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Sevoflurane impairs growth cone motility in dissociated murine neurons

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Abstract

Background—Early postnatal exposure to general anesthetic agents causes a lasting impairment in learning and memory in animal models. One hypothesis to explain this finding is that exposure to anesthetic agents during critical points in neural development disrupts the formation of brain circuitry. Here we explore the effects of sevoflurane on the neuronal growth cone, a specialization at the growing end of axons and dendrites that is responsible for the targeted growth that underlies connectivity between neurons.

Methods—Dissociated neuronal cultures were prepared from embryonic mouse neocortex. Time lapse images of live growth cones exposed to anesthetics were taken using differential interference contrast microscopy, and the rate of change of the area of the lamellipodia and the speed of the filopodial tip were quantified as measures of motility. The involvement of the p75 neurotrophin receptor (p75NTR) was tested using inhibitors applied to the media and via a co-immunoprecipitation assay.

Results—The rate of lamellipodial area change and filopodial tip velocity in both axonal and dendritic growth cones was significantly reduced with sevoflurane exposure between 2% and 6%. Motility could be substantially restored by treatment with Y27632 and TAT-peptide 5, which are inhibitors of Rho Kinase and p75NTR, respectively. Sevoflurane results in reduced co-immunoprecipitation of Rho-GDP dissociation inhibitor after pulldown with p75NTR.

Conclusion—Sevoflurane interferes with growth cone motility, which is a critical process in brain circuitry formation. Our data suggest that this may occur via an action on the p75NTR, which promotes growth inhibitory signaling via the Rho pathway.

Keywords

Anesthetic; Isoflurane; Neurotoxicity; p75NTR; Growth Cone

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INTRODUCTION

Epidemiologic studies show possible deficits in cognitive function in children exposed to anesthesia and surgery in the first four years of life^{1–5}. Experimentation in rodent models conclusively demonstrates that early postnatal exposure to general anesthetic agents causes deficits in performance on a variety of behavioral tests of learning^{6–10}. The convergence of these lines of evidence has raised a concern that anesthetics might have harmful effects on the developing brain that result in lasting impairments in cognitive function, a putative phenomenon that has been called pediatric anesthetic neurotoxicity (PAN). Prospective human trials are underway, but their results are too early to be conclusive¹¹. One primate study has demonstrated changes in behavior after anesthetic exposure¹², but another did not find an effect¹³. While these studies are ongoing, a key question that remains unanswered is what the mechanism of injury is in the animal models that exhibit cognitive deficits after anesthesia exposure.

Growth cones are specializations common to all growing neurites, including pre-polarized neurites, axons, and dendrites. Growth cone motility is a fundamental process in nervous system development¹⁴, and the potential consequences of interrupting this process could include a loss of connectivity and a concomitant loss of brain circuitry¹⁵. The growth cone is a swelling at the end of the neurite that is comprised of two key structures that work in concert to drive growth. The filopodia, which are more distal, are thin, straight rapidly moving transient structures comprised of long bundles of actin¹⁶. The lamellipodia is a flat region emerging from the neurite that has a motile edge¹⁶. They are comprised of microtubules at the core and dense meshworks of actin peripherally. Growth cones have receptors which direct their growth towards appropriate targets¹⁷, and both the filopodia and lamellipodia are critical for this function. These receptors have a broad array of ligands, which include guidance cues and trophic factors, and they set in motion signaling cascades that regulate the dynamic cytoskeletal elements that direct growth¹⁸. The p75 neurotrophin receptor (p75NTR) is one such receptor that has already been implicated in anesthetic toxicity^{19, 20}. This receptor regulates neuronal growth by acting on filamentous actin via the Rho pathway¹⁷.

In this study we have explored the effects of sevoflurane, which is the most commonly used general anesthetic agent in pediatric anesthetic practice, on growth cone motility. We further ask how sevoflurane might exert effects on growth cone motility through the p75NTR-Rho pathway.

METHODS

Neuronal Culture

All animal care and protocols were compliant with the guidelines of Columbia University, Johns Hopkins University, and the National Institutes of Health. Dissociated neurons were prepared from embryonic day 18 C57BL/6 mouse neocortex as previously described^{21–23}. Embryonic day 18 or 19 pups were removed by cesarean section, and the neocortex was dissected out. Tissue was incubated for 15 minutes at 37°C in 320µM papain solution and further dissociated by pipette trituration. The resultant cell suspension was layered on top of

a 20 mg/mL albumin for a discontinuous gradient centrifugation to remove debris. Neuron density was quantified by hemocytometer, and neurons were allowed to settle for three hours in media containing 10% horse serum after plating. For time lapse experiments neurons were plated at a density of 100/mm² in 35 mm glass bottom imaging dishes (MatTek) coated with poly-D-lysine (Sigma). For biochemistry experiments neurons were plated at a density of 1.5 million cells per well in standard 12-well plates. They were maintained in B-27/L-glutamine supplemented neurobasal media (Life Technologies). Experiments were performed on neurons at 1 to 4 days in vitro (DIV), when neuronal growth cones are abundant. Time lapse experiments represent at least three different cultures, and each co-immunoprecipitation experiment was performed with lysates derived from two cultures per condition.

Live Cell Imaging and Anesthesia Exposure

For live cell imaging, glass bottom dishes were enclosed in a stage-top incubator (Tokai Hit), in which temperature was maintained at 36.5°C and a humidified carrier gas mix consisting of 5% CO₂ / 95% air was continuously perfused through the chamber at a flow rate of 2 mL/m and vented into a scavenging system. Sevoflurane was delivered using an in-line vaporizer, and was diluted in 5% CO₂/95% O₂ carrier gas. Controls for these experiments received 5% CO₂/95% O₂ carrier gas only. For experiments conducted on N₂O, cells received 5% CO₂ / 70% N₂O / 25% O₂ and the controls received 5% CO₂/95% O₂ carrier gas. For experiments conducted on propofol both groups received 5% CO₂/95% O₂ carrier gas, and propofol was diluted originally in high concentration in DMSO and then subdiluted twice in media such that DMSO concentrations are negligible. Nitrous oxide was delivered by replacing the carrier gas with 5% CO₂ / 70% N₂O / 25% O₂. All other drugs were dissolved in media and applied directly to the bath. The volume of media in the imaging dish was minimized to improve surface area for efficient gas diffusion. There was a 15-minute equilibration period, developed during pilot work to ensure steady state conditions. Growth cones were imaged via a CCD camera by differential interference contrast on an inverted Ti-E series microscope using a 63×1.4 numerical aperture objective and a perfect focus system (Nikon), and images were captured at a rate of one per second for five minutes. Each growth cone was first pre-imaged for 5 minutes to determine baseline motility, and growth cones that appeared non-motile were excluded as were growth cones which could not be clearly identified as axonal or dendritic (for consistency exclusions were all conducted by a single investigator, CDM). Motile growth cones were then treated with carrier gas alone for control conditions and sevoflurane at 2%, 4%, or 6% propofol at 12 μM (Sigma), N₂O at 70%, Y27632 at 50 μM (Sigma), or TAT Peptide 5 (TAT-Pep5) at 10 μM (Millipore) for experimental conditions. For conditions involving Y27632 or TAT-Pep5 the drugs were applied to the bath for 15 minutes after baseline imaging but prior to co-application of sevoflurane, to allow penetration. In pilot experiments no obvious effects on non-motile growth cones were observed within our experimental parameters.

Movies were imported into ImageJ for analysis by a single investigator (RPM) who was blind to condition. Motility was examined through measurements of lamellipodial area change and filopodial tip velocity adapted from studies of growth cone dynamics^{24, 25} Lamellipodial area change was determined by measuring the area through tracing every 6 seconds. Sampling was between one minute and two minutes. Filopodial tip velocity was

measured by tracing the path of the filopodial tip and using a velocity function in ImageJ. In order to be sampled, filopodia were required to be visible for at least 30 seconds of the movie. Because growth cones have different baseline rates of motility, for each growth cone the data were normalized to lamellipodial area change or filopodial tip velocity that was measured in the pre-imaging time-lapse series taken prior to addition of agent.

Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) was performed using a Thermo Scientific Pierce kit following the manufacturer's instructions. Briefly, p75 antibody (Millipore) was first immobilized for 2 h using AminoLink Plus coupling resin. Neuron lysates were prepared by harvesting primary cultured cells on at 3 DIV, after 1 hr incubation with high dose sevoflurane (2% of total volume, added to media). A total of 1 mg of cell lysate proteins per group was incubated with the resin overnight at 4°C. After incubation, the resin was washed and protein eluted using elution buffer. Upon elution, samples were separated on a 4–20% Mini-Protein TGX precast gel (BioRad) and transferred to polyvinylidene difluoride membrane. Membranes were then incubated with anti-Rho-GDP dissociation inhibitor (Rho-GDI) antibody (Sigma) and developed using enhanced chemiluminescence.

Data Analysis and Statistics

Significance was set a priori at $p < 0.05$. For comparisons of means between two groups the Mann-Whitney test was employed, and for comparisons between three or more groups the Kruskal-Wallis test with Dunn's multiple comparisons test was used. Prism software was used to conduct statistical tests and to design graphical representations of the data. The following key is used to represent significance levels graphically: * <0.05 ; ** <0.01 ; *** <0.001 , **** <0.0001 . Error bars represent standard error of the mean

RESULTS

In initial experiments we exposed neuronal growth cones to 6% sevoflurane and compared their motility to growth cones exposed only to carrier gas. Under control conditions growth cones that were determined to be motile in the initial 5 minute test period invariably continued to be motile during the experimental period that followed (Fig 1A, B). By contrast, neurons exposed to 6% sevoflurane often seemed almost completely stalled, both in their filopodial and lamellipodial domains (Fig 1C, D). Because 6% sevoflurane is not within clinically relevant concentrations, we proceeded to test our effect at lower doses, and quantified both the rate of lamellipodial area change and the tip velocity of filopodia for 2% and 4% sevoflurane exposures. Interestingly, lamellipodial motility decreased in a concentration-dependent fashion over this range of sevoflurane (Fig 1E) (as compared to control: 40% reduction $p < 0.05$ at 6% sevoflurane, 60% reduction $p < 0.0001$ at 4% sevoflurane, 67% reduction $p < 0.0001$ at 6% sevoflurane). Filopodial motility was reduced to similar levels at all concentrations (Fig 1F) (as compared to control: 49%, 55%, 57% reductions for 2%, 4%, 6% sevoflurane respectively, all $p < 0.0001$).

We next asked whether there are differences in the effect on sevoflurane on motility that are apparent between axonal and dendritic growth cones. Axons were identified by standard

length criteria (10 μm longer than next longest process) and non-axonal neurites in polarized neurons were identified as dendrites. When the data were parsed based on these criteria, it is apparent that sevoflurane attenuates motility of lamellipodia and filopodia in both dendrites (Fig 2A, B) and axons (Fig 2C, D). With one exception, all groups and all doses show a significant reduction in motility. Dendrite lamellipodia motility is reduced by 37% with a 2% sevoflurane exposure, but does not meet criteria for significance ($p=0.176$).

A key question about any mechanism of anesthetic toxicity is whether it applies to other commonly used general anesthetic agents that are chemically distinct from sevoflurane. No significant change in either filopodial or lamellipodial motility was observed at a dose of 12 μM (Fig 3A, B). We next tested the effect of N_2O , at 70%, which reduced lamellipodial and filopodial motility by 51% and 52%, respectively ($p<0.0001$ for both) (Fig 3C, D).

Finally, we attempted to identify the mechanism underlying anesthesia-induced suppression of growth cone motility. Previously we identified a GABA-dependent mechanism by which anesthetics interfere with growth cones sensing of guidance cues²⁶, but our finding that propofol does not impair growth cone motility makes a GABA-dependent mechanism unlikely. Previous work by Patel and co-workers showed that isoflurane acts on the p75NTR and the associated Rho signaling pathway²⁷, which regulates actin-dependent motility in growth cones in a growth-inhibitory fashion^{28, 29}. To explore this mechanism we began by conducting time lapse imaging of growth cones using the same paradigm described above while co-administering 6% sevoflurane with 50 μM Y27632, an inhibitor of Rho Kinase, a key effector in the Rho signaling pathway. We found a significant increase in both lamellipodial and filopodial motility ($p<0.0001$ for both) over the sevoflurane treated group, which returned the growth cones to motility levels near that of untreated controls (Fig 4A, B). To determine whether sevoflurane could be acting on the Rho pathway via effects at the p75NTR we co-treated with 6% sevoflurane and 10 μM TAT-Pep5. This cell permeant peptide binds the p75NTR receptor and specifically interferes with Rho pathway signaling by preventing binding of Rho-GDI, which is a necessary step for RhoA activation³⁰. Treatment with TAT-Pep5 significantly increases motility in both filopodia and lamellipodia ($p<0.0001$ for both), and returns it to levels that are slightly elevated above untreated controls (Fig 4A, B). The restoration of growth cone motility that was observed with TAT-Pep5 suggests the p75NTR/Rho-GDI binding event as a likely target for sevoflurane. To test this hypothesis, we immunoprecipitated with an anti-p75NTR antibody using neuronal lysates treated with sevoflurane, and then we immunoblotted for Rho-GDI. A representative example of the blot is shown in 4C. We found that treatment with sevoflurane increases levels of Rho-GDI signal in the pulldown preparation. Taken together, these data suggest that sevoflurane may block growth cone motility by inappropriately activating the P75NTR in such a way as to enhance binding of Rho-GDI.

DISCUSSION

In this manuscript we have used time-lapse imaging of mouse neurons in vitro to show that sevoflurane has a potent inhibitory effect on growth cone motility, which is operative at a dose of 2% and higher. This effect occurs in both dendritic and axonal growth cone. It also occurs with N_2O , but not with propofol at the doses explored in this study. Normal motility

can be restored either by inhibition of Rho Kinase or by disrupting the coupling of the p75NTR with the Rho signaling system. Furthermore, biochemical evidence indicates that sevoflurane acts to increase binding of Rho-GDI to p75NTR. Our study has several limitations which must be acknowledged. It was conducted entirely in primary cell culture, and thus the findings remain to be tested in vivo. Also, conditions examining the effects of TAT-Pep5 and Y27632 alone on growth cone motility were not conducted, and in the nitrous oxide exposure the control carrier gas and the experimental condition had different levels of oxygen, as described in the methods. Finally, a full range of propofol doses was not tested. Nevertheless, based on this evidence we propose that sevoflurane may upregulate p75NTR mediated activity of the Rho pathway, which in turn may cause growth inhibition.

While the literature on this topic is relatively sparse, the studies performed to date indicate that anesthetic agents may interfere with multiple aspects of growth cone function, perhaps via several different mechanisms. The earliest report comes from Uemura et al., who noted an abnormal persistence in growth cone profiles in electron micrographs of the entorhinal cortex and subiculum in animals chronically exposed to low dose halothane (25 to 100 ppm) both during gestation and in early postnatal life³¹. While this finding is open to many possible interpretations, it is at least consistent with our results. Growth cones that are slowed might well persist abnormally, as they would not be expected to reach their targets on the normal developmental timeline, and consistent with this hypothesis, there was a reduction in the number of synapses concomitant with the increase in growth cones³¹. This study demonstrated a delay in the development of appropriate alternation behavior in a T-maze in animals treated with halothane, which indicates that there are consequences in terms of brain function that result from this putative delay. The nature of the exposure here differs very substantially from PAN, but the principle is the same, and it is worth noting that the clinical literature indicates that multiple exposures may be necessary to cause deficits in PAN.

The extant studies most relevant to the direct effects of anesthetics on growth cone motility differ substantially from the findings of this study, but they broadly support the concept that anesthetics have inhibitory effects on growth cone function. Al-Jahdari et al. exposed chick neurons in culture to propofol for 20 hours and found an increase in growth cone collapse and neurite retraction³². Because of the time course of the experiment, it is difficult to relate these findings directly to our observation that anesthetics acutely slow motility. We also found no effects of propofol in our model, but their findings occurred at doses at and above 50 μ M, which is substantially higher than was tested here and may have questionable relevance to clinical practice. Also, there may be substantial differences between the chick culture system and the model used in this manuscript. Working with primary cultured rat neurons, Sepulveda et al. found that ethanol, which has anesthetic properties and shares similarities with the general class of GABAergic anesthetics and sedatives, reduced axon extension, suggesting a possible slowing in motility³³. However, it is difficult to make a close parallel with our findings, given that axon extension takes place over a long time period and typically is the sum of periods of growth cone forward motion, stopping, and retraction that occur over several hours, rather than of slow continuous motion in uniform direction³⁴.

This study is closest in comparison to our own previous work on the effects of anesthetics on growth cone guidance. Previously our group showed that isoflurane can disrupt targeted axon growth in an ex vivo guidance model, and furthermore that midazolam, isoflurane, sevoflurane, and propofol all prevent the growth cone collapse induced Semaphorin 3A and Netrin²⁶. These results mirror a similar phenomenon seen with ethanol³³. We hypothesized that anesthetics that act at the GABA receptor render the growth cones insensitive to repulsive guidance cues. At first glance these results may seem at odds, however the relationship between growth cone guidance systems and growth cone motility is complex. Guidance depends on motility for execution, but growth also occurs in the absence of guidance cues³⁵ and motility driven by cues may require distinct signaling pathways³⁶. Inevitably receptors, signaling molecules, and structural components are shared between the two processes, but they are not entirely alike. It is notable that the effect of anesthetics on growth cone guidance cue sensing was profoundly evident at somewhat lower doses relative to this study – it reached a threshold effect with isoflurane doses of 0.9%. Also, different agents seem to predominate, as guidance effects were seen with low micromolar doses of propofol and while N₂O did block the response to semaphorin 3A, the effect was incomplete at a dose of 70%. Thus we conclude that our finding that pure motility is slowed by anesthetics is distinct from the effects of anesthetics on growth cone guidance sensing.

Our work highlights the likely importance of the p75NTR in mechanisms of anesthetic toxicity in brain development. It has been appreciated for some time that volatile anesthetics can activate RhoA³⁷. Patel and coworkers demonstrated the relevance of this interaction in PAN when they showed first that isoflurane-induced activation of apoptosis pathways could be blocked with the same peptide inhibitor employed in this study¹⁹ and then subsequently extended their results to show that the same paradigm could prevent activation of Rho²⁷. These studies examined neurons at 4 to 7 DIV, a time when growth cones would be sparse and thus they focused on apoptosis and cytoskeletal destabilization as outcomes. Also, the Patel group found a similar effect with propofol²⁰, which differs from our findings. Our study is best understood as the application of the same mechanism in a different context, where it is perhaps equally deleterious. Also, by showing that sevoflurane enhances binding of Rho-GDI to the p75NTR, we have provided the first insight into how for how the receptor's actions is altered by anesthetics. The Patel group has hypothesized that anesthetics exert their effects on p75NTR by generally inhibiting pre-synaptic activity, which results in reduced release of tissue plasminogen activator (tPA), which in turn causes elevated levels of uncleaved brain derived neurotrophic factor (proBDNF) to be available to bind and activate p75NTR¹⁹. BDNF is present in neuronal cultures³⁸ and tPA is expressed in neuronal growth cones³⁹, so this model may be operative in the context of growth cone motility.

Further investigation into how anesthetics act on growth cones is required for a complete understanding of this potentially important mechanism of toxicity in PAN. Our study was conducted solely in primary neuron culture, and thus productive future directions could include testing whether anesthetics act on growth cones in intact tissue and investigating whether these actions have lingering consequences. Also, while the connection between tPA, BDNF, and activation of the p75NTR is logical, it will require substantial further investigation to identify the exact target of anesthetics on growth cones.

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Fig 1. Sevoflurane slows growth cone motility

Representative images of a time-lapse movie for a growth cone exposed only to control gas is shown in **A**. Zoomed image of growth cone shown in **B**. Under control conditions both the lamellipodia and filopodia are constantly changing in position, as is emphasized by an outline of the growth cone profile drawn in red. By contrast motility is nearly arrested in the growth cone shown in **C** which is exposed to 6% sevoflurane (zoomed image with red outline to define profile is shown in **D**). Lamellipodia motility, measured as the rate of area change normalized to pre-treatment values, is reduced in a concentration dependent fashion

by sevoflurane between 2% and 6% (**E**) Filopodial motility, measured as tip velocity normalized to pre-treatment values, is also reduced between 2% and 6% sevoflurane exposure. Scale bar = 5.4 μm for **A**, **C**. Scale bar = 3.7 μm for **B**, **D**. In **E**, n = 64 lamellipodia (14 to 20 per group). In **F**, n = 90 filopodia (37 to 59 per group) * $p < 0.05$, **** $p < 0.0001$. The * shown over each bar indicates significance versus control.

Fig 2. Both dendritic and axonal growth cones exhibit reduced motility with sevoflurane exposure

When only dendritic growth cones are examined, Lamellipodial rate of area change (**A**) and filopodial tip velocity (**B**) are both decreased with sevoflurane exposure between 2% and 6%. Similar results are observed for lamellipodia (**C**) and filopodia (**D**) of axonal growth cones. For **A**, $n = 32$ lamellipodia (7 to 10 per group). For **B**, $n = 67$ filopodia (14 to 24 per group). For **C**, $n = 55$ (9 to 20 per group). For **D**, $n = 119$ (23 to 45 per group). * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. The * shown over each bar indicates significance versus control.

Fig 3. Growth cone motility changes is not equally affected by all anesthetic agents

Propofol given at 12 μM has no significant effect on lamellipodial (A) or filopodial motility (B). In contrast, exposure to 70% nitrous oxide significantly reduces motility of both lamellipodia (C) and filopodia (D). For A, $n = 44$. For B, $n = 87$. For C, $n = 30$. For D, $n = 54$. **** $p < 0.0001$.

Fig 4. Sevoflurane-induced growth cone motility inhibition results from activation of p75NTR-Rho signaling

The significant reduction in motility of lamellipodia (**A**) and filopodia (**B**) is rescued by inhibition of Rho kinase with Y27632 or by inhibition of p75NTR binding to Rho-GTPase Dissociation Inhibitor (Rho-GDI) caused by Tat-Peptide 5. In **C** a representative blot of a co-immunoprecipitation study of p75NTR and Rho-GDI in neuronal lysates is shown. Blotting with anti-Rho-GDI after pulldown with anti-p75NTR shows that exposure to 2% (v/v) sevoflurane enhances binding between p75NTR and Rho-GDI over controls. For **A**, n = 62 (8 to 20 per group). For **B**, n = 180 (23 to 59 per group) **** p<0.0001.