Isoflurane Impairs Learning and Hippocampal Long-term Potentiation via the Saturation of Synaptic Plasticity

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ABSTRACT

Background: General anesthesia induces long-lasting cognitive and learning deficits. However, the underlying mechanism remains unknown. The GluA1 subunit of AMPAR is a key molecule for learning and synaptic plasticity, which requires trafficking of GluA1-containing AMPARs into the synapse.

Methods: Adult male rats were exposed to 1.8% isoflurane for 2 h and subjected to an inhibitory avoidance task, which is a hippocampus-dependent contextual fear learning paradigm (n = 16 to 39). The in vitro extracellular field potential of hippocampal synapses between the Schaffer collateral and the CA1 was evaluated using a multielectrode recorder (n = 6 per group). GluA1 expression in the synaptoneurosomes was assessed using Western blotting (n = 5 to 8). The ubiquitination level of GluA1 was evaluated using immunoprecipitation and Western blotting (n = 7 per group).

Results: Seven days after exposure to 1.8% isoflurane for 2 h (Iso1.8), the inhibitory avoidance learning (control vs. Iso1.8; 294 ± 34 vs. 138 ± 28, the mean ± SEM [%]; P = 0.002) and long-term potentiation (125.7 ± 6.1 vs. 105.7 ± 3.3; P < 0.001) were impaired. Iso1.8 also temporarily increased GluA1 in the synaptoneurosomes (100 ± 9.7 vs. 138.9 ± 8.9; P = 0.012) and reduced the GluA1 ubiquitination, a main degradation pathway of GluA1 (100 ± 8.7 vs. 71.1 ± 6.1; P = 0.014).

Conclusions: Isoflurane impairs hippocampal learning and modulates synaptic plasticity in the postanesthetic period. Increased GluA1 may reduce synaptic capacity for additional GluA1-containing AMPARs trafficking. (Anesthesiology 2014; 121:302-10)

POSTOPERATIVE cognitive dysfunction, including learning deficits, has drawn much attention because it negatively affects the quality of life of patients and increases mortality after surgery. General anesthesia has been postulated as a potential cause of postoperative cognitive dysfunction; however, the evidence regarding this issue is conflicting. Some animal studies have revealed prolonged impairment in learning after general anesthesia, whereas others have shown a lack of effect, and even improvement in learning after general anesthesia. Moreover, the electrophysiological and molecular mechanisms underlying such changes remain unclear.

Contextual learning—an important aspect of cognition—is critically dependent on synaptic plasticity in the hippocampus (e.g., long-term potentiation [LTP] and long-term depression [LTD]), especially regarding synapses established between the Schaffer collateral and the CA1 region (SC/CA1). Excitatory synaptic transmission is regulated mainly by AMPARs. AMPARs are ionotropic glutamate receptors that form various sets of tetramers consisting of a combination of four subunits (GluA1–4). Recently, studies have shown that LTD and contextual learning require the incorporation of GluA1-containing AMPARs into synapses (i.e., trafficking) via the phosphorylation of the cytoplasmic domains of GluA1. Paradoxically, artificial manipulations that promote excessive insertion of AMPARs into synapses interfere with subsequent additional insertion of AMPARs, indicating that animals hardly learn additional information once the learning capacity is saturated.

The purposes of this study were threefold. First, we sought to elucidate the temporal profile of cognitive function up to 28 days after isoflurane anesthesia by using the

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inhibitory avoidance (IA) test in the rat. The IA is a robust test of learning that involves contextual fear conditioning; the hippocampus has been clearly defined as the neural structure that is responsible for this process. Second, we attempted to identify the electrophysiological correlates of the isoflurane-induced impairment in IA learning by examining LTP and LTD in the hippocampus. This was based on a previous report that stated that contextual learning induces synaptic potentiation in the SC/CA1 hippocampal pathway.8 Third, we tested the hypothesis that isoflurane affects GluA1-containing AMPARs in the hippocampus,16 especially their capacity to traffic into synapses, because such trafficking is important for learning and synaptic plasticity.

Materials and Methods

Animals
Male Wistar–Imamichi rats (Japan SLC, Shizuoka, Japan) weighing 250 to 320 g were used in all experiments. Rats were housed in an animal-care facility on a 14/10-h light/dark cycle (lights on from 5:00 AM to 7:00 PM) with ad libitum access to water and food. All animal housing and experimental procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Animal Research Center, Yokohama City University Graduate School of Medicine, Yokohama, Japan (approved protocol F-A-12–033). After purchase, all rats were allowed to rest for at least 1 week before they were used in the experiments.

Exposure to Isoflurane
To induce general anesthesia, rats were placed in a translucent plastic chamber (length, 30 cm; width, 43 cm; and height, 14 cm) within a thermostatic bath (30° ± 2°C). The chamber was continuously flushed with a carrier gas consisting of oxygen and nitrogen (FIO2 = 0.33) at 6 l/min, and rats were allowed to breathe spontaneously.4,17 The rectal temperature of the animals was maintained at 37°C ± 0.5°C. The concentration of isoflurane was maintained at 0.6, 1.2, or 1.8% for 2 h. These concentrations correspond to 0.4, 0.8, and 1.3 minimum alveolar concentration, respectively, because 1 minimum alveolar concentration of isoflurane is 1.4% in the adult rat.18,19 Carbon dioxide in the chamber was maintained at less than 3 mmHg. These gases were monitored using a Capnomac ULTIMA monitor (Datex, Helsinki, Finland). In the control group, one rat at a time was placed in a plastic chamber flushed with the same carrier gas for 5 min, and was then returned to its original cage. This was intended to expose the control animals to the same stress of handling that the isoflurane-treated animals experienced before being anesthetized.

Physiological Monitoring
For the assessment of hemodynamics and arterial blood gases, the femoral artery was catheterized under brief isoflurane anesthesia, as described previously.20 After a 1-h period of recovery from surgery, rats were exposed to 1.8% isoflurane for 2 h. Blood pressure, heart rate, and arterial blood gases were measured immediately before and 60 and 120 min after the start of isoflurane administration. Arterial blood gases were analyzed using a Rapidlab 860 blood gas analyzer (Bayer HealthCare Diagnostics, Tarrytown, NY).

Behavioral Assessment
The IA testing was performed 24 h, 72 h, 7 days, or 28 days after exposure to 1.8% isoflurane for 2 h (n = 16, 16, 28, and 24, respectively). The control rats (n = 39) underwent the IA testing 7 days after brief placement in the anesthetic chamber as described at the first sentence in Exposure to Isoflurane section. Based on the results of these experiments that the effect of 1.8% isoflurane was significant on day 7, the effects of 0.6 or 1.2% isoflurane 7 days before the IA testing were also investigated (n = 24 and 21, respectively) to examine the concentration dependency. Each rat was tested only once and not retested, as described previously.13 On the day of the IA testing, rats were trained and then tested in an IA apparatus (length, 27 cm; width, 45 cm; and height, 25 cm) that was placed in a sound-shielded room during the light phase (9:00 AM to 3:00 PM). A trap door separated a lighted starting box and a dark shock box. Rats were observed using a remote camera recorder.

In the training session, each rat was placed in the lighted box and allowed to explore it for 30 s. After the opening of the trap door, the rat entered the dark box driven by anxiety. Subsequently, we closed the trap door and applied a scrambled electrical foot shock (2 s, 0.8 mA) via the electrified floor by using an SG-1000 shock generator (Melquest, Toyama, Japan). Ten seconds after the cessation of the foot shock, the rat was returned to its original cage. The latency to enter the dark box in this training session was recorded as a measure of the general level of behavioral activity.

The retention trial was performed 30 min after the training session. Each rat was again placed in the lighted box. The latency to reenter the dark box was recorded as a measure of learning performance (i.e., latency after IA learning; the maximum cutoff latency was set at 480 s). Longer latencies were interpreted as better memory retention.

Electrophysiological Recordings
For brain slice preparation, separate groups of rats underwent 1.8% isoflurane anesthesia for 2 h or were handled as controls, and 7 days later, their brains were quickly transferred into ice-cold dissection buffer under brief isoflurane anesthesia.21 Coronal brain slices were cut at 300 μm (Linear Slicer Pro7; Dosaka, Kyoto, Japan) and transferred to artificial cerebrospinal fluid (22°C to 25°C).22 One brain slice was obtained from each animal, and each slice was used only once and discarded. Because each group contains six animals (i.e., six slices) and we used three different simulating...
frequencies (see Electrophysiological Recordings), we used total 36 animals per slices.

The acquisition of data on extracellular field excitatory postsynaptic potentials (fEPSPs) and their analysis were performed using the multielectrode MED64 system (Alpha MED Scientific, Osaka, Japan). Extracellular fEPSPs were evoked by stimulating SCs at 0.067 Hz and were recorded in the CA1 area by using a 64-channel array (150-μm inter-polar distance; MED-P515A; Alpha MED Scientific). The intensity of stimuli was adjusted to produce a 50% maximal response. The slope of fEPSPs was measured at the time at which peak amplitude was observed and 1 ms after the application of the stimulus.

Extracellular fEPSPs were monitored for 30 min before the evoking stimuli, to confirm the stability of the baseline potential. Each slice received evoking stimuli at one of three frequencies: high-frequency stimulus (HFS; 100 Hz, 2 trains of stimulation for 1 s applied 25 s apart), intermediate-frequency stimulus (IFS; 10 Hz, 900 pulses), and low-frequency stimulus (LFS; 1 Hz, 900 pulses). These frequencies were chosen based on the knowledge that HFS, IFS, and LFS induce LTD and LTD, respectively.21,22,24 The averaged fEPSP slopes between 36 and 40 min after the delivery of evoking stimuli were normalized against the averaged fEPSP slopes for the 10 min immediately before the delivery of evoking stimuli, and then they were statistically analyzed.

Western Blotting
The amount of GluA1 was assayed 1, 7, and 28 days after exposure to 1.8% isoflurane for 2 h or after control manipulation. Hippocampal regions were dissected rapidly and stored at −80°C until assayed. Synaptoneurosomal fractions (a fraction enriched in synaptic terminals) were prepared using a filtration method, as described previously.25–28 Briefly, homogenates were filtered through two 100-μm-pore nylon mesh filters and then through a 5-μm-pore polyvinylidene fluoride filter (Millipore, Bedford, MA). Whole lysates were also extracted, according to methods described in previous reports.25–28 Western blotting analyses were performed using primary antibodies against GluA1 (1:1,000; Millipore, Bedford, MA) and β-actin (1:100,000; Sigma, St. Louis, MO), as described previously.25–28

Quantitative Real-time Polymerase Chain Reaction
The levels of expression of GluA1 messenger RNA (mRNA) in the whole lysates of the dorsal hippocampus were assayed 7 days after exposure to 1.8% isoflurane for 2 h or control manipulation by using quantitative real-time polymerase chain reaction, according to the methods described in a previous report.10 The sequence-specific primers used (reported elsewhere) were as follows: GluA1 (5′–CGAGTTCTGCTACAAATCCCG–3′ and 5′–TGTCGCTATGGCTTCTAGTG–3′ (M38060)) and β-actin (which was used as a housekeeping gene, 5′–TGACGTGACATCGTAAAGAC–3′ and 5′–AGAGCCACCAAATCCACA–3′ (NM031144.3)).29

Immunoprecipitation
The ubiquitination levels of GluA1 7 days after exposure to 1.8% isoflurane for 2 h or control manipulation were examined by immunoprecipitating the whole lysates of the dorsal hippocampus with antibodies against GluA1 (Millipore), according to the manufacturer’s instructions and previously described methods.30 Precipitated proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane, and blotted with an antiubiquitin antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA). To normalize ubiquitination levels against GluA1 amount, the precipitated proteins were also blotted with the anti-GluA1 antibody.

Statistical Analyses
All data are presented as the mean ± SEM. Each rat and slice was tested only once and not retested in either the behavioral or electrophysiological assay, respectively. The statistical significance of intergroup differences in the physiological monitoring and the behavioral assessment were determined using a one-way ANOVA followed by a post hoc Dunnett test, using time point or isoflurane concentration as the single factor. For the electrophysiological recordings, we used two-way factorial ANOVA using exposure to isoflurane and stimulus frequency as two factors, followed by between-group (isoflurane and control at each of three frequencies: HFS, intermediate-frequency stimulus, or LFS) post hoc Student t tests with Bonferroni correction. For GluA1 assays, we also used two-way factorial ANOVA using time point and exposure to isoflurane as two factors, followed by between-group (isoflurane and control on day 1, 7, or 28) post hoc Student t tests with Bonferroni correction. The quantification of GluA1 mRNA and GluA1 ubiquitination were compared between groups (isoflurane and control on day 7) by using Student t tests. All analyses were performed using two-tailed tests. Except for post hoc paired comparisons with Bonferroni correction, which were considered significant at 0.05/3 (P = 0.0167), differences between groups were considered statistically significant at P value less than 0.05. All statistical analyses were performed using the SPSS software, version 17.0 (IBM, New York, NY).

Results
Contextual Learning Is Impaired after Exposure to Isoflurane
After anesthesia with 1.8% isoflurane for 2 h, the latencies in the IA test showed a tendency toward prolongation (i.e., improvement in learning) on day 1, but were significantly shortened (impairment in learning) on day 7 (fig. 1, A and B; control [n = 39], 294 ± 34 s; day 1 [n = 16], 379 ± 37 s;
The latencies on day 28 were not different from the control value. The administration of isoflurane at different concentrations 7 days before the IA test led to significantly shortened latencies only in the presence of 1.8% isoflurane (fig. 1, C and D; control [n = 39], 294 ± 34 s; 0.6% isoflurane [n = 24], 181 ± 37 s; 1.2% isoflurane [n = 21], 213 ± 39 s; and 1.8% isoflurane [n = 28], 138 ± 28 s; F[3, 108] = 4.261, P < 0.007; P = 0.003 at control vs. 1.8% isoflurane). The general level of behavioral activity, as reflected in the latencies observed in the training session, remained unchanged during 28 days after anesthetic exposure (data not shown). Hemodynamic and arterial blood gas parameters remained within normal physiological ranges during the 2-h anesthesia with 1.8% isoflurane (table 1). These results indicate that exposure to 1.8% isoflurane for 2 h significantly impairs contextual learning 7 days later.

**Hippocampal Synaptic Plasticity Is Modulated by Previous Exposure to Isoflurane: LTP Impairment and LTD Augmentation**

We assessed the synaptic plasticity in the SC/CA1 hippocampal pathway by using multielectrode array electrophysiology (fig. 2A). Changes of fEPSP slopes between 36 and 40 min after the evoking stimulus were significantly affected by a 2-h exposure to 1.8% isoflurane 7 days before slice preparation and the nature of the stimuli (isoflurane, F[1, 30] = 48.464, P < 0.001; stimuli, F[2, 30] = 50.975, P < 0.001; isoflurane × frequency, F[2, 30] = 4.270, P = 0.023).

High-frequency stimulus induced LTP, the potentiation of fEPSPs, in control animals. However, in the isoflurane group, HFS-induced LTP was significantly impaired 7 days after 2 h of 1.8% isoflurane exposure (fig. 2B; control [n = 6], 125.7 ± 2.5%; isoflurane [n = 6], 105.70 ± 1.35%; P < 0.001). There were no significant

### Table 1. Physiological Variables during Anesthesia

<table>
<thead>
<tr>
<th>Hemodynamic variables</th>
<th>Recovery At 0 h</th>
<th>During Anesthesia At 1 h</th>
<th>At 2 h</th>
<th>P Value</th>
<th>F Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>330 ± 17</td>
<td>351 ± 20</td>
<td>356 ± 15</td>
<td>0.075</td>
<td>F(2, 17) = 3.383</td>
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<tr>
<td>SBP, mmHg</td>
<td>118 ± 9</td>
<td>104 ± 8</td>
<td>104 ± 8</td>
<td>&lt;0.01</td>
<td>F(2, 17) = 19.196</td>
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<tr>
<td>DBP, mmHg</td>
<td>88 ± 7</td>
<td>74 ± 5</td>
<td>72 ± 9</td>
<td>&lt;0.01</td>
<td>F(2, 17) = 8.797</td>
</tr>
<tr>
<td>MBP, mmHg</td>
<td>102 ± 6</td>
<td>90 ± 7</td>
<td>88 ± 10</td>
<td>&lt;0.01</td>
<td>F(2, 17) = 22.399</td>
</tr>
<tr>
<td>ABG analysis</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>pH</td>
<td>7.43 ± 0.07</td>
<td>7.37 ± 0.02</td>
<td>7.39 ± 0.01</td>
<td>0.082</td>
<td>F(2, 17) = 3.228</td>
</tr>
<tr>
<td>PacO2, mmHg</td>
<td>41.3 ± 4.4</td>
<td>46.2 ± 3.3</td>
<td>45.6 ± 2.7</td>
<td>0.098</td>
<td>F(2, 17) = 2.941</td>
</tr>
<tr>
<td>Pao2, mmHg</td>
<td>90.4 ± 13.9</td>
<td>183 ± 16.4</td>
<td>168 ± 5.6</td>
<td>&lt;0.01</td>
<td>F(2, 17) = 66.752</td>
</tr>
<tr>
<td>Sato2, %</td>
<td>94.8 ± 1.5</td>
<td>97.5 ± 0.4</td>
<td>98.1 ± 1.2</td>
<td>&lt;0.01</td>
<td>F(2, 17) = 25.4</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>155 ± 15.9</td>
<td>175 ± 13.8</td>
<td>170 ± 3.7</td>
<td>0.093</td>
<td>F(2, 17) = 3.029</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SEM (n = 6). The significance of differences between data groups was evaluated using ANOVA.

ABG = arterial blood gas; DBP = diastolic blood pressure; HR = heart rate; MBP = mean blood pressure; PaCO2 = partial pressure of carbon dioxide; PaO2 = partial pressure of oxygen; Sato2 = saturation of arterial oxygen; SBP = systolic blood pressure.

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changes between the control and isoflurane groups after intermediate-frequency stimulus (fig. 2C; control [n = 6], 108.9 ± 2.9%; isoflurane [n = 6], 102.7 ± 1.6%; P = 0.086). LFS induced a slight synaptic depression in control animals, however, animals that had been exposed to isoflurane exhibited significantly pronounced fEPSP depression (fig. 2D; control [n = 6], 98.60 ± 1.7%; isoflurane [n = 6], 78.3 ± 4.8%; P = 0.003). Thus, LFS-induced LTD was enhanced by isoflurane. Collectively, these results suggested that a 2-h exposure to 1.8% isoflurane 7 days earlier prevented synaptic potentiation and enhanced synaptic depression (fig. 2E).

Isoflurane-induced Learning Impairment Is Associated with Synaptic Saturation via the Prevention of the Ubiquitination of GluA1

To determine whether isoflurane induces any alterations in the expression levels of the GluA1 subunit in the CA1 region of the hippocampus, we compared the synaptoneurosomal level of GluA1 in control animals with that of animals exposed to 2 h 1.8% isoflurane 1, 7, or 28 days earlier (fig. 3A). Synaptoneurosomal GluA1 levels were significantly affected by exposure to 1.8% isoflurane (isoflurane, F{1, 27} = 48.464, P < 0.001; stimuli, F{2, 30} = 50.975, P < 0.001; isoflurane × frequency, F{2, 30} = 4.270, P = 0.023). High-frequency stimulus–induced long-term potentiation was impaired significantly (n = 6 per group; *P < 0.001). Intermediate-frequency stimulus induced no significant changes (n = 6 per group; P = 0.86). Low-frequency stimulus–induced long-term depression was enhanced significantly (n = 6 per group; #P = 0.003). All statistical analyses were performed using two-way factorial ANOVA (isoflurane × frequency, significance level was set at P < 0.05) followed by Student t tests (significance level was set at 0.05/3 = 0.0167 with Bonferroni correction). Data are shown as mean ± SEM.
was significantly increased at day 7 after isoflurane exposure (fig. 3B; control [n = 6], 100 ± 28.1%; isoflurane [n = 5], 121.5 ± 15.2%; *P = 0.498).

On the contrary, the amount of GluA1 in the whole lysates did not change significantly (isoflurane, F(1, 30) = 0.620, P = 0.437; day, F(2, 30) = 2.279, P = 0.120; isoflurane × day, F(2, 30) = 2.279, P = 0.120). The amount of GluA1 in the whole lysates between days 1, 7, and 28 were as follows: day 1 (fig. 3C; control [n = 6], 100 ± 23.0%; isoflurane [n = 5], 88.2 ± 7.3%; day 7 (fig. 3C; control [n = 6], 100 ± 3.6%; isoflurane [n = 7], 136 ± 1.4%); and day 28 (fig. 3C; control [n = 6], 100 ± 17.7%; isoflurane [n = 5], 100.3 ± 15.5%). To identify the mechanisms underlying the isoflurane-induced increment in synaptoneurosomal GluA1 level, we examined the levels of GluA1 mRNA in the whole lysates (fig. 4A), and we found no difference between the 1.8% isoflurane-exposed and control groups (fig. 4B; control [n = 4], 100 ± 13.5%; isoflurane [n = 5], 99.6 ± 17.9%; *P = 0.014). Ubiquitin was normalized to the levels of β-actin (BA). All statistical analyses were performed using Student t test. Data are shown as mean ± SEM.

Fig. 3. The synaptoneurosomal GluA1 subunit of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA-R) was increased, after the administration of isoflurane. (A) Experimental design. Synaptoneurosomes and whole lysates of GluA1, one of the subunits of AMPAR, of the dorsal hippocampus were measured 1, 7, and 28 days after a 2-h exposure to 1.8% isoflurane (Iso). Comparisons were made between the control and isoflurane groups. (B) The amount of synaptoneurosomal GluA1 was significantly increased on day 7 (day 1, n = 5 or 6; day 7, n = 7 or 8; day 28, n = 5 or 6; isoflurane, F(1, 27) = 6.933, *P = 0.014; day, F(2, 27) = 0.783, *P = 0.783; isoflurane × day, F(2, 27) = 0.783, *P = 0.783; *P = 0.012 at day 7 control vs. isoflurane). (C) The amount of GluA1 in the whole lysate was not significantly changed (day 1, n = 5 or 6; day 7, n = 7 or 8; day 28, n = 5 or 6; isoflurane, F(1, 30) = 0.620, P = 0.437; day, F(2, 30) = 2.279, P = 0.120; isoflurane × day, F(2, 30) = 2.279, P = 0.120). GluA1 was normalized to the levels of β-actin (BA). All statistical analyses were performed using two-way factorial ANOVA (isoflurane × day, significance level was set at P < 0.05) followed by Student t tests (significance level was set at 0.05/3 = 0.0167 with Bonferroni correction). Data are shown as mean ± SEM. ns = absence of significant differences.

Fig. 4. Ubiquitin was significantly decreased, whereas the messenger RNA (mRNA) of GluA1 was not changed, 7 days after the administration of isoflurane. (A) Experimental design. The mRNA of GluA1 and the levels of ubiquitin (Ub) in the dorsal hippocampus were evaluated 7 days after a 2-h exposure to 1.8% isoflurane (Iso). (B) No significant differences (ns) in GluA1 mRNA levels in whole lysates were observed between the control and isoflurane groups (n = 4–5, *P = 0.97). GluA1 mRNA was measured by using quantitative real-time polymerase chain reaction (PCR). Cycle count threshold (CT) is normalized to the levels of β-actin (BA) mRNA. (C) GluA1 subunits were separated from whole lysates by using immunoprecipitation (IP) and Western blotting (WB). The upper panel shows that ubiquitinated GluA1 appears as a smear between 100 and 220 kDa instead of a distinct band. The lower panel shows the GluA1 levels among precipitated proteins in the whole lysates. (D) The ubiquitin level among precipitated proteins in the whole lysates was significantly decreased (n = 7 per group, *P = 0.014). Ubiquitin was normalized to the levels of GluA1. All statistical analyses were performed using Student t test. Data are shown as mean ± SEM.
of GluA1 (100 kDa), to a varying extent (up to as high as 200 kDa), because GluA1 conjugates covalently with a large number of ubiquitin molecules (≥20). Therefore, we regarded the smear observed between 100 and 200 kDa as ubiquinated GluA1. The ubiquitination of GluA1 was decreased in animals exposed to 1.8% isoflurane compared with that in control animals (fig. 4D; control \( n = 7 \), 100 ± 8.7%; and 1.8% isoflurane \( n = 7 \), 71.1 ± 6.1%; \( P = 0.014 \)).

Collectively, these results suggest that isoflurane inhibits the ubiquitination of GluA1, leading to the accumulation of an excessive amount of synaptoneurosomal GluA1. GluA1 accumulation in the hippocampal synaptoneurosomal fraction seems to be independent of increasing GluA1 transcription.

**Discussion**

We performed general anesthesia in rats for 2 h by using isoflurane at normal surgical concentrations.\(^1\)\(^8\),\(^1\)\(^9\) We demonstrated that contextual learning was significantly impaired 7 days after anesthesia with 1.8% isoflurane; the concentration of residual isoflurane in the brain was presumably negligible at this time. This effect was dependent on the concentration of isoflurane because lower concentrations of the anesthetic produced only insignificant effects. Moreover, LTP was impaired, and LTD was enhanced in the SC/CA1 hippocampal pathway 7 days after anesthesia with 1.8% isoflurane, suggesting that hippocampal synaptic plasticity was modulated 7 days after anesthesia.

We also revealed a slowly developing increment of GluA1-containing AMPARs in hippocampal synaptoneurosomes, and this change showed a good correlation with behavioral performance in the contextual learning task. The synaptoneurosomal fraction is a fraction enriched in synaptic proteins; this may be interpreted as evidence of increased synaptic concentration of AMPARs. A possible underlying mechanism for this increment of GluA1-containing AMPARs is delineated by the decline in GluA1 ubiquitination, which is a main degradation pathway of GluA1. The amount of GluA1 mRNA was not increased, but this does not contradict the observed increase in GluA1 because other mechanisms such as increased translation of mRNA are possible. Taken together, the cognitive dysfunction and modulation of synaptic plasticity observed days after the administration of isoflurane were strongly associated with the increment of GluA1-containing AMPARs in the hippocampal SC/CA1 synapses.

Learning and cognitive deficits have been reported after general anesthesia; however, they seem to depend on the type and dose of the anesthetics used, as well as on the time between exposure to the anesthetic and the assessment of its effects. In adult animals, learning performance is improved 1 day after isoflurane-induced anesthesia, and it is associated with the upregulation of N-methyl-D-aspartate receptor (NMDAR) 2B subunit.\(^6\) Conversely, learning performance is impaired 2 weeks after isoflurane anesthesia, and it is accompanied by the upregulation of caspase-3.\(^3\) Our results are consistent with those of these previous studies because we demonstrated a tendency toward improvement in learning 1 day after anesthesia and significant impairment in learning 7 days later. This biphasic change suggests that different mechanisms play a role in these processes, depending on the time after anesthesia. Furthermore, the impairment in learning observed 7 days after anesthesia was reversible because learning ability was improved 28 days after anesthesia to a level that was not significantly different from the control value.

The formation and retention of contextual memory require hippocampal synaptic plasticity. The establishment of LTP in the SC/CA1 hippocampal pathway is necessary for contextual learning, including IA learning. Our findings are in accordance with this because we observed disruption of LTP and enhancement of LTD in the hippocampus 7 days after anesthesia, when impairment in IA learning was maximal.

We have demonstrated that the amount of the GluA1 subunit of AMPAR was reversibly increased in synaptoneurosomal fractions of the rat hippocampus after isoflurane anesthesia. Although this may seem to be at odds with the observed impairment of learning and the change in synaptic plasticity (i.e., suppressed LTP and enhanced LTD), this discordance may be explained by the phenomenon of synaptic saturation. In fact, this phenomenon has been described in, and supported by, previous studies. For example, hippocampus-dependent spatial learning is impaired after HFS delivered directly to the hippocampus \textit{in vivo}.\(^3\)\(^5\) In \textit{in vivo} LTP induced by repetitive HFS is suppressed after IA learning, which drives the synaptic insertion of GluA1-containing AMPARs in the hippocampus.\(^8\) Ongoing single-whisker stimulation suppresses LTP in the barrel cortex, whereas shorter stimulation promotes this phenomenon.\(^1\)\(^5\) In rats, visual deprivation promotes the synaptic insertion of GluA1-containing AMPARs in the barrel cortex, which occludes LTP.\(^3\) The results of these reports suggest that the synaptic capacity for AMPARs is strictly limited, to prohibit additional synaptic incorporation of AMPARs once synaptic AMPARs have been increased to a maximum level, leading to impairment of additional learning. Our results suggest that isoflurane increases synaptic GluA1 excessively, leading to LTP occlusion. Excessive GluA1 may also contribute to the enhancement of LTD by facilitating the turnover of GluA1-containing AMPAR complexes that are trafficked in synapses.\(^3\)\(^5\)

We have also demonstrated that isoflurane inhibits the ubiquitination of GluA1. Because ubiquitination is an important pathway of GluA1 degradation, the GluA1 increments observed after isoflurane anesthesia may be attributable, at least in part, to reduced ubiquitination of this molecule.\(^3\)\(^0\)–\(^3\)\(^2\) The manner \textit{via} which isoflurane reduces the ubiquitination of GluA1 remains to be elucidated.

Our study had several limitations. First, we performed only the IA test to assess learning ability; however, IA is related to only a limited aspect of learning. Second, we
focused on the AMPAR; however, other types of receptors, such as the N-methyl-D-aspartic acid or γ-aminobutyric acid receptors, may be involved in this process. Third, the translation of our findings to clinical situations requires caution because the neural structures that mediate learning in humans are more complex than those of rats.

In summary, this study suggested that exposure to a clinical dose of isoflurane for 2 h induced delayed (7 days after the administration of the anesthetic) impairment of hippocampus-dependent contextual learning in rats, and this phenomenon accounted for the synaptic amount of GluA1 in the hippocampus by inhibiting its ubiquitination, resulting in the saturation of synaptic plasticity. These findings provide behavioral, electrophysiological, and biochemical evidence that inhalational anesthetics produce changes in the function of the brain that greatly outlast the duration of anesthesia. Further studies are warranted to elucidate whether general anesthetics modulate other types of learning and cognitive functions, and to determine whether anesthetics other than isoflurane, especially those with different mechanisms of action (e.g., propofol, nitrous oxide, ketamine, and xenon), exert similar effects on the brain.

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Competing Interests
The authors declare no competing interests.

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