

CRITICAL ROLE OF THE NEURAL PATHWAY FROM THE INTERMEDIATE MEDIAL MESOPALLIUM TO THE INTERMEDIATE HYPERPALLIUM APICALE IN FILIAL IMPRINTING OF DOMESTIC CHICKS (*GALLUS GALLUS DOMESTICUS*)

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Abstract—Filial imprinting in precocial birds is a useful model for studying early learning and cognitive development, as it is characterized by a well-defined sensitive or critical period. We recently showed that the thyroid hormone 3,5,3'-triiodothyronine (T₃) determines the onset of the sensitive period. Moreover, exogenous injection of T₃ into the intermediate medial mesopallium (IMM) region (analogous to the associative cortex in mammals) enables imprinting even on post-hatch day 4 or 6 when the sensitive period has been terminated. However, the neural mechanisms downstream from T₃ action in the IMM region remain elusive. Here, we analyzed the functional involvement of the intermediate hyperpallium apicale (IMHA) in T₃ action. Bilateral excitotoxic ablation of the IMHA prevented imprinting in newly hatched chicks, and also suppressed the recovery of the sensitive period by systemic intra-venous or localized intra-IMM injection of T₃ in day-4 chicks. In contrast to the effect in the IMM, direct injection of T₃ into the IMHA did not enable imprinting in day-4 chicks. Moreover, bilateral ablation of IMHA after imprinting training impaired recall. These results suggest that the IMHA is critical for memory acquisition downstream following T₃ action in the IMM and further, that it receives and retains information stored in

the IMM for recall. Furthermore, both an avian adeno-associated viral construct containing an anterograde tracer (wheat-germ agglutinin) and a retrograde tracer (cholera toxin subunit B) revealed neural connections from the IMM to the IMHA. Taken together, our findings suggest that hierarchical processes from the primary area (IMM) to the secondary area (IMHA) are required for imprinting. © 2015 The Authors. Published by Elsevier Ltd. on behalf of IBRO. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Key words: filial imprinting, critical period, sensitive period, thyroid hormone, memory trace, memory priming.

INTRODUCTION

Filial imprinting in precocial birds (fowl chicks and ducklings) is a useful model for studying early processes in learning, memory consolidation, and cognitive development (Horn, 1998; Matsushima et al., 2003). Newly hatched domestic chicks have specific predispositions and acquire preferences for particular features (e.g. color, shape and configuration) of a conspicuous moving object (Horn, 2004; Rosa Salva et al., 2015). The object is the biological mother under natural conditions, but it can be any other objects in the laboratory (Izawa et al., 2001; Yamaguchi et al., 2012). As the underlying neural substrates, several brain regions have been shown to play critical roles in imprinting (Horn, 1998; Maekawa et al., 2006; Nakamori et al., 2010; Yamaguchi et al., 2012). Among these areas, the intermediate medial mesopallium (IMM) is assumed to be the most critical, as it is necessary for the acquisition of imprinting (McCabe et al., 1981) and also for the 3-h retention from the end of imprinting training (McCabe et al., 1982). In accordance with this, the IMM is one of the poly-sensory areas in the avian isocortex (Delius and Bennetto, 1972; Bonke et al., 1979; Bradley et al., 1985), and it includes neurons that respond to various visual features of the imprinting object after training (McCabe and Nicol, 1999; Horn et al., 2001).

A characteristic feature of filial imprinting is its sensitive period (Hess, 1959). If housed in darkness, chicks can be imprinted only within a few days after hatching. Recently, we discovered that the thyroid hormone 3,5,3'-triiodothyronine (T₃) is the key determinant of the

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Abbreviations: A3V, avian adeno-associated virus; AMHA, anterior medial hyperpallium apicale; ANOVA, analysis of variance; CTB, cholera toxin subunit B; DLA, nucleus dorsolateralis anterior thalami; EGFP, enhanced green fluorescence protein; GFP, green fluorescence protein; HDCo, core region of hyperpallium densocellulare; HDPe, periventricular region of the hyperpallium densocellulare; HSD, honest significant difference; IHA, interstitial nucleus of the hyperpallium apicale; IMHA, intermediate hyperpallium apicale; IMM, intermediate medial mesopallium; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; T2A, thosaesa asigna virus 2A; T₃, 3,5,3'-triiodothyronine; WGA, wheat-germ agglutinin.

sensitive period (Yamaguchi et al., 2012). Level of T_3 in the brain peaks around the peri-natal (peri-hatch) period, and the imprinting training further increases it through the rapid inflow of T_3 into the brain, as T_3 is converted from circulating plasma thyroxine by activity of Dio2 (type 2 iodothyronine deiodinase). It is also shown that the increase of T_3 by imprinting training in the IMM region facilitates the imprinting, making chicks imprintable even on day 4–6 when the sensitive period appears to end. Namely, learning itself triggers the start of the sensitive period of imprinting, and this novel mnemonic function of T_3 was called “memory priming” (Yamaguchi et al., 2012). However, the neural mechanisms downstream from T_3 action in the IMM remain unknown.

The majority of the IMM efferent neurons project to arcopallium (Csillag et al., 1994). The pathway from the IMM to the medial striatum via the arcopallium has been thought to be involved in memory acquisition for passive avoidance learning (Lowndes and Davies, 1994; Csillag, 1999). However, bilateral ablation of the medial striatum does not impair imprinting acquisition (Izawa et al., 2001). Thus, there should be other brain areas that play an important role in imprinting. Previously, we showed that the expressions of brain-derived neurotrophic factor (BDNF) and several immediate early genes increased in the intermediate hyperpallium apicale (IMHA) region immediately after imprinting training (Yamaguchi et al., 2011a). Because neural connections between the IMM and the IMHA are not reported, functional roles of the IMHA for imprinting has remained obscure.

Here, we demonstrated that the IMHA plays crucial roles in the imprinting process; this region is downstream from the IMM in T_3 action, and is connected via neural circuits. Our results suggest that the information of the imprinting object in the primary associative area (IMM) is further modified in the IMHA region to enable imprinting acquisition and recall.

EXPERIMENTAL PROCEDURE

Animals

The experiments were conducted in accordance with the guidelines of the Committee on Animal Experiments at the Teikyo University, from which approval was obtained. The guidelines are based on national regulations for animal welfare in Japan. Newly hatched domestic chicks of the Cobb strain (*Gallus gallus domesticus*) were used. Fertilized eggs were obtained from a local supplier (3-M, Nagoya, Japan) and incubated at 37 °C for 21 days. After hatching, the chicks were placed in dark plastic enclosures in a breeder at 30 °C to prevent exposure to light (Izawa et al., 2001).

Training and test procedures

Training was carried out according to Izawa et al. (2001) after slight modifications. A custom-made I-shaped maze was equipped with a rubber belt (width: 8 cm, length: 43 cm) controlled by a PIC-based sequencer (Tri State Co. Ltd, Hokkaido, Japan). An imprinting object (made of yellow LEGO® blocks, 4.7 cm × 6.2 cm × 5.0 cm) at

one end rotated under illumination, and an infrared sensor was placed near the object. When the chick hit the sensor, the belt moved backward and pulled the chick away from the object. Two 1-h training sessions were conducted, and we counted the number of the sensor hit as a behavioral measure of activity during training. Those chicks that hit more than 500 times during the two training sessions were accepted and subsequently tested. The test was conducted 30 min after the imprinting training.

In choice tests, a cross maze with a pair of 20-cm-long start arms and a pair of 46-cm-long side arms was used. The imprinting object (yellow) was placed at the end of one side arm, and a novel control object of the same shape (but of different color, red) at the end of the other side arm. The successfully trained chick was placed in one start arm while the other was occluded; the start arms were counter-balanced in each test trial. We measured the total time spent near the objects during a 120 s test trial, which was repeated four times and the data were averaged. The preference score was given as (time near the imprinting object) – (time near the control object) in sec.

Bilateral excitotoxic lesion by ibotenic acid and histological localization

Chicks were injected with ibotenic acid as described previously (Yamaguchi et al., 2011b) with a modification. Chicks were anesthetized with a 1% isoflurane/air mixture and fixed on a stereotaxic apparatus. The skin was cut, and a small piece was incised on the skull surface. The dura mater was cut to expose the telencephalon. The stereotaxic coordinates for the IMHA region were 3.0 mm anterior to the bregma, 1.5 mm lateral to the midline, and 0.8 mm in depth. The coordinates for the IMM region were 3.0 mm anterior to the bregma, 1.5 mm lateral to the midline, and 2.5 mm in depth. The coordinates for the anterior medial hyperpallium apicale (AMHA) were 6.0 mm anterior to the bregma, 0.8 mm lateral to the midline, and 0.7 mm in depth. Ibotenic acid (65 mM) in phosphate-buffered saline (PBS) was bilaterally injected slowly (13.4 nl/min) for 35 min using a microinjector (Nanoject I, Drummond Scientific Co., Broomall, PA, USA). Control vehicle was injected into the IMHA of control chicks. After 1–3 days of injection, the chicks were trained and tested for imprinting. The lesions did not affect the locomotor activities measured by the number of sensor hits during training.

After the behavioral experiment, the chicks were given a transcatheter perfusion of a fixative (4% paraformaldehyde in PBS) under deep anesthesia by 1:1 mixture solution of ketamine (10 mg/ml, Ketalar-10, Sankyo Co., Gifu, Japan) and xylazine (2 mg/ml, Sigma-Aldrich, Tokyo, Japan). The brains were dissected, and fixed with the same fixative at 4 °C for 1 day. The brain was then cut into 50- μ m frontal sections using a microslicer (DTK-1000; Dosaka EM Co., Kyoto, Japan) and stained with cresyl-violet.

Systemic and localized T_3 injection

Intra-venous injection or IMM injection of T_3 was carried out as described previously (Yamaguchi et al., 2012). T_3

(10 μM) was dissolved in 0.002 N NaOH and was injected intra-venously before training. The resulting concentration of T_3 in serum was ~ 20 ng/ml. For injection into the IMM or IMHA, T_3 (10 μM) was dissolved in dimethyl sulfoxide (DMSO). Chicks were anesthetized with a 1% isoflurane/air mixture and fixed on a stereotaxic apparatus. T_3 was injected slowly (26.8 nl/ min) for 20 min using a microinjector. The chicks started training 30 min after the injection.

Plasmid constructs

To express Venus which is an improved version of green fluorescent protein (GFP) (Nagai et al., 2002), the construct of pA3V-RSV-Venus was generated. The residues of the Venus were amplified by polymerase chain reaction (PCR) using the Venus of pCSII-CMV-RfA-IRES2-Venus (provided by Dr. Miyawaki, RIKEN, Japan) as a template. The sequence of the sense primer, which includes an Nco1 site at the end was as follows: 5'-GGCCATGGACCATGGTGAGCAAGGGCGAGGAGCT-3'. The sequence of the antisense primer including a BsrG1 site at the end, was as follows: 5'-GTGTACATTACTTGTACAGCTCGTCATGCCGAG-3'. The PCR product was cloned into the Nco1-BsrG1 site of the pA3V-RSV-EGFP construct (Matsui et al., 2012).

An anterograde tracer WGA, which is transferred trans-synaptically (Ohashi et al., 2011), was also used. To express the WGA and enhanced green fluorescence protein (EGFP) bicistronically, a *thoosa asigna* virus 2A (T2A) peptide-linked construct, pA3V-RSV-EGFP-T2A-WGA, was generated. The T2A peptide provides the efficient cleavage site between the WGA and EGFP (Kim et al., 2011). The WGA corresponding residues 47–643 were amplified by PCR using the WGA of pEF-tWGA (Yoshihara et al., 1999) (provided by Dr. Yoshihara, RIKEN, Japan) as a template. The sequence of the sense primer, which includes a T2A sequence and a BsrG1 site at the end, was as follows: 5'-CTGTACAAGGGATCCGGTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCGGGCCCATGAGAAAGATGATGAGCACCATGGCC-3'. The sequence of the antisense primer including BsrG1 site at the end was as follows: 5'-GTGTACATCAGACAGCGTCACAGCCGCCGCTCTGGCA-3'. The PCR product was cloned into the BsrG1-BsrG1 site of the pA3V-RSV-EGFP construct (Matsui et al., 2012).

Generation of recombinant viral particles

The avian adeno-associated virus (A3V) was generated by co-transfection into HEK293T cells (RIKEN BRC, Japan) with a mixture of three plasmids using the calcium phosphate precipitation method (Matsui et al., 2012). Briefly, 293T cells were plated at 4.0×10^5 cells/10-cm dish the day prior to transfection. The medium was replaced with fresh medium 30 min before transfection. The three plasmid mixture consisted of 6.6 μg of pAd12, 6.6 μg of pA3V-RC and 6.6 μg of pA3V-RSV-Venus or 6.6 μg of pA3V-RSV-EGFP-WGA in each of the eight dishes. The 293T cells were co-transfected with the plasmid mixture by calcium phosphate precipitation. Cells were

harvested 48 h after transfection, and pelleted by centrifugation at 2,500 rpm for 5 min. The pellets were lysed in 2.5 mL of 150 mM NaCl and 100 mM Tris-HCl (pH 8.0) by a triple freezing-thaw procedure. The cell lysate was treated with 250 U/mL benzonase (Novagen) for 30 min at 37 °C and centrifuged at 7000 rpm for 60 min. The virus-containing supernatant was further purified by iodixanol (OptiPrep; Axis-Shield) step gradient ultracentrifugation (Zolotukhin et al., 1999). After ultracentrifugation in a HITACHI P40ST rotor at 40,000 rpm for 3.5 h at 18 °C, approximately 1.5 mL of the 40% iodixanol step was obtained. The buffer was then exchanged by dialyzing three times against PBS containing 0.001% pluronic F-68 (Sigma-Aldrich), and the solution was concentrated to 100 μL using a Vivaspinn 20 (GE Healthcare, Tokyo, Japan).

Administration of virus particles to chick brain

The A3V which contains the pA3V-RSV-Venus or the pA3V-RSV-EGFP-T2A-WGA construct was injected into the left or right IMM on day 0. The virus vector solution was injected slowly (13.4 nl/min) for 35 min using a microinjector (Nanoject I, Drummond Scientific Co., Broomall, PA, USA) under anesthesia with a 1% isoflurane/air mixture. The coordinates of the IMM were the same as those in the ibotenic acid injection. Four days to 3 weeks after virus injection, the chicks were transcardially perfused with 4% paraformaldehyde in phosphate buffer under deep anesthesia. Brains were post-fixed with the same fixative for 24 h and immersed in 30% sucrose in PBS. Brain tissues were then cut into 40- μm -thick sections using a cryostat. For fluorescent staining, the sections were blocked with 5% normal goat serum for 1 h and incubated with anti-WGA rabbit polyclonal antibody (1:500, Sigma-Aldrich, Tokyo, Japan) that had been pre-adsorbed with 1% acetone powder from the chick brain and anti-NueN mouse monoclonal antibody (1:100, Merck Millipore, Tokyo, Japan) for 24 h at 4 °C. The sections were then incubated with Alexa Fluor 546-conjugated anti-rabbit antibody (1:200, Life technologies, Tokyo, Japan) and Alexa Fluor 633-conjugated anti-mouse antibody (1:200, Life technologies, Tokyo, Japan). Fluorescent images were obtained by using a confocal microscope (TCS SP-5, Leica, Tokyo, Japan) and a two-photon confocal microscope (FV1000, Olympus, Tokyo, Japan). When we found leakage of the virus vector in the IMHA, the brain was excluded from further analysis. In more than 80% of cases, we successfully injected without leakage.

Counting the EGFP- or WGA-positive cells

Four sections (each 40 μm) corresponding to A7.6, A8.2, A8.8, and A9.4, respectively, were selected by the stereotaxic atlas of the chick brain (Kuenzel and Masson, 1988). Each section was scanned by a virtual slide scanner (NanoZoomer-XR, Hamamatsu Photonics K.K., Hamamatsu, Japan) to obtain the images. One image was divided into 50 parts and magnified. Then, the number of the EGFP- or WGA-positive cells in the IMM, IMHA, and arcopallium was counted. Three brains prepared by independent experiments were used for the

analysis. Mean and total of the EGFP- or WGA-positive cells were calculated.

Administration of a retrograde tracer to chick brain

Cholera toxin subunit B (CTB) conjugated to Alexa Fluor 488 (Life technologies, Tokyo, Japan) was used for retrograde tracing. CTB (0.5%) which was dissolved in the PBS was injected into the left or right IMHA on day 0. The coordinates of the IMHA were the same as those in the ibotenic acid injection. One week after the injection, the chicks were transcardially perfused with 4% paraformaldehyde in phosphate buffer under deep anesthesia. Brains were post-fixed with the same fixative for 24 h. Brain tissues were then cut into 300- μ m thick sections using a cryostat. Fluorescent images were obtained by using a two-photon confocal microscope (FV1000, Olympus, Tokyo, Japan).

Immunoblot analysis

Immunoblot analysis was performed as previously described (Yamaguchi et al., 2007). In brief, brains were cut into 200- μ m frontal sections with a microslicer. Microcapillaries (Harvard Apparatus, Holliston, MA, USA) were used to punch out infected brain tissues in the IMM showing green fluorescence with a fluorescence microscope and brain tissues in the IMHA with an optical microscope. The punched-out brain tissues were later subjected to immunoblotting. For detection of EGFP, anti-EGFP mouse monoclonal antibody was used as the primary antibody (1:1000, Santa Cruz Bio., Dallas, TX, USA), while an anti-mouse horseradish peroxidase-conjugated antibody (1:1000, GE Healthcare, Tokyo, Japan) was used as the secondary antibody. For detection of WGA, an anti-WGA rabbit polyclonal antibody (1:1000, Sigma–Aldrich) that had been pre-adsorbed with 1% acetone powder from the chick brain was used as the primary antibody, whereas an anti-rabbit horseradish peroxidase-conjugated antibody (1:1000, GE Healthcare, Tokyo, Japan) was used as the secondary antibody.

Statistical analysis

All data are expressed as mean \pm standard error. The number of animals used is indicated in each figure. The equality of variance of all data was verified using Bartlett's test. As the variances were equal in all cases, one-way analysis of variance (ANOVA) or two-way repeated measures ANOVA was applied. If necessary, Dunnett's test (many to one comparisons) or Tukey's honest significant difference (HSD) test (pairwise comparisons) was used for *post hoc* multiple comparisons. A p -value < 0.05 was considered significantly different.

RESULTS

Effects of IMHA lesion on imprinting on post-hatch day 1: IMHA is required for imprinting acquisition

In order to examine whether the IMHA region is required for imprinting acquisition, bilateral IMHA regions were

lesioned before imprinting training on day 1 (Fig. 1A, B, D). The preference for the imprinting object was evaluated by the preference score calculated from approach time to the imprinting and control objects. The preference scores of IMHA-lesioned chicks were greatly reduced and were significantly lower than those of the sham control chicks ($p < 0.05$, Fig. 1C). In the case of bilateral AMHA lesions, the preference scores were slightly reduced, but were not significantly different from those of control chicks (Fig. 1C). The IMM receives visual information from the periventricular region of the hyperpallium densocellulare (HDPe) (Nakamori et al., 2010) which is located near the IMHA. We confirmed that the HDPe was not damaged in brain sections of the IMHA-lesioned chicks. Thus, the effect in the lesion experiment is not likely attributable to the blockade of visual information from the HDPe to the IMM. The effect of the surgery on locomotor activity was evaluated by the number of times the chick hit the sensor in the two training sessions. The locomotor activity of the IMHA- or AMHA-lesioned chicks was not different from that of control chicks (IMHA-lesioned chicks: 734.8 ± 131.0 ; AMHA-lesioned chicks: 799.0 ± 155.1 ; control chicks: 846.4 ± 110.0), indicating that the lesioned chicks could approach the imprinting object during training in the same way as the control chicks. Therefore, the walking ability of the lesioned chicks was still intact. This result suggests that bilateral IMHA lesions and bilateral AMHA lesions did not affect locomotor activity of the chicks. These results suggest that the IMHA is critical for imprinting acquisition.

Effects of IMHA lesion after imprinting training: IMHA is required for imprinting recall

In order to examine whether the IMHA region is required for recall of the imprinting object, bilateral IMHA regions were lesioned after imprinting (Fig. 2A, C). The chicks were trained on day 1. On day 2, the chicks were tested (first test), and then bilateral IMHA regions were lesioned. After recovery from the surgery, the chicks were tested again (second test) on day 3. In IMHA-lesioned chicks, the preference scores of the second test were significantly less than those of the first test ($p < 0.05$, Fig. 2B). On the other hand, in both control chicks and IMM-lesioned chicks, the preference scores on the second test were not different from those of the first test (Fig. 2B). These results indicate that the IMHA region is required for recall of imprinting, in addition to acquisition.

Effects of IMHA lesion on recovery of the sensitive period on post-hatch day 4 after the end of the sensitive period: IMHA is required for mediating T_3 action from the IMM

We then examined the involvement of the IMHA region in the recovery of the sensitive period induced by T_3 injection. Bilateral IMHA regions were lesioned on day 0, and T_3 was intra-venously injected 30 min before training on day 4 when the sensitive period ended (Fig. 3A, C). As we reported previously, intra-venous T_3 injection made the chicks imprintable on day 4 (Fig. 3B).

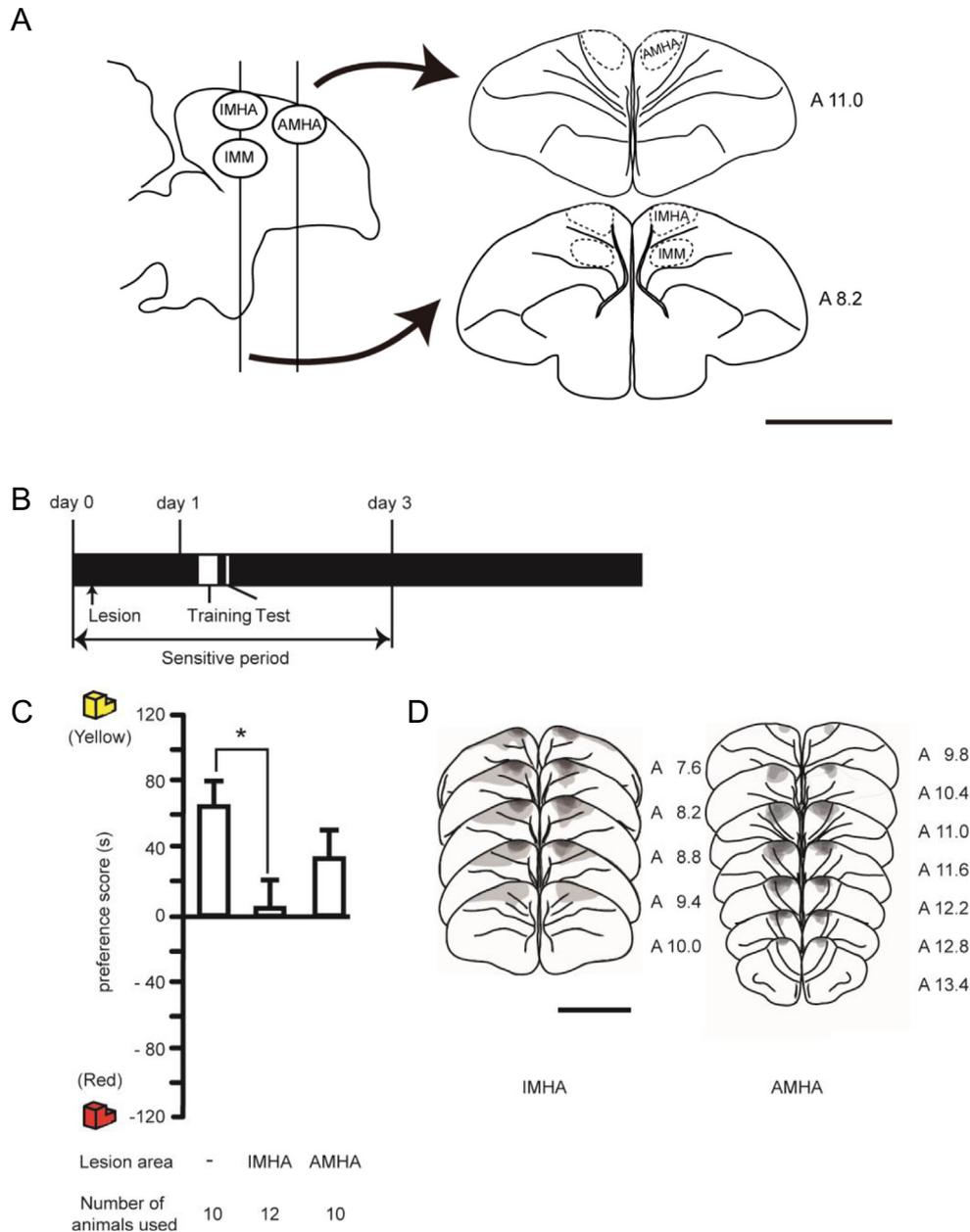


Fig. 1. Effect of bilateral ablation of the IMHA or AMHA region on imprinting acquisition. (A) Brain regions involved in imprinting. Sagittal section showing the IMHA, IMM, and AMHA regions (left). The frontal section 11.0 mm anterior to the bregma including the AMHA (upper right). The frontal section 8.2 mm anterior to the bregma including the IMM and IMHA regions (lower right). (B) A schematic representation of the lesion experiment schedule. Ibotenic acid was injected into the localized regions on day 0, and the chicks were trained and tested on day 1. (C) The preference score was given as (time near the imprinting object, yellow LEGO block) – (time near the control object, red LEGO block) in sec. The preference scores of IMHA-lesioned chicks were greatly reduced and were significantly lower than those of the sham control chicks. The preference scores of AMHA-lesioned chicks were slightly reduced, but were not significantly different from those of the sham control chicks. Mean \pm standard error (one-way ANOVA followed by Dunnett's test, $p < 0.05$). (D) The ablated areas in the IMHA ($n = 12$) or AMHA ($n = 10$) are shown. The ablated areas are superimposed over control sections of the telencephalon. The bars indicate 5.0 mm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

On the other hand, the preference scores of the IMHA-lesioned chicks were markedly lower than those of the T_3 -injected chicks ($p < 0.001$, Fig. 3B). This result indicates that the IMHA is required for the mode of action of T_3 to recover the sensitive period.

Further, we examined whether T_3 injection into the IMHA is sufficient to cause imprinting. T_3 was injected into the IMHA or IMM 30 min before training on day 4 (Fig. 4A). Previously, we showed that T_3 injection into

the IMM region was sufficient to cause imprinting (Fig. 4B) (Yamaguchi et al., 2012). In contrast to T_3 injection into the IMM, T_3 injection into the IMHA did not cause imprinting (Fig. 4B), thereby indicating that the IMHA region is required, but is not sufficient, for imprinting. This suggests that the IMHA receives T_3 signaling and works toward imprinting downstream of the IMM. Therefore, we examined whether T_3 injection into the IMM caused imprinting via the IMHA. Bilateral IMHA regions were

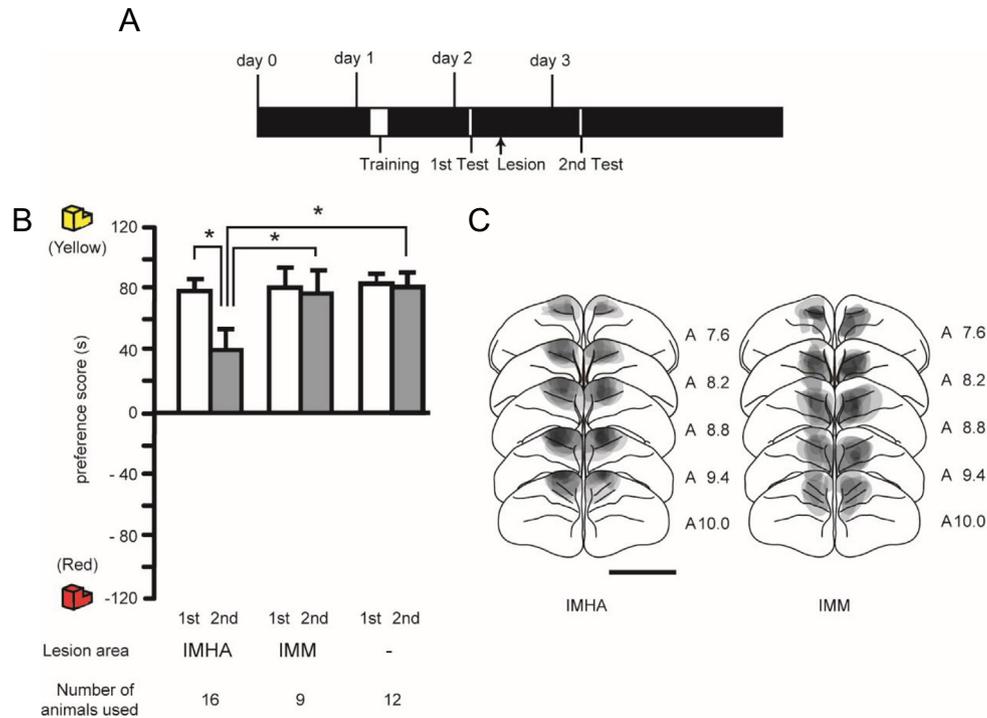


Fig. 2. Effect of bilateral ablation of the IMHA or IMM region on imprinting recall. (A) A schematic representation of the lesion experiment schedule. The chicks were trained on day 1. On day 2, the chicks were tested (first test), and then ibotenic acid was injected into the IMHA or IMM region. After recovery from the surgery, the chicks were tested again (second test) on day 3. (B) In IMHA-lesioned chicks, the preference scores of the second test were lower than those of the first test. In both control and IMM-lesioned chicks, the preference scores of the second test were not reduced. Mean \pm standard error (two-way repeated measures ANOVA followed by Tukey's HSD test, $^*p < 0.05$). (C) The ablated areas in the IMHA ($n = 16$) or IMM ($n = 9$) are shown. The ablated areas are superimposed over control sections of telencephalon. The bars indicate 5.0 mm.

lesioned on day 0, and T_3 was injected into the IMM 30 min before training on day 4 (Fig. 4A, C). The preference scores of the IMHA-lesioned chicks were significantly lower than those of the chicks without the IMHA lesion ($p < 0.01$, Fig. 4B). This result indicates that the IMHA is necessary for the mode of action of T_3 when injected into the IMM, and the IMHA likely has some neuronal connections from the IMM.

Neural connection from the IMM to IMHA: tract-tracing using A3V-Venus vector and A3V-WGA-T2A-EGFP vector

In order to detect the neural connection from the IMM to IMHA, the A3V containing the A3V-RSV-Venus construct was injected into the IMM region on day 0 and the chicks were perfused 4 days after the injection. By using a two-photon confocal microscope, an axon of the transduced neuron in the IMM that projects into the IMHA was observed (Fig. 5A). This suggests that the IMHA receives direct neural connections from the IMM. To confirm the presence of direct connections from the IMM to IMHA, retrograde tracing with CTB conjugated to Alexa Fluor 488 was performed. CTB was injected into the IMHA region, and the chicks were perfused 1 week after the injection. Several CTB-positive cells were observed in the IMM (Fig. 5B). Cell bodies and dendrites of the CTB-positive cells in the IMM were labeled (Fig. 5C).

As the direct connection from the IMM to IMHA seemed rare, the anterograde trans-synaptic tracer,

WGA, transduced by a bicistronic A3V vector was also used to detect the trans-synaptic neural connection. The A3V was injected into the IMM region on day 0, and the chicks were perfused 1–3 weeks after the injection. Fusion proteins consisting of EGFP and WGA are expressed in the transduced neurons. Then, the fusion protein is cleaved in the neurons, and EGFP remains within the cell. The resulting WGA is secreted from the presynapse and transferred to projected postsynaptic cells. As expected, in the immunoblotting, GFP was mostly detected in the IMM (Fig. 5D). The WGA was mainly detected in the IMM, and a small amount of WGA was also significantly detected in the IMHA. Pre-cleaved and partially cleaved GFP-WGA fusion proteins were also detected in the IMM (Fig. 5D). These results indicate that WGA was cleaved from the EGFP-WGA fusion protein in the transduced neurons and transferred to the IMHA, suggesting that the IMHA has neural connections from the IMM.

To confirm this immunohistochemically, the serial sections were stained with the anti-WGA and anti-NeuN antibody. Consistent with the immunoblotting, WGA signals were detected in the cell body of the IMHA neurons, which are NeuN-positive cells (Fig. 5E). This indicates that projected cells from the IMM in the IMHA are neuronal cells. From the stained serial sections, four sections (A7.6, A8.2, A8.8 and A9.4) that contain IMM, IMHA, and arcopallium were selected. The number of EGFP- or WGA-positive cells in the IMM, IMHA and arcopallium of each section were counted.

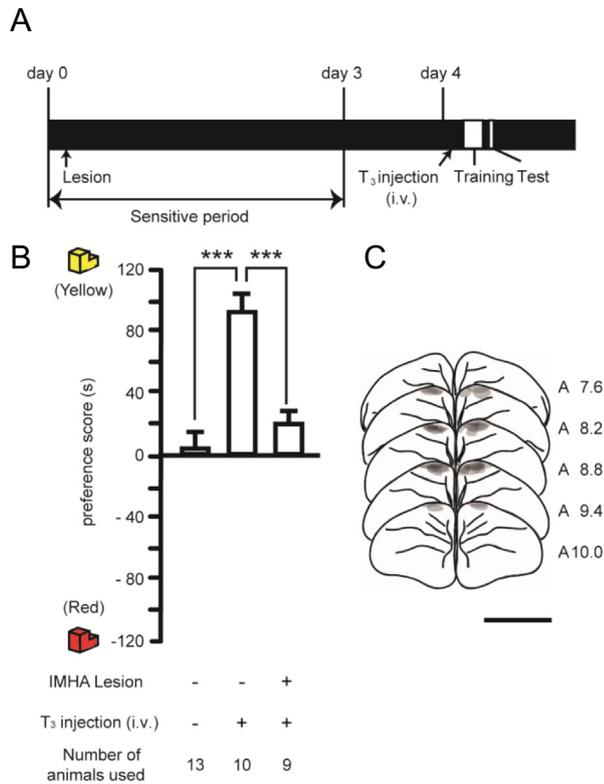


Fig. 3. Effect of bilateral ablation of the IMHA region on the ability of intra-venous injection of T₃ to recover the sensitive period. (A) A schematic representation of the lesion experiment schedule. Ibotenic acid was injected into the IMHA region on day 0, and T₃ was intravenously injected 30 min before training on day 4. (B) Injection of T₃ on day 4 after the end of the sensitive period caused the chick to be imprintable. The preference scores of the IMHA-lesioned chicks were significantly lower than those of the T₃-injected chicks. Mean ± standard error (one-way ANOVA followed by Tukey's HSD test, ****p* < 0.001). (C) The ablated areas in the IMHA region are shown (*n* = 9). The ablated areas are superimposed over control sections of telencephalon. The bar indicates 5.0 mm.

The number of WGA-positive cells in the IMHA is half as much as that in the arcopallium (Table 1). When we injected obliquely with an injection track that did not pass through the IMHA, to help avoid false positives due to leakages, we still found WGA-positive cells in the IMHA region. Taken together, the neural connections from the IMM to the IMHA are thought to be direct and trans-synaptic. The data also suggest that the IMHA has neural connections from the IMM, and that the IMHA receives signals necessary for imprinting from the IMM through these neuronal connections.

DISCUSSION

In this study, we investigated the neural circuits downstream from the IMM for imprinting. We demonstrated that the IMHA region is critical not only for acquisition, but also for recall. We also showed the neural connections from the IMM to the IMHA. From the results of this study, we reason that the IMHA is involved in imprinting acquisition and recall including the extraction of afferent information and execution of imprinting behavior.

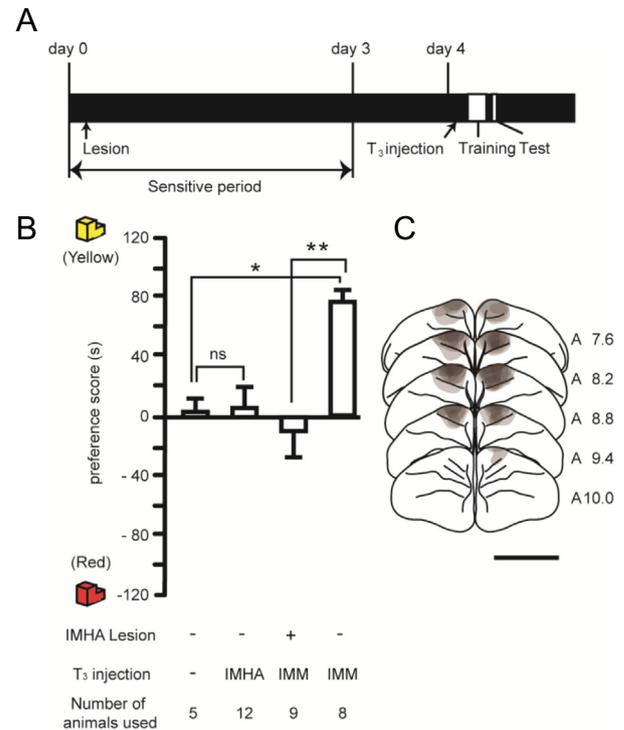


Fig. 4. Effect of bilateral ablation of the IMHA region on the ability of T₃ injection into the IMM region to recover the sensitive period. (A) A schematic representation of the lesion experiment schedule. Ibotenic acid was injected into the IMHA region on day 0, and T₃ was injected into the IMHA or IMM region 30 min before training on day 4. (B) The preference scores of the IMHA-lesioned chicks were significantly lower than those of the chicks injected T₃ into the IMM region, but not into the IMHA region. Mean ± standard error (one-way ANOVA followed by Tukey's HSD test, **p* < 0.05, ***p* < 0.01). (C) The ablated areas in the IMHA region are shown (*n* = 9). The ablated areas are superimposed over control sections of telencephalon. The bar indicates 5.0 mm.

The visual information enters the nucleus dorsolateralis anterior thalami (DLA) of the thalamus, proceeds to interstitial nucleus of the hyperpallium apicale (IHA), a dorsal part of the visual Wulst (Karten et al., 1973; Watanabe et al., 1983) (Fig. 6), which is analogous to the mammalian primary visual cortex. The visual information in IHA then goes into the IMM via the core region of the hyperpallium densocellulare (HDCo) and HDPe (Nakamori et al., 2010). The IMM is the associative area, which retains the sensory information required for imprinting (Bradley et al., 1985). The IMM is indispensable for the memory acquisition and the early phase of the memory retention (McCabe et al., 1981, 1982; Cipolla-Neto et al., 1982). We showed that the IMHA is likely to be required for imprinting downstream from the IMM. This suggests that the hierarchical processing from the IMM to the IMHA is indispensable for imprinting. The IMHA has been classified as the visual Wulst, same as the IHA. However, the IMHA does not receive the neural connections from the DLA of the thalamus (Karten et al., 1973; Watanabe et al., 1983). This suggests that the effects of IMHA lesions in this study were caused by ablation of downstream from the IMM rather than ablation of direct inputs from the thalamus.

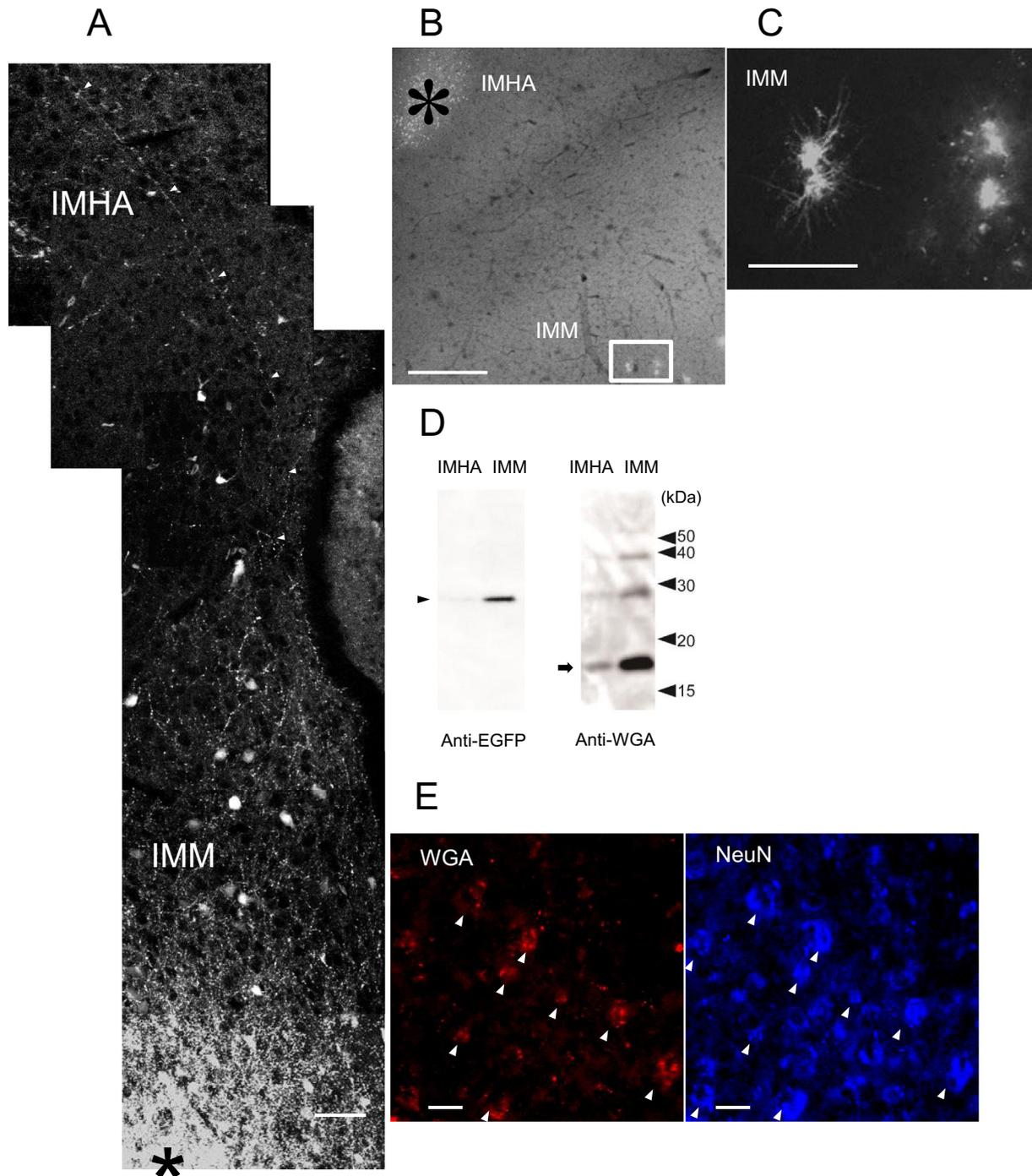
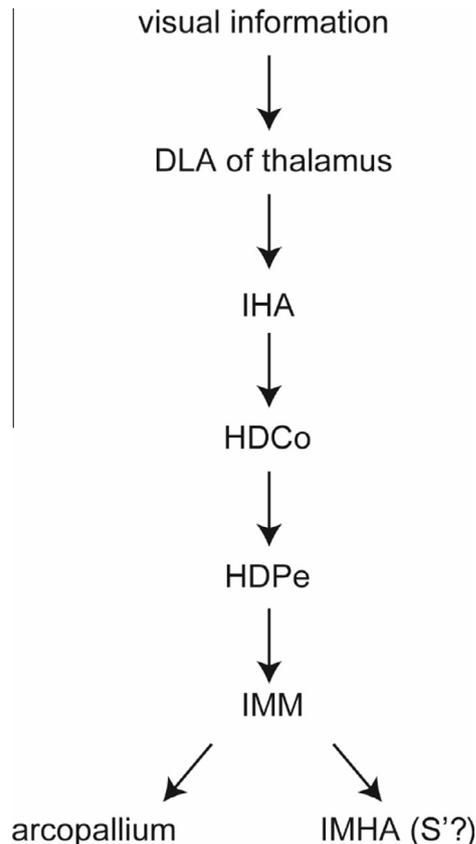


Fig. 5. Neural connection from the IMM to the IMHA region. (A) Viral tracing experiment. The avian adeno-associated virus (A3V) which contains the A3V-RSV-Venus construct was used. The A3V was injected into the IMM region, and the chicks were perfused 4 days after the injection. Fluorescent images (Venus) were obtained by using a two-photon confocal microscope. Asterisk indicates an injection site in the IMM. Arrowheads indicate an axon labeled as a result of injection in the IMM. The bar indicates 50 μm . (B) Retrograde tracing experiment. CTB conjugated to Alexa Fluor 488 was injected into the IMHA region, and the chicks were perfused 1 week after the injection. Fluorescent images were obtained by using a two-photon confocal microscope. Asterisk indicates the injection site in the IMM. A rectangle encloses CTB-positive cells in the IMM and an image of high magnification is shown in (C). The bar indicates 500 μm . For (C), the bar indicates 100 μm . (D) Immunoblotting of EGFP and WGA. The A3V which contains the A3V-RSV-EGFP-T2A-WGA construct was used. The A3V was injected into the IMM region, and the chicks were perfused 3 weeks after the injection. Results from three independent experiments are shown. The predicted molecular sizes of EGFP (arrowhead) and WGA (arrow) are 29 kDa and 16 kDa, respectively. EGFP was detected in the IMM, whereas WGA was detected in the IMHA and IMM. Pre-cleaved and partially cleaved fusion proteins were also detected in the IMM. (E) The WGA was visualized using immunocytochemistry. The WGA signals were detected in the NeuN-positive neuronal cells of the IMHA. Arrowheads indicate WGA-positive cells. The bars indicate 20 μm .

Table 1. The number of the EGFP- or WGA-positive cells in the IMM, IMHA, and arcopallium ($n = 3$).

EGFP	WGA		
	IMM	IMHA	arcopallium
370 ± 140	1630 ± 540	1230 ± 270	2710 ± 850

**Fig. 6.** Visual pathway required for imprinting in chicks. The visual information enters DLA of the thalamus, proceeds to the interstitial nucleus of the IHA, and then goes into the IMM via HDCo and HDPe. The IMHA receives the information required for imprinting from the IMM. The arcopallium also has neural connections from the IMM.

According to previous studies, neural projections of the IMM mainly go to the arcopallium-medial striatum pathway (Csillag et al., 1994), though the medial striatum is not involved in imprinting acquisition (Izawa et al., 2001). From our study, it appears that direct connections from the IMM to the IMHA are not dominant. However, a significant number of WGA-positive cells were detected in the IMHA, suggesting considerable trans-synaptic connections between the IMM and IMHA.

It has been suggested that the memory of the imprinting object is formed in the IMM during and/ or after imprinting training (McCabe et al., 1982; Horn et al., 2001), and that the memory is then established in another area called S' (Cipolla-Neto et al., 1982). The S' is assumed to be somewhere outside the IMM, but its location has not been identified. As previously reported (Cipolla-Neto et al., 1982), the bilateral ablation of the IMM 24 h after imprinting training does not impair recall. In contrast, our present study showed that the bilateral

ablation of the IMHA impaired recall. These suggest that the IMM is necessary in the early phase of acquisition until the memory trace becomes established in the IMHA, while the IMHA is necessary for recall or retention in addition to acquisition. It is possible that the IMHA is the S' or the downstream region of the S'. The IMHA may process the memory trace received from the IMM and then store it as consolidated memory.

The arcopallium which receives massive input from the IMM may also play an important role in imprinting. Lesions of the arcopallium prevent approach behavior to the imprinting object during training (Lowndes et al., 1994), suggesting that the arcopallium is involved in output from the IMM for imprinting, potentially influencing the subject's motivation for approach or locomotor activity. Premotor activities were recorded from neurons of the chick arcopallium (Aoki et al., 2006) and a robust nucleus of the arcopallium works as the premotor nucleus in song birds (Fee et al., 2004). The function of the arcopallium in imprinting is still obscure. We will investigate the functional relationship between the IMHA and the arcopallium in imprinting in the future.

Hierarchical processing from the IMM to the IMHA might be analogous to mammalian intracortical networks. In the mammalian brain, visual information goes from the occipital cortex to the parietal cortex via the dorsal stream and to the temporal cortex via the ventral stream (Goodale and Milner, 1992). The two streams share roles for visual perception. While the dorsal stream is required for navigation and motion perception, the ventral stream is required for recognition and identification of objects. In the chick's brain, a part of the visual Wulst (IHA) which is analogous to the visual cortex in mammals sends visual information to the IMM via the HDCo and the HDPe (Nakamori et al., 2010). The IMM and IMHA then process the visual information and consolidate the imprinting memory. Similar to mammalian intracortical networks, the IMM and the IMHA may have shared roles in imprinting, such as memory acquisition, extraction and storage.

CONCLUSION

We demonstrated that the IMHA is critical not only for imprinting acquisition, but also for recall downstream of the IMM. This suggests that the IMHA is a memory storage region which has been called the S'. We also showed the neural connections from the IMM to the IMHA. Since the IMHA is a downstream region from T₃ action in the IMM, it may also operate as the storage region for "memory priming" to extend the sensitive period for over one week. Whether memory priming is relocated from the IMM to the IMHA will be investigated in future.

CONFLICT OF INTEREST

There are no conflicts of interest.

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