Protection from fatal viral encephalomyelitis: AMPA receptor antagonists have a direct effect on the inflammatory response to infection

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Neuronal cell death during fatal acute viral encephalomyelitis can result from damage caused by virus replication, glutamate excitotoxicity, and the immune response. A neurovirulent strain of the alphavirus Sindbis virus (NSV) causes fatal encephalomyelitis associated with motor neuron death in adult C57BL/6 mice that can be prevented by treatment with the prototypic noncompetitive α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) glutamate receptor antagonist GYKI 52466 [Nargi-Aizenman J, *et al.* (2004) *Ann Neurol* 55:541–549]. To determine the mechanism of protection, NSV-infected mice were treated with 7-acetyl-5-(4-aminophenyl)-8(*R*)-methyl-8,9-dihydro-7*H*-1,3-dioxolo-(4,5-*h*)-benzodiazepine (talampanel), a potent, orally available member of the 2,3 benzodiazepine class of noncompetitive AMPA glutamate receptor antagonists. Talampanel-treated mice were protected from NSV-induced paralysis and death. Examination of the brain during infection showed significantly less mononuclear cell infiltration and no increase in astrocyte expression of glial fibrillary acidic protein in treated mice compared with untreated mice. Lack of CNS inflammation was attributable to failure of treated mice to induce activation and proliferation of lymphocytes in secondary lymphoid tissue in response to infection. Antibody responses to NSV were also suppressed by talampanel treatment, and virus clearance was delayed. These studies reveal a previously unrecognized effect of AMPA receptor antagonists on the immune response and suggest that prevention of immune-mediated damage, in addition to inhibition of excitotoxicity, is a mechanism by which these drugs protect from death of motor neurons caused by viral infection.

alphavirus | paralysis | glutamate | talampanel | Sindbis virus

Arthropod-borne viruses are an emerging worldwide problem. The mosquito-borne alphaviruses Venezuelan equine encephalitis (VEE) and eastern equine encephalitis (EEE) viruses cause outbreaks of encephalomyelitis in the Americas (1). In 1995, VEE reemerged in Venezuela and Colombia, causing an epidemic of 75,000–100,000 cases (2), and in 2005, the number of EEE cases in the United States was the largest since 1964 (3). Increased circulation and spread of these viruses underscores the need for identification of therapeutic interventions.

Sindbis virus (SINV), the prototypic alphavirus, causes rash, fever, and polyarthritis in humans and encephalomyelitis in mice. SINV infects neurons in mice and provides an established model for acute viral encephalitis. The outcome of infection depends on the age and strain of the mouse, the virulence of the virus, and the host immune response (4–6). Neuroadapted SINV (NSV) causes paralysis and death in susceptible strains of adult mice (4, 7) and serves as a model for fatal encephalomyelitis. Hippocampal neurons and motor neurons are particularly targeted for infection (8, 9), and neuronal death can be apoptotic or necrotic (10, 11). Death of uninfected bystander neurons associated with glutamate-induced excitotoxicity also has been observed as a consequence of neuronal infection *in vitro* and *in vivo* (12–14).

Glutamate is an amino acid neurotransmitter that induces neuronal death when present in excess. Glutamate excitotoxicity is mediated by Ca^{2+} influx through iontropic glutamate receptors and has been implicated in acute and chronic neurological disorders (15–19). Three types of receptors have been defined by the drugs that activate them: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainic acid. All can be involved in excitotoxic neuronal damage. NMDA receptor antagonists protect neurons from SINV-induced death *in vitro* (13), but treatment of NSV-infected mice with an NMDA receptor antagonist does not protect against fatal paralytic disease (8, 14). However, treatment with the prototype noncompetitive AMPA receptor antagonist GYKI-52466 prevents damage to spinal cord motor neurons and protects mice from NSV-induced paralysis and death (14).

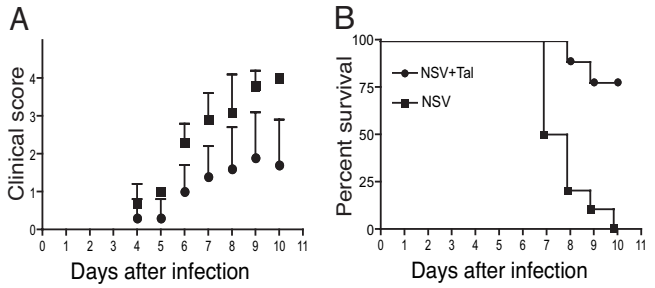

Fig. 1. Morbidity and mortality of untreated and talampanel-treated, NSV-infected mice. B6 mice infected intracerebrally with 10^3 pfu of NSV received either 15 mg/kg talampanel ($n = 20$) or vehicle ($n = 20$) every 12 h for 8 days after infection. (*A*) Paralysis in untreated (NSV) and treated (NSV+Tal) groups. Paralysis scoring was as follows: 0, no signs of illness; 1, weakness; 2, moderate paralysis in one hindlimb; 3, severe paralysis in both hind limbs; and 4, death. The data represent means ± SD. $P = 0.0276$, unpaired Student's *t* test. (*B*) Mortality in untreated and treated mice. $P = 0.0002$, Kaplan–Meier log rank test.

AMPA receptors assemble from subsets of four subunits, GluR1 to GluR4. Ca^{2+} permeability is determined by the GluR2 subunit. GluR2 imparts low Ca^{2+} permeability on AMPA receptors by virtue of an arginine in its pore-forming region that results from RNA editing of GluR2 primary transcripts that code

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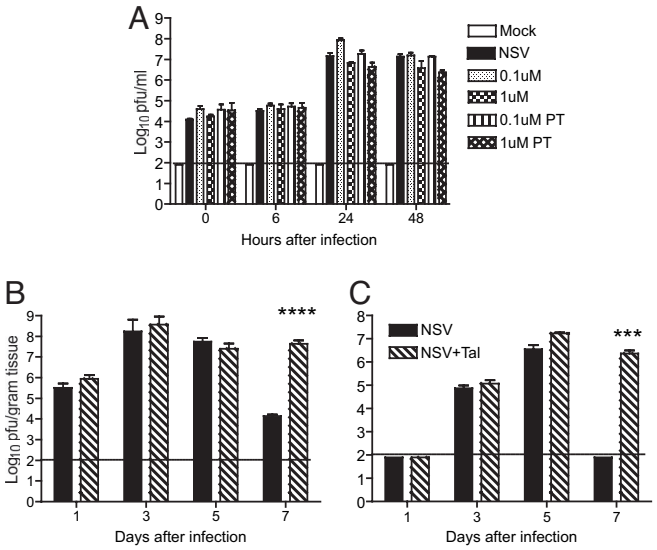

Fig. 2. Effect of talampanel treatment on virus replication. (*A*) Differentiated CSM 14.1 cells were infected with NSV at an multiplicity of infection of 10 and treated with 0.1 or 1 μM talampanel either 1 h before infection (PT) or immediately after infection. Supernatant fluids were assayed for infectious virus by plaque formation. (*B* and *C*) B6 mice were infected with 1,000 pfu of NSV intracerebrally and treated with 15 mg/kg talampanel twice daily (NSV+Tal) or not treated (NSV). Tissue homogenates from brain (*B*) and spinal cord (*C*) were assayed for infectious virus. The bars represent geometric means ± SD of three samples per group. The line indicates the limit of detection. ***, $P < 0.0009$; ****, $P < 0.0001$.

for glutamine (20). Most neurons contain AMPA receptors that include edited GluR2(R) subunits and are resistant to AMPA-induced excitotoxic damage (21, 22). However, motor neurons have a high proportion of Ca^{2+}-permeable receptors and are particularly sensitive to glutamate excitotoxicity mediated through AMPA receptors (16, 18, 21).

To determine the mechanism of protection from fatal viral encephalomyelitis by AMPA receptor antagonists, we studied NSV-induced encephalomyelitis in mice by using a potent, orally available noncompetitive AMPA receptor antagonist, 7-acetyl-5-(4-aminophenyl)-8(*R*)-methyl-8,9-dihydro-7*H*-1,3-dioxolo-(4,5-*h*)-benzodiazepine (talampanel). Talampanel prevented NSV-induced paralysis and death and decreased the inflammation and astrogliosis associated with infection. Virus replication was not affected, but humoral and cellular immune responses were inhibited and viral clearance was delayed. These data suggest that AMPA receptor antagonists protect from fatal alphavirus-induced paralytic disease, in part, by suppressing the immune response to infection.

Results

AMPA Receptor Blockade Protects Against NSV-Induced Fatal Encephalomyelitis. To determine the effect of talampanel treatment on NSV-induced fatal encephalomyelitis, B6 mice infected intracerebrally with NSV were treated for 8 days beginning at the time of infection (Fig. 1). Signs of illness became apparent in untreated animals by days 4–5 and progressed to severe hindlimb paralysis and death by day 10. AMPA receptor blockade delayed the onset of disease and prevented complete hindlimb paralysis in the majority of NSV-infected animals ($P < 0.01$) (Fig. 1*A*). Mortality decreased from 100% in untreated mice to 25% in talampanel-treated mice ($P = 0.0002$) (Fig. 1*B*). There were no deaths after day 10, and treated mice that became paralyzed often survived and recovered motor function over several days.

Effect of Talampanel on Virus Replication and Clearance. To determine whether talampanel had a direct antiviral effect, differentiated CSM 14.1 neuronal cells were infected and treated *in vitro* (Fig. 2*A*). There was no effect on viral replication when added either at the time of infection or before infection. There was also no effect on viral replication in the brains (Fig. 2*B*) or spinal cords (Fig. 2*C*) of treated mice. Maximal levels of virus at day 3 were similar in untreated (4.31×10^8 pfu/g) and treated (1.77×10^8 pfu/g) mice. However, virus clearance differed. In untreated mice, virus clearance was almost complete by day 7, but in treated animals, virus in the brain remained high ($P < 0.0001$) (Fig. 2*B*). This was confirmed by immunohistochemical staining for NSV protein (Fig. 3). Fewer NSV-infected cells were detectable at day 7 in the brains of untreated mice (Fig. 3*A*) than treated mice (Fig. 3*B*) (Fig. 3*C*; $P = 0.0006$). A delay in virus clearance was also evident in the spinal cord ($P = 0.0009$) (Fig. 2*C*).

Effect of Talampanel Treatment on CNS Inflammation. Brains were examined to determine the effect of talampanel treatment on the host inflammatory response to infection (Fig. 4). Treatment was associated with decreased inflammation, as evidenced by less perivascular cuffing and fewer infiltrating mononuclear cells (Fig. 4 *A* and *B*). To quantitate inflammatory cells, sections were stained for CD45 (leukocytes) and CD3 (T cells) (Fig. 4 *C–F*). $CD45^+$ leukocytes were most abundant in the brains of untreated mice at day 5 and began to decrease by day 7 (Fig. 4*I*), whereas $CD3^+$ T cells increased through day 7 (Fig. 4*J*). In treated mice, there were fewer $CD45^+$ cells present at both day 5 ($P < 0.0001$) and day 7 ($P = 0.0022$) and numbers of $CD3^+$ T cells were less abundant at all time points (day 3, $P = 0.0012$; days 5 and 7, $P = 0.0002$).

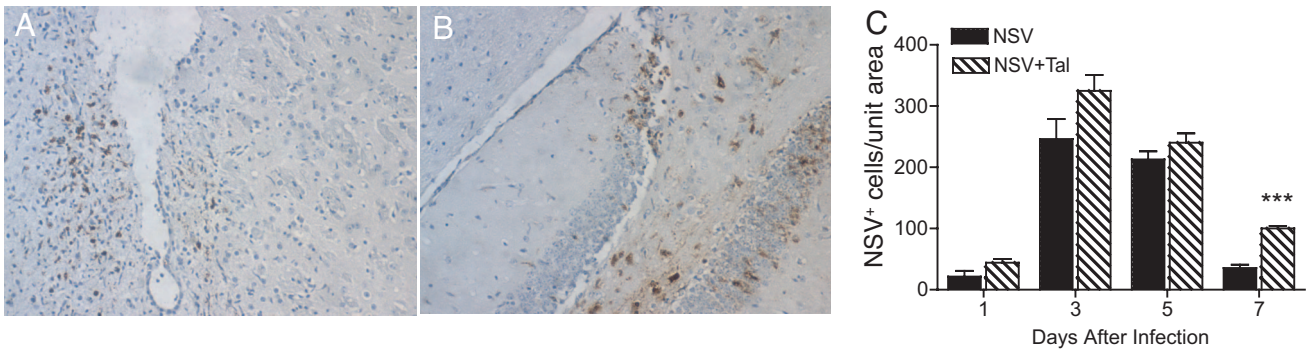

Fig. 3. NSV protein in the brains of untreated and talampanel-treated, NSV-infected mice. (*A* and *B*) Paraffin-embedded sections from the brains of untreated (*A*) and talampanel-treated (*B*) mice 7 days after intracerebral infection with NSV were stained with antibody to SINV. (*C*) Quantitative analysis of numbers of NSV-positive cells. The bars represent means ± SD of positive cells per unit area (0.986 mm^2) for 20 areas per animal for three animals per group. ***, $P = 0.0006$.

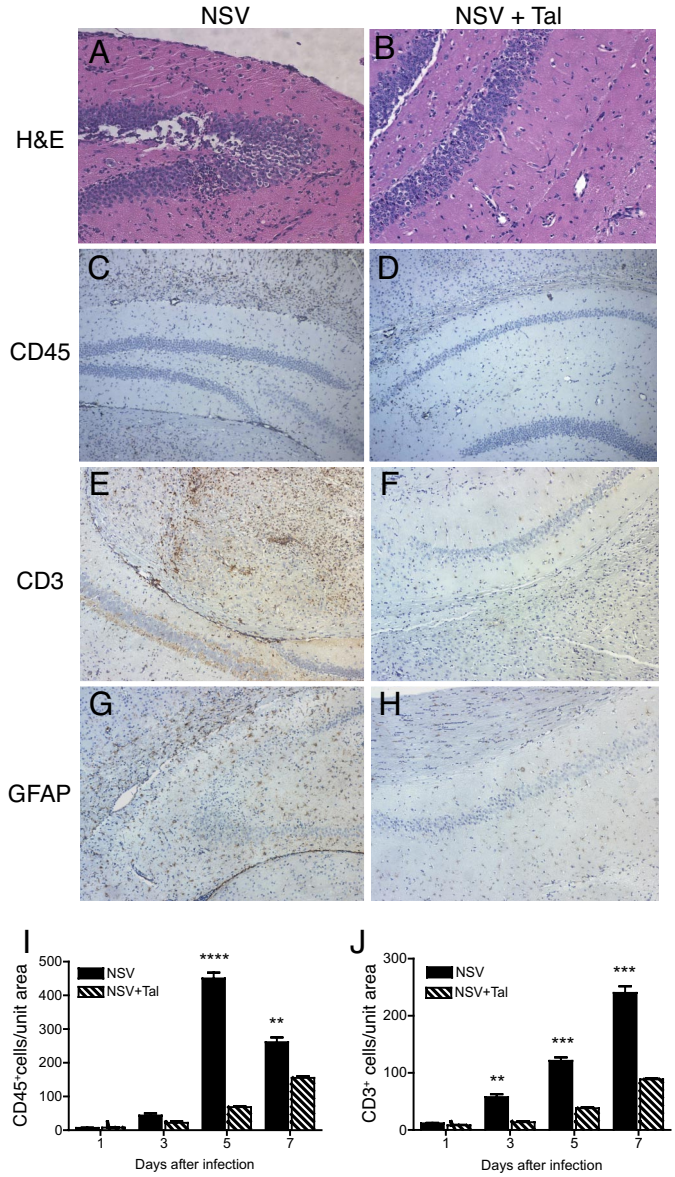

Fig. 4. Histological and immunohistochemical examination of untreated and talampanel-treated, NSV-infected mice. (*A–H*) Paraffin-embedded sections of brain from untreated (NSV) and talampanel-treated (NSV+Tal) mice were stained with H&E (day 7) (*A* and *B*) or antibodies to CD45 (day 5) (*C* and *D*), CD3 (day 7) (*E* and *F*), and GFAP (day 5) (*G* and *H*). (*I* and *J*) Numbers of infiltrating $CD45^+$ cells (*I*) and $CD3^+$ cells (*J*) were quantitated for 20 areas (0.986 mm^2) per animal for three animals per group. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

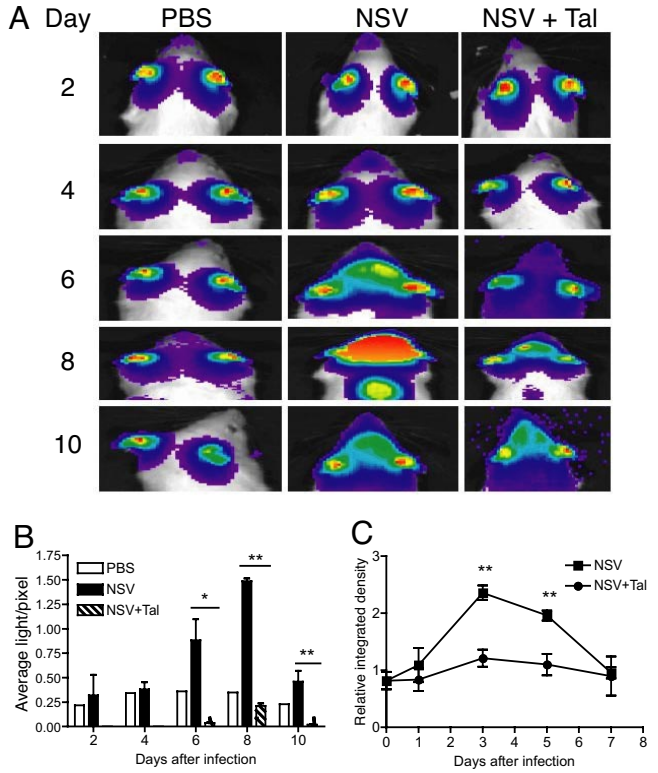

Fig. 5. GFAP promoter activation in untreated and talampanel-treated, NSV-infected GFAP–luciferase FVB/n mice. Transgenic mice expressing luciferase behind the GFAP promoter were mock-infected (PBS) or infected with NSV and not treated (NSV) or infected and treated with talampanel (NSV+Tal). For 10 days after infection, three mice per group were injected with luciferin and imaged. (*A*) Representative images from one mouse per group on days 2, 4, 6, 8, and 10. (*B*) Quantitation of light/pixel of mock-infected (PBS), untreated (NSV), and treated (NSV+Tal) mice. The bars represent means ± SEM for three animals per group per day. *, $P = 0.0257$; **, $P < 0.01$. (*C*) Results of immunoblot analysis of GFAP expression in brains of untreated and treated B6 mice from three independent experiments. The points represent the means ± SEM of three mice per group. **, $P < 0.01$.

Effect of AMPA Receptor Blockade on Astrocyte Activation. Excitotoxic injury and inflammation are accompanied by astrogliosis reflected by astrocyte proliferation and increased expression of glial fibrillary acidic protein (GFAP) (23–25). NSV infection increased GFAP expression, and at day 5, there was substantially more GFAP immunoreactivity in the brain sections of untreated animals than treated animals (Fig. 4 *G* and *H*). To quantify the effect of talampanel on GFAP expression, GFAP–luciferase transgenic mice were imaged over the course of infection (Fig. 5*A*). NSV infection resulted in an increase in GFAP promoter activity from days 6 to 10 (Fig. 5*B*) that was lower in treated mice on days 6 ($P = 0.0257$), 8 ($P = 0.0011$), and 10 ($P = 0.0058$). To confirm a difference in levels of protein, brain lysates were evaluated by immunoblotting (Fig. 5*C*). GFAP was increased in NSV-infected mice within 24 h but was not increased in treated mice. Levels were lower in treated than untreated mice on days 3 ($P = 0.012$) and 5 ($P = 0.0044$).

Effect of Talampanel on T Cell Activation. To determine whether the decrease in inflammation in talampanel-treated mice was attributable to an effect on induction of the cellular immune response to NSV or on entry of activated lymphocytes into the CNS, draining lymph nodes were studied (Fig. 6). Proliferation of cells in secondary lymphoid tissue during the immune response to NSV was greater for untreated mice than treated mice, as evidenced by lymph node weight (Fig. 6*A*) and cell counts (Fig. 6*B*). Percentages of $CD3^+$ cells that were $CD4^+$ (Fig. 6*C*) or $CD8^+$ (Fig. 6*D*) were not markedly different.

Effect of Talampanel Treatment on Antibody Production. Antibody is an important contributor to clearance of SINV from the CNS (26). To determine whether talampanel affected production of antiviral antibody, serum levels of neutralizing and enzyme immunoassay (EIA) binding antibody were assessed (Fig. 6 *E* and *F*). At day 7, levels of virus-neutralizing antibody ($P = 0.0437$) (Fig. 6*E*) and virus-specific IgG, as measured by EIA ($P < 0.0001$) (Fig. 6*F*), were higher in untreated than treated animals.

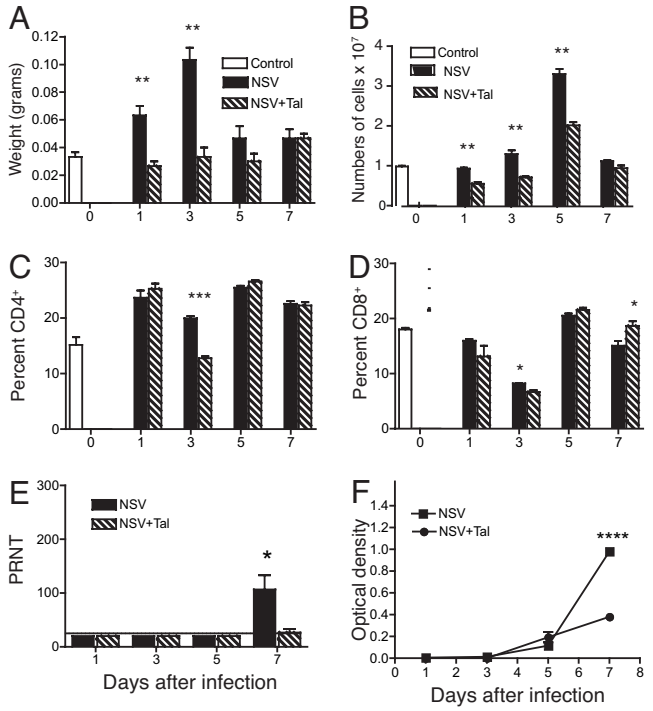

Fig. 6. Immune responses in untreated and talampanel-treated, NSV-infected mice. (*A* and *B*) Draining cervical and deep cervical lymph nodes were collected and weighed (*A*), and cells were counted (*B*). (*C* and *D*) Cells were analyzed by flow cytometry for expression of CD4 (*C*) and CD8 (*D*) on $CD3^+$ cells. (*E* and *F*) Sera were assayed for NSV-specific neutralizing antibody by PRNT (*E*) and for binding IgG antibody by EIA (*F*). The points represent means ± SEM of three samples per group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Discussion

NSV-infected mice treated with the noncompetitive AMPA receptor antagonist talampanel were protected from paralysis and death caused by neuronal infection. Talampanel did not protect by inhibiting virus replication but decreased CNS inflammation, astrogliosis, and antibody responses to infection. Lack of inflammation in the CNS was attributable to a failure of treated mice to induce a cellular immune response in peripheral secondary lymphoid tissue in response to infection. Suppression of immune responses resulted in a delay in virus clearance, indicating that fatal encephalomyelitis was not primarily attributable to virus replication in neurons. Therefore, AMPA receptor antagonists protect from fatal virus-induced motor neuron damage, in part, by preventing inflammatory damage, as well as excitatory damage, to infected neurons. Inhibition of the immune response and prevention of CNS inflammation are previously unrecognized consequences of treatment with noncompetitive AMPA receptor antagonists.

Glutamate-mediated excitotoxicity contributes to the pathogenesis of acute and chronic neurological diseases (27). Motor neurons are particularly prone to AMPA-mediated excitotoxic damage because they are more likely to have AMPA receptors without GluR2 or with unedited GluR2(Q) (16, 28). Previous studies have shown that NSV-induced motor neuron death can be inhibited *in vitro* and *in vivo* by treatment with AMPA receptor antagonists (12, 14). Because damage increases the number of Ca^{2+}-permeable AMPA receptors on neurons (18, 21), virus infection is likely to enhance the intrinsic susceptibility of neurons to excitotoxicity. The current studies have confirmed a protective effect *in vivo* by using talampanel, a noncompetitive AMPA receptor antagonist that protects neurons from excitotoxic damage in animal models of trauma, ischemia, and seizures (19, 27, 29, 30) and has been in clinical trial for treatment of seizures and amyotrophic lateral sclerosis (31–33). Talampanel has not been studied previously for protection from virus-induced neuronal damage and may be especially protective for infected motor neurons. Prevention of Ca^{2+} influx is likely to be one mechanism by which talampanel protects against alphavirus-induced death and paralysis.

Treatment inhibited astrocyte activation in response to infection. The major mechanism for maintenance of homeostatic levels of extracellular glutamate in the CNS is glutamate transport by astrocytes (34). Astrocytes also regulate GluR2 expression in motor neurons (35). Infection of the CNS induces an intense virus-specific inflammatory response, and the relative contributions of inflammation and excitotoxicity to astrocyte activation are not clear (36, 37). Astrocyte transport of glutamate can be decreased, and, thus, glutamate excitotoxicity can be exacerbated, by virus infection and CNS inflammation (12). Activated astrocytes can also contribute directly to neuronal damage through the production of toxic factors such as nitric oxide and CXCL10 (IP-10) (38–40). Talampanel inhibition of astrogliosis in NSV-infected mice likely contributes to a more favorable outcome.

The inflammatory response to SINV infection consists of $CD4^+$ and $CD8^+$ T cells, B cells, and macrophages and infiltration of these cells is coincident with clearance of virus (41, 42). Although talampanel had no direct antiviral affect, treatment did result in delayed viral clearance, which is mediated by antibody to viral glycoproteins and T cell production of IFN-γ (26, 43). Therefore, delayed virus clearance in talampanel-treated mice was most likely attributable to the decrease in production of virus-specific antibody and decreased infiltration of IFN-γ-producing T cells into the CNS.

The T cell response also can contribute to fatal NSV-induced disease. Mortality is reduced in mice deficient in T cells or β_2-microglobulin, the light chain of the class I MHC (44, 45), and in mice treated with drugs that decrease inflammation (37). Neurons are restricted in expression of the MHC proteins needed for antigen-specific interaction with T cells, but activated T cells can cause neuronal death by mechanisms that are independent of MHC (46, 47). For instance, granules from cytotoxic T cells can result in lethal Ca^{2+} influx, which can be prevented by blocking NMDA glutamate receptors (48). Improved survival of mice with a delay in viral clearance suggests that the host immune response to infection contributed to neuronal damage. Therefore, the decrease in T cell infiltration into the CNS during NSV infection is likely to be an important contributor to the protection by talampanel.

Decreased CNS inflammation could be attributable to the inability of virus-specific lymphocytes to enter the CNS or to reduced activation of lymphocytes in the periphery. Lymphocyte proliferation was inhibited by treatment with talampanel, indicating that decreased production of activated cells for entry into the CNS most likely accounts for decreased inflammation. Talampanel, as well as competitive AMPA receptor antagonists, also protects from the inflammatory demyelinating disease experimental autoimmune encephalomyelitis (49) presumably because of inhibition of neuronal glutamate excitotoxicity. However, AMPA receptors are present on many nonneuronal cells, including lymphocytes; therefore, treatment is likely to have effects outside of the CNS (50, 51). Specifically, glutamate and glutamate receptors may play a role in the induction of immune responses. T lymphocytes express glutamate receptors, including ionotropic AMPA receptors (52–56). Dendritic cells, the major cells responsible for antigen presentation, release glutamate when in contact with T cells (57), and T cells respond to low doses of glutamate with increased Ca^{2+} influx, proliferation, migration, and adhesion (52, 53, 58, 59), suggesting an important

role for glutamate in modulating the immune response (60). Treatment of NSV-infected mice with talampanel suppressed the virus-specific immune response and provides convincing evidence of the importance of glutamate and ionotropic AMPA receptors in the adaptive immune response *in vivo*. Thus, AMPA receptor antagonists may act through multiple mechanisms to protect motor neurons from damage during viral infection and deserve consideration as adjunctive therapy in viral encephalomyelitis.

Materials and Methods

Virus, Cells, and Drug. BHK-21 cells were used to grow stocks of NSV (7) and for assay of infectious virus in supernatant fluids and tissue homogenates by plaque formation. CSM 14.1 cells, a rat nigral neuronal cell line immortalized with a temperature-sensitive SV40 T antigen (61, 62), were grown in DMEM supplemented with FBS. Cells were differentiated by shifting from the permissive conditions of 31°C and 10% FBS to the nonpermissive conditions of 39°C and 1% FBS (63, 64).

The AMPA glutamate receptor antagonist talampanel (IVAX Research) was dissolved in DMSO. Three-week-differentiated CSM 14.1 cells were treated with talampanel diluted in MEM/1% FBS to 0.1 or 1 μM (65) for 1 h before or immediately after infection with NSV at a multiplicity of infection of 10 and continued through the course of the experiment.

Animals, Treatment, and Imaging. Four- to 5-week-old female C57BL/6 mice (The Jackson Laboratory) were inoculated intracerebrally with 1,000 pfu of NSV in 20 μl of HBSS. Talampanel was delivered i.p. at a dose of 15 mg/kg every 12 h from the time of infection through 7 days after infection. Control animals were injected every 12 h with diluent. Mice were observed twice daily for signs of disease. Clinical scoring was performed as described in ref 14. For assessment of virus replication, three animals per group were perfused with PBS, tissues were collected, and 10% homogenates were assayed by plaque formation.

For imaging, 5- to 6-week-old female FVB/n mice engineered to express the firefly luciferase gene behind the GFAP promoter (Xenogen) were infected and treated for 12 days after infection. At 2-day intervals, three animals per group were inoculated i.p. with 17 μl/g 2.5% solution of avertin (Sigma), followed by 150 mg/kg luciferin (Xenogen). Images were acquired with the IVIS camera system and analyzed with LivingImage 2.11 software (Xenogen).

All animals were maintained and studies were performed in accordance with experimental protocols approved by the Animal Care and Use Committee of The Johns Hopkins University.

Histology and Immunohistochemistry. On days 1, 3, 5, and 7, three animals per group were perfused with PBS, followed by 4% paraformaldehyde. Brains were postfixed in 4% paraformaldehyde overnight at 4°C and then embedded in paraffin. For immunoperoxidase staining, 6-μm sections of brain were treated with 1% hydrogen peroxide in ice-cold methanol for 30 min to block endogenous peroxidase. Slides were stained, as described in ref. 66, with H&E or antibodies to CD3 (Dako), CD45 (Chemicon International), SINV (8), or GFAP (Chemicon International). Biotin-conjugated secondary antibodies were used. For quantitation, the stained sections were photographed, and the numbers of positive cells per unit area (0.986 mm^2) for 20 areas per animal for three animals per group were counted.

Antibody Assays. Antibody to NSV was measured by EIA and plaque-reduction neutralization (PRNT). Serum was collected from three animals per group, heat-inactivated at 56°C for 1 h, and stored at −80°C. NSV-specific IgG antibody was quantitated by EIA as described in ref. 67. Titers are reported as optical density for serum diluted 1:100. For PRNT, titers are reported as the reciprocal of the highest dilution inhibiting ≥80% plaque formation.

Immunoblotting. Brains from PBS-perfused animals were homogenized in lysis buffer (10 mM Tris, 1% SDS, 1 mM Na orthovanadate) and stored at −80°C. Twenty micrograms of protein was run on 12% SDS/PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk, followed by incubation with primary and secondary antibodies in 1% milk. Primary antibodies against GFAP and actin (Chemicon International) were used. Secondary antibodies were HRP-conjugated ECL anti-rabbit or anti-mouse IgG (Amersham). Membranes were incubated with chemiluminescence reagent (Pierce) and imaged.

Flow Cytometry. Superficial and deep cervical lymph nodes were collected and weighed. Single cells were isolated, counted, and incubated with anti-CD16/CD32 antibody for 15 min at 4°C. Cells were then incubated with FITC-conjugated anti-CD3 and either APC-conjugated anti-CD8 or peridinin chlorophyll A protein-conjugated anti-CD4 (BD Pharmingen). Stained cells were analyzed with a FACS Calibur flow cytometer (Becton Dickinson) and Flowjo version 8.5.2 software.

Statistical Analysis. Data from three independent experiments or at least three mice per group were analyzed by unpaired Student's *t* test. Survival was compared by using Kaplan–Meier survival curves (log rank test). All statistical analysis was performed by using Prism version 4.0b for Macintosh (GraphPad).

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