Dual Effects of Isoflurane on Proliferation, Differentiation, and Survival in Human Neuroprogenitor Cells

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ABSTRACT

Background: Previous studies have demonstrated that isoflurane can provide both neuroprotection and neurotoxicity in various tissue culture models and in rodent developing brains. The cellular and molecular mechanisms mediating these dual effects are not clear, but the exposure level and duration of isoflurane appear to be determinant factors.

Methods: Using the ReNcell CX (Millipore, Billerica, MA) human neural progenitor cell line, the authors investigated the impact of prolonged exposure to varying isoflurane concentrations on cell survival and neurogenesis. In addition, the authors assessed the impact of short isoflurane preconditioning on elevation of cytosolic Ca^{2+} concentration and cytotoxic effects mediated by prolonged isoflurane exposures and the contribution of inositol-1,4,5-trisphosphate or ryanodine receptor activation to these processes.

Results: Short exposures to low isoflurane concentrations promote proliferation and differentiation of ReNcell CX cells, with no cell damage. However, prolonged exposures to high isoflurane concentrations induced significant ReNcell CX cell damage and inhibited cell proliferation. These prolonged exposures suppressed neuronal cell fate and promoted glial cell fate. Preconditioning of ReNcell CX cultures with short exposures to low concentrations of isoflurane ameliorated the effects of prolonged exposures to isoflurane. Pretreatment of ReNcell cultures with inositol-1,4,5-trisphosphate or ryanodine receptor antagonists mostly prevented isoflurane-mediated effects on survival, proliferation, and differentiation. Finally, isoflurane-preconditioned cultures showed significantly less isoflurane-evoked changes in calcium concentration.

Conclusion: The commonly used general anesthetic isoflurane exerts dual effects on neuronal stem cell survival, proliferation, and differentiation, which may be attributed to differential regulation of calcium release through activation of endoplasmic reticulum localized inositol-1,4,5-trisphosphate and/or ryanodine receptors.

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SOFLURANE has shown neuroprotective properties in response to numerous biological stresses *in vitro*[1–5] and *in vivo*.[6–9] However, an increasing number of studies suggest that isoflurane may be neurotoxic *in vitro*[10–13] and *in vivo*[14–19] as well. Isoflurane causes persistent hippocampal-dependent cognitive deficits in rodents,[15,16] but the mechanisms of such deficits are not clear. Neurogenesis in the hippocampus is involved in memory acquisition,[20] suggesting that isoflurane may act on neural progenitor cells (NPCs) to impinge on hippocampal-dependent cognitive functions. Accordingly, emerging studies into the mechanisms of anesthetic-induced cognitive deficits have provided some discrepant results on anesthetic-mediated effects on neurogenesis *in vivo*[17,21] and some consistent results *in vitro*.[22,23] Because of their importance to cognitive functions and regenerative medicine, it is critical to gain more insight into the mechanisms by which general anesthetics affect neurogenesis.

Development of NPCs is regulated by γ-aminobutyric acid and intracellular Ca^{2+} mobilization.[24–27] Ca^{2+} mobilization through inositol-1,4,5-trisphosphate ($InsP_3$) and ryanodine receptors plays important roles in proliferation and differentiation of nonexcitable cells.[28,29] Ca^{2+} is one of the key regulators of cell proliferation, *via* maintaining an oscillatory Ca^{2+} signal, activating the immediate early genes responsible for inducing resting cells (G_0) to reenter the cell cycle, and promoting the initiation of DNA synthesis at the G_1/S transition.[30,31] The Ca^{2+} spiking induced neural cell differentiation by controlling expression of specific neurotransmitters and channels, the behavior of growth cones, and the establishment of the specific connections within neuronal circuits.[30,32] Isoflurane neuroprotective properties in neurons are mediated through an association with smaller isoflurane-evoked Ca^{2+} release *via* $InsP_3$ receptors,[33–35] whereas the cytotoxic properties of this anesthetic are associated with excessive calcium release *via* $InsP_3$ receptors.[12,13,36,37] These results raise the possibility that both isoflurane-mediated cytoprotection and cytotoxicity in neurons occur in NPCs through differential $InsP_3$ or ryanodine receptor–mediated Ca^{2+} mobilization and control of the neurogenesis process. Thus, we hypothesize that isoflurane affects survival, proliferation, and differentiation of NPCs in a dual manner *via* activation of $InsP_3$ or ryanodine receptors. To that end, we used the immortalized human neural progenitor cell line ReNcell, and show that isoflurane exposures promote or inhibit survival, proliferation, and differentiation in a time- or concentration-dependent manner. Preconditioning of these cells with short isoflurane exposures mostly prevented the effects of prolonged exposures to high isoflurane levels on neurogenesis. Pharmacologic and imaging experiments suggest that these effects are likely attributable to differential activation of $InsP_3$ or ryanodine receptors. These results provide some insight into the interaction of anesthetics with neurogenesis and may have implications for studies into cognitive function and transplantation of NPCs under anesthesia.

Materials and Methods

Cell Cultures

ReNcell CX cells (Millipore, Billerica, MA), an immortalized human neural progenitor cell line, were derived from the cortical region of human fetal (14-weeks' gestation) brain tissue obtained from Advanced Bioscience Resources (Alameda CA) following normal terminations and in accordance with nationally (United Kingdom and/or United States) approved ethical and legal guidelines as described previously.[38] They were cultured according to the manufacturer's instructions in ReNcell neural stem cell maintenance medium, supplemented with 20 ng/ml fibroblast growth factor (Millipore) and 20 ng/ml epidermal growth factor (Millipore) as described previously.[39,40] Cells were plated at a density of 1.5 million cells in T75, 75-cm², tissue plastic culture flasks precoated with 20 µg/ml laminin (BD Biosciences, San Jose, CA) in Dulbecco's Modified Eagle Medium/F12 (Gibco, Invitrogen Corp., Grand Island, NY) and maintained as monolayer cultures at 37°C in a humidified incubator with 95% air and 5% CO_2. The culture medium was replaced every 48 h. For consistency and practical reasons, all experiments were carried out on cells between passages of 6 and 15. Proliferation was measured by incorporation of 5-bromodeoxyuridine (1:100; Invitrogen, Grand Island, NY) for 2 h after isoflurane exposure. For the differentiation studies, ReNcell CX cells were cultured for 4 days in maintenance medium devoid of growth factors.

Anesthetic Exposure

ReNcell CX NPCs grown on 96-well plates or culture dishes (30,000 cells/cm²) were exposed to isoflurane in a tight gas chamber (Bellco Glass, Vineland, NJ) placed in a culture incubator (Fisher Scientific, Pittsburgh, PA). Isoflurane was vaporized *via* an agent-specific vaporizer carried by humidified gas consisting of 5% CO_2, 21% O_2, and balanced nitrogen (Boc Gases, Bellmawr, NJ). The flow rate to the tight gas chamber was initially 5 l/min for the first 2 min of the experiment and 0.5 l/min thereafter for the remainder of the experimental period. Pilot studies confirmed that gas flow devoid of isoflurane to the chamber does not affect cell survival. Gas phase concentration in the chamber was checked by infrared absorbance of effluent gas and monitored constantly and maintained at the desired concentration throughout experiments using an infrared Ohmeda 5330 agent monitor (Coast to Coast Medical, Fall River, MA). High-performance liquid chromatography measurement confirmed that isoflurane concentrations of 2.4, 1.2, and 0.6% in the chamber yielded isoflurane concentrations of 0.8, 0.4, and 0.2 mM in the culture medium, respectively. Because isoflurane can pass the blood–brain barrier easily, these isoflurane concentrations in the culture medium are approximately 0.5 to 2 minimal alveolar concentrations (MAC) used in patients and should be considered clinically relevant concentrations. For the experiments on the

impact of exposure duration on ReNcell CX NPC survival, we exposed these cells to 2.4% isoflurane for 6, 12, or 24 h. Although 24-h isoflurane exposure is rarely seen in clinical settings, it has been consistently used to induce cytotoxicity in different *in vitro* systems,[3,36] making it a good model for isoflurane-induced cytotoxicity studies. Control ReNcell CX cultures were placed outside the tight gas chamber but inside the same incubator. Following anesthetic exposures, cells were immediately processed for cytotoxicity assays or immunocytochemistry unless noted otherwise.

Cytotoxicity Assays

Lactate dehydrogenase (LDH) release into the media following isoflurane exposures was detected using an LDH release assay kit (Promega, Madison, WI) as described previously.[3,12,41] Briefly, 50 µl of media was mixed with 50 µl of substrate mix in a 96-well plate and incubated for 30 min at room temperature. The reaction was terminated with 50 µl of stop solution and the sample was quantified spectrophotometrically at 490 nm using a plate reader (Opsys MR Absorbance Reader; DYNEX Technologies, Inc., Chantilly, VA). Background signal from the media was measured and subtracted from control signals. Mitochondrial dehydrogenase activity that reduces 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used to determine cellular redox activity, an initial indicator of cell death, in a quantitative colorimetric assay. Cells were incubated with MTT (125 µg/ml; Sigma-Aldrich, St. Louis, MO) in the growth medium for 1 h at 37°C. The medium was then aspirated and the MTT reduction product, formazan, was dissolved in dimethyl sulfoxide and quantified spectrophotometrically at 570 nm. MTT assay detects early and LDH release assay detects late cell damage. The results of both LDH and MTT reduction assays were from at least three separate experiments and are expressed as percentage of control first and then compared statistically (n ≥ 5) across three separate isoflurane concentrations (0.6, 1.2, or 2.4%) or durations (6, 12, or 24 h).

Cell Proliferation Determined by 5-Bromodeoxyuridine Incorporation and Immunostaining

ReNcell CX NPCs were seeded onto cover glasses precoated with 20 µg/ml laminin (BD Biosciences) in Dulbecco's Modified Eagle Medium/F12 (Invitrogen) overnight in proliferation medium (maintenance medium with 20 ng/ml basic fibroblast growth factor and 20 ng/ml epidermal growth factor). 5-Bromodeoxyuridine was added to the medium at a dilution of 1:100 for 2 h after isoflurane exposure. The cells were then fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After incubation in blocking solution (10% goat serum, 1% bovine serum albumin/phosphate-buffered saline), cells were stained with anti–5-bromodeoxyuridine antibody (1:100; Invitrogen) overnight at 4°C. After washing with Tris-buffered saline, cells were incubated with fluorescein isothiocyanate–goat anti-mouse immunoglobulin G antibody (1:1,000; Jackson

ImmunoResearch Laboratories, Inc., Fairfax, VA) for 1 h. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (1:3,000; Invitrogen) for 2–5 min at room temperature. Cover glasses with immunostained cells were mounted on an IX-70 inverted fluorescence microscope (400×; Olympus USA, Center Valley, PA) and images acquired using IpLab 3.6.5 software (Scanalytics, Inc., Fairfax, VA). 5-Bromodeoxyuridine–positive cells were counted from seven random locations from each slide by two persons blinded to experimental treatments. The percentage of 5-bromodeoxyuridine–positive cells over the total cells was calculated and compared across treatment groups from at least three different cultures.

Cell Differentiation Determined by Immunostaining of Tuj1 and Glial Fibrillary Acidic Protein

ReNcell CX NPCs were cultured as described above for the proliferation experiments. Before differentiation experiments, proliferation medium was replaced with differentiation-conditioned or media devoid of growth factors. For short isoflurane exposure or preconditioning, cells were exposed to 2.4% isoflurane for 1 h. Prolonged isoflurane (2.4%) exposures were for 24 h in either preconditioning or nonpreconditioning experiments. For the preconditioning experiments, prolonged isoflurane (2.4%) exposures began 4 h after initial short isoflurane (2.4%) exposure. After isoflurane exposure, ReNcell CX NPCs were allowed to differentiate for an additional 3 days after completion of isoflurane exposures. At the end of the differentiation period, the cells were fixed with 4% formaldehyde and processed for immunocytochemistry as described above for the proliferation experiments. Incubation of primary antibodies was accomplished with Tuj1 (1:200; Covance, Princeton, NJ) or glial fibrillary acidic protein (GFAP) (1:1,500; Millipore) for 2 h at 37°C for detection of cells with neuronal or glial phenotypes, respectively. Tuj1 has been used successfully as a neuronal marker in the pluripotent human embryonic carcinoma immortalized cell line NTERA2,[42] whereas GFAP has been used as a glial marker in immortalized cell lines.[43,44] For visualization of primary antibody signal, we used the Alexa-488 goat anti-rabbit and Alexa-594 goat anti-mouse immunoglobulin G antibodies (both at 1:1,000; Invitrogen) for 1 h at room temperature. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (1:3,000; Invitrogen) for 2–5 min at room temperature. The cover glasses with immunostained cells were mounted on an IX-70 inverted fluorescence microscope (200× or 600×; Olympus USA) and images acquired with IpLab 3.6.5 software (Scanalytics). Tuj1- or GFAP-positive cells overlapping with 4′,6-diamidino-2-phenylindole signal were counted from seven random locations from each slide by two persons blinded to the experimental treatments. The percentage of Tuj1- or GFAP-positive cells is given over the total cells and compared across treatment groups from at least three different cultures.

Measurement of Isoflurane-evoked Changes in Cytosolic Ca²⁺ Concentration

Changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) were measured using Fura-2/AM fluorescence (Molecular Probes, Eugene, OR) with a photometer coupled to an Olympus 1X70 inverted microscope (Olympus America) and the IPLab v3.7 imaging processing and analysis software (Biovision Technologies, Exton, PA). The procedure for $[Ca^{2+}]_c$ measurement was as described previously.[12,13,18,36] Briefly, coverslips with ReNcell CX human NPCs were washed three times in Ca^{2+}-free Krebs-Ringer buffer and then loaded with 2.5 μM Fura-2/AM in the same buffer for 30 min at room temperature. Cover glasses were then placed in a sealed chamber (Warner Instrument, Inc., Hamden, CT) connected to multiple inflow tubes and one outflow tube, which allowed for constant flow to the chamber. All bubbles in the chamber were flushed out at the beginning so that there was no gas phase in the sealed chamber during measurement of $[Ca^{2+}]_c$ in the buffer. Baseline $[Ca^{2+}]_c$ was first recorded for at least 2 min, and isoflurane-evoked changes were recorded in response to application of isoflurane (0.64 mM or 2 MAC) for at least 15 min in normal Krebs-Ringer buffer. Isoflurane application was through a separate inflow tube driven by a syringe pump (Braintree Scientific, Inc., Braintree, MA). High-performance liquid chromatography (System Gold; Beckman Coulter, Fullerton, CA) was used for measurement of isoflurane concentration in the bath solution as described previously.[12,36] Fluorescence intensities were measured with alternate excitation at 340 and 380 nm and emission at 510 nM for up to 15 min for each treatment. The final results are given as a ratio of fluorescence intensities at 340/380 nm and as an average of at least three separate experiments. The trypan blue exclusion assay was used after each imaging experiment to make sure that $[Ca^{2+}]_c$ measurements were from healthy and living cells.

Statistical Analysis

We used GraphPad Prism 4 software (GraphPad Software, Inc., La Jolla, CA) for all statistical analyses and production of graphs. Data for one-group variables were analyzed using one-way ANOVA followed by Tukey multiple comparisons testing, and those for two-group variables were analyzed using two-way ANOVA followed by the Bonferroni multiple comparison test. The variance factor for one-way ANOVA was group comparisons, whereas those for two-way ANOVA were time or concentration and group comparisons. The significance level for all statistical comparisons was set at $P < 0.05$.

Results
Isoflurane Induced ReNcell CX Cytotoxicity in a Dose- and Time-dependent Manner via Activation of InsP3 and/or Ryanodine Receptors

We determined the dose- and time-dependence of isoflurane exposure on ReNcell CX NPC survival. Our results show that isoflurane induced cell damage in a dose- (fig. 1, A and B) and time-dependent manner (fig. 1, C and D), as we have previously demonstrated for cortical neurons and PC12 cells.[41] Exposure of ReNcell CX NPCs to 0.6% isoflurane for 24h had no effect on survival, but exposure to 1.2% isoflurane, a clinically relevant concentration, resulted in significant cell damage as measured by the LDH assay (fig. 1A). This clinical concentration, however, did not show any significant effects on cytotoxicity as measured by the MTT reduction assay, although a strong trend toward more cytotoxicity was noted (fig. 1B). Exposure to 2.4% isoflurane for 24h induced significant cytotoxicity as determined by both LDH and MTT assays (fig. 1, A–C). In contrast, exposure with the same concentration (2.4%) of isoflurane for 6 or 12h did not result in significant cytotoxicity (fig. 1, C and D). These results suggest that survival of ReNcell CX NPCs depends on both the concentration and duration of isoflurane exposure.

To gain some insight into the mechanisms of isoflurane-mediated cytotoxicity in ReNcell CX NPCs, we investigated the role of calcium release from the endoplasmic reticulum (ER). Pretreatment of ReNcell CX NPCs with the InsP₃ receptor antagonist xestospongin C (Xc) (fig. 2A) or the ryanodine receptor antagonist dantrolene (fig. 2B) significantly inhibited isoflurane-induced early cell damage as determined by the MTT reduction assay. To assess the role of InsP₃ release in isoflurane-mediated cytotoxicity in these cells, exposure to isoflurane (2.4%) was carried out in the presence of the cholinergic agonist carbachol. This treatment condition potentiated isoflurane-induced cytotoxicity as measured by the MTT reduction assay (Fig. 2C). Pretreatment with Xc mostly prevented this effect (Fig. 2C), suggesting that InsP₃ release plays a role in mediating isoflurane-mediated effects on cytotoxicity. Similarly, depletion of ER calcium by the sarcoendoplasmic reticulum calcium ATPase Ca^{2+} pump inhibitor thapsigargin potentiated isoflurane-mediated cytotoxicity in ReNcell CX NPCs (fig. 2D). Overall, these results suggest that isoflurane induced cytotoxicity in ReNcell CX NPCs through disruption of intracellular calcium homeostasis. This disruption in Ca^{2+} homeostasis appears to be mediated through excessive release of Ca^{2+} *via* InsP₃ or ryanodine receptor activation.

Isoflurane Preconditioning Ameliorated ReNcell CX Cell Damage Induced by Prolonged Isoflurane Exposure through Activation of InsP3 or Ryanodine Receptors

We have previously demonstrated that short isoflurane exposure inhibits cytotoxicity in cortical neurons and PC12 cells induced by prolonged exposure to the same anesthetic.[3] Thus, we wondered whether such a preconditioning mechanism operates in ReNcell CX NPCs. Preconditioning with 2.4% isoflurane for short exposure for 1h nearly abolished ReNcell CX NPC cytotoxicity induced by prolonged exposure for 12h to 2.4% isoflurane initiated at 4h after completion of 1-h preconditioning short exposure

(fig. 3, A and B). Pretreatment of cultures with Xc or dantrolene prevented the protection afforded isoflurane-preconditioned ReNcell CX NPCs against toxic insults from prolonged isoflurane exposures (fig. 3, A), suggesting that Ca^{2+} flux through $InsP_3$ or ryanodine receptors plays important roles in isoflurane-mediated preconditioning and cytoprotection. In addition, depletion of ER calcium by thapsigargin not only potentiated the cytotoxicity induced by prolonged isoflurane exposure but eliminated the protection afforded by preconditioning or short isoflurane exposure (fig. 3B). To further understand this dual effect of protection and cytotoxicity by isoflurane, we measured isoflurane-evoked changes in intracellular $[Ca^{2+}]_c$ in preconditioned and control cells (carrier gas exposed). Although our previous studies clearly demonstrated that isoflurane may induce cell apoptosis by overactivation of the $InsP_3$ receptor and subsequent abnormal elevation of cytosolic and mitochondrial Ca^{2+} concentration and decrease of ER Ca^{2+} concentration,[36,37] it is not clear whether preconditioning NPCs with minimal exposures will ameliorate the abnormal elevation of cytosolic Ca^{2+} concentrations induced by

subsequent detrimental isoflurane exposure. Isoflurane-preconditioned ReNcell CX human NPCs showed significantly greater changes in intracellular $[Ca^{2+}]_c$ than control cells in response to isoflurane application than those cells without previous isoflurane preconditioning (fig. 4). These results suggest that the preconditioning mechanism for isoflurane-mediated protection of ReNcell CX human NPCs prevents excessive changes in intracellular $[Ca^{2+}]_c$ in response to isoflurane exposure, possibly *via* calcium release from ER through $InsP_3$ or ryanodine receptors as demonstrated previously.[36,37,41]

Dual Effects of Isoflurane on ReNcell CX Cell Proliferation through Activation of InsP3 or Ryanodine Receptors

Single (4–6 h) or repeated short (45 min/day for 4 days) exposure of rodent NPCs to isoflurane decrease proliferation *in vitro*[22,23] and *in vivo*, albeit with some discrepancies with regard to adult NPCs.[17,21] Thus, we assessed the impact of varying concentrations of isoflurane exposure for different durations on proliferation of ReNcell human NPCs. Compared with control cells (fig. 5A), exposure of these
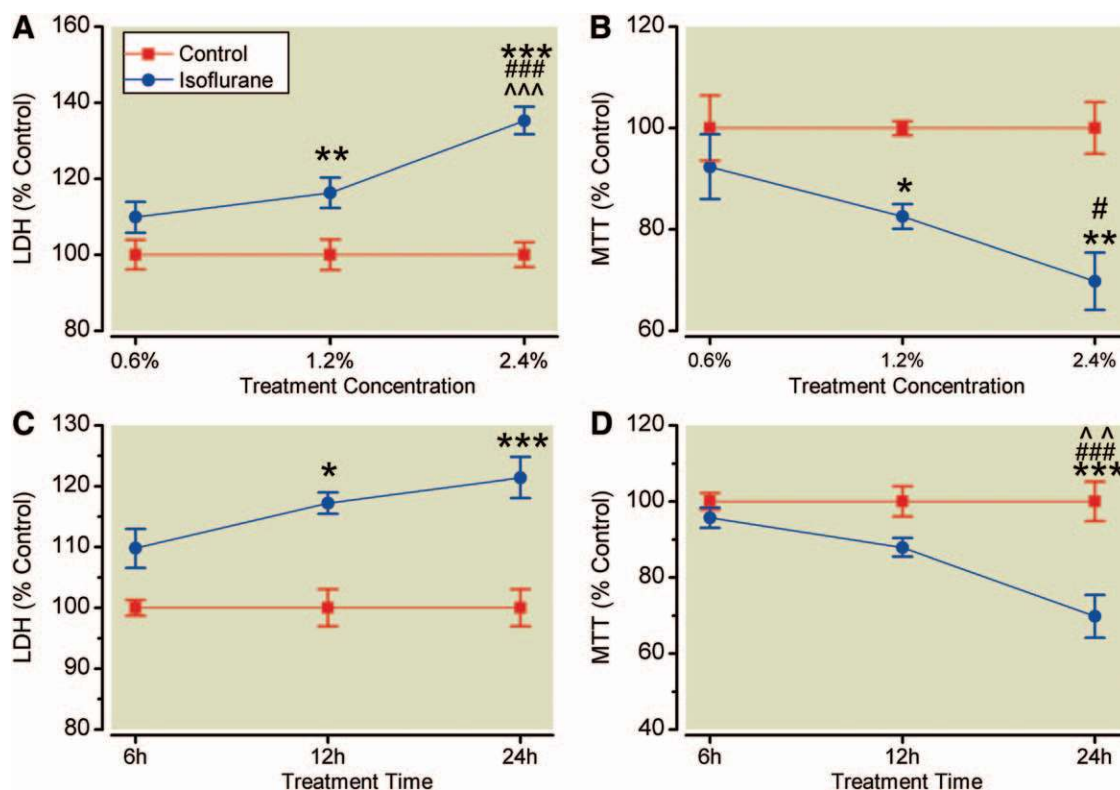

Fig. 1. Isoflurane induces ReNcell CX human neural progenitor cell damage in a dose- and time-dependent manner. Cells were exposed to different concentrations (0.6, 1.2, and 2.4%, respectively) of isoflurane (*A* and *B*) for 24 h or different durations (6, 12, and 24 h, respectively) at 2.4% isoflurane (*C* and *D*). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction and lactate dehydrogenase (LDH) release assays were used to determine early or late cell damage, respectively. Data were obtained from at least three separate cultures, given as mean ± SEM, and analyzed by two-way ANOVA followed by Bonferroni multiple comparison tests (n ≥ 6 for each condition). * $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$, respectively, compared with untreated controls. # $P < 0.05$, ## $P << 0.01$, or ### $P < 0.001$, respectively, compared with 0.6% isoflurane (*A, B*) or 6-h treatment groups (*D*). ^^ $P < 0.01$ or ^^^ $P < 0.001$ compared with 1.2% (*A*) or 12-h treatment groups (*D*). Sample size (n) represents the number of replicates assayed for LDH and MTT experiments.
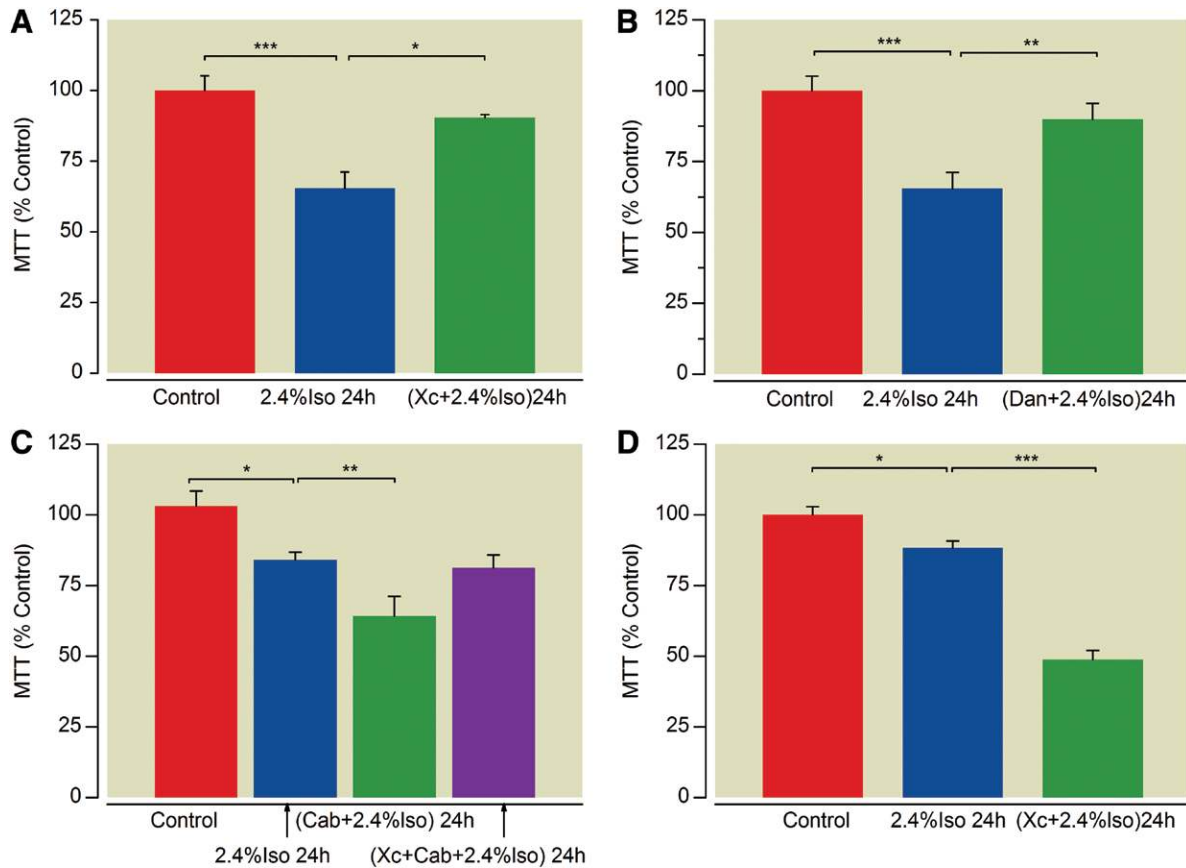
Fig. 2. Isoflurane induces ReNcell CX human neural progenitor cell damage through activation of InsP$_3$ or ryanodine receptors. (*A*) InsP$_3$ receptor antagonist xestospongin (Xc; 200 nM) inhibited ReNcell CX cell damage induced by 2.4% isoflurane (Iso) for 24 h (n = 6 and n = 10, respectively). (*B*) Ryanodine receptor antagonist dantrolene (Dan, 20 μM) inhibited ReNcell CX cell damage induced by 2.4% Iso for 24 h (n = 12 and n = 10, respectively). (*C*) Carbachol (10 μM) enhanced ReNcell CX cell damage induced by 2.4% isoflurane (Iso) for 24 h through activation of InsP$_3$ receptors (n = 6 and n = 18, respectively). (*D*) The sarcoplasmic–endoplasmic reticulum Ca^{2+}–ATPase inhibitor thapsigargin (TG; 100 nM) potentiated ReNcell CX cell damage induced by 2.4% isoflurane for 24 h (n = 6 and n = 12, respectively). All data are given as mean ± SEM from at least three separate experiments, and analyzed by one-way ANOVA followed by Tukey multiple comparison tests. * $P < 0.05$, ** $P < 0.01$, or **** $P < 0.001$. Sample size (n) represents the number of replicates assayed for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction experiments.

cells to isoflurane at different concentrations with different durations, in the presence or absence of isoflurane preconditioning, seemed to not change the shape of cells (fig. 5, B–F). Given that the number of NPCs appears to require modulation of Ca^{2+} influx through interaction with InsP$_3$ receptors,[45] we assessed the impact of prolonged inactivation of InsP$_3$ and ryanodine receptors on ReNcell human NPC proliferation. Indeed, treatment of these cells with varying concentrations of Xc or dantrolene decreased the number of proliferating ReNcell human NPCs, with effective doses of 100 nM and 20 μM for Xc and dantrolene, respectively (fig. 5, G and H). These results suggest that normal Ca^{2+} flux through InsP$_3$ or ryanodine receptors plays a role in the regulation of neurogenesis. A low concentration of 0.6% isoflurane for 1 h enhanced proliferation, but the clinically relevant concentration of 1.2% isoflurane for 1 h had no effects (fig. 5, A, B, and I). However, exposure to a high concentration of 2.4% isoflurane for 1 h decreased proliferation

of ReNcell human NPCs (fig. 5, A and I). To understand the impact of isoflurane exposure on this basal regulation of proliferation *via* modulating activation of InsP$_3$ or ryanodine receptors, we investigated the effects of prolonged isoflurane (2.4%) exposure in the presence of Xc (50 nM) or dantrolene (1 μM) in concentrations that would not induce significant inhibition of ReNcell human NPC proliferation alone as demonstrated in figure 5, G and H. Both Xc and dantrolene significantly inhibited the suppression of ReNcell human NPC proliferation induced by prolonged exposure of 2.4% isoflurane for 24 h (fig. 5, J), suggesting that prolonged use of isoflurane inhibits ReNcell human NPC proliferation by overactivation of InsP$_3$ or ryanodine receptors. Because isoflurane preconditioning protects ReNcell human NPCs from cytotoxicity induced by prolonged isoflurane (2.4%) exposure, we wondered whether this mechanism of cytoprotection has any implication on their proliferation and whether activation of InsP$_3$ or ryanodine receptors plays
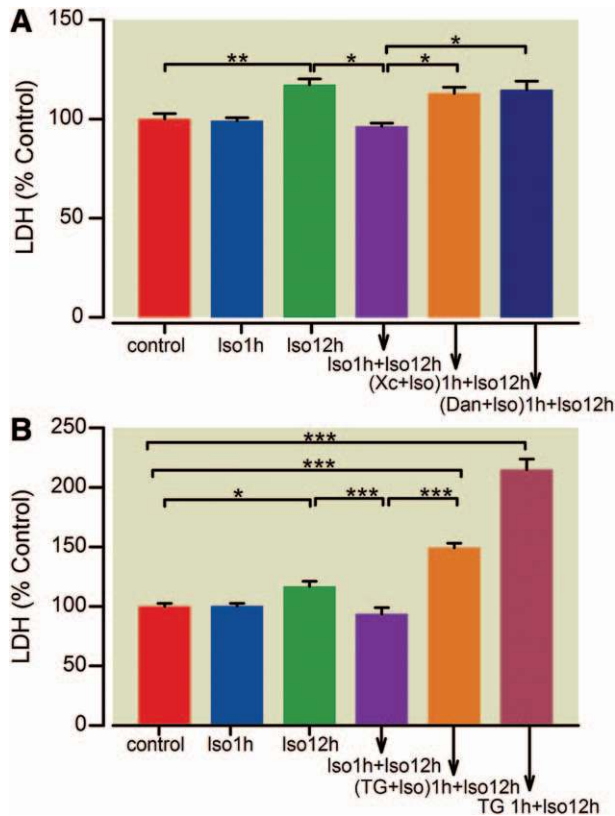
Fig. 3. Preconditioning with short isoflurane exposure inhibits ReNcell CX human NPC damage induced by prolonged isoflurane exposure through activation of InsP$_3$ or ryanodine receptors. Short exposure (1 h) with 2.4% isoflurane (Iso) protected ReNcell CX cell from damage induced by prolonged exposure of 2.4% Iso for 12 h, and this cytoprotective effect was prevented by xestospongin C (Xc; 200 nM) or dantrolene (Dan; 20 µM) (*A*). Thapsigargin (TG; 100 nM) potentiated ReNcell CX cells damage induced by prolonged exposure of 2.4% isoflurane for 12 h and prevented the neuroprotective effects of preconditioning by short exposure to 2.4% isoflurane for 1 h (*B*). All data are given as mean ± SEM from at least three separate experiments and analyzed by one-way ANOVA followed by Tukey multiple comparison tests (n ≥ 16 for each condition). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Sample size (n) represents the number of replicates assayed for lactate dehydrogenase (LDH) release experiments.

a role. Indeed, preconditioning with 0.6% isoflurane for 1 h inhibited suppression of ReNcell CX NPC proliferation induced by 24-h exposure to 2.4% isoflurane (fig. 5, A–D, K). Pretreatment of cultures with Xc (50 nM) or dantrolene (1 µM) prevented the protection afforded isoflurane-preconditioned ReNcell CX human NPCs against toxic insults from prolonged isoflurane exposures (fig. 5, E, F, and K), suggesting that Ca^{2+} flux through InsP$_3$ or ryanodine receptors also plays important roles in the isoflurane-mediated preconditioning effect on proliferation of these cells. Altogether, these results suggest that isoflurane-mediated effects on proliferation of ReNcell CX human NPCs require activation of InsP$_3$ or ryanodine receptors.
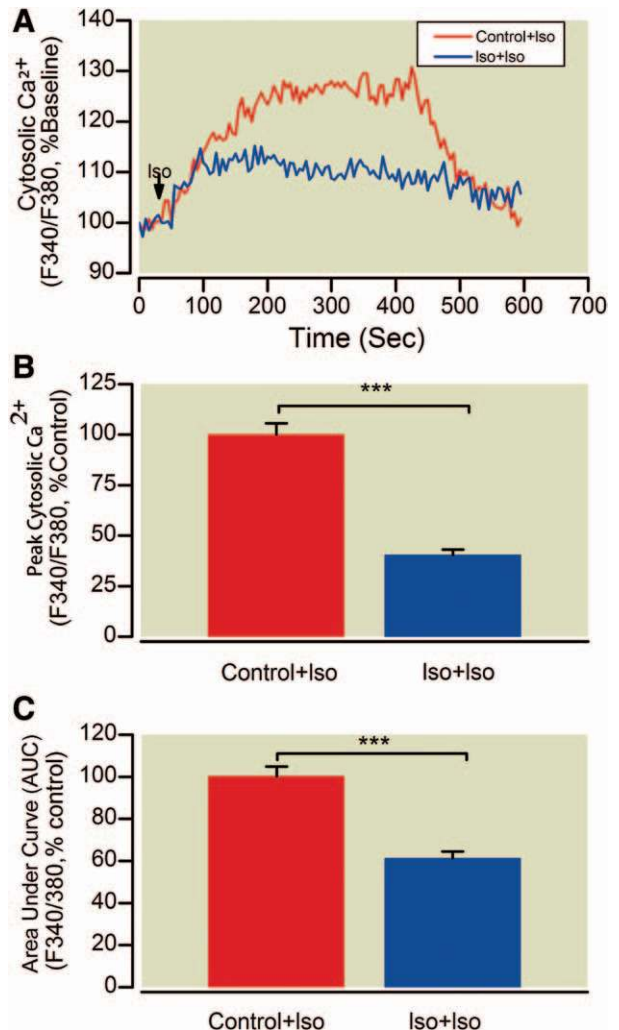

Fig. 4. Isoflurane preconditioning ameliorated isoflurane-evoked elevation in cytosolic calcium concentration in ReNcell CX neuronal progenitor cells. Changes in Fura2-AM intensities were measured in isoflurane (Iso)-preconditioned ReNcell CX neuronal progenitor cells with either 2.4% isoflurane (preconditioned Iso + Iso, 2.4% for 1 h) or carrier gas (Control + Iso) in response to application of isoflurane (0.4 mM). F340/F380 ratio values were normalized to baseline. (*A*) Averaged typical response of cytosolic calcium concentrations to isoflurane. (*B*) Peak elevations of cytosolic calcium level as a percentage of control were compared (n ≥ 38). (*C*) Averaged elevation of cytosolic calcium level was measured using the area under the curve (AUC) as a percentage of control (n ≥ 38). Data are given as mean ±SEM from at least three separate experiments. *** $P < 0.001$ by two-tailed Student *t* test and the sample number (n) represents the number of cells or regions of interest analyzed (*B* and *C*).

Dual Effects of Isoflurane on ReNcell CX Cell Differentiation

Acute isoflurane exposure has been shown to increase differentiation of NPCs *in vivo*[17] and *in vitro*,[23] but the cellular and molecular mechanisms are not clear. Thus, we wondered whether exposure of ReNcell CX NPCs to isoflurane can

affect differentiation in a manner similar to its effects on proliferation as demonstrated in this study (fig. 6). More specifically, we wondered whether isoflurane affects differentiation in a time-dependent manner with preconditioning features. Compared to its control (fig. 6A), differentiation of ReNcell CX human NPCs into neurons or glial fate depended on anesthetic exposure duration (fig. 6, B–D). Exposure to 2.4% isoflurane for 1 h had no effect as measured by Tuj1- (fig. 6, A, B, and E) or GFAP-positive cells (fig. 6, A, B, and F). However, prolonged exposure to the same concentration of isoflurane significantly suppressed neuronal fate and promoted glial fate (fig. 6, A, C, E, and F). Consistent with its dual effects on cell survival (fig. 3) and proliferation (fig. 5), isoflurane-preconditioned ReNcell CX NPCs were protected from the suppression of neuronal fate and promotion of glial cell fate selections induced by prolonged (24 h) isoflurane (2.4%) exposure (fig. 6, A, D–F).

Discussion

We have demonstrated that isoflurane induced cytotoxicity and affected proliferation of ReNcell CX NPCs in a dose- and time-dependent manner. Prolonged isoflurane exposure inhibited neuronal cell fate and promoted glial cell fate. Isoflurane preconditioning abolished cytotoxicity and the effects on neurogenesis induced by prolonged isoflurane exposure. The dual effects on cytotoxicity and proliferation required activation of InsP$_3$ or ryanodine receptors. To our knowledge, this is the first study to demonstrate dual effects of isoflurane on NPC survival and a preconditioning effect on neurogenesis.

Dual effects of cytoprotection and cytotoxicity by general anesthetics have been demonstrated in various *in vitro*[3–5,10,22,35] and *in vivo* model systems.[6,9,15,46–49] In this study, we demonstrated that isoflurane induced cytotoxicity at high doses and cytoprotection at low doses in ReNcell CX NPCs (figs. 1–3). This is remarkably consistent with observations in 7-day-old or in utero developing rat brains.[6,14] The mechanisms of neuroprotection by isoflurane in ReNcell CX NPCs are not clear, but our results suggest a role for ER localized InsP$_3$ or ryanodine receptors.

Isoflurane has been shown to be neurotoxic,[10–19] but rodent NPCs are resistant to its toxic insults.[17,21–23] However, we report that isoflurane induced cytotoxicity in ReNcell CX NPCs in a dose- and time-dependent manner. The difference in exposure time or duration may explain the discrepancies between our study and others. Indeed, we noted significant differences in cytotoxicity only after 24 h of exposure at 1.2% or 2.4% of isoflurane, whereas others have reported data for acute or repeated exposures lasting less than or equal to 6 h.[17,21–23] Nonetheless, the results in this study are consistent with isoflurane-mediated cytotoxicity in cardiac progenitors.[50,51] As demonstrated in cortical neurons and PC12 cells,[3] isoflurane preconditioning of ReNcell CX NPCs protected these cells from cytotoxicity induced

by prolonged isoflurane exposure. This is consistent with the protective effects of isoflurane or sevoflurane noted previously in cardiac[50,51] or endothelial progenitors derived from human embryonic stem cells,[52] respectively. This cytoprotective effect of isoflurane on stem cells has been described in various cell types in response to many biological stresses.[1–5] The InsP$_3$ receptor has been implicated in the maintenance of adult NPC number in Bax knockout mice,[45] suggesting that isoflurane-mediated effects on ReNcell CX NPC survival may require activation of these receptors. We found that to be the case for both isoflurane-mediated protection and cytotoxicity and, most surprisingly, the ryanodine receptor appears to be equally involved in these processes. Interestingly, isoflurane-preconditioned ReNcell CX NPCs are less sensitive to isoflurane-evoked changes in [Ca^{2+}]$_c$, suggesting that the dual effects of isoflurane on cytotoxicity and cytoprotection are possibly mediated through changes in Ca^{2+} homeostatic balance. In support of this notion, depletion of ER Ca^{2+} with thapsigargin exacerbated the cytotoxic effects of isoflurane and prevented its cytoprotective effects.

Proliferation and differentiation of NPCs provide a great opportunity for studies into neurogenesis and replacement therapies under anesthesia. Thus, it is of particular importance to understand the basic mechanisms of anesthetic effects on neurogenesis. Here, we used the human ReNcell CX progenitor line to investigate the hypothesis that isoflurane affects survival and neurogenesis in a dual manner *via* activation of InsP$_3$ or ryanodine receptors. Although immortalized, these cells express the intermediate filament nestin, a marker of NPCs.[38] In addition, they maintain the ability to proliferate and differentiate into astrocytes, oligodendrocytes, and neurons.[38] Immortalized NPCs derived from cortical (ReNcell CX) and midbrain (ReNcell VM) tissues maintain stable phenotypes across passages compared with normal human NPCs,[38] but extrapolation of data from these cells to normal neurons is quite challenging given the paucity of studies into the biochemical and electrophysiologic characterization of these cells. Differentiated ReNcell NPCs express GFAP (astrocyte), βIII-tubulin (neurons), or O1/Gal C (oligodendrocyte), and initial electrophysiologic characterization of voltage-gated potassium (ReNcell CX) and sodium currents or action potentials (ReNcell VM) confirmed the specificity of these markers in these progenitors,[38] making them ideal for mechanistic studies into human neurogenesis.

Recent studies suggest that isoflurane affects proliferation of NPCs in an age-, dose-, and session-dependent manner. Exposure of postnatal rats to isoflurane, above 1 MAC, transiently[17] or persistently[21] decreased proliferation. Single exposure (4 h) of adult rats to 2.4% isoflurane initially decreased (for 1 day) and then increased proliferation of NPCs 5–10 days after anesthesia,[17] whereas short exposure to 1.7% isoflurane had no effect.[21] However, NPCs isolated from embryonic[22] or early postnatal[23] rats consistently exhibited reductions in proliferation following
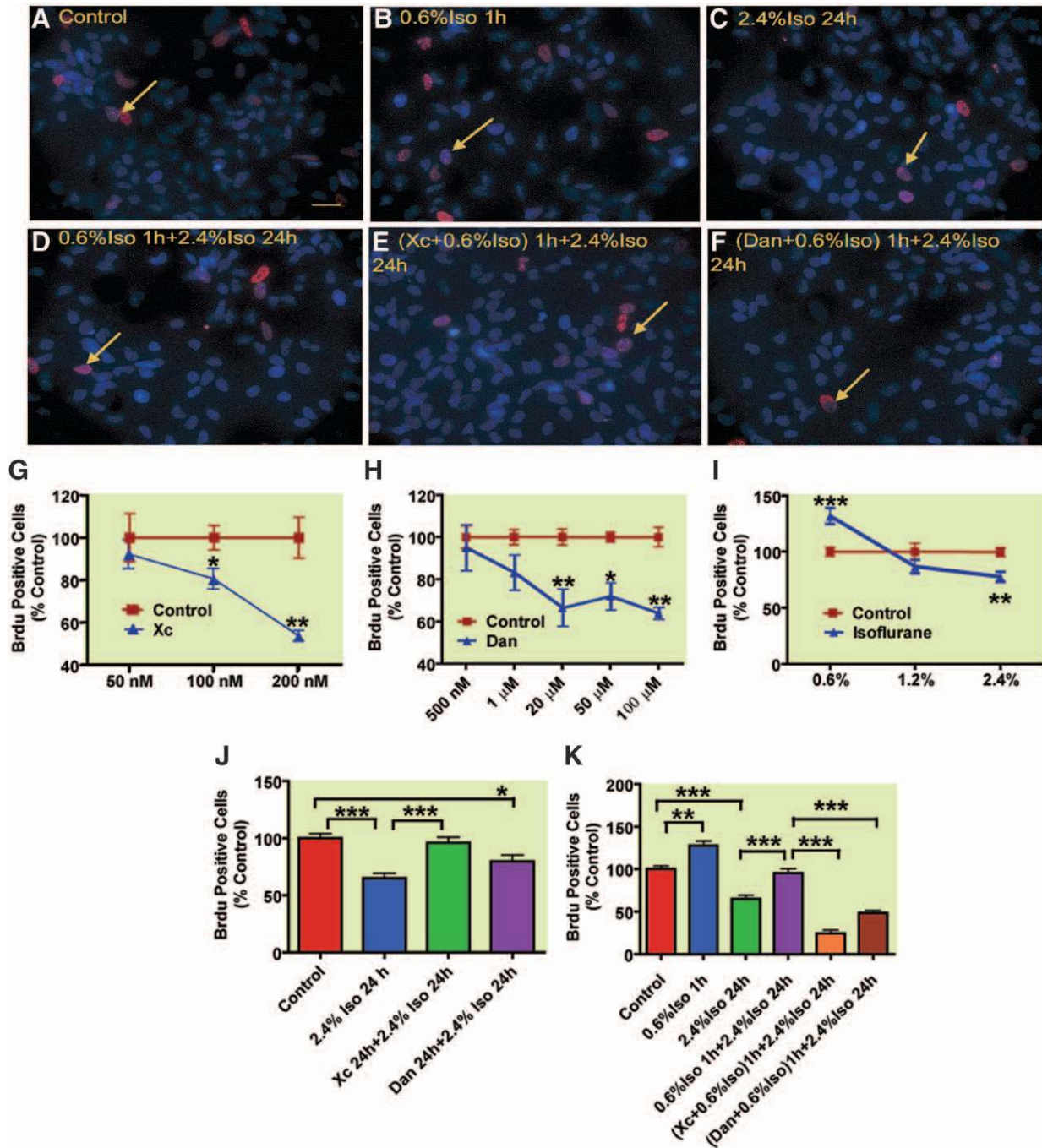
Fig. 5. Dual effects of isoflurane on proliferation and modulation of Ca²⁺ release from the endoplasmic reticulum (ER) *via* activation of InsP₃ or ryanodine receptors in ReNcell CX neural precursor cells. (*A–F*) Representative micrographs of ReNcell neural precursors with incorporated 5-bromodeoxyuridine (Brdu, *arrows*) in the presence or absence of isoflurane (Iso) at various concentrations and durations, with or without the InsP₃ receptor antagonist xestospongin C (Xc) or the ryanodine receptor antagonist dantrolene (Dan) (*scale bar* = 100 μm). Proliferation of ReNcell CX neural progenitors requires calcium release from ER *via* InsP₃ (*G*) or ryanodine (*H*) receptors. Exposure to 2.4% isoflurane for 1 h (*I*) or 24 h (*C, J,* and *K*) significantly suppressed proliferation of ReNcell CX neural progenitors through activation of InsP₃ or ryanodine receptors (*J*). However, exposure of ReNcell CX neural progenitors to 0.6% for 1 h promoted proliferation (*A, B, I,* and *K*) through activation of InsP₃ (*E* and *K*) and ryanodine (*F* and *K*) receptors, whereas exposure to 1.2% isoflurane had no effect. Isoflurane (0.6%) preconditioning of ReNcell CX cells for 1 h mostly prevented the suppression of ReNcell CX neural progenitor cell proliferation induced by prolonged (24 h) isoflurane (2.4%) exposure (*D* and *K*). This preconditioning effect requires activation of InsP₃ (*D, E,* and *K*) or ryanodine receptors (*D, F,* and *K*). All data are given as mean ± SEM from at least three separate experiments and analyzed by one-way (*J* and *K*) or two-way (*G–I*) ANOVA followed by Bonferroni multiple comparison tests (n ≥ 7). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with controls (*G–I*) or as indicated (*J* and *K*). The sample number (n) represents the number of cover glasses used to assess the percentage of 5-bromodeoxyuridine–positive cells from at least seven random locations on each cover glass.
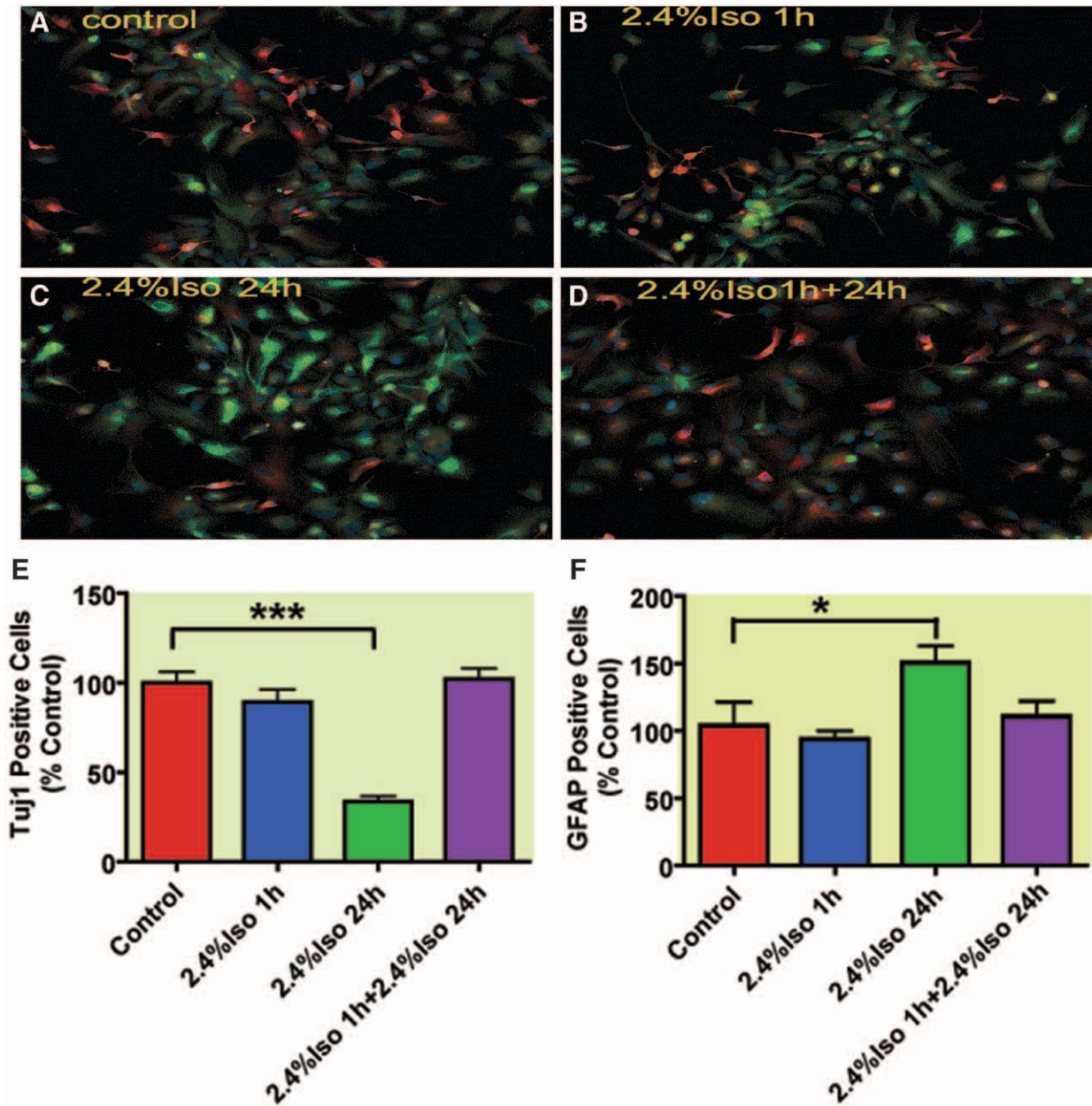
Fig. 6. Isoflurane preconditioning ameliorated the suppression of ReNcell CX neural progenitor cell differentiation induced by prolonged isoflurane exposure. (*A–D*) Representative micrographs of differentiated and nondifferentiated ReNcell CX neural progenitors under various pharmacologic conditions (*scale bar* = 100 μm). ReNcell CX progenitors were exposed to 2.4% iso-flurane (Iso) for 1 h (*B*) or for 24 h (*C*) in the absence or presence of preconditioning with 2.4% isoflurane for 1 h (*D*). Neuronal class III β-tubulin (Tuj1)–positive (*red*) or glial fibrillary acidic protein (GFAP)–positive (*green*) cells (*A–D*) were counted (n ≥ 6) and expressed as a percentage of controls (*E* and *F*). All data are given as mean ± SEM and analyzed by one-way ANOVA followed by Tukey multiple comparison tests (*E* and *F*). * $P < 0.05$, ** $P < 0.001$. The sample number (n) represents the number of cover glasses used to assess the percentage of Tuj1- or GFAP-positive cells from at least seven random locations on each cover glass.

single exposures to isoflurane (4–6 h) in a dose-dependent manner.[22] By contrast, exposure of ReNcell NPCs to 0.6% isoflurane for 1 h enhanced proliferation in this study, but the clinically relevant concentration of 1.2% had no effect. Exposure to 2.4% isoflurane for 1 h, however, decreased proliferation of ReNcell CX NPCs as reported previously for rodent NPCs *in vitro*[22,23] and for young rats *in vivo*.[17,21] Evidently, the duration of isoflurane exposure may influence

proliferation in addition to doses, session number, and age. However, it is clear from this study that activation of $InsP_3$ or ryanodine receptors may be an important modulator of isoflurane-mediated effects on proliferation of ReNcell CX NPCs, regardless of the aforementioned factors. This is further supported by the requirement of the modulatory effect of Ca^{2+} influx and $InsP_3$ receptor activation in regulating NPC numbers in Bax knockout mice.[45]

Cell fate specification is a critical step in the wiring of the central nervous system, and the events underlying this process are under the combinatorial control of intrinsic and extrinsic factors.[53] Indeed, single isoflurane exposure at or above 1 MAC for 4 h promotes neuronal fate selection in primary cultures of early postnatal rat NPCs[23] and in adult rats.[17] In this study, we show that isoflurane exposure affected differentiation in a time-dependent manner. Exposure of ReNcell CX NPCs to 2.4% isoflurane for 1 h had no effect on neuronal or glial cell fate selection. However, prolonged exposure (24 h) suppressed neuronal fate and promoted glial fate. Interestingly, isoflurane preconditioning of ReNcell CX NPCs prevented the suppression of neuronal fate and enhancement of glial fate selections induced by

24-h isoflurane exposure. The suppression of neuronal fate by prolonged isoflurane exposure in this study is inconsistent with previous reports.[17,23] The difference in exposure duration is a possible reason for the discrepancies. Although exposure to 2.4% isoflurane for 24 h is rarely used in clinical settings, it served as a reliable approach for mechanistic insight into neurogenesis and survival of ReNcell CX NPCs. In addition, the contribution of InsP$_3$ and ryanodine receptors to the dual effects of isoflurane on ReNcell CX NPCs is inferred from highly specific pharmacologic antagonists for these receptors, but nonspecific effects occasionally associated with pharmacologic drugs cannot be ruled out completely. Thus, the findings of our study should not be used as a guide directly in anesthesia practice.
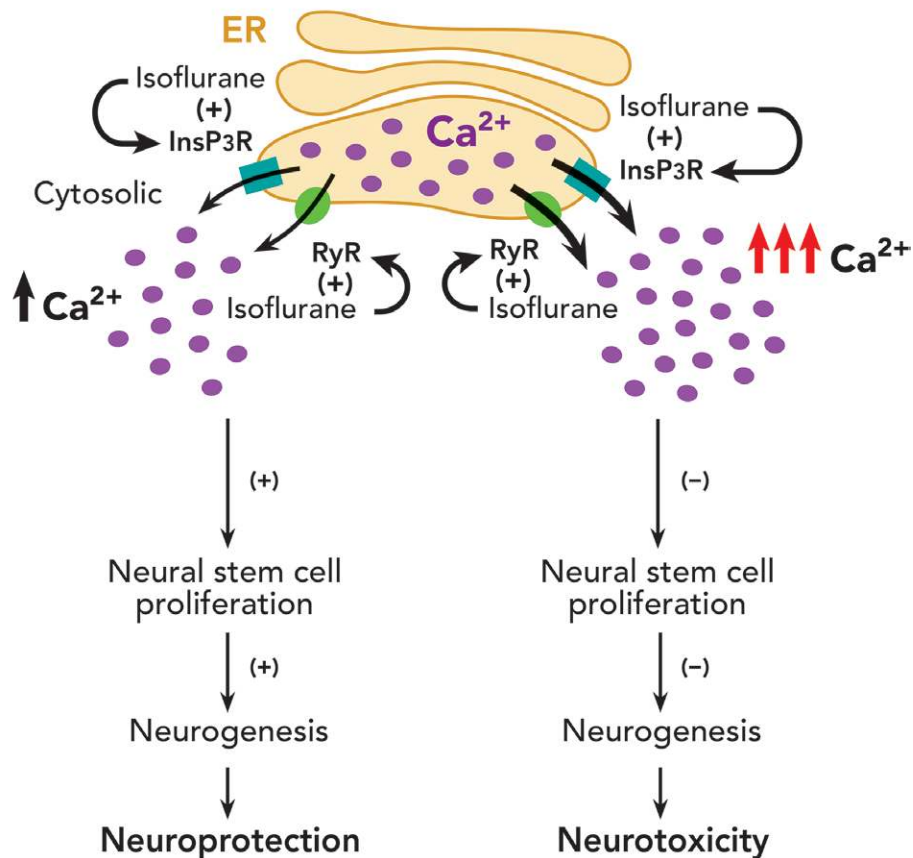

Fig. 7. Model for dual effects of isoflurane on neurogenesis. (*Left side*) Exposure to low and moderate concentrations of isoflurane for short durations induces moderate calcium release from the endoplasmic reticulum (ER) *via* activation of inositol-1,4,5-trisphosphate receptor (InsP$_3$R) and/or ryanodine receptor (RyR) calcium channels on ER membrane. This low or moderate increase in cytosolic calcium concentrations then promotes neurogenesis by stimulating neuronal stem cell (NSC) proliferation, thus providing neuroprotection. (*Right side*) Prolonged exposures to high concentrations of isoflurane induce excessive Ca^{2+} release from the ER *via* overactivation of InsP$_3$R and/or ryanodine receptors. This results in abnormally elevated cytosolic calcium concentrations and inhibition of neurogenesis by suppressing NSC proliferation, thus contributing to isoflurane-mediated neurotoxicity.

Our data suggest a model in which moderate Ca^{2+} release through $InsP_3$ and/or ryanodine receptors promotes neurogenesis (fig. 7). By contrast, excessive Ca^{2+} release caused by prolonged activation of these receptors may suppress neurogenesis (fig. 7). The preconditioning effects of isoflurane are likely attributable to a nondetrimental reduction in ER Ca^{2+} concentration short anesthetic exposure, which then mitigates excessive Ca^{2+} release in response to subsequent and prolonged isoflurane exposures (fig. 4). Indeed, isoflurane-preconditioned ReNcell CX NPCs displayed significantly fewer isoflurane-evoked changes in $[Ca^{2+}]_c$ (Fig 4). Accordingly, neurogenesis and survival of ReNcell CX NPCs are likely to correlate with the duration and level of isoflurane-induced cytoplasmic Ca^{2+} elevation, with short and moderate Ca^{2+} elevation inducing cytoprotection, whereas sustained and excessive Ca^{2+} elevation resulting from prolonged stimulation by isoflurane is expected to induce cytotoxicity (figs. 4 and 7). Given the versatility of Ca^{2+} as a second messenger, isoflurane-induced Ca^{2+} elevation *via* $InsP_3$ or ryanodine receptors may not be sufficient for the noted effects on ReNcell CX NPCs. Additional studies on other signaling pathways upstream and downstream of $InsP_3$ and ryanodine receptor activation should shed more light onto other possible contributing factors into isoflurane-mediated dual effects in these cells. It should be noted that isoflurane has been shown to increase cytoplasmic Ca^{2+} level through *N*-methyl-D-aspartate[1,54] and γ-aminobutyric acid receptors,[55,56] both upstream of ER Ca^{2+} signaling and major players in neurogenesis.[24,26] Isoflurane prolongs γ-aminobutyric acid A receptor activation[57] during the critical period of brain development and disrupts neurogenesis.[24] These results are remarkably consistent with the effects of isoflurane in this study, suggesting that γ-aminobutyric acid may act upstream of the ER to activate $InsP_3$ or ryanodine receptors to impinge on the noted isoflurane dual effects.

In summary, our findings suggest that isoflurane may affect ReNcell CX NPC survival and neurogenesis in a dual manner through differential activation of $InsP_3$ or ryanodine receptors located on the ER membrane. Given the complexity of Ca^{2+} signaling, we cannot attribute these effects solely to levels of Ca^{2+} elevation through $InsP_3$ and ryanodine receptors. However, our results suggest a strong association between isoflurane-induced activity on these receptors and the dual effects on human ReNcell CX NPC survival and neurogenesis.

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