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# *phoenixin(smim20)*, a gene coding for a novel reproductive ligand, is expressed in the brain of adult zebrafish

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) is a highly conserved neuroendocrine decapeptide that is essential for the onset of puberty and the maintenance of the reproductive state. In addition to its role as hypothalamic releasing hormone, GnRH has multiple functions including modulator of neural activity within the nervous system and of resulting behaviors. These multiple functions are reflected by the existence of multiple isoforms. Despite its importance as a critical hypothalamic releasing hormone, the *gnrh1* gene has been lost in zebrafish, and its reproductive function is not compensated for by other GnRH isoforms (GnRH2 and GnRH3), suggesting that, surprisingly, zebrafish do not use any of the GnRH peptides to control reproduction and fertility. Previously we proposed that Phoenixin/SMIM20, a novel peptide identified in mammals and the ligand for the orphan GPR173, is a potential candidate to control the initiation of sexual development and fertility in the zebrafish. Here we confirm the sequence of the zebrafish *phoenixin/smim20* gene and by RT-PCR show that it is expressed early in development through adulthood. Subsequently we show that *phoenixin/smim20* is expressed in the adult brain including the regions of the hypothalamus important in the control of fertility and reproduction.

## 1. Introduction

In vertebrates fertility and reproduction require the release of the neuropeptide gonadotropin releasing-hormone, (GnRH), from the pre-optic area in the hypothalamus where it acts on the anterior pituitary, triggering the onset of puberty and gametogenesis (Gore, 2002). In most vertebrates, there are two isoforms of GnRH: GnRH1 (*gnrh1*) and GnRH2 (*gnrh2)*, where *gnrh1* is the reproductive form expressed in the hypothalamus (Roch et al., 2014). Fish have an additional isoform, GnRH3 (*gnrh3*), where *gnrh1* or *gnrh3* can be the reproductive hypothalamic form. Initially, well before the zebrafish genome sequence was confirmed (Howe et al., 2013), zebrafish were proposed to lack the *gnrh1* gene (Kuo et al., 2005). Genomic analysis of the confirmed genome sequence revealed that the *gnrh1* gene has been lost in the zebrafish lineage (Whitlock et al., 2019). Surprising TALEN-based knockouts of *gnrh3* (Liu et al., 2017; Spicer et al., 2016) and double knockout of *gnrh2* and *gnrh3* (Marvel et al., 2018) do not cause reproductive defects. Furthermore, knockouts of kisspeptin (*kiss1*), a critical peptide responsible for the upstream control of GnRH (Dungan et al., 2006) (Filby et al., 2008), alone (Tang et al., 2015) or in a triple knockout *gnrh3;kiss1;kiss2* (Liu et al., 2017) does not affect reproduction in zebrafish. Thus, these studies suggest that zebrafish control reproduction using mechanisms independent of the GnRH and Kiss peptides.

Recently a bioinformatics based analysis identifying secreted and highly-conserved peptide hormones led to the identification of Phoenixin (PNX) a peptide encoded by the gene *small integral membrane protein 20 (smim20),* (Yosten et al., 2013). This gene is highly conserved in vertebrates and appears to act as a central modulator of GnRH-related hormonal control of reproductive processes (Yosten et al., 2013). The highly conserved pre-propeptide has several predicted cleavage products with two active isoforms cleaved from the C-terminal of small integral membrane protein 20 (SMIM20): a 14 amino acid phoenixin peptide (PNX-14) and a N-terminal extended 20 amino acid phoenixin peptide (PNX-20) (Yosten et al., 2013) (Lyu et al., 2013). These peptides have been detected by immunoassays in peripheral tissues, including heart, thymus, stomach, and spleen, and in the hypothalamus, including the hypothalamic paraventricular and supraoptic nuclei, cells of the median eminence, and the arcuate nucleus, (Palasz et al., 2018; Yosten et al., 2013). In general the PNX-14 isoform has been identified in cardiac tissue and PNX-20 identified in the brain (Clarke and Dhillo, 2019). Physiological analyses of PNX activity using immortalized cell lines expressing GnRH and Kisspeptin confirmed that PNX acts through

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GPR173 to stimulate increases in *Gnrh*, *GnRH-R*, and *Kiss1* gene expression (Treen et al., 2016). Furthermore PNX can stimulate the hypothalamic–pituitary–gonadal (HPG) axis to potentiate GnRH-stimulated LH secretion, and this effect is diminished in pituitary cell lines where the endogenous GPR173 was knocked down (Stein et al., 2016) (McIlwraith and Belsham, 2018). The use of GnRH-R agonists and antagonists can modulate hypothalamic, hypophyseal, and ovarian, levels of *phoenixin/smim2*0 mRNA, suggesting that GnRH analogues are modulators of PNX signaling in the HPG axis (Suszka-Switek et al., 2019). These results support a role for PNX in modulating the reproductive axis in vertebrates through specific interactions with the GnRH and Kiss peptides.

Because zebrafish do not use any of the highly conserved peptides (GnRH/Kiss) to control reproduction and PNX is active in the reproductive axis of mammals (Stein et al., 2016) and fish (Wang et al., 2019) we suggested that PNX could replace GnRH function in zebrafish (Whitlock et al., 2019). Thus, we investigated whether the *phoenixin/smim20* gene is expressed in zebrafish. We confirmed the sequence reported for zebrafish *phoenixin/smim20* and described the expression patterns by RT-PCR, *in situ* hybridization, and immunohistochemistry. We show that the gene encoding the preprohormone *phoenixin/smim20* is widely distributed in the zebrafish brain and that PNX-like immunoreactivity is more restricted, and recognizes a specific group of cells in the hypothalamus. Our data support a potential role for Phoenixin in the control of reproduction in zebrafish.

## 2. Results

*Characterization of zebrafish phoenixin/smim20:* The zebrafish transcript was amplified directly from cDNA and the resulting sequence compared with the corresponding sequence deposited in the NCBI database (*Danio rerio*: NM_001302624.1), revealing 99% sequence identity, with a single nucleotide difference (G/T) at position 182, which resulted in a silent substitution. Analysis of the deduced amino acid sequence of zebrafish phoenixin/smim20 was compared to PNX/SMIM20 in spotted scat, (*Scatophagus argus*, Teleostei: AWM96408.1; 69%), spotted gar, (*Lepisosteus oculatus*, XP_006629716.1; 64%), humans (*Homo sapiens*: NP_001138904.1; 57%), mouse (*Mus musculus*, NP_001138904.1; 54%), chicken, (Gallus gallus: NP_001138902.1; 55%) common lizard, (Zootoca vivipara: XP_034969749.1; 63%), western clawed frog, (Xenopus tropicalis: XP_002939144.1; 66%), Coelacanth, (*Latimer*ia chalumnae: XP_006002723.1; 67%), and

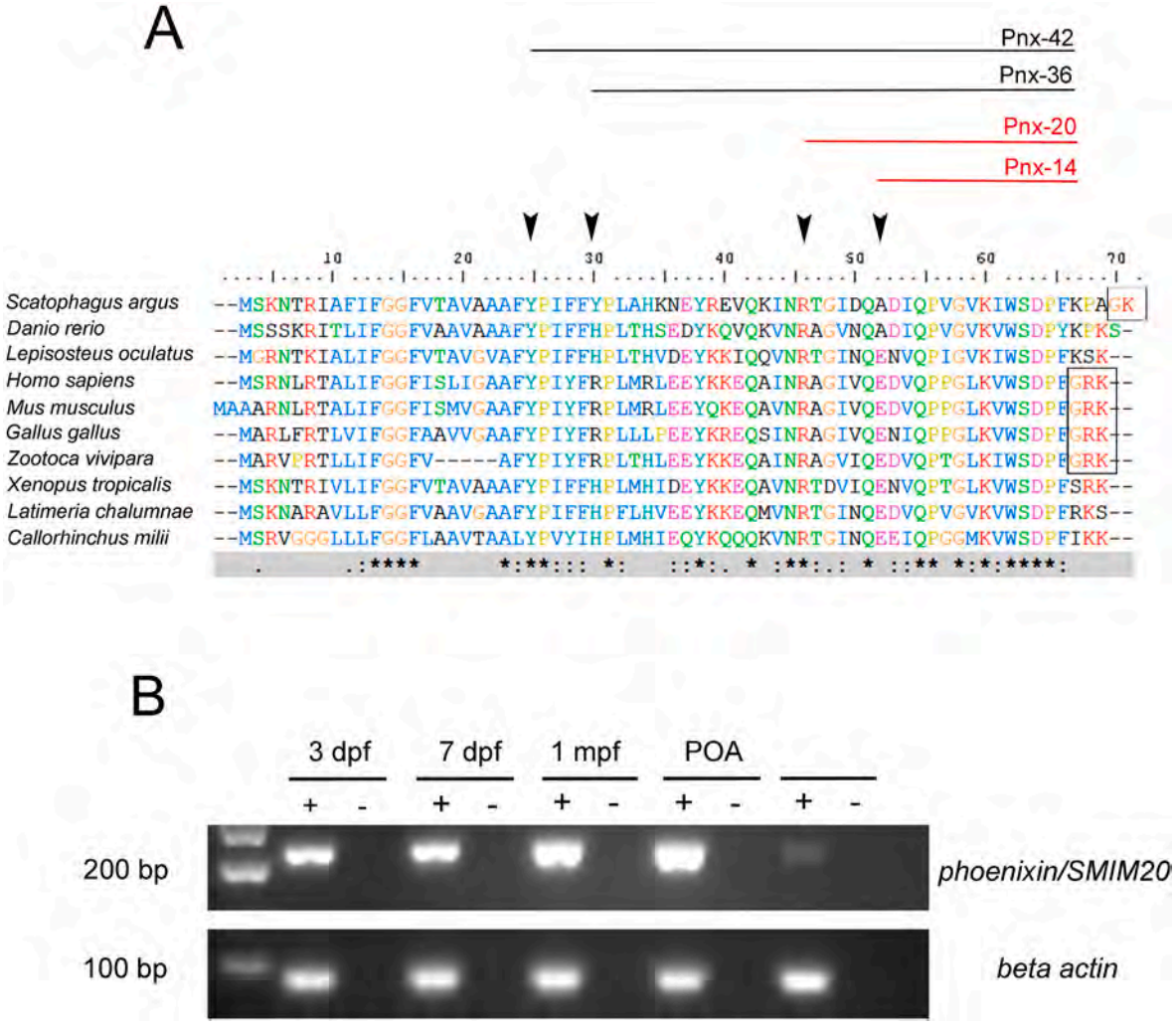


**Fig. 1.** Characterization of zebrafish *phoenixin/smim20*. (A) Amino acid sequence alignment of Phoenixin in spotted scat, (*Scatophagus argus*), spotted gar, (*Lepisosteus oculatus*), humans (*Homo sapiens*), mouse (*Mus musculus*), chicken, (*Gallus gallus*) common lizard, (*Zootoca vivipara*), western clawed frog, (*Xenopus tropicalis*), Coelacanth, (*Latimeria chalumnae*) and elephant shark, (*Callorhinchus milii*). *Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Zootoca vivipara* and *Scatophagus argus* contain amidation sites (rectangles). Within the PNX amino acid sequence cleavage sites are indicated (arrowheads) and predicted (black) and confirmed (red) peptides of varying lengths are indicated (top) (Yosten et al., 2013). (B) *phoenixin/smim20* is expressed in embryos and juvenile zebrafish. RT-PCR from cDNA samples of 3 dpf, 7dpf, 1mpf and of the pre-optic area (POA) of adult fish. +, -: genomic DNA from POA of adult zebrafish. Housekeeping gene: beta actin.

elephant shark, (*Callorhinchus milii*: XP_007887257.1; 61%) (Fig. 1, A). Spotted scat and zebrafish showed the greatest similarity in amino acid sequence (Fig. 1, A), as might be expected since both are teleost fish. Unlike PNX/SMIM20 in other animals (Yosten et al., 2013) (Wang et al., 2018), zebrafish do not have a potential amidation site in the C-terminal, (Fig. 1, A, rectangles). And, while they do not share all cleavage sites with those in previously described in mammalian species (Yosten et al., 2013), (Fig. 1, A), they do share the cleavage site generating the PNX-20 peptide (Fig. 1, A, arrowheads).

In order to determine the developmental profile of *phoenixin/smim20* expression, we performed RT-PCR analysis at 3 dpf, 7 dpf, 1 month post-fertilization (mpf), and in the hypothalamus of the adult (Fig. 1, B). The *phoenixin/smim20* transcript (predicted size: 214 bp) was detected at 3 dpf a developmental stage when the juvenile fish are hatching from their chorion but have not yet resorbed the yolk. *phoenixin/smim20* was expressed at 3 dpf, 7 dpf and 1 mpf (only heads were examined: at one month the brain is still too small to dissect out the hypothalamus). *phoenixin/smim20* was expressed the pre-optic (POA) region of the hypothalamus dissected from female and male fish. Thus, *phoenixin/smim20* is expressed early in development and maintained through adulthood.

*phoenixin/smim20 is expressed in the brain of adult zebrafish*: We analyzed the expression of *phoenixin/smim20* by *in situ* hybridization on cryostat sections of the adult brain. Images of sectioned tissue are oriented relative to the adult brain in sagittal view (Fig. 2, A, diagram). In sections *phoenixin/smim20* expression was observed in the olfactory bulb (OB) where cells where distributed through the OB (Fig. 2, B, inset). Expression in the telencephalon (Fig. 2, C, Tel) was strongest in cells bordering the ascending optic tract (OT) toward the tectum opticum (Fig. 2A, TeO). Within the TeO *phoenixin/smim20* was expressed in a band of cells extending along the ventral posterior border (Fig. 2, D, inset). Cells in the corpus cerebelli (CCe) of the cerebellum also expressed *phoenixin/smim20* (Fig. 2, E) although not as strongly as either cells in the OB or Tel (Fig. 2B and C) when compared within the same sections. Of particular interest were the brain structures controlling reproduction such as the pre-optic area, hypothalamus, and pituitary. Expression of *phoenixin/smim20* was observed in the region of the suprachiasmatic nucleus (Fig. 2 F, SC) of the preoptic area, a region where we also observed anti-PNX-like labeling (see Fig. 3). Labeling was strong in the periventricular hypothalamus (Hv, Fig. 2, G), caudal zone of

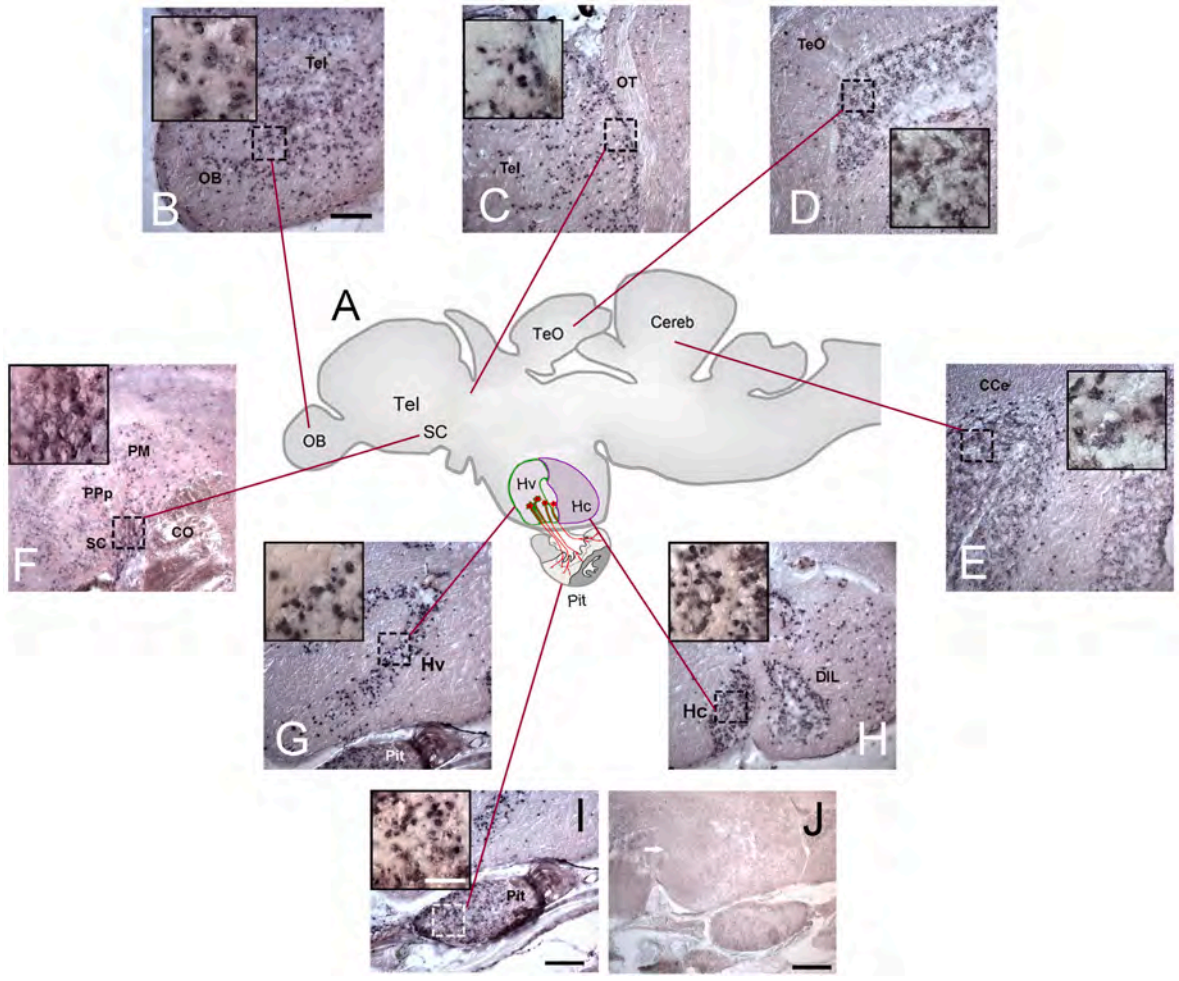


**Fig. 2.** ***phoenixin/smim20*** is expressed in the brain of adult zebrafish. (A) Diagram of sagittal section of generalized adult fish brain, anterior to the left, the location of GnRH cells (red; in fishes with GnRH cells). (B–J) *In situ* hybridization using *phoenixin/smim20* probe, 20 μm sagittal cryosections, anterior to the left. Insets show higher magnification of cells expressing *phoenixin/smim20. phoenixin/smim20* is expressed distinct brain regions outside the hypothalamic-pituitary axis including (B) olfactory bulb (OB), (C) telencephalon (Tel), (D) tectum opticum (TeO), and (E) the corpus cerebelli (CCe) of the cerebellum. Within the preoptic area (F) expression was observed in the magnocellular pre-optic nucleus (PM), posterior parvocellular pre-optic nucleus (PPp), suprachiasmatic nucleus (SC) and chiasma opticum (CO). Strong hypothalamic expression of *phoenixin/smim20* was observed in (G) the ventral zone of the periventricular hypothalamus (Hv), and (H) the caudal zone of periventricular hypothalamus (Hc) and dorsal zones of periventricular hypothalamus, (DIL). Expression was also observed in the pituitary (I). *phoenixin/smim20* sense probe (J) shows no labeling. Scale bars: (B–I) 100 μm; (J) = 50 μm; boxed areas = 20 μm. High magnification views are shown in boxed areas. (A modified from Whitlock et al., 2019).

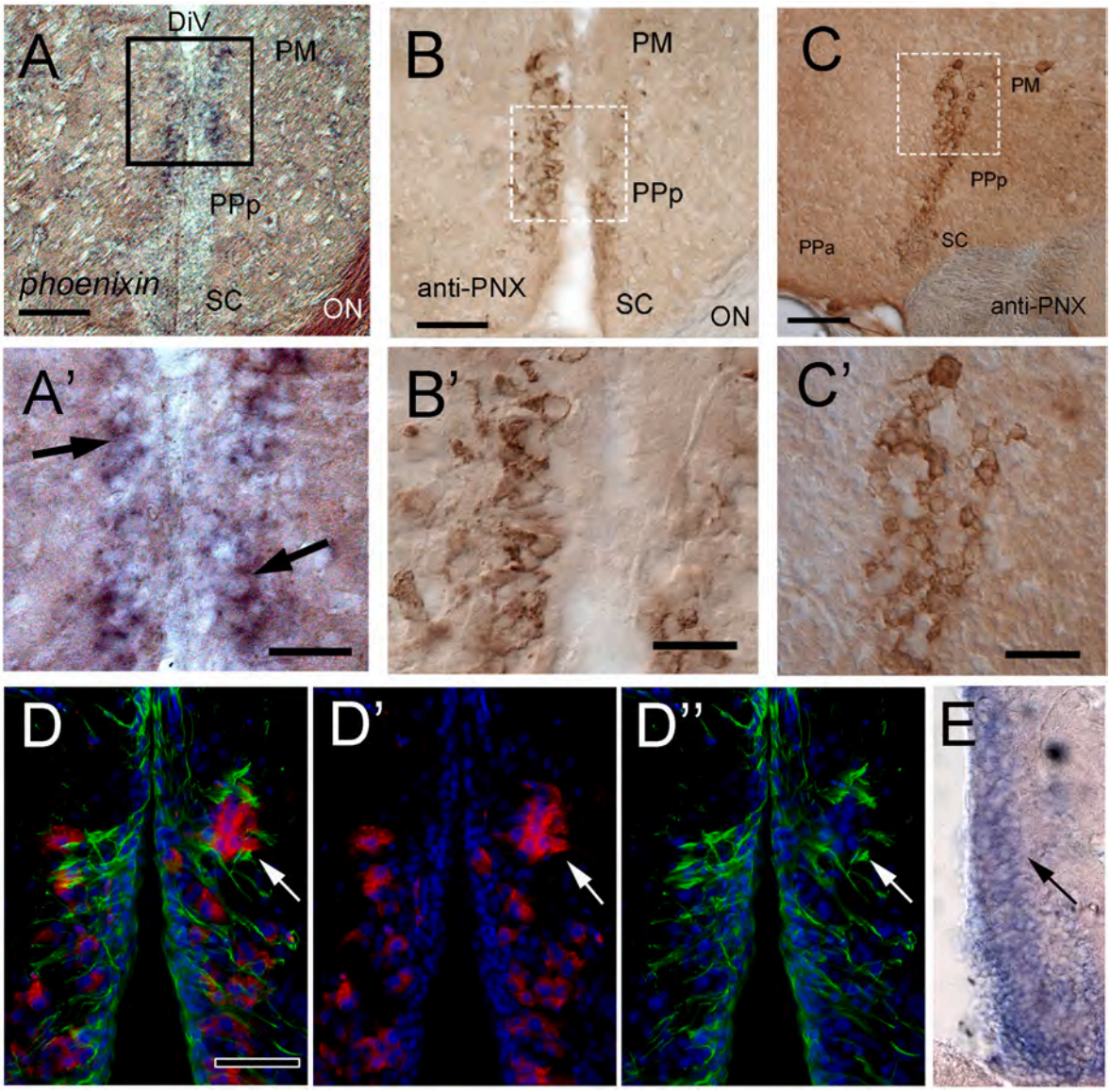


**Fig. 3.** Expression of *phoenixin/smim20* and of PNX-like immunoreactivity in the adult hypothalamus. (A, A′, B, B′: transverse cryosection) (A, A′) *phoenixin/smim20* is expressed in the magnocellular nucleus (PM) of the POA; (A′) Higher magnification of boxed area in A. (B) PNX-like immunoreactivity in cells distributed adjacent to the diencephalic ventricle (DiV) and in the magnocellular pre-optic nucleus (PM) and parvocellular pre-optic nucleus (PPp). (B′) Higher magnification of boxed area in B. (C, C′) Sagittal sections, anterior to the left, labeled with anti-PNX antibody. (C) Immunolabeling of cells in the POA. (C′) Higher magnification view of area boxed in C. (D-D″) Transverse cryosections showing fluorescently labeled PNX-like immunoreactivity (red, D, D′) and ant-Zrf1 (green, D, D″) in the posterior parvocellular preoptic nucleus PPp (see Fig. 2F). (E) *In situ* hybridization showing *phoenixin/smim20* expression in the same region (arrow D-D″, E; transverse cryosection). Abbreviations: PM: magnocellular pre-optic nucleus; PPp: parvocellular pre-optic nucleus, posterior part; SC: suprachiasmatic nucleus, ON optic nerve. A-E. All images are from 20 μm cryostat cross-sections of adult brain. Scale bars: A, B, C = 50 μm; A′, B′, C′ = 25 μm. D-D″, E = 30 μm.

periventricular hypothalamus (Hc), and dorsal zones of periventricular hypothalamus, (DIL: Fig. 2, H). The pituitary also expressed *phoenixin/smim20* (Fig. 2, I) and is shown with control tissue where no specific labeling was detected with the *phoenixin/smim20* sense probe (Fig. 2, J). These results demonstrate that *phoenixin/smim20* is expressed in the adult brain, including the hypothalamus and the pituitary.

*PNX like immunoreactivity:* To detect the Phoenixin (PNX) protein expression we used a commercially available antibody to PNX (https://www.phoenixpeptide.com/topics/detail/192). Previously, the PNX peptide against which the commercially available antibody is made was used in the spotted scat fish (*Scatophagus argus*) to demonstrate that intraperitoneal injection can increase expression of *ghrhr* (growth hormone-releasing hormone receptor) and *gh* (growth hormone) in the pituitary, suggesting PNX is involved in the regulation of food intake (Wang et al., 2018). More recently evidence of a potential role for PNX-20 on the HPG axis in zebrafish has been shown by intraperitoneal injection where PNX-20 up-regulated mRNAs encoding *gnrh3*, *gnrh2*, *kisspeptin* and its receptor in zebrafish hypothalamus (Rajeswari and Unniappan, 2020). The anti-PNX antibody recognizes the amidated carboxy-terminal of human PNX including amino acid residues 53–66. This region of the protein is 100% conserved in mammals (Fig. 1, A (Yosten et al., 2013); and is 71% identical to zebrafish PNX (similarity is 92%; Fig. 1, A). Overall, fewer cells showed anti-PNX-like immunoreactivity when compared to the number of cells labeled by *in situ* hybridization (Fig. 2). The pattern of immunoreactivity in the pre-optic area (Fig. 3) was similar to that obtained by *in situ* hybridization where in both data sets labeled cells were localized to the region of the neurosecretory pre-optic area (Figs. 2F and 3). The cells were found primarily in the region extending ventrally from the magnocellular pre-optic nucleus (PM), adjacent to the diencephalic ventricle (DiV)

(Fig. 3, A, A′, arrows; B, B′ boxed area, cross-sections; C sagittal section) to the suprachiasmic nucleus (Fig. 2F, SC; Fig. 3, A-C, SC). Double labeling with anti-PNX (Fig. 3, D-D′, red) and anti-Zrf-1 [Fig. 3, D-D″, green; marker of radial glia/astroglia in zebrafish (Jurisch-Yaksi et al., 2020);] revealed a close association of the PNX-like labeling with the Zrf-1 positive radial glia lining the third ventricle (Fig. 3, D-D″) in the region that also expresses *phoenixin/smim20* Fig. 3, E arrows). These data demonstrate that, as predicted, the PNX-like labeling has a more restricted pattern than *phoenixin/smim20* gene expression and includes the neurosecretory pre-optic area. The generation of a zebrafish specific antibody will aid in confirming the pattern of PNX protein localization in the developing and adult nervous system.

## 3. Discussion

*Like gnrh3, phoenixin/smim20 is expressed starting in early development.* We have previously shown that *gnrh3* is expressed starting at one day post-fertilization in the zebrafish embryo (Gopinath et al., 2004); (Whitlock et al., 2005) and the neuromodulatory cells containing GNRH3 become active shortly thereafter (Zhao et al., 2013), thus suggesting a potential role for *gnrh3* in establishing this neuromodulatory network. Likewise here we show by RT-PCR that *phoenixin/smim20* expression starts at 3 dpf, thus raising the possibility that PNX has been coopted into the development of the neuroendocrine axis as an important peptide in the circuit controlling fertility and reproduction in zebrafish.

*The phoenixin/smim20 gene is expressed in patterns similar to those reported for the mammalian brain.* The regions expressing the gene are in strong agreement with those reported in the characterization of PNX immunoreactivity in mammals (Palasz et al., 2018; Yosten et al., 2013). Important for the proposed role for PNX in zebrafish reproduction, here we showed that *phoenixin/smim20* is expressed in cells within regions of the hypothalamus analogous to the mammalian supraoptic nuclei, the median eminence, and the arcuate nucleus. Although two active isoforms PNX-14 and PNX-20 have been experimentally identified in rat (Yosten et al., 2013) (Cowan et al., 2015), in zebrafish, these isoforms remain poorly characterized. The cells we identified expressing PNX-like immunoreactivity have morphologies similar to neuropeptide expressing cells that project their axons directly to the neurohypophysis (Eaton et al., 2008) (Almeida and Oliveira, 2015). Because proteins can be cleaved at di-basic, and monobasic residues (Devi, 1991) (Schwartz, 1986) and amidation is not an absolute requirement for biological activity of some neuropeptides (Cuttitta, 1993; Eipper et al., 1992; Kulathila et al., 1999) the immuno-labeling most likely reflects PNX cleavage products. The limited data on the PNX peptide in fishes suggests they only have the cleavage site producing PNX-20 and not PNX-14 (Wang et al., 2018), thus the PNX-like labeling we observed most likely reflects the presence of a PNX-20-like protein. These findings are consistent with observations in mammals that PNX-20 is the peptide form localized predominantly to the brain (Clarke and Dhillo, 2019). Our data showing localization of PNX-like immunoreactivity to a limited group of cells in the hypothalamus and pituitary (Ceriani and Whitlock, unpublished) are in agreement with recently published data from zebrafish. In these studies authors showed that PNX can stimulate hypothalamo-pituitary-gonadal hormones as well as suppress food intake (Rajeswari et al., 2020); (Rajeswari and Unniappan, 2020), both functions of the hypothalamus. The availability of antibodies against the zebrafish PNX peptides will provide additional confirmation for the pattern of immunoreactivity described here.

Previously we proposed that *phoenixin/smim20* could be the reproductive peptide in zebrafish (Whitlock et al., 2019). Here we have shown that zebrafish *phoenixin/smim20* is expressed during development and in the adult brain, including the regions of the hypothalamus controlling reproduction. Future studies characterizing the zebrafish PNX peptides as well as the generation of targeted gene knock-outs of *phoenixin/smim20* will allow us to determine whether this is the mystery neuropeptide that controls fertility and reproduction in zebrafish.

## 4. Experimental procedures

*Animals* Wild-type zebrafish (*Danio rerio*) strains derived from the AB genetic background were used. Zebrafish were generated in the Whitlock laboratory and maintained in a re-circulating system (Aquatic Habitats Inc., Apopka, FL) at 28 °C on a 14 and 10 h light:dark cycle. Adult zebrafish were euthanized submersion in ice water (5 parts ice/1 part water, 0-4 °C) for at least 10 min following cessation of opercular (i.e., gill) movement. In any fish where it was difficult to visualize opercular movement, fish were left in ice water for at least 20 min after cessation of all movement to ensure death by hypoxia. All protocols and procedures employed were reviewed and approved by the Institutional Committee of Bioethics for Research with Experimental Animals, University of Valparaiso (#BA084-2016).

*Cloning of Zebrafish phoenixin/smim20.* RNA from adult brains of zebrafish was isolated using Trizol (Invitrogen) according to the manufacturer's instructions and treated with DNAse I (Amplification grade; Invitrogen). cDNA was synthesized from 1 μg of total RNA (concentration measured using Quant-it RNA BR Assay Kit; Invitrogen) using SuperScript II reverse transcriptase (Invitrogen), Oligo dT (Invitrogen), RNAse Out (Invitrogen), and dNTP mix, in a final volume of 20 μL. PCR amplification was carried out using 1 μL of cDNA, 10 μmol of forward (5′- GTCCTCGAGTGAGGTGAAA-3′) and reverse primer (5′-CGGCAAAACAAAGTTACTCC-3″) (NCBI Reference Sequence: NM_001302624.1), and DreamTaq Green PCR Master Mix (Thermo Fisher Scientific), in a final volume 25 μL. Samples were incubated at 95 °C for 2 min followed by 35 cycles of 30 s at 95 °C, 30 s at 56 °C, and 1 min at 72 °C, followed by a final extension of 10 min at 72 °C. Products were visualized using a 3% agarose gel in TAE buffer. The amplicon was sequenced and the deduced amino acid sequence was obtained using the online software ExPASy translate tool (https://web.expasy.org/translate/). The alignment of the sequences was performed using Clustal Omega https://www.ebi.ac.uk/Tools/msa/clustalo/.

*Expression of phoenixin/smim20* RNA. RT-PCR RNA was isolated using previous published protocol Calfún et al. (2016) (Calfun et al., 2016). RNA was isolated from 3 dpf (days post-fertilization) embryos, the heads of 7 dpf larvae, 1 month-old fish (whole), and from the pre-optic area (POA) of adult zebrafish, and cDNA synthesized (see above) using: forward primer, 5′-CCTCGAGTGAGGTGAAACTGTCAA-3'; reverse primer, 5′-ATCAGACCAGACCTTCACACCAAC-3' (NCBI Reference Sequence: NM_001302624.1).

Single-stranded RNA digoxigenin-UTP (Roche, Mannheim, Germany) labeled probes recognizing *phoenixin/smim20* were generated as described previously (Whitlock et al., 2005). Primers used: forward primer, 5′- GTCCTCGAGTGAGGTGAAA -3'; reverse primer, 5′-CGGCAAAACAAAGTTACTCC- 3' (NCBI Reference Sequence: NM_001302624.1). Anti-sense probe generated with T7 RNA polymerase (Roche, CAT: 10881767001). cDNA from 7 dpf juveniles was used as template and the products were cloned into pGEM-T Easy Vector System I (Promega). Clones were sequenced to confirm identity.

*In situ Hybridization on sections.* Adult females (1 year old) were sacrificed at 10 AM and their heads were fixed in 4% buffered paraformadehyde (PFA), overnight at 4 °C, then decalcified in 0.2 M EDTA solution (pH 7.6) for 48 h at 4 °C, embedded in 1.5% agarose/5% sucrose blocks and submerged in 30% sucrose overnight at 4 °C. Blocks were frozen at −20 °C with O.C.T. Compound (Tissue Tek®) and 20 μm cryostat sections were cut. *In situ* hybridization was performed as described in Thisse et al. (1993) (Thisse et al., 1993), using single-stranded sense and anti-sense RNA probes.

*Immunohistochemistry.* Animals were sacrificed and processed as described above, cryosectioned (40 μm), incubated with rabbit anti-PNX (1:200, Cat N°: G-079-01 Phoenix Pharmaceuticals, Inc.), and processed using either VECTASTAIN elite ABC HRP kit (Rabbit IgG, VECTOR; PK-6101) or ImmPACT® DAB Peroxidase (HRP) Substrate (VECTOR; SK-

4105), following manufacturer's instructions. For double labeling of anti-PNX/anti-Zrf1 sections were incubated with mouse anti-Zrf1 (1:500, Abcam ab154474) and Alexa Fluor 488 and 568 secondary antibodies (1:500; Invitrogen).

*Microscopy and image processing.* Bright field images were obtained using a Leica DMR microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) and a Leica DFC 480 camera (Leica Microsystems Ltd, Heerbrugg, Switzerland) and processed using the Leica Application Suite 2.3.3 software (Leica Microsystems Ltd).

*Sequence alignment.* Information about amino acid sequences was done using ClustalX (2.0) program. The sequences of elephant shark (*Callorhinchus milii*), spotted gar (*Lepisosteus oculatus*), Coelacanth, (*Latimeria chalumnae*) are predicted sequences using Clustal OMEGA program https://www.ebi.ac.uk/Tools/msa/clustalo/

## 5. Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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## Author contributions

K.E.W. designed experiments, analyzed data, wrote the manuscript, and is the guarantor of this work. R.C. performed experiments, analyzed data, and reviewed and edited the manuscript. C.C. performed experiments and reviewed the manuscript. All authors have read and approved the manuscript.

## Declaration of competing interest

The authors have no competing interests.

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