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## Anesthetics interfere with the polarization of developing cortical neurons

C. David Mintz, MD, PhD<sup>1,\*</sup>, Sarah C. Smith, MD<sup>1</sup>, Kendall M.S. Barrett<sup>1</sup>, and Deanna L. Benson, PhD<sup>2</sup>

<sup>1</sup>Department of Anesthesiology, Columbia University, New York, New York, USA

<sup>2</sup>Fishberg Department of Neuroscience, The Mount Sinai School of Medicine, New York, New York, USA

### Abstract

Numerous studies from the clinical and preclinical literature indicate that general anesthetic agents have toxic effects on the developing brain, but the mechanism of this toxicity is still unknown. Previous studies have focused on the effects of anesthetics on cell survival, dendrite elaboration, and synapse formation, but little attention has been paid to possible effects of anesthetics on the developing axon. Using dissociated mouse cortical neurons in culture, we found that isoflurane delays the acquisition of neuronal polarity by interfering with axon specification. The magnitude of this effect is dependent on isoflurane concentration and exposure time over clinically relevant ranges, and it is neither a precursor to nor the result of neuronal cell death. Propofol also appears to interfere with the acquisition of neuronal polarity, but the mechanism does not require activity at GABA<sub>A</sub> receptors. Rather, the delay in axon specification likely results from a slowing of the extension of pre-polarized neurites. The effect is not unique to isoflurane as propofol also appears to interfere with the acquisition of neuronal polarity. These findings demonstrate that anesthetics may interfere with brain development via effects on axon growth and specification, thus introducing a new potential target in the search for mechanisms of pediatric anesthetic neurotoxicity.

### Keywords

Anesthetic; Isoflurane; Neurotoxicity; Axon; Polarity; Neuron

## INTRODUCTION

The evidence from a wide range of clinical and pre-clinical studies suggests that exposure to general anesthetics (GAs) results in harmful effects on brain development. These concerns were first raised in the 1970s and 1980s by studies in which long-term, chronic exposure to low levels of halothane from gestation through the early postnatal period in rodents resulted in poorer performance in behavioral testing as well as disturbances in synaptogenesis and neurite development.<sup>1–3</sup> The clinical question driving this work was a concern that chronic exposure to anesthetic gases in the operating room might pose a risk to operating room personnel who are pregnant. More recently the focus of investigation has been on whether a single GA exposure or several discrete exposures at clinically relevant doses can impair brain development. In a groundbreaking study, Todorovic and coworkers showed that in

\*Correspondence to: C. David Mintz, MD, PhD, Department of Anesthesiology, 622 West 168<sup>th</sup> St., P&S Building, Box 46, New York, NY 10032, USA. Phone: 212.305.0944. Fax: 212.305.3675., cdm2134@columbia.edu.

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early postnatal rodents a single exposure to a combination of nitrous oxide, midazolam, and isoflurane, agents commonly used together in pediatric anesthetic practice, could cause persistent deficits in performance on behavioral tests of learning and memory.<sup>4</sup> Furthermore, clinical studies using epidemiologic techniques have showed a correlation between early pediatric exposure to GA and subsequent learning and/or behavioral disorders.<sup>5-8</sup> Taken together these findings have generated a renewed interest in anesthetic toxicity in the developing brain.<sup>9-12</sup>

The mechanism by which GAs cause clinically relevant alterations in brain development has not been fully elucidated. Numerous studies have shown that commonly used GAs can cause enhanced neuronal apoptosis in the developing brain in animal models.<sup>4, 13-15</sup> However, apoptosis is a normal event in brain development that serves the adaptive purpose of pruning unnecessary elements from neuronal circuits.<sup>16, 17</sup> Thus, it is unclear whether anesthetic-induced apoptosis is a cause of impaired brain function, a consequence of some underlying disruption of circuit formation, or perhaps even an epiphenomenon. A number of groups have begun to investigate alternate mechanisms related to circuit formation and have found disruptions in synapse formation, dendritic development, and astrocyte function.<sup>14, 18-21</sup> There has been relatively little study of the effects of GAs on the generation and development of axons.

The polarization of the neuron that results from the differentiation of the axon is a critical event in brain development, as neuronal function depends on the segregation of axon-specific and dendrite-specific components to their respective compartments.<sup>22-24</sup> Prior to polarization, the developing neuron gives rise to pre-polarized neurites that do not possess axonal or dendritic characteristics. These minor processes alternate between retraction and extension for a period of time, and subsequently one rapidly elongates and begins to acquire axonal characteristics.<sup>25-27</sup> To address the question of whether anesthetic toxicity in brain development might be due in part to effects on the development of axons, we asked whether exposure to GAs interferes with the acquisition of neuronal polarity in cortical neurons in dissociated culture.

## METHODS

### Cultures

Care of animals adhered strictly to the guidelines of the NIH, Columbia University, and the Mount Sinai School of Medicine. Dissociated neurons were prepared from embryonic day 18 C57BL/6 mouse neocortex as previously described,<sup>28</sup> using techniques modified from well-established hippocampal culture protocols.<sup>29</sup> Briefly, neocortex was dissected out into cold PBS and digested for 15 minutes at 37°C in 320 µM papain solution (Sigma-Aldrich, St. Louis, MO). Subsequently the tissue was triturated with a fire-polished Pasteur pipette into a single-cell suspension and layered on top of a 20 mg/mL albumin for a discontinuous gradient centrifugation at 800 rpm for ten minutes. Neurons were then plated at a density of 100/mm<sup>2</sup> on cover slips coated with poly-D-lysine (Sigma-Aldrich), where they were allowed to settle for three hours in media containing 10% horse serum. They were maintained in B-27/L-glutamine supplemented Neurobasal media (Invitrogen, Carlsbad, CA) and co-cultured with a feeder layer of an immortalized astrocytic cell line (gift from JW Jacobberger lab).<sup>30</sup> In the case of L1 immunocytochemistry experiments, cultures were prepared from Sprague-Dawley rats using similar protocols<sup>31</sup> and plated on poly-L-lysine.<sup>28</sup> Experiments on dissociated neurons were performed at one to three days *in vitro* and represent at least two separate cultures.

## Anesthetic exposure

Anesthetic exposures were performed in supplemented Neurobasal media immediately after neurons were allowed to adhere to coverslips. After exposure neurons were placed in fresh media conditioned by the astrocytic feeder layer. For isoflurane exposure, coverslips with adhered neurons were placed in an airtight, humidified modular chamber (Billups-Rothenberg, Del Mar, CA) connected to an agent-specific calibrated vaporizer (Datex-Ohmeda, Madison, WI) that delivered the agent mixed with 5% CO<sub>2</sub>/95% air carrier gas at 12 L/min as previously described.<sup>32</sup> Controls for these exposures consist of carrier gas in the absence of isoflurane. To ensure consistent delivery of the agent, gas composition was periodically measured using a 5250 RGM gas analyzer (Datex-Ohmeda). The standard exposure was 2.4% isoflurane for four hours, except as noted in experiments that tested concentration-response (Fig 1C) and exposure time-response. (Fig 1D) This concentration was chosen in pilot experiments because it produced the maximal effect on axon specification without inducing any measurable cell death. Given that the minimum alveolar concentration (MAC) of isoflurane has been measured at 1.7% in infants,<sup>33</sup> a 2.4% isoflurane exposure is somewhat higher than is used clinically, but significant effects of lesser magnitude were also seen at concentrations of 1.8% and 1.2%. (Fig 1C)

In the case of experiments involving L1 immunocytochemistry, a modified version of methods previously described for exposure in a solution of isoflurane was used.<sup>34</sup> A supersaturated solution of isoflurane was prepared in neurobasal media, which was diluted to 1 mM immediately prior to use. The solution was added to a glass chamber containing coverslips with adhered neurons and sealed tightly without any media-air interface. Isoflurane concentration was verified in pilot experiments using gas chromatography. A 1 mM concentration of isoflurane likely correlates with a dose that is somewhat higher than is used clinically, as 0.4 mM tissue concentrations of isoflurane are required to substantially reduce activity in neuronal networks.<sup>35</sup>

Propofol and muscimol exposures were performed in supplemented Neurobasal media immediately after plating, and neurons were subsequently transferred to dishes with conditioned media. Because the propofol stock solution was dissolved in DMSO, the controls for these exposures contained the appropriate concentration of DMSO in the absence of propofol. Muscimol was dissolved directly in media without a carrier. The concentration of propofol that is clinically relevant is difficult to determine in this model, because propofol is highly protein bound *in vivo* and the culture media does not contain serum. A 3  $\mu$ M concentration of propofol was chosen after pilot experiments, and this is consistent with the levels needed to activate GABA<sub>A</sub> receptors in dissociated rodent neuron culture using serum free media.<sup>36</sup>

## Cell labeling and Immunocytochemistry

For  $\beta$ III tubulin immunocytochemistry, neurons adhered to coverslips were fixed for ten minutes at room temperature in a 4% paraformaldehyde phosphate-buffered saline solution (PBS, pH 7.2) and then permeabilized in a 0.25% Triton-X detergent solution for one minute. After blocking for one hour at room temperature in 10% bovine serum albumin (Sigma), neurons were incubated for 24 hours at 4°C in anti- $\beta$ III tubulin antibody (1/1000, AB9354, Millipore, Billerica, MA) diluted in PBS containing 1% BSA. After washing, neurons were incubated for one hour with a fluorescent secondary antibody and mounted on slides for viewing. Immunolabeling for L1 followed a similar protocol, which differed in that the primary antibody incubation (1/2 ASCS4, Developmental Studies Hybridoma Bank, Iowa City, IA) was done at 37°C prior to fixation and that an incubation with Texas Red-conjugated phalloidin (1/75, Invitrogen) followed the secondary antibody incubation.

## Microscopic Analysis

Fluorescently labeled neurons on coverslips were visualized using a Zeiss Axiophot fluorescence microscope with 40x 1.3 NA and 60x 1.25 NA objectives (Zeiss, Thornwood, NY). Neuronal polarity was assessed using the Banker criterion in which any neurite that has exceeded the length of other neurites by at least 10  $\mu\text{m}$  is considered to have acquired an axonal fate.<sup>27</sup> Neurite length was initially measured by using NeuroLucida imaging software (MicroBrightField, Colchester, VT), and subsequently judged by eye by a single investigator and periodically rechecked for accuracy. The numbers of polarized and unpolarized neurons in a single microscopic field were counted, and the data are reported as the mean percentage of polarized neurons per field, with error bars denoting the standard deviation. For experiments with two groups a student's t-test was used to compare means, and for experiments with more than two groups an ANOVA with Dunnett's multiple-comparison *post hoc* test was performed using Prism 5 software (GraphPad, La Jolla, CA). For measurements of axon or pre-polarized neurite length, tracings were made in NeuroLucida (MicroBrightField), the mean lengths were determined in NeuroLucida, and the results were analyzed as above using Prism 5.

## Cell Death Assay

For the cell viability assay, neurons were cultured as described above and allowed to settle on tissue culture plates treated with poly-D-lysine, with a density of one million cells per well in a standard 12 well plate. Plates were treated with isoflurane in sealed chambers as described above. Cell viability was assessed on DIV1, 2, and 3 using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. MTT was dissolved in media at a concentration of 5 mg/mL and added in an amount equal to 10% of the culture medium volume. The plates with adhered neurons were returned to the incubator for two to four hours at which time the resulting formazan crystals were dissolved in acidified 10% Triton X-100 in isopropanol with 0.1N HCl. Absorbance was measured spectrophotometrically at a test wavelength of 570 nm and a reference wavelength of 690 nm. The data are reported as mean absorbance normalized to control levels, and statistical analysis was performed as described above.

## RESULTS

In order to investigate the effects of anesthetics on neuron development, we treated developing mouse cortical neurons in dissociated culture with isoflurane at a range of clinically relevant concentrations and exposure times, and we examined the effects on neuronal morphology, using  $\beta$ III tubulin labeling to identify neurons and define their structure. We chose to investigate the effects of isoflurane, because it is the most commonly used potent volatile anesthetic agent. We found that neurons treated with isoflurane immediately after plating and examined at one day *in vitro* (DIV1), were considerably less likely to have specified an axon from amongst the pre-polarized pool of neurites (Fig 1A and B, arrowhead indicates neurons that have polarized) as compared to carrier gas treated controls. Using the Banker criteria for neuronal polarization,<sup>27</sup> we quantified the percentage of polarized neurons and found that it was concentration dependent. The mean percentage of polarized neurons was significantly reduced at concentrations of 1.2% and above. (Fig 1C) We found that this effect depended on exposure time as well. At least four hours of isoflurane treatment (2.4%) was required to significantly reduce the mean percentage of polarized neurons. (Fig 1D)

We next asked whether the effects of isoflurane on neuronal polarity are limited to changes in morphology. The L1 cell adhesion molecule (L1CAM), which is highly expressed in axons of the developing nervous system, plays a key role in neuron development including

axon growth, fasciculation, and guidance.<sup>37</sup> L1CAM is concentrated in the axon shortly after axon specification, and it is one of the earliest proteins to adopt a polarized distribution<sup>38</sup> (e.g. Fig 1H–J, arrowhead indicates L1 labeling concentrated in the axon). We treated neurons after plating with isoflurane at one millimolar for six hours, and examined patterns of L1 localization at DIV2. We found numerous neurons that had specified an axon by morphological criteria, but had not completely segregated L1 to the axon (Fig 1H–J, arrows indicate L1 labeling in minor processes, and arrowhead indicates L1 label segregated to the axon). This finding indicates that the effects of isoflurane on neuronal polarity have additional consequences in the axon that may persist beyond DIV1.

Next we sought to examine the time course of the effects of isoflurane on the acquisition of neuronal polarity. To this end, we assayed polarity on DIV1, DIV2, and DIV3 after a four hour exposure to 2.4% isoflurane. (Fig 2A) The mean percent of polarized neurons on DIV1 was 43% for controls as compared to 22% for the isoflurane treated group. This difference was reduced but still significant at DIV2, with 56% polarized in the control group and 44% in the isoflurane group. By DIV3 there was no statistically significant difference and over 90% of neurons assayed were polarized in both groups. Given the association between isoflurane and neuronal cell death,<sup>4</sup> these results raised the question of whether the group of neurons treated with isoflurane that did not specify an axon on time were undergoing cell death or would do so shortly. To address this question, using the same exposure paradigm we examined neuron density at DIV1, DIV2, and DIV3 and found no differences between the isoflurane and control groups at any time point. (Fig 2B) Similarly, we found that an MTT assay of cell viability demonstrated no differences between isoflurane and control groups on DIV1, DIV2, or DIV3. (Fig 2C) The absence of cell death seen in other contexts may relate either to the culture model or the age of the cells, given that another study which examined the effects of isoflurane in early stage dissociated cell culture failed to demonstrate significant cell death resulting from a 3.4% isoflurane exposure for four hours.<sup>39</sup> Taken together our data indicate that isoflurane exposure delays the acquisition of neuronal polarity, but that this process is distinct from the apoptotic mechanism of anesthetic neurotoxicity that has been reported in some animal models.

Given the clinical relevance of anesthetic effects on brain development, we asked whether propofol, the most commonly used general anesthetic alternative to the potent inhalational agents, also had effects on axon specification. We found that a four hour exposure to propofol at three micromolar reduced mean polarity at DIV1 by 26% compared to a vehicle treated control. (Fig 3A) This raised the possibility that the effects of propofol and isoflurane on the acquisition of neuronal polarity are due to activity at the GABA<sub>A</sub> receptor, which is common to both agents.<sup>40</sup> We found, however, that a four hour treatment with the GABA<sub>A</sub> agonist muscimol at a high dose of 10  $\mu$ M did not significantly alter neuronal polarity compared to vehicle controls. (Fig 3B) This finding indicates that activity at GABA<sub>A</sub> receptors, a shared property of isoflurane and propofol, is not likely to be the molecular mechanism by which these GAs inhibit axon specification.

Early work in axonal specification demonstrated that pre-polarized neurites are all equally capable of assuming an axonal fate,<sup>25</sup> and it has been hypothesized that the neurite that grows the longest by chance during the stage prior to polarization will assume an axonal fate.<sup>41</sup> Isoflurane has been shown to interfere with cellular motility in mature fibroblasts and neurons.<sup>42</sup> To test the effects of isoflurane on neurite growth in our model system we performed NeuroLucida tracings of unpolarized neurites in cultured cortical neurons at DIV1 after our standard 2.4% isoflurane treatment after plating. We found that the mean neurite length in the isoflurane treated group was reduced to 11.2  $\mu$ m from 17.9  $\mu$ m in the control group. (Fig 4A–C) These data suggest that the delay neuronal polarization caused by isoflurane treatment is caused by a slowing of pre-polarized neurite growth.



## DISCUSSION

Our data demonstrate that isoflurane retards the acquisition of neuronal polarity in a concentration and exposure time dependent fashion, by both morphological and molecular criteria. The delay in axon specification is neither the result of acute, ongoing cell death nor is it a cause of cell death at later time points. Interestingly, similar effects on polarity are seen with propofol, but not with a specific GABA<sub>A</sub> agonist. The mechanism by which isoflurane affects the development of neuronal polarity likely involves effects on minor process extension, as the neurites of unpolarized neurons were found to be shorter in isoflurane treated groups than in controls. These findings demonstrate that GAs such as isoflurane may interfere with brain development via mechanisms that do not involve cell death, and furthermore that developing axons may be vulnerable to effects of GAs.

Even a transient effect on the acquisition of neuronal polarity in a subset of neurons undergoing axon specification at the time of exposure could have serious consequences for brain development. Brain function depends on the appropriate generation of circuits, and many of the cues that guide axons to their appropriate targets are present for a limited time during specific developmental stages.<sup>43</sup> If the axon “misses” the window when the appropriate cue is present, the parent neuron may not be incorporated into key circuits. Furthermore, a delay in axon specification can also delay dendritic development. In cerebellar neurons when the acquisition of neuronal polarity is delayed by inhibiting tau expression, the growth and development of the remaining neurites is also delayed.<sup>44</sup> Thus, both axonal and dendritic incorporation into neuronal circuits could be altered permanently by delays in polarization.

Several lines of data strongly support that early phases of neuronal development and differentiation are modeled accurately by neuronal cultures, but of course it is not currently possible to draw exact parallels with human exposure in clinical settings. The acquisition of polarity of most neurons in the developing human occurs during gestation, and thus these data may be very relevant to prenatal exposures. Recent research suggests that a single exposure to isoflurane during gestation can cause lasting behavioral changes in rats, including worse performance on hippocampal dependent tasks.<sup>45</sup> However, postnatal neurogenesis of the hippocampal dentate granule cells plays a very important role in learning and memory.<sup>46</sup> In macaque monkeys up to 25% of the cells that will be added over a lifetime proliferate within the first three postnatal months, and thus there is a small, but important cohort of neurons in primates that must generate axons and make synaptic connections during early postnatal life.<sup>47</sup> Thus early postnatal anesthetic exposures that interfere with the incorporation of this population of cells via delays in the acquisition of neuronal polarity could have lasting effects on learning and memory.

Our finding that neurite lengths in unpolarized neurons are shorter in the isoflurane treated group suggests a possible mechanism for the delay in polarity acquisition that we observed. Prior research indicates that polarization occurs when one pre-polarized neurite outgrows the others, and furthermore that if an established axon is transected shortly after polarization then the longest remaining pre-polarized neurite is the one most likely to adopt an axonal fate.<sup>25–27, 48</sup> These findings suggest that there is something fundamental about neurite extension that drives the acquisition of polarity, and it is unsurprising that an insult which slows the growth of pre-polarized neurites also delays the acquisition of neuronal polarity. Our data do not give any further insights into mechanism, but an effect that is mediated via the cytoskeleton is a likely possibility. Isoflurane has been shown to have profound effects on the actin cytoskeleton, thus altering cellular structure and motility. Isoflurane interferes with the formation of actin stress fibers in immature astrocytes<sup>21</sup> and it depolymerizes actin in the dendrites of developing neurons in culture when applied during synaptogenesis,

leading to a loss of dendritic spines.<sup>14</sup> Isoflurane also causes a striking loss of actin based motility both in dendritic spines and in fibroblast lamellipodia.<sup>42</sup> It is possible that the effects we have observed are due to alterations in the actin-based motility of neuronal growth cones that is necessary to establish polarity.<sup>49, 50</sup> Alternately, it is possible that isoflurane disrupts polarization through effects on signaling. Rho GTPases, which are known targets of isoflurane,<sup>51</sup> have been implicated in the acquisition of neuronal polarity.<sup>49, 50</sup> It is also possible that isoflurane might be exerting its effects via as yet undiscovered interactions with other signaling molecules involved in the acquisition of polarity.

Our finding that isoflurane and propofol delay the polarization of neurons represents a new class of putative mechanisms of GA toxicity in brain development. It may point to other effects on axon development that would be relevant at ages that better correspond to pediatric exposures, such as axon tract formation in the hippocampus, an event that occurs postnatally in primate models.<sup>52</sup> Also, effects of GAs on growth cone motility in either axons or dendrites could have profound effects on circuit formation. Finally, effects of GAs on neuronal polarity that involve the dynamic actin cytoskeleton or the molecules that regulate it might suggest effects on axon guidance and synaptogenesis, both of which are highly dependent on actin.

A limitation of this study, as with all *in vitro* work, is that it is difficult to be certain of the clinical relevance of anesthetic concentrations and exposure times that are required to produce measurable effects in dissociated cell culture. However, our finding that a four hour exposure to an isoflurane concentration as low as 0.9%, which is well below the MAC value for infants, significantly reduced neuronal polarization is sufficient to raise concerns that this type of anesthetic toxicity may be relevant to clinical practice. Further study of the effects of anesthetic exposure on the development of axons, including investigations in whole animal models, is a promising direction in pediatric anesthetic neurotoxicity research.

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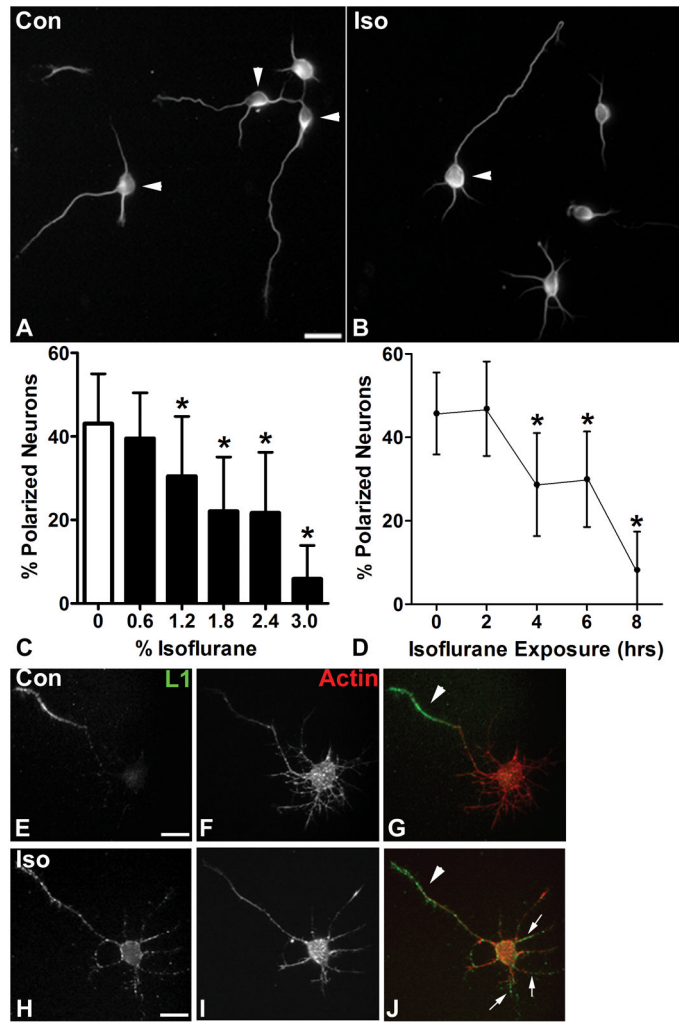
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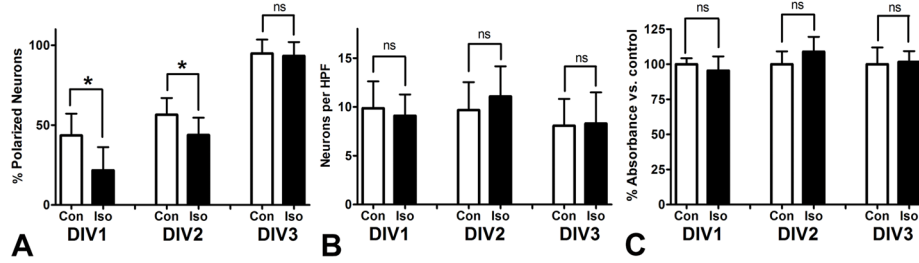


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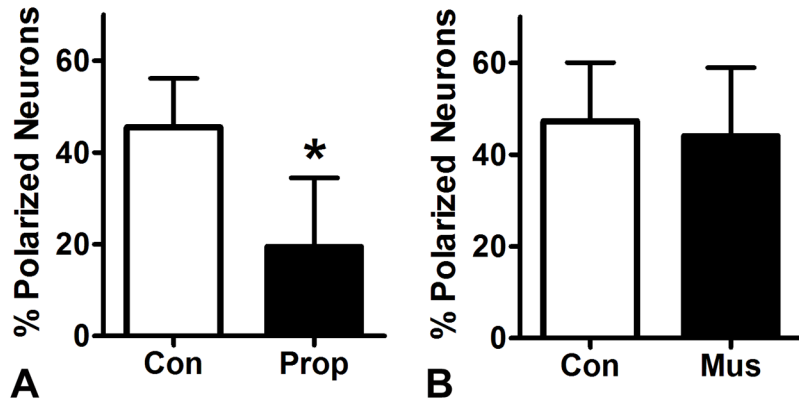


### Figure 1. Isoflurane inhibits axon specification

**A:** On DIV1 under control conditions dissociated cortical neurons, visualized here with immunolabeling for  $\beta$ III tubulin, show a mixed population of polarized (arrowheads) and unpolarized neurons (cell bodies without arrowheads), as assessed by the Banker length criteria. **B:** Cultures treated for four hours with 2.4% isoflurane immediately after plating and assayed at DIV1 demonstrate a reduced percentage of polarized neurons (arrowhead). **C:** Quantitative analysis shows a significant reduction in the percentage of polarized neurons relative to control that is concentration-dependent over a range from 1.2% to 3% isoflurane for four hours. **D:** The magnitude of this effect also varies with exposure time, and there is a significant reduction in neuronal polarity with 2.4% isoflurane treatment in exposures lasting four hours or longer. **E–G:** At DIV2 neurons immunolabeled for the axon-specific protein L1CAM (**E**, green in **G**) and stained for F-actin to define the cell (**F**, red in **G**), show a segregation of L1CAM signal to the axon (arrowhead). **H–J:** By contrast, neurons treated with isoflurane at a one millimolar for six hours and labeled for L1CAM (**H**, green in **J**) and F-actin (**I**, red in **J**) show diffuse labeling for that is seen in the axon (arrowhead) and also in minor processes (arrows). Scale bar in **A** is 20  $\mu$ m for **A** and **B**.  $n = 2151$  cells in 210 fields for **C** and  $n = 1,523$  cells in 150 fields for **D**. Scale bar in **E** is 10  $\mu$ m for **E–J**. \* =  $p < 0.05$ .

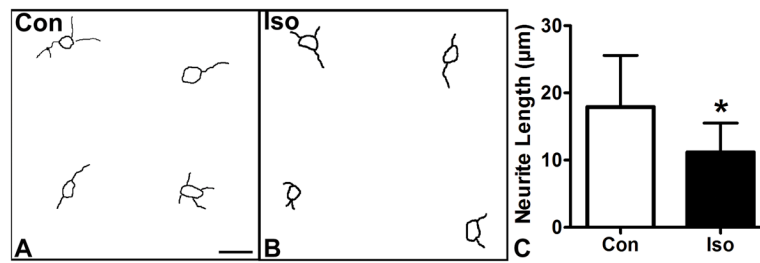


**Figure 2. Isoflurane delays the acquisition of neuronal polarity without causing cell death**  
**A:** Neuronal polarity was assayed at DIV1, DIV2, and DIV3 after a four hour treatment with 2.4% isoflurane at the time of plating. The mean percent of polarized neurons is markedly reduced on DIV1, somewhat reduced on DIV2, and not significantly different on DIV3, when nearly all neurons have polarized. **B:** The number of neurons per microscopic field does not differ significantly between control and isoflurane treated cultures on DIV1, DIV2, or DIV3. **C:** An MTT assay for cell viability shows no significant reduction in absorbance for isoflurane treated cultures over the same time frame, confirming that cell death does not occur with this isoflurane exposure paradigm.  $n = 1,682$  cells in 180 fields for **A** and **B**.  $n = 50$  samples for **C**. \* =  $p < 0.05$ , ns = not significant.



**Figure 3. Propofol inhibits the acquisition of neuronal polarity**

**A:** Cortical neuron cultures were treated with 3  $\mu$ M propofol for four hours immediately after plating and assayed for neuronal polarity on DIV1. Similarly to isoflurane, propofol significantly reduces the mean percentage of polarized neurons. **B:** The GABA<sub>A</sub>R agonist muscimol does not alter mean percentage of polarized neurons in the same exposure paradigm, suggesting that the effects of isoflurane and propofol on axon specification do not occur via activity at GABA<sub>A</sub>Rs. n = 666 cells in 61 fields for **A** and n = 527 cells in 60 fields for **B**. \* = p<0.05



**Figure 4. Isoflurane reduces outgrowth of pre-polarized neurites**

**A, B:** NeuroLucida tracings were made of unpolarized neurons from control (**A**) and 2.4% isoflurane treated cultures (**B**) at DIV1. The isoflurane treated group has similar numbers of neurites, but the lengths appear shorter on average. **C:** Quantitative analysis shows that neurite length is reduced, indicating that the delay in acquisition of neuronal polarity seen with isoflurane treatment is due to a reduction in neurite outgrowth speed. Scale bar in **A** is 25 µm for **A** and **B**. n = 128 neurons and 42 neurons. \* = p<0.05.