

# Inhibitory Effects of Isoflurane and Nonimmobilizing Halogenated Compounds on Neuronal Nicotinic Acetylcholine Receptors

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**Background:** Neuronal nicotinic acetylcholine receptors (nAChRs) are inhibited by low concentrations of volatile anesthetics. However, it is not clear whether this phenomenon contributes to the anesthetic effects of volatile anesthetics. Effects of a volatile anesthetic (isoflurane) and structurally related nonimmobilizers (F6: 1,2-dichlorohexafluorocyclobutane, F8: 2,3-dichlorooctafluorobutane) on the current mediated through neuronal nAChRs were studied.

**Method:** This study investigated neuronal nAChRs in PC12 cells and acutely dissociated rat medial habenula (MHb) neurons. Whole cell currents elicited by 30  $\mu\text{M}$  nicotine were recorded in the absence and presence of the halogenated agents. The minimum alveolar concentrations (MACs) for F6 and F8 were predicted from Meyer-Overtton correlation.

**Results:** All halogenated compounds inhibited the nicotine-induced current in a concentration-dependent manner in PC12 cells. In MHb neurons, while isoflurane and F6 significantly inhibited the nicotine-induced peak current, F8 failed to inhibit it. The peak currents in the presence of isoflurane at 1.7 MAC, of F6 at 2.4 MAC, and of F8 at 2.2 MAC were 12, 31, and 97% of control, respectively.

**Conclusions:** Isoflurane, F6, and F8 inhibited ganglion-type nAChRs in PC12 cells independent from their abilities to produce the anesthetic state. In MHb neurons, isoflurane and F6, which lack the immobilizing effect but has the amnesic effect, inhibited nAChRs. Native brain nicotinic receptors in MHb neurons were almost insensitive to F8, which lacks both the immobilizing and the amnesic effect. These results are consistent with the hypothesis that inhibition of nAChRs in MHb neurons is not important for the anesthetic effect but may contribute to the amnesic effect of these agents.

NEURONAL nicotinic acetylcholine receptors (nAChRs) are expressed widely in the central and autonomic nervous systems.<sup>1-3</sup> Although roles of neuronal nAChRs in the brain are poorly understood, these receptors are reported to be involved in cognitive performance, locomotor activities, nociception, and neurologic degeneration associated with aging.<sup>3-5</sup> It is known that both central and peripheral neuronal nAChRs are very sensitive to volatile anesthetics<sup>6-10</sup>; however, it is not clear whether inhibition of neuronal nAChRs contributes to the anesthetic effect of volatile agents.

A very recent study investigated the effects of immobilizing halogenated agents isoflurane and F3 and structurally related nonimmobilizers F6 and F8 on the recombinant human neuronal nAChRs with subunit compositions putatively expressed in the brain.<sup>11</sup> The study demonstrated that  $\alpha 4\beta 2$ ,  $\alpha 4\beta 4$ , and  $\alpha 3\beta 4$  receptors are very sensitive to isoflurane and F3 but not to the nonimmobilizing halogenated agents. These findings indicate that neuronal nAChRs discriminate anesthetic and nonimmobilizing halogenated compounds, favoring important roles of inhibition of these receptors in the anesthetic action of these agents. However, we cannot automatically extrapolate the findings obtained from the recombinant nAChRs to the native receptors since it is known that native neuronal nAChRs exhibit different electrophysiologic and pharmacologic behaviors from those seen in the recombinant receptors with putatively corresponding subunit composition.<sup>12-14</sup> Furthermore, F6 and F8 are shown to inhibit the current mediated through recombinant mouse muscle nAChRs mainly by open-channel blockade.<sup>15</sup> It is not clear whether inhibitory effects on native neuronal nAChRs correlate with immobilizing effects of halogenated compounds; only one report using the heterologous expression system has studied this issue.

The PC12 cells, derived from the rat pheochromocytoma cell line, express ganglion-type neuronal nAChRs, in which the predominant subunit composition is thought to be  $\alpha 3\beta 4$  or  $\alpha 3\beta 4\alpha 5$ .<sup>16,17</sup> The medial habenula (MHb) nucleus, located in the medial part of the dorsal thalamus, receives cholinergic input from the basal forebrain and the brain stem tegmentum. It is one of the regions in which nAChRs are strongly expressed, and it is a suitable site for studying native central receptors.<sup>18-21</sup> Messenger RNAs detected in MHb neurons are for  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 7$ ,  $\beta 2$ ,  $\beta 3$ , and  $\beta 4$  subunits, and  $\alpha 3\beta 4$ -containing receptors are considered to be predominant on the soma.<sup>22</sup> However, multiple subtypes of the receptors exist and, in addition, the  $\beta 2$  subunits are believed to be expressed in some of these neurons.<sup>22</sup> Although  $\alpha 4\beta 2$  receptors are considered to be a predominant subtype in the brain, other combinations, including  $\alpha 3\beta 4$ -containing receptors, are also identified and play roles in specific areas in the central nervous system.<sup>23,24</sup> In this study, we examined effects of isoflurane, F6, and F8 on neuronal nAChRs in PC12 cells and rat MHb neurons to evaluate the relation between inhibition of the receptors, including the native ones, and immobilizing effects of these agents.

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## Materials and Methods

### Cell Culture

The PC12 cells were cultured as described previously.<sup>25</sup> For the experiment, the cells were plated on cover slips coated with collagen and poly-L-lysine. The cells were used after additional culture of 2–4 days without nerve growth factor (NGF) treatment.

### Acute Isolation of MHB Neurons

This study was approved by our institutional animal care and use committee. The MHB neurons were isolated from the MHB nuclei of rats, using the methods described previously.<sup>26</sup> Briefly, 10- to 25-day-old Sprague-Dawley rats were anesthetized with diethylether and decapitated. Then, the brains were rapidly placed in ice-cold water and equilibrated with 100% oxygen PIPES saline containing (in mM) NaCl: 150; KCl: 5; MgCl<sub>2</sub>: 5; piperazine-N, N'-bis (2-ethansulfonic acid) (PIPES): 25; and D-glucose: 25 (pH was adjusted to 7.4 with NaOH). The transverse slices containing MHB nuclei were made with a Vibratome tissue slicer (DTK-1000; Dosaka, Kyoto, Japan), and then they were incubated in PIPES saline containing papain (7.5 U/ml), bovine serum albumin (1 mg/ml), and L-cysteine (0.2 mg/ml) for 25–35 min at 35°C. The slices were washed twice with PIPES saline containing 1 mg/ml each of bovine serum albumin and trypsin inhibitor. Then, MHB nuclei were dissected out and triturated using a fire-polished Pasteur pipette in Dulbecco modified Eagle medium containing 25 mM HEPES and supplemented with Ultrosor G (2%) (Invitrogen, Carlsbad, CA). Dissociated neurons were plated on polyornitine-coated coverslips in a 35-mm Petri dish, maintained in a humid atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C for more than 1 h, and used up to 8 h after isolation.

### Electrophysiology

Membrane currents were measured by conventional whole cell voltage clamp method.<sup>27</sup> Cells on the coverslips were placed in a recording bath mounted on the stage of an inverted microscope (IX70, Olympus, Tokyo, Japan), with an approximate volume of 1.5 ml and continuously perfused at a rate of 1 to 2 ml/min, with external solution containing (in mM), NaCl: 140; KCl: 5.4; CaCl<sub>2</sub>: 1.8; MgCl<sub>2</sub>: 1; HEPES: 10; and D-glucose: 11.1 (pH was adjusted to 7.4 with NaOH). Bicuculine methbromide (50 μM) was included when we used MHB neurons to prevent γ-aminobutyric acid receptor A (GABA<sub>A</sub>)-mediated chloride current. Heat-polished patch pipettes had a tip resistance of 2–10 MΩ. For the experiments using PC12 cells, which lack GABA<sub>A</sub> receptors, we used an intracellular solution containing (in mM) CsCl: 150; HEPES: 10; ethylene glycol-bis(β-aminoethyl ether) tetraacetic acid (EGTA): 5; and magnesium adenosine triphosphate (Mg-ATP): 2 (pH was adjusted to 7.4

with CsOH). For the experiments using MHB neurons, an intracellular solution contained (in mM) CsF: 140; CsCl: 10; HEPES: 10; EGTA: 5; Mg-ATP 2 (pH was adjusted to 7.3 with CsOH), which brought the reversal potential for Cl close to the holding potential. The junction potential between intracellular and external solutions was corrected, but the series resistance was not compensated for. The series resistance changed within approximately 20% from the initial value. Cells were voltage clamped at –60 mV with a patch clamp amplifier (CEZ 2400, Nihon Koden, Tokyo, Japan). Whole cell currents were filtered at 0.5 kHz with a Bessel filter and digitized at 2.5 kHz. The current readings were stored on a computer using pClamp software (Axon Instrument, Foster City, CA) and analyzed using Axograph 3.5 software (Axon Instruments). All experiments were performed at room temperature (22–26°C).

### Drug Application

A Y-tube method described elsewhere was used for delivery of the nicotine and the volatile compounds.<sup>28</sup> The tip of the Y tube was made by a 2-μl glass micropipette (Microcaps, Drummond, Broomall, PA), with an opening of approximately 100 μm and was positioned approximately 500 μm from the recorder cell. Using this method enabled the complete exchange of the external solution surrounding the cell within approximately 100 ms, as estimated by recording the liquid junction current produced at an open patch pipette. Then, 30 μM nicotine, as an agonist, with or without volatile agents, was applied for 2.5 s to MHB cells and for 5 s to PC12 cells. We halved the application time for MHB cells to minimize the decrease of the response with each application. The volatile compounds in the external solutions were preapplied to the bath for 1 min at the rate of 9 ml/min before coapplication with nicotine. Cells were perfused with the plain external solution at the rate of 5 ml/min for 4 min to wash out the drugs from the bath after the measurements.

### Drugs

Drugs used in the current study included isoflurane, F6 (1,2-dichlorohexafluorocyclobutane), and F8 (2,3-dichlorooctafluorobutane) (PCR, Gainesville, FL, or Lancaster, Morecambe, UK), Mg-ATP (Sigma, St. Louis, MO), nicotine (Wako, Osaka, Japan), and bicuculine methbromide (Research Biochemicals, Cambridge, MA). The saturated solutions of the volatile drugs in the external solution were prepared by stirring in the Teflon-sealed (DuPont, Wilmington, DE) glass tubes for more than 3 h and were centrifuged at 1,000 rpm for 5 min. They were diluted to the designated concentrations with the external solution immediately before use and transferred to the Teflon-sealed glass containers. The Teflon caps were removed immediately before starting the applications. The perfusion system was made of glass and polyethyl-

ene. Gas chromatography (GC-9A, Shimadzu, Kyoto, Japan) was used to assay actual concentrations of the volatile compounds in the saturated solutions and the samples obtained from the bath.<sup>29</sup>

#### Data Analysis

We measured the peak and the nondesensitized current, which was defined as the average amplitude from 2.45 to 2.5 s during agonist application in MHb neurons and from 4.95 to 5 s in PC12 cells. Since nicotine-induced currents decreased with each application of nicotine, the response in the presence of the volatile compounds was compared with the average amplitude of elicited currents before and after the compound application. This procedure was predicated on the finding that the second response was almost the same as the average of the first and third responses when nicotine was applied successively three times at an interval of 4 min (data not shown). The concentration-inhibition curve was fitted to the following equation by a least-squares fit:

$$I = 1 - C^n / (C^n + IC_{50}^n)$$

where  $I$  is the relative current normalized to the control current,  $C$  is the concentration of volatile compounds,  $n$  is the Hill coefficient, and  $IC_{50}$  is the concentration for 50% inhibition. The decaying phases of the nicotine-induced current were fitted either to a single or a double exponential function of the following form by simplex method:

$$I = I_{\text{final}} + \sum I_i \times \exp(-t/\tau_i)$$

where  $I$  is the total peak current,  $I_{\text{final}}$  is the residual current at the steady state condition,  $I_i$  is the peak current amplitude of the each component,  $\tau_i$  is the time constant of the corresponding component. Goodness of fit was compared by chi-square test between single and double exponential models. The time constant ratio was calculated by dividing  $\tau_i$  measured in the presence of the volatile agents by the average of  $\tau_i$ s measured immediately before and after administration of the agents. Desensitization was also evaluated by calculating the percent decay of the current during agonist application (%current decay) defined by the following equation<sup>30</sup>:

$$\% \text{ current decay} = (\text{peak current} - \text{nondesensitized current}) / \text{peak current} \times 100.$$

The %current decay ratio was defined as the ratio of the values in the presence of the drugs relative to the average of pre- and postcontrol values.

#### Statistical Analysis

The data are expressed as mean  $\pm$  SEM. The significance of differences was analyzed using one-way analysis of variance followed by multiple  $t$  tests with Bonferroni

correction for comparison among three or more groups. An unpaired  $t$  test was performed to analyze differences between two groups. A  $P$  value less than 0.05 was considered significant.

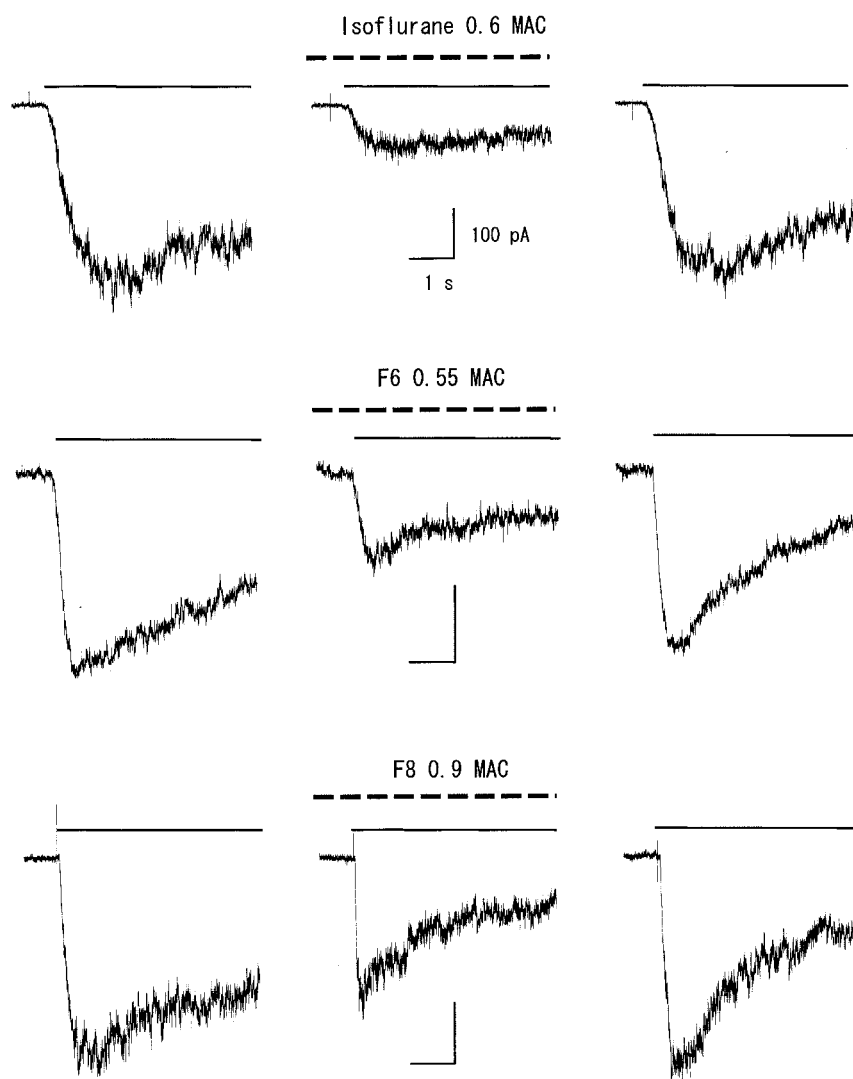
## Results

### Volatile Compounds Concentration

For F6 and F8, predicted minimum alveolar concentration (MAC) was calculated with an oil-water partition coefficient derived from the Meyer-Overton correlation. Then the predicted  $EC_{50}$  at 20°C was calculated as described in the study by Raines *et al.*<sup>31</sup> The aqueous concentrations of the saturated solutions of isoflurane, F6, and F8 were 1,320, 162, and 34  $\mu\text{M}$ , respectively. The concentrations of low doses of isoflurane, F6, and F8 were 85, 3.2, and 1.4  $\mu\text{M}$  and they corresponded to a 0.25 MAC, a 0.2 predicted MAC, and a 0.3 predicted MAC, respectively. The aqueous concentrations of medium doses of isoflurane, F6, and F8 were 201, 8.8, and 4.3  $\mu\text{M}$ , and they corresponded to a 0.6 MAC, a 0.55 predicted MAC, and a 0.9 predicted MAC, respectively. Those of high doses of isoflurane, F6, and F8 were 600, 38.1, and 9.9  $\mu\text{M}$ , and they accounted for a 1.7 MAC, a 2.4 predicted MAC, and a 2.2 predicted MAC, respectively.

### Effect of Volatile Compounds on Nicotine-induced Currents in PC12 Cells

All halogenated compounds reversibly inhibited the nicotine-induced peak and the nondesensitized currents in PC12 cells at the medium doses (fig. 1). These effects were dose-dependent for all three compounds (figs. 2A, 2B). The  $IC_{50}$ s for the peak current inhibition were 0.5 MAC for isoflurane and 0.85 and 20.9 predicted MAC for F6 and F8 (fig. 2A). The  $IC_{50}$ s for the nondesensitized current were 0.5 MAC for isoflurane and 0.45 and 3.5 predicted MAC for F6 and F8 (fig. 2B). The  $IC_{50}$ s for F8 were significantly larger than those for isoflurane and F6 in terms of inhibition of both the peak and the nondesensitized current. There was no significant difference between the  $IC_{50}$ s for isoflurane and F6. The halogenated compounds caused slight increases in % current decay, but the changes were statistically insignificant, except for those at the high doses (fig. 3). Single exponential models gave better fittings than did double exponential models in most cases in the presence or absence of the halogenated agents. The halogenated compounds also induced slight decreases in the time constant ratio; however, the changes were statistically insignificant, except for those at the high dose of F6 (table 1). These three compounds induced no current response when they were applied at high concentrations without nicotine.



**Fig. 1.** Inhibition of the nicotine-induced current by halogenated compounds in PC12 cells. Cells were held at  $-60$  mV, and  $30$   $\mu$ M nicotine was applied for  $5$  s before (left column) and after (right column) coapplication with the halogenated agents, as indicated by a continuous line. Cells were preincubated with the halogenated compounds for  $1$  min before coapplication (middle column), as indicated by a broken line. Isoflurane (top), F6 (middle), and F8 (bottom) reversibly inhibited the nicotine-induced current at  $0.6$  MAC, and  $0.55$  and  $0.9$  predicted MAC.

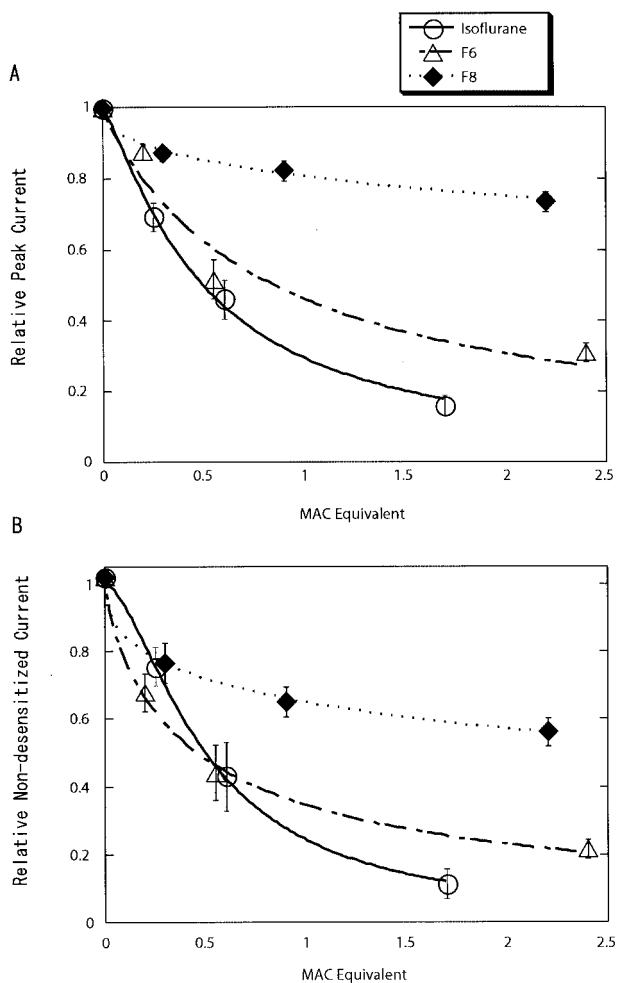
#### *Effect of Volatile Compounds on Nicotine-induced Currents in MHB Neurons*

In MHB neurons, isoflurane and F6 at the medium dose inhibited nicotine-induced current similarly to those observed in PC12 cells. F8 was clearly less effective in inhibiting it than was isoflurane and F6 (figs. 4A, 4B). Although isoflurane and F6 dose-dependently suppressed the peak and the nondesensitized currents, inhibition by F8 was statistically insignificant, except for the reduction in the nondesensitized current at the high concentration (fig. 5A, B).  $IC_{50}$ s for the peak current inhibition were  $0.71$  MAC for isoflurane, and  $1.39$  predicted MAC for F6. F8 caused no significant block of the peak current.  $IC_{50}$ s for the inhibition of the nondesensitized current were  $0.57$ ,  $0.97$ , and  $56.47$  MAC or predicted MAC for isoflurane, F6, and F8. F8 was remarkably less effective in inhibiting the nondesensitized current than were others.  $IC_{50}$ s for isoflurane were significantly less than those for F6 in terms of inhibition of the peak and the nondesensitized components. Neither F6 nor F8 affected % current decay, but it was significantly in-

creased by the high concentration of isoflurane (fig. 6). Isoflurane also significantly decreased the time constant ratio at the high dose, whereas F6 or F8 failed to induce significant changes (table 1). Single exponential fitting gave better results than did double exponential fitting, even in the presence of these agents. The high concentrations of these halogenated agents elicited no current response by themselves.

#### *Comparison Between PC12 Cells and Rat MHB Neurons*

At the medium and high doses of isoflurane, there was no significant difference between the extent of depression of the nicotine-induced response in PC12 cells and in rat MHB neurons. On the contrary, the magnitudes of inhibition by F8 were significantly greater in PC12 cells compared with rat MHB neurons at both concentrations. Although F6 depressed the peak current more strongly in PC12 cells than in MHB neurons at the medium dose, there was no significant difference between the magni-

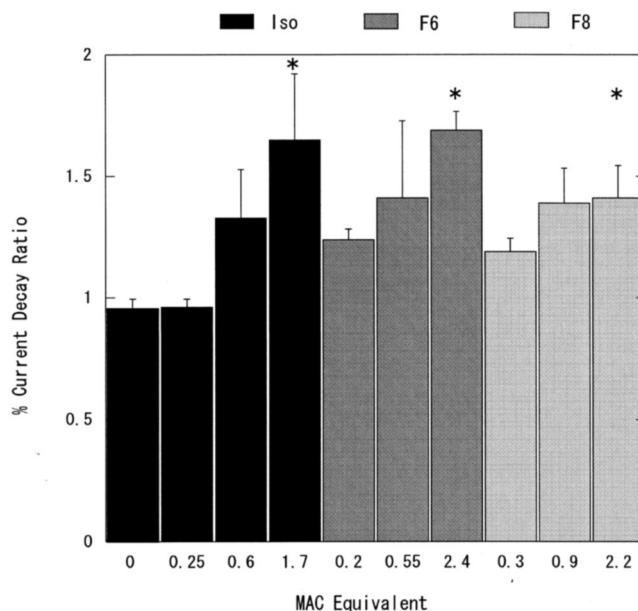


**Fig. 2.** The concentration-inhibition curves for the peak current (A) and the nondesensitized current (B) in PC12 cells. The currents in the presence of the halogenated agents were normalized to the average of the control currents before and after the addition of the halogenated agents and were plotted against the MAC equivalent concentration of the agents. A least-squares fit was performed using the equation described in the Materials and Methods section. The fitting procedure gave  $IC_{50}$  values for the peak current inhibition of  $0.5 \pm 0.02$  MAC for isoflurane ( $R = 1.0$ ) and  $0.85 \pm 0.22$  and  $20.85 \pm 6.57$  predicted MAC for F6 ( $R = 0.985$ ) and F8 ( $R = 0.997$ ). The Hill coefficients were  $1.26 \pm 0.08$ ,  $0.96 \pm 0.27$ , and  $0.46 \pm 0.05$  for isoflurane, F6, and F8, respectively (A). The  $IC_{50}$ s for the nondesensitized currents were  $0.5 \pm 0.01$  MAC for isoflurane ( $R = 1.0$ ),  $0.45 \pm 0.04$  and  $3.53 \pm 0.62$  predicted MAC for F6 ( $R = 0.998$ ) and F8 ( $R = 0.998$ ). The Hill coefficients were  $1.65 \pm 0.08$ ,  $0.83 \pm 0.07$ , and  $0.47 \pm 0.06$  for isoflurane, F6, and F8, respectively (B). Changes in the relative current were statistically significant except for the decrease in the peak current by the low concentration of F6. The marks indicating significance were omitted for clarity. The number of experiments was 4–8 for each condition.

tudes of inhibition by F6 in two preparations in other conditions.

**Discussion**

We studied effects of the immobilizing and nonimmobilizing halogenated compounds on neuronal nAChRs in



**Fig. 3.** Changes in percent of current decay by halogenated compounds in PC12 cells. The percent decay of the current during agonist application defined by the following equation:  $\% \text{current decay} = (\text{peak current} - \text{nondesensitized current}) / \text{peak current} \times 100$ . The percent of current decay in the presence of the halogenated agents was normalized to the average of the values obtained before and after the addition of the agents (% current decay ratio). The ratio for 0 MAC represents control currents measured by comparing the value of the second response, with the average of the first and third responses in the experiments in which nicotine alone was applied successively three times. The number of determination was the same as in figure 2. \*Significant difference from control with  $P < 0.01$ .

PC12 cells and in rat MHB neurons. We found that isoflurane and F6 inhibited the peak and the nondesensitized currents, but F8 caused only a small but significant reduction in the nicotine-induced current in PC12 cells. In rat MHB neurons, we also found that inhibitory effects of F8 were much less extensive than those of

**Table 1.** Changes in the Time Constant Ratio by Halogenated Compounds

|             | Isoflurane        | F6                | F8              |
|-------------|-------------------|-------------------|-----------------|
| PC12 cells  |                   |                   |                 |
| Control     | $0.97 \pm 0.05$   | $0.97 \pm 0.05$   | $0.97 \pm 0.05$ |
| Medium      | $0.86 \pm 0.68$   | $0.96 \pm 0.28$   | $0.87 \pm 0.18$ |
| High        | $0.55 \pm 0.1$    | $0.28 \pm 0.05^*$ | $0.51 \pm 0.18$ |
| MHB neurons |                   |                   |                 |
| Control     | $1.02 \pm 0.07$   | $1.02 \pm 0.07$   | $1.02 \pm 0.07$ |
| Medium      | $0.72 \pm 0.07$   | $0.80 \pm 0.1$    | $0.71 \pm 0.14$ |
| High        | $0.43 \pm 0.11^*$ | $0.70 \pm 0.16$   | $0.60 \pm 0.24$ |

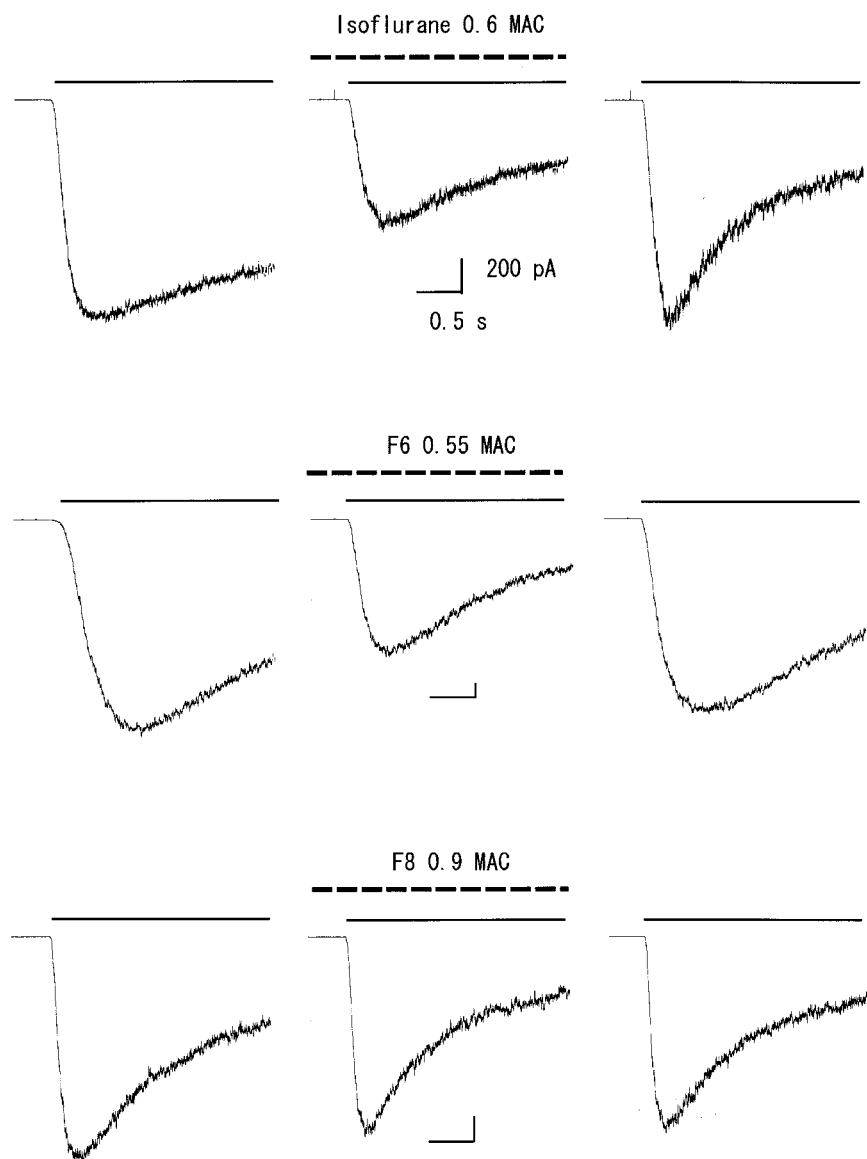
Values are mean  $\pm$  SE of the time constant ratio. Number of experiments was 4 to 9 for each condition. The time constant ratio was calculated by dividing the time constant in the presence of the halogenated agents with the average of the values obtained before and after the agents. Control represents the ratio obtained in control experiments in which we successively applied nicotine alone three times and calculated the ratio of the time constant of the second response relative to the average of the first and third responses. The time constant was derived from fitting the decaying phase of the current with a single exponential function.

\* Significant difference from control with  $P$  less than 0.01.

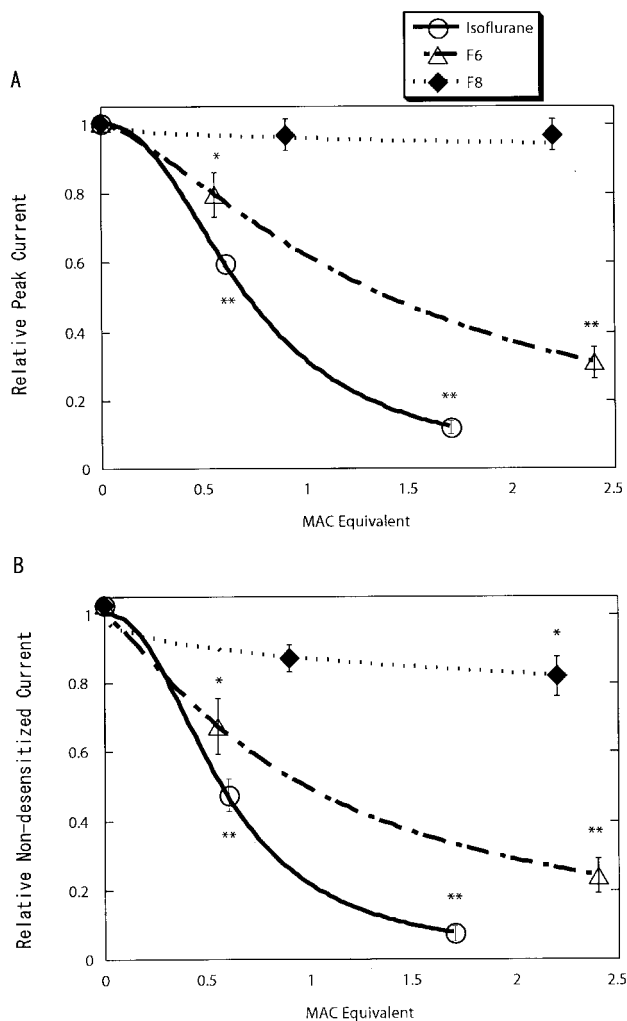
isoflurane and F6 and that depression of the peak current was insignificant for F8, whereas isoflurane and F6 were both effective in inhibiting the peak and the nondesensitized currents. Because it is known that F6 is a nonimmobilizing agent with amnesic activity and F8 lacks immobilizing or amnesic actions,<sup>32</sup> our results suggest that inhibition of neuronal nAChRs in PC12 cells or rat MHB neurons does not correlate with immobilizing activities of the halogenated compounds; however, inhibition observed in MHB neurons may be related to the amnesic actions of these agents.

We compared sensitivities of neuronal nAChRs to three halogenated agents by comparing  $IC_{50}$ s. Although fitting procedures yielded curves with good correlations, the number and range of concentrations studied were limited, especially for MHB neurons, so that reliability of estimation of  $IC_{50}$ s may be compromised. However, there were large differences in the magnitudes of inhi-

bition between F8 and other compounds especially in MHB neurons. Reduction in the peak and the nondesensitized currents accounted for only  $3.8 \pm 4.5$  and  $17.2 \pm 4.1\%$  of control in the presence of a 2.2 predicted MAC for F8, whereas isoflurane at 1.7 MAC reduced the peak and the nondesensitized responses by  $88 \pm 2.1$  and  $92.6 \pm 2.1\%$ , and 2.4 predicted MAC for F6 blocked the responses by  $61.9 \pm 4.5$  and  $76 \pm 5\%$ . Therefore, the receptors in MHB neurons are clearly less sensitive to F8 than are the other two agents. We have chosen  $30 \mu\text{M}$  for the agonist concentration, because it corresponds to the near-saturated dose for the peak and the nondesensitized currents in PC12 cells and MHB neurons both. We limited the duration of nicotine application to 2.5 and 5 s for MHB neurons and PC12 cells to minimize the decrease of the response to the repeated agonist application. The Y-tube system used in this work has a moderately fast exchange time, so that desensitization kinetics



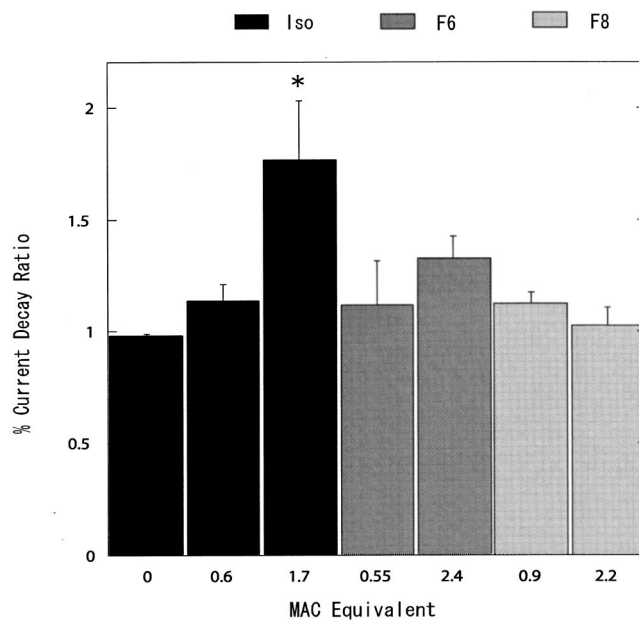
**Fig. 4.** Inhibition of the nicotine-induced current by halogenated compounds in rat medial habenula neurons. Neurons were held at  $-60 \text{ mV}$ , and  $30 \mu\text{M}$  nicotine was applied for 2.5 s before (*left column*) and after (*right column*) coapplication with the halogenated agents, as indicated by a continuous line. Neurons were preincubated with the agents for 1 min before coapplication (*middle column*), as indicated by a broken line. Isoflurane (*top*) and F6 (*middle*) reversibly inhibited the nicotine-induced current at an MAC of 0.6 and a predicted MAC of 0.55, while depression by a 0.9 predicted MAC of F8 was minimal (*bottom*).



**Fig. 5.** The concentration–inhibition curves for the peak (A) and nondesensitized current (B) in rat medial habenula neurons. The currents in the presence of the halogenated agents were normalized to the average of the control currents before and after the addition of the agents and were plotted against the MAC equivalent concentration of the agents. The fitting procedure gave IC<sub>50</sub> values for the peak current inhibition of 0.71 ± 0.002 MAC for isoflurane (R = 1.0) and 1.39 ± 0.01 predicted MAC for F6 (R = 1.0). The Hill coefficients were 2.29 ± 0.01 and 1.47 ± 0.01 for isoflurane and F6. F8 was not effective in inhibiting the peak current (A). The IC<sub>50</sub>s for the nondesensitized current were 0.57 ± 0.03 MAC for isoflurane (R = 0.999) and 0.97 ± 0.08 and 56.47 ± 25.32 predicted MAC for F6 (R = 0.999) and F8 (R = 0.983). The Hill coefficients were 2.32 ± 0.41, 1.27 ± 0.14, and 0.46 ± 0.35 for isoflurane, F6, and F8, respectively (B). The number of experiments was 4–9 for each condition. \*P < 0.05 and \*\*P < 0.01, significant difference from 0 MAC.

can be nearly resolved; however, very fast current changes cannot be studied in our system.

Our finding that the nonimmobilizing halogenated agents F6 and F8 exert inhibitory effects on neuronal nAChRs is inconsistent with that of the earlier study reported by Cardoso *et al.*<sup>11</sup> They investigated effects of the same agents on human neuronal nAChRs expressed in *Xenopus* oocytes and found that isoflurane, but not F6 or F8, depresses the current mediated through human



**Fig. 6.** Changes in percent of current decay by halogenated compounds in rat medial habenula neurons. The percent of current decay ratio was calculated in the same way as in figure 3. Number of determination was the same as in figure 5. \*P < 0.05, significant difference from control.

$\alpha 3\beta 4$  receptors. The large discrepancy between the findings obtained in the studies is intriguing but might be explained by differences in many aspects of the methods used in two studies. 1) Cardoso *et al.*<sup>11</sup> used human receptors, while we used the receptors derived from rats. They used  $\alpha 3\beta 4$  receptors and  $\alpha 2\beta 4$  and  $\alpha 4\beta 2$  subtypes, whereas the receptors in our study may contain  $\alpha 5$  and  $\beta 3$  subunits in addition to  $\alpha 3$  and  $\beta 4$  subunits. 2) They applied acetylcholine at the EC<sub>30</sub> concentration, while we applied nicotine at the near-saturating concentration to induce the responses. 3) They used bath application for the agonist, resulting in the slow responses, whereas the Y-tube system was used in our study resulting in relatively fast responses. The current kinetics, such as the rate of desensitization, is known to be greatly influenced by the concentration of the agonist and the speed and duration of application.<sup>20</sup> The effects of blockers are also influenced by the agonist concentration, depending on the type of inhibition; that is, competitive blockade is reduced, but open-channel block is enhanced by the high agonist concentration. As for inhibition by isoflurane, the results from two studies are qualitatively similar; that is, isoflurane potently inhibited neuronal nAChRs at less than the MAC in both studies, though there are quantitative differences in IC<sub>50</sub>s; IC<sub>50</sub> for human  $\alpha 3\beta 4$  receptors was as low as 0.2 MAC, whereas IC<sub>50</sub> for PC12 cells was 0.5 MAC. In contrast, the difference in the effects of the nonimmobilizers between two studies seems qualitative but not quantitative because F6 was an effective blocker, with the po-

tency close to that of isoflurane in our study but completely ineffective in the earlier report, and slight increases in the current mediated through human receptors by F6 and F8 were never observed in the present study. It is likely that qualitative difference is due to some structural differences of the receptors but not due to differences in the methods of agonist application.

In contrast to the finding of human receptors, Forman and Raines<sup>15</sup> found that F6 and F8 suppress mouse muscle nAChRs expressed in *Xenopus* oocytes using a rapid application system. Enflurane, F6, and F8 were almost equally effective, with IC<sub>50</sub>s ranging from a 1.8- to a 2.4-MAC equivalent in their investigation. As for effectiveness of F6, our results were similar to theirs because differences in IC<sub>50</sub>s for F6 and the anesthetic compounds were few in both studies; although the receptors in PC12 cells and MHB neurons were more sensitive to these compounds than were mouse muscle receptors. As for the inhibitory effects of F8, however, our findings were inconsistent with the findings of Forman and Raines.<sup>15</sup> The effects of F8 were much less effective than effects of F6 and isoflurane in our study, whereas no big difference was seen in their results.

In the study by Forman and Raines,<sup>15</sup> the nonimmobilizers exerted preferential block of open channels resulting in the addition of a new fast-decay phase, while enflurane exhibited no selectivity for open channels. In our study, at the high concentration, three agents similarly accelerated the current decay but added no fast-decay phase in PC12 cells, indicating no distinct blocking mechanisms between anesthetic and nonimmobilizing drugs. However, isoflurane at the high concentration was the sole agent accelerating the current decay in MHB neurons, whereas the nonimmobilizers failed to enhance it. This finding suggests that the different blocking mechanisms might be involved in inhibition of central nAChRs by isoflurane and the nonimmobilizers. It may be possible that desensitization processes of nAChRs in MHB neurons discriminate the anesthetic from the nonimmobilizing agent and are accelerated only by the anesthetic agent. But the significant increase in the current decay might be explained by differences in the magnitudes of the blocking actions of isoflurane and the nonimmobilizing agents. In our experiments, no halogenated compound added a new fast-decay phase observed in the earlier study. This could be due to different properties of blockade caused by the nonimmobilizers in two studies, or slower time resolution of our setting might obscure changes in fast-channel kinetics. Taken together, our results disagree with those by Forman and Raines in terms of effectiveness of F8 and apparent characteristics of blockade by the nonimmobilizers, but the findings in the current decay observed in MHB neurons raised the possibility of different mechanisms of blockade for the anesthetic and nonimmobilizing agents, as Forman and Raines<sup>15</sup> suggested in their study.

Regarding characteristics of neuronal nAChRs from various species, pharmacologic properties of recombinant receptors expressed in various host cells are not necessarily the same as those of native neurons.<sup>12-14</sup> Probable reasons for this include differences in cellular environment, differences in stoichiometry, presence of unidentified subunits in native receptors, and so on.<sup>12</sup> Therefore, the results obtained in the *Xenopus* oocyte-expression system do not necessarily represent the pharmacology of native receptors.

The rank order of potencies of the halogenated compounds was not the same but was similar in MHB neurons and PC12 cells. Isoflurane was most effective, F6 was comparable with or slightly less effective than isoflurane, and F8 was least effective. However, sensitivities of nAChRs to F8 were different in these two preparations, even though both types of cells express mainly  $\alpha\beta\gamma$ -containing receptors. The differences may be due to different stoichiometry, presence of additional subunits, or differences in intracellular modulatory factors, among other factors. Also, the receptors expressed in these cells are heterogeneous and characters of minor subtypes may be different.<sup>16,21</sup> These factors probably account for differences in the effects observed in these preparations.

Our main finding is that inhibition of neuronal nAChRs, especially in rat MHB neurons, correlates with the amnesic effect, but not with the immobilizing effect, of the halogenated agents. This finding implies that neuronal nAChRs in rat MHB neurons may participate in amnesia caused by these agents. It has been reported that destruction of the MHB and its primary efferent pathway, the fasciculus retroflexus, impaired avoidance learning, suggesting that these neurons are responsible for some form of learning and memory.<sup>33,34</sup> Therefore, our finding seems consistent with these reports. However, we cannot extrapolate these results to the receptors of predominant type expressed in the brain because most central nervous system neurons express the  $\alpha\beta\delta$  subunit, and MHB neurons express mainly the  $\alpha\beta\gamma$  subunit, and the pharmacologic properties of these receptors are different.<sup>1,2</sup>

In conclusion, isoflurane, F6, and F8 inhibited ganglion-type nAChRs in PC12 cells independently from their ability to produce anesthesia. Nicotinic receptors in rat MHB neurons were almost insensitive to F8, which lacks the immobilizing or amnesic effect, whereas isoflurane and F6 were effective in inhibiting nAChRs in these neurons. These results are consistent with the hypothesis that inhibition of nAChRs in rat MHB neurons is not important for the anesthetic effect of halogenated compounds but may contribute to the amnesic effect of these agents. Comparison with the earlier studies suggests that there is a large difference in the sensitivities of neuronal nAChRs to the nonimmobilizing halogenated agents among studies. Further investigations are needed to elucidate the reasons for these discrepancies.



## References

- McGehee DS, Role LW: Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. *Annu Rev Physiol* 1995; 57:521-46
- Role LW, Berg DK: Nicotinic receptors in the development and modulation of CNS synapses. *Neuron* 1996; 16:1077-85
- Jones S, Sudweeks S, Yakel JL: Nicotinic receptors in the brain: Correlating physiology with function. *Trends Neurosci* 1999; 22:555-61
- Zoli M, Picciotto MR, Ferrari R, Cocchi D, Changeux JP: Increased neurodegeneration during ageing in mice lacking high-affinity nicotine receptors. *Embo J* 1999; 18:1235-44
- Cordero-Erausquin M, Marubio LM, Klink R, Changeux JP: Nicotinic receptor function: New perspectives from knockout mice. *Trends Pharmacol Sci* 2000; 21:211-7
- Pocock G, Richards CD: The action of volatile anaesthetics on stimulus-secretion coupling in bovine adrenal chromaffin cells. *Br J Pharmacol* 1988; 95:209-17
- Flood P, Ramirez-Latorre J, Role L:  $\alpha 4 \beta 2$  neuronal nicotinic acetylcholine receptors in the central nervous system are inhibited by isoflurane and propofol, but  $\alpha 7$ -type nicotinic acetylcholine receptors are unaffected. *ANESTHESIOLOGY* 1997; 86:859-65
- Violet JM, Downie DL, Nakisa RC, Lieb WR, Franks NP: Differential sensitivities of mammalian neuronal and muscle nicotinic acetylcholine receptors to general anaesthetics. *ANESTHESIOLOGY* 1997; 86:866-74
- Mori T, Zhao X, Zuo Y, Aistrup GL, Nishikawa K, Marszalec W, Yeh JZ, Narahashi T: Modulation of neuronal nicotinic acetylcholine receptors by halothane in rat cortical neurons. *Mol Pharmacol* 2001; 59:732-43
- Salord F, Keita H, Lecharny JB, Henzel D, Desmonts JM, Mantz J: Halothane and isoflurane differentially affect the regulation of dopamine and  $\gamma$ -aminobutyric acid release mediated by presynaptic acetylcholine receptors in the rat striatum. *ANESTHESIOLOGY* 1997; 86:632-41
- Cardoso RA, Yamakura T, Brozowski SJ, Chavez-Noriega LE, Harris RA: Human neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes predict efficacy of halogenated compounds that disobey the Meyer-Overton rule. *ANESTHESIOLOGY* 1999; 91:1370-7
- Sivilotti LG, McNeil DK, Lewis TM, Nassar MA, Schoepfer R, Colquhoun D: Recombinant nicotinic receptors, expressed in *Xenopus* oocytes, do not resemble native rat sympathetic ganglion receptors in single-channel behaviour. *J Physiol (Lond)* 1997; 500:123-38
- Lewis TM, Harkness PC, Sivilotti LG, Colquhoun D, Millar NS: The ion channel properties of a rat recombinant neuronal nicotinic receptor are dependent on the host cell type. *J Physiol (Lond)* 1997; 505:299-306
- Cooper ST, Millar NS: Host cell-specific folding and assembly of the neuronal nicotinic acetylcholine receptor  $\alpha 7$  subunit. *J Neurochem* 1997; 68:2140-51
- Forman SA, Raines DE: Nonanesthetic volatile drugs obey the Meyer-Overton correlation in two molecular protein site models. *ANESTHESIOLOGY* 1998; 88:1535-48
- Rogers SW, Mandelzys A, Deneris ES, Cooper E, Heinemann S: The expression of nicotinic acetylcholine receptors by PC12 cells treated with NGF. *J Neurosci* 1992; 12:4611-23
- Sivilotti L, Colquhoun D: Acetylcholine receptors: Too many channels, too few functions. *Science* 1995; 269:1681-2
- Mulle C, Changeux JP: A novel type of nicotinic receptor in the rat central nervous system characterized by patch-clamp techniques. *J Neurosci* 1990; 10:169-75
- Mulle C, Choquet D, Korn H, Changeux JP: Calcium influx through nicotinic receptor in rat central neurons: its relevance to cellular regulation. *Neuron* 1992; 8:135-43
- Lester RA, Dani JA: Acetylcholine receptor desensitization induced by nicotine in rat medial habenula neurons. *J Neurophysiol* 1995; 74:195-206
- Connolly JG, Gibb AJ, Colquhoun D: Heterogeneity of neuronal nicotinic acetylcholine receptors in thin slices of rat medial habenula. *J Physiol (Lond)* 1995; 484:87-105
- Quick MW, Ceballos RM, Kasten M, McIntosh JM, Lester RA:  $\alpha 3 \beta 4$  subunit-containing nicotinic receptors dominate function in rat medial habenula neurons. *Neuropharmacology* 1999; 38:769-83
- Dineley-Miller K, Patrick J: Gene transcripts for the nicotinic acetylcholine receptor subunit,  $\beta 4$ , are distributed in multiple areas of the rat central nervous system. *Brain Res Mol Brain Res* 1992; 16:339-44
- Winzer-Serhan UH, Leslie FM: Codistribution of nicotinic acetylcholine receptor subunit  $\alpha 3$  and  $\beta 4$  mRNAs during rat brain development. *J Comp Neurol* 1997; 386:540-54
- Andoh T, Furuya R, Oka K, Hattori S, Watanabe I, Kamiya Y, Okumura F: Differential effects of thiopental on neuronal nicotinic acetylcholine receptors and  $P_{2X}$  purinergic receptors in PC12 cells. *ANESTHESIOLOGY* 1997; 87:1199-1209
- Kamiya Y, Andoh T, Watanabe I, Higashi T, Itoh H: Inhibitory effects of barbiturates on nicotinic acetylcholine receptors in rat central nervous system neurons. *ANESTHESIOLOGY* 2001; 94:694-704
- Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ: Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch* 1981; 391:85-100
- Murase K, Ryu PD, Randic M: Excitatory and inhibitory amino acids and peptide-induced responses in acutely isolated rat spinal dorsal horn neurons. *Neurosci Lett* 1989; 103:56-63
- Schlame M, Hemmings HC Jr: Inhibition by volatile anesthetics of endogenous glutamate release from synaptosomes by a presynaptic mechanism. *ANESTHESIOLOGY* 1995; 82:1406-16
- Valenta DC, Downing JE, Role LW: Peptide modulation of ACh receptor desensitization controls neurotransmitter release from chicken sympathetic neurons. *J Neurophysiol* 1993; 69:928-42
- Raines DE: Anesthetic and nonanesthetic halogenated volatile compounds have dissimilar activities on nicotinic acetylcholine receptor desensitization kinetics. *ANESTHESIOLOGY* 1996; 84:663-71
- Kandel L, Chortkoff BS, Sonner J, Laster MJ, Eger EI II: Nonanesthetics can suppress learning. *Anesth Analg* 1996; 82:321-6
- Wirtshafter D: The role of interpeduncular connections with the tegmentum in avoidance learning. *Physiol Behav* 1981; 26:985-9
- Thornton EW, Murray M, Connors-Eckenrode T, Haun F: Dissociation of behavioral changes in rats resulting from lesions of the habenula versus fasciculus retroflexus and their possible anatomical substrates. *Behav Neurosci* 1994; 108:1150-62