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Mini Review

Advanced Techniques to Study Anesthetic Effects on the Nervous System

Review

Recently, there has been increased interest and concern regarding the safety of anesthetics on the long-term impairment of the central nervous system (CNS). The field of anesthesia-related toxicology, therefore, has engaged multiple scientific disciplines in attempt to identify the basic characteristics of the anesthetic agents that produce harmful acute and/or chronic effects on the nervous system. Currently, the clinical relevance of anesthetic neurotoxicity is an urgent matter of public health.

In the preclinical studies [1,2] it has been demonstrated that, at clinically-relevant concentrations, the commonly used general anesthetics including sevoflurane, can effectively induce and maintain a surgical plane of anesthesia in developing nonhuman primates and/or rodents as evidenced by lack of voluntary movement with reduced muscle tone and minimal reaction to physical stimulation.

Significant advances have been made in recent years in the techniques (e.g., molecular imaging, calcium imaging, lipidomics and 3D electron microscopy) that have revolutionized our ability to study anesthetic neurotoxicity. When combined with the classic/traditional methods, such as histo-pathological and biochemical assays, these research approaches make it possible to map and analyze genetic and complex phenotype variations, morphological and biochemical alterations, and dynamic pathological changes, as well as long-term behavioral deficits. These advanced approaches also enable us to dissect mechanisms that underlie anesthetic-induced neurotoxicity, and to develop potential protective strategies. This editorial provides examples of how these approaches have been applied to solve the major problems induced by inappropriate and/or prolonged anesthetic exposure and their underlying mechanisms.

3D Electron microscopy to quantify anesthetic-induced mitochondrial defects

It is known that the mitochondria play a pivotal role in the cell death pathways that are implicated in several types of neurodegeneration [3-5]. Mitochondria form a dynamic network within the cell; continuously fusing and dividing to meet the metabolic demands of the cell. It has been suggested that the topology of the inner mitochondrial membrane represents a balance between mitochondrial fusion and fission with defects corresponding to an imbalance in the process that ultimately may lead to cell death [6]. It has been reported that changes in overall mitochondrial shape and number due to a perturbation in the fusion/fission process appears to accompany inner-membrane remodeling during apoptosis, and administration of anesthetics (e.g., ketamine) significantly decreases both [7].

Mitochondrial ultrastructure is best studied directly using the high resolution afforded by electron microscopy (EM). Although transmission electron microscopy (TEM) has been used to distinguish inner membrane topologies with some success, one of the major drawbacks of conventional TEM is the inability to obtain three-dimensional (3D) information from two-dimensional (2D) micrographs. Recently, a relatively new EM method, serial block-face scanning EM (SBF-SEM), has the potential to bridge this gap [8]. The commercial SBF-SEM system, the Gatan 3View, houses a fully automated ultramicrotome within the specimen chamber of a field emission scanning electron microscope (SEM). Instead of imaging ultrathin sections on grids as in TEM, the 3View images the surface of the bulk sample using backscatter electron detection. After each scan, the sample block advances and a diamond knife removes a thin slice of sample (25-200 nm). The freshly cut block face is then imaged, and the process is repeated. The desired sample depth is *in situ*, building a stack of aligned SEM images that can be used to generate 3D reconstructions of data sets [9]. The data from the control and anesthetic SBF-SEM can be modeled with a single statistical equation that allows for different intercepts and slopes for the two experimental groups. The model is given by

$$V_i = \alpha + \beta SA_i + \gamma I_i + \delta (I * SA)_i + \varepsilon_i ,$$

where V_i, I_i, and SA_i denote the i^{th} observation of volume, treatment indicator with 0=control and 1=anesthetic agent (e.g., ketamine), and surface area, respectively, ε_i is the random error term, and α is the y-intercept. The model reduces to $V_i = \alpha + \beta SA_i + \varepsilon_i$ for the control group. Alternatively, sthe model can be rewritten as $V_i = (\alpha + \gamma) + (\beta + \delta) SA_i + \varepsilon_i$ for the anesthetic group. All data analysis can be performed using Microsoft Excel and R (www.r-project.org).

Taken together, SBF-SEM may help illuminate the link between inner membrane remodeling and mitochondrial fusion/fission; knowledge of the changes occurring at the mitochondrial level may,

therefore, provide important insight into one of the key mechanisms of anesthetic-induced neurotoxicity.

Lipidomics analyses to evaluate anesthetic-induced neurotoxicity

It is known that the primate brain as an organ contains the largest diversity of lipid classes and molecular species, and the largest lipid mass relative to protein in comparison to other organs except adipose tissue. The literature on the frequently used general anesthetics has detailed their effects on synaptogenesis, synaptic networking, neurogenesis, neural cell death and behavioral deficits. However, there has been limited research evaluating whether and/or how anesthetic agents affect lipids, the most abundant component in the brain, other than water.

Thus, identifying biomarkers, especially from samples of brain tissue and blood plasma, may assist in the early detection of neurotoxic effects associated with exposure to general anesthetics and therefore, may prove useful in safety evaluations. Practically, lipid extraction from brain tissue and/or blood can be performed using a modified Bligh and Dyer method [10]. For examples, to confirm the identification of the subclass of phosphatidylethanolamine (PE) species, a portion of lipid extract can be derivatized using fluorenylmethoxycarbonyl (Fmoc) chloride [11]. Precursor ion scanning can then be performed to monitor the fatty acid fragments and identify the aliphatic-chains in each of the PE species [12], followed by lipid analyses using a triple-quadrupole mass spectrometer equipped with a nanomate automated nanospray device [13]. Diluted lipid extract can be directly infused through the nanomate device [13]. For each mass spectrum a 1-minute period of signal averaging in the profile mode is typically employed. Data processing, including ion peak selection, baseline correction, data transfer, peak intensity comparison, ^{13}C de-isotoping, and quantitation is then performed using an in-house programmed Microsoft Excel macro [12], after taking into consideration the principles of lipidomics [14].

Lipids play many roles in cellular functions, from membrane structural components to second messengers. Thus, it is likely that perturbations of the nervous system induced by inappropriate anesthesia and/or prolonged anesthetic exposure will be reflected in changes in lipid content, composition, or both.

Cytokine monitoring to evaluate anesthetic-induced neurotoxicity

It has been reported that disruption of phospholipid integrity in neural membranes causes cytokine secretion from microglia and exacerbated inflammation and neuronal damage in neurodegenerative diseases [15]. Cytokines are signaling molecules that play critical roles in many biological processes. Moreover, neurons express chemokines and their receptors [16-18]. As potential co-biomarkers, the involvement of cytokines and chemokines in anesthetic-induced brain damage can be monitored and/or examined in samples of brain tissue and/or blood plasma.

It has recently been reported that a cytokine monkey (non-human primate) panel was used for analysis of sevoflurane-exposed animals. Protein analysis showed that among the 28 analyzed cytokines, interleukin (IL)-17, macrophage inflammatory protein-1α (MIP-1α), epidermal growth factor (EGF), and monokine induced by gamma interferon (MIG) were all significantly elevated in the anesthetic (sevoflurane)-exposed animals [1]. Thus, elevated ROS production and cytokine secretions could be critical for the development of neuronal damage induced by anesthetics. This was subsequently shown [1], by an increased number of Fluoro-Jade C-positive neurons, a marker for neuronal degeneration, in the frontal cortex. Importantly, sevoflurane-induced neuronal damage in the frontal cortex in studies with infant monkeys is consistent with that observed in previous rodent studies, indicating sevoflurane-induced neurodegenerative effects are dependent on delivered concentrations and exposure duration [2]. Therefore, as biomarkers, it is quite possible that the presence and severity of anesthetic-induced neurotoxicity could effectively be reflected in specific alterations in cytokine/chemokine secretion and the levels of ROS-mediated polyunsaturated fatty acid peroxidative products, such as 4-hydroxynonenal (HNE) in brain tissue, plasma (blood), and cerebral spinal fluid.

Calcium imaging to dissect the underlying mechanisms of anesthetic-induced neurotoxicity

It is known that the most frequently used general anesthetics have either NMDA receptor blocking or GABA receptor enhancing properties. Noncompetitive antagonism of NMDA receptors is thought to be one of the mechanisms by which anesthetics, e.g., ketamine, produces its primary therapeutic effect. It has been reported that NMDA receptor NR1 expression in ketamine-exposed brains is significantly higher than in controls [19,20]. It has been postulated that this up-regulation of the NMDA receptor is responsible for or at least contributes to ketamine-induced neurotoxicity because it allows for a toxic accumulation of intracellular calcium (Ca^{2+}) once ketamine is washed out of the system.

Ca^{2+} is a vital element in the process of neurotransmitter release, and is a common signaling molecule. Calcium can act in signal transduction after influx resulting from activation of ion channels or as a second messenger. Activation of the NMDA-type glutamate receptor increases the concentration of Ca^{2+} in the cell. Ca^{2+} can in turn function as a second messenger in various signaling pathways. It is proposed that activation of up-regulated NMDA receptors results in a calcium overload and/or elevation that exceeds the buffering capacity of the mitochondria and thereby, interferes with electron transport in a manner that results in an elevated production of ROS and subsequent neuronal damage including apoptosis.

Practically, for elucidating the underlying mechanisms associated with anesthetic-induced neuronal toxicity, *cultured* neurons maintained under normal control conditions or exposed to anesthetic can be monitored using a fluorescent calcium indicator, Fura-2-acetoxymethyl (Fura-2 AM), which diffuses across the cell membrane and is de-esterified by cellular esterases to yield Fura-2 free acid [21,22]. It was previously demonstrated that ketamine exposure has a significant impact on intracellular Ca^{2+} homoeostasis: the amplitudes of calcium influx caused by activating concentrations of NMDA were significantly increased in neurons from ketamine-exposed cultures compared with their controls [22]. NMDA-elicited increases in intracellular Ca^{2+} could be blocked by perfusing cultures with Ca^{2+}–

free buffer (e.g., in the presence of EGTA), clearly demonstrating that the NMDA-evoked increases in intracellular calcium originated from an extracellular source, rather than from depletion or release of calcium from the endoplasmic reticulum or intracellular calcium stores [22]. Therefore, calcium imaging (Ca^{2+} influx) techniques can be applied to address the working hypothesis and critical issues related to the potential toxic effects of pediatric anesthetics and a specific involvement of NMDA receptor-mediated excitation in anesthetic-induced neurotoxicity.

Molecular imaging (micropet/ct scanning) to evaluate anesthetic-induced neurotoxicity

Many animal studies have shown that the developing brain is vulnerable to NMDA antagonists and/or GABA agonists: exposure to anesthetics during the period of rapid neuronal growth and synaptogenesis can induce massive neuroapoptosis [23-26]. These effects of general anesthetics are associated with subsequent, long-term behavioral deficits in both rats [26] and nonhuman primates [27]. Recently, several epidemiological studies have indicated a relationship between general anesthesia in childhood and subsequent development of cognitive abnormalities and learning deficits in adolescence [28-32]. Therefore, it is necessary to study both the acute and long-term effects of early exposure to anesthetics on the developing CNS *in vivo*.

Positron emission tomography (PET) is a leading molecular imaging approach that provides quantitative information about biological, biochemical, pathological and pharmacological processes in living animal tissues and organs [33-35].

Radioactive tracers are molecules labeled with short-lived positron-emitting radionuclides such as O-15, N-13, C-11 and F-18. Practically after injection of a radiotracer into an animal subject, the labeled molecule binds to very specific targets and, thus, characterizes the bio-distribution of that specific tracer. By utilizing numerous radioactive tracers, such as *[^{18}F]*-Annexin V, *[^{18}F]*-DFNSH, *[^{18}F]*-FDG, *[^{18}F]*-FEPPA and *[^{18}F]*-FLT, multiple biological or pathological processes can be qualitatively and quantitatively assessed [36-39].

When combined with a CT scanner, PET imaging technologies offer unique opportunities for conducting non-invasive imaging studies. Dynamic imaging also has a great potential for helping advance our understanding of anesthetic related toxic processes, including those associated with neuronal plasticity, neurodegeneration/regeneration and neurotoxicity. The utilization of imaging approaches will provide a framework on which information can be arranged in the form of a biological model for use as a tool in dissecting out mechanisms underlying the toxicological phenomena associated with the use of general anesthetic agents.

Summary

These advanced signatures/techniques/methods discussed in the present paper serve to elucidate the causal biochemical mechanisms underpinning genetic, molecular and cellular changes. The utilization of a highly relevant preclinical models and techniques for assessing the effects of anesthetics is critical to decrease the uncertainty associated with extrapolating the preclinical findings to humans. Moreover, identifying biomarkers, especially from the samples of blood plasma may assist in the early detection of the neurotoxic effects (clinically) associated with exposure to general anesthetics and help with their safety evaluation.

There is no doubt that prolonged bouts of anesthesia in the developing brain may lead to accelerated neurodegeneration. It is proposed that anesthetic-induced neurotoxicity depends upon the concentration of drugs used, the duration of exposure, the route of administration, the receptor subtype activated, the animal species, and the stage of development or maturity at the time of exposure. These facts are important because varying exposure concentrations and durations can be utilized to identify thresholds of exposure for producing neurotoxic effects in the developing nervous system. The application of advanced techniques and/or methods reveal the perspective of extending the utilization of these research approaches, especially the molecular imaging and specific biomarkers, into the detection of neurotoxicity in humans.

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