Developmental neurotoxicity of ketamine: morphometric confirmation, exposure parameters, and multiple fluorescent labeling of apoptotic neurons.*

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*The views presented in this article do not necessarily reflect those of the Food and Drug Administration.

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ABSTRACT

Ketamine is a widely used pediatric anesthetic recently reported (Ikonomidou et al., 1999) to enhance neuronal death in neonatal rats. To confirm and extend these results, we treated 4 groups of PND7 rats with 7 subcutaneous doses, one every 90 minutes, of either saline, 10 mg/kg ketamine, 20 mg/kg ketamine, or a single dose of 20 mg/kg ketamine. The repeated doses of 20 mg/kg ketamine increased the number of silver-positive (degenerating) neurons in the dorsolateral thalamus to a degree comparable to previous results (Ikonomidou et al., 1999), i.e. 28-fold vs. 31-fold respectively. However, blood levels of ketamine immediately after the repeated 20 mg/kg doses were about 14 µg/ml, about 7-fold greater than anesthetic blood levels in humans (Malinovsky et al., 1996; Mueller and Hunt, 1998). Levels of ketamine in blood following exposure to the multiple 10 mg/kg doses of ketamine or to a single 20 mg/kg dose ranged around 2-5 µg/ml; although these blood levels are close to an anesthetic level in humans, they failed to produce neurodegeneration. To investigate the mode of ketamine-induced neuronal death, coronal sections were stained with both Fluoro-Jade B (a green fluorescent stain selective for neurodegeneration) and DAPI (a blue DNA stain), as well as for caspase-3 (using an antisera labeled red with rhodamine). These histochemical results confirmed the developmental neurotoxicity of ketamine, demonstrated that FJ-B, like silver methods, successfully stained degenerating neurons in neonatal rats, and indicated that ketamine acts by increasing the rate of neuronal apoptosis.

KEY WORDS

Neurodegeneration, Apoptosis, Blood Levels, Anesthesia, NMDA, Caspase-3
INTRODUCTION:

Ketamine (\(\pm\)-2-(2-chlorophenyl)-2-(methylamino)cyclohexanone) is a noncompetitive blocker of N-methyl-D-aspartate (NMDA) receptor ion channels (Moffat et al., 2004). It received FDA approval in 1970 for use as an anesthetic. Ketamine is frequently used in infants and toddlers for elective surgeries, as well as for emergency room procedures (Green and Johnson, 1990; Bergman, 1999). It is short acting and provides rapid dissociative anesthesia followed by rapid recovery. In subsequent studies, it was concluded that ketamine could be safely used for anesthesia in infants and children in emergency room settings (McCarty et al., 1999; Green et al., 2000; Green et al., 2001a; Green et al., 2001b). However, adverse events associated with ketamine use have been reported, including emesis, agitation during recovery, apnea, respiratory depression and laryngospasm (Green et al., 1999) (Green et al., 1998b) (Green et al., 1998a) (Green et al., 1996; Ririe et al., 1997). Sometimes, ketamine is used for postoperative analgesia or sedation after cardiothoracic procedures in one-week old infants (Hartvig et al., 1993), and exposure for up to a month has been reported in a 14-month old burn patient (Cederholm et al., 1990).

Recently, NMDA antagonists, whether given alone or in combination with GABA (gamma amino butyric acid) agonists, markedly increased neurodegeneration throughout the developing brain of 7-day-old rat pups (Ikonomidou et al., 1999; Ishimaru et al., 1999; Olney et al., 2000; Ikonomidou et al., 2001; Olney et al., 2002a; Olney et al., 2002b; Olney et al., 2002c). As observed for other NMDA antagonists such as MK-801 (dizocilpine, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine), ketamine produced elevated numbers of apoptotic neurons throughout the 7-day old rat
brain (Ikonomidou et al., 1999). However, while MK-801 neurotoxicity occurred after a single exposure, ketamine neurotoxicity was reported only after a regimen of multiple injections given during a period of about 9 hours (Ikonomidou et al., 1999). The brain region most affected by ketamine was the dorsolateral thalamus, where ketamine-treated animals had a 31-fold increase in neurons stained selectively for neurodegeneration with the De Olmos silver method. These results suggested that NMDA receptor stimulation was a critical factor for neuronal survival during development and that suppression of this activity resulted in an increase in apoptotic cell death (Haberny et al., 2002; Olney, 2002; Olney et al., 2002c).

The present study sought to confirm the finding that multiple doses of ketamine administered over a short period of time to the early postnatal rat pup would result in widespread increases in neurodegeneration, using the same procedures (Ikonomidou et al., 1999). We also wished to include an independent measure of neurodegeneration, the green fluorescent stain Fluoro-Jade B (Schmued and Hopkins, 2000; Ye et al., 2001), which, unlike silver methods, can be used in multiple staining procedures for cellular apoptotic markers. Finally, we wanted to include lower doses and briefer exposures to ketamine, so that we could begin to characterize the blood levels of ketamine associated with its neurotoxicity in perinatal rat pups.

METHODS:

Animals and Dosing

Sprague-Dawley rat pups (Charles River, Wilmington, MA) were sexed, randomized and returned to their dams in sets of 4 females and 4 males on PND 2. On PND 7, rats were
randomly assigned to one of the four dose groups and to either the histology or blood level experiment. Animals were dosed subcutaneously with racemic ketamine hydrochloride (Ketaset™, Wyeth Pharmaceuticals, Madison, N.J.) or saline using a 0.3 ml syringe with a 30 gauge needle. Animals were returned to their dam between injections. Control rats (n=10) received 7 doses of saline, low dose rats (n=14) received 7 doses of 10 mg/kg ketamine, and high dose rats (n=15) received 7 doses of 20 mg/kg ketamine. The interval between repeated injections was 90 minutes for each of these groups. An additional group (n=8), termed “high single”, received six doses of saline followed by a single dose of 20 mg/kg ketamine as the final injection in the series.

**Blood Collection**

Rat pups from each group (n=3-4) were sacrificed by decapitation two minutes after their last injection and their blood was collected for analysis of ketamine levels by HPLC using modified literature methods (Gross *et al.*, 1999). The plasma was isolated from whole blood by centrifugation at 4 °C. The plasma supernate was transferred to plastic cryovials, placed on dry ice until frozen, and then stored in a –80 °C freezer until analysis. Gender was determined at the time of sacrifice.

**Sample Preparation**

Analytes were extracted from rat plasma after application to silica based solid phase extraction (SPE) C-2 cartridges using a modification of a literature SPE method (Mistry *et al.*, 1998). Ketamine and bupivacaine, the internal standard (IS), were added to the plasma samples. Plasma samples and standards (500 uL) were diluted 1:1 with water and vortexed for 30 seconds. The 200 mg C-2 SPE columns (Varian-Harbor City, CA) were conditioned with (1) 3 ml of methanol (MeOH) and (2) 3 mL of water. Plasma samples
were loaded onto the SPE column then rinsed with (1) 3 ml of water and (2) 3 ml of 50:50 MeOH/Water. Samples were eluted with 50:50 acetonitrile/0.2 N hydrochloric acid (HCl). Samples were dried under vacuum in a spin evaporator (Savant-Farmingdale, NY) and re-constituted in 150 uL of the mobile phase.

Plasma Analysis

All reconstituted standards and samples were analyzed on a Hewlett-Packard 1090 (Wilmington, DE) high performance liquid chromatography (HPLC) system equipped with a multi-channel pump, auto-injector, solvent degasser and diode array detector (DAD). Separation was achieved on a Phenomenex C-18 Luna (2), 5 micron (250 x 4.6 mm) reverse phase HPLC column (Torrance, CA) with a Phenomenex C-18 guard cartridge (4.0 x 3.0 mm). The mobile phase was acetonitrile/10 mM phosphate, pH=3.0 (11:89, v/v) delivered isocratically for 22 minutes. The flow rate was 1.0 mL/min. The injection volume for samples and standards was 50 µL. The UV detection wavelength was 205 nm. The observed retention times for ketamine and the internal standard bupivacaine were 9.9 and 15.9 minutes respectively.

Chemicals and reagents

Ketamine and Bupivacaine were purchased from Sigma (St. Louis, Mo). Acetonitrile and Methanol HPLC grade were purchased from Burdick & Jackson (Muskegon, MI). HPLC grade monobasic potassium phosphate and ACS grade hydrochloric acid were purchased from Fisher Scientific (Pittsburgh, PA). Filtered 18 meg-ohm water was supplied in house by a Millipore Milli-Q System (Bedford, MA). Blank rat plasma was purchased from Hilltop Laboratories (Scottsdale, PA).
**Perfusion**

The remaining rat pups (those not sacrificed for blood levels of ketamine) were deeply anaesthetized with sodium pentobarbital 24 hrs after the initial dose (i.e. on PND 8), They were then perfused transcardially, with a saline flush (about 10 ml) followed by about 50 ml of 4% paraformaldehyde fixative in neutral cacodylate buffer. Their brains were then removed and stored in fixative until further processing.

**Histological Processing**

The rat pup brains were then equilibrated with sucrose and embedded in gelatin as batches of 16 brains with all of the groups represented in each batch. They were then frozen prior to serial sectioning on a sledge microtome. Sets of 16 coronal sections, one from each of the 16 brains and each from approximately the same anteroposterior plane of section were then mounted on 2 x 3 inch glass slides. A 1 in 5 series of these slides were then stained with a cupric silver method selective for neurodegeneration (Beltramino et al., 1993).

Certain of the remaining sections were then used for triple fluorescent staining of 1) immunoreactive caspase-3, (using a rabbit polyclonal antisera directed against the active 18 kDa form of caspase-3, Trevigen, Gaithersburg, MD) (Scallet et al., 1988; Scallet, 1995), 2) degenerating neurons using FluoroJade-B (Schmued and Hopkins, 2000; Ye et al., 2001), and 3) nuclear DNA using DAPI (4',6-diamidino-2-phenylindole) (Spackova et al., 2003). Briefly, the procedure involved rinsing the sections 3 times for five-minutes each in 0.1M phosphate buffer (pH 7.4), followed by an overnight incubation in anti-caspase-3 antisera at 5 degrees Celsius. The primary rabbit anti-caspase-3 antisera was
diluted 1:500 in an antibody diluent prepared with 2% normal goat serum and 0.3% Triton X-100 in 0.1M phosphate buffer. The next day, following 3 more five-minute rinses in buffer, sections were incubated for an hour in a 1:100 dilution in antibody diluent of the secondary antibody, a goat anti-rabbit antisera conjugated with rhodamine (Chemicon, Temecula, CA). Following 3 more rinses in buffer, the sections were viewed wet under green excitation for red rhodamine epifluorescence to confirm the labeling of caspase-3 immunopositive apoptotic neurons in the laterodorsal thalamus. Then the sections were rinsed in water for about 2 minutes and in potassium permanganate (0.06%) for 10 minutes with mild agitation. Following another 2 minute rinse in water, the sections were incubated in 0.1% acetic acid containing a mixture of 0.0002% DAPI (Sigma Chemical, St. Louis, MO) and 0.0004% Fluoro-Jade B (Histo-Chem, Jefferson, AR). The resulting sections were then viewed individually and photographed with epifluorescence using green incident light for caspase-3, ultraviolet light for DAPI, and blue light for Fluoro-Jade B.

**Morphometric Methods**

The dosolateral thalamus was previously reported to have sustained the largest fold-increase in degenerating neurons following ketamine exposure (Ikonomidou et al., 1999). Thus the dorsolateral thalamus, with the addition of the medial amygdala, were selected for the purpose of morphometric confirmation of the previous findings (Ikonomidou et al., 1999).

Slides were selected that contained the Lateral Dorsal thalamus, VentroLateral division (LDVL according to the rat brain atlas) at an anteroposterior level of about –2.5 mm from
bregma (Paxinos, 1986). Because the rat brains were embedded in register, usually a single slide contained sections taken from the same anteroposterior level from about 12 of the 16 brains represented on each slide. If a given brain’s sections were a little more anterior or posterior than the others, it was a simple matter to select the preceding or succeeding slide in order to sample the LDVL and the medial amygdala at a comparable anteroposterior level for each brain.

The silver degeneration procedure produced high-contrast staining of dark positive neurons against a light background (see Figure 1b and 1d). Using an image analysis system (MCID5+, Imaging Research, Inc., St. Catherine’s, Ontario, Canada), two investigators unaware of the treatment conditions agreed on an outline of the LDVL and medial amygdala from each brain. The area of the outlined brain nucleus was then provided by the image analysis system. The greyscale thresholds were set midway between the mean signal intensity of a positive neuron and the mean background level for each brain section in order to systematically segment positive neurons from background and provide a total count as previously described (Scallet et al., 2000). Data were then expressed as the mean frequency of degenerating, silver-positive cells per mm² of the brain region and as fold-increases for purposes of comparison with the original report on ketamine neurotoxicity (Ikonomidou et al., 1999).

Statistical Analysis

Statistical analysis was by One-Way Analysis of Variance (Prism Version 3.02, GraphPad Software, San Diego, California) with 4 levels of Dose: Control, Low, High, and High Single. Because there were no differences between the male and female rat
RESULTS:

Frequency of Degenerating Neurons After Exposure to Ketamine

Compared to controls, ketamine exposure increased the number of LDVL thalamic neurons that were positively stained using the De Olmos silver method for detection of neurodegeneration (F(3,32)=7.2, p<0.001, see Figure 1e for quantitation and Figure 1b vs. 1a for appearance. Tukey Multiple Comparisons indicated that the “High Dose” group (7 repeated doses of 20 mg/kg ketamine) was significantly greater (p values < 0.01) than each of the other three groups (Control, Low, and High Single), which were not significantly different from each other. This elevation of the “High Dose” group represented a 28-fold increase over Control levels of degenerating neurons.

Ketamine exposure also increased the number of medial amygdalar neurons positively stained for neurodegeneration (F(3,32)=5.9, p<0.01, see Figure 1f for quantitative comparisons between groups and see Figures 1d vs. 1c for appearance). Tukey Multiple Comparisons indicated that the “High Dose” group (7 repeated doses of 20 mg/kg ketamine) was significantly greater (p values < 0.05) than each of the other three groups (Control, Low, and High Single), which were not significantly different from each other. The elevation of the “High Dose” group, while statistically significant, represents only about a 3-fold increase over Control levels of degenerating neurons. Apparently, the numerically smaller fold-increase is because the Control levels of degenerating neurons...
in the medial amygdala were higher than in the LDVL despite comparable levels between the two regions in the “High Dose” group (see Figure 1e vs. 1f).

*Fluoro-Jade B vs. DeOlmos Silver Neurodegeneration Staining in the LDVL*

To compare Silver and Fluoro-Jade staining results, examine Figure 2 a, b, c, and d. Figure 2a is a silver-stained control, easily distinguished from Figure 2b, a silver-stained section from a ketamine-treated rat pup. Figure 2 c is a Fluoro-Jade-stained control of comparable appearance to the silver-stained control of Figure 2a, while the Fluoro-Jade-stained degenerating “treated” neurons in Figure 2d are quite comparable both in number and pattern to the silver-stained neurons of Figure 2b. Figure 3f is a silver-stained section from the hippocampus of a ketamine-treated rat, while Figure 3e is a nearly adjacent section from the same animal, but stained with Fluoro-Jade-B.

*Multiple fluorescent labeling of neurons in “High Dose” ketamine-exposed rat pups*

To further investigate the mode of death exhibited by these neurons, coronal sections were stained with Fluoro-Jade B (FJ-B, a green fluorescent marker of neurodegeneration, as well as DAPI (4', 6-diamidino-2-phenylindole dihydrochloride, a blue fluorescent DNA stain) and an antibody to the apoptotic marker, caspase-3 (CASP, labeled red with tetramethylrhodamine fluorescence).

The results from the high dose subjects (see Figure 2) reveal that many cells in the LDVL thalamus contained one or more foci of compact, bright DAPI-stained chromatin (Figure 2g), an indication that they were undergoing apoptosis. Most such neurons also stained positively with both FJ-B (Figure 2h) and with the anti-CASP antiserum (Figure 2i),
although some neurons positive for both CASP and FJ-B were not DAPI-positive for apoptosis.

**Blood Levels of Ketamine**

Table 1 indicates that the blood levels achieved by the repeated 20 mg/kg dosing protocol are considerably higher (around 14 µg/ml) than the blood levels in pups receiving either the multiple 10 mg/kg doses or a single dose of 20 mg/kg (each around 2-5 µg/ml).

**DISCUSSION:**

The results indicate that the silver degeneration-selective method was successful at identifying dying cells throughout the brains of 7 day-old rat pups. While a considerable number of silver-positive cells were present even in control brains, treatment with ketamine greatly increased their numbers. The Fluoro-Jade B degeneration-selective method stained populations of cells, on adjacent sections, that were quite comparable to the silver method in distribution and numbers. The silver degeneration methods are useful for quantitative comparisons between animals or between adjacent sections. However, they are not compatible with approaches designed to combine multiple stains applied to the same section, perhaps due to the harsh extremes of pH required (Scallet, 1995). In contrast, Fluoro-Jade and Fluoro-Jade B. (Schmued and Hopkins, 2000) have proven useful when combined with immunohistochemical or other fluorescent stains applied to the same tissue, as we did here for apoptotic markers.

The 7 repeated doses of 20 mg/kg ketamine resulted in a 28-fold increase in the number of degenerating neurons within the laterodorsal thalamic nucleus of the rat pups. The
measured increase in the present study compares very favorably with the 31-fold increase of degenerating neurons in the same region of the thalamus as reported by (Ikonomidou et al., 1999). While that original report is clearly replicated by the present research, we also observed that 7 repeated smaller (10 mg/kg) doses were without measurable effect. Also, a single dose of 20 mg/kg was ineffective at producing neurodegeneration.

Blood was drawn from groups of rats sacrificed on PND-7, two minutes after their last injection. The blood levels of ketamine associated with ineffective exposures were 2-5 µg/ml while the level associated with a neurotoxic exposure was about 14 µg/ml. An anesthetic blood level in humans is about 2 µg/ml (Malinovsky et al., 1996; Mueller and Hunt, 1998), suggesting the possibility that a prolonged supra-anesthetic dose may be needed for neurotoxicity. Our animals at the repeated 10 mg/kg and single 20 mg/kg dose levels appeared anesthetized, but no actual tests of their level of analgesia or anesthesia were conducted. The fact that 7 multiple 20 mg/kg doses produced higher blood levels of ketamine than a single 20 mg/kg dose suggests that repeating the injections every 90 minutes allowed the ketamine to accumulate in blood and/or tissue. The elimination half-life of ketamine in humans is around 2.5 hours (1-2 hours in a pediatric population) (Hartvig et al., 1993; Domino et al., 1997), so accumulation in tissue of ketamine from doses separated by only 90 minutes seems possible. Future work focused on the precise ketamine exposure parameters required to produce analgesia, anesthesia, and neurotoxicity will prove helpful.

Another goal of this research was to use a multiple staining approach to more reliably identify the dying cells as undergoing apoptosis (Dikranian et al., 2001). Caspase-3 is a
cytoplasmic protein which can be activated by cleavage, initiated either by the stimulation of TNF (tumor necrosis factor) receptors on the surface of the membrane or by the release of cytochrome c from mitochondria undergoing calcium-activated pore transition. Following cleavage into an active 18 KDa fragment, caspase-3 is involved as an effector protein degrading downstream targets including structural proteins, inhibitors of apoptosis, DNA repair enzymes, cell cycle proteins, and signal transduction molecules which can then result in the occurrence of apoptosis (Wang, 2000; Ananth et al., 2001; Vyas et al., 2002; Yuan et al., 2003; McLaughlin, 2004). The subsequent research on NMDA antagonist-induced neurotoxicity has identified the presence of apoptotic neurons by electron microscopy and by the use of specific antisera directed against caspase-3 (Olney et al., 2002a). However, the various types of labeled cells noted to respond to NMDA antagonist exposure had to be evaluated on, at best, adjacent brain sections taken from similar regions.

Here, we showed that Fluoro-Jade B could identify apoptotic cells produced after exposure to the NMDA antagonist ketamine. Then, we used Fluoro-Jade B to stain degenerating cells in sections that were also stained for chromatin and caspase-3. We were able to show that in many cases the degenerating cells were triple-labeled; the same neuron revealed bright clumps of DAPI-positive chromatin characteristic of apoptotic nuclei, as well as positive green staining for Fluoro-Jade B and red for Caspase-3. However, there were some DAPI-negative neurons that nevertheless stained positively for both Fluoro-Jade B and Caspase-3 and a few that appeared to be positive for Fluoro-Jade B but not for Caspase-3. Probably, the stains are differentially sensitive to the various stages that apoptotic cells undergo; further studies should be undertaken with
cultured cells, where the occurrence of apoptosis can be synchronized following a toxicant exposure.

The present study confirms previous research indicating the neurotoxicity of ketamine, demonstrates multiple staining approaches for a single section that may be used to identify different stages of apoptosis, and suggests that neurotoxic blood levels of ketamine may be somewhat higher than levels required for anesthesia. Further research will be necessary to resolve concerns regarding the doses and durations of ketamine exposure that are safe for pediatric populations.

ACKNOWLEDGEMENTS

We would like to acknowledge Bob Switzer, Ph.D. and Dana Wheat of NeuroScience Associates, Inc. for preparing the sections and conducting the DeOlmos Silver staining, as well as Joe Bohaganal for assistance with computerized morphometry.
FIGURE LEGENDS:

*Figure 1* illustrates the appearance, distribution, and numerical density of silver-labeled degenerating neurons of the hippocampus, thalamus, and amygdala in control and treated animals. *Figure 1a* (x 2) is from a control 7 day old rat pup sacrificed 24 hours after the first of a series of 7 injections of 0.1 ml/kg of saline, SC. Although the fornix (Fx), hippocampus (Hipp), and thalamus are clearly visible, only a few, sparse silver-labeled cells can be seen scattered through these regions and in the cortex. *Figure 1b* (x 2) is from the comparable areas of a rat that received 7 injections of 20 mg/kg ketamine. Note the dense groupings of silver-positive degenerating neurons in the laterodorsal thalamus, ventrolateral division (LDVL), in the ventromedial thalamus (VM), and the smaller patches of degenerating cells in the striatum. *Figure 1c* (x 10) is from the region of the medial amygdalar nucleus (Med Amyg) of a control rat, with only a few cells stained positively for degeneration. *Figure 1d* (x 10) illustrates the comparable region of a “high dose” (7 x 20 mg/kg) rat; note that considerably more medial amygdalar neurons are silver-positive for degeneration. *Figure 1e* graphs the density of degenerating LDVL cells, identified as apoptotic (See Figure 2), as a function of ketamine exposure. Note that the repeated 20 mg/kg dose has a significantly (* p<0.01) higher density of apoptotic cells than any of the other groups, which do not differ from each other. The elevation of apoptotic cells in the LDVL represents a 28-fold increase, compared to the 31-fold increase in this same region reported by (Ikonomidou et al., 1999). *Figure 1f* indicates a parallel observation for the medial amygdala, with the repeated 20 mg/kg dose group having a significantly (*p<0.05) higher density of apoptotic cells than any of the other groups, which again do not differ from each other.
Figure 2 illustrates comparisons between the appearance, distribution, and approximate density of degenerating neurons as obtained using a cupric silver method (De Olmos) versus the Fluoro-Jade B approach. It also illustrates the results of multiple-staining of a single section through the laterodorsal thalamus (LDVL) of a ketamine-treated pup with anti-caspase-3, DAPI, and Fluoro Jade B. Figure 2a (x 10) shows only a few positive silver-labeled cells in the VLDL of a control 7-day old rat pup, while Figure 2b (x 10) shows many more such cells in the comparable region of a “high dose” (7 x 20 mg/kg) ketamine rat. Figure 2c (x 10) shows a section adjacent to the control pup in Figure 2a, but stained with Fluoro-Jade B. Note the similar numbers and localization of Fluoro-Jade B positive neurons (Figure 2c) as compared to the silver stain (Figure 1a). Figure 2d (x 10) is similar in terms of the number and localization of degenerating neurons stained with Fluoro-Jade B to Figure 2b, stained with the De Olmos silver stain. Figure 2e (x 10) illustrates the hippocampus of a Fluoro Jade B-treated “high dose” (7 x 20 mg/kg) ketamine rat while Figure 2f shows an adjacent section from the same animal stained for degeneration with silver. Ca1= cornu ammonis 1, sl= stratum lacunosum, dg=dentate gyrus. Figure 2g (x 100) is a VLDL thalamic section from a “high dose” animal in which the DNA has been stained with DAPI. The arrows mark 5 cells where the DNA is divided into several “packets” characteristic of apoptosis, while the circle surrounds several neurons with normal-appearing, DNA. Figure 2h (x100) is stained with Fluoro Jade B, indicating that the same 5 apoptotic cells from Figure 2g are also stained with Fluoro Jade B. A few cells (see arrow inside circle) are stained for Fluoro Jade B, but their nuclei do not appear apoptotic with the DAPI stain. Figure 2i (x 100) indicates that there is substantial overlap of staining between anti-caspase-3 (red rhodamine stain in 2i) and the green Fluoro-Jade B stain (2h).
TABLE 1: Blood levels of ketamine two minutes following the last dose.

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<th>±SEM</th>
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<td>3</td>
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<td>Low (10 mg/kg)</td>
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FIGURES

Figure 1:

Laterodorsal Thalamus: **e**

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Medial Amygdala: **f**

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