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KETAMINE PROTECTS HIPPOCAMPAL NEURONS FROM ANOXIA *IN VITRO*

S. M. ROTHMAN,*†‡ J. H. THURSTON,* R. E. HAUHART,* G. D. CLARK*† and J. S. SOLOMON†
Departments of *Pediatrics and †Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, MO 63110, U.S.A.

Abstract—Ketamine, a dissociative, general anesthetic, blocks the excitation produced by activating one class of excitatory amino acid receptors, the *N*-methyl-D-aspartate receptor in the rat. We have found that ketamine can protect hippocampal neurons in culture and slice from anoxia. When added to cultures immediately prior to anoxic exposure, ketamine prevented the neuronal destruction seen after a day of anoxia. Neurons appeared undamaged and had normal resting and action potentials. Adenosine triphosphate levels in ketamine-protected anoxic cultures were approximately two-thirds of normal controls. Ketamine also prevented the irreversible loss of the population spike seen in hippocampal slices after prolonged perfusion with anoxic buffer. These results suggest that ketamine may have therapeutic potential in preventing anoxic damage from stroke in man.

Cerebral hypoxia and ischemia are major causes of irreversible neurological injury in man. Despite extensive basic research, there is still no effective medical or surgical therapy which will preserve human central neurons exposed to an otherwise lethal anoxic or ischemic insult. Recent experiments in a variety of *in vitro* and *in vivo* systems have demonstrated that specific excitatory amino acid antagonists can protect neurons from pure anoxia or ischemia.[26] The amino acid antagonists used in these previous studies cross the blood/brain barrier poorly and are, therefore, unsatisfactory candidates for clinical or even whole animal studies which require systemic drug administration.[17] As the dissociative anesthetic ketamine can block excitatory responses produced by activation of *N*-methyl-D-aspartate (NMDA) receptors,[1,7,30] we investigated whether it was capable of protecting hippocampal neurons from anoxia. We performed four separate types of experiments: morphological, physiological and biochemical observations in tissue culture and physiological observations in tissue slices.

EXPERIMENTAL PROCEDURES

Tissue culture experiments were performed on dissociated rat (Sprague–Dawley) hippocampal neurons 2–3 weeks *in vitro*. Culture preparation has been described in detail in previous publications.[23,24] In order to make cultures anoxic, they were placed into a sealed incubator which was flushed with 95% nitrogen (N_2) and 5% carbon dioxide (CO_2). For intracellular recording, cultures were placed on the stage of an inverted, phase contrast microscope and single cells were impaled under direct vision at ×500 magnification. Standard glass microelectrodes and high-impedance amplifiers were used in these experiments.[25] Drugs were applied with blunt micropipettes positioned close to the impaled neurons, a technique which has been described by Choi and Fischbach.[4]

For adenosine triphosphate (ATP) analyses of the cultures, cells were washed three times with Hanks' balanced salt solution and removed from the bottom of 35 mm culture dishes. After gentle centrifugation they were extracted at 4°C.[14] The cell preparations were then placed into a −10°C alcohol bath and homogenized in approximately 20 volumes of 3 M perchloric acid. They were then diluted with 1 mM ethylenediaminetetra-acetate and again centrifuged. The supernatant was neutralized with 2 M $KHCO_3$. Protein was measured by the method of Lowry.[12] ATP was assayed by measuring light emission in the presence of luciferin/luciferase using a luminometer (Packard 6100).

Our slice experiments used the paradigm described in detail in the preceding publication except that we extended the period of anoxia to 50 min.[5,19] We did not do any intracellular recordings, however. Population spikes were measured before and after anoxia produced by perfusion with buffer equilibrated with 95% N_2/5% CO_2. Control and ketamine experiments were run in random sequence, and the person recording the physiological data did not know whether perfusion solution during anoxia contained ketamine. If population spike return exceeded 100%, return was calculated as 100%.

Materials

Ketamine was supplied by Warner–Lambert (Ann Arbor). The D-2-amino-5-phosphonovalerate was obtained from Cambridge Research Biochemicals (Atlantic Beach, New York). Luciferin/luciferase came from Boehringer Mannheim Biochemicals as a kit.

RESULTS

We verified previous reports that ketamine could block NMDA responses.[1,7,30] Intracellular recording from our cultured neurons showed that the depolarization produced by NMDA (100 μM) alone was reduced by 71 ± 16% (±1 SD) when NMDA was simultaneously applied with ketamine (100 μM, $n = 5$; Fig. 1). In addition, the dramatic neurotoxicity produced by NMDA (100 μM) in our cultures was prevented when ketamine (100 μM) was also present in the medium ($n = 4$; Fig. 2). Ketamine had no effect

‡To whom correspondence should be addressed.
Abbreviations: APV, D-2-amino-5-phosphonovalerate; ATP, adenosine triphosphate; NMDA, *N*-methyl-D-aspartate.

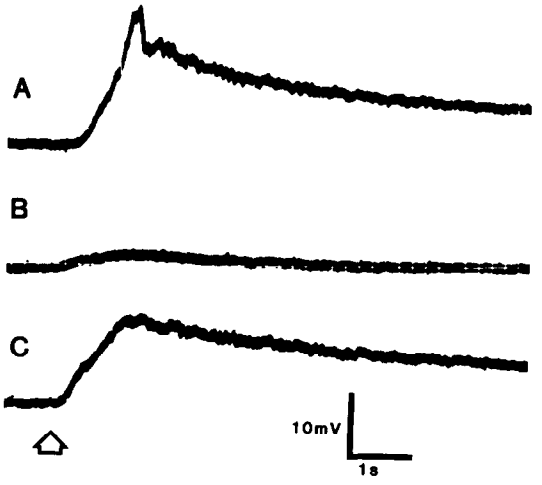

Fig. 1. Antagonism of NMDA by ketamine. Top trace (A) shows the depolarization produced by a 1 s pulse of NMDA (100 μM) close to a neuron impaled with an intracellular microelectrode. Middle trace (B) shows that simultaneous application of ketamine (100 μM) reduces the NMDA response by approximately 80%. Bottom trace (C) shows recovery of NMDA response after ketamine has diffused away. Arrow marks start of drug application for all three traces.

on the toxicity of kainate, another excitatory amino acid.[8]

Our cultured neurons die over approximately 1 day when placed into a sealed incubator with 95% N_2/5% CO_2 (Fig. 3A). At this time virtually no intact neurons are visible in the entire culture dish. Intracellular recording from over 300 cells in culture has shown that cells identical to those dying after anoxia initially possess the physiological properties of neurons.[25] They have action potentials, synaptic activity and transmitter sensitivity typical of neurons. There are some flat cells which form a partial, confluent monolayer beneath the neurons and survive after anoxia. Under the scanning electron microscope, these appear to be glia.[23]

When ketamine was added to the medium before exposing cultures to anoxia, the sensitivity of the neurons to anoxia was markedly reduced (Fig. 3B). After 1 day, neurons were minimally changed when viewed with phase contrast optics. These experiments were replicated nine separate times with 19 cultures. The initial experiments were done with 100 μM ketamine but the concentration was subsequently reduced to 25 and then 10 μM. There was loss of

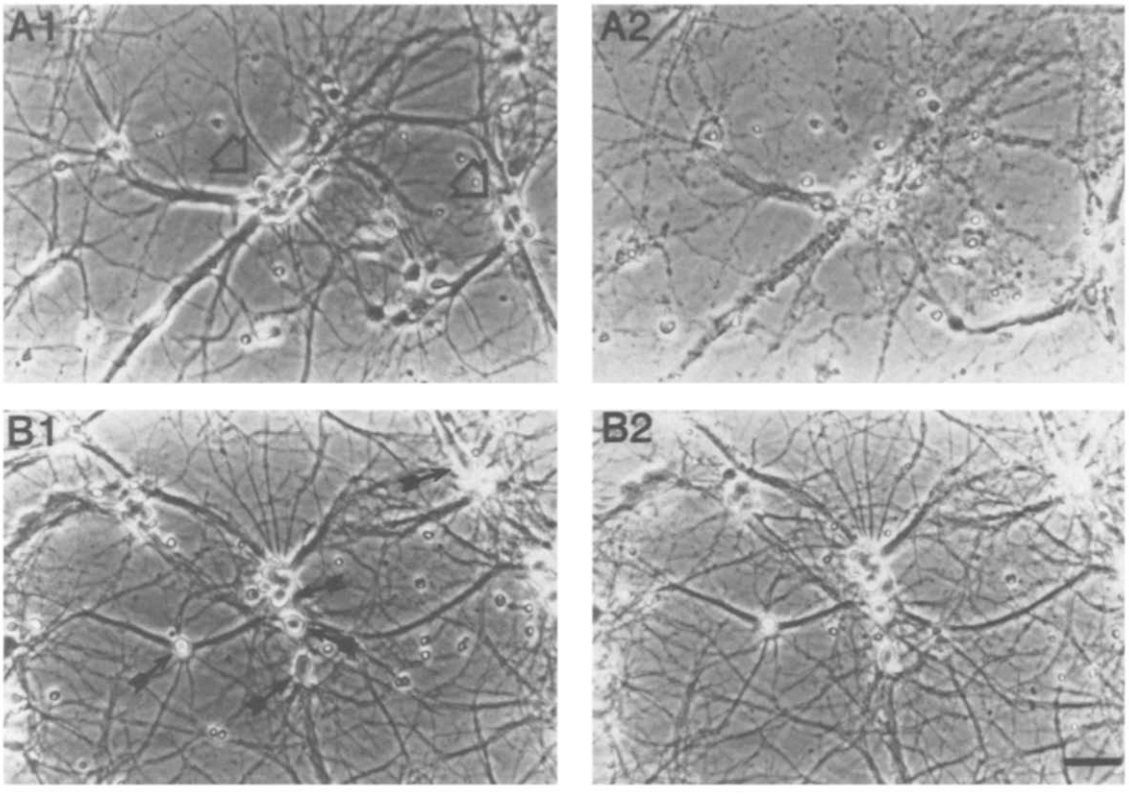

Fig. 2. Ketamine blocks NMDA neurotoxicity. (A) These photomicrographs show the effects of NMDA (100 μM) on hippocampal neurons. Left (A1) shows a field in a culture prior to addition of NMDA. Clusters of neuronal cell bodies are marked by arrowheads. Right (A2) shows the same field after a day exposed to NMDA. Intact neurons are no longer apparent at this time; in fact the neuronal destruction becomes obvious within 1 h of NMDA exposure. (B) Ketamine is capable of blocking NMDA neurotoxicity. When ketamine (100 μM) is added to cultures prior to NMDA (B1), there is no obvious change in neuronal morphology after a day (B2). Representative neuronal cell bodies indicated by arrows in (B1), appear unchanged in (B2). Bar = 50 μm.

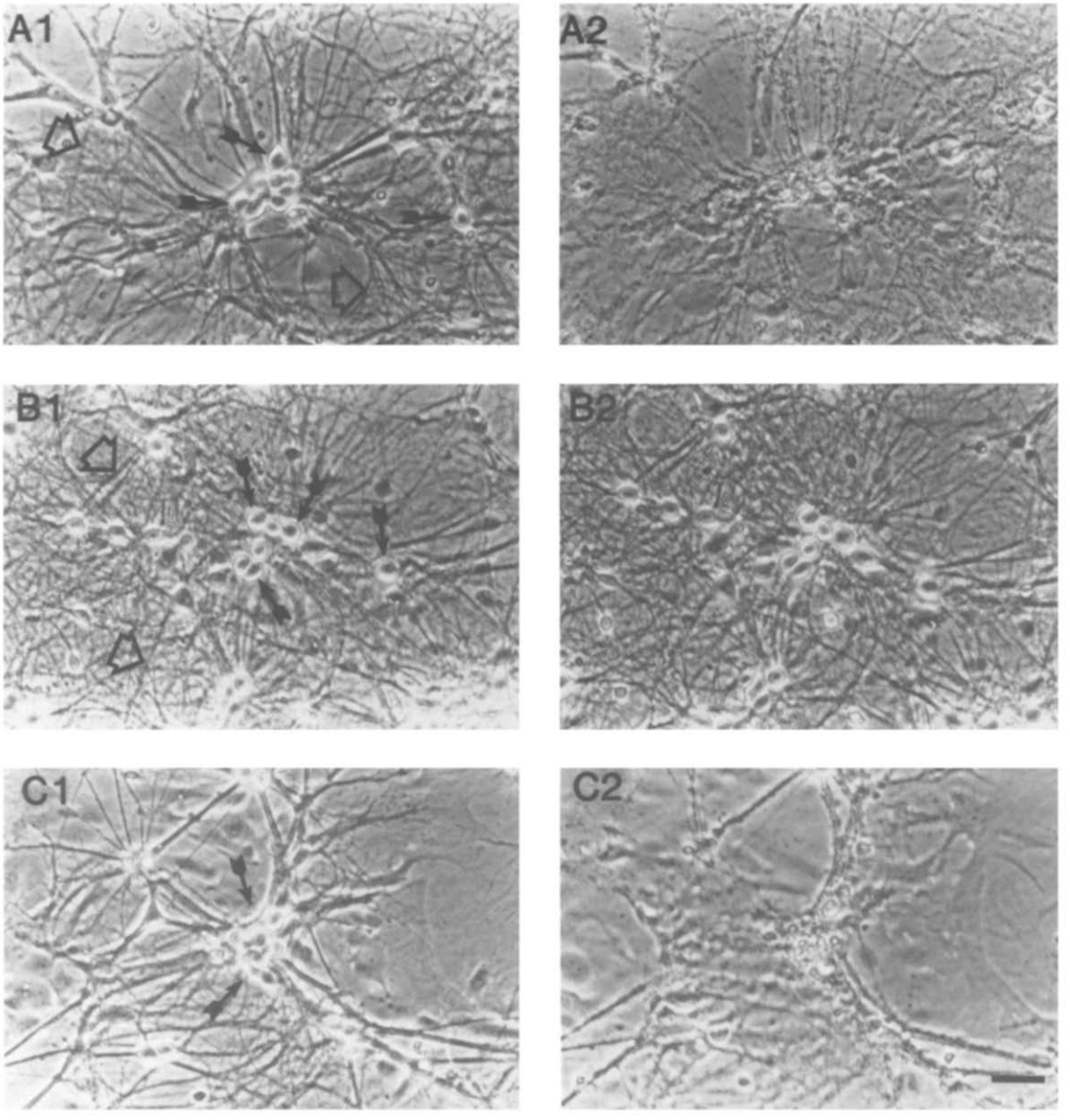

Fig. 3. Effect of ketamine on anoxic hippocampal cultures. (A) Left (A1) is a photomicrograph of a field of hippocampal neurons 18 days in culture. Arrows indicate representative neuronal cell bodies; arrowheads point to portions of the extensive network of neuronal processes. Right (A2) shows the same field after 1 day of anoxia. The cell bodies cannot be easily identified and processes are disintegrating. (B) Left (B1) shows a field in another 18 day culture immediately prior to exposure to an anoxic atmosphere. Arrows mark four neuronal cell bodies and arrowheads point to processes. Ketamine (25 μM) was added to the culture medium and after 1 day without oxygen the cells and processes still appear intact (B2). (C) Left (C1) shows a cluster of neurons (arrows) in a culture treated with pentobarbital (1 mM) immediately prior to anoxia. After 1 day neurons have degenerated (C2). Bar = 50 μm.

neurons in anoxic cultures treated with 1 μM ketamine ($n = 4$), but even these were clearly better preserved than untreated anoxic cultures.

To better document that neurons in ketamine-protected cultures were normal after prolonged anoxia, cells in one such culture were studied with intracellular recording. After 22 h without oxygen, the medium containing 100 μM ketamine was replaced with Hanks' solution and neurons were impaled with microelectrodes. The amplitudes of resting potentials, 57.6 $\pm$ 5.3 mV ($\pm$ 1 SD), and action potentials, 65.1 $\pm$ 8.8 mV, of ten neurons were similar to values found in healthy rodent hippocampal slices (> 50 mV and 64.9 $\pm$ 10 mV for resting and action potentials, respectively).[27]

Over 20 years ago, Lowry and colleagues[13] demonstrated that within a few minutes of irreversible ischemia, ATP levels in adult rat brain become almost undetectable. Therefore, we measured ATP levels in some of our cultures to determine whether

Table 1. Adenosine triphosphate values in anoxic cultures

Treatment	ATP % of control; mean ± SE
Untreated anoxic (7)	17.8 ± 2.6
Ketamine 10 μM (3)	48.3 ± 4.2*†
Ketamine 25 μM (4)	69.4 ± 5.2*‡§
Ketamine 100 μM (4)	64.0 ± 9.9*‡
APV 100 or 200 μM (6)	63.7 ± 3.3*‡
Pentobarbital 1 mM (6)	26.0 ± 1.2

P values versus *anoxia, <0.01; †pentobarbital, <0.05; ‡pentobarbital, <0.01; §ketamine (10 μM), <0.05. Pentobarbital not significantly different from control, anoxic cultures. Statistical significance was determined by using a one-way analysis of variance followed by the honestly significant difference (HSD) method of Tukey[31] to identify statistically significant differences between specific pairs of means. Numbers in parentheses are sample sizes.

energy stores were preserved with ketamine treatment. As control ATP values for different sets of cultures ranged from 0.62 to 2.34 μM/g protein, experimental values were always compared to control values from sister cultures to give a percentage of control. ATP levels in cultures protected with 10, 25 or 100 μM ketamine prior to anoxia were all significantly greater than untreated cultures exposed to identical anoxic treatment (Table 1). Cultures treated with the higher two ketamine concentrations had average ATP levels approximately two-thirds of control, normoxic cultures. The cultures in 10 μM ketamine had lower ATP levels, but these were still about half of control, normoxic values and almost triple control anoxic ATP levels. The residual ATP in control, anoxic cultures may come from surviving glia. The partial conservation of ATP in the ketamine-treated cultures was not due to an elevation of basal ATP levels as neither ketamine nor the other drugs used in these studies affected control ATP concentrations. These results are further evidence that our treated cultures remained viable.

We also felt that it was important to establish that other selective NMDA antagonists could protect the hippocampal cultures from anoxic damage. We tested D-2-amino-5-phosphonovalerate (APV), which has previously been shown to depress NMDA responses.[6] Neurons in cultures pretreated with APV (100 or 200 μM) showed little morphological change after 1 day of anoxia (11 cultures in six separate experiments). ATP levels in these treated cultures were also approximately two-thirds of values seen in control, normoxic cultures (Table 1).

Another control was done to determine if ketamine's protective effects are shared by other general anesthetics. We examined the ability of the anesthetic barbiturate pentobarbital to reduce anoxic neuronal damage. At a high concentration, pentobarbital (1 mM) was ineffective in protecting cultured neurons from anoxia. When pentobarbital was added to cultures immediately prior to anoxia, they did not appear different from untreated anoxic cultures after 1 day (seven cultures in four separate experiments Fig. 3C). ATP levels in pentobarbital-treated cultures were slightly, but not significantly, higher than values in untreated anoxic cultures (Table 1).

A final set of experiments with ketamine and anoxia were done using transverse hippocampal slices.[5] We studied 11 slices, five control and six ketamine-treated, in the current series. After 50 min of anoxia, population spikes failed to return in control slices (Fig. 4A). However, there was a 95 ± 6% return of the population spike in slices treated with ketamine (25 μM) during anoxia (Fig. 4B).

DISCUSSION

Our results in both hippocampal cultures and slices indicate that ketamine was able to protect neurons from anoxia. The preservation of normal neuronal

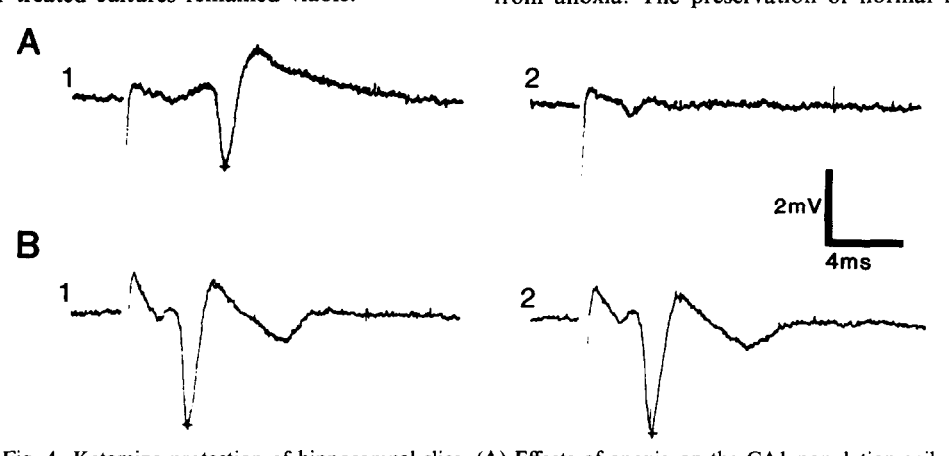

Fig. 4. Ketamine protection of hippocampal slice. (A) Effects of anoxia on the CA1 population spike. (A1) This is the typical appearance of a population spike under control conditions. (A2) After perfusion for 50 min with buffer equilibrated with 95% N_2/5% CO_2 and then 35 min of reoxygenation the population spike failed to return. The downward deflection 2.6 ms after the stimulus has half the latency of the control field potential, so it is not a spike. (B) Ketamine protection of another slice. The population spike (B2) returned to almost control value (B1) after 50 min of anoxia and 45 min of reoxygenation when ketamine (25 μM) was present in perfusate during anoxia.

morphology and physiology argues strongly that the cultured hippocampal neurons were able to tolerate anoxia when pretreated with ketamine. The partial conservation of ATP in ketamine-treated cultures also suggests that they were able to withstand the prolonged absence of oxygen. Finally, the consistent return of population spikes after anoxia in ketamine-treated hippocampal slices provides even more evidence that the drug allows mammalian central neurons to survive without oxygen.[5,18]

These findings are best explained by ketamine's ability to antagonize excitation produced by NMDA receptor activation. Ketamine blocked NMDA-induced depolarization in our cultured neurons, and other investigators have shown that ketamine reduces NMDA responses in spinal cord,[1] cortex [30] and hippocampus.[7] Ketamine prevented the neuronal destruction seen after NMDA, but not kainate, was added to the hippocampal cultures. APV, another selective NMDA antagonist, prevented anoxic neuronal death in the cultures and slices.[5] Finally, pentobarbital, a general anesthetic which has its major action at sites other than excitatory amino acid receptors, could not prevent anoxic neuronal death in the cultures.[11]

The mechanism of NMDA blockade by ketamine is interesting and deserves comment. Ketamine is a non-competitive NMDA antagonist[16] and fails to displace APV from binding sites in brain membranes.[20] In voltage-clamped neurons, its reduction of NMDA-induced currents is dependent on the holding potential.[15] These observations are not consistent with ketamine acting at an NMDA recognition site and suggest that ketamine may actually block ion channels whose opening is triggered by NMDA.

We feel our results have possible therapeutic implications. Simon and co-workers have already shown that direct intracerebral injection of another NMDA antagonist, aminophosphonoheptanoate, protected hippocampal neurons from ischemic injury.[28] However, this compound, like most other excitatory amino acid antagonists, crosses the blood/brain barrier poorly.[17] Ketamine penetrates the brain readily after systemic administration. The level of ketamine measured in the cerebrospinal fluid in man after typical anesthetic doses approaches 1 μM,[3] and experimental animals tolerate doses which should produce concentrations much higher than this. Therefore, the concentrations used in our *in vitro* studies may be tolerated by patients.

Our findings raise questions which have to be answered before ketamine can be tested in clinical trials on patients experiencing acute cerebral hypoxia/ischemia. First, it is not clear if drug administration after an ischemic episode can still protect the brain. However, the recent finding of increased electrical activity in postischemic brain,[29] which only subsequently undergoes infarction, does suggest that transmitter release plays a role in delayed neuronal loss and that there may be a time window for successful intervention with drugs that diminish synaptic activity.

Second, ketamine's activity is probably not restricted to the NMDA receptor/channel complex. Ketamine anesthesia is associated with increased intracranial pressure[9] and an elevated cerebral metabolic rate for glucose.[10] In addition, phencyclidine, another dissociative anesthetic closely related to ketamine,[1] has recently been shown to block presynaptic potassium channels.[2] It is likely that ketamine has similar ability. All of these actions may be deleterious to ischemic brain. If necessary, however, ketamine might be combined with other agents to eliminate these undesirable effects.

Third, these studies have not examined any of the other variables which may be involved in the pathophysiology of hypoxic/ischemic brain damage.[22] As mentioned previously, it is not yet clear why agents affecting only NMDA responses are so effective in protecting against anoxic neuronal damage.[5] The fact that NMDA antagonism did not completely preserve ATP values in the experiments described above suggests that there may have been neuronal damage and that other factors influence the sensitivity of neurons to hypoxia even in our cultures.

None the less, investigators should begin exploring the potential benefits of ketamine therapy in experimental cerebral ischemia. There is now a variety of animal stroke models which would be appropriate preparations in which to test ketamine.[21] As no existent therapies for hypoxic/ischemic brain injury offer much promise of preserving neuronal function, we feel that these *in vivo* studies should be undertaken quickly.

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