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Short communication

Blockade of muscarinic acetylcholine receptor by scopolamine impairs the memory formation of filial imprinting in domestic chicks (*Gallus Gallus domesticus*)

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ABSTRACT

Filial imprinting in precocial birds is a useful model for studying memory formation in early learning. The intermediate medial mesopallium (IMM) in the dorsal telencephalon is one of the critical brain regions where the releases of several neurotransmitters increase after the start of imprinting training. Among the increased neurotransmitters, the role of acetylcholine in imprinting has remained unclear. Acetylcholine in the mammalian brain plays an important role in encoding new memories. The muscarinic acetylcholine receptor subtype 1 (M_1 receptor) and subtype 3 (M_3 receptor) in the hippocampus and cortex of mammalian brain have been shown to be necessary for memory encoding. In this study, we examined whether the imprinting acquisition in chick can be impaired by injecting muscarinic acetylcholine receptor (mAChR) antagonist scopolamine into the bilateral IMM. We show that the injection of scopolamine decreased the preference for the imprinting object in the test, but did not affect the number of approaches to the imprinting object during training. Immunoblotting and immunohistochemistry revealed that M_3 receptors were expressed in the IMM. Our data suggest that acetylcholine is involved in the memory formation of imprinting through M_3 receptors in the IMM. The scopolamine-injected chicks may be useful as an animal model for dementia such as Alzheimer's disease.

1. Introduction

Imprinting occurs when a newly-hatched chick follows a conspicuous moving object, typically a parent, and memorizes it. Neural and molecular mechanisms involved in imprinting have been investigated as a model for memory formation and early learning [1]. The intermediate medial mesopallium (IMM) region in the chick brain that receives multi-modal sensory inputs is the indispensable brain area for imprinting [1]. Bilateral ablation of the IMM before or 3 h after imprinting training causes amnesia, but the ablation of the IMM 24 h after training does not [2,3]. Therefore, it is considered that the IMM is necessary for the early phase of memory formation and that the imprinted memory is subsequently transferred to other brain regions such as intermediate hyperpallium apicale (IMHA) [4]. During the early phase of memory formation, several neurotransmitters increase in the IMM, e.g., glutamate, taurine, GABA, and acetylcholine [5,6]. With regard to GABA, we recently found that the balance of two types of GABA receptor (GABA-A and GABA-B receptors) expression in the chick brain developmentally changed, which determines whether the chicks are able to be imprinted or not [7]. On the other hand, the increased glutamate during imprinting training is suggested to be involved in the early phase of memory formation through αCaMKII and AMPA receptors of the IMM neurons [8]. Until now, the role of acetylcholine in the IMM for memory formation in imprinting has not been revealed.

Acetylcholine has been shown to be one of the key neurotransmitters for the formation of new memories in mammals [9]. There

Abbreviations: αCaMKII, alpha-Ca^{2+}/calmodulin-dependent protein kinase-II; AMPA receptor, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; AD, Alzheimer's disease; GABA, gamma-aminobutyric acid; IMM, intermediate medial mesopallium; IMHA, intermediate hyperpallium apicale; LSt, lateral striatum; mAChR, muscarinic acetylcholine receptor; M_1 receptor, muscarinic acetylcholine receptor subtype 1; M_2 receptor, muscarinic acetylcholine receptor subtype 2; M_3 receptor, muscarinic acetylcholine receptor subtype 3; M_4 receptor, muscarinic acetylcholine receptor subtype 4; M_5 receptor, muscarinic acetylcholine receptor subtype 5; nAChR, nicotinic acetylcholine receptor; PBS, phosphate buffered saline

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are two major types of acetylcholine receptors, metabotropic muscarinic acetylcholine receptor (mAChR) and ionotropic nicotinic acetylcholine receptor (nAChR). In the mammalian brain, mAChR subtype 1 (M_1 receptor) is the most abundant subtype in five subtypes of mAChRs and are necessary for memory formation [10]. However, regarding the evolution of mAChRs, Pedersen et al. noted that the M_1 receptors are not identified in the genome project of domestic chicks and other birds, although the birds possess genes of 4 (M_2-M_5 receptors) of 5 types of mAChR and each gene is highly conserved [11]. Since M_3 receptors in the mammalian hippocampus are also involved in memory formation [12] and M_1 and M_3 receptors share physiological characteristics [13], the M_3 receptors may substitute for the role of M_1 receptors in the avian brains.

An mAChR antagonist, scopolamine, has been used to investigate the effects of a blockade of the mAChR on memory formation. In human subjects, scopolamine impairs the encoding of new memories and working memory [14]. In newly hatched domestic chicks, scopolamine disrupted one-trial passive avoidance learning [15]. In this study, we focused on the function of the mAChRs in the IMM for imprinting, as the mAChRs are expressed abundantly in the IMM [16]. Here, we revealed that the blockade of mAchRs in the IMM neurons by the antagonist scopolamine impaired the memory formation of imprinting and that the IMM neurons expressed the M_3 receptors. These results suggest that acetylcholine plays an important role in memory formation through the M_3 receptors in the IMM.

2. Materials and methods

2.1. Animals

The experiments were conducted under the guidelines of the national regulations for animal welfare in Japan and with the approval of the committee on animal experiments of Teikyo University (approval number: 18-015). In this study, 108 newly-hatched domestic chicks of the Cobb strain (*Gallus gallus domesticus*) were used. Fertilized eggs were obtained from local supplier (3-M, Aichi, 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 light exposure [17].

2.2. In vivo scopolamine injection

The injection was performed as described previously [7] with modifications. Chicks were anesthetized with a 1% isoflurane/air mixture and mounted on a stereotaxic apparatus. The skin and skull above the IMM was incised and the dura mater was cut to expose the telencephalon. Stereotaxic coordinates for the IMM were as follows: 3.0 mm anterior from the bregma, 1.3 mm lateral from midline, and 2.3 mm deep [18]. Scopolamine hydrobromide trihydrate (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) was slowly injected over 25 min (26.8 nL/min) using a nanoliter injector (Nanoject I; Drummond Scientific Co., Broomall, PA, USA). We prepared three doses (2 mM, 20 mM and 200 mM) to examine a dose response. The dosages were determined with reference to Barber et al. in which the injection of 50 mM scopolamine impaired memory formation in the passive avoidance task, but not locomotor activities [19]. Control chicks were subjected to a sham operation in which only the syringe was inserted into the IMM on the stereotaxic apparatus under anesthesia. The chicks were returned to the dark chamber at 30 °C for 30 min to allow them to recover from the anesthesia. To confirm that the neurons around the injection site were not damaged by the scopolamine, we conducted Nissl staining on the brain samples dissected from the injected chicks (Supplementary Fig. 1A), as described in Aoki et al. (2015) [4]. To estimate the drug spread, ibotenic acid was injected at the same volume as the scopolamine into the IMM, as described in Aoki et al. (2015) [4] (Supplementary Fig. 1B).

2.3. Imprinting training and testing

Training for imprinting was performed according to the method of Izawa et al. [17] with the following modifications. A handmade I-shaped training chamber (8 cm wide, 43 cm long, and 15 cm high) was equipped with a rubber belt controlled by a microcomputer (Tri State Co. Ltd, Hokkaido, Japan). Thirty minutes after the injection or sham operation, two 1 h training sessions were conducted as follows. An imprinting object (a blue LEGO block or brown LEGO block) was placed in one side of the training chamber and was rotated clockwise repeatedly for 30 s with pauses of 10 s in between. During the rotation, a fiber optic light was used to illuminate the imprinting object. If the chicks crossed the infrared sensor in front of the imprinting object, the belt moved to the opposite side of the imprinting object. We counted how many times the chicks crossed the sensor during training. If the count was < 100 for the sum of two training sessions, the chicks were not tested. The ratio of chicks that did not reach the criteria were not different among four groups (sham: 6.5%; 2 mM: 7.1%; 20 mM: 0.0%; 200 mM: 5.6%).

In the simultaneous choice test, we used a T-maze with a 20 cm main arm and a 69 cm side arm. The imprinting object and a novel control object (a blue vs. brown block or a brown vs. blue block) were positioned at the end of each side arm of the T-maze. Each object was rotated and illuminated during the test. After a chick started from the main arm, we counted the stay time of the approach area of each object for 120 s. The test was conducted four times and averaged each stay time. We then calculated a preference score by subtracting the stay time for the control object from that of the imprinting object. After the behavioural experiments, the animals were sacrificed with an overdose of isoflurane.

2.4. Statistical analyses for behavioural data

For statistical analyses, we used R software for Windows. Data are presented as box plots or scatter plots. The number of animals used is indicated in figure. We used a one-way ANOVA without repeated measures or Student's *t*-test. When significant difference was detected by ANOVA, we conducted post-poc multiple comparisons by using Tukey's multiple comparison of means. The *p-values* < 0.05 were considered significantly different. We also determined the Cohen's *d* values as the effect size [20]. For testing of the significance of correlations between preference score and number of approach, Pearson's product-moment correlation was applied. The *p*-values and effect sizes are shown in the Table 1.

Table 1
p values and effect sizes.

		p values	effect sizes (*d*)
Fig. 1A	Sham vs 2 mM scopolamine	0.949	0.201
	Sham vs 20 mM scopolamine	0.042	0.914
	Sham vs 200 mM scopolamine	0.020	0.843
	2 mM vs 20 mM scopolamine	0.286	0.732
	2 mM vs 200 mM scopolamine	0.202	0.683
	20 mM vs 200 mM scopolamine	0.999	0.048
Fig. 1B	Sham vs 2 mM scopolamine		−0.127
	Sham vs 20 mM scopolamine		0.027
	Sham vs 200 mM scopolamine		0.447
	2 mM vs 20 mM scopolamine		0.148
	2 mM vs 200 mM scopolamine		0.503
	20 mM vs 200 mM scopolamine		0.411
		p values	effect sizes (*r*)
Fig. 1C	Sham	0.468	0.140
	2 mM scopolamine	0.903	0.038
	20 mM scopolamine	0.215	0.340
	200 mM scopolamine	0.025	0.540

2.5. Immunoblotting

The immunoblot analysis was performed as previously described [4] with modifications. Briefly, one-day-old chicks' brains were cut into 500 μm frontal sections with a microslicer. Microcapillaries (Harvard Apparatus, Holliston, USA) were used to punch out brain tissue from each brain region. The brain regions are shown in Fig. 2A, i.e., lateral striatum (LSt), IMM and IMHA [18]. The punched-out brain tissues were subjected to immunoblotting. For detection of the M_3 receptors, anti- M_3 receptors rabbit polyclonal antibody (ab150480, 1:1000; Abcam plc, Cambridge, United Kingdom; the antigen peptide was made from the C-terminus which is 86% identical with that of chick M_3 receptor in the amino acid sequence according to the manufacturer sheet.) was used as the primary antibody, while an anti-rabbit horseradish peroxidase-conjugated antibody (1:1000, GE Healthcare, Chicago, USA) was used as the secondary antibody. The expression levels of M_3 receptors were normalized by the expression of actin as detected by an anti-actin rabbit polyclonal antibody (AAN01-A, 1:1,000, Cytoskeleton, Inc, Denver, USA). The band intensities were quantified using ImageJ (National Institutes of Health, Bethesda, MD, United States).

2.6. Immunohistochemistry

The immunohistochemistry was performed as described previously [7] with modifications. Chicks on day 4 were transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS) under deep anesthesia using a ketamine-xylazine cocktail. The brains were post-fixed with the same fixative for 24 h and immersed in 20% sucrose in PBS and cut into 10 μm-thick sections using a cryostat. For fluorescent staining, the sections including the IMM [18] were blocked with 3% normal pig serum for 1 h. For detection of the M_3 receptors, the sections were incubated with anti- M_3 receptors rabbit polyclonal antibody (ab150480, 1:250; Abcam plc), then incubated with Alexa Fluor 546-conjugated anti-rabbit antibody (1:300; Thermo Fisher Scientific K.K.). Nuclei of cells were stained by Hoechst 33342 (1: 1000; Thermo Fisher Scientific K.K.). Fluorescent images were obtained using a confocal microscope (FV-10i; Olympus, Tokyo, Japan).

3. Results

3.1. Effects of mAChR antagonist on imprinting

To examine the effects of scopolamine on the memory formation during imprinting, on day 1 chicks were injected with scopolamine (2 mM, 20 mM, or 200 mM) 30 min before the imprinting training. The preference scores of the chicks injected with 20 mM or 200 mM scopolamine were significantly less than those of control chicks (Fig. 1A). And the scores were not significantly different between chicks injected with 20 mM and chicks injected with 200 mM scopolamine. This suggests that these doses of scopolamine equally impaired the memory formation of imprinting. The scores of the chicks injected with 2 mM scopolamine were not significantly different from those of other three groups. While the numbers of approaches during imprinting training was also not significantly different among the four groups (Fig. 1B). This indicates that any doses of scopolamine did not have an effect on locomotor activity. On the other hand, the numbers of approaches during imprinting training in the 200 mM scopolamine injected chicks tended to be lower than the control group (effect size: $d = 0.447$). To investigate the tendency of fewer approaches during the training in the 200 mM scopolamine-injected chicks, we examined whether the correlation between the preference scores and the numbers of approaches are significant. In the sham control chicks and 2 mM scopolamine-injected chicks, the correlation between the scores and the numbers of approaches were not significant (Fig1C). Also in 20 mM scopolamine-injected chick whose score were less than those of control, the correlation was not significant (Fig1C). On the other hand, in the 200 mM scopolamine-injected chicks, the scores were significantly correlated with the numbers of approaches (Fig1C). This suggests that the high doses of scopolamine had effects on locomotor activities in some cases. The weakened locomotor activities may partially contribute to impairment of memory formation. The effect of 20 mM scopolamine was also observed when the brown block was used as the imprinting object (Supplementary Fig. 2). This suggests that the injection of scopolamine does not impair the color perception, but the memory formation of imprinting.

3.2. Expression of M_3 receptors in the IMM region

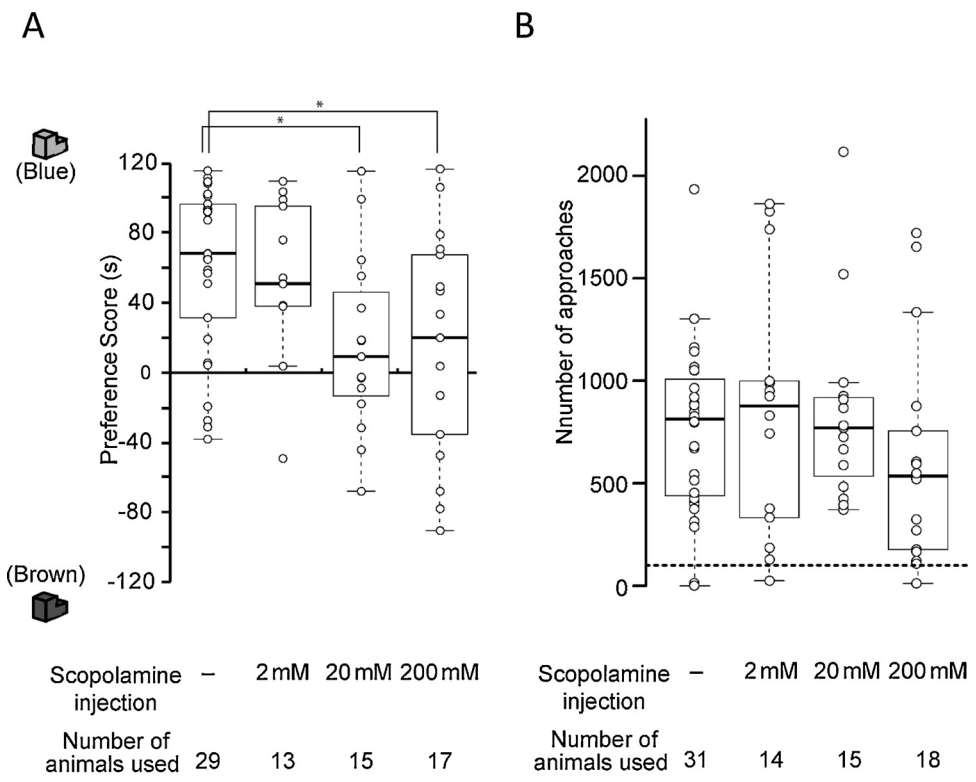
To examine whether the M_3 receptors are expressed in the IMM, IMHA and LSt, we conducted immunoblotting with brain tissues that were punched out from those brain regions. The IMM and IMHA play important roles to acquire imprinting memory [1,4]. The LSt was used as a control region because, as far as we know, the role of the LSt in imprinting has not been reported. Unexpectedly, the M_3 receptors were found to be expressed in all three brain regions (Fig. 2B). The expression levels of M_3 receptors in the IMM were equivalent to those of the IMHA and LSt (Fig. 2C). To confirm that the M_3 receptors were expressed in the IMM neurons, we conducted immunostaining with brain slices containing the IMM region (Fig. 2D). The nuclei of the cells were stained by Hoechst 33342. M_3 receptors were expressed in both the IMM cells and IMHA cells. These findings suggest that the effect of scopolamine injection was attributed to the blockade of M_3 receptors in the IMM.

4. Discussion

In this study, we showed that the blockade of mAChR in the IMM neurons by an antagonist scopolamine impaired the memory formation of filial imprinting, and that the M_3 receptors were expressed in the IMM. Previous studies revealed that the release of acetylcholine increases in the IMM after the start of imprinting training [6] and that the mAChRs are abundant in the IMM [16]. These findings indicate that acetylcholine plays an important role in memory formation through the M_3 receptors.

In the mammalian brain, the M_1 receptors are the most abundant subtype in five subtypes of mAChRs and are necessary for memory formation [10]. And the M_1 receptors are involved in long term potentiation in the hippocampus [21] and cortical plasticity by increasing excitatory- and decreasing inhibitory- post synaptic potential in the cortex [22]. However, previous studies suggested that birds do not have the M_1 receptors [11,23,24], although in zebra finches, the mesopallium, which includes the IMM, expresses M_3, M_4 and M_5 receptors [24]. This suggests that the M_4 and M_5 in the IMM may also be involved in memory formation. To examine the possibilities, we will determine which subtype of mAChR plays a critical role by using subtype-specific blockers for further study.

The injection of 20 mM scopolamine impaired the memory formation of imprinting. And the numbers of approaches to the imprinting object during training were not significantly different among the four groups. The results suggest that the impairment of memory formation observed in the 20 mM scopolamine-injected chicks was not derived from a weakened locomotor activities. However, the number of approaches to the imprinting object during training in the 200 mM group tended to be lower than in the control group. The high doses of scopolamine may have affected the locomotor activities during the imprinting training. Subsequently, the lower approach count may have had an effect on the preference score in the 200 mM scopolamine-injected chicks. In a previous study, injection of another mAChR antagonist, atropine, into the IMM also impaired following behaviour especially within the first 30 min of training [25]. A similar effect was observed with a high dose of scopolamine, indicating that an excessive blockade of mAChRs by high dose scopolamine resulted in reduced



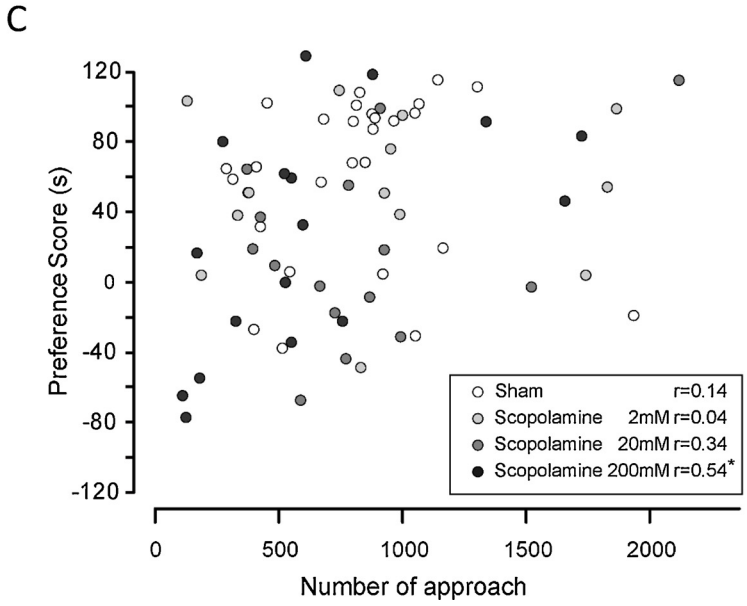


Fig. 1. Effects of mAChR antagonist on imprinting. (A) The scores of either the chicks injected with 20 mM or 200 mM scopolamine were significantly less than those of control chicks (one-way ANOVA, $F = 4.296$. $p = 0.008$; Tukey's multiple comparison of means: Sham vs 20 mM, $p = 0.042$; Sham vs 200 mM, $p = 0.020$). The scores were not significantly different between chicks injected with 20 mM and 200 mM scopolamine. ($p = 0.999$). The scores of the chicks injected with 2 mM scopolamine were not different from other three groups (Sham vs 2 mM, $p = 0.950$; 2mM vs 20 mM, $p = 0.286$; 2mM vs 200 mM, $p = 0.202$). The asterisks (*) indicate significance ($p < 0.05$). (B) The number of approaches to the training object during imprinting training was not significantly different among the four groups (one-way ANOVA, $F = 0.981$. $p = 0.407$). (C) In the 200mM scopolamine-injected chicks, the scores were significantly correlated with the number of approach ($r = 0.540$, $p = 0.025$). In other three groups of chicks, the scores were not significantly correlated with the number of approach during training (sham: $r = 0.140$, $p = 0.468$; 2mM: $r = 0.038$, $p = 0.903$; 20mM: $r = 0.340$, $p = 0.215$). The asterisk (*) indicates significant ($p < 0.05$).

locomotor activities during the imprinting training, but not during the test. From these results, we believe that 20 mM of scopolamine is an appropriate concentration for further analysis as an experimental model for diseases caused by the decrease of acetylcholine.

The effects of scopolamine on memory formation have been investigated using mammals such as mice as an experimental model of Alzheimer's disease (AD), because the low level of acetylcholine of AD patients was mimicked by scopolamine injection. The results of this study suggest that scopolamine injection into the IMM of chicks is potentially a useful animal model of AD patients' dementia that is caused by low acetylcholine. However, to use the scopolamine-injected chicks as AD models, we have to decrease the variability of the scoring of the scopolamine-injected chicks. In order to do that, we will optimize the injection location as well as the number of injections prior to training initiation. Besides the IMM region, the IMHA region also expresses M_3 receptors and has been shown to be necessary for both imprinting acquisition and recall [4]. We will therefore focus future work on the effect of scopolamine injection into the IMHA as another scopolamine-responsive brain region.

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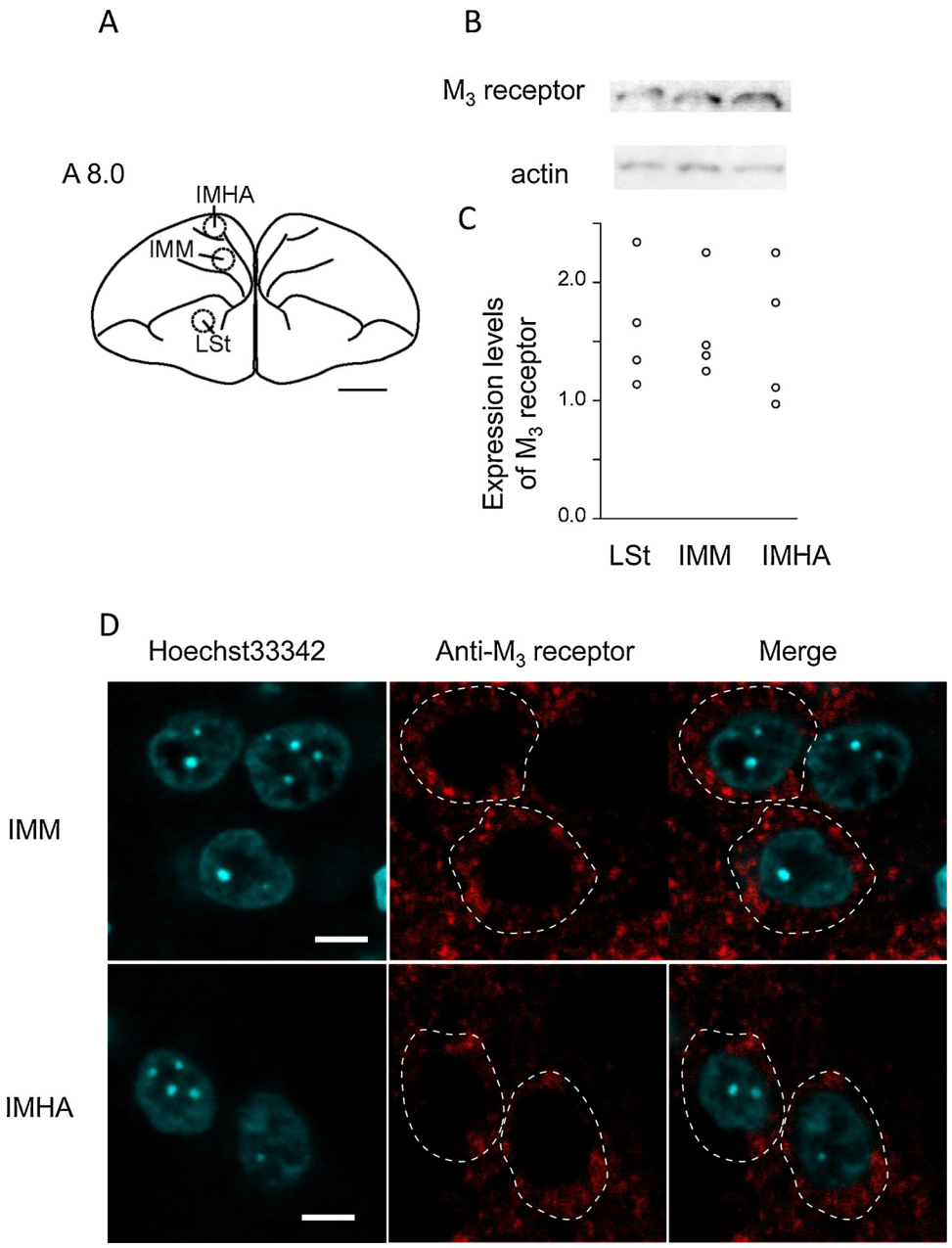

Fig. 2. Expression of M_3 receptors in the IMM region. (A) For immunoblotting analysis, brain tissues including each brain region, the LSt, IMM or IMHA, were punched out from a 500 μm frontal section at A 8.0. The black bar indicates 2.0 mm. (B) An example of immunoblotting results. The M_3 receptors were expressed in the LSt, IMM, and IMHA. (C) Relative expression levels of M_3 receptors in each brain region are plotted (n = 4). The expression levels of M_3 receptors were not different among the three brain regions. The expression level of M_3 receptors were normalized by the expression levels of actin. (D) Both the IMM and IMHA neurons expressed M_3 receptors. Nuclei of cells were stained by Hoechst 33342. Each white bar indicates 5 μm.

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