GnRH neuron type-specific transcriptome analysis by laser captured single-cell microarray in the medaka

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ABSTRACT

Most vertebrates possess at least two gonadotropin-releasing hormone (GnRH) neuron types. To understand the physiological significance of the multiple GnRH systems in the brain, we examined three GnRH neuron type-specific transcriptomes using single-cell microarray analyses in the medaka (*Oryzias latipes*). A microarray profile of the three GnRH neuron types revealed five genes that are uniquely expressed in specific GnRH neuron types. GnRH1 neurons expressed three genes that are homologous to functionally characterised genes, GnRH2 neurons uniquely expressed one unnamed gene, and GnRH3 neurons uniquely expressed one known gene. These genes may be involved in the modulation or maintenance of each GnRH neuron type.

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1. Introduction

Gonadotropin-releasing hormone (GnRH) is a neuropeptide that plays a critical role in the control of reproduction [1]. GnRH is also known as a multifunctional peptide; in addition to controlling the physiology of reproduction, it has a role in cell differentiation [2] as well as functions as a neuromodulator [3] and immuno-modulator [4]. It is now well documented that the brains of most vertebrate species possess two or, as in the case of some recently derived teleosts, three different GnRH types [5]. Each GnRH neuron type exhibits specific spatio-temporal expression patterns in the brain, and they have common as well as distinct functions. GnRH1 is the primary hypophysiotropic hormone, and it stimulates the synthesis and secretion of luteinising hormone (LH) and follicle stimulating hormone (FSH) in the anterior pituitary [6]. GnRH2 is the most evolutionarily conserved form, but little is known about its function, although there have been a few reports on its neuromodulatory function in sexual behaviour and food intake [7]. GnRH3 is uniquely expressed in the forebrain of teleost species, and it plays multiple roles in reproductive and non-reproductive functions [5]. The different functions and distribution patterns of the multiple GnRH neuron types in the brain of single species suggest the existence of unique control mechanisms that are specific to each GnRH neuron type. Although the roles of the different GnRH neuron types in the control of reproduction have been studied, data about the regulatory mechanisms in each GnRH neuron type remain limited. The transcriptome analysis of different GnRH cell populations would provide crucial information about the qualitative or quantitative differences that may exist and how these differences may govern the physiology of specific cell types. We have previously developed a technique using laser capture microdissection coupled with real-time PCR that enables the isolation and gene expression profile analysis of a homogenous cell population that are fixed and labelled using immunocytochemistry and *in situ* hybridisation [8,9]. However, the quantity of RNA samples obtained from fixed and identified cells that are processed using our previously established technique may not be sufficient for transcriptome analysis using microarray-based assay, since this approach requires relatively large amounts of total RNA. Several types of RNA amplification approaches have been developed to enable utilization of low amount of total RNA, including *in vitro* transcription-mediated, isothermal amplification techniques (PCR-based) and *in situ* hybridisation-based signal amplification [10,11]. The PCR-based approach has been the most popular; however, there is always the risk that a bias in the ratios between original transcripts and amplified transcripts will be introduced during the amplification process [12]. To ensure an accurate representation of mRNA levels, linear amplification based on cDNA synthesis and *in vitro* transcription has been widely utilised because this approach theoretically involves an isothermal reaction with linear kinetics [13].

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However, the successful linear amplification of RNA using *in vitro* transcription relies on the quality of the original mRNA, i.e., its integrity, quantity, purity and several other conditions [14]. In the present study, we employed a technique that combines PCR and *in vitro* transcription amplification. This methodology successfully amplifies small amounts of mRNA for microarray analysis [15]. Using this technology, we have successfully isolated and amplified a sufficient amount of cRNA from laser-captured three different GnRH neuron types in the medaka, *Oryzias latipes*. The cRNA samples were further analysed using a medaka brain cDNA/EST microarray to compare the gene expression profiles in the three GnRH types.

2. Materials and methods

2.1. Tissue preparation and in situ hybridisation

The experimental procedures in the present study were performed in accordance with the guidelines of the Animal Care Committee of Nippon Medical School, Tokyo, Japan. Medaka (*Oryzias latipes*, orange-red strain) were maintained in fresh water at 25 ± 1 °C with a natural photo-regimen (10 h light, 14 h dark). Sexually mature males ($n = 5$) were anesthetised by immersion in 0.01% 3-aminobenzonic acid ethyl ester (MS222; Sigma, St. Louis, MO) before they were killed by decapitation. The brains were dissected, fixed in RNase-free 4% buffered paraformaldehyde for 6 h at 4 °C, cryoprotected in 20% sucrose, and embedded in Tissue Tek OCT compound (Sakura Finetek, Tokyo, Japan). The coronal sections (15 µm) were cut on a cryostat and thaw-mounted onto aminopropyl triethoxy-silane-coated glass slides (Matsunami Glass, Tokyo, Japan).

Digoxigenin (DIG)-labelled antisense riboprobes for medaka GnRH1 (medaka fish GnRH), GnRH2 (chicken GnRH-II) and GnRH3 (salmon GnRH) genes (GenBank/EMBL/DDBJ accession numbers; AB041336, AB041334 and AB041335) [16,17] were labelled using DIG-RNA labelling mix (Roche Diagnostics, Penzberg, Germany). Dig-*in situ* hybridisation was performed as described previously [8]. Sections were hybridised with DIG-labelled riboprobes (20 ng/ml) at 42 °C for 12 h in a humidified chamber. The DIG-labelled probes were detected with an alkaline phosphatase-conjugated anti-DIG-antibody (Roche Diagnostics), and the chromogenic reaction was developed using a 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (Roche Diagnostics). DIG-labelled GnRH neurons were laser microdissected using an Arcturus XT system (Molecular Devices, Sunnyvale, CA), and harvested using a heat-pulled glass microcapillary pipette attached with a micromanipulator (Narishige Co. Ltd., Tokyo, Japan). 50 cells of each type were isolated from 5 fish.

2.2. RNA extraction and first-strand cDNA synthesis

The harvested cells were digested with 1 µg of proteinase K supplemented with 300 ng of polyinosinic acid (Sigma) in 17 µl of RLT buffer (Qiagen, Valencia, CA) for 20 min at 42 °C. The crude total RNA extracts were incubated at 90 °C for 10 min followed by purification with phenol–chloroform–isoamino alcohol (PCI). The isolated RNA extracts were further purified using RNeasy columns (Qiagen), followed by DNase digestion and elution in 10 µl solution according to the manufacturer's protocol. The five microlitres of extracted RNA was converted to first-strand cDNA with 0.3 µl of Sensiscript reverse transcriptase (Qiagen) and 0.5 µM of 5′-phosphorylated T7-d(T)$_{24}$ primer (p5′- TCTAGTCGACGGCCAGT-GAATTGTAATACGACTCACTATAGGGAGGCGGTTTTTTTTTTTTTTTTTTTTTTTTT-3′) in a final volume of 10 µl 1× Buffer RT (Qiagen) [15].

2.3. T7 antisense RNA amplification

A modified T7 antisense RNA amplification method [15] was used to amplify the cDNA from GnRH neurons. The second-strand cDNA synthesis reaction was performed in a final volume of 30 µl containing 5× second-strand buffer (Invitrogen, Carlsbad, CA), 3 µl of 10 mM dNTPs, 20 U of *Escherichia coli* DNA polymerase (TOYOBO), 2 U of *E. coli* RNase H (TOYOBO), 10 U of *E. coli* DNA Ligase (TOYOBO), and 10 µl of the first-strand cDNA. The reaction mixture was incubated at 16 °C for 2 h, and further incubated with 10 U of T4 DNA polymerase (TOYOBO) at 37 °C for 10 min. The reaction mixture was purified with PCI and ethanol precipitated.

T7-*in vitro* transcription was performed with 50 U of T7 RNA polymerase (TOYOBO) and 15 U of RNase inhibitor (Eppendorf, Hamburg, Germany). The reaction mixture was incubated at 37 °C for 5 h and then further incubated with 10 U of DNaseI (Promega, Fitchburg, WI) at 37 °C for 20 min. The synthesised cRNA was purified using an RNeasy column (Qiagen).

2.4. Second-round RNA amplification

First-strand cDNA was synthesised using 1 µl of Sensiscript reverse transcriptase (Qiagen) and 1 µg of random hexamer (Takara Bio, Shiga, Japan). The reaction mixture was incubated at 37 °C for 2 h and then further incubated with 2 U of RNase H (TOYOBO) at 37 °C for 20 min. The reaction mixture was purified with PCI and ethanol precipitation. The second-strand cDNA was synthesised with 1 µg of 5′-phosphorylated T7-d(T)$_{24}$ primer as described above without DNA ligase.

To amplify the cDNA by PCR, *Eco*RI–*Not*I–*Bam*HI adaptor (Takara) was ligated to the cDNA using T4 DNA ligase (TOYOBO). The adaptor-ligated cDNA was amplified by PCR using 2.5 U *Taq* polymerase (AmpliTaq Gold, Applied Biosystems) and 3 pmole/µl ER-1 primer (5′-GGAATTCGGCGGCCGCGGATCC-3′). Two-step PCR was performed in 30 cycles of 95 °C for 1 min and 72 °C for 3 min, followed by incubation at 72 °C for 10 min. After PCI extraction, the cDNA was then used as a template for T7 transcription with biotin incorporation for microarray analysis.

2.5. Design and manufacture of a DNA microarray

Preparation of the DNA microarray and hybridization experiment was carried out by GeneFrontier Corporation (Chiba, Japan). The probe sets for the DNA microarray were designed from 5279 medaka brain EST clone sequences available in the NCBI database (Accession nos. AU167026–AU172305). The probe sets consisted of 17 pairs of 24-mer perfect match and mismatch probes for each cDNA clone. The probes were synthesised on a glass slide (SuperClean slides, TeleChem International Inc.) using a Maskless Array Synthesizer (MAS, NimbleGen Systems Inc.)

2.6. Sample labelling and hybridisation

The amplified cDNAs were used to generate biotinylated cRNA probes using T7 RNA polymerase. The cRNAs were analysed with a Bioanalyzer2100 (Agilent Technologies, Santa Clara, CA). The biotinylated cRNAs were fragmented to an average of 200 bases by incubation with 5 × 200 mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc solution at 95 °C for 35 min.

Microarrays were hybridised with the biotinylated cRNA in 300 µl in the presence of 1 × 2-(N-morpholino) ethanesulfonic acid (MES) hybridisation buffer (100 mM MES, 1 M NaCl, 20 mM EDTA, 0.01% [v/v] Tween 20), 100 nM CPK6 oligonucleotide, 10 mg/ml herring sperm DNA, and 50 mg/ml acetylated BSA for
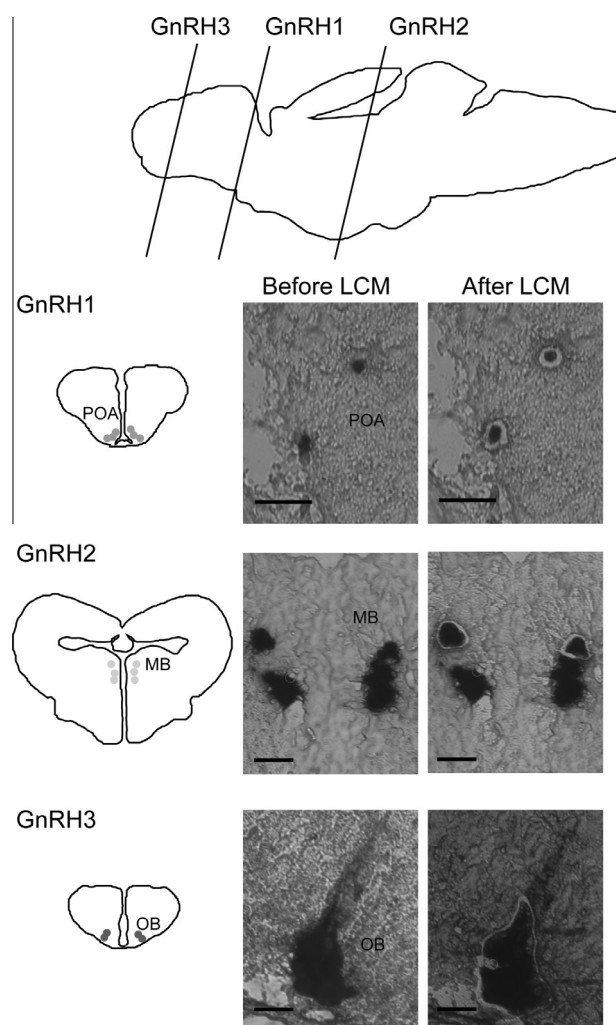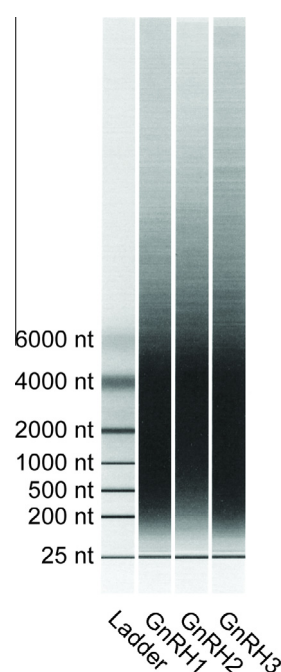
Fig. 1. Identification and laser capture microdissection (LCM) of GnRH neurons. Schematic coronal drawings of the medaka brain show the distribution of GnRH1 (preoptic area (POA)), GnRH2 (midbrain (MB)) and GnRH3 (olfactory bulb (OB)) cell types in the brain. The lines on a schematic sagittal drawing of the brain indicate levels of coronal sections. Scale bar: 25 μm.

Fig. 2. Gel image of amplified cRNAs from the Agilent Bioanalyzer 2100. All cRNAs ranged from 0.2 knt to 5.5 knt. nt: nucleotide.

16–20 h at 45 °C. Before application to the array, the samples were denatured at 95 °C for 5 min, pre-incubated at 45 °C for 5 min, and spun at 14,000g for 5 min. Hybridisation was performed in Nimble-Gen Hybriwheel hybridisation chambers (NimbleGen Systems) in a hybridisation oven with agitation. After hybridisation, arrays were washed in non-stringent buffer (6 × SSPE, 0.01% [v/v] Tween 20) for 5 min at room temperature, followed by washing in stringent buffer (100 mM MES, 0.1 M NaCl, 0.01% Tween 20) for 30 min at 45 °C.

2.7. Data analysis

After hybridisation, the glass slides were scanned with an Axon 4000B 5-μm scanner (Molecular Devices, Sunnyvale, CA). The scanned images were quantitatively analysed with Axon Gene-Pix4.0 (Molecular Devices). The expression data from the microarrays were normalised using robust multi-chip averaging [18], and t-test was then performed. The clones that exhibited a more than 1.5-fold change and a false discovery rate (FDR) lower than 0.2 [19] in expression were isolated.

All of the isolated clones were mapped to the genome, and overlapping clones and clones that could not be mapped to the genome were removed. The EST and amino acid sequences of the genes corresponding to the isolated clones were predicted with Ensembl genome database (http://www.ensembl.org/) and BLAST (http://blast.ncbi.nlm.nih.gov/).

3. Results and discussion

3.1. Medaka GnRH in situ hybridisation and RNA amplification

To identify the three types of GnRH neural populations (GnRH1, GnRH2 and GnRH3), we performed DIG-in situ hybridisation in the medaka brain. DIG-labelled GnRH neuron types were observed in specific regions of the brain: GnRH1 neurons in the preoptic area, GnRH2 neurons in the midbrain tegmentum, and GnRH3 neurons in the terminal nerve, which locates at the caudal-most part of the olfactory bulbs (Fig. 1), consistent with previous observations made using immunohistochemistry and in situ hybridisation [17,20]. The DIG-labelled GnRH neurons were individually microdissected by laser capture microdissection and harvested using a microcapillary pipette attached to a micromanipulator for RNA isolation (Fig. 1).

To evaluate the quality of the biotinylated cRNA, the purity [260/280 ratio = RNA Integrity Number (RIN) value], and cRNA pattern of the purified cRNA products were assessed using a Bioanalyzer 2100 instrument. The RIN values of the cRNA were 2.74, 3.03 and 3.54 for GnRH1, GnRH2 and GnRH3 respectively, which can be considered an acceptable level of RNA purity (RIN, 1.8–5.0) [21]. The sizes of the biotinylated cRNAs in each sample ranged from 0.2 k nucleotides (nt) to 5.0 knt (Fig. 2), which is comparable to the size ranges of cRNA samples amplified with a standard in vitro transcription that have been used for microarray analysis with successful reproducibility [15]. These parameters indicate that the quality of the cRNA samples amplified from GnRH neurons is acceptable for microarray analysis.

Table 1
Genes uniquely express in three GnRH neuron types.

GnRH types	GenBank accession number	Gene name	Successful translation to amino acid sequence	Most homologous sequence by BLAST search	Amino acid identity (E-value) by BLAST	Reported functions	Refs.
GnRH1 (>GnRH2 and GnRH3)	AU167298	Unknown	YES (5'–3' Frame 2)	Tilapia, *Oreochromis niloticus*, Hypothetical protein LOC100699848 (analog to Human jumonji domain-containing 3, JMJD3/KDM6B)	3e−14	Histone methylation during early embryo development, during differentiation of glioblastoma stem cells, neural commitment	[22]
	AU168548	Unknown	YES (5'–3' Frame 3)	Tilapia, *Oreochromis niloticus*, hypothetical protein LOC100705730	2e−103	Unknown	–
	AU170905	Unknown	YES (5'–3' Frame 2)	Mango tilapia, *Sarotherodon galilaeus*, male-specific protein	2e−50	A novel male-specific lipocalin that may function in intra and inter-sex communication.	[29]
GnRH2 (>GnRH3)	AU172107	Unknown	NO	NIL	NIL	NIL	–
GnRH3 (>GnRH2)	AU170758	Liprin alpha-3 (ppfia3)	–	–	–	Trafficking and fusion of synaptic vesicles	[27]

3.2. DNA microarray analysis

Microarray data obtained from the cDNA samples from each GnRH neuron type were compared each other and five genes were identified as the uniquely expressed genes in one of the three GnRH neuron types (Table 1 and Fig. 3).

3.3. GnRH1

Three unknown genes (GenBank accession nos. AU167298, AU168548 and AU170905) were significantly upregulated (>1.5-fold) at FDR of less than 0.2 in GnRH1 neurons compared to GnRH2 and GnRH3 neurons (Table 1 and Fig. 3). BLAST searches with the predicted amino acid sequences showed that two clones (AU167298 and AU170905) exhibited high similarity to functionally known proteins, including human jumonji domain-containing 3 (JMJD3/KDM6B) and male-specific protein (MSP) from the mango tilapia *Sarotherodon galilaeus*, and one clone (AU168548) exhibited high similarity to a functionally unknown protein, *Oreochromis niloticus* hypothetical protein LOC100705730 (Table 1, Supplemental Fig. 1–3). JMJD3/KDM6B is a histone demethylase [22]. Another JMJD, JMJD2B functions as co-factor of estrogen receptor α (ERα) and involves in the ERα signalling pathway in breast cancer [23]. GnRH1 neuronal activity is regulated by estrogen-induced positive and negative feedback [24,25]. Therefore, this JMJD3 analog (AU167298) may be involved in GnRH1 neuronal activity through an estrogen receptor-dependent pathway.

MSP is a functionally uncharacterised protein that was initially identified in the serum of sexually active male but not female cichlid (*Sarotherodon galilaeus*) [26]. MSP is secreted in the urine and seminal fluids and is present in the skin mucus of socially dominant males, and it could be a potential male-specific sex phero-mone-like protein for intra and inter-sex communication [26]. Although the potential role of MSP in GnRH1 neurons is unknown, GnRH1 neurons could be regulated by social and reproductive status through the MSP-like molecule encoded by AU170905 gene in male medaka.

3.4. GnRH2

One gene (GenBank accession no. AU172107) was significantly upregulated (>1.5-fold) at FDR of less than 0.2 in GnRH2 neurons compared to GnRH3 neurons but not GnRH1 neurons, and this gene has no homology to any known genes. This clone does not translate to a valid amino acid sequence (Table 1). However, it is possible that this clone is a functional non-coding RNA, which could be involved in GnRH2 neuron-specific regulation.

3.5. GnRH3

The *Liprin alpha-3* (PPFIA3) gene was significantly upregulated in GnRH3 neurons compared to GnRH2 neurons. PPFIA3 is a member of the Liprin-α protein family that is an essential component of the presynaptic active zone, which plays an important role in the formation and structure of synapses [27]. It is well documented that the secretory activity of the mammalian preoptic GnRH1 system is coordinated through the synaptic interactions and gap junctions between GnRH cells [28]. We have previously observed a heteromeric neural interrelationship between GnRH1- and GnRH3-immunoreactive cells in the tilapia (Ogawa and Parhar, unpublished). Although the potential involvement of the Liprin-α protein in GnRH neurons has not been demonstrated, PPFIA3 may contribute to the neuron-to-neuron communication in GnRH1 and GnRH3 neurons.

In this study, we examined the mRNA expression profiles in homogeneous populations of laser captured DIG-labelled GnRH

Hybridisation Intensity			Fold Change			Accession no.	Predicted genes	Chromosome
GnRH1	GnRH2	GnRH3	$\frac{GnRH1}{GnRH2}$	$\frac{GnRH2}{GnRH3}$	$\frac{GnRH3}{GnRH1}$			
			1.85	-	0.66	AU167298	Unknown	14
			1.80	-	0.58	AU168548	Unknown	9
			-	0.65	-	AU170758	PPFIA3	19
			1.58	-	0.63	AU170905	Unknown	9
			-	1.54	-	AU172107	Unknown	18

Fig. 3. Heatmap of genes that exhibited a significant difference, which a fold-change is more than 1.5 and a false discovery rate is lower than 0.2, in relative expression among GnRH neuron types. The colour gradient from red to green represents increasing ratio of array hybridisation intensity. Fold change indicates the ratio between GnRH neuron types. - indicates no significant difference. PPFIA3: Liprin alpha-3.

neurons using a DNA microarray and identified five genes that are differentially expressed in the three GnRH neuron types. However, the roles of these genes in each GnRH neuron type remain unknown or speculative. Further study is needed to determine the functional significance of these genes in the regulation of each GnRH neuron population.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.004.

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