

Rabies Virus Infection of Primary Neuronal Cultures and Adult Mice: Failure To Demonstrate Evidence of Excitotoxicity†

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Cultures derived from the cerebral cortices and hippocampi of 17-day-old mouse fetuses infected with the CVS strain of rabies virus showed loss of trypan blue exclusion, morphological apoptotic features, and activated caspase 3 expression, indicating apoptosis. The NMDA (*N*-methyl-D-aspartate acid) antagonists ketamine (125 μ M) and MK-801 (60 μ M) were found to have no significant neuroprotective effect on CVS-infected neurons, while the caspase inhibitor Ac-Asp-Glu-Val aspartic acid aldehyde (25 μ M) exerted a marked neuroprotective effect. Glutamate-stimulated increases in levels of intracellular calcium were reduced in CVS-infected hippocampal neurons. Ketamine (120 mg/kg of body weight/day intraperitoneally) given to CVS-infected adult mice produced no beneficial effects. We have found no supportive evidence that excitotoxicity plays an important role in rabies virus infection.

Rabies is an acute viral infection of the central nervous system (CNS) for which there is no effective antiviral therapy in humans (10, 12). The events in rabies virus infection that lead to CNS disease and a fatal outcome are still not well understood (6). Studies with rat cortical neurons and with a rat model suggested the possibility that the noncompetitive *N*-methyl-D-aspartate acid (NMDA) antagonists ketamine and MK-801 might be effective therapeutic agents for human rabies (15, 16, 20) and that ketamine inhibits viral RNA genome transcription (15). Consequently, a working group recommended that ketamine be considered for the therapy of human patients with rabies (12). Recently, a patient survived rabies without administration of rabies vaccine prior to the onset of the disease; her therapeutic regimen included ketamine (48 mg/kg of body weight/day) (21). However, it is unknown if the therapeutic agents she received played an important role in her recovery (8, 9). Excitotoxicity has been implicated in the pathogenesis of virus-induced diseases of the CNS (2, 5, 14, 18, 19). This study examines whether excitotoxicity occurs in rabies virus infection of primary neuronal cultures and evaluates ketamine in experimental rabies in mice.

Primary cultures of mouse cortical and hippocampus neurons were prepared from fetuses of pregnant CD-1 mice at embryonic day 17 (3). Over 95% of the cells expressed the neuronal marker MAP-2. Cells were infected with the CVS strain of rabies virus, and there was expression of rabies virus antigen in over 90% of the cells. CVS- and mock-infected cells were analyzed using trypan blue exclusion for viability (17) on days 1, 2, and 3 postinfection (p.i.). There was a progressive loss in the viability in the cultures over time for the CVS-infected compared with the mock-infected cultures (Fig. 1). By

48 h p.i., infected neurons showed condensations of nuclear chromatin and cytoplasmic shrinkage, indicating apoptosis. Positive TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) staining was observed in CVS-infected neurons by 24 h p.i. and later increased. Expression of activated caspase 3 was demonstrated in CVS-infected cultured cells. Treatment with 25 μ M Ac-Asp-Glu-Val aspartic acid aldehyde (DEVD-CHO), a caspase inhibitor, in CVS infection resulted in significant improvement in viability on days 1 to 3 ($P < 0.002$) (Fig. 1). There was loss of viability on days 1 to 3 p.i. in a dose-dependent manner for both ketamine- and MK-801-treated cortical cells at concentrations of 0 to 2 mM (data not shown), and 125 μ M ketamine and 60 μ M MK-801 were selected for evaluation with CVS-infected cultures. Neither ketamine nor MK-801 was observed to exert a neuroprotective effect on CVS-infected cultures (Fig. 2). At 1 and 2 days p.i. ketamine significantly reduced the viability of CVS-infected hippocampal neurons ($P = 0.019$ and 0.006 , respectively), while MK-801 had no significant effect on CVS-infected neurons.

We measured the relative intracellular calcium concentration ($[Ca^{2+}]_i$) in mock- and CVS-infected cells stimulated by 10 μ M glutamate (13) (Fig. 3). In CVS-infected cortical neurons, glutamate did not induce a change in $[Ca^{2+}]_i$ versus that in mock-infected cultures, except there was a lower response at 24 h p.i. (t test, $P = 0.0022$). There were significant reductions in intracellular Ca^{2+} in CVS-infected hippocampal neurons compared with mock-infected neurons at 24, 48, and 72 h (t test, $P = 0.013$, 0.031 , and 0.029 , respectively).

Mice inoculated in the right hind limb footpad or intracerebrally with CVS that received vehicle or ketamine showed no difference in the time of onset or progression of disease (log rank test, $P = 0.54$ and 0.30 , respectively) or in the mortality rate ($P = 0.53$ and 0.50 , respectively) (Fig. 4A and B). Mice treated with vehicle and ketamine showed similar numbers of infected CNS neurons on days 3 to 6 (Table S1; $P > 0.05$). At day 5 p.i., viral infectivity assays showed a similar amount of infectious virus in the brain and spinal cord with vehicle and

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FIG. 1. Viability of cultured neurons from the cerebral cortex (left) and hippocampus (right) with and without 25 μ M DEVD-CHO, which is a caspase inhibitor, as assessed by trypan blue exclusion. DEVD-CHO exerted a marked neuroprotective effect on the cultures, resulting in viability similar to that in the mock-infected cultures. Error bars represent standard errors of the means.

ketamine treatments (Table S2). In moribund mice inoculated in the footpad, there was no difference in the number of infected neurons in the cerebral cortex or hippocampus, and there were more infected neurons in the midbrains of mice treated with ketamine than of mice treated with vehicle ($P = 0.0009$) (Fig. 4C).

Mice inoculated intracerebrally also showed no difference in the numbers of infected neurons between treatment groups (Table S1; $P > 0.05$ for all brain regions) (Fig. 4D). There were similar amounts of infectious virus in the brain at day 5 p.i. (Table S2). Histopathology of intracerebrally inoculated mice showed neuronal apoptosis and a loss of pyramidal neurons in the CA1 and CA3 regions of the hippocampus in both groups with no significant difference between the groups ($P > 0.05$).

The present study confirms that rabies virus infection of

primary neuron cultures induces caspase-dependent neuronal apoptosis. There was associated expression of the downstream executioner caspase 3, and the caspase inhibitor DEVD-CHO effectively inhibited rabies virus-induced neuronal cell death in the cultures. The noncompetitive NMDA antagonists ketamine and MK-801 did not provide neuroprotection of rabies virus-infected primary neurons. Much lower concentrations of MK-801 (3 μ M) provided effective neuroprotection of Sindbis virus infection of rat primary cortical neurons (18). In our CVS-infected cultures, glutamate stimulation did not result in increased $[Ca^{2+}]_i$ (Fig. 3). Accumulation of intracellular Ca^{2+} plays a critical role in the early stages of glutamate-induced neurotoxicity (4), and NMDA receptors play a key role in glutamate-induced neurotoxicity due to their high Ca^{2+} permeability (1). The $[Ca^{2+}]_i$ indicates that excitotoxicity did not

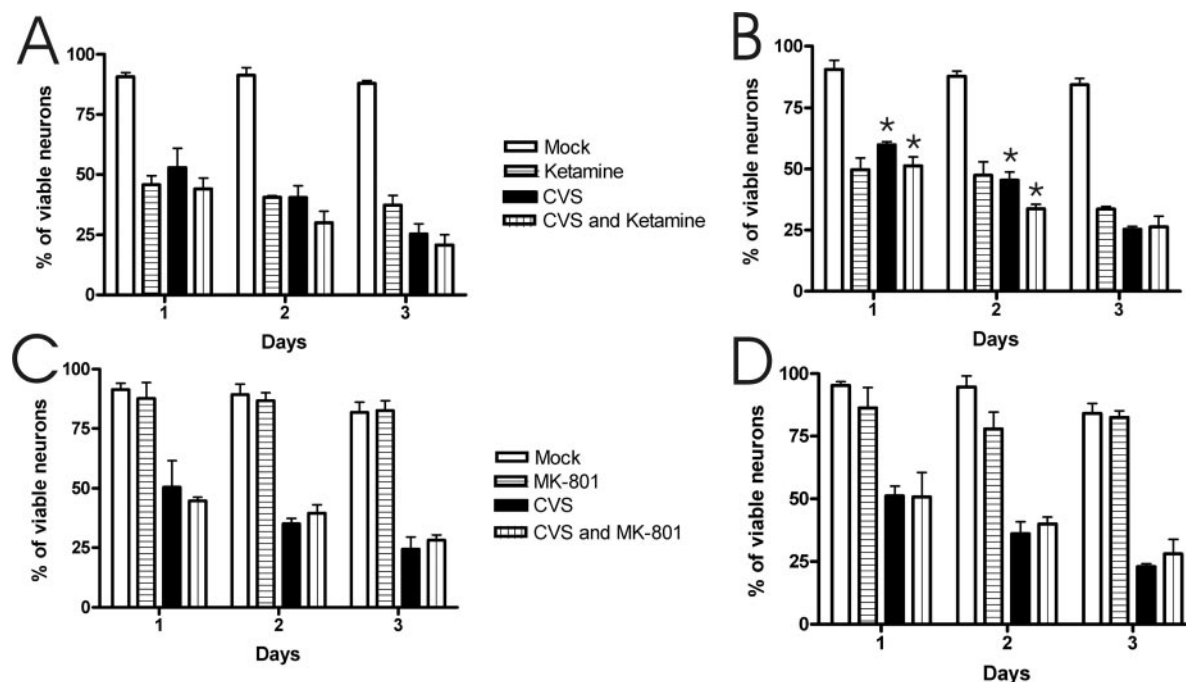


FIG. 2. Viability of mock- and CVS-infected cultured cortical (A, C) and hippocampal (B, D) neurons with and without treatment with 125 μ M ketamine (A, B) and 60 μ M MK-801 (C, D), as assessed by trypan blue exclusion. Ketamine did not improve viability of the CVS-infected neurons and actually reduced viability in CVS-infected hippocampal neurons at 24 and 48 h p.i. (B). MK-801 had no effect on the viability of the CVS-infected neurons. Error bars represent standard errors of the means.

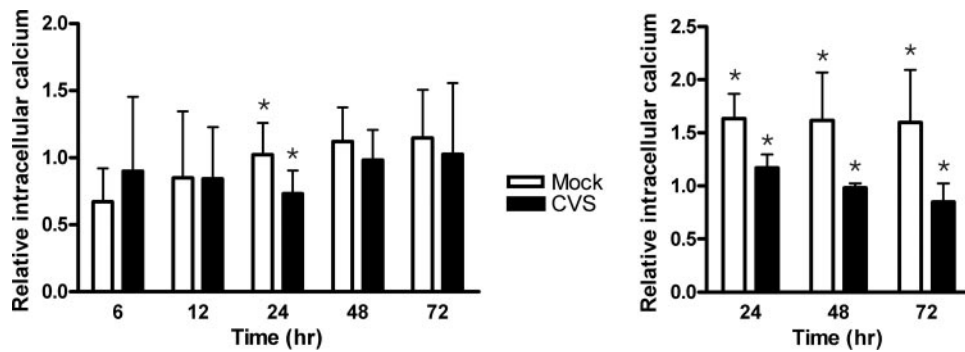


FIG. 3. Quantitative analysis of cytosolic calcium in CVS- and mock-infected cortical (left) and hippocampal (right) neurons in response to 50 mM KCl and 10 μ M glutamate stimulations. Results are shown as glutamate stimulation relative to potassium stimulation. There were significantly lower levels of intracellular Ca^{2+} release in CVS-infected neurons at 24 h in cortical neurons and at 24, 48, and 72 h in hippocampal neurons (*, statistical significance, $P < 0.05$; error bars, standard errors of the means).

play an important role in the CVS-infected primary neuron cultures and indicates functional impairment of rabies virus-infected neurons, which is of unknown cause (6). Sustained glutamate overstimulation was not observed in association with the loss of viability and progressive neuronal death in the infected cultures. In contrast, Sindbis virus infection of rat primary cortical neurons is associated with an increased intracellular calcium concentration (18).

Ketamine (60 mg/kg every 12 h intraperitoneally) did not result in reduced mortality or amelioration of the clinical neurological disease in mice compared to administration of the vehicle, and ketamine did not inhibit viral spread in this model. There was no reduction in the number of infected neurons or the amount of infectious rabies virus in the CNS compared to that in mice receiving only vehicle. In contrast, Tsiang's group previously reported that the same dosage of ketamine inhib-

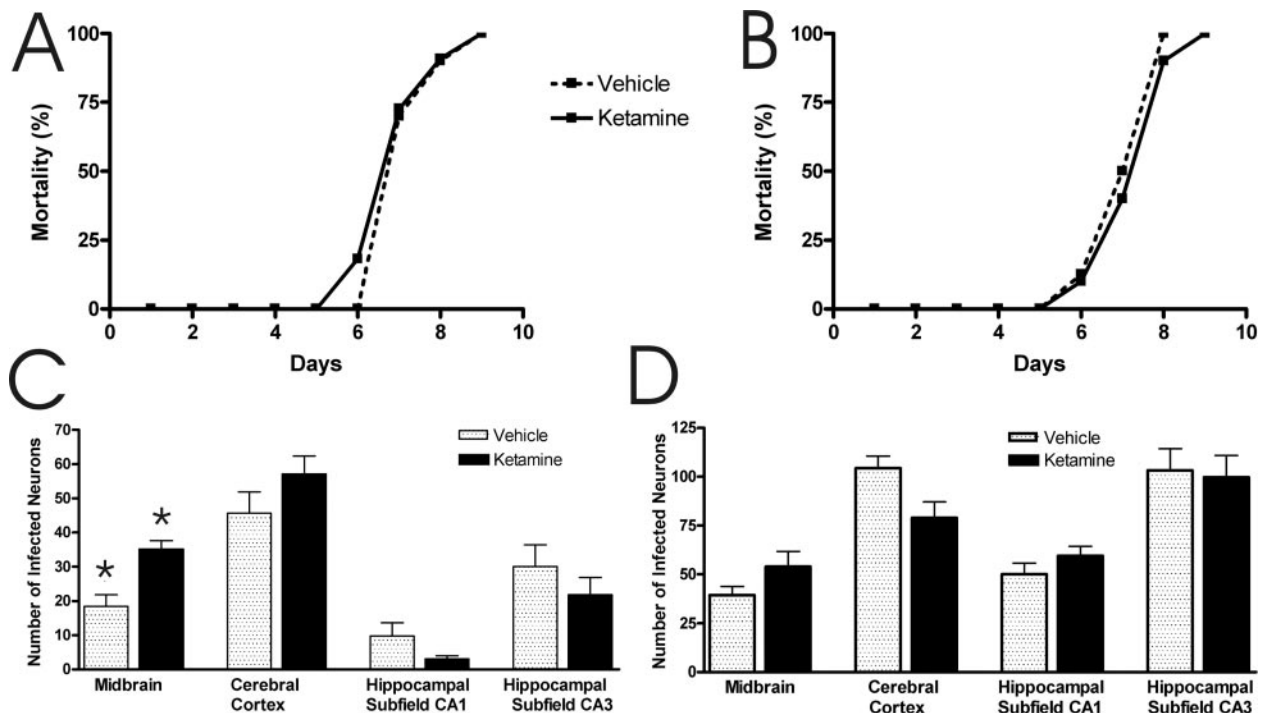


FIG. 4. Kaplan-Meier survival curves of the cumulative mortality in mice inoculated in the right hind limb footpad (A) and intracerebrally (B) with CVS and treated twice daily with vehicle (dashed lines) or ketamine (solid lines). Vehicle (footpad, $n = 10$; intracerebral, $n = 8$) and ketamine (footpad, $n = 11$; intracerebral, $n = 10$) treatment groups were compared using log rank tests, which indicated that there was no difference in the mortality rate between treatment groups (footpad, $P = 0.53$; intracerebral, $P = 0.50$). Counts of the number of infected neurons in various brain regions of moribund mice were taken after right hind limb footpad (C) and intracerebral (D) inoculation of CVS and twice daily treatment with vehicle or ketamine. Slides stained for rabies virus antigen were blinded, and the numbers of infected neurons were counted in three different fields of the same brain region using a high-power (40 \times) objective in areas with the most marked staining. Vehicle (footpad, $n = 8$; intracerebral, $n = 3$) and ketamine (footpad, $n = 11$; intracerebral, $n = 6$) treatment groups were compared using an unpaired t test (*, statistical significance, $P < 0.05$; error bars, standard errors of the means).

ited viral spread after stereotaxic inoculation of rats with CVS (16). Virus entry using footpad inoculation better evaluates viral spread through the neuroaxis, and it is highly doubtful that there is a species-specific difference in the therapeutic effect of ketamine. The more comprehensive analyses used in the present study showed the lack of therapeutic efficacy of ketamine.

CVS infection of the brain is associated with widespread neuronal apoptosis after infection by the intracerebral route (11). Therapy with ketamine was not associated with neuroprotection, as assessed by evaluation of neuronal apoptosis in the brain. No beneficial effects of therapy were observed clinically, histopathologically, or by analysis of the viral spread in the mice.

These studies were performed with CVS, and it is unknown if street rabies virus variants have similar biological properties. The findings indicate that caution should be taken before subjecting future human rabies patients to therapy with ketamine on the basis of the previous experimental work (15, 16, 20). There have been at least four patients treated with ketamine after the survivor received ketamine in 2004, and all of these patients progressed to fatal outcomes (7). Hence, further experimental studies are needed to evaluate the efficacy of ketamine therapy in rabies virus infection before it becomes a standard therapy for human rabies.

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Supplemental Tables

Table S1. Rabies virus infection was evaluated in the midbrain, cerebral cortex and hippocampus (CA1 and CA3 regions) of mice inoculated in the right hindlimb footpad or intracerebrally and treated with either ketamine or vehicle twice daily on days 3 through 6 p.i. (n=4 in each treatment group on each day). Slides stained for rabies virus antigen were masked and the numbers of infected neurons were counted in three different fields with the most marked staining within the same brain region using high power (40×) objective. Counts are expressed as the mean score ± standard error of the mean. Treatment with ketamine or vehicle was compared using an unpaired *t*-test, and a *p* < 0.05 was considered to be significant.

Brain Region	Vehicle	Ketamine	p value
Footpad Inoculation			
Day 3			
Midbrain	0 ± 0	0.3 ± 0.3	0.39
Cerebral cortex	0 ± 0	0 ± 0	1.00
Hippocampal subfield CA1	0 ± 0	0 ± 0	1.00
Hippocampal subfield CA3	0 ± 0	0 ± 0	1.00
Day 4			
Midbrain	2.4 ± 1.8	1.0 ± 0.7	0.49
Cerebral cortex	0.3 ± 0.3	0 ± 0	0.39
Hippocampal subfield CA1	0 ± 0	0 ± 0	1.00
Hippocampal subfield CA3	0 ± 0	0 ± 0	1.00
Day 5			
Midbrain	18.2 ± 1.7	6.8 ± 6.5	0.14
Cerebral cortex	26.0 ± 15.5	5.2 ± 4.0	0.24
Hippocampal subfield CA1	0 ± 0	0 ± 0	1.00
Hippocampal subfield CA3	0 ± 0	0 ± 0	1.00
Day 6			
Midbrain	29.6 ± 4.3	29.1 ± 5.0	0.94
Cerebral cortex	58.7 ± 9.3	53.9 ± 4.6	0.66
Hippocampal subfield CA1	1.9 ± 1.6	0.6 ± 0.6	0.46
Hippocampal subfield CA3	6.4 ± 4.0	6.2 ± 5.8	0.97
Intracerebral Inoculation			
Day 3			
Midbrain	0.5 ± 0.3	0.5 ± 0.3	1.00
Cerebral cortex	0.8 ± 0.4	0.5 ± 0.3	0.79
Hippocampal subfield CA1	1.3 ± 0.7	5.3 ± 1.1	0.13
Hippocampal subfield CA3	6.2 ± 3.2	19.7 ± 4.7	0.24
Day 5			
Midbrain	27.0 ± 3.0	25.3 ± 2.6	0.80
Cerebral cortex	76.3 ± 10.5	76.4 ± 11.6	0.99
Hippocampal subfield CA1	33.6 ± 6.0	32.9 ± 7.3	0.97
Hippocampal subfield CA3	52.8 ± 4.1	68.6 ± 10.5	0.40

Table S2. Viral infectivity assays of brain and spinal cord tissues, expressed as mean titers (ffu/g tissue) \pm the standard error of the mean, of four ketamine- and four vehicle-treated mice at day 5 post-inoculation using the fluorescent focus assay. There was no significant difference in the titers of mice receiving vehicle and ketamine.

CVS	Vehicle	Ketamine	p value
Intracerebral inoculation			
Brain	$1.0 \pm 0.2 \times 10^5$	$83.1 \pm 7.3 \times 10^5$	0.3000
Footpad inoculation			
Brain	$4.0 \pm 0.6 \times 10^4$	$5.6 \pm 2.0 \times 10^4$	0.4557
Spinal cord	$3.8 \pm 0.7 \times 10^4$	$5.0 \pm 0.6 \times 10^4$	0.2643