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Laser-Captured Single Digoxigenin-Labeled Neurons of Gonadotropin-Releasing Hormone Types Reveal a Novel G Protein-Coupled Receptor (Gpr54) during Maturation in Cichlid Fish

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GPR54 is a novel G protein-coupled receptor speculated to be essential for sexual development. However, its role in the regulation of GnRH types is unknown. To address this issue, we cloned GPR54 from the brain of a cichlid fish (tilapia *Oreochromis niloticus*) and determined its expression in immature and mature males using our newly developed technique: laser-captured microdissection of single digoxigenin-labeled GnRH neurons coupled with real-time quantitative PCR. The tilapia GPR54 cDNA contains an open reading frame of 1131 bp encoding 377 amino acids and exhibits 56% identity to human GPR54. Absolute copies of GnRH1 and GnRH3, not GnRH2, mRNAs were significantly high in mature compared with immature males. At the single-cell level, only in mature males, GnRH1 mRNA levels were inversely related to GPR54 mRNA ($P < 0.002$). GPR54 was expressed in a significantly high percentage (45.0–60.0%) of mature GnRH1, GnRH2, and GnRH3 neurons and in immature GnRH3 neurons, which had migrated to the vicinity of their final locations in the brain; on the contrary, only 5.0% of immature GnRH1 and GnRH2 neurons had GPR54 transcripts ($P < 0.001$). Thus, using a novel innovative single-cell gene profiling technique, we provide evidence of the structure of a nonmammalian GPR54, which is highly conserved during evolution and is expressed in GnRH1, GnRH2, and GnRH3 neurons. Furthermore, we propose that the expression of GPR54 is a "stop signal" for GnRH1, GnRH2, and GnRH3 neuronal migration, leading to suppression of cell growth and modulation of GnRH secretion, which is important for normal sexual development. (*Endocrinology* 145: 3613–3618, 2004)

GPR54 IS A NOVEL G protein-coupled receptor recently cloned from the rat brain (1) and thereafter cloned in mouse (2) and humans (3). The rat GPR54 cDNA encodes for a 396-amino-acid protein, which is widely distributed in the brain with highest expression found in the hypothalamus and amygdala (1). GPR54 has been implicated in pubertal maturation and normal sexual development. Mutation in the GPR54 gene in humans and mice causes hypogonadotropic hypogonadism in which pituitary secretion of FSH and LH are decreased, resulting in delayed puberty, small testes, and impaired reproductive functions (4–6); mutation of the gene in mice may be corrected with the administration of exogenous GnRH (5). This phenotype resembles Kallmann's syndrome in humans, which is caused by the failure of GnRH neuronal migration from the olfactory placodes to the hypothalamus (7–9).

GnRH is now recognized as a family of 16 neuropeptides, and it is well documented that all vertebrate species ranging from fish to humans possess two (hypothalamus, GnRH1; midbrain, GnRH2) or three GnRH types (caudal olfactory bulb, GnRH3) (10). The role of GPR54 in the regulation of GnRH1, GnRH2, and GnRH3 or its presence in GnRH neurons is unknown. To address this issue, we used our newly developed innovative technology, integrating laser-captured microdissection (LCM) of single digoxigenin (DIG)-labeled GnRH neurons coupled with real-time quantitative RT-PCR (RT-Q-RT-PCR), which would greatly facilitate our understanding of the complex interactions that exist within individual GnRH neurons. Tilapia *Oreochromis niloticus*, a well-characterized teleost model for studying three GnRH types (10), was used to identify the structure of GPR54 cDNA, to detect GPR54 mRNA in three GnRH types, and to analyze using RT-Q-RT-PCR the functional state of GPR54 and GnRH in individual neurons of GnRH1, GnRH2, and GnRH3 in immature and mature male tilapia.

Materials and Methods

Molecular cloning of GPR54

Total RNA from the brain of juvenile (10 d after hatching) tilapia *O. niloticus* was extracted with ISOGEN (Nippon Gene, Tokyo, Japan) and the poly(A)$^+$ RNAs were purified using Oligotex-dT30 Super (TaKaRa, Tokyo, Japan). First-strand cDNA was synthesized from the poly(A)$^+$ RNA using SuperScript III reverse transcriptase (Invitrogen Corp., Carlsbad, CA) and 50 pmol oligo(dT)$_{21-18}$ primer (Invitrogen) in a thermal cycler (Gene Amp PCR system 9700; PerkinElmer Applied Biosystems, Foster City, CA). All steps were performed according to the manufacturer's instructions. To amplify a fragment of tilapia GPR54 cDNA, degenerate PCR primers (GPR1 and GPR2; Table 1) were designed based on conserved sequences of mammalian GPR54 (GenBank accession numbers are as follows: mouse, NM_053244; rat, AF115516; and human, NM_032551). PCRs were performed in a final volume of 20 μl containing GeneAmp 1× PCR buffer (Applied Biosystems), 160 μM of deoxynucleotide triphosphate, 1.0 U DNA polymerase (AmpliTaq Gold, Applied Biosystems), 10 pmol gene-specific primers, and 1 μl first-strand cDNA or the first-round PCR product. Reaction conditions for PCR were 94 C

Abbreviations: DIG, Digoxigenin; GFAP, glial fibrillary acidic protein; LCM, laser-captured microdissection; RT-Q-RT-PCR, real-time quantitative RT-PCR; TM, transmembrane.

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TABLE 1. Sequences of PCR primers and probes used for PCR

Primer	Sequence (5′–3′)	Code
Degenerate primer		
GPR54-1	GCHAACCTGGCBGCCACDGA	GPR1
GPR54-2	VACRAATTTGCACATGAA	GPR2
RT-PCR		
GPR54-F	GCATTTGGATCGGTTCTTTCG	GPR3
GPR54-R	GGCCAACCCTCTTAACCATCA	GPR4
GnRH1-F	AGAAGCTTTATCCTCAGAAT	G1
GnRH1-R	CGGCCAAACTCGCAAGAA	G2
GnRH2-F	GACTAAGGTGGGAATATCAT	G3
GnRH2-R	GGCTAAGGCATCCAGAAGAATGT	G4
GnRH3-F	TTCTAATGGAAGCAGGCAGC	G5
GnRH3-R	CCTGTGCCCATCATCCTAATG	G6
GFAP-F	TGGTATCGCTCGAAGTTTGC	GP1
GFAP-R	AGGTCCTGGTACTCCTGCA	GP2
RT-Q-RT-PCR		
Q-GPR54-F	CGTGACAGTCTACCCCCTGAA	GPR5
Q-GPR54-R	TCCAAATGCAAATGCTGACAA	GPR6
Q-GPR54 Probe	CGGCACCGAACCCCCAAAGTAGC	GPR7
Q-GnRH1-F	CTCGCAGGGACGGTGTTT	G7
Q-GnRH1-R	CGGCCAAACTCGCAAGAA	G8
Q-GnRH1 Probe	CACAGGGCTGCTGTCAACACTGGTCATA	G9
Q-GnRH2-F	TGGTCCCATGGTTGGTATCC	G10
Q-GnRH2-R	GGCTAAGGCATCCAGAAGAATGT	G11
Q-GnRH2 Probe	AAATCTCTGATGTCCCAAAGGAGTCCAGCT	G12
Q-GnRH3-F	TGCTGGCGTTGGTGGTT	G13
Q-GnRH3-R	CCTGTGCCCATCATCCTAATG	G14
Q-GnRH3 Probe	CAGCACTGGTCCTATGGATGGCTACC	G15

All probes were labeled with 5′ FAM reporter dye and 3′ TAMARA quencher dye. Degenerate primers: H = A + C + T, B = C + G + T, D = A + G + T, V = A + C + G and R = A + G.

for 10 min; 30 cycles of 94 C for 20 sec, 55 C for 20 sec, and 72 C for 20 sec; and 72 C for 7 min. The PCR products were analyzed by 2.0% agarose gels and the bands of expected size were purified and ligated into pGEM-T Easy vector (Promega, Madison, WI) using the DNA ligation kit version 2 (TaKaRa) according to the manufacturer's instructions. The plasmid DNA was purified and both strands of the DNA were sequenced with T7 and SP6 primers using an ABI PRISM 310 DNA sequencer and sequencing analysis software (Applied Biosystems). The sequences of the 5′ and 3′ ends of the cDNAs were obtained using the rapid amplification of cDNA end method. The deduced amino acid sequence of the tilapia GPR54 cDNA was aligned with other known sequences using DDBJ CLUSTAL W SYSTEM (DNA Data Bank of Japan).

Tissue preparation and GnRH in situ hybridization

Experimental procedures in the present study were performed under the guidelines of the Animal Care Committee of Nippon Medical School. Tilapia *O. niloticus* were maintained in fresh water at 27 C with a natural photo regime (10 h of light, 14 h of darkness). Mature (standard length, 11.13 ± 0.68 cm; body weight, 45.73 ± 7.02 g; gonadosomatic index, 1.28 ± 0.39; n = 5) and immature (standard length, 5.25 ± 0.24 cm; body weight, 4.77 ± 0.51 g; gonadosomatic index, 0.032 ± 0.018; n = 5) males were anesthetized by immersion in a 0.01% solution of 3-aminobenzonic acid ethyl ester (MS222; Sigma, St. Louis, MO) before they were killed by decapitation. The brains were dissected and fixed in 4% buffered paraformaldehyde for 6 h at room temperature, cryoprotected in 20% sucrose, and embedded in Tissue-Tek Optimal Cutting Temperature compound (Sakura Finetechnical, Tokyo, Japan). Brain sections were cut in coronal planes (6 μm) and mounted onto aminopropyl triethoxy silane-treated slide glass (Matsunami Glass, Tokyo, Japan) and stored at −80 C until use for DIG *in situ* hybridization.

Sense and antisense tilapia GnRH1, GnRH2, and GnRH3 riboprobes were synthesized using the pGEM-T easy transcription vector (Promega) constructs containing the GnRH coding region (GenBank accession no. AB101665-7) and linearized with *Spe*I or *Nco*I endonuclease (Nippon Gene) as a template for T7 or SP6 RNA polymerase (TOYOBO, Tokyo, Japan). The RNA probes were labeled using DIG-RNA labeling mix (Roche Diagnostics GmbH, Penzberg, Germany).

Twenty-five microliters of hybridization buffer containing 30 ng DIG-labeled GnRH1, GnRH2, and GnRH3 riboprobes were added to each tissue section and incubated in a humidified box at 42 C for 12 h. After hybridization, sections were washed twice in 2× SSC (1× SSC = 0.15 M NaCl, 15 mM sodium citrate) at room temperature for 15 min and in 1× SSC and 0.1× SSC at 55 C for 1 h sequentially. The hybridization signals were detected using anti-DIG conjugated with alkaline phosphatase and visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate solution (Roche). The sections were washed rapidly two times with diethyl pyrocarbonate-H_2O and dehydrated with 70, 95, and 100% ethanol for 30 sec each, incubated in xylene for 5 min, and air-dried. The slides were stored in a desiccating box with silica gel until LCM was performed.

LCM of GnRH neurons

The dehydrated tissue section was overlaid with a thermoplastic membrane mounted on an optically transparent cap (CapSure Macro LCM Caps; Arcturus, Mountain View, CA). Using a Pix Cell II laser capture instrument (Arcturus), DIG-identified GnRH cells were microdissected by focal melting of the membrane through laser activation (laser pulse power, 25–65 mW; laser pulse duration, 1.5 ms; laser spot size, 10–30 μm diameter). A heat-pulled borosilicate glass microcapillary pipette (1.5-mm outer diameter; Harvard Apparatus Ltd., Edenbridge, Kent, UK; micropipette puller, type PE-2, Narishige, Tokyo, Japan) attached to a micromanipulator (Narishige) was used to remove undesirable tissue around the periphery of single GnRH cells. Then, using a negative pressure, single neurons of three GnRH types were harvested from the LCM cap into the micropipette under visual control and subsequently expelled into a sterile 1.5-ml reaction tube containing 50 μl of the lysis buffer and stored at −80 C until total RNA isolation. For unbiased cell sampling, five to six morphologically well-defined cells were harvested at random (~1 cell per alternate section) along the rostral-caudal extent of the whole population of each GnRH type in each animal (n = 5 animals per age group). Cells that were located individually were harvested, and only those cells positive for each GnRH type but negative for glial fibrillary acidic protein (GFAP) and free from genomic contamination were used for RT-Q-RT-PCR analysis (n = 4 cells per animal; 20 cells per GnRH type per age group).

GPR54 expression in GnRH neurons

The harvested single GnRH neuron was digested with 1 μg of proteinase K (Gentra Systems, Minneapolis, MN) and 10 U of Prime ribonuclease inhibitor (Eppendorf, Hamburg, Germany) for an hour at 53 C. The cell lysate was incubated for 1 h at 37 C with 1 U ribonuclease-free deoxyribonuclease I (Promega) to eliminate genomic DNA and was heat denatured at 95 C for 10 min to separate the mRNA from the DIG-labeled riboprobe. Total RNA was extracted from the cell lysate using ISOGEN (Nippon Gene) and Mini RNA Isolation Kit (Zymo Research, Orange, CA) and reverse transcribed to cDNA with 0.1 pmol of random primers (TaKaRa) using 40 U SuperScript III reverse transcriptase (Invitrogen).

To confirm the presence and integrity of GPR54 and GnRH mRNA, the single GnRH neuron's cDNA was subjected to RT-PCR. PCRs were performed in a final volume of 20 μl containing GeneAmp 1× PCR buffer, 160 μM of deoxynucleotide triphosphate, 1 U DNA polymerase (AmpliTaq Gold, Applied Biosystems), 250 nM gene-specific primers (G1–G6, GPR3 and GPR4, GP1 and GP2; Table 1) and one twentieth of a single neuron's reverse transcribed cDNA solution. Forward or reverse primers spanned a predicted intron and cDNA sequence on either side of the splice site (G2, G4, G6, GPR3; Table 1). Reaction conditions for PCR were 94 C for 10 min; 50 cycles at 94 C for 15 sec, 60 C for 15 sec, and 72 C for 15 sec; and 72 C for 7 min. Ten microliters of the reaction mixture were run on a 2% agarose gel and visualized with ethidium bromide. To confirm the sequences, some bands were subcloned into pGEM-T Easy vector (Promega) and both strands of the DNA were sequenced with T7 and SP6 promoter primers (Promega) using an ABI PRISM 310 Genetic Analyzer and Sequence Analysis Software (Applied Biosystems). Several controls were included for the RT-PCR: buffer without harvested cells, no reverse transcription, non-GnRH cells, and tilapia genomic DNA for negative control. For GnRH and non-GnRH cells, GFAP primers were also included in the PCR protocol (GP1 and GP2; Table 1). The GenBank accession numbers of the three GnRH types, GFAP, and the newly cloned GPR54 in tilapia are as follows: GnRH1, AB101665; GnRH2, AB101666; GnRH3, AB101667; GFAP, AB109167; and GPR54, AB162143.

RT-Q-RT-PCR for GnRH and GPR54 in GnRH neurons during maturation

RT-Q-RT-PCR was performed in duplicate in 10-μl reaction volumes consisting of 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM of primers (G7, G8, G10, G11, G13, G14, GPR5, GPR6; Table 1), 200 nM of hybridization probe (G9, G12, G15, GPR7; Table 1), and one twentieth of a single neuron's reverse transcribed cDNA or absolute standard cDNA using the ABI PRISM 7700 Sequence Detection System (TaqMan PCR; PerkinElmer Applied Biosystems). The PCR conditions were 95 C for 10 min followed by 60 cycles at 95 C for 15 sec and 60 C for 1 min. Hybridization primers and fluorogenic probes for RT-Q-RT-PCR were optimized using the ABI PRISM Primer Express Software (Applied Biosystems). The reverse primers (G8, G11, G14, GPR6; Table 1) spanned an intron and complemented the sequence on either side of the splice site of the gene. For each animal and GnRH type, average mRNA levels per cell were determined and these values were combined to give experimental group means. All values are expressed as the mean ± SEM and statistical comparisons were made between different age groups (n = 5, each group) using Student's *t* test or nonparametric ANOVA followed by *post hoc* Dunn's multiple comparison test. $P < 0.05$ was considered statistically significant (see figure legends).

Results and Discussion

Using PCR and the rapid amplification of cDNA end technique, we have cloned and obtained the full-length sequence for a novel receptor in a teleost, designated here as tilapia GPR54. The tilapia GPR54 cDNA contains an open reading frame of 1131 bp encoding 377 amino acids (Figs. 1 and 2; GenBank accession no. AB162143). In the open reading frame, we have identified conserved residues and consensus sequences of seven transmembrane (TM) domains of the rhodopsin superfamily of G protein-coupled receptors (11),

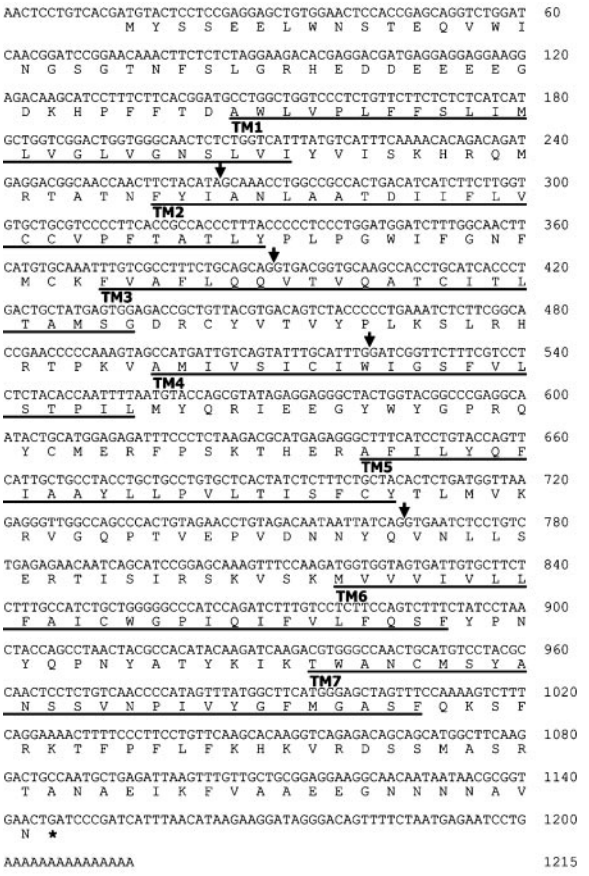

FIG. 1. Nucleotide and deduced amino acid sequence of GPR54 cDNA in tilapia. The *numbers on the right* refer to the nucleotide sequence. Predicted transmembrane regions (TM1–TM7) are *underlined*. The stop codon is marked by an *asterisk*. The predicted exon-intron junctions are marked by *arrowheads*.

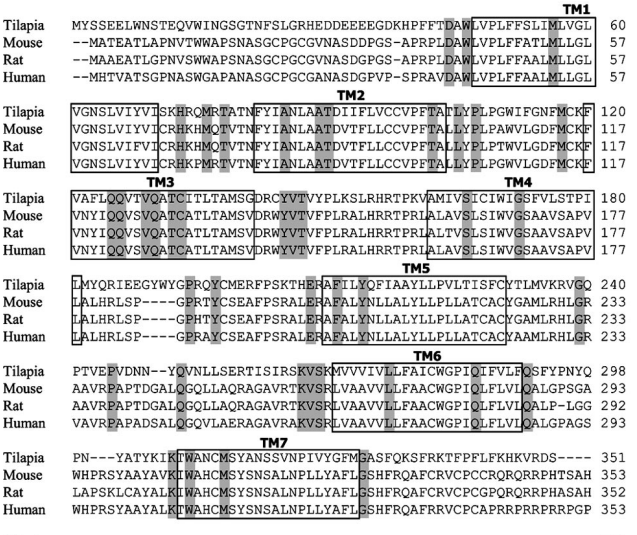

FIG. 2. An alignment of amino acid sequences of the tilapia GPR54 with mammalian GPR54. Conserved amino acid residues among GPR54 sequences are *shaded*. Predicted transmembrane regions are *boxed*.

which include an asparagine in TM1, an aspartate in TM2 and prolines in TMs 4–7 (Fig. 2). The amino acid sequence of tilapia GPR54 has 54, 55, and 56% identity with mouse, rat, and human GPR54, respectively (1–3). Phylogenic analysis also showed close sequence identity with rat galanin receptors (GALR1, GALR2, GALR3; 32~36%) and RFamide-related peptide receptor types (20%).

DIG-labeled *in situ* hybridization using probes against the three GnRH types specifically labeled cells in three brain regions known to express GnRH in tilapia: in the preoptic area (GnRH1), midbrain tegmentum (GnRH2), and at the caudal-most part of the olfactory bulbs (GnRH3) (Fig. 3, A–D). The localization results are consistent with our earlier observations using *in situ* hybridization and immunocytochemistry (12, 13). The present procedure using DIG-labeled *in situ* hybridization to identify neurons is a novel approach that is technically superior to our previous immunocytochemical-based method (13) because it overcomes the problem of cross-reactivity or availability of antiserum and allows single DIG-identified neurons to be harvested routinely for gene profiling and for quantitative studies. Single DIG-labeled GnRH neurons were microdissected using LCM, and the undesired tissue around the periphery of the GnRH neuron was removed using a microcapillary pipette attached to

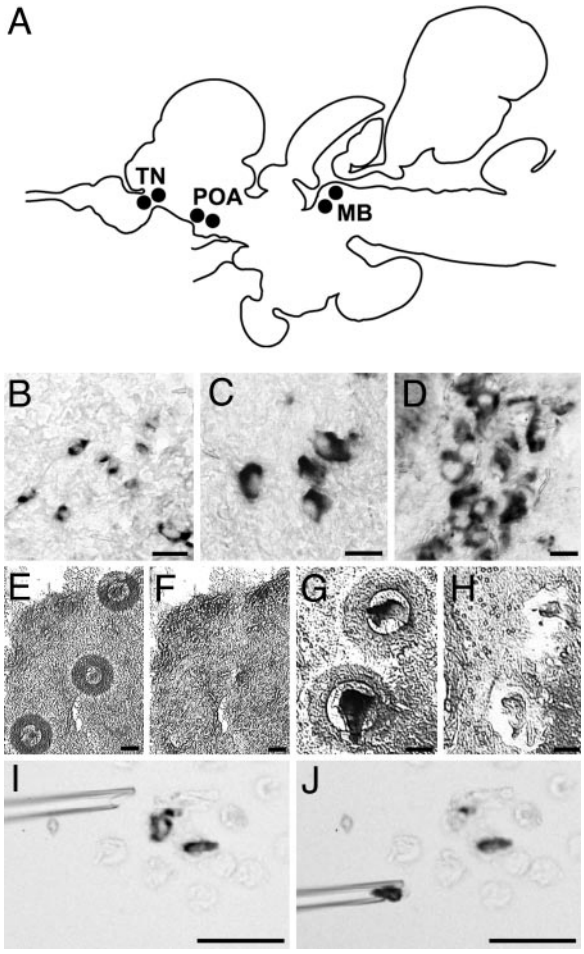

FIG. 3. A, A schematic illustration showing location of GnRH1, GnRH2, and GnRH3 (*black dots*) in a sagittal brain section. B–D, Photomicrographs of DIG-labeled GnRH1, GnRH2, and GnRH3 neurons; B, preoptic neurons (POA, GnRH1); C, midbrain neurons (MB, GnRH2); D, terminal nerve ganglia at the caudal-most olfactory bulbs (TN, GnRH3). E and G, DIG-labeled GnRH1 and GnRH2 neurons before LCM and, F and H, after LCM. I and J, Microdissected cells being harvested from LCM cap using micropipette attached to a micromanipulator. *Scale bar*, 20 μm.

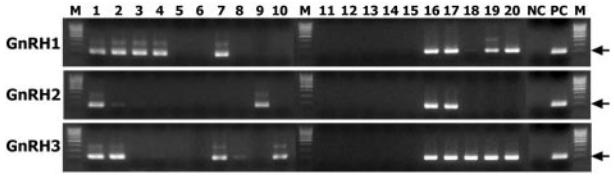

FIG. 4. Composite gel showing amplicons of GPR54 in GnRH1, GnRH2, and GnRH3 neurons taken from mature males (lanes 1–10), without reverse transcriptase (lanes 11–15); and non-GnRH cells surrounding GnRH1, GnRH2, and GnRH3 neurons (lanes 16–20). M, Marker, DNA 100-bp size ladder; NC, tilapia genomic DNA as negative control; PC, whole brain cDNA as positive control for PCR. The *arrowheads* show RT-PCR product of GPR54 at 216 bp.

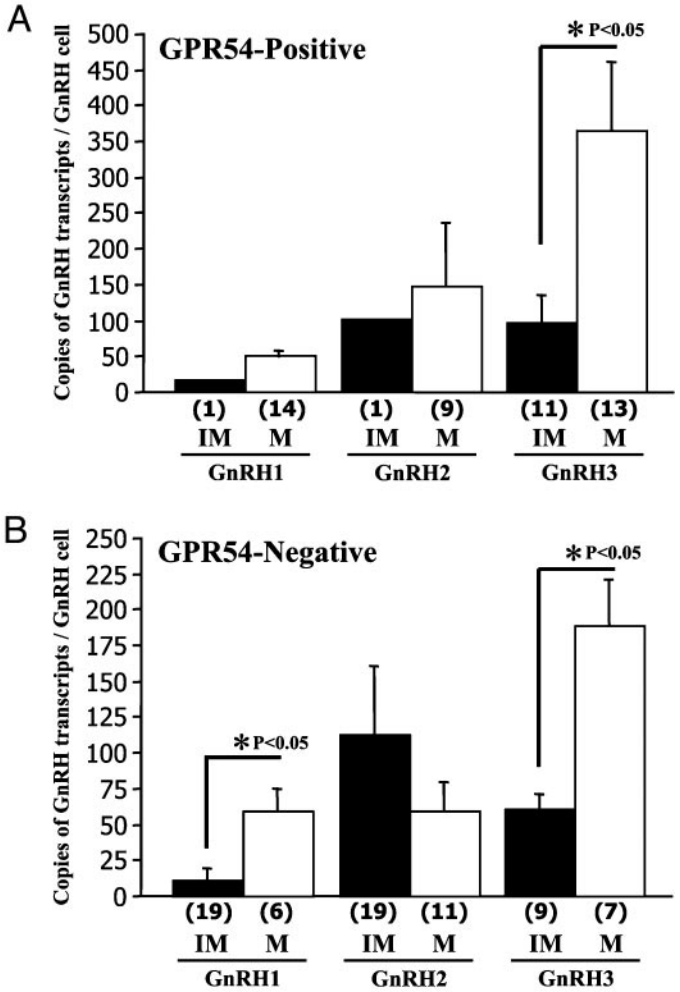

FIG. 5. Histograms showing copies of GnRH1, GnRH2, and GnRH3 mRNA in (A) GPR54-positive GnRH cells and (B) GPR54-negative GnRH cells. The average copies of mRNA transcripts per cell deduced from the total number of individual cells for each GnRH type are given in *parentheses*. Statistical analysis on an animal basis (n = 5, each group) showed significantly more GnRH transcripts in GnRH1 and GnRH3 neurons of mature (M, *white bars*) compared with immature males (IM, *dark bars*) ($P < 0.05$, Student's *t* test).

a micromanipulator (see *Materials and Methods* and Fig. 3, E–J). There was no genomic DNA contamination in the harvested single GnRH neurons (Fig. 4). DIG-labeled *in situ* hybridization had no effect on the integrity of mRNAs of the three GnRH types and other genes (our manuscript in preparation). RT-Q-RT-PCR showed that 100% of DIG-labeled GnRH1, GnRH2, and GnRH3 neurons had GnRH transcripts. The amplicon sizes were approximately 196 (GnRH1), 176 (GnRH2), and 218 bp (GnRH3) and their sequences were identical with tilapia GnRHs (see *Material and Methods* for GenBank accession numbers).

Using RT-Q-RT-PCR, significantly higher absolute copies of GnRH1 and GnRH3 mRNA transcripts were observed in mature (GnRH1, 59.3 ± 13.8; GnRH3, 277.0 ± 57.2 copies per cell) compared with immature males (GnRH1, 18.5 ± 5.9, $P < 0.05$; GnRH3, 81.6 ± 23.7 copies per cell; $P < 0.01$; Fig. 5, A and B). These results are similar to our previous reports suggesting an apparent role of GnRH1 in gonadal (testicular) maturation and the role of GnRH3 in reproductive behaviors (10, 13). In contrast, no difference in GnRH2 mRNA levels between the males (mature males, 98.8 ± 41.9 copies per cell; immature males, GnRH2, 111.1 ± 42.0 copies per cell; Fig. 5, A and B) suggests GnRH2 might be a neuromodulator important for reproductive and nonreproductive functions (10, 13).

In the present study we show, for the first time in a vertebrate species, GPR54 transcripts in GnRH1, GnRH2, and GnRH3 neurons (Fig. 4). At the single-cell level, only in mature males, absolute copies of GnRH1 mRNA transcripts were inversely related to GPR54 mRNA levels ($P < 0.002$; Fig. 6). It is possible that GPR54 might regulate GnRH secretion in mature GnRH neurons. In this context, it is noteworthy that mutations in GPR54 gene in humans and mice causes significant decrease in serum LH levels resulting in hypogonadotropic hypogonadism (4–6). This phenotype resembles Kallmann's syndrome in humans, which is caused by a defect in GnRH neuronal migration from the olfactory placodes to the hypothalamus (7–9). Because hypothalamic GnRH levels were normal in GPR54-deficient mice and serum LH levels were corrected with the administration of exogenous GnRH (5), this suggests GPR54 regulates GnRH secretion. The expression of GPR54 in one GnRH1 and one GnRH2 neuron in two different immature males (Fig. 6) is unlikely to be contamination because of our strict GnRH neuron selection criteria (see *Materials and Methods*). However, there remains a small possibility that a non-GnRH-GFAP-negative fragment could have been harvested along with the GnRH-positive neuron, which cannot be verified. Instead, we speculate that these single GPR54-expressing GnRH1 and GnRH2 neurons in immature males could be early signs of (future) precocious maturation and dominance, which remains an interesting thought.

That GPR54 could be a "stop signal" for GnRH neuronal migration or cell growth is another possibility because GPR54 is expressed in a large number of GnRH1 (71.4%; 14 of 20 cells), GnRH2 (45.5%; 9 of 20), and GnRH3 (66.7%; 13 of 20) in mature males and GnRH3 (55.6%; 11 of 20) neurons of immature males, which have migrated and reached the vicinity of their final location in the brain (present study; Ref. 10) and attained their adult size (immature: GnRH1, 10 μm; GnRH2, 15–18 μm; GnRH3, 20–25 μm; mature: GnRH1, 15–25 μm; GnRH2, 30–35 μm; GnRH3, 30 μm). On the other hand, almost complete lack of GPR54 transcripts in immature GnRH1 (5.0%; 1 of 20) and GnRH2 (5.0%; 1 of 20) neurons suggest that these neurons might be still migrating within the forebrain to their final location. Furthermore, statistical analysis of the percentage of GPR54-positive GnRH neurons on an animal basis (mature: GnRH1, 60.0 ± 10.0%; GnRH2, 45.0 ± 9.4%; GnRH3 65.0 ± 12.7%; immature: GnRH1, 5.0 ± 5.0%; GnRH2, 5.0 ± 5.0%; GnRH3, 65.0 ± 12.7%; n = 5 each group) showed a significantly high percentage of GPR54-positive mature GnRH1, GnRH2, and GnRH3 and immature GnRH3 neurons compared with immature GnRH1 and GnRH2 neurons ($P < 0.001$; Fig. 7). The hypothesis that

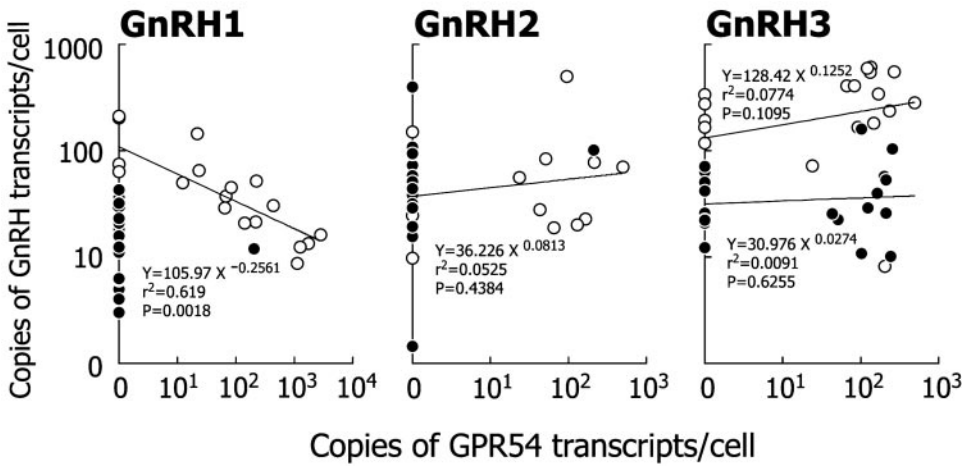

FIG. 6. Scatter diagram showing copies of GnRH and GPR54 mRNA transcripts in individual GnRH1, GnRH2, and GnRH3 neurons of mature (*white circles*) and immature (*dark circles*) males. Each data point represents a single cell (n = 20 cells, each group). Immature GnRH1 and GnRH2 neurons were almost devoid of GPR54 mRNA transcripts, whereas most neurons of mature GnRH types and immature GnRH3 had GPR54 mRNA transcripts. Linear regression statistics performed in mature males (*white circles*) showed inverse relationship between GnRH1 and GPR54 transcripts ($P < 0.002$, Spearman's rank correlation). Regression analysis in immature males *(dark circles*), which was performed only for GnRH3 *vs.* GPR54, showed no correlation.

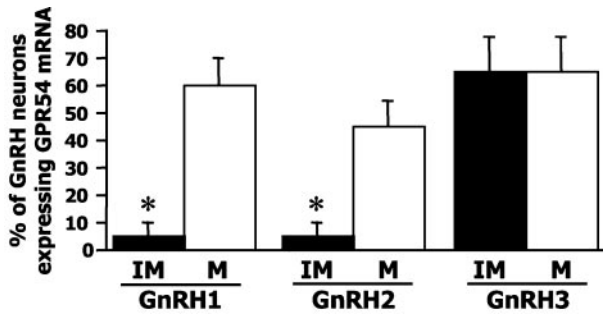

FIG. 7. Histograms showing the frequency of detection of GPR54 transcripts in immature (IM, *dark bars*) and mature male (M, *white bars*) GnRH neurons. Statistical analysis on an animal basis showed a significantly high percentage of GPR54-positive mature GnRH1, GnRH2, and GnRH3 and, immature GnRH3 compared with immature GnRH1 and GnRH2 neurons. *, $P < 0.001$; nonparametric ANOVA ($P = 0.003$) followed by *post hoc* Dunn's multiple comparison test; n = 5, each group.

GPR54 could be a "stop signal" is supported by the fact that metastin, a product of KiSS-1 gene, is the endogenous ligand for GPR54 that has been identified as a metastasis-suppressor gene, which inhibits cell migration and cell growth (2).

In the present study, a laser-captured single-cell gene expression technique was established, which provides the first evidence of a nonmammalian GPR54 in GnRH1, GnRH2, and GnRH3 neurons. Although a physiological role of GPR54 has yet to be ascertained, the present approach will facilitate our understanding of the role of GPR54 in neurons of the three GnRH types and will provide valuable insights into normal sexual development.

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