Activation of cholecystokinin neurons in the dorsal pallium of the telencephalon is indispensable for the acquisition of chick imprinting behavior

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Abstract
Chick imprinting behavior is a good model for the study of learning and memory. Imprinting object is recognized and processed in the visual wulst, and the memory is stored in the intermediate medial mesopallium in the dorsal pallium of the telencephalon. We identified chicken cholecystokinin (CCK)-expressing cells localized in these area. The number of CCK mRNA-positive cells increased in chicks underwent imprinting training, and these cells expressed nuclear Fos immunoreactivity at high frequency in these regions. Most of these CCK-positive cells were glutamatergic and negative for parvalbumin immunoreactivity. Semi-quantitative PCR analysis revealed that the CCK mRNA levels were significantly increased in the trained chicks compared with untrained chicks. In contrast, the increase in CCK- and c-Fos-double-positive cells associated with the training was not observed after closure of the critical period. These results indicate that CCK cells in the dorsal pallium are activated acutely by visual training that can elicit imprinting. In addition, the CCK receptor antagonist significantly suppressed the acquisition of memory. These results suggest that the activation of CCK cells in the visual wulst as well as in the intermediate medial mesopallium by visual stimuli is indispensable for the acquisition of visual imprinting.

Keywords: chick, cholecystokinin, imprinting, learning and memory, plasticity, visual wulst.


In visual imprinting, day-old chicks rapidly form a memory of the moving object to which they were first exposed and show a following response (Lorenz 1937). The importance of the intermediate medial mesopallium [IMM, all nomenclatures are in accordance with Reiner et al. (2004)], which is a subregion of the dorsal pallium region, for the acquisition and/or retention of memory in visual imprinting has been suggested by many studies (Horn et al. 1979; Kohsaka et al. 1979; McCabe et al. 1981, 1982). In addition, we have reported that the visual wulst, which is located dorsorostrally to the IMM, is associated with the acquisition of visual memory in imprinting and exhibits plastic changes (Maekawa et al. 2006). The visual wulst is the highest center
of the thalamofugal pathway in the telencephalon, which is one of two pathways for visual information processing in birds (Karten and Hodos 1970; Karten et al. 1973; Benowitz and Karten 1976).

What are the cellular and molecular mechanisms underlying this plasticity? It is known that learning during the early postnatal periods in Aves induces several biochemical and structural changes in the IMM, including an increase in the size of the post-synaptic densities (Bradley et al. 1981; Horn et al. 1985), learning-related increases in the phosphorylation of the myristoylated alanine-rich C-kinase substrate, an increase in the activation of calmodulin-dependent protein kinase II (Sheu et al. 1993; Zhao et al. 1999), an increase in the number of vesicles near the active release zones in synapses (Ruskov et al. 1995), and an increase in the glutamate, GABA, or taurine release (Tsukada et al. 1999; McCabe et al. 2001; Meredith et al. 2004). All these events may facilitate synaptic transmission, and indeed, the proportion of neurons in the IMM that responded to the imprinted stimulus was increased in imprinted chicks (Nicol et al. 1995).

In addition to glutamate and GABA, several neuropeptides are found in the mammalian neocortex, some of which may play important roles in the learning process. Although their functions have not yet been fully elucidated, these peptides can modulate the activity of neurons both postsynaptically through peptidergic action on receptors and pre-synaptically via the release of glutamate or GABA.

In the present study, we focus on the roles of these peptides in the imprinting behavior in order to reveal a novel regulator of memory acquisition. We attempted to identify the types of neurons in the dorsal pallium that are excited by visual stimuli during the training for visual imprinting, using a combination of c-Fos immunohistochemistry and the detection of various transmitters by immunohistochemistry and/or mRNA in situ hybridization. As a result, we found that more than half of the cholecystokinin (CCK) neurons in the dorsal pallium were also immunopositive for c-Fos after imprinting training. Therefore, we performed detailed examination and characterization of these CCK neurons in order to clarify their roles in inducing visual imprinting.

Materials and methods

Animals

Fertilized eggs of the White Leghorn strain (Gallus domesticus) were obtained from a local supplier (Fukuyama Shukeijo, Hiroshima, Japan) and incubated at 37.7°C under quasi-constant darkness. After hatching, the chicks were placed together in the same incubator. This experiment was approved by the Animal Care and Use Committee and was performed according to the Guidelines for Animal Experimentation of Tokyo Medical and Dental University.

Imprinting apparatus

To evaluate the imprinting behavior in chicks, we used a previously described system (Maekawa et al. 2006). Chicks were placed on a running wheel with a see-through wire net on the floor during training. The running wheel was connected to an angle sensor, which recorded the numbers of forwards and backwards rotations produced by the chick. The lowest-detectable sensitivity of the angle sensor was 22.5°. The data regarding the numbers of rotations in both directions were transmitted every minute to a computer (Muromachi Kikai, Tokyo, Japan) and stored throughout the experiment. A 15-inch thin-film transistor liquid crystal display (TFT-LCD; EIZO FlexScan L367; Nanao, Ishikawa, Japan) with a resolution of 1024 × 767 pixels, driven at a refresh rate of 60 Hz, was set 20 cm away from the edge of the wheel. Two types of image, which were produced by a visual stimulus generator system (VSG; Cambridge Research Systems Ltd., Kent, UK), were used: a red circle of 8.6 cm in diameter and a blue square of 8.6 cm per side (visual fields of 24° × 24°). Both images had a luminance of 10.36 cd/m², and the Commission Internationale de l'Eclairage-xy chromaticity coordinates of these colors were 0.638 and 0.310 for red and 0.15 and 0.062 for blue, and they moved horizontally with a speed of 73 mm/s (20.9°/s).

Training and evaluation

Training and evaluation were performed as described previously (Maekawa et al. 2006). For the imprinting training, chicks were exposed for 30 min to the red circle presented on the left and right TFT-LCD, respectively. Untrained (control) chicks were kept in the incubator without training. The following day, the image preferences of the chicks were evaluated. During the evaluation session, the TFT-LCD was always placed on the left-hand side. After a 5-min-adaptation period, the red circle and blue square were presented sequentially every 5 min. Revolutions of the running wheel towards and away from the TFT-LCD were recorded every minute. To assess imprinting behavior, the preference score (PS), defined by the number of revolutions towards the TFT-LCD divided by the total number of revolutions during the 5-min-test period, was used to compare preferences for each image. Occasionally, when the activity during 5-min-test period for each color resulted in zero counts, the PS was determined as being 0.5.

In situ hybridization

The chicken genes were amplified from chick brain cDNAs using the following primers: for the chick CCK (chCCK; GenBank accession no. NM001001741; nt 102–463) forward 5′-AGCTCCC-TCGCCCAGC-3′ and reverse 5′-CTATCGCTGTCGTCTTTTAGGA-3′ and for the chick CCK receptor (chCCK-R; GenBank accession no. NM001001742; nt 779–1210) forward 5′-GCTTA- CGGGGTATCTTCCCGGA-3′ and reverse 5′-CATTGAAGCGAGTATGAGCCGGGT-3′. The amplified fragments were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA). A 173 bp fragment of the chick glutamic acid decarboxylase 65 (chGAD65) gene was amplified as described previously (Sun et al. 2005), and cloned into the pBSIISK(–) vector (Stratagene, La Jolla, CA, USA). A fragment of the vesicular glutamate transporter 2 (VGLUT2) gene was amplified using two sets of primers: Chvglut2 F1 (5′-AGGCTTTGG TATGGAAACAAT-3′) and Chvglut2R1

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(5′-ATTCTGAGTATGTCCTGCTT-3′); and Chvglut2 F2 (5′-TTTACTGCGGATCCGAGAC-3′) and Chvglut2R2 (5′-A-TACAAAGCCTACGACATGTCAT-3′). The fragment amplified by the latter primer set corresponds to the probe used by Cheng et al. (2004). The chicken VGLUT3 (chVGLUT3) gene fragment (GenBank accession no. XM425451; nt 1290–1849) was amplified using the Chvglut3 F1 (5′-GGCTAAGATTTGTTGGAGCTT-3′) and Chvglut3R1 (5′-GGTAAAAGCTCTTCTTCTGCATGTC-3′) primers. Gene-specific antisense digoxigenin (DIG)-labeled cRNA probes were generated using the Roche DIG RNA labelling kit (F. Hoffmann-La Roche, Basel, Switzerland). In situ hybridization was performed with free-floating sections. Chicks were perfused transcardially with either Zamboni’s fixative, which contained 4% p-formaldehyde and 15% saturated picric acid in 0.05 mol/L phosphate buffer (PB; pH 7.5), or with 4% p-formaldehyde in 0.05 mol/L PB under ether anesthesia. Brains were post-fixed overnight in the same solution as was used for perfusion, cryoprotected with 30% sucrose in 0.05 mol/L PB, and sectioned coronally or sagittally at 30- to 50-μm thickness with a cryostat (CM1900; Leica, Nussloch, Germany). The sections were collected at 180- or 200-μm interval. In situ hybridization was performed according to procedures described previously (Fujiwara et al. 2003; Miyashita et al. 2005), however with slight modifications.

After washing twice with 0.1 mol/L phosphate-buffered saline (PBS; 0.1 mol/L PB; 0.15 mol/L NaCl), the sections were treated with proteinase K (1 μg/mL) in Tris–EDTA buffer (pH 8.0) at 37°C for 10 min and then acetylated with 0.25% acetic anhydride in 0.1 mol/L triethanolamine for 10 min. Hybridization was performed at 60°C for 14–16 h in a solution that contained 50% formamide, 2% blocking reagent (Roche Applied Science, Indianapolis, IN, USA), 5x sodium chloride/sodium citrate buffer (SSC), 0.1% N-laurylsarcosine, 0.1% sodium lauryl sulfate, and DIG-labeled cRNA probe. The sections were washed twice in 50% formamide plus 2x SSC for 15 min at 50°C, treated with 20 μg/mL RNase in Tris–EDTA buffer (pH 8.0) at 37°C for 30 min, washed sequentially with 2x SSC and 0.2x SSC (each for 30 min) at 50°C, and then washed with DIG-1 buffer [0.1 mol/L Tris–HCl (pH 7.5), 0.15 mol/L NaCl, and 0.1% Tween 20]. The sections were incubated in blocking buffer (5% bovine serum albumin and 5% normal goat serum in DIG-1 buffer) for 1 h at 25°C. After blocking, the sections were transferred to alkaline phosphatase-conjugated anti-DIG antibody (1 : 2000 dilution; Roche Applied Science) in blocking buffer and incubated overnight at 4°C. After this incubation, the sections were washed three times with DIG-1 buffer for 15 min, rinsed with DIG-3 buffer [0.1 mol/L Tris–HCl (pH 9.5), 0.1 mol/L NaCl, 50 m mol/L MgCl₂], and incubated in nitro blue tetrazolium chloride 5-bromo-4-chloro-3-indolyl phosphate (1 : 50 in DIG-3 buffer; Roche Applied Science) for 2–10 h at 25°C. The sections were washed in 0.1 mol/L Tris–HCl (pH 8.0) and mounted in aqueous media that contained 14% polyvinyl alcohol and 30% glycerol in PBS.

For double-labeling using in situ hybridization, antisense fluorescein (F) -labeled cRNA probes were synthesized using the fluorescein RNA labeling mix (Roche). DIG- and F-labeled probes were mixed in the hybridization buffer. The sections were first reacted with alkaline phosphatase-conjugated anti-F antibody (1 : 1000; Roche) and visualized as described above. After treatment with 0.1 mol/L glycine (pH 2.2), the sections were incubated with alkaline phosphatase-conjugated anti-DIG antibody, and a chromogenic reaction was performed using a solution of 2-[4-iodophenyl]-3-[4-nitrophenoxy]-5-phenyl-tetrazolium chloride plus 5-bromo-4-chloro-3-indolyl phosphate (1 : 133; Roche).

**Immunohistochemistry**

To visualize the distribution of cell bodies that expressed various neurotransmitters, colchicine treatment was used. Three chicks at post-hatch day 0 (PD0) were injected intracerebroventricularly (i.c.v.) with 4 μL of colchicine solution (10 μg/μL in distilled water; Wako Pure Chemical Industries, Osaka, Japan) using a stereotaxic instrument (Model 900; David Kopf Instruments, Tujunga, CA, USA) with a small bird adaptor under ether anesthesia. The beak bar was lowered 8 mm ventral to the ear bar. The position of the tip of the injector was 2 mm anterior to the ear bar, 3 mm ventral to the surface of the skull, and 0.5 mm lateral to the midline. The injection was executed for 1 min using a multi-syringe infusion pump (KDS 230; KDS Scientific Inc., New Hope, PA, USA). Forty-eight hours after injection, chicks were perfused transcardially with 50 mL 0.05 mol/L PBS following 100 mL ice-cold Zamboni’s fixative under deep pentobarbital anesthesia. The brains were removed, post-fixed using the same fixative overnight at 4°C, and cryoprotected with 30% sucrose in 0.05 mol/L PB for 2–3 days at 4°C.

For c-Fos immunohistochemistry, chicks at PD0 were trained by the method described above and placed back in the incubator at 37.7°C under quasi-constant darkness. One hour after the end of the training, the chicks were perfused as described above. Chicks without training experience were used as controls.

Localization of the target proteins was determined by peroxidase immunohistochemistry. The brains were sectioned coronally or sagittally at 40- to 60-μm thickness with a cryostat (CM1900; Leica). The sections were incubated with: (i) 0.6% H₂O₂ in 0.05 mol/L PBS for 30 min; (ii) 5% normal horse serum and 0.5% Triton X in 0.05 mol/L PBS for 30 min; (iii) rabbit anti-human CCK (peptide 26–33, 1 : 5000; Research Biochemicals International, Natick, MA, USA) that contained 5% normal horse serum and 0.5% Triton X in 0.05 mol/L PBS overnight at 25°C; and (iv) EnVision®/Rabbit-horseradish peroxidase (DAKO, Glostrup, Denmark) for 1 h. The immunoreactivities were visualized using the Vector SG Substrate Kit (Vector Laboratories, Burlingame, CA, USA). Sections were rinsed three times in 0.05 mol/L PBS after each step, except between steps 2 and 3. The sections were mounted on glass slides and covered with mounting medium.

For the double-staining of chCCK by in situ hybridization and the staining of other antigens, immunohistochemistry using peroxidase was performed after the in situ hybridization process. The anti-c-Fos antibody was diluted 1 : 10 000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Mouse monoclonal anti-parvalbumin was diluted 1 : 20 000 (Sigma Chemical co., St Louis, MO, USA), and Triton X was usually excluded from the buffers throughout the staining procedure. The immunoreactivities were visualized using the diamino benzidine Liquid System (DAKO).

**Grouping intensities and quantification**

Images were acquired using an Olympus (BX60) microscope equipped with an Olympus DP50 digital camera and Studio Lite software (Olympus Corporaton, Tokyo, Japan). The light
microscopy images were transferred to a graphics program (Adobe Photoshop 7.0, Adobe Systems Incorporated, San Jose, CA, USA) in which the brightness and contrast were adjusted.

In order to group the intensities, the sections were treated at once, and images were acquired under the constant exposure condition, transferred, and the brightness and contrast were adjusted at the fixed level. Then, from each brain region, the area (0.05 mm²) in which the density of positive cells was the highest was selected. Two observers counted independently the number of positive cells in these areas. The values were averaged, and the averaged values above the 75th, 50th, and 10th percentiles and those below them were assessed as ++, +, and −, respectively. The values (imm²) of the 75th, 50th, and 10th percentiles were 690, 217, and 28 for CCK, respectively, 633, 236, and 47 for CCK-R, and 766, 350, and 0 for c-Fos, respectively.

Counting of single- or double-positive cells was performed as follows. For a coronal section in which the subdivisions of the hyperpallial layers were well segregated, a medial portion (150 × 300 μm) of the three layers, which consisted of the hyperpallium intercalatum (HI), mesopallium dorsale (MD), and mesopallium ventrale (MV), was selected. The HI constitutes one of the layers of the wulst and the wulst subdivisions, and the MV includes the IMM. Positive cells were counted within these areas in three adjacent sections from one chick. Three chicks were used for the trained and untrained groups, respectively. Double-staining was performed simultaneously for all the samples, in order to avoid any staining variations. For the counting of positive cells, only cells that showed staining intensity above the determined level in the grayscale (256 shades) were counted.

Fluorescence real-time RT-PCR for chCCK mRNA measurement

Two-millimeter thick coronal slices of the telencephalon were produced using a brain slicer (Muromachi Kikai). To obtain pallial tissue, two medial sections of the telencephalon that contained the hyperpallium and mesopallium were selected, and the dorsal half region without most of the hyperpallium apicale (HA) was dissected from both sides under a dissection microscope. Half of the cerebellum was also dissected from each brain. Total RNA was extracted using the Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer’s instruction and incubated for 1 h with RNase-free DNase (1 U/tube; Promega) to remove any genomic DNA contamination. After DNase inactivation by heating, cDNA was synthesized using the Superscript II reverse transcription kit with oligo-(dT) primers (Invitrogen). Real-time PCR was performed using gene-specific primers and a Taqman probe. For chCCK mRNA quantification, the forward primer 5′-AAGCAGGAAAGCTTCA-3′, reverse primer 5′-CCAGCCCATGCTCTCTGTCA-3′, and Taqman probe 5′-FAM-AGGGTACAGCATGTCACACACACA-TAMRA-3′ were used. The final amplicon was 100 nucleotides long (GenBank accession no. NM001010741; nt 298–398). For standardization, we also quantified chicken glyceraldehyde-3-phosphate dehydrogenase (chGAPDH) using the forward primer 5′-GGAGCACTGTCAGATCTGAGA-3′, reverse primer 5′-GATCGCTTCGAAAGATATGTT-3′, and Taqman Probe 5′-FAM-TGAGCGTGCCATTGATCTCGAAATGTT-TAMRA-3′. The final amplicon was 75 nucleotides long (GenBank accession no. NM204305; nt 222–295).

Effect of the CCK-R antagonist

The CCK-R antagonist davezepide (L-364 718) was kindly provided by Dr Leslie Iversen (Panos Therapeutics Ltd., Oxford, England). A stock solution of davezepide (4 mg/mL) was prepared in 30% dimethylsulfoxide/70% propylene glycol, and this solution was ultimately diluted to a concentration of 5 μg/mL in saline that contained 60% propylene glycol (Ebenzer 2002). Of this solution 10 μL was administered i.e.v. 5 min before the start of the 30-min training period. For the control group, the vehicle (10 μL) was injected. The intracerebroventricular injection was performed as previously described (Davis et al. 1979; Furuse et al. 1999). Evans Blue (0.1%) was always included in the injected solution to monitor the injection site. At the end of the experiment, the chicks were deeply anesthetized with ether, the brain was dissected, and the blue-staining of the ventricle was inspected. We excluded all data obtained from chicks that lacked staining with the blue dye in the ventricle.

Statistical analysis

All the data are expressed as the mean ± SEM. The number of animals used is indicated in each figure unless otherwise noted. One way ANOVA followed by Fisher’s PLSD was used to compare the PS between images and to compare the total revolutions between the vehicle- and davezepide-treated groups. In addition, a one-sample t-test using the value defined as PS < 0.5 was used for analysis of the PS for each image, to examine whether the PS values that differed from the chance level (0.5) were significant. Two-way ANOVA followed by Fisher’s PLSD was used to compare the number of positive cells between the control and training groups or between brain regions. Relative increases in the numbers of double-positive cells were analyzed by the Mann–Whitney U-test. All statistical analyses were performed using StatView 5.0 (SAS Institute Inc., Cary, NC, USA). Differences were considered significant for p < 0.05.

![Fig. 1](image-url)

**Fig. 1** Imprinting performance in trained and untrained chicks. Post-hatch day 0–2 chicks were exposed to a red circle presented on a thin-film transistor liquid crystal display screen during the training session, which lasted 1 h. Untrained chicks were kept in the incubator. On the following day, the chicks’ preferences for the black screen (No stim.), trained stimulus (Red circ.; red circle), or a new stimulus (blue sq.; blue square) were evaluated. Only those chicks that underwent the training showed a specific preference for the imprinting stimulus. *p < 0.01 (significantly different from the chance level) and *p < 0.01. The number of chicks used is indicated in parenthesis.
Results

Identification of neurons involved in imprinting behavior

The PD0–2 chicks were exposed to the red circle presented on the TFT-LCD for 1 h. The following day, these chicks showed preference for and chased in a specific manner the red circle (Fig. 1). On the other hand, the chicks that were not exposed to the stimulus (untrained) did not display this behavior. Thus, imprinting behavior was successfully established with this training, as we have shown previously (Maekawa et al. 2006). Therefore, we used this training schedule to characterize the neurons activated by imprinting training. As mentioned above, the visual wulst and IMM of the dorsal pallium have been shown to be important for imprinting, so we used immunohistochemical methods to search for neurotransmitters that were expressed abundantly in these regions after imprinting training. Of the immunoreactivities for bombesin, CCK, choline acetyltransferase, enkephaline, neuropeptide Y, neurotensin, somatostatin, and substance P-like protein, we found prominent expression of CCK-like immunoreactive cells in the visual wulst and mesopallium.

To exclude the possibility that the antibody used reacted with an antigen other than CCK, chCCK mRNA-specific in situ hybridization was performed using a DIG-labeled probe. Probe specificity was demonstrated by hybridizing the sections with the same amount of sense riboprobe made from the plasmid used for making the CCK antisense riboprobe. As the in situ hybridization method is more sensitive than immunohistochemistry, chCCK mRNA-containing neurons were found to be more numerous than the CCK-like immunoreactive cells, and the signal intensity varied. This is consistent with the observation made by Schiffmann and Vanderhaeghen (1991) in the rat central nervous system. The chCCK mRNA-positive cells were arranged radially along the lamina in the HI, MD, and MV, and this distribution pattern was similar to that of the CCK-immunoreactive cells. Thus, the distribution pattern of CCK-expressing cells in the chick brain was confirmed (Fig. 2a–d).

The expression of immediate early genes such as c-Fos serves as a marker for neuronal activation. This has also been found in the chick forebrain during visual imprinting (Horn 1998). We compared the distribution of cells that expressed chCCK mRNA and c-Fos antigen in chick telencephalons that were fixed after 1 h of training. As a result, chCCK mRNA-expressing cells and c-Fos-positive cells were found abundantly in the hyperpallium and mesopallium (see details in Fig. 3 and Table 1). The panels are from the stereotaxic atlas of the chick brain by Kuenzel and Masson (1988). Both types of cell were located in the area parahippocampus, hippocampus, and nidopallium intermediate of the pallium and in the nucleus teniae, striatum laterale, striatum mediale, and nucleus striae terminalis lateralis of the subpallium. Thus, most of the areas that contained c-Fos-expressing cells also possessed chCCK mRNA-positive cells. However, although moderate numbers of c-Fos-positive cells were detected in the nidopallium caudale, it was devoid of chCCK mRNA-positive cells. Conversely, in the nucleus basorostralis pallii and globus pallidus, only CCK mRNA was expressed.

**Fig. 2** Cholecystokinin (CCK) expression in the dorsal pallium of the telencephalon of chicks that were subjected to imprinting training. Immunohistochemistry using the anti-CCK antibody (a and c) and in situ hybridization using chick CCK as a probe (b and d) show that the positive cells are distributed in the hyperpallium intercalatum (HI) and mesopallium dorsale (MD). The areas enclosed by rectangles in (a and b) correspond approximately to the regions in (c and d), respectively. Scale bars: 500 μm (a and b) and 100 μm (c and d).
In the untrained control chicks, the distribution pattern of CCK mRNA-expressing cells was similar, although the number of positive cells and the expression level were decreased as compared with trained chicks.

Colocalization of chCCK mRNA and c-Fos expression after training that elicits imprinting behavior

To examine whether the CCK-expressing neurons were really activated by imprinting training, we performed double-staining for chCCK (blue staining; by mRNA in situ hybridization) and c-Fos (red staining; by immunohistochemistry). We calculated the number of single- and double-labeled cells in the dorsal pallium, which has been identified as a region that is essential for the acquisition of visual imprinting (McCabe et al. 1981, 1982; Maekawa et al. 2006).

In all three subdivisions of the pallium (HI, MD, and MV; Fig. 4a and b), the number of c-Fos-positive cells in the chicks trained for visual imprinting was significantly higher than that in the control chicks without training (Fig. 4c–e). The numbers of chCCK mRNA-positive cells in these three areas were also increased following imprinting training (Fig. 4c, d, and f). In addition, the proportion of c-Fos and chCCK double-positive cells in trained chicks was more than threefold higher than in the control chicks (Fig. 4g).

Increase in the chCCK mRNA level in the dorsal pallium after imprinting training

To confirm the results of our histological examinations, we quantified the chCCK mRNA level following imprinting training using real-time PCR and a Taqman Probe. As
Table 1 Distribution of CCK-, CCK-R, and c-Fos-positive cells in the chick telencephalon

<table>
<thead>
<tr>
<th>Pallium</th>
<th>CCK</th>
<th>CCK-R</th>
<th>c-Fos</th>
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<tbody>
<tr>
<td>Area parahippocampus</td>
<td>APH</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>Hippocampus</td>
<td>Hp</td>
<td>+++</td>
<td>++</td>
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<tr>
<td>Cortex piriformis</td>
<td>CPI</td>
<td>+</td>
<td>+++</td>
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<tr>
<td>Hyperpallium apicale</td>
<td>HA</td>
<td>++</td>
<td>+++</td>
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<tr>
<td>Hyperpallium densocellularis</td>
<td>HD</td>
<td>+++</td>
<td>+</td>
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<tr>
<td>Hyperpallium intercalatum</td>
<td>HI</td>
<td>+++</td>
<td>+</td>
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<tr>
<td>Mesopallium</td>
<td>M</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>Nidopallium</td>
<td>N</td>
<td>–</td>
<td>++</td>
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<tr>
<td>Nidopallium intermedium</td>
<td>NI</td>
<td>+</td>
<td>++</td>
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<tr>
<td>Nidopallium caudale</td>
<td>NC</td>
<td>–</td>
<td>++</td>
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<tr>
<td>Entopallium</td>
<td>E</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Nucleus basorostralis pallii</td>
<td>Bas</td>
<td>++</td>
<td>+</td>
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<tr>
<td>Area temporoparieto-occipitalis</td>
<td>TPO</td>
<td>++</td>
<td>+</td>
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<td>Subpallium</td>
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<tr>
<td>Arcopallium anterior</td>
<td>AA</td>
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<tr>
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<td>Nucleus teniae</td>
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<tr>
<td>Striatium laterale</td>
<td>LSt</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Globus pallidus</td>
<td>GP</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Striatium mediale</td>
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<td>–</td>
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<tr>
<td>Nucleus striae terminalis lateralis</td>
<td>BSTL</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

+++ strong; ++; moderate; +, weak; -, none; CCK, cholecystokinin; CCK-R, CCK receptor.

expected based on the in situ hybridization results, the relative concentration of chCCK mRNA in the pallium was increased significantly following imprinting training (Fig. 4h). In contrast, the relative chCCK mRNA level remained unchanged in the cerebellum, which indicates that the regulation of chCCK mRNA is region-specific.

Characterization of chCCK mRNA-expressing cells in the dorsal pallium

Previous studies have demonstrated that the CCK-containing neurons in the mammalian cortex are GABAergic interneurons (Somogyi et al. 1984; Markram et al. 2004; Somogyi and Klausberger 2005). Therefore, we investigated whether this was also true for the chick dorsal pallium. For this purpose, we performed double in situ hybridization for chCCK and chGAD65, which is a marker of inhibitory GABAergic neurons. The chGAD65 mRNA-positive cells were abundant in the subpallium, although some of these cells were scattered uniformly in the pallium (Fig. 6a), as reported previously (Sun et al. 2005). However, in the pallium, the chCCK mRNA-positive cells rarely expressed chGAD65 (Fig. 6b).

We also investigated whether the chCCK-positive cells were glutamatergic. For this purpose, we chose VGLUTs as molecular markers for excitatory neurons. chVGLUT2 has been described previously (Cheng et al. 2004). The gene for chVGLUT3 was found in the database (GenBank accession no. XM425451). We searched without success for a third VGLUT gene, like VGLUT1, in the University of California, Santa Cruz, Genome Browser chicken BLAST-like alignment tool Search database (http://genome.ucsc.edu/cgi-bin/hgBlat). In the in situ hybridization experiment, chVGLUT2 mRNA-expressing cells were densely distributed in the pallium (Fig. 6c), while few cells were detected in the subpallium. We used two probes, both of which gave the same result. In contrast, chVGLUT3 was expressed exclusively in the subpallium, and the pallium was totally devoid of chVGLUT3 mRNA (data not shown).

From these results, we concluded that chVGLUT2 is the major VGLUT expressed in the chicken pallium. Therefore, we performed double in situ hybridization using chCCK and chVGLUT2 as probes. The majority of the chCCK mRNA-positive cells were also chVGLUT2-positive, which indicates that these cells were glutamatergic (Fig. 6d). In contrast, the distribution of parvalbumin-containing cells was distinct from that of chCCK mRNA-positive cells (Fig. 6e). Even at the boundary of their expression patterns in the hyperpallium, there was no superimposition of staining (Fig. 6f). Although a few cells in the mesopallium expressed a low level of chCCK mRNA together with a low level of parvalbumin, in general, the chCCK mRNA-positive cells did not show immunoreactivity for parvalbumin. This indicates that the major population of chCCK mRNA-positive neurons is not a subset of parvalbumin-containing interneurons.

Localization of chCCK-R mRNA in the telencephalon

To identify the target site of chCCK, we performed chCCK-R mRNA in situ hybridization in the telencephalon. Moderate expression levels of chCCK-R mRNA were noted in the HA, area parahippocampus, hippocampus, and mesopallium, and weak expression levels were noted in

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the hyperpallium densocellulare, HI, and nidopallium intermedium. The distributions of chCCK-R mRNA-positive cells were similar to those of chCCK mRNA-positive cells in many regions, reflecting the nature of interneurons. However, chCCK-R-positive cells were more numerous in the HA than in the mesopallium, while chCCK-positive cells were abundant in the mesopallium. In addition, diffuse expression of chCCK-R mRNA was detected throughout the telencephalon, with the exception of the entopallium (Fig. 7 and Table 1). These discrepancies in expression indicate that a subset of CCK-positive cells project their axons to distant areas. In support of this, many CCK-immunoreactive fibers were detected in the telencephalon of colchicine-untreated chicks (data not shown).

**Effect of a CCK receptor antagonist on the acquisition of imprinting in chicks**

The effect of devazepide, which is an antagonist for chCCK-R (Nilsson et al. 2003), on imprinting acquisition was examined. After intracerebroventricular injections of a vehicle or devazepide (50 ng/chick), chicks were exposed to a red circle on PD0, and the chick preference for the red circle or a blue square were assessed on PD1 (Fig. 8a). In both the vehicle- and devazepide-treated groups, chicks with low motor activity during the evaluation session (less than 22.5 revolutions/15 min) were excluded from the analysis. In the vehicle-treated group (n = 13), the chicks approached the TFT-LCD that presented the red circle on PD1, and the mean PS for the red circle was 0.67 ± 0.04. The one-sample t-test demonstrated that this value was significant from the
Fig. 5 Training after the critical period does not increase the number of chick cholecystokinin (chCCK) and c-Fos double-positive cells in the dorsal pallium. A representative brain section from a post-hatch day 6 (PD6) control chick (a) and a PD6 chick that was subjected to imprinting training (b), with staining for chCCK mRNA (blue) and c-Fos (red). Black arrowheads, CCK single-positive cells and red arrowheads, double-positive cells. Scale bars: 20 μm. (c) At PD6, imprinting training with a red circle as stimulus did not induce imprinting behavior. For cell counting, four chicks from each control or trained group were used and three sections from one chick were selected. After imprinting training, the numbers of c-Fos-positive (d), CCK-positive (e), and double-positive cells (f) were not increased in the hyperpallium intercalatum (HI) of the hyperpallium, mesopallium dorsale (MD), and mesopallium ventrale (MV), which includes the intermediate medial mesopallium.

chance level ($p < 0.01$). In contrast, when the blue square, an image the chicks had never seen, was presented, they tended to move in the opposite direction to the image and the PS was $0.41 \pm 0.07$. There was a significant difference in the PS values between these familiar and unfamiliar images ($p = 0.018$). On the other hand, in the devazepide-treated group ($n = 12$), the PS was unchanged (PS = 0.57) regardless of the nature of the presented image (familiar or unfamiliar). This behavioral pattern is reminiscent of that of untrained birds (Fig. 1). Moreover, there was no difference in the general movement levels of the injected and control chicks, as judged from the number of wheel revolution during the training and evaluation sessions (Fig. 8b). Both groups showed elevated levels of motor activities during the evaluation session as compared with during the training session. No other gross behavioral changes were detected in these birds. Therefore, these results indicate that visual imprinting was not acquired during the training on PD0 in CCK antagonist treated-chicks.

Discussion

Visual imprinting, which is a form of filial imprinting in which chicks learn the ensemble of characteristics presented by the mother hen, is an instinctive behavior of ground-nesting fowl that was first analyzed by Konrad Lorenz in the 1930s (Lorenz 1937). Previously, we have shown that the visual wulst, which is located in the dorsal part of the pallium, is critical for the acquisition but not the recall of memory in visual imprinting behavior and exhibits plastic changes after imprinting training (Maekawa et al. 2006). From these results, we postulated that there should be a cellular correlation that supports this plasticity in the dorsal pallium. In the current study, we found that CCK expression and c-Fos immunoreactivity colocalized in the pallium after imprinting training. We confirmed that the CCK mRNA level was increased after imprinting training using both quantitative real-time PCR methods and counting the number of cells that expressed CCK mRNA. These results indicate that many CCK neurons in the dorsal pallium become active following visual imprinting training. Furthermore, we demonstrate that these changes are specifically related to the acquisition of imprinting, as they were not observed in chicks that were trained after closure of the critical period. The indispensability of CCK signaling for the acquisition of visual imprinting was further confirmed using devazepide, which is a potent antagonist of chCCK-R (Nilsson et al. 2003).
We also characterized the CCK neurons in the dorsal pallium. For this purpose, we used GAD65 as a GABAergic marker, VGLUT as a generic glutamatergic marker (Fremeau et al. 2001; Cheng et al. 2004), and parvalbumin, which is a neuropeptide that is known to be expressed in interneurons. Only a small subset of the CCK neurons co-expressed GAD65 mRNA, which indicated that they are inhibitory interneurons. However, most of the CCK neurons were glutamatergic, as judged from VGLUT2 expression, and were devoid of parvalbumin immunoreactivity. The glutamatergic property of most of the CCK neurons was unexpected, as CCK is usually considered to be one of the inhibitory interneuron markers in mammals (Somogyi et al. 1984; Markram et al. 2004; Somogyi and Klausberger 2005). However, other studies have demonstrated that the expression of CCK mRNA is more widespread than was previously assumed and occurs in both the interneurons and the pyramidal cells of the rodent cortex, the latter generally being thought to be glutamatergic (Burgunder and Young 1990; Schiffmann and Vanderhaeghen 1991; Senatorov et al. 1995; Gallopin et al. 2006). Therefore, we assumed that glutamatergic CCK neurons were common in the pallium, and that there may be a species difference in the frequency of occurrence. The precise distribution and functional role of inhibitory CCK neurons in the chick pallium must be determined in future studies.

Although chCCK-R was expressed in the region in which the CCK cells were observed, the precise physiological consequence of CCK release at the cellular level in the chick dorsal pallium is not known at present. In mammals, CCK increases GABA release in the cortex and hippocampus, leading to an increased tonic inhibition of pyramidal neurons (Sheehan and de Belleruche 1983; Perez de la Mora et al. 1993; Miller et al. 1997). Alternatively, activation of the CCK-B receptor, which is a major receptor expressed in the mammalian brain (Gaudreau et al. 1983; Honda et al. 1993), induces phosphatidylinositol hydrolysis and intracellular Ca\(^{2+}\) mobilization (Kopin et al. 1992; Shinohara and Kawasaki 1994; Shigeri et al. 1996). As chCCK-R has a high affinity for sulfated CCK-8, CCK-4, and sulfated gastrin-17 (Nilsson et al. 2003), it may have a function similar to that of the mammalian CCK-B receptor. Therefore, it is possible that these CCK neurons have an excitatory effect on postsynaptic neurons that express chCCK-R. In addition, CCK...
can facilitate glutamate release in rat hippocampal neurons (Migaud et al. 1994; Breukel et al. 1997). These excitatory effects of CCK are consistent with the fact that CCK increases the excitability of pyramidal neurons in the cortex as well as in the hippocampus in mammals (Dodd and Kelly 1981; Jaffe et al. 1987; Boden and Hill 1988; Bohme et al. 1988; Shinohara and Kawasaki 1997; Gallopin et al. 2006). Further physiological characterization at the single cell level is necessary to elucidate the precise role of CCK neurons in the chick dorsal pallium.

A substantial body of evidence suggest that CCK is important in the regulation of anxiety, pain, food intake, memory, and other activities in the central nervous system (Crawley and Corwin 1994). These various biological effects are mediated by the widespread distribution of CCK-R in the brain, although the functional relationships between these various biological effects are not clear at present. However, it is conceivable that CCK, which has a feeding-suppressing effect, facilitates learning and memory, as do other gut-brain peptides, such as bombesin (Ohki-Hamazaki et al. 2005).

To our knowledge, this is the first report to show clearly the involvement of CCK in visual learning. In the murine system, visual learning is difficult to evaluate because of the poor visual discriminating abilities of these animals. However, our results are reminiscent of other studies in rodents. A selective CCK-B agonist improved cognitive performances evaluated by habituation to a novel environment or two-trial recognition memory task in rats (Gerhardt et al. 2006).

Fig. 7 Distribution of chick cholecystokinin receptor (chCCK-R) mRNA in the telencephalon.

Fig. 8 The effect of devazepide, a chick cholecystokinin receptor antagonist, on imprinting learning. (a) Vehicle (left) or devazepide (right) was administered i.c.v. before training. Vehicle treatment did not affect preference for the red circle (Red circ.; stimulus used for training) at evaluation, whereas devazepide completely abolished this preference. (b) Although motor activities increased in the evaluation session as compared with the training session, no difference was detected between the two groups during the training or evaluation sessions (b). *p < 0.01 and #p < 0.01. No stim, black screen; Blue sq., blue square. The number of chicks used is indicated in parenthesis.
In conclusion, CCK neurons are abundant in the chick dorsal pallium and are excited by a visual stimulus that evokes visual imprinting. Inhibition of chCCK-R impairs the acquisition of memory, which is indispensable for visual imprinting. These results indicate for the first time that the activation of chCCK-R is critical for memory formation in imprinting, and CCK may have an important role in shaping neural networks for imprinting.

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