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Three gonadotropin-releasing hormone genes in one organism suggest novel roles for an ancient peptide

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ABSTRACT **Gonadotropin-releasing hormone (GnRH) is known and named for its essential role in vertebrate reproduction. Release of this decapeptide from neurons in the hypothalamus controls pituitary gonadotropin levels which, in turn, regulate gonadal state. The importance of GnRH is underscored by its widespread expression and conservation across vertebrate taxa: five amino acids are invariant in all nine known forms, whereas two others show only conservative changes. In most eutherian mammals, only one form, expressed in the hypothalamus, is thought to exist, although in a recent report, antibody staining in developing primates suggests an additional form. In contrast, multiple GnRH forms and expression loci have been reported in many nonmammalian vertebrates. However, evidence based on immunological discrimination does not always agree with analysis of gene expression, since GnRH forms encoded by different genes may not be reliably distinguished by antibodies. Here we report the expression of three distinct GnRH genes in a teleost fish brain, including the sequence encoding a novel GnRH preprohormone. Using *in situ* hybridization, we show that this form is found only in neurons that project to the pituitary and exhibit changes in soma size depending on social and reproductive state. The other two GnRH genes are expressed in other, distinct cell populations. All three genes share the motif of encoding a polypeptide consisting of GnRH and a GnRH-associated peptide. Whereas the GnRH moiety is highly conserved, the GnRH-associated peptides are not, reflecting differential selective pressure on different parts of the gene. GnRH forms expressed in nonhypothalamic regions may serve to coordinate reproductive activities of the animal.**

In the African cichlid fish *Haplochromis burtoni*, male reproductive behavior is regulated by social interactions. Brightly colored territorial males that vigorously defend territories and court females comprise only 10% of the natural population but account for all the reproductive success while 90% of the males languish in nonterritorial status (1). The differences in body patterns which distinguish a typical territorial male from its nonterritorial counterpart are highlighted in Fig. 1. The external markings of the territorial male enhance both its aggressive postures and its reproductive displays of courtship (2, 3). In contrast, the paler, nonterritorial males are generally less active and mainly school with females except when fleeing the attacks of their conspecifics. Environmental changes can result in a switch in social status such that a territorial male will become nonterritorial or vice-versa, followed, within minutes, by a corresponding switch in their body patterning and behavior.

Within the brain, gonadotropin-releasing hormone (GnRH)-containing neurons in the preopticohypothalamic area (POA) are significantly larger in territorial than in nonterritorial males, as are the gonads (4). These differences are also reversible and dependent upon the social status of the individual (5). Thus, converting a territorial male to nonterritorial status—by, for example, moving it from its home aquarium into another established tank—results in shrinkage of both neurons and gonads. These GnRH-containing neurons integrate convergent sensory and endocrine input to regulate social and reproductive state, so their change in size most likely reflects a change in their regulatory output.

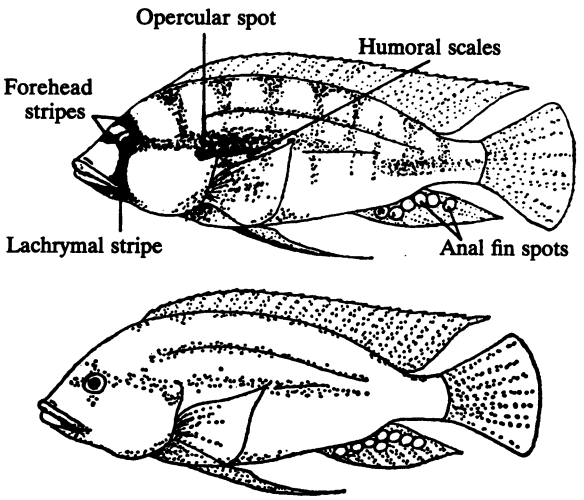

FIG. 1. Schematic illustration of the body patterns for typical territorial and nonterritorial males. (*Upper*) The territorial male possesses distinctive anal fin-spots and dark forehead and lachrymal stripes (eye-bar) and is brightly colored, including orange humeral scales. The overall body color may be either yellow or blue. (*Lower*) Nonterritorial males lack the robust markings of their territorial counterparts and are sand-colored overall. (Adapted from ref. 2.)

In *H. burtoni*, as reported for several other nonmammalian vertebrates (reviewed in ref. 6), three brain regions contain neurons which can be stained with antibodies to GnRH: the terminal nerve at the base of the telencephalon, the mesencephalon, and the POA, whose GnRH-immunoreactive (GnRH-ir) cells directly innervate the pituitary (4, 7, 8). Only the POA GnRH-ir neurons show changes in soma size that correlate with reproductive and social state (4, 8). In our search for the GnRH gene expressed in the POA cells, we have previously cloned and sequenced two GnRH preprohormones: one is expressed only in the GnRH-ir cell population of the terminal nerve (9) and the other is expressed only in the GnRH-ir cell population of the mesencephalon (10). Enigmatically, neither gene is expressed in the POA cell population which is known to control reproduction. Here we use a

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Abbreviations: GnRH, gonadotropin-releasing hormone; ir, immunoreactive; POA, preopticohypothalamic area; RACE, rapid amplification of cDNA ends.

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§The sequence reported in this paper has been deposited in the GenBank database (accession no. U31865).

sequential polymerase chain reaction (PCR; ref. 11) strategy to find the third gene encoding GnRH§ and *in situ* hybridization to show that it is expressed only in the POA.

MATERIALS AND METHODS

cDNA Construction, Reverse Transcription, and Rapid Amplification of cDNA 5′ Ends (5′ RACE), and PCR. *H. burtoni* mRNA was isolated from the POA, which contains the GnRH neurons that project to the pituitary (7). cDNA synthesis was as described (10). In nested PCRs, the downstream primer was bipartite, including a *Hin*dIII recognition site plus $(dT)_{17}$. Upstream primers are shown in Fig. 2*A*. Products were cloned and screened as described (10). Templates were visually compared (color alignment macros; ref. 25) and 5′ RACE primers chosen from consensus regions (11). cDNA synthesis was primed with a randomly generated hexanucleotide. Products were dATP-tailed at their 5′ ends by terminal deoxynucleotidyltransferase (GIBCO/BRL). Nested PCR was performed with a 3′ antisense primer that contained $(dT)_{17}$ plus a *Bam*HI recognition site. The outer 5′ antisense primer for the first round, derived from candidate clone sequence, was 5′-CATATTGCCCAGTGTGTC-3′. The inner primer was 5′-GCACGAATTCGTGTCTGAGAAGTTGTCC-3′, including an *Eco*RI cleavage site plus 18 nt which were 3′ to the first selected sequence. The full preprohormone sequence was obtained by aligning overlapping regions from consensus 5′ RACE templates with those from the primary PCR amplification, and differences attributable to PCR error were assessed. Percent similarity was calculated with the Genetics Computer Group program GAP (version 7.3, 1991).

Northern Analysis. Molecular size was estimated by Northern blot analyses as described (10) with the following modifications. To increase the separation between the similarly sized [Ser^8]GnRH and [Trp^7,Leu^8]GnRH messages, 1.5-μg aliquots of poly(A)$^+$ RNA (FastTrack; Invitrogen) isolated from either the ventral or the dorsal half of *H. burtoni* brains were loaded in replicate lanes of a denaturing 2.5% agarose gel. After electrophoresis and transfer to Nytran membranes (Schleicher & Schuell), RNAs in individual lanes were subjected to both high- and low-stringency hybridizations (50% formamide, 42°C and 62°C, respectively) with the three GnRH RNA probes. RNA probes were synthesized and hybridizations were performed as described above. Transcript lengths were determined by comparison with molecular size standards (100-bp ladder; GIBCO/BRL).

***In Situ* Hybridization.** Social status was determined through behavioral observations (1). After the fish were killed by rapid cervical transection, brain tissue was fixed and sectioned (40 μM) as described (10). The probe for the [Ser^8]GnRH precursor was generated by PCR amplification of a consensus 5′ RACE product cloned into M13 by use of *lac* and *rev-lac* primers. Products were subcloned into pBluescript II SK(+) (Stratagene) and analyzed for the presence of the 5′ RACE insert. A template was chosen for generating an antisense digoxigenin-UTP (Boehringer Mannheim)-labeled RNA probe as described (10). Hybridizations to the [Trp^7, Leu^8]GnRH and [Ser^8]GnRH RNA probes were carried out at high stringency (60°C in 60% formamide) as previously described (9, 10) with these modifications: two washes in 2× standard saline citrate (SSC) were followed by washes in 1.5× and 1× SSC at 60°C and 1× SSC at room temperature; each wash was for 20 min.

Comparison of GnRH Cell Size Between Dominant and Subordinate Fish. Cell areas were measured by an observer under double-blind conditions from video images captured via microscope (Zeiss, ×600) and analyzed by computer (Macintosh, IMAGE 1.55, Wayne Rasband, National Institutes of Health) as described (8).

Statistics. Comparison of mean soma area for preoptic GnRH neurons was computed with STATVIEW II (Abacus Concepts). GnRH-containing neurons located in the POA of one territorial and one nonterritorial animal were compared (n = 54 and 110, respectively) by means of Student's t test, and two-tailed probabilities were calculated. Corrections for the differences in body size were calculated (8) by using a Spearman rank correlation coefficient.

RESULTS

Isolation of Candidate Clones by 3′ and 5′ RACE PCR. We isolated the novel GnRH gene by using enriched target tissue, by exploiting key differences among GnRH peptides in selecting PCR primers, and by using a nested PCR strategy. In *H. burtoni*, as in other teleost fish, there is no portal vasculature. Instead, the pituitary receives direct projections from POA neurons and, consequently, has high concentrations of releasing hormones. Of the three GnRH-ir neuronal populations in *H. burtoni*, only those in the POA project to the pituitary (7). Therefore, PCR primers were based on the GnRH decapeptide form isolated from dissected pituitary tissue. This sequence was found to be QHWSYGLSPG (26) and is referred to as [Ser^8]GnRH (see ref. 10). Correspondingly, PCR amplification was performed on template cDNAs derived from a restricted pool of mRNA—specifically, those messages expressed in POA tissue microdissected from the brain.

Our RT–PCR strategy was similar to that previously described (10) in that it targeted sequences coding for regions 3′ to the GnRH-encoding region of the precursor molecule (see Fig. 2*A*) and that it was nested (see *Materials and Methods*). To maximize our chances of finding the third GnRH gene, two sets of degenerate upstream primers were synthesized whose 3′ ends terminated in codons for residues which differ among the three forms of GnRH in *H. burtoni*. In contrast, in screening for clones, we exploited the similarity among the GnRH genes by assessing the presence of codons for conserved residues including the 10th amino acid in the decapeptide followed by three amino acids which serve as posttranslational substrates for proteolytic processing (27). Three separate first-round and seven second-round PCRs yielded candidate clones which contained these hallmarks and had an open reading frame of 210 nt beyond the primer.

Regions of complete consensus were chosen from these candidate clones to construct primers for 5′RACE (11). 5′RACE products were analyzed for the antisense codons of the GnRH decapeptide. Approximately 80% included the [Ser^8]GnRH-encoding region and extended some 140 nucleotides further before ending in the upstream primer sequence.

Comparison of [Ser^8]GnRH Preprohormone with the [Trp^7,Leu^8]GnRH and [His^5,Trp^7,Tyr^8]GnRH Precursors. The predicted amino acid sequence of the novel form ([Ser^8]-GnRH) is shown in Fig. 2*B* aligned with the [Trp^7,Leu^8]GnRH and [His^5,Trp^7,Tyr^8]GnRH prohormones. Consistent with previously reported GnRH preprohormone sequences (10), each gene codes for a signal sequence (≈63 nt), the conserved GnRH decapeptide (30 nt), and a less conserved, larger peptide known as GnRH-associated peptide (GAP; ≈185 nt; Fig. 2*C*). This strongly suggests that the portion of the gene encoding GnRH has been subjected to greater selective pressure by functional constraints than the portion encoding GAP.

Northern blot analysis was performed (10) with probes for the three GnRH precursors in *H. burtoni* and mRNA from brains transected along their rostral–caudal axis. Like the RNA probes for the [Trp^7,Leu^8]GnRH and [His^5,Trp^7,Tyr^8]-GnRH transcripts, the [Ser^8]GnRH RNA probe detected a single band, even at the lower hybridization stringency. This band was localized to ventral brain mRNA. Comparison with molecular size markers revealed that the [Ser^8]GnRH mRNA precursor was ≈550 bases, intermediate in size between the

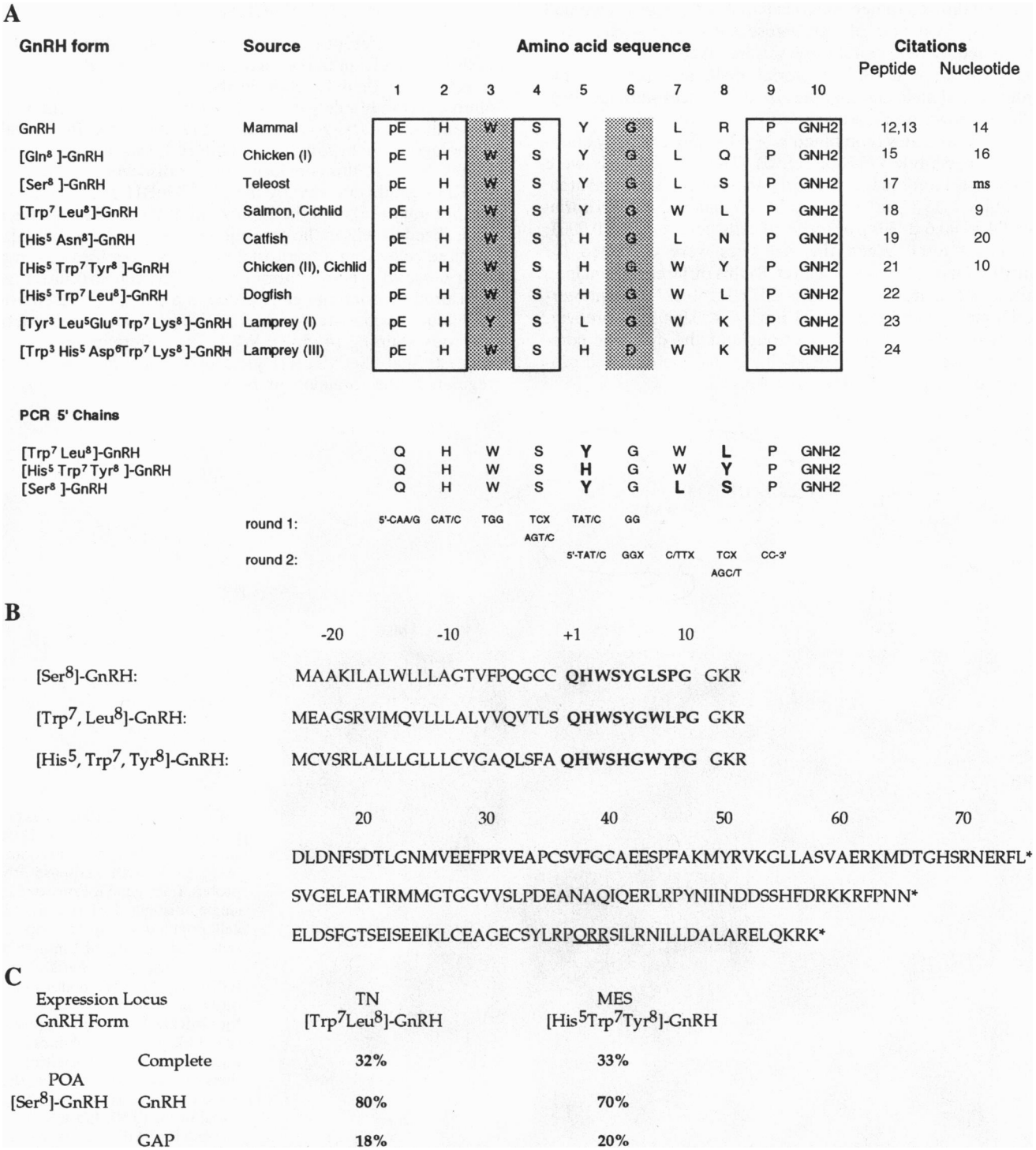

FIG. 2. (*A*) Primary sequences for the nine known GnRH decapeptides are shown with reference to the mammalian sequence. Positions 1, 2, 4, 9, and 10 are invariant, whereas 3 and 7 show conservative changes. The newly identified third form in *H. burtoni* is referred to as [Ser^8]GnRH. Below, the [Ser^8]GnRH decapeptide is compared with the other GnRH forms found in *H. burtoni*. Residues which vary are shown in boldface type. Degenerate 5′ oligonucleotides used for the initial PCR are shown below the peptides. ms, This manuscript. (*B*) The predicted amino acid sequence of the [Ser^8]GnRH preprohormone shown aligned with those for the other two GnRH preprohormones in *H. burtoni*. Each gene codes for a signal sequence followed by the respective GnRH decapeptide, a proteolytic processing site, and a unique GnRH-associated peptide (GAP). (*C*) Homology among predicted amino acid sequences of the three GnRH preprohormones shown as percent identity. The [Ser^8]GnRH preprohormone is compared with the other two forms as a complete molecule, only the GnRH moiety, and only the GAP moiety (see text). The GnRH portion of the amino acid sequence has 3–4 times more similarity than GAP. TN, terminal nerve; MES, mesencephalon.

[Trp^7,Leu^8]GnRH and [His^5,Trp^7,Tyr^8]GnRH transcripts (data not shown).

[Ser^8]GnRH mRNA Is Expressed Solely Within the Preoptic Area in Cells That Show Socially Regulated Changes in Soma Size. We confirmed that this [Ser^8]GnRH precursor encodes the functional homolog of mammalian hypothalamic GnRH by examining its expression in the only GnRH-containing cells that project to the pituitary (7). *In situ* hybridization of *H. burtoni* brains ($n = 7$) localized expression of the [Ser^8]GnRH precursor to large cells within the POA. We

measured the two-dimensional sizes of the digoxigenin-labeled cell somas in a pair of fish whose social states had been determined by behavioral observations. We found that these cells were large in the territorial male and small in the nonterritorial male and that the size differences were identical to those shown on measurements of multiple territorial and nonterritorial males (combined $n > 40$) using antibody staining of the peptide (Fig. 3; ref. 5). The labeled cells were significantly larger in the territorial male (cross-sectional area, $A = 214.8 \pm 15.1\ \mu m^2$; mean ± SEM) than the nonterritorial male ($A = 90.6 \pm 3.6\ \mu m^2$; $n > 50$ cells per fish; $P < 0.0001$, Student's t test). When the cell sizes were corrected for contribution of body size (e.g., ref. 8) this difference remained significant (territorial male, $A = 207.63 \pm 14.57\ \mu m^2$; nonterritorial male, $A = 93.66 \pm 3.74\ \mu m^2$; $P < 0.0001$). Moreover, the larger cells were labeled throughout the cytosolic compartment, suggesting that social change had upregulated production of the [Ser^8]GnRH transcript.

DISCUSSION

That three different forms of GnRH exist and are expressed in distinct nuclei in the brain of *H. burtoni* poses an interesting puzzle about their function in the organism. [Ser^8]GnRH is almost certainly solely responsible for regulation of the hypothalamic–pituitary–gonadal axis and therefore functionally homologous to hypothalamic GnRH forms in other vertebrates. We base this conclusion on the discovery that only the GnRH peptide encoded by the [Ser^8]GnRH gene is found in the pituitary (26), that the expression of the [Ser^8]GnRH gene is localized to cells in the preoptic area, and that the soma sizes of these cells from a territorial male were significantly larger than those of a nonterritorial male. This cell size difference measured by using the gene probe in a pair of representative individuals is consistent with extensive previous evidence from antibody staining (4, 5, 7). While the difference in cell size suggests that [Ser^8]GnRH gene expression may be socially regulated, this remains to be formally tested. Such social

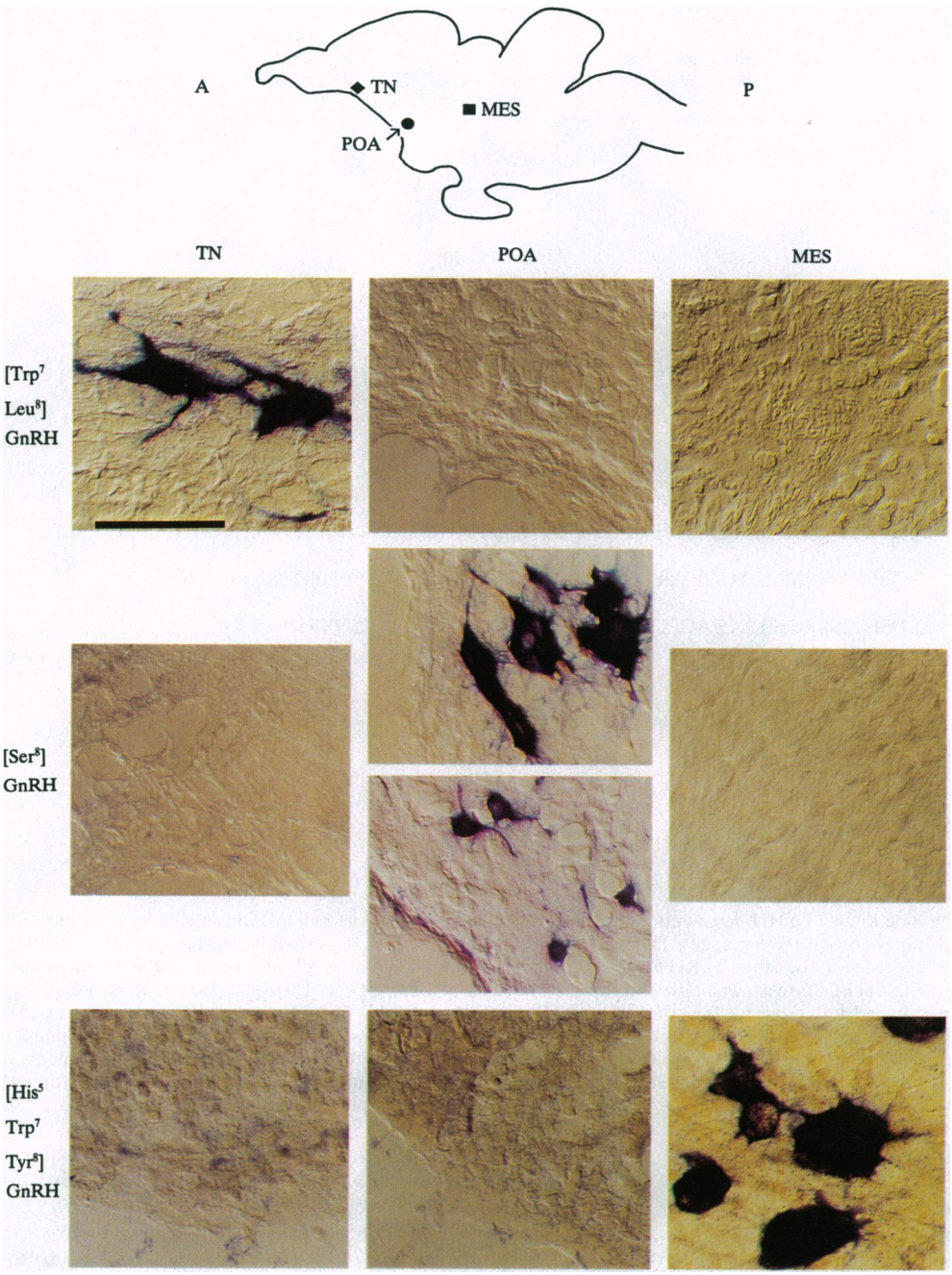

FIG. 3. Localization of expression for each of the three GnRH genes by *in situ* hybridization of brain tissue with antisense RNA probes. Each gene is expressed in a single, distinct GnRH-containing cell population. At the top is a schematic drawing of a midsagittal section through *H. burtoni* brain indicating the three regions previously shown to be immunoreactive for GnRH (A, anterior; P, posterior). Below, three columns show micrographs of parasagittal sections of *H. burtoni* brain from these three regions; left to right: the terminal nerve (TN), POA, and mesencephalon (MES). Each row pictures sections hybridized to a different GnRH RNA probe. Top row: the [Trp^7,Leu^8]GnRH RNA probe labels cells only in the TN. (Bar = 50 μm.) Middle row: the [Ser^8]GnRH RNA probe hybridizes specifically within the POA; the center panel is split, showing a brain section from a dominant, territorial male (upper half) which has larger labeled cells than a subordinate, nonterritorial male (lower half). Bottom row: only the mesencephalic GnRH cell population is recognized by the [His^5,Trp^7, Tyr^8]GnRH RNA probe. This row is adapted from ref. 10.

regulation makes sense, given the reproductive strategy of *H. burtoni* males that switch between two states with radically different behavioral and physiological requirements, depending on reproductive opportunity.

Demonstration that the terminal nerve and POA express different GnRH genes in *H. burtoni* suggests that perplexing GnRH expression patterns in these regions reported in other fish species (e.g., ref. 28) arise because of the different specificities of antibodies as compared with gene probes. In *H. burtoni*, an anti-mammalian antibody (Incstar, Stillwater, MN) and an anti-salmon antibody (GF4; courtesy of N. Sherwood) each label both of these cell populations despite the differences in the GnRH forms they contain (8).

Developmentally, terminal nerve and POA GnRH cells share a common embryonic origin in the olfactory placode: In rats, as these cells migrate into and through the brain, some stop in the terminal nerve area of the telencephalon while others continue into the diencephalon (29, 30). The GnRH peptide contained in these mammalian cell populations is thought to be identical and encoded by the same transcript (31, 32). Recently, however, antibody staining of the developing terminal nerve population in primates has suggested an additional GnRH form, distinct from that in the hypothalamus (33). If this form is developmentally regulated, then its expression may have been missed in previous studies of adult mammals. Thus, there may be multiple GnRH transcripts in the terminal nerve and hypothalamic regions of other species as seen in *H. burtoni*. Whether these cells originate from a single source and switch gene expression along their migration, similar to neural crest cells (34), or whether they arise initially as separate populations remains to be discovered.

It seems likely that GnRH forms have proliferated in the service of reproduction. In the dwarf gourami, the terminal nerve GnRH-containing cell population has been shown to project widely throughout the brain and exhibit an internal rhythmicity (35). The location of these cells along olfactory pathways as well as projections to the retina suggests that [Trp^7,Leu^8]GnRH might coordinate sensory input with reproductive requirements (e.g., ref. 36).

In contrast, [His^5,Trp^7,Tyr^8]GnRH is implicated in the control of reproductive motor behavior (e.g., ref. 37) and is localized to midbrain and caudal brain regions of metatherian and nonmammalian vertebrates. This form is a robust releaser of pituitary gonadotropins (e.g., ref. 38) despite the fact that in many species, including *H. burtoni*, it is not found in the pituitary. [His^5,Trp^7,Tyr^8]GnRH is also the most effective GnRH form in eliciting the late slow excitatory postsynaptic potential in bullfrog sympathetic ganglia (39). The remarkable potency of this form augurs for its potential effectiveness at distant targets, including peripheral ones. A second form of GnRH has been reported by use of antibodies in the midbrain of a musk shrew (40), providing further evidence that even eutherian mammals may have co-opted the GnRH motif for multiple functions in distinct brain regions.

In sum, sensory, motor, and humoral systems might each utilize distinct but related forms of GnRH: the terminal nerve form, perhaps coordinating the sensory and motivational systems; the mesencephalic form, modifying motor action in the service of reproductive behavior; and the hypothalamic form, causing the release of gonadotropins to permit reproduction. Coordination of the activities of these forms could result from shared signaling pathways and regulatory motifs. How and why three GnRH different genes evolved, however, remains to be discovered.

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