

# Propofol at Clinically Relevant Concentrations Increases Neuronal Differentiation But Is Not Toxic to Hippocampal Neural Precursor Cells *In Vitro*

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## ABSTRACT

**Background:** Propofol in the early postnatal period has been shown to cause brain cell death. One proposed mechanism for cognitive dysfunction after anesthesia is alteration of neural stem cell function and neurogenesis. We examined the effect of propofol on neural precursor or stem cells (NPCs) grown *in vitro*.

**Methods:** Hippocampal-derived NPCs from postnatal day 2 rats were exposed to propofol or Diprivan. NPCs were then analyzed for bromodeoxyuridine incorporation to measure proliferation. Cell death was measured by lactate dehydrogenase release. Immunocytochemistry was used to evaluate the expression of neuronal and glial markers in differentiating NPCs exposed to propofol.

**Results:** Propofol dose dependently increases the release of lactate dehydrogenase from NPCs under both proliferating and differentiating conditions at supraclinical concentrations (more than 7.1  $\mu\text{M}$ ). Both Diprivan and propofol had the same effect on NPCs. Propofol-mediated release of lactate dehydrogenase is not inhibited by blocking the  $\gamma$ -aminobutyric acid type A receptor or extracellular calcium influx and is not mediated by caspase-3/7. Direct  $\gamma$ -aminobutyric acid type A receptor activation did not have the same effect. In differentiating NPCs, 6 h of propofol at 2.1  $\mu\text{M}$  increased the number

### What We Already Know about This Topic

- Propofol has been shown to kill immature neurons in various cellular and animal models; whether it is also toxic to neural progenitor cells is unknown
- The effect of propofol on hippocampal-derived neural progenitor cell division, death, and differentiation was studied *in vitro*

### What This Article Tells Us That Is New

- Propofol toxicity was observed, but only at concentrations above those relevant to anesthesia, although clinical concentrations increased neuronal differentiation
- Effects of propofol on developing neurons do not appear to involve toxicity to neural progenitor cells

neurons but not glial cells 4 days later. Increased neuronal differentiation was not blocked by bicuculline.

**Conclusions:** Only supraclinical concentrations of propofol or Diprivan kill NPCs in culture by a non- $\gamma$ -aminobutyric acid type A, noncaspase-3 mechanism. Clinically relevant doses of propofol increase neuronal fate choice by a non- $\gamma$ -aminobutyric acid type A mechanism.

**P**ROPOFOL (2,6-diisopropylphenol) is a sedative and anesthetic drug commonly used for induction and intravenous maintenance in the operating room, for short-term procedural sedation (such as magnetic resonance imaging or endoscopy), and for long-term sedation in the intensive care unit. It is favored for its rapid onset, antinausea effects, and short recovery time. Like many other anesthetics, propofol has been shown to cause immediate brain cell degeneration in neonatal but not adult rodents.<sup>1-4</sup>

Propofol has a wide variety of potential targets, most obvious being the  $\gamma$ -aminobutyric acid type A receptor (GABA<sub>A</sub> receptor), but also including glycine, glutamate, nicotinic, and muscarinic receptors.<sup>5,6</sup> The mechanism of cell death that has been suggested after propofol exposure in neurons isolated from very young rodents is early GABA<sub>A</sub> receptor activation leading to a rise in calcium concentration<sup>7</sup> that is followed by activation of calpain and caspase-3.<sup>2,3,7</sup> An outstanding recent study by Pearn *et al.* clearly identifies an important early role for the p75 neurotrophin receptor followed by activation of cleaved caspase-3 both *in vitro* and *in vivo*, although the role of the GABA<sub>A</sub> or other receptor in these effects on neurons from young animals was not determined.<sup>8</sup>

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It is not known if propofol has the same effect on neural stem or precursor cells (NPCs) that it does on immature neurons. Previous studies using isoflurane have identified cell death in neurons.<sup>9,10</sup> But in NPCs, which are abundant in the rodent brain during the early postnatal period, no cell death was observed, although changes in both proliferation and differentiation were identified.<sup>11–13</sup>

The aim of this study is to determine the effect of propofol on hippocampal-derived NPCs grown in culture. This model was selected because it allows direct observation of the action of propofol on NPCs in isolation from effects it may have on the surrounding brain tissue, as well as the ability to more directly investigate a mechanism for its actions. To that end, we evaluated the effect of propofol on hippocampal-derived NPC cell division, cell death, and differentiation.

## Materials and Methods

### *Isolation and Culture of Hippocampal Precursor Cells*

All animals were cared for following procedures approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco (UCSF), San Francisco, California. NPCs were isolated following methods previously described<sup>11</sup> with slight modification. Unanesthetized postnatal day 2 Sprague-Dawley rats were separated from the dam and decapitated using a guillotine. Hippocampi were immediately dissected out and placed in 10 ml ice-cold Hanks Balanced Salt Solution without calcium (Hanks) (UCSF Cell Culture Facility, San Francisco, California). Whole hippocampi were pooled from 5–10 animals and washed two times with Hanks. The supernatant was discarded and the hippocampi were gently triturated 10 times in 1 ml of Hanks using a Rainin P1000 pipette before adding another 9 ml of Hanks. The solution was then centrifuged at 600 rcf for 3 min. The pellet was resuspended gently in 1 ml of prewarmed proliferation medium consisting of 3:1 Dulbecco modified Eagle medium: Ham's F12 (UCSF cell culture facility), 1% penicillin and streptomycin, 1 × B-27 supplement (Invitrogen, Carlsbad, CA), 20 ng/ml basic fibroblast growth factor (Chemicon, Temecula, CA), and 0.75 units heparin/ml (Abraxis, Schaumburg, IL). Hippocampal precursor cells were then plated in additional prewarmed medium in T-25 flasks and grown in 5% carbon dioxide in air, at 37°C with 80% media exchange three times per week. Cells were triturated and transferred to new flasks every 7 days so that adherent cells were left behind and nonadherent proliferating NPCs were moved to the new flask. NPCs were grown in culture for 13 or 14 days at the time of experimentation.

### *Propofol Concentration*

Propofol concentration in cell culture medium for pure propofol diluted in dimethylsulphoxide was determined at multiple time-points spectrophotometrically. Culture medium was extracted 5:1 with hexane and absorbance was measured at 270 nm using a Smart Spec Plus spectrophotometer (BioRad,

Philadelphia, PA). The propofol concentration was found to decrease over time. The area under the curve (concentration across time) was determined and the concentration reported is relative to freshly extracted medium that was not incubated in plastic cell culture dishes. The concentration of propofol reported for experiments using Diprivan (APP Pharmaceuticals, Schaumburg, IL) is the nominal concentration based only on the amount of drug added to the medium and its expected concentration. No spectrophotometric analysis was performed.

### *BrdU Incorporation*

NPCs were grown in proliferation medium for 14 days.  $1.5 \times 10^4$  cells per chamber were added to a coated 8-chamber microscope slide in proliferation medium and treated with propofol (Aldrich Chemical Company, Milwaukee, WI) in the carrier solution 0.4% (v/v) dimethyl sulfoxide (DMSO) (UCSF cell culture facility) for the time indicated. During the last hour of propofol exposure, 5-bromo-2-deoxyuridine (BrdU), a thymidine analog, was added to the medium. Slides were fixed and stained as described previously<sup>11</sup> with 4',6-diamidino-2-phenylindole (DAPI) or antibody to BrdU. Slides were then photographed using the x40 objective on an E400 fluorescence microscope equipped with filters at 385, 490, and 570 nm (Nikon, Melville, NY). Ten sets of images were acquired at different locations and were subsequently merged using NIH Image J software (National Institutes of Health, Bethesda, MD), and the total number of cells (DAPI) as well as the number of BrdU-positive cells was determined.

### *Lactate Dehydrogenase Release Assay*

NPCs were grown as neurospheres in suspension for 13 days as described in Isolation and Culture of Hippocampal Precursor Cells. On day 13, cells were triturated with a P1000 pipette and counted. On day 14 they were resuspended in fresh medium and plated at  $1 \times 10^4$  cells per well in 96-well plates in proliferation medium. NPCs were then treated with propofol in DMSO, Diprivan (APP Pharmaceuticals), Nifedipine (Sigma-Aldrich, St. Louis, MO), midazolam (Hospira, Lake Forest, IL) staurosporine (Axxora, San Diego, CA), PicROTOXIN (Sigma-Aldrich), or Muscimol (MP Biomedicals, Solon, OH) as indicated. Experiments carried out in calcium-free conditions were plated in 96-well plates using proliferation medium in which Dulbecco modified Eagle medium was replaced with Hanks and supplemented with 3.35 g/L sodium bicarbonate. Cells were then analyzed for release of lactate dehydrogenase (LDH) using the Cyttox-96 assay kit (Promega, Madison, WI) following the manufacturer's directions. Incubation at -80°C for 30 min followed by 15 min at 37°C was considered complete lysis and called 100%. All data are presented as percent of complete lysis for each cell line. Plates were analyzed using a Fluostar Optima plate reader (BMG Lab Tech, Cary, NC) by measuring absorbance at 490 nm.

### Caspase Activation

NPCs were grown as neurospheres in suspension in proliferation medium. On day 13 cells were triturated with a P1000 pipette and counted. On day 14 they were resuspended in fresh medium and plated at  $1 \times 10^4$  cells per well in proliferation medium and grown in opaque 96-well plates. The following day cells were exposed to propofol or staurosporine. Cells were then lysed and analyzed for caspase-3/7 activity following the manufacturer's instructions (Promega, Caspase-Glo 3/7, Promega). Fluorescence activity was analyzed using a Fluostar Optima plate reader and recording luminescence. Staurosporine is a known activator of caspase-mediated cell death and was used as a positive control.

**Western Blot Analysis.** Total protein was collected from whole brain or cell lines by homogenization in ice-cold RIPA lysis buffer (Boston Bioproducts, Boston, MA), and protein concentration determined by Bradford analysis (Pierce, Rockford, IL). Proteins were separated by electrophoresis in 10% Tris-HCl polyacrylamide gels (BioRad, Hercules, CA) at 100 V for 1.5 h in Tris-glycine-SDS buffer (TGS) (BioRad, Hercules, CA), and electrically transferred to polyvinylidene fluoride membranes (BioRad, Hercules, CA) in Novex TGS-methanol transfer buffer (Invitrogen). Size was determined using Precision Plus dual color protein standards (BioRad, Hercules, CA). Membranes were blocked with 4% nonfat dry milk in Tris-buffered saline for 45 min at room temperature. GABA<sub>A</sub> receptor  $\alpha$ -1 (1:500) and  $\beta$ -3 (1:1,000) antibodies (Antibodies Inc., Davis, CA) and glyceraldehyde phosphate dehydrogenase antibody (1:1,000, Cell Signaling, Danvers, MA) were incubated overnight at 4°C. Blots were washed three times in Tris-buffered saline, then incubated with goat antimouse and donkey antirabbit conjugated horseradish peroxidase secondary antibodies (1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 45 min. After washing five times in Tris-buffered saline, membranes were treated with 2 ml SuperSignal West Pico Chemiluminescent Substrate (Pierce) for 3 min before imaging with a Gel Logic 2200 Carestream Molecular Imaging System (Carestream, Rochester, NY).

### Differentiation

NPCs growing in suspension in proliferation medium for 13 or 14 days were collected, triturated, and plated at 20,000 cells per well in differentiation medium consisting of Neurobasal-A (Invitrogen), B27 supplement, 1% penicillin-streptomycin (UCSF cell culture facility), L-glutamine (Invitrogen, Carlsbad CA), and with or without 5% fetal bovine serum (UCSF cell culture facility). The next day, medium was replaced with differentiation medium lacking serum, if it was included. Microscope chamber slides were precoated with poly-L-ornithine (Sigma-Aldrich) in water overnight and then with laminin (Sigma-Aldrich) in phosphate-buffered saline (PBS), pH 7.4, for 6–18 h. After 4 days, the slides were fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS for 15 min at room temperature

and blocked with 10% goat serum and 0.03% triton X-100 in PBS for 2 h at room temperature. Anti-Tuj1 (Covance, Billerica, MA) diluted 1:500, and antigial fibrillary acidic protein (GFAP) (Chemicon, Emeryville, CA) diluted 1:2,000 with PBS, were added for overnight incubation at 4°C. Slides were washed five times with PBS before adding goat antimouse Alexa 488 and goat antirabbit Alexa 594 (Invitrogen, Grand Isle, NY) diluted 1:2,000 in PBS for 2 h at room temperature. Nuclei were labeled with DAPI for 10 min at room temperature. Slides were washed with PBS five times, coverslips applied with Aquapolymount (Polysciences, Warring, PA), and left overnight to dry. All slides were then photographed using x20 objective on a Nikon E400 fluorescence microscope with filters at 385, 490, and 570 nm; 10–14 sets of images were acquired at random locations and were subsequently merged using NIH Image J software. The total number of nuclei (DAPI) and the ratio of cells positive for Tuj1 or GFAP was determined by counting.

**Statistical Analysis.** All statistical analyses were performed and all graphs produced using Prism 4 (GraphPad Software, LaJolla, CA). Data are expressed as mean  $\pm$  SE. The "n" for each group represents the number of experiments carried out on independently isolated groups of neural precursors that are derived from by combining hippocampal isolates from 5–10 animals, then grown in culture for 13 or 14 days. BrdU incorporation was analyzed using a two-way ANOVA to determine the contribution of both time and treatment to the variance and Bonferroni correction was performed to compare specific groups. All other data were analyzed using repeated measures one-way ANOVA, except when unequal numbers of replicates would not allow for repeated measures in all conditions. This occurred because of bacterial contamination or lack of enough total cells to repeat all conditions on each cell line. Data lost is noted in the Results section when it occurred. Post testing was performed using Dunnett test to compare all groups to a control, or Bonferroni correction to compare specific groups. Repeated measures analysis were used when exposing cells from a single isolation to different treatments. All analyses were two-tailed and were considered to be statistically different when a *P* value < 0.05 was determined.

## Results

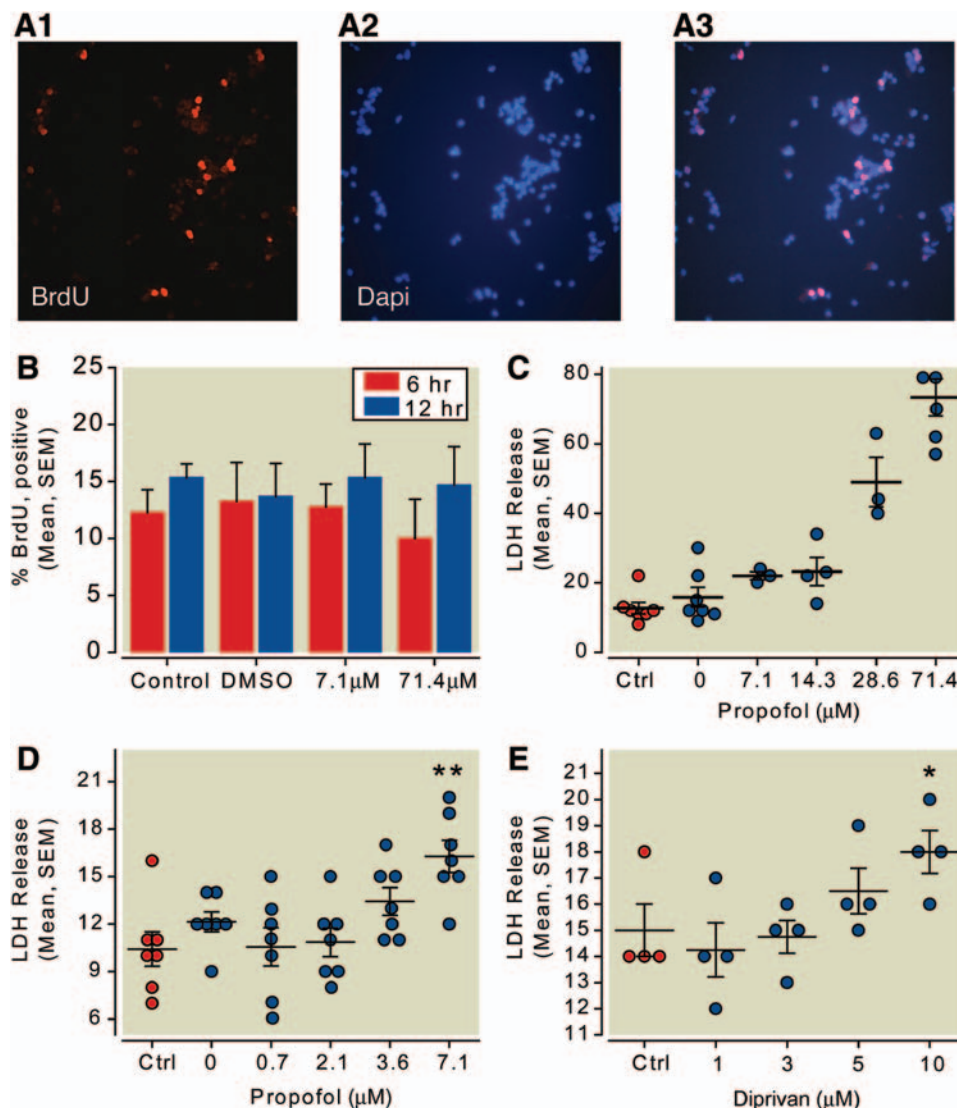
### ***Propofol Does Not Alter BrdU Incorporation, but at High Doses Causes LDH Release in Proliferating Neural Precursor Cells***

A single pulse of BrdU was given to precursor cells after exposure to 7.1 or 71.4  $\mu$ M propofol for 6 or 24 h. Cells were counted and the proportion of nuclei that incorporated BrdU was determined (fig. 1A). The percentage of cells incorporating BrdU was not different at either dose or time-point (fig. 1B) (two-way ANOVA: interaction *P* = 0.88, time: *P* = 0.16, treatment: *P* = 0.92). Propofol had no effect

on the proportion of NPCs in s-phase in the cell cycle. One data point was lost for all conditions because of bacterial contamination at the 24-h time-point.

Exposing NPCs grown in proliferation conditions to propofol from 0–71  $\mu\text{M}$  for 6 (fig. 1C) or 24 h (data not shown) produced a dose-dependent increase in LDH released into the media. The low end of the dose–response curve was further evaluated and found to significantly increase LDH release at 7.1  $\mu\text{M}$ . Lower doses of propofol had no effect and DMSO carrier (labeled 0  $\mu\text{M}$ ) was not different from

the untreated control (fig. 1D) (repeated measures ANOVA:  $P < 0.0001$ , Dunnett test: 0  $\mu\text{M}$ ,  $12.3 \pm 0.58$  vs. 7.1  $\mu\text{M}$ ,  $16.4 \pm 1.0$ ;  $P < 0.01$ ). The dose–response curve was also repeated using Diprivan, a clinically used preparation of propofol, and 10  $\mu\text{M}$  was found to be different from control (fig. 1E) (repeated measures ANOVA:  $P < 0.05$ ; Dunnett test: control,  $15.8 \pm 0.84$  vs. 10  $\mu\text{M}$ ,  $17.8 \pm 0.85$ ;  $P < 0.05$ ). The control in this experiment did not contain the lipid carrier found in all clinical formulations of propofol. However, a similar dose–response curve is seen with propofol in DMSO or with



**Fig. 1.** Propofol did not change the proportion of cells in S-phase, but did cause dose-dependent toxicity in proliferating neural precursor or stem cells (NPCs). (A) NPCs were given a pulse of 5-bromo-2-deoxyuridine (BrdU) after 6 or 24 h exposure to propofol then fixed for immunocytochemistry with anti-BrdU antibody (A1) or 4',6-diamidino-2-phenylindole (A2). Overlaid images (A3) were then photographed and the nuclei were counted. (B) The number of cells that incorporated BrdU was not different in cells treated with propofol versus dimethyl sulfoxide carrier only. (C) NPCs were exposed to increasing concentrations of propofol for 6 h then assayed for lactate dehydrogenase release into the media. All values are relative to complete lysis of cells, which was set at 100%. Increasing the concentration of propofol increased the amount of lactate dehydrogenase released in the media. (D) The low end of the dose–response curve was further investigated, and 7.1  $\mu\text{M}$  propofol was found to be different from 0  $\mu\text{M}$  propofol (carrier only) (Dunnett test). (E) Diprivan, a clinically used formulation of propofol, produced a similar result when compared with control (Dunnett test). \* $P < 0.05$ , \*\* $P < 0.01$ . DMSO = dimethyl sulfoxide; LDH = lactate dehydrogenase.

Diprivan, suggesting that it is not the carrier but the drug that induces LDH release from NPCs. These results demonstrate that propofol at high concentration can dose-dependently kill precursor cells grown in proliferating conditions, but low clinically relevant concentrations of propofol do not.

### High Concentration Propofol Causes Cell Damage and LDH Release but Does Not Increase Caspase-3/7 Activity in Proliferating Precursors

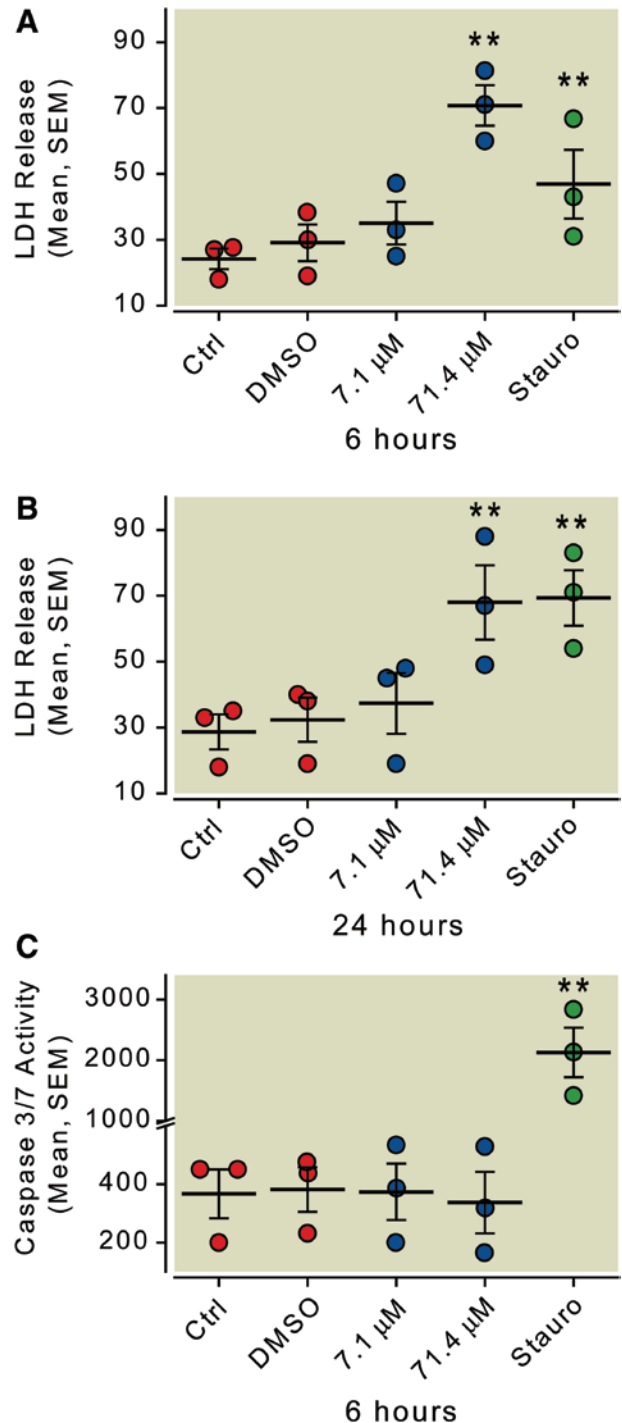
To determine the role of caspase-3 in propofol-mediated cell damage, we compared propofol with staurosporine, a known activator of caspase-mediated apoptosis. Both propofol and staurosporine caused LDH release after 6 h exposure (fig. 2A), with staurosporine-mediated LDH release increasing by 24 h of exposure (fig. 2B) (repeated measures ANOVA, Dunnett test *vs.* control, 6 h: control,  $24.2 \pm 3.1$ , propofol,  $70.8 \pm 6.1$ ,  $P < 0.01$ , staurosporine,  $46.9 \pm 10.5$ ,  $P < 0.01$ ; 24 h: control,  $28.6 \pm 5.4$ , propofol,  $68.0 \pm 11.3$ ,  $P < 0.01$ , staurosporine,  $69.3 \pm 8.4$ ,  $P < 0.01$ ). Caspase-3/7 activity was not changed by propofol at either concentration but was induced by the staurosporine-positive control, as expected (fig. 2C). Precursor cells are capable of caspase-mediated cell death, but propofol does not activate this pathway in NPCs. Similar results were seen at an earlier time-point; after 2 h of propofol exposure, there was also no increase in caspase-3/7 activity (data not shown).

### Cultured Neural Precursor Cells Express the GABA<sub>A</sub> Receptor $\beta$ -3 Subunit

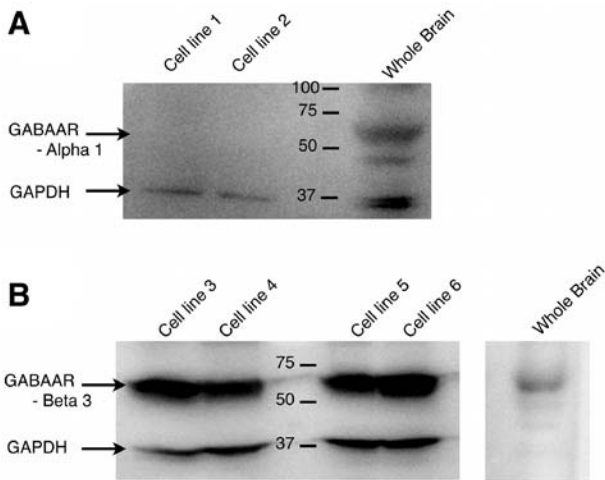
The GABA<sub>A</sub> receptor is one of the primary targets of propofol,<sup>5</sup> and it has previously been shown to cause cell death in immature neurons *via* this pathway.<sup>7</sup> Specifically, the  $\beta$ -3 subunit is known to mediate some of the anesthetic action of propofol.<sup>14</sup> Western blot analysis of cultured NPCs for the GABA<sub>A</sub> receptor  $\alpha$ -1 subunit was negative (fig. 3A). By contrast, the GABA<sub>A</sub> receptor  $\beta$ -3 subunit is expressed in all NPC lines analyzed (fig. 3B). Both the  $\alpha$ -1 and  $\beta$ -3 subunit are seen in extracts of whole brain (figs. 3A and B).

### High-dose Propofol-mediated Cell Damage in Proliferating Precursors Is Not Mediated by the GABA<sub>A</sub> Receptor

Propofol both potentiates and directly opens GABA<sub>A</sub> receptors.<sup>15</sup> To test the role of GABA<sub>A</sub> receptor activation in propofol-mediated cell death, cultured precursor cells were pretreated for 15 min with the GABA<sub>A</sub> receptor antagonist bicuculline (50  $\mu$ M) before the addition of propofol. Bicuculline did not decrease propofol-mediated LDH release in precursor cells grown in proliferation medium with either 14.3 or 71.4  $\mu$ M propofol. Bicuculline or DMSO carrier alone had no effect on LDH release. One data point with bicuculline and propofol was lost because of bacterial contamination (fig. 4A; ANOVA,  $P < 0.01$ , Bonferroni



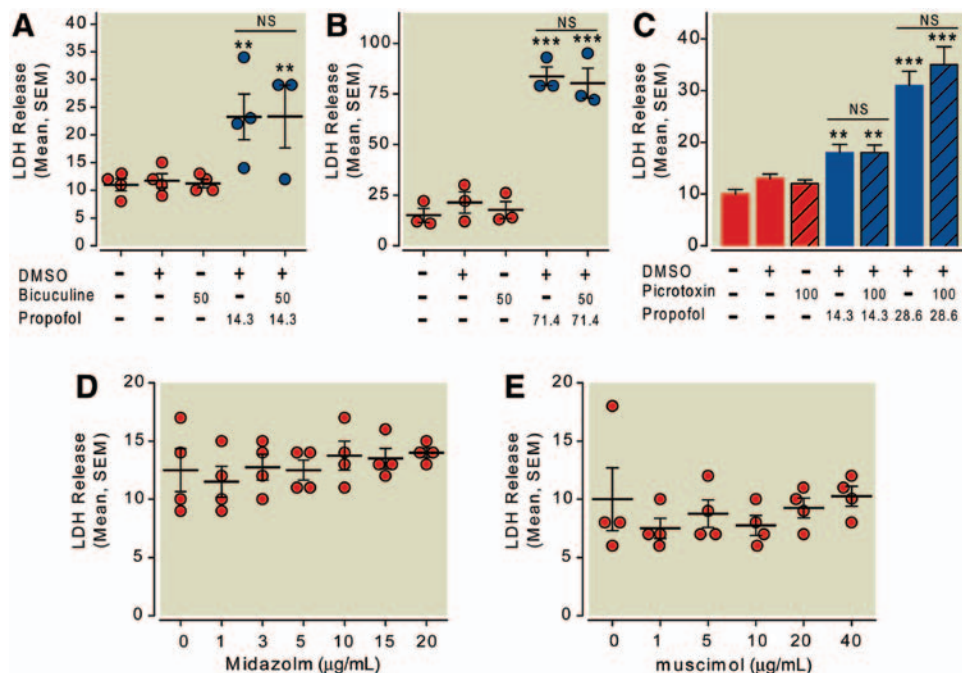
**Fig. 2.** Toxicity is not mediated by caspase-3/7 activation. Neural precursor cells grown in proliferation medium were exposed to propofol or staurosporine as a positive control for caspase activation. Like propofol, staurosporine caused some lactate dehydrogenase release after 6 h (A), and more lactate dehydrogenase release 24 h later (B). However, only staurosporine induced caspase-3/7 activation in neural precursor cells (C) (Dunnett test,  $**P < 0.01$ ). Ctrl = control; DMSO = dimethyl sulfoxide; LDH = lactate dehydrogenase; Stauro = staurosporine.



**Fig. 3.** Neural precursor cells grown in culture express the  $\alpha$ -aminobutyric acid type A (GABA<sub>A</sub>) receptor. Western blot analysis reveals that 55 kD GABA<sub>A</sub> receptor  $\alpha$ -1 subunit is expressed in whole brain but not in independently isolated cultured neural stem cell lines (A). The GABA<sub>A</sub> receptor  $\beta$ -3 subunit was found in all stem cell lines that were analyzed as well as in whole brain (B). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a control. GABA<sub>AR</sub> =  $\gamma$ -aminobutyric acid type A receptor.

correction: control,  $11.0 \pm 1.08$  vs.  $14.3 \mu\text{M}$  propofol,  $23.2 \pm 4.1$ , or propofol and bicuculline,  $23.3 \pm 5.7$ ,  $P < 0.05$ ; no difference between groups with and without bicuculline) (fig. 4B: repeated measures ANOVA,  $P < 0.0001$ , Bonferroni correction: control,  $15.0 \pm 3.5$  vs.  $71.4 \mu\text{M}$  propofol,  $83.7 \pm 4.7$ , or propofol and bicuculline,  $80.3 \pm 7.4$ ,  $P < 0.001$ ; no difference between groups with and without bicuculline). Similarly, when using  $100 \mu\text{M}$  picrotoxin, there was no difference in the amount of LDH release following exposure to propofol at  $14.3$  or  $28.6 \mu\text{M}$  (fig. 4C: repeated measures ANOVA,  $P < 0.0001$ , Bonferroni correction: control,  $9.8 \pm 0.9$  vs.  $14.3 \mu\text{M}$  propofol,  $17.5 \pm 1.6$ ,  $P < 0.01$ , or  $28.6 \mu\text{M}$  propofol,  $31.0 \pm 2.7$ ,  $P < 0.001$ ; no difference between groups with and without picrotoxin).

Next, we tested the effect of other GABA<sub>A</sub> agonist drugs on NPCs. The clinically used drug, midazolam, which binds the benzodiazepine site of the GABA<sub>A</sub> receptor, produced no increase in LDH release following treatment with  $0$ – $20 \mu\text{g/ml}$  (fig. 4D). Similarly, the direct GABA<sub>A</sub> agonist muscimol from  $0$ – $40 \mu\text{g/ml}$  did not increase LDH release from proliferating NPCs (fig. 4E). This provides further evidence that propofol-mediated toxicity in proliferating NPCs is not because of potentiation or direct opening of GABA<sub>A</sub>



**Fig. 4.** Propofol toxicity is not mediated by  $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>) receptor activation. Neural precursor cells in proliferation media were exposed to propofol in the presence or absence of the GABA<sub>A</sub> receptor antagonists bicuculline or picrotoxin. Six hours of  $14.3 \mu\text{M}$  (A) or  $71.4 \mu\text{M}$  (B) propofol induced lactate dehydrogenase (LDH) release that was not inhibited by addition of bicuculline. Similarly, the channel blocker picrotoxin did not block LDH release after propofol exposure for 6 h (C). Treating neural precursor cells with GABA<sub>A</sub> receptor agonists midazolam (D) or muscimol (E) did not cause release of LDH from neural precursor cells over a wide range of concentrations (Bonferroni correction vs. control or treatment pair). \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . DMSO = dimethyl sulfoxide; LDH = lactate dehydrogenase; NS = not significant.

receptors because it cannot be blocked by an antagonist, and a pure GABA<sub>A</sub> agonist does not cause LDH release.

### High-dose Propofol Toxicity in Proliferating Precursors Is Not Mediated by Extracellular Calcium

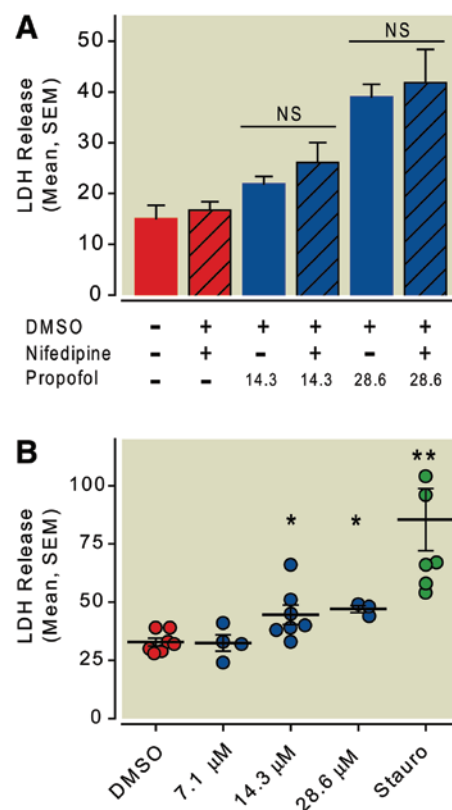
GABA activation leading to calcium influx through voltage-dependent calcium channels has been demonstrated in NPCs.<sup>16</sup> It has also been shown in immature neurons, where it leads to cell death after propofol exposure.<sup>7</sup> To determine if this was the mechanism of toxicity in NPCs grown in proliferating conditions, we exposed them to propofol in the presence or absence of the calcium channel blocker nifedipine (fig. 5A). Nifedipine did not block or decrease LDH released from the cells (repeated measures ANOVA,  $P < 0.0001$ , Bonferroni correction showed no difference between propofol-treated groups with or without nifedipine). We next assessed whether extracellular calcium entry *via* some other mechanism was responsible for NPC death by plating the cells in calcium-free media for the 6 h of propofol exposure (fig. 5B). Despite a slightly higher baseline when NPCs were switched to this medium, 14.3 or 28.6  $\mu\text{M}$  propofol still led to a significant increase in LDH release (ANOVA,  $P < 0.05$ , Dunnett test: DMSO,  $32.8 \pm 1.6$  *vs.* 14.3  $\mu\text{M}$  propofol,  $44.6 \pm 4.1$ , or 28.6  $\mu\text{M}$  propofol,  $46.9 \pm 1.6$ ,  $P < 0.05$ ). Not enough cells were available to analyze all cell lines at each dose in this experiment, so repeated measures were not used.

### High-dose Propofol Is Also Toxic to Neural Precursor Cells in Differentiating Conditions

To determine whether precursor cells might be protected from the effect of propofol after they begin to differentiate, we exposed precursors in differentiating medium to propofol from 0–71.4  $\mu\text{M}$ . Baseline values for experiments performed in differentiation medium were somewhat higher because of the serum in the medium. Larger doses of propofol caused a greater release of LDH into the medium (fig. 6A: repeated measures ANOVA,  $P < 0.001$ , Dunnett test: 0  $\mu\text{M}$   $17.5 \pm 0.6$  *vs.* 28.6  $\mu\text{M}$  propofol,  $36.2 \pm 4.4$ , or 71.4  $\mu\text{M}$  propofol,  $58.7 \pm 5.0$ ,  $P < 0.01$ ; 0  $\mu\text{M}$  *vs.* 14.3  $\mu\text{M}$  propofol,  $26.7 \pm 3.2$ ,  $P < 0.05$ ). This response at 14.3 and 28.6  $\mu\text{M}$  was not inhibited by the GABA<sub>A</sub> antagonist bicuculline (fig. 6B). Precursor cells were then allowed 24 h in differentiating medium before addition of propofol. Again a dose response was seen from 14.3 to 71.4  $\mu\text{M}$  propofol, suggesting no protective effect of differentiation for 24 h (fig. 6C: repeated measures ANOVA,  $P < 0.0001$ , Dunnett test: 0  $\mu\text{M}$ ,  $17.5 \pm 4.4$  *vs.* 28.6  $\mu\text{M}$  propofol,  $35.7 \pm 2.1$ , and 71.4  $\mu\text{M}$  propofol,  $40.5 \pm 1.7$ ,  $P < 0.01$ ; or 0  $\mu\text{M}$  *vs.* 14.3  $\mu\text{M}$  propofol,  $25.5 \pm 1.4$ ,  $P < 0.05$ ,  $n = 4$ ).

### Differentiating Precursors Express Neuronal Markers More Often When Treated with Propofol, an Effect Not Mediated by the GABA<sub>A</sub> Receptor

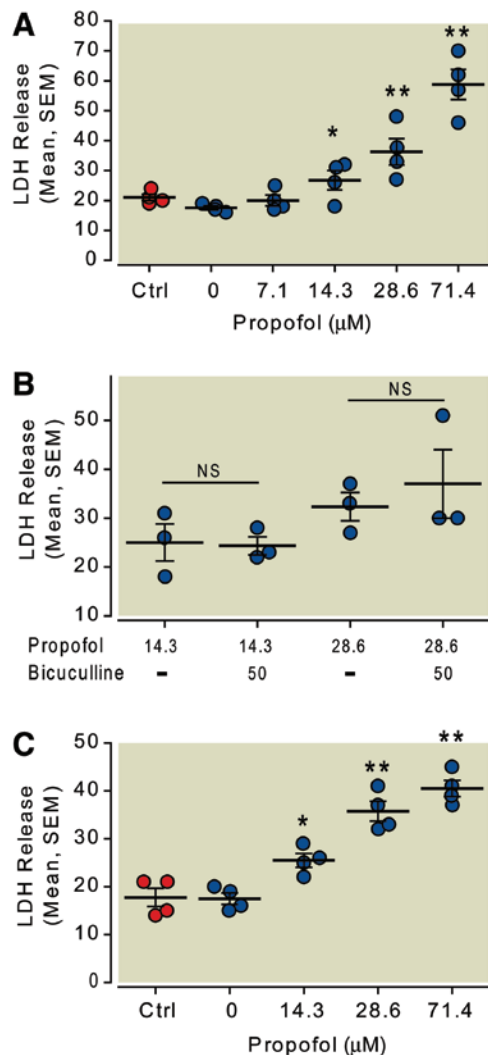
Precursor cells were induced to make a fate choice by switching to differentiation medium with propofol for 6 h. All wells



**Fig. 5.** Propofol toxicity is not mediated by extracellular calcium. Neural precursor cells growing in proliferation media were exposed to propofol in the presence or absence of the calcium channel blocker nifedipine. (A) Six hours of propofol exposure induced lactate dehydrogenase (LDH) release. Propofol-mediated LDH release was not inhibited by addition of 10  $\mu\text{M}$  nifedipine (Bonferroni correction). (B) Neural precursor cells grown in proliferation media were transferred to calcium-free media and treated with various concentrations of propofol. The media change caused an increased baseline value but propofol exposure still led to increased LDH release into the media (Bonferroni correction *vs.* dimethyl sulfoxide or DMSO control). The positive control staurosporine, which induced caspase-mediated cell death, also caused cell death and LDH release under these calcium-free conditions. \* $P < 0.05$ . DMSO = dimethyl sulfoxide; LDH = lactate dehydrogenase; NS = not significant; Stauro = staurosporine

were then changed to standard differentiation medium for 4 days with no propofol. Under these conditions, propofol significantly increased the number of Tuj1 positive cells (fig. 7A: repeated measures ANOVA,  $P < 0.001$ , Dunnett test: control *vs.* 7.1  $\mu\text{M}$  propofol,  $1.43 \pm 0.06$ ,  $P < 0.05$ , or *vs.* 71.4  $\mu\text{M}$  propofol,  $1.77 \pm 0.14$ ,  $P < 0.01$ ; 1,563  $\pm$  536 cells counted per data point). No statistical difference was seen in the proportion of GFAP-positive cells (fig. 7B).

Propofol is highly protein-bound in blood,<sup>17</sup> and likely some portion is protein-bound in medium that contains fetal bovine serum, which was used in the differentiation medium in the experiments in figures 7A and B. Because the concentrations of propofol used in figures 7A and B



**Fig. 6.** Neural precursor cells in differentiation medium are not protected from propofol toxicity. (A) Neural precursor cells growing in proliferation medium were switched to differentiation conditions at the time of propofol exposure. Increasing doses of propofol exposure for 6 h under differentiating conditions caused cell damage and release of lactate dehydrogenase (LDH) into the medium (Dunnett test vs. 0  $\mu\text{M}$ ). (B) LDH release after 6 h of propofol was not inhibited by addition of bicuculline to the medium (Bonferroni correction). (C) Neural precursor cells were switched to differentiation conditions 24 h before addition of propofol. Increasing doses of propofol still caused a rise in LDH release (Dunnett test vs. 0  $\mu\text{M}$ ). \* $P < 0.05$ , \*\* $P < 0.01$ . Ctrl = control; NS = not significant.

also induced LDH release, we repeated these experiments in serum-free medium (a standard condition for differentiating NPCs) with lower doses of propofol. NPCs in serum-free differentiation medium were exposed to 2.1  $\mu\text{M}$  propofol in DMSO or to Diprivan, the clinical formulation of propofol, for 24 h. After 24 h, medium on all samples was changed to remove the propofol and DMSO. Cells were then left in culture for the next 3 days before fixation and cell counting.

All nuclei are stained blue with DAPI, GFAP-positive glial cells are red, and Tuj-1-positive neurons are green (fig. 7C). The control for Diprivan did not contain lipid carrier solution. Both Diprivan and propofol in DMSO increased the proportion of cells that expressed Tuj-1 after 4 days (figs. 7C and D: repeated measures ANOVA,  $P < 0.05$ , Bonferroni correction vs. Diprivan,  $1.42 \pm 0.06$ ,  $P < 0.05$ , DMSO,  $1.0 \pm 0.07$  vs. propofol,  $1.41 \pm 0.19$ ,  $P < 0.05$ ; 1,828  $\pm$  337 observations per data point). No difference was seen in the expression of GFAP (figs. 7D and E).

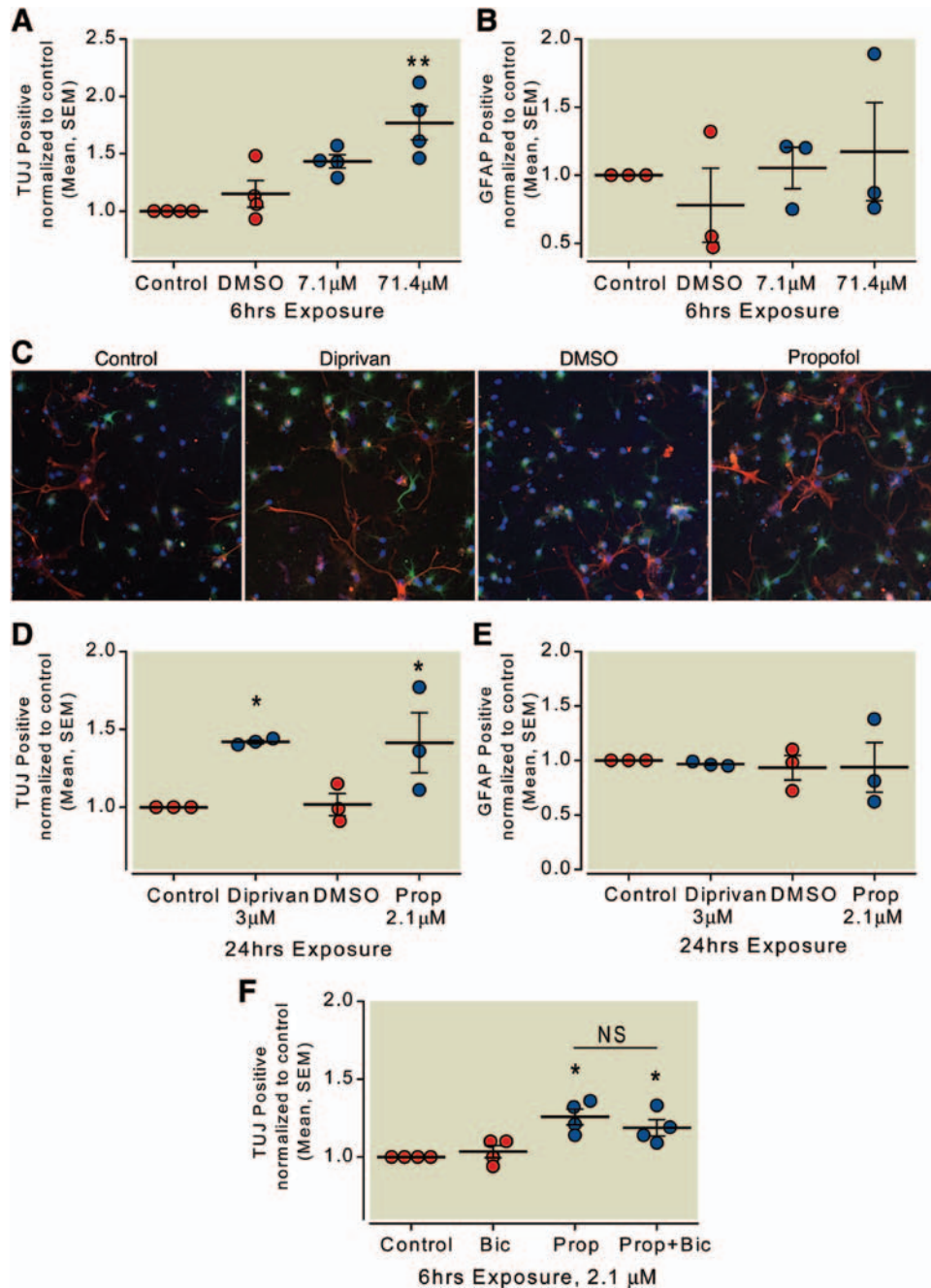
To determine the role of the GABA<sub>A</sub> receptor in the effect of propofol on differentiation, we exposed NPCs to 2.1  $\mu\text{M}$  propofol or propofol plus bicuculline for 6 h. Medium on all samples was changed to remove the propofol, DMSO, and bicuculline. Four days after plating, the cells were fixed and analyzed for GFAP or Tuj-1 expression. Six hours of low-dose propofol increased the ratio of cells expressing Tuj-1 (fig. 7F: repeated measures ANOVA,  $P = 0.0019$ , Bonferroni correction vs. propofol,  $1.3 \pm 0.05$ ,  $P < 0.01$ , control vs. propofol and bicuculline,  $1.2 \pm 0.05$ ,  $P < 0.05$ , propofol vs. propofol and bicuculline,  $P > 0.05$ ; 1,915  $\pm$  139 observations per data point). This increase was smaller than that observed after 6 h of exposure at a higher concentration (7.1  $\mu\text{M}$ , fig. 7A) and smaller than that observed after 24 h of exposure at the same concentration (fig. 7D).

## Discussion

We examined the effect of a wide range of propofol doses on NPCs *in vitro*. The results show that propofol-induced toxicity in NPCs only occurs at concentrations above those observed clinically in humans. At these supraclinical concentrations, cell death was not mediated by GABA<sub>A</sub> receptors and did not require extracellular calcium or activation of caspase-3. Propofol at lower, clinically relevant concentrations led to increased expression of a neuronal marker in differentiating NPCs exposed to propofol at the onset of differentiation.

Determining the appropriate dose of propofol to use in an *in vitro* study is difficult. Propofol in whole blood is largely bound to red blood cells and albumin, leaving only about 1–3% free in the plasma for diffusion and potential interaction with cell surface receptors.<sup>17,18</sup> However, propofol is very lipophilic and the brain tissue concentration may be considerably higher than the plasma or extracellular fluid concentration.<sup>19</sup> In addition, the dose of propofol required in rodents is higher than that required in humans to achieve an equal level of anesthesia. All of this confounds the process of determining what concentration to use for an *in vitro* study, but 1–3  $\mu\text{M}$  is generally considered to be a clinically relevant concentration. The exact site of action of propofol was not identified in this study, but it is not the GABA<sub>A</sub> receptor at the cell surface that led to cell death with high doses of propofol or to altered differentiation with low doses of propofol. If it has an intracellular site of action, then the potential concentration seen at that site may be much higher





**Fig. 7.** Propofol increases expression of neuron specific Class III  $\beta$ -tubulin (Tuj1) in differentiating precursor cells. Neural precursor cells were plated in differentiation medium with propofol for 6 h, then washed to remove propofol and serum. Four days later, the expression of Tuj1 and glial fibrillary acidic protein (GFAP) was evaluated by immunocytochemistry using fluorescence microscopy. (A) Neuronal precursors differentiated in the presence of propofol were more likely to express the early neuronal marker Tuj1 (Dunnett test vs. control). (B) No difference was seen in expression of GFAP. (C) Representative photomicrographs of neural precursor cells that were differentiated under serum-free conditions with 3  $\mu$ M Diprivan or 2.1  $\mu$ M propofol for 24 h, then washed to remove the propofol. After 4 days, cells were evaluated for expression of Tuj1 (green) or GFAP (red) and 4',6-diamidino-2-phenylindole (blue) to mark the nuclei. (D) Both the clinical preparation, Diprivan, and propofol in dimethyl sulfoxide increased the number of cells expressing Tuj1 4 days later (Bonferroni correction). (E) No difference was seen in the number of cells expressing GFAP. (F) Neuronal precursor cells were exposed to 2.1  $\mu$ M propofol for 6 h in the presence or absence of bicuculline. Media was changed to remove propofol or bicuculline and cells were allowed to differentiate. Four days after low-dose propofol exposure, Tuj1 expression was increased and this was not blocked by addition of bicuculline to the medium (Bonferroni correction). \* $P$  < 0.05, \*\* $P$  < 0.01. Bic = bicuculline; DMSO = dimethylsulfoxide; NS = not significant; Prop = propofol.

than the free plasma concentrations measured *in vivo*,<sup>19</sup> as the drug becomes concentrated in lipophilic environments.

Our results on hippocampal-derived precursor or stem cells identify several differences from previous *in vitro* studies of neurons. Pearn *et al.*<sup>8</sup> recently reported 6 h of 3  $\mu\text{M}$  propofol induced cell death in neurons isolated from the brain of 1- or 2-day-old mice that were grown in culture for 4–7 days, although the brain region the neurons were isolated from was not reported and the role of the GABA receptor or other targets of propofol was not evaluated.<sup>8</sup> Kahraman *et al.*<sup>7</sup> isolated hippocampal neurons from post natal day 0 rats and grew them *in vitro* for 4 or 8 days. They found 5 h of 5  $\mu\text{M}$  propofol led to calcium influx and caspase-mediated cell death that was inhibited by addition of bicuculline or nifedipine in neurons grown for 4 days. When neurons were grown for 8 days *in vitro*, neither calcium influx or cell death were observed.<sup>7</sup> Slight differences in these results may be because of age or species of the animal, duration in culture, and source of the cells that were isolated.

Unlike these studies performed on cultured neurons,<sup>7,8</sup> using hippocampal-derived NPCs, we did not find toxicity in the clinically relevant range. High-dose propofol-induced toxicity in NPCs was not GABA<sub>A</sub>, caspase-3, or calcium mediated, like that seen in young neurons by Kahraman<sup>7</sup> or Pearn.<sup>8</sup> Vutskits *et al.* report findings similar to ours using a model of neuroblasts isolated from the subventricular zone and plated immediately in differentiating conditions. Here, only very high doses of propofol caused cell death. Lower doses of propofol (5–28  $\mu\text{M}$ ), but not midazolam, altered dendritic development and architecture.<sup>20</sup> Lack of a change with midazolam suggests the action of propofol may be mediated by some mechanism other than the GABA<sub>A</sub> receptor. The present model of hippocampal-derived NPCs showed an increase in neuronal fate selection after 6 h exposure to 2.1  $\mu\text{M}$  propofol, a finding that was not blocked by the addition of bicuculline – further evidence that propofol may act at sites other than the GABA<sub>A</sub> receptor. The effect of propofol on neural stem cells is different from its effect on neurons. *In vitro* and *in vivo* studies have shown caspase-3 activation and neuronal cell death following administration of propofol, whereas NPCs or neuroblasts display altered differentiation but not cell death.

A difference between precursor cells and neurons has previously been demonstrated with the volatile anesthetic isoflurane. Caspase-mediated cell death was reported in immature neurons after exposure to isoflurane.<sup>9</sup> Isoflurane, even at high doses, does not induce cell death in precursor cells, but similar to the results reported in this study, it does alter cell fate decisions by increasing neuronal fate selection as well as decreasing the rate of proliferation.<sup>11,13</sup>

A similar change in susceptibility with the age of cultured neurons has been reported in response to isoflurane, where caspase is activated in mixed hippocampal and cortical mouse neurons after 5 or 14 days *in vitro* but not after 21 days *in vitro*.<sup>9</sup> Together these findings fit well with *in vivo* results

demonstrating neuroapoptosis during the early postnatal period that decreases as the animal ages (and has fewer new neurons) until little or no cell death is seen following an anesthetic after 4 weeks of age.

The exact cell type and the age of the cell that is lost after a propofol anesthetic *in vivo* has not been identified, but widespread cell death occurs even at very low doses.<sup>1</sup> *In vitro* data from this and others studies would suggest that cell death does not occur in NPCs or in well-differentiated and connected neurons, but perhaps newly differentiated cells that are destined to become neurons.<sup>7,8</sup> Careful attention to the age of cells grown in culture and the brain region they are isolated from will be important in defining the effect of propofol on different cell types.

Further *in vivo* studies to determine the age and kind of cells that undergo apoptosis will help define the exact injury caused by propofol anesthesia in young animals and may improve our understanding of developmental differences in cell death patterns caused by other anesthetics as well.<sup>21</sup> The age of the neuron (days since fate selection) and not the age of the animal (days since conception) is likely a critical factor in which cells die after anesthetic exposure.

The implications of a change in phenotype selection by precursor or stem cells is not clear. Although it is tempting to assume additional neurons to be beneficial, this is not always the case, and this may even be detrimental, as has been reported in autism.<sup>22,23</sup> Increased neuronal selection *in vivo* might be assumed to occur as a result of anesthetic-induced cell death and a subsequent compensatory response from the stem cell pool. However, these studies demonstrate that even in the absence of other signals from the brain or any surrounding cell death, there is an increase in neuronal differentiation after propofol exposure.

### Limitations

The limitations of this study are that it was performed on NPCs grown in culture. This makes it difficult to extract the findings to an *in vivo* setting and even more difficult or impossible to relate them to the changes in cognitive function or drug response that are found in adult animals that were anesthetized with propofol as neonates. A further limitation is that the site of action for the effect of propofol on NPC cell death and differentiation was not identified. Despite these limitations, the data add to our knowledge of the direct action of propofol on one of several different cell types in the brain during early development and suggest a nonGABA<sub>A</sub> mechanism for these effects.

### Conclusion

Propofol likely has effects on the developing brain beyond neuronal cell death. Very low doses of propofol inhibit neuronal arborization *in vitro*,<sup>20</sup> and increase the number of neuronal spines on differentiated cells *in vivo*.<sup>24</sup> In the data presented here, low doses of propofol that were not

toxic to NPCs led to an increase in neuronal fate selection during differentiation. Propofol at clinically relevant doses may have a variety of different effects on cells at different stages of differentiation in the developing brain, such as increased neuronal fate selection by precursor cells, cell death of immature neurons, and inhibited arborization of differentiated neurons, to list a few. These data help to broaden our understanding of the effects of propofol on NPCs in the developing brain. Future studies will require careful attention to different brain cell types and different brain regions in order to eventually understand the link between cellular level findings and behavioral alterations that occur after anesthesia.

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