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Expression of Transcription Factor *ZENK* (*zif/268*) in Telencephalon of Quail Chicks after Induced Seizure and Passive Avoidance Training

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ABSTRACT—Functional significance of an immediate early gene *ZENK* (*zif/268*) was examined in telencephalic regions (homologues of neocortex and basal ganglia) of newly-hatched quail chicks; hyperstriatum accessorium (HA), hyperstriatum ventrale (HV), neostriatum (N) and lobus parolfactorius (LPO). Chicks were trained by a green bead soaked either in a strong aversant (methylanthranilate, MeA), in a weak aversant (MeA diluted by ethanol, 1/3MeA), or in water. Chicks were then tested at 45–50 min post-training, and immediately processed for *ZENK* immunostaining. Neither the training condition (MeA, 1/3MeA, or water) nor the responses at test (recall or amnesia) significantly contributed to the immunopositive cell densities in all of these regions. On the other hand, single intraperitoneal injection of metrazole (CNS convulsant) induced a transient epileptiform seizure, and caused significantly enhanced *ZENK* expression in HV and LPO but not in HA and N. However, the metrazol-induced seizure did not interfere with the following passive avoidance training, and chicks successfully learned to avoid the aversive bead when tested at 24 hr subsequently. Among three groups of chicks (metrazol-treated, saline control, and untreated chicks), no significant differences were found in their responses at test (recall, generalized avoidance, or amnesia). These results suggest that enhanced *ZENK* expression may represent lasting neural activities, but may not be involved specifically in the passive avoidance memory formation.

INTRODUCTION

Brain mechanisms of memory formation in newly-hatched poultry chicks have been intensively studied as an ideal model system for understanding cellular and molecular basis of learning (see reviews by Horn 1985, 1998 for filial imprinting; see also Rose 1991, 1995 for one-trial passive avoidance learning). Of the telencephalic regions involved, intermediate medial hyperstriatum ventrale (IMHV; functional analogue of visual association cortex in mammals such as infero-temporal cortex in primates) is supposed critical for initial registration of memory for both imprinting and avoidance task (Buttler, 1999; Cipolla-Neto *et al.*, 1982; Davies *et al.*, 1988; Gilbert *et al.*, 1991; Patterson and Rose 1992). On the other hand, lobus parolfactorius (LPO; homologue of caudate-putamen in mammals) is involved in long-term storage of passive avoidance memory (see Csillag 1999 for review). In both of IMHV and LPO, cascades of cellular and subcellular events have been shown to occur subsequent to training. These changes include; enhanced glucose uptake (indicative of an enhanced neural activity; Rose and Csillag 1985), through enhanced expression of immediate early genes (IEGs such as c-*fos* and c-*jun*), and enhanced synthesis of a wide variety of proteins (Rose 1995), leading to permanent changes in synaptic and dendritic ultrastructures (Bradley *et al.*, 1981; Stewart *et al.*, 1984; Patel *et al.*, 1988).

Of these cascades, enhanced expression of c-*fos* is supposed a key process that bridges between neuronal activity and memory consolidation. The enhanced c-*fos* is supposed sufficient, since expression was positively correlated with the degree of learning (Anokhin *et al.*, 1991, McCabe and Horn 1994, Ambalavanar *et al.*, 1999). The enhanced c-*fos* expression also proved necessary, since suppression of c-Fos protein synthesis by using antisense nucleotide prevented long-term memory formation in the avoidance task (Mileusnic *et al.*, 1996). However, we have recently reported in IMHV slices *in vitro* that the c-*fos* expression and synaptic long-term potentiation (LTP; a favorable candidate of cellular memory device) were based on distinct mechanisms and not causally linked (Yanagihara *et al.*, 1998). Low-frequency tetanic stimulation induced LTP, but it was not sufficient for enhancing the density of c-Fos immunopositive cells around the site of stimulation. Furthermore, disinhibition of the IMHV network by bicuculline ($GABA_A$ receptor antagonist) enhanced the c-*fos*

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expression, but effectively suppressed the induction of LTP. Although a weak link was suggested between enhanced neuronal activity and the c-*fos* expression, both of these events are not directly related with fast plastic changes in synapses.

Here, we analyzed *ZENK* as another candidate of molecular machinery that could functionally link the neuronal activity with the memory consolidation. The *ZENK* is an inducible transcription factor known also as *zif/268*, *egr-1*, *NGFI-A*, or *Krox-24*, induction of which is involved in memory formation in several vertebrates. At the cellular level, in rat hippocampus, the expression of *zif/268* was correlated with induction and stabilization of LTP (see review by Abraham *et al.*, 1992). At the behavioral level, in temporal lobe of Macaque monkey, an enhanced *zif/268* expression was found in a patchy fashion during visual paired associate learning task (Okuno and Miyashita, 1996). Also in songbirds, *ZENK* is induced in the auditory forebrain area when conspecific song was given, suggesting an involvement in memory-based cognitive process (Mello *et al.*, 1992).

In this study, we investigated whether *ZENK* expression is functionally linked with passive avoidance learning in chick telencephalon by examining 1) if the training enhances *ZENK* expression, 2) if *ZENK* expression is correlated with memory formation, and 3) if a preceding induction of *ZENK* interferes with the training.

MATERIALS AND METHODS

Subjects and housing conditions

Newly hatched quail chicks (*Coturnix japonica*) were used. Breeding and housing conditions were identical to those reported previously (Yanagihara *et al.*, 1998; Sakai *et al.*, 2000; Aoki *et al.*, 2000). Fertilized eggs were obtained from our outbred colony of wild-type plumage, and incubated for 16–17 days at 37.6°C. For 12–24 hr after hatch, chicks were left in a dark incubator. On the next day, chicks were randomly paired in small transparent plastic cages (12.5×8.5 cm^2, 8.2 cm high) and raised in a breeder. We identified each individual by using leg-rings. Inside of the breeder was illuminated by a pair of 60W white light bulbs (12L:12D with the light period starting at 9:00), highly moisturized, and kept at ca. 35°C. To reduce the handling stress, we transferred, trained, and tested chick pairs in the same plastic cage except for treatments such as metrazol injection. Chicks were not fed but given a petri-dish of water-soaked cotton, so that they could drink freely.

One-trial passive avoidance task

In Experiment-1, we examined the effects of passive avoidance training in terms of the *ZENK* expression, or the density of *ZENK*-product-like immunopositive cells (ZENK-li cells), in telencephalic regions involved in passive avoidance task. For the avoidance training and tests, we used the same procedure as our previous studies (Sakai *et al.*, 2000; Aoki *et al.*, 2000). Briefly, a pair of chicks in cage was placed in an experimental chamber, which was illuminated at 900–1,800 lux and kept at 31–35°C. After a 5-min equilibration, chicks were presented with a bead for 30 sec. The bead (plastic ball, 2.5 mm in diameter) was painted in green (type X-28 acrylic paint, Tamiya Co. Japan), glued to a transparent rod (1.5 mm thick) and manipulated by experimenter's hand from outside of the chamber. Behavior of chicks was observed through a Plexiglass one-way window. The bead was coated with either methylanthranilate (MeA, Sigma Co.; strong aversive stimulus), MeA diluted in ethanol of twice as much volume (1/3 MeA; weakly aversive stimulus), or water (W; neutral stimulus). We recorded number of pecks, and noted if chicks showed disgust responses such as head shaking or bill wiping. Chicks were trained by single presentation of a green bead, and no differential experience was given as pre-training trials. Chick pairs were then placed back to the breeder for 40 min.

At test, after a 5-min equilibration in the chamber, chicks were presented with a green bead (type X-28 acrylic paint, Tamiya Co. Japan), and then with a yellow bead (type X-8) each for 30 sec at a 5-min interval. Both beads at tests were dry, and these tests were performed at 45 and 50 min respectively after the training. Chicks behaved in one of the following patterns; (1) chick avoided the green bead and pecked at the yellow (recall-and-discrimination), (2) chick avoided both beads (generalized avoidance), (3) chick pecked at both beads (amnesia), (4) chick pecked at the green bead and avoided the yellow (paradoxical amnesia). In this study, we trained 6 pairs of chicks with MeA, 8 pairs with 1/3 MeA, and other 8 pairs with W. A total of 44 chicks (12, 16, and 16 chicks for each group) were thus trained, tested and classified as below.

Out of 12 chicks trained by MeA, we rejected two chicks that failed to peck, or pecked but failed to show disgust responses at training (training failure). We further rejected 1 chick that showed generalized avoidance for both beads at test, because this chick might have been generally depressed for pecking. Out of the remaining 9 chicks, we randomly selected 6 chicks that showed recall-and-discrimination for ZENK immunostaining, one from each of the 6 pairs. Out of the 16 chicks trained by 1/3MeA, we similarly rejected 2 chicks of generalized avoidance, and selected 4 chicks of recall-and-discrimination and 4 chicks of amnesia; one from each of the 8 pairs. No chicks showed training failure or paradoxical amnesia in this group. Out of the 16 chicks trained by W, we rejected 2 chicks of training failure, and 2 other chicks of paradoxical amnesia. Of the remaining 12 individuals, 4 chicks showed recall-and-discrimination, and 8 chicks showed amnesia; of these 12 chicks, we randomly selected 2 and 5 chicks respectively for ZENK immunostaining. At 1 hr after the training session, or 10 min after the test trial, the selected chicks were deeply anesthetized and transcardially perfused as below.

In Experiment-3, we examined the effects of induced epileptiform seizure on the passive avoidance task. Initially, 30 pairs of chicks were prepared. One chick of the pair was randomly selected and allocated to the metrazol treatment (see below), and the other chick to the saline control. Chicks were then trained by using a green ball coated with MeA. In a pilot experiment, where chicks were trained at 60–90 min after the metrazol injection, more than half of the chicks showed training-failure. We therefore trained chicks at 2 hr post-treatment. Basically the same procedure was adopted as in Experiment-1, except that chicks were tested at 24 hr subsequently. During the retention period, chick pairs were placed in the breeder. Chicks were similarly classified based on the behavior patterns at test. We successfully trained and tested 21 chicks out of 30 metrazol-treated chicks, and 22 chicks out of 30 saline-treated control chicks; the remaining chicks showed training-failure and discarded. Another group of intact (untreated) 40 chicks (20 pairs) were similarly trained and tested, and 32 out of these 40 were successfully tested. Statistic comparisons were made among these three groups of chicks by using chi-square test of independent multiple sets of data (two-tailed) at the significance level of 0.05.

Epileptiform seizure induced by metrazol injection

Metrazol is a potent convulsant, which passes through blood-brain-barrier and depolarizes CNS neurons by blocking $GABA_A$-ergic inhibitory synaptic transmissions (Saffen *et al.*, 1988). In Experiment-2, we examined the effects of metrazol-treatment and the accompanying epileptiform seizure on the *ZENK* expression. In order to deprive chicks of any visual experiences, we treated chicks in the dark incubator until anesthesia. A total of 8 chicks were randomly selected and allocated to one of the following two groups; one group

of 4 chicks were injected with metrazol at the concentration of 7.5 mg / ml saline (0.1 ml i.p. injection / chick) and the other group of 4 chicks were left untouched in the incubator. Behaviors of the treated chicks were not recorded. At 60 min after the injection, when the induced seizure was supposed to fade almost completely (see Fig. 3), we anesthetized chicks and processed the brain specimens for ZENK immunostaining.

In Experiment-3, we analyzed strength and time course of the induced epileptiform seizure, and examined the effects on passive avoidance training. We evaluated the seizure by recording 8 behavior patterns during a 30-sec observation period; each of 3 characteristic seizure patterns was scored as +1, and each of 5 normal behavior patterns was scored as –1. The seizure patterns included: continuously shivering its body (*shivering*), continuously stretching its wings upright (*upright wing posture*), and lying down on the floor with its feet stretched and shaking (*feet shaking*). The normal behavior patterns included: ability to maintain a standing posture (*standing still*), to walk spontaneously and straightforward (*walking*), to peck at objects spontaneously (*pecking*), to emit distress calls (*calling*), and the ability to pick itself up immediately after the experimenter put the chick with its back on the floor (*upright reflex*). The *upright reflex* was examined at the last step of the observation. For example, assume a chick that showed *shivering* and *upright wing* posture, but was able to keep its *standing-still* posture, to *walk*, and to show *upright reflex*, without spontaneous *pecking* and *distress call*. In this case, we scored the chick as +2 plus –3, yielding a total score of –1. In this manner, each chick was given a score ranging between +3 (very strong seizure) to –5 (normal and actively behaving). Behaviors were scored at 5 min, 10 min, 30 min, and 90 min after injection, and comparisons between two groups were made at corresponding post-injection time by using chi-square test of median values (two-tailed, significance level of 0.05). Chick pairs were trained and tested as described above.

Immunohistochemical staining

Immunohistochemical procedures for *ZENK* product basically followed the protocol by Ball *et al*. (1997). Chicks were deeply anesthetized by xylazine (Sigma, 2 mg / ml saline, 0.3 ml i.p. injection), and then perfused transcardially with Zanbomi's fixative; 4% paraformaldehyde in 0.15 M phosphate buffer, pH = 7.4 containing 15% of a saturated picric acid solution. Brain was dissected and immersed in the same fixative for 2–3 hr at room temperature, cryoprotected in increasing concentrations (10, 20, and 30%) of sucrose phosphate buffer for 2 overnights at 4°C. Brains were then coded, and the following staining, sampling and counting procedures were performed

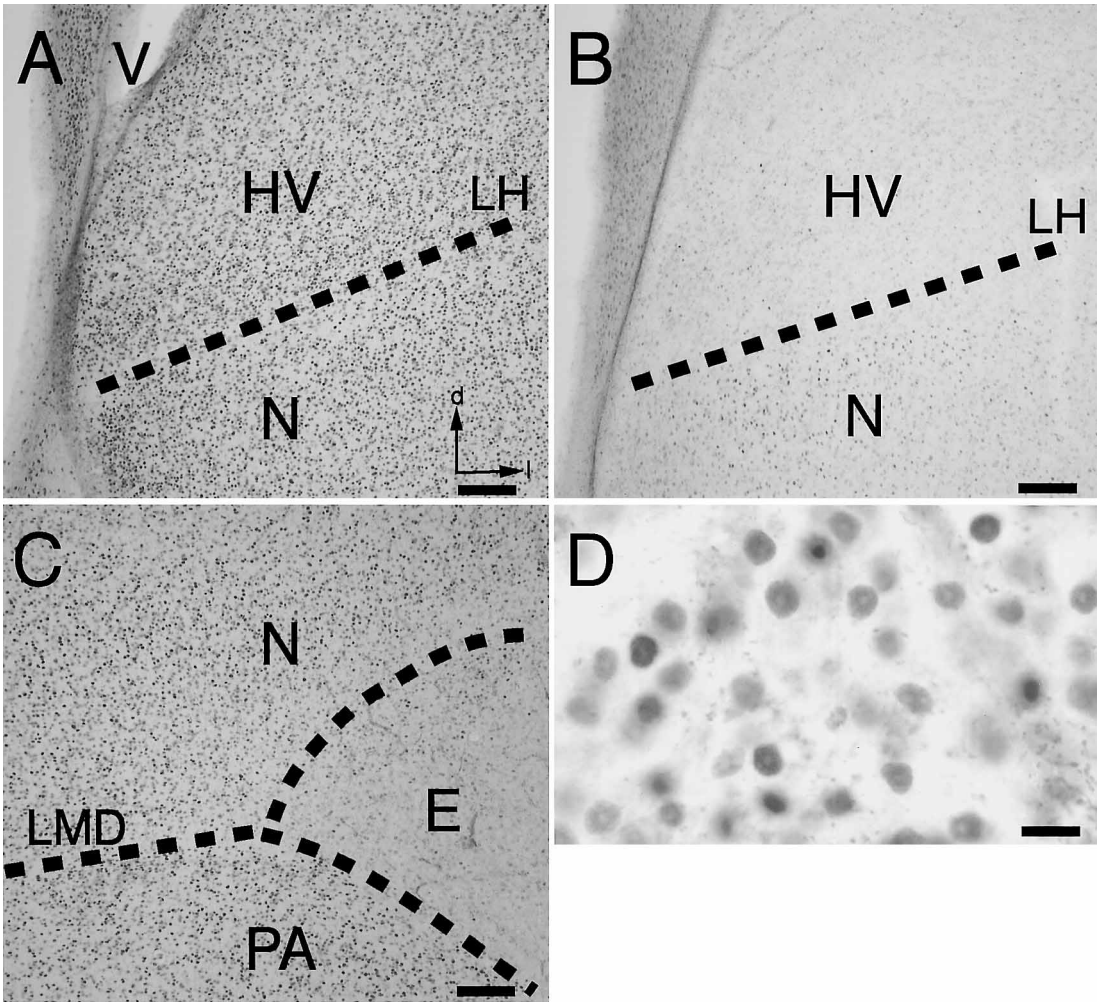

Fig. 1. Photomicrographs of frontal sections of quail chick telencephalon through hyperstriatum ventrale (HV), neostriatum (N), ectostriatum (E), and paleostriatum augmentatum (PA). Lamina hyperstriatica (LH) and lamina medullaris dorsalin (LMD) are stressed by dashed lines. V indicates ventricle. **A** and **C** are from a metrazol-treated chick, and **B** from an intact control chick. **A**, **B** and **C** are obtained from sections that contained tractus septo-mesensecphalicus (TSM-sections). **D** shows a part of neostriatum in **C** at a higher magnification. Scales are 100 μm for **A**, **B**, and **C**, while it is 10 μm for **D**. In **A**, *d* and *l* mean dorsal and lateral direction, respectively.

blind. The codes were broken after data were obtained for each brain specimen.

Free-floating frontal sections (50 μm thick) were cut by cryostat. Every third section was processed for immunostaining, and a neighboring section was mounted on gelatinized slide for Nissl staining. Sections were incubated with a polyclonal antibody (dilution 1:3,000, 2 overnights at 4°C) raised against the C-terminal of Egr-1 peptide of human (Santa Cruz Biotech, Egr1 (C-19)). In songbird, Mello and Ribeiro (1998) have shown that this antibody recognizes the avian ZENK protein. The immunoreaction was visualized by streptoavidin-biotin-peroxidase (Histofine SAB-PO, Nichirei Co.) with DAB (3,3'-diaminobenzidine tetrahydrochloride, Wako Co.) as chromogen. After staining, sections were mounted on slides in a serial order according to the corresponding Nissl preparations. Slides were then dehydrated through aceton series, cleared in xylene, and coverslipped. Fig. 1 shows photomicrographs obtained from a chick after metrazol-induced epileptic seizure (A, C and D), and from a naive control (B). Boundaries between hyperstriatum ventrale (HV) and neostriatum (N) (lamina hyperstriatica, LH), and between N and lobus parolfactorius (LPO) (lamina medullaris dorsalis, LMD) are stressed by dashed lines. Fig. 1D shows a higher magnification of N of under an oil-immersion objective lens (×100); note that cell nuclei were strongly stained but nucleoli were immunonegative. A preliminary experiment showed that no stained cells appeared without incubation of the first antibody.

Sampling frames and counting immunopositive cells

Among a series of immunostained sections (150 μm apart each other) obtained from a single brain specimen, we defined one section that contained tractus septo-mesencephalicus; this section is referred to as TSM section. Four successive sections were thus selected (i.e., 150 μm rostral to TSM, TSM, 150 μm and 300 μm caudal to TSM), and frames were sampled from these 4 sections for measuring HV and N. Similarly two successive sections were selected (i.e., 300 μm and 450 μm rostral to TSM), and frames were sampled for measuring HA and LPO.

In order to measure the density of immunopositive cells, we analyzed microscopic photo images of rectangular sampling frames. Images were digitized by using Nikon E600 with an objective lens (CFI Plan 20×) and a high-resolution video camera (HQ-130Ci; Hamamatsu Photonics, Japan), and stored as Adobe Photoshop files (Adobe Co.) in computer (Power Macintosh, Apple Co.). Single frame consisted of 1920 x 1035 pixels in a 807×436 μm rectangle (i.e., 0.2 μm^2 per pixel) at a resolution of 256-steps of gray scales. Fig. 2 shows the spatial arrangement of frames on representative sampling sections. For HV, a frame was set on each sampling section with its ventral line aligned with LH and its dorso-medial corner attached to ventricle. For N, a frame of the same orientation was set ventral to the LH with its dorsal line aligned with the HV frame. A total of 4 HV frames and 4 N frames were thus sampled. For LPO, two neighboring frames (dorsal and ventral LPO frames) were set for each sampling section with dorsal line of the dorsal LPO frame aligned with LMD, and its dorso-medial corner attached to ventricle. For HA, a frame of the same orientation was set with its ventro-medial corner attached to the dorsal edge of ventricle. A total of 4 LPO frames and 2 HA frames were thus sampled.

Number of immunopositive cells was counted for each frame under visual inspection of the digitized images. Criteria of immunopositive cells were; 1) single immunonegative nucleolus was clearly seen in each nucleus, 2) staining of nucleus clearly exceeded that of surrounding cytoplasm. As shown in Fig. 1, immunopositive cells were uniformly distributed in each frame and regional bias was not found between sections, so that data from all sampling frames were collected for HV, N, LPO and HA. Densities of ZENK-like immunopositive cells were thus calculated per square millimeter of frontal sections (50 μm thick) in each brain specimen (ZENK-like immunopositive cells/mm^2). Data from multiple independent groups were compared by Kruskal-Wallis test; when significantly different, two of these groups were further compared using Mann-Whitney U-test. Chi-square test of median values was not applicable because of relatively small samples obtained in this study. Level of significance was set at 0.05. All tests were two-tailed.

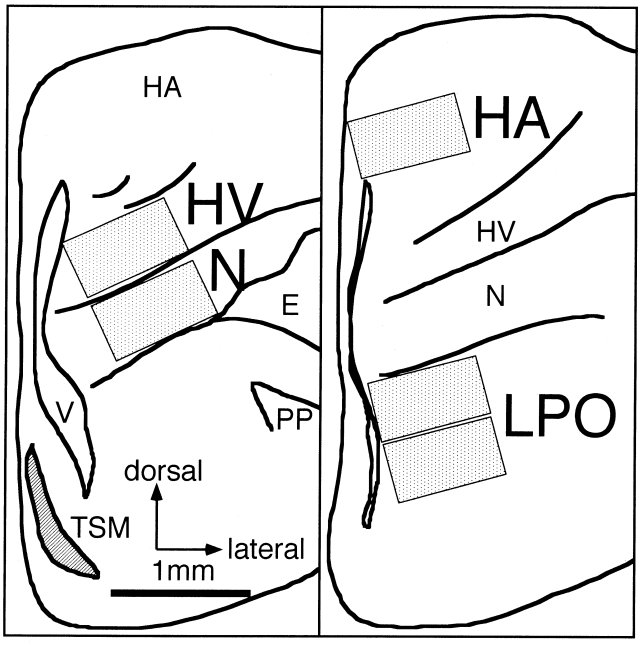

Fig. 2. Schematic drawings of two sections of chick telencephalon indicating the location of sampling frames for HV, N, HA and LPO. For abbreviations of neural nuclei, see the legend of Fig. 1. Left: TSM section. Right: section that is 300 μm rostral to the TSM-section.

RESULTS

Experiment-1: Effects of passive avoidance training on *ZENK* expression

In N and HV, densities of *ZENK*-product-like immunopositive cells (ZENK-li cells) did not differ among 3 experimental groups (Table 1-1). In HA and LPO, on the other hand, both of Kruskal-Wallis test and Mann-Whitney U-test revealed significant differences. In HA, differences proved significant between MeA- and 1/3MeA-trained chicks (U_{cal}=6, 0.01<p<0.05), and between W- and 1/3MeA-trained chicks (U_{cal}=10, 0.01<p<0.05), but not between MeA- and W-trained chicks (U_{cal}=20, N.S.). In LPO, differences proved significant similarly between MeA- and 1/3MeA-trained chicks (U_{cal}=6, 0.01<p<0.05), and between W- and 1/3MeA-trained chicks (U_{cal}=10, 0.01<p<0.05), but not between MeA- and W-trained chicks (U_{cal}=18, N.S.). We further examined the differences among those chicks that showed the same response at test (i.e., recall-and-discrimination), and found no statistical significance among groups of different training conditions in all of the brain regions (Table 1-2). It might be concluded that the experience of pecking at a bitter bead at training *per se* does not enhance the ZENK-li cell density. Similarly, no significant difference was found between two groups of chicks that showed recall-and-discrimination and those that showed amnesia after the same training condition of 1/3MeA (Table 1-3). It is thus suggested that the memory formation does not

Table 1. Densities of ZENK-li cells in neostriatum (N), hyperstriatum accessorium (HA), hyperstriatum ventrale (HV) and lobus parolfactorius (LPO) in groups of chicks that were trained by using MeA, diluted 1/3MeA and W (*Experiment-1*). Mean±S.E.M. of counts ($\times 10^3$ cells / $mm^2 \times 50$ μm thick sections) are shown. Statistical comparisons were made for each of the brain regions among three groups irrespectively of the behavior patterns at test (**table 1-1**), and among populations of chicks that showed recall-and-discrimination at test (**table 1-2**). Comparisons were also made between two populations of chicks in the 1/3 MeA-trained chick group; i.e., 4 chicks of recall-and-discrimination vs. 4 chicks of amnesia (**table 1-3**). N.S. means that no significant differences were found at the level of 0.05. When significant, the corresponding *p*-value is shown.

Table 1-1

	N	HA	HV	LPO
MeA-trained chicks (n=6)	2.45±0.32	1.13±0.14	1.50±0.15	2.01±0.13
1/3MeA-trained chicks (n=8)	2.88±0.18	1.53±0.11	1.91±0.12	2.56±0.12
W-trained chicks (n=7)	2.76±0.26	1.11±0.13	1.50±0.18	2.08±0.18
Kruskal-Wallis H-value	1.33	6.84	4.79	6.82
	N.S.	$0.02<p<0.05$	N.S.	$0.02<p<0.05$

Table 1-2

	N	HA	HV	LPO
Chicks that showed recall-and-discrimination after...				
MeA-training (n=6)	2.45±0.32	1.13±0.14	1.50±0.15	2.01±0.13
1/3MeA-training (n=4)	2.80±0.21	1.42±0.15	1.75±0.17	2.37±0.18
W-training (n=2)	3.37±0.46	1.12±0.50	1.51±0.26	2.32±0.51
Kruskal-Wallis H-value	3.00	2.90	1.67	1.80
	N.S.	N.S.	N.S.	N.S.

Table 1-3

	N	HA	HV	LPO
Chicks that were trained by 1/3MeA and showed...				
recall-and-discrimination (n=4)	2.80±0.21	1.42±0.15	1.75±0.17	2.37±0.18
amnesia (n=4)	2.96±0.33	1.64±0.15	2.08±0.15	2.74±0.11
Mann-Whitney U-cal	6	5	3	2
	N.S.	N.S.	N.S.	N.S.

Table 2. Densities of ZENK-li cells in neostriatum (N), hyperstriatum accessorium (HA), hyperstriatum ventrale (HV) and lobus parolfactorius (LPO) in metrazol-treated chicks and naive control chicks (Experiment-2). Mean±S.E.M. of ZENK-li cell counts ($\times 10^3$ cells / $mm^2 \times 50$ μm thick sections) are shown. Statistical comparisons were made between the two groups for each brain region by using Mann-Whitney U-test. N.S. means that no significant differences were found at the level of 0.05; the corresponding *p*-value is shown, when significant.

	N	HA	HV	LPO
Metrazol-treated chicks (n=4)	5.92±0.29	3.40±0.35	5.26±0.13	5.29±0.17
Naive control chicks (n=4)	4.27±0.40	2.66±0.13	2.50±0.16	3.01±0.21
Mann-Whitney U-cal	1	2	0	0
	N.S.	N.S.	$0.01<p<0.05$	$0.01<p<0.05$

enhance the ZENK-li cell density in chick telencephalon.

Experiment-2: Effects of intraperitoneal injection of metrazol on *ZENK* expression

Between metrazol-treated and naive control chicks, significant difference of ZENK-li cell densities were found in HV and LPO, but not in N and HA (Table 2). In each brain region, comparison was made between chicks that received single injection of metrazol and those that were left untouched. Chicks of both groups were placed together in the same dark incubator, so that the environmental conditions, such as visual experiences, should have been identical.

Experiment-3: Effects of induced epileptiform seisure on passive avoidance learning

Single injection of metrazol induced a transient epileptiform seizure that decayed slowly within 90 min post-treatment (Fig. 3). At 5, 10 and 30 min, metrazol-treated chicks showed significantly higher scores than the saline-treated sham control chicks, whereas no difference was found at 90 min. The induced seizure did not interfere with the following passive avoidance training in terms of the response patterns at test. The training was performed at 2 hr after the metrazol injection, i.e., after the induced seizure had faded away completely. This point of time is just 1 hour after the enhanced *ZENK* expression was found in HV and LPO (Experiment-2).

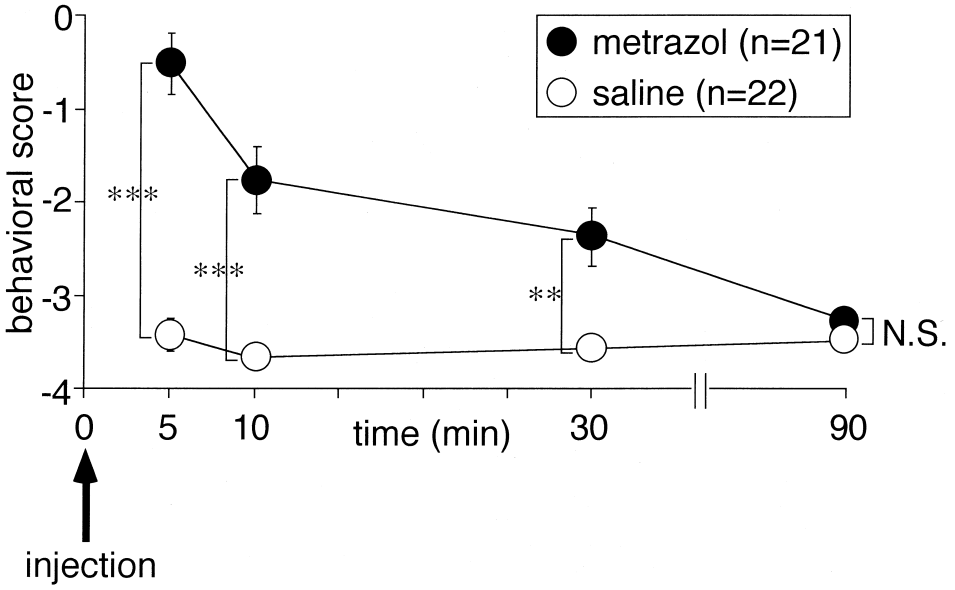

Fig. 3. Time-course of epileptiform seizure induced by intra-peritoneal injection of metrazol (Experiment-3). The mean±S.E.M. of behavioral score (see text for definition) were plotted against time (min) after the injection of metrazol (7.5 mg / ml saline, 0.1 ml i.p. injection: filled circles) or saline alone (0.10 ml: open circles). Statistically significant differences were found between the two groups at 5 min, 10 min and 30 min, but not at 90 min after the injection. Asterisks indicate the level of significance, e.g., ***: $p < 0.001$, **: $0.001 \leq p < 0.01$, by using chi-square test (two-tailed). N.S. means that the difference was not significant.

Table 3. Effects of metrazol-treatment (and the accompanying transient epileptiform seizure) on the passive avoidance learning (Experiment-3). Numbers of chicks at test (recall-and-discrimination, generalized avoidance, or amnesia) are shown; those chicks that showed paradoxical amnesia are included in the amnesia (#). Comparisons were made among the three independent groups of chicks, and no statistical significance was found.

	recall-and-discrimination	generalized avoidance	amnesia#	total
Metrazol-treated chicks	11	3	7	21
Saline-treated chicks	7	10	5	22
Intact control chicks	16	8	8	32

(chi-square value = 5.689, d.o.f = 4, $0.2 < p < 0.3$)

When tested 24 hr post-training, chicks of different pre-treatments showed basically identical response patterns, and no significant differences were found (Table 3).

DISCUSSIONS

In this study, we initially hypothesized a causal link between *ZENK* and memory formation in neonatal chick telencephalon, but found no supporting evidence. In Experiment-1, single training session using MeA-coated bead failed to enhance *ZENK* expression in all of the brain regions examined. The *ZENK* expression did not differ among groups of different training experience (Table 1-2), nor among groups of different responses at test (Table 1-3). We conclude that, at least in the present behavioral protocol, memory formation does not accompany an enhanced *ZENK* expression. Next, we examined the metrazol-induced epileptiform seizure (Experiment-2). Metrazol treatment, or the accompanying seizure *per se*, increased the ZENK-li cell densities in all regions examined, though statistical significance was found only in HV and LPO. Finally, we examined if the metrazol treatment (and the *ZENK* expression that followed) could interfere with the passive avoidance training (Experiment-3), and found no effects. Most simple and straightforward conclusion could be that *ZENK* expression may be induced after lasting activities in a mass of neurons, but is not specifically involved in the memory formation.

Experimental procedures for unmasking a functional link

The negative arguments drawn from the present study should well be criticized, since the present protocol might simply fail to unmask a functional link. First, we ought have examined more chicks in Experiment-1. We might have found a statistically significant correlation with more specimens of chicks. Secondly, we counted number of immunopositive cells irrespectively of the degree of staining. We might have found a positive correlation if we had quantified the immunopositivity in each cell. However, immunostaining proved variant, possibly depending on subtle experimental conditions such as penetration of antibody molecules into the tissue, concentration

of DAB, temperature, and so forth. We tried our best to stabilize the conditions, but the variation was inevitable and quantification of immunostaining did not seem realistic. Third, we should consider a possibility that the antibody used in this experiment might have recognized other proteins rather than ZENK. As mentioned in Materials and Methods, we did a negative control experiment and confirmed that the immunopositivity was due to specific reaction. However, we cannot absolutely deny the possibility of nonspecific binding. The preabsorption experiments should also be done.

Identities of immunopositive cells

Fourth, but most seriously, we did not characterize the immunopositive cells. We might have found a functional link if we had compared only in a certain subpopulation of ZENK-li cells. In this study, most immunopositive cells seemed neurons because of their morphology of nucleoli (Fig. 1D), but we did not identify them. In domestic chicks, McCabe and Horn (1994) have shown that successful imprinting procedure caused more Fos-immunopositive cells in IMHV. Ambalavanar *et al*. (1993, 1999) further revealed that most of these Fos-positive cells were also immunopositive for GABA and parvalbumin, and for gamma isoform of protein kinase. In songbird, Kimpo and Doupe (1997) have found that singing, but not hearing *per se*, enhanced Fos-li cells in HVc, one of the major song sensori-motor nuclei. They found also that the expression was highly specific to a subpopulation of HVc neurons which project to the next motor nucleus RA; another population of area X-projecting HVc neurons failed to show such an enhanced Fos expression after singing. Most probably, immediate early genes (IEGs) expression may represent neural activities, but is confined to a certain population of neurons in a highly specific manner, as has been pointed out by Chaudhuri (1997).

Context dependency of IEGs expression

Fifth, expression of IEGs might be coupled with certain aspects of behavior, but in a highly context-dependent manner that did not fit in the present protocol. In songbird, Jarvis *et al*. (1998) examined *ZENK* expression in song-controlling nuclei, and found that the expression depended on *for whom the male sang*. In most of the song controlling nuclei, significant positive correlation appeared between *ZENK* mRNA expression and the number of emitted song bouts. The context-dependent differences in *ZENK* expression were found in parts of the song-controlling nuclei such as RA, *l*MAN and *l*Area X. In a condition where a male sang alone (undirected songs) or toward other conspecific males, significantly positive correlation appeared between *ZENK* expression and the number of emitted song bouts. On the other hand, when sang toward females as courtship behavior (directed songs), the correlation was weaker than in the context of undirected songs.

In our case of chick memory, similarly, significant coupling might have appeared between *ZENK* and memory formation, if we had trained chicks with appetitive paradigm instead of the present aversive one. Actually, with 24 hr of mild water deprivation, domestic chicks can be trained to peck at bead of specific colors if rewarded by a few drops of water (Yanagihara, unpublished data). The reinforced pecking is based on memorized color cue of the bead, which lasts long and stable as with the passive avoidance memory using a bitter bead. It should also be stressed that we trained chicks with single MeA-training session, but a pre-training procedure might have unmasked a significant link. As has been reported previously (Sakai *et al*., 2000), a differential experience of pecking at a neutral bead of different shape successfully gave rise to more chicks that discriminated beads by the shape-cues at test. Similar facilitation of memorized color cues by differential pre-training has been shown in domestic chicks by using beads pained in other colors (Burnes and Rose 1997; Yanagihara unpublished). Subtle contextual factors of experiemntal procedures could be of a critical significance in controlling IEGs expression relevant for memory.

Forced expression of *ZENK* and memory formation

The enhanced *ZENK* expression after metrazol-induced epileptiform seizure (Table 2) would be reasonable, if we assume that *ZENK* expression represents a tonic increase in neuronal activities. Our recent single unit recording from freely behaving chicks actually revealed that the metrazol-induced seizure was accompanied by 2 to 3-folds increase in spontaneous firing rates of LPO neurons (Yanagihara and Izawa, unpublished). The increased firing lasted as the seizure, in contrast to the neuronal activities observed during passive avoidance training; lasting facilitation has never been found in spontaneous firing rates of IMHV and LPO neurons during and immediately after the training. Phasic neuronal activities may be sufficient for registrating memory, but not for drastic changes in IEGs expressions.

On the other hand, several points should be seriously examined before a conclusion is drawn from the results of Experiment-3. First, we trained chicks at 2 hours after metrazol treatment, whereas the enhanced *ZENK* expression was confirmed at 1 hr post-treatment in Experiment-2. Our excuse is that we tried but failed to train chicks at this point of time, because of generalized depression after the seizure. Another argument is that *ZENK* lasts long in general. In hippocampus of awake rats, electrical stimulations applied through chronically implanted electrodes enhanced *zif/268* or *Krox 24* product, which remained its peak for 2 hours or longer after tetanization (Richardson *et al*., 1992; Abraham *et al*., 1992; Williams *et al*., 1995). We may well expect a long-lasting expression of *ZENK* even at 2 hours after the metrazol injection, if the avian *ZENK* lacks the negative feedback feature of the c-*fos* expression, similarly to the mammalian *zif/268* (Chaudhuri 1997). Secondly, the population of ZENK-li cells might not be involved in the memory formation. In other words, metrazol might have enhanced *ZENK* expression, but in a population of neurons not relevant for the memory.

Functional link among IEGs, synaptic plasticity and memory formation

It remains yet unanswered as to whether IEGs such as c-*fos* or *ZENK* are linked with synaptic plasticity, and thus specifically with memory formation. In rat hippocampus, IEGs are shown to be significantly involved in stabilization, or slow phase of synaptic long-term potentiation (LTP; Abraham *et al.*, 1992). As far as the chick memory is concerned, we are not convinced at all that plastic changes of synapses constitute the critical cellular machinery of memory. We can simply say that both IMHV and LPO, two major critical regions for passive avoidance memory, also contain excitatory glutamatergic synapses that undergo LTP under certain experimental conditions (Bradley *et al.*, 1991; Matsushima and Aoki 1995). We may further stress a close parallelism between memory formation and LTP in terms of common pharmacological treatments effective in suppressing both events; such as selective blockers of NMDA-type glutamate receptors or dopamine D1 receptors (Bradley *et al.*, 1995; Matsushima and Yanagihara 1998; Kabai *et al.*, unpublished data). Causal link among molecular, cellular and behavioral events awaits direct answers.

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