

# GABAergic Mechanism of Propofol Toxicity in Immature Neurons

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**Abstract:** Certain anesthetics exhibit neurotoxicity in the brains of immature but not mature animals. Gamma-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the adult brain, is excitatory on immature neurons via its action at the GABA<sub>A</sub> receptor, due to a reversed transmembrane chloride gradient. GABA<sub>A</sub> receptor activation in immature neurons is sufficient to open L-type voltage-gated calcium channels. As propofol is a GABA<sub>A</sub> agonist, we hypothesized that it and more specific GABA<sub>A</sub> modulators would increase intracellular free calcium ( $[Ca^{2+}]_i$ ), resulting in the death of neonatal rat hippocampal neurons. Neuronal  $[Ca^{2+}]_i$  was monitored using Fura2-AM fluorescence imaging. Cell death was assessed by double staining with propidium iodide and Hoechst 33258 at 1 hour (acute) and 48 hours (delayed) after 5 hours exposure of neurons to propofol or the GABA<sub>A</sub> receptor agonist, muscimol, in the presence and absence of the GABA receptor antagonist, bicuculline, or the L-type  $Ca^{2+}$  channel blocker, nifedipine. Fluorescent measurements of caspase-3,-7 activities were performed at 1 hour after exposure. Both muscimol and propofol induced a rapid increase in  $[Ca^{2+}]_i$  in days in vitro (DIV) 4, but not in DIV 8 neurons, that was inhibited by nifedipine and bicuculline. Caspase-3,-7 activities and cell death increased significantly in DIV 4 but not DIV 8 hippocampal neuronal cultures 1 hour after 5 hours exposure to propofol, but not muscimol, and were inhibited by the presence of bicuculline or nifedipine. We conclude that an increase in  $[Ca^{2+}]_i$ , due to activation of GABA<sub>A</sub> receptors and opening of L-type calcium channels, is necessary for propofol-induced death of immature rat hippocampal neurons but that additional mechanisms not elicited by GABA<sub>A</sub> activation alone also contribute to cell death.

**Key Words:** calcium, apoptosis, anesthetic, caspase

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The immature brain differs from the adult brain in its enhanced neuronal excitability. In the adult rat brain, the neurotransmitter  $\gamma$ -amino butyric acid (GABA), acting at the GABA<sub>A</sub> receptor, induces chloride influx resulting in membrane hyperpolarization and inhibition of neuronal excitability. In contrast, in the immature brain, activation of the GABA<sub>A</sub> receptor results in chloride efflux and membrane depolarization due to a reversed chloride gradient compared with the adult.<sup>1,2</sup> The resultant membrane depolarization activates voltage-dependent calcium channels, in particular the L-type, increasing intracellular  $Ca^{2+}$  levels. The elevated intracellular  $Ca^{2+}$ , due to spontaneous depolarizing GABAergic as well as glutamatergic activity, is required for both neuronal maturation and synaptogenesis during brain growth maturation. In the rat pup, the critical stage for brain growth takes place at an early postnatal period, associated with neuronal depolarization by GABA during the first postnatal week. For the human infant, the critical time when the  $Cl^-$  transport is as immature as in the rat spans the third trimester of pregnancy into the first 6 months after birth.<sup>3</sup> Disturbance of  $Ca^{2+}$  homeostasis during brain maturation, either by pharmacologic blockade of glutamatergic neurotransmission or the activation of GABA<sub>A</sub> receptors, leads to neurodegeneration in the developing brain.<sup>4–9</sup> Recent data from Jevtovic-Todorovic and coworkers<sup>10–13</sup> revealed that general anesthetics modulating both GABAergic and glutamatergic neurotransmission (isoflurane, alone or in combination with midazolam and nitrous oxide, and ketamine) trigger widespread apoptotic neurodegeneration in many regions of developing rat brain. In addition, Warner and coworkers demonstrated that isoflurane induces a time-dependent and maturity-dependent neuronal degeneration in vitro.<sup>14</sup> However, the relative contribution of inhibition of glutamatergic neurotransmission or activation of depolarizing GABAergic neurotransmission to the anesthetic-induced neurotoxicity is unclear.

Propofol (2, 6-diisopropyl phenol), an intravenous hypnotic-sedative agent that selectively modulates GABA<sub>A</sub> receptor function by interacting with  $\beta$  subunits, is widely used in pediatric anesthesia and intensive care practice. To reveal the GABAergic mechanism of anesthetic-induced neurotoxicity in the developing brain, we studied comparatively the effect of propofol and GABA<sub>A</sub> modulators on cytoplasmic free calcium ( $[Ca^{2+}]_i$ ) and acute/delayed death of rat hippocampal neurons before and after the conversion of GABA receptor activity from depolarizing to hyperpolarizing.

## MATERIALS AND METHODS

### Materials

All cell culture reagents were from GIBCO-BRL, except B-27 supplement (Invitrogen, Carlsbad, CA). Pure propofol was purchased from Alexis Biochemicals (San Diego, CA) and freshly diluted in artificial cerebrospinal fluid (aCSF). Fura-2 AM was obtained from Molecular Probes (Eugene, OR). Unless otherwise stated, all other chemicals were obtained from Sigma-Aldrich Inc (St. Louis, MO).

### Cell Preparation

Subjects were first-generation descendants of Sprague-Dawley albino rats from Charles River Laboratory (Wilmington, MA). Female rats were bred in the University of Maryland School of Medicine animal colony. Animals were housed under 12 hours light/dark cycle with unlimited access to food and water. All animal procedures were carried out according to the University of Maryland, Baltimore IACUC and adhered to the NIH guidelines for the care and use of laboratory animals. The cages of pregnant females were checked daily for the presence of pups and the day of birth was designated as PND0. Hippocampi from PND0 male pups were used for primary cultures of hippocampal neurons. Pups are easily sexed at birth by examination of the external genitalia, providing a 99% degree of accuracy in correctly identifying the sex. Hippocampi were dissected into Hank's balanced salt solution (HBSS+) (88 mL sterile H<sub>2</sub>O, 10 mL HBSS (Ca<sup>2+</sup>-free and Mg<sup>2+</sup>-free) 10×, 1 mL HEPES buffer, 1.0 M, pH 7.3, 1 mL antibiotic/antimycotic 100× liquid), then additional HBSS+ was added to the tube to a volume of 4.5 mL, with 0.5 mL trypsin (2.5%), and incubated in a 37°C water bath for 15 minutes. The supernatant was discarded and the tissue washed with HBSS+. This procedure was repeated a second time. Cells were dissociated by trituration, with cell number, and viability determined by trypan blue exclusion. Cells were plated on 25-mm poly-L-lysine (0.1 mg/mL, Sigma, St. Louis, MO)-coated coverslips at a density of 30,000 cells per coverslip, and placed in 60-mm dishes containing 4 mL plating medium [86 mL MEM, 10 mL horse serum, 3 mL glucose (filter sterilized, 20%), 1 mL of 100 mM pyruvic acid]. Cells were allowed 4 hours to adhere to the coverslips in a 37°C, 5% CO<sub>2</sub> incubator. The coverslips were removed from the plating dishes and placed into 60-mm dishes filled with 3 mL neurobasal media, made by adding 1 mL B-27 supplement with 1 mL antibiotic/antimycotic 100×, with 125 μL L-glutamine and filled to 50 mL with neurobasal (phenol red free). One third of the neurobasal media was removed every other day and replaced with fresh media. Cells were used at 4 or 8 days in vitro (DIV).

### Fluorescence Microscopy

Intracellular free Ca<sup>2+</sup> was monitored in hippocampal neurons by epifluorescence microscopy. Neurons were loaded with 2 μM concentration of Fura-2 AM

(Molecular Probes, Eugene, OR) for 30 minutes followed by a 15 minutes dye deesterification period at 37°C. The coverslips were mounted in the chamber of a Nikon Eclipse TE2000-S inverted microscope (SFluor 20 × 0.75 N.A.) in aCSF containing 120 mM NaCl, 3.5 mM KCl, 1.3 mM CaCl<sub>2</sub>, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 5 mM NaHCO<sub>3</sub>, 10 mM HEPES (pH 7.4), and 15 mM glucose. Single cell fluorescence of Fura-2 AM was imaged by alternate excitation at 340 and 380 nm (Polychrome IV, Till, Munich, Germany), and measurement of emission at 510 nm. Image sequences (10 s/frame, 50 ms exposure time, 2 × 2 binning) were acquired by an ORCA-ER cooled digital CCD camera (Hamamatsu Photonics, Hamamatsu, Germany) and imaged with Metafluor 6.3 (Universal Imaging, West Chester, PA) imaging software.

All experiments were performed at 37°C. In-line heating (Harvard Apparatus Inc, Holliston, MA) of perfusate flow, solution reservoir heating by separate syringe warmers (Harvard Apparatus Inc, Holliston, MA), and chamber platform heating were all used in combination to provide efficient thermal regulation.

### Cell Death and Caspase-3,-7 Staining

Cell death was assessed at either 1 hour (acute) or 48 hours (delayed) after 5 hours of treatment with propofol and other GABA<sub>A</sub> modulators. Propidium iodide (50 μM) (Sigma-Aldrich, St. Louis, MO) was used to label plasma membrane-permeable cells and Hoechst 33258 (40 μM) (Sigma-Aldrich, St. Louis, MO) to label all cell nuclei. Both propidium iodide-positive cells and fragmented/condensed nuclei were considered dead cells. Dead cells were scored by counting 3 random fields per slide. Green fluorescent inhibitor of caspases reagent (Molecular Probes, Eugene, OR) was used 1 hour after the 5 hours treatments to detect caspase-3,-7 activation.

### Data Analysis

All data are expressed as means ± SEM of n = 3 to 9 coverslips. Statistical significance was assessed by 1-way analysis of variance (ANOVA) with Dunnett post-hoc test. Data with a heterogeneous variance were log-transformed. For data that were not normally distributed, Kruskal-Wallis nonparametric ANOVA with Dunn's post-hoc test for multiple comparisons of unbalanced data was used. *P* < 0.05 was considered to be statistically significant.

## RESULTS

### Muscimol and Propofol Elevate Intracellular Calcium in Immature (DIV 4) Hippocampal Neurons

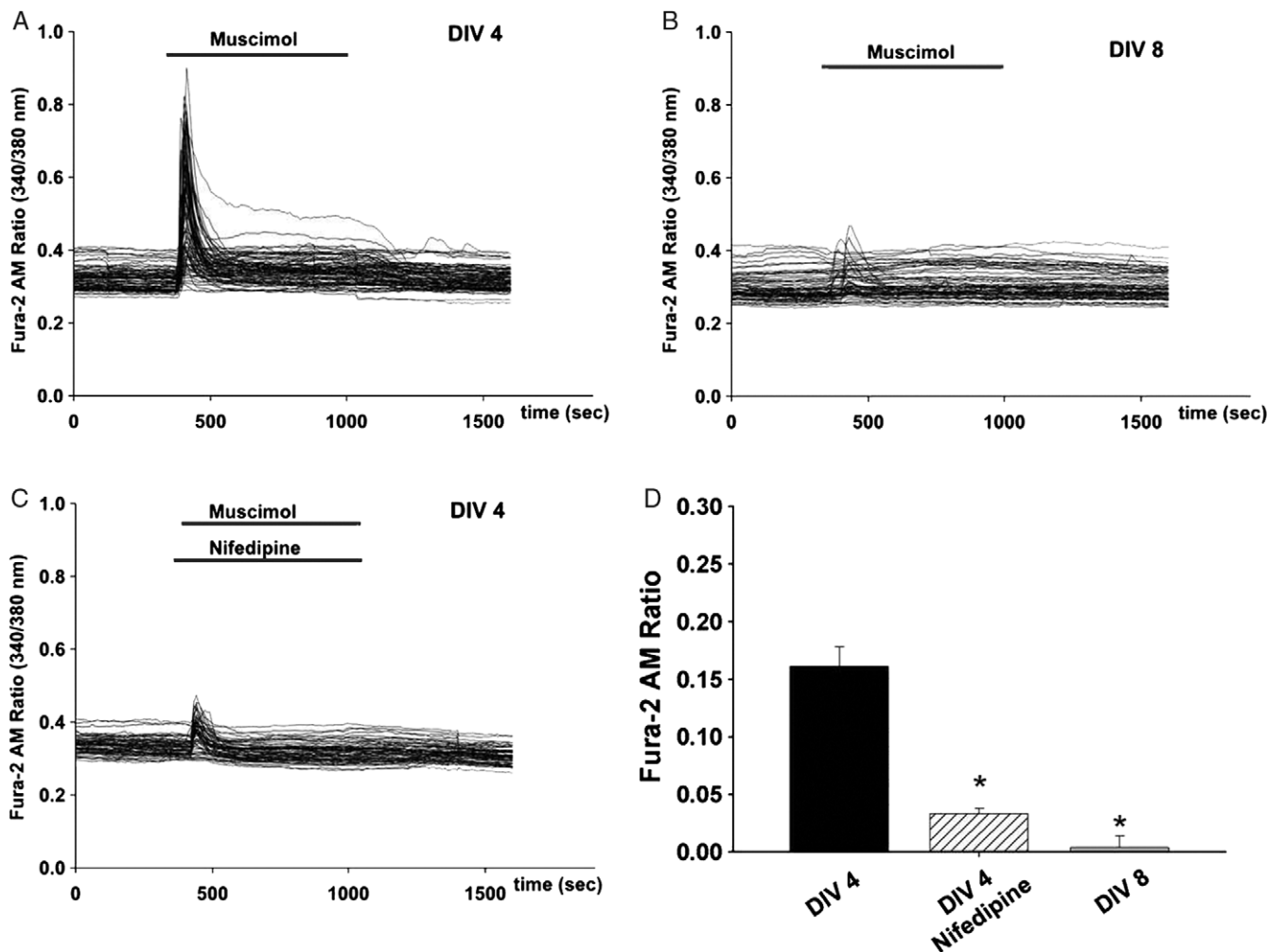
Changes in [Ca<sup>2+</sup>]<sub>i</sub> elicited by the GABA<sub>A</sub> agonist muscimol and propofol were measured in the absence and presence of the GABA<sub>A</sub> antagonist bicuculline using rat hippocampal neurons at both 4 and 8 DIV. Baseline [Ca<sup>2+</sup>]<sub>i</sub> level of DIV 4 and DIV 8 hippocampal neurons was the same. Exposure of neurons to muscimol (10 μM), induced a transient peak in [Ca<sup>2+</sup>]<sub>i</sub> that remained

elevated in DIV 4 neurons at the end of the 10 minutes exposure period [ANOVA  $F = (2,8) 48.3, P < 0.001$ ; Fig. 1A]. Perfusion of DIV 8 neurons with muscimol for 10 minutes did not cause a significant elevation in  $[Ca^{2+}]_i$ , and therefore remained significantly less than those observed with DIV 4 neurons ( $P < 0.001$ ; Fig. 1B, D). The increase in  $[Ca^{2+}]_i$  elicited by muscimol in DIV 4 neurons was significantly reduced by concurrent treatment with the L-type voltage-gated  $Ca^{2+}$  channel inhibitor, nifedipine,  $10 \mu M$  ( $P < 0.001$ ; Fig. 1C, D). Perfusion of DIV 4 neurons with  $5 \mu M$  propofol also caused a significant transient increase in  $[Ca^{2+}]_i$  [ $F = (3,11) 65.7, P < 0.001$ ] to a level that was not different than that observed upon exposure to muscimol (Fig. 2A). In contrast to DIV 4 neurons, propofol infusion did not lead to a significant change in  $[Ca^{2+}]_i$  in DIV 8 neurons,

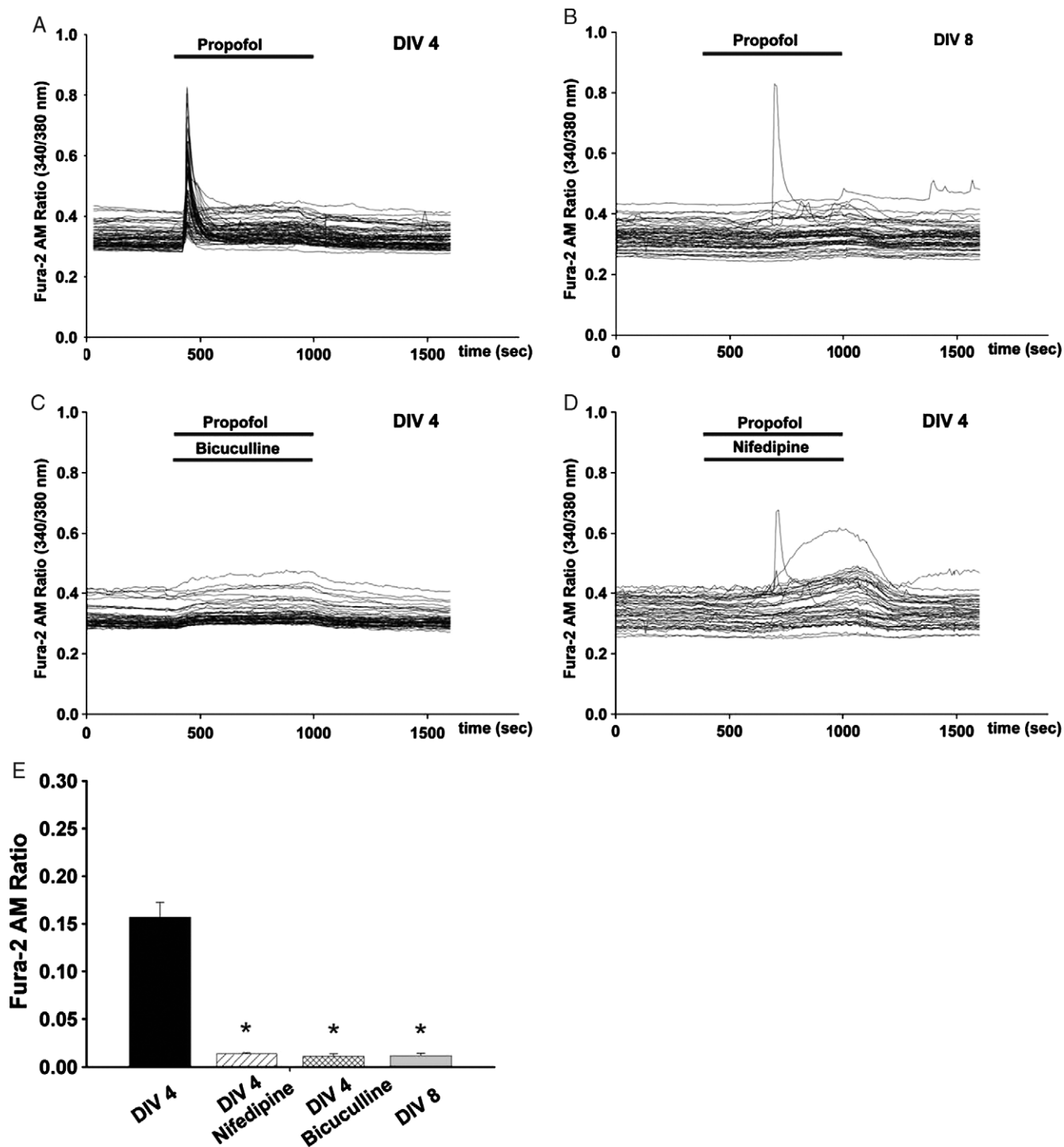
resulting in fluorescent ratios that were significantly lower than those obtained during perfusion of DIV 4 neurons ( $P < 0.001$ , Fig. 2B, E). In DIV 4 cultures, simultaneous application of nifedipine with propofol perfusion inhibited the propofol-induced rise in  $[Ca^{2+}]_i$  ( $P < 0.001$ , Fig. 2D, E). Perfusion of DIV 4 neurons with the GABA<sub>A</sub> antagonist bicuculline ( $10 \mu M$ ) completely abolished the propofol-induced initial rise in  $[Ca^{2+}]_i$  ( $P < 0.001$ , Fig. 2C, E).

### In Vitro Age-dependent Propofol-induced Neuronal Death

Treatment of DIV 4 hippocampal neuronal cultures for 5 hours with propofol induced a rapid and dose-independent increase in percentage of dead cells at 1 hour



**FIGURE 1.** GABA<sub>A</sub> receptor activation increases intracellular free  $Ca^{2+}$  in immature hippocampal neurons. Fura-2 AM fluorescence (ratio at 340/380 nm excitation) measured in hippocampal neurons of different age in culture, DIV 4 (A) versus DIV 8 (B) induced by  $10 \mu M$  muscimol applied for 10 minutes. (Each trace represents fluorescence monitored in single neuron somata in the field). The muscimol-induced rise in the fluorescence ratio for DIV 4 hippocampal neuronal cultures was suppressed by the presence of nifedipine ( $10 \mu M$ ) in the perfusate during muscimol perfusion (C). Values shown in (D) represent the means  $\pm$  SEM for 3 to 4 independent experiments where fluorescence was quantified 40 seconds after addition of muscimol using a total of 66 to 85 cells per group. \* $P < 0.001$  when compared with control,  $n = 3$  to 4 per group. DIV indicates days in vitro; GABA,  $\gamma$ -aminobutyric acid.



**FIGURE 2.** Propofol increases intracellular free Ca<sup>2+</sup> in immature hippocampal neurons. Fura-2 AM fluorescence ratio in hippocampal neurons of different ages in culture, DIV 4 (A) versus DIV 8 (B) induced by 5 μM propofol applied for 10 minutes. The presence of 10 μM bicuculline (C) and 10 μM nifedipine (D) in the perfusate inhibited the propofol-induced increase in Fura-2 fluorescence ratio in DIV 4 hippocampal neuronal cultures. Values shown in (E) represent the means ± SEM for 3 to 4 independent experiments where fluorescence was quantified 30 seconds after the addition of propofol using a total of 58 to 73 cells per group. \*P<0.001 when compared with control, n= 3 to 4 per group. DIV indicates days in vitro.

posttreatment (Fig. 3A, C). There was 94.4% more cell death in cultures treated with propofol 5  $\mu$ M and 80.8% more in cultures treated with 50  $\mu$ M propofol compared with untreated controls (K-W  $P = 0.004$ ). However, the extent of the cell death induced by muscimol 10  $\mu$ M was not significantly different than controls (23.4% more than control; Fig. 3A). Simultaneous incubation of bicuculline 10  $\mu$ M or nifedipine 10  $\mu$ M with propofol prevented the propofol-induced increase in acute cell death (Fig. 3A). The increase in delayed cell death in DIV 4 neuronal cultures at 48 hours after treatment with propofol 5 and 50  $\mu$ M was not significant (K-W  $P = 0.05$ ; Fig. 3B). Muscimol treatment for 5 hours did not cause an increase in delayed cell death. Neither propofol nor muscimol induced acute or delayed cell death in DIV 8 hippocampal neuronal cultures (Fig. 4A, B). Concurrent treatment of DIV 8 neuronal cultures with propofol and bicuculline or nifedipine affected neither acute nor delayed cell death percentage.

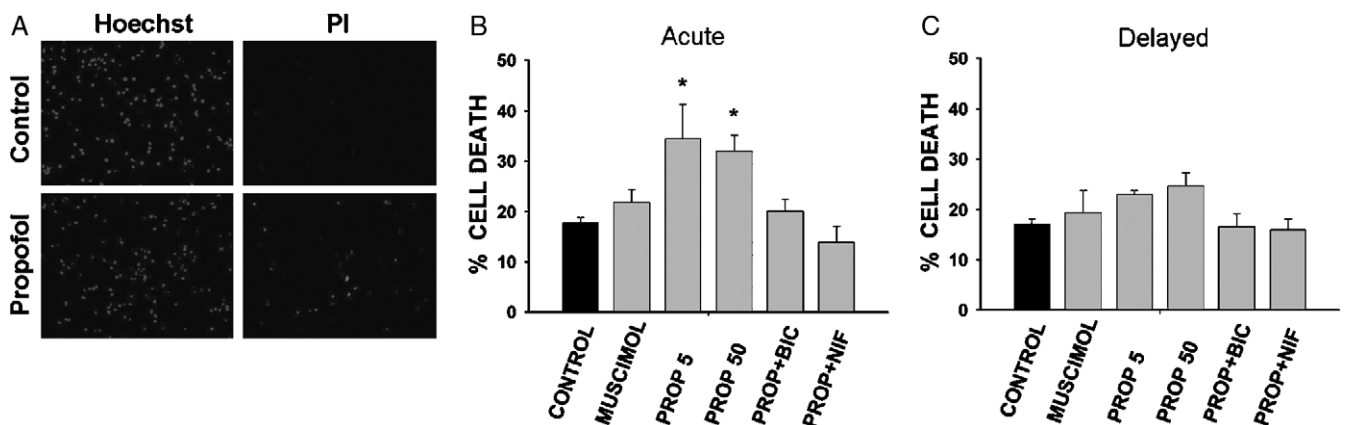
### Propofol-induced Caspase-3,-7 Activation in DIV 4 but not in DIV 8 Hippocampal Neurons

Baseline level of caspase-3,-7-positive cells was 10.4%  $\pm$  2.2% in untreated DIV 4 cultures and 6.5%  $\pm$  3.6% in DIV 8 untreated cultures. In propofol-treated DIV 4 cultures, the percentage of caspase-3,-7-positive cells increased 110.6% at 1 hour posttreatment, when compared with untreated control cultures [21.9%  $\pm$  2.3% vs. 10.4%  $\pm$  2.2%,  $F = (5,21) 3.39$ ,  $P = 0.028$ ; Fig. 5]. Treatment of DIV 8 hippocampal cultures for 5 hours with propofol had no effect on caspase-3 and -7 activities, nor did muscimol treatment change the percentage of caspase-3,-7 positive cells in cultures of either age.

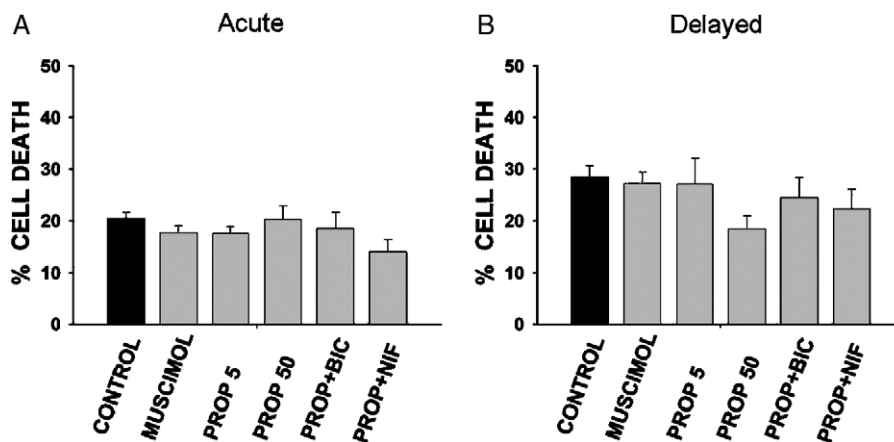
### DISCUSSION

The most important observation of this study is that 5 hours of exposure to propofol, at a pharmacologically relevant dose of 5  $\mu$ M, stimulates caspase activation and promotes acute death of neurons at an in vitro developmental age (DIV 4) when the propofol-sensitive GABA<sub>A</sub> receptor elicits cellular depolarization. GABA<sub>A</sub>-dependent propofol-induced depolarization was verified by detection of bicuculline and nifedipine inhibitable elevation of neuronal [Ca<sup>2+</sup>]<sub>i</sub>. Parallel inhibition of propofol toxicity by both bicuculline and nifedipine provides evidence that the rise in [Ca<sup>2+</sup>]<sub>i</sub> is necessary to induce death. The rise in [Ca<sup>2+</sup>]<sub>i</sub> does not, however, seem to be a sufficient explanation for propofol toxicity as a comparable elevation generated upon exposure of DIV 4 neurons to the GABA<sub>A</sub> agonist, muscimol, did not result in significant death. One limitation of this study is that changes in [Ca<sup>2+</sup>]<sub>i</sub> were measured during a 10 minutes exposure to propofol or muscimol and for 10 minutes thereafter whereas caspase activation and cell death were measured 1 hour after 5 hours of continuous exposure to these GABAergic agents. It is therefore possible that a delayed Ca<sup>2+</sup> deregulation occurs in response to the early rise in [Ca<sup>2+</sup>]<sub>i</sub>, such as what is well established for glutamate excitotoxicity,<sup>15</sup> and that this event is more robust with propofol than with muscimol.

Other known effects of propofol on cell physiology could contribute to the neuronal toxicity observed in our experiments. Clinically relevant concentrations of propofol induce a calcium-dependent change in the actin-cytoskeletal organization of neurons and glial cells,<sup>16,17</sup> that is inhibited by bicuculline and the Ca<sup>2+</sup> channel blocker, verapamil.<sup>17,18</sup> Whereas propofol stimulates tyrosine phosphorylation of actin, this effect is not observed with muscimol, possibly due to the different



**FIGURE 3.** Acute and delayed death of hippocampal neurons (4 d in vitro) after exposure to propofol and other GABA<sub>A</sub> receptor modulators. Neurons were stained with propidium iodide (PI) and Hoechst at 1 hour (acute), and 48 hours (delayed) after treatment for 5 hours. Sample images of Hoechst and PI staining in untreated (control) and propofol-treated DIV 4 hippocampal neuronal cultures (A). The acute (B) and delayed (C) cell death percentage in neuronal cultures of untreated (control) and treated with muscimol, 10  $\mu$ M (MUSCIMOL); propofol, 5  $\mu$ M (PROP 5); propofol, 50  $\mu$ M (PROP 50); combined propofol 50  $\mu$ M and bicuculline 10  $\mu$ M (PROP+BIC); and combined propofol, 50  $\mu$ M and nifedipine, 10  $\mu$ M (PROP+NIF) for 5 hours. \* $P = 0.004$  when compared with control,  $n = 3$  to 9. GABA indicates  $\gamma$ -aminobutyric acid

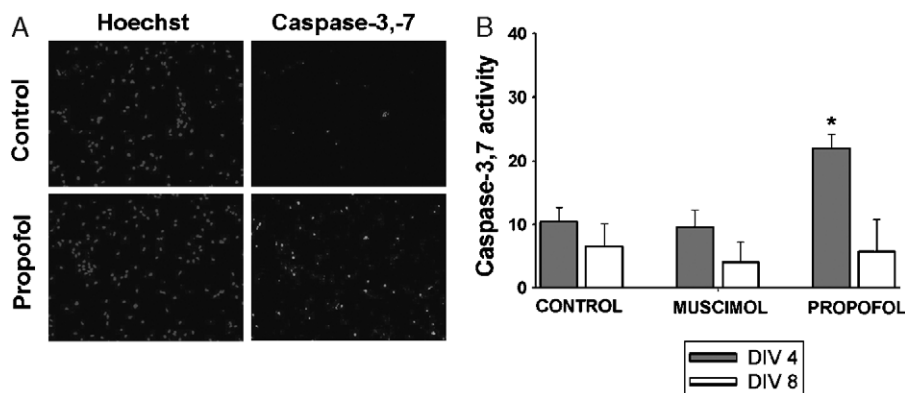


**FIGURE 4.** Acute and delayed death of hippocampal neurons (8 d in vitro) after exposure to propofol and other GABA<sub>A</sub> receptor modulators. Cell death in DIV 8 hippocampal neuronal cultures treated with the artificial CSF vehicle (control); muscimol, 10 μM (MUSCIMOL); propofol, 5 μM (PROP 5); propofol, 50 μM (PROP 50); combined propofol 50 μM and bicuculline 10 μM (PROP+BIC); and combined propofol, 50 μM and nifedipine, 10 μM, (PROP+NIF) for 5 hours. Neurons were stained with propidium iodide (PI) and Hoechst at 1 hour (A), and 48 hours (B) after treatment for 5 hours, n=4 to 6. CSF indicates cerebrospinal fluid; DIV, days in vitro; GABA, γ-aminobutyric acid.

GABA receptor β subunits with which these agents interact.<sup>19</sup> As cell death can be induced by alterations in actin dynamics,<sup>20</sup> the differential sensitivity of actin to propofol and muscimol might explain the selective propofol-effects on viability observed in our study. Another effect of propofol that could promote Ca<sup>2+</sup> dependent neuronal death is inhibition of respiration, as reported for macrophages,<sup>21</sup> hepatocytes,<sup>22–24</sup> cardiomyocytes,<sup>25,26</sup> and brain synaptosomes.<sup>27,28</sup> Effects of muscimol on neuronal respiration have not been reported, however. Because the toxicity of elevated intraneuronal Ca<sup>2+</sup> is often ascribed to the multiple effects it has on mitochondrial bioenergetics,<sup>15</sup> any additional impairment of mitochondrial respiration by propofol could therefore promote Ca<sup>2+</sup>-induced neurotoxicity.

Additional studies support the concept that propofol can be toxic to neurons within the immature brain.

In aggregating cell cultures of fetal rat telencephalon, Honegger and Matthieu<sup>29</sup> report that application of > 10 μM propofol for 8 hours selectively produces dose-dependent irreversible structural changes in GABA<sub>A</sub> receptor containing GABAergic neurons; however, no protection was observed with GABA<sub>A</sub> receptor blockers.<sup>29</sup> Moreover, exposure of immature GABAergic neurons to propofol (> 5 μM) but not midazolam, induces a dose-dependent decrease in dendritic growth under physiological conditions.<sup>30</sup> To more fully define the role of GABA<sub>A</sub> receptor modulation by propofol in immature neurons, we tested the effects of propofol and muscimol on DIV 4 hippocampal neurons compared with those observed with neurons cultured for 8 days, when the chloride gradient reverses and GABA receptor activation causes hyperpolarization instead of depolarization.<sup>1,31,32</sup> As predicted, neither agent caused an increase in [Ca<sup>2+</sup>]<sub>i</sub> or cell death in DIV 8 neurons. Although there are no



**FIGURE 5.** Caspase-3,-7 activity in hippocampal neurons after exposure to propofol and muscimol. Sample images of Hoechst and green caspase-3,-7-stained DIV 4 neurons (A). Mean percentage of caspase-3,-7-positive cells in DIV 4 and DIV 8 cultures at 1 hour after treatment with the artificial CSF vehicle (control); muscimol, 10 μM; and propofol, 5 μM, for 5 hours (B). \*P=0.028 when compared with control group, n=3 to 5. CSF indicates cerebrospinal fluid; DIV, days in vitro.

reports of toxicity to immature neurons by propofol administration in vivo, Nuñez et al<sup>7</sup> reported that administration of muscimol twice a day for 2 days in newborn rats increases cell death in the hippocampus measured on postnatal day 7, suggesting that other GABAergic stimuli such as propofol could also be toxic in vivo.

The degree to which our results obtained in vitro can be translated to the clinical scenario of propofol anesthesia is limited by several factors, including the relevance of propofol concentrations used in our experiments. Propofol is highly lipophilic and therefore concentrates in lipid-rich tissues such as brain.<sup>33</sup> Thus, while the concentration of propofol in the CSF of patients is < 2  $\mu\text{M}$ , studies in animals<sup>33</sup> and humans<sup>34,35</sup> indicate the measured/predicted brain concentration of propofol during maintenance of surgical anesthesia is above 22  $\mu\text{M}$  (4  $\mu\text{g}/\text{mL}$ ), and as high as 73  $\mu\text{M}$ . The brain:blood partition coefficient for propofol of greater than 1.2 in both human and animals is in agreement with its high lipophilicity.<sup>33,35–39</sup> The 5 to 50  $\mu\text{M}$  concentration range used in our experiments are therefore within the range of concentrations that exist in the human brain during anesthesia.

Although the results of our in vitro studies and those of other investigators suggest the possibility of propofol neurotoxicity to the immature brain, only careful dose-response experiments performed in vivo with more than one animal species, including primates, will resolve this question. The significance of our findings is therefore limited to the conclusion that pharmacologically relevant concentrations of propofol can be toxic to immature neurons in vitro and that this toxicity requires a GABA receptor-mediated rise in intracellular  $\text{Ca}^{2+}$ . Additional studies are underway to determine what targets in addition to GABA receptors are responsible for the selective toxicity of propofol toward immature neurons.

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