The gonadotropin-releasing hormones: lessons from fish.

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Abstract

Fish have been of paramount importance to our understanding of vertebrate comparative neuroendocrinology and the mechanisms underlying the physiology and evolution of gonadotropin-releasing hormones (GnRH) and their genes. This review integrates past and recent knowledge on the GnRH system in the fish model. Multiple GnRH isoforms (two or three forms) are present in all teleosts, as well as multiple GnRH receptors (up to five types), which differ in neuroanatomical localization, pattern of projections, ontogeny and functions. The role of the different GnRH forms in reproduction seems to also differ in teleost models possessing two *versus* three GnRH forms, GnRH3 being the main hypophysiotropic hormone in the former and GnRH1 in the latter. Functions of the non-hypothalamic GnRH isoforms are still unclear, although under suboptimal physiological conditions (e.g. fasting), GnRH2 may increase in the pituitary to ensure the integrity of reproduction under these conditions. Recent developments in transgenesis and mutagenesis in fish models have permitted the generation of fish lines expressing fluorophores in GnRH neurons and to elucidate the dynamics of the elaborate innervations of the different neuronal populations, thus enabling a more accurate delineation of their reproductive roles and regulations. Moreover, in combination with neuronal electrophysiology, these lines have clarified the GnRH mode of actions in modulating LH and FSH activities. While loss of function and genome editing studies had the premise to elucidate the exact roles of the multiple GnRHs in reproduction and other processes, they have instead evoked an ongoing debate about these roles and opened new avenues of research that will no doubt lead to new discoveries regarding the not-yet-fully-understood GnRH system.

1. Introduction

The expression, secretion and activity of pituitary gonadotropins in fish are controlled by multiple neurohormonal factors that are synthesized in specific neuronal populations of the brain and are released directly into the pituitary to trigger a series of endocrine cascades that lead to reproduction (Zohar et al., 2010). Elucidation of the nature and origin of these neurohormones has provided substantial information on how the brain controls the reproductive process, with the neuroanatomical organization of the preoptic-hypothalamic-pituitary complex being a significant contributor.

Although the number of neuroendocrine factors implicated in the control of reproduction in fish has continuously increased during the last decades, there are gaps in our understanding of when and how their effects are exerted and how they are used in diverse fish species. In this review, we will focus on one neuroendocrine system that has been studied in depth and whose actions on the reproductive axis seem well established: the 10 amino acid long neuropeptide, gonadotropin-releasing hormone (GnRH).

Originally, it was thought that this decapeptide is released by a single population of hypophysiotropic neurons that stimulate the secretion of pituitary gonadotropins. This population represents the principal neuroendocrine complex triggering the hormonal cascade that controls reproduction along the hypothalamus-pituitary-gonad (HPG) axis. Early characterizations and physiological studies performed in cyprinids and salmonids (Breton et al., 1972; Sherwood et al., 1983), have since been expanded to cover the GnRH system in a wide variety of fish. As will be laid out in this review, we now know that the GnRH system in fish is much more complex than originally believed, and all fish species express multiple forms (two or three) of GnRH in cells located in different brain areas and tissues. These GnRH isoforms can exert pleiotropic actions mediated by different types of GnRH receptors (Zohar et al., 2010).

In general, and as will be reviewed here, fish have played a crucial role in advancing our understanding of how the functions of the different forms and neurons of GnRH have evolved and diversified in vertebrate species (Lethimonier et al., 2004; Guilgur et al., 2006; Kah et al., 2007). Initially, the intense interest in studying the multi-faceted GnRH system in fish stemmed from the fact that a malfunctioning GnRH system was shown to be responsible for the lack of ovulation and spawning in commercially important aquaculture species when raised in captivity (Zohar and Mylonas, 2001; Mylonas and Zohar, 2001, 2009). Understanding the nature of this failure and the effort to overcome it by administering exogenous GnRH and its analogs, led to focused research efforts of the GnRH system in key aquaculture species such as salmonids, cyprinids and many perciform species. With the recent establishment of fish like zebrafish and medaka as vertebrate models, for all their known benefits, along with breakthrough developments in molecular platforms such as genomics, transgenesis and loss of function, new insights and paradigm shifts have been achieved in our understanding of the GnRH system. A striking example, which will be discussed in more detail below, comes from recent knockout studies in zebrafish, which suggested that GnRH could be dispensable for normal reproduction (after decades of scientific consensus that it was indispensable), and that in its absence the functions of GnRH may be replaced by other neuroendocrine factors through a compensatory mechanism (Spicer et al., 2016; Trudeau, 2018; Whitlock et al., 2019; Marvel et al., 2019).

This review describes the history of the scientific progress in the field of vertebrate GnRHs, focusing on fish models, from their initial discovery to our current state of understanding of the biologically critical and highly elaborate GnRH system. For convenience, all over this review we have adopted the gene/mRNA/protein nomenclature conventions for zebrafish (<https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Conventions>).

2. How the neuroendocrine brain message reaches the fish pituitary

The existence of vascular contact between the hypothalamus and the pituitary was demonstrated in the 1930's, but it was not until the 1940's and 1950's that Geoffrey Harris, one of the pioneers of neuroendocrinology, began to consolidate the concept of neurohormonal regulation of adenohypophyseal functions. This concept was initially in conflict with the existing research paradigm, whereby it was held that neurons only communicated with each other by means of electrical signals, and that the anterior lobe of the adenohypophysis of mammals was not innervated. The experiments of Geoffrey Harris provided confirmation that sectioning the pituitary stalk interrupted ovarian cyclicity in the rat, and that the reinstitution of the hypothalamus-hypophyseal portal system restored this cyclicity. These studies paved the way for the discovery of the brain factors, which are transported by this vascular system to the pituitary, to regulate the secretion of gonadotropins and development of the gonads (Donovan and Harris, 1954).

In fish, the pituitary is attached to the hypothalamus by a slender stalk called the neurohypophysis, which consists of brain origin axons of neurosecretory cells that penetrate the pituitary. The pituitary of fish is peculiar in that it lacks the median eminence and the portal vasculature system, characteristic of tetrapods, which connect the hypothalamus with the pituitary. As a result, the neurohormones that act on the pituitary are released from the neurosecretory terminals more or less directly into the surroundings of the target cells (Batten and Ingleton, 1987). In some species of fish (salmonids, eels), these neurosecretory terminals are separated from the adenohypophyseal cells by a double basal membrane into which the neurosecretory products are released; these are then diffused across this membrane and through the intercellular spaces, to reach their target cells. In other species (goldfish and other cyprinids), the basal membrane is discontinuous and allows the nerve terminals to penetrate into the adenohypophysis and to innervate the pituitary secretory cells by means of direct

contacts that are similar to synaptic contacts. This direct innervation of the pituitary of teleost fish, together with the identification of specific neurosecretory terminals in the vicinity of a particular hypophyseal cellular type, has enabled researchers to characterize those brain factors that are involved in controlling the secretion of the hypophyseal hormones. Thus, the presence of terminals that secrete GnRH, dopamine, Neuropeptide Y (Npy), gamma-aminobutyric acid (GABA) or Gonadotropin-inhibitory hormone (GnIH) in the proximity of the gonadotropic cells constitutes extremely useful neuroanatomical evidence that has led to the determination of the actions of these neurohormones on the secretion of gonadotropins and on the reproductive cycle of fish (Kah et al., 1987, 1989, 1992; Paullada-Salmerón et al., 2016). Recently however, using combined GnRH3/LH and FSH transgenic zebrafish, an intricate vasculature network, populating the entire pituitary, was shown to actively transmit GnRH3 signaling to the pituitary (Golan et al., 2015). This study suggests that, in addition to direct GnRH neuron innervation of the gonadotropes, the vascular system plays a part in the neurohormonal signaling in teleosts, despite the absence of a median eminence-like structure.

The direct innervations in the adenohypophysis of fishes have allowed the application of neuronal tracing techniques to identify, by means of retrograde transport, the brain areas that innervate the pituitary. In these studies, the use of a fluorescent marker such as DiI (1-1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine) has been especially useful; this marker is transported retrogradely across the membranes of axons fixed with formaldehyde. The employment of DiI has allowed the identification of hypophysiotropic areas in species like the goldfish (Anglade et al., 1993), the electric fish *Apteronotus leptorhynchus* (Johnston and Maler, 1992), the African catfish *Clarias gariepinus* (Dubois et al., 2001) and the sea bass (García-Robledo and Muñoz-Cueto, unpublished) among others. Most of the innervations of the pituitary originate from neurons located in brain nuclei at the preoptic area (periventricular preoptic nucleus, parvocellular preoptic nucleus, magnocellular preoptic

nucleus, anterior periventricular nucleus, suprachiasmatic nucleus) and the mediobasal hypothalamus (lateral tuberal nucleus, anterior tuberal nucleus, posterior periventricular nucleus, nucleus of the lateral recess, nucleus of the posterior recess), known as the main neuroendocrine regions involved in the control of reproduction in vertebrates. Additional hypophysiotropic cells have been described in the olfactory bulb, the ventral telencephalon, the thalamus and the mesencephalic tegmentum (Anglade et al., 1993; Kah et al., 1993).

More recently, the use of transgenic zebrafish and medaka lines that express fluorophores, under the control of promoters for *gnrh1*, *gnrh2* and/or *gnrh3*, have permitted the detection and characterization of GnRh neurons from early developmental stages and facilitated the distinction of the different GnRh neuronal populations (Abraham et al., 2008; Palevitch et al., 2009; Takahashi et al., 2016, see the sections 4 and 6 of this review).

3. History and functional significance of GnRH multiplicity

3.1. The discovery of GnRH isoforms

Nearly two decades after the finding that brain-pituitary input is essential for reproduction (Donovan and Harris 1954), the first GNRH peptide was discovered in pig and sheep hypothalamus, and its staple sequence of 10 amino acids was revealed (Amoss et al. 1971; Burgus et al. 1971; Matsuo et al. 1971). Based on its LH release activity, it was initially termed luteinizing hormone releasing-hormone (LHRH), which was later revised to gonadotropin-releasing hormone (GNRH) following evidence of its FSH stimulatory activity. Extensive studies that followed have established GNRH's critical role in controlling reproduction in mammals through its pulsatile secretion in the median eminence. The amplitude and frequency of these GNRH pulses control the synthesis and release of FSH and LH (Counis et al., 2005). Soon after the discovery of mammalian GNRH, it was demonstrated that GNRH was also able to stimulate the release of gonadotropins in the carp

(Breton et al., 1972). Fish GnRh acts on pituitary gonadotropes to induce the synthesis and release of Lh and Fsh to the bloodstream which, in turn, regulate all aspects of gonadal activities, including the onset of gonadal development, germ cell development (oogenesis and spermatogenesis), gonadal steroid production, ovulation, spermiation and spawning (Zohar et al., 2010). In contrast to mammals, no clear indication that GnRh release in the pituitary is pulsatile has been reported in fish (Kah and Dufour, 2011). However, the existence of GnRh pulsatility in teleost fish deserves further investigation because regular pacemaker activities have been identified in GnRh3 and GnRh2 neurons of medaka and dwarf gourami by single cell electrophysiological recording (Abe and Oka, 2002; Kanda et al., 2010). In addition, GnRh has been implicated in controlling the release and expression of Growth hormone and Somatolactin (Grey and Chang 2013), and prolactin (Weber et al., 1997; Seale et al., 2013).

The amino acid sequence of the first-discovered GNRH was conserved in most mammalian species and, hence, for many years it was considered a common form among vertebrates (Okubo and Nagahama, 2008). During the '80s and '90s, with the development of specific GNRH anti-sera and advanced molecular techniques, a growing number of GNRH forms were identified in the brain of different vertebrate species, in parallel with the realization that at least two isoforms co-exist in the brain of many vertebrate species (Muske, 1993; Kasten et al., 1996; Lescheid et al., 1997; White et al., 1998). The first variant form of GNRH, chicken GNRH-I (cGNRH-1), was discovered in chickens (King and Millar, 1979, 1982a, 1982b; Miyamoto et al., 1982, 1983), followed by salmon GnRh (sGnRh), the first form of GnRh characterized in teleost fish (Sherwood et al., 1983). A second form of GNRH in a single species was found in chicken, hence named chicken GNRH-II (cGNRH-II, Miyamoto et al., 1984) and later found in other species including fish (Amano et al., 1991), amphibians (Iela et al., 1991), reptiles (Masucci et al., 1992) and, based on these findings, also in primates and humans (Lescheid et al., 1997; White et al., 1998). In fish, the first variant *gnrh*

cDNA was detected and sequenced in the Atlantic salmon (Aleström et al., 1992). A short time later, three distinct Gnrh isoforms were discovered and identified in the brain of gilthead seabream, a perciform fish (Powell et al., 1994). This was the first time three forms were found in a single species. This discovery was followed by the characterization of three corresponding cDNAs (Gothilf et al., 1996; Chow et al., 1998). It is now widely recognized that while less evolved teleost species such as salmonids and cyprinids possess two Gnrh isoforms, the brain of most teleost species, including Perciformes, Pleuronectiformes and many other fish orders, exhibit three distinct forms of Gnrh (Kah et al., 2007; Zohar et al., 2010; Choi, 2018). Interestingly, three isoforms of Gnrh were found in the superclass Agnatha and also recently across the non-teleost, cartilaginous fish (Gaillard et al., 2018).

For many years, the multiple GnRH variants were named after the species in which they were first discovered, despite the fact that they can be present in other species. With the increasing number of GnRH variants discovered, and in order to standardize GnRH nomenclature and avoid confusion, a new classification of the GnRH forms based on the phylogenetic analysis of known sequences and their respective sites of expression was proposed (Fernald and White, 1999; Kah et al., 2007; Zohar et al., 2010). Early phylogenetic analysis from diverse vertebrate species segregates the GnRHs into three branches termed type 1, type 2, and type 3, regardless of the actual number of GnRH isoforms in a species (White et al., 1998; Fernald and White, 1999). The GnRH type 1 branch includes cDNAs encoding for variable species/family-specific peptides that are expressed in the hypophysiotropic preoptic region, which area now commonly termed GnRH1. All terrestrial vertebrates and teleosts with three GnRH isoforms possess this form. The type 2 branch includes sequences corresponding to the single peptide, cGnRH-II, which has now been termed GnRH2, found in most tetrapods and teleosts (White et al., 1998). The type 3 branch only contains teleost sequences, all generating the same fish-specific peptide, salmon Gnrh

(referred to now as GnRH3), which is always located in the olfactory bulbs/telencephalon nuclei (Sherwood et al., 1983) but may be located in other places as well.

3.2. Structure of the GnRHs

Molecular cloning of the cDNAs and genes encoding GnRHs from a variety of vertebrate species has revealed the overall organization to be well conserved among all forms. Each form of GnRH is encoded by its cognate gene as a precursor polypeptide termed prepro-GnRH. Prepro-GnRH consists of a signal peptide, the GnRH decapeptide followed by a proteolytic processing site (Gly-Lys-Arg), and another peptide referred to as GnRH-associated peptide (GAP) (Figure 1) (Seeburg and Adelman, 1984). Each *GnRH* gene comprises four exons (denoted exons 1, 2, 3 and 4) and three introns. Exon 1 encodes the 5'-untranslated region (5'-UTR). Exon 2 encodes the N-terminus of the prepro-GnRH polypeptide, including a signal peptide, the GnRH decapeptide, a Gly-Lys-Arg site, and the N-terminus of GAP. Exon 3 encodes the middle portion of GAP, whereas exon 4 encodes the C-terminus of GAP and the 3'-UTR (Seeburg and Adelman, 1984). The physiological function of GAP is still unclear; human GAP peptide had an inhibitory effect on the secretion of prolactin (Nikolics et al. 1985; Yu et al., 1988a), whereas sheep GAP did not (Thomas et al., 1988). Given the high degree of sequence divergence in GAP peptide, it was assigned no conserved function. However, despite low amino acid sequence conservation, recent prediction of the 3D structure revealed a striking conservation in all species amongst GAP1 (GAP associated with the hypophysiotropic type 1 GnRH), and GAP2 in some vertebrates, of a typical helix-loop-helix (HLH). Interestingly, none of the GAP3 analyzed featured this HLH structure (Pérez Sirkin et al., 2017). Because 3D structure often conveys biological activity regardless of the amino acid sequence, the authors propose that the conservation of the HLH motif, known to confer biological activity to various proteins, grants GAP1 peptides

possible hypophysiotropic biological functions across vertebrates. It remains to be revealed what biological activity is carried out by GAP1 and GAP2 HLHs.

Of the three GnRH isoforms, only GnRH1 decapeptide exhibits species-specific structural diversification. The N-terminal begins with aromatized glutamine (pyroglutamate, pGlu), and the C-terminal glycine is amidated. For example, the amino acid sequence of human GNRH1 is pGlu-His-Trp-Ser-Tyr-GlyLeu-Arg-Pro-Gly-NH₂ (QHWSYGLRPG in one letter code) and seabream GnRH (sbGnRH1), which is found in most perciforms, is QHWSYGLSPG. The N- and C-termini of the amino acid sequence of GnRH1 are fairly conserved at positions 1-4, 9, and 10. N-terminals generally consist of pGlu-His-Trp-Ser and C-terminals are fixed to Pro-Gly-NH₂. Position 8 is the most variable amino acid, followed by positions 6, 5 and 7. The N- and C-termini are key for receptor binding and biological activity (Millar et al., 2004; Schally et al., 2017). Specifically, pGlu, His and Trp at the N-terminal are essential to the physiological activity of GnRH1 and the arginine residue at position 8 strengthens receptor binding (Flanagan and Manilall, 2017). Glycine at position 6 serves as a hinge allowing the folding and formation of a β II type compacted conformation, and thus more efficient and tighter binding to the receptor (Millar, 2005).

3.3. Evolution of GnRH isoforms

Genome synteny and phylogenetic analyses in a variety of vertebrate species have provided insights into the evolutionary origin of and relationship between paralogous *GnRH* genes.

The classification of the GnRH isoforms into three groups, i.e. 1, 2 and 3, is supported by syntenic analyses of the different GnRH isoforms (Okubo and Nagahama, 2008). For example, the *GnRH1* genes of both medaka and human lie adjacent to a gene for a potassium channel tetramerization domain containing 9 (*KCTD9*) and *GnRH2* genes are adjacent to a

264 gene for protein tyrosine phosphatase A (*PTPRA*) (Okubo et al. 2002). The syntenies in the
 265 *GnRH* gene loci are also preserved in several other vertebrate species, including
 266 chimpanzees, mice, chickens, the pufferfish *Tetraodon nigroviridis* and *Takifugu rubripes*,
 267 zebrafish, lamprey and sharks (Morgan and Millar, 2004; Kuo et al., 2005; Ikemoto and Park,
 268 2006; Tostivint, 2011; Decatur et al. 2013; Gaillard et al., 2018). It is now suggested that
 269 *GnRH1*, *GnRH2* and *GnRH3* genes represent three paralogous genes resulting from the two
 270 rounds (2R) of tetraploidization that took place early in vertebrate evolution, followed by two
 271 separate deletion events (Figure 2) (Kim et al. 2011; Tostivint, 2011; Roch et al., 2014).
 272 According to this hypothesis, the common ancestor of all extant jawed vertebrates already
 273 possessed these three *GnRH* genes, e.g. the two wide genome duplication events (both 1R
 274 and 2R) that generated the different fish and tetrapod paralogs took place before the
 275 divergence of the ancestral jawless vertebrates (agnathans) and jawed vertebrates
 276 (gnathostome, e.g. cartilaginous and teleosts) lineages (Decatur et al., 2013; Roch et al.,
 277 2014). The fourth *GnRH* gene, generated in the 2R has been lost soon after, and later, the
 278 teleost specific *gnrh3* gene has most likely been deleted during the early development of the
 279 tetrapod lineage and thus is not a result of the teleost specific 3R (Figure 2) (Okubo and
 280 Nagahama, 2008; Tostivint, 2011; Kim et al., 2012). Despite early suggestions that lamprey
 281 *Gnrh-I* and *-III* emerged from type 4 *Gnrh* (Silver et al., 2004; Kavanaugh et al., 2008),
 282 syntenic analysis demonstrated that *lGnrh-II* groups with type 2 *GnRH* and *lGnrh-I* and *-III*
 283 share a common ancestry with type 3, and hence are no longer considered type 4 *GnRH*s
 284 (Figure 2) (Decatur et al., 2013; Roch et al., 2014). Recent phylogenetic analysis of 73 fish
 285 species clusters the newly discovered three cartilaginous *Gnrhs* within this general scheme
 286 (Figure 2) (Gaillard et al., 2018), and further supports the proposed agnathans orthology
 287 (Gwee et al., 2009; Decatur et al., 2013). A teleost-specific third genome-wide duplication
 288 likely occurred as well, which was followed by selective immediate loss due to 50-80%

genome deletion, as suggested by Lynch and Force (Figure 2) (Lynch and Force, 2000). For example, in goldfish, zebrafish and salmon, the type 1 *gnrh* gene is lost, whereas in catfish or eel, type 3 *gnrh* is missing. In some cases, no loss occurred and two copies of the type 1 *gnrh* gene (catfish and elephant shark; Roch et al., 2014; Gaillard et al., 2018) or type 3 *gnrh* gene (salmon, trout) are found (Kah et al., 2007). With regard to *Gnrh2*, most species possess only a single *gnrh2* gene, however cyprinids and salmonids display two orthologues.

3.4. Localization of *Gnrh* neurons in the brain

Early studies describing the neuroanatomical distribution and organization of the *Gnrh* system in fish mainly utilized histological techniques like immunohistochemistry and *in situ* hybridization. These studies provided great detail on the different neuronal populations, subsets and projections (Kah et al., 1986, 2007). As indicated above, later studies utilizing gene transfer technologies to generate transgenic fish expressing fluorescent proteins driven by *gnrh* promoters in zebrafish, medaka and tilapia, corroborated these findings. Moreover, they provided additional valuable information on the early development of the *Gnrh* system and its regulation (Palevitch et al., 2007; Abraham et al., 2008; Takahashi et al., 2016), elaborate and precise details of neuronal innervations (Golan et al., 2015, 2016), synapse connections (Ma et al., 2015), and network neuronal interactions (Spicer et al., 2017; Marvel et al., 2018). Importantly, these transgenic fish allowed the tracking of neuronal paths and real time plasticity, as well as electrophysiological activities, in live animals (Kargo and Oka 2013; Ma et al., 2015).

The distribution of neurons producing non-distinguishable forms of *Gnrh* was initially reported in goldfish (Kah et al., 1986) and catfish (Subhedar and Krishna, 1988), showing immunoreactive cells within the olfactory nerves and bulbs, along the ventromedial telencephalon, the ventrolateral preoptic area and the latero-basal hypothalamus, as well as

large perikarya present in the dorsal midbrain tegmentum. A decade later, the availability of specific riboprobes in the gilthead seabream, following the discovery of seabream *Gnrh1* in this species (Powell et al., 1994), enabled the revelation, for the first time in fish, of the brain distribution of three *Gnrhs* in discrete sites of expression, i.e., *gnrh1* neurons in the preoptic area, *gnrh2*-producing cells in the midbrain tegmentum and *gnrh3*-expressing neurons in the ventromedial olfactory bulbs and the terminal nerve (Gothilf et al., 1996). This basic scheme was later elucidated in other teleosts including the European sea bass (González-Martínez et al., 2001, 2002a) and many more (Zohar et al., 2010), in which *Gnrh2* soma were localized exclusively in the dorsal synencephalon/midbrain tegmentum, while *Gnrh1* and *Gnrh3* perikarya overlapped in the olfactory bulbs/terminal nerve (OB/TN), ventral telencephalon (vTEL) and preoptic area (POA) (Figure 3B). Interestingly, *Gnrh* neurons in fish species that possess only two *Gnrh* paralogs share the same distribution as in three-isoform species (Figure 3A). Following the loss of a paralog, the remaining isoform is expressed in the neurons of the lost form, and takes over its function in what is termed “functional compensation” (Karigo and Oka, 2013). For example, *gnrh1* gene is lost in the zebrafish, but the hypophysiotropic function is performed by the *gnrh3*-expressing GnRH neurons in the POA (Steven et al., 2003; Abraham et al., 2010). Conversely, eels and catfish appear to have lost the *gnrh3* gene, but *gnrh1*-expressing neurons found in the OB/TN seem to compensate for the function of the *Gnrh3* system (King et al. 1990; Ngamvongchon et al. 1992; Montero et al., 1994; Zandbergen et al. 1995).

Preoptic *Gnrh* neurons directly innervate and penetrate the pituitary and *Gnrh* is released by nerve endings located in close proximity to their target cells, the gonadotropes (Ball, 1981; Kah et al. 2007; Golan et al., 2015). In some species, true *Gnrh* synaptic-like contacts appear to be present on gonadotropes. A recent work using transgenic zebrafish expressing multiple variable fluorescent proteins in Lh, Fsh and *Gnrh3* cells, as well as in blood vessel

cells has demonstrated that the majority of Lh cells are not in direct contact with GnRH axon terminals but rather GnRH peptide reaches these cells through an intricate vasculature network populating the entire pituitary (Golan et al., 2015).

3.5. Presence of GnRHs in the pituitary, hypophysiotropic roles and functional significance

Because GnRH neurons are innervating the pituitary directly, the presence of the GnRH peptides in the pituitary conveys a strong indication of their hypophysiotropic function. In fish with two GnRH forms, one GnRH variant (GnRH3 in zebrafish, cyprinids and salmonids and GnRH1 in catfish and eel), which is expressed both in the OB/TN and POA, is the more abundant form in the pituitary and its levels correlate with gonadal development (see for review Goos et al., 1997; Amano et al., 1997; Kah et al., 2007; Zohar et al., 2010; Choi, 2018). Nevertheless, evidence obtained in several species suggests that OB/TN cells rarely project to the pituitary (Umatani and Oka, 2019).

In fish with three GnRH forms, the situation seems to be more diverse. In the gilthead seabream (*Sparus aurata*), GnRH1 and GnRH2 are present in the pituitary throughout the gonadal cycle but GnRH3 levels remained below the detection level of the assay (Holland et al., 1998). However, while pituitary levels of GnRH1 and GnRH2 were similar in sexually immature fish, only the levels of GnRH1 increased significantly in sexually mature fish and in correlation with gonadal development and the rise of pituitary and plasma LH levels (Holland et al., 1998). Similarly, in the turbot (*Scophthalmus maximus*), GnRH1 levels were markedly higher in the pituitary and increased in correlation with the increase in oocyte diameter (Anderson et al., 2001). This data strongly indicated that, in these two species, GnRH1 is the most relevant form for the control of LH release and reproduction. A more detailed study through development of the striped bass (*Morone saxatilis*), from juvenile to adulthood, demonstrated that like in the gilthead seabream, GnRH1 was about 10 times more abundant

than Gnrh2 in the pituitary of sexually maturing fish (Holland et al., 2001). However, both forms displayed a similar spatio-temporal pattern of increase during gonadal recrudescence, peaking prior to (Gnrh2) or during (Gnrh1) the spawning season in correlation with changes in gonadosomatic index (GSI), oocyte diameter, Lh pituitary content (Holland et al., 2001) and pituitary levels of the Gnrh receptor (Alok et al., 2000). Moreover, in the closely related European sea bass (*Dicentrarchus labrax*), all three forms of Gnrh were detected in the pituitary by ELISA and increased during sexual maturation, although Gnrh1 was much more abundant than the other two forms (Rodríguez et al., 2000). However, an immunohistochemical study performed in the same species using specific antibodies against the most divergent Gnrh-associated peptides (anti-Gap1, 2 and 3; Figure 1) revealed that only Gap-Gnrh1 and Gap-Gnrh3 fibers are present in the sea bass pituitary, where the innervations of Gnrh1 are much more abundant than that of Gnrh3 (González-Martínez et al., 2002a).

Unlike the POA Gnrh isoform that is consistently found in the pituitaries, the relative content and presence of the OB/TN and midbrain isoforms varies between species. In addition to the three-Gnrh species described above, in fish with two Gnrh isoforms, Gnrh2 is found in the pituitary of catfish (Ngamvongchon et al., 1992; Schulz et al., 1993), cyprinids such as goldfish and zebrafish (Yu et al., 1988b; Steven et al., 2003), and in the pituitary of salmonids (Okuzawa et al., 1990; Amano et al., 1991).

Temporal expression profiles of the three Gnrh variants during the reproductive cycle corroborated the dominant relevance of the POA Gnrh isoform to reproduction. However, they also demonstrated species-specific patterns. In the gilthead seabream (*Sparus aurata*), a species undergoing daily cycles of final oocyte maturation, ovulation and spawning, mRNA levels of all three *gnrh* forms peaked at 8 hours prior to spawning, concomitant with the pre-ovulatory Lh surge (Gothilf et al., 1997). In the closely related red seabream (*Pagrus major*)

however, *gnrh1* and *gnrh2* mRNA levels gradually increased during gonadal development before peaking at spawning time, while *gnrh3* remained unchanged (Okuzawa et al., 2003). In the grass puffer (*Takifugu niphobles*), *gnrh1* and *gnrh3* mRNA levels increased in the spawning season with no change in those of *gnrh2* (Shahjahan et al., 2017). An interesting situation was described in the cod (*Gadus morhua*), a species with three Gnrh forms, where the *gnrh1* gene was suggested to be a pseudogene and *gnrh3* was demonstrated to take over the reproduction-related hypophysiotropic role (Hildahl et al., 2011, 2013). Altogether, these studies strongly indicate the reproductive relevance of the POA Gnrh form, while also suggesting a possible reproductive-relevance (that is not yet fully understood) and a role for the non-POA forms as well.

Supporting this relevance, recent studies in zebrafish, a two-Gnrh model species exhibiting two Gnrh variants into the brain (i.e., Gnrh2 and Gnrh3), with a dominant pituitary presence of the hypophysiotropic GnRH3 (Stevens et al., 2003), showed that under fasting conditions, the POA Gnrh3 disappeared from the pituitary, while the levels of Gnrh2 increased (Marvel et al. 2019; Xia et al., 2014). These results indicate that the non-POA Gnrh forms may serve as a “backup” system to ensure the integrity of reproduction under suboptimal or other specific physiological conditions. Additional, non-hypophysiotropic potential roles of the OB/TN Gnrh form are further discussed below.

However, the dominant presence of the POA Gnrh form in the pituitary and its correlation to changes in the endocrine-reproductive axis, are not always reflected in its gonadotropin-releasing potency when tested *in vivo* or *in vitro*. In the two- Gnrh goldfish model, Gnrh2 elicited a more robust Lh secretion compared to the POA’s Gnrh3 in sexually mature, pre-spawning fish, while in sexually regressed animals, Gnrh3 had potent Lh-releasing activity and Gnrh2 had no effect (Khakoo et al. 1994; Murthy and Peter, 1994). Soon after three Gnrh variants were discovered in the perciform gilthead seabream (Powell et

al., 1994), it was demonstrated that all three GnRHs were potent stimulators of Lh release *in vivo* (Zohar et al., 1995). However, GnRH2 was found to be the most potent Lh releaser while the hypophysiotropic form (GnRH1) was the least potent (Zohar et al., 1995). This unexpected result may be explained by the fact that GnRH2 has the highest affinity to the pituitary GnRH receptor (Alok et al., 2001), indicating that the physiological *in situ* gonadotropin-releasing activity of the GnRH1 in perciform fish, such as seabream and striped bass, is determined by its dominant presence in the pituitary when it is needed. Interestingly, a similar situation was described in the catfish, a fish with 2 GnRH forms (Schultz et al., 1993), where GnRH2, which is barely present in the pituitary, has a much higher Lh-releasing potency (100-fold) and receptor affinity than the POA GnRH form (GnRH1), the dominant form in the pituitary (Schultz et al., 1993; Goos et al., 1997). The same group also concluded that the GnRHs in the African catfish can, in addition to their direct stimulatory action on Lh release, modulate each other's Lh releasing activity. This mutual modulation can be both stimulatory and/or inhibitory, the final result depending on the relative concentrations of the two GnRH forms (Bosma et al., 2000). These results collectively demonstrate that the relative functional/reproductive significance and effects of GnRH on gonadotropin expression and release depend upon the species, sex and reproductive status of the fish, as well as complex endocrine interactions along the brain-pituitary axis (Zohar et al., 2010).

More recently, additional conclusive evidence of the hypophysiotropic action of the POA GnRH1 came from studies in the medaka. The use of calcium imaging in GnRH1 transgenic medaka demonstrated GnRH1 peptide release from the nerve terminals upon high frequency neuronal firing which, in turn, resulted in a direct activation of Lh cells (Hasebe and Oka, 2017). Using on-cell patch-clamps of GnRH1 neurons in transgenic medaka, *Tg(gnrh1:eGFP)*, Karigo et al. were able to compile a daily timeline of events preceding spawning and describe how increased spontaneous GnRH1 neuronal firing in the afternoon results in the release of

Gnrh and the immediate secretion of Lh surge needed for nocturnal ovulation and, at the same time, conditions the nighttime ('lights-off') elevated expression of *lh* in the pituitary to replenish Lh levels for ovulation the next day (Karigo et al., 2012).

3.6. Possible additional roles of non-POA Gnrh isoforms

Gnrh2 is synthesized in the midbrain tegmentum near the third ventricle. Gnrh2 neurons project throughout the brain, but are primarily confined to the midbrain and hindbrain with their axon terminals in the third ventricle (Amano et al., 1997; Kah et al., 2007). Using eGFP-labeled Gnrh2 neurons in transgenic zebrafish, extensive Gnrh2 projections were described throughout the brain, with massive extensions into and along the spinal cord and a few projections in the pituitary (Xia et al., 2014). Together with the report of the anorexigenic action of Gnrh2 (Nishiguchi et al., 2012), these findings led to a recent study in zebrafish that implicated Gnrh2 in the control of both reproduction (mainly egg quality) and feeding, suggesting a role for Gnrh2 in mediating these two processes (Marvel et al., 2018, 2019). In addition, Gnrh2 neurons were observed innervating the pineal gland (Figure 3) in the European sea bass (Servili et al., 2010) and in zebrafish (Marvel et al., in preparation). Servili et al. have also shown the presence of pinealofugal projections in the midbrain tegmentum of the European sea bass, where Gnrh2 cells were previously reported (Servili et al., 2010, 2011), reinforcing the idea that connections between the pineal organ and Gnrh2 cells could be bidirectional. This body of evidence suggests a possible regulation of circadian activities and photosensory transduction functions by Gnrh2.

Interestingly, with the advancement of imaging technologies, using the same transgenic fish, Gnrh2 neuronal soma were found alongside, and sometimes co-localized with, Gnrh3 neurons in the nasal placode and olfactory bulbs (Marvel et al., unpublished). The exact role of Gnrh2 neurons in these regions is not yet known. It is very possible though that Gnrh2,

like the other GnRH isoforms in the olfactory region, plays a role in social and pheromonal cue perception (Forlano and Bass, 2011; Li, et al., 2017).

GnRH3 neurons in three- GnRH species or a subset of GnRH1 neurons in two- GnRH species are localized in the OB/TN and their projections are distributed throughout the whole brain (Chiba et al., 1996). Recent studies indicate that the OB/TN- GnRH3 system is most likely engaged in several neuromodulatory actions throughout the brain. Measurements of single neuron firing activity demonstrated that social cues, comprised of chemosensory and visual cues from conspecific medaka, alter electrical activity of the OB/TN- GnRH3 neurons (Ramakrishnan and Wayne, 2009). Indeed, OB/TN- GnRH3 neurons receive strong afferent somatosensory and visual synaptic inputs from a tegmental nucleus, as well as olfaction information from the olfactory bulb and some telencephalic areas (Yamamoto and Ito, 2000). In zebrafish, activation of the terminal nerve in the olfactory bulb increases GnRH signaling in the brain and triggers mating-like behaviors in males (Li et al., 2017), more notably in the final stages of oocyte maturation (Corchuelo et al., 2017). GnRH3 in the OB/TN region was also implicated in the homing migration to spawning grounds in salmon, as gene expression and peptide levels of GnRH3 increased in the adult salmon brain during this period (Onuma et al., 2005; Onuma et al., 2010; Ueda, 2011; Ueda et al., 2016). Recently, a co-localization of GnRH 3 and GnIH peptides in the same OB/TN neurons has been found in *Cichlasoma dimerus*, pejerrey and seabass (Di Yorio et al., 2019a, b). Interestingly, modulatory effects of GnIH on *gnrh3* expression and/or GnRH3-stimulated LH secretion have been reported in goldfish (Moussavi et al., 2013, 2014; Qi et al., 2013), orange-spotted grouper (Wang et al., 2015), zebrafish (Spicer et al., 2017) and Senegalese sole (Aliaga-Guerrero et al., 2018). These evidences reinforce the existence of GnRH3-GnIH interactions in fish, although the inhibitory or stimulatory nature of these actions seem to be dependent on the species, sexual stage, route of administration and sampling times after treatment.

4. The ontogenic development of fish GnRHs

In the late twentieth and early twenty-first centuries, there was controversy concerning the origins of the forebrain GnRH system in fish, particularly in those species expressing three *gnrh* forms. Based on the fact that the three GnRH forms appeared to exhibit different patterns of expression, the first ontogenetic studies in fish suggested that GnRH3 (TN/OB GnRH cells) and GnRH1 (POA GnRH cells) neurons had different embryonic origins (Northcutt and Muske, 1994; Parhar et al., 1997, 1998; Pandolfi et al., 2002; Amano et al., 2004). However, it is now generally accepted that GnRH neurons located in the TN/OB and POA originate peripherally in the olfactory placode and subsequently migrate during early development to their final positions (González-Martínez et al., 2002b, 2004a; Okubo et al., 2006; Palevitch et al., 2007; Okubo and Nagahama, 2008; Takahashi et al., 2016). Pioneering immunohistochemical and *in situ* hybridization (ISH) ontogenetic studies of the three GnRH populations performed in sea bass using specific antibodies and riboprobes against GnRH-associated peptide (GAPs) showed that both GnRH1 and GnRH3 neurons were first detected in the olfactory region, and then moved caudally through the ventral telencephalon and preoptic area (González-Martínez et al., 2002b, 2004a), with GnRH3 cells appearing much earlier than GnRH1 neurons (Figure 4). The same pattern of origin and migration along the telencephalon and hypothalamus was previously reported in other vertebrates (Schwanzel-Fukuda, 1990; Muske and Moore, 1994; Wiren et al., 2004). Using transgenic medaka (*Oryzias latipes*) expressing eGFP driven by *gnrh1* and *gnrh3* promoters, Okubo and colleagues corroborated these observations and provided strong evidence supporting the premise that both GnRH1 and GnRH3 cell populations emerge from the olfactory region (Okubo et al., 2006). New transgenic lines recently developed in medaka showed the presence of two early GnRH1 neuronal populations in the ventral POA with hypophysiotropic and retinopetal projections, respectively, suggesting that GnRH1 plays a significant role in the development and maturation of the gonadotrope cells, as

well as in the modulation of visual/photosensory functions, from early developmental stages (Takahashi, et al., 2016). Furthermore, three *Gnrh3* neuronal populations were also identified in medaka during early development in the terminal nerve (TN), thalamic area and bigeminal ganglion (TG) with different origins and migratory pathways (Takahashi, et al., 2016).

In mammalian species, compromised gonadal development and impaired fertility is evoked by abnormal GNRH neuronal migration (MacColl et al., 2002). CXCL12A has been involved in guiding the accurate migration of GNRH neurons and axonal direction in the developing nervous system (Toba et al., 2008; Tiveron and Cremer, 2008). In this sense, Palevitch and collaborators studied the involvement of the chemokine receptor *Cxcr4b* and its cognate ligand *Cxcl12a* in the neural migration of *Gnrh3* in zebrafish (Palevitch et al., 2010). In this study, the authors found that *Cxcr4b* and *Cxcl12a* were expressed along the *Gnrh3* migration pathway. Moreover, knockdown of *Cxcr4b* or *Cxcl12a* in *Tg(gnrh3:egfp)* zebrafish provoked an abnormal pattern of *Gnrh3* axonal projections and cell migration, implicating *Cxcl12a*-*Cxcr4b* ligand-receptor pair in the regulation of *Gnrh3* neuronal development and migration in zebrafish (Palevitch et al., 2010). Similarly, in an attempt to understand when and how newborn *Gnrh* neurons are distributed to form a functional network in the developing brain, Onuma and coworkers used zebrafish to study whether insulin-like growth factor (Igf) signaling plays a role in *Gnrh* neuronal development during embryogenesis (Onuma et al., 2011). Depending on the developmental stage, the disruption of Igf signaling provoked a delay in the timing of *Gnrh2* and *Gnrh3* emergence in the midbrain tegmentum and olfactory bulbs, respectively, as well as the appearance of ectopic *Gnrh3* cells outside the OB/TN region (Onuma et al., 2011). Immunoglobulin superfamily member 10 (*Igsf10*) has also been involved in the early migration of *Gnrh3* neurons to the hypothalamus, as shown in knockdown experiments that impaired the migration of immature *Gnrh* neurons *in vitro*, and perturbed migration and extension of *Gnrh* neurons in a *gnrh3:egfp* zebrafish model (Howard

et al., 2016). This Igsf10-mediated dysregulation of GnRH neuronal migration leads to hypogonadotropic hypogonadism as well as delayed puberty (Howard et al., 2016).

According to studies in most vertebrates, the origin and structure of GnRH2 are highly conserved, as well as its location in the synencephalic/mesencephalic area (Roch et al., 2011). GnRH2 neurons arise in a midbrain germinative primordium and are the earliest detectable GnRH cell population in the brain of sea bass (Figure 4) (González-Martínez et al., 2002b, 2004a), barfin flounder (Amano et al., 2004) and zebrafish (Xia et al., 2014). The ontogenic pattern characterized by the increasing number of GnRH2 cells during development and the significant decrease at adulthood represents a common feature in numerous fish species analyzed, suggesting an important role of this neuropeptide at early stages (Parhar et al., 1998; White and Fernand 1998; González-Martínez et al., 2002b, 2004a). The analysis of the distribution of GnRH2 at different developmental stages has revealed an intricate projection pattern of this neurohormone system within the fish brain (González-Martínez et al., 2004a). Immunohistochemical studies performed in developing sea bass identified GnRH2-immunopositive fibers throughout the entire brain, e.g. in almost all forebrain areas (olfactory bulbs, dorsal and ventral telencephalon, periventricular preoptic area and hypothalamus, thalamus, pretectum, posterior tuberculum) and, particularly, in the midbrain (mesencephalic tectum and tegmentum) and hindbrain (granular layer of the valvula, corpus and vestibulolateral lobe of the cerebellum and rhombencephalon) from 26 days after hatching onwards (González-Martínez et al., 2002b, 2004a). However, in this species, GnRH2 fibers were not detected in the pituitary of developing or adult animals (Figure 4) (González-Martínez et al., 2002a, 2004a). As indicated above, a detailed study of GnRH2 using the *Tg(gnrh2:egfp)* zebrafish line showed that GnRH2 fibers are widely distributed in the central nervous system from early stages, originating in tegmental cells and extending posteriorly up

to the caudal spinal cord and anteriorly up to the telencephalic hemispheres and olfactory bulbs, with only a few axons reaching the pituitary (Xia et al., 2014).

5. The GnRH receptors

5.1. Structure and types of GnRH receptors

The action of GnRHs on target cells is mediated by binding to specific membrane GnRH receptors (GnRHRs). Corresponding to the major function of GnRH in the control of reproduction, GnRHRs are mainly localized in the brain to mediate the GnRH neuromodulatory actions on other neuronal systems, and in the pituitary gonadotropic cells to regulate gonadotropin secretion. In addition, GnRHRs, like GnRHs, are found in the gonads and other peripheral tissues, exerting multiple physiological actions in a wide variety of targets (Lethimonier et al., 2004).

The first GNRHR was identified in mammals in the early 1990's (mouse, Tsutsumi et al., 1992) and a few years later the first fish Gnrhrs were described in the gilthead seabream (Pagelson and Zohar, 1992) and African catfish (Tensen et al., 1997). Since then, Gnrhrs have been characterized in many fish species (Lethimonier et al., 2004; Weltzien et al., 2004; Levavi-Sivan and Avitan, 2005; Kah et al., 2007), including the goldfish (Illing et al., 1999), Japanese eel (Okubo et al., 2000), rainbow trout (Madigou et al., 2000), striped bass (Alok et al., 2000), medaka (Okubo et al., 2001), salmon (Jodo et al., 2003), sea bass (González-Martínez et al., 2004b; Moncaut et al., 2005), fugu (Moncaut et al., 2005), spotted green pufferfish (Ikemoto and Park, 2005), cobia (Mohamed et al., 2007), zebrafish (Tello et al., 2008), Atlantic cod (Hildahl et al., 2011a,b), European eel (Peñaranda et al., 2013), Japanese grenadier anchovy (Duan et al., 2016), mackerel (Lumayno et al., 2017) and ricefield eel (Chen et al., 2018). However, the available knowledge on the distribution of Gnrhrs in cells and tissues, regulatory mechanisms and biological functions is still limited in fish.

The GnRHRs belong to the rhodopsin family of G protein-coupled receptors (GPCRs) (Sealfon et al., 1997). Structurally, GnRHRs share a common overall structure, typical of the GPCRs, with an N-terminal extracellular region, the characteristic hydrophobic seven transmembrane domains (TMD) connected by intra- and extracellular hydrophilic loops, and a cytoplasmic C-tail (Millar et al. 2004). The N-terminal extracellular region is important for receptor ligand selectivity, whereas the C-tail, present in non-mammalian but absent in mammalian GnRHRs, is important for the expression and regulation of the receptor, susceptibility to desensitization and subsequent internalization (Tensen et al., 1997; Wang et al., 2001; Pawson et al., 2003).

Similar to GnRHs, multiple forms of GnRHRs have been identified in vertebrates. One or two GnRHRs have been identified in mammals, whereas up to five GnRHs have been recognized in fish species (Hapgood et al., 2005; Ikemoto and Park, 2005; Moncaut et al., 2005). Based on structural characteristics and phylogenetic analysis, it is now accepted to group GnRHRs into two types: type I grouping denotes the receptors lacking an intracellular C-tail and type II signifies those possessing the C-terminal tails (Sefideh et al., 2014; Roch et al., 2014; Flanagan and Manilall, 2017; Chang and Pemberton, 2018). Type I GnRHRs are present in mammalian and lower vertebrates, including coelacanth and some cartilaginous fishes, but appear to have been lost in the ray-finned fish, amphibian, reptile and bird lineages (Roch et al., 2014). Type II GnRHRs are further divided in two subtypes, GnRHR-IIA (consisting of IIA-1, IIA-2 and IIA-3 forms) and GnRHR-IIB, and are present in some mammals (although absent or non-functional in others like human and rat) as well as in non-mammalian tetrapods and fish (Hildahl et al., 2011a, b; Roch et al., 2014; Williams et al., 2014). Several studies in fish have suggested that the action of GnRH on reproduction, and particularly on gonadotropin release, is mediated by the activation of one type of GnRH-IIa, while activation of GnRH-IIb would mediate neuromodulatory GnRH actions on systems related

to locomotion, sensory perception and metabolism (Soga et al., 2005; González-Martínez et al., 2004b; Servili et al., 2010; Lumayno et al., 2017).

5.2. Ligand selectivity of GnRH receptors

In fish, *in vitro* studies have revealed that different GnRHs can activate the GnRHs, with a clear preference for GnRH2, followed by GnRH3 and GnRH1 (Figure 5). However, each receptor subtype displays different relative potencies of the different GnRH ligands (Okubo et al., 2001; Lethimonier et al., 2004; Flanagan et al., 2007; Servili et al., 2010). The GnRHr-II cloned in chub mackerel, *Scomber japonicus*, which was implicated in reproduction and, specifically, in the regulation of puberty, is activated by all three GnRHs to stimulate LH release from cultured pituitary cells *in vitro* (Lumayno et al., 2017). As indicated above, five type II GnRHs have been identified in the European sea bass (González-Martínez et al., 2004b; Moncaut et al., 2005). One of these GnRHr-II subtypes (*gnrhrr-II-1a*) was highly expressed in the pituitary gonadotropes (notably in LH cells) and exhibited marked variation in expression in the brain and pituitary along the reproductive cycle (González-Martínez et al., 2004b). Although the potency of GnRH2 and GnRH3 in the activation of GnRHr-II-1a was higher, this receptor was the only one sensitive to the hypophysiotropic GnRH1 in sea bass (Figure 5A) (Servili et al., 2010), suggesting its relevance to the mediation of GnRH effects on reproduction. In turn, another type II GnRHr (i.e., *gnrhrr-II-2b*) was strongly expressed in the sea bass pineal gland and exhibited a clear selectivity for GnRH2 (Figure 5B), reinforcing the suggested role of this receptor in sensory functions (Servili et al., 2010). Binding studies of the GnRHr have also been extensively performed in goldfish using perfused fragments of pituitary *in vitro*. These studies showed that both GnRH2 and GnRH3 activated the goldfish GnRHs to stimulate LH and GH release, but their potencies were very different, i.e., GnRH2 was somewhat more active than GnRH3 in eliciting the release of gonadotropins, whereas GnRH3 appeared to have a greater potency than

Gnrh2 in stimulating Gh release (Habibi et al., 1987, 1989, 1990, 1992; Cook et al., 1991), suggesting that different Gnhrhs are present in gonadotropes and somatotropes. In zebrafish, both Gnhrh1 and Gnhrh3 (Gnhrh-IIb subtypes) showed a greater preference for Gnrh2 compared with Gnrh3, whereas Gnhrh2 and Gnhrh4 (Gnrh-IIA subtypes) showed equal potency with both native ligands and represented the only Gnhrhs that showed a distinct response to the non-native Gnrh1 (Tello et al., 2008).

5.3. Intracellular signal transduction pathways

The intracellular signal transduction pathways associated with Gnhrh activation have been recently reviewed by Chang and Pemberton (2018). Studies in several species have demonstrated that binding of Gnrh to the Gnhrhs probably activates multiple signaling pathways, such as protein kinase A (Pka), protein kinase C (Pkc) and mitogen-activated protein kinase (Mapk) (Kraus et al., 2001; Naor, 2009; Naor et al., 1998; Naor and Huhtaniemi, 2013; Gopurappilly et al., 2013; Chang and Pemberton, 2018).

The elucidation of Gnrh-stimulated signal transduction pathways in fish pituitary has been derived primarily from studies in goldfish and, to a lesser extent several other species (i.e., tilapia, African catfish, medaka, zebrafish), but was mainly focused on the regulation of Lh release because the availability of Fsh detection assays was restricted to only a few species (Chang et al., 2000, 2009; Chang and Pemberton, 2018). In goldfish, pituitary Lh release is induced by the two endogenous Gnrrhs (Gnrh2 and Gnrh3) via Ca^{++} - and Pkc-dependent signaling, which also involves $\text{Na}^{+}\text{-H}^{+}$ exchange (Chang et al., 2000, 2009; Chang and Pemberton, 2018). Likewise, Pi3k and Mek-Erk signaling participate in Gnrh2 and Gnrh3 stimulation of Lh release in goldfish pituitary cells (Pemberton et al., 2013, 2015). Results obtained with dispersed Atlantic cod pituitary cell cultures suggest that, as has been shown for Lh release, Gnrh-induced Ca^{++} signaling may be important for Fsh release (Hodne et al., 2013).

Nevertheless, differences in the expression of Ca^{++} -activated K^+ channels may be involved in seasonal regulation of hormone secretion as well as the differential response to GnRh in Lh- and Fsh-producing gonadotropes. As reported for gonadotropins, GnRh1 and GnRh3 regulation of Gh secretion in fish pituitary somatotropes also implies involvement of the Plc/IP3/Pkc and Ca^{++} pathways, but does not seem to involve the cAMP-Pka pathway (Chang et al., 1993; Melamed et al., 1995; Levy et al., 2009; Chen et al., 2018).

Intracellular signal transduction pathways mediating GnRh effects on gonadotropin gene expression and synthesis have also been studied in fish (Chang and Pemberton, 2018). In tilapia pituitary cells, GnRh regulated *G-α* subunit expression through a Pkc-dependent Erk activation and stimulation of *lhβ* transcription concerned Pkc and/or Pka-dependent Erk pathways, whereas Pka-dependent Erk signaling was involved in *fshβ* transcription (Melamed et al., 1996; Gur et al., 2002). This regulation seems to differ between species, because Mek-Erk signaling, but not Pkc activation, is required for eliciting the GnRh2 and GnRh3 stimulation of transcription of all three gonadotropin subunits in goldfish pituitary cells (Klausen et al., 2005, 2008).

5.4. Localization of GnRh receptors

The expression and distribution of GnRhR isoforms has been studied by immunohistochemistry, RT-PCR and/or ISH in many fish species, including the goldfish (Illing et al., 1999), rainbow trout (Madigou et al., 2000), striped bass (Alok et al., 2000), African catfish (Bogerd et al., 2002), the European sea bass (González-Martínez et al., 2004b; Moncaut et al., 2005; Servili et al., 2010), tilapia *Oreochromis niloticus* (Soga et al., 2005; Parhar et al., 2005), the spotted green pufferfish (Ikemoto and Park, 2005), *Astatotilapia burtoni* (Chen and Fernald, 2006; Flanagan et al., 2007), zebrafish (Tello et al., 2008), pejerrey (Guilgur et al., 2009) and ricefield eel (Chen et al., 2018), among others. As has been indicated before, the nomenclature of GnRhRs remains inconsistent between studies, although all

receptors identified in fish appear to represent Gnhrh-II types (Roch et al., 2014). Localization studies have been commonly focused in the brain and the pituitary and have shown that the expression of different Gnhrh subtypes varies greatly between species, depending on the tissue and the reproductive status of the fish. In the pituitary, most Gnhrh types have mainly been detected in the proximal *pars distalis*, where the gonadotropic cells are located, as corresponds to the major function of Gnhrh in the regulation of Lh and Fsh secretion (Parhar et al., 2002; González-Martínez et al., 2004).

In situ hybridization studies in the cichlid *Astatotilapia burtoni* showed a wide distribution of *gnhrh2* (*gnhrh-IIb*) expression through the brain, while *gnhrh1* (*gnhrh-IIa*) expression was restricted to specific brain regions, such as the preoptic area, ventral hypothalamus, telencephalon and olfactory bulbs, as well as to the pituitary (Chen and Fernald, 2006). In tilapia, Gnhrh1 and Gnhrh3 (both classified as Gnhrh-IIa) immunoreactive cells were found in the forebrain and midbrain, and two different Gnhrhs were evident in Lh cells (Parhar et al., 2002; Soga et al., 2005). In the eel, *Anguilla anguilla*, expression of all 3 Gnhrhs was detected in the pituitary and all parts of the brain by PCR, one of them showing increased gene expression in pituitary during gonad maturation (Peñaranda et al., 2013). The four Gnhrhs identified in zebrafish were also profusely expressed in the brain and eye, as shown by PCR (Tello et al., 2008). In Atlantic cod brain (*Gadus morhua*), *gnhrh1b* (classified as type IIa receptor) and the *gnhrh2a*, *gnhrh2b* and *gnhrh2c* (classified as type IIb receptors) were all expressed in the pituitary and the brain, with most of them (except *gnhrh2a*) displaying highest levels in the brain compared to the pituitary (Hildahl et al., 2011b). In the European sea bass, *in situ* hybridization localized *gnhrh-II-1a* expression in the forebrain and the midbrain and, notably, in the pituitary (González-Martínez et al., 2004b). qPCR analysis showed that all five *gnhrhs* identified in the sea bass *Dicentrarchus labrax*, are differentially expressed in the brain (Servili et al., 2010, 2013). Daily variations in brain expression were only detected for the

gnrhr-II-1c and *gnrhr-II-2a* receptor subtypes, with higher diurnal transcript levels, and melatonin injection affected the expression of *gnrhr-II-1c*, *gnrhr-II-2a* and *gnrhr-II-2b* receptors (Servili et al., 2013). Whether these melatonin actions on Gnhrs are direct or indirect remain to be elucidated, although overlapping of brain areas and/or cell masses expressing *gnrhrs* and *melatonin receptors*/melatonin binding sites has been observed in sea bass (González-Martínez et al., 2004b; Bayarri et al., 2004; Herrera-Pérez et al., 2010). Additionally, as reported above, *gnrhr-II-2b* and *gnrhr-II-1a* were found to be expressed in the sea bass pineal gland, the former at higher levels than the latter (Servili et al., 2010), indicating not only variations in the expression patterns but also, as was suggested for Gnhr2, the possible involvement of the Gnhr system in the transduction of photoperiodic signals.

Spatial and temporal variations in the expression levels of different *gnrhr* subtypes may reflect a functional specialization of these receptors in mediating Gnhr actions (Robison et al., 2001). Several studies in fish have suggested that the Gnhr-IIa type represents the most likely hypophysiotropic receptor form. This is supported by binding studies demonstrating the potency of the hypophysiotropic Gnhr isoforms in the activation of Gnhr-IIa receptor subtypes, as well as by anatomical and physiological evidence (González-Martínez et al., 2004b; Flanagan et al., 2007). The Gnhr-IIa has been localized in the pituitary gonadotropic cells of *Astatotilapia burtoni* (Flanagan et al., 2007) and sea bass (González-Martínez et al., 2004) and, as demonstrated in several fish species, expression levels of the *gnrhr-IIa* in the brain and pituitary are highly correlated with gonad development and reproduction (González-Martínez et al., 2004b; Hidahl et al., 2013; Guilgur et al., 2009; Lin et al., 2010). In Atlantic cod, the four identified *gnrhrs* are expressed in the brain during sexual maturation. However, one variant, the *gnrhr2a* (a Gnhr-IIa subtype) is highly expressed in the pituitary, has significantly increased expression during the spawning season and is likely the main receptor for mediating Gnhr3 function during spawning (Hidahl 2011a, 2011b, 2013). In the

clupeiforme *Coilia nasus*, the expression levels of a *gnrhr-IIa* subtype in the brain, pituitary and gonad, as well as plasma circulating levels of the Gnrhr-IIa protein, were highly correlated with migration, gonad maturation and spawning, indicating central and peripheral actions of the Gnrhr-IIa in regulating reproductive processes (Duan et al., 2016). The studies on the Gnrhr-IIb showed that the expression levels of these types of receptors are in many cases low or undetectable in the pituitary and do not correlate with reproductive processes, thus suggesting no direct actions on reproduction (Guilgur et al., 2009; Ikemoto and Park, 2005; Lin et al., 2010; Hidahl et al., 2011, 2013).

In fish pituitary, *gnrhirs* are most abundant in the gonadotropic cells, but they are also expressed in other adenohypophyseal cell types (Cook et al., 1991; Illing et al., 1999; Parhar et al., 2002, 2005; Stefano et al., 1999). The *gnrhr-II* (*gnrhr-IIb*) has been localized in somatotropes of *Astatotilapia burtoni* (Flanagan et al., 2007), indicating that this receptor could be relevant for regulation of growth by mediating the known stimulatory actions of GnRh on Gh secretion (Klausen et al., 2001). The expression of several *gnrhr* isoforms has also been detected simultaneously in certain single cell types of the tilapia pituitary (lactotropes, somatotropes, thyrotropes, melanotropes, corticotropes and somatolactin cells) by using laser capture microdissection followed by RT-qPCR analysis (Parhar et al., 2005). In contrast, Gh cells of the European sea bass do not express the *gnrhr-II-1a* receptor (González-Martínez et al., 2004b). It should be noted that the sea bass possesses five GnRhirs and, therefore, it cannot be ruled out that one of remaining four GnRhirs is expressed in somatotropes and/or other adenohypophyseal cells.

Apart from the brain and the pituitary, *gnrhirs* have been detected in the gonads and other peripheral tissues of many fish species (Dufour et al., 1993; Okubo et al., 1999a,b; Robison et al., 2001; Lethimonier et al., 2004; Weltzien et al., 2004; Levavi-Sivan and Avitan, 2005; Ikemoto and Park, 2005; Tello et al., 2008; Lin et al., 2010; Peñaranda et al., 2013; Chen et al.,

2018). The ovary and testis of zebrafish expressed four *gnrhrs*, two of them are Gnhrh-IIa subtype. Some of these receptors are also expressed in gill, skeletal muscle, intestine and heart (Tello et al., 2008). In the gonads, Gnhrs may mediate GnRh action related to the regulation of apoptosis, as suggested in goldfish (Andreu-Vieyra et al., 2005). In the European sea bass, three of the five Gnhrh isoforms are restricted to the central nervous system, while the other two receptor isoforms exhibited a wide distribution in reproductive and non-reproductive tissues, including the eye, kidney, gut, liver and gill (Moncaut et al., 2005). Similarly, the *gnrhr-Ia* and *gnrhr-Ib* in the European eel had a wider distribution among non-reproductive tissues than the *gnrhr-II*, whose expression was restricted to the brain, pituitary and gonad (Peñaranda et al., 2013). Gene expression of *gnrhrs* in the gills and kidney has been reported in several teleost species, suggesting a possible function of these receptors in the mediation of GnRh actions in osmoregulation (Ikemoto and Park, 2005; Lin et al., 2010; Robison et al., 2001; Tello et al., 2008; Peñaranda et al., 2013).

6. Genome editing of the GnRH system for the study of GnRH functions

The emergence of gene-editing technologies has allowed for a myriad of permanent or temporary loss-of-function studies to clearly deduce the biological functions of the different GnRH isoforms and other neuropeptides thought to be involved in reproductive regulation in vertebrates. Gene-editing techniques were first developed using murine models but have recently been adapted and optimized to be used in teleost models as well (Zhu and Ge, 2018). These gene-editing methods include gene knockouts (KO), induced by gene-editing nuclease systems such as zinc-finger nucleases (ZFN) (Doyon et al., 2008), transcription activator-like effector nucleases (TALEN) (Sander et al., 2011), and clustered regularly interspaced palindromic repeats/Cas9 (CRISPR/Cas9) (Hwang et al., 2013), to induce permanent mutations in the targeted genes-of-interest, and gene knockdowns (KD), such as the

temporary silencing of genes induced by antisense morpholino oligomers (MOs) (Zhu and Ge, 2018). Many of these techniques have recently been used to study the *Gnrh* isoforms in zebrafish, a cyprinid with two *Gnrh* isoforms, and in medaka, a teleost with three *Gnrh* isoforms, as will be discussed below. The genetic study of the biological roles of GnRH first began with looking at natural mutations in the human and mouse models and have recently expanded to include teleost models.

The importance of gonadotropin-releasing hormones, their upstream regulators, and the gonadotropin-releasing hormone receptors in maintaining reproductive competence has been well-established in mammalian models, as many cases of hypogonadotropic hypogonadism, which results in failed pubertal development and infertility, were found to be caused by genetic mutations in GNRH1 (Mason et al., 1986; Bouligand et al., 2009), the GNRH receptor, GNRHR1 (Tello et al., 2012), and upstream GNRH regulators, KISS1 and TAC3, along with their receptors (Topaloglu et al., 2012). The first loss-of-function experiment conducted in zebrafish did not entail genetic manipulation, but instead, the targeted ablation of the hypophysiotropic *Gnrh3* neurons at early development using focused laser beams (Abraham et al., 2010). This *Gnrh3* neuron ablation resulted in all-female fish with arrested oocyte development at the pre-vitellogenic stage and infertility (Abraham et al., 2010). These results clearly demonstrated that *Gnrh3* in zebrafish is involved in both sex determination and oogenesis and support the notion that the importance of *Gnrh* in reproduction is conserved from teleosts to mammals. Additionally, as discussed in previous sections, knockdown of *gnrh3* (Abraham et al., 2008) and knockdown of a factor controlling the migration of *Gnrh3* neurons in embryonic stages, nasal elongation *Lhrh* factor (*nelf*) (Palevitch et al., 2009), both resulted in mis-migrated *Gnrh3* neurons in zebrafish. This is a similar phenomenon to that found in mammals, in which NELF mutations result in mis-migrated GNRH1 neurons and

idiopathic hypogonadotropic hypogonadism, suggesting a similar ontogeny for mammalian GNRH1 and teleost Gnrh3.

The paradigm of the importance of the Gnrh system in teleost reproductive control has been recently challenged, however, by the unexpected results of multiple gene knockout studies. Surprisingly, zebrafish *gnrh3* knockouts (*gnrh3*^{-/-}) generated by multiple laboratories have resulted in fish with normal reproduction, displaying no changes in gonadal morphology, reproductive competence, or gonadotropin levels in sexually mature adults (Figures 6A, D) (Spicer et al., 2016; Liu et al., 2017). A single *gnrh2* knockout was also generated in the zebrafish model, as the Gnrh2 neuronal projections also reach the pituitary and therefore may be a regulator of gonadotropin release (Xia et al., 2014). However, like the *gnrh3*^{-/-} fish, the *gnrh2*^{-/-} fish had overall normal reproduction (Marvel et al., 2019; Figures 6B, E), despite minor reproductive differences such as slightly reduced *lhb* expression and oocyte diameters. These findings suggest a minor hypophysiotropic role for Gnrh2 in *lhb* synthesis and release, and reinforce the notion that Gnrh2 is not the primary regulator of reproduction (Marvel et al., 2019). However, because Gnrh2 neurons also project to the pituitary (Xia et al., 2014), and Gnrh2 is able to activate all four Gnrh receptors with the same potency as Gnrh3 (Tello et al., 2008), Gnrh2 may be compensating for the loss of Gnrh3 in *gnrh3*^{-/-} fish. Nevertheless, the additional knockout of *gnrh2* in the *gnrh3*^{-/-} background (*gnrh2*^{-/-};*gnrh3*^{-/-}) resulted in reproductively normal fish (Figures 6C, F) (Marvel et al., 2018). Additionally, the triple knockout zebrafish, with mutations in *gnrh3*, *kisspeptin1*, and *kisspeptin2* genes (*gnrh3*^{-/-};*kiss1*^{-/-};*kiss2*^{-/-}) also displayed normal reproduction and gonadotropin expression (Liu et al., 2017). The reproductive phenotype of *gnrh* knockouts appears, however, to be dependent on whether the fish endogenously possess two or three Gnrh isoforms. In medaka, a teleost species with three Gnrh isoforms, of which Gnrh1 is the hypophysiotropic form, the effects of *gnrh1* knockout are both similar and

different. Medaka *gnrh1* knockout males are fertile, with normal gonadal development, similar to zebrafish. However females, despite normal gonadal development, exhibited a failure of ovulation, potentially as a result of reduced *lh* expression (Takahashi et al., 2016). The phenotypic differences of *gnrh* knockouts in medaka and zebrafish suggest that Gnrh1 is still a critical reproductive regulator in species which possess it, but the evolutionary loss of Gnrh1 in some teleost species, like the zebrafish (and potentially Cyprinidae and Salmonidae species), may have led to the emergence of additional neuropeptides outside the Gnrh system that can facilitate Gnrh3's actions in taking over the roles of the Gnrh1 isoform. Alternatively, in fish with three Gnrh isoforms, the continued presence of the Gnrh3 soma and neurons in the olfactory bulb/terminal nerve after Gnrh1 knockout may have prevented a compensatory response from being activated, resulting in the failure of ovulation.

The previous knockout studies suggest that either Gnrh is dispensable for normal reproduction in species with two Gnrh isoforms, due to functional redundancy by other reproductive regulators capable of maintaining gonadotropin synthesis and release (Figure 7A) (Trudeau, 2018; Whitlock et al., 2019), or that Gnrh is still an important reproductive regulator but the genetic loss and presence of mutated mRNA transcripts initiate a compensatory response, which is activated to maintain reproduction (Figure 7D) (Liu et al., 2017; Kanda, 2018; Marvel et al., 2019). There is supportive evidence for both hypotheses. The previous finding that neuronal ablation of Gnrh3 in zebrafish resulted in infertile females, suggests that at the very least, functional Gnrh3 neurons are necessary for maintaining reproductively competent females (Abraham et al., 2010). However, as neurons are known to contain multiple neuropeptides and neurotransmitters, there could also be other factors besides Gnrh3 produced in these neurons that act similar to Gnrh in stimulating gonadotropin release (Figure 7B), as suggested by previous reviews (Trudeau, 2018). In support of the redundancy hypothesis, a peptide belonging to the RFamide family, termed

862 PQRamide, was found to be expressed in GnRH3 neurons and detected in the terminal nerve
863 of zebrafish, suggesting this factor may be functioning alongside GnRH3 to regulate
864 reproduction. However, its presence was restricted to the olfactory region, with no expression
865 in the preoptic area GnRH3 neurons that innervate the pituitary (Oehlmann et al., 2002), and
866 studies in lamprey showed that PQRamide could modulate GnRH2 but had no effect on the
867 gonadotropins (Dauks et al., 2012). Further research on other factors expressed in GnRH3
868 neurons will need to be conducted in order to test if these factors have gonadotropin-releasing
869 abilities. The possibility of multiple neurons synapsing near the gonadotropes and
870 coordinately stimulating gonadotropin synthesis and release has also been proposed (Figure
871 7A) (Trudeau, 2018; Liu and Lin, 2017). Other factors, including GnIH, PACAP, and
872 Secretogranin II (Scg2) type a, are known to be hypophysiotropic and can stimulate
873 gonadotropin release *in vitro* (Trudeau, 2018; Liu and Lin, 2017; Spicer et al., 2017; Marvel
874 et al., 2018). These factors may be non-canonical regulators, acting in concert to coopt
875 gonadotropin synthesis and release, which is unaffected when some of these players are lost.
876 There is also the possibility of a single major non-GnRH hypophysiotropic factor controlling
877 reproduction that has yet to be discovered (Figure 7C) (Whitlock et al., 2019). In support of
878 this hypothesis, knockout of the *scg2a* and *scg2b* genes in zebrafish that encode for the
879 gonadotropin enhancer, Secretoneurin, resulted in drastically reduced oviposition rates
880 (Mitchell, 2018), suggesting Secretogranin-II may be an important regulator of reproduction
881 in teleosts. Other neuropeptides, including GnIH or Phoenixin, which were previously shown
882 to modulate gonadotropin release in some animals, could also be potential candidates
883 (Amano et al., 2006; Stein et al., 2016; Spicer et al., 2017; Muñoz-Cueto et al., 2017;
884 Whitlock et al., 2019). In order to conclusively determine if these systems are working alone
885 (solo) or in tandem with GnRH to mediate gonadotropin release, single knockouts as well as

multiple knockouts of these hypophysiotropic candidates in multiple teleost species must be generated.

Conversely, it may be the case that the gene expression of unknown neuropeptides and/or neurotransmitters in the GnRH neurons, or other neurons, exhibit plasticity and are only activated in response to the genetic loss of *gnrh* (i.e., they are needed to take over the role of gonadotropin release in teleosts) (Figure 7D). Activation of compensation is documented for several other gene knockouts in zebrafish and genetic compensation has been increasingly noted to occur subsequent to deleterious mutations, potentially induced by the presence of truncated or mutated mRNA transcripts. For instance, knockout of *egf17* resulted in the upregulation of other extracellular matrix genes that share a similar motif, whereas temporary knockdown resulted in no compensation, and knockout, but not knockdown, of *vegfaa* resulted in the compensatory upregulation of *vegfab* (Rossi et al., 2015). Therefore, it is possible that a similar compensation may be occurring in single and double *gnrh* knockouts in zebrafish as well due to the presence of deleterious mutations. Whole transcriptome and differential gene expression analysis of *gnrh3* knockout zebrafish showed the upregulation of many neuropeptides, some of which may be hypophysiotropic and function similarly to GnRH (Zohar lab, unpublished data). This upregulation included factors such as Vasoactive intestinal peptide (Vip), Secretogranins, and Gnih (Zohar lab, unpublished data). Additionally, the double *gnrh* knockout (*gnrh2*^{-/-}; *gnrh3*^{-/-}), as well as the triple *gnrh* /*kisspeptin* knockout (*gnrh3*^{-/-}; *kiss1*^{-/-}; *kiss2*^{-/-}) resulted in upregulation of several genes, including *tachykinin 3a* (*tac3a*), that encodes for Nkb, and *secretogranin-IIa* (*scg2a*), that encodes for Secretoneurin a (Liu et al., 2017; Marvel et al., 2018). These factors, together with other upregulated genes in the single *gnrh3* knockout fish, may all be part of the compensatory mechanism activated by the loss of GnRH. Nkb, Vip, Secretoneurin, and Gnih have been shown to be hypophysiotropic in some animals and able to stimulate gonadotropin

release (Zhou et al., 2012; Biran et al., 2012; Moussavi et al., 2013; Canosa et al., 2011; Trudeau et al., 2012; Paullada-Salmerón et al., 2016). The nature of the reproductive roles of these neuropeptides in wild-type and knockout zebrafish will need to be further determined to solidify whether they are compensating for the loss of *Gnrh3* (Figure 7D), or have major and shared reproductive roles in wild-type fish (Figures 7A, C).

The timing of genetic loss appears to be an important factor in inducing compensation and plasticity of neuronal systems in the brain. For instance, laser ablation of *Gnrh3* neurons in zebrafish at 2 dpf resulted in the rapid regeneration and migration of *Gnrh3* neurons, whereas laser ablation at later stages of 4 and 6 dpf resulted in the permanent disruption of the neuronal migration of *Gnrh3*, suggesting a time-sensitive period of plasticity (Abraham et al., 2010). Additionally, genetic knockdown of *gnrh3* (Abraham et al., 2008) or genetic knockdown of the migratory factor expressed in *Gnrh3* neurons, *nelf*, (Palevitch et al., 2009) results in mis-migrated *Gnrh3* neurons, whereas inherited *gnrh3* knockout has no effect on neuronal migration patterns (Spicer et al., 2016). Therefore, a *gnrh* genetic mutation that is inherited, unlike KD methods, may lead to the activation of compensation by other factors due to the absence of *Gnrh* prior to cell differentiation. This suggests that there is a critical time-sensitive period in which *Gnrh* must be absent in order to induce compensation, as suggested by the *Gnrh3* laser ablation experiments. In order to test if this time-sensitive theory is true, experiments that silence *Gnrh* at later stages in development, such as conditional *gnrh* knockout in adult fish, must be conducted. These experiments may also prevent the early genetic compensation induced by the mutated *gnrh* transcripts, and allow for a more careful analysis of *Gnrh* function.

Furthermore, compensation or redundancy of reproductive regulators appear to be dependent on the specific level of the HPG axis that is affected. For instance, knockout of some factors at the level of the brain, such as the *gnrhs* and *kisspeptins* in zebrafish, results in

no reproductive abnormalities (Tang et al., 2014; Spicer et al., 2016; Liu et al., 2017; Zhu and Ge, 2018; Marvel et al., 2018), whereas knockout of factors at the pituitary level, such as the *lh* and *fsh* gonadotropins in zebrafish, results in some reproductive abnormalities (Chu et al., 2015; Zhang et al., 2015a; Zhu and Ge, 2018; Li and Cheng, 2018), and double knockout of the gonadotropin receptors in the gonads results in infertility (Chu et al., 2015; Zhang et al., 2015b, Zhu and Ge, 2018; Li and Cheng, 2018). This suggests that at the level of the brain, there is higher plasticity and functional redundancy of multiple reproductive neuropeptides, whereas downstream in the HPG axis, factors at the pituitary and gonadal level may be more specialized. In order to test if this is the case and to add information on another level along the HPG axis, knockout of the four *gnrh* receptors in zebrafish is currently underway to determine the reproductive phenotype.

As noted earlier in this review, an abundance of previous studies on the roles of GnRh in teleost reproduction suggest that this neuropeptide is indeed a critical regulator of reproductive processes, but the extremely plastic nature of teleost brains may be able to compensate for the genetic loss of this peptide to ensure reproduction and the survival of the fish lineage. Future studies will need to determine whether the importance of GnRh is species-specific or universal and elucidate the nature of the compensation that may occur with natural or induced mutations in the GnRh system of teleosts.

7. Future perspectives

The decades-long journey in GnRH research has taken us from the discovery of a brain factor that controls reproduction, to the identification of a single GnRH form thought to be responsible for overall control of the HPG axis, and ultimately to the recognition that there are multiple GnRH variants that may be responsible for more than just controlling reproduction. During the research process, and largely due to the fact that rodents have only

one GnRH form, fish became a recognized vertebrate model for the study of the GnRH system and for exploring the functional significance of GnRH multiplicity. Initial intensive research in commercially important aquaculture species, conducted in an effort to understand and overcome the GnRH failure responsible for the lack of captive finfish ovulation and spawning, has more recently shifted to fish models such as zebrafish and medaka. These powerful fish models, together with rapid advancement in molecular, genomic, post-genomic and genome editing platforms, led to the more recent groundbreaking discoveries reviewed in this paper. Future research will continue to benefit from these useful fish models, representing a species with 2 GnRH variants (zebrafish) and 3 variants (medaka), and should explore the possible additional roles of GnRHs, such as coordinating feeding, body metabolism and reproduction, transducing external and environmental cues such as pheromones and photoperiod through the olfactory and pineal systems, respectively, and reproductive behavior. The role of GnRH2, which is ubiquitous to most vertebrates, continues to be elusive in all vertebrates from fish to humans, and the small fish models provide a unique opportunity to unravel its significance and explain its wide distribution in the brain. The surprising discovery of the lack of reproductive phenotypes when the *gnrh*s are knocked out should be further explored in the context of possible redundancy or compensation mechanisms to ensure that reproduction and the survival of the animal lineage are maintained, even when the main GnRH players are impaired.

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1935 **Figure legends**

1936 **Figure 1.** Organization of the three *prepro-gnrh* cDNAs showing the *signal peptide*, the *gnrh*
1937 *decapeptide*, the *processing peptide (pp)*, and the *gnrh-associated peptide (gap)*. The
1938 sequence identity among the different *gaps* and *gnrhs* sequences in a perciform fish is
1939 depicted through the *gnrh* sequences of the European sea bass.

1940 **Figure 2.** Phylogenetic tree showing the putative evolution of the *GnRH* gene family in
1941 vertebrates. *GnRH1*, *GnRH2* and *GnRH3* paralogous genes are indicated in green, purple and
1942 red boxes, respectively. Lost genes are represented by grey crossed-out boxes. 1R, 2R and 3R
1943 represent different rounds of whole genome duplication. Duplications of *GnRH3* and *GnRH1*
1944 genes in Cyclostomes and Holocephalans, are indicated by double-headed red and green
1945 arrows, respectively. Adapted from Gaillard et al., 2018.

1946 **Figure 3. (A).** Graphical depiction of the general localization of *Gnrh1/3* (green) and *Gnrh2*
1947 (purple) neuronal soma (circles) and projections (dashed lines) throughout a typical two-
1948 *GnRH* isoform teleost brain. **(B).** General localization of *Gnrh1* (green), *Gnrh2* (purple), and
1949 *Gnrh3* (red) neuronal soma (circles) and projections (dashed lines) throughout a typical three-
1950 *Gnrh* isoform teleost brain. Black and grey circles represent pituitary gonadotropes (Fsh and
1951 Lh cells). Cer = cerebellum; Hypo = hypothalamus; MO = medulla oblongata; OB =
1952 olfactory bulb; OT = optic tectum; POA = preoptic area; Tel = telencephalon; TN = terminal
1953 nerve.

1954 **Figure 4.** Chronogram summarizing the temporal and regional expression of prepro-*Gnrh1*-
1955 (green circles), prepro-*Gnrh2*- (purple circles) and prepro-*Gnrh3*- (red circles)
1956 immunoreactive cells in the brain of developing European sea bass. The temporal pattern of
1957 arrival of prepro-*Gnrh3* (dotted red line) and prepro-*Gnrh1* (squared green line) fibers to the

1958 sea bass pituitary is also reflected. The thickness of the lines is proportional to the amount of
 1959 entering GnRh fibers. In the bottom of the table, the apex of triangles marks the brain area
 1960 exhibiting the highest number of each GnRh-immunoreactive cell type. Abbreviations: OLN,
 1961 olfactory nerve; OB, olfactory bulbs; Vv, ventral nucleus of the ventral telencephalon; POA,
 1962 preoptic area; MBH, mediobasal hypothalamus; Pit, pituitary; MT, midbrain tegmentum.
 1963 Modified from González-Martínez et al., 2004a.

1964 **Figure 5.** Hormonal responsiveness of the GnRh-II-1a and GnRh-II-2b receptors of the
 1965 European sea bass. COS-7 cells were transfected with the GnRh-II-1a (**A**) and GnRh-II-2b
 1966 (**B**) expression vectors independently. At 48 h after transfection, cells were incubated for 6 h
 1967 with increasing concentrations of endogenous sea bass GnRh peptides and inositol
 1968 triphosphate production was monitored. Results are expressed as percentage of maximal
 1969 stimulation obtained for GnRh2 and represent the mean of five independent experiments in
 1970 triplicate. EC50 values (M) of GnRh forms for IP3 production of COS-7 cells transfected with
 1971 GnRh expression vectors are also shown. Note the high selectivity of the GnRh-II-2b for the
 1972 GnRh2 form. Modified from Servili et al., 2010.

1973 **Figure 6.** Comparison of the average number of eggs per spawn (fecundity) of wild-type
 1974 (WT) spawning pairs compared to mixed *gnrh3*^{-/-} (G3 KO) spawning pairs (**A**), *gnrh2*^{-/-} (G2
 1975 KO) spawning pairs (**B**), and *gnrh2*^{-/-};*gnrh3*^{-/-} (DKO) spawning pairs (**C**). Comparison of the
 1976 average percentage of eggs fertilized per spawn (fertility) of wild-type (WT) spawning males
 1977 compared to *gnrh3*^{-/-} (G3 KO) males (**D**), *gnrh2*^{-/-} (G2 KO) males (**E**), and *gnrh2*^{-/-};*gnrh3*^{-/-}
 1978 (DKO) males (**F**). All data were expressed as means ± S.E.M. Modified from Spicer et al.,
 1979 2016, Marvel et al., 2018, Marvel et al., 2019.

1980 **Figure 7. (A-D).** Different theories on the compensation and dispensability of
1981 hypophysiotropic Gnrh in zebrafish, integrating studies cited in the main text (Trudeau, 2018,
1982 Whitlock et al., 2019, Liu and Lin, 2017, Kanda, 2018, and Marvel et al., 2019). Npx, Npx1,
1983 Npx2 = putative neuropeptides, one or more of which may stimulate gonadotropin release.

Figure 1

Sequences

	<i>gaps</i>	<i>gnrhs</i>
<i>prepro-gnrh1</i> vs. <i>gnrh2</i>	36%	70%
<i>prepro-gnrh1</i> vs. <i>gnrh3</i>	42%	80%
<i>prepro-gnrh2</i> vs. <i>gnrh3</i>	41%	80%

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7