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A Novel Form of Gonadotropin-Releasing Hormone in the Medaka, *Oryzias latipes*

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The present study has identified three molecular forms of gonadotropin-releasing hormone (GnRH) in the brain of a teleost, the medaka, by isolation of their cDNAs. This species has a novel GnRH, which is here named medaka-type GnRH (mdGnRH), in addition to two characterized forms, chicken-II-type GnRH (cGnRH-II) and salmon-type GnRH (sGnRH). Phylogenetic analysis showed that mdGnRH is a medaka homolog of and seabream-type GnRH (sbGnRH) and mammalian-type GnRH (mGnRH) in other species, and suggested that all vertebrates have three distinct GnRHs. Furthermore, *in situ* hybridization revealed that the mdGnRH gene is expressed only in neurons clustered within the preoptic area as sbGnRH and mGnRH genes in other species are, while the genes for cGnRH-II and sGnRH are only in the midbrain tegmentum and nucleus olfactoretinalis, respectively. This result suggested that mdGnRH is a hypophysiotropic factor and the other two forms are involved in other physiological events as neuromodulators or neurotransmitters.

Key Words: **gonadotropin-releasing hormone (GnRH); molecular forms; mdGnRH; cGnRH-II; sGnRH; phylogeny; gene expression; medaka.**

Gonadotropin-releasing hormone (GnRH) was originally isolated from mammalian hypothalamus and named for its role as the physiologic regulator of gonadotropin release from the pituitary (1, 2). To date, eleven molecular forms of the neuropeptide have been identified in various vertebrates (3, 4). The length of GnRH, however, has been conserved to be 10 amino acids during evolution, and 4 out of the 10 residues are identical among known forms. Although it is accepted that at least two molecular forms of GnRH exist within the brain of a single vertebrate species, the number of GnRH forms in one organism is a matter of controversy. Moreover, physiological role for each form of GnRH is still unclear.

One teleost species, the medaka *Oryzias latipes*, was chosen as an experimental model for elucidation of the physiological functions, regulatory mechanisms, and phylogeny of GnRHs. This fish is an ideal model system for reproductive, developmental, and genetic studies because of its well-established reproductive biology and genetic analysis, its short generation time, and the availability of a large number of inbred strains and mutants (5–7). In the medaka brain, two forms of GnRH, chicken-II-type GnRH (cGnRH-II) and salmon-type GnRH (sGnRH), have been detected by HPLC, radioimmunoassay (RIA), and immunocytochemistry (8, 9).

As a first step, this study has identified the molecular forms of GnRH in the medaka brain. Isolation and characterization of their cDNAs revealed the presence of a novel form of GnRH in addition to two characterized forms in the medaka. The transcripts for three GnRHs in the brain were characterized by Northern blot analysis, and their localizations were examined by *in situ* hybridization.

MATERIALS AND METHODS

Animals and sample preparation. The medaka purchased from a local dealer were kept in freshwater under controlled photoperiod (14 h of light, 10 h of darkness) with commercial fish chow daily. The water temperature was maintained at 25°C. Sexually mature males and females, which spawned every morning, were used in this study. When sampled, fish were anesthetized in 0.06% 2-phenoxyethanol and sacrificed by decapitation. Total RNA was extracted with ISOGEN (Nippongene, Tokyo, Japan) from the brains of fifty fish. Subsequently, poly(A)$^+$ RNA was purified with Oligotex-dT 30 (Takara, Shiga, Japan).

Molecular cloning. Rapid amplification of cDNA ends (RACE) were carried out to identify molecular forms of GnRH in the medaka brain using Marathon cDNA amplification kit (Clontech, Palo Alto, CA) (10). Six degenerate sense primers were designed for isolation of 3′-ends of the cDNAs encoding three forms of GnRH. MD-F1 (5′-CAGGG(A/C)TGCTGTCAGCACTGGTC-3′) and MD-F2 (5′-CAG-

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CACTGGTCITA(C/T)GGICT-3′) were based on conserved nucleotide sequences among mammalian-type GnRH (mGnRH) cDNA of the eel *Anguilla japonica*, seabream-type GnRH (sbGnRH) cDNA of the seabream *Chrysophrys major*, and the sbGnRH cDNA of the cichlid *Haplochromis burtoni*. CII-F1 (5′-CA(A/G)CA(C/T)TGGTCICA(C/T)GG-ITGGTA-3′) and CII-F2 (5′-TGGTCICA(C/T)GGITGGTA(C/T)CCIGG-3′) were based on the amino acid sequence of cGnRH-II. S-F1 (5′-CA(A/G)CA(C/T)TGGTCITA(C/T)GGITGGCT-3′) and S-F2 (5′-TGGTCITA(C/T)GGITGGCTICCIGG-3′) were based on sGnRH sequence. First PCRs were carried out with MD-F1, CII-F1, or S-F1 in combination with adaptor primer 1 (AP1) (Clontech). The first PCR solutions with MD-F1, CII-F1, and S-F1 served as templates for the nested PCRs with MD-F2, CII-F1, and S-F2, respectively, in combination with adaptor primer 2 (AP2) (Clontech). After the nucleotide sequences of the 3′-ends of the cDNAs encoding three GnRH forms were determined, six gene-specific antisense primers were designed for isolation of 5′-ends of these cDNAs. MD-R1 (5′-TAAAGATGACTCCTCCAGGTGGCTC-3′) and MD-R2 (5′-CTC-AAGTCACTGCAGGGTGTATTGC-3′) were based on the sequence of the obtained cDNA encoding a novel form of GnRH (medaka-type GnRH; mdGnRH). CII-R1 (5′-CTCCTCTGAGGTCTCATGTAGC-TGC-3′) and CII-R2 (5′-TCTGAAACCTCAAAGGAGTCTAGCTCC-3′) were based on the sequence of the cGnRH-II cDNA. S-R1 (5′-AGACACCACTCTTCCTGTGCCCATC-3′) and S-R2 (5′-ATCCTG-ATGGTTGCCTCGAGCTCTC-3′) were based on the sequence of the sGnRH cDNA. First PCRs were carried out with MD-R1, CII-R1, or S-R1 in combination with AP1. The first PCR solutions with MD-R1, CII-R1, and S-R1 served as templates for the nested PCRs with MD-R2, CII-R2, and S-R2, respectively, in combination with AP2. An additional confirmation was performed by amplification of cDNA fragments containing full-length open reading frames (data not shown).

Northern blot analysis. Four micrograms of poly(A)$^+$ RNA from the brain were subjected to electrophoresis on a 0.9% agarose gel and transferred to membranes (Hybond-N$^+$; Amersham Pharmacia Biotech, Buckinghamshire, UK). Plasmid DNAs containing three GnRH DNAs were used as templates to generate probes labeled with [α-^{32}P]dATPs and [α-^{32}P]dTTPs (Amersham Pharmacia Biotech) by PCR amplification. The cDNA probes specific to mdGnRH (225 bp), cGnRH-II (217 bp), and sGnRH (256 bp) were amplified with MD-F4 (5′-GGGAAGCGAGAACTGAAATAC-3′)/MD-R4 (5′-AATATCCCA-TACAGTAGAGACAC-3′), CII-F4 (5′-AATGCAGCTACATGAGAC-CTC-3′)/CII-R4 (5′-TTTGCAACAGAACAATATTGCAATG-3′), and S-F4 (5′-AAGAAGTGTGGGAGAGCTCG-3′)/S-R4 (5′-AACACATA-AAGCTTTGTTAGTTGTG-3′) primer pairs, respectively. After prehybridization in hybridization buffer (PerfecHyb; Toyobo, Tokyo, Japan) at 68°C for 30 min, the membranes were hybridized with cDNA probes for mdGnRH, cGnRH-II, and sGnRH in the hybridization buffer at 68°C for 14 h. The membranes were washed twice in 2× SSC containing 0.1% SDS at 68°C for 5 min, and then washed twice in 1× SSC containing 0.1% SDS at 68°C for 5 min. The membranes were exposed to Fuji X-ray film (Fuji Film, Tokyo, Japan) at −80°C for 3 days.

Sequence analysis. Amino acid identities between prepro-GnRHs of the medaka and those of other species were calculated by a Mac Vector version 6.0 (Oxford Molecular, OR) with default setting. Full-length prepro-GnRH proteins in the medaka and other species were aligned to each other by CLUSTAL W (11) with default setting. After the alignment, a phylogenetic tree was generated by PHYLIP (12) using neighbor-joining method (13).

In situ hybridization. In situ hybridization was performed as described by Amano *et al.* (14). The brains of twelve fish of both sex were removed and fixed. Antisense oligonucleotide probes for a novel GnRH (5′-GACTCCTCCAGGTGGCTCAAGTCACTGCAGGGTGTA-TTGCTGTT-3′), cGnRH-II (5′-GTCGCAGTGTAGAAAAGCATGAC-AGGTGAAGGGTCACTTCC-3′), and sGnRH (5′-TCCATCAGTAG-TGCTGAGATTCAAGGCGACTTCTTCACTGACTC-3′) were labeled with [α-^{35}S]dATP (Dupont/NEN, Boston, MA) on their 3′-ends. The tissue sections were covered with hybridization buffer with the labeled probes. In addition to the labeled probes, excessive unlabeled probes (200 times as much as the labeled probes) were added to the hybridization buffer for alternate serial sections in order to validate the specificities of the probes.

RESULTS AND DISCUSSION

Three GnRHs, Including a Novel Form, Exist in the Medaka

The present study has identified the molecular forms of GnRH in the medaka brain by molecular cloning of their cDNAs. A 429 bp cDNA isolated encodes a GnRH which is different from any other forms of GnRH characterized so far (Fig. 1). Its deduced amino acid sequence is pGlu-His-Trp-Ser-Phe-Gly-Leu-Ser-Pro-Gly-NH_2. Here this novel form is named medaka-type GnRH (mdGnRH) (Fig. 2). A cDNA of 630 bp encodes a characterized form of GnRH, cGnRH-II (Fig. 1). sGnRH is encoded by two cDNAs of 418 and 781 bp, resulting from alternative polyadenylation-signal usage (Fig. 1). Each form of the three GnRHs is encoded as part of prepro-GnRH protein, which is composed of a signal peptide, the GnRH decapeptide, a Gly-Lys-Arg processing site, and a GnRH-associated peptide. Northern blot analysis revealed that the three GnRH genes are indeed expressed in the brain, and each of mdGnRH and cGnRH-II is encoded by a single gene transcript, but two transcripts are present for sGnRH (Fig. 3).

The Number of Molecular Forms of GnRH in One Organism

It is still a matter of controversy whether the presence of three distinct forms of GnRH in a single species is characteristic of limited some teleost groups. In this context, the sequence data of prepro-GnRHs in the medaka is of some interest. mdGnRH decapeptide has the highest sequence identities with sbGnRH and herring-type GnRH (hrGnRH): They differ by one amino acid in position 5 (Fig. 2). Although mdGnRH did not show remarkable identities to any other forms at the prepro-protein level, it also has the highest identity with 46% and 49% to prepro-sbGnRHs of the cichlid and the seabream, respectively (Table 1). Also, mdGnRH differs from catfish-type GnRH (cfGnRH) and mGnRH by only two residues in positions 5 and 8 (Fig. 2), and shows relatively high identity with 32% to prepro-cfGnRH of the catfish *Clarias gariepinus* and prepro-mGnRH of the eel (Table 1). These lines of evidence indicate that mdGnRH is a medaka homolog of sbGnRH, hrGnRH, cfGnRH, and mGnRH in other species. On the other hand, this also suggests that each of cGnRH-II and sGnRH have been evolved separately

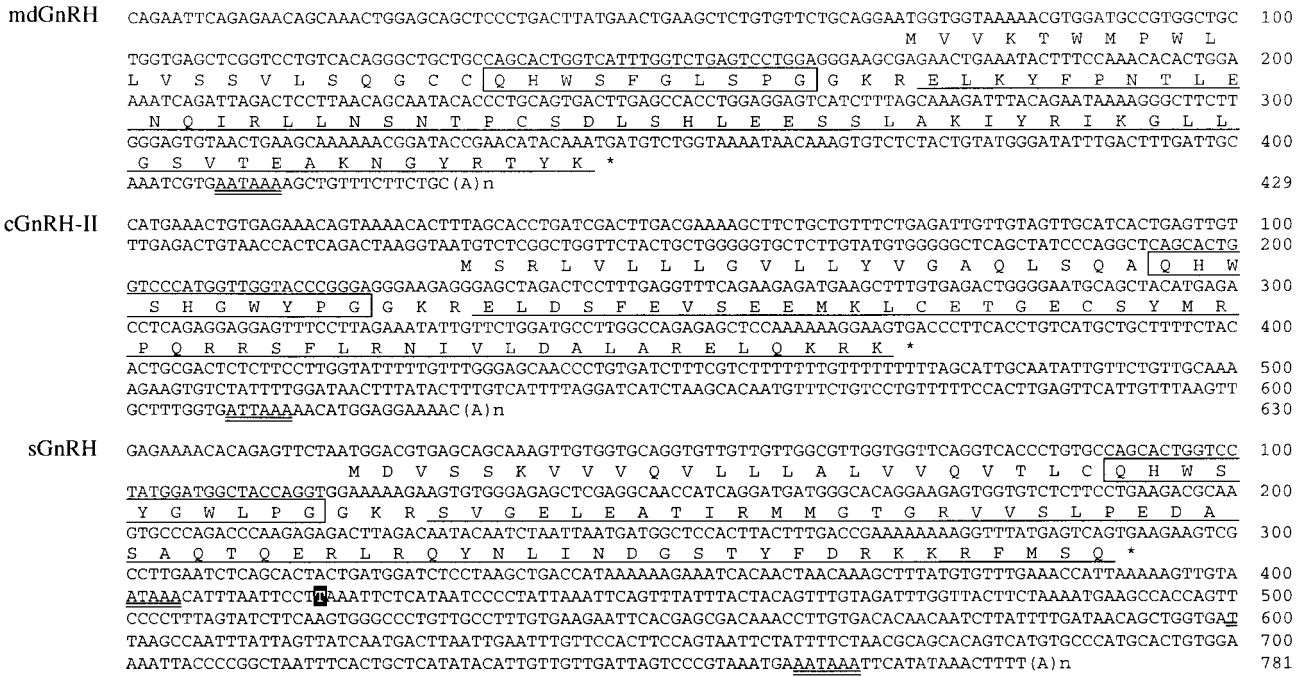

FIG. 1. Nucleotide and deduced amino acid sequences of the cDNAs for medaka-type GnRH (mdGnRH), chicken-II-type GnRH (cGnRH-II), and salmon-type GnRH (sGnRH) in the medaka. Each of mdGnRH and cGnRH-II is encoded by a single type of transcript, but sGnRH is encoded by two transcripts, which are generated through alternative polyadenylation-signal usage. GnRH decapeptide and GnRH-associated peptide regions are boxed and single underlined, respectively. Stop codons are denoted by asterisks and polyadenylation signals (AATAAA) are double underlined. The last nucleotide of the shorter sGnRH cDNA is in black box. The sequences of cDNAs and their deduced proteins for mdGnRH, cGnRH-II, and sGnRH (short and long transcripts) have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the Accession Nos. AB041333, AB041330, AB041331, and AB041332, respectively.

from these forms. In this case, all species in the teleost would have three distinct forms of GnRH; cGnRH-II, sGnRH, and a third diversified form (mdGnRH, sbGnRH, hrGnRH, cfGnRH, or mGnRH). This idea is supported by the phylogenetic analyses in this and previous studies (Fig. 4) (15, 16). The phylogenetic tree indeed divided multiple molecular forms of GnRH in vertebrate into three groups as described above. As to the tetrapod, only two molecular forms of GnRH have been isolated from one organism: One form is cGnRH-II and the other is mGnRH, guinea pig-type GnRH, or chicken-I type GnRH (17, 18). Although sGnRH has not yet been isolated in the tetrapod, the recent studies using HPLC and RIA detected sGnRH-like form in the brain of several mammals (19–21). Therefore sGnRH-like form may be a universal form in all vertebrates, and it is possible that the existence of three forms of GnRH in one organism is a general condition of both the teleost and tetrapod.

Gene Expression Sites of Three GnRHs in the Brain

The localization of mRNAs for three GnRHs within the medaka brain was determined by *in situ* hybridization (Fig. 5). Each of three GnRH genes had a clear region-specific pattern of expression in the brain. Hybridization signals for mdGnRH mRNA were observed only in the cell bodies of several neurons within the preoptic area (POA). These neurons form a cluster. Signals for cGnRH-II mRNA were present only in the cell bodies of a cluster of neurons found in the midbrain

	1	2	3	4	5	6	7	8	9	10
mdGnRH	pGlu	His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	Gly NH_2
sbGnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly NH_2
hrGnRH	pGlu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	Gly NH_2
mGnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly NH_2
cfGnRH	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly NH_2
cGnRH-I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly NH_2
gpGnRH	pGlu	Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	Gly NH_2
sGnRH	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly NH_2
dfGnRH	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly NH_2
cGnRH-II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly NH_2
lGnRH-I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly NH_2
lGnRH-III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly NH_2

FIG. 2. Comparison of mdGnRH with eleven GnRHs identified from vertebrates so far. Shaded areas indicate the regions of identity with mdGnRH. mdGnRH, medaka-type GnRH; sbGnRH, seabream-type GnRH; hrGnRH, herring-type GnRH; mGnRH, mammalian-type GnRH; cfGnRH, catfish-type GnRH; cGnRH-I, chicken-I-type GnRH; gpGnRH, guinea pig-type GnRH; sGnRH, salmon-type GnRH; dfGnRH, dogfish-type GnRH; cGnRH-II, chicken-II-type GnRH; lGnRH-I, lamprey-I-type GnRH; lGnRH-III, lamprey-III-type GnRH.

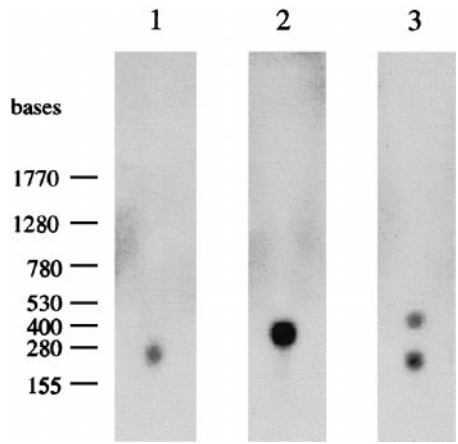

FIG. 3. Northern analysis of three GnRHs in the medaka brain. Four micrograms of poly(A)$^+$ RNA from the brain were hybridized with labeled probes specific for the cDNAs encoding mdGnRH (lane 1), cGnRH-II (lane 2), and sGnRH (lane 3). Single transcripts of approximately 550 and 700 bases were obtained in lanes 1 and 2, respectively. Two transcripts of approximately 500 and 850 bases were detected in lane 3. The positions of RNA size markers are shown on the left.

tegmentum (MT). sGnRH mRNA signals were present only in the nucleus olfactoretinalis (NOR) neuron cell bodies which formed a dense cluster. In contrast, absorption experiments by addition of excessive unlabeled probes revealed no hybridization signals. Cross-reaction of the probes is unlikely, because signals obtained showed very high region specificity.

This result supports the hypothesis that mdGnRH is a medaka homolog of sbGnRH and mGnRH, the genes of which are also detected in the POA (22). In the herring, the content of hrGnRH is much higher than the other forms in the pituitary, suggesting that hrGnRH is produced in the POA (4), suggesting that mdGnRH and hrGnRH are homologous.

Additionally, this result indicates that respective forms play distinct roles within the organism and their gene expressions are differentially regulated. GnRH-producing neurons in the POA are known to project to the pituitary, and responsible for regulation of reproductive endocrine system through acting on the pituitary to stimulate the synthesis and release of gonadotropins. In the medaka, therefore, mdGnRH would be the molecular form playing the role as a hypophysiotropic factor, while the other two GnRHs produced in the MT and NOR would function as neuromodulators or neurotransmitters.

TABLE 1

Amino Acid Identity (%) between Prepro-GnRHs of the Medaka and Those of Other Teleosts

	Medaka		
	mdGnRH	cGnRH-II	sGnRH
Cichlid sbGnRH	49	28	25
Seabream sbGnRH	46	24	24
Catfish cfGnRH 1/2	32/33	28/31	25/25
Eel mGnRH	32	26	30
Cichlid sGnRH	25	28	77
Seabream sGnRH	21	26	82
Goldfish sGnRH 1/2	23/26	33/30	41/47
Salmon sGnRH 1/2	25/23	27/28	63/69
Cichlid cGnRH-II	29	81	29
Seabream cGnRH-II	28	80	30
Catfish cGnRH-II	27	58	31
Goldfish cGnRH-II 1/2	21/30	60/56	25/30
Eel cGnRH-II	30	71	29

Note. For abbreviations, see the legend to Fig. 2.

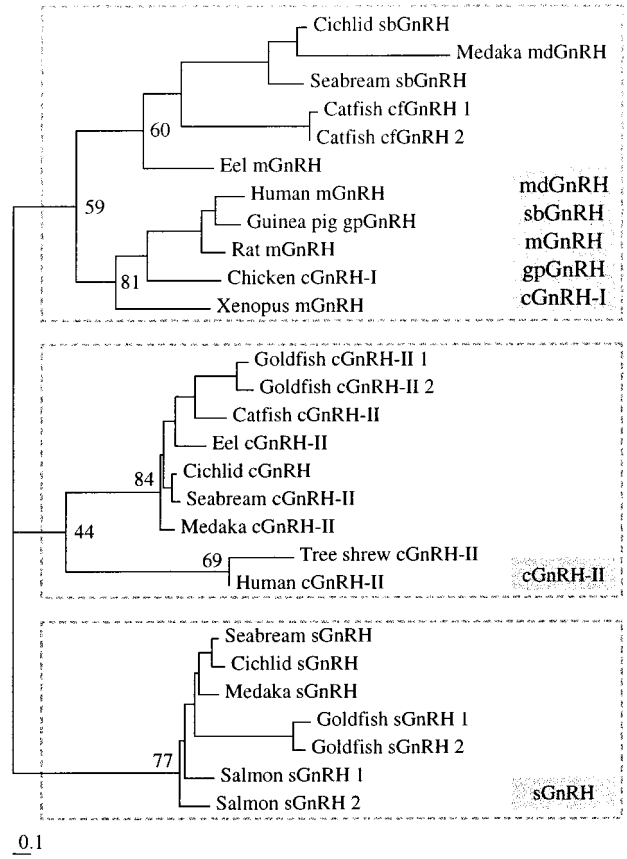

FIG. 4. A phylogenetic tree of prepro-GnRHs in vertebrates. Phylogenetic analysis divided multiple molecular forms of GnRH into three groups: the first group including mdGnRH, sbGnRH, mGnRH, gpGnRH, and cGnRH-I; the second contains only cGnRH-II; the third contains only sGnRH. This unrooted tree was constructed by neighbor-joining method (13) using PHYLIP software (12), based on the alignments of the amino acid sequences of whole prepro-GnRHs using CLUSTAL W (11). The values at the nodes are bootstrap probabilities (%) estimated by 100 times replications. The scale bar corresponds to estimated evolutionary distance units. For abbreviations, see the legend to Fig. 2.

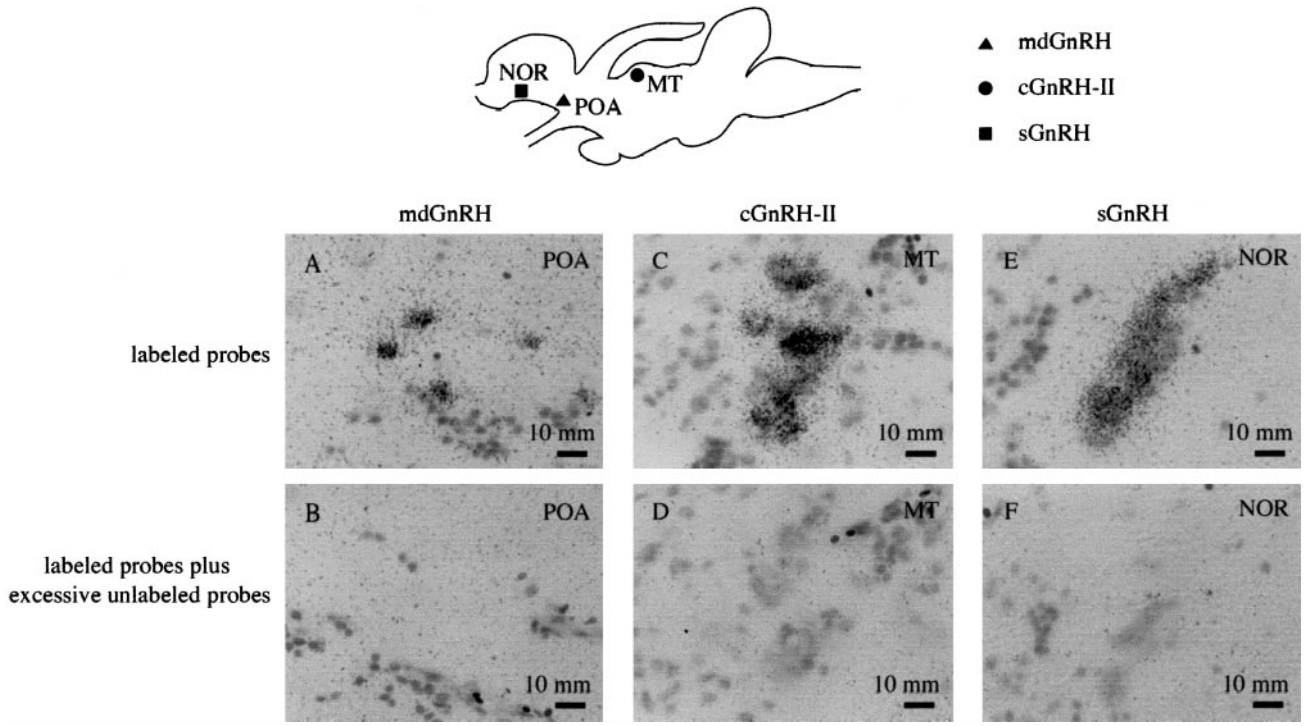

FIG. 5. Localization of the mRNAs for three GnRHs in the medaka brain, determined by *in situ* hybridization. (Upper) Schematic drawing of a midsagittal section of the medaka brain showing that the mRNAs for mdGnRH, cGnRH-II, and sGnRH are present in the preoptic area (POA), midbrain tegmentum (MT), and nucleus olfactoretinalis (NOR), respectively. (Lower) Micrographs of sagittal sections of the medaka brain, resulting from *in situ* hybridization with the probe specific for three GnRH mRNAs. (A) Hybridization signals for mdGnRH mRNA over the neurons within the POA. (B) Addition of excessive unlabeled probes specific for mdGnRH mRNA to hybridization buffer revealed no signals for mdGnRH mRNA in the POA. (C) cGnRH-II mRNA signals over a cluster of neurons in the MT. (D) Addition of excessive unlabeled probes for cGnRH-II mRNA gave no signals for cGnRH-II mRNA in the MT. (E) Signals for sGnRH mRNA over the NOR neurons forming a cluster. (F) Addition of excessive unlabeled probes for sGnRH mRNA gave no signals for sGnRH mRNA in the NOR. Scale bars = 10 μm.

The Medaka As an Ideal Model System for Study of GnRH

We have chosen the medaka as a model system, since this species has a number of useful characteristics. The study presented here strengthens the advantage of this species as a model system for understanding the physiological function, regulatory mechanism, and phylogeny of GnRH for the following reasons: (i) The medaka is one of few species in which three molecular forms of GnRH were determined, while it is possible that all vertebrates have three molecular forms of GnRH; (ii) The cDNAs for all three forms of GnRH were characterized; (iii) Different forms of GnRH are uniquely expressed by separate neuronal populations; and (iv) The neural populations producing GnRHs constitute clusters. The latter two characteristics of the medaka make it easy to analyze the roles of respective forms of GnRH. This model system will provide new insight into the physiological function and genetic regulation of GnRHs.

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