

# Apoptotic Neurodegeneration in the Developing Human Brain: Possible Role of General Anesthetics in its Genesis, and of L-Carnitine in its Reversal

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## Abstract

Recent studies suggest that general anesthetic (GA) agents administered to developing rats, through its mechanism as an NMDA antagonist or a GABA<sub>A</sub> mimetic, may damage developing neural cells by inducing a higher rate of programmed cell death (apoptosis). Similar heightened degeneration was also apparent in higher primates such as the monkey. This warrants strong concern, as every year thousands of pregnant women and children below 1 year of age undergo a surgical procedure in which GAs are used. A spike in neuroapoptosis may lead to long term cognitive deficiencies lingering into adulthood. Are humans vulnerable to these affects? Different pathways are under investigation as to the cause of the damage in animals, but humans have different metabolic pathways than even higher primates, and the basic mechanism by which GAs take affect is not well understood. Scientists continue to unravel the underlying mechanism, seeking to stop the apoptotic cascade, all the while maintaining the benefits of the sedative effect. Promising new hope comes

## Introduction

### Anesthetic Pathway / Mechanism

For conformation to occur between an anesthetic agent and its target, it would be expected that the agent would be highly specific. But the broad arrays of such agents, with their varied structural and chemical compositions seem to belie such a nature. Nevertheless, all general anesthetics work through either of two primary transmission mechanisms: NMDA (N-methyl-D-aspartate) receptor antagonists or GABA mimetic receptors (gamma-aminobutyric acid).

GABA receptors, a type of ligand-gated ion channel, are the main inhibitory neurotransmitter in the central nervous system (Colquhoun et al. 2004). The sedative effect of the GABA<sub>A</sub> agonists is caused by the inhibition of firing new action potentials. When molecules bind extracellularly, a selective Cl ion pore is opened. This increased accessibility of the Cl ion drives it towards -65mV, a membrane potential at which there is no overall change in the ion concentration on either side of the membrane, a situation called reversal potential.

NMDA receptor antagonists block the glutamate and glycine from binding to the receptor, blocking signal

transmission between the spinal cord and the brain (Olney et al. 1991). NMDA receptors are important in the function of neuronal migration and differentiation, synaptic plasticity, and in promoting learning and memory. Glutamate receptors are the most common excitatory neurotransmitter found in mammals. NMDA glutamate receptor antagonists include ketamine and nitrous oxide, both widely used in pediatric anesthesia. The collective effect of GABA<sub>A</sub> agonists and NMDA antagonists is to block synaptic transmissions from occurring between neurons and the spinal cord.

## Methods

Data presented in this paper was mined from case studies, review articles, online sources, and scientific journals using search engines of the Touro College Library database, EBSCO, Pubmed, and Google Scholar.

## Discussion

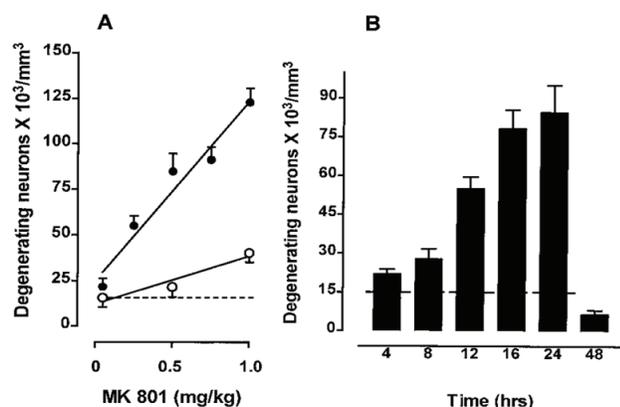
### Effects on lower mammals

Deleterious effects of anesthetics on developing neural cells was first observed in extensive exposure of rat pups on postnatal days 0,3,7, and 21 to Dizocilpine [(+)MK 801], an NMDA receptor antagonist (Ikonomidou et al. 1999). NMDA receptor activation promotes survival of their re-

spective cells. Even brief periods of inhibition were shown to cause brain-wide apoptosis. Rats were injected variously with .5mg, .75, and 1mg per kg of body weight and apoptosis was measured after 24 hours using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). TUNEL is a common technique for identifying DNA fragmentation representative of apoptotic signaling cascades by marking the terminal end of nucleic acids.

Pervasive apoptosis was perceived throughout the brain including within the hippocampus, dentate gyrus, thalamus, hypothalamus, frontal cortex, parietal cortex, cingulate cortex, and retrosplenial cortex, as evidenced by decreased cell density. The apoptotic effect was most pronounced in 7 day old rats when expression of NMDA receptors peak. The neural deletion was commensurate with the influence of both dosage and time factors (Figure 1A). The enantiomer, (-)MK 801, known to be less effective in its ability to inhibit NMDA receptor signal transmission, was also tested. The effects of (-)MK 801 remained negligible relative to the vehicle treated rats (the control; rats just given the solvent without the active ingredient, MK801) up to a dose of 1.0 mg/kg. Electron microscopy demonstrated that these degenerate cells showed no physiological or ultrastructural distinction from corresponding apoptotic cells.

**Figure 1:** Part (A) shows the detrimental effect of MK 801 is proportional to the dosage. Solid circles denote (+)MK 801; Empty circles represent (-)MK 801. The dashed line represents vehicle treated rats. Part (B) demonstrates a relationship between the degeneration and the time of exposure. Only (+)MK 801 was used. The dashed line shows the level of spontaneous apoptosis. (Source: Ikonomidou et al.1999)



Embryonic rats in utero were also given 0.5mg (+)MK 801 at embryonic days 17, 19, and 21 for 8 or 16 hours, and were examined after 24 hours. Significant apoptosis occurred in the embryonic 21 day old rats in the dentate gyrus, hippocampus and hypothalamus. Embryonic day 19 rats showed some modest apoptosis while embryonic day 17 rats showed no sign of apoptosis.

At certain points in the postnatal period rats were more vulnerable to neural degeneration than during the fetal period. Increased vulnerability in the postnatal period may result from the subunit composition of the NMDA receptor which is modified in the transition from the embryonic to the postnatal stage. Higher expression of NMDA receptors at certain periods would make the cells more vulnerable to the NMDA antagonist.

Most concerning was that ketamine, a common pediatric anesthetic, was also shown to cause neuroapoptosis in the developing rat brain. Humans are more developmentally advanced than rats and these results are difficult to extrapolate, but these findings suggest a period of susceptibility in humans during the corresponding period. This time frame is controversial, and an early postnatal study in rats may compare to late gestation or the newborn stage. Perhaps the danger exists throughout the corresponding brain growth spurt period (synaptogenesis) which lasts from mid-gestation to several years after birth.

GAs were also shown to have a time dependent effect with apoptosis shown to continually accentuate when rats were given 0.5mg/kg and were killed variously at 4,8,12,16,24, and 48 hours after being subjected. With the passage of just 4 hours apoptotic effects were apparent. A peak effect was reached after 24 hours while after 48 hours the effects mostly subsided (Figure 1B).

### Apoptosis - Essential for Life

During normal development of the central nervous system apoptosis plays an integral part in the development. Signals that proliferate or restrain the apoptotic program using a precise biochemically guided system controls the number of cells extant and are important in shaping controlling and establishing the development of cells. When apoptotic caspases were eliminated to block the apoptotic mechanism in rats, abnormalities such as

increased subpopulations in the brain occurred and in some cases resulted in lethality. Apoptosis further aids in sexual differentiation, development of olfactory and thermoregulatory systems, and processing of pheromones (Broad et al. 2009). While a moderate amount of neural cells undergo apoptosis to support healthy development, the number of cells undergoing apoptosis in regions throughout the brain upon administration of general anesthetics was excessive.

Do disturbances in other major excitatory transmitter systems of the brain also cause widespread neuroapoptosis? No, it is not apparent in antagonists of excitatory pathways such as the muscarinic or non-NMDA glutamate antagonists. Nor does it occur in agonists of the inhibitory dopaminergic system. Only with the use of NMDA antagonists and GABA<sub>A</sub> agonists is widespread apoptosis apparent (Ikonomidou et al. 1999). Because of these discoveries, researchers set out to discover the effects of GABA<sub>A</sub> mimetics, NMDA antagonists, and the combined neurotoxic effects of the two.

### Permanent Effects on Intelligence

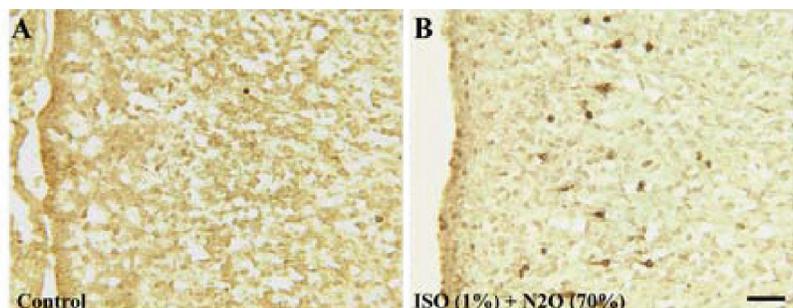
Even presuming robust apoptotic effects upon subjection to anesthetics, what practical significance would it have? Jevtovic-Todorovic et al. (2003) showed that exposure of developing rodents to anesthetics results in a decline in brain density leading to a long-term decline in cognitive function. Seven day old rats were anesthetized for 6 hours with N2O, isoflurane (ISO), and midazolam (triple anesthetic cocktail). They were put in the Morris Water maze and trained to swim to an observable stand. Their performance concerning path length and vigor in the Morris Water maze was on a par with that of the control. When the stand was submerged in the same location, the triple anesthetic cocktail rats showed substantial learning deficits in the middle trials, although they performed at the same level as the controls by the test's conclusion. When retested as adults, their lower memory capacity was evident when they spent less time searching for the submerged stands in the area of the pool in which it was had formerly been raised. Control rats spent more time searching the appropriate pool area for the stand's location. Spatial memory was shown to be im-

paired in the anesthetic cocktail rats by use of a radial arm maze, with a significant learning deficit as compared to the performance of the control.

Ikonomidou et al. (1999) learned that NMDA receptor antagonists can cause apoptosis. Wang et al. (2012) however contends that rats subjected to a dosage commonly used in clinical settings [NMDA receptor antagonist N2O (70%), or to the GABA<sub>A</sub> agonists ISO (1%)] showed no significant measure of apoptosis upon harvest and TUNEL examination after 6 hours. Rather, they needed to be subjected to prolonged and substantial doses of the NMDA receptor antagonist N2O or ISO for any measurable damage (Figure 2).

Separate administration did not cause significant apoptosis, but when administered concurrently, apoptotic levels were accentuated. While gene expression was altered when N2O and ISO was delivered independently the pathways of these genes are not closely associated with neurons, suggesting that cascades are inducing apoptosis rather than the anesthetic doing so directly. This provides hope to researchers. If the anesthetic itself does not directly cause the damage but instead generates a cascade to do so, scientists may be able to determine the point at which the damage occurs. A drug can then be developed to act in concert with the anesthetic and inhibit propagation of the damaging pathway. Accordingly, pediatric surgeons can continue to benefit from the sedative effects without concern for the health of the developing brain.

**Figure 2.** The apoptotic damage in the ISO + N2O group relative to the control is apparent (Source: Wang et al. 2012)



### Widespread Effects Observed

How serious are the effects generated by neuroapoptosis? Huang et al. (2012) proposed that ketamine causes permanent learning and memory impairment. Postnatal day 7 rats were given doses of 25, 50, and 75 mg/kg of ketamine for 3 days. At two months old, the group subjected to 75 mg/kg had a diminished capacity for memory and learning, as measured by the Morris water maze. Behavioral issues were observed as well. These effects were not apparent in the groups exposed to the smaller dosages.

Groups of rats were given the same treatment as described previously but dispatched after 3 days. TUNEL-stained hippocampal neurons were compared between the hippocampal sub-regions cornu ammonis 1 (CA1), CA2, and CA3, and the dentate gyrus in rat pups. Groups subject to lower doses of 25 or 50 mg/kg did not have higher rates of apoptosis while a group subjected to 75 mg/kg showed significant apoptosis in these regions. Protein expression of PKC $\gamma$ , ERK1/2 and Bcl-2 in the hippocampus was measured by western blot. The greater dose of ketamine inhibited p-PKC, p-ERK1/2 and Bcl-2 manifestation but not that of t-PKC or t-ERK. These findings corroborate the position that the malign effects of ketamine are associated with p-PKC, p-ERK1/2 and Bcl-2 expression, which in turn is related to persistent destructive cognitive effects.

Dikranian et al. (2001) suggested perhaps scientists were mistaken in comparing neuronal cells believed to be undergoing apoptosis to non-neuronal cells. Maybe the detailed biological structure of a neuronal cell undergoing apoptosis would have a different ultrastructure than a corresponding cell in the rest of body. NMDA antagonists, and GABA $\alpha$  agonists were used to effect neuroapoptosis and the cellular ultrastructure of apoptotic cells was compared to that of a physiologically dying cell as it occurs naturally in the brain. It was verified to have similar properties to that set forth by the Kerr/ Wyllie team, the scientists who originally discovered and formulated the word apoptosis and classified the structure and formation of such a cell.

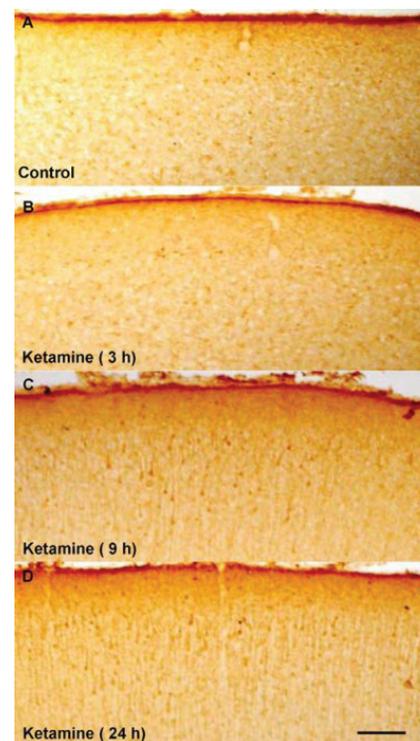
### Apoptosis Seen in Higher Primates

Perhaps only such an effect is observed in rats but higher non-human primates such as the monkey would be

impervious to their affects. Zou et al. (2009) demonstrated this was not so. However, in contrast with the earlier rat studies, the primate monkeys needed to be overwhelmed by a high dosage of ketamine (20 mg/kg) for degeneration to occur. An initial intramuscular injection of 20 mg/kg was followed by continuous intravenous administration of 20-50 mg/kg h for 3, 9, or 24 hours. The monkeys were dispatched after a 6 hour period to allow the anesthetics to take effect. Ketamine was confirmed with liquid chromatography and mass spectrometry. Brain slices of 40 micrometers were prepared using a microtome and a polyclonal antibody that detects cleaved caspase-3, an effector of apoptosis. Out of all sections of the brain- the hippocampus, amygdala, cerebellum, cerebrum, thalamus, and striatum, apoptosis was concentrated mainly in the frontal cortex.

How long does a monkey need be exposed to ketamine for neurodegeneration to occur? Although three hours did not seem to cause neuroapoptosis, exposure for more than nine hours did, while exposure for twenty-four hours resulted in long term cognitive deficits (Figure 3). Most welcome is the knowledge that the 3 hour period did not cause significant apoptosis, as this is the time frame for an average surgery.

**Figure 3.** The effect of Ketamine over time. Note the insignificant effect over 3 hours. (Source: Zou et al. 2009)



Prolonged subjection to a high dosage of ketamine in postnatal day 3 perinatal rhesus monkeys for 24 h, with 1, 10, or 20  $\mu\text{M}$  of ketamine, an NMDA antagonist, caused neurodegeneration in the frontal cortical area (Wang et al. 2006). Significant change in mitochondrial metabolism, DNA fragmentation, and the release of lactate dehydrogenase were also observed. These phenomena are all characteristic of apoptosis.

Even when ISO or ketamine was administered to 6 day old rhesus monkeys for just 5 hours to maintain a light surgical plane and all physiological parameters were controlled as they would be for a neonate patient, neurodegeneration ensued (Brambrink et al. 2010). Pronounced apoptotic neurodegeneration was especially apparent among the immature oligodendrocyte glial cells participating in myelination as well as neurons. ISO, a widely used anesthetic for maintaining a prolonged stable surgical plane, was shown to cause approximately 4 times more degeneration than ketamine.

Other studies discussed which regions of the brain were affected but Creeley et al. (2013) pinpoints the specific cells - neurons and oligodendrocytes just beginning to myelinate their axons - that were deleted. In this experiment, 120 day old fetuses of rhesus monkeys, (comparable to a late third trimester human fetus) and 6 day old neonates (similar to the 4-6 month old human brain) were subjected to propofol for 5 hours. A pronounced effect was detected in the subcortical and caudal areas in the fetus. The neonates caudal brain regions were affected less and neurocortical regional damage formed distinct laminar patterns.

Sun et al. (2012) showed that even adolescent monkeys, when administered ketamine regularly, developed permanent damage. This was evident from the apoptotic effectors present in the prefrontal cortex. Macaque monkeys demonstrated abnormal behavior in their walk, jump, climb, and general movements. The damage appeared permanent in the 6 month treated monkeys, while 1 month treatment and control monkeys had no such display upon TUNEL testing.

### Can These Results be Extrapolated to Humans?

What can be done to determine definitively if this

issue applies to the human fetus and neonates? How can such studies be done non-invasively in humans? While studies can be done retrospectively on humans who have already undergone treatment based on the incidence and statistical distribution of apoptotic neurodegeneration, there are many confounding factors such as the dosage amount and duration, acid-base disturbances, hypoxia, starvation, route of administration, developmental period, and the subtype receptors activated. There are many cofactors present in humans absent in animals. To further muddle the matter, pediatric patients typically are administered a combination of anesthetic agents such as benzodiazepines and/or anticholinergics, possibly reducing the amount of ketamine needed to maintain a surgical plane. Due to all these confounding factors, it is not clear if results from these studies can be extrapolated to humans. Perhaps if the epidemiological studies were well-constructed and accounted for various variables by setting up groups of similar circumstances and arrange for a control for each group, it would aid in determining whether the human brain is vulnerable to apoptotic devastation by anesthetics. Or, methods can be developed for noninvasive techniques to determine if *in vivo* neurodegeneration takes place in the developing brain.

### Anesthetics - Similar to Ethanol?

Because of the similarities in the molecular pathways of intoxication and general anesthetic agents-ethanol is both an NMDA antagonist and a GABA agonist-Ikonomidou et al. (2000) suspected that that studies on the effects of ethanol on fetuses would be applicable to general anesthetics. Seven day old rats were given 2.5 g/Kg of 20% ethanol in saline, and compared to the control only given saline. TUNEL and silver staining showed only modest physiological cell death in the control while the subject group showed condensed pervasive sections of degeneration. Creeley et al. (2013) presented the same effect on neurons and oligodendrocytes in the fetal macaque brain.

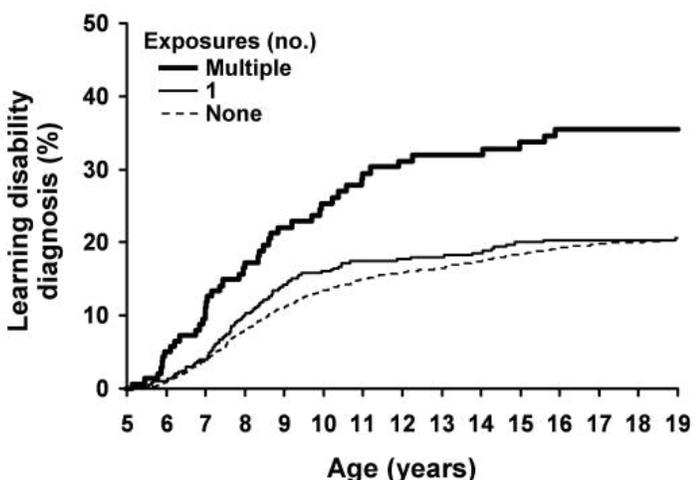
Ethanol has a debilitating effect at the molecular level. Above a 12 mol % (30.5 v/v %) threshold, desorption results from breaks in the lipid-water interface of the bi-membrane, some of the lipid fragments amalgamate within the cell (Gurtovenko, Anwar, 2009). At the same time, components of the inner and outer leaflets of the lipid bi-

layer substitute one another's position, permanently upsetting the membrane structure. And this suggests that anesthetics may cause neurological damage just as ethanol causes developmental neurological damage to fetuses (fetal alcohol syndrome). Neurobehavioral deficiencies perceived to persist into adulthood include psychosis, hyperactivity, learning disabilities, and depression.

**Anesthetics' Affects Persist into Maturity**

Wilder et al. (2009), in a population based cohort study demonstrated retrospectively how children under 4 years of age subjected to an incidence of anesthesia were unlikely to be affected. However, 2 or more periods of general anesthetic application revealed a cognitive deficit as revealed by inferior scores on IQ and standardized achievement tests. Learning disabilities as characterized by poor math, reading and writing skills were apparent and they lagged continually further behind their peers as they advanced through school. By 19 years of age, 35% had a learning disability, representing a 15% rise against the general population (Figure 4). Because of the controversy regarding how to relate the vulnerable developmental stage of animals to their corresponding human stage, the experiment was repeated for children subjected to anesthesia for two or more instances. Similar results were generated. It is not clear if the anesthetics themselves or other factors were the cause.

**Figure 4:** A population based cohort study demonstrating the long-term affect of multiple anesthetic incidence. (Source: Wilder et al. 2009)



But even if anesthetics are shown to definitively cause neurodegeneration, clinical medicine is not much further advanced. How can children who need surgery safely receive treatment? Anesthetic application to children during surgery cannot simply be abandoned; its use prevents neurotoxicity from developing in the brain as well as the harmful effects of stress, anxiety and chronic pain disorders. Exposure to prolonged or repeated painful stimuli lowers the threshold of pain processing, and alters development of the brain, cognitive functioning, and behavior. Moreover, surgical patients who were not administered anesthetics exhibited the very same pervasive neurodegeneration; nothing was gained by avoiding it. Anesthetic treatment resulted in attenuating the pain and protecting the developing brain. (Anand et al. 2004). Anesthetics also protect the brain from damage by preventing hypoxic and ischemic incidents, allowing the necessary oxygen-carrying blood flow to continue to nurture the brain.

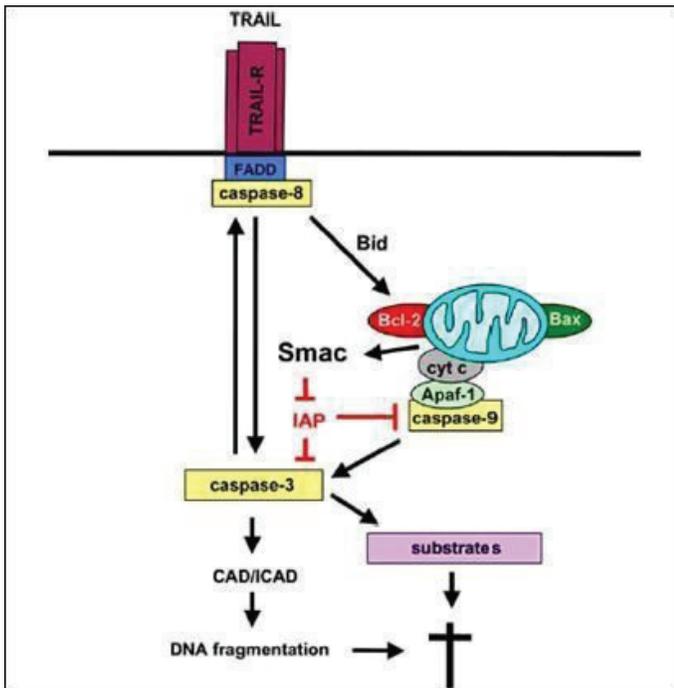
**Apoptotic Mechanism**

A possible mechanism for GA induced neuroapoptosis is via the mitochondrial pathway (Figure 5). Molecular stimulation prompts association of Bcl-2 with mitochondrial Bcl-2, leading to the release of cytochrome c. Cytochrome c in turn binds Apaf-1, effecting a change in its conformation. Apaf-1 association with procrease-9 allosterically activates it, in turn stimulating procaspase-3 and procaspase-7. Perhaps a cascade induced by GAs cause the mitochondria to release more cytochrome c, leading to activation of more caspases. Sanchez et al. (2011) demonstrated support for the theory of GAs affecting mitochondria when he discovered that long term use of GAs compromised its structure and function as well as lingering dysfunction in inhibitory synapses in neural signaling.

**Figure 5 :** (Printed on next page) A schematic summary of the apoptotic pathway. A stimulant triggers association of Bcl-2 with mitochondrial Bcl-2. The ensuing series of reactions activates caspases. (Source: Kalantri 2010)

Alternatively, Kuwana and Newmeyer (2003) suggests that GAs spur a release of a large concentration of Cl<sup>-</sup> (2+) from the ER lumen which can in turn lead to an increase in permeability of mitochondria. The mitochondria

swells and the external membrane may burst. The cytochrome c will then disperse throughout the cell and the apoptotic cascade will begin. Bcl-2 can bring about apoptosis by controlling the release of Ca(2+), thereby regulating whether mitochondria will burst and discharge cytochrome c.



**Possible Protection Against Apoptosis**

A viable alternative to GAs must be developed or the underlying mechanisms better studied and understood so as to address the specific toxic pathway. When ISO and N2O were administered together, differential gene expression was observed in 45 pathways related to the brain, highlighting the difficulties researchers have in trying to pinpoint the exact pathway by which the apoptotic damage is caused and developing a drug to neutralize its affects (Wang et al. 2012).

Once the exact apoptotic pathway is determined, drugs can be developed to act in concert with the anesthetic to prevent the apoptotic cascade while maintaining the benefits of a sedative effect. Progress in labs has already been made in identifying a possible “antidote,” L-carnitine. L-carnitine is an amino protein that supports

oxidation of fatty acids and is essential in skeletal muscle metabolism. L-carnitine, when administered concurrent with GAs, significantly minimized the damage even for long periods under high dosages (Zou et al. 2008).

Di Marzio et al. (1997) reports that sphingolipids play a key role in apoptosis; Apoptosis may be arrested by inhibition of a sphingolipid activation cascade. L-carnitine in AIDS patients blocked sphingomyelinase, thus preventing sphingomyelin breakdown into phosphocholine and ceramide, an intracellular apoptotic effecting molecule. Ceramide exists in the mitochondrial pathway. When high levels are present, the respiratory chain is prevented and cytochrome c is released (Ghafourifar et al. 1999).

Mechanisms in neuropathic diseases also lead to initiation of the apoptotic cascade with cytochrome c released into the cytoplasm (Di Cesare Mannell et al. 2007). Treatment of rats with acetyl-L-carnitine reduced the amount of cytochrome c present in the cytosol and suppressed the apoptotic pathway. Unfortunately, L-carnitine, when tested, did not provide successful protection. Acetyl-L-carnitine also reduced the number of chromatin undergoing the irreversible condensation of chromatin characteristic of apoptotic cells (karyopyknosis).

While the exact apoptotic effecting pathway of GAs is uncertain, it is probable that it is related to both sphingomyelinase and the release of cytochrome c into the cytoplasm. Consequently, it is likely that both L-carnitine and acetyl-L-carnitine would aid in preventing apoptosis. It is premature to declare whether one would be more successful than the other in preventing neuroapoptosis while the basic pathways GAs trigger remain a mystery. Perhaps L-carnitine and acetyl-L-carnitine’s shielding capacity is applicable to only specific mechanisms initiated by a specific anesthetic or a specific form of the anesthetic’s administration.

The X-chromosome linked inhibitor of apoptosis protein (XIAP) acts as an important factor in the apoptotic pathway, inhibiting apoptosis by blocking the activity of caspase-3. When the GA sevoflurane was delivered to lung carcinoma cells for up to 6 hours, apoptosis resulted, blocking cell proliferation (Liang et al. 2011). Researchers observed down-regulation of XIAP expression while caspa-

se-3 levels rose. But administration of Acetyl-L-carnitine was shown to parallel a reduction of karyopyknosis, suggesting the induction of XIAP to block this occurrence (Di Cesare Mannelli et al. 2007).

## Conclusion

While recent studies of rodents and primates suggest that GAs may cause apoptosis in the developing brain by its dual mechanism as an NMDA antagonist and GABA<sub>A</sub> agonist, whether this applies to the developing human brain remains inconclusive. Better constructed epidemiological studies would probably be the best route to determine unequivocally if the developing human brain is vulnerable. Should it prove to be vulnerable, protection in the form of L-carnitine and Acetyl-L-carnitine may be used to neutralize the malevolent affects in future pediatric anesthesiology.

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