Regular Article*Highlighted Paper selected by Editor-in-Chief***Nicotine Enhances Object Recognition Memory via Stimulating $\alpha 4\beta 2$ and $\alpha 7$ Nicotinic Acetylcholine Receptors in the Medial Prefrontal Cortex of Mice**Hirohito Esaki,[#] Shoma Izumi,[#] Akari Fukao, Shiho Ito, Naoya Nishitani, Satoshi Deyama, and Katsuyuki Kaneda**Laboratory of Molecular Pharmacology, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University; Kanazawa, Ishikawa 920–1192, Japan.*

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Nicotine has been known to enhance recognition memory in various species. However, the brain region where nicotine acts and exerts its effect remains unclear. Since the medial prefrontal cortex (mPFC) is associated with memory, we examined the role of the mPFC in nicotine-induced enhancement of recognition memory using the novel object recognition test in male C57BL/6J mice. Systemic nicotine administration 10 min before training session significantly enhanced object recognition memory in test session that was performed 24 h after the training. Intra-mPFC infusion of mecamylamine, a non-selective nicotinic acetylcholine receptor (nAChR) antagonist, 5 min before nicotine administration blocked the effect of nicotine. Additionally, intra-mPFC infusion of dihydro- β -erythroidine, a selective $\alpha 4\beta 2$ nAChR antagonist, or methyllycaconitine, a selective $\alpha 7$ nAChR antagonist, significantly suppressed the nicotine-induced object recognition memory enhancement. Finally, intra-mPFC infusion of nicotine 1 min before the training session augmented object recognition memory in a dose-dependent manner. These findings suggest that mPFC $\alpha 4\beta 2$ and $\alpha 7$ nAChRs mediate the nicotine-induced object recognition memory enhancement.

Key words nicotinic acetylcholine receptor (nAChR); $\alpha 4\beta 2$ nAChR; $\alpha 7$ nAChR; medial prefrontal cortex; nicotine; novel object recognition test

INTRODUCTION

Nicotine affects attention, working memory, decision-making, as well as recognition.^{1–4)} Acute administration of nicotine has been known to enhance object recognition memory in the novel object recognition (NOR) test.⁵⁾ Nicotine also improves visual recognition memory in rhesus monkeys.⁶⁾ Additionally, nicotine improves recognition deficits in patients with Alzheimer's disease and schizophrenia.^{7,8)} $\alpha 4\beta 2$ and $\alpha 7$ -containing nicotinic acetylcholine receptors (nAChRs) are two major subtypes in the brain, and play a crucial role in these beneficial nicotine effects.^{9–11)} The heteromeric $\alpha 4\beta 2$ nAChRs exhibit a high affinity to nicotine and desensitize slowly, whereas the homomeric $\alpha 7$ nAChRs show a low affinity for nicotine and desensitize rapidly.^{12–15)} Both subtypes are expressed in pre- and postsynaptic membranes throughout the brain, although the precise localization patterns depend on brain areas and cell types.^{16–18)}

The medial prefrontal cortex (mPFC) is considered to be critically involved in attention, working memory, decision-making, and reward processing.^{19–23)} However, it is still controversial whether this brain region is also associated with recognition memory, although other brain areas, including the hippocampus, entorhinal cortex, and perirhinal cortex, which comprise neural circuits with the mPFC, are associated with this type of memory.^{24–30)} Lesions or pharmacological inhibition of the mPFC failed to affect the performance of rodents in the NOR test, suggesting that the mPFC is dispensable for the recognition memory.^{24,26,31)} On the other hand, several stud-

ies demonstrated that chemogenetic inactivation of excitatory neurons, blockade of dopamine D1 or *N*-methyl-D-aspartate (NMDA) receptors, or protein synthesis inhibition in the mPFC disrupt the object recognition memory consolidation.^{29,32,33)} Additionally, the expressions of neuronal activity markers, c-Fos and Zif-268, are increased in the mPFC after the NOR test.^{28,33,34)} These findings imply the requirement of the mPFC for object recognition memory. Therefore, we hypothesized that the mPFC is involved in nicotine-induced enhancement of object recognition memory. To test this hypothesis, in this study, we examined whether systemic nicotine administration-induced object recognition memory enhancement is suppressed by local infusion of a non-selective nAChR antagonist, or an antagonist selective for $\alpha 4\beta 2$ or $\alpha 7$ nAChR using the NOR test in mice. Then, we tested whether intra-mPFC infusion of nicotine could enhance object recognition memory.

MATERIALS AND METHODS

Animals Male C57BL/6J mice (7–12 weeks old, $n = 258$) were obtained from the animal facilities of Kanazawa University and group-housed in plastic cages with sawdust bedding at a constant ambient temperature ($22 \pm 2^\circ\text{C}$) under a 12–12 h light/dark cycle with free access to food and water. All experiments were carried out with the approval of the Institutional Animal Care and Use Committee at Kanazawa University.

Drugs (–)-Nicotine liquid was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.), and diluted with sterile saline and stored at -20°C . Mecamylamine hydrochloride (Mec; Sigma-Aldrich), dihydro- β -erythroidine hydrobromide (DH β E;

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Tocris, Bristol, U.K.) and methyllycaconitine citrate (MLA; Sigma-Aldrich) were dissolved in sterile saline and stored at -20°C . All stock solutions were diluted with saline just before use.

Surgery and Drug Treatments for Behavioral Analyses and Subsequent Histology Drug infusions into the mPFC were performed as described earlier with some modifications.^{35–38} Under anesthesia with chloral hydrate (400 mg/kg, intraperitoneal), mice were implanted bilaterally with 25-gauge stainless-steel guide cannulae (o.d., 0.51 mm; i.d., 0.26 mm) above the mPFC infusion sites (coordinates from bregma: AP 1.8 mm, ML ± 1.3 mm, DV -1.5 mm, approached at a 20° lateral angle).³⁹ Following the surgical procedure, mice were singly housed and left to recover for at least 7 d. Mice were infused bilaterally into the mPFC with vehicle (saline), nicotine (0.1 or 0.3 $\mu\text{g}/\text{side}$), Mec (0.25 $\mu\text{g}/\text{side}$), DH β E (2.5 $\mu\text{g}/\text{side}$) or MLA (10 $\mu\text{g}/\text{side}$) in a 0.2- μL volume at a rate of 0.2 $\mu\text{L}/\text{min}$, using 33-gauge stainless-steel infusion cannulae (o.d., 0.2 mm; i.d., 0.08 mm) that protruded 1.1 mm beyond the tip of the guide cannulae. The infusion cannulae were kept in place for another 1 min to allow for diffusion. Subcutaneous (s.c.) injection of saline or nicotine (0.1 mg/kg) was performed 10 min prior to the training session of the NOR test. Mec, DH β E and MLA were infused into the mPFC 5 min before s.c. injection of saline or nicotine. Intra-mPFC infusion of nicotine was performed 1 min before the NOR training session. The doses of these drugs used in the current study were determined based on previous reports.^{40–43}

Histological analyses were performed after the behavioral tests. Mice were sacrificed and decapitated, and the brains were rapidly removed, frozen in powdered dry ice, and stored at -80°C until use. Coronal sections (50 μm) were prepared on a cryostat, thaw-mounted on slides, and stained with thionin to confirm the location of the infusion sites within the mPFC. Mice with incorrect infusion placements were excluded from the analyses ($n = 107$).

Novel Object Recognition Test The NOR test was conducted according to our previous study with minor modifications.³⁶ Briefly, mice were firstly habituated to a testing chamber (L38 $\times$ W26 $\times$ H24 cm) under dim illumination (5 ± 1 lx) by allowing them to freely explore this chamber for 10 min on two consecutive days. During the training session, two identical objects (transparent plastic bottles) were placed symmetrically in two adjacent corners 7 cm from the walls, and each mouse was allowed to freely explore in the chamber for 10 min and videotaped. The exploration time for each object was measured in a blinded manner. Exploration was defined as sniffing or touching the objects with the nose and/or forepaws when the nose was in contact with or directed at the object at a distance of ≤ 1.5 cm. Twenty-four hours after the training session, the test session was performed, and one of the objects used in the training session (familiar objects) was exchanged by a novel one (a brown glass bottle). Each animal was placed back in the same testing chamber for 10 min, videotaped, and the exploration time for each object was measured in a blinded manner. Mice that climbed the objects ($n = 5$) and that explored the objects < 20 s ($n = 4$) during the training or test sessions were excluded from the analysis. The discrimination index (DI) was computed as follows: $\text{DI} = (\text{exploration time for the novel object} - \text{exploration time for the familiar object}) / (\text{total exploration time during the test session})$,

and used to evaluate memory retention.

Statistical Analyses Data are presented as mean $\pm$ standard error of the mean (S.E.M.), and were analyzed by one-way or two-way ANOVA followed by the Holm–Sidak's *post hoc* test, when comparing more than two groups, or the Student's *t*-test, when comparing two groups using the GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, U.S.A.). Differences with $p < 0.05$ were considered statistically significant.

RESULTS

Systemic Administration of Nicotine Enhances Object Recognition Memory Because a previous study reported that the systemic nicotine administration enhances object recognition memory,⁵ we first confirmed this effect of nicotine (0.1 mg/kg, s.c.) using the NOR test (Fig. 1A). Total exploration time during the test session was not significantly different between saline- and nicotine-injected groups (Fig. 1B). Mice that had received an s.c. injection of nicotine 10 min before the training session showed a significantly higher DI than saline-injected mice (Fig. 1C). These results indicate that systemic nicotine administration before the training session enhances object recognition memory.

nAChRs in the mPFC Mediate the Object Recognition Memory Enhancement by Systemic Nicotine Administration To investigate the role of nAChRs in the mPFC in nicotine-induced object recognition memory enhancement, mice were infused with vehicle (saline) or the non-selective nAChRs antagonist Mec (0.25 $\mu\text{g}/\text{side}$) into the mPFC 5 min before s.c. injection of saline or nicotine (Figs. 2A, D). Total exploration time during the test session was not significantly different among all groups (Fig. 2B). In intra-mPFC vehicle-infused mice, nicotine significantly increased the DI compared

Fig. 1. Systemic Nicotine (Nic) Administration Enhances Object Recognition Memory

(A) Experimental timeline. (B) Total exploration time of saline (Sal)- ($n = 6$) and Nic-treated ($n = 6$) mice during the test session ($t_{10} = 0.181$, $p = 0.860$, Student's *t*-test). (C) Discrimination index (DI) during the test session ($t_{10} = 2.35$, $p = 0.0405$). * $p < 0.05$ (Student's *t*-test).

Fig. 2. Intra-mPFC Infusion of a Non-selective Nicotinic Acetylcholine Receptor Antagonist Blocks Enhancement of Object Recognition Memory Induced by Systemic Nicotine (Nic) Administration

(A) Experimental timeline. (B) Total exploration time of vehicle (Veh) + saline (Sal)- ($n = 9$), Mec + Sal- ($n = 6$), Veh + Nic- ($n = 10$) and Mec + Nic-treated ($n = 6$) mice during the test session (interaction, $F_{1,27} = 0.00993$, $p = 0.921$; Nic, $F_{1,27} = 2.11$, $p = 0.158$; Mec, $F_{1,27} = 0.101$, $p = 0.753$). (C) Discrimination index (DI) during the test session (interaction, $F_{1,27} = 9.41$, $p = 0.00490$). * $p < 0.05$ (two-way ANOVA followed by the Holm-Sidak's *post hoc* test). (D) Locations of the injection cannula tips. Numbers indicate the approximate AP distance (mm) from bregma.

to saline, and this effect was blocked by intra-mPFC infusion of Mec (Fig. 2C). In saline-treated mice, intra-mPFC infusion of Mec did not affect object recognition memory compared with vehicle infusion (Fig. 2C). These results indicate that the systemic administration of nicotine enhances object recognition memory *via* nAChRs in the mPFC.

$\alpha 4\beta 2$ and $\alpha 7$ nAChRs in the mPFC Mediate the Object Recognition Memory Enhancement by Systemic Nicotine Administration We next investigated the role of $\alpha 4\beta 2$ nAChRs in the mPFC in nicotine-induced object recognition memory enhancement. Mice were infused with vehicle (saline) or DH β E (2.5 μ g/side), the $\alpha 4\beta 2$ nAChR antagonist, into the mPFC 5 min before s.c. injection of saline or nicotine (Figs. 3A, D). Total exploration time during the test session did not significantly differ among all experimental groups (Fig. 3B). In intra-mPFC vehicle-infused mice, nicotine significantly increased the DI compared to saline, and this effect was blocked by intra-mPFC infusion of DH β E (Fig. 3C). In saline-treated mice, intra-mPFC DH β E infusion did not affect object recognition memory compared with vehicle infusion (Fig. 3C). These results indicate that nicotine enhances object recognition

memory *via* stimulating $\alpha 4\beta 2$ nAChRs in the mPFC.

Furthermore, to investigate the involvement of $\alpha 7$ nAChRs expressed in the mPFC in the nicotine-induced object recognition memory enhancement, mice were infused with vehicle (saline) or the $\alpha 7$ nAChRs antagonist MLA (10 μ g/side) into the mPFC 5 min before s.c. injection of saline or nicotine (Figs. 3E, H). A nonsignificant difference in total exploration time during the test session was observed among these four groups (Fig. 3F). In intra-mPFC vehicle-infused mice, nicotine significantly increased the DI compared to saline, and this effect was blocked by intra-mPFC MLA infusion (Fig. 3G). In saline-treated mice, intra-mPFC MLA infusion did not affect object recognition memory compared with vehicle infusion (Fig. 3G). These results indicate that nicotine enhances object recognition memory *via* stimulation of $\alpha 7$ nAChRs in the mPFC.

Intra-mPFC Infusion of Nicotine Enhances Object Recognition Memory To directly examine the involvement of the mPFC in nicotine-induced object recognition memory enhancement, vehicle (saline) or nicotine (0.1 or 0.3 μ g/side) was infused into the mPFC before the training session (Figs.

Fig. 3. Intra-mPFC Infusion of an $\alpha 4 \beta 2$ or $\alpha 7$ Nicotinic Acetylcholine Receptor Antagonist Blocks the Nicotine (Nic)-Induced Enhancement of Object Recognition Memory

(A) Experimental timeline. (B) Total exploration time of vehicle (Veh)+ saline (Sal)- ($n=13$), DHβE + Sal- ($n=8$), Veh + Nic- ($n=11$) and DHβE + Nic-treated ($n=9$) mice during the test session (interaction, $F_{1,37}=2.50$, $p=0.122$; Nic, $F_{1,37}=3.92$, $p=0.0552$; DHβE, $F_{1,37}=1.89$, $p=0.177$). (C) Discrimination index (DI) during the test session (interaction, $F_{1,37}=5.52$, $p=0.0243$). * $p<0.05$, ** $p<0.01$ (two-way ANOVA followed by the Holm-Sidak's *post hoc* test). (D) Locations of the injection cannula tips. Numbers indicate the approximate AP distance (mm) from bregma. (E) Experimental timeline. (F) Total exploration time of Veh + Sal- ($n=8$), MLA + Sal- ($n=11$), Veh + Nic- ($n=9$) and MLA + Nic-treated ($n=10$) mice during the test session (interaction, $F_{1,34}=1.39$, $p=0.247$; Nic, $F_{1,34}=0.501$, $p=0.484$; MLA, $F_{1,34}=4.29$, $p=0.517$). (G) Discrimination index (DI) during the test session (interaction, $F_{1,34}=6.60$, $p=0.0148$). ** $p<0.01$ (two-way ANOVA followed by the Holm-Sidak's *post hoc* test). (H) Locations of the injection cannula tips. Numbers indicate the approximate AP distance (mm) from bregma.

Fig. 4. Intra-mPFC Infusion of Nicotine (Nic) Enhances Object Recognition Memory

(A) Experimental timeline. (B) Total exploration time of vehicle (Veh)- ($n=7$) and Nic- ($0.1 \mu\text{g}/\text{side}$, $n=7$; $0.3 \mu\text{g}/\text{side}$, $n=6$) treated mice during the test session ($F_{2,17}=1.80$; $p=0.194$). (C) Discrimination index (DI) during the test session ($F_{2,17}=4.67$, $p=0.0242$). * $p<0.05$ (one-way ANOVA followed by the Holm-Sidak's *post hoc* test). (D) Locations of the injection cannula tips. Numbers indicate the approximate AP distance (mm) from bregma.

4A, D). A nonsignificant difference in the total exploration time during the test session was observed among these groups (Fig. 4B). Mice infused with the higher, but not the lower, nicotine dose exhibited a significantly increased DI compared to vehicle-infused mice (Fig. 4C). These findings indicate that intra-mPFC infusion of nicotine dose-dependently enhances object recognition memory.

DISCUSSION

The main findings of the present study are as follows: (1) systemic or intra-mPFC nicotine administration increased object recognition memory in mice; and (2) the enhancement of object recognition memory by systemic administration of nicotine was suppressed by the intra-mPFC infusion of Mec, DH β E, and MLA. These findings suggest that nicotine-induced object recognition memory enhancement is mediated by $\alpha 4\beta 2$ and $\alpha 7$ nAChRs in the mPFC.

Previous studies reported that the hippocampus, perirhinal cortex, and mPFC comprise neural networks critical for the acquisition, consolidation, and reconsolidation of object recognition memory.^{24,26,28,29,44–47} Thus, we hypothesized that the mPFC could be one of the brain regions relevant to the nicotine-induced enhancement of object recognition memory. Consistent with a previous study,⁵ the systemic nicotine administration before the training session significantly increased the DI. The intra-mPFC infusion of Mec, DH β E, or MLA suppressed nicotine-induced DI increases, indicating that the sites of action of nicotine are $\alpha 4\beta 2$ and $\alpha 7$ nAChRs expressed in the mPFC. Additionally, intra-mPFC nicotine infusion increased the DI, suggesting that the neural activation through the stimulation of nAChRs in the mPFC is sufficient to enhance object recognition memory. This suggestion is further supported by the finding that the expression of c-Fos and

Zif-268, neuronal activity markers, is increased in the mPFC after the NOR test,^{28,33,34} implying a close relationship between mPFC neuronal activity and the enhancement of object recognition memory.

Systemic and intra-mPFC administration of nicotine may affect the activity of almost all types of neurons and synaptic transmissions within the mPFC. Thus, it would be difficult to determine the exact layer(s) and cell type(s) at which nicotine acts in the mPFC to enhance object recognition memory. A previous study demonstrated that nicotine stimulation results in the inhibition of layer 2/3 pyramidal neurons while layer 5 pyramidal neurons are activated by nAChR stimulation due to the specific expression pattern of nAChRs in the mPFC.⁴⁸ Additionally, our recent electrophysiological study revealed that nicotine increases firing activity of layer 5 pyramidal neurons by stimulating $\alpha 4\beta 2$ and $\alpha 7$ nAChRs.⁴⁹ Given that the interaction of mPFC layer 5, but not layer 2/3, pyramidal neurons with the perirhinal cortex and hippocampus has been considered critical for object recognition memory acquisition and/or consolidation,⁵⁰ it is most likely that nicotine-induced activation of mPFC layer 5 pyramidal neurons may be involved in the enhanced object recognition memory observed in the present study. Future studies will be needed to assess the cellular mechanisms underlying the enhancing effect of nicotine on recognition memory.

It has been reported that nicotine enhances object recognition memory through the stimulation of nAChRs expressed in the hippocampus and the perirhinal cortex,⁴⁶ which form a neural circuit with the mPFC. This circuit plays a critical role for object recognition memory.²⁸ Thus, the disruption of the neural circuit by inhibiting the nicotine-induced mPFC activation might account for the finding that intra-mPFC infusion of the nAChR antagonists inhibited the nicotine-induced enhancement of object recognition memory.

We found that the intra-mPFC infusion of either the $\alpha 4\beta 2$ or the $\alpha 7$ nAChR antagonist inhibited the nicotine-induced enhancement of object recognition memory, suggesting that stimulation of both nAChR subtypes is required for the effect of nicotine. At present, mechanisms underlying this requirement is unclear. Because we have recently revealed that nicotine-induced increase in firing activity of mPFC layer 5 pyramidal neurons is also blocked by either an $\alpha 4\beta 2$ or an $\alpha 7$ nAChR antagonist,⁴⁹⁾ it is possible that simultaneous activation of intracellular signaling mediated by these nAChR stimulation might be necessary for the increased activity of mPFC layer 5 pyramidal neurons, and thus, for enhanced object recognition memory. Alternatively, since $\alpha 4\beta 2$ and $\alpha 7$ nAChRs have been known to regulate the release of different neurotransmitters, such as dopamine, glutamate, and noradrenaline,^{12,17,48,51,52)} simultaneous and complementary modulation of neurotransmitter release might be required to the effect of nicotine. In either case, further studies are necessary to address this issue.

Since we injected nicotine before the training session, the nicotine-induced object recognition memory enhancement may be exerted by affecting object recognition memory acquisition. However, it is not clear whether the injected nicotine is washed out from the mPFC during the training session. Thus, we could not exclude the possibility that nicotine affects object recognition memory consolidation. Indeed, a previous study demonstrated that the systemic nicotine administration immediately after training enhances object recognition memory.⁵³⁾ Thus, further studies are necessary to address this point by examining whether the infusion of nicotine into the mPFC immediately after the training session enhances the object recognition memory.

In conclusion, the present results demonstrated that nicotine enhances object recognition memory *via* $\alpha 4\beta 2$ and $\alpha 7$ nAChRs in the mPFC. Our data suggest that mPFC $\alpha 4\beta 2$ and $\alpha 7$ nAChRs might be potential targets to treat cognitive disorders such as dementia and schizophrenia, in which the expression of $\alpha 4\beta 2$ and $\alpha 7$ nAChRs are declined.^{54,55)}

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Conflict of Interest The authors declare no conflict of interest.

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