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Research report

Up-regulation of microtubule-associated protein 2 accompanying the filial imprinting of domestic chicks (*Gallus gallus domesticus*)

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Abstract

Using cDNA microarrays, we have identified elsewhere the genes of microtubule-associated proteins as a group up-regulated in newly hatched chick brains after filial imprinting training. Here we show by *in situ* hybridization that the mRNA for the microtubule-associated protein 2 (MAP2) gene was enriched in the mesopallium and the hippocampus in the trained chick brain. The regionally specific enrichments of MAP2 mRNA were not observed in the brain of dark-reared or light-exposed chick as controls, implying an association between the degree of expression and the strength of the learned preference. In agreement with the gene expression, MAP2 protein was accumulated in the mesopallium of the trained chick brain, but not in the brains of the controls. The accumulation of MAP2 was found in the cytosol of neurons and co-localized with β-tubulin, suggesting a change in microtubule assembly. Our results suggest a postnatal reorganization of cytoskeleton following filial imprinting.

Keywords: Filial imprinting; MAP2; *Gallus gallus domesticus*; Chick; Microtubule; Behavior

1. Introduction

It has been proposed that the nervous system is so plastic that new experiences after birth can modify the original set of neuronal connections, causing the creation of new neurons or the formation of new synapses between existing neurons [12]. Birds have a varied behavioral repertoire that is associated with neural and behavioral plasticity, thus offering an ideal model system for experimental analysis of learning such as imprinting [13]. On the day of hatching, visually naive chicks (*Gallus gallus domesticus*) quickly learn visual characteristics of a conspicuous moving object, and subsequently form a social attachment to it. Imprinted chicks show a clear behavioral preference for the object, and selectively approach it rather than unfamiliar items [7].

To elucidate the molecular processes underlying the neural mechanism of filial imprinting in newly hatched chicks, we identified candidate genes preferentially expressed in the brain of imprinted chicks using cDNA microarrays. Those genes included that encoding MAP2, microtubule-associated protein 2 as a group up-regulated in the newly hatched chick brain after filial imprinting training [22].

MAPs, a group of filamentous proteins, have been demonstrated to promote assembly of tubulin, to bind and stabilize microtubules, and to form cross-bridge structures between microtubules [6]. MAP2 is a major member of neuronal MAPs and is found specifically in dendrites. MAP2 protein is known for its microtubule-stabilizing activity and for regulating microtubule networks in the dendrites of neurons, resulting in dendrite elongation [5]. The ability of MAP2 to interact with microtubules might be critical for neuromorphogenic processes, such as neuronal migration and outgrowth of neurites, during which networks of microtubules are reorganized in a coordinated manner [4]. There is some evidence that MAP2 is associated with learning and memory. The deletion of the N-terminus of murine

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MAP2 containing the binding site for regulatory subunit II PKA by gene targeting, disrupts hippocampal CA1 neuron architecture and alters contextual memory [3]. The up-regulation of hippocampal MAP2 appears to be highly correlated with contextual memory as measured by significantly heightened fear responses [20]. Thus, the up-regulation of the MAP2 gene in the imprinted chick brain raised the possibility that MAP2 has some role in the memory formation through cytoskeletal organization.

Here we show by *in situ* hybridization that the mRNA for the MAP2 gene was enriched in the mesopallium and the hippocampus of the chick brain following imprinting training. In agreement with the expression of its gene, the MAP2 protein was accumulated in the cytosol of the neuronal cell in the mesopallium of the trained chick brain. Our findings suggest that MAP2 is involved in the regulation of microtubule networks in neurons which might be critical for memory formation.

2. Materials and methods

2.1. Animals

Newly hatched domestic chicks of the Cobb strain (*Gallus gallus domesticus*) were used. Fertilized eggs were obtained from a local supplier, and incubated at 37 °C. After hatching, chicks were placed in a breeder at 30 °C in dark plastic enclosures to exclude light [9].

2.2. Training and test procedures for imprinting

Training and testing were carried out by the method of Izawa et al. [9]. We used three training groups of chicks in this experiment. The first group comprised chicks subjected to imprinting training ($n = 38$). The second group contained dark-reared controls not subjected to imprinting training and dark-reared until the test ($n = 15$). The third group comprised light-exposed control chicks in a training chamber in which the light was turned on and off but there was no training object and the running belt was turned off ($n = 22$). Three hours after training, simultaneous choice tests were carried out to test whether the chicks showed a preference for the training object [9].

2.3. Cloning of MAP2 cDNA from the chick brain

Total RNA (2 μg) extracted from chick brains was treated with 2 U of RNase-free DNase I and then reverse-transcribed with 200 U of SuperScriptII RT (Invitrogen, Tokyo, Japan). Using these cDNAs as templates, we obtained a DNA fragment corresponding to +3 to +504 of partial MAP2 cDNA (GenBank accession number; BU203845) with primers 5′-cgtacccatggtagatacaagc-3′(sense) and 5′-cttctgcggtctctctgtcc-3′(antisense). The resulting PCR product was subjected to agarose gel electrophoresis, purified by the Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA), and cloned into pGEM-T easy vector (Promega, Madison, Wisconsin, USA). The nucleotide sequence of the insert completely matched that of the partial MAP2 cDNA clone [2].

2.4. In situ hybridization

Chicks were anesthetized 3 h after training by an i.p. injection (0.40 ml) of a 1:1 mixture solution of ketamine (10 mg/ml, ketalar-10, Sankyo Co., Tokyo, Japan) and xylazine (2 mg/ml, Sigma, St. Louis, Missouri, USA). When necessary, supplementary injections (0.15 ml each) were given to maintain constant anesthesia. Then chicks were perfused intraventricularly with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.5), and paraffin coronal sections (10 μm thick) of brains were prepared with microtome (Leica, Tokyo, Japan). Serial coronal sections were prepared from a level A7.6 to a level A9.0 of Kuenzel and Masson's atlas [11]. The digoxigenin (DIG)-labeled RNA probes were prepared by *in vitro* transcription using cloned MAP2 partial cDNA as template. Hybridization was performed as described previously [18]. Detection was performed by the method of the tyramide signal amplification (TSA) detection system using TSA Biotin system (NEN Life Science Products, Boston, MA, USA) to amplify the weak signals [23]. After hybridization and washes with TNT buffer (0.1 M Tris–HCl [pH 7.5], 0.15 M NaCl, 0.05% Tween 20), the brain sections were incubated with TNT buffer containing a 0.5% blocking reagent for 15 min, rinsed briefly with TNT buffer and were incubated with the anti-DIG-peroxidase antibody (1:250, Roche, Tokyo, Japan) at room temperature for 30 min. Then the sections were washed by immersion in TNT buffer for 5 min at room temperature, incubated with biotinyl-tyramide (1:250, NEN Life Science Products) for 10 min at room temperature, rinsed with TNT buffer for 5 min, and streptavidin–alkaline phosphatase (1:250, Roche) was added. After 30 min incubation at room temperature, the sections were rinsed with TNT buffer and immersed in the same buffer for 5 min.

2.5. Quantitative analysis of MAP2 mRNA-positive cells by in situ hybridization

The numbers of MAP2 mRNA-positive cells in IMM (or hippocampus) on brain sections were counted at a level A7.6 in each group of training conditions (trained ($n = 5$), dark-reared ($n = 5$), light-exposed ($n = 5$)). Images of sections were captured by digital camera, DP-70 (Olympus, Tokyo, Japan). The regional position of the IMM was determined following the description of McCabe and Horn [14]. To evaluate the enhancement of MAP2 gene expression in IMM (or hippocampus) associated with filial imprinting, MAP2 mRNA-positive cells were counted in a rectangular sampling box placed over IMM (or hippocampus) and in a rectangular reference box placed over nidopallium on the diagram of brain sections shown in Fig. 2E. To normalize the number of MAP2 mRNA-positive cells in IMM (or hippocampus) in each brain section, the number of MAP2 mRNA-positive cells in the sampling box over the IMM (or hippocampus) was subtracted by that in the reference box over nidopallium. The number of MAP2 mRNA-positive cells in the reference box over nidopallium was less than 20% in average of that in sampling box over IMM (or hippocampus) in each group of training condition. The Kruskal–Wallis test was performed to analyze the difference among three groups of training conditions in the number of MAP2 mRNA-positive cells.

2.6. Immunocytochemistry

Immunocytochemistry was performed as previously described [21]. Trained chicks were perfused intraventricularly with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.5) under deep anesthesia 3 h after training (24 h after hatch). Dark-reared chicks were perfused 24 h after hatching as controls. Paraffin coronal sections (10 μm thick) of chick brains were then prepared for immunostaining. Mouse anti-MAP2 monoclonal antibody (Abcam, Tokyo, Japan, 1:1500) and rabbit anti-β-tubulin III antibody (Abcam, 1:500) were used as primary antibodies, respectively. Anti-mouse Alexa 546-conjugated antibody and anti-rabbit Alexa 488-conjugated antibody (Invitrogen, Tokyo, Japan, 1:200) were used as secondary antibodies. Alexa 633-conjugated Phalloidine (Invitrogen, 1:200) was used to stain F-actin. Fluorescence images of brain sections were obtained using a Leica TCS-SP5 confocal fluorescence microscope.

3. Results

We found, using cDNA microarrays, that the MAP2 gene was up-regulated in the newly hatched chick brain after filial imprinting training [22]. To test whether the MAP2 gene expression is associated with filial imprinting, we first examined the relationship between the strength of preference for imprinting objects and training conditions. As shown in Fig. 1A, we prepared three groups of chicks with different training conditions. Three hours after training, simultaneous choice tests were carried out to test whether chicks showed a preference for the training object. In the first test, we used a control object (Toy A) which had the same