PERSPECTIVE

The changes of oligodendrocytes induced by anesthesia during brain development

With the advent of modern techniques, drugs, and monitoring, general anesthesia has come to be considered an unlikely cause of harm, particularly for healthy patients. While this is largely true, newly emerging clinical and laboratory studies have suggested that exposure to anesthetic agents during early childhood may have long-lasting adverse effects on cognitive function. This concern has been the focus of intense study in the field of anesthesia research. A recent high-profile review by Rappaport et al. (2015) concluded that while many questions remain unanswered, there is strong evidence from laboratory studies that commonly used anesthetics interfere with brain development and that clinical studies suggest a correlation between early childhood exposure to these agents and subsequent effects on learning and cognition. The issue is of sufficient public health importance that a public-private partnership known as SmarTots (Strategies for Mitigating Anesthesia-Related Neurotoxicity in Tots) was developed by the FDA to study pediatric anesthetic neurotoxicity. The mechanism of injury underlying this phenomenon has yet to be fully elucidated, and there is evidence to suggest that anesthetics may have direct cytotoxic effects on neurons leading to cell death or suppressed neurogenesis (Stratmann et al., 2010) and that they may interfere with key processes in neuronal growth and development that underlie brain circuit development (Wagner et al., 2014).

Nearly all of the focus of basic science investigations of mechanisms of anesthetic toxicity has fallen on the effects of anesthetics on neurons, and strikingly little attention has been paid to possible mechanisms of injury mediated through effects on glial cells. Both the development and mature function of neurons depends heavily on the two principal species of glia: astrocytes, which support neuronal function *via* a variety of biochemical and structural roles and oligodendrocytes, which provide myelin to electrically insulate neuronal transmission. This article highlights evidence that anesthetic exposure during development may have toxic effects on astrocytes and oligodendrocytes, including recent evidence that these effects could lead to impairments in the function of the neuronal circuitry that underlies cognition.

Currently, only a few studies exist which have examined the effects of anesthetic exposure on astrocytes in the context of brain development. Todorovic and co-workers first opened this line of investigation by describing injury to developing astrocytes that resulted from a 4 hour exposure to 3% isoflurane in a primary culture model. The authors describe a substantial disruption of the actin cytoskeleton, particularly evident in examination of the stress fibers, that is accompanied by a reduction in paxillin, a marker of focal adhesions, and that correlates with reduced levels of RhoAGTPase, a signaling molecule that is critical for the development of normal glial morphology (Lunardi et al., 2011). Another study of the effects of isoflurane on astrocytes *in vitro* that used a lower dose, a 4 hour exposure to 1.4% isoflurane, revealed no changes in, phalloidin binding, a gross measure of F-actin levels, but instead showed a decrease in glial fibrillary acid protein expression and a decrease in alpha tubulin levels (Culley et al., 2013). These data provide strong evidence that isoflurane, the benchmark potent volatile anesthetic, impairs the cytoskeleton in developing astrocytes. The cytoskeletal effects do not appear to be lethal for astrocytes, as assays of astrocyte survival after anesthesia exposure show no significant change from controls. Given the fundamental nature of the cytoskeleton for cellular function, it is likely that such a disruption would have some effect on glial activity, but neither of these manuscripts uncovered a functional consequence related to brain development.

In a study by Ryu et al., (2014), we asked whether isoflurane exposure in astrocytes impaired the ability of astrocytes to support neuronal growth, one of their key roles in early development. Culley et al. (2013) had previously addressed this question by asking whether neurons grown in conditioned media from astrocytes treated with isoflurane manifested a change in synapse number compared to neurons growth in control conditioned media. They found no change, and thus concluded that isoflurane treatment did not alter the ability of astrocytes to support neuronal growth. We took a different approach, in which we measured axonal growth in naïve neurons co-cultured with astrocytes that had been previously exposed to isoflurane or control conditions (Ryu et al., 2014). We found that axon length was reduced in the isoflurane treated co-culture group in a dose dependent manner over a range from 2.4% to 3.6% of 5 hour treatment with isoflurane, and furthermore that isoflurane treated astrocytes remained less capable of supporting axonal growth for up to 24 hours. The cues that guide targeted axonal growth are only transiently expressed, and thus axons must grow at the correct speed in order to encounter the appropriate cues for the duration of their extension towards synaptic targets.

Our findings could not be explained by reduced numbers of astrocytes since we did not observe any cell death resulting from isoflurane exposure, so we examined other putative mechanisms. We found that levels of brain derived neurotrophic factor (BDNF) were reduced in isoflurane treated co-culture media and that axon outgrowth in the isoflurane treated group could be rescued to normal levels by adding exogenous BDNF to the co-culture (Ryu et al., 2014). BDNF is also critical for synaptogenesis, and thus it is likely that the absence of an effect on synapses seen previously was due to the difference in paradigm. Our effect was only evident at 2.4%, and not at a lower dose of 1.2% that is more consistent with the 1.4% dose used by Culley and co-workers. Alternately, and perhaps equally likely, the effect may be due to the relationship between BDNF and astrocytes. In conditions of injury and inflammation, astrocytes can produce high levels of BDNF, but in normal development, the predominance of BDNF is secreted by neurons, and the role of astrocytes is thought to be in uptake and recycling of this growth factor (Bergami et al., 2008), a process that would not have been observed in a conditioned media model, but which would have been evident in a co-culture paradigm. As astrocytes co-exist with neurons in the developing brain *in vivo*, these data suggest that isoflurane may inhibit neuronal growth indirectly *via* impairment of astrocyte effects on BDNF levels. The mechanism by which isoflurane might act on astrocytes to impair BDNF recycling is unclear, and we speculate that the structural deficits discussed above may contribute to an inability of the astrocytes to project processes into appropriate proximity with neurons to serve this function. Alternately, both the cytoskeletal disruption and their reduced capacity to support neuronal growth may both be consequences of metabolic impairment caused by isoflurane, which has been shown to cause mitochondrial injury during development (Sanchez et al., 2011).

The effects of developmental exposure to anesthetics on oligodendrocytes appear to reflect the potential cytotoxicity properties of anesthetics. Seminal work in pediatric anesthetic

toxicity by Jevtovic-Todorovin et al. (2013) showed that commonly used anesthetics cause an increase in neuronal apoptosis in rodents. Depending on the dose and timing of exposure, anesthetics have been shown to cause cell death, but the literature on this topic is complex and highly dependent on context and cell type. None of the studies of the effects of anesthetics on astrocytes *in vitro* discussed above revealed any cytotoxicity, and similar work *in vivo* support this conclusion. Brambrink and co-workers exposed postnatal day 6 rhesus macaques to five hoursof isoflurane administered between 0.7% and 1.5% to ablate movement and physiologic response to painful stimulus, using physiologic monitoring and support consistent with standards applied in the clinical practice of anesthesia (Brambrink et al., 2012). Brains were harvested 3 hours after the termination of anesthesia exposure, and a substantial increase in apoptosis relative to unexposed controls was appreciated as measured by quantitative microscopy for activated caspase and Hoechst labeling in serial sections throughout the brain. Unlike astrocytes and microglia, the oligodendrocyte population showed a marked increase in apoptosis after isoflurane treatment (Brambrink et al., 2012). Using histological analysis, they estimated that this effect amounted to a loss of 6.3% of total population of myelinating oligodendrocytes. These degenerative changes were not confined to any single anatomical location, but rather were diffusely present throughout the brain. Interestingly, the same group of researchers reported a similar effect with propofol, an intravenous anesthetic commonly used in clinical practice (Creeley et al., 2013). In a study of postnatal day 6 and fetal gestational day 120 rhesus macaques using the same methodology discussed above, a substantial increase in oligodendrocyte apoptosis was detected in isoflurane exposed brains. Deletion of myelinating oligodendrocytes could cause pathological changes in axonal structure or function. In addition, given that one oligodendroglial cell will contribute myelin to several axons, a loss of myelinating oligodendrocytes could be expected to broadly disrupt myelination and nerve conduction for multiple axons within the field of a single oligodendrocyte. However, these studies were conducted only at a single, early time-point, without any long term follow-up. Thus, it is possible that a recovery from injury might occur, particularly as the authors note that oligodendrocyte precursors in the isoflurane exposed groups did not undergo increased apoptosis, but rather appeared to be engaged in reactive proliferation.

The evidence strongly suggests that glia are a possible target of anesthetic toxicity. Astrocytes clearly undergo cytoskeletal disruption as a result of early anesthetic exposure and their capacity to support neuronal growth is transiently impaired. These results have been obtained using dissociated culture models, and must still be translated to the context of the intact brain. Anesthetics induce apoptotic cell death in a substantial population of oligodendrocytes in the developing brain, but the significance of this finding will be greatly enhanced if it is shown to be persistent. While clear effects of anesthetics on astrocytes and oligodendrocytes have been demonstrated, a possible role for microglia has not been explored to date. Recent evidence has shown that microglia play an important role in normal brain development (Michell-Robinson et al., 2015), and anesthetics have been shown to modulate microglial cytokine release in an in vitro model of inflammation (Ye et al., 2013). This finding suggests the possibility that anesthetics might alter brain development *via* effects on microglia. The currently available literature on the effects of anesthetics on glia in the developing brain is fairly sparse compared to studies of developmental anesthetic toxicity in neurons, and we conclude that this is a promising area for further study in the field of pediatric anesthetic neurotoxicity.

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