene/) provided by CBS Prediction Service. Putative signal peptides were predicted using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). Gene names used for data analysis are shown in Supplemental Tables 1 and 2, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. The amino acid sequences of the mature peptides and receptors were aligned using MUSCLE as implemented in MEGA 5.1. The default alignment parameters were applied. Alignments were bootstrapped 100 times, and a maximum likelihood phylogenetic tree was constructed using the Jones-Taylor-Thornton model. Trees were plotted using MEGA 5.1.

Synteny analysis and tracing the evolutionary history of the gene families

The synteny analysis was performed by comparing the Contig Views that display genomic regions containing genes surrounding the peptide and receptor loci. The information for chromosome localization of orthologs/paralogs of neighboring genes was provided from the Ensembl Genome Browser. Chromosome fragments with reliable synteny were matched with the reconstructed *VAC* model by Nakatani et al (17) as previously described (14, 15). The Nakatani model suggests the presence of 10–13 pre-2R *VAC*s, defined as the *A-J* linkage groups. These *VAC*s then underwent 2R to generate approximately 40 post-2R gnathostome ancestor chromosomes (*GACs A0-J1*), resulting in up to 4 copies of paralogous genes. Because chromosomal segments of medaka, chicken, mouse, and human have been matched to these reconstructed linkage groups, the location of a current gene can be traced back to the linkage groups.

Chemicals and peptides

All chemicals were obtained from Sigma-Aldrich unless otherwise stated. Restriction enzymes were obtained from New England BioLabs. Human GAL, SPX1, and zebrafish SPX2 peptides were synthesized by AnyGen. The purity of the synthesized peptides was greater than 98% as determined by HPLC analysis. All peptides were dissolved in dimethyl sulfoxide and then diluted in media to the desired working concentrations.

Plasmid constructs

The pcDNA3.1 expression vector was purchased from Invitrogen Corp. The serum-response element (SRE)-driven luciferase activity (SRE-luc) vector was purchased from Stratagene. The cDNAs for human GALR1, GALR2, GALR3, and KISSR were from BRN SCIENCE, Inc. The cDNA genes were inserted into the *Eco*RI and *Xho*I sites of pcDNA3.1. The cDNAs for *Xenopus* GALR1a, GALR2a, GALR2b, GALR3, and zebrafish GALR1a, GALR1b, GALR2a, and GALR2b were obtained by RT-PCR. All genes were constructed at the *Eco*RI and *Xho*I sites of pcDNA3.1 by PCR using appropriate primers from CosmogenTech, Inc. The identity of each gene was verified by sequencing.

Cell transfection and luciferase assay

HEK293 cells stably expressing $G\alpha_{qi}$ that can mediate $G\alpha_i$-coupled receptor activation by stimulating $G\alpha_q$-dependent signaling pathways (18) were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 μg/mL streptomycin (Invitrogen). Cells were seeded in 48-well

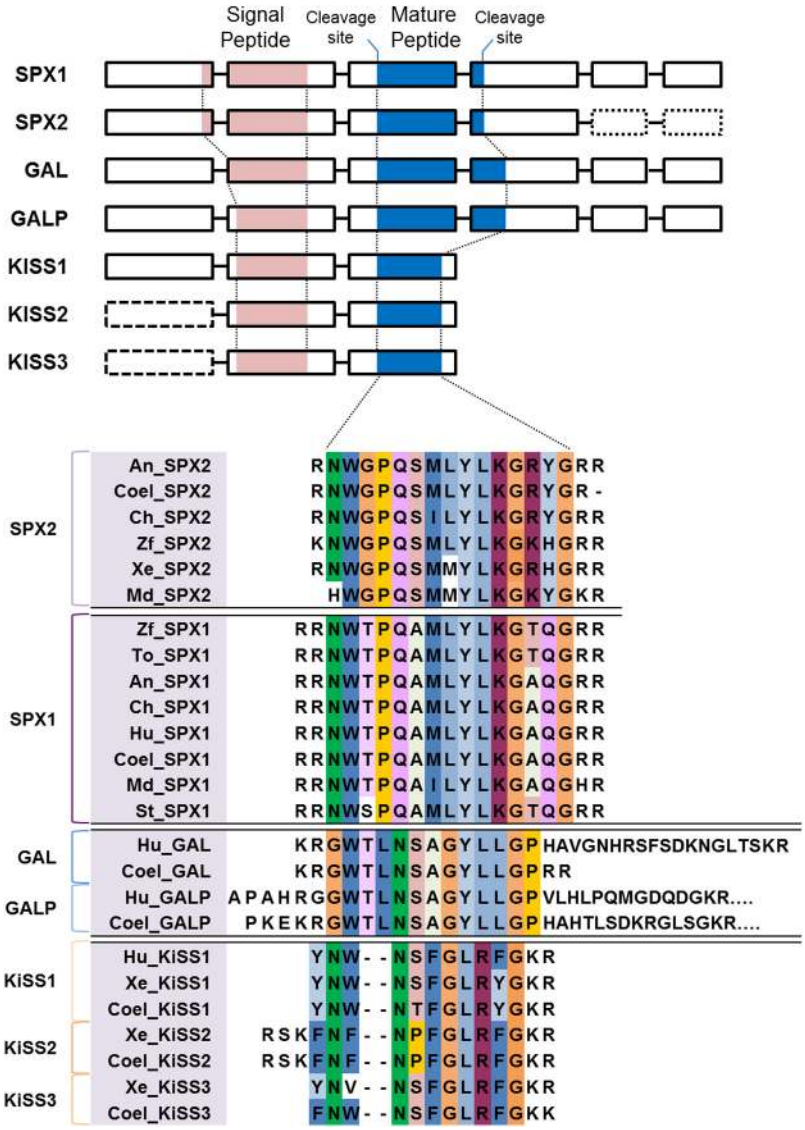

Figure 1. The general structure of exon and amino acid sequence alignment of KISS, GAL, and SPX family peptides. The mature peptide flanked by cleavage sites and signal peptide region is represented within the coding exon structure. Because the presence of noncoding exons in *SPX2*, *KISS2*, and *KISS3* is uncertain, the dashed boxes were used. The mature peptide sequences along with monobasic or dibasic sites were aligned, and conserved residues among the peptides are indicated by different colors as proposed by ClustalX-2.1. Variable regions of GAL and GALP were not aligned. The predicted mature peptide sequences were retrieved from human (Hu), anole lizard (An), chicken (Ch), *Xenopus* (Xe), coelacanth (Coel), zebrafish (Zf), medaka (Md), stickleback (St), and *Tetraodon* (To) genomes.

plates at a density of 2×10^4 cells per well, 1 day before transfection. A mixture containing 75 ng of the SRE-luc reporter construct, 75 ng of expression plasmid, and 2 μL of Effectene reagent (QIAGEN) was prepared and added to each well according to the manufacturer's instructions. Cells were then maintained in serum-free DMEM for 16–18 hours before treatment with the ligands. Approximately 48 hours after transfection, cells were treated with the respective ligands for 6 hours. Cells were lysed by adding 100 μL lysis buffer. The luciferase activity in 50 μL of cell extract was determined by a luciferase assay system according to the standard protocol for the Synergy 2 Multi-Mode Microplate Reader (BioTek).

Binding assay

GAL and SPX1 was radioiodinated by the chloramine-T method and then purified by chromatography on Sephadex G-25 (for ^{125}I-GAL) and G-10 (for ^{125}I-labled SPX1) columns (Sigma-Aldrich) in 0.01M acetic acid and 0.1% BSA (19). HEK293T cells were transfected with human GALR1, GALR2, or GALR3 (300 ng of DNA/well in 12-well plates) with Effectene (QIAGEN). Cells were washed after 48 hours and incubated for 1 additional hour with binding buffer (serum-free DMEM with 0.1% BSA; pH 7.4) containing 100 000 cpm of ^{125}I-labeled ligand in the absence or presence of various concentrations of unlabeled peptides. Cells were washed twice with ice-cold Dulbecco's PBS and dissolved in 1% sodium dodecyl sulfate and 0.2M NaOH. Radioactivity was measured with a Wallac 1489 Wizard 3 γ-counter (PerkinElmer Life Science).

Data analysis

Data analysis was performed by nonlinear regression with a sigmoidal dose response. The agonist concentrations that induced half-maximal stimulation (EC_{50}) or half-maximal inhibition of binding (IC_{50}) were calculated with GraphPad PRISM4 software (GraphPad Software, Inc). All data are presented as mean ± SE of at least 3 independent experiments.

Results

Presence of *SPX2* in vertebrate genomes

Using genome BLAST search information, we cloned partial cDNAs encoding SPX2 from the brain of *X. tropicalis* and zebrafish (Supplemental Figure 1). We also identified putative *SPX2* genes from chicken, coelacanth, anole lizard, and medaka (Supplemental Figure 2). Mammals may not have *SPX2*. The coding exon structure of *SPX2* is quite similar to that of *SPX1*. The first and second exons encode the signal peptide, and the third and fourth exons encode the mature peptide sequences flanked by conserved monobasic and dibasic cleavage sites (Figure 1). Like *SPX1*, *SPX2* encodes a mature peptide containing 14 amino acids that are amidated at the C terminus. The amino acid sequence of SPX2 differs from that of SPX1 at positions 3 (Gly vs Thr), 6 (Ser vs Ala), 13 (Arg

vs Thr or Ala), and 14 (Tyr or His vs Gln) (Figure 1). Sequence alignment of mature peptides for SPX, GAL, and KISS revealed conservation of several residues. For example, Trp^2, Thr^3, Tyr^9, Leu^{10}, and Gly^{12} of SPX1 are identical to those at the corresponding positions of GAL. It is of noteworthy that Trp^2, Thr^3, and Tyr^9 of GAL are critical determinants for receptor binding and activation (20). Although SPX and KISS do not share a high degree of sequence identity, GAL and KISS do share sequence similarity. For example, Asn^4, Ser^5, and Gly^7 of KISS are commonly observed in positions 5, 6, and 8, respectively, of GAL (Figure 1).

SPX, *GAL*, and *KISS* genes on the same *VAC* linkage group

The evolutionary relationship among *SPX*, *GAL*, and *KISS* genes was examined by synteny of vertebrate chromosomes containing these peptide genes (Supplemental Figure 3 and Supplemental Table 1). *SPX1* localizes closely with *KISS2* on the same chromosomes of coelacanth, anole lizard, zebrafish, and *Tetraodon*, although the corresponding human chromosome lacks *KISS2*. *SPX2* and *GAL* are on the same chromosome of coelacanth, anoles, chicken, *Xenopus*, zebrafish, and medaka. *GAL-like peptide* (*GALP*) and *KISS3* reside in the near vicinity in coelacanth and *Xenopus* chromosomes. To determine the mechanism of gene/genome duplication, we traced the locations of the peptide genes along with their neighbor genes on the ancient vertebrate gene linkage groups reconstructed by Nakatani et al (17). According to the Nakatani model, approximately 40 post-2R reconstructed gnathostome ancestor chromosomes (*GACs, A0* -J1) are derived from the pre-2R *VAC*s, which are denoted as the *A-J* linkage groups (17). Tracing the genome fragment on the ancestral genome revealed that all the *SPX*, *KISS*, and *GAL* genes are closely located in the *VAC D* (*GACs D0*, *D1*, *D2*, and *D3*) linkage group that commonly contains the *SYT*, *PLEKHA*, and *TEAD* gene families (Figure 2A). These results suggest that the ancestral form of *SPX*, *GAL*, and *KISS* arose by tandem local duplications before 2R.

Phylogenetic analysis was performed using the mature peptide sequences of SPX, GAL, and KISS from representative vertebrate taxa, allatostatin-4 (a GAL/KISS-like peptide in *Drosophila*), and 2 KISS peptides from lamprey. Each peptide group in the phylogenetic tree was matched on the *GAC* blocks. The SPX family is phylogenetically closer to the GAL family than to the KISS family (Figure 2B). Phylogeny showed 2 distinct groups for SPX1 and SPX2 on *GAC D0* and *D1*, respectively. The absence of *SPX* genes on *GAC D2* and *D3* suggests the loss of *SPX* on *GAC D2/3* after the first round of WGD. Thus, *SPX1* and *SPX2* are likely to have emerged through a second round of WGD of *GAC D0/D1* (Figure 2C). Two GAL groups are matched on *GAC D1* and *D3*, indicating a loss of the third and fourth *GAL* genes (on *GAC D0* and *GAC D2*, respectively) after the second round of WGD. Three branches of the *KISS* groups are mapped on *GAC D0* (*KISS2*), *D2* (*KISS1*), and *D3* (*KISS3*). Thus, *KISS1* and *KISS3* emerged through a second round of WGD of *GAC D2/D3*, and the fourth *KISS* on *GAC D1* was lost after duplication of *GAC D0/D1* (Figure 2C).

Presence of *GALR1*, *GALR2/3*, and *KISSR* ancestors before WGD

Amino acid sequences of GALR and KISSR from vertebrates, lamprey, *Ciona*, amphioxus, *Drosophila*, and *C. elegans* were retrieved by the ENSEMBL and NCBI genome browsers (Supplemental Tables 1 and 2). Synteny of vertebrate chromosomes containing *GALR* and *KISSR* were carried out, and these chromosomal fragments were matched on the ancestral chromosomes (Supplemental Figure 4). The phylogenetic tree shows 3 distinct lineages for *GALR1*, *GALR2/3*, and *KISSR* that are on the 3 independent linkage groups *VAC B*, *I*, and *A*, respectively (Figure 3). Thus, the human *GALR1* is from a different lineage than that of *GALR2* and *GALR3*. The *GALR1* subfamily splits into 2 branches (*GALR1a* and *GALR1b*) that locate on *GAC B0* and *B4*, respectively, indicating that the third and fourth *GALR1s* were lost after 2R. The *GALR2/3* family is divided into the 3 members *GALR2a*, *GALR2b*, and *GALR3*, which are on *GAC I1*, *I0*, and *I2*, respectively. According to the Nakatani model, there are only 3 paralogons for the *GAC I* linkage group (17). Thus, the complete set of *GALR2/3* exists after 2R and before the divergence of the teleost and tetrapod lineages. *GALR2a* and *GALR2b* are phylogenetically closer to each other than to *GALR3*; thus, they are likely to have emerged through a second round of WGD. Teleosts lack *GALR3*. The KISSR family has 4 members that are on *GAC A0* to *A3*. *KISSR1* and *KISSR3* are closer to each other than to *KISSR2* and *KISSR4*, and vice versa, which is consistent with the Nakatani model for *VAC A* chromosome duplication. Some genes have been lost during evolution. For example, whole *KISS/KISSR* pairs are completely absent in chicken (21). However, coelacanth, which is considered as an ancestor of tetrapods, has all isoforms of *KISSRs* (22) and *GALRs*. The basal lamprey contains 1 *KISSR*, 2 *GALR1s*, and 1 *GALR2/3*. In invertebrate species, *Ciona* has only 1 form of *GALR* but lacks *KISSR*, and the early deuterostome amphioxus possesses 6 forms of *KISSR* and 6 forms of *GALR2/3* (23). In protostomes, *Drosophila* contains 2 forms of allatostatin receptor, and *C. elegans* has 1 form of *NPR-9* (24, 25). The

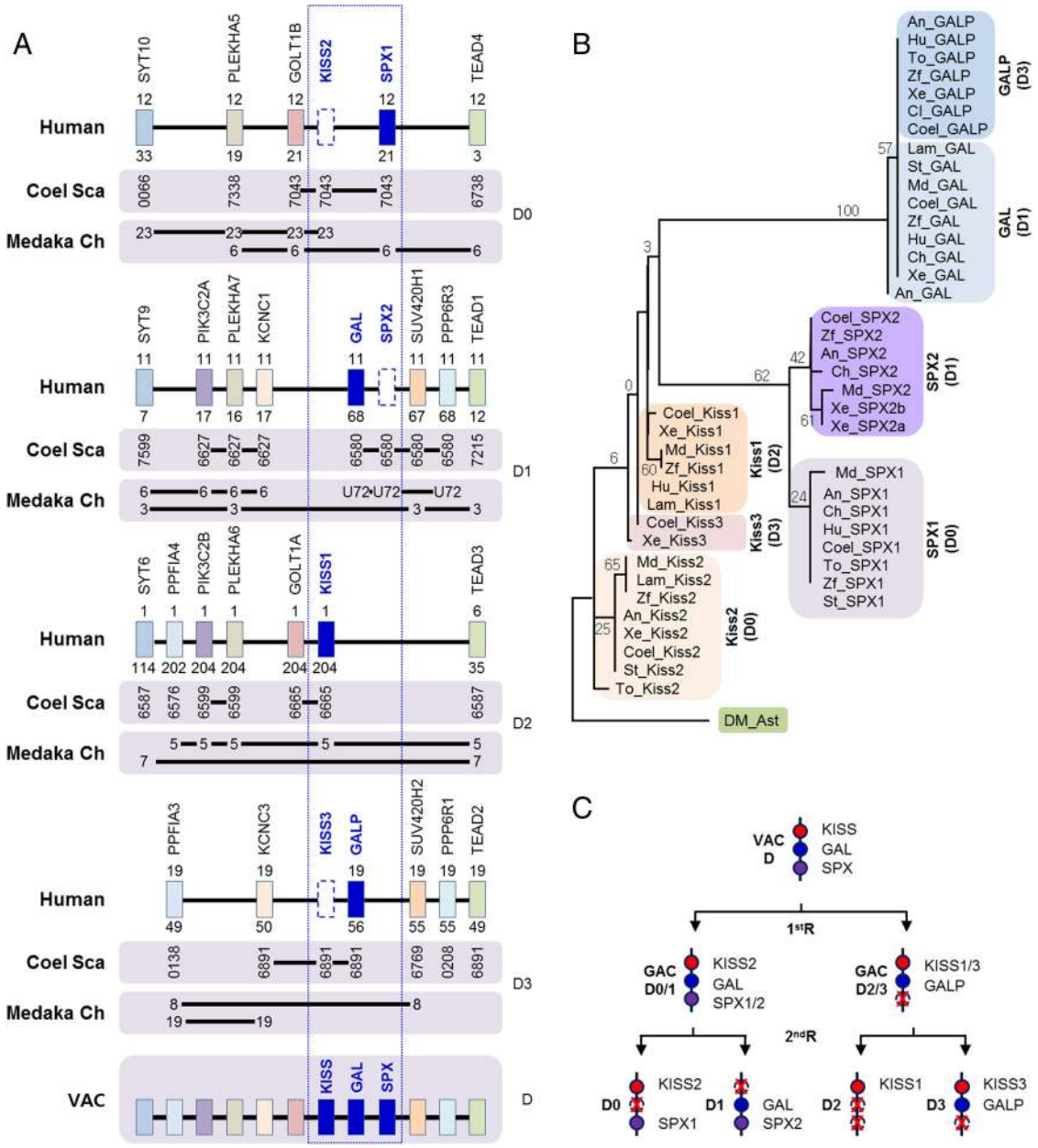

Figure 2. Evolutionary relationships among KISS, GAL, and SPX. A, Synteny for chromosomal regions containing the peptide genes. The peptides and their neighboring genes on human, coelacanth, and medaka genome fragments are matched on the *GAC D0 ~ D3* linkage groups. Paralogous genes are aligned on the same column with the same color. For humans, chromosome numbers are indicated above the gene, and gene locations (megabase) are indicated below the gene. The broken boxes indicate the absence of these genes on the human chromosomes. For coelacanth and medaka, only chromosome or scaffold numbers of orthologs for the human gene are presented in the horizontal bars. For coelacanth scaffold numbers, only the last 4 digits are shown (eg, JH127043 is indicated as 7043). For medaka, genes duplicated by teleost-specific 3R are indicated in 2 different lines. Ancestral genes are aligned on the pre-2R *VAC D* linkage group. The clustered location of KISS, GAL, and SPX is indicated by a vertical dashed box. B, Maximum likelihood phylogenetic tree for mature KISS, GAL, and SPX from vertebrates is constructed by MEGA5.1. The lamprey (Lam) GAL and KISS peptides are included. allatostatin-4, a GAL/KISS-like peptide in *Drosophila* (DM_Ast), is used for the out-group. Bootstrap values represent 100 replicates; the locations of the genes on the GAC linkage group are represented next to colored boxes. C, Proposed duplication scheme for KISS, GAL, and SPX during the first (firstR) and second (secondR) round of WGD. *VAC D* containing the ancestors for *KISS*, *GAL*, and *SPX* is duplicated to produce *GAC D0 ~ D3*. Gene loss occurs during each step of WGD.

receptors in these protostomes are phylogenetically more related to *KISSR* than *GALR* (Figure 3).

SPX specifically activates GALR2 and GALR3

The amino acid similarity between SPX and GAL suggests the possibility that SPX can activate GALR. Because each GALR subtype is coupled to different G proteins (G_i for GALR1 and GALR3; $G_{q/11}$ for GALR2) (26), we employed the HEK293-G_{qi} stable cell line for these experiments. Treatment of HEK293-G_{qi} cells expressing each GALR with GAL increased a SRE-luc in a concentration-dependent manner. GAL exhibited the strongest induction

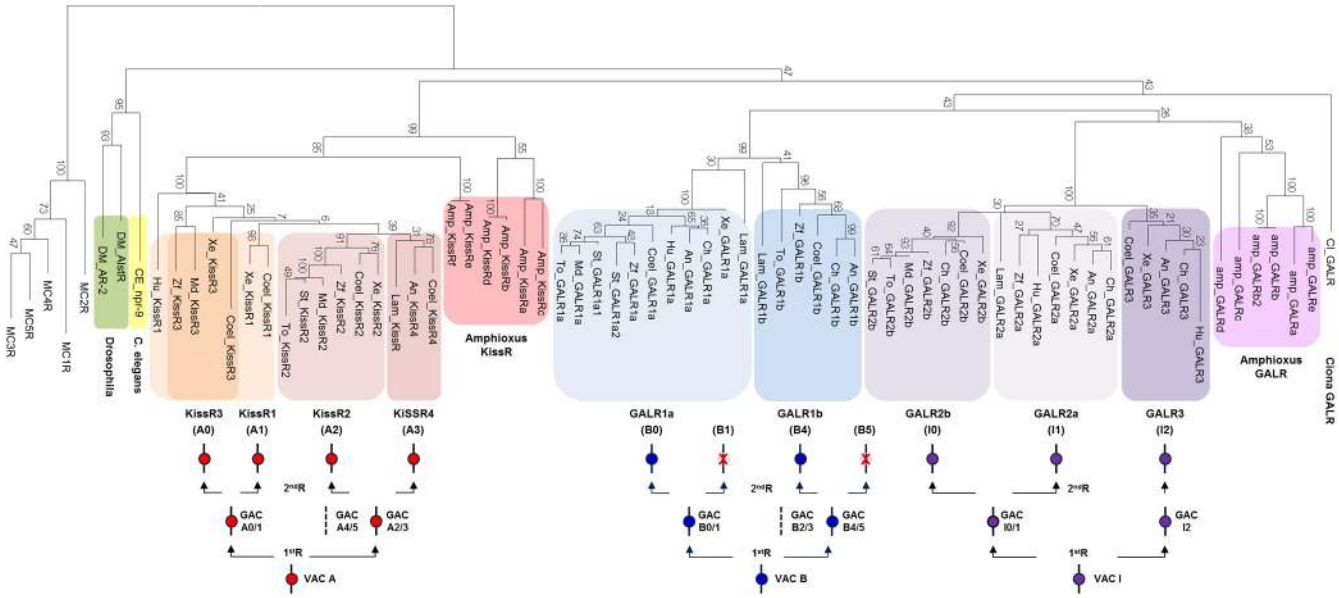

Figure 3. Maximum likelihood phylogenetic tree for KISSR, GALR, ALSTR, and NPR-9. The receptor amino acid sequences of human (Hu), anole lizard (An), chicken (Ch), *Xenopus* (Xe), zebrafish (Zf), medaka (Md), *Tetraodon* (To), stickleback (St), coelacanth (Coel), lamprey (Lam), amphioxus (Amp), *Ciona intestinalis* (CI), *C. elegans* (CE), and *Drosophila melanogaster* (DM) are aligned using MUSCLE, and tree is constructed by MEGA 5.1. Receptors are grouped in areas with different colors. Bootstrap values represent 100 replicates. Melanocortin receptors are used as the out-group. The proposed duplication scheme for KISSR on *VAC A*, GALR1 on *VAC B*, and GALR2/3 on *VAC I* during the first (firstR) and second (secondR) round of WGD is shown. According to the Nakatani model, *GAC A4/5* and *GAC B2/3* are generated through chromosome fission after the first round of WGD. Because these linkage groups do not contain KISSR or GALR1, these groups are excluded from the genome duplication scheme. The Nakatani model also proposes the absence of genome duplication of *GAC I2* during the second round of WGD. Gene loss occurs during each step of WGD.

of human GALR1 activity and moderate induction of human GALR2 activity. High concentrations of GAL were required to activate human GALR3. Human SPX1 and zebrafish SPX2 elevated SRE-luc activity in cells expressing human GALR2 and GALR3 but not in cells expressing human GALR1 (Figure 4, A–C). The potencies of SPXs toward human GALR2 were slightly less than that of GAL (for SPX1, EC_{50} = 161nM; for SPX2, EC_{50} = 317nM; and for GAL, EC_{50} = 88nM). The potencies of SPXs toward human GALR3 was greater than that of GAL (for SPX1, EC_{50} = 626nM; for SPX2, EC_{50} = 724nM; and for GAL, EC_{50} = 1383nM). SPX1 was not able to activate KISSR (data not shown).

The binding affinity of SPXs to human GALRs was determined using a competitive displacement binding assay. ^{125}I-labled GAL was used for human GALR1 and GALR2, and ^{125}I-labled SPX1 was used for human GALR3. SPXs displaced ^{125}I-labled ligands in cells expressing human GALR2 and GALR3 but not in cells expressing human GALR1, in a manner similar to that shown in the receptor activation study (Figure 4, D–F). Together, these results indicate that SPXs are a functional agonist for human GALR2 and GALR3 but not for human GALR1.

To further corroborate the interaction between SPXs and GALR2/3, cells expressing nonmammalian GALRs (from *Xenopus* and zebrafish) were treated with GAL or SPXs. Like human GALR1, *Xenopus* GALR1 and zebrafish GALR1a and GALR1b did not respond to SPX but were activated by GAL (Figure 5, A, E, and F). *Xenopus* GALR2a showed a highest response to GAL but poor response to SPXs (Figure 5B). In cells expressing *Xenopus* GALR2b and GALR3, SPX1 and SPX2 exhibited higher potency than GAL in inducing SRE-luc activity (Figure 5, C and D). In zebrafish, GALR2a responded to all 3 ligands with similar potency (Figure 5G). SPXs have higher potencies toward zebrafish GALR2b than GAL (Figure 5H). These results indicate that SPXs can activate GALR2/3 in the nonmammalian system.

Discussion

The evolutionary relationship and history of a gene family can be explored by the analysis of phylogeny and synteny of chromosomal fragments containing the gene family members (11–13). Recent work showed that relocating the family genes on the reconstructed vertebrate ancestral linkage groups is an additional analytical tool (14, 15). This method is particularly useful for a gene family with a large number of paralogs that arose before WGD. Because this method provides a WGD scheme for each linkage

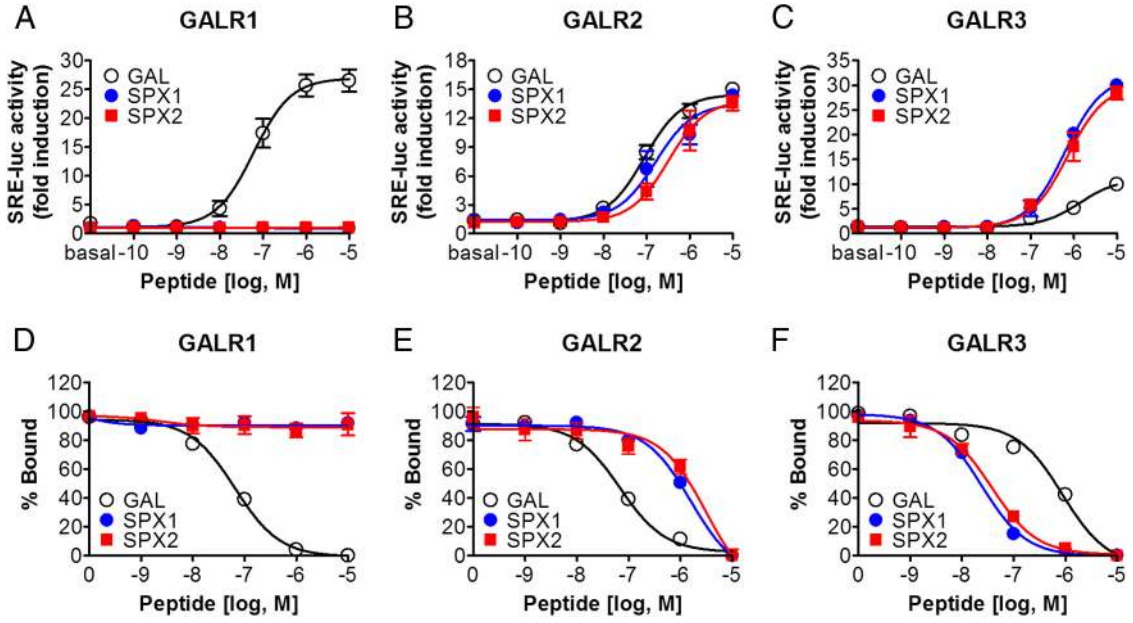

Figure 4. SPX activates human GALR2/3. Plasmids containing human cDNAs for GALR1 (A), GALR2 (B), and GALR3 (C) were cotransfected with the SRE-luc reporter vector into HEK293-G_{qi} cells. Forty-eight hours after transfection, cells were incubated with increasing concentrations of the human peptides GAL (○), SPX1 (●), and SPX2 (■). Luciferase activity was then determined. For the displacement binding assay, cDNAs for GALR1 (D), GALR2 (E), and GALR3 (F) were transfected into HEK293T cells. Cells were then incubated with ^{125}I-GAL (D and E) or ^{125}I-SPX1 (F) in the presence of various concentrations of unlabeled GAL (○), SPX1 (●), and SPX2 (■) ligands. All experiments were performed in triplicate and repeated at least 3 times.

group, gene duplication and/or loss of a gene during each step of genome duplication can be traced (15). However, some ancestral linkage groups are inaccurately reconstructed because of massive chromosome rearrangements in these regions, or because of single gene translocation that caused it to move from its authentic chromosomal fragments after 2R. Thus, the combination with phylogenic and syntenic analyses provides a better understanding of the evolutionary relationship and history of a gene family (14, 15).

Tracing the evolutionary history of a peptide gene family is particularly difficult due to several reasons. Evolutionary pressure acts only on functional mature peptide sequences; the precursor sequences, including the signal peptide sequence, are highly variable even among vertebrate orthologs (12, 14). The mature peptide sequences are also highly variable among the paralogs that arose before WGD (14, 27). For instance, the peptide families for class B GPCRs evolved to preserve the 3-dimensional structure of the mature peptide, rather than to conserve the

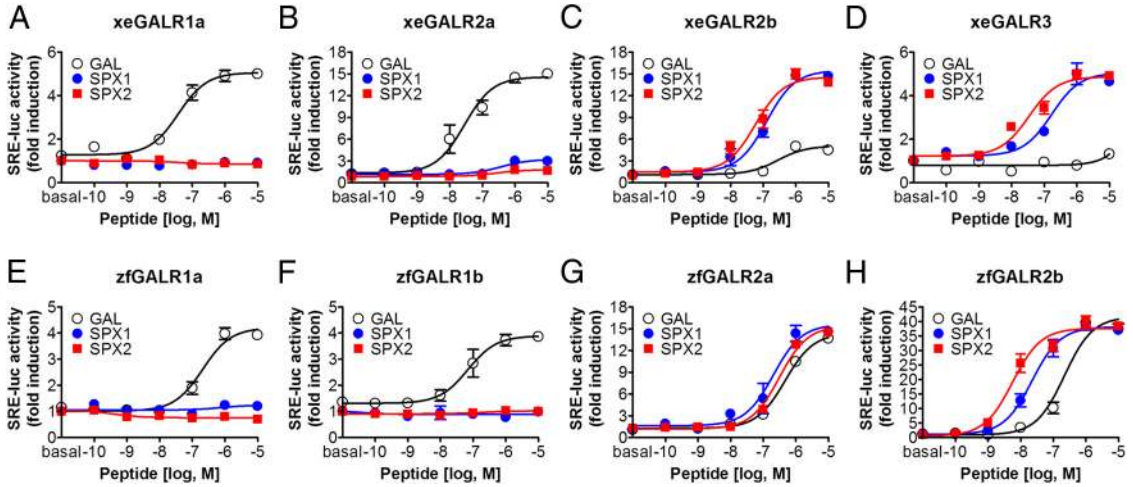

Figure 5. SPX activates nonmammalian GALR2/3. Plasmids containing *Xenopus* cDNAs for GALR1a (A), GALR2a (B), GALR2b (C), and GALR3 (D) and zebrafish cDNAs for GALR1a (E), GALR1b (F), GALR2a (G), and GALR2b (H) were cotransfected with the SRE-luc reporter vector into HEK293-G_{qi} cells. Forty-eight hours after transfection, cells were incubated with increasing concentrations of the human peptides GAL (○), SPX1 (●), and SPX2 (■). Luciferase activity was then determined. All experiments were performed in triplicate and repeated at least 3 times.

amino acid sequences (28). Thus, their evolutionary relationships can be postulated based on the relationships among their corresponding receptor family members (14, 27). Alternatively, the distance in the genome among the peptide family genes can account for their evolutionary relationship. For example, paralogs that emerged through local tandem duplications before WGD often remain closely localized on the same chromosomes (13, 14).

On the reconstructed ancestral chromosome, *SPX* genes are closely localized with *KISS* and *GAL*. Although the sequence similarity among SPX, GAL, and KISS was not noticed before our analysis, KISS1R was found to have a considerably high degree (>44%) of amino acid sequence identity with GALRs (16). Our phylogenic and syntenic analyses indicated the presence of the 3 distinct gene groups *KISSR*, *GALR1*, and *GALR2/3* that likely correspond to the 3 peptide gene lineages *KISS*, *GAL*, and *SPX*, respectively. The ligand-receptor interaction assay revealed that SPXs can activate GALR2 and GALR3 but did not activate GALR1 or KISSR. Thus, SPX1 may be a GALR2/3-specific ligand in mammals, which may further facilitate our knowledge of the physiological function of SPX1 and GALR2/3. SPX2 is found in a variety of vertebrate species but not in mammals. Considering the presence of the 3 GALR2/3 members GALR2a, GALR2b, and GALR3 in vertebrates, the function of SPX2 could be mediated through one of these receptors in vertebrates. It is of interesting to note that SPX2 exhibited a highest potency toward *Xenopus* GALR3 and zebrafish GALR2b in a heterologous expression system. These results support the coevolution of these 3 sets of ligand-receptor pairs during early vertebrate evolution.

Because *SPX*, *GAL*, and *KISS* genes arose from a common ancestor, they may share similar functions. For example, several reports suggest a functional relationship among SPX, GAL, and KISS, particularly in reproduction and feeding behaviors. GAL and KISS exert a stimulatory effect on secretion of GnRH and LH in mammals (29–32). SPX, however, suppresses LH secretion from the gonadotrope of sexually mature female goldfish (4). KISS1R and GALRs are commonly expressed in the hypothalamus (33–35). Neurons that secrete GnRH express both KISS1R (33) and GALR (36). Subsets of KISS neurons also express GAL in the anteroventral periventricular nucleus of the preoptic region, periventricular area of the third ventricle, and arcuate nucleus (37). The 2 distinct neuropeptides KISS and GAL are directly involved in the control of GnRH release (33, 36, 38), which suggests a functional conservation of the KISS/KISSR and GAL/GALR systems in GnRH regulation. The involvement of GALP and KISS in feeding behavior has been demonstrated (30, 39–41). Centrally administered GALP and KISS may eventually induce a reduction of food intake (39, 42). Both central and peripheral injections of SPX1 into goldfish result in the prompt inhibition of food intake and feeding behavior (5). It is of interest to note that GALR2 and GALR3 exist in the arcuate, dorsomedial, and ventromedial nuclei of the hypothalamus, where regulation of appetite takes place (43).

SPX may have additional roles in peripheral tissues and the brain regions other than the hypothalamus. Exposure of rat stomach explants to SPX1 induces rapid smooth muscle contraction (1). SPX1 can increase the proliferation of rat adrenocortical cells (7). SPX1 induces an immediate decrease in heart rate and urine flow rate, increases mean arterial pressure and urinary sodium excretion, and produces an antinociceptive effect in rats (9). The function of SPX in brain tissues other than the hypothalamus can be postulated based on observations of *GALR2* knockout (or knockdown) mice and animal models treated with GALR2/3-specific agonists and antagonist. For example, *GALR2* knockdown in the dentate gyrus causes serious seizures in rats (42). Activation of dentate gyrus GALR2 by a specific agonist causes transient attenuation of long-term potentiation (44). *GALR2* null-mutant mice exhibit anxiety-like behavior (45). Microinjection of a GALR2-specific agonist into the spinal cord induces allodynic effects (46). GALR3-specific antagonists increase anxiety and induce depressant-like behavior in rats (47). Overall, these observations suggest the possible involvement of SPX, in conjunction with GALR2 and GALR3, in learning and memory, seizure, pain, anxiety, and mood disorder.

In summary, syntenic and evolutionary genomics tools are useful to explore the evolutionary relationship of a peptide gene family that appeared to be unrelated because of amino acid sequence differences within short mature peptides. When this gene family arose before 2R, variations among the mature peptide sequences were much greater than those that occurred through 2R. Therefore, conventional phylogenetic tools often fail to delineate the relationships among this gene family. This study shows that *SPX*, *GAL*, and *KISS* genes are closely localized on the same ancestral chromosome, suggesting that these 3 neuropeptide genes arose through local duplications before 2R. SPX was found to activate GALR2/3 receptors in a heterologous expression system; thus, our study may facilitate the understanding of the physiological functions of SPX in relation with GALR2/3.

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References

1. **Mirabeau O, Perlas E, Severini C, et al.** Identification of novel peptide hormones in the human proteome by hidden Markov model screening. *Genome Res*. 2007;17:320–327.
2. **Sonmez K, Zaveri NT, Kerman IA, et al.** Evolutionary sequence modeling for discovery of peptide hormones. *PLoS Comput Biol*. 2009;5:e1000258.
3. **Wan B, Wang XR, Zhou YB, Zhang X, Huo K, Han ZG.** C12ORF39, a novel secreted protein with a typical amidation processing signal. *Biosci Rep*. 2010;30:1–10.
4. **Liu Y, Li S, Qi X, et al.** A novel neuropeptide in suppressing luteinizing hormone release in goldfish, *Carassius auratus*. *Mol Cell Endocrinol*. 2013;374:65–72.
5. **Wong MK, Sze KH, Chen T, et al.** Goldfish spexin: solution structure and novel function as a satiety factor in feeding control. *Am J Physiol Endocrinol Metab*. 2013;305:E348–E366.
6. **Porzionato A, Rucinski M, Macchi V, Stecco C, Malendowicz LK, De Caro R.** Spexin expression in normal rat tissues. *J Histochem Cytochem*. 2010;58:825–837.
7. **Rucinski M, Porzionato A, Ziolkowska A, et al.** Expression of the spexin gene in the rat adrenal gland and evidences suggesting that spexin inhibits adrenocortical cell proliferation. *Peptides*. 2010;31:676–682.
8. **Porzionato A, Rucinski M, Macchi V, et al.** Spexin is expressed in the carotid body and is upregulated by postnatal hyperoxia exposure. *Adv Exp Med Biol*. 2012;758:207–213.
9. **Toll L, Khroyan TV, Sonmez K, et al.** Peptides derived from the prohormone proNPQ/spexin are potent central modulators of cardiovascular and renal function and nociception. *FASEB J*. 2012;26:947–954.
10. **Fredriksson R, Lagerström MC, Lundin LG, Schiöth HB.** The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol*. 2003;63:1256–1272.
11. **Larsson TA, Olsson F, Sundstrom G, et al.** Early vertebrate chromosome duplications and the evolution of the neuropeptide Y receptor gene regions. *BMC Evol Biol*. 2008;8:184.
12. **Lee YR, Tsunekawa K, Moon MJ, et al.** Molecular evolution of multiple forms of kisspeptins and GPR54 receptors in vertebrates. *Endocrinology*. 2009;150:2837–2846.
13. **Kim DK, Cho EB, Moon MJ, et al.** Revisiting the evolution of gonadotropin-releasing hormones and their receptors in vertebrates: secrets hidden in genomes. *Gen Comp Endocrinol*. 2011;170:68–78.
14. **Hwang JI, Moon MJ, Park S, et al.** Expansion of secretin-like G protein-coupled receptors and their peptide ligands via local duplications before and after two rounds of whole-genome duplication. *Mol Biol Evol*. 2013;30:1119–1130.
15. **Yegorov S, Good S.** Using paleogenomics to study the evolution of gene families: origin and duplication history of the relaxin family hormones and their receptors. *PLoS One*. 2012;7:e32923.
16. **Lee DK, Nguyen T, O'Neill GP, et al.** Discovery of a receptor related to the galanin receptors. *FEBS Lett*. 1999;446:103–107.
17. **Nakatani Y, Takeda H, Kohara Y, Morishita S.** Reconstruction of the vertebrate ancestral genome reveals dynamic genome reorganization in early vertebrates. *Genome Res*. 2007;17:1254–1265.
18. **Conklin BR, Farfel Z, Lustig KD, Julius D, Bourne HR.** Substitution of three amino acids switches receptor specificity of Gq α to that of Gi α. *Nature*. 1993;363:274–276.
19. **Moon MJ, Kim HY, Park S, et al.** Evolutionarily conserved residues at glucagon-like peptide-1 (GLP-1) receptor core confer ligand-induced receptor activation. *J Biol Chem*. 2012;287:3873–3884.
20. **Carpenter KA, Schmidt R, Yue SY, et al.** The glycine residue in cyclic lactam analogues of galanin(1–16)-NH2 is important for stabilizing an N-terminal helix. *Biochemistry*. 1999;38:15295–15304.
21. **Um HN, Han JM, Hwang JI, Hong SI, Vaudry H, Seong JY.** Molecular coevolution of kisspeptins and their receptors from fish to mammals. *Ann NY Acad Sci*. 2010;1200:67–74.
22. **Pasquier J, Lafont AG, Tostivint H, Vaudry H, Rousseau K, Dufour S.** Comparative evolutionary histories of kisspeptins and kisspeptin receptors in vertebrates reveal both parallel and divergent features. *Front Endocrinol (Lausanne)*. 2012;3:173.
23. **Liu Z, Xu Y, Wu L, Zhang S.** Evolution of galanin receptor genes: insights from the deuterostome genomes. *J Biomol Struct Dyn*. 2010;28:97–106.
24. **Lenz C, Søndergaard L, Grimmelikhuijzen CJ.** Molecular cloning and genomic organization of a novel receptor from *Drosophila melanogaster* structurally related to mammalian galanin receptors. *Biochem Biophys Res Commun*. 2000;269:91–96.
25. **Bendena WG, Boudreau JR, Papanicolaou T, Maltby M, Tobe SS, Chin-Sang ID.** A *Caenorhabditis elegans* allatostatin/galanin-like receptor NPR-9 inhibits local search behavior in response to feeding cues. *Proc Natl Acad Sci USA*. 2008;105:1339–1342.
26. **Webling KE, Runesson J, Bartfai T, Langel U.** Galanin receptors and ligands. *Front Endocrinol (Lausanne)*. 2012;3:146.
27. **Cardoso JC, Vieira FA, Gomes AS, Power DM.** The serendipitous origin of chordate secretin peptide family members. *BMC Evol Biol*. 2010;10:135.
28. **Neumann JM, Couvineau A, Murail S, Lacapère JJ, Jamin N, Laburthe M.** Class-B GPCR activation: is ligand helix-capping the key? *Trends Biochem Sci*. 2008;33:314–319.
29. **Castellano JM, Navarro VM, Fernández-Fernández R, et al.** Effects of galanin-like peptide on luteinizing hormone secretion in the rat: sexually dimorphic responses and enhanced sensitivity at male puberty. *Am J Physiol Endocrinol Metab*. 2006;291:E1281–E1289.
30. **Taylor A, Madison FN, Fraley GS.** Galanin-like peptide stimulates feeding and sexual behavior via dopaminergic fibers within the medial preoptic area of adult male rats. *J Chem Neuroanat*. 2009;37:105–111.
31. **Choe HK, Kim HD, Park SH, et al.** Synchronous activation of gonadotropin-releasing hormone gene transcription and secretion by pulsatile kisspeptin stimulation. *Proc Natl Acad Sci USA*. 2013;110:5677–5682.
32. **Roseweir AK, Kauffman AS, Smith JT, et al.** Discovery of potent kisspeptin antagonists delineate physiological mechanisms of gonadotropin regulation. *J Neurosci*. 2009;29:3920–3929.
33. **Messager S, Chatzidaki EE, Ma D, et al.** Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proc Natl Acad Sci USA*. 2005;102:1761–1766.
34. **Waters SM, Krause JE.** Distribution of galanin-1, -2 and -3 receptor messenger RNAs in central and peripheral rat tissues. *Neuroscience*. 2000;95:265–271.
35. **Mitchell V, Bouret S, Prévot V, Jennes L, Beauvillain JC.** Evidence for expression of galanin receptor Gal-R1 mRNA in certain gonadotropin releasing hormone neurones of the rostral preoptic area. *J Neuroendocrinol*. 1999;11:805–812.
36. **Mitchell V, Bouret S, Howard AD, Beauvillain JC.** Expression of the galanin receptor subtype Gal-R2 mRNA in the rat hypothalamus. *J Chem Neuroanat*. 1999;16:265–277.
37. **Kalló I, Vida B, Deli L, et al.** Co-localisation of kisspeptin with

galanin or neurokinin B in afferents to mouse GnRH neurones. *J Neuroendocrinol*. 2012;24:464–476.
38. **Tillet Y, Tourlet S, Picard S, Sizaret PY, Caraty A.** Morphofunctional interactions between galanin and GnRH-containing neurones in the diencephalon of the ewe. The effect of oestradiol. *J Chem Neuroanat*. 2012;43:14–19.
39. **Shiba K, Kageyama H, Takenoya F, Shioda S.** Galanin-like peptide and the regulation of feeding behavior and energy metabolism. *FEBS J*. 2010;277:5006–5013.
40. **Stengel A, Wang L, Goebel-Stengel M, Taché Y.** Centrally injected kisspeptin reduces food intake by increasing meal intervals in mice. *Neuroreport*. 2011;22:253–257.
41. **Luque RM, Kineman RD, Tena-Sempere M.** Regulation of hypothalamic expression of KiSS-1 and GPR54 genes by metabolic factors: analyses using mouse models and a cell line. *Endocrinology*. 2007;148:4601–4611.
42. **Mazarati A, Lu X, Kilk K, Langel U, Wasterlain C, Bartfai T.** Galanin type 2 receptors regulate neuronal survival, susceptibility to seizures and seizure-induced neurogenesis in the dentate gyrus. *Eur J Neurosci*. 2004;19:3235–3244.
43. **Qi Y, Henry BA, Oldfield BJ, Clarke IJ.** The action of leptin on appetite-regulating cells in the ovine hypothalamus: demonstration of direct action in the absence of the arcuate nucleus. *Endocrinology*. 2010;151:2106–2116.
44. **Badie-Mahdavi H, Lu X, Behrens MM, Bartfai T.** Role of galanin receptor 1 and galanin receptor 2 activation in synaptic plasticity associated with 3′,5′-cyclic AMP response element-binding protein phosphorylation in the dentate gyrus: studies with a galanin receptor 2 agonist and galanin receptor 1 knockout mice. *Neuroscience*. 2005;133:591–604.
45. **Bailey KR, Pavlova MN, Rohde AD, Hohmann JG, Crawley JN.** Galanin receptor subtype 2 (GalR2) null mutant mice display an anxiogenic-like phenotype specific to the elevated plus-maze. *Pharmacol Biochem Behav*. 2007;86:8–20.
46. **Liu HX, Brumovsky P, Schmidt R, et al.** Receptor subtype-specific pronociceptive and analgesic actions of galanin in the spinal cord: selective actions via GalR1 and GalR2 receptors. *Proc Natl Acad Sci USA*. 2001;98:9960–9964.
47. **Swanson CJ, Blackburn TP, Zhang X, et al.** Anxiolytic- and antidepressant-like profiles of the galanin-3 receptor (Gal3) antagonists SNAP 37889 and SNAP 398299. *Proc Natl Acad Sci USA*. 2005;102:17489–17494.