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Neuroendocrinology of reproduction: Is gonadotropin-releasing hormone (GnRH) dispensable?

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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. First identified in mammals, the GnRH signaling pathway is found in all classes of vertebrates; homologues of GnRH have also been identified in invertebrates. In addition to its role as a hypothalamic releasing hormone, GnRH has multiple functions including modulating neural activity within specific regions of the brain. These various functions are mediated by multiple isoforms, which are expressed at diverse locations within the central nervous system. Here we discuss the GnRH signaling pathways in light of new reports that reveal that some vertebrate genomes lack GnRH1. Not only do other isoforms of GnRH not compensate for this gene loss, but elements upstream of GnRH1, including kisspeptins, appear to also be dispensable. We discuss routes that may compensate for the loss of the GnRH1 pathway.

1. Introduction

Gonadotropin-releasing hormone (GnRH) is the pivotal neuropeptide regulating fertility and reproduction in vertebrates (Gore, 2002). In vertebrates, at least fifteen GnRH isoforms have been identified (Lethimonier et al., 2004), where any given species has two or three forms of GnRH, each encoded by different genes. The different forms of GnRH are expressed in both neuronal and non-neuronal tissues and serve a number of distinct functions. Historically, GnRH has been known primarily as a hypothalamic hormone controlling the onset of puberty and the maintenance of fertility through the release of the gonadotropes, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), from the pituitary. For the purposes of this review the name "hypophysiotropic" will be used to refer to the hypothalamic reproductive form of GnRH.

GnRH has been extensively studied in mammals and fishes and as different isoforms of GnRH were discovered they were named according to the animal in which they were found, thus leading to a confusing body of literature. However, when the corresponding genes were identified, the different GnRH peptides could be classified based on the sequence of the corresponding genes and their location in the genome, clarifying the naming of GnRH isoforms.

In considering the function of the hypothalamic GnRH neurons, it is important to note that there are significant differences between the anatomy of fish and mammalian brains, both important model systems used to study the neuroendocrinology of GnRH. In mammals, GnRH is released from nerve endings into the hypophyseal portal system in a pulsatile manner to stimulate the synthesis of the gonadotropes, FSH and LH, from the anterior pituitary. Thus, the communication between the hypothalamic GnRH cells and the pituitary occurs *via* a localized circulatory system called the median eminence in which hypothalamic-releasing and -inhibiting hormones converge onto the portal capillary system of the ventral hypothalamus (Fig. 1A) (Kitahashi et al., 2013; Biran et al., 2015). However, this elaborate pituitary vasculature connecting the hypothalamus to the anterior pituitary gland typical of tetrapods is notably lacking in teleost fish (Peter et al., 1990), in which axons of the GnRH neurons project directly to post-synaptic sites in the pituitary (Fig. 1B). In addition, the neurohypophysis is greatly reduced in size. While structurally different from mammals, the teleost fish brain contains all the hypothalamic cell types identified in mammals (Machluf et al., 2011), including the GnRH containing cells localized to the parvocellular nucleus (Gomes et al., 2013). The lack of a median eminence in teleost fish is believed to be a derived trait because primitive fishes such as coelacanth, sturgeons, and lungfish (Ball, 1981; Gorbman, 1995), as well as cartilaginous fishes (Gaillard et al., 2018), contain a median eminence. Although hypothalamic structures differ between teleosts and mammals, it is generally thought that GnRH has remained the principal peptide controlling fertility, reproduction, and

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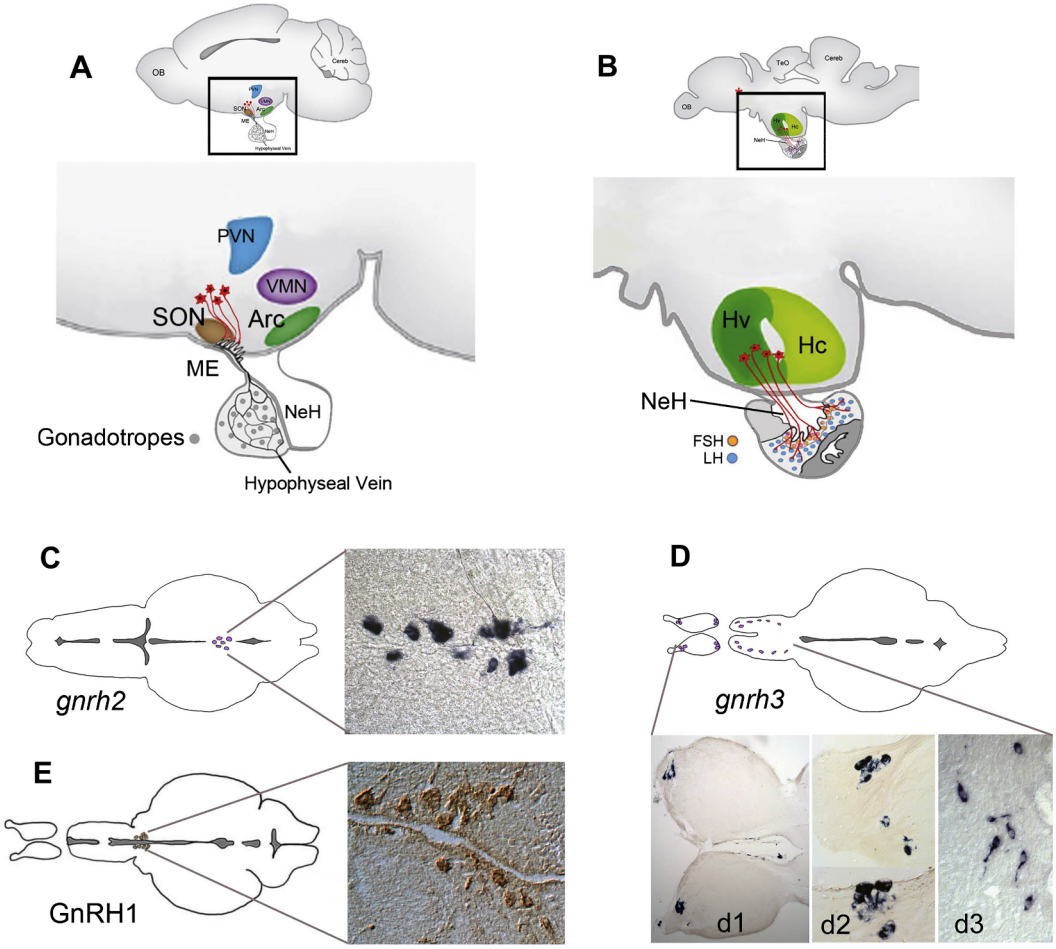

Fig. 1. Structure of the vertebrate hypothalamus and description of *gnrh*/GnRH containing cells in the adult zebrafish brain. (A, B) Schematic diagram of a lateral view of the mouse (A) and the zebrafish (B) brain. In mammals (A), the GnRH cells (red) project to the median eminence, a highly vascularized connection to the pituitary. In teleosts (B), the neurohypophysis (NeH) is reduced and the GnRH cells make neural connections with the pituitary. (C-E) *gnrh2* and *gnrh3* expression detected by *in situ* hybridization in the midbrain (C), and terminal nerve (D, d1, d2) and ventral telencephalon (D, d3) (Whitlock, 2005) In contrast, GnRH cells in the parvocellular nucleus of the hypothalamus can be detected only by immunohistochemistry (E from Cortes-Campos et al., 2015). There are no convincing data supporting cellular expression of any *gnrh* gene in the parvocellular nucleus of the adult zebrafish. Abbreviations: Arc, arcuate nucleus; Hv, ventral zone of periventricular hypothalamus; Hc, caudal zone of periventricular hypothalamus; (red asterisk), neurosecretory preoptic area; ME, median eminence; NeH, neurohypophysis; OB, olfactory bulb; PVN, paraventricular nucleus; SON, supraoptic nucleus; TeO, tectum opticum; VMN, ventromedial nucleus. (A, B modified from Kitahashi et al., 2013; Biran et al., 2015).

behaviors associated with reproduction, in these groups of vertebrates.

In addition to playing a crucial role in the regulation of reproduction, GnRH also has a variety of functions outside of the hypothalamic-pituitary-gonadal axis as reflected by the variety of isoforms and receptors that are expressed throughout the brain. GnRH-containing neurons are located in the terminal nerve (cranial nerve zero) (Whitlock, 2004), the ventral telencephalon, the hypothalamus, and the midbrain. Although the general function of GnRH is highly conserved across vertebrates, there are notable differences in the locations and functions of specific GnRH isoforms in the brain. In most vertebrates, the hypophysiotropic form (the form essential for puberty that controls the release gonadotropes from the pituitary) is called GnRH1, and its primary site of synthesis and release is from neurons in the hypothalamus. A second more highly conserved isoform, GnRH2, is found in neurons of the midbrain tegmentum. This form of GnRH is generally thought to be involved in the regulation of behaviors associated with reproduction (Desaulniers et al., 2017); in some animals it has been coopted as the hypothalamic releasing hormone acting on the pituitary (Yu et al., 1998; King and Millar, 1982b). A third isoform, GnRH3, is specific to fish and its cell bodies are located in the terminal nerve and the ventral telencephalon. GnRH3 is usually neuromodulatory and does not control puberty and reproduction through the liberation of pituitary gonadotropes. However it may be indirectly linked to the reproductive neuroendocrine axis by modulating functions of the nervous system that relate to social behaviors important for reproduction (Oka, 2009).

Several groups have reported that the gene encoding GnRH1 does not exist in the zebrafish genome and, based on a limited number of analyses, *gnrh1* has been suggested to also be absent from the genome of other cyprinid fish (Lin and Peter, 1996; Penlington et al., 1997; Steven et al., 2003; Filby et al., 2008). Here we discuss the implications of this finding by considering which peptide might replace GnRH1 in the hypothalamus. We also consider the possibility that the loss of GnRH1 in laboratory strains of zebrafish is a consequence of domestication. Indeed, domesticated (i.e., laboratory) zebrafish have acquired a mechanism of sex determination that is not found in wild zebrafish (Wilson et al., 2014), and this change could affect the hypothalamic-pituitary axis, including the functions normally subserved by GnRH1. Finally, we consider the mechanisms by which puberty and reproduction might be controlled in zebrafish and possibly in other cyprinid fishes.

1.1. GnRH isoforms and their functions

Mammalian brains typically express two different forms of GnRH (GnRH1 and GNRH2), whereas fish typically express three isoforms

(GnRH1-3) (Choi, 2018). GnRH was first discovered in the mammalian brain (Matsuo et al., 1971; Burgus et al., 1972; Tan and Rousseau, 1982) and its sequence shown to be: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2. This isoform is universally called GnRH1 with the understanding that this is the hypothalamic version of the hormone. Following the initial discovery of GnRH1, other forms of GnRH were isolated and characterized in a variety of vertebrates. Thus, a second form of GnRH, which is universally present from jawed fish to humans and is also highly conserved between these groups, is the form originally named chicken GnRH II (cGnRH: pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH2) (Sherwood, 1986; King and Millar, 1982a, 1982b; Miyamoto et al., 1982, 1983, 1984), which is now called GnRH2. Finally GnRH3 (pGlu-His-Trp-Ser-Try-Gly-Trp-Leu-Pro-Gly-NH2) (Sherwood, 1986) is an isoform specific to fishes, which has been proposed to be a form extant in early vertebrates and secondarily lost in tetrapods (Tostivint, 2011).

All GnRHs are encoded as a preprohormone whose cleavage product generates the GnRH decapeptide and the GnRH Associated Protein (GAP). The genes encoding the GnRH preprohormone share the same basic structure that was originally described for mammalian GnRH (Seeburg and Adelman, 1984): They all contain four exons where the first exon encodes only the 5 prime–UTR; exon 2 encodes the signal peptide, the GnRH decapeptide, the proteolytic cleavage site, and the N-terminus of the GAP; exons 3 encodes the central portion and the C terminus of the GAP; and exon 4 encodes the 3 prime-UTR. Five of the ten amino acids present in the mature GnRH hormone are invariant whereas two other positions show conservative changes. An unusual aspect of the GnRH preprohormone is that in spite of extremely high conservation of the GnRH gene structure and peptide sequences, the GAP coding sequences are highly divergent even within closely related groups of animals (White et al., 1995; Kasten et al., 1996; White and Fernald, 1998). The high level of conservation of GnRH peptide sequence and gene structure suggests that all GnRH genes may have arisen through gene duplication of a single ancestral GnRH whose origin predates the diversification of vertebrates.

1.1.1. GnRH as a neuromodulator

The reproductive, releasing, form of GnRH is expressed in the hypothalamus but the various GnRH isoforms and their receptors are found throughout the brain where they may act to control multiple higher functions such as feeding and reproductive behaviors as well as learning and memory (Okuyama et al., 2017; Hough et al., 2017). The majority of the literature concentrates on the role of GnRH as the pivotal peptide controlling reproduction in vertebrates. Often overlooked is the role of GnRH as a neuromodulatory peptide in the brain. In all vertebrates, GnRH-containing cells can be found not only in the hypothalamus but also in the terminal nerve, the ventral forebrain, and the midbrain (Fig. 1C–E) to mention the most common regions when comparing across species.

1.1.2. GnRH containing neurons of the terminal nerve (cranial nerve 0)

The GnRH isoform expressed within the hypothalamus *versus* the terminal nerve/ventral telencephalic networks can vary depending upon the animal, but is generally either GnRH1 or GnRH3; GnRH2, the form with the most conserved amino acid sequence, is consistently expressed in neurons of the midbrain in most vertebrate groups. The terminal nerve/ventral telencephalic network is part of the centrifugal visual system, a visually driven retinal feedback projection (Reperant et al., 2006 for review) that is present in tetrapods and teleosts, reflecting the importance of GnRH not only as a hypophysiotropic releasing hormone but also as a neuromodulatory peptide within the brain. In fishes, the GnRH3 positive neurons of the terminal nerve (Fig. 1D, d1, d2) send their axons to a variety of regions within the central nervous system (Whitlock, 2004), most notably the retina, where they synapse in the inner plexiform layer (IPL) (Zucker and Dowling, 1987). In the white perch, for example, they have been shown to stimulate the release of dopamine from interplexiform cells (Umino and Dowling, 1991). Interestingly, FMRFamide, which is also present in the terminal nerve neurons terminating in the retina, has no physiological effects on its own yet has been reported to block the effects of GnRH on the retina (Umino and Dowling, 1991). In zebrafish, the terminal nerve contains both GnRH3 and a homologue of the PQRF subfamily of RFamides, which is expressed in the same neurons that express GnRH3 (Oehlmann et al., 2002). In zebrafish and medaka, GnRH3 is present not only in the terminal nerve, but also in the trigeminal nerves (cranial nerve V) of developing embryos (Okubo et al., 2006; Abraham et al., 2008). In zebrafish a *Tg(gnrh3:emd)* reporter line was used to show that, starting at two days post-fertilization, the neuromodulatory GnRH3:EMD cells of the terminal nerve initiate spontaneous firing, followed by a bursting pattern of activity that matures into a final tonic firing pattern by three days post-fertilization (Ramakrishnan et al., 2010).

The GnRH population expressed in neurons of the terminal nerve has also been identified in a variety of mammals including rats (Zheng et al., 1988; Merchenthaler et al., 1989) and dolphins (Demski et al., 1990). In the big brown bat (*Eptesicus fuscus*), GnRH and FMRFamide have been detected in the terminal nerve, and their projections extend throughout the basal forebrain with additional intense labeling in cell bodies within the arcuate nucleus (Oelschlager et al., 1998). Thus, the GnRH positive cells in the brain form a complex consisting of two principal GnRH neuronal networks: one associated with the POA/hypothalamus, which projects to the pituitary (hypophysiotropic function), and a second terminal nerve/ventral telencephalic population, which send axons to peripheral targets and multiple regions of the brain, but never do so to the pituitary (Wirsig-Wiechmann et al., 2002).

The different physiological roles of GnRH are correlated with the developmental origin of the GnRH cells (Whitlock et al., 2003, 2006). Indeed, the GnRH3 cells of the hypothalamus do not originate from the region of the terminal nerve/olfactory sensory system, but from within the hypothalamus (Zhao et al., 2013). This suggests a separate developmental origins for the neuromodulatory *vs.* the reproductive GnRH cells, as has been suggested for other animals including chick (el Amraoui and Dubois, 1993; Witkin et al., 2003), medaka (Parhar et al., 1998), and monkey (rhesus macaque), where two distinct GnRH cell populations have been reported, one associated with the olfactory system and a second arising independently from within the central nervous system and which populates the hypothalamus (Quanbeck et al., 1997).

1.1.3. The role of GnRH in longevity

More recently, an additional role for GnRH has been added to the already extensive list of GnRH functions, and that is as a peptide important for longevity. Linked to the control of reproduction, hypothalamic GnRH appears to prevent the onset of ageing. Recent studies in mouse have examined the effects of GnRH on ageing and uncovered an intriguing potential pathway. Normally proteins involved in hypothalamic immunity, IκB kinase-β (IKK-β) and nuclear factor κB (NF-κB), inhibit GnRH during the ageing process, and recently it has been shown that blocking this pathway results in the restoration of GnRH in the hypothalamus in both male and female mice (Zhang et al., 2013). Additionally, treatment with GnRH to compensate for the aging-related decline in GnRH resulted in neurogenesis in brain regions including the hypothalamus and interestingly, the hippocampus (Tang and Cai, 2013).

1.2. Evolutionary genomics of GnRH

The number of *gnrh* genes varies across genomes of modern-day vertebrates. The *gnrh1* gene exists broadly across species but has been lost in certain teleostean clades within the Cyprinidae and Salmonidae families (see below). The *gnrh2* gene is consistently found in most vertebrate groups ranging from fish to humans, but has been lost in

rodents (Desaulniers et al., 2017). Finally, *gnrh3* appears to be a teleost-specific gene. In species with three GnRH isoforms, *gnrh1* is expressed in the hypothalamus and is the hypothalamic releasing hormone. Overall at least thirteen different GnRH isoforms and up to four GnRH receptors have been identified in vertebrates. Genome sequencing has revealed the genomic location of these genes in both model and non-model systems, providing an opportunity to independently confirm the sequence of various reported GnRH peptides and that of their receptors. In mammals no gene encoding for GnRH3 has been identified thus far, but both *gnrh1* and *gnrh2* genes have been identified in most mammalian genomes. A notable exception to the highly conserved presence of *gnrh2* is the lack of a *gnrh2* gene in the mouse genome (Desaulniers et al., 2017). The majority of fishes have three genes encoding GnRH: *gnrh1* (the hypophysiotropic form), *gnrh2*, and *gnrh3* (the neuromodulatory form). In some species, such as the goldfish (*Carassius)*, which has no GnRH1, either GnRH2 or GnRH3 is the hypothalamic releasing form (Kobayashi et al., 1997; Yu et al., 1998). Yet, in other species of fishes, such as the zebrafish, GnRH1 appears to be absent and, furthermore, no hypophysiotropic form can be detected in the hypothalamus.

1.3. GnRH and the zebrafish genome

The three extant *gnrh* genes are proposed to have arisen from a common ancestor where, at the origin of vertebrates, two rounds of genome duplication produced four paralogous chromosomal regions, each with a GNRH gene, followed by loss of *GNRH3* and *GNRH4* in the human lineage (Tostivint, 2011). The gene encoding GnRH1 (the hypothalamic isoform) is found in the majority of fishes and is encoded by a single gene, in spite of the genome duplication that occurred at the radiation of the teleosts (Amores et al., 1998). Based on the identification of *gnrh2* (Gopinath et al., 2004); *gnrh3* (Torgersen et al., 2002; Gopinath et al., 2004), and the presence of GnRH-immunolabeling in the hypothalamus (Whitlock et al., 2006; Cortes-Campos et al., 2015), it was proposed that zebrafish would have three forms of GnRH (Fig. 1C–E). In zebrafish, the *gnrh* genes were initially localized to LG21 (*gnrh2*) and LG17 (*gnrh3*), whereas the ortholog to human *GNRH1* appeared to be lacking (Kuo et al., 2005). Subsequent synteny analyses confirmed the presence of *gnrh2* on (now) chromosome 21 and *gnrh3* on chromosome 17, as well as the lack of *gnrh1*, in spite of retaining neighboring syntenic genes (Kuo et al., 2005; Kim et al., 2011) (Fig. 2A).

Because of the gaps in the zebrafish genome sequence at the time of previous publications (the zebrafish genome was only officially released in 2013; Howe et al., 2013), there existed the possibility that the small *gnrh1* gene does exist in the zebrafish genome but was missing from the zebrafish genome assembly due to incomplete coverage. For this reason, we reanalyzed the *gnrh1* region of the zebrafish genome using the most recent release (Z11) of the zebrafish genome. Fig. 2A shows the genomic region surrounding *GNRH1* gene orthologs in humans, among lobe fin vertebrates (e.g., the coelacanth *Latimeria chalumnae*), and in fugu (*Takifugu rubripes*), tilapia (*Oreochromis nitoticus*), spotted Gar (*Lepisosteus oculatus*), medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*) among ray fin vertebrates. In all species of the latter group except zebrafish, *gnrh1* is flanked by the *kctd9* gene on the left (Fig. 2A, blue), and (where annotated) by the *npy8br* gene on the right (Fig. 2A, yellow). In contrast, no *gnrh1* is present between *kctd9* and *npy8br* in the zebrafish genome. Furthermore, in zebrafish the *kctd9* gene is in the opposite orientation in comparison to the other genomes, which is the signature of a small inversion in the *gnrh1* syntenic region. In the zebrafish genome database this region was notated as having less than optimum coverage. To confirm the genomic situation in zebrafish, we re-sequenced the BAC containing the *Ankrd9-npy8br* region (CH211-178D20). Our data showed that the reference sequence contains the same sequence as the BAC, confirming that the zebrafish genome lacks the ortholog of *gnrh1* found in tetrapods and other fishes. We conclude that *gnrh1* disappeared from its ancestral location due to a genomic rearrangement, including at least one inversion, at the position of the ancestral *gnrh1* gene. The loss of *gnrh1* in zebrafish was further confirmed by analyses of RNA-seq data from our lab (Calfun et al., 2016) as well as from data deposited at NCBI (https://www.ncbi.nlm.nih.gov/) for zebrafish brains. These analyses readily detected transcripts for *gnrh2* and *gnrh3* but failed to pick up transcripts for *gnrh1*.

Despite the lack of a *gnrh1* gene, zebrafish do show GnRH immunoreactivity in the hypothalamus (Fig. 1E) in the region known to contain GnRH-positive cells in other animals. In order to investigate the origin of this immunoreactivity we performed a proteome/peptidome analysis of tissues extracted from the adult hypothalamus of zebrafish. Consistent with the genomic analysis, GnRH1 was not detected in MALDITOF or Orbitrap analyses. Furthermore, this same analysis failed to reveal the presence of GnRH2 or GnRH3 peptides in these tissues (Christian Wegener, Neurobiology and Genetics, University of Würzburg, Germany personal communication). Thus, the origin of GnRH immunoreactivity in the zebrafish hypothalamus remains unresolved (Fig. 1E). Indeed, this immunoreactivity is detected using an antibody that recognizes GnRH1 and GnRH3, yet, to date, there are no reports of cell bodies expressing detectable levels of *gnrh1* or *gnrh3* in the parvocellular nucleus of the hypothalamus of adult zebrafish. Previously, GnRH3:GFP positive fibers were reported in the pre-optic area (POA) of developing zebrafish (Zhao et al., 2013) and GnRH3-immunoreactive fibers were described in the POA of adult zebrafish using an antibody produced against the GnRH-associated peptide (GAP) fragment of the preproGnRH3 of sea bass (which shows 80% identity with the GAP fragment of zebrafish GnRH3, (Servili et al., 2011). Taken together, these results suggest the presence of low levels of GnRH3 peptide in the hypothalamus. Yet, elimination of GnRH3 does not affect zebrafish fertility (see Section 1.5, below), leaving unanswered the question of which would be this fish's hypophysiotropic releasing hormone.

1.4. The effects of domestication on the genome

The modification of the genome by directed mutagenesis or through persistent selection for a given trait creates invisible ripples through the genome that can manifest themselves in unintended ways. The consequences of human intervention on animal genomes are most notable in the intentional domestication of plants and animals for horticulture and agriculture. As observed by Darwin (1875), the domestication of animals leads to a striking increase in variability, yet many features associated with domestication are similar in different animals, such as the white star/blaze and floppy ears observed in domesticated mammals (Trut et al., 2009). The process of domestication also often decouples reproduction from the environmental photoperiod (Asher et al., 1999). This change enables animals such as chickens, for instance, to mature their gametes throughout the year, and at the same time tends to attenuate the role of the hypothalamic-pituitaryadrenal (HPA) axis (Rauw et al., 2017). A particularly striking example of the effects of selective domestication is the case of the silver fox of Russia. These initially aggressive animals were domesticated for the fur industry. Selection for submissive behaviors led to dramatic changes in other features of these animals, including their physical appearance, physiology, and genome. Thus, the domesticated foxes have white markings, curled tails, droopy ears, and behave much like a dog (Trut et al., 2009). Importantly, this selection also resulted in dramatic changes in endocrine profiles of male and female silver foxes where levels of *proopiomenanocorticotropin* (*POMC*) (anterior pituitary) and corticotropin releasing hormone (hypothalamus) gene expression were reduced (Gulevich et al., 2004; Trut et al., 2009), as were the levels of pituitary and plasma adrenocorticotropic hormone (ACTH) (Hekman et al., 2018). Finally, the selection for tame foxes was associated with increased level of neurogenesis in the hippocampus of adult foxes (Huang et al., 2015).

Domestication-induced genomic alterations have also been

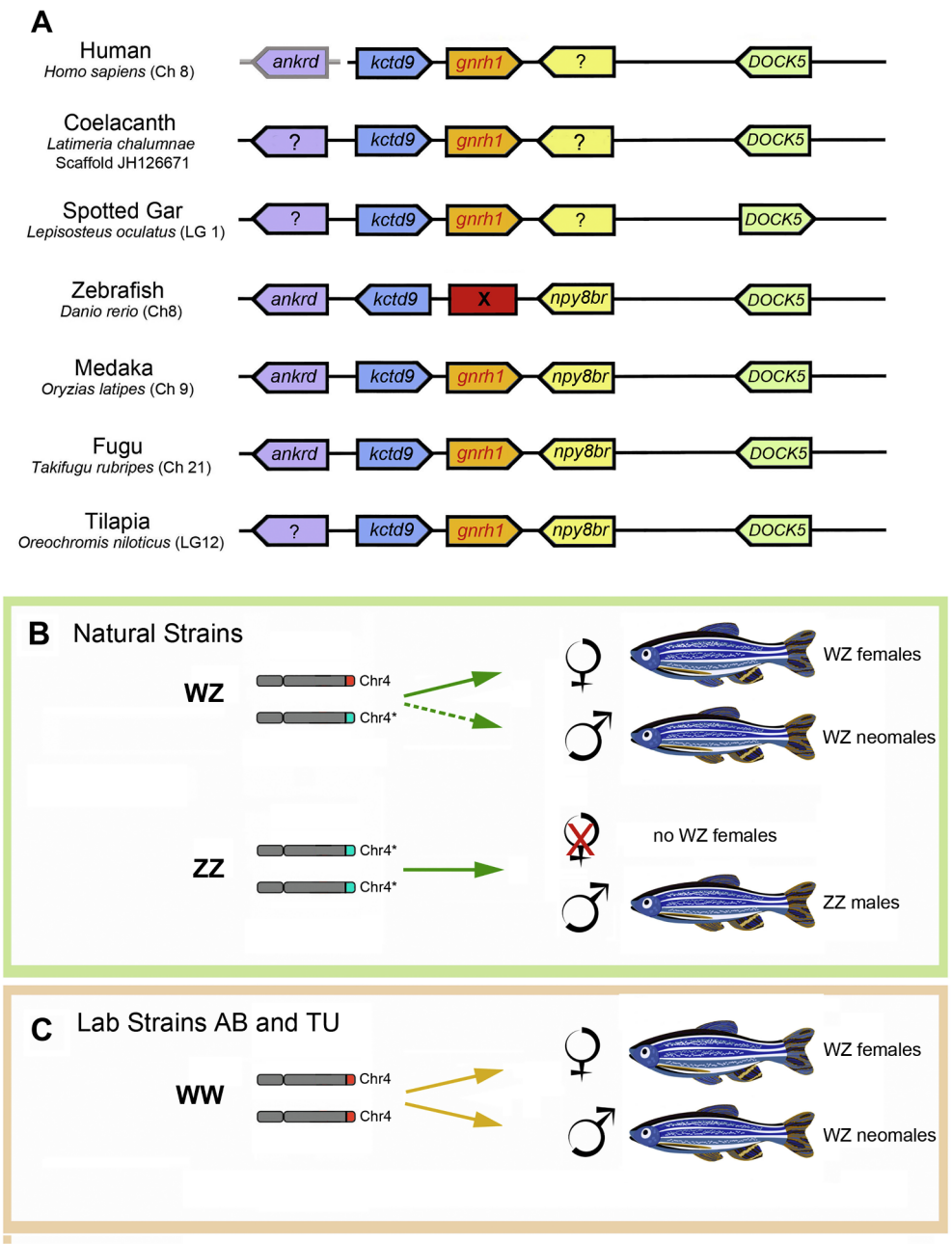

Fig. 2. Genomic rearrangements in the syntenic region of GnRH1 shows a loss of *gnrh1* in zebrafish and effects of domestication on zebrafish sex determination. (A) Analysis of conserved syntenies in the region of the *gnrh1* gene reveals an inversion of the *kctd9* gene in zebrafish relative to the other species, suggesting a re-arrangement in this region (see text for details). Human *ankrd* is boxed in grey because it is located on a separate chromosome. (B, C) Genes controlling sex determination in zebrafish have been altered by domestication. In wild-caught zebrafish (B), the majority of fish heterozygous at the Z locus (Chr4*/Chr4) become females (solid arrow). Animals with two copies of the Z locus (light blue band) on chromosome 4 (Chr4*/Chr4*) become males. Under specific conditions Chr4*/Chr4 animals can become neomales (dashed arrow, see text). In contrast, laboratory strains AB and TU (C) have lost this locus (Chr4*) on chromosome 4 and the population exists as Chr4/Chr4 where both females and neomales are generated (solid yellow arrows) albeit often in skewed sex-ratios.

observed in aquaculture, where the collection of salmon from the wild for hatchery stocks has led to changes in their genome within the first three generations (Bicskei et al., 2014). In research animals, inbred lab strains generally show genomic variations that correlate with the number of generations maintained in captivity.

A dramatic example of genomic effect of domestication in a model system is evident in the sex determination system of the zebrafish. First introduced to the lab setting by Dr. George Streisinger (Varga, 2018), the lines of zebrafish most researchers use (called AB and TU, Mullins et al., 1994) have been maintained in closed breeding populations with no introduction from wild populations. It is known that stress can affect the sex ratio in zebrafish, such that reduced food, high temperatures, and low oxygen, can drive the sex ratio towards an increased production of males (Shang et al., 2006; Villamizar et al., 2012). Furthermore, zebrafish can change sex under conditions in which germ cell signaling is disrupted (Dranow et al., 2016). Juvenile animals that lack oocytes will develop as phenotypic males (Slanchev et al., 2005; Siegfried and Nusslein-Volhard, 2008). And strikingly, the depletion of oocytes, but retention of germline stem cells, causes adult females to sex-revert to sperm-producing males (Rodriguez-Mari et al., 2010; Dranow et al., 2013). These studies were performed using lab strains, yet cytogenetic studies on zebrafish captured in India (this fish is native of India and Southeast Asia; Engeszer et al., 2007; Parichy, 2015); have revealed females to be the heterogametic sex with a defined female-specific chromosome (Sharma et al., 1998).

Research into the mechanisms that control sex determination in zebrafish have reconciled these conflicting results and led to the surprising finding that domestication of zebrafish has led to the loss of sex-determining genetic loci (Fig. 2B, C). Studies comparing wild-caught zebrafish *versus* zebrafish maintained for generations in captivity has revealed that the natural strains (named EKW, Nadia, WIK, and Cooch Behar), have retained a region of chromosome 4 that is genetically different between phenotypic females and most phenotypic males, consistent with a ZZ(male)/ZW(female) sex determination system (Wilson et al., 2014) (Fig. 2B). All phenotypic females have a W allele, no ZZ individuals ever become females, but some ZW fish sex-reverse to

become males (neo-males). In contrast, lab strains AB and TU lack the alleles strongly associated with sex determination (Wilson et al., 2014), although there is evidence that weak or polymorphic sex biasing genes are present in other parts of the genome in some lab strains (Orban et al., 2009; Siegfried, 2010; Tong et al., 2010; Bradley et al., 2011; Anderson et al., 2012; Liew and Orban, 2014; Liew et al., 2012) (Fig. 2C). The AB lab strain originated from pet store animals (1970 s, Oregon, USA) that were selected to produce many offspring and were subjected to several rounds of gynogenesis to make them free of lethal mutations, thereby creating a genetic bottleneck (Streisinger et al., 1981). Likewise, the TU lab strain originated from a fish pet store in Germany, and was also heavily selected to produce large numbers of offspring free of lethal mutations (Mullins et al., 1994). By contrast, the EKW strain has been maintained since 1962 in large populations in outdoor ponds without selection for specific characteristics (EkkWill Waterlife Resources).

These observations on the altered genome of "domesticated" zebrafish raise the possibility that domestication itself may have led to the loss of the *gnrh1* gene in this species. Indeed, some of the aforementioned changes seen in domesticated mammals, such as the de-coupling of reproduction from the natural cycles, increased fecundity, and the effects on the HPA axis, suggest that the loss of the *gnrh1* gene in zebrafish (and potentially also in other domesticated Cyprinids) may have resulted from the selective pressures imposed by human desires. This hypothesis predicts that *gnrh1* would be found in the genome of the unselected EKW line and absent from the reference zebrafish genome (currently GRCz11), since this latter sequence was obtained from lab-selected fish. Nevertheless, we found that the genome of the unselected EKW strain of zebrafish also lacks a *gnrh1* gene (Fig. 3), indicating that the absence of a *gnrh1* gene in the reference zebrafish genome sequence is not a consequence of domestication (Fig. 3A, B). In contrast, Medaka, (*Oryzias latipes*) another fish used in biomedical research (Kirchmaier et al., 2015) retains the *gnrh1* gene (Okubo et al., 2006); Fig. 3C). Yet, the analysis of other fish genomes revealed that the loss of the *gnrh1* gene is not exclusive to zebrafish but also occurred in other fishes, such as the Mexican cavefish (*Astyanax mexicanus*; Fig. 3D). A finer analysis of this species' *Ankrd9-npy8br* genomic region is not yet possible given the current quality of the cavefish genome sequence, but it will be interesting to determine if the loss of the *gnrh1* gene is associated with the same inversion observed in zebrafish, as this would suggest that gene loss occurred before the divergence of the *Astyanax* (Order Characiformes, Family Characidae) and zebrafish (Order Cypriniformes, Family Cyprinidae) lineages (Fig. 3E).

1.5. Potential alternative pathways to GnRH in zebrafish

1.5.1. Loss of gnrh1 and/or gnrh3 does not result in infertility

The absence of the *gnrh1* gene in the zebrafish (and likely in cypriniformes in general) raises the possibility that GnRH3 may be the hypophysiotropic form in these fish. In a study to determine the potential role of GnRH3 in zebrafish reproduction, cells containing GnRH3, as identified *in vivo* using a *Tg(gnrh3:egfp)* reporter line (Abraham et al., 2008), were laser ablated during early development (Abraham et al., 2010). The resulting adult fish lacked GnRH3:GFP positive neurons and showed arrested oocyte development and reduced average oocyte diameter, suggesting that GnRH3 might play a role in reproduction. However, because these ablations eliminated neurons that may contain not only GnRH3 and its GAP but potentially also other peptides such those in the FMRFamide family (which are known to be co expressed with GnRH; Oehlmann et al., 2002) the possibility existed that it was the loss of other peptides (alone or in combination with GnRH3) that may have caused an unintended trophic effect on the development of the HPA axis (Zhao et al., 2013). Consistent with this possibility, two different groups recently reported that the deletion of the *gnrh3* gene using TALEN technologies did not affect the fertility of adult zebrafish. One group (Spicer et al., 2016) reported limited effects on mRNA expression of *follicle-stimulating hormone beta* (*fshb*), *luteinizing hormone beta* (*lhb*), and *chorionic gonadotropin subunit alpha* (*cga*) during early development. Yet, these changes in expression were not present in the adult animals and there were no adverse effects on reproduction in either male or female *gnrh3-/-* adult fish (Spicer et al., 2016). These data are consistent with those reported by a second study showing that *gnrh3-/-* mutants retained full reproductive capacity, although in this second study (Liu et al., 2017), unlike Spicer et al. (Spicer et al., 2016) no changes in expression of the pituitary gonadotropins *fshb, lhb*, and *cga* were found in female or male *gnrh3* mutant fish but it is unclear whether they were sampling the same stages of development. Thus, knocking-out *gnrh3* in zebrafish, which already lack the *gnrh1* gene, resulted in animals that were fertile, with normal gametogenesis and reproductive performance in both males and females.

1.5.2. Loss of gnrh2 does not affect fertility

If zebrafish lacking GNRH3 are fertile, the only GnRH hormone left that could play a role in reproduction is GnRH2. The role of GnRH2 in reproduction and behavior is relatively unknown, including its potential relationship with the GnRH3 network within the HPA axis. Recently, transgenic reporter lines, *Tg(GnRH2:eGFP)* and *Tg (GnRH3:tdTomato; GnRH2:eGFP*), were used to visualize the GnRH2 and GnRH3 networks in intact developing zebrafish. These analyses showed that GnRH2 neurons have extensive projections in the brain and spinal cord with potential projections to the pituitary (Xia et al., 2014). Zebrafish have four GnRH receptors (zfGnRHR1, zfGnRHR2, zfGnRHR3, zfGnRHR4; (Roch et al., 2014; Tello et al., 2008) where zfGnRHR1 and zfGnRHR3 have a greater affinity for GnRH2 than for GnRH3, and zfGnRHR2 and zfGnRHR4 have the same affinity for GnR2 and GnRH3 (Tello et al., 2008). Since the various GnRH receptors are expressed in the brain, eye, and ovaries and testes (although zfGnRHR3 shows very reduced expression in the brain and the ovaries; Tello et al., 2008) the possibility exists that GnRH2 could replace GnRH3 as the hypophysiotropic hormone controlling reproduction in zebrafish. Because of the promiscuity of ligand/receptor interactions and the reported GnRH2 projections to the pituitary, the *gnrh2* gene was knocked out in a *gnrh3-/-* background to determine whether this peptide could compensate for the loss of the *gnrh3* gene function. Surprisingly, whereas age-matched adult male and female *gnrh2-/-; gnrh3-/-* double knockout fish exhibited up-regulation of several genes in the brain, including the *gonadotropin inhibitory hormone* (*gnih*; also known as Lpxrfa), they showed no reproductive defects (Marvel et al., 2018). Since these mutant animals lack all forms of GnRH, these results show that in zebrafish, neither GnRH2 nor GnRH3 replaces the function of GnRH1, raising the possibility that other, non-GnRH, peptides replace GnRH1 as the hypophysiotropic releasing hormone.

1.5.3. Loss of gnrh3 and kisspeptin does not affect reproduction

An essential hypothalamic peptide that regulates GnRH is kisspeptin (Kiss), an obligate upstream regulator of gonadotropin-releasing hormone secretion in mammals. Kiss is so important in this process that it has been proposed to be the "master molecule" in reproductive events, not only during puberty but also in adulthood (Dungan et al., 2006; Seminara and Crowley, 2008). In teleosts there are two kiss genes, called *kiss1* and *kiss2* (Kanda et al., 2008), and two Kiss receptors encoded by *kiss1ra* and *kiss1rb* (van Aerle et al., 2008). Subsequent analysis has shown that in zebrafish, Kiss2 is important for reproductive events, whereas the function of Kiss1 remains to be established (Servili et al., 2011). The Kiss peptides are part of the GnRH pathway in animals in which GnRH acts as the hypophysiotropic hormone. In zebrafish, animals homozygous for TALEN-generated knockouts of both *kiss1* and *kiss2* genes showed normal puberty and gonadal development (Servili et al., 2011). Furthermore, the inactivation of the *kiss* genes in a *gnrh3-/-* mutant background did not result in impaired puberty or reproductive defects. Interestingly, the expression of *neuropeptide Y* (*npy*), *tachykinin 3* (*tac3*), and *secretogranin-II* (*sgII*), all neuropeptides reported

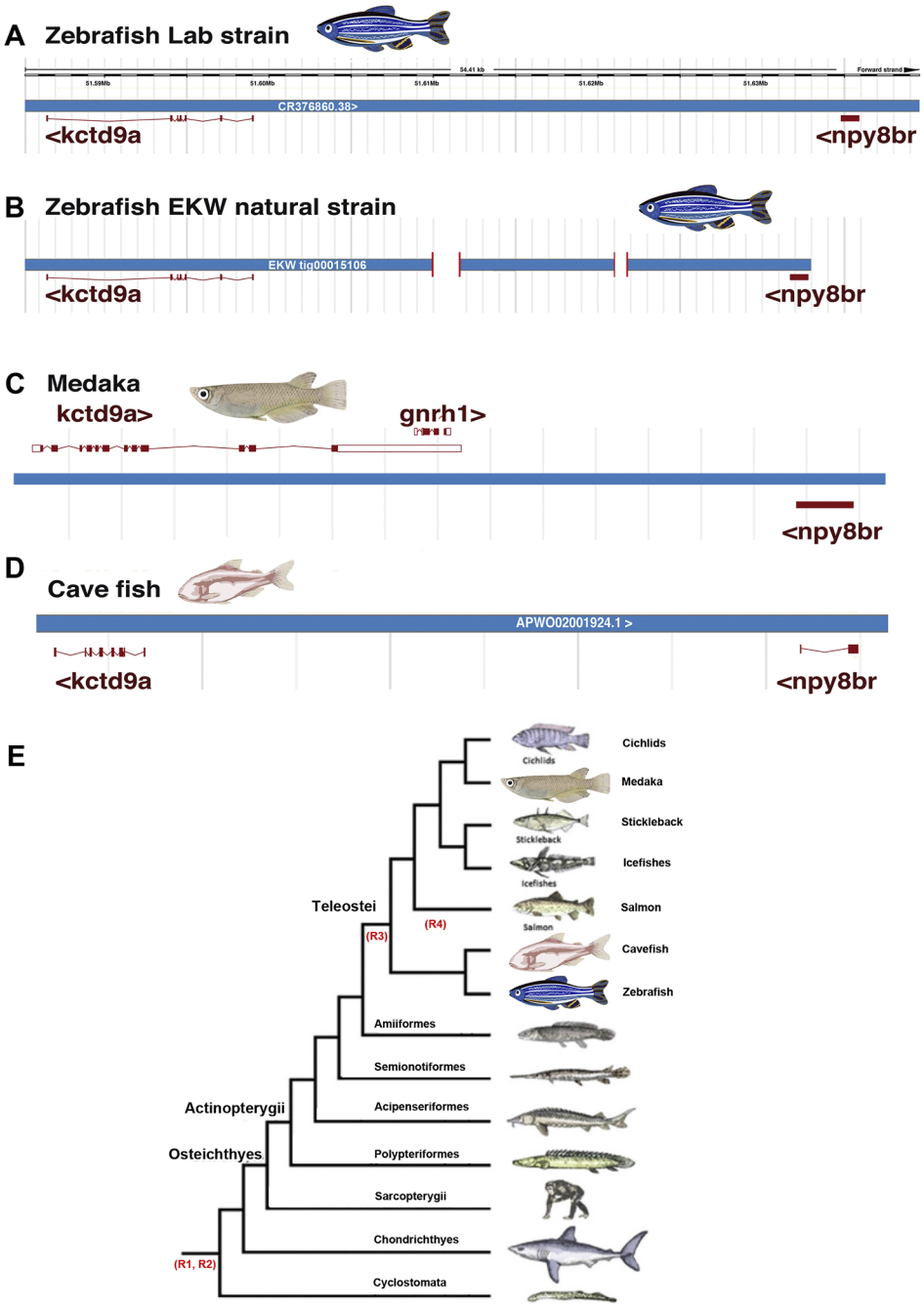

Fig. 3. Loss of *gnrh1* pre-dates the domestication of zebrafish. (A-D) neither lab strains (A) nor wild-caught (B) zebrafish contain the *gnrh1* gene. In wild-caught zebrafish this interval contains small deletions not present in the lab stains (indicated as gaps in the schematic). In contrast to zebrafish, medaka contains *gnrh1* (C). Similar to zebrafish, the cavefish *Astyanax mexicanus* lacks the *gnrh1* gene (D). (E) Simplified lineage tree of fishes. Similarly to zebrafish (*Danio rerio*, Order Cypriniformes, Family Cyprinidae; panels C and D), the genome of the cavefish (*Astyanax mexicanus*, Order Characiformes, Family Characidae; panel B) lacks *gnrh1* (E adapted from Casane and Retaux, 2016).

to stimulate gonadotropin release, were significantly increased in the triple knockout mutant (Liu et al., 2017). These results suggest that zebrafish must use a different non-Kiss- and non-GnRH-based mechanism to regulate reproduction. If GnRH1 does not exist in zebrafish, and its function has not been replaced by GnRH2, GnRH3, Kiss1, or Kiss2, which peptide has taken its place?

1.6. Potential alternative pathways to GnRH in zebrafish

Approximately half of the extant vertebrates are fish and, of this group, greater than 95% are teleosts (numbering over 30,000 species), thus making teleosts the dominant class of not only fish but of vertebrates on the Earth (Harvey Pough et al., 2005). Teleosts have secondarily lost the median eminence characteristic of vertebrate ancestors and of other extant vertebrates, which allows for direct neuroendocrine regulation of the brain-pituitary axes. The list of potential regulators of reproduction is long, including neuropeptides such as gonadotropin inhibitory hormone (GnIH), aminergic neurotransmitters such as dopamine and serotonin, and amino acid neurotransmitters such as glutamate and GABA (for review see Trudeau, 2018). Thus, the perplexing situation of the reproductive control in zebrafish could possibly be explained by a variety of pathway modifications, such as *a)* Cooption of a known peptide, such as neuropeptides or neurotransmitter, in the reproductive cascade, or *b)* Use of an unrelated and as yet unidentified peptide.

1.6.1. Duplication of one of the other forms of GnRH

With the diversity of the teleost fishes also comes a diversity in the mechanisms of sexual plasticity and their mechanics of gametogenesis and reproduction (Kitahashi et al., 2013) and underlying this diversity is the evolution of a fantastically diverse genome. Goldfish, one of the earliest domesticated fish (Komiyama et al., 2009), also lacks the *gnrh1*

gene, and has undergone an additional round of whole genome duplication (WGD) relative to zebrafish, although both are cyprinid fishes (Kuang et al., 2016). Interestingly, this WGD appears to have arisen through allopolyploidzation (a process in which chromosomes are derived from one of two or more species) of hybrid offspring of ancestors of common carp (*Cyprinus carpio*) and goldfish (*Carassius* sp.) (Ma et al., 2014). In goldfish (*Carassius auratus*), there are two isoforms of GnRH3 (originally named sGnRH-I and II) and two *gnrh2* genes (originally named cGnRH-I and II) (Lin and Peter, 1996; Yu et al., 1998), and both GnRH2 and GnRH3 can trigger spawning in female goldfish (Volkoff and Peter, 1999). Like goldfish, salmonids (genera of the subfamily Salmoninae), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), and Arctic charr (*Salvelinus alpinus*), lack *gnrh1* and their genome has also undergone an additional WGD compared to that of the zebrafish (Leder et al., 2004). The salmonid genome duplication resulted in a duplication of the *gnrh3* gene (each copy encoding GNRH3A and GNRH3B) (formerly known as sGnRH1 and sGnRH2, (Leder et al., 2004) and in this group of fishes, GnRH3A appears to be the hypophysiotropic form used to control spawning (Ando and Urano, 2005). Thus, to date the only fishes where a specific GnRH has been duplicated (cf, GNRH3A and GNRH3B) are those that have undergone an additional round of WGD (R4: Fig. 3E). Yet, in spite of a duplication at the base of the teleost lineage (R3: Fig. 3E), zebrafish do not have duplicates of GnRH genes, unlike other genes in their genome.

1.6.2. Coopting a peptide known to be part of the reproductive cascade

One neurohormone proposed to potentially modulate the reproductive axis in the zebrafish is GnRH-Inhibitory hormone (Fig. 4A, B; GnIH, also called LPXFR), a hypothalamic peptide first discovered in the Japanese quail, where it inhibits gonadal development and gonadotropin release (Tsutsui et al., 2018; Ubuka et al., 2003). GnIH belongs to the RFamide family and inhibits the brain-pituitary reproductive axis in mammals and birds (Osugi et al., 2014; Tsutsui and Ubuka, 2016). In addition, GnIH can have a stimulatory effect on gonadotropin secretion in some fish, including tilapia (Ogawa et al., 2016) and goldfish (Moussavi et al., 2014), as well as in a variety of other fishes (for review see Munoz-Cueto et al., 2017). The GnIH orthologs in fish are often referred to as "LPXRFamide (Lpxrfa) peptides", of which zebrafish has three: Lpxrfa-1, Lpxrfa-2, and Lpxrfa-3. Lpxfra peptides are cleaved from a 198-amino-acid precursor peptide, and act on three GPCRs (Lpxrf-R1, Lpxrf-R2, and Lpxrf-R3) (Zhang et al., 2010). Most recently, the Lpxfra-3 peptide was shown to reduce the expression of *lsh* and *cga* in pituitary explants of zebrafish, thereby inhibiting the reproductive axis, and also reduced the expression of *gnrh3* in neurons in the brain (Spicer et al., 2017). Therefore, reproductive inhibitory neuropeptides acting through Lpxrfa may interact with GnRH3 neurons in the brain and with pituitary gonadotropes, while also potentially utilizing the Kiss2/Kiss1ra pathway (Spicer et al., 2017). To date, these data do not support a potential role for GnIH/Lpxfra in the stimulation of the reproductive axis in the zebrafish, although the functions of Lpxfra-1 and Lpxfra-2 need to be better defined because they could be bypassing the kisspeptin pathway (Fig. 4B, red).

1.6.3. Use of an unrelated peptide

It is often overlooked that there are still many "orphan" receptors, which suggests the existence of uncharacterized ligands with potentially new functions. One such recently discovered protein is phoenixin (Fig. 4C), originally identified using a bioinformatic approach to screen

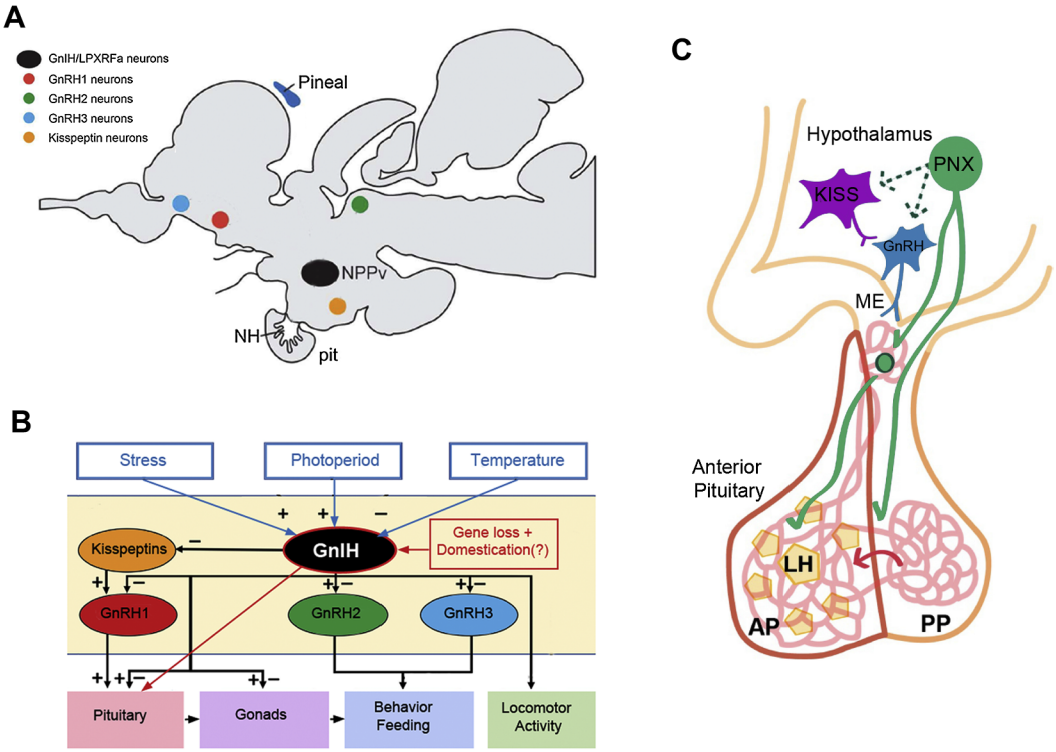

Fig. 4. Potential alternative pathways replacing GnRH1 signaling in zebrafish. (A) Cells containing GnRH-Inhibitory hormone (GnIH) (black) are shown relative to the populations of cells with which GnIH might interact: GnRH1 (red), GnRH2 (green), and GnRH3 (blue), and Kisspeptins (orange). (B) GnIH signaling pathways in the brain pituitary axis. In zebrafish, the loss of *gnrh1* and loss-of-function mutations in *gnrh2, gnrh3*, *kiss1,* and *kiss2*, do not result in infertility, thus suggesting a potential direct interaction of GnIH on the pituitary gonadotropes (red arrow). This unusual loss of signaling may be due to intense selective pressures (red box: gene loss + domestication) (A modified from Ogawa and Parhar, 2014; B modified from Munoz-Cueto et al., 2017). (C) The recently discovered reproductive peptide, phoenixin (PNX), can stimulate kisspeptin (purple) and GnRH (blue) release in hypothalamic cell lines. In intact animals PNX can stimulate LH release from the hypothalamus (green cell with solid arrows) although the exact details have yet to be uncovered. PNX is conserved in fish and is a candidate peptide for hypothalamic-pituitary interactions in zebrafish (C reprinted from The phoenixins: From discovery of the hormone to identification of the receptor and potential physiologic actions, Stein et al., 2018 with permission from Elsevier).

the human genome for sequences predicted to encode previously unrecognized secreted peptides (Yosten et al., 2013). Phoenixin was shown to be highly conserved from humans to zebrafish (Yosten et al., 2013), is cleaved from the C-terminus of the so-called small integral membrane protein 20 (SMIM20). The 14 and 20 amino acid cleavage products, PNX-14 and PNX-20, have been shown in rats to act on gonadotropes through the orphan GPCR, Gpr173 (Stein et al., 2016), and thus appear to affect gonadotropin release from the pituitary via modulation of GnRHR expression (Yosten et al., 2013; Stein et al., 2016). Furthermore, the presence of PNX peptides has been confirmed in different regions of the mammalian brain including the hypothalamus, sensory ganglia, and spinal cord (Lyu et al., 2013). Because PNX can regulate the expression of kisspeptin, GnRH, GnRH receptor, and LH (see for review Yuan et al., 2017), PNX peptides have been proposed to be hypothalamic factors that could potentiate the action of pituitary gonadotropes (Treen et al., 2016; Palasz et al., 2018), thereby acting as reproductive peptides. Because of the potential reproductive functions of PNX, we have confirmed the expression of PNX in developing and adult zebrafish (Ceriani, Calfun and Whitlock, *in preparation*), which opens the door for the analysis of this peptide as a potential regulator of the brain-pituitary axis in the zebrafish.

A final part of the GnRH mystery, yet to be solved, is a strikingly specific GnRH immunoreactivity pattern in the parvocellular nucleus of the adult zebrafish (Fig. 1E, Cortes-Campos et al., 2015). Previously, we used antibodies recognizing GnRH1 (Park and Wakabayashi, 1986) to localize cells in the hypothalamus (Cortes-Campos et al., 2015). Because of their location and the fact that to date there is no convincing evidence showing expression of *gnrh3* in the parvocellular region of hypothalamus in the adult zebrafish, we classified the immunopositive cells as GnRH1 cells. In light of the overwhelming evidence that a *gnrh1* gene does not exist in the genome of the zebrafish and that neither MALDITOF nor Orbitrap analyses of hypothalamic tissues detected any GnRH-like peptide in this tissue, the anti-GnRH antibodies must be recognizing a protein immunologically similar to, but unrelated to, GnRH1. We hope that future analysis will uncover the basis for this perplexing, highly specific expression pattern.

2. Conclusions

Here we have presented an overview of the fascinating situation of the brain-pituitary axis in zebrafish. In spite of an extra round of duplication relative to mammals, the genome of zebrafish lacks the gene encoding GnRH1 and this loss is not compensated for by either *gnrh2* or *gnrh3*. Furthermore, knocking out genes encoding several key peptides involved in reproduction, most importantly *kisspeptin*, does not cause defects in fertility in the adult fish. Interestingly, we now know that the zebrafish genome has been highly modified by domestication to the extent of altering the genetic mechanisms of sex determination relative to the wild populations. Although domestication does not explain the loss of the *gnrh1* gene, it must be kept in mind when working with highly selected lines developed for medical research, aquaculture, or the pet trade industry, especially when investigating the endocrine control of the reproductive axis.

Here we have shown that the “usual suspects” are not involved in regulating the hypothalamic-pituitary-gonadal axis in zebrafish, and there are hints that a similar situation may occur in other fishes that also lack GnRH1. It is interesting to note that in humans the availability of genome-wide analyses has revealed that only ~30% of the cases of idiopathic hypogonadic hypogonadism, which was once considered a monogenic disorder, are explained by mutations in one of eleven known genes (Pitteloud et al., 2010). This suggests that there may be more flexibility than expected in this pathway even beyond fishes; it could also be that physiological and environmental factors may also be playing a role.

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