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Anesthetics disrupt growth cone guidance cue sensing through actions on the $GABA_A$ α2 receptor mediated by the immature chloride gradient

Jing Xu[1,2], **Michael Xu**[2], **YuChia Wang**[2], **R. Paige Mathena**[2], **Jieqiong Wen**[1,2], **Pengbo Zhang**[1], **Orion Furmanski**[2], **C. David Mintz**[2]

[1] Department of Anesthesiology, The Second Affiliated Hospital of Xi'an Jiaotong University School of Medicine, Xi'an, Shaanxi, 710004, China.

[2] Department of Anesthesiology and Critical Care Medicine, the Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

Abstract

Background—General anesthetics (GAs) may exert harmful effects on the developing brain by disrupting neuronal circuit formation. Anesthetics that act on γ-aminobutyric acid (GABA) receptors can interfere with axonal growth cone guidance, a critical process in the assembly of neuronal circuitry. Here we investigate the mechanism by which isoflurane prevents sensing of the repulsive guidance cue, Semaphorin 3A (Sema3A).

Methods—Growth cone sensing was assayed by measuring growth cone collapse in dissociated neocortical cultures exposed to recombinant Sema3A in the presence or absence of isoflurane and/or a panel of reagents with specific actions on components of the GABA receptor and chloride ion systems.

Results—Isoflurane exposure prevents Sema3A induced growth cone collapse. A $GABA_A$ α2 specific agonist replicates this effect (36.83±3.417% vs 70.82±2.941%, in the Sema3A induced control group, $p<0.0001$), but an α1-specific agonist does not. Both a Na-K-Cl cotransporter 1 antagonism (bumetanide, BUM) and a chloride ionophore (IONO) prevent isoflurane from disrupting growth cone sensing of Sema3A. (65.67±3.775% in Iso+BUM group vs 67.45± 3.624% in Sema3A induced control group, 65.34±1.678% in Iso+IONO group vs 68.71± 2.071% in Sema3A induced control group, no significant difference) (n=96 growth cones per group).

Conclusion—Our data suggest that the effects of isoflurane on growth cone sensing are mediated by the α2 subunit of the $GABA_A$ receptor and also that they are dependent on the

Correspondence to: C. David Mintz, MD, PhD, Department of Anesthesiology, Ross Bldg. 370, 720 Rutland Ave, Johns Hopkins School of Medicine, Baltimore, MD 21205, USA. **Phone:** 917-733-0422, cmintz2@jhmi.edu.

Conflicts of Interest

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developmental chloride gradient, in which Cl^- exhibits a depolarizing effect. These findings provide a rationale for why immature neurons are particularly susceptible to anesthetic toxicity.

Keywords

Anesthesia; Neurotoxicity; $GABA_A$Rs; Chloride Gradient; Growth Cone

Introduction

Commonly used anesthetic and sedative agents now carry U.S. Food and Drug Administration mandated 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 2017). These concerns arise from epidemiologic studies showing a correlation between cognitive deficits and repeated or lengthy exposure to anesthesia and surgery in early life (DiMaggio, et al. 2012; Flick, et al. 2011; Ing, et al. 2012; Jevtovic-Todorovic, et al. 2003; Wilder 2010; Wilder, et al. 2009). While results from the only two clinical trials that have reached endpoints give reassurance that short, single exposures in healthy children are benign (Davidson, et al. 2016; McCann, et al. 2019; Sun, et al. 2016), whether longer or repeated exposures are potentially harmful remains an open question. Multiple studies conducted in the intact rodent model have found that early postnatal exposure to anesthetics without surgery can lead to deficits in learning and memory (Kodama, et al. 2011; Lee, et al. 2014; Ramage, et al. 2013; Satomoto, et al. 2009; Wang, et al. 2013; Zheng, et al. 2013). Several unavoidable confounds related to the difficulty in anesthetizing neonatal rodents and the substantial differences in timeline and complexity of rodent versus human brain development complicate the interpretation of these findings, but recently reported primate studies which represent a more faithful model of human anesthesia and brain development have also found cognitive and behavioral changes after early developmental anesthesia exposure (Talpos, et al. 2019).

The potential mechanism by which a limited developmental exposure to anesthetic or sedatives could have a lasting effect on cognitive function remains unclear, but most data point to one of two possibilities, which are not mutually exclusive. Anesthetics may have direct cytotoxic effects in developing brain cells (Jackson, et al. 2016; Yang and Wei 2017) and/or they may alter the formation of brain circuitry (Xu, et al. 2018a). In previous work, we have found that anesthetic agents have the potential to interfere with the development of connectivity in the brain by disrupting axon guidance (Mintz, et al. 2013), the process by which developing axons grow towards the appropriate dendritic targets to establish appropriate circuitry (Blanquie and Bradke 2018; Russell and Bashaw 2018). We found that the process by which growth cones, the specialized structures that direct growth of the leading edge of a developing axon (Gasperini, et al. 2017), sense chemotropic guidance cues is disrupted by a wide range of anesthetic agents with GABA agonist activity (Mintz, et al. 2013).

Signaling via GABA is increasingly recognized as a key determinant of neuronal development (Fritschy 2015; Namba, et al. 2017). The GABA system changes notably between developing and mature states (Gonzalez-Burgos, et al. 2015; Oh and Smith 2019),

including changes in receptor subunit composition and in membrane polarization resulting from GABA receptor activity that is due to a switch in chloride ion transporter expression. This is of great interest as the most commonly used general anesthetics have actions at GABA receptors (Brohan and Goudra 2017; Hayashiuchi, et al. 2017; Woll, et al. 2018), and it appears that developing neurons are subject to toxic effects of anesthetics that are not manifested in mature neurons. Here we employ a dissociated neuron culture model to ask whether the effects of isoflurane, a canonical volatile anesthetic, on axonal growth cone guidance cue sensing, are dependent on features of the immature GABA transmission system.

Methods

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 company protocols. Neurons were plated on 12 mm polylysine coated glass coverslips at 16,000 cells/cm^2 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 2 and 4 days *in vitro* (DIV), during which time axonal growth cones are easily distinguished and highly active. All experiments incorporated coverslips drawn from a minimum of 3 separate cultures.

Anesthetic Agents Exposure and Drug Treatments

For volatile anesthetic treatment, coverslips in 12-well plates with a low volume of culture media (500μl to facilitate gas diffusion, were placed in airtight, humidified modular chambers (Billups-Rothenberg, Del Mar, CA, USA) as previously described (Mintz, et al. 2013; Xu, et al. 2018b). The chamber was connected to an agent-specific calibrated vaporizer (SuperaVet, Vaporizer Sales and Services Inc, Rockmart, GA, USA) that delivered 2.4% isoflurane mixed with 5% carbon dioxide / 95% air carrier gas at 12 L/min. Carrier gas alone was used as for controls. Gas composition was measured periodically using a 5250 RGM gas analyzer (Datex-Ohmeda, Madison, WI, USA). In some cases, co-treatment or pre-treatment with pharmacologic compounds was performed. These compounds included: TCS 1205 (10 nM, 100 nM, 1μM, Tocris), TCS 1105 (10 nM, 100 nM, 1μM, Tocris), Zolpidem (10 nM, 100 nM, 1μM, Tocris), Bumetanide (10μM, Tocris), and Chloride Ionophore I (3μM, Millipore). After a 15-min equilibration, the sealed chambers with dissociated cultures were placed in an incubator to maintain temperature at 37°C for 1 hour, followed by 20 min exposure to either vehicle control or a recombinant soluble axon guidance cue, Semaphorin 3A (R & D Systems, Bend, OR) at 100 ng/ml to induce growth cone collapse (Figure 1A, B).

Cell Labeling and Immunocytochemistry

Fluorescent immunocytochemistry and labeling with fluorescently tagged F-actin were conducted as previously described (Mintz, et al. 2012). Dissociated neurons were fixed with

4% paraformaldehyde at room temperature for 10 min, then permeabilized and blocked for 1 hour at room temperature in 5% donkey serum with 0.1% Triton X-100. After rinsing with PBS, neurons were incubated for 20 min with Alexa 488-conjugated phalloidin (1:50, Invitrogen). Subsequently, neurons were mounted on coverslips using 2.5% PVA/DABCO Mounting Media.

Growth Cone Analysis

A Leica TCS light microscope was applied for imaging. The axons were identified as the longest neurite, a consistent morphological determinant that is readily apparent at this timepoint (Dotti, et al. 1988). Axonal growth cones were classified morphologically by fluorescence microscopy as “extended” or “collapsed” based on standard criteria (collapsed growth cones are defined as lacking a full lamellipodia and/or exhibiting two or fewer filopodia in the direction of growth) (Mintz, et al. 2013). Coverslips were divided into quadrants for analysis, and the classification and counting of growth cones was done in real-time using a 63× 1.4NA objective by an investigator who was blind to the experimental condition, as previously described (Mintz, et al. 2013). Each experiment represents 3 separate cultures and 12 coverslip quadrants with no less than 8 growth cones per field. Representative images were captured as photomicrographs and Photoshop CC2014 (Adobe Inc. San Jose, CA, USA) was used for resizing and to make minor alterations in contrast and brightness to best represent what was visible to the microscopist. Data are reported as the mean percentage of collapsed axonal growth cones per field, and the error bars denote standard error of the mean from the mean.

Statistical Analysis

All statistical analysis was conducted using Prism 6.0 (Graphpad, San Diego, CA, USA). For all collapse assays we tested the hypothesis that mean percentage of growth cones, which collapsed in response to Sema3A was reduced by the specified pharmacologic treatment. Data sets were confirmed to have normal distribution, and one-way ANOVA with multiple comparisons (Dunnett’s multiple-comparison post hoc tests) was conducted to assess the differences in mean collapse between groups. Statistical significance for all tests was set *a priori* at $p<0.05$.

Results

Growth cones exist in either extended (Fig. 2A) or collapsed (Fig. 2B) states in dissociated neuron culture, as they do *in vivo*. The repulsive guidance cue, Sema3A, at 100ng/ml treatment for 20mins, causes collapse (70.82±2.94%) of axonal growth cones (AGCs) in cortical neurons in culture compared to the naïve control group (29.20±3.23%) (Fig 2E). Our previous work has shown that treatment with 2.4% isoflurane for one hour results in inhibits of the growth cone collapse in this system (Fig 2C, D), and this effect is dependent on $GABA_AR$ activation by anesthetics and that it can be replicated with other $GABA_AR$ agonists (Mintz, et al. 2013).

In order to determine whether the effect of anesthetics on guidance cue sensing is dependent on immature $GABA_AR$ subunit composition, we employed pharmacologic agents that have

differential specificity for the α1 and α2 subunits, which are, respectively, associated with the mature and immature $GABA_AR$ phenotype. The agonist and antagonist compounds were employed at concentrations from the nanomolar to micromolar range in order. Neurons were treated with TCS 1205, an agonist with greater specificity for receptors containing the α2 subunit than for those containing the α1 subunit (Primofiore, et al. 2001). The AGC collapse response to Sema3A is fully inhibited even at the lowest tested dose of 10 nM (36.83±3.417% vs 70.82±2.941% in the Sema3A induced control group, $p<0.0001$) (Fig. 2E). Next, we treated cultures with TCS 1105, which acts as an agonist at α2-containing $GABA_ARs$ and an antagonist at α1-containing $GABA_ARs$ (Taliani, et al. 2009). The 100 nM and 1 μM TCS 1105 dose groups exhibited significant inhibition of the growth cone collapse response compared to the control treated with Sema3A group (39.58±3.554% in 100nM and 34.33±3.063 in 1μM vs 65.67± 4.091% in the Sema3A induced control group, $p<0.0001$) (Fig. 2F). Finally, we also tested zolpidem, a $GABA_A$ agonist with greater activity at α1 rather than α2-containing receptors. With zolpidem, there was no significant effect on Sema3A induced collapse at 10 nM and 100 nM concentrations, and significant inhibition only at partial levels at 1 μM (47.83±4.121 in 1μM vs 72.18± 5.231% in Sema3A induced control group, $p<0.001$) (Fig. 2G).

Next, we sought to determine whether the chloride gradient associated with immature neurons is required for anesthetic inhibition of growth cone cue-sensing. To this end we pre-treated neurons for 24 hours in culture with bumetanide, which is an inhibitor of NKCC1 (Kahle and Staley 2008), the developmentally predominant ion transporter that is thought to be responsible for the high intracellular concentration of chloride ions that is measured in immature neurons (Watanabe and Fukuda 2015). We found that bumetanide pre-treated cultures exhibited no significant inhibition of Sema3A growth cone collapse response after treatment with isoflurane (65.67±3.775% in Iso+BUM group vs 67.45± 3.624% in Sema3A induced control group, no significant difference) (Fig 3A). To confirm that the isoflurane mediated inhibition of growth cone sensing is dependent on the chloride gradient, we next pre-treated the cultured neurons for 24 hours with chloride-specific ionophore, which allows chloride to move freely through the membrane and prevents the formation of a gradient (Kalueff 2007). After pre-treatment with the ionophore, isoflurane did not prevent Sema3A dependent AGC collapse (65.34±1.678% in Iso+IONO group vs 68.71± 2.071% in Sema3A induced control group, no significant difference) (Fig. 3B).

Discussion

In this study, we asked whether the anesthetic-induced disruption of normal growth cone guidance is dependent on features of the GABA system that are unique to developing neurons. We found that agonist activity directed at the α2 but not the α1 subunit of the $GABA_A$ receptor is required to inhibit the collapse response to Sema3A in dissociated cortical neurons. Our data also indicates that the inverted chloride gradient that is dependent on NKCC1 activity is required for isoflurane-induced failure of response to Sema3A (a repulsive guidance cue) in the dissociated immature cortical neurons.

These findings more broadly provide a framework to explain why mature, developed neurons are relatively resistant to anesthetic-induced toxicity, whereas developing neurons are more vulnerable.

The mechanism by which anesthetics could act on the developing brain to have a lasting impact remains unclear, although numerous candidate targets, many of which are not mutually exclusive, have been proposed (Kang, et al. 2017; Mintz, et al. 2013; Xu, et al. 2018a; Xu, et al. 2018b). The GABA receptor is an intriguing target, as activity at this receptor is one of the few things that is common to many general anesthetics agents (Franks and Lieb 1994; Jones, et al. 1992; Kapfhammer, et al. 2007). Our previous work in this area had three key findings: 1. GABA agonists that are not used as anesthetics could mimic the inhibitory effects of isoflurane on growth cone guidance cue sensing; 2. Anesthetics, sedatives, and other compounds with actions on the GABA receptor inhibited growth cone guidance cue sensing; and 3. Compounds with GABA antagonist activity negated the inhibitory effects of isoflurane on growth cone guidance cue sensing. In this manuscript, we have extended these results by identifying the α2 subunit of the $GABA_A$ receptor complex as a key target. Our work on the effects of isoflurane on growth cone guidance cue sensing is not the only instance in literature in which GABA receptors have been implicated in anesthetic toxicity. GABA antagonist treatment has been shown to reverse the effects of propofol on both proliferation and survival of embryonic neurons in culture (Wang, et al. 2015). In a study of embryonic neurons in culture, Fiskum and co-workers showed that either propofol or a non-anesthetic GABA agonist induced neurotoxicity could be reversed by either a chloride or calcium channel blocker (Kahraman, et al. 2008). The authors concluded that depolarization due to activation of $GABA_A$ receptors in the setting of the developmental chloride gradient cause activation of L-type calcium channels, which in turn caused cellular apoptosis, a mechanistic hypothesis for anesthetic toxicity that has been dubbed the “Calcium Overload Hypothesis” (Bosnjak, et al. 2016). Electrophysiologic studies of neurons in culture suggest that isoflurane can also cause apoptosis that is dependent on a calcium influx due to a depolarizing chloride current (Zhao, et al. 2011). Our results support these findings by demonstrating that the effects of isoflurane on growth cone guidance cue sensing are definitively dependent on the developmental gradient of chloride which is required for a depolarizing effect of GABA, although we have not specifically tested whether this is a calcium dependent phenomenon.

Traditionally GABA has been viewed as an inhibitory neurotransmitter at synapses in the adult CNS, but there is now a large body of work which suggests in the developing brain GABA functions both as a paracrine trophic factor to regulate neural proliferation and growth and as an excitatory neurotransmitter to mediate activity-dependent aspects of development (Represa and Ben-Ari 2005; Wang and Kriegstein 2009). The excitatory actions of GABA result from an outwardly directed chloride gradient that is maintained by NKCC1, which is highly expressed in developing neurons, and as neuronal maturation progresses, NKCC1 is largely replaced by KCC2 which results in a switch to an inwardly directed chloride gradient (Alvarez-Leefmans, et al. 2001; Ben-Ari 2002; Yamada, et al. 2004). Depolarizing GABA has been shown to play an important role in growth cones. Exogenously applied GABA results in an efflux of chloride and an influx of calcium in isolated growth cones in culture (Hong, et al. 2000; Zheng, et al. 1994). It is well established

that calcium signaling in growth cones mediates the growth response to guidance cues (Henley and Poo 2004; Zheng 2000). These results support our interpretation that isoflurane interferes with growth cone guidance cue sensing via depolarizing GABA actions that are dependent on the immature chloride gradient. This effect would be predicted to apply to other GABAergic anesthetics and also for ethanol, which also interferes with Sema3A mediated growth cone function in a model of fetal alcohol toxicity (Sepulveda, et al. 2011). The extent to which disruptions of growth cone function could interfere with brain development are unknown, but they would presumably increase with multiple or lengthy exposures and they would have greater impact at earlier ages when a higher proportion of neurons are immature.

Our study has several limitations that we hope will be addressed in future work. First, it was conducted entirely in dissociated neuron culture. While this is a good model to understand the fundamental processes that govern neuronal development, the absence of patterned input to drive development and the two-dimensional geometry of culture are limitations of the model that must ultimately be overcome by testing in the intact brain. We see disruption of growth cone sensing over a short period of a single hour, but it has yet to be determined what consequences might result in terms of development of brain circuitry from the lengthy and/or multiple exposures that are required to disrupt brain development in an intact animal. Second, we employed only pharmacologic approaches, rather than genetic ones. We did this in order to achieve acute effects, as perturbation of the GABA system has the potential to massively disrupt development, but confirmation using conditional knockouts would be useful. Ultimately, we believe the most useful direction suggested by our work would be to use *in vivo* mouse models to test the hypothesis that individual neurons are rendered vulnerable or protected from anesthetic effects based on the status of the chloride gradient and to determine whether bumetanide administration at the time of anesthetic exposure can prevent or mitigate the lasting deficits in learning and memory associated with developmental anesthetic neurotoxicity in this model system..

Conclusions

In summary, we found that the α2 subunit of the $GABA_A$ receptor is involved in the effects of isoflurane on growth cone sensing, and this phenomenon is also affected by the developmental chloride gradient, in which Cl^- exhibits a depolarizing effect. According to our data, we provided one of the mechanisms about why the immature neurons are vulnerable to anesthetic exposure.

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Abbreviations

GAs	General anesthetics
GABA	γ-aminobutyric acid

Sema3A	Semaphorin 3A
BUM	Bumetanide
IONO	Ionophore.
DIV	day *in vitro*
AGCs	axonal growth cones

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Highlights

1. Growth cone sensing of guidance cue is a critical process in the development of brain circuitry which can be disrupted by anesthetics.
2. Anesthetic interference with growth cone sensing is mediated via the $GABA_A$ α2 subunit, which predominates in immature neurons.
3. Anesthetic actions on growth cone sensing are dependent on the inverted developmental chloride gradient, which is present only in immature neurons.

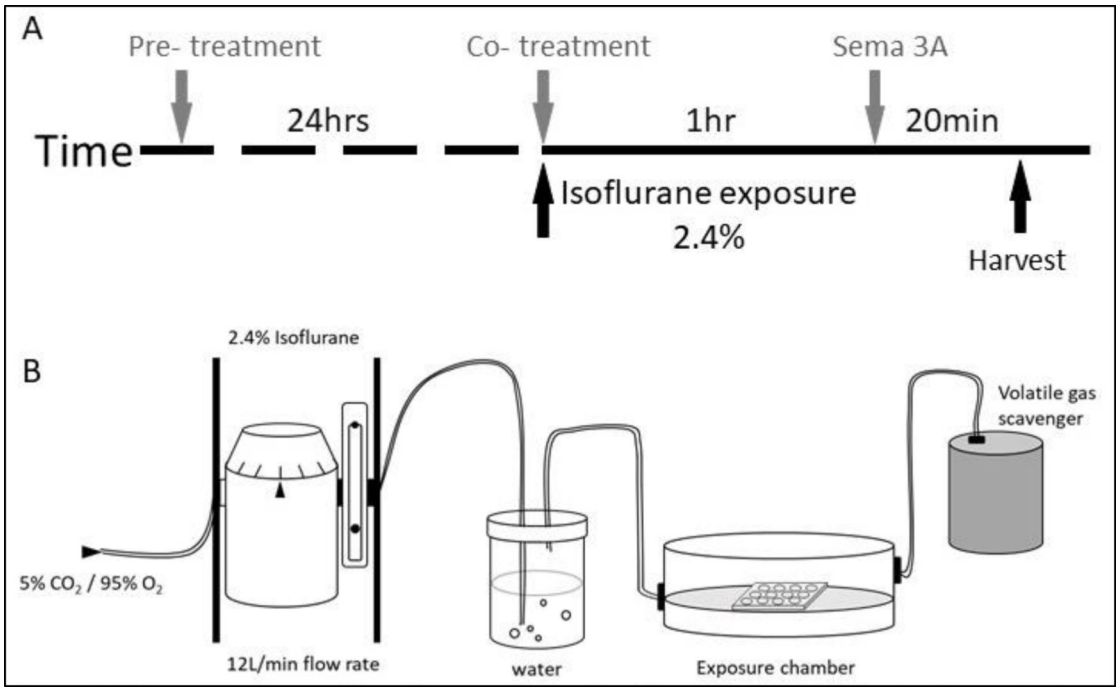

Figure 1. Schematic representation of the experimental timeline and exposure induction diagram *in vitro*.

(A). The general experiment timeline *in vitro*. The neurons were exposed to 2.4% isoflurane for 1hr on their 2–4DIV, followed by 20 min with recombinant guidance cues to induce collapse. In some cases co-treatment or pre-treatment with other soluble agents was performed. The cells were fixed for immunohistochemistry after the incubation.

(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-min equilibration period, the sealed chambers were placed in an incubator to maintain temperature at 37°C for the duration of anesthesia exposure.

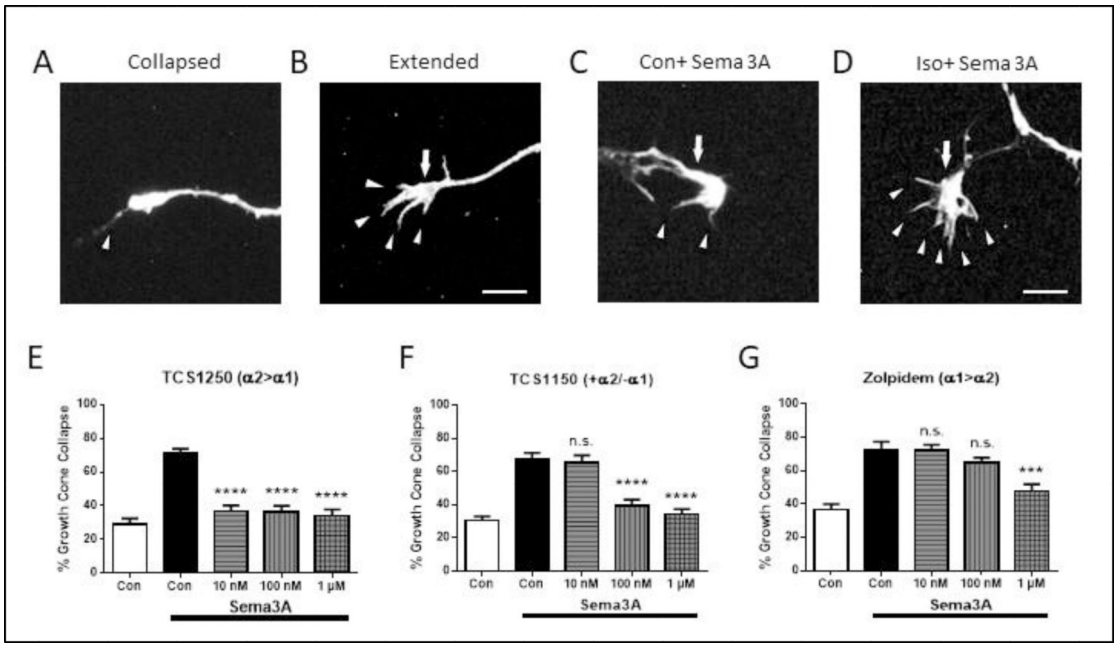

Figure 2. Inhibition of Sema3A induced AGC collapse is caused by activity at $GABA_A$ α2 receptors subunits, but is not dependent on $GABA_A$ α1 activity.

(A-B) Representative images of extended (**A**) and collapsed (**B**) AGCs. Sema3A treatment induces a collapsed morphology in AGCs characterized by a reduced lamellipodia (A, arrow) and two or fewer filopodia extending in the direction of growth (A, arrowhead). Extended growth cone shows a large lamellipodia (B, arrow) and multiple filopodia (B, arrowheads) predominates. (Scale bar =10 μm).

(C-D) Representative images of control (**C**) and isoflurane treated (**D**) AGCs. A 1 hour treatment with isoflurane 2.4% prevents collapse, and shows the extended morphology (**D**), while the control group shows collapsed morphology (**C**). (Scale bar =10 μm).

(E) TCS 1205, a $GABA_A$ α2 agonist and α1 partial agonist, blocks AGC collapse at low doses, which suggests that anesthetic interference with ACG guidance cue sensing is more likely to occur via the α2 rather than the α1 subunit.

(F) TCS 1105 acts as an agonist at α_2 and antagonist at α_1 benzodiazepine receptors on $GABA_A$. This drug also blocks Sema3A induced AGC collapse in a concentration-dependent fashion, further suggesting a particular affinity for the α2 subunit.

(G) Zolpidem, a $GABA_A$ α1 subunit agonist, causes only a partial effect on AGC collapse at a high dose, suggesting that anesthetic disruption of AGC guidance cue sensing is not an α1 subunit dependent effect. (***p<0.001, ****p<0.0001 compared to control treated with Sema3A (black bar) One-way ANOVA and multiple comparisons).

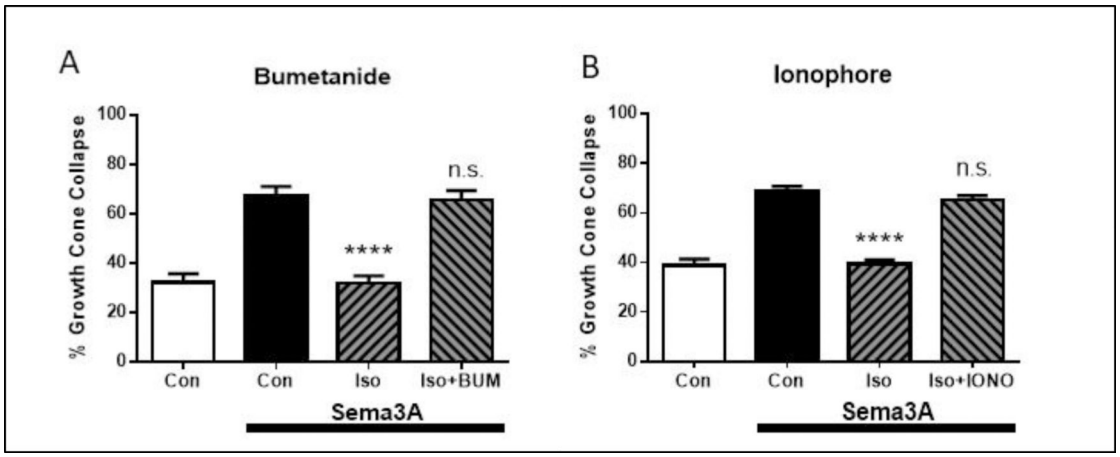

Figure 3. Inhibition of Sema3A induced AGC collapse is dependent on an immature chloride gradient.

(A) Bumetanide, a NKCC1 transporter blocker, prevents the intracellular concentration of Cl^- ions. When the neurons were pre-incubating for 24hrs with bumetanide, the percentage of growth cone collapse did not decrease after isoflurane exposure.

(B) Chloride ionophore is a mobile ion carrier that shields the charge of the chloride, and therefore enables the ions to penetrate the lipid layer of the cell memberance. When the neurons were pre-incubating for 24hrs with chloride ionophore, isoflurane did not prevent Sema3A dependent AGC collapse. (****p<0.0001 compared to control treated with Sema3A (black bar) One-way ANOVA and multiple comparisons).