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The gonadotropin-inhibitory hormone system of fish: The case of sea bass (*Dicentrarchus labrax*)

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ABSTRACT

Gonadotropin-inhibitory hormone (GnIH) is a hypothalamic neuropeptide belonging to the RFamide peptide family that was first discovered in quail by Tsutsui and co-workers in the year 2000. Since then, different GnIH orthologues have been identified in all vertebrate groups, from agnathans to mammals. These GnIH genes synthesize peptide precursors that encompass two to four C-terminal LPXRFamide peptides. Functional and behavioral studies carried out in birds and mammals have demonstrated a clear inhibitory role of GnIH on GnRH and gonadotropin synthesis and secretion as well as on aggressive and sexual behavior. However, the effects of Gnih orthologues in reproduction remain controversial in fish with both stimulatory and inhibitory actions being reported. In this paper, we will review the main findings obtained in our laboratory on the Gnih system of the European sea bass, *Dicentrarchus labrax*. The sea bass *gnih* gene encodes two putative Gnih peptides (sbGnih1 and sbGnih2), and is expressed in the olfactory bulbs/telencephalon, diencephalon, midbrain tegmentum, rostral rhombencephalon, retina and testis. The immunohistochemical study performed using specific antibodies developed in our laboratory revealed Gnih-immunoreactive (ir) perikarya in the same central areas and Gnih-ir fibers that profusely innervated the brain and pituitary of sea bass. Moreover, *in vivo* studies revealed the inhibitory role of centrally- and peripherally-administered Gnih in the reproductive axis of male sea bass, by acting at the brain (on *gnrh* and *kisspeptin* expression), pituitary (on *gnrh* receptors and gonadotropin synthesis and release) and gonadal (on androgen secretion and gametogenesis) levels. Our results have revealed the existence of a functional Gnih system in sea bass, and have provided evidence of the differential actions of the two Gnih peptides on the reproductive axis of this species, the main inhibitory role in the brain and pituitary being exerted by the sbGnih2 peptide. Recent studies developed in our laboratory also suggest that Gnih might be involved in the transduction of photoperiod and temperature information to the reproductive axis, as well as in the modulation of daily and seasonal rhythmic processes in sea bass.

1. Introduction

In fish, reproductive physiology and behavior are controlled by complex environmental and hormonal interactions that occur along the brain-pituitary-gonadal axis (BPG). This axis is entrained by external cues such as light and temperature (Pankhurst and Porter, 2003; Migaud et al., 2010; Carrillo et al., 2015) that control the secretion of neurohormones and neurotransmitters in the brain, which in turn, modulate the synthesis and release of gonadotropins (Gths, follicle stimulating hormone, Fsh and luteinizing hormone, Lh) in the pituitary (Zohar et al., 2010).

Since pioneer studies in early 1970's (Matsuo et al., 1971; Burgus et al., 1972) discovering the role of gonadotropin-releasing hormone (GnRH) as a central regulator of reproductive axis in mammals, important progress in this field has been made to understand and improve our current knowledge on reproductive neuroendocrinology. Nowadays, it is well established that Gnrh is a key player in the control of reproduction because it integrates both internal and external signals and constitutes the main neuroendocrine factor stimulating the secretion of gonadotropins in fish (for review see Zohar et al., 2010). In 1980, Peter and Paulencu published an elegant study reporting that electrolytic preoptic lesions in female goldfish caused an increase in

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plasma GTH levels. These results suggested the existence of a brain factor that inhibited GTH release in this brain area (Peter and Paulencu, 1980). Afterwards, Chang and co-workers revealed that dopamine (DA), a monoamine secreted in the preoptic region, inhibited GTH release from dispersed pituitary cells or pituitary fragments, suggesting a direct action of DA at the pituitary level (Chang et al., 1983). Subsequently, different studies performed in several fish species demonstrated that DA inhibited the reproductive process by acting on the synthesis and/or release of gonadotropins through dopamine D2-like receptors (De Leeuw et al., 1986; Yu and Peter 1990; Chang et al., 1990; Vacher et al., 2000; Yaron et al., 2003). This role of DA was confirmed in representative species of some teleost orders such as Cypriniformes, Salmoniformes, Siluriformes, Cichliformes and Mugiliformes, among others (for review see Dufour et al., 2010). However, this dopaminergic inhibition has not been demonstrated in most perciforms species studied up to date, including the European sea bass (*Dicentrarchus labrax*) (Zohar et al., 1995; Holland et al., 1998; Prat et al., 2001; Kumakura et al., 2003). Consequently, as a dopaminergic inhibition on reproduction does not seem to operate in the European sea bass, it is likely that another brain factor antagonizing Gnrh actions could exist in this species.

Eighteen years ago, Tsutsui and colleagues identified in the hypothalamus of the quail brain, using an antibody raised against Arg-Phe-NH_2 peptide, a novel RFamide peptide that inhibited the gonadotropin release in the pituitary (Tsutsui et al., 2000). As a result, it was termed as gonadotropin-inhibitory hormone or GnIH (also known as LPXRFamide peptide). Since then, several studies demonstrated the inhibitory role of GnIH on gonadotropin synthesis and/or release in other avian species, including sparrow, chicken, starling and zebra finch (Tsutsui et al., 2000; Bentley et al., 2003; Ciccone et al., 2004; Osugi et al., 2004; Ikemoto and Park, 2005; Ubuka et al., 2006, 2008; Tobari et al., 2010). Following these initial discoveries in birds, further research was directed to characterize this inhibitory neuroendocrine system in different vertebrate groups including mammals (RFRP, Fukusumi et al., 2001; Ukena et al., 2002), amphibians (FGRP, Chartrel et al. 2002; Koda et al. 2002; Sawada et al. 2002a; Ukena et al. 2003b), teleosts (Lpxrfa, Sawada et al., 2002b; Amano et al., 2006; Zhang et al., 2010; Shahjahan et al., 2011) and agnathans (LPXRFa, Osugi et al., 2012). All the identified GnIH orthologues possessed a C-terminal Leu-Pro-X-Arg-Phe-NH_2 (X = Leu or Gln) motif and, thus, they were termed as LPXRFamide peptides. Generally, the GnIH precursors encode two to four LPXRFa peptides, depending on the species (for review see Ubuka et al., 2016).

The neuroanatomical studies have provided relevant information to understand the role of GnIH system across vertebrates. Different researches localized GnIH peptides in the brain of vertebrates using immunohistochemistry and *in situ* hybridization. Birds contained GnIH cells in the paraventricular hypothalamic nucleus (PVN) as well as in the septal area (Tsutsui et al., 2000; Bentley et al., 2003; Ukena et al., 2003a; Osugi et al., 2004). In mammals, GnIH cells were restricted in the dorsomedial (DMH), paraventricular (PVN) and ventromedial (VMH) nuclei of the hypothalamus (Kriegsfeld et al., 2006; Dardente et al., 2008; Clarke et al., 2008; Soga et al., 2014). In amphibians, GnIH neurons were identified in the anterior preoptic area (POA), the suprachiasmatic nucleus (SCN), and the dorsal and the ventral hypothalamic nuclei (Koda et al., 2002; Chowdhury et al., 2008; Chartrel et al., 2002). In the majority of vertebrates studied to date, GnIH fibers were widely distributed throughout the brain and with the main projections extending to the medium eminence (ME) to control anterior pituitary functions (Tsutsui et al., 2000; Koda et al., 2002; Bentley et al., 2003; Ukena et al., 2003a; Osugi et al. 2004; Chowdhury et al., 2008; Chartrel et al., 2002; Ubuka et al., 2009a,b; Clarke et al., 2008). Additionally, immunohistochemical studies have shown that GnIH cells projected to GnRH neurons in birds and mammals, which also expressed GnIH receptors (Bentley et al., 2003; Kriegsfeld et al., 2006; Ubuka et al., 2008; Ubuka et al., 2009b, 2012a). According to the fiber projections pattern, it has been proposed that GnIH might be involved in functions other than reproduction and could exert pleiotropic actions in vertebrates.

So far, there is consensus that in most tetrapod species GnIH inhibits the reproductive process by decreasing gonadotropin synthesis and release through its inhibitory actions on GnRH neurons and/or pituitary gonadotropes via specific GnIH receptors (Kriegsfeld et al., 2006; Murakami et al., 2008; Kadokawa et al., 2009; Ubuka et al., 2012a). These GnIH receptors have been named as GPR147 in birds (Yin et al., 2005) and neuropeptide FF receptor 1 (NPFF1) or OT7022 in mammals (Hinuma et al., 2000; Bonini et al., 2000), and are typical G-protein coupled receptors (GPCR) that inhibit the AC/cAMP/PKA-mediated signaling pathway in GnRH neurons as well as in gonadotropes (Son et al., 2012; Ubuka et al., 2013). Nonetheless, the GnIH actions seem more complex in fish and although most of studies showed the inhibitory effects of GnIH on BPG axis in this group of vertebrates, several *in vivo* and *in vitro* studies have also reported a stimulatory effect (Muñoz-Cueto et al., 2017; Ubuka and Parhar, 2018).

Recently, we have compared Gnih precursor sequences from multiple fish species with other vertebrate GnIH orthologues, showing that fish precursors encode for two to three Gnih-like peptides. Whereas fish belonging to orders Lepisosteiformes, Anguilliformes, Siluriformes, Characiformes, Cypriniformes, Salmoniformes, Beloniformes, Cyprinodontiformes and Labriformes analyzed up to date exhibited 3 Gnih-like sequences, most species from modern teleost orders such as Perciformes, Pleuronectiformes or Tetraodontiformes seem to show only two RFamide peptides (Muñoz-Cueto et al., 2017). On the other hand, studies developed in fish have revealed that Gnih neurons are consistently localized in the posterior periventricular nucleus (NPPv) of the diencephalon (Sawada et al., 2002b; Amano et al., 2006; Biswas et al., 2015; Paullada-Salmerón et al., 2016a; Ogawa et al., 2016; Aliaga-Guerrero et al., 2018). However, recent research has reported the presence of Gnih cells in other brain regions (Biswas et al., 2015; Paullada-Salmerón et al., 2016a; Aliaga-Guerrero et al., 2018).

The European sea bass (*Dicentrarchus labrax*) belongs to the order Perciformes, the most abundant group of teleosts encompassing more than 28.000 species (Nelson 2006). This species is very important for marine aquaculture in Europe and also represents an interesting fish model for the study of environmental and neuroendocrine control of different physiological processes, including reproduction (Carrillo et al., 1995, 2009; Bayarri et al., 2004; Herrera-Pérez et al., 2010; Servili et al., 2014). However, sea bass still presents problems related to reproduction such as early puberty and unbalanced sex proportions that compromise the efficiency of its aquaculture (Carrillo et al., 2009, 2015). Although stimulatory Gnrh system has been studied in depth in this species (González-Martínez et al., 2001, 2002a,b, 2004; Rodríguez et al., 2001a,b; Servili et al., 2014), a functional inhibitory system such as the dopaminergic system does not seem to operate in the control of the reproductive process in the European sea bass (Prat et al., 2001). For this reason, we decided in our laboratory to characterize and elucidate the reproductive role of Gnih in this species. In this review, we highlight the main results obtained in several studies performed in our group during the last years, in which we have addressed different molecular, neuroanatomical, physiological and behavioral aspects of the Gnih system in the European sea bass.

2. Structure and distribution of Gnih in sea bass

The cloning strategy allowed us to characterize a sea bass Gnih precursor that encoded a 200 amino acids protein with two putative RFamide peptides, termed sbGnih1 and sbGnih2, respectively (Fig. 1) (Paullada-Salmerón et al., 2016a). The phylogenetic analysis showed that sea bass Gnih precursor shares a high identity (up to 80%) with perciforms (Paullada-Salmerón et al., 2016a) but also with pleuronectiforms (Aliaga-Guerrero et al., 2018) and lower identity (30%) with cypriniforms (Sawada et al., 2002b). Like in sea bass, some teleost species such as stickleback, tetraodon, takifugu and tongue sole (Zhang

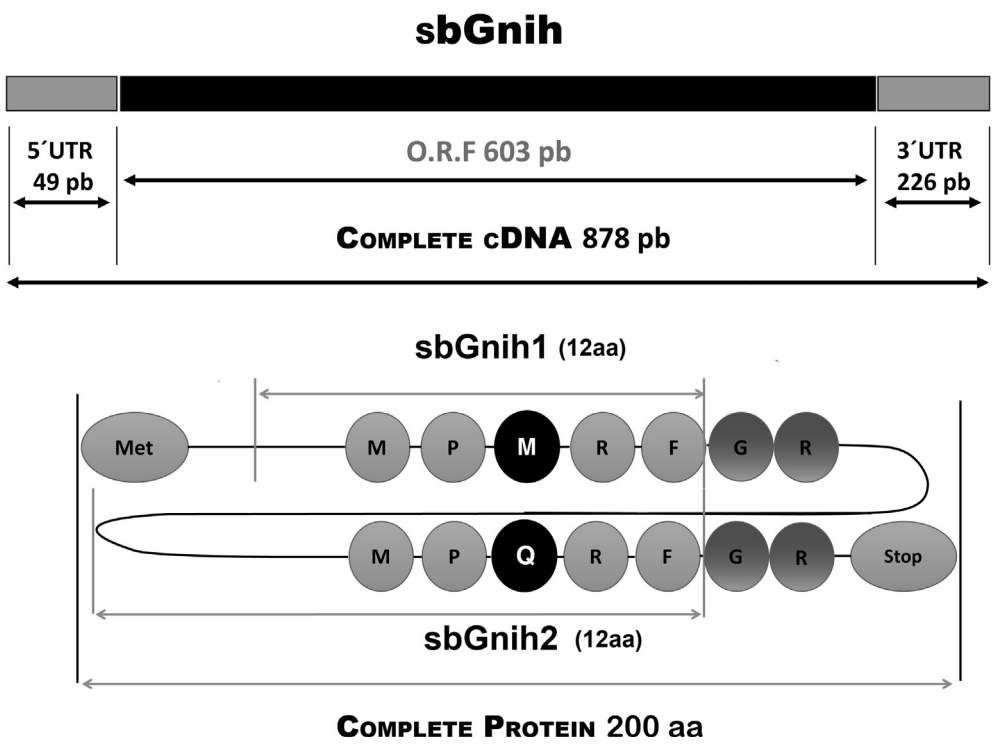

Fig. 1. Schematic representation of the cloned sea bass Gnih cDNA showing product sizes of complete cDNA obtained and deduced protein sequence with C-terminal RFamide motifs. The differing amino acid between sbGnih1 and sbGnih2 in the C-terminal motif is identified by a black circle.

et al., 2010; Shahjahan et al., 2011; Wang et al., 2018) also presented only two putative Gnih-like peptides that exhibited MPMRF (Gnih1) and MPQRF (Gnih2) residues at their C-termini. By contrast, Gnih precursors encode three putative RFamide peptides in other fish species as goldfish, zebrafish, sockeye salmon, tilapia, medaka, grouper, cichlid *Cichlasoma dimerus* and Senegalese sole (Sawada et al., 2002b; Amano et al., 2006; Zhang et al., 2010; Shahjahan et al., 2011; Biran et al., 2014; Wang et al., 2015; Di Yorio et al., 2016; Aliaga-Guerrero et al., 2018). In fish species having 3 different RFamide peptides, Gnih1 and Gnih2 showed a much higher structural diversity at the C-terminal end, whereas Gnih3 exhibited consistently the LPQRF motif (Muñoz-Cueto et al., 2017). Whether this structural diversity of Gnih peptides observed in fish (and, consequently, in their affinities for Gnih receptors) are responsible of different physiological actions of Gnih reported in this group of vertebrates remains to be elucidated.

Expression of *GnIH* gene in the diencephalon is a common feature in all vertebrate species studied up to date. However, studies performed in fish species have reported some discrepancies in the localization of *gnih* expression. In goldfish (Sawada et al., 2002b), grouper (Wang et al., 2015) and tilapia (Biran et al., 2014), *gnih* transcripts appear to be restricted to the diencephalon. In contrast, *gnih* expression was profusely distributed in the CNS (anterior brain, midbrain, hindbrain and retina), as well as in the pituitary, kidney and spleen of grass puffer (Shahjahan et al., 2011). Specific expression pattern of *gnih* gene was also determined in sea bass, showing that *gnih* transcripts were present not only in the diencephalon but also in other brain areas, as well as in some peripheral tissues. In addition to the diencephalon, *gnih* expression was also remarkable in olfactory bulbs and cerebral hemispheres, mesencephalon, rhombencephalon, retina, testis, ovary and intestine (Paullada-Salmerón et al., 2016a). The expression of the *gnih receptor* in the brain and peripheral tissues of sea bass (Fig. 2) was highly consistent with the distribution of its ligand, reinforcing the importance of this neuropeptidergic system in this species. A similar *gnih* expression pattern was observed in the central nervous system of Senegalese sole (Aliaga-Guerrero et al, 2018).

Since its first identification in goldfish (Sawada et al., 2002b), the precise brain localization of Gnih has been addressed in a significant number of teleost species (Muñoz-Cueto et al., 2017). Nevertheless, specific antibodies against endogenous Gnih peptides have been developed only in three fish species, the sea bass (Paullada-Salmerón et al., 2016a), tilapia (Ogawa et al., 2016) and Senegalese sole (Aliaga-Guerrero et al., 2018). In our laboratory, we developed specific antibodies against sea bass Gnih peptide (sbGnih1 and sbGnih2), for the first time in teleost fish (Paullada-Salmerón et al., 2016a). For Gnih immunostaining, sections were incubated overnight with rabbit sbGnih2 antibodies (1:500), followed by goat anti-rabbit secondary antibodies coupled to Alexa Fluor 488 (green fluorescence) or 594 (red fluorescence) diluted 1:200 for 2 h, at room temperature and dark conditions, as indicated in Paullada-Salmerón et al. (2016a). Using these specific antibodies, we reported the presence of Gnih-immunoreactive cells in the diencephalic posterior periventricular nucleus (NPPv) as well as in the olfactory bulbs, corresponding to the terminal nerve ganglion cells (TNgc), in the lateral nucleus of the ventral telencephalon (Vl), in the mesencephalic dorsal tegmentum (MT) and in the secondary gustatory nucleus (NGS) of the rhombencephalic isthmus (Fig. 3) (Paullada-Salmerón et al., 2016a). The distribution of immunolabelled cells in the brain of sea bass was highly consistent with the results obtained in our previous RT-PCR study. To confirm the validity of these results, we performed laser capture microdissection followed by quantitative real-time PCR in the immunolabelled Gnih cells, evidencing that *gnih* transcripts can also be detected in Gnih-immunoreactive cells (Paullada-Salmerón et al., 2016a). A similar distribution of Gnih cells was obtained in a subsequent study performed in Senegalese sole using specific antibodies (Aliaga-Guerrero et al., 2018). However, previous immunohistochemical studies only found Gnih cell bodies in the posterior periventricular nucleus (NPPv) of the caudal preoptic area in other fish species including sockeye salmon (Amano et al., 2006) and Indian major carp (Biswas et al., 2015), as well as in the ventral zone of the periventricular hypothalamus of zebrafish (Spicer et al., 2017) and in the periventricular preoptic nucleus (NPP) and magnocellular preoptic nucleus (NPOm) of Indian major carp (Biswas et al., 2015). In goldfish, *Carassius auratus,* immunostained

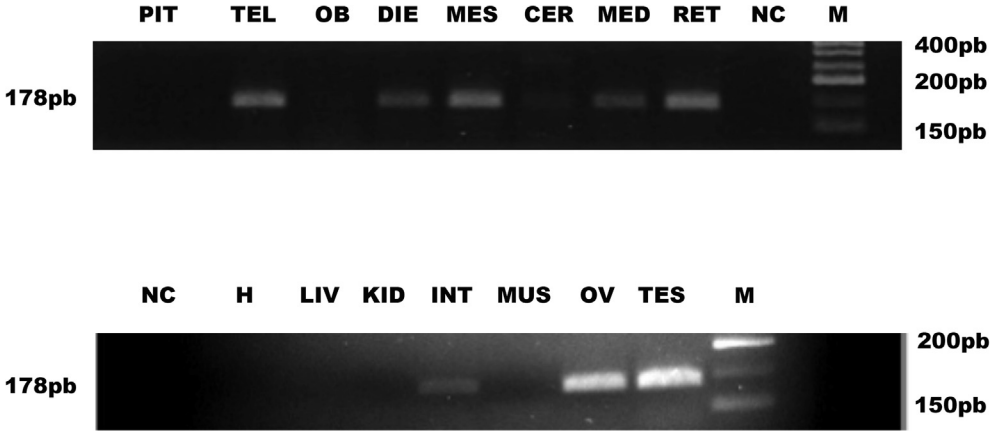

Fig. 2. RT-PCR expression of Gnih receptor (GenBank accession no. LN681208) in central (upper panel) and peripheral (lower panel) tissues of sea bass. PIT, pituitary; TEL, telencephalon; OB, olfactory bulbs; DIE, diencephalon; MES, mesencephalic optic tectum and tegmentum; CER, cerebellum; MED, medulla oblongata and spinal cord; RET, retina; H, heart; LIV, liver; KID, kidney; INT, intestine; MUS, muscle; OV, ovary; TES, testis; NC, non-template control; M, marker. Primer sequences (5′ to 3′): F: GTACGGAAGCATCGGAGTCAAAC; R: CCA GGACAGCATGAAAAGCAAAG.

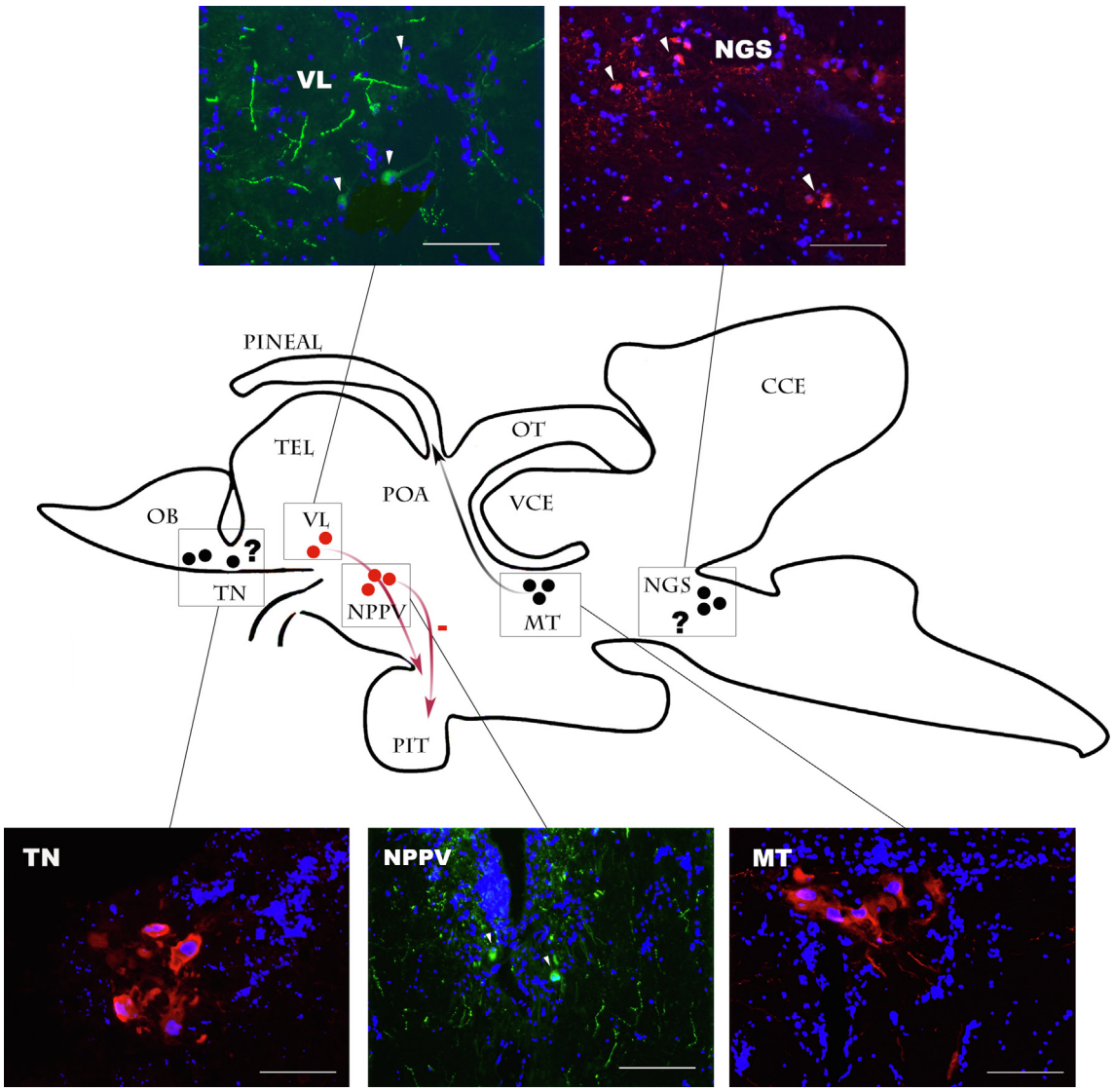

Fig. 3. Schematic representation of a sagittal section of the sea bass brain showing the localization of the different Gnih cell populations reported in this species (circles). Immunolabelled cells from different brain areas are also detailed in pictures (white arrowheads). The red circles represent diencephalic groups of Gnih cells projecting to the pituitary (red arrows). The projection of dorsal tegmental Gnih cells to the pineal organ (black arrow) is also shown. Abbreviations: CCE, corpus of the cerebellum; MT, dorsal mesencephalic tegmentum; NGS, secondary gustatory nucleus; NPPV, posterior periventricular nucleus; OB, olfactory bulbs; OT, optic tectum; PIT, pituitary gland; POA, preoptic area; TEL, telencephalon; TN, terminal nerve; VCE, valvula of the cerebellum; VL, lateral nucleus of the ventral telencephalon. The immunostaining for sbGnih was performed according to Paullada-Salmerón et al. (2016a).

Gnih cells were present in the olfactory terminal nerve area and NPPv, but labelled cells were only detected in NPPv by using *in situ* hybridization (Sawada et al., 2002b). These contradictory results might suggest that Gnih transcripts could exhibit low levels in the terminal nerve cells of goldfish or reflect a possible cross-reactivity of the antibody used in this study (anti-fGRP) with another unknown peptide presents in these olfactory cells. Similarly, an *in situ* hybridization study performed in tilapia revealed *gnih* mRNA-expressing cells only in the NPPv (Ogawa et al., 2016). In general, our results have shown that the distribution of Gnih neurons in the brain of sea bass is much more profuse than that reported in other teleost fish, suggesting that species-specific differences could also exist.

As stated above, a common feature between analyzed species is the abundant Gnih innervation within the brain in all vertebrate groups. In sea bass, Gnih-immunoreactive cells profusely innervated the preoptic area, hypothalamus, optic tectum, semicircular torus, and caudal midbrain tegmentum, but we also observed conspicuous projections that reached the olfactory bulbs, ventral and dorsal telencephalon, habenula, pineal organ, ventral thalamus, vascular sac, pretectum, rostral midbrain tegmentum, posterior tuberculum, reticular formation and facial-vagal sensory lobe (Paullada-Salmerón et al., 2016a). A comparable Gnih innervation pattern was found in goldfish (Sawada et al., 2002b), sockeye salmon (Amano et al., 2006), Indian major carp (Biswas et al., 2015), tilapia (Ogawa et al., 2016), *Cichlasoma dimerus* (Di Yorio et al., 2016) and Senegalese sole (Aliaga-Guerrero et al., 2018). These results, together with the presence of Gnih-immunoreactive fibers in the proximal pars distalis (PPD) of the sea bass pituitary, reinforced the involvement of this neuropeptide in the regulation of pituitary hormone secretion in sea bass. In addition, carbocyanine dye (DiI) tract-tracing methods combined with immunohistochemical staining revealed that ventral telencephalic (Vl) and preoptic (NPPv) cells might represent the source of Gnih projections reaching the sea bass pituitary and innervating gonadotropin cells (Paullada-Salmerón et al., 2016a). The wide distribution of Gnih cells and the extensive innervation of these neuropeptidergic fibers in non-reproductive related areas of the sea bass brain suggest that Gnih could be implicated in some physiological functions other than reproduction in this species.

The characterization, binding affinity, signaling and localization of Gnih receptors (Gnih-r) in the brain and peripheral tissues are also important to gain understanding about the potential targets and functions of Gnih (Biran et al., 2014; Wang et al., 2019). So far, information on the distribution of Gnih-r is still scarce and only a few studies have used riboprobes and antibodies to address its localization in fish (Qi et al., 2013; Biran et al., 2014; Ogawa et al., 2016). Tilapia Gnih-r immunoreactive cells are widely distributed in the preoptic area, hypothalamus, optic tectum, semicircular torus, and caudal midbrain tegmentum, as well as in the olfactory bulbs, ventral/dorsal telencephalon and rhombencephalon (Ogawa et al., 2016). Moreover, Gnih-r is also expressed in Lh, Acth and α-Msh cells in the pituitary of this species (Ogawa et al., 2016). In goldfish, *in situ* hybridization technique showed that three Gnih-r subtypes were extensively localized to the preoptic area and hypothalamus, whereas in pituitary only two Gnih-r were observed (Qi et al., 2013). Unfortunately, the precise localization of Gnih-r in the brain and pituitary of sea bass is still unknown, but research in progress in our laboratory is being directed to elucidate its distribution in this species.

3. Physiological actions of Gnih in the reproductive axis of sea bass

The main function of GnIH in tetrapods is to inhibit the synthesis and release of gonadotropins, thus affecting reproduction. As we mentioned above, the physiological action of Gnih on gonadotropin release is not clear in fish and contradictory findings have been reported to date. For instance, Amano and coworkers reported that goldfish Gnih peptides stimulated the Lh, Fsh and Gh release, whereas they did not affect the secretion of prolactin and somatolactin from pituitary cells of sockeye salmon (Amano et al. 2006). Similarly, incubation of grass puffer pituitaries with goldfish Gnih1 increased *lhβ* and *fshβ* mRNA levels (Shahjahan et al., 2011). In addition, Biran and colleagues reported that tilapia Gnih increased the release of Lh, Fsh and Gh both *in vivo* and *in vitro* (Biran et al., 2014). In contrast, intraperitoneal administration of three Gnih forms in grouper decreased *gnrh1* mRNA expression levels in the hypothalamus, whereas Gnih2 reduced pituitary *lhβ* transcript levels (Wang et al., 2015). cdLPQRFa-1, the cichlid fish Gnih, also inhibited Lhβ and Fshβ release and stimulated Gh secretion in intact pituitary cultures of *Cichlasoma dimerus* (Di Yorio et al., 2016). More recently, we have reported that intramuscular injection of sole Gnih3 provoked a significant reduction in *gnrh3* and *lhβ* expression in this Pleuronectiform species (Aliaga-Guerrero et al., 2018). In other cases, for instance in goldfish, Gnih exerts stimulatory or inhibitory effects on gonadotropin synthesis or secretion, depending on the reproductive stage of animals (Moussavi et al., 2012, 2013, 2014). These discrepancies might also be explained by the nature of Gnih peptide or assay (homologous or heterologous) used, the type of receptor to which this neurohormone binds and/or a downregulation of Gnih receptors provoked when high concentration of Gnih is administered. In addition, it is also possible that Gnih effects are dependent on the species, the sex of the animals and/or the route of administration of the neuropeptides (Muñoz-Cueto et al., 2017).

3.1. Gnih actions in the sea bass brain

All the previous functional studies addressing the effects of Gnih in fish used *in vitro* approaches or intraperitoneal injections. Considering that Gnih is a neuropeptide that is mainly synthesized and delivered into the brain, we decided to analyze, for the first time in fish, the effects of intracerebroventricular (icv) injections of sbGnih1 and sbGnih2 peptides, at three different doses, 1, 2 and 4 Âµg per animal in male sea bass (Paullada-Salmerón et al., 2016b). At the brain level, Gnih1 was able to decrease the expression of Gnrh1 isoform at the three doses tested. These effects were consistent with immunohistochemical studies that revealed an intense Gnih innervation in the ventral telencephalic and preoptic regions, where Gnrh1 cells are found (Paullada-Salmerón et al., 2016a; González-Martínez et al., 2001; 2002a). In order to identify putative contacts of Gnih fibers on Gnrh1cells, Gnih immunostaining was combined with Gnrh1 immunofluorescence. The specificity of these antibodies was demonstrated in previous studies in sea bass (Paullada-Salmerón et al., 2016a; González-Martínez et al., 2002a). For sbGnih immunostaining, sections were incubated with rabbit sbGnih2 antibody (1:500), followed by goat anti-rabbit secondary antibody coupled to Alexa Fluor 488 (1:200). Second immunostaining was performed by incubating the same brain sections with guinea pig sbGnrh1 antiserum (1:1000), followed by goat anti-guinea pig secondary antibody coupled to Alexa Fluor 594 (1:200). Double immunostaining followed by confocal analysis showed that Gnih axons established contacts with cell bodies and dendrites of preoptic Gnrh1 cells (Fig. 4A and C). Some kind of plasticity in this innervation seems to exist because the number of Gnih axons and their contacts on preoptic Gnrh cells appears higher during the resting season (summer, Fig. 4A and C) compared to the reproductive season (winter, Fig. 4B), reinforcing the inhibitory nature of Gnih in the brain of sea bass (Paullada-Salmerón et al., 2016a,b). Interestingly, an *in situ* hybridization study revealed the presence of *gnih-r* expressing cells in preoptic and hypothalamic cell nuclei of goldfish also known for containing hypophysiotropic Gnrh cells (Qi et al., 2013), suggesting that Gnih may act directly on Gnrh cells of this species through specific Gnih-rs. In this context, on-going studies in our laboratory are being directed to elucidate whether Gnih-r are present in Gnrh1 cells of sea bass. In contrast, no apparent contacts between Gnih and Gnrh1 cells were detected in the neotropical cichlid fish *Cichlasoma dimerus* or

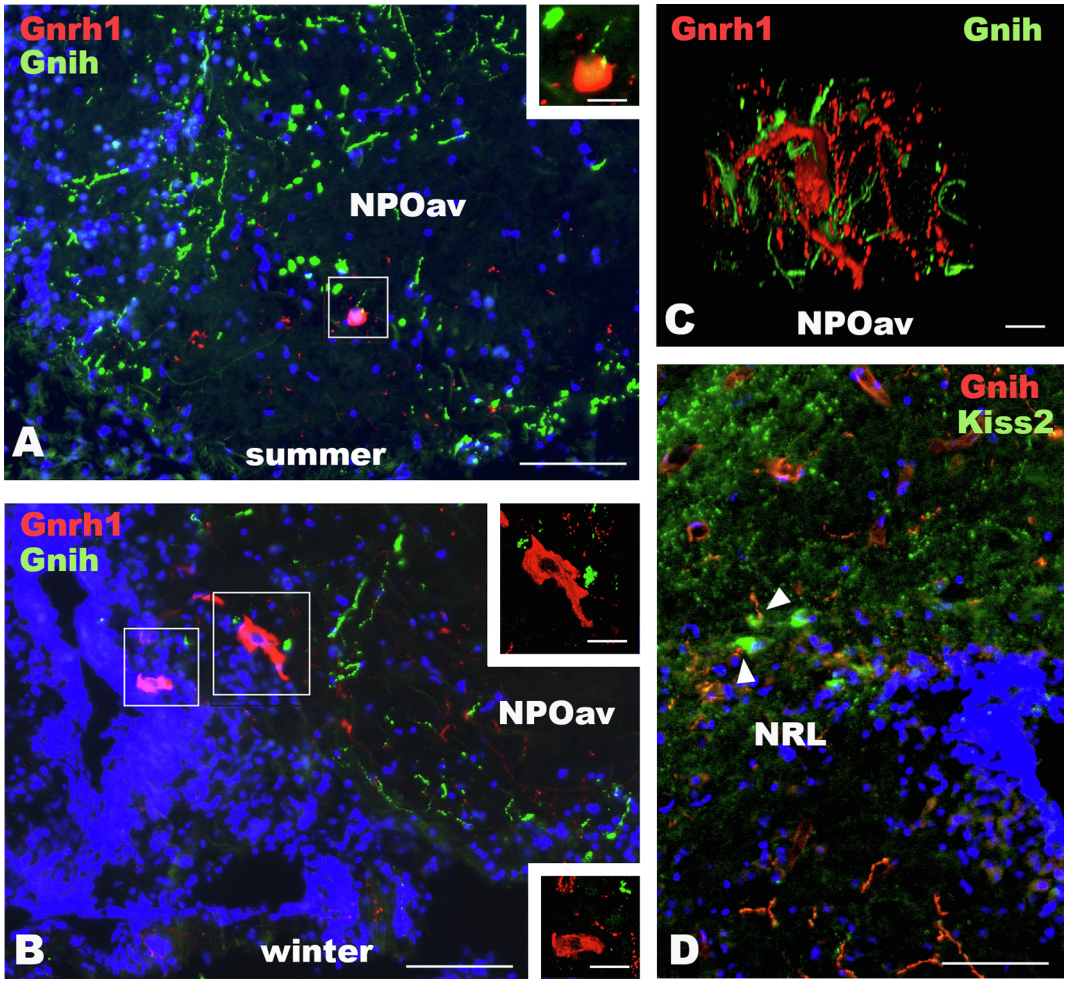

Fig. 4. Gnih innervation in the preoptic area and hypothalamus of sea bass. **A.** Gnih fibers (green fluorescence) establishing contacts with a Gnrh1 cell (red fluorescence) in the anteroventral part of the parvocellular preoptic nucleus (NPOav). Note the profuse Gnih innervation in the preoptic area of immature sea bass (summer, resting season). **B.** Gnih fibers (green fluorescence) in the anteroventral part of the parvocellular preoptic nucleus (NPOav) close to two Gnrh1 cells (red fluorescence). Note that Gnih innervation is markedly reduced in the preoptic area of mature sea bass (winter, reproductive season) compared to resting specimens at summer (A) and that Gnih fibers do not contact the Gnrh1 cells (see insets for detail). **C.** Confocal image of a Gnrh1 cell (red fluorescence) surrounded by a profuse Gnih innervation (green fluorescence) in the preoptic area of a resting specimen (summer). **D.** Gnih fibers (red fluorescence) in close contact with Kiss2 cells (green fluorescence, white arrowheads) of the hypothalamic nucleus of the lateral recess (NRL). Squared area containing Gnrh1 cells in A and B are magnified in insets. Scale bars = 50 μm in A, B and D, 20 μm in C and 10 μm in insets. The immunostaining for sbGnih was performed according to Paullada-Salmerón et al. (2016a). Gnrh1 and kiss2 immunostainings were performed according to González-Martínez et al. (2002a) and Escobar et al. (2013b), respectively.

tilapia (Ogawa et al., 2016; Di Yorio et al., 2018), and *gnih-r* expression was not evident in Gnrh1 neurons of tilapia (Ogawa et al., 2016). Whether this discrepancy reveals species-specific differences or reflects a transient plasticity of the Gnih innervation remains to be deciphered. In turn, the three doses tested of Gnih2 reduced the transcripts levels of the Gnrh2 form, revealing that Gnih can also control the synthesis of non-hypophysiotrophic Gnrhs in this species (González-Martínez et al., 2002a; Paullada-Salmerón et al., 2016b). In sea bass, many brain areas, and notably sensorimotor regions, exhibit a dense innervation originating in tegmental Gnrh2 cells (González-Martínez et al., 2002a). Furthermore, Gnih is also produced in tegmental midbrain cells of sea bass, and innervated profusely sensory-motor areas and the spinal cord (Paullada-Salmerón et al., 2016a). Interestingly, GnIH participates not only in neuroendocrine functions related to reproduction processes but also in behavioral control in birds and mammals (for review see Ubuka et al., 2016). Therefore, both neuropeptides could interact in the modulation of sensory-motor activity and behavior of sea bass.

We also provided physiological evidence of the action of Gnih on Kisspeptin system of sea bass. In our study, the animals treated with sbGnih2 showed a decrease of *kiss1* and *kiss2* expression, as well as in transcripts levels of the *kiss1* receptor (Paullada-Salmerón et al., 2016b). In sea bass, both *kiss1* and *kiss2* mRNAs were detected by RT-PCR in the brain and gonads, and their stimulatory role in the control of reproduction have also been revealed (Felip et al., 2009; Espigares et al., 2015; Alvarado et al., 2013). Additionally, recent studies reported the presence of conspicuous population of Kiss2 cells surrounding the extensions of the hypothalamic lateral recess as well as a population of Kiss1 neurons in the habenula (Escobar et al., 2013a). We have recently revealed that sbGnih-immunoreactive fibers innervated profusely the different subdivisions of the nucleus of lateral recess of sea bass and the habenula, where Kiss2 and Kiss1 cells, respectively, are located (Escobar et al., 2013a; Paullada-Salmerón et al., 2016a). As reported previously for interactions between Gnih and Gnrh1 systems, double immunostaining has shown the presence of Gnih fibers in close contact with Kiss2 neurons of sea bass (Fig. 4D). Taken together, these results suggest that Gnih can regulate the reproductive process of sea bass by acting on brain Gnrh and Kisspeptins systems. In contrast, no apparent interactions between Gnih and Kiss2 cells, nor the presence of Gnih receptors in Kiss2 cells was evident in tilapia (Ogawa et al., 2016).

It has been reported that GnIH plays a relevant function in the

modulation of socio-sexual behavior in birds (Bentley et al., 2006; Ubuka et al., 2012b) and mammals (Johnson et al., 2007; Piekarski et al., 2013). It is interesting to note that the European sea bass is a species that exhibits diurnal feeding and locomotor activity patterns during the resting season, but switches to nocturnal in winter, corresponding with its reproductive season (Sánchez-Vázquez et al., 1998; Villamizar et al., 2012). According to these results, in a recent study performed in our laboratory we found that control animal increase progressively their nocturnal behavior as they approach the reproductive season, whereas peripherally Gnih-implanted sea bass specimens exhibited a significant increase in diurnal activity during the same period (Paullada-Salmerón et al., 2016c). Whether these actions are determined by Gnih itself or are mediated by the effects of peripheral Gnih in the feedback of gonadal steroids into the brain remains to be elucidated. All together, these findings suggest that Gnih might be regulating the reproductive process of sea bass by acting at the neuroendocrine brain but also by modulating the sea bass behavior.

3.2. Gnih actions in the sea bass pituitary

At the pituitary level, our results showed that treatment with sbGnih2 decreased both *lhβ* and *fshβ* mRNA levels and diminished Lh plasma level. Likewise, the sbGnih1 peptide reduced the Lh plasma level in sea bass (Paullada-Salmerón et al., 2016b). A recent study performed in tilapia revealed the presence of Gnih-immunoreactive fibers ending in the vicinity of pituitary Fsh and Lh cells (Ogawa et al., 2016). In sea bass, Gnih-immunoreactive fibers originated in telencephalic and preoptic Gnih cell populations ran through the ventral hypothalamus and innervated the pituitary (Fig. 3). As in tilapia, these Gnih-positive fibers were also observed in close proximity to the Fsh and Lh cells of the proximal pars distalis, supporting the neuroendocrine effects of Gnih on gonadotropin synthesis and/or secretion observed in sea bass (Paullada-Salmerón et al., 2016a,b). At present, five Gnrh receptor subtypes have been characterized in sea bass but only one of these receptors (known as Gnrhr-II-1a) is strongly expressed in the pituitary (González-Martinez et al., 2004) and has some affinity for the hyphophysiotropic Gnrh1 isoform (Servili et al., 2010). Hence, Gnih actions on gonadotropin synthesis and release could also be exerted by modulating Gnrh signaling in the pituitary. Interestingly, sea bass treated with sbGnih2 peptide exhibited decreased *gnrhr-II-1a* mRNA levels in the pituitary (Paullada-Salmerón et al., 2016b).

3.3. Gnih actions in the sea bass testis

In male fish, both pituitary gonadotropins, Lh and Fsh, regulate sex steroid production in Leydig cells and Sertoli cell activities required for proper spermatogenesis. During male gametogenesis, androgens gradually increase their levels as spermatogenesis proceeds, decreasing thereafter at spermiogenesis and spermiation, which are associated with the increase in progestin levels, and all these sequential changes drive the proliferation, development and maturation of gametes (Schulz et al., 2010). In turn, gonadal steroids are also used by the brain and the pituitary as indicators of the sexual status via short and long regulatory feedback loops (Yaron et al., 2003). In addition to the brain, gonads have been reported as a source of Gnih in birds, mammals and fish (Bentley et al., 2008; Singh et al., 2011; Zhang et al., 2010; Biran et al., 2014; Paullada-Salmerón et al., 2016a; Corchuelo et al., 2017). However, research focused on Gnih actions in gametogenesis and steroidogenesis in fish is rather scarce (Qi et al., 2013; Wang et al., 2017). Therefore, we next explored the possibility that Gnih may also act on the regulation of gonadal development and steroidogenesis in male sea bass (Paullada-Salmerón et al., 2016c). For this study, we performed monthly peripheral implants of sbGnih (sbGnih1 and sbGnih2) during critical periods of the reproductive cycle, from October (pre-spermatogenesis) to February (spermiation). We found that both sbGnih1 and sbGnih2 caused an inhibition of the testicular steroidogenesis (testosterone and 11-ketotestosterone levels) at early- and mid-spermatogenesis (November and December) and also induced a delay in development of gonads, which exhibited partial spermatogenesis with abundant type A spermatogonia and scattered isolated clusters of spermatozoids. In contrast, a previous study performed in male goldfish showed that implantation of gfGnih2 or gfGnih3 increased serum testosterone levels (Qi et al., 2013). Whether or not this disparity between species is determined by differences in doses and/or time of administration of Gnih peptides will require further investigation in the future.

4. Regulation of Gnih system in sea bass.

So far, many evidences obtained in tetrapods have shown that GnIH system is mediating the effects of photoperiod on different physiological processes, including reproduction, suggesting that its expression could be modulated through a melatonin-dependent process (Kriegsfeld et al., 2015; Tsutsui et al., 2013). As mentioned in the introduction, the reproductive axis of fish is controlled by environmental factors such as photoperiod and temperature, which display daily and seasonal changes. In fish, the pineal gland plays a key role in transducing both light and temperature cues into a neuroendocrine signal represented by melatonin, that acts on the regulation on reproduction at all levels of the reproductive axis (Bayarri et al., 2003; Falcón et al., 2010). Indeed, many evidences have shown that melatonin effects on the reproductive axis of fish can be stimulatory (Carrillo et al. 1995; Khan and Thomas 1996, Amano et al. 2000) or inhibitory (Amano et al. 2004; Ghosh and Nath 2005) depending on the species. To date in fish, only a few studies have approached the links between the Gnih system with photoperiod and/or melatonin (Shahjahan et al., 2011; Choi et al., 2016; Cowan et al., 2017a; Yumnamcha et al., 2017; Ando et al., 2018). Particularly, it was shown that Gnih was co-localized with the melatonin receptor MT1 in the diencephalon of the cinnamon clownfish (Choi et al., 2016). Further evidence was obtained in zebrafish, in which brain *gnih* mRNA levels were high under constant light conditions and low in constant darkness, and melatonin exogenous treatment reduced *gnih* expression in cultured brain in a dose-dependent manner (Yumnamcha et al., 2017). What is more, in the grass puffer the expression of *gnih* and its receptor showed seasonal oscillations and a diurnal and circadian rhythmicity at the spawning stage, suggesting the possibility that Gnih is regulated by melatonin in this species (Shahjahan et al., 2011; Ando et al., 2018). These authors have proposed that the periodic regulation of *gnih* and its receptor genes by melatonin and circadian clock may be important in the precisely-timed spawning of the grass puffer (Ando et al., 2018). Nevertheless, this regulation seems also to operate in immature fish because we found daily variation in the expression of *gnih* and *gnih receptor* in developing sea bass, with higher diurnal mRNA levels at early stages, until 25 days post-fertilization (dpf), and a shift to higher nocturnal expression levels at 300 dpf (Paullada-Salmerón et al., 2017). These results reinforce the proposed role of melatonin in the modulation of Gnih system sea bass.

As in birds and mammals, the Gnih peptide emerged as a candidate for guiding the seasonal reproduction in fish, and some evidences support this assumption. However, how this photoperiod information is linked to the response of the neuroendocrine systems that control the reproductive process is still to be deciphered. In sea bass, removal of the major site of melatonin production, the pineal gland, decreased *sbgnih* precursor mRNA levels in the midbrain/hindbrain (Cowan et al., 2017a), a region with bilateral connections to the pineal organ (Servili et al., 2010, 2011) and that seems to represent the origin of Gnih fibers reaching the pineal organ in this species (Paullada-Salmerón et al., 2016a). Interestingly, we recently reported that the midbrain and hindbrain of sea bass contained two Gnih cells population, located in the dorsal mesencephalic tegmentum and the secondary gustatory nucleus (NGS) of the rostral rhombencephalon, two regions that exhibit abundant melatonin-binding sites (Herrera-Pérez et al., 2010; Paullada-Salmerón et al., 2016a). In contrast, there was no effect of pinealectomy

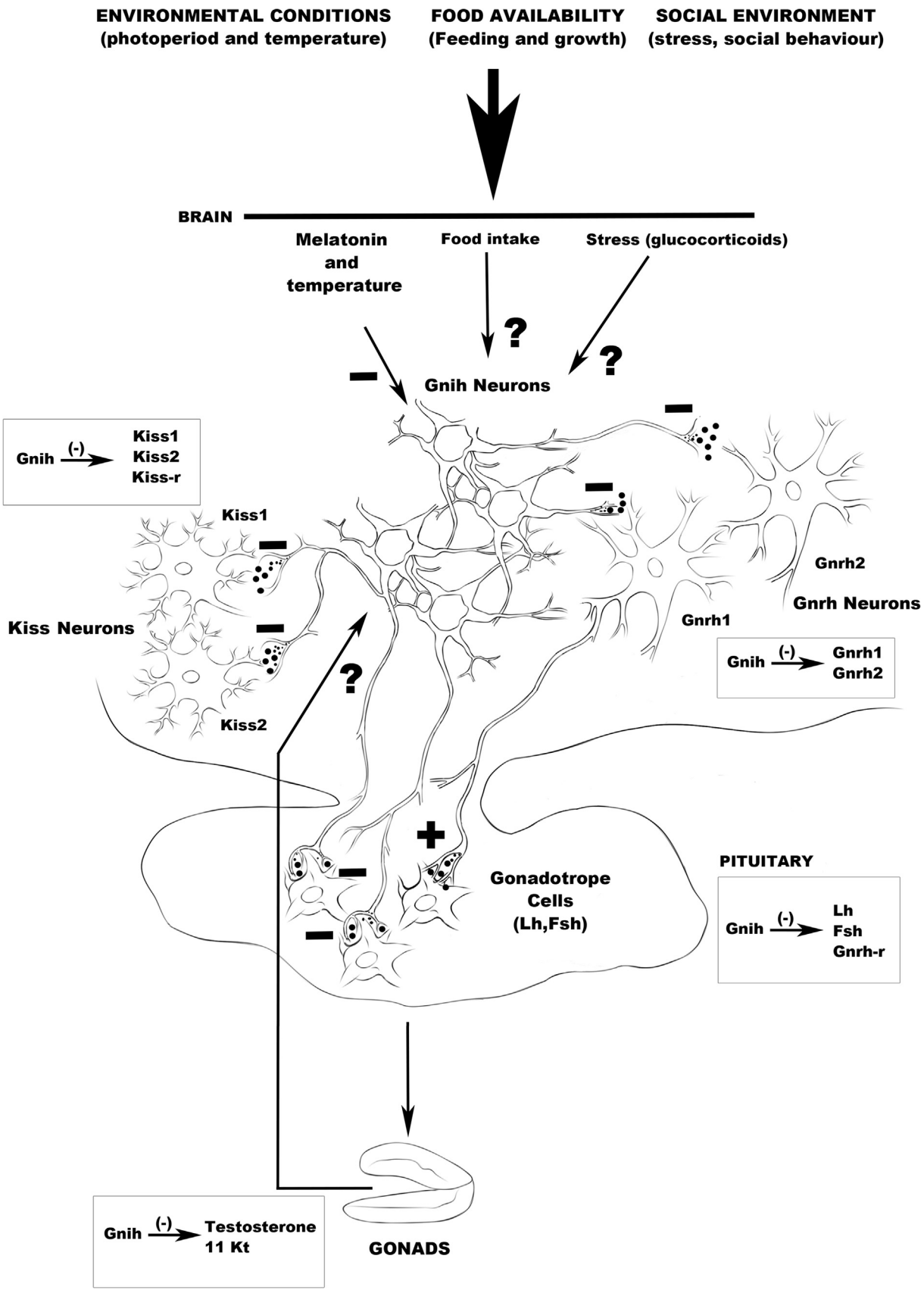

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Fig. 5. Schematic model of the neuroendocrine actions of sbGnih on the reproductive brain-pituitary-gonad axis and its regulation in sea bass. Environmental cues such as daily/seasonal photoperiod and temperature, as well as social behaviour, stress or food availability, might influence on Gnih neurons, which could be mediating these environmental effects on reproduction and other physiological axes in sea bass. Gnih plays an inhibitory role in the reproductive axis of sea bass by acting at the brain (inhibiting GnRH and kisspeptin synthesis), pituitary (inhibiting gonadotropin synthesis and release) and gonads (decreasing androgen secretion and delaying gametogenesis). Abbreviations: Fsh, follicle-stimulating hormone; Gnih, gonadotropin-inhibitory hormone; Gnrh, gonadotropin-releasing hormone; Gnrh-r, Gnrh receptor; Lh, luteinizing hormone; kiss1, Kisspeptin type 1; Kiss2, kisspeptin type 2; Kiss-r, kisspeptin receptor; 11Kt, 11-ketotestosterone. Inhibitory and stimulatory actions are indicated by symbols – and +, respectively. The symbol ? reflects unknown actions that require further research.

on *sbgnih* expression in the telencephalon and diencephalon of sea bass (Cowan et al. 2017a). However, the transcripts of diencephalic *sbgnih* and its receptor exhibited marked seasonal differences in expression, i.e. higher levels in August (resting season) and lower levels in March (reproductive season), suggesting that this Gnih system could also be transducing seasonal information in sea bass (Cowan et al., 2017a). It should be noted that this seasonal *gnih* expression pattern in the diencephalon of sea bass is consistent with our morphofunctional study showing a seasonal plasticity in the Gnih innervation of preoptic Gnrh1 cells (i.e., more Gnih fibers in resting season and less Gnih axons during the reproductive season, see Fig. 4). Taken together, our results suggest that different Gnih cell populations can have different roles in sea bass. Thus, midbrain/hindbrain Gnih cell population may be involved in perceiving and integrating photoperiod/pineal information, while the diencephalic Gnih cell masses could have a more direct role in the seasonal neuroendocrine control of reproduction by modulating Gnrh and Kisspeptins secretion.

Temperature also represents an important environmental factor that exhibits daily and seasonal variations and entrains the rhythms in endocrine systems controlling reproduction (Cowan et al., 2017b). In fish, temperature also has remarkable effects during sensitive periods of early development, acting on sex determination, sex differentiation and puberty. For example, in the South American pejerrey (*Odontesthes bonariensis*), a low incubation temperature of 19 °C caused functional feminization with 0% males, and an elevated temperature of 29 °C produced a 100% functional masculinization, while intermediate temperature (25 °C) applied for 8 weeks post-hatching gave balanced sex ratios (Strüssmann et al., 1996; Strüssmann and Patiño, 1999). In addition, temperature seems to be implicated indirectly in the onset of puberty through its effects on somatic growth and energy storage (Svasand et al., 1996).

Rearing temperature also affected sex differentiation in sea bass, in which exposure to high temperature during larval stages provoked a masculinization of the population mediated by an inhibition of aromatase gene expression that seems responsible of biased sex ratio (≥75% of males) found in the aquaculture of this species (Piferrer et al., 2005). In chicks, heat stress caused by the exposition to a high ambient temperature increased the expression of diencephalic GnIH mRNA (Bahry et al., 2018). Recently, it has been suggested in the orange-spotted grouper that the Gnih/Gnih receptor signal might be involved in the regulation of the reproductive function of sex differentiation, gonadal development and sex reversal via regulating the synthesis of both Gnrh and gonadotropins (Wang et al., 2015). Nonetheless, how temperature is modulating the Gnih system of fish at early ontogenetic stages and whether this neuroendocrine system could be mediating the effects of temperature on sex differentiation remained unaddressed in fish. For this purpose, in a recent study we analyzed the *gnih* and *gnih receptor* (*gnih-r*) expression in developing sea bass reared under different temperature regimes, i.e., high temperature (21 °C) and low temperature (15 °C), from early larval stages up to one year of age (Paullada-Salmerón et al., 2017). Our results showed that both *gnih* and *gnih-r* expression increased significantly from hatching to the second/third week of life and decreased thereafter until 120 dpf, to later increase at the onset of sex differentiation at 150 dpf. Subsequently, *gnih* transcript levels dropped significantly during the sex differentiation period (150–240 dpf) until 360 dpf. The different rearing temperature used in this experiment also affected the ontogeny of *gnih* and *gnih-r* gene expression during the thermosensitive period (5–60 dpf) in sea bass. Exposure to high temperature during early development caused a decrease in *gnih* and *gnih-r* transcript levels, which was also evident at the end of the sex differentiation period (240–300 dpf). An interesting work performed in sea bass reported that higher water temperature increased methylation of a *cyp19a1a* promoter, which inhibited aromatase expression and resulted in masculinization of genotypic females (Navarro-Martín et al., 2011). The remarkable changes in the expression of *gnih* and *gnih-r* genes during the sex differentiation period and their sensitivity to temperature regimes suggest that Gnih could be involved in this relevant process in sea bass. Whether high temperature is also inducing the methylation of *gnih* and *gnih-r* promoters in this species will require further research in the near future.

5. Conclusions and future directions.

The present overview points to GnIH system as an important actor in the reproductive process of the European sea bass (Fig. 5). Summarizing, our findings have shown that sbGnih precursor encoded two RFamide peptides termed sbGnih1 and sbGnih2. The molecular tools and antibodies against sbGnih orthologues obtained as a result of this work, have allowed us to determine that Gnih cells are not restricted to the diencephalon but can also be found in different telencephalic, mesencephalic and rhombencephalic areas of the sea bass brain. Likewise, the functional studies carried out in this species revealed that both Gnih peptides, but notably sbGnih2, play an inhibitory role in the reproductive axis, by acting on the brain (on Gnrh and kisspeptin expression) and pituitary (on Gnrh receptors and gonadotropin synthesis and release), but also at gonadal level (on testicular steroidogenesis and gametogenesis). As dopamine does not seem to have a relevant inhibitory effect on the reproductive axis of sea bass, our findings make Gnih prime candidate to exert this neurohormonal inhibitory role in this species. However, additional exploration of the relationship between Gnih and dopamine remains necessary to clarify whether this RFamide neuropeptide might have a role on dopaminergic system and function in sea bass.

Our pinealectomy results and the existence of day-night variations in the expression of *gnih* and its receptor point towards a role of Gnih (mainly, Gnih cells localized in the midbrain-hindbrain regions) in perceiving and integrating photoperiodic information. In turn, diencephalic Gnih cell populations (telencephalic and preoptic Gnih cells) seem more plausible candidates to be involved in the neuroendocrine control of reproduction and gonadotropin synthesis and secretion. Finally, the marked changes in gene expression of *gnih* and *gnih receptor* around the sex differentiation period, and the differential effects of rearing temperature during the thermosensitive period on their transcript levels, could be indicating a role of Gnih in the mediation of the effects of temperature on sex differentiation and puberty in sea bass.

Since its discovery, almost two decades ago, most studies to date have examined the impact of GnIH on neuronal functioning, physiology and behavior across species. However, whereas last studies addressed in fish have shed some light on the different mechanisms of Gnih actions on the reproductive process, there are numerous findings that evidence the role of this peptide in functions other than reproduction -such as feeding, stress response and behavior-, and the existence of regulatory mechanisms (e.g., steroid feedback on Gnih cells) that have not been approached in this group of vertebrates (Fig. 5). These new findings

represent a big challenge for researchers working in fish Gnih that could help us to approach some relevant problems arising in the aquaculture industry of sea bass and other fish species.

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