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Rapid communication

Bioluminescence imaging of c-fos gene expression accompanying filial imprinting in the newly hatched chick brain

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ABSTRACT

Bioluminescence imaging is a powerful tool for examining gene expression in living animals. Previously, we reported that exogenous DNA could be successfully delivered into neurons in the newly hatched chick brain using electroporation. Here, we show the *in vivo* bioluminescence imaging of c-fos promoter activity and its upregulation, which is associated with filial imprinting. The upregulation of c-fos gene expression correlated with both the strength of the chicks' approach activity to the training object and the acquisition of memory. The present technique should be a powerful tool for analyzing the time changes in neural activity of certain brain areas in real-time during memory formation, using brains of living animals.

Bioluminescence enables us to perform whole-animal imaging for monitoring gene expression. This technique can be used to study behaviors and their related genes in an animal (Welsh and Kay, 2005). Thus, bioluminescence imaging is a promising tool for studying gene expression *in vivo* associated with learning and memory. Until now, bioluminescence imaging has been used for studying gene expression in mice that express the luciferase reporter gene under the control of various promoters or transcription factor binding sites *in vivo* (Honigman et al., 2001; Martin, 2008; Zhang et al., 2001). In the brain, it has been mainly used to track and quantify luciferase-tagged tumor cells (Uhrbom et al., 2004) or to trace the migration of neural progenitor cells (Tang et al., 2003). However, bioluminescence imaging has not yet been applied for analyzing the relationship between the acquisition of memory and gene expression *in vivo*.

Birds have a varied behavioral repertoire, including memory formation, and can thus serve as an ideal model system for the experimental analysis of learning such as filial imprinting (Matsushima et al., 2003). Chicks (*Gallus gallus domesticus*) have been intensively studied as a model for filial imprinting (Horn, 2004). Filial imprinting is a behavior in which a young animal learns the characteristics of its parent. It is most obvious in precocial birds, who imprint on their parents and then follow them around (Lorenz, 1937). It has been shown that total RNA synthesis increases in the intermediate medial mesopallium (IMM) region which appears to correspond to the part of the mammalian association cortex (Horn, 2004; Horn et al., 1979). Accordingly, the expression of several genes and proteins including the immediate-early gene (IEG), c-fos in the IMM region, are suggested to have a significant correlation between the degree of expression and the strength of preference to a training object by immunocytochemistry using the fixed brain slices (McCabe and Horn, 1994). The expression of IEGs is induced by neural activity associated with learning; moreover, IEGs have been widely used as neural activity markers to study the neural circuits involved in learning and behavior (Guzowski, 2002). In mice, the expressions of several IEGs, including c-fos, Zif268/Zenk, and Arc/Arg3.1, are known to be strongly upregulated by the induction of long-term potentiation (LTP) and by the behavioral training (Guzowski et al., 2005). These evidences suggest that the induction of IEGs associated with filial imprinting is involved in a key process in the initial course of the memory formation.

Here, we demonstrate bioluminescence imaging of c-fos promoter activity associated with filial imprinting. Bioluminescence

Abbreviations: IMM, intermediate medial mesopallium; LTP, long-term potentiation; IEG, immediate-early gene; BLI, bioluminescence imaging; GAPDH, glyceraldehyde phosphate dehydrogenase.

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imaging showed that the upregulation of c-fos gene expression correlated with both the strength of the approach activity to the training object and the acquisition of memory. As far as we know, our study is the first report showing the real-time change of neural activities during memory formation.

The experiments were conducted with the approval of and in accordance with the guidelines of the Committee on Animal Experiments of Teikyo University based on the national regulations for animal welfare in Japan. Fertilized eggs of domestic chicks of the Cobb strain (*Gallus gallus domesticus*) were obtained from a local supplier (3-M, Nagoya, Japan), and incubated at 37 °C for 21 d. After hatching, chicks (unsexed) were placed in dark plastic enclosures in a breeder at 30 °C to prevent exposure to light. The imprinting training was initiated approximately 18 h after the hatching. The training and the test for imprinting were carried out by the method described by Izawa et al. (2001) with minor modifications. Briefly, the training chamber was equipped with a computer-controlled rubber belt (8 cm wide, 43 cm long) and an infrared sensor. A microcomputer (RCX2.0; Robotics Invention System, LEGO Co., Tokyo, Japan) commanded the running belt to move backward when a chick interrupted the beam of an infrared sensor. The training object (yellow LEGO block, 4.7 cm × 6.2 cm × 5.0 cm high) was placed 20 cm in front of the infrared sensor, and illuminated from above by a 100 W fiber optic light (LG-PS2, Olympus, Tokyo, Japan). Each chick was exposed to the training object for 15 min. During training, the number of interruptions of the infrared sensor beam was counted in order to measure the "approach activity" toward the training object. The training session consisted of 3 successive runs (4 min each) at 1 min intervals. In each run, the object was rotated for 30 s six times, with 10 s pauses between each rotation. After the training, the chicks were tested by a simultaneous choice test. For the test, a stationary runway (8 cm wide, 43 cm long) with 13-cm long approach areas on both sides was used. The chick was given the training object and a novel object. Both objects were placed at the ends of the approach areas and programmed to move synchronously according to the same schedule used in the training. Then, each chick was gently placed at the mid-point of the runway, facing the sidewall, and then released, allowing it to walk freely. During 120 s, the total time that the chick spent in each approach area near the objects was measured. The difference in the approach time was calculated to evaluate the preference for the training object in order to rate it as "acquisition of memory," i.e., the total time that the chick spent near the training object was subtracted from the total time chick spent near the control object.

The MluI/HindIII fragment of pdfGH (Gift from Dr. Goodall, G.J. (Lagnado et al., 1994)) containing c-fos promoter sequence was cloned into the cDNA for firefly luciferase in the pGL3-Basic plasmid (Promega). *In vivo* electroporation was performed 3 h after the hatching as described previously (Yamaguchi et al., 2007).

Before the imprinting training, we measured the basal level of the bioluminescence 15 h after the electroporation of the plasmid for evaluating the expression of c-fos in the IMM region of one of the hemispheres. Chicks were anesthetized with 2% isofluorane/air mixture and given a single i.p. dose of 150 mg/kg of D-luciferin in saline. Bioluminescence imaging (BLI) was initiated 10 min after luciferin administration using the Xenogen Ivis Imaging Series 100 system (Xenogen, Alameda, CA). During image acquisition, isofluorane anesthesia was maintained by using a nose cone delivery system, and the animal body temperature was regulated with a temperature-controlled stage. After the measurement of the basal level of bioluminescence for 100 min, chicks were returned to the dark breeder to recover from the anesthesia for 30 min. Then, the chicks were subjected to the training for 15 min each. Immediately after the training, the chicks were tested for evaluating the preference for the training object and then subjected to BLI. For data collection, a gray-scale body image was collected (field of view, B; exposure, 0.2 s; binning, small (high resolution); and f/stop, 16) followed by the acquisition and the overlay of a pseudocolor image representing the spatial distribution of the detected photons emitted from the animal (field of view, B; exposure, 300 s; binning, large (high sensitivity)), acquired as photons per s/cm^2 per steridian. The signal intensity was quantified as the sum of all the detected photon counts within a region of interest (ROI) over individual uniform-sized ROIs for 3 min that were manually placed during the post-data acquisition image analysis.

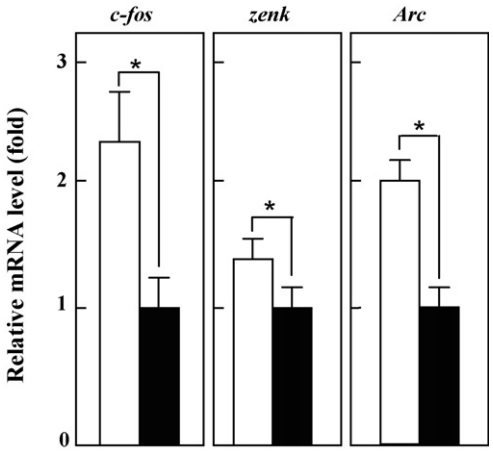

Fig. 1. Upregulation of IEGs (c-fos, zenk, and Arc/Arg3.1) associated with filial imprinting. The brains of the imprinted chicks and the dark-reared chicks were analyzed by quantitative RT-PCR. The relative IEGs mRNA levels of the imprinted chicks ($n = 5$, white bars) were higher than those of the dark-reared chicks ($n = 5$, black bars). Results are expressed as means ± S.E.M. An asterisk indicates significant differences between the imprinted group and the dark-reared group as shown by the Mann–Whitney test ($p < 0.05$). The relative expression levels were normalized by GAPDH.

Real-time quantitative reverse transcriptase (RT)-PCR was performed as described previously (Yamazaki et al., 2006). The sequences of the primers used were as follows: GAPDH sense, TGGAGCCCCTGCTCTTCA; GAPDH antisense, GGAACAGAACTGGCCTCTCACT; c-fos sense, TGTTCCTGGCAATATCGTGTTC; c-fos antisense, CTTTCCCCCCCACGTAAGA; Arc/Arg3.1 sense, GAGGACCATGTGCCAACTGA; Arc/Arg3.1 antisense, GCCAGGCAGGCCTTGAT; zenk sense, TGTGACCGGCGATTTTCAC; and zenk antisense, GTCCAGTGTGGATGCGAATG. The total RNA used for quantitative RT-PCR was extracted from chick whole brains excluding hypothalamus using Trizol (Invitrogen).

Because the upregulation of the c-fos gene was suggested to correlate significantly with the strength of filial imprinting (McCabe and Horn, 1994), we examined whether other IEGs (Zenk and Arc/Arg3.1) were similarly upregulated by such imprinting behavior. We compared the expression levels of IEGs between the trained chicks and the dark-reared chicks by real-time RT-PCR. As shown in Fig. 1, the IEGs, including c-fos, were upregulated 3 h after the training for imprinting (c-fos: 2.4 ± 0.41; zenk: 1.3 ± 0.11; Arc: 1.9 ± 0.18). These differences between imprinted and dark-reared birds may be associated with memory formation or with concomitant events such as changes in locomotion and stress. As the c-fos transcripts were maximally upregulated, we selected the c-fos gene for monitoring the *in vivo* gene expression associated with filial imprinting by using bioluminescence. In order to monitor the c-fos gene expression, we transfected the luciferase cDNA under the control of the c-fos promoter into the IMM region, which has a critical role in imprinting (Fig. 2D). The transfected chicks were then examined by the Ivis bioluminescence imaging system to quantify the c-fos promoter activity (Fig. 2A and B). After 15 min of training for imprinting, there was a significant increase in the c-fos promoter activity. A typical example was shown in Fig. 2C. The peak

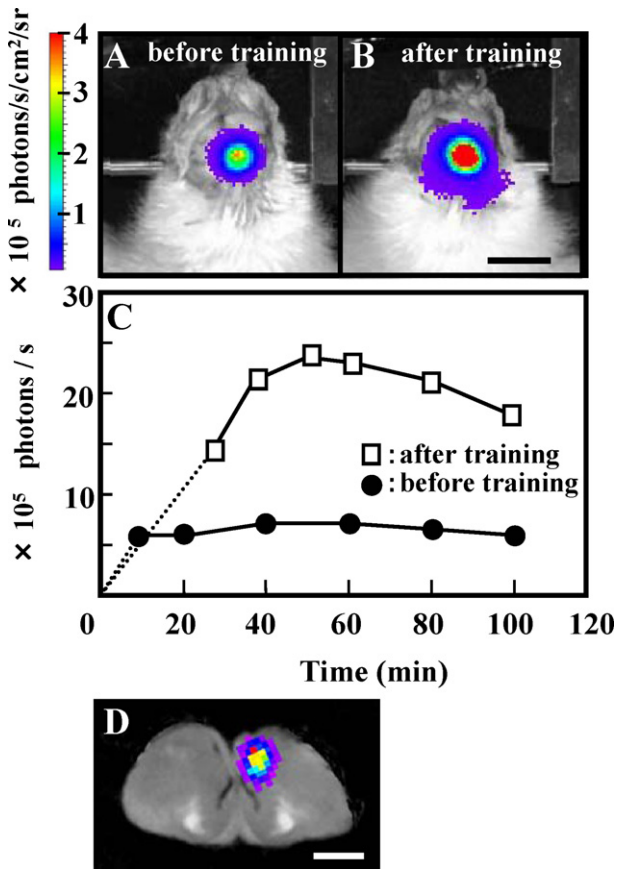

Fig. 2. Upregulation of the c-fos gene expression associated with filial imprinting. (A–C) BLI was initiated 10 min after the injection of luciferin. Before imprinting training, basal level of BLI was measured for 100 min. Then, chicks were returned to the breeder for 30 min to recover from anesthesia. After 15 min of training, chicks were injected with luciferin and subjected to BLI for 100 min. The representative chick depicted before (A) and after (B) the imprinting training at peak bioluminescence induction. Scale bar, 15 mm. (C) Quantification of bioluminescence signals associated with filial imprinting. A typical example was shown. (D) The plasmid containing c-fos gene promoter was transfected to IMM region by *in vivo* electroporation. Whole brains were dissected and transverse sections (300 μm thick) were collected without fixation. Detectable bioluminescence signal was shown to be derived from the IMM region. Scale bar, 3 mm.

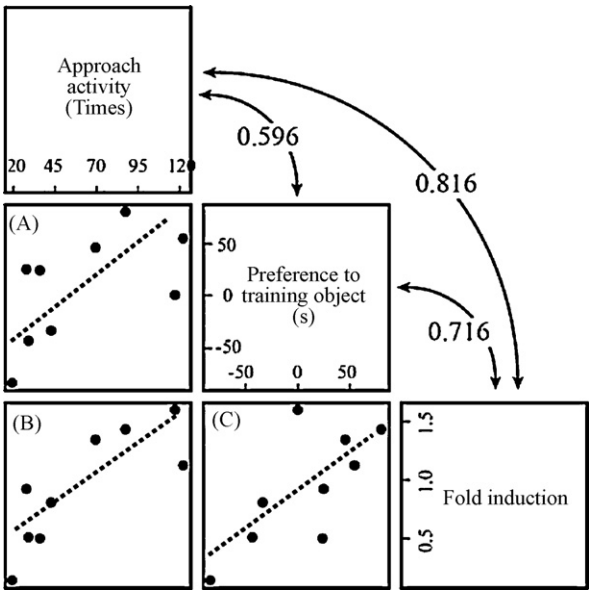

Fig. 3. The correlation among the chicks' approach activity, acquisition of memory and c-fos gene expression. During the training, the number of interruptions of the beam of the infrared sensor was counted in order to quantify the approach activity toward the training. The difference in the approach time was calculated to evaluate the preference for the training object in order to rate it as "acquisition of memory". The average of differences in approach time from two successive experiments was shown. The c-fos gene expression was quantified by the c-fos promoter activity using BLI.

BLI was 3.6-fold higher than the baseline following the training, coinciding with the result of quantitative real-time RT-PCR. From this data, we consider that it is possible to quantify endogenous c-fos gene expression *in vivo* by measuring the promoter activity by assessing the bioluminescence accompanying the filial imprinting.

The chicks trained for 3 h mostly established a robust preference to the training object (28 out of 35 chicks) as compared to the control toy object (Yamaguchi et al., 2008). However, among the chicks trained for 15 min, the degree of preference varied with individuals. We evaluated the correlation between the approach activity and preference to the training object ($r = 0.596$, Fig. 3A). As previously reported, the degree of preference depends upon the effort exerted by the chicks approaching to the training object (Hess, 1959; Zajonc et al., 1973). We therefore evaluated the correlation between the chicks' approach activity and their c-fos expression. As shown in Fig. 3B, there was a significant correlation between the approach activity and the c-fos gene expression ($r = 0.816$); the chicks showing greater approach activity showed increased c-fos gene expression, while the chicks with lesser approach activity showed decreased gene expression. To evaluate the correlation between the c-fos gene expression and the acquisition of memory, chicks were subjected to BLI before and after 15 min-training, and the preference for the imprinting object was tested. As shown in Fig. 3C, there was a significant correlation between the degree of c-fos gene expression and the strength of the acquisition of memory ($r = 0.716$); the chicks with a stronger preference to the training object showed increased c-fos expression, while the chicks with a poorer preference demonstrated decreased c-fos gene expression. We assume that the upregulated c-fos protein would transcribe the downstream target genes, leading to structural modifications in neurons that are important for memory formation. On the other hand, we found that the chicks with lesser approach activity and weaker preference to the training object showed decreased c-fos expression, suggesting that c-fos expression was positively suppressed when chicks did not attempt to actively participate in the process of learning, resulting in the acquisition of poor memory.

Based on our findings, we consider that bioluminescence imaging may be a powerful tool for studying the real-time change of neural activities (c-fos gene expression) during memory formation. This system can easily be applied in analyzing the expressions of genes of interest *in vivo* accompanying the filial imprinting. This bioluminescence system based on *in vivo* electroporation will also be applicable in different areas of the chick brain; for example, since the avian pallium is organized to form a largely continuous field of nuclei and appears to be structurally homogeneous, electric square pulses may run in any area of the avian pallium, resulting in similar transfection efficiencies. In our current system, the intervals between the quantifications of promoter activity were restricted by the pharmacokinetics of the substrate (D-luciferin). For accurate measurement, a stable concentration of D-luciferin needs to be maintained in the blood in order to sustain a saturated condition for the continued enzymatic activity of luciferase. To eliminate these constraints, a recent report has proposed the administration of a slow infusion of luciferin using a small capsule buried in the body cavity that allows the continuous sampling of bioluminescence data (Gross et al., 2007); further, the generation of luciferase molecules with a shorter half-life will also enable researchers to acquire data with shorter intervals (Leclerc et al., 2000). These

improvements may aid in obtaining continuous bioluminescence imaging for longer periods and might eventually provide us with real-time bioluminescence imaging.

In conclusion, we have described here the bioluminescence imaging of the *in vivo* promoter activity of the c-fos gene associated with filial imprinting. BLI showed a significant correlation between the approach activity to the training object and the gene expression as well as that between the acquisition of memory and c-fos gene expression. The chicks with greater approach activity and stronger preference to the training object showed increased c-fos expression, suggesting that BLI in living chicks can be a powerful tool for studying the real-time changes of neural activity (c-fos gene expression) during memory formation.

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