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Research Report

Differential co-localization with choline acetyltransferase in nervus terminalis suggests functional differences for GnRH isoforms in bonnethead sharks (*Sphyrna tiburo*)

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ABSTRACT

The nervus terminalis (NT) is a vertebrate cranial nerve whose function in adults is unknown. In bonnethead sharks, the nerve is anatomically independent of the olfactory system, with two major cell populations within one or more ganglia along its exposed length. Most cells are immunoreactive for either gonadotropin-releasing hormone (GnRH) or RF-amide-like peptides. To define further the cell populations and connectivity, we used double-label immunocytochemistry with antisera to different isoforms of GnRH and to choline acetyltransferase (ChAT). The labeling patterns of two GnRH antisera revealed different populations of GnRH-immunoreactive (ir) cell profiles in the NT ganglion. One antiserum labeled a large group of cells and fibers, which likely contain mammalian GnRH (GnRH-I) as described in previous studies and which were ChAT immunoreactive. The other antiserum labeled large club-like structures, which were anuclear, and a sparse number of fibers, but with no clear labeling of cell bodies in the ganglion. These club structures were choline acetyltrasferase (ChAT)-negative, and preabsorption control tests suggest they may contain chicken-GnRH-II (GnRH-II) or dogfish GnRH. The second major NT ganglion cell-type was immunoreactive for RF-amides, which regulate GnRH release in other vertebrates, and may provide an intraganglionic influence on GnRH release. The immunocytochemical and anatomical differences between the two GnRH-immunoreactive profile types indicate possible functional differences for these isoforms in the NT. The club-like structures may be sites of GnRH release into the general circulation since these structures were observed near blood vessels and resembled structures seen in the median eminence of rats.

1. Introduction

The nervus terminalis (NT), which is found in all vertebrate groups, is the most rostral cranial nerve. Although its function has yet to be determined, cells and fibers within the NT contain the hormone gonadotropin-releasing hormone (GnRH; Phillips et al., 1980; Schwanzel-Fukuda and Silverman, 1980; Munz et al., 1982; Wirsig and Getchell, 1986; Wirsig and Leonard, 1986b; White and Meredith, 1995; Kim et al., 1999). In elasmobranchs, the NT contains a higher concentration of GnRH than any other part of the forebrain (Nozaki et al., 1984; Demski et al., 1987),

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suggesting that NT may be important for the reproductive function of GnRH.

The central trunk of the NT extends as a bundle of GnRH-positive and -negative fibers from the peripheral NT ganglion to ventral–caudal regions of the telencephalon, towards the hypothalamus (Stell, 1984; Wright and Demski, 1991; Lovejoy et al., 1992a; Oka and Matsushima, 1993; Forlano et al., 2000). There is no pituitary portal system in elasmobranchs (Dodd, 1983), and it is unknown where GnRH is released or how it reaches gonadotropes in the ventral lobe of the pituitary. It has been proposed that some of the NT fibers lead to the ventral telencephalon or septal area where they might release GnRH (Nozaki, 1985; Lovejoy et al., 1992a). Numerous GnRH-immunoreactive (ir) fibers also extend throughout the forebrain, suggesting a neuromodulatory action on other neural systems (Pfaff et al., 1987; Oka and Matsushima, 1993; Millar, 2003). In Atlantic stingrays, stimulation of the peripheral trunk of NT led to an increase in measurable levels of GnRH in the cerebrospinal fluid, presumably by synaptic release from NT fibers in the brain (Moeller and Meredith, 1998).

The NT has also been suspected of having a chemosensory component since it connects the nose and the brain, and fibers from the peripheral trunks extend into the nasal epithelium (Demski and Northcutt, 1983; Wirsig and Leonard, 1986a; Demski et al., 1987; Koza and Wirsig-Wiechmann, 2001). However, an intrinsic chemosensory function has not been demonstrated and NT fibers appear not to influence olfactory sensory signals directly, at least in elasmobranchs (Meredith and White, 1987). GnRH itself does modulate some olfactory responses in axolotls (Park and Eisthen, 2003) and mudpuppies (Zhang and Delay, 2007). NT is present in mammals, and in male golden hamsters, NT damage caudal to the olfactory bulb leads to some deficits in mating behavior (Wirsig and Leonard, 1987; Wirsig-Wiechmann, 1997). A response generally considered to be GnRH-dependent, the testosterone secretion induced by odors from female hamsters in estrus was not decreased by NT lesions (Wirsig-Wiechmann, 1993), although a NT-dependent pathway might have survived, via NT connections to the accessory olfactory bulb (Wirsig and Leonard, 1986a) and on to medial amygdala.

It is now understood that GnRH decapeptides are produced from three genes (in fish) and can be classified into three corresponding families (GnRH-I, -II, and -III) (Fernald and White, 1999; Millar et al., 2004). Differences in anatomical location suggest functional differences in the release and action of different GnRH isoforms (Phillips et al., 1987; Muske, 1993; Sherwood et al., 1993; Muske et al., 1994; Rissman et al., 1995; King and Miller, 1995; Latimer et al., 2000; Millar, 2003; Millar et al., 2004; Temple et al., 2003). The putative ancestral form, GnRH-II (His^{5}Trp^{7}Tyr^8-GnRH; formerly chicken-GnRH-II; Millar, 2003), generally expressed in a cluster of cells in the midbrain, is completely conserved across species. It can act as a neuromodulator (Troskie et al., 1998) and can facilitate reproductive behavior in birds (Maney et al., 1997) and food-restricted musk shrews (Temple et al., 2003). At least one additional isoform is expressed elsewhere in the brain (Millar et al., 2004), generally a GnRH-I or GnRH-III-family peptide responsible for LH release from the pituitary. GnRH-I peptides are quite variable in structure and are most visible in a separate population of cells along the NT–septum–preoptic axis (Jennes and Stumpf, 1986; Peter et al., 1987; Lehman et al., 1987; Silverman et al., 1987; Forlano et al., 2000; Dubois et al., 2002). The originally identified peptide, mammalian GnRH-I (mGnRH), is the releasing peptide in most mammals, amphibia, and probably in some teleost fish (Dubois et al., 2002). A variant GnRH-I (Gln^8-GnRH; formerly chicken-I GnRH) is the releasing peptide in birds and reptiles, and other variants have been identified in the preoptic area of several teleosts and one mammal (Grove-Strawser et al., 2002). Most teleosts have the (well-conserved) GnRH-III peptide (Trp^7-Leu^8-GnRH, originally named salmon GnRH) replacing GnRH-I as the LH-releasing peptide (Palevitch et al., 2009), but some also express additional isoforms (Pandolfi et al., 2005). Mammalian GnRH-I (mGnRH) can also act as a neuromodulator if applied exogenously (Pan et al., 1988) and has been suggested to mediate a neural (intracerebral) facilitation of reproductive behavior in male and female mammals (Dorsa and Smith, 1980; Moss and Dudley, 1989; Fernandez-Fewell and Meredith, 1995; Blake and Meredith, 2010) independently of its LH-releasing function.

Elasmobranchs, which lack a developed pituitary portal system, express GnRH-II in the midbrain, and often two or more other isoforms, concentrated more in neurons of the NT nerve and ganglia than in the preoptic area, and generally considered to be involved in gonadotropin release.

Within the NT, a close relationship has been shown in elasmobranchs and some other species, between cells expressing GnRH and fibers immunoreactive to antisera raised against RF-amide (RFa) peptides, e.g., FMRF-amide (Phe-Met-Arg-Phe-NH_2; Stell, 1984; Muske and Moore, 1988; Wirsig-Wiechmann and Basinger, 1988; Chiba, 2000) or LPLRF-amide (Leu-Pro-Leu-Arg-Phe-NH_2; White and Meredith, 1995).

A similar close relationship has recently been demonstrated in the ventral forebrain of birds and mammals (see Tsutsui et al., 2010; Johnson et al., 2007; Simonneaux et al., 2009). The RF-amide family peptides, including kisspeptin, gonadotropin inhibitory peptide (GnIH) and RFRP1/3, are critically involved in regulating release of GnRH acutely and according to season in birds, mammals, and reptiles and probably in teleosts (Johnson et al., 2007; Kriegsfeld et al., 2006; Simonneaux et al., 2009; Clarke et al., 2009; Oka, 2009). In elasmobranchs, the function of the RFa-ir cell groups in NT is unknown, but they are placed in an ideal location to influence GnRH release from the GnRH system(s) in NT.

In bonnethead sharks (*Sphyrna tiburo*), one or more ganglia are situated along the exposed length of the NT nerve between the olfactory bulbs and the nerve's entry into the forebrain. Cells within the large main ganglion can be classified into two types. One cell class is GnRH-ir as described above and is co-localized with a plexus of catecholamine-ir fibers. A second, distinct class is immunoreactive to antibodies raised against RFa peptides (including LPRFa). Both were acetylcholinesterase-positive (White and Meredith, 1995).

Here we report on two classes of GnRH-ir cell profiles in the NT ganglion in addition to the RF-amide-ir cells. They express different isoforms of GnRH, and one (only) shows co-localization of choline acetyltransferase (ChAT) immunoreactivity. Their anatomical and cytochemical differences suggest potential differences in function. Early parts of this study were published in abstract form (Moeller et al., 1997).

2. Results

The GF-5 antiserum, raised against salmon GnRH, labeled large clusters of cells distributed throughout the NT ganglion (Figs. 1A and 3A). Cells tended to be oval and near 20 μm in diameter. The antiserum also labeled bundles of fibers, some of which can be seen traveling through both the peripheral and central nerve trunks. Surrounding the labeled cells were areas, generally similar in size, with no labeled cell bodies. Tracts of labeled fibers, however, traversed clusters of these unlabeled cell bodies.

The 7CR-10 antiserum, raised against dogfish GnRH (His^5, Trp^7, Tyr^8 GnRH), showed a strikingly different labeling pattern (Figs. 1B and 3F). Compared to the GF-5 pattern, overall labeling was extremely sparse. Cell bodies were rarely labeled: only two ganglia out of twelve had one or two faintly stained structures, which appeared cell-like (Fig. 3F). A few isolated fibers with varicosities traversed all segments of the ganglion in no apparent overall pattern. Occasionally larger club-like structures capped the ends of these isolated fibers. Some were up to 20 μm in diameter, the size of cell bodies. In sections of the NT ganglion double labeled with 7CR-10 antiserum and Hoechst dye, there was clearly no co-localization of the blue fluorescent nuclear marker with the large club-like structures labeled with this GnRH antiserum (Fig. 2A). Moreover, these club-like structures were often observed near blood vessels. With the confocal microscope, it was possible to image blood vessels with DIC transmission optics and then take a single 1-μm optical section of fluorescence at the same focal plane and position. The micrograph in Fig. 2B, for example, shows a blood vessel traversing the section surrounded by cell structures labeled with the 7CR-10 antiserum. A club-like structure (arrow) can be seen near the cross-section of a blood vessel (arrowhead), as it runs perpendicular to the plane of the section. Numerous other club-like structures were also observed adjacent to blood vessels. In fact, whenever these structures were investigated by confocal sectioning, they were located next to blood vessels. This examination included one or two clubs in at least four ganglia acquired from separate specimens. However, a quantitative and systematic study of this relationship was not undertaken here.

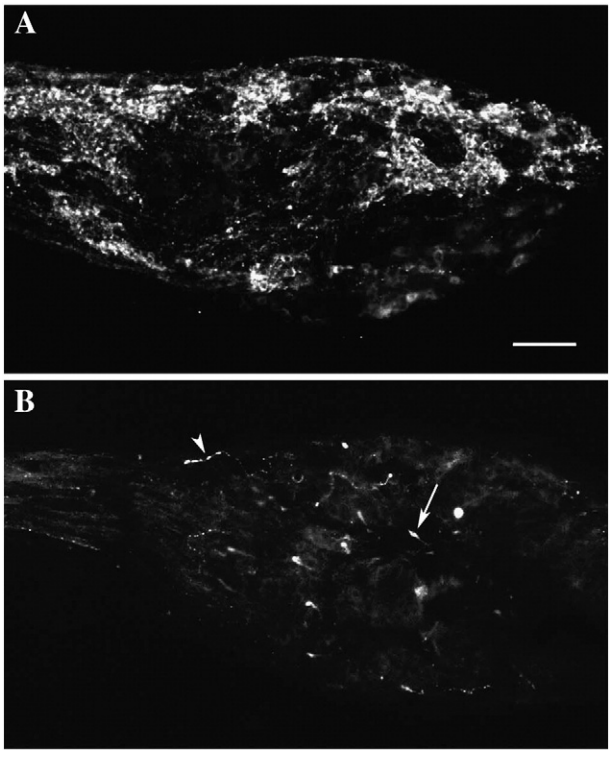

Fig. 1 – Examples of differences in GnRH immunocytochemistry in the ganglion of bonnethead NT. (A) Labeling with GF-5 antiserum showing large clusters of GnRH-ir cells and tracts of fibers. (B) Labeling with 7CR-10 antiserum showing relatively sparse labeling of varicose fibers (arrowhead) and a few club-like structures (arrow). Scale bar = 100 μm.

We were also unable to determine the cellular origin of these neural fibers. Two cells displayed faint labeling with 7CR-10, but this may have been nonspecific background signal, especially since no fibers were seen extending from these cells. Labeled fibers, however, passed through both nerve trunks (Figs. 1B and 2F). Elasmobranchs are particularly difficult for tracing neural connections, and we have not yet succeeded in labeling cells in the brain or periphery with tracers placed in the main ganglion.

To control for cross-reactivity and to provide a tentative identification of the GnRH isoforms present, normal patterns of immunoreactivity with the two GnRH antisera were compared to those observed after preabsorption of the antisera with an excess of one of four isotypes of GnRH. Labeling with GF-5 antiserum (Fig. 3A–E) was clearly blocked by preabsorption with mammalian (mGnRH) GNRH-I or salmon (sGnRH) GnRH-III but not with lamprey GnRH (lGnRH). Preabsorption with chicken-II (cGnRH-II) (GnRH-II) decreased the intensity of GF-5 labeling, especially in the fiber bundles, but it did not completely block labeling. The percent cross-reactivities for the GF-5 antiserum reported by Lescheid et al. (1997) were 100% (mammalian), 68.8% (salmon), 3.9% (chicken-II), and <0.03% (lamprey). Labeling with 7CR-10 antiserum (Fig. 3F–J) was completely blocked with cGnRH-II) and sGnRH. There was strong 7CR-10 labeling (no apparent block) after preabsorption with mGnRH, but only a faint signal with lGnRH preabsorption. The percent cross-reactivities for the 7CR-10 antiserum reported by Lescheid et al. (1997) were 100% (chicken-II), 84.8% (salmon), 6.0% (lamprey), and <0.03% (mammalian).

The ChAT antiserum labeled distinct clusters of cells within the NT ganglion, and only GF-5 labeling was specifically co-localized with ChAT-positive cells in double-label experiments (Fig. 2C). This co-localization was seen in every cell that was ChAT-positive. Correspondingly, there was no GF-5-ir cell that was not ChAT-positive. Preabsorption with an excess of ChAT protein inhibited all labeling with ChAT antiserum (not shown). Neither FMRFa-ir cells (Fig. 2D) nor 7CR-10-labeled cell structures (Fig. 2E) were ChAT-positive. Club-like structures labeled with 7CR-10 were often located among ChAT-positive cells but were not labeled with ChAT antiserum. We attempted to double-label sections with the GF-5 and 7CR-10 antisera, to determine if their targets were co-localized. These antisera were both raised in rabbits so we used techniques for enzyme precipitation and antibody fragments that were designed to

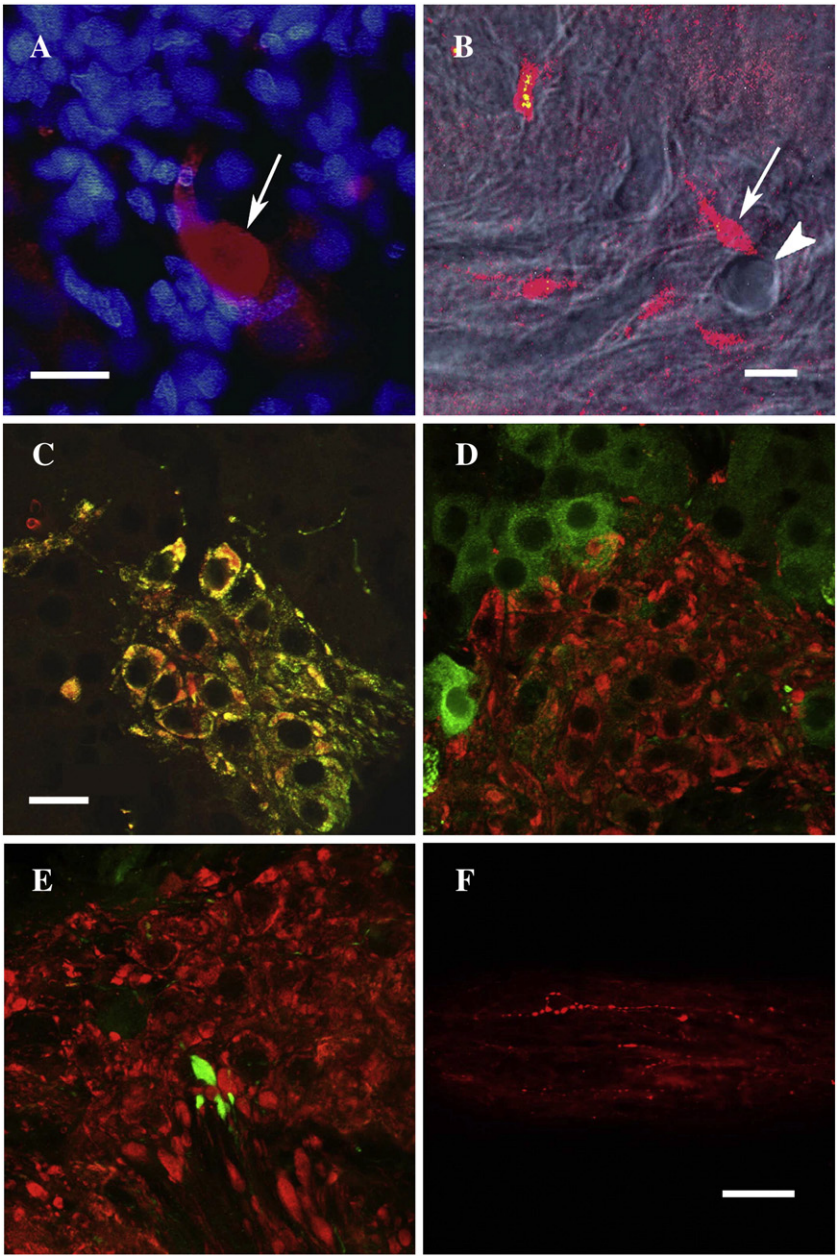

Fig. 2 – Confocal micrographs of GnRH and ChAT immunocytochemistry in NT ganglion. (A) Large club-like structure labeled with 7CR-10 (arrow) contains no blue-stained nuclear material (Hoechst dye). (B) Combined DIC/single-confocal slice image: a club-like structure (arrow) is adjacent to a blood vessel (arrowhead) that traverses the plane of section, exiting the section near the club. (C) A projected stack of confocal images showing ChAT-ir (red) and GnRH/GF-5-ir (green) are co-localized resulting in clusters of cells labeled yellow. All single confocal images also showed co-localization in all cells. (D) FMFRa-ir (green) and ChAT-ir (red) are not co-localized. (E) Club-like structures labeled with 7CR-10 (green) are not co-localized with ChAT-ir (red). Small areas of yellow in panels D and E are an artifact of stacked confocal images from adjacent optical sections superimposed on one another and thus do not indicate co-localization. (F) Central nerve trunk labeled with 7CR-10. Scale bar = 20 μm for panels A–E (D and E taken at same magnification as C). Scale bar = 50 μm for panel F.

shield the first primary antiserum from further processing (Beltz and Burd, 1989). None of these techniques were successful in this tissue. Profiles resembling the club structures were not seen in GF-5 single-labeled tissue, although these might possibly have been missed among the many GF-5-labeled somata. More cogently, there were no profiles in double-labeled tissue that were immunoreactive for GF-5 and not for ChAT.

3. Discussion

This study found that two different GnRH isoforms are expressed in distinct neuronal processes in the nervus terminalis ganglion of bonnethead sharks. One isoform, which from its immunocytochemical profile is probably

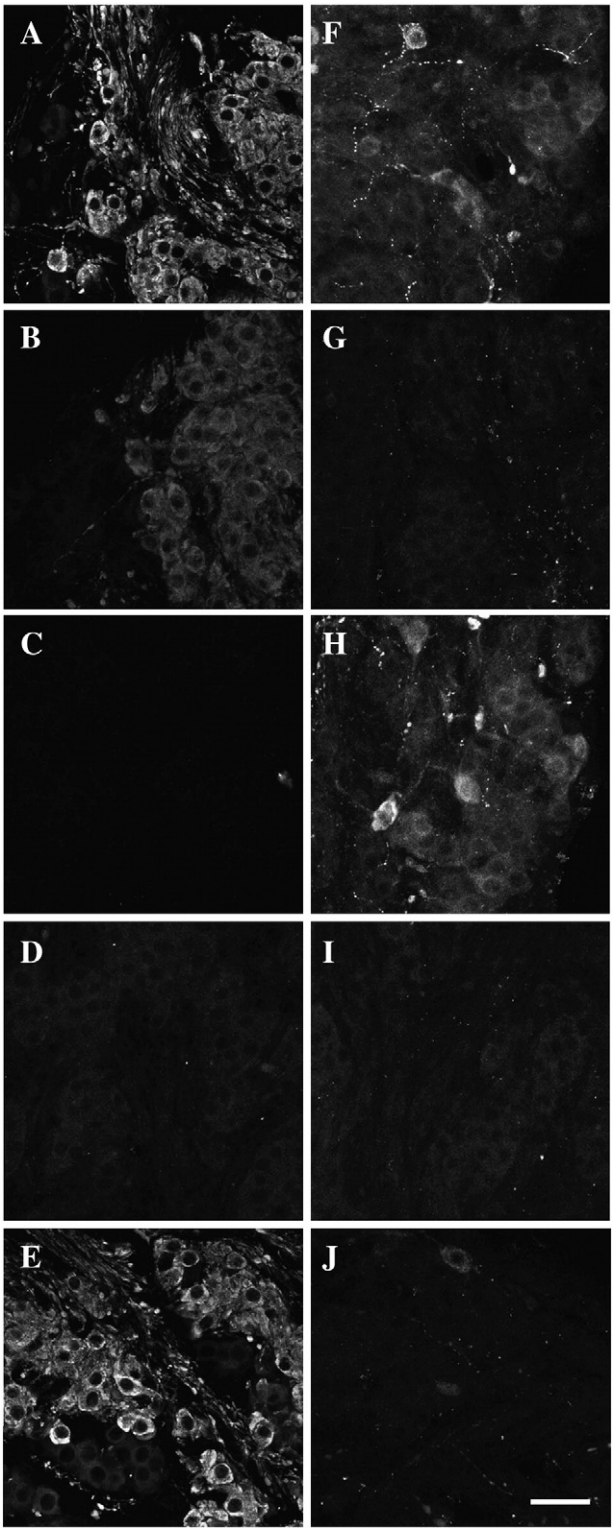

Fig. 3 – Examples of control studies using different isoforms of the GnRH peptide to block labeling with the GF-5 (B–E) and 7CR-10 antisera (G–J) compared to the normal labeling patterns of GF-5 (A) and 7CR-10 (F). Control sections were preabsorbed with either chicken-II (B and G), mammalian (C and H), salmon (D and I), or lamprey GnRH (E and J). Scale bar=50 μm.

GnRH-II or dogfish (d)GNRH, is restricted to fibers and club-like anuclear profiles resembling enlarged nerve terminals, many of which abut blood vessels within the ganglion. The other isoform, probably mammalian (m)GNRH-I, is expressed in cells in the NT ganglion, which also express choline acetyltransferase (ChAT). Together with previously reported synaptic responses within the NT ganglion, these findings suggest possible functional interactions, involving GnRH, RF-amide peptides, and acetyl choline, that may influence GnRH release.

It has been known for some time that the NT ganglion of bonnethead sharks contains two cell classes, delineated best by antibodies to either GnRH or FMRF-amide/LPLRF-amide-like (RF-amide) peptides (White and Meredith, 1995). Labeling with these two peptide markers has been described in the NT system of other elasmobranchs (Stell, 1984; Lovejoy et al., 1992a,b; D'Antonio et al., 1995; Chiba, 2000; Forlano et al., 2000) as well as other vertebrate groups (Muske and Moore, 1988; Wirsig-Wiechmann and Basinger, 1988; Fisher et al., 1996; Wright and Demski, 1996). Less well known are the functional interactions of these two cell types, even though there are extensive synaptic connections within NT ganglia (White and Meredith, 1987; Zeng et al., 1990; Oka and Ichikawa, 1991). It seems possible that RF-amide peptides regulate GnRH release from the GnRH cells in NT as they do in the ventral forebrain of mammals and birds (Johnson et al., 2007; Simonneaux et al., 2009; Clarke et al., 2009). Although not conclusive of the relationship, there is a striking similarity between the anatomy of RF-amide-ir fibers and GnRH-ir cells in bonnethead NT ganglion and mammalian forebrain (compare Fig. 2b of White and Meredith, 1995 and Fig. 3 of Johnson et al., 2007). Little is known about other possible classes of cells influencing NT activity in elasmobranchs, although the two known cell types seem to account for the vast majority of cells within the ganglion in bonnetheads (White and Meredith, 1995).

The labeling with antiserum GF-5 illustrates the pattern and distribution of GnRH cells and fibers in the NT ganglion similar to those of other studies using different GnRH antisera (White and Meredith, 1995; Chiba, 2000). Principally, large clusters of labeled GnRH-ir cells were distributed throughout the ganglion, interspersed with other clusters of the RFa-ir cell type. Fibers of each these two immunoreactivities come into close contact with cells of the other type and form terminal or en-passant swellings that may include synaptic connections (White and Meredith, 1995). On the basis of indirect but convergent information from anatomical and electrophysiological studies, we had earlier suggested that the RFa-ir cells were cholinergic and the GnRH-ir cells were not (White and Meredith, 1995). The present results show clearly that the opposite is the case (see below).

The sparse labeling pattern revealed with the second GnRH antiserum, 7CR-10, was very surprising. The unusual labeling pattern suggested a possible functional division of GnRH cell types in the NT, which is supported by other immunocytochemical differences. Labeling of cell bodies within the ganglion by 7CR-10 antiserum was unconvincing. Strongly labeled varicose fibers, however, were observed scattered throughout each individual ganglion, as solitary fibers. The most unusual feature was the club-like structures. These structures were sometimes the size of cells but were clearly

anuclear (Fig. 2A). Careful reconstruction of these structures from confocal-microscope optical sections suggested that they were nerve terminals. In shape, they were similar to GnRH nerve terminals seen in the median eminence of rats (King and Rubin, 1995). Moreover, like those nerve terminals in rats, the GnRH-ir terminals in bonnethead NT ganglia were associated with blood vessels (Fig. 2B), suggesting a site of GnRH release into the general circulation. Further studies at the ultrastructure level will be needed to demonstrate that this association is consistent and has the appropriate microstructure for such a function.

The differences in GnRH-ir patterns are likely due to expression of two distinct GnRH isoforms. At least four isoforms of GnRH have been characterized in elasmobranchs (Lovejoy et al., 1992b; Calvin et al., 1993; D'Antonio et al., 1995). The preabsorption controls using commercially available GnRH peptides indicate that GF-5 antiserum may be labeling either sGnRH (GnRH-III), which the antibody was raised against, or mGnRH-I, with which it shows high cross-reactivity (Lescheid et al., 1997). Other antisera that were raised against mGnRH (a GnRH-I isoform) gave similar labeling patterns to that of GF-5 (White and Meredith, 1995). Furthermore, the cellular structures labeled by this antiserum and those labeled with 7CR-10 are separate. All the GF-5-ir cell structures and none of the 7CR-10-ir structures co-localized with ChAT antiserum labeling. Thus, they appear to be separate cellular components of the bonnethead NT and not a single cell type co-expressing two different GnRH isotypes. Since 7CR-10 cross-reacts well with sGnRH but does not label the GF-5-ir cells, the likely isoform in GF-5-ir cells is mGnRH (mammalian GnRH-I) and not sGnRH (GNRH-III). The data concerning the GnRH isoform labeled with 7CR-10 are less clear. This antiserum has a much narrower specificity (Lescheid et al., 1997; Forlano et al., 2000) and cross-reacted primarily with cGnRH-II (GnRH-II) and with dGnRH, which 7CR-10 was raised against. The dGnRH peptide was not available for this study and was not tested. Either one of these isoforms is a possible candidate for the GnRH in the club-like terminals, but it does seem clear that it is not mGnRH. Forlano et al. (2000) have demonstrated a lamprey-like GnRH (lGnRH) immunoreactivity in a peripheral NT ganglion in the Atlantic Stingray. Here, the residual staining after preabsorption with lGnRH and the low cross-reactivity of 7CR-10 with lGnRH (Lescheid et al., 1997; Forlano et al., 2000) make it less likely that the structures stained with 7CR-10 in bonnethead shark contain a lamprey-like peptide. All the antibodies were purified as supplied, so artifacts due to cross-reactivity with the conjugated peptides used to produce the antibodies are unlikely.

The two distinct GnRH isoforms and the anatomical difference of their distribution in the NT ganglion are presumably related to differences in function of these two cell types. It is now clear that most vertebrate groups express two or more isoforms of GnRH arising from two or three different embryonic origins (Schwanzel-Fukuda and Pfaff, 1989; Muske, 1993; Whitlock, 2004), with different anatomical distributions in the brain (see Introduction). The highly conserved chicken-GnRH-II isoform (GnRH-II) in the midbrain is widely distributed, whereas other forms, including mGnRH (mGnRH-I), are more variable across taxa. The variable isoform, generally a GnRH-I or GnRH-III isoform, distributed within the NT and preoptic systems, appears to be the one associated with gonadotropin release from the pituitary, where there is a pituitary portal system (Jennes and Stumpf, 1986; Lehman et al., 1987; Silverman et al., 1987; Forlano et al., 2000, see Introduction). In elasmobranchs, where there is no pituitary portal system, there are at least four isoforms of GnRH that can be expressed but it is not clear which forms are playing an endocrine role or where they are released (Lovejoy et al., 1992a).

The 7CR-10-ir club-like structures in the NT ganglion may be a potential site for GnRH-II release into the general circulation. It is unknown where the cell bodies of these fibers are located, although several species of elasmobranchs express GnRH-II in a cluster of cells in the midbrain (Wright and Demski, 1991; Forlano et al., 2000, see Introduction). The GnRH-II isoform has an influence on reproductive physiology (Phillips et al., 1987; Millar, 2003) and behavior (Maney et al., 1997), possibly through synaptic release as a neuromodulator (Jones, 1987). GnRH-II-ir cells within the midbrain in elasmobranchs may influence the mechanics of mating by exerting some control over motor neurons of the male claspers (Demski, 1984), and these cells also have widespread connections with brainstem sensory nuclei (Forlano et al., 2000). GnRH-II (cGnRH-II) may play also an endocrine role (Miyamoto et al., 1984; Khakoo et al., 1994; Zohar et al., 1995). It does not appear to be the major form causing pituitary LH release in most vertebrates (Sharpe et al., 1990; Muske, 1993) but may help modulate relative LH and FSH release in mammals (Niell et al., 2001).

If, as in most species, the GnRH-I isoform (GF-5-ir) is the primary hypophysiotropic peptide, bonnethead sharks may possibly release two isoforms of GnRH into the general circulation: GnRH-I (mGnRH) somewhere in the ventral telencephalon and GnRH-II (or dGnRH) from the NT ganglion. Control of reproductive development and behaviors may be enhanced by variations in the amount and timing in endocrine release of these two hormones, either by acting on the pituitary or directly on the reproductive organs (Millar, 2003). Numerous GnRH-I-ir fibers from the NT cells extend throughout the forebrain so the mGnRH-I, as well as GnRH-II, is likely acting also as a neuromodulator on neural systems within the brain (Oka and Matsushima, 1993; Moeller and Meredith, 1998). There are extensive synaptic connections within NT ganglia, and neural activity in NT cells is influenced by efferent activity from the brain (White and Meredith, 1987, 1993, 1995), so both GnRH release from NT GnRH cells and their neuromodulatory influence in the brain could be regulated both locally and centrally.

The two types of cell somata in bonnethead shark NT ganglia exhibit a difference in ChAT immunoreactivity. Clearly the large clusters of GnRH-ir cells labeled with GF-5 contain ChAT-ir, and the clusters of LPLRFa-ir/FMRFa-ir (RFa-ir) cells do not. Thus GF-5-ir cells are likely to be cholinergic, as ChAT is one of the essential enzymes in the production of acetylcholine (ACh). In the dogfish also, there is widespread distribution of ChAT-ir, including cells in the retrobulbar region (Anadon et al., 2000) that may belong to the same population, and in rats, ChAT immunoreactivity is also localized in the nervus terminalis (Schwanzel-Fukuda et al., 1986). In previous studies in the NT ganglia of bonnethead

sharks, both the GnRH-ir and RFa-ir somata displayed reactivity for acetylcholinesterase (AChE) (White and Meredith, 1995). The RFa-ir cells were more strongly reactive and were previously (incorrectly) considered to be cholinergic. Other NT systems also show a distinction between cells that are strongly AChE-ir and those that are GnRH-ir (Wirsig and Leonard, 1986b; Caldani et al., 1987). AChE markers are clearly useful in delineating cell populations in the NT and may indicate important functional relationships. Markers for AChE and ChAT labeling often co-localize in various neural tissues and sometimes within individual cells (Mesulam, 1988; Illing, 1990; Chriswell and Brandon, 1993). Both enzymes appear to be expressed by the prominent GnRH-ir cell population in bonnethead NT. There are also numerous examples where AChE is synthesized by non-cholinergic, non-ChAT-ir cells (Godfrey et al., 1984; Levey et al., 1984; Pourcho and Osman, 1986 including the RFa-ir cells in the NT. Such cells are generally considered to be cholinoceptive (LeJeune and Jourdan, 1994; Mangoura et al., 1988) when associated with cholinergic cells. Thus, the RFa-ir cells could be responsive to ACh released by the GF-5-ir GnRH cells.

Whether GnRH-ir cells synapse with RFa-ir cells or with each other to influence cell activity remains to be determined, but ACh inhibits one population of NT cells *in vitro*, possibly the RFa-ir cells, and excites another, possibly the GnRH cells (White and Meredith, 1995). These interactions may be important in regulating differences in endocrine release of the various isoforms of GnRH. In isolated GnRH producing cells from the hypothalamus, ACh will regulate the secretion of GnRH (Krsmanovic et al., 1998). In bonnethead sharks, the 7CR-10-ir club-like structures are usually observed in a cluster of GF-5-ir mGnRH-like cells, so there is the potential for direct interaction via ACh between the different GnRH-ir cell populations as well as between GF-5-ir cells and RFa-ir cells. If RF-amides are involved in regulating GnRH release in elasmobranchs as they are in mammals and birds (Johnson et al., 2007; Simonneaux et al., 2009), the RFa-ir cells may influence GnRH release from processes immunoreactive for either GnRH isoform, and there is also the possibility of an ACh-mediated feedforward effect on 7CR-10-ir processes or a feedback effect on GF-5-ir cells. Both RFa-ir cells and GnRH-ir cells also appear to be responsive to efferent control signals (White and Meredith, 1987, 1993, 1995), so GnRH functions may be influenced by both intra- and extra-ganglionic circuits. GF-5-ir cells may regulate GnRH secretion from the NT by influencing activity or peptide release of 7CR-10-ir cells directly via ACh, or indirectly via RFa, or they may affect GnRH secretion from their own cell type through an autocrine-like effect or, via a feedback loop, from RFa cells. Further studies will be needed to flesh out these potential interactions, both in their synaptic connections and potential hormonal activities.

4. Experimental procedures

Adult bonnethead sharks, *S. tiburo*, were captured with a 100 m trammel net along the Northern Gulf coast of Florida, near shore in 2-m depth. Live specimens were quickly brought back to the Florida State University Marine Laboratory and maintained in a 6-m annular tank. The FSU Animal Care and Use Committee, in accordance with NIH guidelines, approved all animal treatments.

For immunocytochemistry studies, sharks were anesthetized with tricaine methanesulfonate (MS222 at 1:1000), sexed, and weighed. The animals were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) after injection with heparin. The nervus terminalis, along with the brain and olfactory tract, were dissected out and placed into the same fixative overnight. The ganglia of the NT were later placed into gelatin and sectioned with a vibratome at 25–35 um.

All immunocytochemical procedures involved a standard two-step process with fluorescent markers (Beltz and Burd, 1989). Primary antibodies included GF-5 (1:5000), raised in rabbit against salmon GnRH and 7CR-10 (1:3000), raised in rabbit against dogfish GnRH (gifts from Dr. Nancy Sherwood). Antibodies to FMRF-amide (DiaSorin, Inc.) were also raised in rabbit and used at a 1:100 dilution. Cholinergic cells were labeled with an antiserum (144P; 1:100) raised in goat against ChAT (Chemicon, Inc.). Some sections were double labeled with 7CR-10 and Hoechst dye (for DNA) at 10 μg/ml (Molecular Probes, Inc.). All antibodies were purified, as supplied. All sections were first presoaked in PBS containing 3% normal donkey serum and 0.4% Triton X-100. All subsequent steps involved solutions in 0.1 M PBS at room temperature. Sections were incubated in primary antiserum for 24 h at room temperature, washed, and then incubated for an additional 18–24 h with donkey anti-rabbit or donkey anti-goat secondary antibody conjugated to either Cy3 or FITC. For double-labeled procedures, the two primary antisera were incubated sequentially. Washed sections were mounted on slides and allowed to dry overnight. Slides were coverslipped with vectashield (Vector Laboratories, Inc.) and sealed with nail polish for later examination with a Zeiss LSM 410 scanning confocal microscope. All images were digitally collected with Zeiss software and later printed using Adobe Photoshop and a Kodak dye sublimation printer. Some minor manipulations of contrast and brightness were employed during this final stage, but all such manipulations were applied uniformly to the entire micrograph.

As a control for nonspecific binding, incubation with each primary antiserum reported here was omitted during the processing of some tissue sections. No fluorescent signals could be detected in any of these sections. To test for antiserum specificity, some sections were processed with primary antiserum that was first incubated for 24 h with the target protein (10–100× the molar concentration of the primary antisera). For both GnRH antisera, this involved incubation with several isoforms of GnRH (Sigma): mammalian (m=mGnRH-I), chicken-II (c=GnRH-II), salmon (s=GnRH-III), and lamprey (l). Sections were then processed as above with these preabsorbed antisera and with normal antisera, in parallel. During documentation of GnRH preabsorption controls (Fig. 3), settings on the confocal microscope (e.g., brightness and contrast) were adjusted appropriately to show GnRH-ir for sections that were processed normally (no preabsorption). These settings were then maintained when documenting all preabsorption sections of a given primary antiserum, to minimize biases during capture and printing of digital images.

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