Clinically Relevant Concentrations of Propofol but Not Midazolam Alter In Vitro Dendritic Development of Isolated γ-Aminobutyric Acid–positive Interneurons

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Background: Recent laboratory studies showed that exposure to supraclinical concentrations of propofol can induce cell death of immature neurons. However, no data are available regarding the effects of clinically relevant concentrations of this agent on neuronal development. The authors addressed this issue by evaluating the effect of propofol on dendritic growth and arbor expansion of developing γ-aminobutyric acid–positive (GABAergic) interneurons.

Methods: Immature neuroblasts were isolated from the newborn rat subventricular zone and differentiated into GABAergic interneurons in culture. In addition to cell death, the effects of increasing concentrations and durations of propofol exposure on neuronal dendritic development were evaluated using the following morphologic parameters: total dendritic length, primary dendrites, branching point, and Scholl analysis.

Results: The authors demonstrate that propofol induced cell death of GABAergic neurons at concentrations of 50 μg/ml or greater. As little as 1 μg/ml propofol significantly altered several aspects of dendritic development, and as little as 4 h of exposure to this agent resulted in a persistent decrease in dendritic growth. In contrast, application of midazolam did not affect neuronal development.

Conclusion: Short-term exposure of immature developing GABAergic neurons to clinically relevant concentrations of propofol can induce long-term changes in dendritic arbor development. These results suggest that propofol anesthesia during central nervous system development could interfere with the molecular mechanisms driving the differentiation of GABAergic neurons and thus could potentially lead to impairment of neural networks.

IN humans, the brain growth spurt period, including dendritic development and synaptogenesis, starts at the beginning of the third trimester of pregnancy and is thought to end only several years after birth. This issue is of particular interest in anesthesia practice because a significant number of patients require general anesthesia for surgical or diagnostic interventions during this period. It is now well established that interference with the finely tuned molecular mechanisms, guiding the formation of neural circuits in the developing brain, can lead to persistent dysfunction of the central nervous system.2 Dendrites represent the primary sites of synaptic contacts in developing neurons. Appropriate processing of synaptic inputs is critically determined by the highly dynamic structure and electrical properties of the dendritic arbor.5 Dendritic development continues in virtually all types of central nervous system neurons for years in postnatal life, and pharmacologic interactions with this process might lead to impairment of higher cognitive functions.6

Recent experimental evidence indicates that interference with γ-aminobutyric acid type A (GABA_A) and N-methyl-D-aspartate receptor signaling pathways during synaptogenesis can lead to apoptotic degeneration of neurons in the developing brain.5,6 Exposure of the human fetus to ethanol, an agent acting through both GABA_A and N-methyl-D-aspartate receptors, causes a well-described neurotoxic syndrome, fetal alcohol syndrome.6 In this context, administration of currently used anesthetics might exert adverse effect on neuronal development because these drugs principally act by either increasing γ-aminobutyric acid–mediated inhibitory tone via GABA_A receptors or decreasing excitatory glutamatergic activity by blocking N-methyl-D-aspartate receptors. Exposure of 7-day-old rats to a combined midazolam–nitrous oxide–isoflurane anesthesia for 6 h led to widespread neurodegeneration in the developing brain.7 However, the relevance of these findings in higher primates and humans remains to be determined.8,9

Propofol (2,6-diisopropyl phenol) is an alkyl phenol derivative dissolved in a lipid emulsion. Although controversy exists,10,11 this agent is commonly used in young children, including neonates.12,13 The current study was undertaken to evaluate the effect of low, clinically relevant concentrations of propofol on the development of GABAergic neurons. To achieve our aim, we isolated immature neuroblasts from the postnatal rat subventricular zone as previously described.14 This purified cell population developed into GABAergic interneurons in low-density cultures with a nice dendritic arbor. Application of as low as 1 μg/ml propofol significantly altered dendritic development and as short as 4 h exposure to clinically relevant concentrations of this agent led to a substantial decrease in dendritic growth. In contrast, application of midazolam did not affect neuronal development.
Materials and Methods

Cell Culture and Reagents

After obtaining approval from the Animal Care Committee of the University of Geneva (Geneva, Switzerland), cell cultures were prepared from newborn Sprague-Dawley rats. Animals were killed by decapitation, and the brain was carefully removed and transferred into an ice-cold Hank’s magnesium- and calcium-free solution. Two coronal cuts were then made to expose the anterior horn of the lateral ventricles, and the subventricular zone was microdissected. Successively, the small tissue pieces obtained were dissociated mechanically and digested with trypsin (Invitrogen, Life Technologies, Paisley, United Kingdom) in PBS and centrifuged 10 min at 37°C. The trypsin reaction was stopped with 1 ml cold fetal calf serum, and cells recovered after 10 min centrifugation at 300g. To eliminate cell debris, the pellet was resuspended into 1 ml phosphate-based saline (PBS) and layered onto a 22% Percoll (Amersham Pharmacia, Little Chalfont, United Kingdom) in PBS and centrifuged 10 min at 500g. Cells were washed three times with culture medium before plating onto polyornithin (Sigma, Saint Louis, MO)–coated coverslips in 35-mm Petri dishes (Falconn, Plymouth, United Kingdom). Seeding density was 5,000 cells/cm². Cells were cultured for 5 days in neurobasal medium (Invitrogen, Life Technologies) supplemented with 2% B27 (Invitrogen, Life Technologies), 200 mM l-glutamine (Invitrogen, Life Technologies), and 1 mM Na pyruvate (Sigma).

To test the effect of propofol on neuronal survival and development, cultures were exposed to propofol (Disoprivian; Astra-Zeneca, London, United Kingdom). The amount and the duration of propofol treatment are indicated in detail for each experiment. An intralipid emulsion was used as a control for the propofol vehicle (20% Lipoveineuse; Fresenius, Bad Homburg, Germany). Midazolam (Roche Pharma, Basel, Switzerland) was used in a concentration range between 0.25 and 25 μg/ml.

Immunocytochemistry

Cells were fixed with cold (4°C) paraformaldehyde 4% in phosphate buffer (pH 7.4). Then, they were rinsed three times in PBS and incubated overnight at 4°C with the primary antibody diluted in 0.5% PBS containing bovine serum albumin (0.3%) and Triton X-100 for intracellular antigens. For γ-aminobutyric acid (GABA) staining, in addition to this protocol, a prefixation in 2% paraformaldehyde–2% glutaraldehyde in phosphate buffer (pH 7.4) for 30 min was performed. After fixation, cells were in addition rinsed three times for 20 min with PBS and incubated 30 min in a solution of 0.1 M NH₄Cl. The following primary antibodies were used: (1) A mouse monoclonal antibody directed against β-tubulin isotype III (Sigma; 1:400 dilution) was used to identify neurons. (2) The monoclonal antibody anti-GABA 3D5 was used to detect GABA-immunopositive neurons (gift from Peter Streit, M.D., Professor, Brain Research Institute, University of Zurich, Zurich, Switzerland; dilution: 1:2,000). Bound antibodies were revealed with rhodamine- or fluorescein-conjugated sheep anti-mouse immunoglobulin G (Boehringer, Mannheim, Germany; dilution: 1:40 for rhodamine and 1:80 for fluorescein) diluted in PBS containing 0.5% bovine serum albumin. Immunostained cultures were examined with an Axioskop fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Cell Counts, Statistical Analysis, Image Acquisition, and Processing

Cultures were examined using an Axioskop fluorescence microscope. Cells were counted with the help of a square grid placed into the ocular of the microscope. On each coverslip, 30 samples were randomly taken, and then samples were pooled. Data are expressed as number of neurons/mm² ± SEM and reflect the results obtained from at least three independent experiments.

For quantitative analysis of dendritic arbors, cells were stained with the monoclonal GABA antibody and photographed. Before the analysis, brightness and contrast were optimized with Adobe Photoshop program (Adobe Systems Incorporated, San Jose, CA). The following parameters of dendritic shape and extent were then determined: number of primary dendrites, length of dendrites, and number of dendritic branches. Total dendritic length was measured drawing all visible processes with Scion software.||The remaining parameters were manually scored on the image. Processes shorter than 5 μm were excluded from the analysis. To examine whether alterations in dendritic parameters were localized to a specific portion of the dendritic arbor, we used a modified Sholl analysis in which the amount of dendritic arbor was calculated within concentric radii drawn at 10-μm intervals, centered in the cell body. Values were expressed as mean ± SEM and analyzed for statistical significance.

Differences between groups were first discriminated by one-way analysis of variance, and then an unpaired t test was performed, where t was corrected for multiple comparisons against the untreated group using the Bonferroni test. * P < 0.05 compared with the untreated control group. (The asterisk indicates significance, defined as P < 0.05.)

Results

We have previously shown that exposure of dissociated primary cultures from the newborn rat cerebral cortex to supraclinical concentrations of propofol leads...
to massive cell death of GABAergic neurons. In that study, GABAergic neurons were cultured on the top of a glial monolayer at relatively high densities. That model allowed us to measure cell death, but the quantitative assessment of other morphologic parameters, which describe development and differentiation of these neurons, was difficult. In the current study, to circumvent this problem, we took advantage of our recently developed culture model, where purified neuronal precursors from the subventricular zone differentiate into GABAergic neurons and elaborate dendritic arbors under serum-free conditions. In addition, to test the direct effect of anesthetic agents on cell survival, this model also allowed us to quantitatively track the temporospatial development of the neuronal dendritic arbor. Figures 1A and B show that under defined culture conditions, these neurons survived and developed an extensive dendritic arborization pattern. All neurons in the culture expressed the neurotransmitter GABA.

To evaluate the effect of propofol on the development and survival of these neurons, first, we exposed these cultures to increasing concentrations of this agent and assessed cell survival as well as morphology over time. As seen in figures 1C–F, low, clinically relevant concentrations of propofol (1–20 μg/ml) or midazolam (0.25–25 μg/ml) did not affect cell survival up to 5 days in culture. However, similar to our findings using mixed cortical cultures, we found that high concentrations of propofol (≥ 50 μg/ml) led to a rapid and massive loss of GABAergic neurons (fïgs. 1D and F). In contrast to propofol, an intralipid vehicle alone did not affect survival and differentiation of neurons.

Interestingly, while cell survival remained unaltered, clinically relevant concentrations of propofol strongly altered the dendritic development of GABAergic neurons (fïgs. 1C and F). In contrast to propofol, midazolam up to a concentration of 25 μg/ml did not affect differentiation and survival of these cells (fïgs. 1E and F). Figure 2 shows representative examples of the effect of clinically relevant concentrations of propofol and midazolam on dendritic arbor development (see fig. 2A for experimental protocol). Before exposure of cultures to these agents for 48 h (i.e., 24 h after seeding), neurons in culture exhibited a rather immature morphology with short dendritic processes (fig. 2B). Under defined serum-free conditions, these cells developed a nice arborization pattern by 48 h in culture (fig. 2C). In contrast, cells cultured in the presence of propofol (1–20 μg/ml) showed severely impaired neuronal differentiation (fïgs.
Exposure to midazolam (0.25–25 μg/ml) did not seem to affect dendritic development (fig. 2G).

We measured four parameters to quantitatively describe dendritic development: (1) the total dendritic length; (2) the number of primary dendrites, defined as those arising from the cell body; (3) the number of branching points; and (4) the spatial distribution of dendrites (Scholl analysis15). Figure 3A shows that, under defined serum-free conditions, total dendritic length increased approximately 10 times during the 48 h culture period. In contrast, exposure to propofol resulted in a decreased dendritic length at concentrations as low as 1 μg/ml (E) or 20 μg/ml (F). (G) In the presence of midazolam, neuronal arbor development was comparable to control conditions. Cells were stained with an anti-γ-aminobutyric acid antibody. Correction bar (B–G): 75 μm.

Next, we wanted to know how short-term exposures to propofol influence subsequent long-term dendritic development. Cultures were grown for 2 days in serum-free medium and then exposed to 5 μg/ml propofol for 4 or 8 h. Cells were then washed extensively to remove propofol, and serum-free medium was added again for an additional 24–72 h (see fig. 4A for experimental protocol). Corresponding sister cultures were then fixed and analyzed at 0, 24, 48, and 72 h. Figure 4B shows that GABAergic neurons developed a nice dendritic arbor in control, nontreated cultures. On the contrary, both 4- and 8-h exposure to propofol (5 μg/ml) had a negative effect on subsequent dendritic development (figs. 4C and D). Although there were no differences in any of the examined morphologic parameters between experimental groups at the end of the 4- to 8-h propofol treatment (i.e., at 0 h), quantitative dendritic analysis revealed that even this short-term exposure to this agent could significantly influence ulterior arbor development (figs. 5 and 6). The number of primary dendrites did not differ between experimental groups (fig. 5C), whereas both the total dendritic length (fig. 5A) and arborization pattern, expressed as the number of branching points (fig. 5B) and Scholl analysis (fig. 6) significantly decreased compared with controls as soon as 24 h after propofol exposure, and these differences remained significant at all time points examined. We also found an important difference between the 4-h-long and the 8-h-long propofol treatments on ulterior dendritic development (figs. 6B–
D). At time points of 24 and 48 h, both total dendritic length and the number of branching points decreased, although not significantly, in the 8-h propofol treatment group compared with the 4-h exposure, while Scholl analysis, describing the overall spatial dendritic arborization pattern, revealed a significant difference between these two groups (fig. 6). Finally, it is important to note that both total dendritic length and the complexity of the arborization pattern increased by time in propofol treated groups; however, there was an important delay compared with control cultures (figs. 5 and 6).

**Discussion**

Despite the gap between the perception of teratogenic risk and its confirmation in clinical practice, *in vitro* models are useful tools to draw the attention to the potential adverse effects of pharmacologic agents during development. The current study was designed to investigate the effect of propofol on the differentiation and survival of isolated GABAergic interneurons in culture. We showed that supraplacental concentrations of propo-
fol led to a rapid cell death of these cells. Clinically relevant concentrations of this agent did not affect cell survival but had an important, dose- and time-dependent effect on neuronal differentiation. Propofol, at a concentration as low as 1 μg/ml, significantly decreased dendritic arborization pattern, and a 4-h exposure to this agent was sufficient to induce long-term changes in dendritic development. In contrast to propofol, midazolam did not affect GABAergic neuronal development. These results thus raise the theoretical possibility that propofol anesthesia during the third trimester of pregnancy and up to the first years of life could interfere with the molecular mechanisms governing the differentiation of GABAergic neurons and might lead to long-term impairment of central nervous system function.

High concentrations of propofol were reported to produce toxic effects on neurons in aggregated cell cultures of the fetal rat telencephalon by Honegger and Mat-thieu. Accordingly, in a recent study, we provided morphologic evidence that supraclinical doses of propofol led to cell death of GABAergic interneurons in dissociated cultures of the newborn rat cerebral cortex. These studies were hindered by the methodologic disadvantage that important morphofunctional parameters, other than cell death, could not be adequately assessed.

Fig. 5. Quantitative assessment of dendritic development after short-term propofol exposure. For experimental protocol, see figure 4A. Both 4-h-long and 8-h-long exposures of neurons to propofol (5 μg/ml) resulted in an altered ulcerior dendritic growth, as expressed by the total dendritic length (A) and branching (B). In contrast, the number of branching points did not significantly differ between experimental groups (C). Results are presented as mean ± SEM; n = 3 independent experiences (3 culture dish per experiment, 30 neurons per culture dish) for each time point and each treatment expressed. * P < 0.05 compared with the untreated control group.

Fig. 6. Spatial expansion of dendritic arbor after short-term propofol exposure. For experimental protocol, see figure 4A. Scholl analysis revealed that administration of propofol (5 μg/ml) for 4–8 h did not affect the spatial distribution of the dendritic tree (A). After exposure to propofol, subsequent dendritic arborization pattern was impaired at time points of 24 h (B), 48 h (C), and 72 h (D). Results are presented as mean ± SEM; n = 3 independent experiences (3 culture dish per experiment, 30 neurons per culture dish) for each time point and each treatment expressed. * P < 0.05 compared with the untreated control group.
Hence, to describe the effect of propofol on more subtle morphologic parameters, such as dendritic arbor development during neuronal differentiation, we took advantage of our recently developed in vitro system, where isolated and purified neuronal precursors from the subventricular zone were cultured at low seeding density and differentiated into GABAergic neurons. In these cultures, direct interactions with other cell types were absent, allowing us to investigate the direct effect of propofol on GABAergic neuronal differentiation. This model permitted to demonstrate that even concentrations of propofol used to provide sedation (1 μg/ml) are sufficient to alter dendritic development in these neurons. Most importantly, we also showed that the effect of propofol on dendritic expansion persisted even after removal of the drug. To our knowledge, this is the first demonstration that propofol at clinically relevant concentrations can impair adequate neuronal development.

In our study, propofol but not midazolam altered dendritic growth. The reason for this difference is unclear but might be explained by the different sites of action of these molecules on the GABAA receptor complex. These agents potentiate the effect of GABA or, at higher concentrations, directly activate the GABAA receptor complex, but while benzodiazepines attach selectively to the α subunits of the GABAA receptors, propofol tyrosine phosphorylates the β and γ subunits. Stimulation of different receptor subunits would thus initiate different intracellular signaling pathways. Recent evidence indicates that propofol but not midazolam tyrosine phosphorylates actin, leading to a rapid restructuring of actin in neurons. Therefore, reorganization of the actin cytoskeleton in developing GABAergic neurons would provide a plausible explanation of the effect of propofol on dendritic development. Propofol is also known to act on various glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxalylone prionerate, kainate, and N-methyl-D-aspartate receptors as well as through various sodium channels in the brain. Hence, alternatively, it is also possible that modifications in dendritic growth, induced by propofol, are the results of a complex molecular interplay between various intracellular signaling systems. Clearly, further studies are needed to answer these questions.

The clinical significance of our data remains to be determined. Although an increasing number of in vitro and in vivo studies show that anesthetics could potentially exert adverse effects on the developing brain, extrapolation of these findings to humans is extremely difficult and controversial. Our in vitro study explored the impact of propofol on the differentiation of immature neuroblasts into GABAergic interneurons. In humans, such dendritic development is an ongoing process from the last trimester of pregnancy up to the first years of life, with a peak corresponding to the last month of gestation and the first 6 months of the postnatal period. Data presented in this study suggest that propofol could interfere with dendritic development and thus might lead to long-term impairment of higher-order central nervous system functions. Future in vitro experimental and clinical studies should be conducted to further elucidate this important issue.

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References