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Neurotoxicity of Anesthetics: Mechanisms and Meaning from Mouse Intervention Studies

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Abstract

Volatile anesthetics are widely used in human medicine and generally considered to be safe in healthy individuals. In recent years, the safety of volatile anesthesia in pediatric patients has been questioned following reports of anesthetic induced neurotoxicity in pre-clinical studies. These studies in mice, rats, and primates have demonstrated that exposure to anesthetic agents during early post-natal periods can cause acute neurotoxicity, as well as later-life cognitive defects including deficits in learning and memory. In recent years, the focus of many pre-clinical studies has been on identifying candidate pathways or potential therapeutic targets through intervention trials. These reports have shed light on the mechanisms underlying anesthesia induced neurotoxicity as well as highlighting the challenges of pre-clinical modeling of anesthesia induced neurotoxicity in mice. Here, we summarize the data derived from intervention studies in neonatal mouse models of anesthetic exposure and provide an overview of mechanisms proposed to mediate anesthesia induced neurotoxicity in mice based on these reports. The majority of these studies implicate one of three mechanisms: reactive oxygen species (ROS) mediated stress and signaling, growth/nutrient signaling, or direct neuronal modulation.

Introduction

Volatile anesthetics are widely used in human medicine, and routine anesthesia is generally considered to be safe in healthy individuals. In recent years, the use of anesthetic in neonates and children has been questioned following reports of anesthetic induced neurotoxicity (AIN) in pre-clinical studies, as discussed in various reviews and commentaries (for representative examples, see (Lin et al., 2017; Vutskits and Davidson, 2017; Walters and Paule, 2017)). These studies in mice, rats, and primates have demonstrated that exposure to anesthetic agents during early post-natal periods can lead to acute neurotoxicity and later-life

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defects in learning and memory. Defects in subtler behavioral outcomes, such as fear responses and social interactions, have also been reported in both rodents and primates (Alvarado et al., 2017; Coleman et al., 2017; Lei et al., 2013; Raper et al., 2015; Shi et al., 2017). Acute effects of AIN have generally been quantified by staining for neurodegeneration and apoptotic cells, and cell death appears to occur in both neurons and glia.

The precise period of neonatal sensitivity to anesthesia is controversial. Some rodent studies suggest that neuronal progenitor cells remain sensitive to anesthesia throughout life, and that the period of neonatal sensitivity is primarily defined by the relative fraction of progenitors involved in rapid brain post-natal rodent brain development (Hofacer et al., 2013). However, the period of neonatal rodent hypersensitivity is generally thought to peak around post-natal day 7, with anesthetics causing little or no degeneration or behavioral defects when administered after post-natal day 10 (Jevtovic-Todorovic, 2012; Yon et al., 2005). The putative window of sensitivity is less clear in non-human primates, and there is no consensus in humans.

It is worth noting that AIN appears to occur at both extremes of life – in neonates and in the elderly (Figure 1). In contrast with pediatric studies, neurocognitive complications are well-documented in geriatric patients, involving a range of symptoms collectively referred to as ‘post-operative cognitive dysfunction’, POCD (Johnson et al., 2002). The mechanisms of POCD are unclear, but they appear to be at least partly distinct from those in the very young (Canet et al., 2003; Moller et al., 1998; Newman et al., 2001; Rasmussen et al., 2003). This review will focus on data from models of pediatric AIN models in mice.

The substantial literature surrounding the phenomenon of AIN involves mouse, rat, non-human primate, and invertebrate models of exposure. This literature is the subject of multiple reviews which outline the evidence for or against the hypothesis that exposure to anesthetics during early post-natal development has the potential for acute neurotoxicity and long-term adverse cognitive effects in humans (Lin et al., 2017; Vutskits and Davidson, 2017; Walters and Paule, 2017). Here, we summarize intervention studies in neonatal mouse models of anesthetic exposure, i.e. studies where pharmacological, dietary, or genetic manipulations were used to target putative pathways of AIN, providing an overview of proposed mechanisms underlying AIN in mice based on these reports.

Neurotoxicity of Anesthetics - Mechanisms Implicated through Rodent Intervention Studies

Early reports describing the neurotoxic effects of anesthesia were primarily descriptive, but current literature in rodents is now dominated by intervention studies. Mechanisms of AIN are inferred through manipulation of candidate pathways (see Table 1, Figure 2). The majority of putative targets fall into one of three general categories: 1) Reactive oxygen species (ROS) mediated stress and signaling; 2) growth/nutrient signaling; and 3) direct neuronal modulation. A comprehensive assessment of available rodent data provides a landscape of putative mechanisms of AIN.

Oxidative Stress and ROS Signaling

Oxidative stress and ROS signaling are the most widely reported therapeutic targets in rodent models of AIN. The most recognizable chemical antioxidant applied to this paradigm is ascorbic acid (vitamin C), which has been shown to attenuate many cellular, molecular, and behavioral endpoints of AIN *in vitro* and *in vivo*, including induction of cleaved caspase 3, increased ROS, mitochondrial permeability transition pore (MPTP) opening (a step in mitochondria mediated cell death), reduced ATP levels, and 'freezing time' in a fear conditioning assay, a cognitive behavioral phenotype (Cheng, B. et al., 2015; Xu, K.X. et al., 2015). Similarly, Trolox, a water soluble vitamin E analog, prevented neuron death in vitro in a study implicating mitochondrial ROS induced apoptosis in AIN (Bai et al., 2013).

Attenuation of AIN has also been reported in mice exposed to carbon monoxide (CO) or 'hydrogen rich saline' (HRS), two treatments shown to have general antioxidant effects (Cheng and Levy, 2014; Li et al., 2017). CO has been found to reduce mitochondrial ROS production in vitro, reportedly by binding to and inhibiting the peroxidase activity of cytochrome C, a source of mitochondrial ROS that is reportedly enhanced by isoflurane (Cheng and Levy, 2014; Kapetanaki et al., 2009). CO provided a dose dependent attenuation of molecular endpoints associated with anesthesia, with 5 ppm attenuating, and 100 ppm fully abrogating acute molecular and cellular markers of AIN. In agreement with these in vitro effects, CO treatment also partially attenuated behavioral defects induced by isoflurane (Wang, L. et al., 2017a). Similarly, HRS has been found to act as a potent antioxidant in vivo with, apparently, little in the way of off-target effects on redox or signaling (reviewed in Ohta, 2015 (Ohta, 2015)). The CO data are fascinating but have yet to be independently reproduced, while others have shown low dose CO can lead to neurodevelopmental defects in mice which are reminiscent of AIN (Trentini et al., 2016). Clearly, more work is needed to identify the ideal concentrations and exposure times of CO to alleviate AIN, and further characterize the mechanisms underlying the benefits of CO.

Curcumin and rutin, 'nutraceutical' compounds associated with many putative bioactive functions, have been used to prevent AIN in vivo (Ji et al., 2015; Man et al., 2015). IP injection of curcumin prior to sevoflurane exposure was reported to attenuate an array of outcomes including induction of cleaved caspase 3, expression of NADPH Oxidase 2 (Nox2, involved in cell non-autonomous ROS signaling), expression of brain derived neurotrophic factor (BDNF), expression of tumor necrosis factor alpha (TNFα), and prevented fear response defects. Similarly, rutin, provided orally, was found to prevent induction of cleaved caspase 3, circulating S100B, and Morris Water Maze defects. While the purported bioactive functions of these compounds are diverse, the putative antioxidant effects were implicated as mediating their benefits in the setting of AIN.

Each of these studies reported attenuation of AIN using general approaches to targeting oxidative stress through modulation of ROS levels. In contrast, a recent report by Makita et al. found that the specific NADPH oxidase inhibitor apocynin protected against AIN, as measured by the lipid peroxidation marker 4-HNE, the ROS indicator dye DHE, cleavage of caspase 3, and behavioral defects (Sun, Z. et al., 2016). NAPDH oxidases are membrane bound enzymes which produce superoxide in neutrophils and in cells involved in ROS mediated signaling, such as vascular smooth muscle cells (Garcia-Redondo et al., 2016;

Prieto-Bermejo and Hernandez-Hernandez, 2017). The apocynin results implicate ROS signaling, rather than oxidative stress, in mediating the benefits of antioxidants in AIN models, as inhibition of membrane bound NADPH oxidases would not be expected to impact intracellular ROS.

Attenuation of ROS levels is generally thought to protect cellular energetic status by preventing ROS induced damage to mitochondrial macromolecules, such as subunits the electron transport chain. Targeting energetics more directly has also been attempted. Ubiquinone (coenzyme Q10 or CoQ10), a vitamin-like cofactor which carries electrons from complexes I and II to III in the mitochondrial electron transport chain, rescued ATP levels, mitochondrial membrane potential, and Morris Water Maze performance defects resulting from anesthesia exposure. Ubiquinone did not, however, attenuate increased ROS levels induced by sevoflurane exposure (Xu et al., 2017). The authors concluded energetics directly mediate the benefits of CoQ10 in this paradigm.

Studies involving ROS are mired with several caveats. Methodological approaches to measuring ROS production, or even net ROS damage, are technically challenging, and prone to false positive findings (Egea et al., 2017; Gorlach et al., 2015; Griendling et al., 2016). Compounds with antioxidant effects demonstrated via chemical analysis do not necessarily act as antioxidants in vivo, and antioxidant therapies have proven ineffective in multiple clinical settings where ROS were thought to play a causal role (Goszcz et al., 2015; Sawyer, 2011). In recent years it has become apparent that ROS act as potent intracellular and extracellular signaling molecules, and modulation of ROS levels or production can also lead to unexpected changes in signaling. Finally, even when changes to ROS production and/or oxidative damage are clearly demonstrated, causality is extremely difficult to establish in the context of ROS. For these reasons, the role of ROS in AIN must be assessed with great caution. These pitfalls of ROS assays are reviewed extensively elsewhere (Egea et al., 2017; Gorlach et al., 2015; Griendling et al., 2016) and should be carefully considered in designing experiments aimed at determining the role of ROS in AIN. Even in the best designed study, it is extremely difficult to demonstrate causality between ROS and disease. State of the art methodologies such as single cell RNA sequencing, in situ RNA sequencing, and in vivo detection of oxidative stress and redox status may provide new temporal and spatial evidence linking ROS and CNS apoptosis (Bacic et al., 2016; Lee, 2017a, b; Zhu et al., 2017). In addition, future experiments where both the timing and cellular location of antioxidants are carefully controlled may help resolve the current uncertainty in the roles of ROS in causing AIN.

Growth and nutrient sensing signaling

Growth, differentiation, viability, and survival of neurons depends on the interactions of numerous extra-and intra-cellular signaling cascades, together often referred to as growth signaling or nutrient sensing signaling (see Figure 3). Extracellular growth factors include insulin, insulin-like growth factor 1 (IGF-1), growth hormone (GH), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and platelet derived growth factor (PDGF). Many of these systemic factors are directly or indirectly regulated by nutritional status, and each acts on one or more cell surface receptor tyrosine kinases (RTK's) or G-

protein coupled receptors (GPCR's) (Lemmon and Schlessinger, 2010; Wauson et al., 2013). Transduction to intracellular signaling is mediated by membrane associated factors such as phosphatidyl inositol 3 kinase (PI3K), which activates intracellular mediators of growth/nutrient signaling (Paez and Sellers, 2003; Piwien-Pilipuk et al., 2002). The mechanistic target of rapamycin (mTOR) is a hallmark intracellular mediator of nutrient growth signaling (reviewed in (Saxton and Sabatini, 2017)). mTOR activation tunes up anabolic processes, such as mRNA translation, and dampens catabolic processes, such as autophagy. mTOR is positively regulated by cell receptor/PI3K signaling via AKT mediated inactivation of the mTOR inhibitors TSC1/TSC2.

Growth/nutrient signaling pathways are also highly regulated intracellularly. AMP activated kinase (AMPK) is an mTOR inhibitor that is activated by low cellular ATP status. REDD1/2 are hypoxia sensors that inhibit mTOR in low oxygen conditions. mTOR is also regulated by amino acid levels at the lysosome and modulated by ribosome status, linking mTOR activity to functional capacity for mRNA translation. Together, the intra- and extra-cellular regulation of growth/nutrient sensing signaling provides for cellular adaptation to a variety of stressful conditions. Various reviews detail the role of growth/nutrient signaling in neuronal development (Switon et al., 2017; Takei and Nawa, 2014).

Nutrient sensing and signaling pathways are critical to neurogenesis, synaptogenesis, and neuron development, migration, and survival (Alsina et al., 2012; Lee, 2015; Nieto-Estevez et al., 2016). Genetic defects in growth/nutrient signaling cause overt neurological conditions including epilepsy, while subtler abnormalities in nutrient signaling are implicated in neurological disorders ranging from autism to Alzheimers's (Adachi et al., 2018; Bedse et al., 2015; Borrie et al., 2017; Wang, L. et al., 2017b). Given their importance to neurodevelopment, it is unsurprising that these pathways have also been implicated in the pathogenesis of AIN through both in-vivo and in-vitro studies. Here, we focus on in-vitro evidence supporting a role for nutrient signaling in AIN (*in vitro* models are reviewed elsewhere, for example see (Wang, 2012; Wang and Slikker, 2008; Wang, C. et al., 2017)).

In some studies, isoflurane has shown to reduce levels of activating phosphorylation on AKT, GSK3B, and ERK through unknown mechanisms in the setting of both neonatal (Tao et al., 2016; Wang et al., 2012) and adulthood (Liu et al., 2014) exposures in mice. In mouse neonates, treating isoflurane anesthetized mice with lithium chloride (a poorly understood but broadly neuroactive compound) attenuated isoflurane induced decreases in AKT and GSK3B phosphorylation, and prevented neuronal death and learning/memory defects (Morris Water Maze, MWM) associated with AIN in neonatal mice (Tao et al., 2016; Wang et al., 2012). Roscovitine, an inhibitor of the cell-cycle regulator cyclin dependent kinase 5 (CDK5), was recently reported to prevent AIN through activation of the ERK pathway (Liu et al., 2017a). The reported benefits of CO have been ascribed to antioxidant effects (as mentioned above) (Levy, 2017) as well as to inhibitory effects on intracellular signaling. Specifically, CO has been found to modulate cAMP, p38 MAPK, and PKB/AKT signaling, increasing mitochondrial biogenesis, inhibiting inflammation, and preventing anesthesia induced apoptosis. The mechanistic underpinnings of these 'signaling' functions are yet to be defined, but the authors suggest that CO prevents anesthesia related damage at least partly through these intracellular signaling modulations.

The observation that anesthetics alter signaling is consistent with reports finding modest or no increase in cell death following anesthesia but observing behavioral defects nonetheless. Changes to signaling pathways could modify neuronal development and function independently of cell loss. Signaling may also provide a reasonable model for neonatal hypersensitivity to AIN (compared to adult animals) given the role of PI3K/AKT pathways in nervous system development, as discussed. It has been suggested that volatile anesthetic induced alterations in signaling impact neurogenesis and reduce neural stem cell pools in young, but not adult, animals. This is suggested by observed reductions in BrdU/NeuN positive (proliferating) and Sox-2/GFAP (neural progenitor) cells in young rodents exposed to volatile anesthetics (Zhu et al., 2010). A similar model supported by Hofacer et al suggests that the age of neurons, not the age of the organism, underlies sensitivity to volatile anesthetic toxicity, and that young animals are more sensitive overall simply because they have a greater pool of ‘young’ neurons (Hofacer et al., 2013). Voluntary exercise and environmental enrichment are both neurogenesis promoting interventions which have been reported to function through growth signaling, and both have been shown to attenuate AIN in mice (Zheng et al., 2013).

A number of *in vitro* studies of cultured neurons have found that anesthetic exposure alters neuronal survival, morphology, and function through activation of the neurotrophin receptor (p75NTR) and subsequent downstream activation of the actin cytoskeleton regulating kinase RhoA. In addition, inhibition of p75NTR can prevent apoptotic neuron death and cytoskeletal depolymerization resulting from anesthetics (Head et al., 2009; Lemkuil et al., 2011; Schallner et al., 2014). Inhibition of this p75NTR/RhoA pathway *in vivo* with the p75NTR inhibitor TAT—Pep5 did not attenuate memory defects induced by anesthesia exposure, suggesting an uncoupling of this apoptosis-mediating pathway from cognitive effects (Schilling et al., 2017). While intriguing, this finding is difficult to interpret, however, due to the absence of molecular data or a positive control, specifically the lack of apoptosis data derived from the in vivo model.

The precise role of growth and nutrient signaling remains controversial, however, as some reports suggest signaling *activation*, not inhibition, by anesthetic exposure (Liu et al., 2015). In line with this view, neonatal hyper-nutrition (accomplished through a neonatal ‘high-fat’ paradigm) has been reported to worsen AIN, although the authors attributed the effects to oxidative stress in the ‘obese’ pups (Xu, K.X. et al., 2015). In mouse models of Alzheimer’s disease, anesthetics have been reported to increase the phosphorylation of various signaling factors including PI3K, AKT, MAPK, and JNK, with some work suggesting that anesthetics directly inhibit the phosphatase PP2A, thereby increasing phosphorylation status among PP2A targets. Such a change would broadly disrupting nutrient signaling through inhibition of a negative regulator of activation (Le Freche et al., 2012; Tao et al., 2014). Other reports indicate that ER calcium release induced by ryanodine receptors (RYR’s), downstream of PI3K, mediates the neurotoxicity of anesthetics (Liang et al., 2010; Wang et al., 2014). Blocking RYR’s with the antagonist Dantrolene was found to reduce cell death and ER stress induction in cultured mouse brain slices treated with isoflurane. ER stress has also been shown to mediate isoflurane induced neurotoxicity during developmental stages in the nematode *C. elegans* in an mTOR dependent pathway (Na et al., 2017).

Conflicts in reported pathway directionality may be at least partly the result of the transient nature of intracellular signaling cascades and the timing of measurement. For example, one study indicates that GSK3 and AKT phosphorylation is increased by short term exposure to volatile anesthetics but decreased by long term exposure (Zhang et al., 2014). The variability is also likely to be at least partly a result of differing anesthetic protocols (see Figure 4). Accordingly, while nutrient signaling appears critically important, new approaches with careful experimental design, execution, and interpretation are needed to resolve these controversies. Most pressing, perhaps, are the need for standardization of anesthetic conditions in neonatal mouse AIN models and an effort to carefully model clinical exposures. Careful temporal dissection of the impact of anesthetics on nutrient signaling in rigorously monitored anesthetic exposures may provide a unifying model for the role of signaling in AIN. As in the case of ROS, state of the art techniques assessing single cell changes in signaling may provide direct causal evidence linking nutrient signaling to apoptotic death or altered neuronal function. Finally, experiments aimed at defining mechanistic links between nutrient signaling and other putative pathways of AIN (ie ROS, neuronal modulation, etc) will clarify the relationship between nutrient signaling and other pathways of damage in relation to the overall pathogenesis of AIN.

Additional Targets

Direct neuronal modulation—AIN has also been attenuated through direct modulators of neuronal activity, though much of the data comes from non-neonatal settings. In one study, acute treatment with dexmedetomidine, an alpha-2 agonist, prevented anesthesia induced inflammation and cognitive defects in geriatric mice (Qian et al., 2015). Attenuation of AIN by dexmedetomidine implicates excitotoxicity in anesthesia related neurotoxicity, suggesting that select non-volatile anesthetics may actually prevent the neurotoxicity of volatile agents. Consistent with this idea, Hispudilin, a nutraceutical with ‘potent anti-antiepileptic activity in rats’ and putative a $GABA_AR$ agonist, attenuated anesthetic neurotoxicity in vitro (see also above) (Niu et al., 2014). The most directly supportive data is provided by memantine, a clinically approved drug for Alzheimer’s disease which targets excitotoxicity through uncompetitive inhibition of glutamatergic AMPA. Memantine was found to attenuate the induction of an epigenetic biomarker associated with anesthesia induced neurotoxicity in P7 neonatal mice sedated with sevoflurane (Han et al., 2015). Direct modulation of neuronal signaling can also exacerbate AIN; caffeine, an adenosine receptor antagonist, substantially worsened AIN phenotypes, although the paradigm (P3 mouse pups), the exceptionally high dose of caffeine (80 mg/kg, roughly 50% of the LD50 for subcutaneous caffeine in adult mice according to Caymen chemicals), and lack of a caffeine-only control cohort greatly limit the interpretability of this report (Cabrera et al., 2017).

Central nervous system metabolism and peripheral metabolism are both acutely altered by some anesthetics, but the precise impact of any given anesthetic on individual metabolic pathways and the role of metabolic shifts in AIN are unclear (Yamada et al., 2009). Serum metabolites have, remarkably, been generally ignored in AIN studies. When circulating metabolites have been considered, hypoglycemia has been repeatedly identified as a physiological consequence of volatile general anesthetic use. 3% isoflurane induces

substantial hypoglycemia by 90 minutes in 10 day old neonatal mice, and extended (6 hour) exposure to 1.5% isoflurane induces hypoglycemia in P7 mice (Loepke et al., 2009; Loepke et al., 2006). Furthermore, 6 hours of equipotent 1.5% isoflurane, 2.9% sevoflurane, or 7.4% desflurane all resulted in significantly reduced blood glucose in neonates compared to fasted controls in CD1 x C57Bl/6 hybrid mice, a background specifically selected for its 'low mortality' (of 20%) during 6 hours of treatment (Istaphanous et al., 2011). One report has suggested that dextrose administration fails to prevent neuronal apoptosis or learning and memory defects in isoflurane anesthetized pups. The same report presented data showing that 6 hours of 0.75% isoflurane induced neuronal apoptosis and cognitive defects without leading to marked hypoglycemia (Johnson et al., 2008). Circulating metabolites therefore appear important, but glucose alone may not fully explain AIN in mice. It is worth noting that no study to date has provideed a rigorous assessment of the physiological impact of extended anesthesia in neonatal mice.

Inflammation—Genetic deletion of FAS and FASL, two factors involved in extrinsic apoptotic signaling, prevented induction of apoptosis related to anesthesia exposure in neonatal mice (Song et al., 2015). This result suggests that the majority of AIN related apoptosis results from extrinsic, rather than intrinsic (mitochondrial), apoptotic pathways. Knockout of the receptor for IL-1B, a major cytokine involved in inflammatory signaling, was shown to prevent post-operative cognitive dysfunction in mice (Cao et al., 2012; Cibelli et al., 2010). Together, these findings suggest that neuronal apoptosis and behavioral defects associated with volatile anesthetic exposure require, and can be greatly attenuated by targeting, cell-extrinsic factors. These data are at odds with studies reporting that apoptosis results from cell intrinsic inducers such as mitochondrial oxidative stress, reduced mitochondrial membrane potential, or cellular energetic stress, and discussed above.

Epigenetics—Epigenetic and miRNA targets have also been studied in the context of AIN, though the results are difficult to interpret. Knockdown of miR-124 and miR-210 have been reported to attenuate anesthesia induced neuronal death in vitro or in vivo (Wang et al., 2016; Xu, H. et al., 2015). miR-124 was shown to activate the PKC/ERK pathway in brain and upregulate AMPA receptor phosphorylation, but direct targets and a clear mechanism were not identified. Daily oral administration of SAHA (suberanilohydroxamic acid), a histone deacetylase inhibitor with broad and non-specific actions on epigenetic regulation through histone deacetylation, was reported to modify Morris Water Maze performance in mice exposed to sevoflurane as neonates (Lin et al., 2014). While intriguing, each of these studies lack clear rationale for their epigenetic target of interest, and with such broadly acting regulatory factors, particularly histone acetylation, it is entirely unclear what the mechanisms or off-target effects of these approaches might be.

Limitations of Pre-Clinical Studies

Any discussion of the phenomena of AIN would be incomplete without a critical evaluation of the pre-clinical literature. While, as stated above, it is clear that anesthetics can cause neurotoxicity under various experimental conditions, a multitude of factors have muddied the AIN field. AIN studies tend to lack sufficiently detailed methodological reporting, often have inappropriately low sample numbers, and are missing appropriate controls.

Furthermore, the high variability in anesthetic protocols and conditions largely precludes comparisons between individual reports (see Table 2). These issues have led to highly discordant body of research that is not easily extrapolated to the clinical setting. Finally, it remains unclear whether AIN models in mice are relevant to human anesthesia exposure. As noted elsewhere, mouse exposures are poorly monitored when compared to human anesthesia, and a detailed assessment of the physiological impact of anesthesia on neonatal mice is lacking (Van Biesen et al., 2015).

The lack of standardization in anesthetic protocols and conditions is arguably one of the greatest limitations of the AIN field. Even among only neonatal mouse studies it is immediately clear that there are is no standardization in pre-clinical models (Table 2, Figure 4). Chosen anesthetic agents include volatile compounds such as isoflurane, desflurane, and sevoflurane, as well as injectable drugs compounds like midozalam, ketamine, and propofol, and AIN studies sometimes include complex combinations of drugs. There is no consensus on effective or clinically relevant doses or exposure time, even in studies using single agents. Regarding duration, some laboratories report AIN phenotypes only when anesthetic exposure is long enough to induce significant mortality from cardio-respiratory failure, a situation which seems unlikely to model human clinical anesthesia. Others suggest that even very short term anesthesia can have overt effects on neuron viability and lasting effects on learning and memory. There is disagreement over whether single or repeat exposures are necessary to induce behavioral outcomes, a question of particular significance to the interpretation of human clinical trials.

The variation in timing, dose, and anesthetic choice is mirrored by variability in other conditions. Oxygen levels, temperature, humidification, flow rate, ventilation method, animal genetic background, age, gender, and physiological maintenance (i.e. dextrose infusion) may all be critically important to the outcome, but no consensus exists. In fact, many studies simply ignore these factors in their methodology, data, and discussions, leaving the reader to guess at how the animals were maintained. Finally, there is little overlap between molecular, cellular, and behavioral endpoints; even commonly used behavioral studies such as Morris water maze very widely when the details of implementation are examined. Simply put, the extraordinary number of known variables which are uncontrolled between published datasets completely precludes any broad cross comparisons or large-scale data reduction efforts.

The complexity of Figure 2 may be reasonable if each pathway or process played a partial role in the overall outcome, but published reports tend to show complete or nearly complete prevention in almost every case. Because of this, it is difficult to imagine a cohesive model for published findings in AIN. Published data can only be judged on statistical rigor, appropriate controls, and cautious interpretation of results. Some discrepancies may result from unstated caveats. For example, antibody based detection methods provide powerful tools but as reagents they vary greatly in quality and benefit from the inclusion of additional controls, such as secondary-only staining in IHC (see ref's (Gorr and Vogel, 2015; Ivell et al., 2014; McDonough et al., 2015)). Similarly, litter-effects are of particular importance in neonatal AIN research. Litter-effects are known to invalidate standard statistical assumptions of sample independence and normal distribution and have recently been shown to be a

frequent cause of false positives in mouse behavioral studies (see (Williams et al., 2017)). Population variability should be carefully assessed, with considerations given to both total sample size and litter distribution between treatments. These issues are not unique to the AIN field, but may be contributing to the complexity of the pre-clinical AIN literature.

Current perspective and additional avenues

Recent evidence from large-scale clinical trials now indicates that short duration routine anesthetic exposure in pediatrics is generally safe and without overt neurological risks, but the risks of multiple or long-term exposures remain unclear (Chinn et al., 2016; Davidson et al., 2016; Ing et al., 2016; Miller et al., 2016; Sun, L.S. et al., 2016; Vutskits and Davidson, 2017). While these unanswered questions warrant further study, the continued relevance of pre-clinical research on AIN will depend on how researchers model these clinical paradigms and whether the quality and comparability issues in the AIN field can be resolved. One of the most pressing issues in pre-clinical AIN studies is arguably the need for some form of standardization in experimental approaches, as discussed above. The intervention literature has provided a variety of intriguing targets for attenuating off-target toxic effects of anesthetics, but until these studies are convincingly validated they are unlikely to impact clinical practice.

It is clear that anesthetic agents, as any chemical compound, can cause cell death under certain conditions. While the *potential* for anesthetic exposures to cause damage to the neonatal brain is well-supported, the relevance of pre-clinical models to patient care remains unclear. While pathways underlying the molecular, cellular, and behavioral outcomes in pre-clinical models have been extensively probed. Data derived from neonatal mouse models of AIN strongly implicate ROS, nutrient/growth signaling, and neuronal activity in the pathogenesis of neurotoxicity resulting from anesthetic exposure in neonates, but individual reports have varied widely and the relative importance of each of these mechanisms is unclear. The risks of anesthesia in pre-clinical models is supported by a variety of data, but it is not yet clear which animal model approaches best reflect or advise the use of anesthesia in human patients. These models generally utilize long duration or repeated exposure to anesthetics under only partially controlled experimental conditions, a setting which are unlikely to fully mirror human pediatric clinical exposures. Addressing these questions will require a critical re-evaluation of the primary pre-clinical AIN literature in addition to carefully constructed clinical trials.

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Highlights

- Anesthetic exposure has been reproducibly shown to induce central nervous system cell death in neonatal mouse models
- Anesthesia exposure in neonatal mice is also reported to result in long-term neurocognitive defects, such as defects in learning and memory
- Intervention studies in the neonatal mouse model have identified multiple putative mechanistic pathways underlying anesthesia induced neurotoxicity; the major mechanistic targets implicated are oxidative stress, altered nutrient/growth signaling, and direct neuromodulation
- A lack of standardization between studies and technical issues surrounding the study of anesthesia induced neurotoxicity in neonatal rodents complicate interpretation of the this field

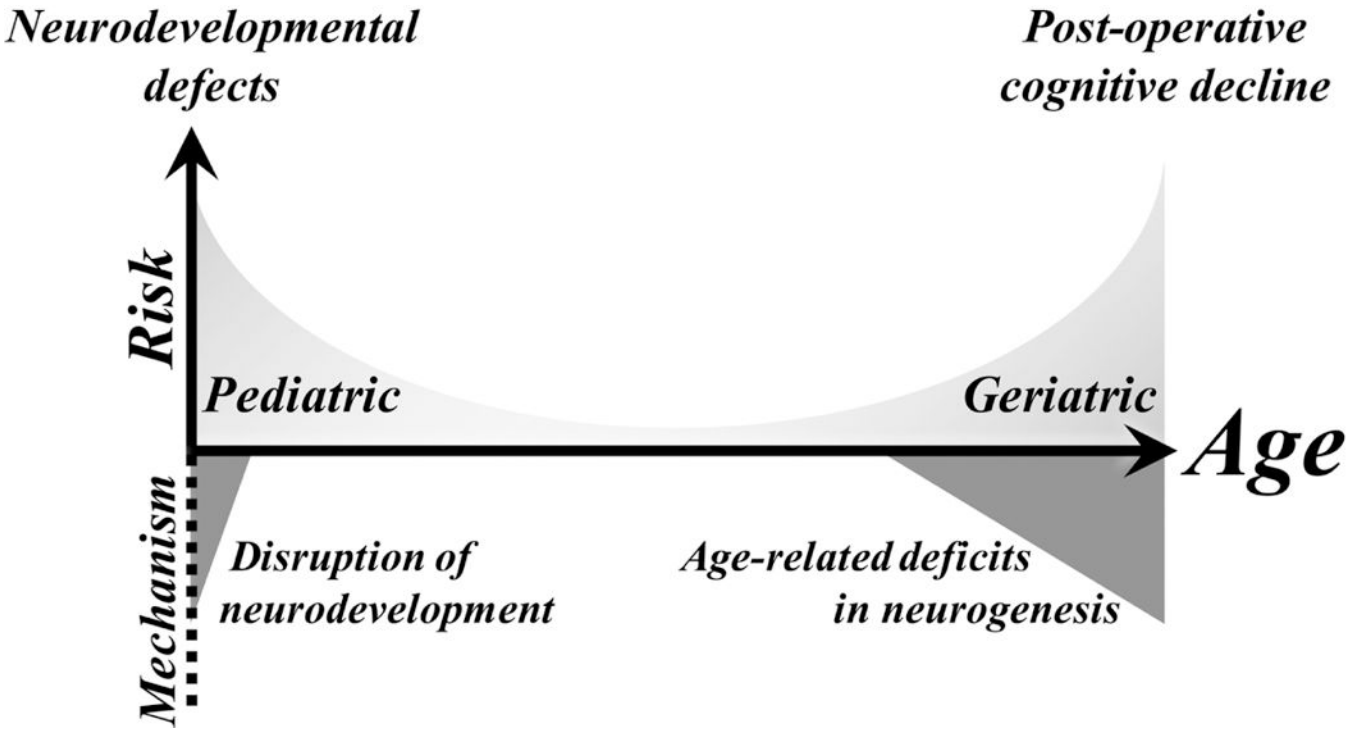

Figure 1: Neurotoxicity of anesthesia appears to occur at both extremes of age.

Anesthetics are associated with neuronal death and adverse cognitive effects in both pediatric and geriatric populations. While the precise mechanisms of anesthesia induced neurotoxicity are unclear, data suggests that anesthetics have some neurotoxicity at all ages. In pediatric patients, this neurotoxicity disrupts normal neurodevelopment, and neonates are highly sensitive to as a result of their relatively high number of young neurons. Conversely, sensitivity to anesthesia in geriatric patients appears to result from age-related deficits in neurogenesis which exacerbate the functional impact of neuron loss due to anesthesia exposure.

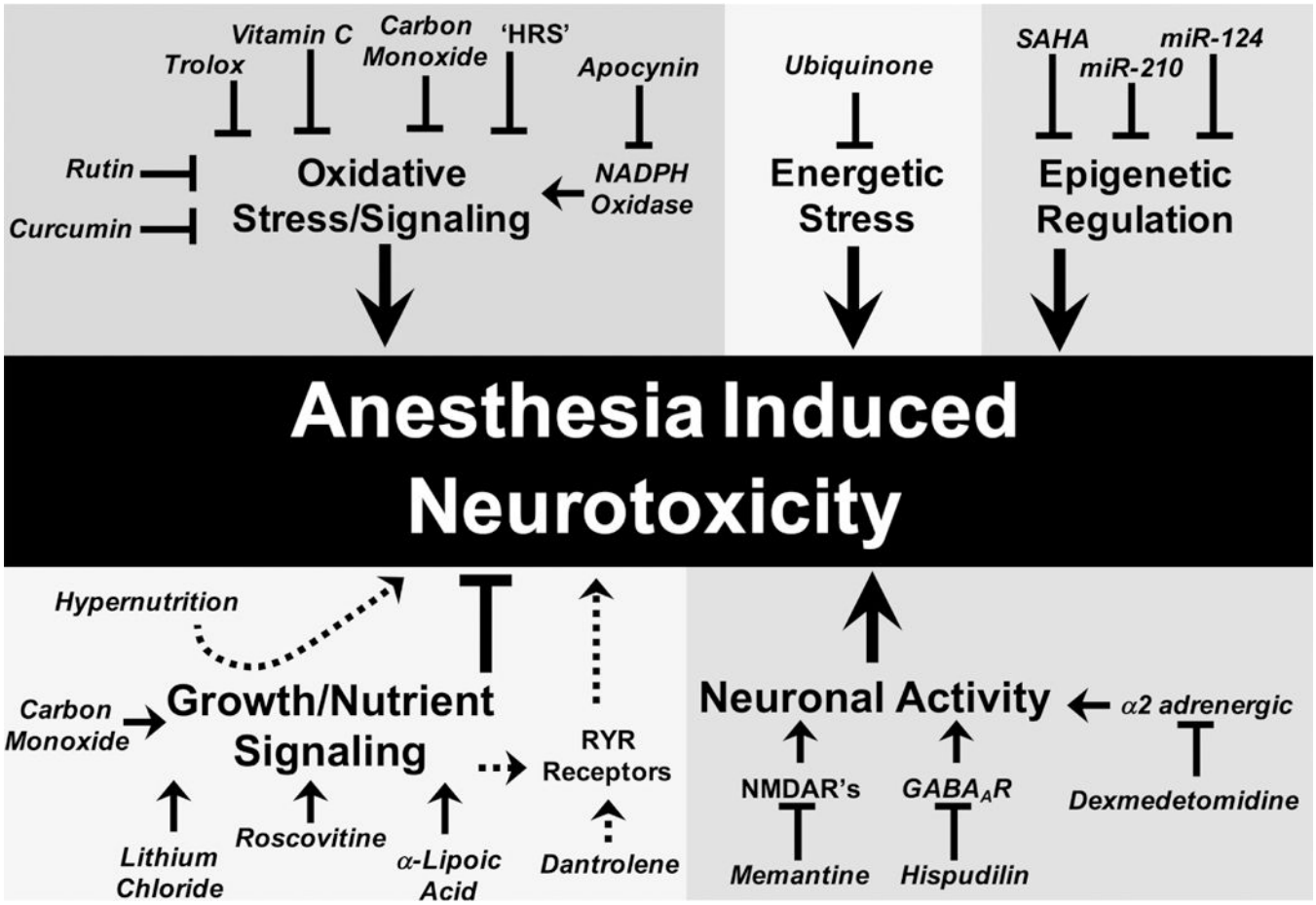

Figure 2: Interventions and mechanisms reported in mouse models of AIN.

Intervention studies in mouse AIN models have identified a wide variety of candidate targets and compounds. The majority of these can be grouped into one of three major categories: oxidative stress, ROS signaling, and energetics; growth/nutrient signaling; and direct modulation of neuronal activity. Additional candidates, such as epigenetic factors, are less clearly defined.

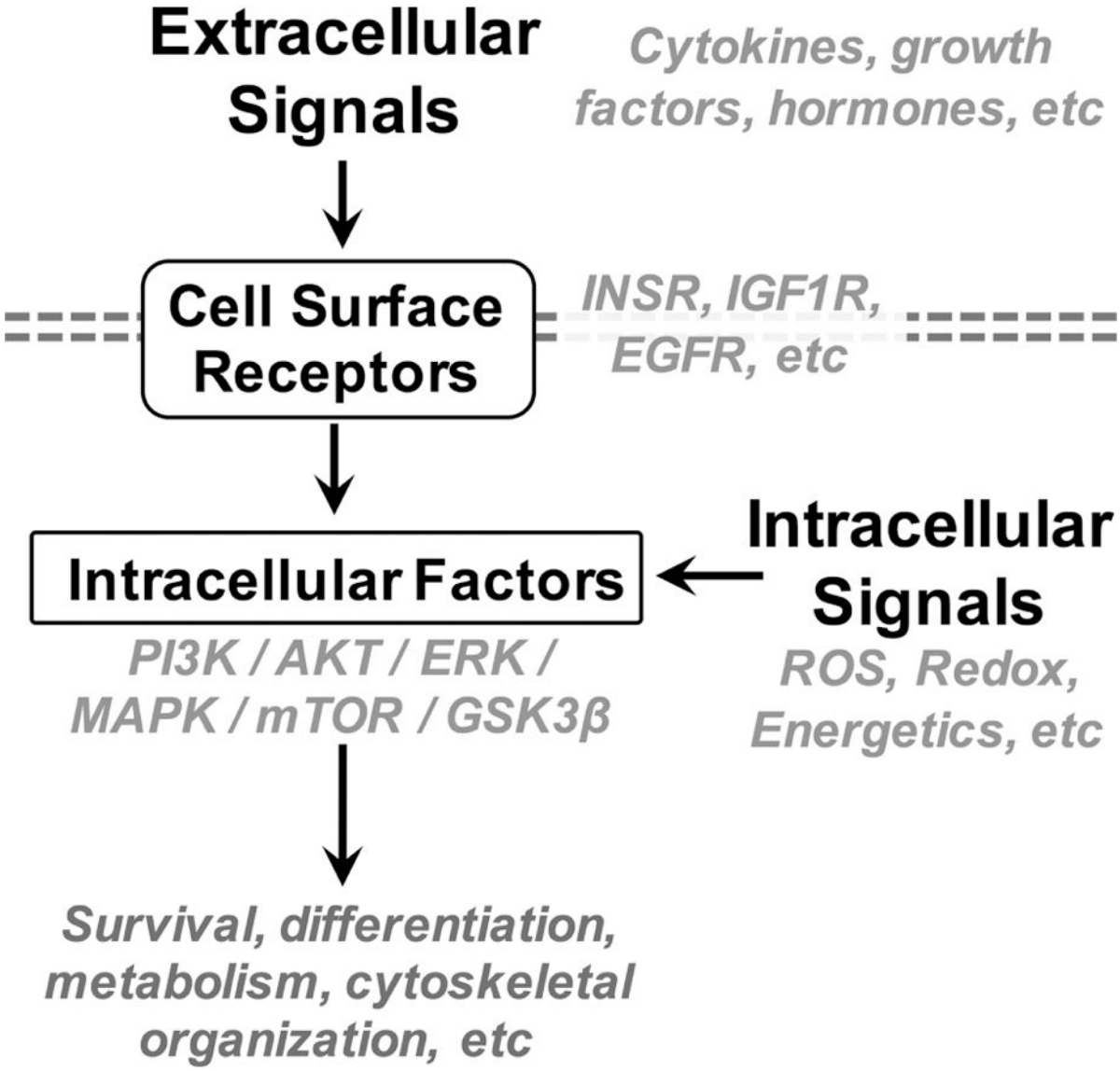

Figure 3: Growth and nutrient sensing signaling pathways at the interface between intra- and extra-cellular stimulus.

Growth and nutrient signaling pathways involve soluble signaling factors, membrane bound receptors at the cell surface, intracellular sensors, and intracellular kinases which mediate signal transduction and amplification. Growth and nutrient signaling pathways, such as the canonical PI3K/AKT/mTOR pathway, play critical roles in neuron survival, differentiation, metabolism, and cellular organization.

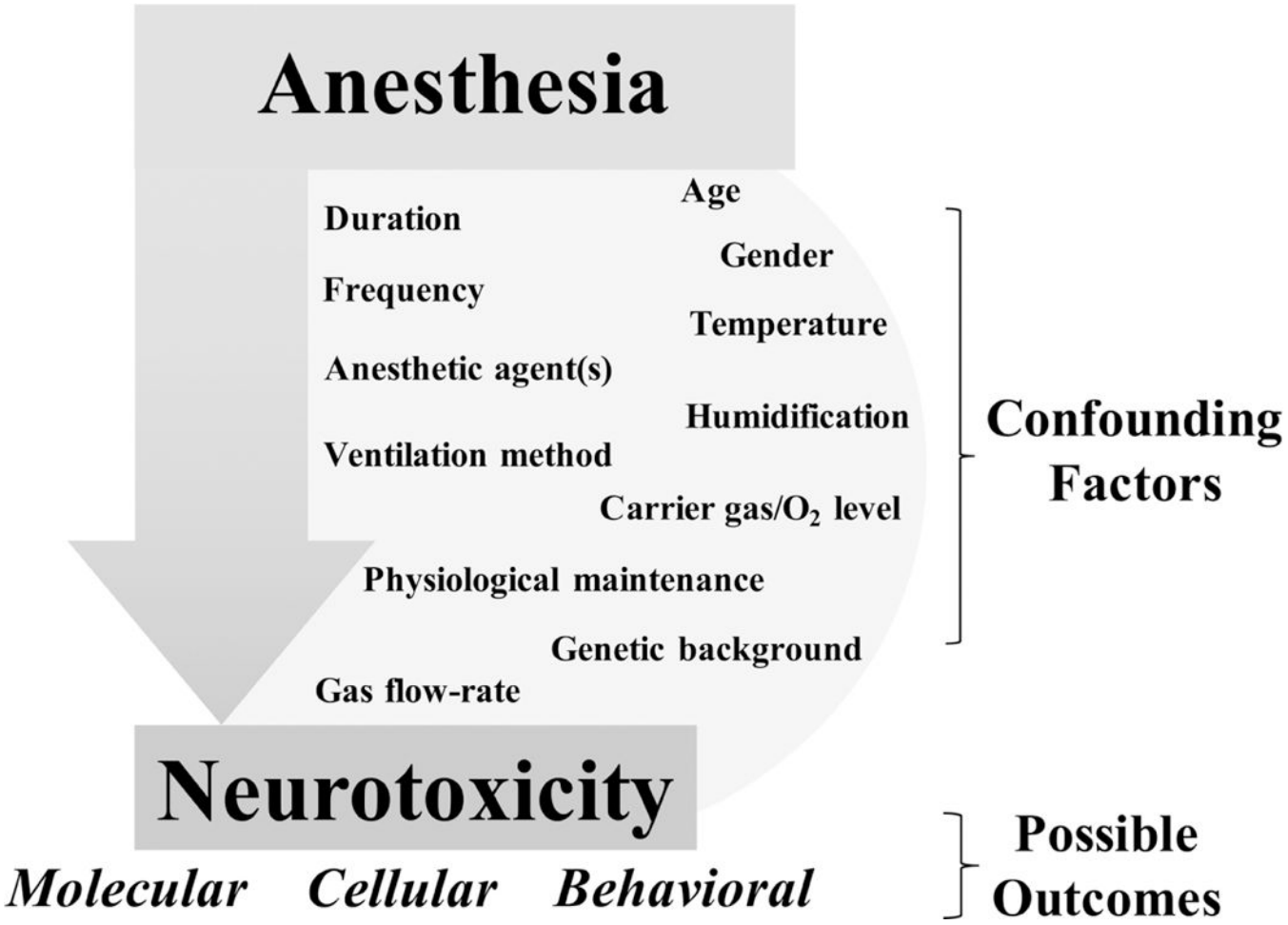

Figure 4: Sources of variability in pre-clinical anesthesia induced neurotoxicity literature.

Pre-clinical models of anesthesia induced neurotoxicity have identified a variety of pathways involved in AIN, but a global assessment of these studies is hampered by the high variability in experimental conditions. Confounding factors include anesthetic dose, frequency, duration, and various aspects of physiological maintenance. In addition, measured outcomes vary widely between studies. Future work in pre-clinical AIN would benefit from standardization of anesthetic protocols and experimental methods used to assess AIN.

Table 1 –

AIN Interventions Tested *In Vivo* in Mice

Compound	Dose	Mechanism(s)* Known (k), reported (r), or putative (p)	Report(s)
Apocynin	50 mg/kg	NADPH oxidase inhibitor (k); reduces oxidative stress (r)	(Sun, Z. et al., 2016)
Carbon Monoxide	5 ppm; 100 ppm	Reduced oxidative stress (r)	(Cheng and Levy, 2014; Cheng, Y. et al., 2015; Wang, L. et al., 2017a)
Coenzyme Q10	50 mg/kg	Electron transport chain component (k);antioxidant (r)	(Xu et al., 2017)
Curcumin	20 mg/kg; 40 mg/kg	Antioxidant (p)	(Ji et al., 2015)
Dexmedetomidine	15 g/kg; 25 g/kg	2 adrenergic receptor agonist (k)	(Qian et al., 2015)
Environmental enrichment	2 hrs/day from P7 to P31	Increased neurogenesis (p)	(Zheng et al., 2013)
'Hydrogen rich saline'	n/a	Antioxidant (p)	(Li et al., 2017)
Lithium Chloride	100 mg/kg	AKT/GSK3/ERK activator (p)	(Tao et al., 2016)
Memantine	1 mg/kg	NMDA glutamate inhibitor (k)	(Han et al., 2015)
Roscovitine	25 mg/kg/day	CDK5 inhibitor, action through ERK (p)	(Liu et al., 2017a)
Rutin	25 mg/kg 50 mg/kg	Unknown; nutraceutical, putative antioxidant (p)	(Man et al., 2015)
Suberanilohydroxamic acid (SAHA)	25 mg/kg	Histone deacetylase inhibitor (k)	(Lin et al., 2014)
Vitamin C	80 mg/kg	Multiple; antioxidant (k,p)	(Cheng, B. et al., 2015; Xu, K.X. et al., 2015)
Contraindicated (worsens AIN)	**Dose**	**Mechanism(s)**	**Report(s)**
Caffeine	80 mg/kg	Adenosine receptor agonist (k)	(Cabrera et al., 2017)
High-fat diet	Induced by reducing litter size and maternal high fat chow	Unknown in this paradigm	(Xu, K.X. et al., 2015)
Genetic	**Manipulations**	**Gene Product Function**	**Report**
FAS or FASL	KO	Ligand and receptor for extrinsic apoptosis (k)	(Song et al., 2015)
IL-1B	KO	Inflammatory cytokine (k)	(Cao et al., 2012)
miR-124	Knockdown	Unknown; most highly expressed miRNA in neurons	(Xu, H. et al., 2015)
miR-34a	Knockdown	Regulates FGFR1 (p)	(Jiang et al., 2014)

*Known – generally accepted function supported by robust published pharmacological data; reported – mechanism of action proposed in the setting of AIN if distinct from known mechanisms of action (or if none known); putative – the mechanism suggested by the authors when little mechanistic data is available (includes commonly reported compounds with poorly described mechanisms of action, for example nutraceuticals).

Table 2 –

AIN Paradigms in Neonatal Mice

Age	Background/Gender	Drug(s)	Dose and time	Other Conditions	Endpoints	Report
P6–8	C57Bl/6; gender not indicated	Sevoflurane	3% 2hr/day for 3 days	60% oxygen	Morris water maze at P30–34; assays for apoptosis following treatment	(Song et al., 2015)
P7	C57Bl/6; both genders	Propofol	30 or 60 mg/kg IP injected	37 degrees C	Apoptosis and neurogenesis at P8 or P17; AKT/ERK signaling	(Huang et al., 2016)
P6	C57Bl/6; male only for behavior, both genders for molecular endpoints	Sevoflurane	3% for 6 hours	40% oxygen, 38 degrees C	Apoptosis related endpoints immediately after treatment	(Sun, Z. et al., 2016)
P7	C57Bl/6; both genders	Isoflurane; Lidocaine; Lidocaine plus midazolam	Isoflurane: 0.75% for 6 hours; Lidocaine: 4 mg/kg subcutaneous; midazolam: 9 mg/kg subcutaneous	Warmed with heat lamp, agitated as needed to increase respiratory rate	Apoptosis related endpoints from samples 6 hours after exposure	(Lee et al., 2014)
P7	(F) C57Bl/6 x (M) CD-1 hybrid offspring; gender not indicated	Isoflurane	1.5% for 6 hours	30% oxygen, 35.5 degrees C	Apoptosis related endpoints after exposure; Morris water maze and spontaneous activity at P70	(Loepke et al., 2009)
P5–7	C57Bl/6, gender not indicated	Isoflurane; isoflurane plus midazolam	0.75% 4 hours 0.75% plus midazolam 6 hrs; 1.5% 2 hours; 2% 1 hour	30 degrees C	Apoptosis related endpoints	(Johnson et al., 2008)
P14	C57Bl/6; males	Isoflurane	1.7%, 35 min/day for 4 days	50% oxygen, 37 deg C	Apoptosis related endpoints; neurogenesis	(Zhu et al., 2010)
P6–8	C57Bl/6; both genders	Sevoflurane	3% isoflurane, 2 hours/day for 3 days	60% oxygen, 37 degrees C	pGSK3, pAKT	(Zhang et al., 2014)
P6	C57Bl/6; both genders	Sevoflurane	2.2%, 2 hours per day for 3 days	37–38 deg C	Morris water maze at P40	(Liu et al., 2017a)
P6	C57Bl/6; both genders	Isoflurane, desflurane	2% isoflurane or 8% desflurane (0.7 MAC) 2 hrs/day for 3 days	60% oxygen, 37 deg C	pAKT, pGSK3 after exposure; Morris water maze P31–37	(Tao et al., 2016)
P10	129T2/SvEvMsJ x C57BL6/J F1 hybrid, both genders	Isoflurane	3% isoflurane, 90 minutes	Not specified; mechanical ventilation for some at 300 breaths/min	Metabolic parameters	(Loepke et al., 2006)
P6	C57Bl/6; both genders	Sevoflurane	2%, 6 hours	40% oxygen, 1L/min flow, humidified	Apoptosis related parameters	(Satomoto et al., 2016)
P7	C57Bl/6; both genders	Isoflurane; propofol	1.5% isoflurane or 150 mg/kg propofol	Isoflurane: in 30% oxygen	Apoptosis related parameters; Morris water maze at P39	(Yang et al., 2014)

Age	Background/Gender	Drug(s)	Dose and time	Other Conditions	Endpoints	Report
P6-P8	C57Bl/6; males	Sevoflurane	3% 2 hours/day for 3 days	40% oxygen, 37 degrees C	Apoptosis related parameters after exposure	(Ji et al., 2015)
P7	C57Bl/6; both genders	Sevoflurane; propofol	2.9% sevoflurane 6 hours or 150 mg/kg propofol	Sevoflurane in 30% oxygen, 38 deg C, humidified	Apoptosis related parameters after exposure; morris water maze at P31–34	(Man et al., 2015)
P7	C57Bl/6; not indicated	Sevoflurane; sevoflurane plus propofol or thiopental	3% for 6 hours with or without 5 mg/kg thiopental or 10 mg/kg propofol	Not available	Apoptosis related parameters after treatment	(Tagawa et al., 2014)
P6–8	C57Bl/6	Sevoflurane	3%, 2 hours daily for 3 days	60% oxygen, 37 degrees	Apoptosis related parameters; Morris water maze at P31–37	(Lin et al., 2014)
P7	C57Bl/6; both genders	Sevoflurane, isoflurane, desflurane	Isoflurane: 1.5% 6 hours; sevoflurane 2.9% 6 hours; desflurane: 7.4% 6 hours	30% oxygen, 38 degrees, humidified	Apoptosis related parameters; various behavior tests at P35	(Xu, K.X. et al., 2015)
P7	C57Bl/6CR; both genders	Isoflurane, sevoflurane	0.75% isoflurane for 6 hours; 1.1% sevoflurane 6 hours	30% oxygen, 38 degrees C, humidified	Apoptosis related parameters at 2 hours post exposure; Morris water maze at P42	(Liang et al., 2010)
P7	C57Bl/6; male mice	Sevoflurane	1.5%, 2 hours	Air oxygen, 37 degrees C	Protein/signaling changes	(Han et al., 2015)
P6–8	C57Bl/6J; both genders	Sevoflurane	3%, 2 hours daily for 3 days	60% oxygen, 37 degrees C	Synaptic protein levels; Morris water maze at P31–37	(Xu et al., 2017)
P14–21	C57Bl6; gender not indicated	Ketamine	50 mg/kg/day for 6 days	n/a	Morris water maze at 2 months; molecular endpoints	(Xu, H. et al., 2015)
P7	CD-1, male	Isoflurane	2%, 1 hour	Air oxygen	Retinal cell apoptosis immediately and 5 hours after anesthesia	(Cheng, Y. et al., 2015)
P7	CD-1, males	Isoflurane	2%, 1 hour	Air oxygen	Apoptosis related endpoints	(Cheng and Levy, 2014)
P6–7	C57Bl/6J; both genders for biochemical endpoints, males only for behavior	Sevoflurane	Titrated dose for 6 hours: 3.5% for 90 min, 3% for 90 min, then 2.5% for the final 3 hours.	30% oxygen	Autism related behaviors, memory assessed by fear related assays; apoptosis related endpoints	(Chung et al., 2015)
P7–9	C57Bl/6	Isoflurane,sevoflurane	1.5% isoflurane or 2.2% sevoflurane, 2 hours/day for 3 days	37–38 degC	Morris water maze; apoptosis related endpoints; expression of BDNF and synaptic proteins	(Liu et al., 2017b)
P7–8	CD-1 male x C57Bl/6 hybrid, both genders	Isoflurane, sevoflurane, desflurane	6hrs 1.5% isoflurane, 2.9% sevoflurane, or 7.4% desflurane	30% oxygen, 35.5 deg C	Apoptosis related endpoints	(Istaphanous et al., 2011)

Age	Background/Gender	Drug(s)	Dose and time	Other Conditions	Endpoints	Report
P7, P21, or P49	C57Bl/6J, both genders	Isoflurane	1.5%, 6 hours	30% oxygen, 35.5 deg C	Apoptosis related endpoints, neurogenesis	(Hofacer et al., 2013)
P7	C57Bl/6CR, both genders	Isoflurane, sevoflurane	0.75% isoflurane or 1.1% sevoflurane, 6 hours	30% oxygen, 38 deg C	Apoptosis, serum S100B, Morris water maze at P42	(Liang et al., 2010)
Prenatal – dams at gestation day 14	C57Bl/6J; pregnant females, both genders of pups used	Sevoflurane	2.5%, 2 hours	100% oxygen	Apoptotic, inflammatory, and synaptic markers; Morris water maze at P31	(Zheng et al., 2013)