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Article

Early developmental exposure to general anesthetic agents in primary neuron culture disrupts synapse formation via actions on the mTOR pathway

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Abstract: Human epidemiologic studies and laboratory investigations in animal models suggest that exposure to general anesthetic agents (GAs) have harmful effects on brain development. The mechanism underlying this putative iatrogenic condition is not clear and there are currently no accepted strategies for prophylaxis or treatment. Recent evidence suggests that anesthetics might cause persistent deficits in synaptogenesis by disrupting key events in neurodevelopment. Using an in vitro model consisting of dissociated primary cultured mouse neurons we demonstrate abnormal pre- and post-synaptic marker expression after a clinically relevant isoflurane anesthesia exposure conducted during neuron development. We find that pharmacologic inhibition of the mechanistic target of rapamycin (mTOR) pathway can reverse the observed changes. Isoflurane exposure increases expression of phospho-S6, a marker of mTOR pathway activity, in a concentration-dependent fashion and this effect occurs throughout neuronal development. The mTOR 1 complex (mTORC1) and the mTOR 2 complex (mTORC2) branches of the pathway are both activated by isoflurane exposure and this is reversible with branch-specific inhibitors. Upregulation of mTOR is also seen with sevoflurane and propofol exposure, suggesting that this mechanism of developmental anesthetic neurotoxicity may occur with all the commonly used GAs in pediatric practice. We conclude that GAs disrupt the development of neurons during development by activating a well-defined neurodevelopmental disease pathway and that this phenotype can be reversed by pharmacologic inhibition.

Keywords: Anesthesia; Neurotoxicity; Synapse; mTOR; Neurodevelopment

1. Introduction

The United States Food and Drug Administration has recently required that 12 commonly used anesthetic and sedative agents with mechanisms of action on NMDA and GABA receptors carry labels warning that repeated or lengthy exposure to these drugs between the third trimester and the first three years of life may result in adverse consequences for brain development (FDA Drug Safety Communication). An estimated 115,000 children each year are anesthetized for surgery and other procedures in the U.S. alone, suggesting that millions of children are exposed to anesthesia each year worldwide [1]. It is not yet clear which patients are potentially at risk of cognitive dysfunction

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related to this exposure, but early results from the only two clinical trials that have reached endpoints give reassurance that short, single exposures in healthy children do not have deleterious effects [2, 3]. This finding is consistent with data from large epidemiologic studies showing no effect of short, single early life exposures to surgery and anesthesia, but a correlation between long or multiple exposures and reduced scores on cognitive testing, worsened scores in educational testing assessments and increased billing codes indicates developmental or behavioral disorders [4-6]. Numerous studies have found that early postnatal exposure to GA in rodents results in deficits in performance on tests of learning and memory [7-15], but rodent anesthesia models introduce a confound of physiologic perturbation that is hard to measure and also the short timeline of rodent brain development might exaggerate the consequences of a toxic developmental exposure. However, recent data in non-human primates have provided definitive evidence that early postnatal GA exposure can have lasting effects on cognition, including deficits in socioemotional and learning function [16-19].

The mechanism by which a transient exposure to GA could have lasting consequences on brain development has been the subject of considerable investigation, but no clear conclusion has been reached [20, 21]. We have found evidences in an *in vivo* mouse model that early postnatal exposure to isoflurane causes a lasting increase in activity in the mTOR pathway in the hippocampal dentate gyrus. Inhibition of mTOR upregulation with rapamycin reversed a loss of neuronal spines in dentate gyrus granule neurons and also restored performance on hippocampal-dependent learning tests that are impaired by isoflurane exposure [8]. The mTOR pathway is a complex and heterogeneous signaling system that integrates intra- and extracellular cue sensing and links to numerous other signaling pathways in order to regulate metabolism, growth, and homeostasis [22]. A lasting anesthetic action on mTOR function is an intriguing potential mechanism of developmental anesthetic neurotoxicity. The mTOR system is critical for neuronal development [23] and a causative role of mTOR system dysfunction has been proposed for better understood neurodevelopmental disorders, such as Fragile X, autism, schizophrenia, and drug addiction [24]. However, mTOR has not been extensively studied in this context, and the evidence linking it to anesthetic toxicity is mixed [25].

Here we use an *in vitro* primary rat neuron culture system to further explore the hypothesis that GAs disrupt neuron development via an upregulation of mTOR signaling. To this end we employ quantitative immunohistochemistry to examine the effects of anesthetic-induced mTOR changes on synapse development. We also test for contributions of the mTOR1 and mTOR2 complexes, which represent a divergence in the pathway. Finally, we ask whether effects on the mTOR pathway generalize to multiple anesthetic agents.

2. Results and Discussion

2.1. Effects of 1.8% Isoflurane Exposure for 6hrs on Synaptogenesis

Our previous work in newborn dentate gyrus granule neurons in the intact mouse showed that isoflurane could act via an mTOR-mediated mechanism to cause a lasting reduction in the numbers of dendritic spines, which represent a morphological marker for excitatory post-synaptic elements.

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To determine whether this effect is an acute one that occurs during neuron synapse development and to test whether it generalizes to multiple neuronal types, we explored the effects of isoflurane administered during the period of ongoing synaptogenesis in cultured neocortical neurons, a population that is both heterogeneous and distinctly different from dentate gyrus neurons. Exposures consisting of 1.8% isoflurane for 6hrs were performed at 7 days *in vitro* (DIV) when synaptogenesis is ongoing, and results were assayed at 10 DIV when it is largely complete [26] (Figure 1). Double immunofluorescence staining was performed using MAP-2 as a dendritic marker to define the area over which synaptic markers were measured, and either Synapsin-1 to identify pre-synaptic elements or Homer-1 to identify excitatory post-synaptic elements. The locations of the images taken for analysis were 50μm from the nuclear, representative images showed in Figure 2A (Scale bar: 50μm) and Figure 2B (Scale bar: 2μm).

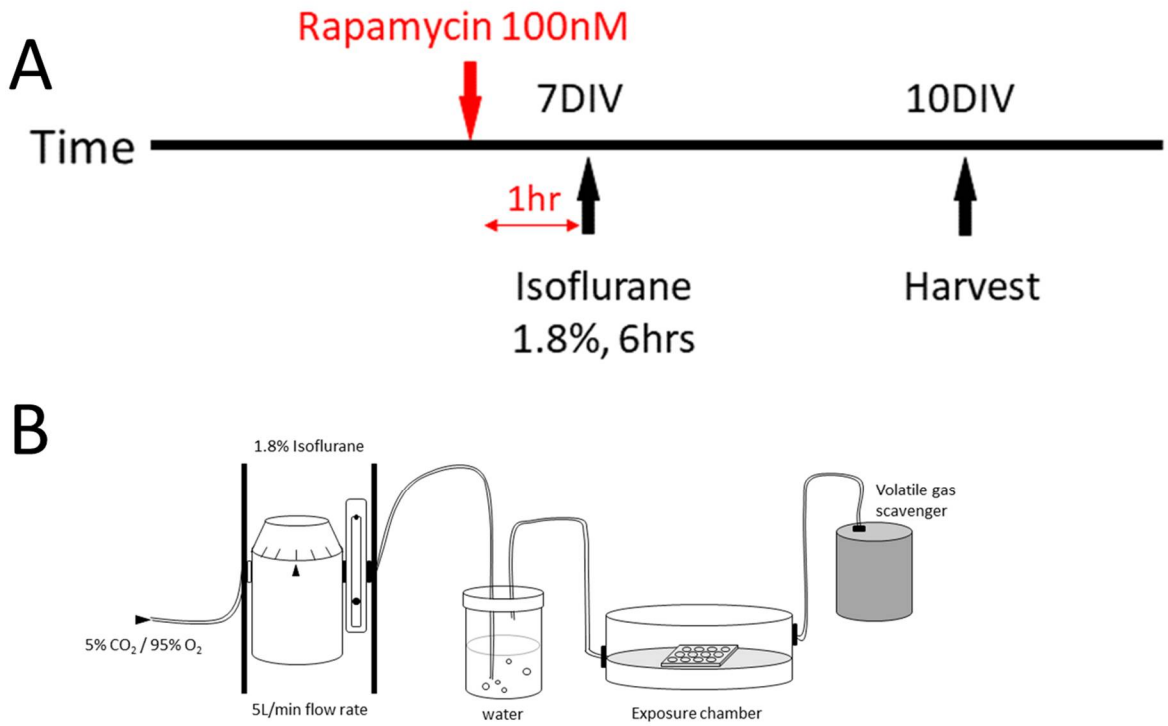

Figure 1. Schematic representation of the experimental timeline and exposure induction diagram *in vitro*.

(**A**). The general experiment timeline *in vitro*. The neurons were exposed to 1.8% isoflurane for 6hrs on their 7DIV, and 100nM rapamycin was added into the media 1hr before the exposure according to the experiment design. The fresh media change was done regularly. The cells were fixed for immunohistochemistry on 10DIV.

(**B**). Coverslips in 12-well plates were placed in identical air-tight, humidified chambers. Isoflurane was delivered using an agent-specific, calibrated inline and was diluted in 5% CO_2 / 95% O_2 carrier gas. Controls for these experiments received 5% CO_2 / 95% O_2 carrier gas only. After a 15-minute

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equilibration period, then the sealed chambers placed in an incubator to maintain temperature at 37 ℃ for the duration of anesthesia exposure.

We found that 6hrs of isoflurane treatment at a concentration of 1.8% resulted in a significant decrease in the intensity of Synapsin-1 immunoreactivity (20.46± 7.33%) compared to the control group (48.95± 19.02%, $p<0.001$) (Figure 2C). Rapamycin treatment results in Synapsin-1 intensity levels (32.11± 9.10%) that are not significantly different from the control plus rapamycin treatment group (36.13± 11.70%), suggesting a rescue effect of rapamycin (Figure 2D). Carrier gas and isoflurane treatment were also used in the presence of the rapamycin diluent, dimethyl sulfoxide (DMSO), and the results did not differ from the same experiment performed without DMSO, indicating that the diluent has no independent effect. Isoflurane treatment at 1.8% for 6hrs resulted in a significant reduction in intensity of Homer-1 immunoreactivity (30.47± 5.22%) compared to the control group (68.46± 11.18%, $p<0.0001$) (Figure 2E). As was found with Synapsin-1, rapamycin treatment after isoflurane exposure prevented the effects of isoflurane. Homer-1 immunoreactivity after rapamycin treatment did not differ significantly between the isoflurane (49.33± 7.32%) and carrier gas groups (56.14± 8.91%) (Figure 2F). Taken together, these data indicate that isoflurane interferes with the formation of excitatory synapses in developing cultured neocortical neurons and that this effect may be due to actions on the mTOR pathway.

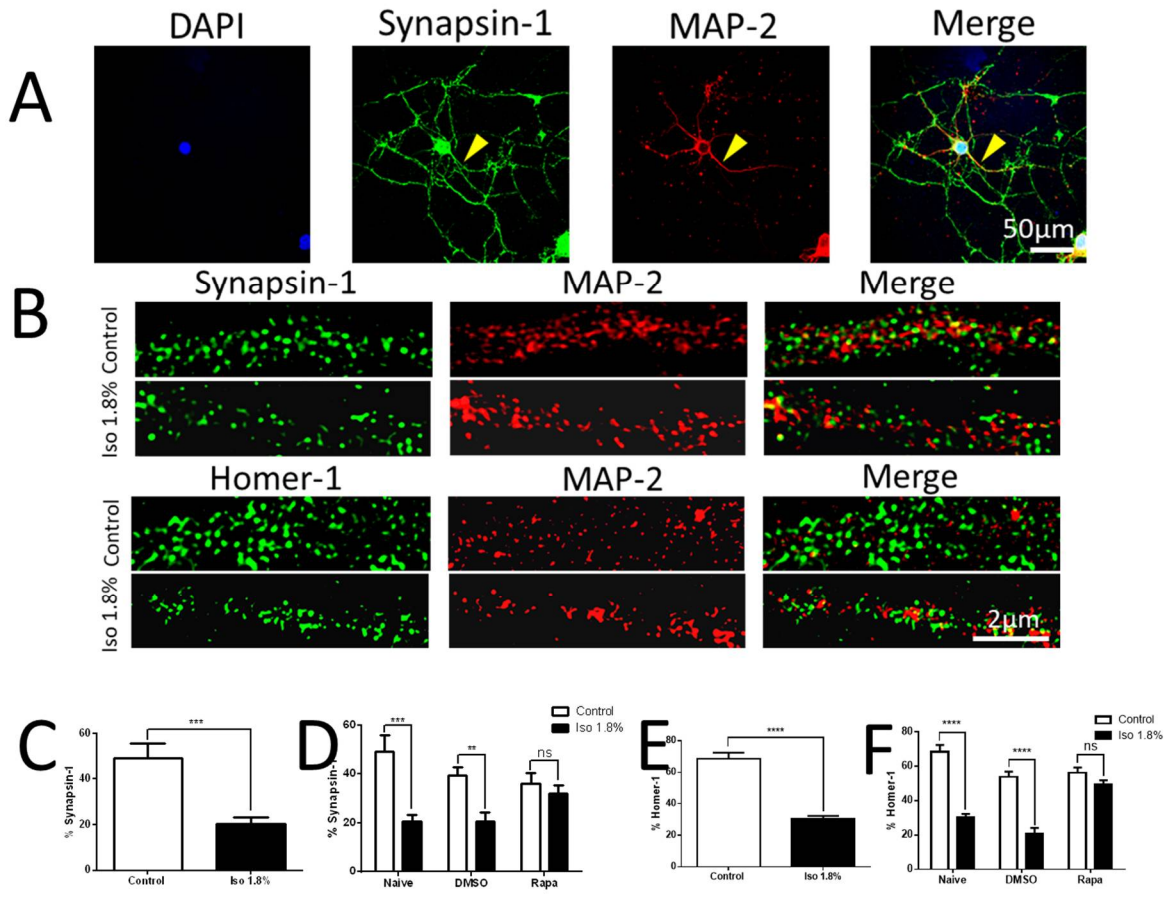

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Figure 2. 1.8% isoflurane exposure for 6hrs decreases pre- and post-synaptic marker intensity *in vitro*.

(**A-B**). Representative images of Synapsin-1/ Homer-1 (green), Map-2 (red), DAPI (blue) immunofluorescence in neurons in dissociated culture at 10DIV are shown. The segment for the dendrite was picked according to MAP-2 staining from each neuron and the locations for image taken were defined as 20-30μm from the nuclear according to DAPI (shown as the yellow arrow pointing in A).

(**C-F**). 6hrs of isoflurane exposure on 7DIV caused a significant difference in the intensity decrease of Synapsin-1 compared to the control group (**C**), while rapamycin treatment before the isoflurane exposure reversed the Synapsin-1 intensity to normal compared to the control with rapamycin treatment group (**D**). The intensity of Homer-1 also decreased compared to the control group (**E**), while rapamycin treatment before the isoflurane exposure reversed the Homer-1 intensity to normal compared to the control with rapamycin treatment group (**F**). (n=30 per group, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n.s. indicates no significant difference, t-test)

Our own work *in vivo* shows that newborn dentate gyrus neurons in mice exposed to GA with isoflurane are found to have reduced numbers of spines overall and profoundly reduced numbers of mushroom morphology spines over a month later [8]. As in our culture model, we found that this effect was reversible by treatment with rapamycin not acutely, but for a week after the exposure. While dendritic spines are generally the sites of excitatory post-synaptic elements, the correlation is imperfect, and our finding of reduced Homer-1 immunoreactivity in culture lends weight to our previous findings *in vivo*, particularly as we also found a decrease in expression of a pre-synaptic marker as well. However, our results in this manuscript differ in some important ways. Our anesthesia exposure occurred during synaptogenesis, rather than at the point of generation, and also the neurons observed in a cortical culture differ morphologically and functionally from dentate gyrus granule cells, which have many unusual features compared to other neurons. Thus, we predict based on our findings that mTOR-mediated effects on synapse formation are likely to generalize across a broad range of contexts. The current literature does not have any other studies of mTOR and anesthetic effects on synapses, but the preponderance of evidence suggests at least that GA exposure during development can disrupt synapse formation or maintenance. Two in vivo rodent studies using electron microscopy to identify synapses found decreased synaptic density in the hippocampus of young adult mice that had been exposed to GAs during the early postnatal phase [27, 28]. Interestingly, when this phenomenon was studied in the rodent pre-frontal cortex using light microscopy to quantify spine numbers, it was found that a P5 exposure reduced spine number but a P15 exposure actually increased spine number [29], suggesting that the state of neuron at the time of exposure is critically important to determine the effect of GAs. Our findings in this manuscript support the conclusion that GA exposure prior to stabilization of synapses leads to a failure of synapse formation.

2.2. Parameters of Activation of mTOR by Isoflurane in Cultured Neurons

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We have previously shown that isoflurane exposure causes a lasting increase in expression of phospho-S6 (pS6), a commonly used marker of activity in the mTOR pathway [8, 25]. However, the constraints of *in vivo* experimentation are such that we were unable to determine at what stage of development neurons are subject to this phenomenon, and we were also unable to test the minimum time of exposure and exposure dose required. To address these questions we stained for DAPI (grey) to define cell bodies and immunolabeled for βIII-tubulin (blue) to verify neuronal cell type. To measure the activity in the mTOR pathway, we co-labeled for unphosphorylated-S6 (red) and phosphorylated-S6 (green) to assess mTOR activation. A representative example of control and isoflurane 1.8% for 6hrs treatment on 7DIV with harvest on 10DIV is shown (Figure 3A, Scale bar: 50μm).

We first tested the effects of varying the time of exposure to isoflurane on pS6 expression. We found that 6hrs of 1.8% isoflurane treatment on 3DIV caused a significant increase in the percentage of pS6 positive neurons (as the yellow arrows pointed out in Figureure 3A) compared to the control group with harvest at 5DIV (64.25± 15.95% vs. 17.22± 10.15%, $p<0.0001$), 7DIV (54.33± 37.69% vs. 23.98± 11.54%, $p<0.0001$), 10DIV (65.53± 15.26% vs. 23.73± 9.60%, $p<0.0001$), and 14 DIV (64.17± 21.40% vs. 28.01± 11.92%, $p<0.0001$) (Figure 3B). Isoflurane treatment at 5DIV caused a significant increase in the percentage increase of pS6+ neurons compared to the control group at 7DIV (48.00± 11.43% vs. 23.60± 11.33%, $p<0.05$), 10DIV (36.65± 14.74% vs. 25.13± 9.63%, $p<0.05$), and 14 DIV (44.36± 15.36% vs. 26.65± 9.57%, $p<0.05$) (Figure 3C). Exposure at 7DIV caused a significant increase in pS6 positive neurons compared to the control group on 10DIV (79.21± 16.54% vs. 23.86± 18.39%, $p<0.0001$), but no difference was detected at the 14 DIV (42.51± 12.51% vs. 32.74± 7.70%) harvest time point (Figure 3D). These findings suggest that isoflurane exposure causes pS6 to increase at any early developmental time point, but that the effect is reduced as the neuron approaches maturity.

Next, we tested the effects of different concentrations of isoflurane delivered at 7DIV and assayed for pS6 on 10DIV. There was a significant difference between the 1.2% isoflurane group (67.33± 22.31%, ANOVA, $p<0.01$), 1.8% isoflurane group (79.20± 16.53%, ANOVA, $p<0.01$) and 2.4% isoflurane group (71± 32.31%, ANOVA, $p<0.05$), compared to the control group (23.86± 18.39%), while there is no significant difference between the 0.6% isoflurane group (37.80± 11.13%), 0.9% isoflurane group (29.65± 13.18%) and the control group (Figure 3E). This represents a clear inflection point at a value corresponding to one adult minimum alveolar concentration (MAC), which is a clinically reasonable dose in pediatric setting.

Then we sought to determine the minimum duration of exposure to isoflurane that is required to cause an increase in mTOR signaling. We exposed P7 neurons to 1.8% isoflurane with varying durations and measured pS6 levels on 10DIV. There was a significant difference between the 0.5h isoflurane group (67.28± 26.06%) compared to the control group (21.40± 10.43%, $p<0.0001$), 1h isoflurane group (58.00± 10.62%) compared to the control group (35.07± 19.39%, $p<0.0001$), and 6hrs isoflurane group (79.20± 16.53%) compared to the control group (23.86± 18.39%, $p<0.0001$) (Figure 3F). Half an hour exposure is the shortest practical duration to measure in our model, and we conclude that even brief exposures have the potential to act on the mTOR pathway.

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In order to further confirm that the increase in pS6 labeling that we observe is in fact evidence of mTOR pathway activation we treated the cultures with rapamycin as in Figure 2. We found that there was a significant increase of the percentage of pS6 positive cells among all the DAPI/ Tubulin neurons between the isoflurane + vehicle (DMSO) group (44.49± 9.73%) compared to the control + vehicle (DMSO) group (19.44± 16.86%, $p<0.01$). Rapamycin treatment prevented the increase of pS6 immunoactivity in the isoflurane group (21.00± 23.25%) compared to the isoflurane group without rapamycin (44.49± 9.73%, $p<0.05$), and there was no significant difference between isoflurane+ rapamycin group compared to the control+ rapamycin group (27.52± 23.06%) (Figure 3G).

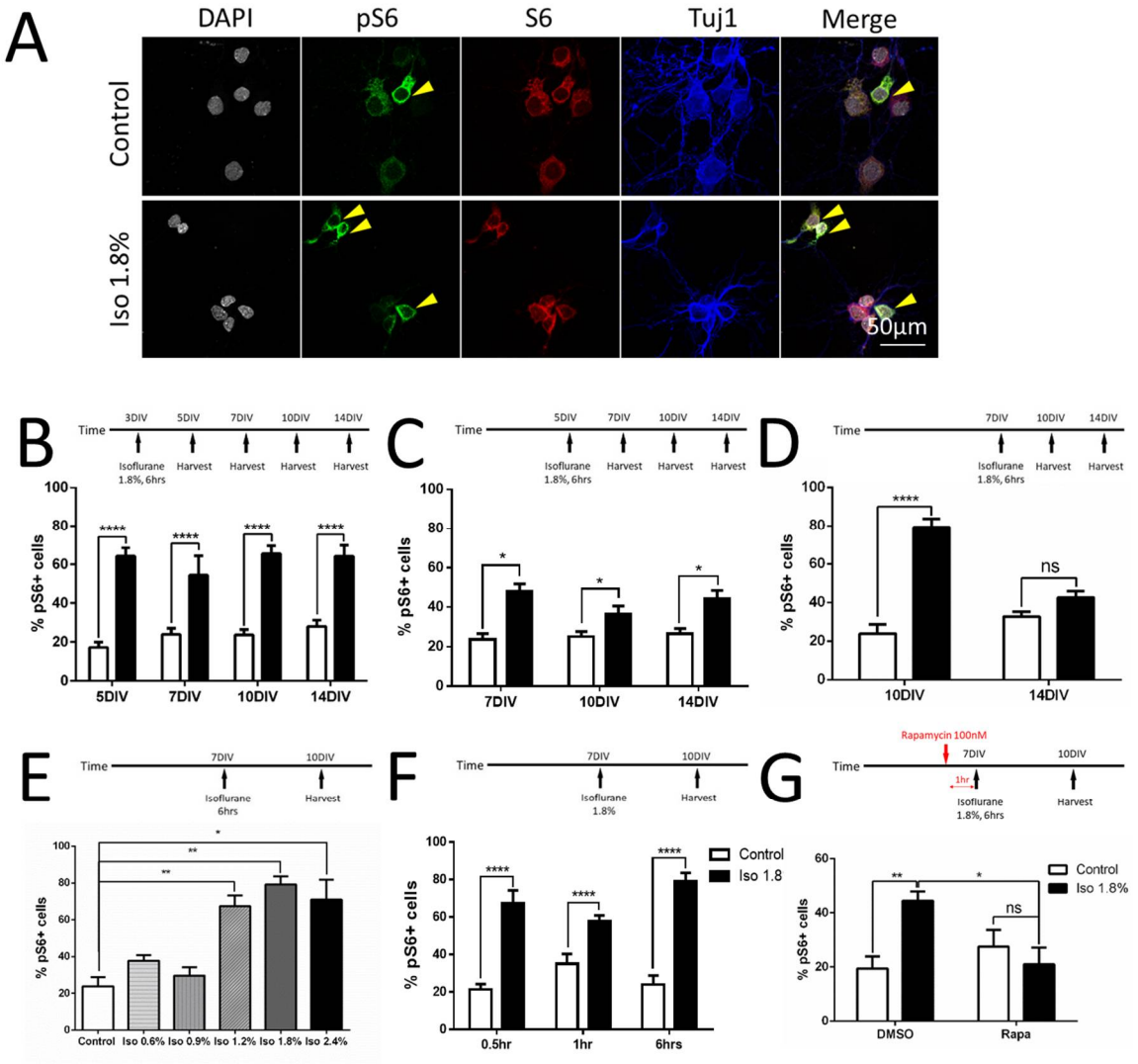

Figure 3. Isoflurane exposure at different time points showed effects on the downstream marker of mTOR pathway.

(**A**). Representative images of DAPI (grey), pS6 (green), S6 (red), beta III Tubulin (blue) immunofluorescence in the dissociated neurons at 10DIV.

(**B-G**). 6hrs of 1.8% isoflurane treatment on different early time points caused significant increases in the percentage of pS6 positive cells among all the DAPI/ Tubulin neurons compared to the control group at late time points except the ones exposed on 7DIV and tested on 14DIV (**B-D**). The effect on pS6 levels at 10DIV varied depending on the doses of isoflurane. There was a significant increase in

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immunoactivity starting from the 1.2% isoflurane group to the 2.4% isoflurane group, while lower doses (0.6% and 0.9%) remained at control levels of pS6 immunoactivity (**E**). Different exposure durations (0.5hr, 1hr and 6hrs) of 1.8% isoflurane also resulted in increased pS6 immunoactivity at all exposure times compared to control (**F**). Adding rapamycin, the mTOR pathway inhibitor reversed the increase of pS6 after isoflurane exposure on 7DIV (**G**). (n=15 per group, *p<0.05, **p<0.01, ****p<0.0001, n.s. indicates no significant difference, ANOVA, t-test)

The use of a dissociated culture model presents a substantial advantage for studying the pharmacology of anesthetic toxicity as compared to *in vivo* models, as the short timeline of experiments and the lesser requirements for resources allow for the study of a broad range of doses and exposure paradigms. The general consensus in the literature is that the period of synaptogenesis represents the peak window of vulnerability to developmental anesthetic neurotoxicity *in vivo* [30, 31], but *in vivo* synaptogenesis is a heterogeneous process that occurs over long periods of time as different cohorts of neurons mature over widely variable timelines. Using the culture model, in which synaptogenesis is synchronous starting from 5DIV and ending about 14DIV [32], we asked which stages of synaptogenesis are vulnerable to a potentially harmful increase in mTOR pathway in response to isoflurane exposure to gain a clearer understanding of the potential window of vulnerability. The only time point we studied at which pS6 upregulation due to isoflurane exposure was at all abated was the P7 exposure with measurement of pS6 at 14DIV. Synapses are highly dependent on filamentous actin for stability during the first week in culture, but during the second week there is a marked shift towards persistence of synapses even when actin is perturbed [33]. Several previous studies have suggested that isoflurane toxicity during development may be mediated in part via effects on the actin cytoskeleton [34, 35], and our results are consistent with the period of actin-dependent synapse formation as the window of vulnerability to mTOR mediated effects on synaptogenesis. One of the principal concerns in the study of developmental anesthetic toxicity is that many reported phenomenon may lack clinical relevance as they are reported by studies that use only supra-therapeutic doses, sometimes in excess of 2 adult MAC, or unrealistically long exposure times, which in some cases are as much as 24 hours [36]. Our findings in cultured neurons show that the vulnerability of neurons to isoflurane-induced mTOR activation appears to have a threshold between 0.9% and 1.2% isoflurane, which is a dose that is clinically realistic as it represents less than 1 MAC for pediatric patients [37]. Furthermore, the duration of exposure required to generate a significant effect is strikingly short at 30 minutes, the briefest exposure that is practical in our system. This finding does call into question the clinical relevance of mTOR activation as the evidence from clinical trials suggests that anesthetic exposures under an hour do not have measurable effects on children [38, 39]. However, it is reasonable to suppose that *in vivo*, particularly in the setting of a complex brain with a long developmental timeline, there may be a high threshold for phenotypically detectable events, which exceeds the threshold for detectable change at the cellular and molecular level. Nevertheless, the discrepancy between thresholds of toxicity in rodent models and human and non-human primates remains an unsolved problem in the field of anesthetic toxicity in neuro-development [40].

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2.3. Effects of Isoflurane Exposure on the mTORC1 and mTORC2 Pathway

The mTOR pathway has two principal branches, which arise from mTORC1 and mTORC2. These pathways perform biologically distinct functions in some settings, but there is substantial communication between them [41]. We next sought to determine whether the effects of isoflurane are mediated through one branch of the pathway. This was accomplished via a series of experiments using mTOR pathway inhibitors with differential effects between mTORC1 and mTORC2, and by measuring levels of immunoreactivity of downstream phospho-proteins that are activated differentially between the pathway branches. Inhibitors were added into the media one hour before the 1.8% isoflurane/ carrier gas exposure at 7DIV for a harvest at 10DIV (Figure 4A). The concentrations of the inhibitor were maintained after the exposure by media change with fresh inhibitor on 8DIV and 9DIV. The branch specific inhibitor and readout strategy (shown in Figure 4B) is as follows: PP242 was used as an inhibitor to block both mTORC1 and mTORC2 pathways simultaneously. Rapamycin was used as an mTORC1-specific pathway inhibitor. Ser473 phosphorylated Akt (pAkt, Ser473) was used as an mTORC2 downstream activity marker while Thr389 phosphorylated 70S6 (p70S6, Thr389) was used as an activity marker downstream from mTORC1. The combination of these inhibitors and markers has been shown to be effective in differentiating activity in between the mTORC1 and mTORC2 branches [42].

We found a significant difference in the percentage of pAkt positive neurons between the isoflurane + vehicle (DMSO) group (30.19± 6.12%) and the control + vehicle (DMSO) group (11.45± 11.71%, $p<0.0001$). As expected, rapamycin treatment did not change pAkt levels which was shown in the isoflurane + rapamycin group (26.09± 7.04%) compared to the isoflurane + DMSO group, but there was a significant difference between the isoflurane+ PP242 group (14.60± 14.50%) compared to the isoflurane+ DMSO group ($p<0.01$). While comparison between the isoflurane+ PP242 group and the control+ PP242 group (4.16± 5.27%) showed no significant difference. Taken together, the mTORC2 was affected during the isoflurane exposure to the neurons. There was a significant increase in the percentage of Thr-389 positive cells among all the DAPI/ Tubulin neurons between the isoflurane + vehicle (DMSO) group (54.88± 10.56%) compared to those of the control+ vehicle (DMSO) group (24.67± 10.19%, $p<0.0001$) (Figure 4D). Adding rapamycin before the exposure prevented the changes in Thr-389 levels (45.37± 6.09%) seen with the isoflurane + DMSO group ($p<0.05$), and there was a significant difference between isoflurane+ PP242 group (24.22± 13.66%) compared to the isoflurane + DMSO group ($p<0.0001$). While comparing the isoflurane+ PP242 group and the control+ PP242 group (34.15± 16.55%), no significant difference was measured. Taken together, these data indicate that isoflurane acts on both the mTORC1 and mTORC2 branches. This is principally significant because it shows that therapeutic strategies cannot be designed around only one pathway branch or the other, unless it can be determined that the deleterious effects occur downstream of only one of the two branches.

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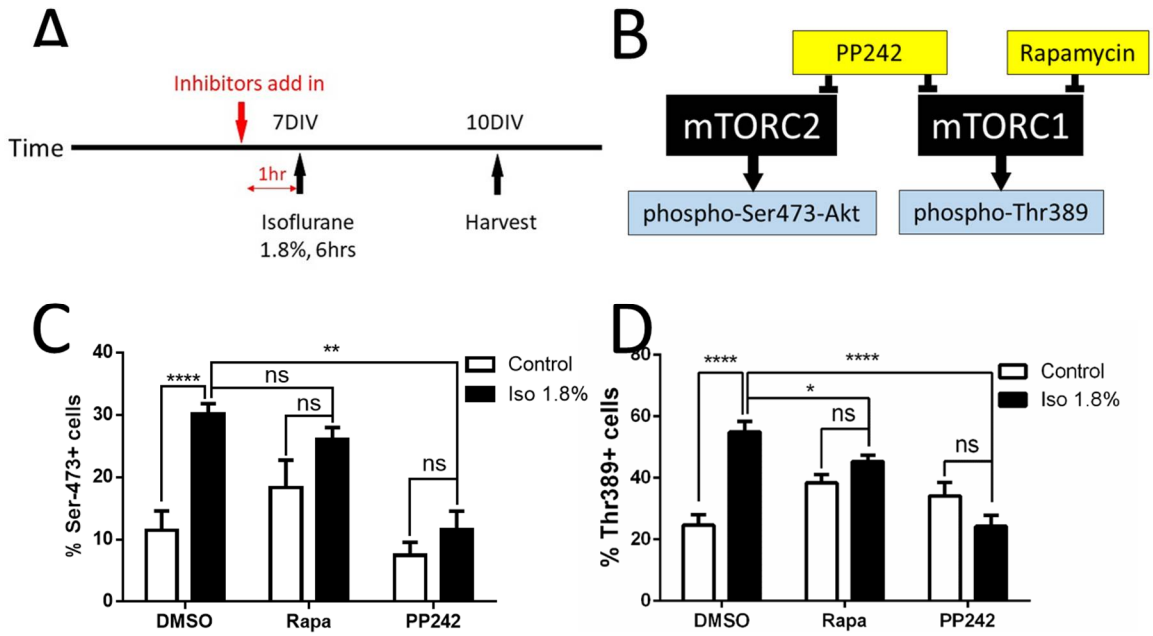

Figure 4. Effects of 1.8% isoflurane exposure for 6hrs on the downstream marker of mTORC1 and mTORC2 pathway.

(**A**). The timeline for adding mTORC1 / mTORC2 inhibitors. The inhibitors were added to the media 1 hour before the 1.8% isoflurane/ carrier gas exposure on the 7DIV. The cells were fixed for immunohistochemistry on 10DIV.

(**B**). A visual diagram showing the inhibition of PP242 and rapamycin on mTORC1 and mTORC2 pathways.

(**C-D**). For the mTORC2 downstream marker Ser473-Akt, there is a significant increase after 1.8% isoflurane exposure for 6hrs on 7DIV compared to the control group. Adding rapamycin did not fully reverse it back to normal, but adding PP242 made a significant difference between the isoflurane+PP242 and isoflurane+ DMSO groups, while the positive Ser-473 cells returned back to normal compared to the control+ PP242 group. This indicates that mTORC2 pathway is involved in the isoflurane neurotoxicity changes (**C**). For the mTORC1 downstream marker Thr389, isoflurane exposure increased its immunoactivity significantly, while adding either rapamycin or PP242 reversed its immunoactivity back to normal. This indicates that mTORC1 pathway is also involved in the deficiency of neuron growth caused by isoflurane as well (**D**). (n=15 per group, *p<0.05, **p<0.01, ****p<0.0001, n.s. indicates no significant difference, ANOVA, t-test)

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2.4. Effects of Sevoflurane and propofol on the Downstream Marker of mTOR Pathway.

A key question in developmental anesthesia toxicity is whether unwanted effects of anesthetic agents could be avoided through different choices of the primary anesthetic drug used. Thus, we asked what the effects of sevoflurane, the most commonly used volatile agent in pediatric anesthesia practice, and propofol, which is an intravenous agent that serves as the next likely alternative to isoflurane or sevoflurane, are on the mTOR pathway. Sevoflurane exposure in cultured neurons was accomplished using the same methods used for isoflurane exposure. Propofol exposure was accomplished by adding propofol in a carrier to the culture media, followed by media replacement at the appropriate time to terminate the exposure.

We measured the effect of a range of clinically relevant concentrations of sevoflurane and propofol delivered at 7DIV on pS6 levels measures at 10DIV. We found no significant difference in the percentage of neurons positive for pS6 between the 0.9% sevoflurane group (22.29± 14.86%) or the 1.8% sevoflurane group (26.03± 10.52%) and the control group (23.85± 18.39%) (Figure 5A). However, at 2.7% sevoflurane (59.00± 12.11%, $p<0.0001$), 3.6% sevoflurane (71.35± 21.27%, $p<0.0001$) and 4.5% sevoflurane group (42.39± 20.91%, $p<0.05$), there was a significant increase in the percentage of pS6+ neurons over control (Figure 5A). Rapamycin treatment prevented the increase in pS6 labeling with 3.6% sevoflurane exposure (45.13± 8.77% for sevoflurane plus rapamycin compared to 39.42± 10.10% for rapamycin plus carrier gas, no significant difference.) (Figure 5B). One adult MAC of sevoflurane is approximately 1.8%, and thus compared to isoflurane, a higher dose of sevoflurane, which is at the high end of a clinically reasonable concentration, is required to show an increase in pS6 expression.

Next, we tested the effects of propofol on pS6 expression. There was a significant increase in the percentage of pS6 positive cells measured in the 10nM propofol group (29.57± 6.05%, $p<0.001$), the 20nM propofol group (48.26± 10.98%, $p<0.0001$), and the 40nM propofol group (74.42± 17.78%, $p<0.0001$), compared to the control group (11.22± 6.94%). Adding rapamycin 1 hour before the 20nM propofol exposure decreased the pS6 immunoactivity (22.02± 10.63%) compared to the ones without rapamycin treatment (48.26± 10.98%, $p<0.01$), and there was no significant difference between the 20nM propofol+ rapamycin group and the control+ rapamycin group (18.29± 7.50%) (Figure 5D). These data indicate that propofol may also mediate its effects though the mTOR pathway, although there is no clear way to draw equivalence in dosing between isoflurane or sevoflurane and propofol. One of the most practical strategies to potentially avoid anesthetic toxicity would be to choose drugs that do not activate pathways that result in toxic effects related to neural development. While numerous studies have identified mechanisms specific to either the potent volatile agents or to propofol [20], relatively few studies have conducted head to head comparisons between these two principal approaches to general anesthesia. Our data suggest to the extent that mTOR is a key mechanism in developmental anesthetic neurotoxicity, the choice of the agent may not be protective.

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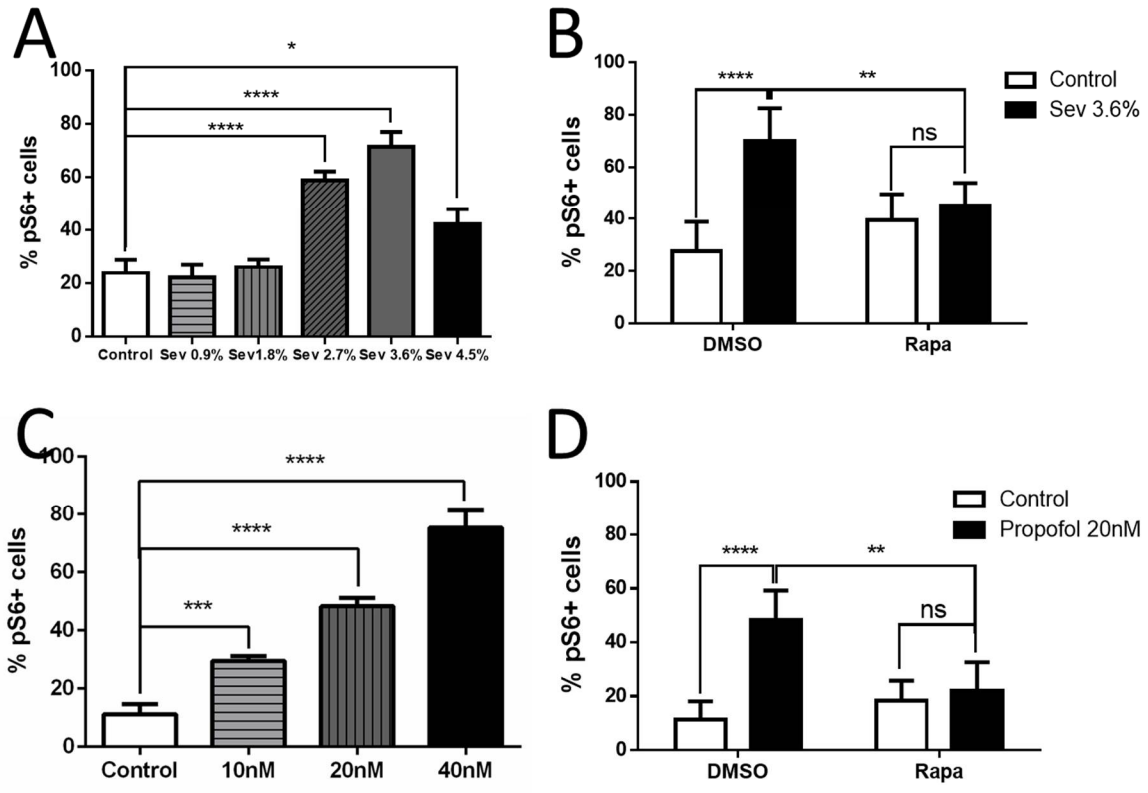

Figure 5. Effects of sevoflurane and propofol on the downstream marker of the mTOR pathway. (**A-B**). The effect on pS6 levels at 10DIV varied depending on the doses of sevoflurane at 7DIV. There was a significant increase in immunoactivity starting from the 2.7% sevoflurane group to the 4.5% sevoflurane group, while lower doses (0.9% and 1.8%) remained at control levels of pS6 (**A**). Rapamycin treatment prevented the increase in pS6 labeling with 3.6% sevoflurane exposure (**B**). (**C-D**). Different doses of propofol at 7DIV had similar effects on pS6 levels at 10DIV. There was a significant increase in pS6 immunoactivity starting from the 10nM propofol group to the 40nM propofol group (**C**). Rapamycin treatment prevented the increase in pS6 labeling with 20nM propofol exposure (**D**). (n=15 per group, **p<0.01, ***p<0.001, ****p<0.0001, ANOVA, t-test)

3. Materials and Methods

3.1. Neuronal Cultures

Primary neuron cultures were obtained from BrainBits, LLC (Springfield, IL, USA). Cultures consisted of dissociated neurons obtained from neocortex dissected from E18 Sprague Dawley rat embryos according to the company protocols. Neurons were plated on 12 mm glass coverslips at 16,000 cells/cm² and maintained in NbActiv4 medium (BrainBits, Springfield, IL, USA) with half media changes conducted three times per week. Pilot experiments showed over 95% of cells from these cultures are immunopositive for β-tubulin, suggesting a high degree of purity. Experiments were performed on neurons between 3 and 14DIV, and all experiments incorporated coverslips from a minimum of three separate cultures.

3.2. Anesthetic Agent Exposure

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Coverslips in 12-well plates were placed in identical air-tight, humidified chambers (Billups-Rothenberg, Del Mar, CA, USA) as previously described [43]. Isoflurane (Baxter Healthcare Cooperation, Deerfield, IL, USA) or sevoflurane (AbbVie Inc., North Chicago, IL, USA) was delivered using an agent-specific, calibrated inline vaporizer (SuperaVet, Vaporizer Sales and Services Inc., Rockmart, GA, USA), and was diluted in 5% CO_2 / 95% O_2 carrier gas. Controls for these experiments received 5% CO_2 / 95% O_2 carrier gas only. There was a 15-minute equilibration period, which was required to achieve the correct concentration of isoflurane or sevoflurane as measured by a 5250 RGM gas analyzer (Datex-Ohmeda, Madison, WI, USA). Then the sealed chambers were placed in an incubator to maintain temperature at 37°C for the duration of anesthesia exposure. Isoflurane / sevoflurane concentration was periodically measured at the end of the experimental period to verify that it was appropriately maintained throughout the exposure. The propofol exposure was done by adding pure 2, 6-diisopropylphenol (Sigma Aldrich, Saint Louis, MO, USA) into experiment wells, and incubated at 37°C for the duration of anesthesia exposure. The exposure was terminated by removing all the media and by adding a combination of previously removed media without propofol and fresh media.

3.3. The mTOR Pathway Inhibition

The mTOR inhibitors used in this study were as follows: PP242 at 1μM (EMD Millipore, Billerica, MA, USA), and rapamycin at 100nM (Sigma Aldrich, Saint Louis, MO, USA). They were used to inhibit mTORC1 or mTORC2, which are distinct functional pathways of the mTOR pathway. The neurons were pretreated with inhibitors 1 hour before isoflurane or carrier gas exposure. The inhibitor concentration was maintained until the time of fixation by incorporating inhibitor in media changes.

3.4. Immunocytochemistry

Fluorescent immunocytochemistry and labeling with fluorescently tagged F-actin were conducted as previously described [44]. Neurons on coverslips were briefly fixed with 4% paraformaldehyde at room temperature for 10 minutes, then permeabilized and blocked for 1 hour at room temperature in 5% donkey serum with 0.1% Triton X-100. Neurons were incubated overnight at 4°C in using the following antibodies: rabbit-anti-Synapsin-1 (1:200, EMD Millipore, Burlington, MA, USA), chicken-anti-Homer-1 (1:400 , Synaptic Systems, Goettingen, Germany), mouse-anti-MAP-2 (1:200, Abcam, Cambridge, MA, USA), rabbit anti-human phospho-p70S6K (Thr-389, 1:1000, EMD Millipore, Billrecia, MA, USA), rabbit anti-human phospho-AKT (Ser-473,1:500, Cell Signaling Technologies, Danvers, MA, USA), rabbit anti-human S6 (1:100, Cell Signaling Technologies, Danvers, MA, USA), rabbit anti-human phospho-S6 (Ser-235/236, Cell Signaling Technologies, Danvers, MA, USA), and chicken-anti-human anti-β-III Tubulin (1:1000, EMD Millipore, Billrecia, MA, USA). All the antibodies were diluted in phosphate-buffered saline solution containing 0.1% Triton X-100. After rinsing, neurons were incubated for 2hrs with a fluorescent secondary antibody and 4', 6-diamidino-2-phenylindole (DAPI) at the manufacturer's recommended concentration (Jackson Immuno Research Labs, West Grove, PA, USA). Subsequently, neurons were mounted on coverslips using 2.5% PVA/ DABCO Mounting Media.

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3.5. Imaging and Microscopic Analysis

A Leica SP8 confocal microscope was used to capture all microscopic images. Cell counting analyses were conducted manually. In these experiments, the counting field was conducted by capturing five 63x fields that were selected to represent all four quadrants and the center of the coverslip. Neuronal cell bodies were identified as those positive for both β-III Tubulin and DAPI, and representative images were taken using a 63x 1.0 N.A. objective with an additional 1.0x magnification lens in line. For the synaptic marker analysis, five neurons from each sample were evenly distributed throughout the coverslip to represent all four quadrants and the center was randomly selected for analysis. Images were taken using a 63x 1.0 N.A. objective with an additional 5x magnification lens in line. One dendrite was picked according to MAP-2 staining from each neuron and the locations for image taken were defined as 20-30μm from the nuclear according to DAPI. Synaptic puncta were quantified using ImageJ software. The dendrite segment outline was traced and the area quantification was done according to the MAP-2 channel, and the threshold was maintained the same for the synaptic marker channel. The intensity of Synapsin-1/ Homer-1 puncta inside the dendrite outline was measured and recorded. Both imaging and analysis were conducted by an investigator blind to condition.

3.6. Statistical Analysis

Results are expressed as mean ± SEM. All statistical analysis was conducted using Prism 6.0 (GraphPad, San Diego, CA, USA). Student's t-test was used for determine statistical differences between each experiment group and the control-group data. One-way ANOVA with multiple comparisons for the data with group number over three. Multiple t-test were used between the groups and have the same exposure condition but different inhibitor treatments. All data examined with parametric tests were determined to be normally distributed and was done by an investigator blind to condition. Statistical significance for all tests was set a priori at $p<0.05$.

4. Conclusions

In summary, we conclude that the potent volatile anesthetics and propofol, which are the mainstays of nearly all pediatric anesthetics, all have the capacity to upregulate signaling in both branches of the mTOR pathway in neurons during synaptogenesis. Anesthetic exposure in this setting inhibits synaptogenesis, and this effect is reversible with the mTOR inhibitor, rapamycin. Our study has several limitations, principally related to study of neural development in culture, where there is no patterned activity. In addition, because manipulation of mTOR via genetic means is problematic, only pharmacologic inhibition was used. Nevertheless, we believe that future study of mTOR as a putative mechanism for developmental anesthetic neurotoxicity in dissociated culture will prove informative, and that questions about which types of neurons and synapses are at risk and what the effects on neural function could be successfully addressed in this model system.

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Abbreviations

GA	General Anesthetics
mTOR	mechanistic target of rapamycin
mTOR C1	the mTOR 1 complex
mTOR C2	the mTOR 2 complex
DIV	days *in vitro*
DAPI	4′,6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
pS6	phosphorylated S6
MAC	minimum alveolar concentration
Ser473	Ser473 phosphorylated Akt
Thr389	Thr389 phosphorylated 70S6

References

1. Tzong, K. Y.; Han, S.; Roh, A.; Ing, C., Epidemiology of pediatric surgical admissions in US children: data from the HCUP kids inpatient database. *Journal of neurosurgical anesthesiology* **2012,** 24, (4), 391-5, DOI: 10.1097/ANA.0b013e31826a0345.

2. Davidson, A. J.; Disma, N.; de Graaff, J. C.; Withington, D. E.; Dorris, L.; Bell, G.; Stargatt, R.; Bellinger, D. C.; Schuster, T.; Arnup, S. J.; Hardy, P.; Hunt, R. W.; Takagi, M. J.; Giribaldi, G.; Hartmann, P. L.; Salvo, I.; Morton, N. S.; von Ungern Sternberg, B. S.; Locatelli, B. G.; Wilton, N.; Lynn, A.; Thomas, J. J.; Polaner, D.; Bagshaw, O.; Szmuk, P.; Absalom, A. R.; Frawley, G.; Berde, C.; Ormond, G. D.; Marmor, J.; McCann, M. E., Neurodevelopmental outcome at 2 years of age after general anaesthesia and awake-regional anaesthesia in infancy (GAS): an international multicentre, randomised controlled trial. *Lancet (London, England)* **2016,** 387, (10015), 239-50, DOI: 10.1016/s0140-6736(15)00608-x.

3. Sun, L. S.; Li, G.; Miller, T. L.; Salorio, C.; Byrne, M. W.; Bellinger, D. C.; Ing, C.; Park, R.; Radcliffe, J.; Hays, S. R.; DiMaggio, C. J.; Cooper, T. J.; Rauh, V.; Maxwell, L. G.; Youn, A.; McGowan, F. X., Association Between a Single General Anesthesia Exposure Before Age 36 Months and Neurocognitive Outcomes in Later Childhood. *JAMA* **2016,** 315, (21), 2312-20, DOI: 10.1001/jama.2016.6967.

4. DiMaggio, C.; Sun, L. S.; Li, G., Early childhood exposure to anesthesia and risk of developmental and behavioral disorders in a sibling birth cohort. *Anesthesia and analgesia* **2011,** 113, (5), 1143-51, DOI: 10.1213/ANE.0b013e3182147f42.

5. Ing, C.; Dimaggio, C.; Whitehouse, A.; Hegarty, M. K.; Brady, J.; von Ungern-Sternberg, B. S.; Davidson, A.; Wood, A. J.; Li, G.; Sun, L. S., Long-term Differences in Language and Cognitive

Function After Childhood Exposure to Anesthesia. *Pediatrics* **2012,** 130, (3), e476-85, DOI: peds.2011-3822.

6. Wilder, R. T.; Flick, R. P.; Sprung, J.; Katusic, S. K.; Barbaresi, W. J.; Mickelson, C.; Gleich, S. J.; Schroeder, D. R.; Weaver, A. L.; Warner, D. O., Early exposure to anesthesia and learning disabilities in a population-based birth cohort. *Anesthesiology* **2009,** 110, (4), 796-804, DOI: 10.1097/01.anes.0000344728.34332.5d.
7. Jevtovic-Todorovic, V.; Hartman, R. E.; Izumi, Y.; Benshoff, N. D.; Dikranian, K.; Zorumski, C. F.; Olney, J. W.; Wozniak, D. F., Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2003,** 23, (3), 876-82.
8. Kang, E.; Jiang, D.; Ryu, Y. K.; Lim, S.; Kwak, M.; Gray, C. D.; Xu, M.; Choi, J. H.; Junn, S.; Kim, J.; Xu, J.; Schaefer, M.; Johns, R. A.; Song, H.; Ming, G. L.; Mintz, C. D., Early postnatal exposure to isoflurane causes cognitive deficits and disrupts development of newborn hippocampal neurons via activation of the mTOR pathway. *PLoS Biol* **2017,** 15, (7), e2001246, DOI: 10.1371/journal.pbio.2001246.
9. Lee, B. H.; Chan, J. T.; Hazarika, O.; Vutskits, L.; Sall, J. W., Early exposure to volatile anesthetics impairs long-term associative learning and recognition memory. *PLoS One* **2014,** 9, (8), e105340, DOI: 10.1371/journal.pone.0105340.
10. Levin, E. D.; Uemura, E.; Bowman, R. E., Neurobehavioral toxicology of halothane in rats. *Neurotoxicology and teratology* **1991,** 13, (4), 461-70.
11. Sanders, R. D.; Xu, J.; Shu, Y.; Januszewski, A.; Halder, S.; Fidalgo, A.; Sun, P.; Hossain, M.; Ma, D.; Maze, M., Dexmedetomidine attenuates isoflurane-induced neurocognitive impairment in neonatal rats. *Anesthesiology* **2009,** 110, (5), 1077-85, DOI: 10.1097/ALN.0b013e31819daedd.
12. Satomoto, M.; Satoh, Y.; Terui, K.; Miyao, H.; Takishima, K.; Ito, M.; Imaki, J., Neonatal exposure to sevoflurane induces abnormal social behaviors and deficits in fear conditioning in mice. *Anesthesiology* **2009,** 110, (3), 628-37, DOI: 10.1097/ALN.0b013e3181974fa2.
13. Stratmann, G.; Sall, J. W.; May, L. D.; Bell, J. S.; Magnusson, K. R.; Rau, V.; Visrodia, K. H.; Alvi, R. S.; Ku, B.; Lee, M. T.; Dai, R., Isoflurane differentially affects neurogenesis and long-term neurocognitive function in 60-day-old and 7-day-old rats. *Anesthesiology* **2009,** 110, (4), 834-48, DOI: 10.1097/ALN.0b013e31819c463d.
14. Fang, F.; Xue, Z.; Cang, J., Sevoflurane exposure in 7-day-old rats affects neurogenesis, neurodegeneration and neurocognitive function. *Neurosci Bull* **2012,** 28, (5), 499-508, DOI: 10.1007/s12264-012-1260-4.
15. Gonzales, E. L.; Yang, S. M.; Choi, C. S.; Mabunga, D. F.; Kim, H. J.; Cheong, J. H.; Ryu, J. H.; Koo, B. N.; Shin, C. Y., Repeated neonatal propofol administration induces sex-dependent long-term impairments on spatial and recognition memory in rats. *Biomol Ther (Seoul)* **2015,** 23, (3), 251-60, DOI: 10.4062/biomolther.2014.120.
16. Alvarado, M. C.; Murphy, K. L.; Baxter, M. G., Visual recognition memory is impaired in rhesus monkeys repeatedly exposed to sevoflurane in infancy. *Br J Anaesth* **2017,** 119, (3), 517-523, DOI: 10.1093/bja/aew473.
17. Coleman, K.; Robertson, N. D.; Dissen, G. A.; Neuringer, M. D.; Martin, L. D.; Cuzon Carlson, V. C.; Kroenke, C.; Fair, D.; Brambrink, A. M., Isoflurane Anesthesia Has Long-term Consequences on Motor and Behavioral Development in Infant Rhesus Macaques. *Anesthesiology* **2017,** 126, (1), 74-84, DOI: 10.1097/ALN.0000000000001383.

18. Raper, J.; Alvarado, M. C.; Murphy, K. L.; Baxter, M. G., Multiple Anesthetic Exposure in Infant Monkeys Alters Emotional Reactivity to an Acute Stressor. *Anesthesiology* **2015,** 123, (5), 1084-92, DOI: 10.1097/ALN.0000000000000851.
19. Raper, J.; De Biasio, J. C.; Murphy, K. L.; Alvarado, M. C.; Baxter, M. G., Persistent alteration in behavioural reactivity to a mild social stressor in rhesus monkeys repeatedly exposed to sevoflurane in infancy. *Br J Anaesth* **2018,** 120, (4), 761-767, DOI: 10.1016/j.bja.2018.01.014.
20. Vutskits, L.; Xie, Z., Lasting impact of general anaesthesia on the brain: mechanisms and relevance. *Nat Rev Neurosci* **2016,** 17, (11), 705-717, DOI: 10.1038/nrn.2016.128.
21. Yu, D.; Li, L.; Yuan, W., Neonatal anesthetic neurotoxicity: Insight into the molecular mechanisms of long-term neurocognitive deficits. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* **2017,** 87, 196-199, DOI: 10.1016/j.biopha.2016.12.062.
22. Laplante, M.; Sabatini, D. M., mTOR signaling in growth control and disease. *Cell* **2012,** 149, (2), 274-93, DOI: 10.1016/j.cell.2012.03.017.
23. Takei, N.; Nawa, H., mTOR signaling and its roles in normal and abnormal brain development. *Frontiers in molecular neuroscience* **2014,** 7, 28, DOI: 10.3389/fnmol.2014.00028.
24. Costa-Mattioli, M.; Monteggia, L. M., mTOR complexes in neurodevelopmental and neuropsychiatric disorders. *Nature neuroscience* **2013,** 16, (11), 1537-43, DOI: 10.1038/nn.3546.
25. Xu, J.; Kang, E.; Mintz, C. D., Anesthetics disrupt brain development via actions on the mTOR pathway. *Communicative & Integrative Biology* **2018**, e1451719, DOI: 10.1080/19420889.2018.1451719.
26. Goslin, K.; Banker, G., Experimental observations on the development of polarity by hippocampal neurons in culture. *The Journal of cell biology* **1989,** 108, (4), 1507-16, DOI:
27. Amrock, L. G.; Starner, M. L.; Murphy, K. L.; Baxter, M. G., Long-term effects of single or multiple neonatal sevoflurane exposures on rat hippocampal ultrastructure. *Anesthesiology* **2015,** 122, (1), 87-95, DOI: 10.1097/ALN.0000000000000477.
28. Lunardi, N.; Ori, C.; Erisir, A.; Jevtovic-Todorovic, V., General anesthesia causes long-lasting disturbances in the ultrastructural properties of developing synapses in young rats. *Neurotox Res* **2010,** 17, (2), 179-88, DOI: 10.1007/s12640-009-9088-z.
29. Briner, A.; Nikonenko, I.; De Roo, M.; Dayer, A.; Muller, D.; Vutskits, L., Developmental Stage-dependent persistent impact of propofol anesthesia on dendritic spines in the rat medial prefrontal cortex. *Anesthesiology* **2011,** 115, (2), 282-93, DOI: 10.1097/ALN.0b013e318221fbbd.
30. Jevtovic-Todorovic, V., General Anesthetics and Neurotoxicity: How Much Do We Know? *Anesthesiology clinics* **2016,** 34, (3), 439-51, DOI: 10.1016/j.anclin.2016.04.001.
31. Lei, X.; Guo, Q.; Zhang, J., Mechanistic insights into neurotoxicity induced by anesthetics in the developing brain. *International journal of molecular sciences* **2012,** 13, (6), 6772-99, DOI: 10.3390/ijms13066772.
32. Banker, G.; Goslin, K., *Culturing nerve cells*. 2nd ed.; MIT Press: Cambridge, Mass., 1998; p xii, 666 p., 11 p. of plates.
33. Zhang, W.; Benson, D. L., Stages of synapse development defined by dependence on F-actin. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2001,** 21, (14), 5169-81.
34. Lemkuil, B. P.; Head, B. P.; Pearn, M. L.; Patel, H. H.; Drummond, J. C.; Patel, P. M., Isoflurane neurotoxicity is mediated by p75NTR-RhoA activation and actin depolymerization. *Anesthesiology* **2011,** 114, (1), 49-57, DOI: 10.1097/ALN.0b013e318201dcb3.

35. Lunardi, N.; Hucklenbruch, C.; Latham, J. R.; Scarpa, J.; Jevtovic-Todorovic, V., Isoflurane impairs immature astroglia development in vitro: the role of actin cytoskeleton. *J Neuropathol Exp Neurol* **2011,** 70, (4), 281-91, DOI: 10.1097/NEN.0b013e31821284e9.
36. Brown, R. E., Jr., Safety considerations of anesthetic drugs in children. *Expert opinion on drug safety* **2017,** 16, (4), 445-454, DOI: 10.1080/14740338.2017.1295037.
37. Murray, D. J.; Mehta, M. P.; Forbes, R. B., The additive contribution of nitrous oxide to isoflurane MAC in infants and children. *Anesthesiology* **1991,** 75, (2), 186-90.
38. Sun, L., Early childhood general anaesthesia exposure and neurocognitive development. *Br J Anaesth* **2010,** 105 Suppl 1, i61-8, DOI: 10.1093/bja/aeq302.
39. Sanders, R. D.; Hassell, J.; Davidson, A. J.; Robertson, N. J.; Ma, D., Impact of anaesthetics and surgery on neurodevelopment: an update. *Br J Anaesth* **2013,** 110 Suppl 1, i53-72, DOI: 10.1093/bja/aet054.
40. Rappaport, B. A.; Suresh, S.; Hertz, S.; Evers, A. S.; Orser, B. A., Anesthetic neurotoxicity--clinical implications of animal models. *N Engl J Med* **2015,** 372, (9), 796-7, DOI: 10.1056/NEJMp1414786.
41. Dowling, R. J.; Topisirovic, I.; Fonseca, B. D.; Sonenberg, N., Dissecting the role of mTOR: lessons from mTOR inhibitors. *Biochimica et biophysica acta* **2010,** 1804, (3), 433-9, DOI: 10.1016/j.bbapap.2009.12.001.
42. Warren, K. J.; Fang, X.; Gowda, N. M.; Thompson, J. J.; Heller, N. M., The TORC1-activated Proteins, p70S6K and GRB10, Regulate IL-4 Signaling and M2 Macrophage Polarization by Modulating Phosphorylation of Insulin Receptor Substrate-2. *The Journal of biological chemistry* **2016,** 291, (48), 24922-24930, DOI: 10.1074/jbc.M116.756791.
43. Mintz, C. D.; Barrett, K. M.; Smith, S. C.; Benson, D. L.; Harrison, N. L., Anesthetics interfere with axon guidance in developing mouse neocortical neurons in vitro via a gamma-aminobutyric acid type A receptor mechanism. *Anesthesiology* **2013,** 118, (4), 825-33, DOI: 10.1097/ALN.0b013e318287b850.
44. Mintz, C. D.; Smith, S. C.; Barrett, K. M.; Benson, D. L., Anesthetics interfere with the polarization of developing cortical neurons. *Journal of neurosurgical anesthesiology* **2012,** 24, (4), 368-75, DOI: 10.1097/ANA.0b013e31826a03a6.