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Research paper

# Morphological relationship between GnIH and GnRH neurons in the brain of the neotropical cichlid fish *Cichlasoma dimerus*

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ABSTRACT

Reproduction is regulated by the hypothalamic-pituitary-gonadal axis. The first neuropeptide identified that regulates this function was the decapeptide gonadotropin-releasing hormone (GnRH). Nowadays, in gnatostomates, a number of GnRH variants have been identified and classified into three different types: GnRH1, GnRH2, and GnRH3. Almost 30 years later, a new peptide that inhibits gonadotropin synthesis and secretion was discovered and thus named as gonadotropin-inhibitory hormone (GnIH). In avians and mammals, the interaction and regulation between GnRH and GnIH neurons has been widely studied; however, in other vertebrate groups there is little information about the relationship between these neurons. In previous works, three GnRH variants and a GnIH propeptide were characterized in *Cichlasoma dimerus*, and it was demonstrated that GnIH inhibited gonadotropins release in this species. Because no innervation was detected at the pituitary level, we speculate that GnIH would inhibit gonadotropins via GnRH. Thus, the aim of the present study was to evaluate the anatomical relationship between neurons expressing GnIH and the three GnRH variants by double labelling confocal immunofluorescence in adults of *C. dimerus*. Our results showed no apparent contacts between GnIH and GnRH1, fiber to fiber interactions between GnIH and GnRH2, and co-localization of GnIH and GnRH3 variant in neurons of the *nucleus olfacto-retinalis*. In conclusion, whether GnIH regulates the expression or secretion of GnRH1 in this species, an indirect modulation seems more plausible. Moreover, the present results suggest an interaction between GnIH and GnRH2 systems. Finally, new clues were provided to investigate the role of *nucleus olfacto-retinalis* cells and putative GnIH and GnRH3 interactions in the modulation of the reproductive network in teleost fish.

## 1. Introduction

In vertebrates, reproduction is regulated by the hypothalamic-pituitary-gonadal axis, which senses different internal and external signals. The first neuropeptide identified in this pathway was the decapeptide gonadotropin-releasing hormone (GnRH), discovered in the 70s in mammals, where it was demonstrated its key role in the control of pituitary gonadotropins and reproduction (Burgus et al., 1971; Matsuo et al., 1971). In all vertebrates, GnRH is synthetized as a prepro-hormone containing the decapeptide itself and a GnRH-associated peptide named GAP (Seeburg and Adelman, 1984; Roch et al., 2014). Nowadays, a number of variants of GnRH have been identified in vertebrates, and multiple GnRH variants are found in the brain of a single species (Guilgur et al., 2006; Roch et al., 2014). These GnRH variants are currently classified into three different types, according to their amino-acid sequence, neuroanatomical localization, embryological origin, and synteny: GnRH1, GnRH2, and GnRH3 (Guilgur et al., 2006; Kim et al., 2011; Tostivint, 2011; Decatur et al., 2013; Plachetzki et al., 2016). In vertebrates, GnRH1 is the most variable GnRH type according to its amino-acid sequence and it is expressed in neurons originated from the olfactory placode during embryogenesis (González-Martínez et al., 2004; Kah et al., 2007). This variant plays the classical

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hypophysiotropic function in mammals, as well as in most other gnathostomes. GnRH2, originally discovered in chicken (Miyamoto et al., 1984), was then found in almost all gnathostomates and it is mainly expressed by midbrain neurons. It has been proposed that this variant plays a key role on reproductive behavior (revised in Parhar et al. (2016)). Finally, GnRH3, originally discovered in salmon (Sherwood et al., 1983), is expressed in ventral forebrain neurons. Although it was considered to be present only in teleost fish species, some reports suggested a more ancient origin (Decatur et al., 2013; Roch et al., 2014). This variant is important as a neuromodulator of olfactory and visual information related to reproduction (Kawai et al., 2010; Umatani et al., 2015) and also plays hypophysiotropic functions, particularly in those teleost species expressing only GnRH2 and GnRH3 as most Cypriniformes and Salmoniformes (Guilgur et al., 2006; Zohar et al., 2010).

Even though for almost 30 years GnRH was considered the main neuropeptide regulating gonadotropin release in vertebrates, Tsutsui et al. (2000) discovered, in the Japanese quail, *Coturnix japonica*, a hypothalamic neuropeptide that directly inhibits gonadotropin release, and thus named as gonadotropin-inhibitory hormone (GnIH). GnIH is a member of the RFamide peptide family that includes among others, kisspeptin and neuropeptide FF (NPFF). In the last years, GnIH orthologs have been identified in tetrapods, fish and prothocordates (Sawada et al., 2002; Ubuka et al., 2009; Osugi et al., 2015; Muñoz-Cueto et al., 2017; Tsutsui et al., 2017). However, in fish, the function of GnIH as an inhibitory factor of reproduction is far from being understood (Muñoz-Cueto et al., 2017); for this reason, some authors prefer to name it as LPXRFamide peptide. The location of GnIH neurons is variable among different vertebrate species, although the presence of a population of GnIH neurons in the periventricular region of the preoptic/hypothalamic area and a profuse innervation of almost all brain regions are common characteristics of all studied vertebrates (Muñoz-Cueto et al., 2017). In avians and mammals, an interaction between GnIH and GnRH neurons was demonstrated, as well as GnIH fiber projections to the median eminence, evidencing that this peptide may affect the reproductive axis either directly on gonadotrops or indirectly via GnRH neurons (Kriegsfeld et al., 2006; Ubuka et al., 2008; Tsutsui and Ubuka, 2016; Peragine et al., 2017). In fish, pituitary innervation by GnIH fibers has not been demonstrated to be a general characteristic, and different scenarios have been observed with respect to the relationship between GnIH and GnRH neurons. While in zebrafish GnIH fibers reach the pituitary, and contact GnRH3 neurons, the hypophysiotropic variant in this species (Spicer et al., 2017), in tilapia, GnIH fibers also reach the pituitary, but no association was observed between GnIH fibers and GnRH1 nor GnRH3 cells (Ogawa et al., 2016).

In our experimental species, the South American cichlid fish *Cichlasoma dimerus*, three GnRHs variants have been identified: GnRH1 (sbGnRH), GnRH2 (cGnRH2) and GnRH3 (sGnRH). The distribution pattern was previously determined, showing that neurons expressing GnRH1 are mainly localized in the preoptic area (POA), while GnRH3 mostly in the forebrain, but some overlapping pattern is observed. These two variants were shown to innervate the pituitary gland, being GnRH1 the most prominent on this respect. As in any other bony fish, GnRH2 expressing cells, are located in the midbrain and their fibers are mainly distributed along the mid and hindbrain but they do not innervate the pituitary gland (Pandolfi et al., 2005). GnIH neurons are located in two discrete nuclei: the nucleus *posterioris periventricularis* (NPP) of the hypothalamus and in the nucleus *olfacto-retinalis* (NOR). GnIH-immunoreactive fibers are present in all brain regions, presenting high density in the nucleus *lateralis tuberis* (NLT) at both sides of the third ventricle, but no fibers were observed at the pituitary level in adult fish (Di Yorio et al., 2016).

Taking into account the scarce information on the relationship between GnIH and GnRH neurons in fish, and considering that morphological associations allow us to infer physiological interactions between these systems, the aim of the present study was to evaluate the anatomical relationship between neurons expressing GnIH and the three GnRH variants in *Cichlasoma dimerus*.

## 2. Materials and methods

### 2.1. Animals

*C. dimerus* adults of both sexes were captured in *Esteros del Riachuelo*, Corrientes, Argentina, and then transferred to the laboratory where they were maintained. Fish were acclimated to a constant temperature at 25 ± 2 °C and photoperiod (14 L:10 D) in 130 l fresh water aquaria prior to the experiments and they were daily fed with commercial pellets (#310; *Mixes del Sur*, *provincia de Buenos Aires*, *Argentina*). Animals were handled according to the Principles of Laboratory Animal Care (guidelines on the care and the use of fish in research, teaching and testing, Canadian Council on Animal Care, 2005), which were approved by the *Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina* (Protocol #26).

### 2.2. Antisera

The following antisera were used: antisera against *Dicentrachus labrax* GnRH3-GAP (sGAP), GnRH1-GAP (sbGAP) and GnRH2-GAP (cGAP2) (González-Martínez et al., 2002). These antisera produced against GAPs instead of GnRH decapeptides, generate a specific immunostaining avoiding cross-reactivity among GnRH variants. The immunostaining corresponding to GAP antisera co-localized with the ones obtained by antisera against the GnRH decapeptides (González-Martínez et al., 2002). Also, in a previous work it was demonstrated that these antisera recognized *C. dimerus* GAPs and co-localized with GnRH-expressing neurons (Pandolfi et al., 2005). In order to label GnIH neurons, an antiserum raised against a bullfrog peptide, SLKPAANLPQRFa (Koda et al., 2002) was used. This peptide presents a 67% of identity with *Cichlasoma dimerus* GnIH (cdGnIH) and its use was validated in *C. dimerus* in a previous work (Di Yorio et al., 2016). Finally, for preadsorbtion test, a monoclonal antibody, LRH13 (Park and Wakabayashi, 1986), was used in order to label GnRH3 neurons.

### 2.3. Double-labelling immunofluorescence

Fish were anaesthetized with benzocaine 0.1% (w/v) and euthanized by decapitation, and then brains with the pituitary attached were fixed for 18 h in Bouin's solution as previously described by Pérez Sirkin et al. (2012). Next, they were embedded in Paraplast® (Leyca Biosystems, Germany) and cut in parasagittal or coronal sections at 10 μm intervals. Sections were mounted on gelatin-coated slides, deparaffinized in xylene and rehydrated through a graded ethanol gradient with phosphate-buffered saline (PBS) (pH 7.4), and incubated with PBS containing 5% non-fat dry milk at room temperature (RT). Then, they were incubated with the following antisera: guinea pig anti-sGAP, -sbGAP or -cGAP2 (González-Martínez et al., 2002) overnight at 4 °C, at a 1:500 dilution in PBS. After washing sections in PBS, they were incubated with biotinylated anti-guinea pig IgG (1:1000 in PBS; Vector laboratory, Burlingame, California, USA) at RT for 1 h. Later, they were incubated with streptavidin-Alexa 647 (infrared)-conjugated (1:200 in PBS, Invitrogen) at RT for 1 h. Afterwards, sections were incubated with rabbit anti-GnIH antisera (Koda et al., 2002) overnight at 4 °C, at a 1:500 dilution in PBS. After washing the slides in PBS, they were incubated with an anti-rabbit IgG-Alexa 594 (red)-conjugated (1:150 en PBS, Invitrogen) at RT for 1 h. Samples were finally mounted in PBS-glycerin (1:1) and analyzed with a confocal laser microscopy (Olympus FV-30 attached to an Olympus Bx61 microscope).

### 2.4. Specificity of the antisera

Specificity of anti-GAPs and anti-GnIH antisera used in the present study has already been determined in previous works in our biological model (Pandolfi et al., 2005; Di Yorio et al., 2016). Since co-localization between GnRH3 and GnIH was observed, the following tests were performed in order to discard possible cross-reactions:1) Replacement of the sGAP primary antiserum in the first step of the double immunofluorescence procedure by sGAP antiserum preadsorbed with an excess (5 µg/500 µl) of the cdGnIH (Di Yorio et al., 2016), 2) Replacement of the GnIH primary antiserum in the second step of double immunofluorescence procedure by anti-GnIH preadsorbed with an excess (5 µg/500 µl) of sGnRH. In this particular case, the GnRH3 neurons were labeled using LRH13 antibody. In parallel, each antiserum was preadsorbed with an excess of its respective antigen in order to corroborate the process of preadsortion itself.

## 3. Results

Taking into account the distribution of GnRHs and GnIH neurons previously described in Pandolfi et al. (2005) and Di Yorio et al. (2016), the analysis was focused in those areas were somas and a high density of fibers were observed (Fig. 1).

### 3.1. Morphological relationship between GnRH1 and GnIH

Double-labelling immunofluorescence did not show axo-somatic, or fiber-fiber contacts in any of the analyzed areas (Fig. 2); though some GnIH-ir axons were observed close to sbGAP-immunoreactive (-ir) fibers (Fig. 2F, inset, L). It is interesting to remark that sbGAP-ir and GnIH-ir fibers exhibited a different arrangement: while sbGAP-ir fibers were restricted to a ventral position, the highest density of GnIH-ir fibers was detected in a more dorsal position. This is clearly observed in the NOR (Fig. 2A–C), NPP (Fig. 2G–I) and in the NLT (Fig. 2J–L).

### 3.2. Morphological relationship between GnRH2 and GnIH

Double-labelling immunofluorescence did not show axo-somatic contacts in the NOR (Fig. 3A–C), NPP (Fig. 3D–F), nor in midbrain *tegmentum* (MBT) (Fig. 3J–L). However, some fiber-fiber contacts were observed in the NLT (Fig. 3G–I, inset) and the MBT (Fig. 3J–L, inset).

### 3.3. Morphological relationship between GnRH3 and GnIH

Co-localization of sGAP-ir and GnIH-ir was observed in NOR neurons (Fig. 4A–C). Interestingly, no co-localization was observed for GnIH-ir somas of the NPP (Fig. 4D–F). In all analyzed regions there were fibers presenting immunoreactivity to both antisera (Fig. 4F and I, insets), together with other fibers that were labeled with only one of the antisera (Fig. 4).

Specificity controls were performed, in order to discard cross-reactivity of both antisera. Preadsorption tests (anti-sGAP preadsorbed with an excess of cdGnIH peptide and anti-GnIH preadsorbed with an excess of GnRH3 decapeptide) resulted in no loss of immunostaining in the somata and fibers (Supplementary Fig. S1).

## 4. Discussion

In the present work, the anatomical relationship between neurons and fibers expressing GnIH and GnRH variants were studied in the cichlid fish, *C. dimerus*. This study showed that GnIH-ir neurons co-expressed GnRH3 in the NOR and GnIH-ir fibers presented contacts with GnRH2-ir fibers but not with GnRH1-ir perikarya and cell processes.

Generally speaking, the regulation of GnIH on gonadotropins synthesis and release, although controversial, could be direct and/or indirect via GnRH, among others peptides. In this sense, different scenarios have been reported in vertebrates. In mammalian and avian species, GnIH fibers reach the median eminence and contact with GnRH neurons, suggesting both direct and indirect effects on gonadotropin regulation (Bentley et al., 2006). This is in accordance with some reports where GnIH receptors were detected in the gonadotropes and in GnRH1 neurons in the POA (Tsutsui, 2016; Tsutsui and Ubuka, 2016). However, in amphibians and fish, this does not seem to be so clear. In fish, GnIH fibers were found in the neurohypophysis of goldfish (Sawada et al., 2002), sockeye salmon (Amano et al., 2006), sea bass (Paullada-Salmerón et al., 2016a), Nile tilapia (Ogawa et al., 2016), zebrafish (Spicer et al., 2017) and sole (Aliaga-Guerrero et al., 2018). However, in Indian major carp (Biswas et al., 2015), as in *Cichlasoma dimerus* (Di Yorio et al., 2016) adult fish, no fibers were detected at this level. This indicates that in these species, the effect of GnIH on pituitary hormones could be indirect. Alternatively, plasticity in the GnIH innervation could operate in the pituitary of these species depending on the sexual stage. In a previous work, it was demonstrated by immunohistochemistry that GnRH1, originated from preoptic area, represents the main hypophysiotropic variant in *C. dimerus*. GnRH1 expressing neurons are localized along the ventral surface of the forebrain and present an overlapping distribution with GnRH3 neurons (Pandolfi et al., 2005), as reported in other fish species (González-Martínez et al., 2002; Mohamed et al., 2005). In order to clarify the interactions between GnIH and GnRH1 neurons in this species, double labelling

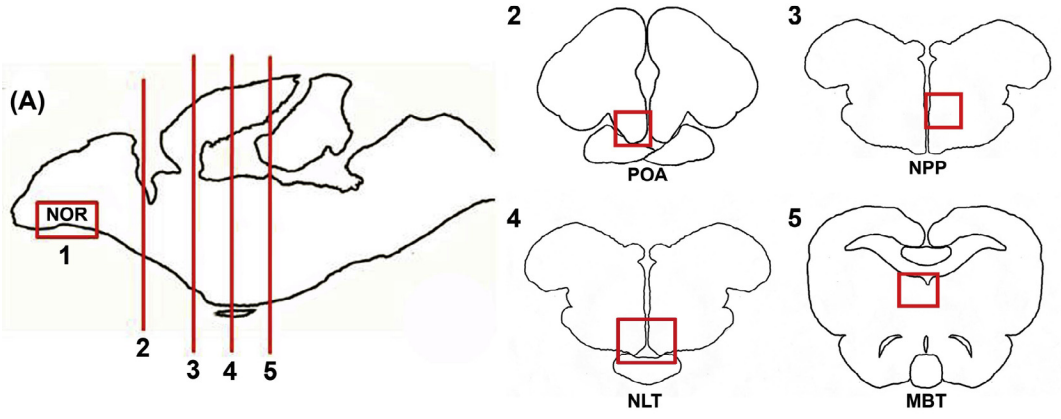


**Fig. 1.** Schematic representation of the regions of *C. dimerus* brain analyzed by double-labelling immunofluorescence. (A) Schematic parasagittal section of *C. dimerus* brain. Boxed area (1) indicates the area where microphotographs were taken at the *nucleus olfacto-retinalis* level. Lines show the position of the coronal sections represented in the *camera lucida* drawing of the telencephalon (2), diencephalon (3–4) and mesencephalon (5). Boxes indicate the areas where microphotographs were taken at the preoptic area (POA) (2), *nucleus posterioris periventricularis* (NPP) (3), *nucleus lateralis tuberis* (NLT) (4), and midbrain *tegmentum* (MBT) (5) level.

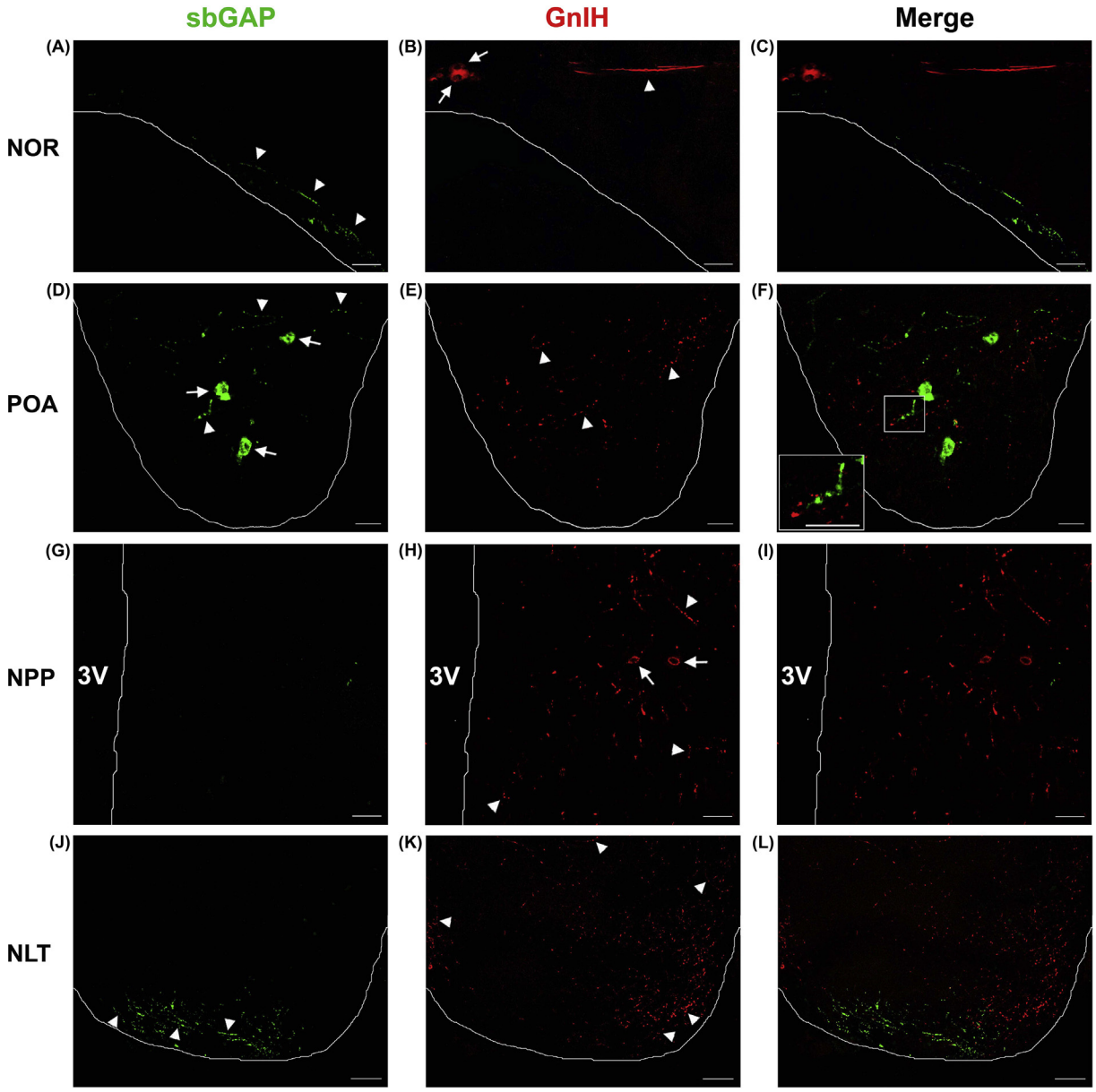


**Fig. 2.** Double-labelling immunofluorescence using GnIH and GnRH1-GAP (sbGAP) antisera in sections of *C. dimerus* brain. Microphotographs present sbGAP-immunoreactive (sbGAP-ir) neurons in green and GnIH-immunoreactive (GnIH-ir) neurons in red. (A–C) Parasagittal sections in the *nucleus olfacto-retinalis* (NOR) showing sbGAP-ir fibers (A) and GnIH-ir somata and fibers (B), and overlay of A and B (C). (D–F) Coronal sections in the preoptic area (POA) showing sbGAP-ir somata and fibers (D) and GnIH-ir fibers (E), and overlay of D and E (F). The squared area containing GnIH-ir axon in close proximity to sbGAP-ir fiber is magnified in the inset. (G-I) Coronal sections in the *nucleus posterioris periventricularis* (NPP) showing no sbGAP-ir fibers (G) and GnIH-ir somata and fibers (H), and overlay of G and H (I). (J–L) Coronal sections in the *nucleus lateralis tuberis* (NLT) showing sbGAP-ir (J) and GnIH-ir (K) fibers and overlay of J and K (L). Immunoreactive somata are indicated with arrows and some representative fibers with arrowheads. A–C and J–L: Scale bar: 50 μm. D–I: Scale bar: 20 μm. Third ventricle: 3 V.

immunohistochemistry was performed. Surprisingly, the results obtained did not show axo-somatic, or fiber-fiber contacts in any of the analyzed areas, although GnIH-ir axons were observed close to GnRH1-ir fibers. Based on these results, it is suggested that GnIH does not directly interact with GnRH1 to inhibit gonadotropins release in this species. This is in agreement with the observed in the POA of tilapia (Ogawa et al., 2016), but contrast with the obtained with the hypophysiotropic variant (GnRH3) in zebrafish (Spicer et al., 2017). Taking into account that in our model species GnIH was not found innervating the pituitary neither GnRH1 neurons, we could speculate that GnIH potentially uses other pathways (kisspeptin, dopamine, neuropeptide Y, etc.) of the reproductive network to control gonadotropin secretion, as suggested in zebrafish (Spicer et al., 2017). Other possibility is that the interaction of GnIH-GnRH1 fibers show plasticity depending on the sexual stage. Finally, considering that a neurovascular regulation plus to the classical innervation of gonadotropes was demonstrated in zebrafish (Golan et al., 2015), it is possible that GnIH reaches the gonadotropes in *C. dimerus* via the vascular system, which is an interesting issue to be evaluated in future studies.

GnRH2, also known as the “midbrain variant,” was described in almost all gnathostomes groups (Kavanaugh et al., 2008). Although it is not present or functional in some mammalian species, it is the most conserved GnRH variant in vertebrates (Pawson et al., 2003; Morgan and Millar, 2004; Kah et al., 2007). Studies in different vertebrate groups proposed that this variant is involved in the regulation of sexual and feeding behavior (Matsuda et al., 2008; Tostivint, 2011). On the other hand, several lines of evidence indicate that GnIH can also modulate behavior and feeding (Tsutsui and Ubuka, 2016). For

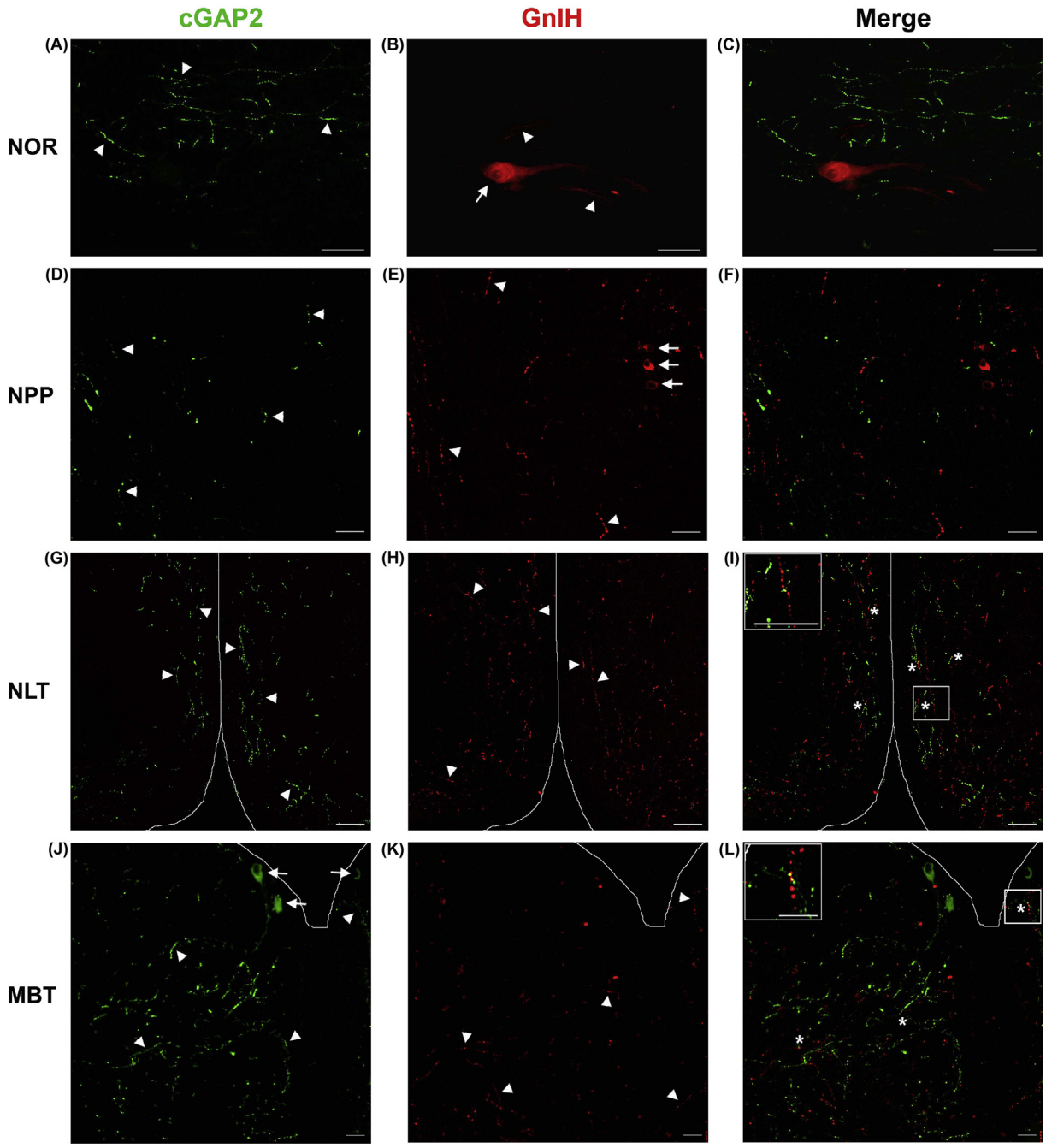


**Fig. 3.** Double-labelling immunofluorescence using GnIH and GnRH2-GAP (cGAP2) antisera in sections of *C. dimerus* brain. Microphotographs present cGAP2-immunoreactive (cGAP2-ir) neurons in green and GnIH-immunoreactive (GnIH-ir) neurons in red. (A–C) Parasagittal sections in the *nucleus olfacto-retinalis* (NOR) showing cGAP2-ir fibers (A) and GnIH-ir somata and fibers (B), and overlay of A and B (C). (D–F) Coronal sections in the *nucleus posterioris periventricularis* (NPP) showing cGAP2-ir fibers (D) and GnIH-ir somata and fibers (E), and overlay of D and E (F). (G–I) Coronal sections in the *nucleus lateralis tuberis* (NLT) showing cGAP2-ir (G) and GnIH-ir (H) fibers and overlay of G and H (I). (J–L) Coronal sections in the midbrain tegmentum (MBT) showing cGAP2-ir somata and fibers (J) and GnIH-ir fibers (K) and overlay of J and K (L). The squared areas containing contacts between fibers are magnified in the insets. Immunoreactive somata are indicated with arrows and some representative fibers with arrowheads. Contacts are indicated with asterisks. A–C and G–I: Scale bar: 50 μm. D–F and J–L: Scale bar: 20 μm.

example, in avians and mammals, intracerebroventicular injections of GnIH stimulates food intake (Tachibana et al., 2005; Johnson et al., 2007; Clarke et al., 2012; Tsutsui and Ubuka, 2016) and in hamsters, food restriction activates GnIH neurons (Klingerman et al., 2011). Also, it was demonstrated that GnIH administration can inhibit sexual motivation in hamsters (Klingerman et al., 2011; Piekarski et al., 2013) and rats (Johnson et al., 2007). However, very little is known about the role of GnIH in fish reproductive behavior. There is only one study regarding the administration of GnIH in sea bass that affects the diurnal to nocturnal ratio to locomotor activity along the reproductive cycle (Paullada-Salmerón et al., 2016c). In this context, the neuroanatomical and functional relationships between both neuropeptidergic systems were reported in vertebrate species from different groups. For example, in song birds GnIH neurons terminated in close proximity to GnRH2 neurons, which express GnIH receptors (Ubuka et al., 2008). Recently, a study performed in a teleost, the European sea bass, has revealed an inhibitory role of sea bass GnIH2 form on brain GnRH2 expression (Paullada-Salmerón et al., 2016b). In Indian major carp (Biswas et al., 2015) and sea bass (Paullada-Salmerón et al., 2016a) GnIH somas were localized in the midbrain, but in our model species, no GnIH somas

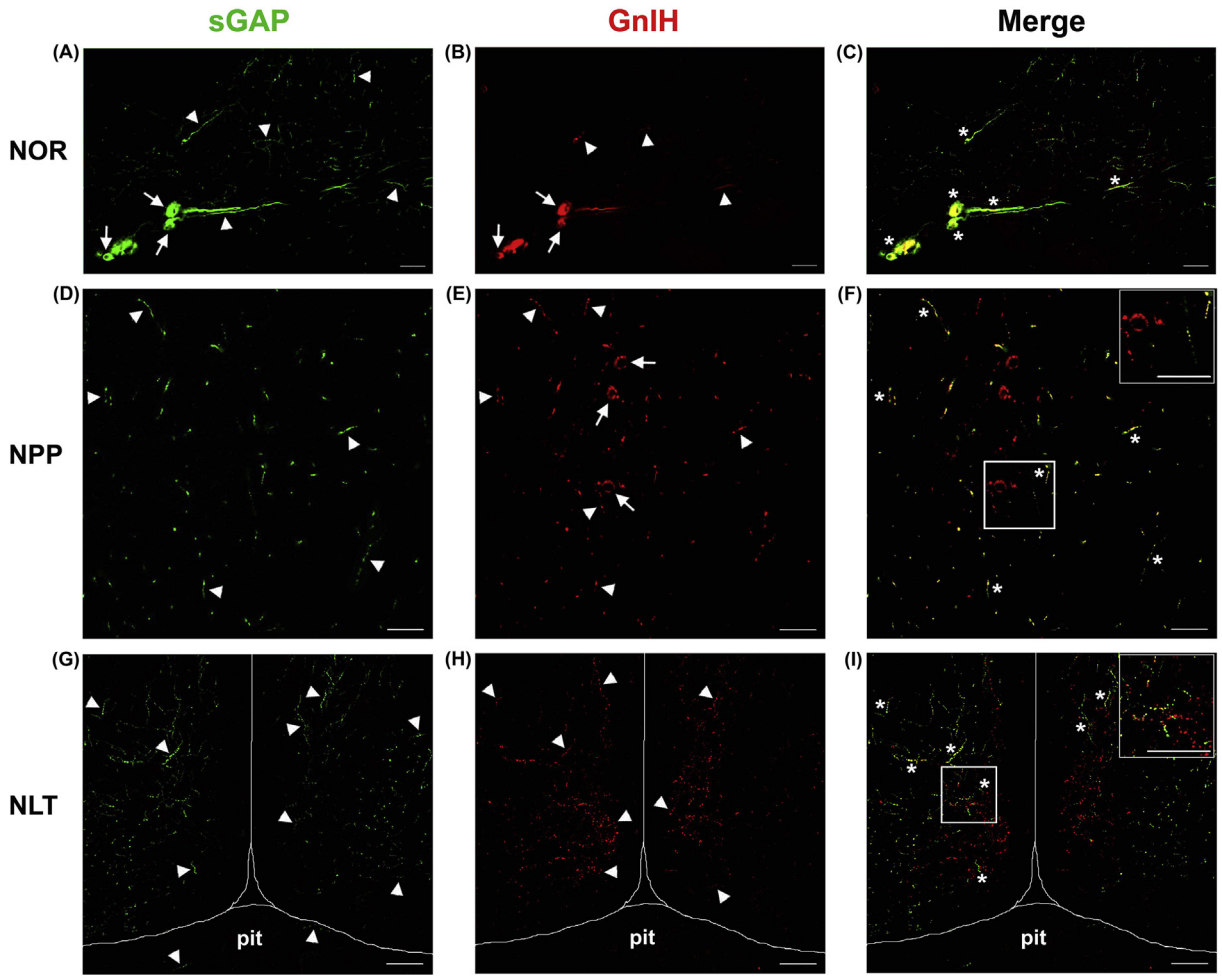


**Fig. 4.** Double-labelling immunofluorescence using GnIH and GnRH3-GAP (sGAP) antisera in sections of *C. dimerus* brain. Microphotographs present sGAP-immunoreactive (sGAP-ir) neurons in green and GnIH-immunoreactive (GnIH-ir) neurons in red. (A–C) Parasagittal sections in the *nucleus olfacto-retinalis* (NOR) showing sGAP-ir (A) and GnIH-ir (B) somata and fibers, and overlay of A and B (C). (D–F) Coronal sections in the *nucleus posterioris periventricularis* (NPP) showing sGAP-ir fibers (D) and GnIH-ir somata and fibers (E), and overlay of D and E (F). (G–I) Coronal sections in the *nucleus lateralis tuberis* (NLT) showing sGAP-ir (G) and GnIH-ir (H) fibers, and overlay of G and H (I). The squared areas containing co-localization and also single label somata or fibers are magnified in the insets. Immunoreactive somata are indicated with arrows and some representative fibers with arrowheads. Co-localization is indicated with asterisks. A–C and G–I: Scale bar: 50.0 μm. D–F: Scale bar: 20.0 μm.

were observed in this region where GnRH2 perikarya are localized. Despite this fact, we could demonstrate that several GnIH fibers in the NLT and in the MBT are in contact with GnRH2 fibers, suggesting a possible regulation. In fish, it has been evidenced that midbrain GnRH2 cells are bidirectionally connected to the pineal organ and modulate melatonin secretion (Servili et al., 2010, 2011), suggesting its involvement in the transduction of environmental effects in reproduction. Interestingly, the GnIH system of sea bass (Paullada-Salmerón et al., 2016a) and sole (Aliaga-Guerrero et al., 2018) also projects to the pineal organ. In addition, in birds, it was demonstrated that GnIH mediates the effects of photoperiod, as GnIH neurons express melatonin receptors and melatonin stimulates hypothalamic GnIH expression and release (Ubuka et al., 2005; Chowdhury et al., 2010). Thus, it remains to be elucidated in future studies whether the interactions between midbrain GnRH2 and GnIH reported in the present study represent a piece of a network involving the pineal organ and mediating the transduction of environmental information to the reproductive axis.

*Cichlasoma dimerus*, as most teleost fish species, present neurons expressing GnRH3 (Pandolfi et al., 2005). As in most bony fish species, the neurons expressing GnRH3 are mainly located in the NOR, although few neurons were detected in the olfactory bulb, ventral telencephalon and the POA (González-Martínez et al., 2002; Somoza et al., 2002; Kah et al., 2007). In *Cichlasoma dimerus*, fibers from these neurons are mainly located in the forebrain and contribute to pituitary innervation (Pandolfi et al., 2005). In those fish where the three GnRHs are present, the function of GnRH3 is not completely understood. There are some reports where this variant is considered as a neuromodulator of reproductive behavior (Yamamoto et al., 1997; Ogawa et al., 2006). In addition, in *C. dimerus* females, the somatic and nuclear area of GnRH3 neurons varies according the reproductive phase suggesting that they are also related to reproduction (Tubert et al., 2012). In previous studies, we observed that GnIH-ir neurons were localized in the NOR and the morphology of these cells was similar to GnRH3 neurons located in this area (Di Yorio et al., 2016). In the present study, we corroborated that both peptides were co-expressed in the same neurons. Similar results were previously obtained in larvae of the same species (Di Yorio et al., 2018). It is interesting to note that in the forebrain some fibers co-expressed both peptides whereas some other fibers only express GnRH3 or GnIH, suggesting that these fibers correspond to neurons located in other brain areas like the NPP (for GnIH) or from the OB, ventral TEL and POA (for GnRH3). On the other hand, at the NPP no contacts between GnRH3 fibers and GnIH neurons were observed, but co-expression was observed in fibers in the NLT and NPP. Although the probability of a cross-reaction was very low, we decided to perform

preadsortion tests where each antiserum was preadsorbed with the respective and the other peptide and the obtained results discarded this possibility. Despite that GnIH neurons in the forebrain were described in many fish species (Muñoz-Cueto et al., 2017), we cannot rule out the possibility that the antiserum used recognized other LPXRFamide peptide as suggested by Sawada et al., (2002) and Ogawa et al., (2016). The present results provide new clues to investigate the possible co-operation or interaction between both peptides in the modulation of the reproductive network in teleost fish.

In summary, we described here the neuroanatomical relationship between GnIH and GnRH neurons in *C. dimerus*. To our knowledge, this is the first report analyzing such relationship considering neurons expressing the three GnRH variants in one species. Thus, we can highlight the following facts: 1) GnIH-ir neurons co-expressed GnRH3 variant in the NOR; 2) As fiber-to-fiber contacts between GnIH-ir and cGAP2-ir were detected, a direct modulation between them can be proposed; 3) Whether GnIH regulates the expression of GnRH1 and/or its secretion in this species, an indirect modulation or neuronal plasticity can be proposed since no contacts between GnIH-ir and sbGAP-ir cell and processes were observed.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ygcen.2018.06.010.

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