Propofol Affects Neurodegeneration and Neurogenesis by Regulation of Autophagy *via* Effects on Intracellular Calcium Homeostasis

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ABSTRACT

Background: In human cortical neural progenitor cells, we investigated the effects of propofol on calcium homeostasis in both the ryanodine and inositol 1,4,5-trisphosphate calcium release channels. We also studied propofol-mediated effects on autophagy, cell survival, and neuro- and gliogenesis.

Methods: The dose–response relationship between propofol concentration and duration was studied in neural progenitor cells. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and lactate dehydrogenase release assays. The effects of propofol on cytosolic calcium concentration were evaluated using Fura-2, and autophagy activity was determined by LC3II expression levels with Western blot. Proliferation and differentiation were evaluated by bromodeoxyuridine incorporation and immunostaining with neuronal and glial markers.

Results: Propofol dose- and time-dependently induced cell damage and elevated LC3II expression, most robustly at 200 μM for 24 h (67 ± 11% of control, n = 12 to 19) and 6 h (2.4 ± 0.5 compared with 0.6 ± 0.1 of control, n = 7), respectively. Treatment with 200 μM propofol also increased cytosolic calcium concentration (346 ± 71% of control, n = 22 to 34). Propofol at 10 μM stimulated neural progenitor cell proliferation and promoted neuronal cell fate, whereas propofol at 200 μM impaired neuronal proliferation and promoted glial cell fate (n = 12 to 20). Cotreatment with ryanodine and inositol 1,4,5-trisphosphate receptor antagonists and inhibitors, cytosolic Ca^{2+} chelators, or autophagy inhibitors mostly mitigated the propofol-mediated effects on survival, proliferation, and differentiation.

Conclusions: These results suggest that propofol-mediated cell survival or neurogenesis is closely associated with propofol's effects on autophagy by activation of ryanodine and inositol 1,4,5-trisphosphate receptors. **(Anesthesiology 2017; 127:490-501)**

PROPOFOL, the most commonly used intravenous anesthetic agent, has been reported to cause brain cell degeneration,[1] as well as learning and behavior deficits,[2] in neonatal rodents. These studies have raised significant safety concerns over the administration of anesthetics in the pediatric population, and we propose that propofol may regulate neurogenesis when administered early in life. Neural progenitor cells (NPCs), which are plentiful in the postnatal developing rodent brain, are capable of differentiating into neurons and glial cells and provide a promising cell model to probe the underlying mechanisms governing anesthetic-induced neurotoxicity. Previous studies testing the administration of isoflurane have identified neuronal apoptosis in immature neurons[3] but not in NPCs.[4,5] However, changes in both proliferation and differentiation of NPCs were identified.[4,5] It remains unknown whether propofol has the same effect on NPCs as isoflurane. The aim of this study is to determine propofol's effects on and the role of autophagy activity in cortical-derived NPC viability, proliferation, and differentiation.

What We Already Know about This Topic

- In neonatal rodents, propofol has been reported to cause brain cell degeneration and learning and behavior deficits
- In neural progenitor cell cultures, isoflurane was reported to cause changes in cell proliferation and differentiation

What This Article Tells Us That Is New

- In neural progenitor cell cultures, only extremely high concentrations of propofol for 24 h caused cell damage and impaired neuronal proliferation
- Propofol effects appeared related to autophagy

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Materials and Methods

NPC Cultures

ReNcell CX cells, an immortalized human NPC line obtained from human fetal cortex (Millipore, USA), were cultured following the manufacturer's protocol. For all experiments, cells frozen between passages 6 and 15 were thawed and resuspended in laminin-coated (Sigma-Aldrich, USA) T75-cm^2 tissue culture flasks in ReNcell NSC maintenance medium (Millipore). To ensure that the cells remained in a proliferative state, 20 ng/ml of basic fibroblast growth factor (Sigma-Aldrich) and epidermal growth factor (Millipore) were added to the medium. The cell cultures were maintained in an incubator at 37°C, 95% humidity, and 5% CO_2. Culture medium was replaced every 24 h. Differentiation was induced by withdrawal of both growth factors (basic fibroblast growth factor and epidermal growth factor) at a confluence of approximately 70%.

Determination of Cytotoxicity

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) reduction assays were used to measure the cellular redox activity, a relatively early cell damage indicator. The assay measured the activity of mitochondrial dehydrogenase, which reduces MTT to formazan. MTT (0.5 mg/ml) was added to the growth medium in 96-well plates and incubated with NPCs for 4 h at 37°C. Formazan was solubilized from the medium in 150 μl of dimethyl sulfoxide, and the optical density was measured at 540 nm (Synergy™ H1 microplate reader; BioTek, USA). A lactate dehydrogenase (LDH) release assay (Thermo Scientific, USA) was used to quantify disruption of membrane integrity, an indicator of later stage cell damage, as described previously,[6] by measuring lactate dehydrogenase released by the cells into the medium. Briefly, 50 μl of the medium was added to 96-well plates with the reaction mixture for 30 min at room temperature. The reaction was stopped, and the mixture solution was measured at 490 and 680 nm (Synergy™ H1 microplate reader; BioTek). Background signal of the medium was deducted from control signals. The mean signal was determined from 6 to 10 wells per condition from three or four separate cultures for each condition (n = 18). The data were presented as percentages of vehicle control.

Cell Proliferation Assays

ReNcell CX cells were plated onto laminin-coated coverslips for 4 h in the proliferation medium containing the growth factors (see NPC Cultures). 5-Bromodeoxyuridine (Invitrogen, USA) at a concentration of 10 μM was mixed with the medium 24 h before the end of the propofol treatment. Then the cells were fixed (4% paraformaldehyde) and permeabilized (0.1% Triton X-100 in phosphate-buffered saline [PBS]). The cells then underwent acid treatment (1 N HCL on ice for 10 min followed by 2 N HCL at room temperature for 10 min), which separated the DNA into single strands so that the primary antibody could detect the 5-bromodeoxyuridine. After incubation with blocking serum (1% bovine serum and 10% goat serum in PBS), the cells were incubated overnight with rat monoclonal anti-5-bromodeoxyuridine primary antibody (1:100) (Santa Cruz Biotechnology, USA) at 4°C. The cells were washed (0.1% Triton X-100 in PBS), and 5-bromodeoxyuridine was detected with fluorescently labeled secondary antibody conjugated with anti-rat IgG (1:1,000 for 2 h) (Invitrogen). The immunostained cells were mounted on microscope slides with Prolong Gold Antifade Reagent containing 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) for visualization of cell nuclei. The cells were imaged on an Olympus BX41TF fluorescence microscope (200×; Olympus, USA) equipped with iVision v10.10.5 software (Biovision Technologies, USA). The number of DAPI-labeled cells and the number of 5-bromodeoxyuridine-labeled cells were counted, and the mean number of cells was calculated from 5 random areas of each coverslip, with 5 to 10 coverslips per condition, from 3 to 4 different cultures. The experimental n equals the number of coverslips. The data are expressed as the percentages of the number of 5-bromodeoxyuridine-positive cells to the total number of cells.

Cell Differentiation Assays

ReNcell CX cells were initially cultured with the same proliferation medium containing growth factors (see NPC Cultures) until just before the differentiation experiments, when the medium was replaced with medium devoid of growth factors. After the propofol treatment, the cells were allowed to differentiate for an additional 3 days, fixed (4% formaldehyde), and processed for immunocytochemistry overnight at 4°C using mouse monoclonal antibody reactive to the neuronal marker Tuj1 (1:200) (Covance, USA) or the rabbit polyclonal antibody reactive to astrocytes, glial fibrillary acidic protein (GFAP; 1:1,500) (Millipore). Thereafter, cells were incubated with Alexa 594 goat anti-mouse (1:1,000) and Alexa 488 goat anti-rabbit IgG antibodies (1:1,000) (Invitrogen) at room temperature for 1 h to visualize the primary antibody signal. The slides with immunostained cells were coverslipped with Prolong Gold antifade reagent containing DAPI to visualize cell nuclei, examined on an Olympus BX41TF fluorescence microscope (200×; Olympus), and the images were acquired using iVision v10.10.5 software (Biovision Technologies). Tuj1- or GFAP-positive cells overlapping with DAPI signal were analyzed at 10 random locations on each slide. The number of DAPI-labeled cells and the number of Tuj1- or GFAP-positive cells were counted, the mean number of Tuj1- or GFAP-positive cells were calculated for each slide, and the mean as determined for three slides per condition. The n equals the number of slides. The data are expressed as the percentages of the number of Tuj1- or GFAP-positive cells relative to the total number of cells.

Western Blot Analysis

A 6-well cell culture plate on ice was washed once with ice-cold PBS. After aspiration of PBS, 100 μl of ice-cold lysis buffer (1% Triton X-100, 150 mM NaCl, and 50 mM

Tris-HCl) was added to each well and maintained on ice for 5 min. The homogenate was collected with a cell scraper and then centrifuged at 4°C in a microcentrifuge at 10,000*g* for 30 min. The supernatant was gently collected and preserved at −70°C for future use. Bicinchoninic acid assay determined the protein concentration with a bicinchoninic acid kit (Thermo Scientific). For electrophoresis, 25 to 30 μg of protein from different samples were loaded on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and run with a constant current, and then the protein was transferred to nitrocellulose membranes (Bio-Rad, USA) using a wet transfer system (Bio-Rad). The membranes were blocked with 5% nonfat dry milk dissolved in PBS with 0.2% Tween 20 for 1 h at room temperature and incubated overnight at 4°C with the primary rabbit monoclonal antibody against LC_3 (1:1,000) (Cell Signaling Technology, USA) or primary mouse monoclonal antibody against β-actin (1:3,000) (Santa Cruz Biotechnology). This was followed by a wash with secondary antibody conjugated with anti-rabbit and anti-mouse IgG conjugated with horseradish peroxidase (1:10,000) (Bio-Rad) at room temperature for 1 h. The protein on the membranes was detected in a Kodak Image Station 4,000 MM Pro (Kodak, USA) ECL Prime Western blotting detection reagent (GE Healthcare, UK), and images were acquired with Carestream imaging software v5.3.4 (Carestream Health, USA). Signal intensity was quantitatively analyzed with ImageJ v1.49 software (https://imagej.nih.gov/ij/download.html), and the β-actin loading control was used for normalization. The n equals the number of wells for each condition.

Measurement Cytosolic Ca^{2+} Concentration

The NPC cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_c$) were measured by Fura-2 acetoxy methylester (AM) fluorescence (Invitrogen) on an Olympus IX70 inverted system microscope (Olympus) equipped with a photometer and IPLab v3.71 software (Scientific Instrument Company, USA), as previously described.[6] Briefly, NPCs were plated onto 35-mm laminin-coated culture dishes for 4 h, washed three times in Ca^{2+}-free Dulbecco's modified Eagle's medium (Gibco, USA), loaded with 2.5 μM Fura-2 AM in the same buffer at 37°C for 30 min, then washed, and incubated in Ca^{2+}-free Dulbecco's modified Eagle's medium for another 30 min at 37°C. The cells were exposed to propofol (200 μM) in minimum essential medium (Gibco), and fluorescence intensities were determined by recording at 340- and 380-nm excitation and at 510-nm emission for up to 5 min for each treatment. The data are presented as ratios of 340/380 nm of fluorescence intensity normalized to baseline. After each imaging experiment, the trypan blue exclusion assay was routinely used to ensure that the cells for $[Ca^{2+}]_c$ measurements were healthy and living.

Dose Selection of the Pharmacologic Agents

The doses of dantrolene and xestospongin C used in this experiment were the same as those used in our previous study with the same cell line.[6] In a pilot study, we conducted dose responses for *bis-N,N,N′,N′*-tetraacetic acid-acetoxy methylester (BAPTA-AM) and bafilomycin (data not shown). The maximum concentrations that did not induce cytotoxicity (5 μM and 400 nM, respectively) were chosen for this study. The doses of lithium used (2 and 10 mM) were based on previous studies.[7,8]

Statistical Analysis

The statistical analyses were performed, and graphs were created using GraphPad Prism 6 software (GraphPad Software, Inc., USA). The sample sizes we used were based on previous publications.[6,9,10] One set of MTT and LDH data for the dose response was excluded for suspicious contamination. Parametric variables are expressed as the means ± SD with a Gaussian distribution. The data were analyzed using one-way ANOVA followed by Tukey multiple comparisons testing or two-way ANOVA using propofol concentration and exposure duration as the between-group factors followed by the Bonferroni multiple comparisons test, as detailed in the figure legends. The results were considered statistically significant with $P < 0.05$, based on two-tailed analysis.

Results

Propofol Induces NPC Cytotoxicity through Excessive Autophagy by Overactivation of Inositol 1,4,5-Trisphosphate and/or Ryanodine Receptors

ReNcell CX NPCs were exposed to different concentrations of propofol (1 to 300 μM) for 2, 6, or 24 h. MTT assays determined that treatment with 10 μM propofol, a clinically relevant concentration, increased cell viability after the 6- and 24-h exposures. On the contrary, propofol at 200 or 300 μM for the same duration (6 h and 24 h) decreased cell viability, whereas 100 μM propofol had no effect on cell survival throughout the experiment (fig. 1A). However, LDH assays showed that significant cytotoxicity was only observed with the 200 μM propofol treatment for 24 h, indicating late-stage cell death (fig. 1B). Our results suggested that the concentration and exposure duration of propofol were the determining factors for the survival of ReNcell CX NPCs.

Previous studies suggested that propofol impacted cellular biology through an autophagy-involved regulatory mechanism.[11,12] In this study, we proposed that autophagy plays a role in the cytotoxicity induced by propofol. Western blots showed a time- and concentration-dependent increase in the level of the autophagy biomarker LC3II during propofol treatment, peaking at 24 h with 100 μM and at 6 h with 200 μM propofol (fig. 2A). However, exposure to 10 μM propofol, a clinically relevant concentration, did not show a significant difference in LC3II (fig. 2A) in ReNcell CX NPCs. To differentiate between the induction of autophagy from the impairment of autophagy flux, bafilomycin was added to the culture medium at 22 h of the 24-h propofol exposure period. Further increases in the LC3II level after the bafilomycin treatment indicated the induction of autophagy by propofol (fig. 2B).

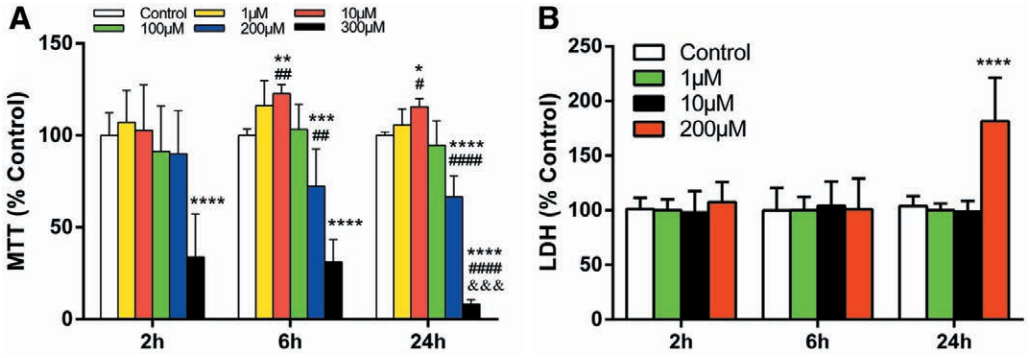

Fig. 1. Propofol induces neural progenitor cell cytotoxicity in a time- and dose-dependent manner. ReNcell CX human neural progenitor cells were exposed to different concentrations of propofol (1 to 300 μM) for 2, 6, or 24 h. (*A*) The 3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction assay was used to determine relatively early cell damage. Treatment with 10 μM propofol increased, whereas 200 μM propofol decreased cell viability after 6- and 24-h exposures. 300 μM propofol induced remarkable cell damage at all three time points relative to vehicle control. #$P < 0.05$, ##$P < 0.01$, ####$P < 0.0001$ compared with treatment for 2 h with the same concentration; &&&$P < 0.001$ compared with treatment for 6 h; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ compared with vehicle controls at corresponding time points (n = 19 at 2 h; n = 12 at 6 h; n = 17 at 24 h). (*B*) Relatively late cell damage was determined by lactate dehydrogenase (LDH) release assay. Only 200 μM propofol treatment for 24 h exhibited significant cell damage. ****$P < 0.0001$ compared with vehicle controls (n = 18). All data are expressed as the means ± SD from at least three separate experiments in duplicate or triplicate and analyzed by two-way ANOVA followed by Tukey multiple comparison tests.

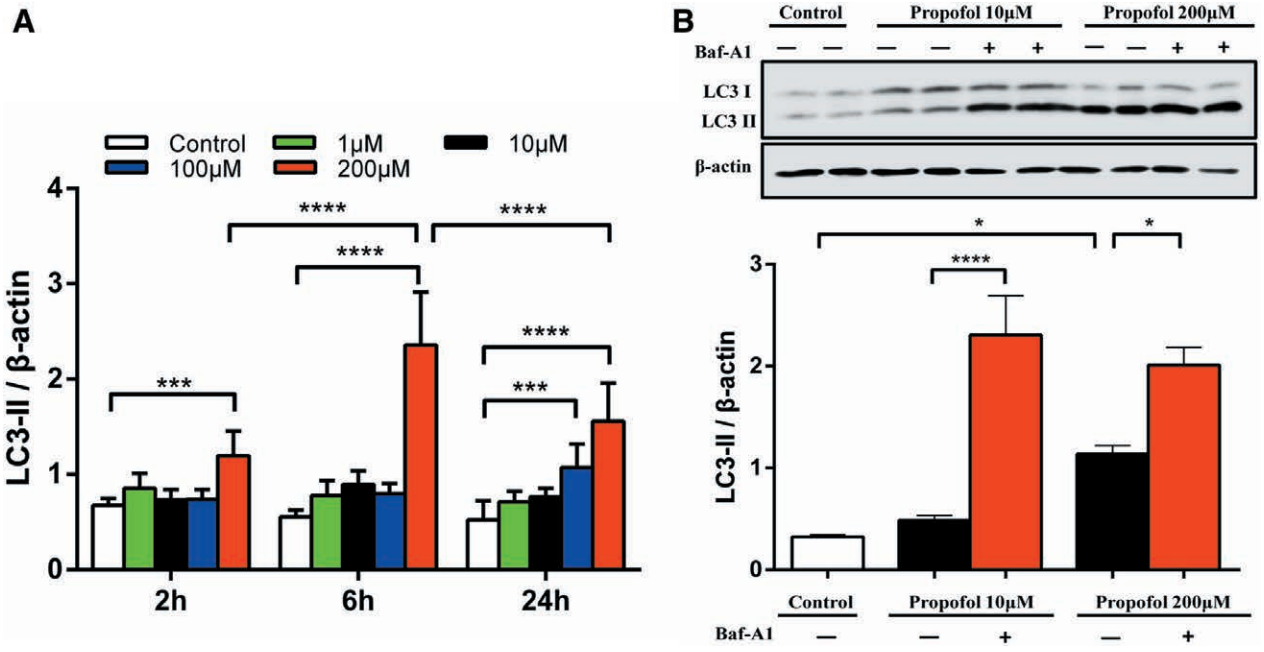

Fig. 2. Propofol induced autophagy in neural progenitor cells. ReNcell CX human neural progenitor cells were exposed to propofol concentrations ranging from 1 to 200 μM for 2, 6, or 24 h. (*A*) Quantitative analysis of Western blots of the autophagy biomarker, LC3II. The LC3-II/β-actin ratio showed that 200 μM propofol increased the levels of LC3-II at exposure durations at all time points (n = 7). (*B*) A representative immunoblot of LC3II from three separate experiments from neural progenitor cells cultured with 10 or 200 μM propofol for 24 h and the densitometric analysis of the LC3II/β-actin ratio. Further increases in the LC3II level after bafilomycin (Baf-A1; 400 nM) treatment indicated induction of autophagy by propofol (n = 6). All bar graphs are presented as means ± SD from three separate experiments with duplicate samples and analyzed by two-way ANOVA followed by Tukey multiple comparison tests. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$ compared with corresponding time control or as indicated.

Previously, we detected that isoflurane induced ReNcell CX cytotoxicity by Ca^{2+} release from the endoplasmic reticulum (ER) through inositol 1,4,5-trisphosphate ($InsP_3$) and/or ryanodine receptors (RyRs).[6] To evaluate the role that Ca^{2+} release from $InsP_3$ and RyRs may have in the propofol-mediated cytotoxicity, the intracellular Ca^{2+} concentrations were measured after propofol treatments in the presence of the inositol 1,4,5-trisphosphate receptor ($InsP_3R$) antagonist xestospongin C, the ryanodine receptor antagonist dantrolene, the intracellular Ca^{2+} chelator BAPTA-AM,

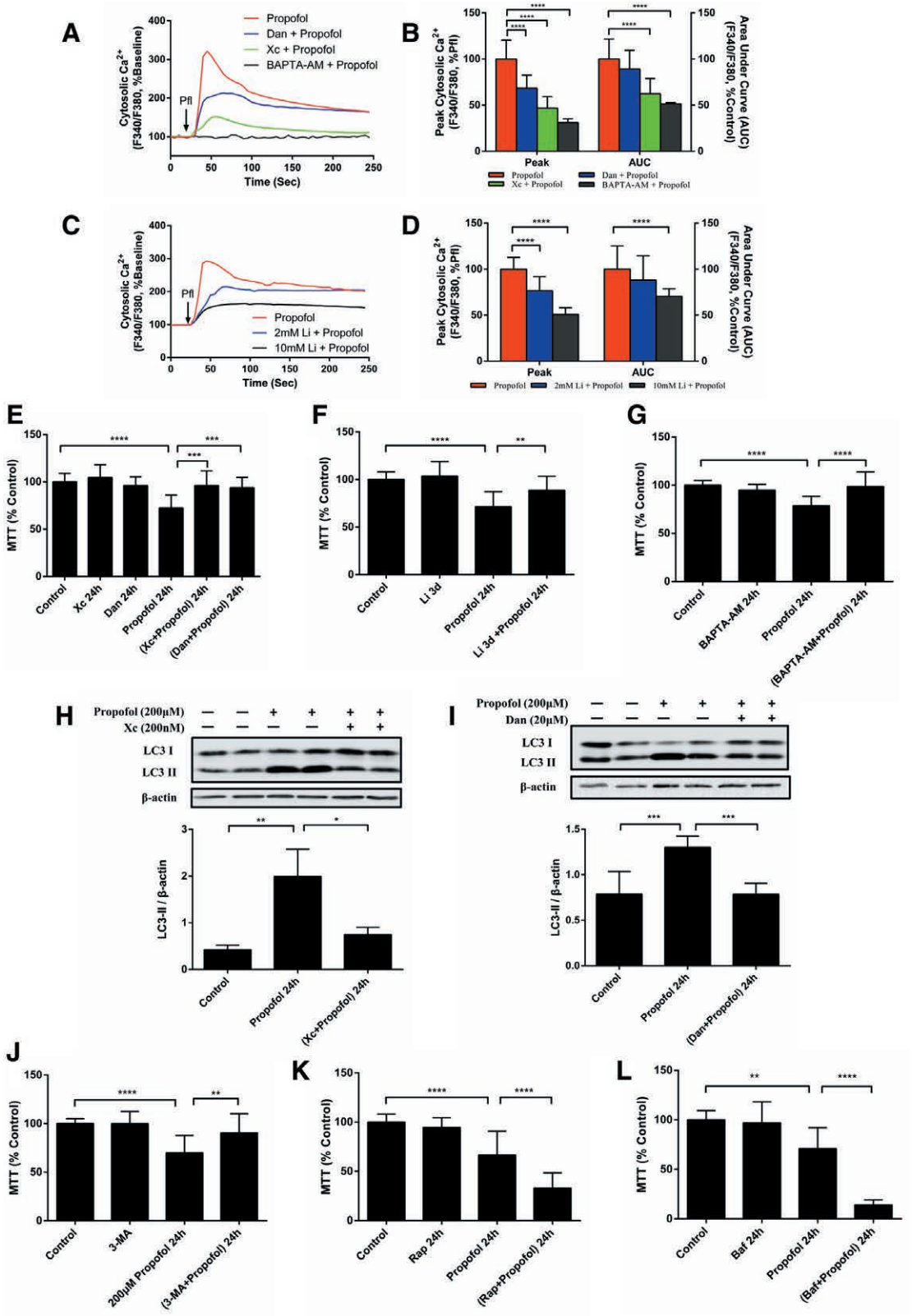

Fig. 3. (*Continued*)

Fig. 3. Propofol induces neural progenitor cell (NPC) damage through excessive autophagy by inositol 1,4,5-trisphosphate ($InsP_3$) and/or ryanodine receptors. (*A*) Average calcium response to 200 μM propofol (Pfl, *arrow*) in NPCs cultured in normal medium or medium pretreated with 200 nm of the inositol 1,4,5-trisphosphate receptor antagonist, xestospongin C (Xc), 20 μM of the ryanodine receptor antagonist, dantrolene (Dan), or 5 μM of the intracellular calcium chelator, *bis-N,N,N',N'*-tetraacetic acid-acetoxy methylester (BAPTA-AM). Changes in Fura-2 AM intensities from at least 15 single cells in three separate experiments were measured to assess the F_{340}/F_{380} ratio, a representative indicator for the cytosolic calcium concentration ($[Ca^{2+}]_c$). The F_{340}/F_{380} ratios were normalized to baseline. (*B*) Pretreatment with Xc, Dan, or BAPTA-AM decreased the peak amplitudes and the areas under the curve (AUC) of the propofol-evoked $[Ca^{2+}]_c$ signals. ****$P < 0.0001$ compared with propofol administration alone. (*C*) Average calcium response to 200 μM propofol (*arrow*) in NPCs cultured in normal medium or medium pretreated with inositol 1,4,5-trisphosphate production inhibitor lithium (Li; 2 or 10 mM). (*D*) Pretreatment of 2 or 10 mM Li significantly decreased the peak amplitudes and AUC of propofol-evoked $[Ca^{2+}]_c$ signals. ****$P < 0.0001$ compared with propofol administration alone. (*E–G*) Cell viability, determined with the 3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction assay, detected inhibition of cell damage induced by 200 μM propofol for 24 h after cotreatment with Xc and Dan (*E*), pretreatment with Li for 3 days (*F*), and cotreatment with BAPTA-AM (*G*). The data in (*E*, *F*) are expressed as the means ± SD from at least three separate experiments and analyzed by one-way ANOVA followed by Tukey multiple comparison tests. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. (*H*) A representative immunoblot of LC3II of three separate experiments from cells cultured with 200 μM propofol for 24 h with or without Xc. (*I*) Dan and the quantitative analysis of LC3II/β-actin ratio (means ± SD). Cotreatment with Xc or Dan alleviated the propofol-induced elevation in the autophagy biomarker, LC3II. (*J*) Similarly, the NPC cytotoxicity induced by 200 μM propofol for 24 h was mitigated by cotreatment with 5 nM of the autophagy inhibitor 3-methyladenine (3-MA). (*K*, *L*) Cotreatment with (*K*) autophagy inducer rapamycin (Rap; 100 nM) or (*L*) autophagy flux inhibitor bafilomycin (Baf; 1 nM) promoted NPC damage induced by 200 μM propofol for 24 h. The data in (*I*, *J*) are expressed as the means ± SD from at least three separate experiments and analyzed by one-way ANOVA followed by Tukey multiple comparison tests. **$P < 0.01$, ****$P < 0.0001$.

or the $InsP_3$ production inhibitor lithium. Propofol alone induced a greater elevation in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) than in the presence of xestospongin C, dantrolene, or BAPTA-AM (fig. 3, A and B). Pretreatment with 2 or 10 mM lithium significantly decreased the peak amplitudes and the area under curve of propofol-evoked $[Ca^{2+}]_c$ signals (fig. 3, C and D). These results suggested that propofol possibly increased the $[Ca^{2+}]_c$ by Ca^{2+} release from the $InsP_3$ or RyRs into the ER. Thus, we propose that the cytotoxicity induced by propofol was mediated by these ER membrane receptors' excessive release of calcium into the cytosol. The MTT reduction assays showed that cotreatment with xestospongin C, dantrolene, BAPTA-AM, or lithium inhibited ReNcell CX cell damage induced by 200 μM propofol for 24 h (fig. 3, E–G).

We further questioned whether $InsP_3Rs$ and/or RyRs were also involved in the induction of autophagy flux stimulated by propofol. Blockage of $InsP_3Rs$ or RyRs significantly alleviated propofol-induced elevation of the autophagy biomarker LC3II (fig. 3, H and I). ReNcell CX cell damage induced by 200 μM propofol for 24 h was mitigated by cotreatment with autophagy inhibitor 3-methyladenine and promoted by cotreatment with the autophagy inducer rapamycin or the autophagy flux inhibitor bafilomycin (fig. 3, J–L), meaning that excessive autophagy activity was associated with propofol-induced cell damage, which may have resulted in type II autophagic cell death.[13]

Propofol Affects Proliferation in NPCs and Regulation of Autophagy

Because autophagy is involved in differentiation and development,[14,15] we investigated whether autophagy plays a role in the effects of propofol on proliferation. The impact of propofol on the proliferation of ReNcell NPCs was assessed at various concentrations and exposure times. Compared with control cells, exposure to the clinical concentration of propofol (10 μM) for 24 h promoted cell proliferation, whereas 200 μM propofol treatment significantly decreased proliferation (fig. 4, A and B). Interestingly, cotreatment with xestospongin C or dantrolene alleviated the dual effects of propofol on NPC proliferation, except for the dantrolene treatment with 10 μM propofol (fig. 4, C and D). These results suggest that the dual effects of propofol on cell proliferation is mediated by calcium released from the ER, whereas the distinctly opposing impact of differing concentration may be due to the amount of calcium released. Propofol at 200 μM for 24 h significantly impaired proliferation, but this could be inhibited by the autophagy inhibitor 3-methyladenine (3-MA) and potentiated by the autophagy inducer rapamycin (fig. 4, E and F). However, cotreatment with 3-MA or rapamycin had no effect on the 10 μM propofol-induced proliferation increase (fig. 4G). These results suggest that suppression of proliferation induced by propofol at 200 μM for 24 h was related to increased autophagy activity, whereas the clinical concentration of propofol accelerated cell proliferation *via* a nonautophagy mechanism.

Propofol Affects NPC Differentiation via Activation of $InsP_3Rs$

Exposure to 10 μM propofol for 24 h promoted neuronal fate as measured by the increase in Tuj1-positive cells and suppressed glial fate as determined by the decrease of GFAP-positive cells (fig. 5, A and B). On the contrary, 200 μM propofol decreased the percentage of Tuj1-positive cells and increased GFAP-positive cells, indicating an enhancement of glial fate. Consistent with propofol's effects on cytotoxicity and proliferation, cotreatment with the $InsP_3R$ antagonist xestospongin C mitigates these dual effects of propofol, suggesting the prominent role of $InsP_3Rs$ in these processes (fig. 5, C and D).

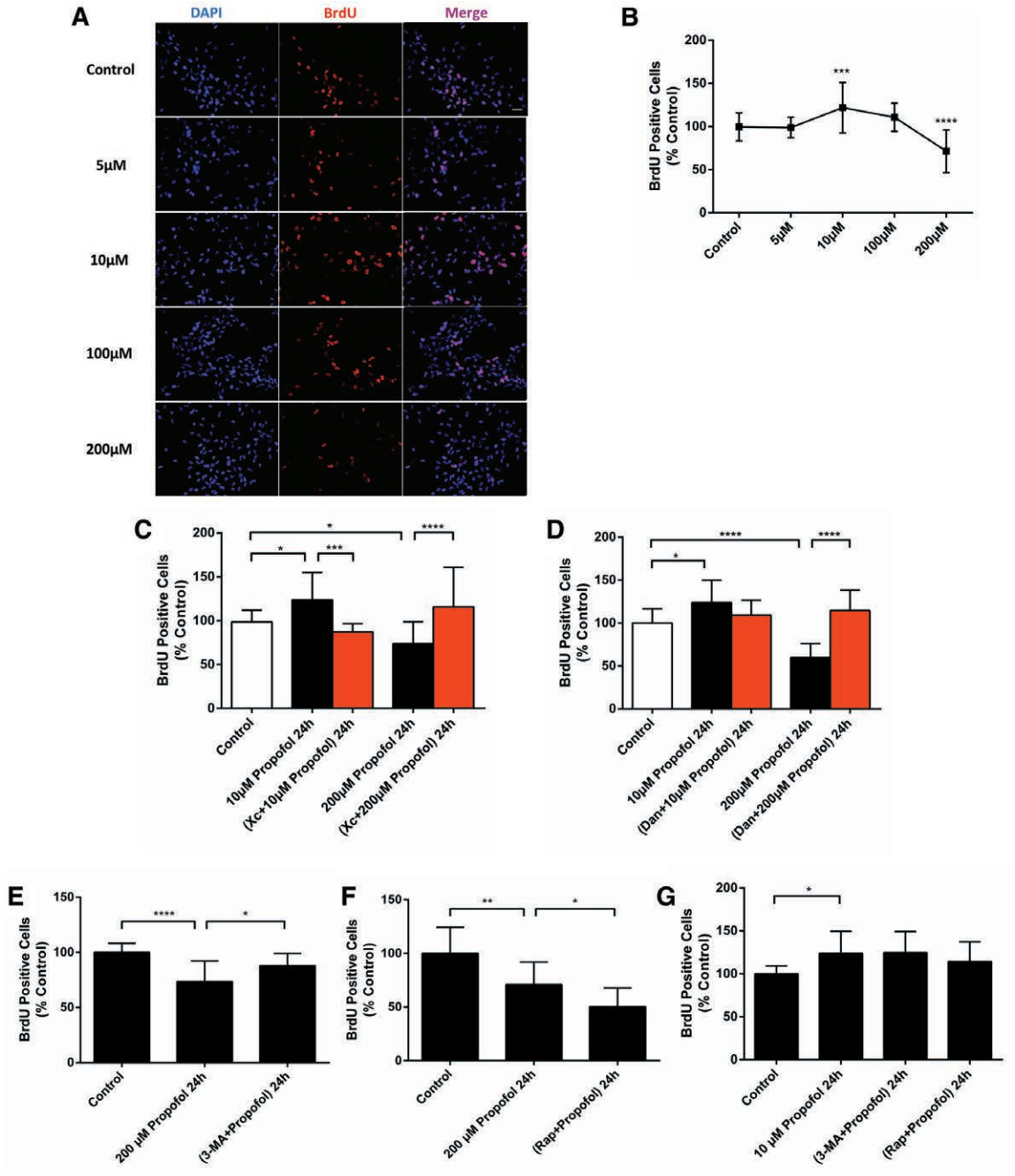

Fig. 4. Propofol affects neural progenitor cell proliferation and regulation of autophagy. (*A*) Representative images showing the double immunostaining of cell nuclei with 4′,6-diamino-2-phenylindole (DAPI, *blue*, *arrows*) and 5-bromodeoxyuridine (BrdU, *red*, *arrows*) in ReNcell CX neural progenitor cells (NPCs) with or without propofol (10, 200 μM) for 24 h. *Scale bar* = 50 μm. (*B*) Quantitative analysis of the effect of a 24-h exposure to 10 or 200 μM propofol on the proliferation of BrdU-positive NPCs. Exposure to 10 μM propofol increased the percentage of BrdU-positive cells, whereas 200 μM propofol resulted in significantly decreased proliferation (n = 30). (*C*, *D*) Quantitative analysis of the effect of a 24-h exposure to 10 or 200 μM propofol on the proliferation of BrdU-positive NPCs, with or without the inositol 1,4,5-trisphosphate receptor antagonist, xestospongin C (Xc, 50 nm) (*C*) or the ryanodine receptor antagonist, dantrolene (Dan, 1 μM) (*D*). Cotreatment with xestospongin C (n = 20) or dantrolene (n = 14) alleviated the dual effects of propofol on NPC proliferation. (*E*) Quantitative analysis of the effect of a 24-h exposure to 10 or 200 μM propofol on the proliferation of BrdU-positive NPCs, with or without the autophagy inhibitor 3-methyladenine (3-MA, 5 mM) or the autophagy inducer rapamycin (Rap; 100 nM). (*F*) Propofol significantly impaired proliferation at 200 μM for 24 h, which was inhibited by 3-MA (n = 15) and potentiated by Rap (n = 15). (*G*) Cotreatment with 3-MA or Rap had no effect on 10 μM propofol-induced increase of proliferation (n = 17). The above data are expressed as the means ± SD from at least three separate experiments and analyzed by one-way ANOVA followed by Tukey multiple comparison tests. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, or *****P* < 0.0001, compared with vehicle control or as indicated.

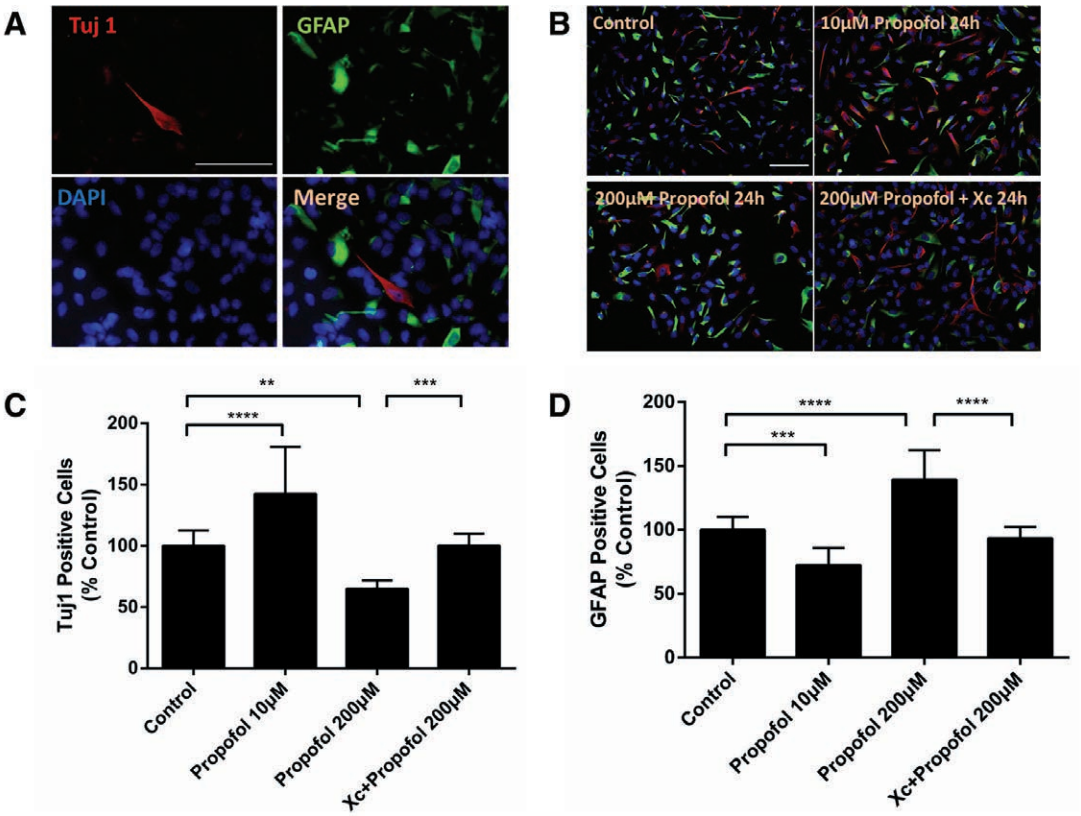

Fig. 5. Propofol affects neural progenitor cell differentiation *via* inositol 1,4,5-trisphosphate receptors. (*A*) Representative micrographs showing immunostaining for Tuj1, neuronal class III β-tubulin (*red*), glial fibrillary acidic protein (GFAP; *green*), and 4′,6-diamino-2-phenylindole (DAPI; *blue*) of ReNcell neural progenitor cells cultured in differentiation medium for 3 days. (*B*) Representative micrographs showing immunostaining of Tuj1 (*red*), GFAP (*green*), and DAPI (*blue*) of neural progenitor cells cultured with 10 or 200 μM propofol, with or without the inositol 1,4,5-trisphosphate receptor antagonist, xestospongin C (Xc; 50 nm), followed by differentiation medium for 3 days. (*C*, *D*) Tuj1-positive (*C*) or GFAP-positive (*D*) cells were counted, and the data expressed are as percentages of controls. Propofol at 10 μM increased Tuj1-positive neuronal cells but decreased the GFAP-positive glial cells. Propofol at 200 μM had the opposite effect of deceasing Tuj1-positive cells and increasing GFAP-positive cells. These effects could be mitigated by cotreatment with Xc. *Scale bar* = 50 μm. The data are expressed as the mean ± SD and analyzed by one-way ANOVA followed by Tukey multiple comparison tests. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Discussion

This study examined the effects of propofol on human cortex-derived NPCs and the role of calcium-mediated autophagy pathway in neurogenesis and neurodegeneration. We found that propofol induced Ca^{2+} release from the endoplasmic reticulum of NPCs by activation of the $InsP_3R$ and/or RyR. A clinically relevant dose of propofol promoted cell proliferation and favored neuronal cell fate *via* adequate activation of the $InsP_3R$ and adequate autophagy. However, a high dose of propofol induced cell damage and favored glial cell fate through excessive autophagy by overactivation of $InsP_3Rs$ and RyRs.

Previous studies have shown that propofol can induce both excitotoxic and apoptotic cell death[16] in cell cultures[17,18] and in animal models,[19,20] although the mechanism is not clear. Most of the previous studies have focused on the anesthetic-induced type 1 cell death (apoptosis),[21,22] but cells can also die from type 2 (autophagic cell death) or type 3 cell death (necrosis). Palanisamy *et al.*[23] recently demonstrated that propofol, at approximately 15 and 30 μM for a 4-h exposure, did not induce rat primary NPC necrotic cell death and apoptosis. However, prolonged use of propofol for 24 h transiently impaired proliferation. Consistent with these results, we detected significant NPC cytotoxicity at an extremely high pharmacologic concentration (200 μM) but not at clinically relevant concentrations (1, 10 μM). In contrast, in this study, propofol at 10 μM for 24 h promoted NPC proliferation, whereas in the previous study,[23] propofol at about 15 μM (2.5 μg/ml) transiently impaired rat primary NPC proliferation. The discrepancy between these two studies is likely due to the different types of cells, primary *versus* immortalized NPCs. Future studies in rat primary NPCs at clinically relevant concentrations will be needed to sort out these differences.

There is a consensus that autophagy is required for cell survival and is involved in various physiologic events, including immune responses, cancer, aging, and neurodegeneration,[24,25] as well as brain development and differentiation.[14,15,26] A recent study has shown that knockdown of the

of the autophagy-related gene Atg5 inhibited cortical NPC differentiation but increased proliferation during embryonic brain development.[27] The exact role of autophagy during neurogenesis, as well as the consequences of pharmacologic stresses, such as anesthetics, on autophagy, are unclear.

Like most anesthetics including pentobarbital, ketamine, and isoflurane, propofol exhibited up-regulation of autophagy in skeletal muscles[28] and human umbilical vein endothelial cells.[11] This dose-dependent stimulatory effect of propofol on autophagy flux was also observed in our experiments. To implicate autophagy as a possible contribution to neurogenesis, an autophagy inducer or inhibitor was applied, together with propofol. As confirmed by MTT and proliferation assays, cell damage or decreased proliferation correlated with excessive induction of autophagy flux by propofol exposure. Consistent with our results, autophagy is believed to be a double-edged sword in ischemia and preconditioning.[29] Physiologic autophagy eliminates harmful protein aggregates and damaged organelles in cells and thus limits the transmission of harmful signaling; excessive or pathologic autophagy, however, induces type II autophagy cell death and causes irreversible injury.

We also explored changes in cytosolic calcium concentrations $[Ca^{2+}]_c$ as a possible mechanism for our observed effects. Previously, propofol has been reported to increased $[Ca^{2+}]_c$ in glia, neurons,[30] and neural stem/progenitor cells[31] and that an increase in $[Ca^{2+}]_c$ within the physiologic range promotes cell proliferation, differentiation, synthesis, and catabolism.[32] Similarly, we found that $[Ca^{2+}]_c$ almost tripled after propofol treatment, and we posit that propofol initiated a Ca^{2+}-dependent signaling pathway.

The γ-aminobutyric acid type A receptor is one of the likely targets for propofol. Previous research has shown that activation of γ-aminobutyric acid receptor type A receptors by isoflurane during brain development may cause cell membrane depolarization, Ca^{2+} influx through voltage-dependent Ca^{2+} channels, and increased $[Ca^{2+}]_c$.[33] Recently, $InsP_3Rs$ have been implicated as a molecular target for isoflurane and to be involved in anesthetic-mediated neurotoxicity.[10] The $InsP_3Rs$ and RyRs are the main channels through which Ca^{2+} leaves the ER, although a small amount of Ca^{2+} leaves the ER through the so-called leak channel. The increase in the $[Ca^{2+}]_c$ after isoflurane administration in mature neurons was caused primarily by calcium release from intracellular stores, most likely from the ER. Therefore, we propose that the mechanism of propofol's impact on neurogenesis on NPCs is $InsP_3R$ activation, followed by a rise in the $[Ca^{2+}]_c$, and finally either the induction or an overactivation of autophagy, depending on the amount of Ca^{2+} released (fig. 6). Indeed, we found that $InsP_3R$ inhibition prevented most of the effects of propofol on cell viability, autophagy, proliferation, and differentiation. Although the mechanisms underlying

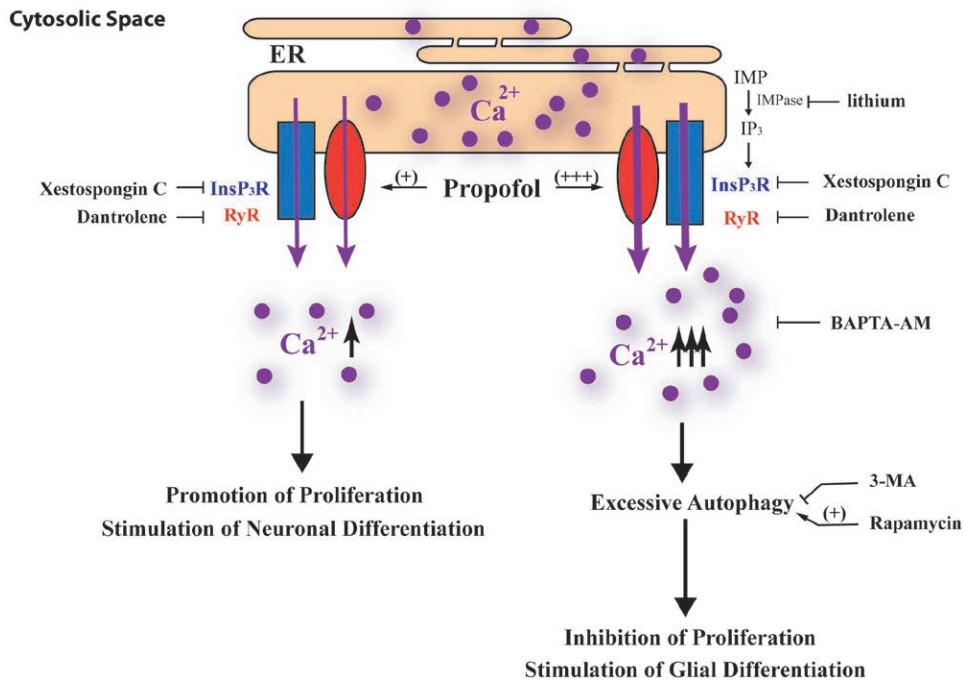

Fig. 6. Summary of the effects of propofol on neuronal progenitor cell (NPC) proliferation and differentiation. Exposure to a clinically relevant concentration of propofol induces calcium release from the endoplasmic reticulum (ER) through the activation of the calcium channels, inositol 1,4,5-trisphosphate receptors ($InsP_3R$), and/or ryanodine receptors (RyR). This results in a moderate increase in the concentration of cytosolic calcium that stimulates NPC proliferation and neuronal and glial cell differentiation. These effects involve a nonautophagy mechanism. On the other hand, a prolonged exposure to a high concentration of propofol induces the overactivation of $InsP_3Rs$ and/or RyRs on the ER membrane, thereby allowing the buildup of intracellular Ca^{2+}. This elevated concentration of cytosolic calcium leads to excessive autophagy and the inhibition of NPC proliferation, which contributes to the propofol-mediated neurotoxicity. BAPTA-AM = *bis*-*N*,*N*,*N*',*N*'-tetraacetic acid-acetoxy methylester; IMP = inositol monophosphatase; IP_3 = inositol 1,4,5-trisphosphate; 3-MA = 3-methyladenine.

these observed results by propofol in human cortical NPCs are not fully apparent, our results suggest the involvement of $InsP_3Rs$ and RyRs. Because the mitochondria and the ER have intracellular communication sites, they may also regulate Ca^{2+} homeostasis by uptake of calcium released by the ER,[34] especially when intracellular calcium concentrations are high, such as after exposures to anesthetics. A previous study[35] demonstrated that mitochondrial Ca^{2+} concentrations are increased after isoflurane-mediated Ca^{2+} release from the ER *via* activation of $InsP_3R$, followed by transfer into mitochondria. It is important that future studies investigate whether propofol has similar effects, because mitochondria play important roles in ATP production and autophagy regulation.[36]

The outcome of either physiologic autophagy or pathologic autophagy may be explained by $InsP_3Rs$ and intracellular Ca^{2+} signaling. Although apparently contradictory, both autophagic flux[37] and inhibition[38] result from autolysosomal formation from an increase in autophagosomes to lysosomes. Our experimental data provide compelling evidence for both the propofol-mediated dose-dependent increase in $[Ca^{2+}]_c$ and the subsequent induction of autophagy. The different outcomes of autophagy are possibly due to a divergent role of the $InsP_3R$ with respect to basal *versus* stress-induced autophagy and the different spatiotemporal characteristics of Ca^{2+} signals that can be generated, each having distinct impacts on different steps in the autophagy pathway.

There is evidence, from *in vitro*[39] and *in vivo*[40] studies, that lithium, a common drug for the treatment of neuropsychiatric disorders, has facilitated neurogenesis and reduced the neurotoxicity induced by certain stressors, including anesthetics.[41] It has been proposed that the mechanism for the neuroprotective properties of lithium result from its interactions with cell survival and cell death pathways. Lithium can affect intracellular Ca^{2+} homeostasis by inhibiting *N*-methyl-D-aspartate receptor-mediated Ca^{2+} entry into the cell, as well as reducing $InsP_3$ production by inhibiting inositol monophosphatase. Lithium can also affect autophagy *via* its regulation of intracellular Ca^{2+} homeostasis,[42] inhibition of GSK-3β, and activation of the inhibitory regulator mammalian target of rapamycin.[43] Thus, the effects of lithium observed in our experiment may be explained by its effects on the regulation of calcium homeostasis and the associated autophagy process. In addition, lithium may affect propofol's effects due to their mutual interaction at the voltage-gated Na^+ channel, because both have been demonstrated to affect these Na^+ channels.[44] Thus, the effects of lithium appear to outweigh the effects of propofol at lower concentrations than at higher anesthetic concentrations.

Propofol exposure during the period of rapid brain development in neonatal animals has negatively impacted hippocampal structure and function, resulting in subsequent cognitive impairment.[2,45] The mechanisms for propofol's deleterious effects during development are conflicting, including interference with n eural stem cell function, neurogenesis, and synapse formation.[4,46] Such controversy may be due to confounding variables in the use of animal models.[47,48] In an attempt to reduce such variables, we chose to study NPCs *in vitro*. Our results suggested that at clinically relevant concentrations of propofol, proliferation contributed more to the increased cell viability than differentiation. Although the increased cell viability may also be due to suppressed apoptosis, a previous study indicated that apoptosis was not a major factor associated with improved viability when neural stem cells were treated with propofol.[9] Propofol administration is generally considered to be excitotoxic neurodegeneration, which is fundamentally different, both in type and sequence, from that of the apoptotic phenomenon.[16] Propofol toxicity was only observed at concentrations that exceeded clinically relevant concentrations. Corresponding with previous results, propofol at clinical concentrations increased NPC differentiation into neurons,[49] whereas differentiation into astrocytes was decreased.[50]

When interpreting the data presented in our study, some limitations must be considered. Our investigations into the mechanisms of the propofol-induced changes on calcium homeostasis and autophagy, and thus NPC fate, were not exhaustive. Recent studies indicated that enhanced cAMP response element binding protein (CREB) phosphorylation may play a role in the Ca^{2+}-mediated pathway[9] and in both proliferation and differentiation of neural stem cells.[51] Furthermore, the protective effect of xenon preconditioning for asphyxia is thought to involve pCREB-regulated protein synthesis in rat brain.[52] Downstream signaling after CREB activation may be the key to direct stem cell fate, and further study is required to elucidate the mechanisms.

In conclusion, only extreme, high pharmacologic concentrations of propofol adversely affect NPC cell viability *in vitro* by excessive autophagy through a Ca^{2+}-mediated pathway. Clinically relevant doses of propofol enhance proliferation of NPCs and increase neuronal differentiation by a Ca^{2+}-related nonautophagic mechanism.

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Competing Interests

The authors declare no competing interests.

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