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REVIEW

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Preclinical Assessment of Ketamine

Cheng Wang,[1] Fang Liu,[1] Tucker A. Patterson,[1] Merle G. Paule[1] & William Slikker Jr[2]

1 Division of Neurotoxicology, National Center for Toxicological Research (NCTR)/FDA, Jefferson, AR, USA
2 Office of the Director, National Center for Toxicological Research (NCTR)/FDA, Jefferson, AR, USA

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Correspondence
Cheng Wang, M.D., Ph.D., National Center for Toxicological Research, HFT-132, Food and Drug Administration, Jefferson, AR 72079-9501, USA.
Tel.: +870-543-7259;
Fax: +870-543-7745;
E-mail: Cheng.Wang@fda.hhs.gov
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SUMMARY

Ketamine is used as a general anesthetic, and recent data suggest that anesthetics can cause neurodegeneration and/or neuroprotection. The precise mechanisms are not completely understood. This review is to examine the work on ketamine and to address how developmental biology may be utilized when combined with biochemical, pathological, and pharmacokinetic assessments to produce a bridging model that may decrease the uncertainty in extrapolating preclinical data to human conditions. Advantages of using preclinical models to study critical issues related to ketamine anesthesia have been described. These include the relationships between ketamine-induced neurotoxicity/protection and the preclinical models/approaches in elucidating mechanisms associated with ketamine exposure. The discussions focus on the following: (1) the doses and time-course over which ketamine is associated with damage to, or protection of, neural cells, (2) how ketamine directs or signals neural cells to undergo apoptosis or necrosis, (3) how such exposures can trigger mitochondrial dysfunction, (4) how antioxidants and knockdowns of specific transcription modulators or receptors affect neurotoxicity induced by ketamine, and (5) whether the potential neural damage can be monitored after ketamine exposure in living animals using positron emission tomography.

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Introduction

Ketamine, a noncompetitive NMDA receptor antagonist, is used primarily in adult, pediatric [1], and veterinary [2] medicine. It is also highly abused for its hallucinogenic and out-of-body experiences [3] and has proven useful in the study of psychiatric disorders [4–6]. At clinically relevant concentrations, ketamine acts to block the NMDA receptor, a subtype of glutamate receptor which is involved in a variety of processes including the following: development and differentiation of the nervous system; learning and memory; and synaptic plasticity [7–10]. NMDA receptors, along with other glutamate receptors, are abundantly expressed in the developing brain and are excitatory on neurons that play key roles in many physiological and pathological processes.

Noncompetitive antagonism of NMDA receptors is thought to be the mechanism by which ketamine produces its primary therapeutic effect: It is also thought to result in ketamine's antiinflammatory activity [11]. Interest in ketamine has increased in recent years as it has been shown to block neuronal death caused by excitotoxic mechanisms [12].

In contrast, these receptors are well-known mediators of neuronal cell death that occurs in a variety of neuropathological conditions [4,13–15]. A growing body of data indicates that ketamine may cause neuronal damage in several major brain regions in animal models including rodents and nonhuman primates, during certain periods of development, particularly the brain growth spurt [16–19]. It has been reported that NMDA receptor NR1 expression in ketamine-treated rat pup brains is significantly higher than in controls [18,20,21]. 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 once ketamine is washed out of the system.

This review will focus on the characteristics of the toxic and protective effects of ketamine as a function of concentration, duration, and route of administration, receptor subtypes activated, cell type affected, and stage of development at the time of exposure. Also, this review will address the issue of how the tenets of biology can be combined with biochemical, pathological, and pharmacokinetic assessments to produce a bridging model that may decrease the uncertainty associated with the process of extrapolating preclinical ketamine data to the clinical situation.

Potential Roles of Ketamine in Neuroprotection and Anti-Inflammation

Cognitive deficits are commonly observed following premature birth [22–24]. However, it remains unclear how these early neuroanatomical alterations in the immature brain lead to the cognitive differences observed between preterm and term infants [25,26]. A recent publication explored whether ketamine's noncompetitive antagonism of the NMDA receptor could be the

underlying mechanism for its primary therapeutic and/or antiinflammatory effects [27]. In these experiments, neonatal rats were repeatedly exposed to subcutaneous injections: 4% formalin into the forepaws to induce inflammatory pain, ketamine prior to formalin injections, ketamine alone, or left untreated (naïve) [27]. Subsequently, when animals were adults, cognitive testing using a delayed nonmatch to sample paradigm for the assessment of working memory in the 8-arm radial maze was performed. The data indicated that ketamine attenuated the impaired cognitive function resulting from repetitive, neonatal inflammatory pain, presumably by attenuating the associated cell death in the cortex and hippocampus. Therefore, it was concluded that the analgesic and antiinflammatory effects of ketamine can be neuroprotective in a setting of neonatal inflammatory pain.

Also, in a recent clinical study [28], 24 infants were randomized to receive either ketamine (2 mg/kg) or placebo (saline) before cardio-pulmonary bypass for repair of ventricular septal defects. Plasma markers of inflammation and central nervous system injury were then compared at the end of surgery. Spectroscopy before cardio-pulmonary bypass and at the time of hospital discharge was performed in a subset of cases and controls. Cerebral hemodynamics were monitored postoperatively using near-infrared spectroscopy, and neurodevelopmental outcomes were assessed using the Bayley Scales of Infant Development (BSID)-II before and 2–3 weeks after surgery.

Although ketamine treatment was associated with some changes in the magnetic resonance spectroscopy findings and decreases in C-reactive protein levels, no discernible effects on other plasma proteins were observed. No significant differences in BSID-II scores were detected between the ketamine and control groups, although slightly better scores were seen in the ketamine group. Based on the data from this study, it was concluded that there was no convincing evidence that ketamine was either neuroprotective or neurotoxic.

While the above-mentioned findings, particularly those from the clinical study, were not overtly suggestive of either a neuroprotective or neurotoxic effect of ketamine, elements of the study designs and outcomes may provide a framework around which to design future studies. Only an adequately powered, randomized, placebo-controlled study focusing on long-term cognitive and developmental follow-up will be able to definitively answer the questions concerning the effects of pediatric anesthesia on the developing CNS. As it is not likely that such studies will be conducted in populations of human infants, much of this work will necessarily be carried out in animal models. Due to the complexity of the primate brain, the monkey is often the model of choice for neurological and behavioral experiments [18,19,28].

Ketamine-Induced Neurotoxicity During Development

There is mounting and convincing evidence from animal models that anesthetics in common clinical use are neurotoxic to the developing brain; accumulated data indicate the involvement of NMDA-type glutamate receptors in these effects [16,29–32]. Glutamatergic transmission is mediated by receptor families that are classified as ionotropic (iGluRs) or metabotropic (mGluRs) receptors. iGluRs are ligand-gated ion channels that are subclassified into the following groups based upon their ligand-binding properties: N-methyl-D-aspartate (NMDA); alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA); kainate (KA). NMDA receptors are heteromeric complexes composed of obligatory NR1 subunits as well as subunits from the NR2 subfamily (NR2A, NR2B, NR2C, NR2D) or the NR3 subfamily (NR3A or NR3B) [33–36]. Various combinations of subunits generate a large number of different NMDA receptors with differing pharmacological and biological properties. NMDA receptors are excitatory on neurons that play key roles in many physiological and pathological processes and are well-known mediators of neuronal cell death in numerous neuropathological conditions [4,13–15].

Ketamine is distributed rapidly after administration with a bioavailability of 93% [37]. In a previous study in the rat [20], brain and plasma ketamine levels were found to be highest 5 min after ip administration, after which concentrations decreased to undetectable levels approximately 6 h after the last ketamine injection. Ketamine levels in brain tissue were generally lower than those seen in plasma. These data demonstrated that ketamine clearance from plasma and brain is very rapid; findings which are consistent with previous reports that ketamine is rapidly distributed [37] with a mean terminal half-life of 186 $\pm$ 10 min [38]. Although the concentrations of ketamine in plasma and brain were maintained at relatively stable levels over the course of several injections repeated every 2 h, no significant neuroapoptotic effects were detected for up to 4 h after the last ketamine injection. In contrast, beginning approximately 6 h and lasting for up to 18 h following the last ketamine injection, when plasma and brain levels had decreased to virtually zero, enhanced apoptosis was apparent. These data suggest that the enhanced apoptotic cell death associated with ketamine exposure is not directly associated with the *in situ* blood and brain ketamine levels at the time of cell death, but likely reflects some indirect or compensatory mechanisms.

Anesthetic drugs also produce other dose-dependent cellular effects [31,32,39]. Ketamine may act as an antiinflammatory agent at sub-anesthetic concentrations [40,41], whereas higher concentrations produce nonspecific cytostatic effects [42]. High doses of ketamine can also promote seizures [43], a property shared by other anesthetics [44]. In an *in vivo* rodent study [20], no significant increase in apoptotic neurodegeneration was detected in animals exposed to single or multiple (three or six) injections of 5 or 10 mg/kg ketamine. However, significant increases in apoptotic neurodegeneration were observed in layers II and III of the frontal cortex and several other major brain regions including the striatum, hippocampus, thalamus, and amygdala in animals treated with six injections of 20 mg/kg ketamine, as revealed by caspase-3 immunostaining. These data are consistent with other reports that exposure of the developing brain to NMDA antagonists results in widespread and dose-dependent apoptotic neurodegeneration [16,30,45,46]. These data also suggested that the frontal cortex is the brain region most vulnerable to ketamine-induced neurotoxicity.

The first report demonstrating ketamine-induced neuronal cell death in nonhuman primates exposed perinatally to anesthetics was published in 2007 [18]. The neurotoxic effects of ketamine were assessed several hours after the end of hours-long

intravenous infusions. The findings were interpreted in the context of the hypothesis that prolonged exposure to ketamine induces a compensatory up-regulation of the NMDA receptor, causing neurons to be more vulnerable to the excitotoxic effects of endogenous glutamate after ketamine has been cleared from the system. A 24-hour ketamine infusion was shown to produce a large increase in the number of TUNEL-positive cells in monkeys exposed on postnatal day (PND) 5. The degree to which the nervous system is susceptible or resistant to neurotoxic insults is highly dependent upon its stage of development. In addition to assessing the neurotoxic effects of ketamine in PND 5 monkeys, gestational day (GD) 122 and PND 35 monkeys were also evaluated [18]. As seen with the PND 5 monkeys, GD 122 fetuses also showed clear ketamine-induced neuronal cell damage, whereas PND 35 monkeys did not. GD 122 fetuses and PND 5 neonates, thus, are more sensitive to ketamine-induced cell death than PND 35 monkeys, an age at which less synaptogenesis is occurring. Although a complete understanding of the developmental stages during which nervous system cells are sensitive to ketamine in the primate is not possible from these few early studies, it is apparent that rhesus monkeys are sensitive during the last 25% of gestation (term is 165 days) to sometime before PND 35. While determining equivalent stages of development between humans and animal models is critical for the extrapolation of safety assessment data, it is also not an easy or exact process. It is generally believed that the nonhuman primate fetus and the human fetus are more similar with respect to stage of maturation at birth than are the rat and the human: Rats are much more immature at birth. Both humans and rhesus monkeys are born with their eyes open, whereas newborn rat pups are not. At PND 7, the rat pup is more similar in maturation to a monkey late in gestation than to a neonatal monkey. According to a recent review [47], the GD 123 monkey fetus is roughly equivalent to a GD 199 human fetus—as determined by cortical development—and both are in the range of 75–80% of normal term. NMDA receptor-binding sites in the human fetal brain are present by GD 115, increase until GD 140–150 and then decrease slightly by GD 168–182 [48]. The localization of NMDA receptors in monkey cortex is also similar to that observed in humans [49].

There is no doubt that prolonged bouts of anesthesia in perinatal rat pups or neonatal monkeys lead to accelerated neurodegeneration. It is proposed that the 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 exposure concentrations and durations can be utilized to identify thresholds of exposure for producing neurotoxic effects in the developing nervous system.

Advanced Preclinical Research Models and Approaches for Studying the Adverse Effects of Ketamine Anesthesia

Several studies have demonstrated that ketamine causes neuronal cell death in important brain areas in animals during the brain growth spurt [16–19]. Apoptosis is a common mechanism underlying ketamine-induced neuronal cell death in rodents [20,50]. Previous work based on mRNA levels showed that NMDA receptor NR1 expression in ketamine-treated rat pup brains was significantly higher than in controls [18,20,21] and subsequent work also showed altered expression levels of the NMDA receptor NR2 family, including NR2A and NR2C [21], after repeated ketamine exposure. This evidence of NMDA receptor upregulation suggests that, upon removal of ketamine from the extracellular milieu, the now upregulated NMDA receptor population (a probable compensation for prolonged NMDA receptor blockade) will "over" respond to normal levels of extracellular glutamate, resulting in glutamatergic excitotoxicity.

To determine whether the upregulated NMDA receptor expression in ketamine-exposed neurons is of functional significance, a recent study [51] examined cytosolic calcium concentrations 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 [52]. Utilizing a primary neuronal culture system, it was demonstrated that ketamine exposure has a significant impact on subsequent intracellular Ca^{2+} homoeostasis: The amplitude of calcium influx caused by activating concentrations of NMDA was significantly increased in neurons from ketamine-exposed cultures compared with neurons from control cultures [51]. The NMDA-elicited increases in intracellular Ca^{2+} were blocked by chelation of extracellular Ca^{2+} with ethylene glycol tetraacetic acid (EGTA), clearly demonstrating that the NMDA-evoked increases in intracellular calcium originated from an extracellular source, rather than from a depletion or release of calcium from the endoplasmic reticulum. Although calcium is necessary for cell growth, survival, and normal functioning, it can be neurotoxic when present in excess [53]. As ketamine has a well-defined effect on the NMDA receptor at anesthetic concentrations [54], these data provide further support for the hypothesis that enhanced NMDA-type glutamate receptor expression (compensatory up-regulation after prolonged NMDA receptor blockade) promotes the specific signal transduction that plays a critical role in ketamine-induced neurotoxicity.

Meanwhile, advances in our understanding of stem cell biology and neuroscience have opened up new avenues of research for detecting early-life, stress-induced neurotoxicity and for developing potential protective strategies against anesthetic-induced neuronal injuries. Stem cell–derived models, especially human embryonic neural stem cells with their capacity for proliferation and potential for differentiation, provide a great advantage for identifying potential ketamine-induced neurotoxicity. The use of neural stem cell models, especially those of human origin, when combined with calcium imaging and molecular biology approaches, holds great promise for helping to elucidate relevant mechanisms underlying the etiology of the neurotoxicity associated with developmental exposures to general anesthetics and may also help identify ameliorative strategies.

Because the brain growth spurt in both human and nonhuman primates extends over a much longer time period than in the rat, matching the timing of a developmental event in humans and nonhuman primates is less problematic than matching the same between primates and rodents. No other commonly used research animal has a functional fetal–placental unit, a propensity for

single births and a fetal-to-maternal weight ratio comparable to that of humans. Due to the complexity of the primate brain, the monkey is often the animal of choice for neurotoxicology experiments and, given the protracted period of brain development, the monkey is arguably the very best model for studies of developmental neurotoxicity. The phenomenon of interest in the present discussion has been previously observed in the nonhuman primate, *Macaca mulatta* [18,19,28]. Thus, the relevance of anesthetic-induced neuronal cell death observed in rodent models to children is inferred because similar effects occur in the developing nonhuman primate.

There are two key bridging approaches (cognitive behavioral tests and molecular imaging) being used in efforts to try to predict, in humans, what might be the consequences associated with anesthetic exposure. The National Center for Toxicological Research (NCTR) Operant Test Battery (OTB) has been used in our animal and human research laboratories for a number of years in translational studies of cognitive function [55]. The OTB contains several complex positively reinforced tasks in which correct performance is thought to depend on relatively specific and important brain functions, which include learning, color, and position discrimination, motivation, and short-term memory. Previous experiments from our laboratory have shown that the tasks in the OTB are differentially sensitive to the acute effects of a variety of drugs from different pharmacological classes and that OTB performance by children is not generally distinguishable from that of well-trained rhesus monkeys [55]. The similarity in OTB performance between monkeys and children [56] is of particular importance with regard to extrapolating to humans the neurobehavioral (and possibly neurotoxic) effects of drugs and toxicants as determined in the monkey model. Also, we have demonstrated that early ketamine exposure, in the rhesus model, results in long-term functional deficits [57]. Additionally, the demonstration that several measures of OTB performance correlate highly with measures of intelligence in children [58] serves to highlight the relevance of such measures.

In vivo imaging of rodents, nonhuman primates, and human (both in adults and children) using positron emission tomography (PET) allows for objective and quantitative assessments of functional and molecular targets in a longitudinal manner. When combined with behavioral assessments such as the OTB, PET offers a unique bridging approach allowing insight into "structure and function" issues that are not accessible via other methods. As an extensively studied apoptotic tracer, $^{[18]}$F-annexin V has been used to label apoptotic neurons in the anesthetic-exposed brain of rodents [59]. PET imaging utilizing the smaller size probe for apoptosis; $^{[18]}$F-DFNSH (5-(dimethylamino)-N′-(4-fluorobenzylidene) naphthalene-1-sulfonohydrazide) [60] provides results similar to those seen for $^{[18]}$F-annexin V with a higher signal-to-noise ratio. $^{[18]}$F-FEPPA ($^{[18]}$F-N-2-(2-fluoroethoxy)benzyl)-N-(4-phenoxypyridin-3-yl)acetamide), a recently employed marker of activated microglia, also appears to be suitable for visualizing and quantifying aspects of neurotoxicity (neuroinflammation) and is also applicable to the primate [61]. Utilization of this and hopefully other tracers capable of providing measures of neurotoxicity in human PET imaging will allow investigators to determine whether similar phenomenon occurs in humans.

Antioxidants and Ketamine Neurotoxicity

It is becoming increasingly apparent that mitochondria lie at the center of the cell death regulation process. In a recent *in vitro* mechanistic study [51], an increase in the generation of reactive oxygen species (ROS) was associated with the increased Ca^{2+} influx seen in ketamine-exposed neurons in culture. These ROS appear to originate in mitochondria. Recent evidence suggests that general anesthetics, administered during the peak of synaptogenesis, cause protracted injury to mitochondria including significant enlargement, impairment of their structural integrity, and a decrease in their regional distribution [62]. Along with morphological changes, the general anesthetics also cause functional impairment of immature neuronal mitochondria [63]. Injured mitochondria may be a significant source of ROS [63]. In a mechanistic study [51], ketamine administration markedly elevated both nuclear and mitochondrial levels of 8-oxoguanine. The concordance between elevated 8-oxoguanine levels, enhanced DNA fragmentation and increases in the number of cells with DNA strand breaks following ketamine exposure, suggests key roles for calcium homeostasis and mitochondrial ROS production in inducing neuronal DNA damage and ketamine-induced cell death via apoptotic pathways.

ROS generated by mitochondria are not just toxic by-products of respiration; they are also important for cell signaling [64,65]. Calcium homeostasis is one determinant of ultimate cell survival: Excessive calcium sequestration by mitochondria can produce injury leading to respiratory inhibition, uncoupling of oxidative phosphorylation, and ultimately, the release of cytochrome *c* from the mitochondrial membrane into the cytosol [66–68]. Most of the mitochondrial effects of Ca^{2+} require its entry across the mitochondrial double membrane into the matrix. Although the mitochondrial outer membrane was thought to be permeable to Ca^{2+}, recent studies suggest that the outer membrane voltage-dependent anion channel is a ruthenium red (RuRed)-sensitive Ca^{2+} channel that serves to regulate Ca^{2+} entry into the mitochondrial inter-membrane space [69]. To test whether mitochondrial function is affected by disturbed Ca^{2+} influx, L-carnitine, an antioxidant dietary supplement, was utilized [51]. L-carnitine plays an integral role in attenuating brain injury associated with mitochondria-related oxidative stress [70]. Cells from forebrain cultures were exposed to ketamine or ketamine plus L-carnitine. After removal of ketamine, cultures were assayed using 8-oxoguanine and Cell Death Detection ELISAs and the Comet (Single-Cell Gel Electrophoresis) assay. The data from all three of these assays indicated that aspects of ketamine-induced DNA damage and neurotoxicity can be effectively blocked by L-carnitine. The neuronal protective mechanism of L-carnitine is very complicated and still unclear. It may involve cell membrane stabilization, increased heat shock protein and superoxide dismutase production and/or decreased expression of iNOS. It is proposed that the protective effects of L-carnitine are likely due to membrane modulation, presumably by reducing ROS generation or increasing ROS scavenging, to preserve mitochondrial membrane integrity, a process thought to be downstream of ketamine-induced receptor alterations and disturbed Ca^{2+} homeostasis.

Little is known about the signaling pathway(s) that mediate the postulated roles of altered Ca^{2+} influx. Ca^{2+} may enhance ROS output by making the entire mitochondrion work faster and consume more O_2. Indeed, mitochondrial ROS generation correlates well with metabolic rate, [71,72] suggesting that faster metabolism simply results in more leakage of electrons from the respiratory chain. In addition, Ca^{2+} stimulation of nitric oxide synthase [73] generates NO, which inhibits complex IV [74], thus enhancing ROS generation. Importantly, several recent studies using blockers of oxidative stress such as melatonin [75], the superoxide dismutase mimetic, M40403 [76], the NOS inhibitor, 7-nitroindazole [77], hypothermia [78], EUK-134, a synthetic ROS scavenger, or R(+) pramipexole (PPX), a synthetic aminobenzothiazol derivative that restores mitochondrial integrity [63], have indicated that reduction in oxidative stress may protect the developing animal from anesthetic-induced brain cell death. In addition, Trolox, a ROS scavenger, has also been demonstrated to significantly attenuate ketamine-induced increases in ROS formation, caspase-3 activity, and cell damage [67].

Conclusion

This review has described both the toxicity and protective effects of ketamine and indicated that the involvement of ketamine in either toxicity and/or protection may be determined or directed by the dose of ketamine used, the duration of exposure, the route of administration, and the stage of development at the time of exposure.

This review discussed the proposed mechanisms by which prolonged ketamine exposure produces an increase in NMDA receptor expression that allows for a toxic influx of calcium into neurons once ketamine is removed from the system, leading to elevated ROS generation and neuronal cell death. The application of antioxidants such as L-carnitine appears to be promising for preventing or reversing the toxic effects of ketamine.

Application of sophisticated research models such as the nonhuman primate or stem cell–derived models, when combined with advanced research approaches, holds promise for elucidating relevant mechanisms underlying the etiology of the neurotoxicity associated with developmental exposures to ketamine.

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Conflict of Interest

The authors declare no conflicts of interest.

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