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# New evidence for SPX2 in regulating the brain-pituitary reproductive axis of half- smooth tongue sole (*Cynoglossus semilaevis*)

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Spexin (SPX) is an evolutionarily conserved neuropeptide, which was first identified in human proteome by data mining. Two orthologs (SPX1 and SPX2) are present in some non-mammalian species, including teleosts. It has been demonstrated that SPX1 is involved in reproduction and food intake, whereas the functional role of SPX2 is still absent in any vertebrate. The aim of the current study was to evaluate the actions of intraperitoneal injection of endogenous SPX2 peptide on the expression levels of some key reproductive genes of the brain-pituitary axis in half-smooth tongue sole. Our data showed an inhibitory action of SPX2 on brain *gnih*, *spx1*, *tac3* and pituitary *gth $\alpha$* , *lh $\beta$*  mRNA levels. However, SPX2 had no significant effect on brain *gnih*, *gnrh2*, *gnrh3*, *kiss2*, *kiss2r*, *spx2* expression or pituitary *gh* expression. On the other hand, SPX2 induced an increase in pituitary *fs $\beta$*  expression. Taken together, our results provide initial evidence for the involvement of SPX2 in the regulation of reproduction in vertebrates, which is in accordance with previous studies on SPX1.

## KEYWORDS

spexin, GnIH, GnRH, kisspeptin, gonadotropin, reproduction

## Introduction

Spexin (SPX), also termed neuropeptide Q (NPQ) or C12ORF39, is a novel hypothalamic neuropeptide that was first identified by bioinformatics approach (1, 2), and subsequently its orthologs have been found from fish to mammals (3–5). The SPX mature peptide is a tetradecapeptide that is flanked by two dibasic protein cleavage sites (RR and GRR), and its amino acid sequence is highly conserved in various vertebrates, with only one amino acid substitution at position 13 between tetrapods and teleosts (3–5). Consistent with its widespread distribution in different tissues of teleosts and other vertebrates, SPX participates in a variety of physiological functions, such as glucose homeostasis, lipid metabolism, feeding, digestion, reproduction, among others (6–10). Preliminary evidence in teleosts and other vertebrates have indicated that SPX binds to the membrane galanin receptor 2 (Galr2) and Galr3, but not Galr1, to exert its functions (11–13).

Based on data acquisition and comparative synteny analysis, a novel SPX form, namely SPX2, has been identified in a few non-mammalian species, such as chicken, anole lizard, *Xenopus tropicalis*, zebrafish, medaka, and coelacanth (11). However, SPX2 is absent in mammals and the initial SPX is designated as SPX1 now (11). Interestingly, Nile tilapia and other cichlid fish species have two SPX1 paralogs (SPX1a and SPX1b) but have no SPX2 (12). In teleosts, the physiological functions of SPX are just emerging, and mainly focus on the control of reproduction and appetite (3–5). For instance, *in vivo* and *in vitro* administration of SPX1 suppress LH secretion in goldfish (14), and both LH and FSH plasma levels are significantly reduced after a single intraperitoneal injection of SPX1a or SPX1b in Nile tilapia (12). However, the reproductive capability is not impaired in SPX1 mutant zebrafish, suggesting that SPX1 is not essential for reproduction in this species (15).

SPX1 expression can be altered by nutritional status in several fish species (12, 16–22), and SPX1 has been shown to act as a satiety factor in goldfish (16) and zebrafish (15). Moreover, overexpression of SPX1 in the dorsal habenula reduces anxiety in zebrafish (23). On the other hand, no information is available regarding the biological role of SPX2 in any vertebrate, other than two studies on the SPX2-Galr2/3 interaction and detailed brain distribution of SPX2 in zebrafish (3–5). The serum response element-driven luciferase (SRE-luc) activity is significantly elevated by zebrafish SPX2 in HEK293 cells expressing zebrafish, *Xenopus*, and human Galr2 or Galr3, suggesting that SPX2 is an endogenous ligand for Galr2/3 (11). Recent data in zebrafish have revealed that SPX2 expression is restricted in the preoptic area of the hypothalamus by whole-mount *in situ* RNA hybridization, implying that SPX2 is implicated in reproduction and feeding control in this species (24). Accordingly, further investigation is urgently needed to clarify the potential role of SPX2 in vertebrates.

In all vertebrates, reproduction is mainly regulated by the brain-pituitary-gonadal (BPG) axis. A plethora of neuropeptides are involved in the control of reproduction, including gonadotropin-releasing hormone (GnRH), kisspeptin (Kiss), gonadotropin-inhibitory hormone (GnIH), neurokinin B (NKB), among others (25–35). Half-smooth tongue sole (*Cynoglossus semilaevis*) is an economically important marine flatfish that is widely cultured in China, and this species needs approximately 3 years of sexual maturation. In nature, the body length of mature females is twice larger and the body weight is over six times greater than those of mature males, exhibiting a sexual dimorphism of growth (36). Genes encoding these key factors have been cloned in half-smooth tongue sole, namely *gnrh2* (37), *gnrh3* (38), *kiss2* (39), Kiss2 receptor (*kiss2r*) (36), *gnih* (40), GnIH receptor (*gnih*) (41), and *tac3* (42). Furthermore, growth hormone (*gh*) and three gonadotropin subunits (*gth $\alpha$* , *lh $\beta$* , and *fsh $\beta$* ) are also available in this species (43, 44). Previous studies have indicated the existence of SPX1 and SPX2 in half-smooth tongue sole, and SPX1 exerts an action on the expression levels of brain and pituitary reproductive genes (20, 45). Herein, this study aimed to further clarify the possible role of SPX2 in the regulation of reproduction in this flatfish species.

## Materials and methods

### Animals

Approximately 2-year-old immature female tongue sole (body weight (BW), total length (TL) and gonadosomatic index (GSI) of  $772.61 \pm 25.69$  g,  $49.97 \pm 0.51$  cm and  $2.66 \pm 0.25\%$ , respectively) were purchased from Haiyang Yellow Sea Aquatic Product Co., Ltd. (Haiyang, China), and maintained in an indoor concrete tank with recirculating seawater (water temperature 21–23°C and dissolved oxygen > 6 mg/L). Fish specimens were acclimatized for one week under a cyclical light–dark photoperiod (12 h: 12 h) and fed to satiation twice daily with commercial dry pellets. The animal study protocol was approved by the Animal Care and Use Committee of Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (ID Number: YSFRI-2021025).

### Peptide synthesis

The tongue sole SPX2 mature peptide (45) with amidation at the C-terminus (LNIHWGPQSMYLGKGY-NH<sub>2</sub>) was synthesized by ChinaPeptides Co., Ltd. (Shanghai, China) with a purity of 95%, as determined by HPLC. The SPX2 peptide was dissolved in phosphate-buffered saline (PBS) just before the intraperitoneal injection experiments.

## In vivo effects of SPX2 on the brain-pituitary reproductive axis

SPX2 *in vivo* treatment experiments were generally performed as the previous study on tongue sole SPX1 (20). After acclimatization for one week as mentioned above, the fish were divided into three groups, anesthetized with MS222 (Sigma, 200 mg/L), weighed, and injected intraperitoneally with SPX2 peptide at two doses (100 ng/g BW and 1000 ng/g BW) or PBS alone ( $n = 8$  fish/group). The injection volume of each dosage varied depending on the body weight of each fish. The whole brain and pituitary tissues were collected 6 h after the injection, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until use.

## RNA extraction, reverse transcription, and real-time quantitative PCR

All experiments were performed as described previously (46). Total RNA was isolated using the RNAiso Plus reagent (Takara),

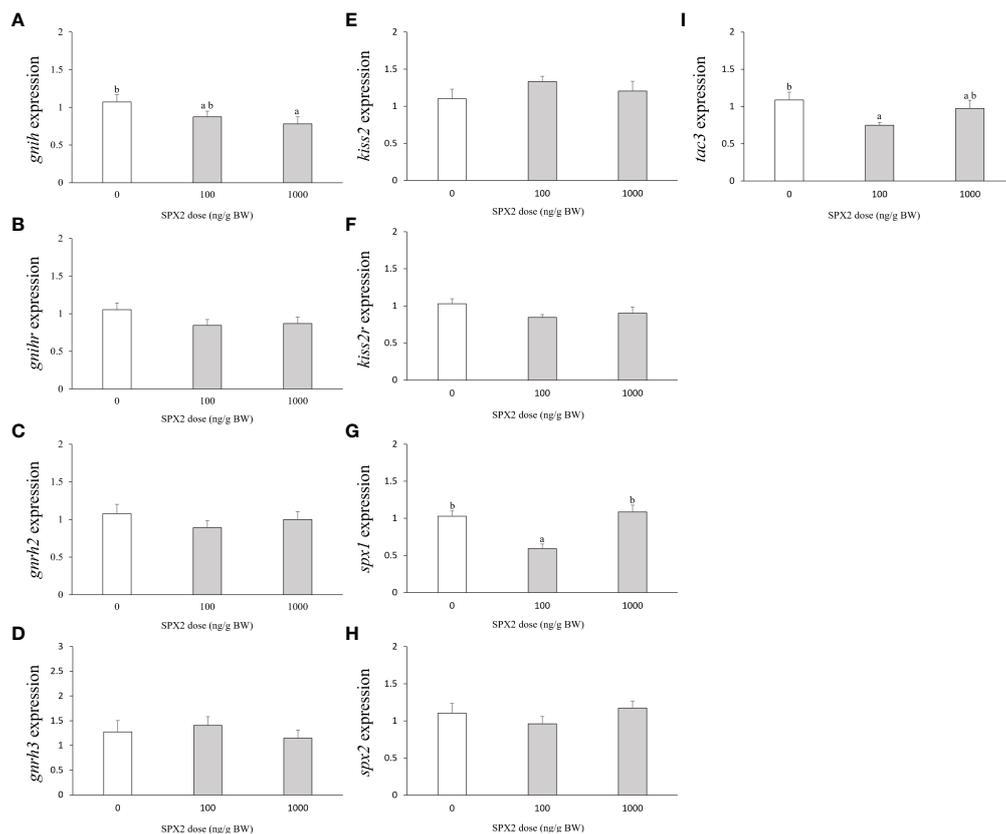
and 1  $\mu\text{g}$  of total RNA was used as a template for the first-strand cDNA synthesis using the PrimeScript<sup>TM</sup> RT reagent Kit with gDNA Eraser (Takara). Real-time quantitative PCR analysis was performed on the LightCycler<sup>®</sup> 96 PCR Instrument (Roche) using TB Green<sup>®</sup> Premix Ex Taq<sup>TM</sup> II (Takara) and the specific primers (Table 1). The thermal cycling profiles were as follows:  $95^{\circ}\text{C}$  for 30 s, and 40 cycles of  $95^{\circ}\text{C}$  for 5 s,  $60^{\circ}\text{C}$  for 20 s, and  $72^{\circ}\text{C}$  for 10 s. Melting curve analysis was performed in order to confirm the specificity of each product. 18s ribosomal RNA was used as the internal reference for data normalization. The relative expression levels of each gene were normalized against those of the housekeeping gene and calculated by the comparative Ct method.

## Statistical analysis

Data were analyzed by one-way ANOVA followed by Duncan's multiple comparison test using SPSS17.0, and are presented as mean  $\pm$  SEM. Differences were considered statistically significant at  $p < 0.05$ .

TABLE 1 List of primers for real-time quantitative PCR.

Name	Primer sequence (5'-3')	Amplicon size (bp)	GenBank accession NO.
<i>gnih-F</i>	GGAAATCAGCCTACAGTGACAAAA	120	KU612223
<i>gnih-R</i>	GCCTCTCCAAGTCCAAACTCC		
<i>gnihr-F</i>	GCTTTTCATGTTGTCCTGGTTG	147	KX839491
<i>gnihr-R</i>	GGGTTGATGCTTGAGTTGGAG		
<i>gnrh2-F</i>	GGAATCTGAACTGGAGAAGCTGCT	121	KX090947
<i>gnrh2-R</i>	TGGCTGCTCACAACCTTATCAC		
<i>gnrh3-F</i>	AGGCAGCAGAGTGATCGTG	92	JQ028869
<i>gnrh3-R</i>	CACCTGGTAGCCATCCATAAGAC		
<i>kiss2-F</i>	GGCAACTGCTGTGCAACGA	133	KX090946
<i>kiss2-R</i>	AAGACAGAAAGCGGGGAGAAC		
<i>kiss2r-F</i>	AGTTGTGATCGTCCTCCTTTTG	92	KX685668
<i>kiss2r-R</i>	AGTTGGGTTGGTATTGGGATG		
<i>spx1-F</i>	GTCCTAAGGGTTCGTTCCA	185	MG775238
<i>spx1-R</i>	AGTATGGTGGCTGCCTGGTC		
<i>spx2-F</i>	TGTTAATCGCCTCCCTGTT	137	MH782165
<i>spx2-R</i>	AGTGGTGCCTTGTTGTTCTCCT		
<i>tac3-F</i>	TCTGGTCCTCGTCGTCAAAC	175	MK336423
<i>tac3-R</i>	CGTGTTCCTTCTGCCCATC		
<i>gh-F</i>	TTATAGACCAGCGCGTTTC	179	HQ334196
<i>gh-R</i>	ATGCTTGTGTGCGGGGATG		
<i>gth<math>\alpha</math>-F</i>	TTCCCCACTCCTCTAACGACA	116	JQ364953
<i>gth<math>\alpha</math>-R</i>	ACCACAATACCAGCCACCACTAC		
<i>lh<math>\beta</math>-F</i>	TCCACCTGACACTAACGCTG	191	JQ277934
<i>lh<math>\beta</math>-R</i>	GTTTGGTTCCTTTGTTCTGC		
<i>fsh<math>\beta</math>-F</i>	TGATGGGTGTCAGAGGAAG	95	JQ277933
<i>fsh<math>\beta</math>-R</i>	CAACAAACCGTCCACAGTCC		
<i>18s-F</i>	GGTCTGTGATGCCCTTAGATGTC	107	GQ426786
<i>18s-R</i>	AGTGGGGTTCAGCGGGTTAC		



**FIGURE 1**  
Effects of intraperitoneal injection of SPX2 peptide on brain *gnih* (A), *gnihr* (B), *gnrh2* (C), *gnrh3* (D), *kiss2* (E), *kiss2r* (F), *spx1* (G), *spx2* (H) and *tac3* (I) transcript levels in tongue sole. Data are presented as mean  $\pm$  SEM (n = 7–8). Different letters indicate significant differences between groups ( $p < 0.05$ ).

## Results

### Effects of SPX2 peptide on the brain gene expression

First of all, we studied the *in vivo* effects of tongue sole SPX2 peptide on the expression levels of *gnih* and its cognate receptor *gnihr* genes in the brain (Figures 1A, B). Intraperitoneal injection of SPX2 at 1000 ng/g BW significantly inhibited *gnih* mRNA levels when compared to the control group (Figure 1A). However, no apparent variation in *gnihr* expression was noticed after administration of SPX2 at any of the two doses (Figure 1B).

Second, to investigate whether the GnRH system is a target of SPX2 action, brain expression levels of *gnrh2* and *gnrh3* were examined after treatment with SPX2 peptide (Figures 1C, D). Neither *gnrh2* nor *gnrh3* mRNA transcripts were altered by administration of SPX2 at the two doses tested (Figures 1C, D).

Third, we further evaluated the central action of SPX2 on the kisspeptin system (*kiss2* and its cognate receptor *kiss2r*). Similarly, SPX2 had no significant effects on brain *kiss2* and *kiss2r* mRNA levels compared to the control group (Figures 1E, F).

Fourth, to analyze the autocrine regulation of the spexin system, we examined the brain expression levels of *spx1* and *spx2* after administration of SPX2 peptide (Figures 1G, H). Only the fish treated with SPX2 at 100 ng/g BW showed an evident reduction in *spx1* mRNA levels (Figure 1G). However, *spx2* mRNA levels were not modified by the SPX2 peptide at the two doses tested (Figure 1H).

Finally, we detected the effects of SPX2 on the expression levels of *tac3* expressed in the brain (Figure 1I). Only SPX2 at the dose of 100 ng/g BW exerted an inhibitory action on *tac3* expression levels (Figure 1I).

### Effects of SPX2 peptide on the pituitary gene expression

As shown in Figure 2A, *gh* mRNA levels were not significantly altered by SPX2 at the two doses tested when compared to the control group. For *gth $\alpha$*  and *lh $\beta$* , a significant suppression in their mRNA levels was observed by SPX2 at 1000 ng/g BW (Figures 2B, C). In contrast, SPX2 markedly stimulated *fsh $\beta$*  mRNA levels with the lower dose of 100 ng/g BW when compared to the control group (Figure 2D).

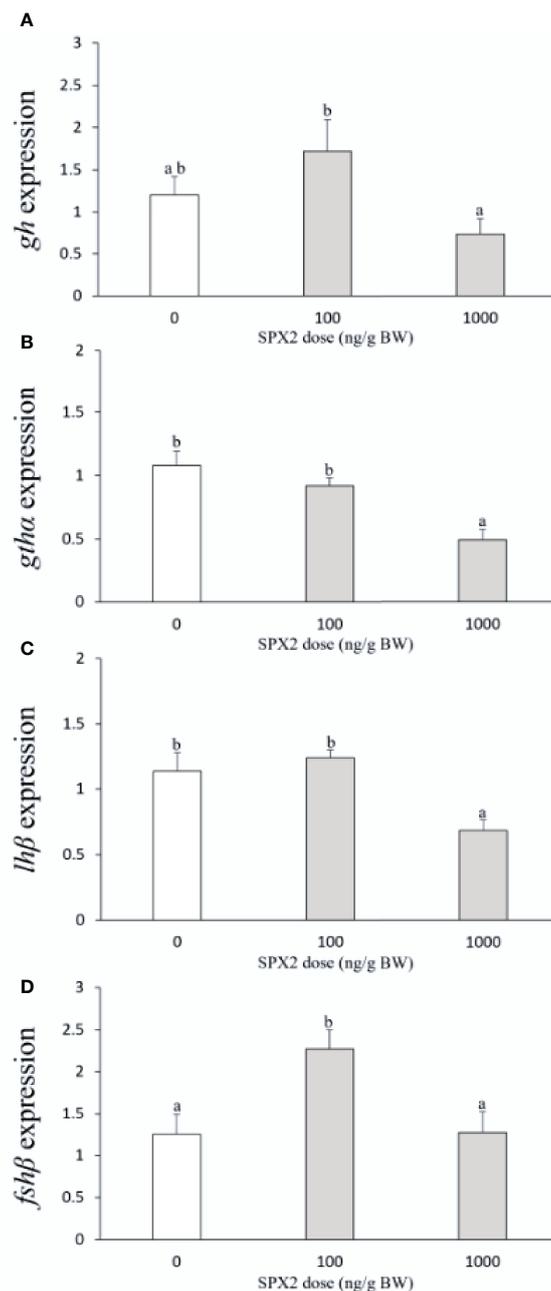


FIGURE 2

Effects of intraperitoneal injection of SPX2 peptide on pituitary *gh* (A), *gtha* (B), *lhβ* (C) and *fshβ* (D) transcript levels in tongue sole. Data are presented as mean  $\pm$  SEM (n = 6-8). Different letters indicate significant differences between groups ( $p < 0.05$ ).

## Discussion

SPX, which was first discovered by bioinformatics tools, is a newly described neuropeptide with pleiotropic functions in mammals (3, 4). Two SPX orthologs (SPX1 and SPX2) have been reported in some non-mammalian species, while the physiological functions of SPX are still largely unknown and

remain to be investigated in this group of vertebrates. In bony fish, SPX1 exerts an inhibitory effect on reproduction (12, 14) and food intake (15, 16). However, no information exists about the potential biological functions of SPX in any vertebrate (3, 4). In the current study, therefore, half-smooth tongue sole was used as a model to investigate the *in vivo* actions of SPX2 on expression levels of reproductive genes in the brain-pituitary axis.

There is compelling evidence supporting that GnIH plays a critical role in the regulation of reproduction by acting at three levels of the BPG axis from fish to humans *via* its cognate receptor GnIHR (25, 27, 47). Our previous studies also have revealed that GnIH1 and GnIH2 peptides encoded by the same precursor exert a direct action on mRNA levels of pituitary hormones through the PKA and PKC signaling pathways in half-smooth tongue sole (41, 46). Results obtained in the present study indicated that intraperitoneal injection of SPX2 reduced *gnih* mRNA levels, without any effect on *gnih*r expression. In contrast, administration of SPX1 provoked an increase of *gnih* mRNA levels in immature females of the same species (20). Overall, these data suggest that SPX2 is implicated in the control of reproduction, while SPX1 and SPX2 may have different biological roles in half-smooth tongue sole. It is worth mentioning that Galr2 is an alternative endogenous receptor for SPX in zebrafish and Nile tilapia (11, 12). However, the morphological relationship between SPX and GnIH neurons is very limited in fish, thus much more studies need to be done to unveil whether Galr2 exists in GnIH neurons of half-smooth tongue sole and other species.

GnRH has been well demonstrated to be a master stimulator of the reproductive axis in vertebrates, and two or three distinct GnRH isoforms (GnRH1, GnRH2, and GnRH3) exist in all teleosts investigated so far. The brain distribution and physiological functions of these three GnRH variants are quite different. In a teleost species possessing all three GnRH types, GnRH1 is the main hypophysiotropic hormone regulating the BPG axis. However, GnRH3 takes over the role of GnRH1 in other teleost species that have GnRH2 and GnRH3 only (28, 29). One of the other key hypothalamic neuropeptides established in the control of reproduction is kisspeptin, which can exhibit potent action on pituitary directly or on GnRH neurons indirectly to regulate LH and FSH synthesis and secretion (30, 32, 33). In this study, none of *gnrh2*, *gnrh3*, *kiss2* or *kiss2r* expression levels were altered by SPX2 injection, indicating that the GnRH and kisspeptin systems may be not the central targets of SPX2 action on reproduction. Similarly, there is no significant effect of SPX1 on *gnrh1* and *gnrh2* mRNA levels in orange-spotted grouper (17) and immature females of half-smooth tongue sole (20), respectively. However, *gnrh3* expression is evidently elevated after SPX1 administration in the latter (20). Therefore, SPX1 and SPX2 might regulate different aspects of fish physiology.

In the present study, we evaluated the effects of SPX2 on the autocrine and paracrine regulation of spexin system. Peripheral injection of SPX2 suppressed *spx1* expression, without any effect on *spx2* mRNA levels. It has been demonstrated that SPX1 is involved in feeding, reproduction, and other functions in fish (3, 4), and these data indicate that SPX2 may participate in these physiological processes *via* SPX1 indirectly. Whether SPX1 has any effect on *spx2* expression is still unknown, which warrants further studies in various vertebrates. On the other hand, NKB encoded by the *tac3* gene has emerged as a key regulator of

reproduction in mammals (34) and several teleost species, including zebrafish (48, 49), Nile tilapia (50), goldfish (51, 52), striped bass (53), European eel (54), and half-smooth tongue sole (42). Results obtained in this study indicated that SPX2 can reduce brain *tac3* mRNA levels, suggesting the regulation of reproduction by SPX2 *via* NKB indirectly.

In addition to its effects on brain functions, SPX can also modulate the synthesis and release of pituitary hormones. On one hand, both *gth $\alpha$*  and *lh $\beta$*  expression were down-regulated after intraperitoneal injection of SPX2, whereas *fsh $\beta$*  mRNA levels were up-regulated in half-smooth tongue sole. On the other hand, SPX1 suppressed the expression levels of *gth $\alpha$*  and *fsh $\beta$* , without affecting *lh $\beta$*  expression in immature females of the same species (20). Neither *lh $\beta$*  nor *fsh $\beta$*  transcripts were modified after SPX1 treatment in orange-spotted grouper (17). For hormone secretion, an inhibitory action of SPX1 on the plasma LH level was observed in goldfish and Nile tilapia (12, 14) along with a reduction of plasma FSH level in the latter. Interestingly, SPX1 evoked a decrease in the serum LH level, but an increase in the serum FSH level in mature female rats (55). Of note, SPX2 had no effect on *gh* expression in this study. However, SPX1 reduced *gh* mRNA levels in orange-spotted grouper and half-smooth tongue sole immature females (17, 20). It is worth mentioning that sexually immature female specimens were used in this study, and sexual maturity could be a contributing factor influencing the obtained results. Accordingly, further studies in sexually mature females during the seasonal reproductive cycle will contribute to a more complete picture of these two SPX peptides in this species. Taken together, SPX2 can modulate the reproduction of half-smooth tongue sole through actions on the expression of the components of brain-pituitary reproductive axis, and SPX1 and SPX2 seem to have divergent roles in the same species.

Despite its functional significance, the molecular mechanisms of SPX actions are incipient in vertebrates. A ligand-receptor interaction study has revealed that both SPX1 and SPX2 could increase SRE-luc activity in HEK293 cells expressing zebrafish Galr2a and Galr2b (11). Both SRE-luc and cAMP-response element luciferase (CRE-luc) activities are significantly elevated after SPX1a or SPX1b treatment in COS-7 cells expressing tilapia Galr2b (12). These data indicate that SPXs are a functional agonist for Galr2, and both PKC and PKA pathways mediate SPX functions. It is worth mentioning that clarifying the intricate web of intracellular pathways in response to SPX and its interaction with GnRH (28, 56), GnIH (57, 58), and kisspeptin (32, 35), is a promising area for future research not only in fish but also in other vertebrates.

## Conclusions

In summary, this study provides preliminary evidence for the involvement of SPX2 in the regulation of reproduction in

vertebrates by acting at the brain and pituitary levels. Combined with previous studies on SPX1, it appears that some functional divergences exist between SPX1 and SPX2 peptides, perhaps due to the differences in their structures and binding affinity to their cognate receptors. Further studies on the molecular mechanisms involved in SPX actions on the target cells would contribute to the knowledge of the functional significance and divergence of this emerging neuropeptide in vertebrate species.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## Ethics statement

The animal study was reviewed and approved by the Animal Care and Use Committee of Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences.

## Author contributions

BW: conceptualization, validation, investigation, writing—original draft preparation, funding acquisition; KW: validation, investigation; ZT: investigation; AC: investigation; XiL: investigation; ZJ: investigation; XuL: resources, writing—review

and editing; YJ: formal analysis; YX: validation, writing—review and editing, supervision, funding acquisition.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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