Published in final edited form as:

Brain Res. 2009 March 31; 1263: 143–154. doi:10.1016/j.brainres.2009.01.040.

Tumor necrosis factor- α modulates glutamate transport in the CNS and is a critical determinant of outcome from viral encephalomyelitis

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Abstract

Neuroadapted Sindbis virus (NSV) is a neuronotropic virus that causes a fulminant encephalomyelitis in susceptible mice due to death of motor neurons in the brain and spinal cord. We and others have found that uninfected motor neurons die in response to NSV infection, at least in part due to disrupted astrocytic glutamate transport, resulting in excitotoxic motor neuron death. Here, we examined the mechanisms of astrocyte dysregulation associated with NSV infection. Treatment of organotypic slice cultures with NSV results in viral replication, cell death, altered astrocyte morphology, and the downregulation of the astrocytic glutamate transporter, GLT-1. We have found that TNF- α can mediate GLT-1 downregulation. Furthermore, TNF- α deficient mice infected with NSV exhibit neither GLT-1 downregulation nor neuronal death of brainstem and cervical spinal cord motor neurons and have markedly reduced mortality. These findings have implications for disease intervention and therapeutic development for the prevention of CNS damage associated with inflammatory responses.

Keywords

Astrocyte; TNF- α ; Motor neuron; GLT-1; Glutamate; Virus

1. Introduction

Sindbis virus (SV) is an alphavirus, similar to eastern, western, and Venezuelan encephalitis viruses, arboviruses that are naturally transmitted by mosquitoes. Intracranial inoculation of susceptible adult mice with the neuroadapted strain (NSV) of Sindbis virus results in substantial neuronal death in the brain, brainstem and spinal cord, and high animal mortality and flaccid hindlimb paralysis in survivors (Jackson et al., 1987, 1988). NSV is a neuronotropic virus productively infecting only neurons since non-neuronal cells in the central nervous system (CNS) do not support viral replication (Jackson et al., 1988). We have demonstrated that motor

neuron loss exceeds the number of neurons actually infected with NSV and that non-infected bystander neurons die because the NSV-induced inflammatory response triggers excitotoxic death (Darman et al., 2004; Nargi-Aizenman and Griffin, 2001; Nargi-Aizenman et al., 2004).

The NSV-elicited inflammatory response involves the recruitment of activated macrophages, CD4⁺, and CD8⁺ T cells and results in the secretion of many cytokines (Binder and Griffin, 2003) (Binder and Griffin, 2001; Wesselingh et al., 1994). NSV infection of CD4⁺ and CD8⁺ deficient mice results in reduced paralysis, and NSV-infected interferon- γ deficient mice are protected from NSV-mediated mortality highlighting the potentially destructive role of the inflammatory response in NSV infection (Rowell and Griffin, 2002). We have demonstrated that NSV-infected mice treated with an inhibitor of microglial activation are protected from paralysis and mortality as well as from the downregulation of GLT-1 expression (Darman et al., 2004).

Excitotoxic motor neuron cell death may be one of the consequences of inflammation within the spinal cord and may be caused by failure of appropriate astrocytic glutamate transport away from neuronal synapses (Anderson and Swanson, 2000; Bal-Price and Brown, 2001; Bal-Price et al., 2002; Bezzi et al., 2001, 2004; Darman et al., 2004; Mander et al., 2005; Rossi et al., 2000). Additionally, cytokines have been shown to predispose neurons to excitotoxic death (Drachman et al., 2002; Fernandez-Ortega et al., 2004; Huang and O'Banion, 1998). TNF- α , in particular, has been shown to downregulate astrocyte-mediated glutamate transport either by repression of enzymes that astrocytes use to process glutamate (Chakrabarti, 1998; Huang and O'Banion, 1998) or by the direct downregulation of GLT-1 (Fine et al., 1996; Su et al., 2003; Szymocha et al., 2000; Zou and Crews, 2005).

In this study we used spinal cord organotypic slice cultures and found that NSV infection in this culture system recreates the downregulation of astrocytic GLT-1 and excitotoxic motor neuron death seen in adult rodents. TNF- α is secreted within spinal cord cultures following NSV infection and correlates with the repression of GLT-1 expression and activity. GLT-1 levels are maintained in NSV-infected cultures deficient in TNF- α demonstrating a role for TNF- α in the repression of GLT-1. Infection of TNF- α deficient mice results in neither GLT-1 downregulation nor significant motor neuron death in the brainstem and cervical spinal cord, and these animals exhibit markedly reduced mortality. These data emphasize the potentially deleterious effects of CNS inflammation and implicates TNF- α as a critical mediator of neuron death in viral encephalomyelitis.

2. Results

To verify that NSV would behave in spinal cord organotypic slice cultures as it does in susceptible mice (Darman et al., 2004; Jackson et al., 1987; Nargi-Aizenman et al., 2004), we have characterized NSV infection of these cultures. After 12 h of incubation in serum-free media, each well (containing 5 $\times$ 250 μ m thick slices) of spinal cord organotypic cultures was inoculated with 1 $\times$ 10⁷ PFU/mL administered directly to the tissue slices. Culture supernatants were collected from at least 3 individual wells at each time point. Over a 48-hour period virus levels increase in culture supernatants by several log units (Fig. 1A) confirming that there is viral replication within this culture system. Since NSV is a neuronotropic virus *in vivo*, we investigated whether exclusively neurons were infected *in vitro* as well. We found that though astrocytes and microglial cells were not productively infected by a recombinant NSV-GFP construct (Figs. 1B, C), motor neurons (Figs. 1D, E) were infected, leading to the expression of GFP within these cells. Additionally, NSV infection of organotypic sections led to cellular injury as defined by LDH release (Fig. 1F) and loss of motor neurons (Fig. 1G). These findings suggested that this culture system replicated the *in vivo* tropism and neuronal injury seen with

NSV infection *in vivo* and that because of the absence of the acquired immune system would allow us to determine intrinsic neural determinants of neural injury following viral infection.

We investigated the humoral inflammatory response within organotypic cultures following NSV infection from the supernatants of at least 3 wells of slice cultures using multiple cytokine/chemokine protein arrays. We found TNF- α and IL-6 consistently elevated at early time points post infection (data not shown). We have previously used cytokine/chemokine arrays to screen for inflammatory factors upregulated in NSV-infected spinal cord lysates and found that there was no elevation of multiple factors including IL-2, IL-4, IFN- γ , RANTES and IL-17. We also performed quantitative ELISA for nitric oxide metabolites and MCP-1 and did not see elevation (data not shown).

We then quantitatively defined the time course and extent of this upregulation after NSV infection (Figs. 2A, B). While TNF- α could not be detected at time 0 of infection, it became significantly elevated at 12 and 24 h post infection. Peak TNF- α levels were approximately 900 ± 0.202 pg/mL in the supernatant of infected cultures at 24 h post infection. These studies confirm that an endogenous inflammatory response is initiated within infected organotypic cultures characterized by a cytokine response similar to what has been observed *in vivo* (Binder and Griffin, 2001, 2003; Wesselingh et al., 1994). TNF- α became significantly elevated through time at 12 and 24 h following NSV infection ($p < 0.01$, Figs. 2A, B).

We previously showed that downregulation of the astrocytic glutamate transporter GLT-1 was an important determinant in the outcome from NSV infection *in vivo* (Darman et al., 2004) and so we chose to investigate whether NSV infection alters astrocyte glutamate transport *in vitro*. We generated at least 3 pooled tissue lysates from NSV-infected spinal cord organotypic culture slices and carried out immunoblot analyses against GLT-1. As observed in lysates from NSV-infected adult mice (Darman et al., 2004), there was a significant decrease in GLT-1 protein expression through time over a 24-hour period of NSV-infection of spinal cord organotypic cultures (Figs. 3A, B; $p < 0.05$). In order to determine if this decline in GLT-1 protein correlated with a decline in functional GLT-1-mediated glutamate transport, membrane preparations were generated from pooled tissues of organotypic slice cultures in triplicate (Fig. 3C). Membrane preparations from at least 3 sample pools were exposed to radioactive glutamate in an assay that primarily tests the ability of GLT-1 to mediate glutamate transport (Darman et al., 2004; Sepkuty et al., 2002). GLT-1-mediated glutamate transport was significantly reduced in cultures 24 h post-NSV infection (61.727 pmol/mg/min ± 11.8), compared to mock-treated controls (139.70 pmol/mg/min ± 15.7 ; $p < 0.05$). We conclude that NSV infection of spinal organotypic cultures results in neuronal specific infection, neuron death, and repression of GLT-1 expression and function.

Since TNF- α has been shown to directly downregulate the expression (Fine et al., 1996; Su et al., 2003; Szymocha et al., 2000) and function (Fine et al., 1996; Zou and Crews, 2005) of GLT-1, we determined whether purified, recombinant rat TNF- α was capable of mimicking the observed downregulation of GLT-1 seen after NSV infection in spinal organotypic cultures. TNF- α treatment of spinal cord organotypic slice cultures results in a transient increase in GLT-1 protein expression, followed by a repression at 24 h post-treatment (Figs. 4A, B, $p < 0.05$). GLT-1-mediated glutamate uptake was also significantly reduced ($p < 0.05$) upon treatment with TNF- α at 24 h post treatment (46.89 pmol/mg/min ± 5.80) compared to mock treatment (139.7 pmol/mg/min ± 15.7 , Fig. 4C). These data suggest that TNF- α is sufficient to induce a reduction in GLT-1 expression in spinal cord organotypics and may be a critical inflammatory mediator of excitotoxic neuron death following NSV infection.

In order to determine if TNF- α is necessary for the reduction in GLT-1 expression following NSV infection, we used TNF- α -/- spinal organotypic cultures infected with NSV. Cultures

generated from TNF- α deficient mice were susceptible to NSV-infection and replicate virus similarly to cultures generated from wild type mice (Fig. 5A). Expression of GLT-1 was preserved in pooled spinal cord organotypic slice cultures deficient in TNF- α (Figs. 5B, C). Resolved bands represent comparisons of tissue collected from individual TNF- α -/- animals that were either mock treated (0) or infected (24, Fig. 5B). Relative ratios of GLT-1 expression in TNF- α -/- (0.9088 ± 0.031) compared to WT (0.6071 ± 0.061 , $p < 0.05$) 24 h after NSV infection (Fig. 5C) reveal preservation of GLT-1 expression in the absence of TNF- α . We found that functional glutamate transport from mouse organotypic sections was unreliable and therefore presented these data in the form of persistence of GLT-1 by immunoblot analyses.

To test the importance of TNF- α in NSV pathogenesis *in vivo*, 5–6 week old TNF- α deficient mice were infected with NSV and the animals were monitored daily for mortality. TNF- α -/- mice were significantly protected from NSV-mediated mortality (mortality of 33% vs 91% for TNF- α -/- vs WT at 7 days post infection, $p < 0.05$, Fig. 6A). All of the WT control mice were dead by 9 days post infection which is typical for NSV infection (Thach et al., 2000), however, after 14 days, only 30% of TNF- α KO mice had succumbed to NSV-mediated mortality. Peak virus replication in these animals is similar, though virus persists in the TNF- α deficient mice compared to surviving WT mice (Fig. 6B). This reduction in mortality was associated with a paradoxical upregulation of GLT-1 expression at days 3–5 post infection as defined by immunoblot analyses with a return to baseline levels by 8 days post infection (Fig. 6C). Consistent with these findings there was a near complete preservation of GLT-1 mediated glutamate transport at 6 days post infection (Fig. 6D). These data suggest that TNF- α is necessary to induce the downregulation of GLT-1 expression and function *in vivo* following NSV. While we are not sure why there was a paradoxical upregulation of GLT-1 expression at days 3–5 post infection, we have previously reported similar findings when microglial activation was inhibited during NSV infection (Darman et al., 2004).

In order to investigate the disparity in mortality observed between the wild type and TNF- α -deficient mice infected with NSV, 5 animals were given intravenous injections of hydro-ethium (HEt) prior to sacrifice. In dying cells, HEt is taken up and oxidized to a red fluorescent dye, ethidium (Murakami et al., 1998). Tissues collected from HEt injected mice were cryosectioned and counter stained for neurons with Nissl green. Because the difference in mortality may be related to differential survival of motor neuron populations in the brain stem and cervical spinal cord, we looked for HEt incorporation in these regions and in the hippocampus of NSV-infected WT and TNF- α -/- mice (Figs. 7A–C). There were more HEt-positive cells in the cortex, hippocampus, brain stem and cervical spinal cord of wild type mice compared to TNF- α deficient mice at 5 days post infection (Figs. 7A–C).

To determine whether brainstem and cervical motor neurons were resistant to NSV-induced death in the TNF- α -/- mice, we examined and quantified surviving motor neurons in a series of brainstem nuclei and the rostral cervical spinal cord. Neurons in these regions are important in cardiovascular and respiratory control. We reasoned that resistance of these neurons to NSV-induced excitotoxic death might correlate with the enhanced survival of these animals in response to NSV. The ventral respiratory group (VRG) nucleus, hypoglossal (XII) nucleus and the nucleus tractus solitarius (NTS) were chosen both because they are glutamatergically innervated (Schlenker et al., 2001) and regulate cardiovascular and pulmonary function (Bradley et al., 1996; Lawrence and Jarrott, 1996; Smith et al., 1991; Withington-Wray et al., 1988). The VRG and XII include neurons responsible for respiration-related motor output and the VRG also includes the pre-Botzinger complex, a likely site for automatic rhythmic breathing (Smith et al., 1991; Withington-Wray et al., 1988). The area postrema, a primarily noradrenergic non-glutamatergic system, was used as a control nucleus which would not be expected to be differentially resistant to NSV in WT and NSV animals since it does not receive glutamate as a neurotransmitter.

We found that at baseline (day 0) the number of neuronal nuclei in the area postrema, VRG, XII, NTS and cervical spinal cord did not differ between WT and TNF- α -/- mice. At day 7, there was a significantly reduced number of surviving neurons in the VRG, XII NTS and cervical spinal cord, but not in the non-glutamatergic neurons of the area postrema (Table 1). Remarkably, in the TNF- α -/- mice, there was no decline in the number of surviving neurons in any of the four examined brainstem nuclei or the cervical spinal cord. This confirms that there is a significant correlation between animal mortality and death of motor neurons involved in respiratory and cardiovascular control in WT mice. Further, this confirms that TNF- α is required for the death of motor neurons that receive glutamatergic input and that protection of these neurons by the absence of TNF- α correlates with enhanced survival of these mice in response to NSV infection.

We conclude, therefore, that both *in vitro* and *in vivo*, infection with NSV results in upregulation of TNF- α that results in downregulation of GLT-1 expression and function. In the absence of TNF- α , GLT-1 expression is preserved and critical neuronal populations do not die resulting in markedly reduced mortality. We further conclude that excess TNF- α , produced from CNS cells, is critical in NSV pathogenesis and potentiates motor neuron loss.

3. Discussion

In this study we have investigated the intrinsic neural inflammatory response to NSV infection and have defined the upregulation of TNF- α in NSV-infected neural tissue to be a critical mediator of neural death by modulation of astrocyte-mediated glutamate transport. These studies were performed in organotypic slice cultures so that we could observe the interactions between the endogenous cell types present in the CNS independent of infiltrating cells. Additionally, investigation of GLT-1 expression in dissociated primary astrocyte cultures is difficult because they do not readily express GLT-1 unless supplemented with cAMP analogs or cultured with neurons or in neuron conditioned media (Schlag et al., 1998; Zelenai et al., 2000). We have found that treatment of rat spinal cord organotypic cultures with NSV results in a productive infection exclusively of neurons. The increase in viral titer, the decrease in motor neuron counts, and increase in LDH release (cell death) were all predicted and validated the use of this model system for study of the innate inflammatory response to NSV infection in CNS tissue.

Since astrocytes are not productively infected by NSV either *in vivo* or in organotypic culture slices, we reasoned that a component of the intrinsic neural inflammatory response to NSV may be contributing to the altered astrocyte function and capacity to remove glutamate from neuronal synapses. Although the primary source of TNF- α secretion in neural culture slices is not known, we suspect that astrocytes (Bezzi et al., 2001; Brouwer et al., 2004; Fernandes et al., 2004; Johnstone et al., 1999; Ransohoff and Tani, 1998) or microglial cells (Darman et al., 2004; Tikka et al., 2001; Wu et al., 2002) are the most likely sources. A potential trigger for TNF- α release in these organotypic slice cultures could be the activation of microglial cells by the presence of the virus. Microglial cells, as macrophage lineage cells, have the capacity to recognize viral RNA either by toll-like receptors (TLRs, Akira and Takeda, 2004) or dsRNA dependent RNA polymerase (PKR, Steer et al., 2003), or retinoic acid inducing gene-1 (RIG1, Onomoto et al., 2007; Yoneyama et al., 2004). Whatever the source, the release of inflammatory cytokines into the supernatant of spinal organotypic cultures undeniably signifies an important role for glial cells in the immune response to NSV.

There is evidence in the literature suggesting that IFN- γ , and not TNF- α , is important for the clearance of NSV from infected neurons (Binder and Griffin, 2003). However, in our organotypic spinal cord slice culture system, we have isolated the CNS tissue in order to study the effects of the innate immune response (endogenous, tissue-specific cells such as astrocytes

and microglia) independent of the adaptive immune response (infiltrating T-cells and macrophages). Accordingly, we did not anticipate a release of IFN- γ (secreted by T-cells) in our organotypic cultures and instead focused on TNF- α . Although, TNF- α has not been shown to be protective in prior studies and that we find it protective from NSV-mediated fatality in TNF- α -deficient mice is likely due to the fact that TNF- α is pleiotropic serving both as a mediator of intrinsic neural effects and as a critical component of a complex inflammatory cascade. The lack of TNF- α in the system could shift the entire inflammatory cascade resulting in altered viral clearance.

Our findings are consistent with evidence in the literature suggesting that cytokines can disrupt the expression of glutamate transporters (Prow and Irani, 2008). Specifically, TNF- α expression can disrupt the expression and function of GLT-1 (Fine et al., 1996; Sitcheran et al., 2005; Su et al., 2003; Zou and Crews, 2005). Additionally, TNF- α has also been shown to modulate AMPA receptor expression (reviewed in Bains and Oliet, 2007). TNF- α has been shown to increase the surface expression of AMPA receptors as well as overall synaptic strength in rat hippocampus slice cultures (Stellwagen and Malenka, 2006). Therefore, in addition to disrupting astrocyte-mediated clearance of synaptic glutamate, TNF- α could also render neurons to be hypersensitive to extracellular glutamate via the increased expression of AMPA receptors. Treatment of NSV-infected mice with AMPA receptor antagonists has been shown to protect from virus-mediated mortality (Darman et al., 2004; Greene et al., 2008; Nargi-Aizenman and Griffin, 2001; Nargi-Aizenman et al., 2004) indicating the importance of excitotoxicity in the pathogenesis of NSV infection.

At the protein level, TNF- α has the expected effect of decreasing GLT-1 expression at 24 h. However, we consistently observed an increase in GLT-1 protein at 6 and 12 h after TNF- α , suggesting that factors such as protein stabilization or increased translation may be important in this regard. Furthermore, the biphasic nature of TNF- α -mediated GLT-1 repression may be caused by the recruitment of distinct cofactors to the GLT-1 promoter at different times, resulting in either repression or enhancement of expression (Sitcheran et al., 2005). NSV-infection of the cultures results in a gradual, consistent decline in GLT-1 protein expression while TNF- α treatment initially caused an increase in GLT-1 protein, followed by a decrease. The disparity of GLT-1 repression between NSV-infection and TNF- α treatment indicates that while TNF- α is a major contributor to GLT-1 repression, it may not be the only factor involved in NSV-mediated GLT-1 repression. To test the significance of TNF- α -mediated repression of GLT-1 in the NSV model, spinal organotypic cultures from TNF- α -/- mice were infected and found to be resistant to the downregulation of GLT-1. Additionally, glutamate transport levels were protected from repression in NSV-infected TNF- α deficient cultures. Although multiple factors likely regulate astrocyte function and the repression of GLT-1, TNF- α is most prevalent contributor to GLT-1 repression in NSV-infected neural tissue.

The route of viral entry and infection *in vivo* following intracerebral inoculation involves the spread of virus via cerebral spinal fluid as evidenced by the presence (by immunohistochemistry) of viral particles in the ependymal cells along the central canal in the spinal cord; ultimately infecting motor neurons in the ventral horn of the lumbar spinal cord. This has been contrasted with the infection of polio virus in which the virus spreads via rapid axonal transport resulting in the infection of cervical spinal cord prior to lumbar (Jackson et al., 1987). Therefore, the death of motor neurons in the brain stem and cervical cords of WT mice is not due to the infection of these cells, rather these cells are dying due to a secondary effect of the disease pathology. The fact that there is little to no neuron death in the brain stem and spinal cord of TNF KO mice further supports the notion that death and paralysis are separable in this system and indicates that TNF- α may play a role in the spread of virus-mediated pathology. It is likely that the death of motor neurons in the brain stem and cervical cord renders NSV infection lethal, despite the fact that these cells are not directly infected by the virus.

There is an increasing appreciation of the role of endogenous inflammatory processes in the modulation of neuronal function. For example, a positive correlation has been shown between neural patterning and intrinsic neurotrophins (Barde, 1994) and major histocompatibility molecules (Tonelli et al., 2005). However, inflammatory molecules generated by endogenous cells in the CNS have often been shown to have deleterious effects on neurons. The generation of reactive oxygen species (Bal-Price and Brown, 2001), the downregulation of glutamate catabolizing enzymes in astrocytes (Belin et al., 1997; Hardin-Pouzet et al., 1997; Huang and O'Banion, 1998), and the repression of astrocyte-mediated glutamate transport (Darman et al., 2004; Fine et al., 1996; Sitcheran et al., 2005; Su et al., 2003) all contribute to neuronal loss. In instances of distress in the CNS, proper functioning of astrocytes is imperative for the survival of neurons.

4. Experimental procedures

4.1. Organotypic slice cultures

Spinal cord organotypic slice cultures were prepared from 8-day-old Sprague Dawley rats. Animals were euthanized according to Johns Hopkins Animal Care and Use Committee guidelines and spinal cords were removed under sterile conditions and placed in Gey's balanced salt solution (Sigma #G9779). Spinal cords were cut into 250 μ m sections using a McIlwain Tissue Chopper. Freshly cut spinal cords were placed into 0.4 μ m tissue culture plate inserts (Millicell #PICM03050) inside 6 well plates pre-incubated with 1 mL of organotypic growth media [50% MEM (Gibco #11575-032), 25% Hanks balanced salt solution (Gibco #24020-117), 25% heat-inactivated horse serum (Gibco #26050-088), 0.8% Hepes Buffer Solution [(Gibco #15630-080) and 1% penicillin/streptomycin (Gibco #15140-122)]. Growth media were changed one day after culturing and twice more before using. Cultures were used no sooner than 7 days after culturing. Cultures were changed to serum-free organotypic media [30mL MEM (Gibco #11575-032), 75 μ L 200 mM L-glutamine (Gibco #25030-081), 60 μ L B-27 supplement (Gibco #17504-044)] at 12 h prior to infection. For NSV infection, cultures were infected with neuroadapted Sindbis virus (NSV) or NSV expressing GFP (NSV-GFP) at time 0. One hour after infection, the inoculum was washed away and cultures were replenished with fresh serum-free media. For TNF- α treatment, cultures were treated with 1 μ L of 0.1 μ g/ μ L [100 ng, 20 μ g of TNF (Peprotech #400-14) reconstituted in 200 μ L of sterile, distilled water]. Culture slices and/or supernatants were pooled from 2 wells at time points indicated and at least 3 pooled samples were generated for each time point. Cell death was determined by lactate dehydrogenase (LDH) release using the Panvera (#11901) kit according the manufacturer's protocol.

4.2. Animal infections

C57BL/6 mice and TNF- α deficient mice on the B6 background (Tnf^{tm1Gkl}) were purchased from Jackson Laboratories. Male animals between 5 and 6 weeks of age were used for these studies. Mice were anesthetized by inhalation of isoflurane and injected intracranially with 1000 plaque forming units (PFUs) of NSV.

4.3. Tissue preparation

All tissues taken for time points are from animals that were terminally anesthetized by isoflurane and then transcardially perfused with cold 1 $\times$ PBS and 4% paraformaldehyde (PFA) for histological samples. Tissues were harvested and either used immediately for glutamate uptake studies, snap frozen for fresh frozen Western blot samples, or post-fixed overnight in 4% PFA. Finally histological samples were either soaked in 1 $\times$ PBS for paraffin embedding or 30% sucrose for cryosectioning. Paraffin blocks and frozen tissues embedded in OTC compound were cut into thin (<10 μ m) slices and placed on glass slides for immunostaining.

4.4. Glutamate uptake assay

Glutamate uptake assays were performed with slight modification to previously published protocols (Darman et al., 2004; Sepkuty et al., 2002). Briefly, fresh lumbar spinal cord tissue or 15 (a pooling of 3 wells containing 5 slices each) lumbar spinal cord organotypic culture tissue slices were homogenized in buffer (10mM Tris pH7.4, 5mM EDTA, 1 mini protease inhibitor tab from Roche Diagnostics, Mannheim, Germany) in order to obtain a fresh membrane preparation. The membrane pellets were clarified twice, resuspended in tissue buffer (0.05 M Tris, 0.32 M sucrose, pH 7.4), spun down and the resulting pellet was resuspended in 250 μ L of Na⁺-Krebs (120 mM NaCl, 25 mM NaHCO₃, 5 mM KCl, 2 mM CaCl₂, 1 mM KH₂PO₄, 1 mM MgSO₄; pH 7.4). Duplicate samples were generated in which Na⁺-free Krebs (120 mM Choline CL, 25 mM Tris, 5 mM KCl, 2 mM CaCl₂, 1 mM KH₂PO₄, 1 mM MgSO₄; pH 7.4) was used as the blanking control. Samples were incubated with ³H-glutamate for 4 minutes at 37 °C and immediately transferred to ice. Membrane-bound labeled glutamate was adsorbed to filter paper while free labeled glutamate passed through the filter. Filters were incubated in scintillation fluid overnight, and total counts per minute (cpm) measured. The amount of labeled glutamate bound by each sample (pmol/mg protein/min) was calculated using the total incorporated cpm and concentration of protein in each sample. Tissue samples were processed in duplicate and at least three independent experiments were carried out for each experimental condition.

4.5. Viral growth curve

Supernatant fluids from infected cultures were frozen at -80 °C until ready to use. Supernatants were then thawed and serially diluted in DMEM (Gibco #11885-084) supplemented with 1% fetal bovine serum (Gibco #1009901). 200 μ L of the diluted supernatants was plated onto confluent BHK-21 cells for 1 h. Infected cells were then overlaid with 2 mL of molten agar. Once hardened, the cultures were then incubated at 37 °C for 48 h. Cells were stained in a 10% solution of neutral red and plaques visualized and counted.

4.6. Immunofluorescence

Cultures to be stained were first fixed at indicated time points in 4% PFA for 1 hour at 4 °C. Cultures were washed 3 times in 1 $\times$ phosphate-buffered saline (PBS). Tissues were then transferred from the plate inserts into wells of a 24-well plate as floating sections. Tissues were blocked and permeabilized for 3 h [1 $\times$ PBS in 1% of 10 $\times$ Trypsin stock (Gibco #15400-054) and 5% normal goat serum (Vector Laboratories #S-1000)] on an orbital shaker at room temperature. Tissues were then washed 3 times in PBS and incubated with rabbit anti-mouse GFAP (Chemicon #MAB360, 1:1000), mouse anti-rat GFAP (Chemicon #AB5804, 1:1000), or anti-synaptophysin (Chemicon #MAB5258, 1:10,000) where indicated for 48 h at 4 °C. Tissues were then washed 3 times at room temperature for 30 min each and then incubated with the secondary antibodies Alexa-Fluor-594 goat anti-rabbit (Molecular Probes #A11012, 1:1000) and Alexa-Fluor-488 goat anti-mouse (Molecular Probes #A11008, 1:1000) for 24 h at 4 °C. Tissues from HET injected animals were post-fixed for 30 min in ice cold methanol and then counter stained with Nissl Green (Molecular Probes #N-21480). Confocal fluorescent images were captured with two-color imaging with a Zeiss (LSM510) confocal microscope. Images were acquired in green and red emission channels by an argon-krypton laser with a single-channel, line-switching mode.

4.7. Protein expression analysis

Tissues were sonicated in lysis buffer (10 mM Tris, 1% SDS, 1 mM sodium orthovanadate, pH 7.6), proteins quantitated by BCA assay (Pierce BCA Protein Assay Kit #23227), and 5 μ g of each sample subjected to SDS-PAGE and resolved in 12% acrylamide gels (Bioexpress). Proteins were transferred to polyvinylidene membranes (Millipore Immobilon-P) in 10%

methanol transfer buffer. Freshly transferred membranes were stained with Ponceau S Solution (Sigma #P-7170) and imaged to ensure equal loading. Membranes were blocked in 5% milk buffer and stained in 1% milk buffer using rabbit anti-ratGLT-1 (courtesy of Dr. Jefferey Rothstein, 1:10,000) and horseradish peroxidase conjugated anti-rabbit secondary antibody (Amersham Biosciences #RPN 4301, 1:15,000). Blots were developed with the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce #35095) and visualized using a Fuji Luminescent Image Analyzer (LAS-1000 plus camera). The intensity of each band was determined using Image Gauge software (version 3.4). Protein expression in infected tissue is normalized to levels in uninfected controls by dividing measured band intensities. Because virus infection variably alters the expression of several housekeeping genes making control immunoblots (GAPDH or actin) unreliable, Ponceau S staining of immunoblots is performed to ensure equal loading of lanes.

4.8. Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels in 3 samples of clarified slice culture supernatants were determined by ELISA. Rat tumor necrosis factor- α (TNF- α , Biosource # KRC3012) and mouse TNF- α (Biosource #KMC3012) were used following the manufacturer's protocols and analyzed with Softmax Pro3 software and cytokine concentrations determined by standard curve.

4.9. Quantification of brainstem and cervical spinal cord motor neuron death

Three WT and TNF- α -/- mice were used per group. For the NSV-infected animals, tissues were harvested at day 7 post infection. Brains were harvested from all animals after 4% PFA perfusion and 5 μ m paraffin coronal sections were generated. Sections were stained with hematoxylin and eosin. Every 6th section was scanned from rostral to caudal until the rostral-most section that contained the nucleus of interest was included. Individual sections were then examined rostral to that section in order to define the most rostral section that included neuronal nuclei from that nucleus. The number of neuronal nuclei was then quantified from the left brainstem nucleus on each of four consecutive slides. Microscopy was performed using a 100 $\times$ magnification lens after outlining the nucleus. Three animals were used per group and all the cells within the left outlined nucleus were counted per section.

4.10. Statistical analysis

SPSS 12.0 was used for all the statistical analyses. Due to the non-parametric nature of the data (as determined by using tests of normality), nonparametric equivalent tests of ANOVA and repeated measures ANOVA were used to increase the robustness of the results. The Kruskal-Wallis test was performed to analyze differences between groups at each time point, and Friedman's nonparametric repeated measures comparison was used to analyze differences across time within a group. Mann-Whitney *U* test was used for the comparison of two independent samples. Significance was assessed at the 0.05 level. These tests were used because they make no assumptions about the distribution of the data such as normality.

Abbreviations

NSV	neuroadapted Sindbis virus
GLT-1	glutamate transporter-1
TNF- α	tumor necrosis factor- α
HEt	hydroethidium bromide

Acknowledgments

We thank Irina Shats and Sonny Dike for their help in troubleshooting some of the experiments in this manuscript. We thank the Johns Hopkins PROJECT RESTORE for funding this research.

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Fig. 1.

NSV infection of spinal organotypic cultures induces astrocytic changes and motor neuron death. Spinal cord organotypic cultures were generated from 8-day old Sprague-Dawley rats and infected 1 week after culturing. (A) Viral titers in culture supernatants collected at the indicated time points. Viral titers are expressed as the LOG of plaque forming units (PFUs) (B) GFAP (red) staining for astrocytes in cultures infected with NSV-GFP construct (C) Iba-1 (red) staining for microglia in cultures infected with NSV-GFP virus (D, E) ChAT (blue) staining for motor neurons and NF (neurofilament, red) staining for neurons in cultures infected with NSV-GFP virus (F) LDH is measured as relative cytotoxicity of NSV-infected cultures compared to trypsin treated cultures for a measure of maximal death. There is a significant increase in cellular death of NSV-infected cultures through time ($p < 0.05$). Cultures 24 h post treatment (G) Mean motor neuron counts in tissue slices before infection (time 0) and 48 h post infection. There is significantly ($p < 0.05$) fewer motor neurons 48 h post NSV-infection.

Fig. 2.
ñ NSV infection of spinal organotypic cultures elicits and innate inflammatory response. Culture supernatants were sampled at the indicated time points. Supernatants were clarified to eliminate cellular debris and frozen down until used for ELISA. Both TNF- α (A) and IL-6 (B) increased in NSV-infected spinal organotypic culture supernatants through time ($p < 0.01$).

Fig. 3. ñ NSV infection of spinal organotypic cultures induces the downregulation of astrocyte mediated glutamate transport. Samples were collected from NSV-infected cultures at the indicated time points. (A, B) GLT-1 protein expression, normalized to uninfected cultures. Ponceau S staining of the membrane prior to Western blotting serves as loading control (LC). NSV infection of cultures results in a decline in GLT-1 protein expression through time, specifically at 12 and 24 h post infection ($p < 0.05$). (C) The glutamate uptake assay was used to study the ability of membrane preparations generated from infected cultures to transport radiolabeled glutamate. NSV-infection of spinal organotypic cultures induced a significant drop in glutamate transport at 24 h post infection ($p < 0.05$).

Fig. 4.

ñ TNF- α treatment of spinal organotypic cultures is sufficient to induce downregulation of astrocyte mediated glutamate transport. (A, B) GLT-1 protein expression in TNF- α -treated spinal organotypic cultures at the indicated time points. Protein expression is normalized to untreated cultures. Ponceau S staining of the membrane prior to Western blotting serves as loading control (LC). Treatment of spinal organotypic cultures results in a change in GLT-1 protein expression through time ($p < 0.05$) and is significantly increased at 6 and 12 h and decreased at 24 h post ($p < 0.05$). (C) Transport of radiolabeled glutamate by membrane preparations generated from TNF- α -treated cultures. Glutamate uptake is significantly reduced at 24 h post TNF- α treatment ($p < 0.05$).

Fig. 5.

ñ TNF- α is necessary for NSV-induced downregulation of astrocyte mediated glutamate transport. Spinal organotypic slice cultures were generated from 8-day old TNF- α -deficient mice and infected 1 week after culturing. (A) Viral titers are similar in cultures generated from both TNF- α deficient and WT mice. (B) GLT-1 protein expression in TNF- α -deficient spinal organotypic cultures at 24 h post infection. Protein expression is normalized to uninfected cultures. (C) There is a statistically significant decline in GLT-1 expression in WT cultures ($p < 0.05$) that is not observed in TNF- α deficient cultures. Viral titers in culture supernatants collected at the indicated time points.

Fig. 6.

ñ TNF- α deficient mice are resistant to NSV-induced glutamate downregulation and are protected from NSV-induced death. (A) Survival curves of both TNF- $\alpha^{-/-}$ (n=13) and WT C57BL/6 (n=15) mice demonstrate a protection from mortality of the TNF- α -deficient mice compared to age-matched WT controls. (B) Viral titers in TNF- α deficient mice suggest that the virus persists in these animals. By day 8 no detectable virus is observed in the surviving WT mice, however viral titers are still elevated in the remaining TNF- α deficient mice. (C) GLT-1 protein expression in TNF- α -deficient mice at the indicated time points reveals a constitutive expression of GLT-1 in TNF- $\alpha^{-/-}$ mice. (D) Glutamate uptake is significantly

reduced ($p < 0.05$) in the spinal cords of wild type mice 6 days post NSV infection while glutamate uptake in TNF- α -deficient mice is not significantly lowered after NSV infection.

Fig. 7.
 ñ Increased in neuronal death in WT compared to TNF- α deficient mice. Animals were treated with hydroethidium (HET) prior to sacrifice in order to detect the presence of dying cells. There is more neuronal HET incorporation in the cervical spinal cord (A), hippocampus (B), and brain stem (C) of WT mice compared to TNF- α -/- mice.

Table 1

Mean number of neurons in each of the brainstem nuclei (the area postrema, the ventral respiratory group, cranial nerve XII and the nucleus tractus solitarius) and motor neurons in the cervical spinal cord

	WT-Day 0	TNF-Day 0	WT-Day 7	TNF-Day 7	Significance
Area postrema	53 ± 3.8	55.5 ± 8.2	49.8 ± 8.7	54.1 ± 18.1	NSS
VRG	97.3 ± 27	92.3 ± 19.4	49.3 ± 22.1	70.5 ± 4.4	p<0.05 WT vs TNF day 7
XII	71.7 ± 10.3	70.7 ± 16.2	50.7 ± 22.5	76.4 ± 10.9	NSS
NTS	72.1 ± 12.9	75.7 ± 4.8	30.41 ± 19.8	65.1 ± 26.6	NSS
Cervical MN	30.7 ± 1.86	33.8 ± 4.0	13.2 ± 6.2	29.4 ± 1.8	p<0.05 WT vs TNF day 7

Abbreviations: VRG = ventral respiratory group; XII = hypoglossal nucleus, cranial nerve XII; NTS = nucleus tractus solitarius, dorsomedial nucleus. Shown above is the mean number of neuronal nuclei ± S.E. counted per section counted in a blinded manner in the three animals per group. Data were analyzed using a two way ANOVA with repeated measures design.