Molecular cloning and effect of c-*fos* mRNA on pharmacological stimuli in the goldfish brain.

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Abstract

c-*fos* is an immediate early gene, and is rapidly and transiently induced in neurons of the central nervous system according to their activities. To investigate neuronal activities in the brain of the goldfish (*Carassius auratus*), we considered that expression of c-*fos* mRNA would be an available marker for the neuronal activities. Therefore, we firstly isolated a cDNA clone encoding c-Fos from the goldfish brain by RT-PCR and RACE methods. A full length cDNA of the goldfish c-*fos* was composed of 1,044 bp open reading frame. The amino acid sequence of the goldfish c-Fos was approximately 56-90 % identical to those of teleost fish c-Fos. Northern blot analysis showed that the expression of c-*fos* mRNA was rapidly and transiently induced in the brain of the goldfish by the intraperitoneal administration of kainic acid. We also showed that the identification of the c-*fos* mRNA expression site by *in situ* hybridization will be able to use as an anatomical marker for identification of the activated neuronal region in the goldfish brain.

Keywords:

c-fos; immediate early gene; goldfish; kainic acid

1.Introduction

Expression of the proto-oncogene c-*fos*, a member of the immediate early gene family, is rapidly and transiently induced in neurons of the central nervous system (CNS) depending on their activities(Cirelli and Tononi 2000; Sabban and Kvetnansky 2001). c-Fos and c-Jun form a heterodimer binding to AP-1 binding site on the promoter region of many cellular genes regulating the transcription of other genes necessary for long-term alterations in neuronal functions(Bohmann et al., 1987; Angel et al., 1988; Chiu et al., 1988; Franza et al., 1988; Curran et al., 1990; Macgregor et al., 1990; Cui et al., 1999). The expressions of c-*fos* mRNA and the c-Fos protein possibly reflect the genomic response and neuronal activity of cells to environmental stimuli at cellular levels(Bosch et al., 1995). In the mammalian and bird, it has been revealed that the expression regions of the c-*fos* mRNA and c-Fos protein in the brain are central sites of the sensory perception for light, odor, or tastant, and of pharmacological, hormonal and electrical stimuli(Guthrie et al., 1993; Hess et al., 1995; Koistinaho and Sagar 1995; Harrer and Travers 1996; DiNardo and Travers 1997; Schafe et al., 2000). The c-*fos* mRNA has been used as a marker for mapping functionally activated neurons(McCabe and Horn 1994; Ueta et al., 2001). As we has investigated neural activity underlying fish behavior with goldfish (*Carassius auratus*), the expression of c-*fos* mRNA will be useful marker for identification of the activated neuron in the goldfish brain. We anticipate that brain areas showed c-*fos* mRNA expression are associated with central neurons involved in processing sensory information from environments, learning and particular behaviors in teleost fish

brain. Therefore, we firstly tried to isolate a cDNA clone encoding c-Fos from the brain of goldfish treated with kainic acid, which is known as an agonist of glutamate receptors to enhance neural excitability in the brain(Zhang et al., 2002). We further investigated the effect of kainic acid on the expression of the c-*fos* mRNA in the goldfish brain by northern blotting analysis and *in situ* hybridization.

2. Materials and Methods

2.1. Animal and tissue preparation

Goldfish were obtained from a commercial supplier. Three adult goldfish ranging from 106 to 127 mm in body length were used for cDNA cloning, twenty two goldfish weighing from 52 to 68 mm were for northern blotting analysis, and five adult goldfish weighing from 60 to 66 mm were for *in situ* hybridization.

2.2. RNA preparation

Goldfish were deeply anesthetized with 150 ppm tricaine methanesulphonate solution at 60 min after intraperitoneal administration of kainic acid (6 mg/kg of body weight), and then were sacrificed. Total RNA was extracted from the brain using Isogen (Nippon Gene), and poly(A)$^+$RNA was isolated using QuickPrep micro mRNA Purification Kit (Amersham Biosciences Corp), according to the manufacturer's protocol.

2.3. RT-PCR

A fist strand (1st) cDNA was synthesized from poly(A)$^+$RNA (1 μg) of the goldfish brain, using oligo(dT)$_{20}$ adaptor primer (5'-GTTTTCCCAGTCACGACdT$_{20}$-3') and M-MLV Reverse transcriptase (Promega). Primers were designed according to the nucleotide sequence (forward primer, RTs1;5'-CCCATCTGCAAGATCCC-3' identical to nucleotides (nt) 663-679 and reverse primer, RTa1; 5'-AGTCAGTTCCATAGCCCTG-3' complementary to nt 1239-1257) of carp c-*fos* (Accession no U81595). PCR was carried out using 1st strand cDNA, KOD Dash DNA polymerase (Toyobo), and primers (RTs1 and RTa1). Amplification was performed using following cycle conditions: 94 °C for 120 s, 35 cycles 94 °C 30 s 56 °C 5 s 74 °C 30 s. PCR product was subcloned into pGEM-T easy vector (Promega), and sequenced by ABI 373A DNA sequencer (Applied Biosystems) using DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences Corp). Based on the determined nucleotide sequence of the internal cDNA, primers (3Rs1 for 3'-RACE; 5'-CGGGATCCATAAAATGGTATTGCAGGGC-3' identical to nt 1231 to 1250 , and 5Rs1 and 5Ra1 for 5'-RACE, 5'-CAGCAATGACCAATCATCCG-3' identical to nt 1231-1250 and 5'-TGATGCTGTGGATCTCAGGG-3' complemntary to nt 847-855 in accession no. AB058417) were designed for 5'- and 3'-end amplifications. The 3'-end amplification of the goldfish c-*fos* cDNA was carried out using 1st strand cDNA described above, and primers (adaptor primer; 5'-GTTTTCCCAGTCACGAC-3' and RTs1). Amplification was performed using

following cycle conditions; first 10 cycles 94 °C 30 s 50 °C 10 s 74 °C 30 s, next 10 cycles temperature of annealing to 53 °C, last 20 cycles annealing to 56 °C. A second PCR was carried out using the first PCR products and primers (3Rs1 and adaptor primer), and cycles condition was the same as that of the first PCR. The PCR product was subcloned and sequenced as described above. For 5'-end amplification of the goldfish c-*fos* cDNA, a first strand cDNA was synthesized from the poly(A)$^+$RNA (1 μg) of the goldfish brain, using M-MLV Reverse transcriptase and 5'-end phosphorylated oligo(dT)$_{16}$ primer (5'-p-TTCTAGAATTCAGCGGCCGCTdT$_{16}$-3'). After hydrolysis of poly(A)$^+$RNA with RNase H (Takara Bio) at 30 °C for 60 min, the cDNA was ligated to circularize cDNA by T4 RNA ligase (Takara) at 16 °C overnight. PCR was performed with the circularized cDNA for a template and primers (5Ra2 and 5Rs1). Cycle condition was following; 35 cycles 94 °C 30 s 57 °C 10 s 72 °C 30 s. The PCR product was subcloned and sequenced as described above.

2.4. Northern blot analysis

To investigate the expression level of c-*fos* mRNA in the goldfish brain, northern hybridization was carried out as described previously(Moon et al., 2005). Digoxigenin (DIG)-labeled cRNA probe was prepared with DIG RNA Labeling Kit (Roche Diagnostics KK). The sequence of goldfish c-*fos* cDNA corresponding to nt 368-675 in Acc. No. AB058417 was used for DIG-labeled cRNA probe. The total RNA from the goldfish brain was hybridized with the DIG-labeled antisense cRNA probe at 65 °C overnight. After hybridization, the nylon membrane was washed at

65 °C. Hybridization signals were detected by using anti-DIG-AP-fragments (1:5000 v/v) and CDP-Star (Tropix Inc). The analysis of signals was performed with NIH Image (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Statistical analysis of the data was performed with Stat View J-5.0 (SAS Institute Inc.).

2.5. In situ hybridization

To determine the specific localization of the c-*fos* mRNA expression in the brain from kainic acid-treated goldfish, *in situ* hybridization was performed on 8 μm sections of the goldfish brain, referred to(Uchiyama et al., 2002). The brain from the kinic acid-treated goldfish was fixed in Bouin's solution at 4 °C up to 6 h. Fixed tissue were dehydrated and embedded in paraffin. The tissue was transversely sectioned at 8 μm and attached onto 3-aminopropyltriethoxysilane coated glass slides (Matsunami glass). The deparaffiend serial sections were hydrated and treated with 4 μg/ml Proteinese K (Takara) for 15 min at 37 °C. The sections were hybridized with 100 ng/ml DIG-labeled cRNA antisense probe at 45 °C overnight. The sections were washed for 30 min at 55 °C and treated with 50 μg/ml ribonuclease A (Roche Diagnostics) for 30 min at 37 °C. Signal was detected by using NBT/BCIP. The treated sections were dehydrated and coverslipped with Entellan new (Merck KGaA). The sections were observed under Eclipse E600 microscope (Nikon) attached to a digital microscopic camera (Nikon, Tokyo).

3. Results and Discussion

The primers were designed based on the nucleotide sequence of carp c-*fos*. Poly(A)+RNA was prepared from the brain of the goldfish treated with kainic acid for c-*fos* mRNA exprssion. From RT-PCR method, we isolated a partial cDNA clone with homology to carp c-*fos*. Based on the sequence, gene-specific primers for 3'-, and 5'-RACE was designed to yield a full length cDNA encoding the goldfish c-Fos and succeeded in isolating cDNA by the RACE methods. The cDNA sequence has been registered in GeneBank under an accession number AB058417. The nucleotide sequence of the goldfish c-*fos* cDNA includes a 1,044 bp open reading frame. The calculated molecular mass and isoelectric point of the c-*fos* protein were 437,854 Da and 4.79, respectively. An alignment of amino acid sequence for the goldfish (goldfish1, this study;AB058417) c-Fos with those of goldfish (goldfish2, Acc. No. BAC77043), carp (Acc. No. AAB39938), zebrafish (Acc. No. CAI11634), grass carp (Acc. No. AAK58088), fugu(Acc. No. AAC59778), green puffer (Acc. No. AAB07359), cherry salmon (Acc. No. AB111054), sockeye salmon (Acc. No. BAC77045), Rivulus marmoratus (Acc. No. AAQ16633), human (Acc. No. AAA52471), mouse (Acc. CAA24105), chick (Acc. No. AAA48670) c-Fos was shown in figure 1. The basic region leucine zipper (bZIP) domain (Glu140-Ala204 in Fig.1), which mediates the dimerization and DNA binding properties, is highly conserved among all species. The amino acid sequence of the goldfish c-Fos bear higher similarity to those of cyprinids including carp (89 %), zebrafish (85 %), and grass carp(88 %) c-Fos (data not shown). The sequence of goldfish c-Fos (goldfish1 in Fig.1) determined in this study showed the highest similarity to that of another

goldfish c-Fos(Ogawa et al., 2002) (goldfish2 in Fig.1). The two goldfish c-*fos* would be derived from the common ancestral gene. In the rainbow trout, two partial cDNA clone encoding c-*fos* had been also isolated(Matsuoka et al., 1998). The partial sequences of the two c-Fos showed higher similarity with each other. A phylogenetic tree was derived from an alignment of the amino acid sequences, using the CLUSTAL W program (Thompson et al., 1994) and Tree view (Page 1996)(Fig. 2). Fish c-Fos would be classified into two groups; cyprinids and one of puffer and salmon c-Fos, based on their primary structures. The multiplicity of the c-*fos* gene would be a characteristic of fish c-Fos. It must be needed to further comprehensive and prudent investigation to verify the finding.

We next investigated the effect of kainic acid on the expression of the goldfish c-*fos* mRNA in the brain by the northern blotting analysis. The behavior of the goldfish treated with kainic acid showed the seizures consisted of convulsion and malrotation (data not shown). It is known that kainic acid, which is a glutamate analog, elicits seizures directly by stimulating kainate-type glutamate receptors(Zhang et al., 2002). From the analysis, the intense signal indicating expression of the c-*fos* mRNA was observed in the brain of the kainic acid treated goldfish, compared to that of the non-treated goldfish brain (Fig. 3). We further investigated the time course of the induction of the c-*fos* mRNA in the goldfish brain after the administration of kainic acid. Total RNA (15 μg) were prepared from the goldfish brains sacrificed at 15, 30, 60, 90, and 120 min after the administration of kainic acid. To quantify the induced expression rate of c-*fos* mRNA, the intensities of signals were calculated by the NIH image software. The expression of c-*fos* mRNA was induced significantly as early as 30 min after the

administration of kainic acid to the goldfish, compared with the initial level (Fig. 4). The level of the c-*fos* mRNA expression was slightly decreased at 90 min after the administration. It was suggested that c-*fos* in the goldfish brain also participate in transcriptional regulation of gene in neural activity implicated in activation of glutamate receptors. Taken together, these results showed the expression of goldfish c-*fos* mRNA reflects neuronal activity after stimulation in the brain of goldfish. Therefore, expression region of c-*fos* mRNA in the goldfish brain will provide us with useful information on the neural activities at a cellular level. We further investigated the regional and cellular distribution of c-*fos* mRNA in the goldfish brain by *in situ* hybridization. In the brain of goldfish un-treated with kainic acid, no signal was observed with an antisense probe (Fig. 5A-1, -2 and -3). According to results of the northern blot analysis, the brains of the fish of 30 min after administration of kainic acid was investigated. Hybridization signals of c-*fos* mRNA in the goldfish brain were detected in the telencephalon, diencephalon, cerebellum and modellua oblongata (Fig. 5B-1, -2 and -3, respectively). In telencephalon, the signals were mainly localized in the ventral nuclei of the ventral telencephalic area (Vv). On the other hands, the signals were detected in some particular region in diencephalon. Moderate signals were observed in the dorsal and central posterior thalamic nuclei (DP and CP, respectively), the periventricular nucleus of posterior tuberculum (TP), the posterior tuberal nucleus (PTN) and the preglomerular nuclei (PG). The most intense signals were detected in the hypothalamic nucleus (PH). In the cerebellum and modellua oblongata, the signals was mainly detected within the corpus cerebelli (CC) and the eminentia granularis (EG).

In the mammalian brain, it has been reported that kainic acid induced an early neuronal activation in the hippocampal-entorhinal axis and the expression level of the c-*fos* mRNA was largely elevated especially within the hippocampus, limbic lobe and amygdala after administration of kainic acid(Hiscock et al., 2001; Zhang et al., 2002). These regions belong to the cerebrum in mammals, and are considered to be extremely related to declarative memory, autonomic nervous system and instinctive behavior. Though, in the fish brain, it remains to be revealed where the region of the brain equivalent to these three regions, it might be that the brain areas detected the signal for the expression of c-*fos* mRNA in telencephalon and diencephalon have a relationship with the three regions (the hippocampus, limbic lobe and amygdala) of the mammalian brain.

In this study, we isolated the cDNA clone encoding goldfish c-*fos* and investigated the effect of the kainic acid on the c-*fos* mRNA expression in the goldfish brain. As described above, another c-*fos* isotype has been found in the goldfish, as well as in the rainbow trout(Matsuoka et al., 1998). Furthremore, it has been also reported that the expression patterns of the two rainbow trout c-*fos* mRNA were extremely similar. The sequence of the DIG-labeled cRNA probe used in this study was complementary to the conserved domain, bZIP domain high identical to each sequence of goldfish c-*fos* isotypes (Fig. 1). Thus, the DIG-labeled cRNA probe detected both mRNA expression of c-*fos* isotypes by northern blot and *in situ* hybridization. It might be needed to investigate the specificity of the probe, but our purpose of this study was to identity the neural active regions in the goldfish brain through the induced expression of c-*fos* mRNA. Furthermore, the results of a wide-ranging and dramatic induction pattern of the c-*fos* mRNA in the goldfish

brain were consistent with the results reported in other vertebrates treated with kainic acid(Hiscock et al., 2001; Silveira et al., 2002; Zhang et al., 2002). Therefore, the DIG-labeled cRNA probe used in this study is useful tool to investigate the neural activities in the goldfish brain. We hope that future studies using the probes of the goldfish c-*fos* will reveal the central sites involved in processing sensory information from environments, learning and particular behaviors such as swimming in the teleost brain.

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Figure 1

Alignment of the amino acid sequences of c-Fos of various animals, including goldfish1 and 2 (Acc Nos. AB058417 and BAC77043, respectively), carp (Acc No. AAB39938), zebrafish (Acc No. CAI11634), grass carp (Acc. No. AAK58088), fugu (Acc. No. AAC59778), green puffer (Acc. No. AAB07359), cherry salmon (Acc. No. AB111054), sockeye salmon (Acc. No. BAC77045), Rivulus marmoratus (Acc. No. AAQ16633), human (Acc. No. AAA52471), mouse (Acc. CAA24105), chicken (Acc. No. AAA48670). Dots indicate the conserved amino acid residues identical to those of goldfish1 c-Fos. Amino acid residues conserved in every animal are entangled with black boxed. The sequences corresponding to the basic region and the leucine zipper region are underlined.

Figure 2

A phylogenetic tree of various animal c-Fos. For the accession numbers see Fig.1

Figure 3

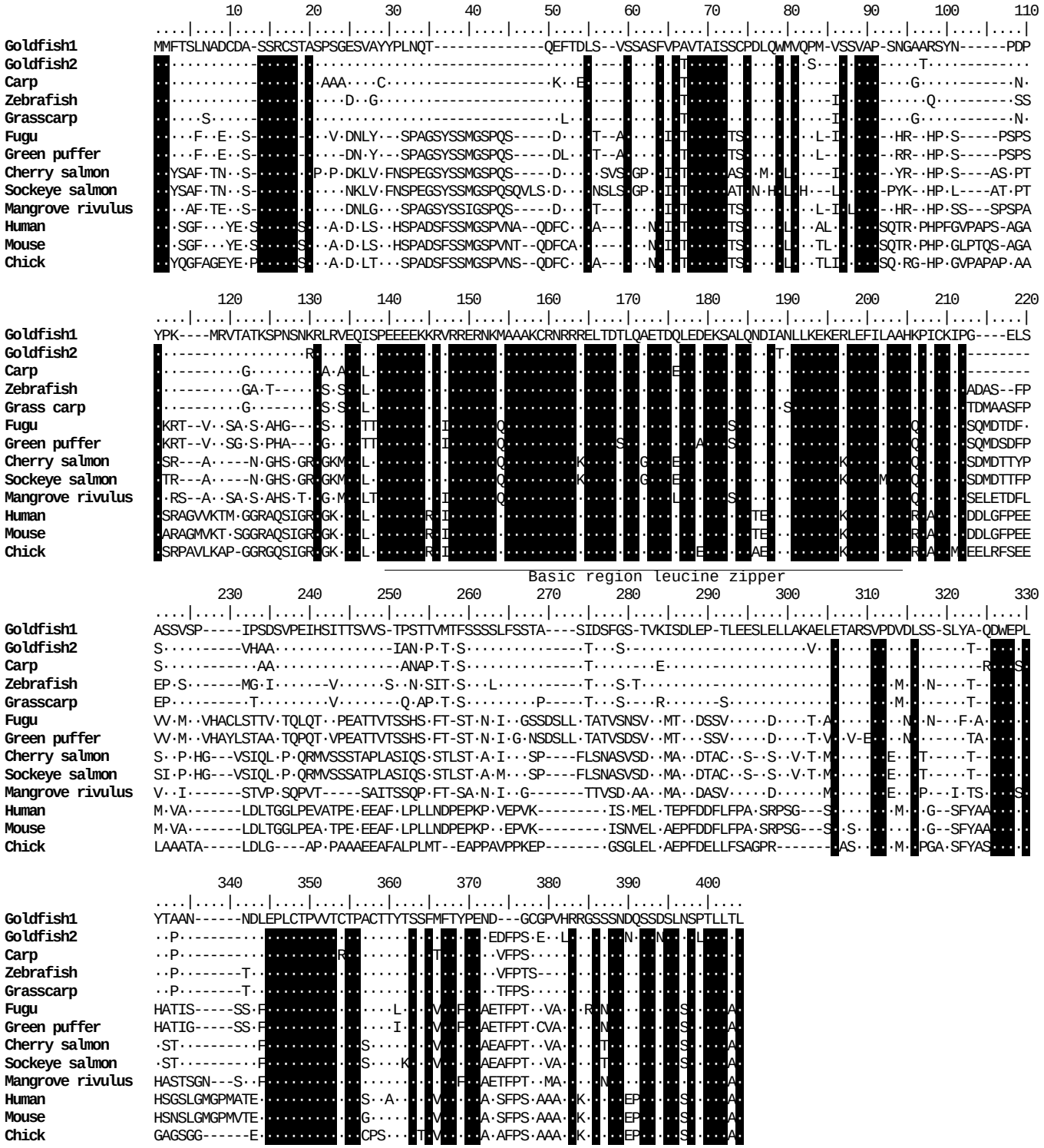
Northern blotting analysis of the goldfish c-fos mRNA in the brain. Total RNA (20μg) extracted from the non-(lane1) or kainic acid (lane 2)-treated brain were separated in 1% denaturing agarose gel electrophoresis, blotted onto nylon membrane and hybridized with DIG-labeled cRNA probes for the goldfish c-fos (upper panel). Lower panel showed 28S and 18S rRNA by staining with ethidium bromide.

Figure 4

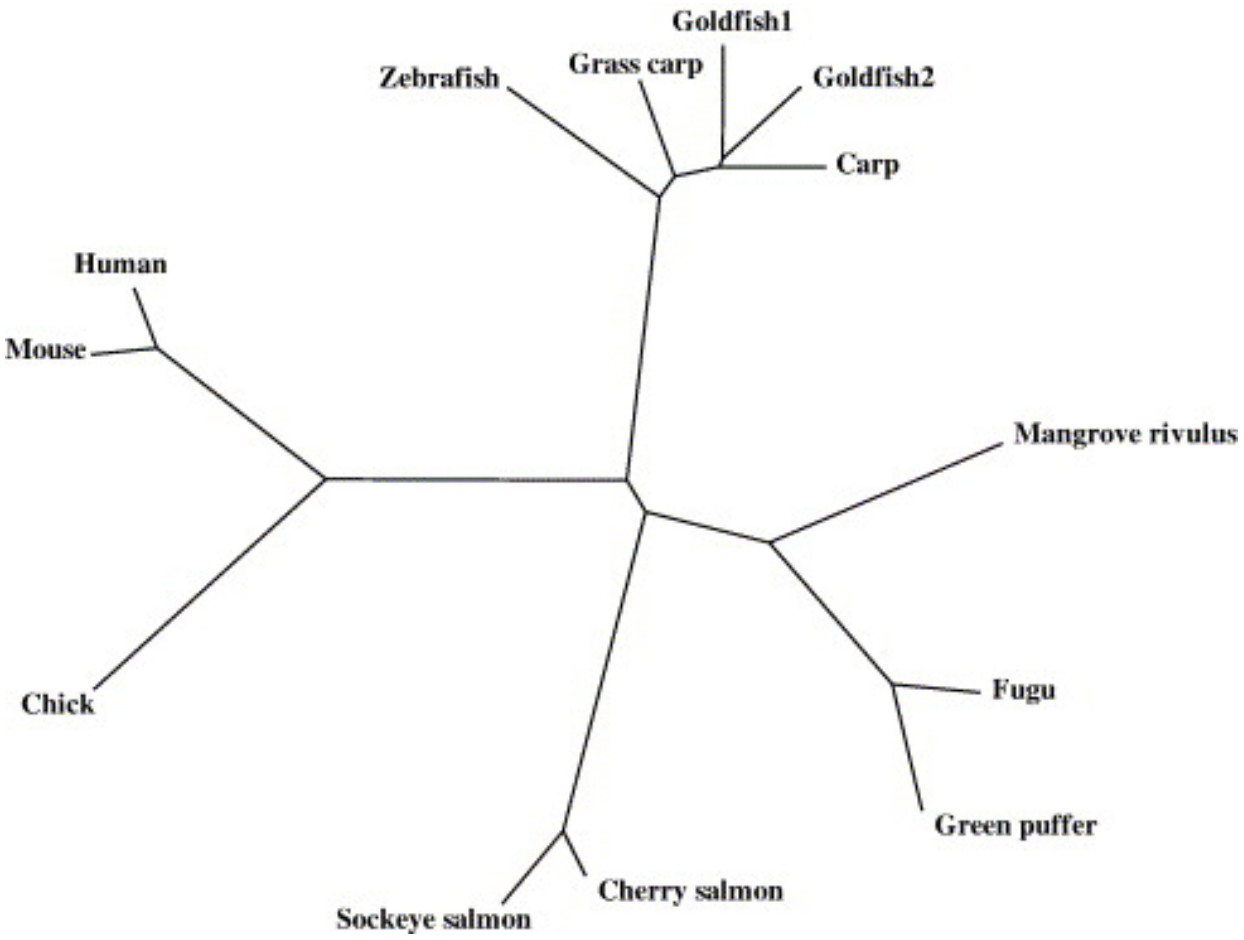
Northern blotting analysis of the effect on the expression of the golfish c-fos mRNA in the brain by kanic acid. Total RNA (15 μg) were separated and hybridized with DIG-labeled cRNA probes for the goldfish c-fos. The signals was analyzed by NIH Image and StatView J-5.0. *Significantly different from the 0 min level ($p<0.05$)

Figure 5

In situ hybridization of the goldfish c-fos mRNA in the brain of the goldfish treated with kainic acid. Sections of the goldfish brain were incubated with DIG-labeled cRNA probes for the goldfish c-fos mRNA and reacted with alkaline phosphatase-conjugated antidigoxygenin antibodies. The brain sections were prepared from the goldfish administered without (A) or with (B) kainic acid. Bar indicates 500 μm. CC; corpus cerebelli, CP; central posterior thalamic nucleus, DP; dorsal posterior thalamic nucleus, EG; eminentia granularis, MON; medial actovolateralis nucleus, OT; optic tectum, PG; preglomerular nucleus, PH; posterior hypoth alamus, PTN; posterior tuberal nucleus, RF; reticular formation, TL;torus longitudinalis, TP posterior tuberculam, V; ventral telencephalic area, VCL; lateral lobe of valvula cerebelli, Vv; ventral nuclei of the ventral telencephalic area.



Fujikawa et al. Fig.1



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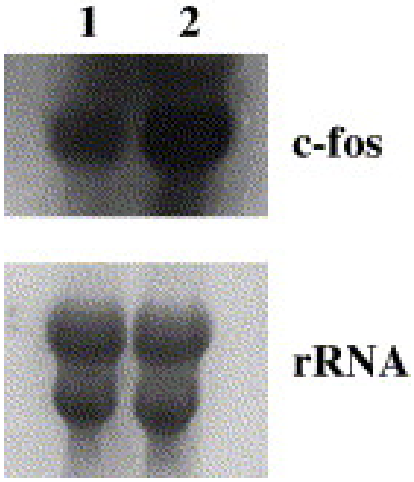

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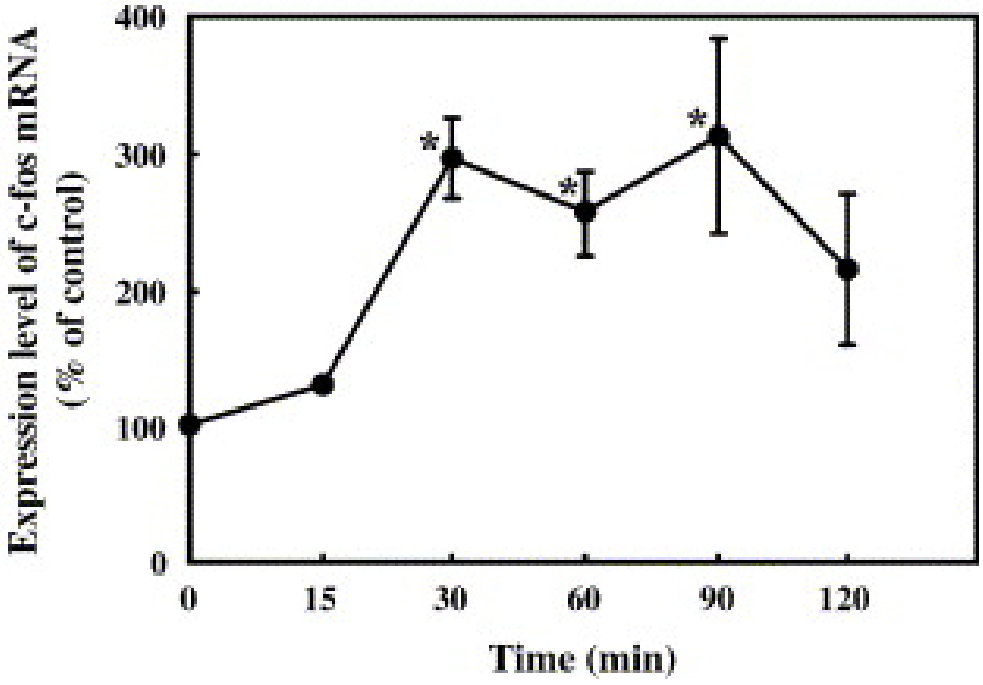
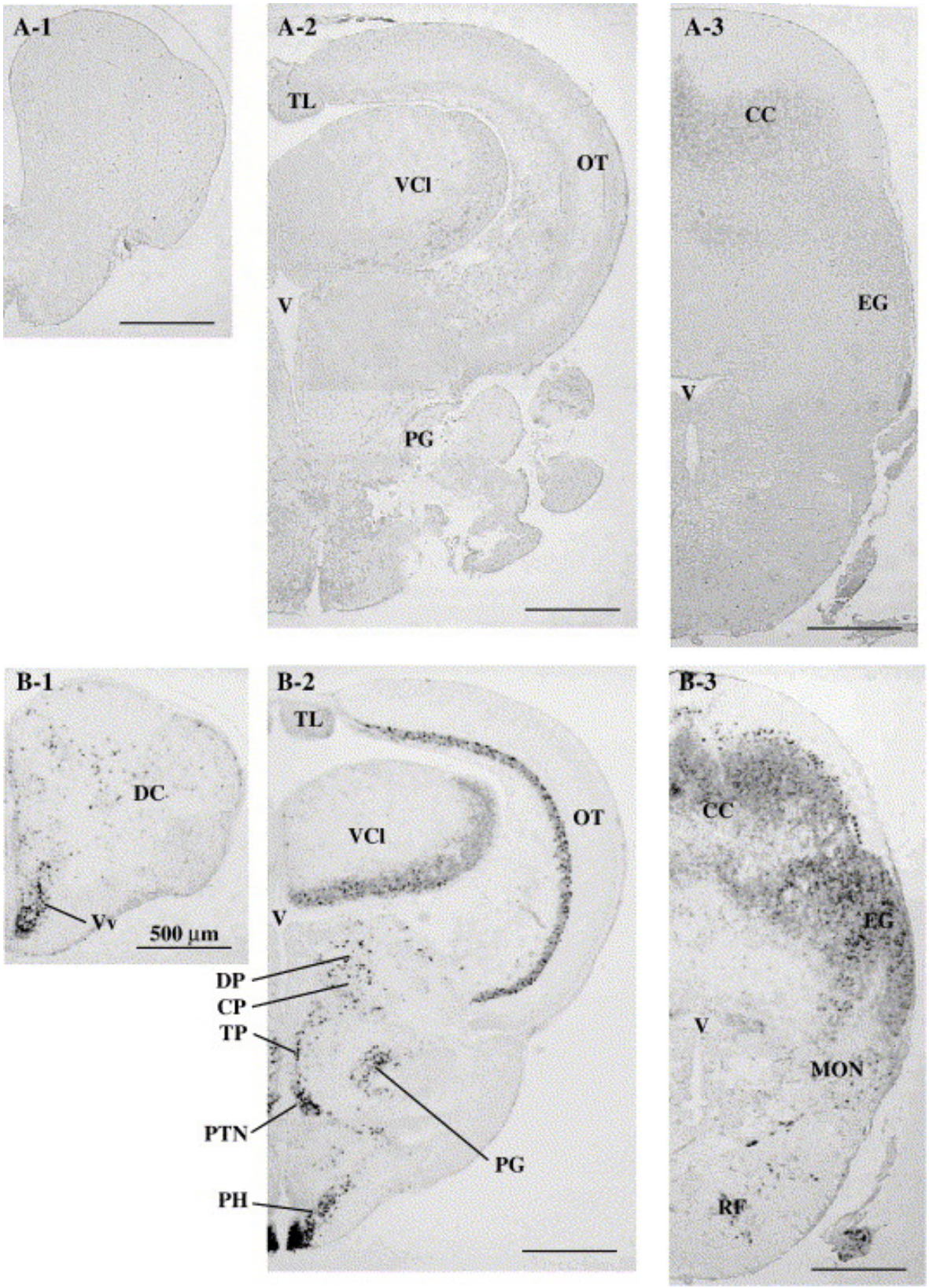


Fujikawa et al. Fig. 4



Fujikawa et al. Fig.5