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Induction of *c-fos* transcription in the medaka brain (*Oryzias latipes*) in response to mating stimuli

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ABSTRACT

Immediate-early genes (IEGs) are useful for mapping active brain regions in various vertebrates. Here we identified a *c-fos* homologue gene in medaka and demonstrated that the amounts of *c-fos* transcripts and proteins in the medaka brain increased in relation to an artificially evoked seizure, suggesting that the homologue gene has the characteristics of IEGs, which are used as markers of neural activity. Next, quantitative reverse-transcription-polymerase chain reaction revealed that female mating behaviors upregulated *c-fos* transcription in some brain regions including the telencephalon, optic tectum, and cerebellum. In addition, we performed *in situ* hybridization with a *c-fos* intron probe to detect the de novo synthesis of *c-fos* transcripts and confirmed induction of *c-fos* transcription in these brain regions after mating. This is the first report of IEG induction in response to mating stimuli in teleost fish. Our results indicated that *c-fos* expression was induced in response to behavioral stimuli in the medaka brain and that medaka *c-fos* could be a useful marker of neural activity.

1. Introduction

The medaka (*Oryzias latipes*) is a freshwater teleost fish native to East Asia that has long been an ornamental fish in Japan. Medaka exhibit various social interactions, such as schooling behavior [1,2], aggressive behavior [3], and a female mating preference for large males [4]. The medaka mating behaviors, for example, comprise a series of behavioral steps. First, the male medaka approaches the female and swims underneath her [5,6]. Then the male swims rapidly in a small circle, which is called a "quick circle". If the female is receptive to the male courtship display, the fish copulate by crossing their cloaca. As the medaka is a model organism for molecular genetics [7], functional analysis of the neural circuits involved in social interactions using advanced genetic methods will contribute to a better understanding of the neural/molecular basis underlying vertebrate social interactions.

Immediate-early gene (IEG) expression is induced in neurons by stimuli naturally associated with behaviors and the localization of IEG expression is a useful marker of neural activity. Based on IEG expression, brain regions that are active in response to (social) behavioral stimuli have been identified in vertebrate brains, such as rodents [8], songbirds, [9], and anurans [10]. Brain regions associated with mating behavior have been successfully mapped. For example, mating in rodent significantly increases the number of Fos-immunoreactive neurons in several brain regions, including the medial preoptic area, bed nucleus of the stria terminalis, medial amygdala, hypothalamic ventromedial nucleus, subparafascicular thalamic nucleus, and midbrain central tegmental field [8,11]. Although IEG mapping studies have been extensively performed in mammalian and avian model systems, similar analyses in fish have been limited to a few studies in teleost fish [12–14]. Here we identified a medaka *c-fos* homologue gene and demonstrated that IEG expression was induced in response to mating stimuli.

2. Materials and methods

2.1. Fish

Medaka fish (*O. latipes*, dr-R strain) were maintained in like groups in plastic aquariums (12 cm × 13 cm × 19 cm). Sexually mature male and female adult medaka fish (more than 3 months after hatching) were used for the cDNA cloning, real-time PCR, Western blotting, and *in situ* hybridization studies.

2.2. Mating condition

The adult female and male medaka fish were separated by two tanks overnight, prior to mating (Fig. 4A). The next morning, the male and female were placed together in a single tank and then the pair began to exhibit mating behavior within 5-min. We confirmed that the females exhibited normal mating behavior,

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including crossing and spawning, and the males exhibited normal mating behavior, including approach, courtship, and ejaculation.

2.3. Quantification of the c-fos transcript

Real-time RT-PCR was performed with Light Cycler-DNA master hybridization probes (Roche) according to the manufacturer's protocol, using gene-specific primers (c-fos; and 5′-TTCAGAAGAAGCG CTCAAGGA-3′ and 5′-AAGAGCAAGCCTTGGATGAAG-3′; actin; 5′-C TGTCTTTCCCTCCATCGTT-3′ and 5′-TGAGGTAGTCTGTAAGGTCG-3′). The amount of *c-fos* transcript was normalized with that of actin. No significant difference was detected in the levels of actin transcripts [15] between pentylenetetrazole (PTZ) -exposed, control medium-exposed, and mating-stimulated medaka.

2.4. In situ hybridization

In situ hybridization of tissue sections was performed as described previously with some modifications [15,16]. Paraffin-embedded coronal brain sections (10-μm thick) were fixed in 4% paraformaldehyde in phosphate buffered saline, pretreated, and hybridized with digoxigenin (DIG)-labeled riboprobes. The *c-fos* first intron fragment was amplified with forward primer: 5′-GT AAATTGAAACGACGATTGCTTAGATG-3′ and reverse primer: 5′-CT GAGAGAAAGAGGGAGGG-3′ using a genome DNA BAC plasmid ola1-200A07 (National BioResource Project) as a template. The DIG-labeled riboprobes were synthesized by T7 or SP6 polymerase with a DIG labeling mix (Roche) from a template containing the *c-fos* first intron fragment. After stringent washes, DIG-labeled riboprobes were detected immunocytochemically with peroxidase-conjugated anti-DIG antibody (1:500; Roche) and TSA Biotin System (Perkin Elmer). Sense probes were used as negative controls and the signals were confirmed to be antisense probe-specific in every experiment. Micrographs of section *in situ* hybridization were taken using a BX50 optical microscope (Olympus). Intensity and brightness of the micrographs were processed with Photoshop software (Adobe, San Jose, CA).

2.5. Western blotting

Protein detection was performed with antibodies against c-fos (anti-c-Fos (K-25): sc-253, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and β-actin (mouse anti-actin monoclonal antibody: MAB1501, Chemicon International). Blots were simultaneously incubated with differentially labeled species-specific secondary antibodies after transfer to membranes [anti-rabbit IgG conjugated with HRP and anti-mouse IgG conjugated with HRP].

3. Results

3.1. Medaka c-fos homologue has characteristics of an immediate-early gene

To search for active regions in the medaka brain, we focused on the *c-fos* gene as an IEG that is transiently expressed in active neuronal cells. We found an exon encoding a putative medaka *c-fos* homologue in the medaka genome database and isolated a full-length cDNA using the 5′-and 3′-rapid amplification of the cDNA ends (RACE) method (Genbank No. AB572350). The open reading frame encoded 364 amino acids, which had the highest identities (57% and 60%) with fos homologues in mouse (Genbank No. NM_010234) and zebrafish (Genbank No. NM_205569), respectively, which have IEG characteristics [17,18]. First we examined whether medaka *c-fos* homologue also has IEG characteristics. In some rodents, the expression of IEGs is induced in brain regions corresponding to sites of seizure initiation [19,20]. Exposure to a common convulsant agent (PTZ; a GABA-A receptor antagonist) induces stereotyped seizure behavior and leads to *c-fos* expression in zebrafish [18].

First, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to demonstrate the temporal induction of *c-fos* transcription following PTZ exposure. Medaka fish were transferred from a tank with normal medium to a tank with PTZ (10-mM)-containing medium. Adult medaka was exposed to PTZ for 0, 10, 20, 30, 40, 60, and 90 min and then total RNA from the whole brain was isolated. We also isolated total RNA from medaka that was transferred to normal bathing medium as controls. An increase in *c-fos* transcripts levels was detected within minutes after initiating the stimulus, peaked around 30 min, and returned to basal levels within 60 min (Fig. 1A). Next, we analyzed c-Fos protein expression levels using Western blotting with an anti-c-Fos antibody, whose epitope mapped within an internal region of c-Fos of human origin (datasheet from Santa Cruz Biotechnology). Total protein from the whole brain in which adult medaka fish were exposed to 10-mM PTZ for 0, 30,60, 90, 120, and 150 min was isolated. Western blot analysis using the medaka brain lysate showed a major band at approximately 63 kDa (Supplementary Fig. S1), whose molecular weight is consistent with that of homolog proteins in mouse and zebrafish [21,22]. Further, using normal IgG as the primary antibody instead of the c-Fos antibody, we confirmed that there was no nonspecific labeling at bands of the same size as the c-Fos immunoreactive protein (Fig. 1B, lower panel). In medaka exposed to PTZ for 60 min, the level of c-Fos immunoreactive protein was significantly upregulated in comparison with medaka exposed to PTZ for 30 min or untreated control medaka (Fig. 1B, upper panel). Taken together, we concluded that the medaka *c-fos* homologue had IEG activity.

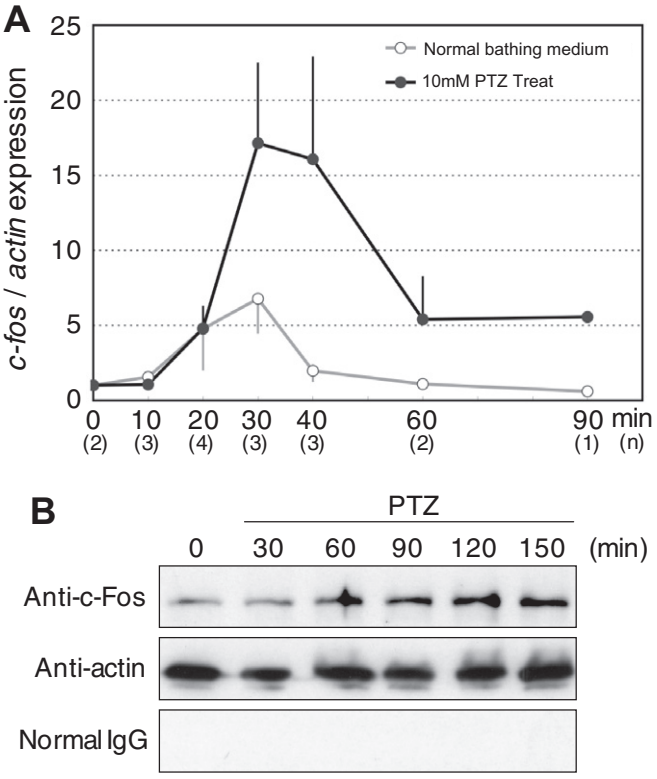

Fig. 1. Immediate early induction of medaka c-fos homologue in the whole brain in response to seizure. (A) The time course of the *c-fos* transcript level revealed by qRT-PCR. All data are shown as the means ± SEM. (B) The time course of the amount of c-Fos protein using Western blotting analysis.

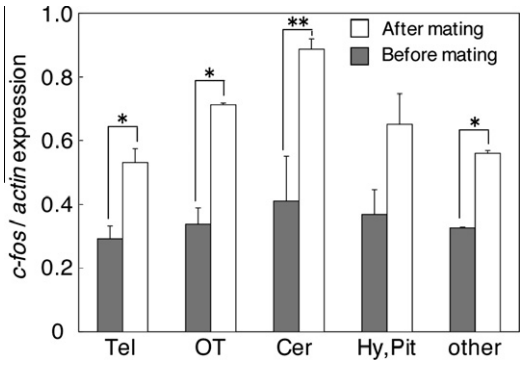

Fig. 2. Mating stimulation induced *c-fos* transcription in various regions of the female brain. Comparison of *c-fos* mRNA expression investigated by qRT-PCR in The telencephalon (Tel), optic tectum (OT), cerebellum (Cer), hypothalamus and pituitary gland (Hy, Pit), and other regions. ($^{*}P < 0.05$; $^{**}P < 0.01$; Student's *t*-test) All data are shown as the means ± SEM.

3.2. Mating stimulation induced c-fos transcription in various brain regions of the female brain

To examine whether *c-fos* expression is induced in response to behavioral stimuli, we investigated changes in *c-fos* expression in the female brain during mating behavior. The female brains were sampled 30 min after mating behavior, because *c-fos* transcripts were sufficiently increased within 30 min of neural stimulation (Fig. 1).

First, we quantitatively analyzed *c-fos* transcripts in the female brain using qRT-PCR. We compared the amount of *c-fos* transcripts in five regions of the female brain before and after mating: the telencephalon, optic tectum, cerebellum, hypothalamus and pituitary gland, and other regions that mainly contained the medulla oblongata and the anterior part of the spinal cord. (Fig. 2). Mating behavior significantly increased *c-fos* transcripts in the telencephalon, optic tectum, cerebellum, and other regions. In the hypothalamus with pituitary gland, the level of *c-fos* transcripts after mating tended to be higher than that before mating, although the difference was not significant. These results suggest that mating stimulation induced *c-fos* transcription in very broad areas of the female brain.

3.3. Detection of localization of c-fos expression in response to mating stimulation using in situ hybridization

To detect only the immediate early induction of *c-fos* transcription (the de novo synthesis of *c-fos* transcripts), we performed *in situ* hybridization using a probe corresponding to the first intron sequence of the *c-fos* gene, which generally improves the temporal and spatial resolution of IEG mapping [23]. To examine whether immature *c-fos* transcripts can be detected using the *c-fos* intron probe, we prepared paraffin sections of brain using medaka exposed to 10-mM PTZ for 30 min, and then performed *in situ* hybridization. Dot-like signals were detected in the telencephalon and hypothalamus (Fig. 3A and Supplementary Fig. S2). In addition, very few dot-like signals were observed in medaka without PTZ treatment. Therefore, we concluded that the de novo synthesis of *c-fos* transcripts could be detected using *in situ* hybridization method with a *c-fos* intron probe.

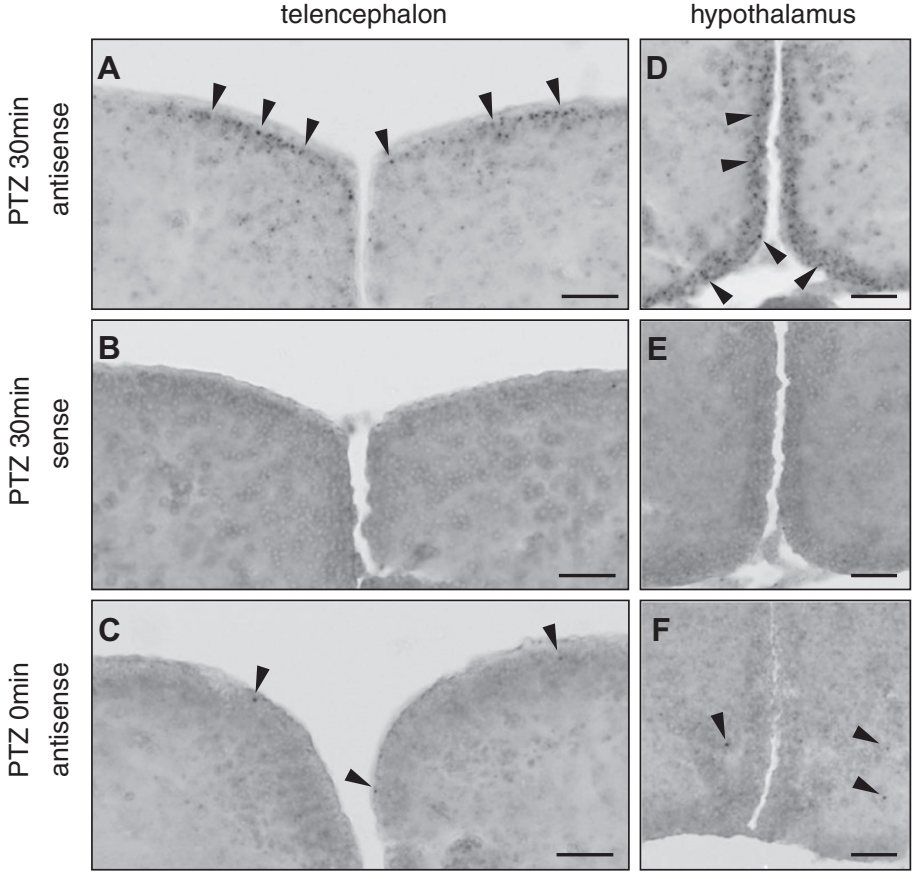

Fig. 3. Detection of the de novo synthesis of *c-fos* transcripts using *in situ* hybridization method. In the brains of medaka treated with 10-mM PTZ, dot-like signals were detected in the telencephalon (A; arrowhead) and hypothalamus (D; arrowhead). No significant signal was detected using the sense probe as a negative control (B and E) in cell nuclei. The number of dot-like signals increased depending on PTZ-treatment both in the telencephalon (C) and the hypothalamus (F). Scale bar, 50 mm.

Next, using this method, we compared the *c-fos* transcription before and after mating behavior in the female brain. We prepared a series of paraffin sections from the female brains 30 min after spawning and ejaculation. In the female brain, dot-like signals were detected in the preoptic area, optic tectum, and cerebellum (Fig. 4C–E). A large number of dot-like signals were detected in the dorsomedial telencephalon of females (Fig. 4B). The induction was not detected in the dorsolateral region of telencephalon (Supplementary Fig. S2B). The expression pattern was clearly different from that of PTZ-exposed medaka, where dot-like signals were detected in the dorsomedial and dorsolateral regions of telencephalon (Fig. 3A and Supplementary Fig. S2B). In contrast, a small number of dot-like signals were detected in the female brains before mating behavior (Fig. 4F–I and Supplementary Fig. S2B), and no dot-like signals were observed when the *c-fos* sense probe was used as a negative control (data not shown and Supplementary Fig. S2B).

4. Discussion

Here we identified a *c-fos* homologue gene in medaka and characterized the time course of its gene expression after pharmacologic stimulation. The *c-fos* expression peaked at 30-min post-induction and was markedly reduced at 90 min (Fig. 1A). The time course of *c-fos* mRNA induction was similar to that in mammals

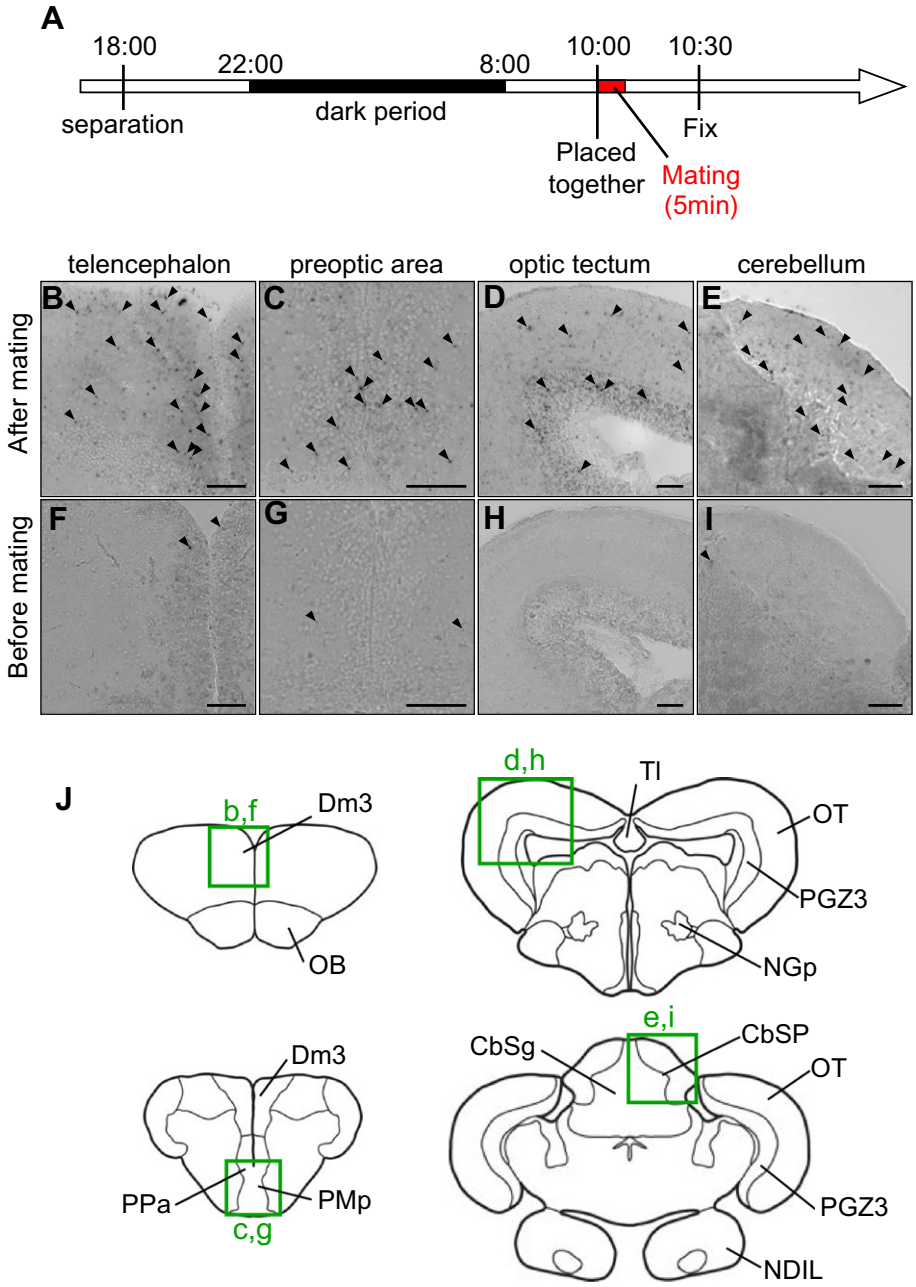

Fig. 4. Induction of the de novo synthesis of *c-fos* transcripts during mating. (A) Time course of the experiment. *c-fos* expression was detected by *in situ* hybridization using coronal brain sections after mating (B–E) and before mating (F–I). The dot-like signals (black arrowhead) were detected by *in situ* hybridization using coronal brain sections of female brain; medial telencephalon (B and F), preoptic area (C and G), optic tectum (D and H), and cerebellum (E and I). Scale bar, 50 mm. (J) Schematic presentation of the telencephalon, preoptic area, optic tectum and cerebellum of medaka brain. Areas corresponding to panels (B–I) are boxed in (J). The positions of the coronal sections are indicated in Supplementary Fig. S2. Dm3, area medialis3 of D; OB olfactory bulb; PPa, nucleus preopticus parvocellularis pars; PMp, nucleus preopticus magnocellularis pars parvocellularis; Tl torus longitudinalis; OT, tectum opticum; PGZ3 periventricular grey zone3; NGp nucleus glomerulosus posterioris; CbSg, stratum granulare of corpus cerebelli; CbSP, stratum Purkinje of corpus cerebelli; NDIL, nucleus diffuses of lobus inferioris.

[24]. A small increase in *c-fos* expression was also detected in control fish (Fig. 1A), which might be due to handling during transfer. Furthermore, qRT-PCR and *in situ* hybridization revealed that *c-fos* expression was induced in broad brain areas, including the telencephalon, optic tectum, and cerebellum in response to mating stimuli. In the present study, we performed *in situ* hybridization using a *c-fos* intron probe to detect only the immediate early induction of *c-fos* transcription (the de novo synthesis of *c-fos* transcripts). Generally, the use of intron-specific IEG riboprobes provides a marker of activated neuronal nuclei, without contamination by mature mRNA that can be encountered when using cDNA-based probes containing exonic sequences. Thus, use of the IEG intron probes can improve the temporal and spatial resolution of IEG mapping [23]. Furthermore, *in situ* hybridization signals using the IEG intron probes were detected in discrete intranuclear foci, which represented newly transcribed RNA at allelic sites [23]. In the present study, consistent with previous reports, the dot-like signals were detected using a *c-fos* intron probe.

The behavioral and pharmacologic induction of *c-fos* expression in medaka brain suggested that neural activity induces *c-fos* expression in medaka in a similar manner to that in mammals and birds. The merit of IEG mapping is that it allows us to simultaneously examine the response of multiple brain regions that are involved in natural behaviors under free-moving conditions. The present findings suggested widespread activation in the female brain following mating and we consider these results an initial step toward identifying the specific brain region involved in a behavioral element of female mating behavior, such as female choice and spawning. The medaka is a model organism for molecular biology and genetics [25] and efficient methods of generating both transgenic and knockout medaka are available [26,27]. Modulation of neural activity in target activated neurons using molecular techniques such as optogenesis [28] will lead to fine mapping of the neural circuit underlying mating behaviors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.143.

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