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Changes in the Effect of Isoflurane on N-*methyl-*D*-aspartic Acid-gated Currents in Cultured Cerebral Cortical Neurons with Time in Culture*

Evidence for Subunit Specificity

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Background: **Developmental changes in NR1 splice variants and NR2 subunits of the *N*-methyl-D-aspartate (NMDA) receptor have been associated with changes in the sensitivity of NMDA receptors to agonists, antagonists, and pharmacologic modulators. The authors have investigated changes in the effect of isoflurane on NMDA-gated currents from cultured cortical neurons with time in culture and related these changes to the subunit composition of the NMDA receptors.**

Methods: ***N*-methyl-D-aspartate–gated currents were measured using whole-cell voltage clamp recording in cortical neurons cultured for 1–4 weeks and HEK 293 cells transiently expressing NR1–1a + NR2A or NR1–1a + NR2B subunit-containing receptors. NMDA alone or NMDA with treatment agents (isoflurane or ifenprodil) was applied to cells using a U tube.**

Results: **The effect of isoflurane and the NR2B selective antagonist ifenprodil on NMDA-gated currents from cortical neurons decreased significantly with time in culture. NMDA-gated currents mediated by NR2A-containing receptors were less sensitive to isoflurane than those mediated by NR2B-containing receptors. Tachyphylaxis to repeated application of isoflurane was found in cortical neurons and HEK 293 cells with recombinant NMDA receptors. Hooked tail currents were induced by isoflurane in cultured cortical neurons and HEK 293 cells with expressed NMDA receptors.**

Conclusions: **Isoflurane inhibits NMDA-gated currents at concentrations well below 1 minimum alveolar concentration (MAC). This effect of isoflurane was subunit dependent with the NR2B-containing receptors more sensitive to isoflurane than the NR2A-containing receptors. A potent tachyphylaxis occurred after brief exposure to isoflurane.**

SEVERAL lines of evidence suggest interactions between volatile anesthetic agents and *N*-methyl-D-aspartate (NMDA) receptors. Intrathecally or intravenously administered NMDA receptor antagonists reduce the minimum alveolar concentration (MAC) of isoflurane with diverse potency.[1,2] Further, isoflurane blocks NMDA-stimulated currents in cultured hippocampal and cerebral cortical neurons.[3,4] Likewise, volatile anesthetic agents depress NMDA-gated currents in frog oocytes expressing NMDA receptors[5] and depress glutamate stimulation of MK-801 binding to the open channel of the NMDA receptor in a concentration-dependent manner.[6] Finally, excitatory transmission mediated by NMDA receptors is depressed by isoflurane.[7]

The rat and human genes encoding subunits of NMDA receptor have been identified and classified into two related groups: the NMDAR1 (NR1) subunit with eight splice variants, and the NMDAR2 (NR2) subunits A, B, C, and D.[8,9] Functional NMDA receptors are assembled as a tetramer containing an NR1 and one or more of the NR2 subunits in mammalian expression systems.[10] Receptors comprising different NR1 splice variants and NR2 subunits have different sensitivities to agonists, antagonists, and pharmacologic modulators.[10,11] There have been relatively few reports of anesthetic action on NMDA receptors in expression systems. Hollmann *et al.*[5] found that, although several volatile anesthetic agents antagonized NMDA receptor function, the effectiveness of the anesthetic was not subunit dependent. However, modulation of the function of NMDA receptors by other depressant inhalants, including ethanol, depends on NR1 splicing and the composition of NR2 subunits.[12,13]

Developmental changes in NR1 splice variants and NR2 subunits of NMDA receptors in different regions of the brain have been widely studied.[14–21] Similar developmental changes in NR2 subunits of NMDA receptors in rat brain and in cultured rat neurons have been documented.[15–21] In addition, developmental changes in the sensitivity of NMDA receptors to various agonists, antagonists, and pharmacologic modulators have been reported.[22–25]

Based on evidence that there are developmental changes in the subunit composition of NMDA receptors in neurons and that there are subunit-dependent pharmacologic properties of NMDA receptors, we hypothesized that the sensitivity of NMDA receptors in cultured neurons to a volatile anesthetic agent might change with time in culture. Thus, the present study examines changes in the effect of isoflurane on NMDA-gated currents from cultured cerebral cortical neurons with time in culture and the relationship between sensitivity to isoflurane and NMDA-subunit composition of the neurons across time.

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Materials and Methods

Primary Culture of Cortical Neurons

After approval by the institutional Animal Care and Use Committee (University of North Carolina, Chapel Hill, NC), cortical tissue was removed from the brain of newborn (less than 8 h) rat pups and placed in an isotonic salt solution containing 100 U/l of penicillin G (Pfizer, New York, NY), 100 μg/l of streptomycin (Pfizer, New York, NY), and 0.25 μg/l of amphotericin B (Fungizone, Gibco, Grand Island, NY; pH, 7.4). Blood vessels and pia mater were removed, and the cortical tissue was chopped into approximately 2-mm chunks. The cortex pieces were then suspended in 25 ml of 0.25% trypsin (wt/vol, Worthington Biochemicals, Freehold, NJ) in an isotonic salt solution (pH, 7.4) and placed in a shaking water bath for 10 min at 37°C to dissociate the cells. The cell suspension was then removed and combined with 10 ml of Dulbecco modified eagle's medium (DMEM, GibcoBRI, Grand Island, NY) containing 10% platelet-depleted horse serum (PDHS, Sigma, St. Louis, MO), and any undissociated chunks were triturated until the cells dissociated. The dissociated cell suspensions were combined, centrifuged at 500 × *g* for 5 min, and the resulting pellet was washed with 50 ml DMEM containing 10% PDHS. Cells were resuspended in DMEM containing 10% PDHS and plated at a density of 4×10^6 cells/well on poly-L-lysine precoated 12 × 12 mm glass cover slips placed in culture wells and incubated at 37°C in a humidified incubator with 5% CO_2–95% air. On day 3, cells were treated with 10 μM β-cytosine arabinoside in DMEM containing PDHS. After 2 days of β-cytosine arabinoside treatment, culture medium was replaced with DMEM containing 10% PDHS.

Transfection of HEK 293 Cells

HEK 293 cells were cultured on poly-L-lysine (0.04 mg/ml) coated, 12 × 12 mm glass coverslips in a standard 12-well plate. Cells were grown in feeding media containing DMEM and 10% fetal bovine serum (FBS) in an incubator with 5% CO_2–95% air. Cells were allowed to reach 50% coverage of coverslips before transfection. Transient expression of functional receptors was accomplished by transfection of HEK 293 cells with cDNA encoding NR1–1a + NR2A or NR1–1a + NR2B receptor subunits using the lipofectamine method. Cells on coverslips were rinsed once with phosphate buffered saline (PBS) and then transfected for 5 or 6 h with 750 μl/well of Opti-MEM1 (GibcoBRL) containing a mixture of 3 μl/well LipofectAMINE Reagent (GibcoBRL) and 1.3 μg/well plasmid DNA. The plasmid DNA mixture consisted of 0.1 μg/well pEGFPN1 (GFP), 0.3 μg/well pRc/CMV-NR1–1a, and 0.9 μg/well of a single plasmid DNA encoding the NR2 subunit (pRc/CMV-NR2A or pRc/CMV-NR2B). NR1 and NR2 plasmid DNA was a generous gift from David Lovinger, Ph.D. (Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN). After 5 or 6 h incubation, the transfection mixture was removed, and the cells were fed with 1 ml media (DMEM with 10% FBS). DL-2-amino-5-phosphonovaleric acid (1 mM, Sigma) or (+)-MK-801 (100 μM) was added to the media to prevent glutamate toxicity. Transfected cells were used for electrophysiologic recording for 1–3 days after transfection. Positively transfected cells were identified by GFP fluorescence.

Current Recordings

Standard whole-cell voltage clamp recordings were made with glass electrodes, fire-polished to a resistance of 2–5 MΩ, and filled with internal solution: KCl, 150 mM; $MgCl_2$, 3 mM; HEPES, 15 mM; K-ATP, 2 mM; EGTA, 5 mM; phosphocreatine, 15 mM; and 50 U/ml creatine phosphokinase (adjusted to pH 7.4 with KOH). The recording chamber was perfused at 0.5–1 ml/min with Mg^{2+} free external solution: NaCl, 145 mM; KCl, 5 mM; HEPES, 10 mM; $CaCl_2$, 2 mM; and glucose, 10 mM (adjusted to pH 7.4 with NaOH). When NMDA-gated currents were studied in cultured cortical neurons, 0.2 μM tetrodotoxin (Sigma), a sodium channel blocker, and 5 μM glycine (Sigma), a coagonist of NMDA receptor, were included in the perfusion solution. When the currents from transfected HEK 293 cells were studied, only 5 μM glycine was included in the perfusion solution, and KCl was replaced by CsCl (150 mM) in the internal solution. Cells were voltage clamped at a holding potential of −60 mV. Currents were recorded using an Axopatch-1D patch clamp amplifier with an 80% series resistance compensation and collected with a computer. Capacitance of the neurons was measured by "whole cell compensation" on the amplifier on breaking the neuronal membrane.

N-methyl-D-aspartate (Sigma), 100 μM, alone (control) or with various concentrations of isoflurane (Baxter, Deerfield, IL) was applied for 8–12 s by a U tube placed 100–200 μm from the cells. This technique allowed a brief cellular application and removal of drugs. The interval between applications was 1.5 min. The activation time from 10 to 90% of peak currents gated by 100 μM NMDA alone was 568 ± 45 ms (n = 26). Recordings were performed at room temperature (22 or 23°C). Glycine, 5 μM, was included in the control and isoflurane-containing solutions. For most neurons, the desensitization of NMDA-gated currents reached steady state after 8-s exposure to NMDA. Each experimental treatment was preceded and followed by an application of NMDA alone as a control. The drug effects were expressed as the percent change from the average of the pre- and posttest control values.

The experimental protocol for testing tolerance to isoflurane in cultured cortical neurons was as follows. Ninety seconds after the application of 100 μM NMDA alone (pre-control), 100 μM NMDA with 0.3 mM isoflurane (ISO1) was applied. Then, 100 μM NMDA with 0.3 mM isoflurane

(ISO2) was applied again, which was followed by 100 μM NMDA alone (postcontrol) 1.5 min later. The intervals between ISO1 and ISO2 were 15 s, 30 s, 45 s, and 3 min. The currents induced by repeated application of 100 μM NMDA alone (N1 and N2) at the same interval of ISO1 and ISO2 were also recorded in the same cell. Compared with the precontrol steady state current, if there was more than a 10% increase or decrease (mostly decrease) in postcontrol steady state current, the data were discarded.

Peak current refers to the initial peak amplitude of the NMDA-gated current. The steady state current refers to the amplitude of NMDA-gated current after 8 s of drug delivery. Because slow delivery of NMDA by U tube indicated by long activation time of the currents, peak currents measured in this study are likely to be underestimated. Amplitude of the hooked tail current was measured from the point where the hooked tail current started to the peak of the current. The density of peak or steady state current was calculated by dividing the amplitude of peak or steady state current with the capacitance of the cell. Percent desensitization refers to percent decrease in steady state current compared with the peak current.

Preparation of the Treatment Solutions

For preparing treatment solutions containing varying concentrations of isoflurane, the amount of isoflurane was calculated from its specific gravity (1.45) and molecular weight (184.5), and 7 μl was injected into 7 ml of NMDA- and glycine-containing external solution in a tightly capped small glass bottle to prevent exposure to air. The mixture was sonicated until the isoflurane droplets completely dissolved. This procedure produced a 7.86 mM solution of isoflurane. Varying volumes of the isoflurane stock solution were then combined with the NMDA- and glycine-containing external solution to prepare the desired treatment solution concentrations. The treatment solutions were delivered through Teflon lines from glass syringes with plungers to retard evaporative loss of isoflurane. To minimize the possible absorption of isoflurane by Teflon tubing, the lengths of tubing used were as short as possible. Thus, the solutions between the glass syringes and the orifice of the U tube were completely exchanged within the 1.5-min interval between applications. To ascertain the degree of loss of isoflurane during preparation and handling of isoflurane solution, gas chromatography was used to assay solution samples collected at the beginning and end of a 4-h period. Losses of the volatile agent from the delivery system were found to be less than 10% (fig. 1). Because the lowest concentration of isoflurane measured by gas chromatography was 30 μM, the samples with concentrations lower than 30 μM were not measured. When the tolerance of NMDA-gated currents to isoflurane was tested in cultured cortical neurons, 0.3 mM isoflurane was applied twice with the intervals up to 3 min. To determine whether the concentration of isoflurane changed during the 3-min interval, two samples were collected from the U-tube opening at an interval of 3 min, and the isoflurane concentration was measured by gas chromatography. There was no significant difference between the concentration of isoflurane from the first and second collections (Student *t* test, $P > 0.05$; fig. 1).

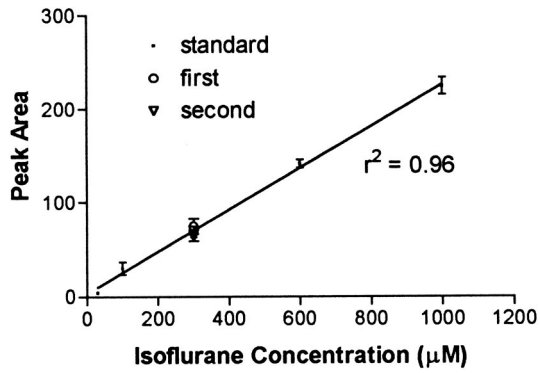

Fig. 1. Isoflurane concentrations measured with gas chromatography are shown. A standard curve was constructed by measuring the area under the peak for samples of isoflurane in sealed glass syringes. The measured concentrations, expressed as integrated peak area, were plotted, and a linear curve, standard, was obtained (r^2 = 0.96; n = 4 at each concentration). Two samples were collected from the U-tube opening with a 3-min interval. The peak areas of the samples are shown (for detail, see text). In the legend, Standard = samples for standard line; First = the first collection; Second = the second collection. The n is 4 at each concentration for standard line and 6 for either the first or second collection.

Analysis

The EC_{50} values were generated from the least squares fit to the equation [Y = Bottom + (Top − Bottom)/(1 + $10^{\blacktriangle}$ (LogEC_{50} − X) × Hill slope))]. X is the log of the concentration, and Y is the response. Y starts at Bottom and goes to Top with sigmoid shape. Data were expressed as mean ± SE, and n refers to the number of cells tested. Where appropriate, Student *t* test, analysis of variance (ANOVA), and correlation were performed as indicated. Calculated *P* values of less than 0.05 were accepted as evidence of a significant difference.

Results

Effect of Time in Culture on NMDA-gated Currents in Cultured Cortical Neurons

To understand the change in the effect of isoflurane on NMDA-gated currents over time in culture, we first looked for corollary changes in other parameters related to NMDA receptor function with time in culture. Figure 2A shows the lack of a significant change in capacitance of cortical neurons as a function of time in culture (one-way ANOVA, $P > 0.05$), suggesting that the size of the neurons did not change significantly over time. However, when the amplitudes of steady state NMDA-gated

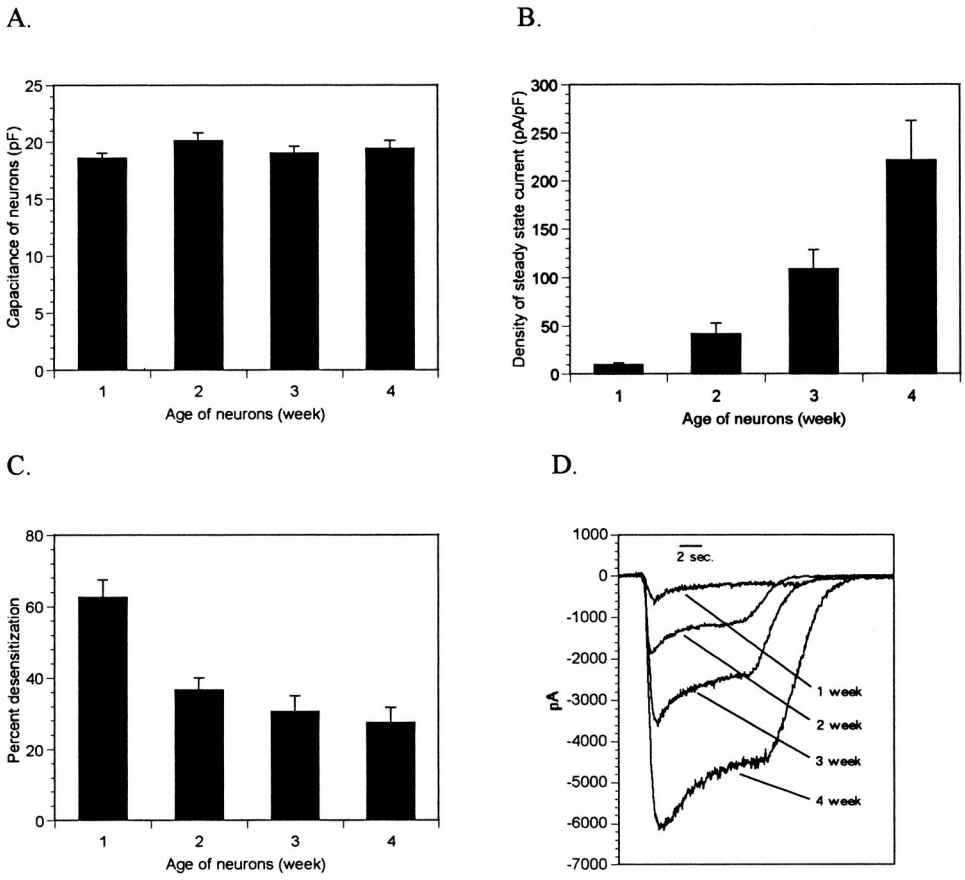

Fig. 2. Effect of time in culture on NMDA-gated currents in cultured cortical neurons is shown. (*A*) The capacitance of cortical neurons cultured for 1–4 weeks. The n in each group is 14 for 1 and 2 weeks, 12 for 3 weeks, and 10 for 4 weeks. (*B*) Changes in the density of NMDA-gated steady state currents with time in culture for cortical neurons. The n in each group is as the same as that in *A*. (*C*) Age-dependent changes in percent desensitization of NMDA-gated currents in cultured cortical neurons. The n for each group is 16 for 1 week, 20 for 2 weeks, 17 for 3 weeks, and 13 for 4 weeks. (*D*) Representative currents activated by 100 μM NMDA from cortical neurons cultured for the indicated number of weeks. Note the changes in amplitudes and desensitization of currents during time in culture.

currents induced by 100 μM NMDA were normalized by capacitance (steady state current density), the current density increased strikingly with time in culture (fig. 2B; one-way ANOVA, $P < 0.001$), suggesting an increase in receptor density. The density of the peak currents also increased significantly with time in culture (data not shown; one-way ANOVA, $P < 0.001$). Because of the space clamp limitation of the whole-cell patch, the dendrites may not be well voltage clamped. Because the dendrites may grow with time in culture, the capacitance of currents from neurons with greater age may be underestimated. Nevertheless, this is the best effort to estimate the size of neurons. The increase in the density of NMDA-gated currents during time in culture is consistent with the increase in density of NMDA receptors on neurons with time in culture.[23]

The percent desensitization of NMDA-gated control currents is plotted in figure 2C. A decrease in percent desensitization with time in culture was observed (one-way ANOVA, $P < 0.001$). The representative control NMDA-gated current traces obtained from neurons cultured for 1–4 weeks are shown in figure 2D.

Concentration–response curves for steady state NMDA-gated currents from cortical neurons cultured for 1–4 weeks are plotted in figure 3. The response to each concentration of NMDA for each cell has been normalized to the maximum response by that neuron. The curves for neurons cultured for 1 and 2 weeks reach the plateaus at 30 μM and 100 μM NMDA, respectively. The curves for neurons cultured for 3 and 4 weeks reach the peaks at 100 μM NMDA and then decrease at higher concentrations of NMDA. Thus, 100 μM NMDA, the concentration used in this study, produced a maximum response in all neurons with different culture time. Because the curves for the neurons cultured for 3 and 4 weeks are not in sigmoid shape, the curves in this figure are not fitted by least squares fit.

Effect of Time in Culture on Sensitivity of NMDA-gated Currents to Isoflurane in Cultured Cortical Neurons

When NMDA (100 μM) with various concentrations of isoflurane was applied to the neurons cultured for 1–4 weeks, isoflurane concentration dependently inhibited

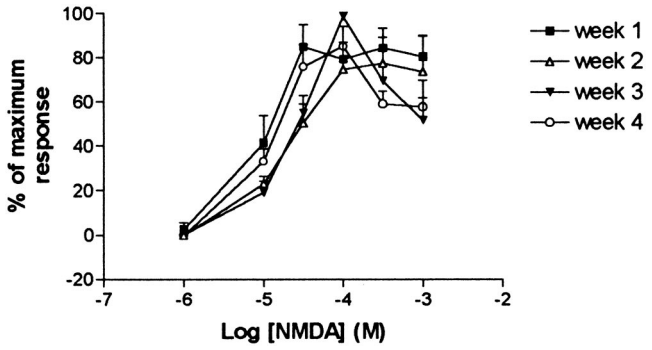

Fig. 3. Concentration–response curves for steady state NMDA-gated currents from cortical neurons cultured for 1–4 weeks. The response to each concentration of NMDA for each cell has been normalized to the maximum response by that neuron. Note the curves either reach the plateaus (week 1 and 2) or the peaks (week 3 and 4) at 100 μM NMDA. The n is 4 for 1 week, 5 for 2 and 4 weeks, and 6 for 3 weeks.

peak and steady state NMDA-gated currents. As shown in figure 4A, the concentration–response curve for steady state currents from 1-week-old neurons was shifted markedly to the left when compared with older cells, reaching a plateau at 10 μM isoflurane. The estimated EC_{50} values for neurons cultured for 1 and 2 weeks were 0.1 and 42.5 μM, respectively. EC_{50} values for neurons cultured for 3 and 4 weeks were not estimated because the concentration–response curves for those neurons did not reach a plateau. This decrease in sensitivity to isoflurane with time in culture was statistically reliable when the data obtained at concentrations of isoflurane applied to all ages of neurons were analyzed (two-way ANOVA, $P < 0.001$). Tukey HSD tests showed that the 1-week and 2-week curves differed from all others ($P < 0.05$), whereas there was no difference between weeks 3 and 4 ($P > 0.1$). The responses of the peak NMDA-gated currents to isoflurane are presented in figure 4B. The curves for peak currents were similar to those for steady state currents, except that the inhibition of peak currents by isoflurane was less than that of steady state currents (two-way ANOVA, $P < 0.001$ for each age). Representative current traces for the effect of various concentrations of isoflurane on NMDA-gated currents from individual neurons cultured for 1 and 2 weeks are illustrated in figures 4C and 4D, respectively.

Rapid Tolerance (Tachyphylaxis) to the Effect of Isoflurane on NMDA-gated Currents in Cultured Cortical Neurons

The NMDA-gated currents were relatively stable with a 1.5-min interval between applications; however, when the same concentration of isoflurane was applied repeatedly, tachyphylaxis to the effect of isoflurane occurred. This tachyphylaxis to isoflurane was determined on cortical neurons cultured for 12–17 days at different intervals of repeated application of isoflurane (ISO1 and ISO2). As a control, the currents induced by repeated application of NMDA alone (N1 and N2) at the same

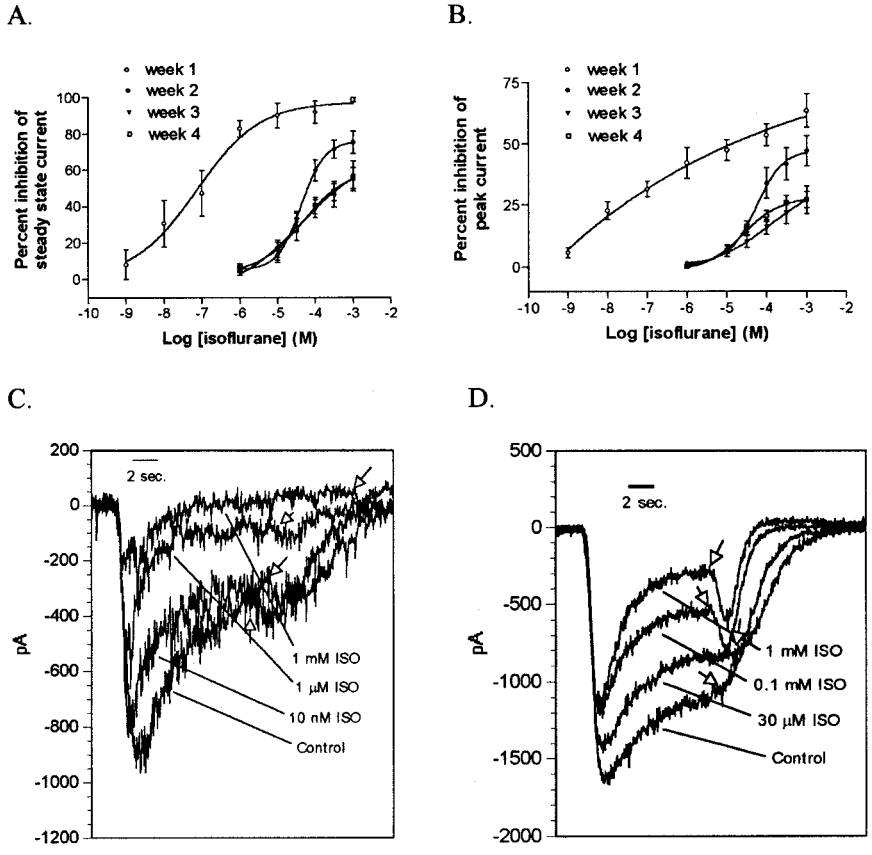

Fig. 4. Changes in the response of NMDA-gated currents to isoflurane in cultured cortical neurons with time in culture. (*A*) Inhibition by isoflurane of steady state currents as a function of the concentration of isoflurane in neurons cultured 1–4 weeks. The n for each group is 5 for 1 week, 8 for 2 weeks, 9 for 3 weeks, and 6 for 4 weeks. (*B*) Concentration-dependent inhibition of NMDA-gated peak currents by isoflurane. The n for each group is as the same as that in *A*. (*C* and *D*) Currents activated by 100 μM NMDA alone (control) or 100 μM NMDA + the indicated concentrations of isoflurane (ISO) from two neurons cultured for 1 (*C*) or 2 (*D*) weeks. The arrows indicate the termination of NMDA or NMDA + isoflurane. Note the hooked tail currents on the termination of NMDA + isoflurane in *D*.

interval between ISO1 and ISO2 were also recorded in the same neuron. Tachyphylaxis was considered to have occurred when the following criteria were met. First, percent inhibition by ISO2 of steady state currents was significantly less than that by ISO1. Second, the ratio of the amplitudes of steady state currents induced by NMDA and ISO1 (SS_{ISO1}) to those induced by NMDA and ISO2 (SS_{ISO2}) was significantly less than the ratio of the currents induced by N1 (SS_{N1}) to those induced by N2 (SS_{N2}). Judged by these criteria, tachyphylaxis did not occur at shorter intervals of ISO1 and ISO2 (15 and 30 s), but it occurred when the intervals were 45 s or longer. For example, when the interval between ISO1 and ISO2 was 3 min, which is the interval of application of isoflurane in the concentration–response measurements, the inhibition of steady state NMDA-gated currents by ISO2 was significantly less than that by ISO1 (59.2 ± 8.0% and 29.3 ± 8.0% for ISO1 and ISO2, respectively; paired Student *t* test, $P < 0.01$; n = 4; fig. 5A). In this group of neurons, SS_{ISO1}/SS_{ISO2} was significantly less than SS_{N1}/SS_{N2} (0.60 ± 0.03 and 1.04 ± 0.01 for SS_{ISO1}/SS_{ISO2} and SS_{N1}/SS_{N2}, respectively; paired Student *t* test, $P < 0.01$; n = 4). Normalized SS_{ISO1}/SS_{ISO2} is presented in figure 5B, where SS_{ISO1}/SS_{ISO2} was normalized by SS_{N1}/SS_{N2}. Although there is no significant difference between SS_{ISO1}/SS_{ISO2} and SS_{N1}/SS_{N2} at the intervals of 15 and 30 s, SS_{ISO1}/SS_{ISO2} is significantly less than SS_{N1}/SS_{N2} at intervals of 45 s and 3 min (paired Student *t* test, $P < 0.01$). Thus, when normalized by the control, the amplitudes of the currents induced by NMDA and 0.3 mM isoflurane virtually did not change over 15 and 30 s, whereas the amplitudes increased over 45 s and 3 min, indicating tolerance of the currents to isoflurane (fig. 5B). Because of possible tachyphylaxis, the order of drug application in the experiments determining concentration–response curves was low to high. Nevertheless, the responses of NMDA-gated currents to isoflurane after the first application of isoflurane may be underestimated. The effect of time in culture on tachyphylaxis was not studied.

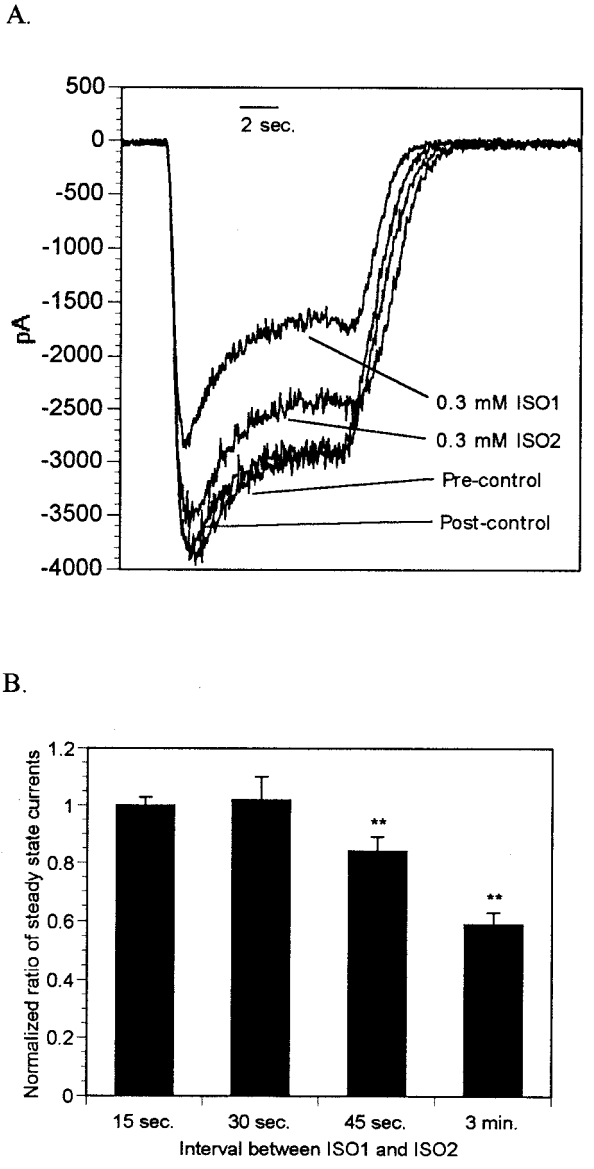

Fig. 5. Tolerance of NMDA-gated currents to repeated application of isoflurane in cultured cortical neurons. (*A*) Representative current traces are shown from a cultured cortical neuron with a 3-min interval between ISO1 and ISO2. Note less inhibition during application of ISO2. (*B*) Normalized ratios of steady state currents induced by ISO1 to those induced by ISO2. The ratios were normalized by the ratios of steady state currents gated by two presentations of NMDA alone (N1 and N2) at the same intervals of ISO1 and ISO2. The n is 6 for an interval of 15 s; 9 for a 30-s interval, 8 for a 45-s interval, and 4 for a 3-min interval. **$P < 0.01$.

Effect of Time in Culture on Hooked Tail Currents Induced by Isoflurane in Cultured Cortical Neurons

In some cortical neurons, a transient inward tail current appeared immediately on termination of coapplication of NMDA and the higher concentrations of isoflurane (see fig. 4D), which is interpreted as being the result of rapidly reversible channel blockade.[26] Occasionally, much smaller tail currents appeared on the termination of NMDA alone. At the highest concentration of isoflurane, hooked tail currents appeared in about a half of the tested neurons (16 of 28). The incidence of hooked tail currents decreased linearly with age of the cultured neurons (r = 0.98; $P < 0.05$) as shown in figure 6. These transient inward currents on termination of coapplication of NMDA and isoflurane were similar to the hooked tail currents induced by some NMDA open channel antagonists.[26–28]

Effect of Time in Culture on Sensitivity of NMDA-gated Currents to Ifenprodil in Cultured Cortical Neurons

The decreased desensitization to NMDA shown above is consistent with a decreased proportion of the NR2B subunit in NMDA receptors during development.[15–18]

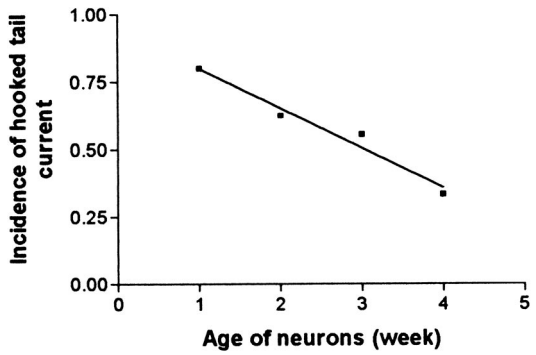

Fig. 6. Relationship between the incidence of hooked tail currents and time in culture for cortical neurons is shown. The incidence of hooked tail currents is calculated by the following equation: incidence of hooked tail currents = (number of neurons with hooked tail current/number of total neurons tested) × 100%.

Given that ifenprodil inhibits the function of heteromeric NMDA receptors that contain the NR2B subunit with a potency higher than that for receptors containing the other subunits,[23,24] the response of NMDA-gated currents to ifenprodil was investigated across time in culture. First, a concentration of ifenprodil selective for NMDA receptors containing the NR2B subunit under our experimental conditions was established by examining the effect of ifenprodil on NMDA-gated currents from HEK 293 cells expressing the NR1–1a and NR2A or NR2B subunits. Ifenprodil, 10 μM, inhibited steady state currents from the cells containing NR2B receptors by 93.2 ± 4.0%, whereas the inhibition in the cells containing NR2A receptors was only 5.7 ± 2.9% (fig. 7A). Thus, the effect of 10 μM of ifenprodil on NMDA-gated currents was used to identify the presence of the NR2B subunit in cultured cortical neurons.

The effects of ifenprodil on steady state NMDA-gated currents from neurons cultured for 1–4 weeks are presented in figure 7B. Time in culture significantly decreased the sensitivity of NMDA-gated currents to ifenprodil (one-way ANOVA, $P < 0.01$). Figures 7C and 7D show representative current recordings activated by 100 μM NMDA in the absence or presence of 10 μM ifenprodil from neurons cultured for 1 (fig. 7C) or 4 weeks (fig. 7D).

Differential Modulation by Isoflurane of NMDA-gated Currents Mediated by Recombinant NMDA Receptors

The findings of a decrease in the sensitivity of NMDA-gated currents to ifenprodil and a decrease in inhibition of NMDA-gated currents by isoflurane as a function of time in culture in the above studies would be consistent with an increased sensitivity of NMDA receptors containing NR2B subunits to isoflurane. Therefore, the effects of isoflurane on NMDA-gated currents from HEK 293 cells transiently transfected with cDNAs encoding NR1–1a + NR2A or NR1–1a + NR2B subunits were studied. NR1–1a

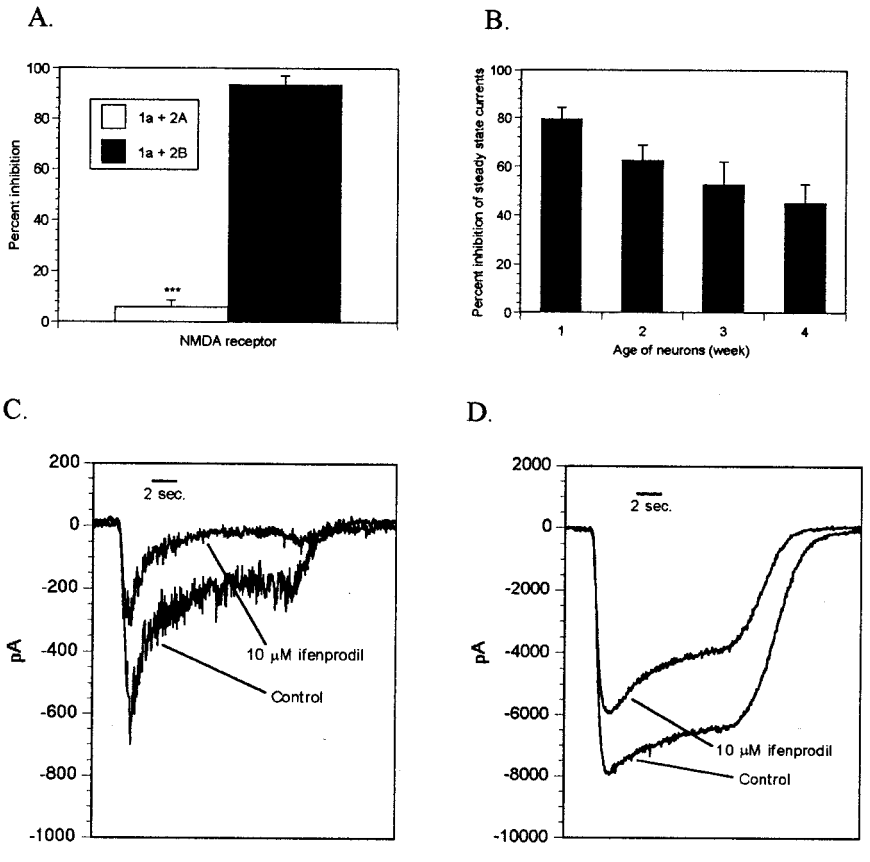

Fig. 7. Effect of time in culture on responses of NMDA-gated currents to ifenprodil in cultured cortical neurons. (*A*) Responses of NMDA-gated currents to 10 μM ifenprodil in HEK 293 cells expressing NR1–1a + NR2A (1a + 2A; n = 6) and NR1–1a + NR2B (1a + 2B; n = 9) receptors (Student *t* test, $P < 0.001$). ***$P < 0.001$. (*B*) Percent inhibition of steady state NMDA-gated currents by 10 μM ifenprodil in neurons cultured for 1–4 weeks with a significant linear trend ($P < 0.05$). The n is 5 for 1, 3, and 4 weeks; 7 for 2 weeks. (*C* and *D*) Illustrations showing inhibitory effect of ifenprodil on NMDA-gated currents in two neurons cultured for 1 week (*C*) or 4 weeks (*D*).

was chosen for this study because it is one of the most common splicing isoforms of NR1 subunit in the neocortex of the rat brain.[29] Concentration–response curves for the effect of isoflurane on HEK 293 cells transfected with cDNA for NR1–1a + NR2A or NR1–1a + NR2B receptor subunits are plotted in figure 8A. NR2B-containing NMDA receptors exhibited extremely high sensitivity to isoflurane. The curve for NR2B-containing receptors reached a plateau at 100 pM isoflurane, and the estimated EC_{50} was 2.04 pM. In contrast with the potent effect of isoflurane on NR2B subunit-containing receptors, the inhibition of steady state NMDA-gated currents mediated by NR1–1a + NR2A subunit-containing receptors was much less. The concentration–response curves for isoflurane in cells with NR1–1a + NR2A receptors did not reach a plateau. However, the maximum effect of 3 mM isoflurane was nearly a 100% inhibition of the steady state currents (92.9 ± 5.4%). Therefore, EC_{50} values were estimated based on the concentrations of isoflurane, which produced half of the maximum measured response. The EC_{50} value for isoflurane inhibition of NMDA responses in cells with NR1–1a + NR2A receptors was estimated as 29.5 μM. Thus, the NR2B subunit-containing receptors showed a markedly greater sensitivity to isoflurane when compared with receptors containing NR2A subunits.

A concentration-dependent tachyphylaxis was found in the responses of NR2B- and NR2A-containing receptors to a second application of isoflurane 1.5 min after the first application of the same concentration of isoflurane (two-way ANOVA, $P < 0.001$; figs. 8B and 8C). However, the maximum concentration of isoflurane produced no tachyphylaxis in either receptor subtype. The degree of tolerance was greatest at low concentrations for both receptor subtypes. The responses to either the first or the second application of isoflurane, at concentrations applied to both recombinant receptors, in HEK 293 cells expressing NR2A-containing receptors are less than those in cells with NR2B subunits (two-way ANOVA, $P < 0.001$).

Figure 9A and 9B illustrate current traces recorded during application of 100 μM NMDA alone and the application of 100 μM NMDA with various concentrations of isoflurane from HEK 293 cells expressing NR1–1a + NR2A or NR2B receptors, respectively.

In the absence of isoflurane, the percent desensitization of NMDA-gated currents mediated by NR2B-containing receptors (n = 19) was significantly greater than that mediated by NR2A-containing receptors (n = 16) (7.8 ± 2.3% and 17.5 ± 2.9% for NR2A and NR2B, respectively; Student *t* test, $P < 0.05$).

Hooked Tail Currents Induced by Isoflurane from HEK 293 Cells with NR2A- or NR2B-containing Receptors

As seen in cultured cortical neurons, hooked tail currents appeared on the termination of coapplication of NMDA and isoflurane in HEK 293 cells transiently transfected with cDNAs encoding either NR1–1a + NR2A or NR1–1a + NR2B subunits (fig. 9). The incidence of hooked tail currents mediated by receptors assembled from NR1–1a + NR2A subunits was significantly less

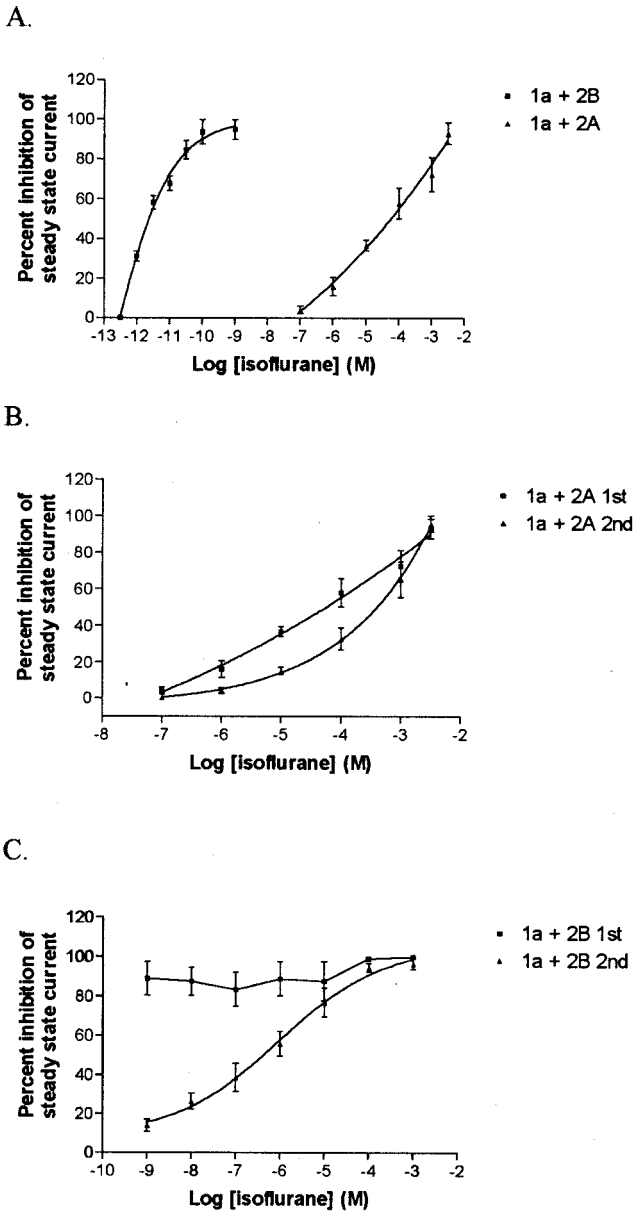

Fig. 8. Effect of subunit composition on isoflurane inhibition of NMDA-gated currents is shown. (*A*) Concentration–response curves for effects of isoflurane on NMDA-gated currents mediated by NR1–1a + NR2A (1a + 2A, n = 7) and NR1–1a + NR2B (1a + 2B, n = 6) subunits. (*B*) Concentration–response curves for the effect of repeated application of isoflurane on NMDA-gated currents mediated by NR1–1a + NR2A receptors (n = 7). The first application of isoflurane to cells expressing NR1–1a + NR2A subunits (1a + 2A 1st) produced a greater effect than the second application (1a + 2A 2nd). (*C*) Concentration–response curves for the effect of repeated application of isoflurane on NMDA-gated currents mediated by NR1–1a + NR2B receptors (n = 7). The first application of isoflurane to cells expressing NR1–1a + NR2B subunits (1a + 2B 1st) produced a greater effect than the second application (1a + 2B 2nd).

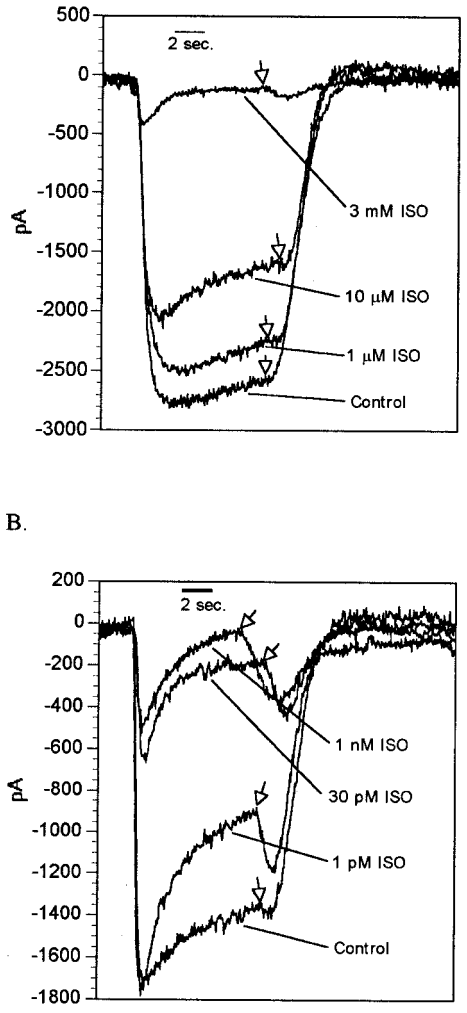

Fig. 9. Effect of isoflurane on NMDA-gated currents from HEK 293 cells expressing either the NR2A or NR2B subunit is shown. Current traces recorded during the application of NMDA alone and the first application of isoflurane from two HEK cells expressing NR1–1a + NR2A (*A*) or NR1–1a + NR2B subunits (*B*). The arrows indicate the termination of NMDA alone or NMDA + isoflurane. Note hooked tail currents on the termination of coapplication of NMDA and isoflurane, and the difference in the amplitudes of hooked tail currents from NR2A and NR2B subunit-containing receptors.

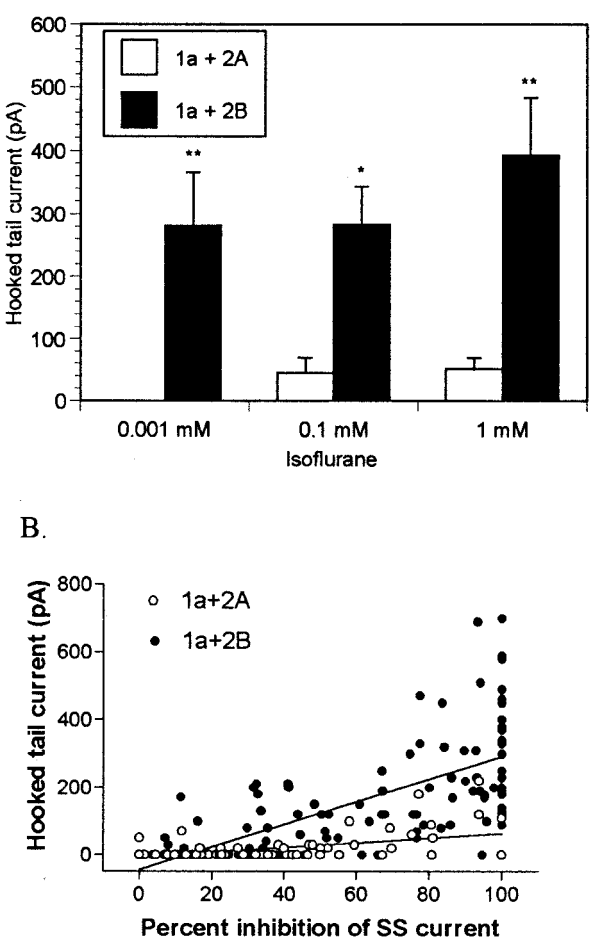

Fig. 10. Effect of subunit composition on hooked tail currents induced by isoflurane. (*A*) The amplitudes of hooked tail currents induced by isoflurane from NR1–1a + NR2A (1a + 2A, n = 7) and NR1–1a + NR2B (1a + 2B, n = 7) receptors (two-way ANOVA, $P < 0.001$ for NR2A *vs.* NR2B). Isoflurane, 0.001 mM, did not induce hooked tail current in cells expressing NR1–1a + NR2A receptors (0 pA for the current). *$P < 0.05$; **$P < 0.01$ (*post hoc* Tukey HSD test). (*B*) Correlation of the amplitudes of hooked tail currents and percent inhibition of steady state currents produced by isoflurane from recombinant NMDA receptors. The abscissa is percent inhibition of steady state current. Linear regression lines are shown for the data from both receptors.

than that by receptors assembled from NR1–1a + NR2B subunits (6 of 14 and 17 of 18 for NR2A- and NR2B-containing receptors, respectively; Fisher exact test, $P < 0.01$). Further, the amplitudes of hooked tail currents induced by given concentrations of isoflurane in the cells with NR2A-containing receptors were lower than those with NR2B-containing receptors (fig. 10A; two-way ANOVA, $P < 0.001$). There were no significant differences between the mean amplitudes of control NMDA-gated currents for each concentration of isoflurane in cells with NR2A- and NR2B-containing receptors (data not shown). Thus, it is unlikely that the amplitudes of control currents contributed to the difference in hooked tail currents mediated by two subtypes of NMDA receptors.

The amplitude of hooked tail current depends on the degree of inhibition of NMDA-gated currents by channel blockers.[26–28] Thus, the smaller hooked tail currents mediated by NR1–1a + NR2A receptors compared with those mediated by NR2B-containing receptors could be secondary to the decreased inhibition of NMDA-gated currents by isoflurane in cells with NR2A-containing receptors. To answer this question, the correlation of the amplitudes of hooked tail currents and percent inhibition of steady state currents by isoflurane was plotted in figure 10B. Significant correlation between amplitudes

of the tail currents and inhibition of steady state currents was found for both receptor subtypes ($r = 0.5$, $P < 0.05$, for NR1–1a + NR2A receptors; $r = 0.67$, $P < 0.05$, for NR1–1a + NR2B receptors). The correlation indicates an inhibition dependence of the amplitudes of hooked tail currents mediated by both receptors. However, the slope of linear regression for the correlation points from NR2A-containing receptors was significantly less than that for the points from NR2B-containing receptors (0.69 ± 0.14 and 3.37 ± 0.40 for NR2A and NR2B, respectively; Student *t* test, $P < 0.001$). The difference in slopes indicates that a smaller hooked tail current would be induced in a cell with NR2A-containing receptor than that with NR2B-containing receptor at a given percent inhibition of steady state current.

Discussion

Sensitivity of NMDA-gated Currents to Isoflurane

The inhibition of NMDA-gated currents by isoflurane in the present study is in agreement with previous work showing effects of volatile anesthetics on NMDA-gated currents in oocytes transiently expressing NMDA receptors,[5] in brain slice preparations,[7] and in cultured hippocampal and cortical neurons.[3,4] However, although the EC_{50} value in the low μM range is consistent with a previous report of cultured cortical neurons,[4] it is much lower than that reported in oocytes[5] and in cultured hippocampal neurons, in which the effect of isoflurane on NMDA-stimulated increase in intracellular calcium was studied.[30] The most likely explanation for the discrepancy is that in the studies in which higher EC_{50} values were found, isoflurane was delivered in the bath.[5,30] Thus, the tachyphylaxis observed in the present study would have occurred before the NMDA was applied, resulting in decreased receptor sensitivity.

The present study is the first, to our knowledge, to show tachyphylaxis to the effect of a volatile anesthetic on NMDA-gated currents. In cultured neurons, the second application of 0.3 mM isoflurane produced approximately one half of the inhibition of the initial application when the interval between the first and second application was 3 min. Tachyphylaxis was not present 15 or 30 s after a brief application of NMDA and isoflurane, but it was present at 45 s and was greater at 3 min. Thus, the onset of tachyphylaxis was delayed for several seconds and did not require the continued presence of either drug. This delayed development of tolerance suggests that a cascade of events is initiated by isoflurane that requires seconds to complete. A logical candidate for such a cascade is activation of a second messenger mechanism. Further investigations will be required to assess this phenomenon.

Changes in Sensitivity of NMDA Receptors to Isoflurane with Time in Culture

Sensitivity to the effect of isoflurane on NMDA-gated currents decreased with time in culture. The decrease in sensitivity to isoflurane was observed during a developmental period associated with a shift from a predominance of NR2B subunit-containing receptors (early in development) to an increased proportion of NR2A receptor subunits (2 or 3 weeks) observed *in vivo* and *in vitro*.[19–21] The changes in desensitization rate of NMDA-gated currents and sensitivity to the NR2B selective antagonist ifenprodil observed in the present study are consistent with this view. The high degree of ifenprodil sensitivity in the 1-week-old neurons indicated that the majority of the receptors contained the NR2B subunit. The decrease in ifenprodil sensitivity with time in culture could be the result of replacement of the NR2B subunit with any of the other NR2 subunits, as a lack of response to ifenprodil does not distinguish between these latter subunits. Thus, one possible source of the changes in sensitivity to isoflurane over time in culture is the change from receptors composed primarily of NR2B subunits to those containing other NR2 subunits. Although developmental changes in the splice variants of NR1 have been reported,[14] ifenprodil inhibition appears independent of the NR1 isoforms.[31] As a test of the hypothesis that changes in NR2 subunits may play a role in the changes in sensitivity to isoflurane in cultured neurons over time, the sensitivity to isoflurane of HEK 293 cells expressing known combinations of specific NR1 and NR2 subunits was examined. In HEK 293 cells expressing NR2A or NR2B subunits with the NR1–1a subunit, NMDA-gated currents mediated by NR2B-containing receptors were more sensitive to isoflurane than currents mediated by NR2A-containing receptors. This difference in isoflurane sensitivity of NMDA-gated currents is consistent with the hypothesis that changes in NMDA receptor subunit composition during time in culture mediate at least a part of the change in responses to isoflurane over time from cultured neurons. However, the sensitivities to isoflurane of NMDA-gated currents in the neurons cultured for 1 week (EC_{50}, 0.1 μM) and in HEK 293 cells expressing NR1–1a + NR2B receptors (EC_{50}, 2.04 pM) are different. The 100,000-fold difference in sensitivity between the cultured cortical neurons and HEK 293 cells cannot be explained by a simple dilution of the NR2B-containing subunits in the neurons by a few NR2A-containing subunits. One possible explanation for this difference in sensitivity to isoflurane would be that the NR1–1a subunit used in the HEK 293 cells might produce an unusually sensitive receptor when coassembled with an NR2B subunit. This explanation is unlikely because the NR1–1a subunit would have to be almost completely absent in the neurons to cause such a large effect. It has been demonstrated that the NR1–1a and NR1–1b subunits are present in roughly equal

amounts at 1 week.[14] Finally, HEK 293 cells are not neurons and may have elements that alter the sensitivity of NMDA receptors to isoflurane that differ from cultured cortical cells. Such inconsistencies are not uncommon when comparing the pharmacologic and biophysical properties of recombinant and native NMDA receptors.[10,32] Nevertheless, the robust difference in sensitivity to isoflurane by the NR2A and NR2B subunit-containing receptors suggests that there may be regional differences in the effect of isoflurane based on the distribution of these subunits.

There was a robust decrease in the effectiveness of a second application of isoflurane in HEK 293 cells transfected with either NR2A or NR2B subunits just as was observed in cultured cerebral cortical neurons. This tachyphylaxis is demonstrated in the "second dose" concentration–response curves by shifting the curves to the right approximately an order of magnitude for the NR2A-containing receptors and up to four orders of magnitude for the NR2B-containing receptors. For both receptor subtypes, the degree of tachyphylaxis was concentration dependent, with tolerance most evident at low concentrations. The greater degree of tachyphylaxis associated with presence of the NR2B subunit partially offsets the increased sensitivity of receptors containing this subunit to isoflurane. This may explain the failure of previous studies using a slow bath application of isoflurane to the oocyte preparation to detect a differential effect of isoflurane on NR2A and NR2B subunit-containing receptors.[5]

Implications for Isoflurane Anesthesia

At first glance, the robust effect of isoflurane on NMDA-gated currents at concentrations well below 1 MAC suggests that this action may be responsible for the mental confusion observed during recovery from anesthesia. However, this low-dose effect of isoflurane is likely present only transiently. In a clinical situation where anesthesia is delivered by inhalation, the low-dose effects would be lost because of tachyphylaxis. Thus, even if the results from cultured rat cerebral cortical neurons transfer directly to the intact human, it is unlikely that the effect of low concentrations of isoflurane on NMDA-gated currents plays a major role in the recovery room.

In the present study, sensitivity of the NMDA receptor to isoflurane decreased with time in culture. This finding may suggest a greater sensitivity of the fetus or of neonates to the effect of isoflurane on NMDA-gated currents. Because the NMDA system is involved in neuronal development, such an effect could be deleterious. However, the degree to which time in culture translates to development *in situ* is not known. Primary cultured neurons have been used to determine changes in the effects of physiologic or pharmacologic agents on NMDA receptors during neuronal development.[22–25] The increase in density of NMDA receptors and the change from a uniformly high affinity for ifenprodil to progressively lower affinity during development of rat brain were also found in cultured neurons.[23] Similarly, the developmental progression from a predominance of NR2B subunits to an increased proportion of NR2A subunits has been detected in rat brain and cultured rat cortical neurons.[14–21] However, the developmental change in the sensitivity of NMDA receptors to spermine that was observed *in vivo* was not seen in cultured cortical neurons,[33] suggesting that some aspects of development of the glutamate receptor system do not occur in culture as they do *in situ*. Taken together, although caution should be taken to explain developmental changes *in situ* based on the data obtained from cultured neurons, time in culture appears to model developmental changes resulting from alteration in NR2A and NR2B subunits. Therefore, the effects of time in culture on sensitivity of NMDA-gated currents to isoflurane and ifenprodil observed in the present study suggest that similar changes may occur during *in situ* development.

Implications for a Mechanism of the Effect of Isoflurane on NMDA-gated Currents

The mechanism underlying the effect of volatile anesthetics on ligand-gated ion channels is not presently known. However, progress is being made using mutated receptor subunits to locate molecular sites related to anesthetic action.[34] The present work offers some hints into the mechanism of anesthetic action on the NMDA receptor. First, the action is rapid. When isoflurane and NMDA were applied simultaneously, there was a decrease in the peak NMDA-gated current observable in a millisecond time frame. This observation rules out many indirect modes of action of isoflurane on the NMDA receptor. Second, the robust difference between the response of NR2A and NR2B subunit-containing receptors points to a high degree of receptor specificity for an important factor in transduction of the effect of isoflurane on NMDA receptor function. However, it does not indicate whether that factor is a site of direct interaction of isoflurane with the receptor. Third, the high degree of tachyphylaxis indicates that there is a plasticity of the response of the NMDA receptor to isoflurane. The difference in the degree of tachyphylaxis between the NR2A- and NR2B-containing receptors indicates a possible relation between variables determining sensitivity to isoflurane and variables determining the degree of tachyphylaxis observed.

Hooked tail currents were observed on cotermination of NMDA and isoflurane. The occurrence of these currents has been interpreted as being the result of either rapidly reversible channel blockade[26] or prevention of channel closure by an open channel "foot-in-the-door" mechanism.[28] Because the open channel blockers are charged,[27,28] whereas isoflurane is uncharged, it is likely that the observed hooked tail currents are the result of rapid dissociation of isoflurane from the NMDA receptor

allowing the still bound NMDA to gate a current.[26] The observed decrease in the incidence of tail current with time in culture and the increased tail currents when the NR2B subunits were compared with NR2A subunits would be consistent with this latter interpretation because receptors containing the NR2B subunit have a higher affinity for NMDA.[35] However, the increased sensitivity to isoflurane conferred by the NR2B subunit would counteract this effect if the isoflurane acted according to a standard high-affinity binding model. Because hooked tail currents are related to gating kinetics of the channel and the interactions of agonist, antagonist, and receptor,[26,28] the mechanism underlying the difference in hooked tail currents mediated by two subtypes of NMDA receptors remains to be clarified.

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