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Is age-dependent, ketamine-induced apoptosis in the rat somatosensory cortex influenced by temperature?

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Abstract

General anesthetics have long been thought to be relatively safe but recent clinical studies have revealed that exposure of very young children (4 years or less) to agents that act by blocking the *N*-methyl-D-aspartate receptor (NMDAR) can lead to cognitive deficits as they mature. In rodent and non-human primate studies, blockade of this receptor during the perinatal period leads to a number of molecular, cellular and behavioral pathologies. Despite the overwhelming evidence from such studies, doubt remains as to their clinical relevance. A key issue is whether the primary injury (apoptotic cell death) is specific to receptor blockade or due to non-specific, patho-physiological changes. Principal to this argument is that loss of core body temperature following NMDAR blockade could explain why injury is observed hours later. We therefore examined the neurotoxicity of the general anesthetic ketamine in P7, P14 and P21 rats while monitoring core body temperature. We found that, at P7, ketamine induced the pro-apoptotic enzyme activated caspase-3 in a dose-dependent manner. As expected, injury was greatly diminished by P14 and absent by P21. However, contrary to expectations, we found that core body temperature was not a factor in determining injury. Our data imply that injury is directly related to receptor blockade and is unlikely to be overcome by artificially changing core body temperature.

Keywords

glutamate; rat; anesthesia; neonatal; cooling; heating; cell death

Introduction

Blockade of the NMDAR can lead to induction of apoptosis throughout the CNS that is age-dependent and observed in many species, including non-human primates (Ikonomidou et al., 1999a, Wang et al., 2001, Olney et al., 2002, Turner et al., 2002, Bittigau et al., 2003, Wang et al., 2004, Slikker et al., 2007, Turner et al., 2007b, Wang and Johnson, 2007, Turner et al., 2009a). Since this discovery, many groups have sought to understand mechanisms or expand on the pathological outcomes (McInnis et al., 2002, Olney et al., 2004, Wang et al., 2005, Yon et al., 2005, Kaindl et al., 2006, Lu et al., 2006, Turner et al., 2007b, Stefovskaja et al., 2008,

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Turner et al., 2009a). Indeed, in rodents, injury from agents such as MK801, ketamine, or phencyclidine, leads to short-term and long-term molecular changes in functionally diverse protein families (Wang et al., 2004, Lema Tomé et al., 2006, Turner et al., 2007b, Kaindl et al., 2008, Ringler et al., 2008, Turner, 2009, Turner et al., 2009c) that suggest the still developing CNS has been permanently altered (Abekawa et al., 2007, Wang et al., 2007, Lunardi et al., 2009). These changes are not without behavioral consequence, as learning deficits and diminished sensory processing are also observed following NMDAR blockade at or around P7 (Wang et al., 2001, Jevtovic-Todorovic et al., 2003, Wang et al., 2003, Fredriksson et al., 2004, Abekawa et al., 2007, Fredriksson et al., 2007, Boctor et al., 2008, Lyall et al., 2009).

Despite the original discovery being made a decade ago (Ikonomidou et al., 1999b), the clinical relevance of age-dependent, drug-induced programmed cell death has often been challenged (Soriano and Loepke, 2005, McGowan and Davis, 2008). For example, earlier studies were criticized because they could not rule out non-specific effects of NMDAR antagonists, such as diminished caloric intake, loss of body temperature, changes in blood pressure or other physiological factors. More recent studies now suggest there is no link between these parameters and the injury observed (Rizzi et al., 2008). Further, with respect to temperature, hypothermia is considered to be therapeutically beneficial to ongoing head trauma or stroke (Rosomoff, 1959, Ikonomidou et al., 1989). However, the benefit of cooling the brain may be limited to mild or modest hypothermia, as more severe reductions may actually induce injury (Steen et al., 1979, Ditsworth et al., 2003). These mixed experimental outcomes have their clinical correlates (Yager et al., 2009), suggesting that lowering temperature to control ongoing brain injury is a more complex issue than some studies might indicate.

To more fully understand how temperature may influence NMDAR blockade-induced neonatal brain injury, we measured the core body temperatures of P7, P14 and P21 animals exposed to the general anesthetic and NMDAR antagonist ketamine and determined apoptotic injury by estimating the number of cells positive for the pro-apoptotic marker activated caspase-3 (AC3). If, as suggested in other models, lowering temperature during NMDAR blockade is beneficial then reducing the temperature of animals treated with ketamine should reduce or prevent injury. On the contrary, if (as some anesthesiologists have argued) that loss of temperature is a cause of brain injury, then raising the core temperature in animals treated with ketamine should defeat this injury. Thus, temperatures were either artificially raised or lowered (while simultaneously exposing animals to ketamine) to determine how changes in this physiological parameter influences NMDAR blockade-induced AC3 expression. We now describe our results and discuss these outcomes in the context of their clinical relevance.

Experimental Procedures

Treatments groups

All *in vivo* procedures used in these studies were approved by the Wake Forest University Animal Care and Use Committee. All pups were maintained in the cage with the mother until the day of the experiment (water and food was available *ad libitum*). For stereological AC3 estimations we used a total of 57 Sprague-Dawley rat pups of various postnatal ages (P7, P14, and P21; Charles River Laboratories, Charlotte, NC). For the Western blot analyses, we used a total of 34 P7 rat pups. For group sizes, gender distribution, and body mass (before and after treatments) please see Supp. Table I.

At P7, animals were gender-typed and randomly assigned to a given treatment group. Animals were injected subcutaneously with vehicle (sterile PBS) or ketamine (various doses; see below) for a total of 4 times. The time of the first injection was defined as time = 0 (t_0) and subsequent injections were made at 1, 2, and 3 hours thereafter. For comparison, we also injected MK801

(1 mg/kg, one injection only, at t_0). Unless otherwise stated, all animals were returned to the mother. At t_0 , and again at 1, 2, 3, 4, and 8 hours thereafter, the core body temperature (CBT) of each animal was determined using a rectal thermocouple probe (RET-3, Physitemp Instruments Inc, Clifton, NJ). In addition, to determine the general level of sedation at each of these time points, we also scored animals on the following righting reflex scale (RSS): 3 – returns to upright position immediately; 2 – returns to upright position only after a delay (5 seconds or greater); 1 – unable to respond (remains on back). Body mass of each animal was recorded at the start and end of the study period (8 hours after t_0). At this time, induction of apoptosis was determined by immunohistochemistry for AC3 (see under “Tissue Processing”) and AC3 numbers estimated (see under “Stereological Quantification”). In a parallel study, we also performed Western blot analysis for AC3 expression of brain tissue from similarly treated animals (see under “Western Blot”).

In our first study, ketamine-injected animals were divided up into 4 different dose groups: 2, 5, 10, or 20 mg/kg (K2, K5, K10, or K20, respectively). Vehicle-injected animals served as controls. RSS scores and CBT were determined as described above and apoptosis assessed at 8 hours (see below). We also compared outcomes in the vehicle and K20 groups with those found in similarly treated P7 pups that were placed in an insulated environment, within which the ambient temperature was precisely controlled at 25°C (veh-25C or K20-25C, respectively). Thus, whereas vehicle and K20 animals were kept with the mother after each injection (and were therefore exposed to temperatures closer to the maternal temperature of 34-37°C), veh-25C and K20-25C animals were kept in an insulated environment for the entire 8 hour study period (and were thus exposed to a constant ambient temperature of 25°C).

In a parallel experiment, we examined the effects of 40 mg/kg ketamine on temperature and apoptosis. Pilot studies had revealed that at this dose of ketamine the CBT was substantially lower than vehicle controls. Thus, we referred to this group as the hypothermic group (K40-HT). We compared outcomes with animals that were also treated with 40 mg/kg ketamine but whose temperature was artificially maintained at normal body temperature (33-34°C for P7 animals; (Liu et al., 2006, Rogalska et al., 2006)). We referred to this group as the normothermic group (K40-NT). In this group, CBT was maintained by placing the pups in an insulated environment within which the temperature was precisely controlled (range was from 32-34°C). Vehicle-treated animals served as controls. RSS scores and CBT were determined as above and apoptosis assessed at 8 hour (see below).

Finally, in a separate study we also examined the effects of ketamine on CBT and apoptosis in P14 and P21 rat pups. To maximize neurotoxicity, we employed the highest dose of ketamine (40 mg/kg; K40) and compared outcomes with vehicle-injected animals. We injected at t_0 and again at 1, 2, and 3 hr. RSS scores and CBT were recorded at 0, 1, 2, 3, 4, and 8 hr (see above). After 8 hr, AC3 levels were determined by immunohistochemistry and quantified (see below).

Tissues processing and Immunohistochemistry (IHC)

At 8 hours following the first injection (see above), animals were anesthetized with 2% isoflurane and perfused with PBS followed by 4% paraformaldehyde in PBS (4% PFA). Brains were then dissected, further fixed in 4% PFA for another 24 hr and equilibrated in 10, 20, and 30% sucrose-PBS solutions (total time was 3-4 days). Brains were cut into 100 μ m thick coronal sections using a sliding (freezing) microtome and stored in 24-well plates (containing PBS) until further processed. Thus, each well contained serially cut sections that were 3 $\times$ 100 μ m apart from the next section in each well. The first section collected was just anterior to the genu of the corpus callosum and the last was collected at the level of superior colliculus.

Sections were washed (in PBS) and then reacted with 10 mM sodium citrate (pH 6.0) at 95°C for 3-5 min. After rapid cooling and washing (PBS, room temperature), free-floating sections

were incubated with the primary antibody (rabbit cleaved Caspase-3 (Asp175), 1:2000; R&D Systems) in IHC buffer (1% BSA, 0.1% TX100, PBS; pH 7.4) at 4°C overnight. Following PBS washes, sections were then reacted with biotinylated goat anti-rabbit secondary antibody (1:200 in IHC buffer; Vector Lab, Burlingame, CA) for 2 hours at room temperature. After further PBS washes, sections were then exposed to ABC Elite solution (1-2 hours; Vector Labs) and VIP chromagen solution (5 min; Vector Labs). Sections were then washed, mounted onto glass slides (Superfrost Plus; Fisher, Pittsburgh, PA), air-dried, dehydrated in ascending ethanol concentrations and coverslipped using Depex mounting media (Fisher). Assay controls were performed by omitting the primary antibody.

Stereological quantification

In this study we sampled from the anterior somatosensory cortex (specifically S1/S2 sub-regions), starting from the first section that showed the emergence of the caudate-putamen and terminating at the first section that showed the emergence of the hippocampus (Paxinos and Watson, 1998). We referred to this sampling territory as the SSC_{ANT}. Numbers of AC3-positive neurons were estimated within lamina IV and V of the SSC, as previous studies have reported reliable and robust injury in these layers (Turner et al., 2007b, Turner et al., 2009a, Turner et al., 2009b). For comparison, we also qualitatively evaluated AC3-positive profiles in the superficial portion of layer II/III in the same sections we sampled layers IV and V.

The stereology procedure was performed using an optical fractionator workflow (StereoInvestigator 7.0; MicroBrightField Inc, Williston, VT) that was supported by an Olympus BX51 microscope with a 100x oil immersion objective (PlanApo 1.40 NA, Nikon), an X-Y motorized stage (MicroBrightField Inc), and a digital camera (Microfire A/R, Optronics, Goleta, CA). Pilot studies were first performed to optimize sampling parameters. We determined antibody penetration by estimating the frequency of AC3-positive profiles throughout the entire depth of randomly selected sections (N = 4). We found that staining was detectable at all planes of focus throughout the section (Supp. Fig. 1). From these pilot studies we also determined that the average section thickness (after tissue processing) was 25 µm. We therefore set guard zones of 3 µm and a disector height of 19 µm.

Following these pilot studies, AC3-positive profiles were then estimated for each animal as follows. In each section, a region of interest (contour) was mapped onto the SSC_{ANT}-IV-V at 4x magnification and the counting grid size and the number of count boxes established. We used a counting frame size of 75 × 75 µm and an average sampling grid area of 9630 µm². The section interval was 3 and we sampled from 4-7 sections per animal. We then switched to 100x magnification and estimated mean section thickness before counting. To determine total AC3-positive cells per animal, we used the “estimated total by number weighted section thickness” (StereoInvestigator 7.0; MicroBrightField Inc). The mean estimated number (± SE) was then determined across all animals in a given treatment group. We wish to emphasize that we are not attempting to estimate absolute numbers of AC3 cells for a given brain region but only wish to determine (using a non-biased, stereological approach) the relative differences between the vehicle and experimental groups.

Western Blot Analysis

P7 rat pups were subcutaneously injected with vehicle (N = 5) or MK801 (1 mg/kg; N = 5), or ketamine (2, 5, 10, 20 mg/kg; K2-K20; N = 6). Pups were anesthetized after 8 hr (2% isoflurane) the brains quickly removed. Each brain was micro-dissected (on ice) into various brain regions and tissue was placed into microfuge tubes, frozen on dry ice and stored at -80°C until needed. Following protein extraction, a small volume of each sample (each containing 25 µg of protein) was mixed with loading buffer and loaded into a single well of a polyacrylamide gel (12%). Separated proteins were transferred to nitrocellulose membranes (Trans Blot Transfer Medium,

BioRad, Hercules, CA), which were then probed for AC3 expression (rabbit anti-AC3, 1:1000, Cell Signaling; donkey HRP-conjugated anti-rabbit secondary, 1:5000, GE Healthcare, UK). Membranes were stripped and probed for β -actin as an internal control (mouse-anti- β -actin, 1:5000, Sigma, St Louis, MO; donkey HRP-conjugated anti-mouse, 1:5000, Jackson ImmunoResearch Laboratories, PA). Visualization of bands was performed using ECL solution (Amersham, UK) and protein levels were determined based on their band density on exposed film (Biomax XAR, Kodak, Rochester, NY). The density for AC3 was normalized to the corresponding β -actin for that lane and the mean band density ($\pm$ SE) across all animals in a given treatment group determined.

Statistical Analyses

For temperature estimations, we compared mean values at each time point by ANOVA. For AC3 counts and Western blot observations, we compared means across all groups by ANOVA. Significant differences between the groups were determined using a Bonferroni post-test comparison of means. With respect to both stereological cell counts and Western blot band densities, we also performed regression analysis using the individual observations from vehicle, K2, K5, K10, and K20 groups. All statistical analyses were performed using GraphPad Prism 4.0 (GraphPad, San Diego, CA).

Results

Is temperature a factor in determining ketamine-induced apoptosis?

As described in the Experimental Procedures, we injected ketamine (1 injection at 0, 1, 2 and 3 hours; 4 total) using 2, 5, 10, or 20 mg/kg doses (K2, K5, K10, or K20, respectively). The time of the first injection was defined as time = 0 (t_0). For comparison with this and previous studies, we also injected MK801 at 1 mg/kg (one injection only at t_0), as this dose produces robust AC3 induction that is reliable and widespread (Turner et al., 2002, Turner et al., 2007b, Turner et al., 2009a). Sterile PBS (1 injection at 0, 1, 2 and 3 hours; 4 total) served as vehicle controls.

We first asked how ketamine exposure affected core body temperature (CBT; see Experimental Procedures). Temperatures were measured at various times (0, 1, 2, 3, 4, and 8 hr) after the first injection (t_0). We found that in all ketamine treatment groups (K2, K5, K10 and K20), the CBT did not significantly differ from that found for vehicle controls (Fig. 1A), which was around the normal value for P7 pups (32-34°C (Rogalska et al., 2006)). In contrast, MK801-treated animals showed significant loss in core temperature (around 4-5°C) at 1, 2, 3, and 4 hours but not at 8 hours. Based on RSS scores (see Experimental Procedures), whereas MK801 animals were rapidly and deeply sedated, ketamine-treated animals showed dose-dependent levels of sedation that approached or matched MK801 levels only at the 10 and 20 mg/kg doses (Supp. Fig. 2).

We next asked what effect ketamine had on apoptosis 8 hr after t_0 by examining brain sections for the pro-apoptotic enzyme AC3 in layers IV and V of the anterior somatosensory cortex (SSC_{ANT}; this region corresponds to the S1/S2 sub-regions, spanning the rostro-caudal extent of the caudate putamen through to the emergence of the hippocampus (Paxinos and Watson, 1998)). Previous studies have shown this region (specifically layers IV and V) is especially sensitive to NMDAR blockade at this age and that AC3 induction peaks at 8-16 hours (Lema Tomé et al., 2006, Turner et al., 2007b, Turner et al., 2009a). For ease of comparison, we remained focused on this region (and these layers) in the present study. Compared to vehicle controls, we found that AC3 levels were elevated in a dose-dependent manner in the ketamine groups (Fig. 1B; reaching significance at 20 mg/kg; $p < 0.001$) and that the 20 mg/kg dose compared well with what was found in the MK801 group. Regression analysis of dose vs AC3

counts (vehicle, K2, K5, K10 and K20) yielded a “goodness of fit” (r^2) of 0.7624 ($p < 0.0001$), suggesting that ketamine dose was a reasonable predictor of injury (at least using the 4-injection strategy employed in this study).

Western blot analyses of similarly treated P7 pups (vehicle, K2-K20, MK801) confirmed the dose-dependent nature of the ketamine effect (Fig. 2), reaching significance at 10 mg/kg ($p < 0.001$) and that 20 mg/kg was required to induce AC3 expression levels that compared well with those found following MK801 injection. Regression analysis of the Western blot data (vehicle, K2, K5, K10 and K20) yielded an r^2 of 0.8716 ($p < 0.0001$), indicating even greater correlation between ketamine dose and apoptosis than was found for cell counts. It should be noted however, that the Western blot data reflects AC3 levels within the entire somatosensory cortex and (perhaps for this reason) AC3 induction in the K20 group was greater than it was in the MK801 group (compare Fig. 2 with Fig. 1). Indeed, a qualitative analysis of immunoreactivity in layer II/III of the SSC_{ANT} revealed a greater number of AC3-positive profiles in the K20 animals compared to MK801-treated animals (Supp. Fig. 3). We wish to emphasize that this explanation is only tentative and definitive proof will require more studies.

Collectively, we show that over the 8 hour period, anesthesia-like levels of sedation were observed (at least at K10 and K20), CBTs of the K2-K20 animals remained relatively constant and normothermic, and that ketamine dose was a reasonable predictor of injury. Further, despite a dramatic loss of CBT, MK801 produced similar levels of injury to K20.

Lowering temperature from normothermic levels did not alter apoptosis

Despite robust injury being observed in the high dose ketamine group (K20), CBTs were at normothermic levels throughout the 8 hour period. In P4 mice, lowering the axial temperature to 25°C during the period of NMDAR blockade is thought to protect against injury (Creeley et al., 2008). We therefore wondered if artificially lowering the temperature of ketamine-exposed animals would influence injury in our model. Thus, we treated P7 pups with vehicle or 20 mg/kg ketamine (a dose that was substantially neurotoxic but did not by itself produce hypothermia) and kept them in an environment that maintained the temperature at 25°C (veh-25C, K20-25C, respectively). We compared outcomes in these animals with the same vehicle and K20 animals that we already have found to be normothermic (see Fig. 1). Please note, for greater visual clarity we have elected to show comparisons between vehicle, K20, veh-25C and K20-25C, in a separate figure.

Compared to the normothermic K20 animals, the CBT in the K20-25C group was generally lower (by about 1-2°C) at 1-4 hours but substantially less (by 4-5°C) at 8 hours (Fig. 3A). Likewise, when we compared the normothermic vehicle group with the veh-25C group, the CBT was generally the same for the first 4 hours and substantially less (by 3-4°C) at 8 hours. As indicated by RSS scores, veh-25C animals were alert and functional whereas K20-25C animals were fully sedated for much of the 8 hour study period (Supp. Fig. 2). When we examined AC3 levels, mean cell counts were very similar in both the normothermic K20 and K20-25C groups (Fig. 3B). Likewise, in both the normothermic vehicle and veh-25C groups, mean AC3 cell counts were statistically no different. As expected, mean AC3 counts in the normothermic K20 and the K20-25C groups were significantly different from their respective vehicle control group. These data suggest that lowering the external temperature to 25°C influences neither endogenously regulated cell death nor injury resulting from exposure to the general anesthetic ketamine.

We had noted earlier that layer II/III may be more sensitive to ketamine than layers IV and V. Thus, it is possible that layer II/III may exhibit a different outcome with respect to exposing P7 pups to a constant external temperature of 25°C. However, we found no evidence that AC3

levels in the K20-25C pups were different than in the K20 pups ($p > 0.05$, ANOVA; Supp. Fig. 3).

It could be argued that in the K20-25C group, cell death could have been due to loss of calories or dehydration but we should have observed greater cell death in the veh-25C group (compared to the normothermic vehicle controls), which was not the case. Further, other labs that use a similar model to the one used here have eliminated nutritional or cardio-pulmonary factors as potential confounds (Olney et al., 2004, Rizzi et al., 2008). We therefore feel that brain injury following exposure to MK801 or ketamine is a direct result of NMDAR antagonism and not a non-specific effect following changes in general physiology.

Raising CBT from hypothermic levels did not alter apoptosis

In contrast to the K2-K20 groups, pilot studies had revealed that the CBT of P7 animals treated with 40 mg/kg ketamine was substantially lower than vehicle-treated animals. As such, we designated these animals as K40-hypothermic (K40-HT). For example, at 1 hr after the first injection the CBT of the K40-HT group had plummeted to well below vehicle levels (4-5°C difference) and remained low (by as much as 6-7°C) for the rest of the study period (Fig. 4A). We compared this group with P7 pups injected with the same dose of ketamine whose CBT was artificially maintained at normothermic levels throughout the 8 hr period (K40-NT; see Experimental Procedures). In contrast to the K40-HT animals, the CBT of the K40-NT group remained stable at vehicle levels (32-34°C) throughout the same period. In both the K40-HT and K40-NT groups, all animals were rapidly and deeply sedated and remained so for the duration of the study period (see Supp. Fig. 2). When we examined brain sections for signs of injury at 8 hr, the K40-HT animals displayed robust AC3 in the SSC_{ANT} (Fig. 4B & C). In the K40-NT group, AC3 levels were essentially no different from that found in the K40-HT group, suggesting that raising the internal body temperature (at least to normothermic levels) does not alter pathological outcome.

Core body temperature and injury vary with age

We next asked if ketamine could induce changes in CBT in older animals and what relationship this had with AC3 expression. From our P7 studies, we found that induction of injury was maximal in the K40 group. Thus, P14 or P21 animals were injected with vehicle or 40 mg/kg ketamine (4 times total; see Experimental Procedures) and CBT (as well as RSS) was estimated at 0, 1, 2, 3, 4, and 8 hr (see Experimental Procedures). As with the P7 animals, there was substantial loss of CBT in P14 pups in the K40 group (Fig. 5A). Likewise, levels of sedation in the P14 K40 group were comparable to the P7 K40 group (Supp. Fig. 2). In contrast to P14 animals, the CBT of K40 animals at P21 displayed normothermic temperatures throughout the 8 hour period. In addition, levels of sedation in the P21 K40 group were light and more transitory compared to the K40 groups at both P14 and P7 (Supp. Fig. 2). Thus, the effect of ketamine (with respect to both CBT and RSS) was age-dependent. P14 animals had a pre-injection mean CBT of 34.8 ± 0.4 °C (range: 34.2 - 35.2) compared to 37.3 ± 0.2 °C (range: 37.1 - 37.5) in P21 pups. Given that the pre-injection, mean CBT of P7 pups was 34.7 ± 0.3 °C (range 34.0 - 35.5), our data suggest that adult CBT levels are achieved only during the last postnatal week, in close agreement with findings elsewhere (Liu et al., 2006, Rogalska et al., 2006).

When we examined induction of apoptosis in the K40 group of P14 animals, AC3 levels were no more than 2-fold higher than vehicle controls (Fig. 5B), indicating that despite a dramatic and sustained loss of CBT, apoptosis was substantially less than that found at P7. In the P21 animals, there was no evidence of any injury 8 hr after t_0 (Fig. 5C). Collectively, these data show that P7 and P14 animals display similar thermoregulatory behavior (in the absence or presence of ketamine) yet significant injury was only observed at P7. Further, in P21 animals,

we observed higher CBTs before exposure to ketamine (compared to P7 and P14 ages), no decline in CBT after ketamine injection, and an absence of apoptotic injury after NMDAR blockade.

Discussion

Both experimentally and clinically, reducing body temperature has been shown to be neuroprotective in the immature brain (Busto et al., 1987, Yager et al., 1993, Yager et al., 2009). Thus, a critical question in the model we present here was whether altering body temperature could influence ketamine-induced apoptosis. Perhaps key to such studies, we measured CBT as this has been shown to be a more accurate indicator of brain temperature in rats (Ohmura et al., 2005). Our main observation was that CBT was not correlated with apoptotic injury. For example, in P7 pups, we observed a dose-dependent increase in AC3 (from 2-20 mg/kg ketamine) even though these animals were able to maintain their CBT at normothermic levels throughout the 8-hr study period. In contrast, in MK801-treated P7 animals, we noted that there was an abrupt drop in CBT that was maintained for at least 4 hr after the time of injection, yet AC3 levels were robust. At P14, CBT in K40 animals also dropped substantially but AC3 levels were not significantly different from vehicle. Perhaps most revealing was that artificially lowering or raising CBT did not influence pathological outcome (compare normothermic K20 with K20-25C or compare K40-HT with K40-NT).

Is cooling the body the same as cooling the brain?

Lowering temperatures is thought to prevent NMDAR blockade-induced injury in P4 mice (Creeley et al., 2008, Yuede et al., 2009). On closer examination however, a key difference between these studies and our own was how body temperature was reported. In our study we measured the CBT (a direct estimation of the internal environment) whereas ambient or axillary temperatures were reported elsewhere (Creeley et al., 2008, Yuede et al., 2009), giving no (direct) information on the internal status of each animal. Indeed, when we kept animals in a 25°C environment for 8 hours, even though CBTs were slightly reduced for the first 4 hours they did not reach hypothermic levels until 8 hours (Fig. 3). Given that our data more likely represent actual brain temperatures (see (Ohmura et al., 2005)), and that our data are consistent with studies elsewhere that also measured CBT (Rizzi et al., 2008), we believe that changes in CBT are not a factor in age-dependent, anesthesia-induced brain injury (at least at P7). However, perhaps in the mouse studies (Creeley et al., 2008, Yuede et al., 2009) thermoregulation is more difficult at P4 compared to P7 (in rats), in which case axillary or ambient temperature may well be a reasonable index of brain temperature, though this will have to be empirically determined. If age (P4 vs P7) is truly a factor across these studies, it would suggest that a narrower window exists wherein body cooling is protective against iatrogenically-induced neonatal brain injury than some studies might indicate.

Despite the above argument, body cooling has been shown to prevent injury in other models of neonatal brain injury, such as hypoxia-ischemia (Busto et al., 1987, Yager et al., 1993, Yager et al., 2009). It must be considered however, that traumatic brain injury invokes a number of highly toxic responses (loss of blood flow, inflammation, hemorrhage, blood brain barrier breakdown) that are not found in the NMDAR blockade-induced injury model. Further, head trauma or stroke is often a mix of necrotic and apoptotic injury, with the former predominating in the earlier stages. In contrast, our model of neonatal brain injury is purely apoptotic in nature. Further, given that other groups suggest the relationship between cooling and protection is complex (too severe a drop in CBT and apoptosis can be induced independent of any ongoing injury (Yager et al., 2009)), future studies must address the effects of mild, moderate as well as severe hypothermia, both in the context of ongoing injury as well as in the non-injured brain.

With respect to ongoing neonatal brain injury (from hypoxia-ischemia, for example), it has often been pointed out that ketamine (and other NMDAR antagonists) is neuroprotective (Proescholdt et al., 2001, Engelhard et al., 2003). However, we and others have clearly demonstrated that age-dependent injury is to be expected following exposure to such agents (Ikonomidou et al., 1999a, Turner et al., 2002). We have explained this enigmatic outcome using a Calcium Set Point hypothesis (Turner et al., 2002, Lema Tomé et al., 2006, Turner et al., 2007a, Turner et al., 2007b, Ringler et al., 2008, Turner et al., 2009a). Briefly, immature neurons unable to buffer abrupt changes in calcium are vulnerable to such changes. If, following brain trauma, calcium levels become pathologically high, then blocking calcium entry would be a logical choice. However, in the absence of ongoing injury reducing neuronal calcium is a death sentence (for some neurons at least). As neurons mature they acquire greater sophistication in handling abrupt changes in calcium and are subsequently far less vulnerable.

Layer-specific effects

In deeper cortical layers (layer VI), it has been reported that NMDAR activation is anti-apoptotic whereas in the more superficial layers (layer II-IV) NMDAR blockade is pro-apoptotic (Desfeux et al., 2009). In our own study we noted apparent differences between layers IV and V and layer II/III, with respect to the neurotoxicity produced by ketamine. Given that these data were qualitative, we feel it is premature to speculate as to why this might be so. However, the study by Desfeux et al suggests that the maturation state of the neurons and the manner in which they handle calcium flux may be critical factors. This is exactly what we have argued for some time ourselves, using the Calcium Set Point hypothesis to explain why apoptosis following NMDAR blockade is so age-dependent (see above). Indeed, the findings of Desfeux et al dovetail well our own observations. For example, Desfeux et al show that NMDAR blockade-induced injury is not associated with glial cells, is bax dependent and targets GABAergic interneurons. We have shown that, following loss of calcium, AC3 is induced in neurons not glia, that AC3 induction does not occur in bax knockout animals (unpublished observations), and that AC3 immunoreactivity is colocalized with GABAergic terminals (Turner et al., 2007b, Turner et al., 2009d). A potentially crucial difference may be that in our P7 *in vivo* model, injury from NMDAR blockade occurs in what are clearly clusters of pyramidal cells in layers IV and V (Turner et al., 2009a, Turner et al., 2009c), whereas in the Desfeux et al P2 slice model, injury occurs in more uniformly distributed GABAergic interneurons of layers II-IV. Regardless, both models offer mostly convergent evidence regarding potential mechanisms behind NMDAR blockade induced death in the immature brain.

Are multiple ketamine exposures clinically relevant?

In this study we show a strong correlation exists between ketamine dose and AC3 induction. However, a single injection of ketamine does not reliably promote apoptotic injury (Lyll et al., 2009) (see also (Mellon et al., 2007)). Likewise, a recent clinical study has shown that a single exposure to general anesthetics is not nearly as effective as multiple or prolonged exposures, with respect to causing learning deficits later in life (Wilder et al., 2009). Thus, our experience with the general anesthetic ketamine closely mirrors what is found clinically. Regardless, the lack of a consistent outcome following a single exposure to ketamine requires some explanation. There are a number of factors that may determine pathological outcome: gender, physiological parameters (weight, CBT, blood pressure, caloric status), pharmacodynamics (receptor occupancy, drug clearance or metabolism). Across littermates these factors may differ substantially, generating robust injury in one animal but not the next. This may be more than just an esoteric lab issue, as even a single exposure to anesthetics in young children can lead to mild learning deficits in some but not all children (Wilder et al., 2009). Further, it has been argued that multiple or prolonged exposures to ketamine is unusual, or that ketamine can easily be substituted with another anesthetic, and as such, concerns about

ketamine-induced injury in humans may be unfounded (Bhutta, 2007). However, this anesthetic is used in a number of situations where exposure is prolonged, when there is no other choice, and is deemed to be safe (see (Vardi et al., 2002, Tomatir et al., 2004, Yamamoto, 2008, Bazin et al., 2009, Bredmose et al., 2009)). Clearly, from a clinical perspective, not only must we identify what factors will trigger injury even after a single exposure to general anesthetics but we must also determine effective therapeutic strategies that account for the various factors that influence pathological outcome.

Summary

Debate over whether anesthesia-induced brain injury is clinically relevant has raised questions about whether the neurotoxicity observed is a direct result of NMDAR blockade in the brain or due to indirect changes in physiological parameters, including temperature. In this study, we have found no correlation between anesthesia-induced apoptosis and core body temperature. Even when temperatures were artificially lowered or raised, pathological outcomes remained unaffected. These studies suggest that (at least in P7 rats) anesthesia-induced injury is a centrally driven event and that the internal temperature of subjects is not a primary factor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

NMDAR	N-methyl-D-aspartate receptor
AC3	activated caspase-3
CBT	core body temperature
RSS	righting reflex scale
PFA	paraformaldehyde
IHC	Immunohistochemistry
SSC	somatosensory cortex
SSC _{ANT} -IV-V	layers IV and V of the anterior somatosensory cortex
t ₀	the time of the first injection
V	vehicle
Veh-25C	V placed in an insulated environment at 25°C
K2	2 mg/kg ketamine
K5	5 mg/kg ketamine
K10	10 mg/kg ketamine
K20	20 mg/kg ketamine

K20-25C	K20 placed in an insulated environment at 25°C
K40	40 mg/kg ketamine
K40-HT	K40 hypothermic group
K40-NT	K40 normothermic group

References

- Abekawa T, Ito K, Nakagawa S, Koyama T. Prenatal exposure to an NMDA receptor antagonist, MK-801 reduces density of parvalbumin-immunoreactive GABAergic neurons in the medial prefrontal cortex and enhances phencyclidine-induced hyperlocomotion but not behavioral sensitization to methamphetamine in postpubertal rats. *Psychopharmacology (Berl)* 2007;192:303–316. [PubMed: 17340116]
- Bazin V, Bollot J, Asehnoune K, Roquilly A, Guillaud C, De Windt A, Nguyen JM, Lejus C. Effects of perioperative intravenous low dose of ketamine on postoperative analgesia in children. *Eur J Anaesthesiol*. 2009
- Bhutta AT. Ketamine: a controversial drug for neonates. *Semin Perinatol* 2007;31:303–308. [PubMed: 17905185]
- Bittigau P, Siffringer M, Felderhoff-Mueser U, Hansen HH, Ikonomidou C. Neuropathological and biochemical features of traumatic injury in the developing brain. *Neurotox Res* 2003;5:475–490. [PubMed: 14715432]
- Boctor SY, Wang C, Ferguson SA. Neonatal PCP is more potent than ketamine at modifying preweaning behaviors of Sprague-Dawley rats. *Toxicol Sci* 2008;106:172–179. [PubMed: 18667523]
- Bredmose PP, Grier G, Davies GE, Lockey DJ. Pre-hospital use of ketamine in paediatric trauma. *Acta Anaesthesiol Scand* 2009;53:543–545. [PubMed: 19226295]
- Busto R, Dietrich WD, Globus MY, Valdes I, Scheinberg P, Ginsberg MD. Small differences in intraschemic brain temperature critically determine the extent of ischemic neuronal injury. *J Cereb Blood Flow Metab* 1987;7:729–738. [PubMed: 3693428]
- Creeley, CE.; Strajko, MMW.; Cattano, D.; Olney, JW. Annual Society for Neuroscience. Washington, DC: 2008. Hypothermia prevents spontaneous and anesthesia-induced neuroapoptosis in the infant mouse brain; p. 355-356.
- Desfeux A, El Ghazi F, Jegou S, Legros H, Marret S, Laudenbach V, Gonzalez BJ. Dual Effect of Glutamate on GABAergic Interneuron Survival during Cerebral Cortex Development in Mice Neonates. *Cereb Cortex*. 2009
- Ditsworth D, Priestley MA, Loepke AW, Ramamoorthy C, McCann J, Staple L, Kurth CD. Apoptotic neuronal death following deep hypothermic circulatory arrest in piglets. *Anesthesiology* 2003;98:1119–1127. [PubMed: 12717133]
- Engelhard K, Werner C, Hoffman WE, Matthes B, Blobner M, Kochs E. The effect of sevoflurane and propofol on cerebral neurotransmitter concentrations during cerebral ischemia in rats. *Anesth Analg* 2003;97:1155–1161. table of contents. [PubMed: 14500174]
- Fredriksson A, Archer T, Alm H, Gordh T, Eriksson P. Neurofunctional deficits and potentiated apoptosis by neonatal NMDA antagonist administration. *Behav Brain Res* 2004;153:367–376. [PubMed: 15265631]
- Fredriksson A, Ponten E, Gordh T, Eriksson P. Neonatal exposure to a combination of N-methyl-D-aspartate and gamma-aminobutyric acid type A receptor anesthetic agents potentiates apoptotic neurodegeneration and persistent behavioral deficits. *Anesthesiology* 2007;107:427–436. [PubMed: 17721245]
- Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler J, Dikranian K, Tenkova TI, Stefovskva V, Turski L, Olney JW. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. *Science* 1999a;283:70–74. [PubMed: 9872743]

- Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler J, Dikranian K, Tenkova TI, Stefovskaja V, Turski L, Olney JW. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. *Science* 1999b;283:70–74. [PubMed: 9872743]
- Ikonomidou C, Mosinger JL, Olney JW. Hypothermia enhances protective effect of MK-801 against hypoxic/ischemic brain damage in infant rats. *Brain Res* 1989;487:184–187. [PubMed: 2546648]
- Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW, Wozniak DF. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. *J Neurosci* 2003;23:876–882. [PubMed: 12574416]
- Kaindl AM, Koppelstaetter A, Nebrich G, Stuwe J, Siffringer M, Zabel C, Klose J, Ikonomidou C. Brief alteration of NMDA or GABAA receptor-mediated neurotransmission has long term effects on the developing cerebral cortex. *Mol Cell Proteomics* 2008;7:2293–2310. [PubMed: 18587059]
- Kaindl AM, Siffringer M, Zabel C, Nebrich G, Wacker MA, Felderhoff-Mueser U, Endesfelder S, von der Hagen M, Stefovskaja V, Klose J, Ikonomidou C. Acute and long-term proteome changes induced by oxidative stress in the developing brain. *Cell Death Differ* 2006;13:1097–1109. [PubMed: 16254572]
- Lema Tomé CM, Bauer C, Nottingham C, Smith C, Blackstone K, Brown L, Hlavaty C, Nelson C, Daker R, Sola R, Miller R, Bryan R, Turner CP. MK801-induced caspase-3 in the postnatal brain: inverse relationship with calcium binding proteins. *Neuroscience* 2006;141:1351–1363. [PubMed: 16782280]
- Lema Tomé CM, Nottingham CU, Smith CM, Beauchamp AS, Leung PW, Turner CP. Neonatal exposure to MK801 induces structural reorganization of the central nervous system. *Neuroreport* 2006;17:779–783. [PubMed: 16708014]
- Liu Q, Lowry TF, Wong-Riley MT. Postnatal changes in ventilation during normoxia and acute hypoxia in the rat: implication for a sensitive period. *J Physiol* 2006;577:957–970. [PubMed: 17038423]
- Lu LX, Yon JH, Carter LB, Jevtovic-Todorovic V. General anesthesia activates BDNF-dependent neuroapoptosis in the developing rat brain. *Apoptosis* 2006;11:1603–1615. [PubMed: 16738805]
- Lunardi N, Ori C, Erisir A, Jevtovic-Todorovic V. General Anesthesia Causes Long-Lasting Disturbances in the Ultrastructural Properties of Developing Synapses in Young Rats. *Neurotox Res*. 2009
- Lyll A, Swanson J, Liu C, Blumenthal TD, Turner CP. Neonatal exposure to MK801 promotes prepulse-induced delay in startle response time in adult rats. *Exp Brain Res* 2009;197:215–222. [PubMed: 19565228]
- McGowan FX Jr, Davis PJ. Anesthetic-related neurotoxicity in the developing infant: of mice, rats, monkeys and, possibly, humans. *Anesth Analg* 2008;106:1599–1602. [PubMed: 18499584]
- McInnis J, Wang C, Anastasio N, Hultman M, Ye Y, Salvemini D, Johnson KM. The role of superoxide and nuclear factor-kappaB signaling in N-methyl-D-aspartate-induced necrosis and apoptosis. *J Pharmacol Exp Ther* 2002;301:478–487. [PubMed: 11961046]
- Mellon RD, Simone AF, Rappaport BA. Use of anesthetic agents in neonates and young children. *Anesth Analg* 2007;104:509–520. [PubMed: 17312200]
- Ohmura A, Nakajima W, Ishida A, Yasuoka N, Kawamura M, Miura S, Takada G. Prolonged hypothermia protects neonatal rat brain against hypoxic-ischemia by reducing both apoptosis and necrosis. *Brain Dev* 2005;27:517–526. [PubMed: 15899566]
- Olney JW, Wozniak DF, Jevtovic-Todorovic V, Farber NB, Bittigau P, Ikonomidou C. Drug-induced apoptotic neurodegeneration in the developing brain. *Brain Pathol* 2002;12:488–498. [PubMed: 12408236]
- Olney JW, Young C, Wozniak DF, Ikonomidou C, Jevtovic-Todorovic V. Anesthesia-induced developmental neuroapoptosis. Does it happen in humans? *Anesthesiology* 2004;101:273–275. [PubMed: 15277906]
- Paxinos, G.; Watson, A. The rat brain in stereotaxic coordinates. Academic Press; San Diego: 1998.
- Proescholdt M, Heimann A, Kempfski O. Neuroprotection of S(+) ketamine isomer in global forebrain ischemia. *Brain Res* 2001;904:245–251. [PubMed: 11406122]
- Ringler SL, Aye J, Byrne E, Anderson M, Turner CP. Effects of disrupting calcium homeostasis on neuronal maturation: early inhibition and later recovery. *Cell Mol Neurobiol* 2008;28:389–409. [PubMed: 18196452]

- Rizzi S, Carter LB, Ori C, Jevtovic-Todorovic V. Clinical Anesthesia Causes Permanent Damage to the Fetal Guinea Pig Brain. *Brain Pathol.* 2008
- Rogalska J, Danielisova V, Caputa M. Effect of neonatal body temperature on postanoxic, potentially neurotoxic iron accumulation in the rat brain. *Neurosci Lett* 2006;393:249–254. [PubMed: 16289321]
- Rosomoff HL. Protective effects of hypothermia against pathological processes of the nervous system. *Ann N Y Acad Sci* 1959;80:475–486. [PubMed: 14439121]
- Slikker W Jr, Zou X, Hotchkiss CE, Divine RL, Sadovova N, Twaddle NC, Doerge DR, Scallet AC, Patterson TA, Hanig JP, Paule MG, Wang C. Ketamine-induced neuronal cell death in the perinatal rhesus monkey. *Toxicol Sci.* 2007
- Soriano SG, Loepke AW. Let's not throw the baby out with the bath water: potential neurotoxicity of anesthetic drugs in infants and children. *J Neurosurg Anesthesiol* 2005;17:207–209. [PubMed: 16184066]
- Steen PA, Soule EH, Michenfelder JD. Deterimental effect of prolonged hypothermia in cats and monkeys with and without regional cerebral ischemia. *Stroke* 1979;10:522–529. [PubMed: 116394]
- Stefovska VG, Uckermann O, Czuczwar M, Smitka M, Czuczwar P, Kis J, Kaindl AM, Turski L, Turski WA, Ikonomidou C. Sedative and anticonvulsant drugs suppress postnatal neurogenesis. *Ann Neurol* 2008;64:434–445. [PubMed: 18991352]
- Tomatir E, Atalay H, Gurses E, Erbay H, Bozkurt P. Effects of low dose ketamine before induction on propofol anesthesia for pediatric magnetic resonance imaging. *Paediatr Anaesth* 2004;14:845–850. [PubMed: 15385013]
- Turner CP. Perfect storm in a baby's brain. *Annual Society for Neurosciences Meeting Chicago* 2009;39:413.10.
- Turner CP, Connell J, Blackstone K, Ringler SL. Loss of calcium and increased apoptosis within the same neuron. *Brain Res* 2007a;1128:50–60. [PubMed: 17125751]
- Turner CP, DeBenedetto D, Liu C. NMDAR blockade-induced neonatal brain injury: Reversal by the calcium channel agonist BayK 8644. *Neurosci Lett* 2009a;450:292–295. [PubMed: 19070650]
- Turner CP, DeBenedetto D, Walburg C, Ware E, Lambert A, Lee A, Swanson J, Stowe R, Lyle M, Desai P, Johnson R, Liu C. MK801-induced activated caspase-3 exhibits selective co-localization with GAD67. *Neurosci Lett* 2009b;462:152–156. [PubMed: 19596402]
- Turner CP, DeBenedetto D, Ware E, Stowe R, Lee A, Swanson J, Walburg C, Lambert A, Lyle M, Desai P, Liu C. Postnatal exposure to MK801 induces selective changes in GAD67 or parvalbumin. *Exp Brain Res.* 2009c In Press.
- Turner CP, DeBenedetto D, Ware E, Walburg C, Lee A, Stowe R, Swanson J, Lambert A, Lyle M, Desai P, Johnson R, Liu C. MK801-induced activated caspase-3 exhibits selective co-localization with GAD67. *Neurosci Lett* 2009d;462:152–156. [PubMed: 19596402]
- Turner CP, Miller R, Smith C, Brown L, Blackstone K, Dunham SR, Strehlow R, Manfredi M, Slocum P, Iverson K, West M, Ringler SL, Berry ZC. Widespread neonatal brain damage following calcium channel blockade. *Dev Neurosci* 2007b;29:213–231. [PubMed: 16921238]
- Turner CP, Pulciani D, Rivkees SA. Reduction in intracellular calcium levels induces injury in developing neurons. *Exp Neurol* 2002;178:21–32. [PubMed: 12460605]
- Vardi A, Salem Y, Padeh S, Paret G, Barzilay Z. Is propofol safe for procedural sedation in children? A prospective evaluation of propofol versus ketamine in pediatric critical care. *Crit Care Med* 2002;30:1231–1236. [PubMed: 12072673]
- Wang C, Anastasio N, Popov V, Leday A, Johnson KM. Blockade of N-methyl-D-aspartate receptors by phencyclidine causes the loss of corticostriatal neurons. *Neuroscience* 2004;125:473–483. [PubMed: 15062989]
- Wang C, Fridley J, Johnson KM. The role of NMDA receptor upregulation in phencyclidine-induced cortical apoptosis in organotypic culture. *Biochem Pharmacol* 2005;69:1373–1383. [PubMed: 15826608]
- Wang C, McInnis J, Ross-Sanchez M, Shinnick-Gallagher P, Wiley JL, Johnson KM. Long-term behavioral and neurodegenerative effects of perinatal phencyclidine administration: implications for schizophrenia. *Neuroscience* 2001;107:535–550. [PubMed: 11720778]

- Wang C, McInnis J, West JB, Bao J, Anastasio N, Guidry JA, Ye Y, Salvemini D, Johnson KM. Blockade of phencyclidine-induced cortical apoptosis and deficits in prepulse inhibition by M40403, a superoxide dismutase mimetic. *J Pharmacol Exp Ther* 2003;304:266–271. [PubMed: 12490600]
- Wang CZ, Johnson KM. The role of caspase-3 activation in phencyclidine-induced neuronal death in postnatal rats. *Neuropsychopharmacology* 2007;32:1178–1194. [PubMed: 16985504]
- Wang CZ, Yang SF, Xia Y, Johnson KM. Postnatal Phencyclidine Administration Selectively Reduces Adult Cortical Parvalbumin-Containing Interneurons. *Neuropsychopharmacology*. 2007
- Wilder RT, Flick RP, Sprung J, Katusic SK, Barbaresi WJ, Mickelson C, Gleich SJ, Schroeder DR, Weaver AL, Warner DO. Early exposure to anesthesia and learning disabilities in a population-based birth cohort. *Anesthesiology* 2009;110:796–804. [PubMed: 19293700]
- Yager J, Towfighi J, Vannucci RC. Influence of mild hypothermia on hypoxic-ischemic brain damage in the immature rat. *Pediatr Res* 1993;34:525–529. [PubMed: 8255688]
- Yager JY, Armstrong EA, Black AM. Treatment of the term newborn with brain injury: simplicity as the mother of invention. *Pediatr Neurol* 2009;40:237–243. [PubMed: 19218037]
- Yamamoto LG. Initiating a hospital-wide pediatric sedation service provided by emergency physicians. *Clin Pediatr (Phila)* 2008;47:37–48. [PubMed: 17766577]
- Yon JH, Daniel-Johnson J, Carter LB, Jevtovic-Todorovic V. Anesthesia induces neuronal cell death in the developing rat brain via the intrinsic and extrinsic apoptotic pathways. *Neuroscience* 2005;135:815–827. [PubMed: 16154281]
- Yuede, CM.; Wang, S.; Qin, Y-Q.; Creeley, CE.; Olney, JW. Annual Society for Neuroscience. Chicago: 2009. Hypothermia is protective against PCP-induced apoptosis in the developing mouse brain; p. 509.502

Fig. 1. Ketamine induces changes in core body temperature (CBT) and activated caspase-3 (AC3) expression in P7 neonates

A. CBT (mean $\pm$ SE) was measured at various times (0-8 hours) following injection of vehicle (V; $n = 7$), MK801 (1 mg/kg; $n = 4$) or ketamine (2-20 mg/kg; K2-K20; $n = 4-5$). **B.** Quantitative analysis of AC3 positive cells (mean $\pm$ SE) in layers IV and V of the anterior somatosensory cortex across all treatments (see Experimental Procedures). In A and B, differences in the means determined by ANOVA using a Bonferroni post-test comparison; *** $p < 0.001$ compared to control. **C1-C6** Expression of AC3 in the somatosensory cortex of vehicle (V), MK801 or ketamine (K2-K20) treated animals; scale bar is 25 μ m. Roman numerals indicate approximate location of layers and arrows point to AC3-positive cells.

Fig. 2. Ketamine induces activated caspase-3 (AC3): Western blot analysis

P7 rat pups were injected with vehicle (Veh; $n = 5$), MK801 (MK; 1 mg/kg; $n = 5$) or various doses of ketamine (K2, 2mg/kg; K5, 5 mg/kg; K10, 10 mg/kg or K20, 20 mg/kg; $n = 6$ for all doses). See Experimental Procedures for details. At 8 hr, proteins were extracted from the anterior somatosensory cortex and processed for expression of the 17 kDa band for the cleaved (activated) form of caspase-3 (AC3) **A**. Representative Western blot for Veh, MK801, or ketamine (K20)-treated rats. **B**. Mean band density ($\pm$ SE) for AC3 (relative to β -actin); *** $p < 0.001$ compared to vehicle controls; ANOVA, Bonferroni post-test comparison of means.

Fig. 3. Lowering CBT does not alter ketamine-induced changes in AC3 expression in layers IV and V of the SSC in P7 neonates

P7 rat pups were injected with vehicle or ketamine (20 mg/kg) and were housed in an environment that was kept at a constant 25°C (veh-25C; K20-25C). For comparison, we included a vehicle and a K20 group that were returned to the mother after each injection. To account for litter-litter variations (as well as to increase sample size), these data were combined with those derived from the vehicle and K20 groups described in Fig. 1 and for greater visual clarity are present as separate figure here. **A.** CBT (mean $\pm$ SE) was measured at various times (0-8 hours). V - vehicle (with mother; n = 9), K20 - ketamine (20 mg/kg; with mother; n = 7); veh-25C – vehicle (kept at 25°C; n = 3); K20-25C – 20 mg/kg ketamine (kept at 25°C; n = 3).. **B.** Quantitative analysis of AC3 positive cells (mean $\pm$ SE) in layers IV and V of the anterior somatosensory cortex across all treatments (see Experimental Procedures). In both A and B, differences in the means determined by ANOVA using a Bonferroni post-test comparison; **p<0.01, ***p<0.001 compared to control.

Fig. 4. Elevation of CBT does not alter ketamine-induced apoptosis in layers IV and V of the somatosensory cortex in P7 rats

Animals were injected with vehicle or 40 mg/kg ketamine (K40) as described (see Experimental Procedures). For some K40 animals, CBT was not artificially maintained (K40-HT) whereas for other K40 animals CBT was artificially maintained (K40-NT; see Experimental Procedures) **A**. Changes in CBT (mean $\pm$ SE) after injection of vehicle (V; $n = 7$) or 40 mg/kg ketamine in the absence or presence of an artificial heat source (K40-HT, $n = 4$ or K40-NT, $n = 4$). **B**. Quantitative analysis of AC3 positive cells within layers IV/V of the anterior somatosensory cortex (see Experimental Procedures). In A and B, differences in the means determined by ANOVA using a Bonferroni post-test comparison; * $p < 0.05$, *** $p < 0.001$ compared to control; all other comparisons not significant. **C1-C2**. Expression of AC3 within layers IV/V of the anterior somatosensory cortex in V, NT and HT animals; scale bar in C2 is 25 μm . Roman numerals indicate approximate location of layers and arrows point to AC3-positive cells.

Fig. 5. Ketamine-induced changes in CBT are age-dependent

A. CBT changes (mean $\pm$ SE) were measured in P14 and P21 animals after injection of vehicle (V; $n = 2$ both ages) or ketamine (K40; $n = 3$ both ages). **B** Quantitative analysis of AC3 positive cells within layers IV/V of the anterior somatosensory cortex (see Experimental Procedures) in P14 and P21 animals. In A and B, differences in the means determined by ANOVA using a Bonferroni post-test comparison; * $p < 0.05$, ** $p < 0.01$, compared to control; all other comparisons not significant. **C1-C4** Expression of AC3 within layers IV/V of the anterior somatosensory cortex in NT and HT animals; scale bar in C2 is 25 μ m. Roman numerals indicate approximate location of layers and arrows point to AC3-positive cells.